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AVAILABILITY AND USE OF ORGANIC MATTER IN STREAM ECOSYSTEMS: THE ROLE OF BIOFILMS

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PhD Thesis

AVAILABILITY AND USE OF ORGANIC MATTER IN STREAM ECOSYSTEMS: THE ROLE OF BIOFILMS

Irene Ylla i Monfort

2010

PROGRAMA DE DOCTORAT D' ECOLOGIA FONAMENTAL I APLICADA

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Memòria presentada per a optar al títol de Doctora per la Universitat de Girona

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Institut d'Ecologia Aquàtica

La Dra. Anna Maria Romaní i Cornet, professora titular del departament de Ciències Ambientals de la Universitat de Girona i el Dr. Sergi Sabater i Cortés, catedràtic d'ecologia del departament de Ciències Ambientals de la Universitat de Girona,

CERTIFIQUEN:

Que aquest treball titulat "Availability and use of organic matter in stream ecosystems: the role of biofilms", que presenta Irene Ylla i Monfort, per a l'obtenció del títol de Doctora, ha estat realitzat sota la seva direcció i que compleix els requeriments per poder optar a Menció Europea.

Dra. Anna Maria Romaní i Cornet

Dr. Sergi Sabater i Cortés

Girona, novembre 2010

L’aigua que toques en la superfície d’un riu, és l’última gota que acaba de passar i la primera que ve: així és l’instant present.

Leonardo Da Vinci

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En primer lloc voldria agrair als meus directors de tesi (Anna i Sergi) per confiar en mi i donar-me l'oportunitat d'embrancar-me en aquesta aventura de fer un doctorat. Vaig començar rondant en el departament col·laborant en la tesi d'un company i tot plegat un bon dia em van proposar de tirar endavant la meva pròpia tesi. Encara no havia tingut temps ni de pair-ho que ja em vaig trobar amb unes botes d'aigua posades i mostrejant en una riera anomenada Fuirosos (anomenada en els meus primers dies, Furiosos) rascant pedres del riu amb un raspall de dents per extreure'n unes algues llefiscoses a les quals anomenaven biofilm.

En segon lloc voldria donar les gràcies als meus pares (Mayra i Josep) l'ajuda i suport dels quals ha estat imprescindible per tirar endavant en segons quins moments del doctorat, perquè s'ha de reconèixer que això de fer una tesi té els seus punts negres!

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La tesi presentada a continuació té un format de tesi com a compendi d'articles ja que compleix els requisits establerts per la Comissió d'Autorització de Defensa de Tesis Doctorals en la sessió de 16 de desembre de 2009. En base a les condicions establertes en l'esmentada sessió, tot seguit es mostra que es compleixen els mínims de qualitat i quantitat d'articles sol·licitats per presentar la tesi com a compendi d'articles. En el meu projecte de tesi presento dos articles publicats dels quals en ambdós en sóc la primera autora, dos que es troben en revisió i també en sóc la primera autora i un tercer en preparació el qual en sóc coautora. Les referències dels articles, i en cas que ja hagin estat publicats, les àrees temàtiques, factors d'impacte (FI) i el rang de les revistes segons l'ISI web de l'any 2009 són les següents:

- Referència: Ylla, I., Sanpera-Calbet, I., Vázquez, E., Romaní, A. M., Muñoz, I., Butturini, A. & Sabater S. 2010. Organic matter availability during pre- and post-drought periods in a Mediterranean stream. Hydrobiologia 657: 217-232.
    - Àrea temàtica: *Marine and Freshwater Biology*
    - FI: 1.754
    - Rang dins la categoria: 27/88

- Referència: Ylla, I., Sanpera-Calbet, I., Muñoz, I., Romaní, A. M. & Sabater, S. 2010. Organic matter characteristics in a Mediterranean stream through amino acid composition: Changes driven by intermittency. *Submitted to Aquatic Sciences.*

- Referència: Ylla, I., Borrego, C., Romaní, A. M. & Sabater, S. 2009. Availability of glucose and light modulates the structure and function of a microbial biofilm. FEMS-Microbiology Ecology 69: 27-42.
    - Àrea temàtica: *Microbiology*
    - FI: 3.598
    - Rang dins la categoria: 26/94

- Referència: Ylla, I., Romaní, A. M. & Sabater, S. 2010. Differential effects of increasing water temperature on labile versus recalcitrant biofilm dissolved organic carbon use. *Submitted to Limnology and Oceanography.*

- Referència: Peter, H., Ylla, I., Gudasz, C., Romaní, A. M., Sabater, S., & Tranvik, L. 2010. Multifunctionality is affected by loss of diversity in microbial biofilm communities. *In prep*.

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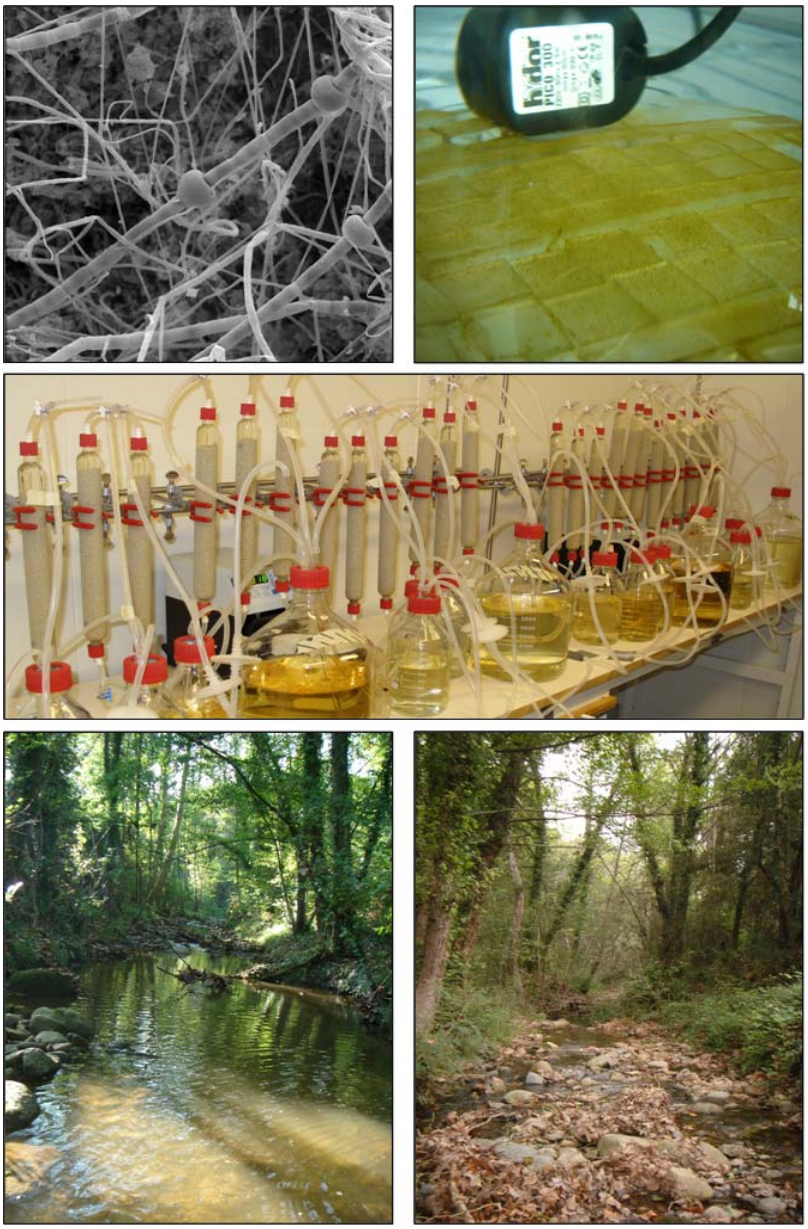


SUMMARY; RESUMEN; RESUM

SUMMARY (English)

The benthic community in river ecosystems processes a large amount of organic matter entering the river. The input sources (autochthonous and allochthonous) as well as their chemical composition and amount (frequency of inputs and concentrations reaching the river), determine the structure of the autotrophic and heterotrophic benthic community, their trophic relationships and their potential interactions (competition, synergism). It is the hypothesis of this thesis that the amount as well as the quality and availability of materials will favour the relative abundance of autotrophic/heterotrophic microorganisms as well as their diversity, which at the end will determine the overall efficiency in the use of the flowing materials.

Mediterranean stream ecosystems, mainly at their headwaters and middle sections, show a high hydrological variability. Floods and droughts are common events that shape the biological and biogeochemical processes. Global changes (including climate change) may favour a higher intensity and frequency of droughts and floods, as well as a potential rise of stream water temperature. Understanding the effects of these disturbances on materials entering the river and on the biofilm community could significantly contribute to knowing the functioning of the biological communities in these specific periods in Mediterranean systems. This includes the benthic microbial loop, through which flows an important quantity of the organic matter and energy processed by the river, hence becoming the base of the autodepurative fluvial processes.

The aim of this thesis is to elucidate the use of dissolved organic matter (DOM) by the benthic stream microbial biofilms and determine the efficiency of the fluvial system in the use of the different materials that circulate through it. Different experiments were developed in the field and others in laboratory conditions to control the respective effects of organic matter availability (quantity) and quality (chemical composition and lability), as well as the effects of increasing river water temperature.

CHAPTER I: ORGANIC MATTER AVAILABILITY DURING PRE- AND POST-DROUGHT PERIODS IN A MEDITERRANEAN STREAM

Mediterranean streams ecosystems are characterized by water flow changes caused by floods and droughts. To characterize the changes in the stream organic matter quantity and quality throughout a drying and rewetting process, a field monitoring (Fuirosos stream) was done. Organic matter and enzyme activities were analyzed in the benthic accumulated material (biofilms growing on cobbles, leaves, and sand) and in flowing water (dissolved and particulate fractions). The total

polysaccharide, amino acid and lipid content in the benthic organic matter were on average higher in the drying period than in the rewetting period. However, during the drying period, peptide availability decreased, as indicated by decreases in leucine aminopeptidase activity as well as amino acid content in the water and benthic material, except leaves; while polysaccharides were actively used, as indicated by an increase in β-glucosidase activity in the benthic substrata and an increase in polysaccharide content of the particulate water fraction and in leaf material. During this process, microbial heterotrophs were constrained to use the organic matter source of the lowest quality (polysaccharides, providing only C), since peptides (providing N and C) were no longer available.

During the flow recovery phase, the microbial community rapidly recovered, suggesting the use of refuges and/or adaptation to desiccation during the previous drought period. The scouring during rewetting was responsible for the mobilization of the streambed and loss of benthic material, and the increase in high quality organic matter in transport. The dynamics of progressive and gradual drought effects, as well as the fast recover after rewetting, might be affected by the interaction of the individual dynamics of each benthic substratum: sand sediments and leaves providing refuge for microorganisms and organic matter storage, while on cobbles an active bacterial community is developed in the rewetting. The maintenance of benthic substrata heterogeneity within the stream may be important for stream recovery after droughts.

CHAPTER II: ORGANIC MATTER CHARACTERISTICS IN A MEDITERRANEAN STREAM THROUGH AMINO ACID COMPOSITION. CHANGES DRIVEN BY INTERMITTENCY

As a detailed follow-up of the first chapter, the stream amino acid composition was studied during the wet-drought-wet periods occurring in Mediterranean streams. The amino acid composition (quality) and abundance (quantity) was analyzed in epilithic and epipsammic biofilms and in leaf material; as well as in the DOM. The amino acid content and composition analyzed at the different compartments were highly dependent on the organic matter source as well as on its diagenetic state. Amino acids were sensitive enough to detect changes in organic matter characteristics. The highest-quality organic matter (high degradation index and high N content) occurred on cobble biofilm substrata, whereas the most degraded and lowest-quality organic matter accumulated in sand sediments. Relevant differences in amino acid quantity and quality between the pre- and post-drought periods occurred in the DOM, with a large peak of fresh organic matter in the rewetting day. The amino acid composition of DOM reflects the heterogeneous mixture of source inputs: autochthonous inputs prevailed

during the pre-drought while allochthonous inputs dominated during the post-drought period. In contrast, the steadier composition of the benthic compartments indicates organic matter of in situ origin, which quality progressively decreasing throughout the drought period.

CHAPTER III: DIFFERENTIAL EFFECTS OF INCREASING WATER TEMPERATURE ON LABILE VERSUS RECALCITRANT BIOFILM DISSOLVED ORGANIC CARBON USE

Dissolved organic carbon (DOC) inputs shifts between periods in temperate streams, from high quality DOC when primary productivity is high to recalcitrant DOC after long periods of organic matter (OM) accumulation. Climate change may reinforce these shifts in DOC quality and concentration. A laboratory experiment mimicking these different OM sources was performed under two water temperatures (14 and 18ºC). It is aimed to determine whether a temperature increase could differentially affect the degradation of labile versus recalcitrant DOC by microbial biofilms. The potential combination of OM pulses and higher water temperature may provide different outcomes depending on the OM source. The microbial community was clearly affected by higher water temperature: bacterial cell densities, respiratory activity (ETS) and bacterial metabolism (enzyme activity) were all higher. The addition of labile DOC (dipeptide plus cellobiose) caused a further augmentation of heterotrophic biomass and respiratory activity. The fluorescence index and the ratio Abs_{250}/total DOC indicated that recalcitrant DOC was degraded by higher temperatures. The experiment showed that naturally occurring pulses of highly available carbon, together with higher water temperature, might have consequences on the processing of the flowing DOC. The more bioavailable OM will be rapidly cycled irrespective of higher temperature, while degradation of recalcitrant substances will be enhanced by warming.

CHAPTER IV: AVAILABILITY OF GLUCOSE AND LIGHT MODULATES THE STRUCTURE AND FUNCTION OF A MICROBIAL BIOFILM

The differences in the organic matter processing and biofilm composition and structure between auto-heterotrophic and heterotrophic biofilm communities was analysed in a laboratory experiment. Microbial communities grown on artificial biofilms were monitored following incubation under light and dark conditions and with or without the addition of glucose as labile organic compound. Glucose addition greatly affected the microbial biofilm composition as shown by differences in 16S rRNA gene fingerprints. A significant increase in β-glucosidase and peptidase enzyme activities were also observed in glucose amended biofilms incubated in the dark, suggesting an

active bacterial community. Light enhanced the algal and bacterial growth, as well as higher extracellular enzyme activity, thereby indicating a tight algal-bacterial coupling in biofilms incubated under illumination. In these biofilms, organic compounds excreted by photosynthetic microorganisms were readily available for bacterial heterotrophs. This algal-bacterial relationship weakened in glucose-amended biofilms grown in the light, probably because heterotrophic bacteria preferentially use external labile compounds. These results suggest that the availability of labile organic matter in the flowing water and the presence of light may alter the biofilm composition and function, therefore affecting the processing capacity of organic matter in the stream ecosystem.

CHAPTER V: MULTIFUNCTIONALITY IS AFFECTED BY LOSS OF DIVERSITY IN MICROBIAL BIOFILM COMMUNITIES

Bacteria comprise the majority of biodiversity and at the same time drive the bulk of ecosystem processes, but the basic understanding about the connections of biodiversity and ecosystem functioning of aquatic microbial communities remains limited. In this chapter, the effects of loss of bacterial species richness on degradation of labile and recalcitrant DOC were examined by means of a bioreactor experiment. DOC concentrations, oxygen consumption rates, bacterial abundances and activities of five extracellular enzymes were measured over 10 days in reactors colonized for 4 and 7 weeks, respectively. Gradients in bacterial community composition from the inflow to the outflow of the reactors were sampled for molecular fingerprinting by Terminal Restriction Fragment Length Polymorphism (T-RFLP). The bacterial communities were able to rapidly degrade a substantial fraction of the labile DOC, while DOC concentration remained unchanged and oxygen consumption rates were lower in the recalcitrant treatments. The intentional decrease in bacterial diversity was linked to a decrease in the enzyme multifunctionality. The multifunctionality decrease occurring at lower diversity was larger under recalcitrant DOC conditions and in older biofilms. The loss of functionality was not “equal” for all functions; those enzymes linked to the use of complex compounds were the first to be lost. The results show the implications of bacterial biodiversity in the maintenance of ecosystem functioning.

RESUMEN (Castellano)

La comunidad bentónica de los ecosistemas fluviales procesa una gran cantidad de la materia orgánica que llega a los ríos. El origen de las entradas de material (autóctonas o alóctonas), su composición química y su cantidad (frecuencia de las entradas y concentración existente en el río), determinan la estructura de la comunidad bentónica autotrófica y heterotrófica, sus relaciones tróficas y sus interacciones potenciales (competencia, sinergismo). En esta tesis se contrasta la hipótesis de que tanto la cantidad, como la calidad y la disponibilidad de los materiales favorecen la abundancia relativa de microorganismos autótrofos y/o heterótrofos, así como también su diversidad, la cual determina a su vez la eficiencia en la utilización de los materiales circulantes.

Los ecosistemas fluviales mediterráneos, principalmente en sus cabeceras y cursos medios, exhiben una gran variabilidad de caudal. Las avenidas y las sequías son fenómenos frecuentes que condicionan los procesos biológicos y biogeoquímicos. Como consecuencia del cambio climático global, la intensidad y la frecuencia de los períodos de sequía y de intensas precipitaciones, así como también la temperatura del agua, podrían aumentar. La comprensión de los efectos que estas perturbaciones pueden tener sobre los materiales que llegan al río y la comunidad del biofilm podrían contribuir significativamente al conocimiento del funcionamiento de las comunidades biológicas durante estos períodos concretos de los sistemas mediterráneos. Esto incluye el conocimiento del bucle microbiano bentónico, a través del cual circula una importante cantidad de la energía y la materia orgánica procesada por el río y que constituye la base de los procesos de autodepuración fluvial.

El objetivo de la presente tesis es poner de manifiesto la utilización del carbono orgánico disuelto (COD) por parte de los biofilms bacterianos bentónicos fluviales y determinar la eficiencia del sistema fluvial en el uso de los distintos materiales circulantes. Con esta finalidad se han llevado a cabo distintos experimentos, tanto de campo como de laboratorio, para conocer los efectos de la disponibilidad de la materia orgánica (cantidad) y su calidad (composición química y biodegradabilidad) y los efectos debidos al aumento de temperatura del agua del río.

CAPÍTULO I: DISPONIBILIDAD DE MATERIA ORGÁNICA ANTES Y DESPUÉS DE LOS PERÍODOS DE SEQUÍA EN UN RÍO MEDITERRÁNEO

Los ríos de los ecosistemas mediterráneos se caracterizan por las oscilaciones de caudal debidas a episodios de inundación seguidos por episodios de sequía. Con el fin de caracterizar los cambios en la calidad y la cantidad de la materia orgánica circulante durante uno de estos períodos de disminución de caudal (hasta llegar a la sequía) y su posterior recuperación, se llevó a cabo un trabajo de campo en la riera de Fuirosos. Se analizó la materia orgánica y la actividad enzimática en el material bentónico acumulado (biofilms de los cantos rodados, hojas y arena) y en el agua corriente (fracción disuelta y particulada). La cantidad total de polisacáridos, aminoácidos y lípidos en la materia orgánica bentónica fue, de media, más elevada durante el período de sequía que durante el período de recuperación del caudal. No obstante, los péptidos disponibles disminuyeron durante el período seco, tal y como lo indicó la disminución de la actividad leucino-peptidasa y también del contenido de aminoácidos en el agua y en el material bentónico (exceptuando las hojas). Al mismo tiempo los polisacáridos eran activamente utilizados, como se dedujo por el incremento de la actividad β-glucosidasa en los sustratos bentónicos y por el incremento en la cantidad de polisacáridos en la fracción particulada del agua y de la hojarasca. Durante esta época de sequía la comunidad microbiana heterotrófica se vio forzada a utilizar la materia orgánica de menor calidad (polisacáridos, sólo como fuente de C), ya que los péptidos (fuente de C y N) no estaban disponibles.

Durante la fase de rehidratación la comunidad microbiana se recuperó con rapidez, hecho que sugiere la utilización de refugios y/o adaptaciones a la desecación durante los períodos previos de sequía. En el momento de la recuperación del caudal, el barrido del lecho fluvial fue el responsable de la movilización y pérdida de una gran cantidad de material bentónico aguas abajo, consecuentemente la calidad de la materia orgánica transportada aumentó. La dinámica de los efectos de una sequía gradual y progresiva, así como la rápida recuperación después del retorno del caudal, podrían verse afectados por la dinámica propia de cada sustrato bentónico: los sedimentos arenosos y las hojas proporcionan refugio a los microorganismos y constituyen un reservorio de materia orgánica, mientras que en los cantos rodados tiene lugar la implantación de una activa comunidad bacteriana durante la rehidratación. En un río, la preservación de la heterogeneidad de los sustratos bentónicos es un factor determinante para su recuperación después de un episodio de sequía.

CAPÍTULO II: CARACTERÍSTICAS DE LA MATERIA ORGÁNICA EN UN RÍO MEDITERRÁNEO MEDIANTE EL ESTUDIO DE LA COMPOSICIÓN DE AMINOÁCIDOS. CAMBIOS OCASIONADOS POR LA DESECACIÓN Y LA REHIDRATACIÓN

Como continuación del capítulo 1 se estudiaron los aminoácidos de un río mediterráneo durante un ciclo "húmedo-seco-húmedo". Se analizó la composición de los aminoácidos (calidad) y su abundancia (cantidad) en los biofilms epilíticos y epipsámicos, así como también en la hojarasca y en la materia orgánica disuelta (MOD). El contenido y la composición de los aminoácidos analizados en los distintos compartimentos fueron enormemente dependientes del origen de la materia orgánica así como también de su estado diagenético. Los aminoácidos fueron suficientemente sensibles como para detectar cambios en las características de la materia orgánica. La materia orgánica de más alta calidad (elevado índice de degradación y elevado contenido de N) se halló en el biofilm de los cantos rodados, mientras que la más degradada y de menor calidad se acumuló en los sedimentos arenosos. Además, en la MOD se encontraron diferencias relevantes en la cantidad y calidad de los aminoácidos comparando los períodos anteriores y posteriores a la sequía, con un gran pico de materia orgánica fresca el día en que se recuperó el caudal. La composición en aminoácidos de la MOD reflejó la mezcla heterogénea de entradas de material: los materiales autóctonos predominaron durante la pre-sequía, mientras que los alóctonos predominaron durante la post-sequía. Contrariamente, la mayor estabilidad en la composición de los compartimentos bentónicos indicaba una materia orgánica originada *in situ*, cuya calidad decrecía progresivamente durante el período de sequía.

CAPÍTULO III: EFECTOS DEL AUMENTO DE LA TEMPERATURA DEL AGUA SOBRE LA UTILITZACIÓN, POR PARTE DE LOS BIOFILMS, DEL CARBONO ORGÁNICO DISUELTO LÁBIL VERSUS EL RECALCITRANTE

En los ríos de las zonas templadas, las entradas de carbono orgánico disuelto (COD) varían en función del período, oscilando entre el COD de alta calidad cuando la producción primaria es elevada y el COD recalcitrante después de largos períodos de acumulación de materia orgánica (MO). Estas oscilaciones, tanto en la calidad como en la cantidad del COD, se pueden acentuar debido al cambio climático. Con la finalidad de determinar si un incremento de temperatura del agua podría afectar de forma diferencial la degradación del COD lábil *versus* el recalcitrante por parte de los biofilms microbianos, se llevó a cabo un experimento de laboratorio mimetizando los

distintos orígenes de la MO bajo dos temperaturas del agua (14 y 18ºC). La combinación potencial de entradas de MO y una mayor temperatura del agua puede proporcionar resultados diferentes en función del origen de dicha MO. Un incremento en la temperatura del agua afectó claramente la comunidad microbiana: la densidad bacteriana, la actividad respiratoria (ETS) y el metabolismo bacteriano (actividades enzimáticas) aumentaron. La adición de COD lábil (dipéptidos y celobiosa) ocasionó un incremento aun mayor de la biomasa heterotrófica y de la actividad respiratoria. El índice de fluorescencia y la relación Abs_{250}/COD total indicaron que el COD recalcitrante se degradaba por efecto del incremento de la temperatura. El experimento evidenció que entradas de grandes cantidades de carbono, que se producen de manera natural, juntamente con una temperatura del agua más alta podrían tener consecuencias en el procesado del COD circulante. La MO más biodisponible se utilizará con rapidez independientemente de la temperatura, mientras que la degradación de las sustancias recalcitrantes se incrementará por el calentamiento.

CAPÍTULO IV: LA DISPONIBILIDAD DE GLUCOSA Y LUZ MODULAN LA ESTRUCTURA Y LA FUNCIÓN DEL BIOFILM MICROBIANO

En un experimento *in vitro* se analizaron las diferencias en el procesamiento de la materia orgánica y la composición y estructura del biofilm entre las comunidades auto-heterotróficas y heterotróficas presentes en el mismo. Para ello se hizo un seguimiento de las comunidades microbianas desarrolladas sobre biofilms artificiales bajo condiciones de luz u oscuridad y con o sin adición de glucosa como compuesto orgánico lábil. La adición de glucosa afectó marcadamente la composición microbiana del biofilm, tal y como se evidenció por las diferencias en los patrones resultantes del gen 16S rRNA. Se observó también un incremento significativo de la actividad enzimática β-glucosidasa y peptidasa en los biofilms enriquecidos con glucosa e incubados en la oscuridad, hecho que sugería la presencia de una activa comunidad bacteriana. La disponibilidad de luz indujo el aumento del crecimiento de las algas y las bacterias, al mismo tiempo que también provocó una mayor actividad enzimática extracelular, indicando una fuerte relación entre algas y bacterias en los biofilms incubados bajo iluminación. En estos biofilms, los compuestos orgánicos excretados por los microorganismos fotosintéticos eran fácilmente disponibles por las bacterias heterótrofas. Esta relación algas-bacterias se debilitó en los biofilms enriquecidos con glucosa y crecidos bajo luz, debido probablemente a que las bacterias heterótrofas utilizaban preferiblemente los compuestos lábiles externos. Los anteriores resultados

sugieren que la disponibilidad de materia orgánica lábil en el agua circulante y la presencia de luz pueden alterar la composición y la función del biofilm, afectando la capacidad de procesamiento de la materia orgánica en los ecosistemas fluviales.

CAPÍTULO V: EFECTO DE LA PÉRDIDA DE DIVERSIDAD DE LAS COMUNIDADES MICROBIANAS DE LOS BIOFILMS SOBRE LA MULTIFUNCIONALIDAD

Es sabido que las bacterias representan la mayor parte de la diversidad, al mismo tiempo que dirigen la mayoría de los procesos que tienen lugar en los ecosistemas. De todas formas, el conocimiento de las relaciones entre la biodiversidad acuática bacteriana y las funciones de los ecosistemas fluviales es aún muy limitado. En este capítulo se presentan los resultados de un experimento llevado a cabo mediante bioreactores, investigándose los efectos de una pérdida de diversidad bacteriana sobre la degradación del COD lábil y recalcitrante. Se determinó la concentración de COD, la tasa de consumo de oxígeno, la abundancia bacteriana y la actividad de cinco enzimas extracelulares a lo largo de 10 días en reactores colonizados durante 4 y 7 semanas respectivamente. Los gradientes de las comunidades bacterianas formadas desde la entrada hasta la salida de los reactores, fueron analizados mediante la técnica molecular del T-RFLP (Terminal Restriction Fragment Length Polymorphism). Las comunidades bacterianas fueron capaces de degradar con rapidez una fracción sustancial del COD lábil, mientras que la concentración del carbono recalcitrante se mantuvo invariable y las tasas de consumo de oxígeno también fueron inferiores en estos tratamientos con COD recalcitrante. La disminución intencionada de la diversidad bacteriana fue paralela al decrecimiento de la multifuncionalidad enzimática. La disminución de la multifuncionalidad tuvo lugar en las diversidades más bajas y fue más importante en los tratamientos de COD recalcitrante y en biofilms más viejos. La pérdida de funcionalidad no fue “igual” para todas las funciones: la actividad de los enzimas relacionados con el uso de compuestos complejos fue la primera en perderse. Los resultados muestran las implicaciones de la biodiversidad bacteriana en el mantenimiento del funcionamiento del ecosistema.

RESUM (Català)

La comunitat bentònica dels ecosistemes fluvials processa una gran quantitat de la matèria orgànica que arriba als rius. L'origen de les entrades de material (autòctones o al·lòctones), la seva composició química i la seva quantitat (freqüència de les entrades i concentració assolida en el riu), determinen l'estructura de la comunitat bentònica autotròfica i heterotròfica, les seves relacions tròfiques i les seves interaccions potencials (competència, sinergisme). En aquesta tesi es contrasta la hipòtesi de que tant la quantitat com la qualitat i la disponibilitat dels materials afavoreixen l'abundància relativa de microorganismes autòtrofs i/o heteròtrofs així com també la seva diversitat, la qual determina l'eficiència en la utilització dels materials que hi flueixen.

Els ecosistemes fluvials mediterranis, principalment a les seves capçaleres i cursos mitjos, presenten una gran variabilitat de cabal. Les avingudes i les sequeres són fenòmens freqüents que condicionen els processos biològics i biogeoquímics. Com a conseqüència del canvi climàtic global, la intensitat i freqüència dels períodes de sequera i d'intenses precipitacions, així com també la temperatura de l'aigua poden augmentar. La comprensió dels efectes que aquestes pertorbacions poden tenir sobre els materials que entren al riu i la comunitat del biofilm podria contribuir significativament al coneixement del funcionament de les comunitats biològiques durant aquests períodes concrets dels sistemes mediterranis. Això inclou el coneixement del bucle microbià bentònic, a través del qual circula una quantitat important de l'energia i la matèria orgànica que processa el riu i que esdevé la base dels processos d'autodepuració fluvial.

L'objectiu d'aquesta tesi és posar de manifest la utilització del carboni orgànic dissolt (COD) per part dels biofilms bacterians bentònics fluvials i determinar l'eficiència del sistema fluvial en l'ús dels diferents materials que hi circulen. Amb aquesta finalitat s'han portat a terme diversos experiments, tant de camp com de laboratori, per tal de conèixer els efectes de la disponibilitat de la matèria orgànica (quantitat) i la seva qualitat (composició química i biodegradabilitat) i els efectes deguts a l'augment de temperatura de l'aigua del riu.

CAPÍTOL I: DISPONIBILITAT DE MATÈRIA ORGÀNICA ABANS I DESPRÉS DELS PERÍODES DE SEQUERA EN UN RIU MEDITERRANI

Els rius dels ecosistemes mediterranis es caracteritzen per les oscil·lacions de cabal ocasionades per episodis d'inundació seguits per episodis de sequera. Amb la

finalitat de caracteritzar els canvis en la qualitat i quantitat de la matèria orgànica circulant durant un d'aquests períodes de disminució del cabal (fins arribar a la sequera) i posterior recuperació, es va dur a terme un treball de camp en la riera de Fuirosos. Es va analitzar la matèria orgànica i l'activitat enzimàtica en el material bentònic acumulat (biofilms dels còdols, fulles i sorra) i en l'aigua corrent (fracció dissolta i particulada). La quantitat total de polisacàrids, aminoàcids i lípids en la matèria orgànica bentònica va ser més alta de mitjana durant el període de sequera que durant el període de recuperació del cabal. No obstant, els pèptids disponibles van disminuir durant el període sec, tal i com ho indicaren la disminució en l'activitat leucino-aminopeptidasa i també en el contingut d' aminoàcids a l'aigua i al material bentònic (exceptuant les fulles). Al mateix temps, els polisacàrids eren activament utilitzats, com ho indicaren l'increment de l'activitat β-glucosidasa en els substrats bentònics i l'increment en el contingut de polisacàrids en la fracció particulada de l'aigua i en la fullaraca. Durant aquesta època de sequera, la comunitat microbiana heterotròfica estava forçada a utilitzar la matèria orgànica de menor qualitat (polisacàrids, com a font només de C), ja que els pèptids (font de N i C) no eren disponibles.

Durant la fase de rehidratació la comunitat microbiana es va recuperar ràpidament, fet que suggereix la utilització de refugis i/o adaptacions a la dessecació durant els períodes previs de sequera. En el moment de la recuperació del cabal, l'escombrat del llit del riu va ser el responsable de la mobilització i la pèrdua d'una gran quantitat de material bentònic aigües avall, conseqüentment la qualitat de la matèria orgànica transportada va incrementar. La dinàmica dels efectes d'una sequera gradual i progressiva, així com la ràpida recuperació després de la tornada del cabal, podrien veure's afectats per la pròpia dinàmica de cada substrat bentònic: els sediments sorrencs i les fulles proporcionen refugi als microorganismes i constitueixen un reservori de matèria orgànica, mentre que damunt els còdols s'hi desenvolupa una activa comunitat bacteriana durant la rehidratació. En un riu, el manteniment de la heterogeneïtat del substrats bentònics és un factor determinant per la seva recuperació després d'un episodi de sequera.

CAPÍTOL II: CARACTERÍSTIQUES DE LA MATÈRIA ORGÀNICA EN UN RIU MEDITERRANI MITJANÇANT L'ESTUDI DE LA COMPOSICIÓ D'AMINOÀCIDS. CANVIS OCASIONATS PER L'ASSECAMENT I LA REHIDRATACIÓ

Com a continuació del capítol 1, ens centrarem en l'estudi detallat de la composició dels aminoàcids d'un riu mediterrani durant un cicle "humit-sec-humit". S'analitzaren la composició dels aminoàcids (qualitat) i la seva abundància (quantitat)

en els biofilms epilítics i epipsàmics, així com també en la fullaraca i en la matèria orgànica dissolta (MOD). El contingut i la composició dels aminoàcids analitzats en els diferents compartiments foren altament dependents de l'origen de la matèria orgànica així com també del seu estat diagenètic. Els aminoàcids van ser suficientment sensibles per detectar canvis en les característiques de la matèria orgànica. La matèria orgànica de més alta qualitat (índex de degradació elevat i alt contingut de N) es va trobar en el biofilm dels còdols, mentre que la més degradada i de més baixa qualitat es va acumular en els sediments sorrencs. A més, en la MOD es van trobar diferències rellevants en la quantitat i la qualitat dels aminoàcids comparant els períodes anteriors i posteriors a la sequera, amb un gran pic de matèria orgànica fresca el dia que es va recuperar el cabal. La composició en aminoàcids de la MOD és un reflex de la barreja heterogènia d'entrades de materials: els materials autòctons predominaren durant la pre-sequera, mentre que els al·lòctons predominaren durant la post-sequera. Contràriament, la major estabilitat en la composició dels compartiments bentònics indicava una matèria orgànica originada *in situ*, la qualitat de la qual decreixia progressivament durant el període de sequera.

CAPÍTOL III: EFECTES DE L'AUGMENT DE LA TEMPERATURA DE L'AIGUA SOBRE LA UTILITZACIÓ, PER PART DELS BIOFILMS, DEL CARBONI ORGÀNIC DISSOLT LÀBIL VERSUS EL RECALCITRANT

En els rius de les zones temperades, les entrades de carboni orgànic dissolt (COD) varien en funció del període, oscil·lant entre el COD d'alta qualitat quan la producció primària és elevada i el COD recalcitrant després de llargs períodes d'acumulació de matèria orgànica (MO). Aquestes oscil·lacions, tant en la qualitat com en la quantitat del COD, es poden accentuar degut al canvi climàtic. Amb la finalitat de determinar si un increment de temperatura de l'aigua podria afectar de manera diferencial la degradació del COD làbil *versus* el recalcitrant per part dels biofilms microbians, es va dur a terme un experiment de laboratori mimetitzant els diferents orígens de la MO sota dues temperatures de l'aigua (14 i 18ºC). La combinació potencial d'entrades de MO i una temperatura de l'aigua més alta pot proporcionar diferents resultats en funció de l'origen d'aquesta MO. Un increment en la temperatura de l'aigua va afectar clarament la comunitat microbiana: la densitat bacteriana, l'activitat respiratòria (ETS) i el metabolisme bacterià (activitats enzimàtiques) van augmentar. L'addició de COD làbil (dipèptids i cel·lobiosa) va ocasionar un increment encara més fort de la biomassa heterotròfica i l'activitat respiratòria. L'índex de fluorescència i la relació Abs_{250}/CODtotal indicaren que el COD recalcitrant era degradat per efecte de l'increment de temperatura. L'experiment mostrà que entrades

de grans quantitats de carboni, que tenen lloc de manera natural, juntament amb una temperatura de l'aigua més alta podrien tenir conseqüències en el processat del COD circulant. La MO més biodisponible s'utilitzarà amb rapidesa independentment de la temperatura, mentre que la degradació de les substàncies recalcitrants s'incrementarà per l'escalfament.

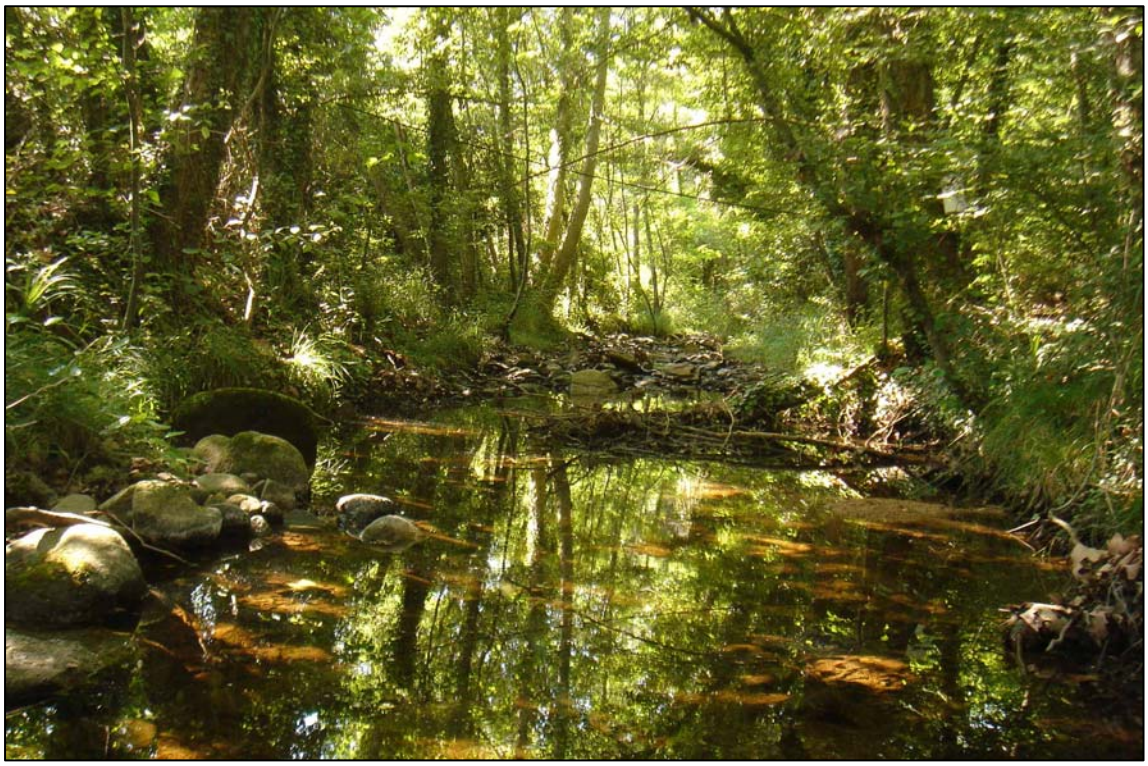
CAPÍTOL IV: LA DISPONIBILITAT DE GLUCOSA I LLUM MODULEN L'ESTRUCTURA I LA FUNCIÓ DEL BIOFILM MICROBIÀ

En un experiment de laboratori s'analitzaren les diferències en el processament de la matèria orgànica i la composició i estructura del biofilm, entre les comunitats auto-heterotròfiques i heterotròfiques del mateix. Es va fer un seguiment de les comunitats microbianes crescudes sobre biofilms artificials sota condicions de llum o foscor i amb o sense addició de glucosa com a compost orgànic làbil. L'addició de glucosa va afectar enormement la composició microbiana del biofilm, tal com mostraren les diferències en els patrons resultants del gen 16S rRNA. Es va observar també un increment significatiu de l'activitat enzimàtica β-glucosidasa i peptidasa en els biofilms enriquits amb glucosa i incubats a les fosques, fet que suggeria la presència d'una activa comunitat bacteriana. La disponibilitat de llum va fer augmentar el creixement de les algues i els bacteris alhora que també va provocar una activitat enzimàtica extracel·lular més gran, indicant una forta relació entre algues i bacteris en els biofilms incubats sota il·luminació. En aquests biofilms, els compostos orgànics excretats pels microorganismes fotosintètics eren fàcilment disponibles pels bacteris heteròtrofs. Aquesta relació algues-bacteris es va debilitar en els biofilms enriquits amb glucosa i crescuts amb il·luminació, probablement degut a que els bacteris heterotròfics utilitzaven preferentment els compostos làbils externs. Aquests resultats suggereixen que la disponibilitat de matèria orgànica làbil en l'aigua circulant i la presència de llum poden alterar la composició i la funció del biofilm, afectant la capacitat de processament de la matèria orgànica en els ecosistemes fluvials.

CAPÍTOL V: EFECTE DE LA PÈRDUA DE DIVERSITAT DE LES COMUNITATS MICROBIANES DELS BIOFILMS SOBRE LA MULTIFUNCIONALITAT

Es coneix que els bacteris representen la major part de la diversitat, al mateix temps que dirigeixen la majoria dels processos que es donen en els ecosistemes. Tanmateix, el coneixement de les relacions entre la biodiversitat aquàtica bacteriana i les funcions dels ecosistemes fluvials és encara molt limitat. En aquest capítol es presenten els resultats d'un experiment el qual es va desenvolupar mitjançant l'ús de bioreactors. Es van investigar els efectes d'una pèrdua de diversitat bacteriana sobre

la degradació del COD làbil i recalcitrant. Es van mesurar la concentració de COD, la taxa de consum d'oxigen, l'abundància bacteriana i l'activitat de cinc enzims extracel·lulars al llarg de 10 dies en reactors colonitzats durant 4 i 7 setmanes respectivament. Els gradients de les comunitats bacterianes formats des de l'entrada fins la sortida dels reactors, van ser analitzats mitjançant la tècnica molecular del T-RFLP (Terminal Restriction Fragment Length Polymorphism). Les comunitats bacterianes van ser capaces de degradar amb rapidesa una fracció substancial del COD làbil, mentre que la concentració del COD recalcitrant va romandre invariable i les taxes de consum d'oxigen també van ser inferiors en aquests tractaments amb COD recalcitrant. La disminució intencionada de la diversitat bacteriana va anar lligada amb un decreixement de la multifuncionalitat enzimàtica. La disminució de la multifuncionalitat es va donar en les diversitats més baixes i va ser més important en els tractaments de COD recalcitrant i en biofilms més vells. La pèrdua de funcionalitat no va ser "igual" per totes les funcions: l'activitat dels enzims lligats a l' ús de compostos complexes va ser la primera de perdre's. Els resultats mostren les implicacions de la biodiversitat bacteriana en el manteniment del funcionament de l'ecosistema.



GENERAL INTRODUCTION

GENERAL INTRODUCTION

An observer that gazes upon a river may be impressed by the strength of the current, the dimensions of the channel, and perhaps the occurrence of boulders in the streambed or the shape of the banks. This spectator might also note whether this waterway can be suitable for various activities, such as the passage of boats, recreational uses and whether it poses a hazard to humans. However, might be less apparent to him/her that these channel and flow characteristics likely influence the functioning of running water ecosystems, and the biota found therein (Allan & Castillo, 2007).

Stream ecosystems are open systems with inputs and outputs of organic matter. The organic matter fluxes through the trophic chain and the microbial loop or simply through physical transport. Nowadays, human activities as well as climate change exert a high pressure on the whole fluvial ecosystem as well as on the organic matter suspended and transported in its waters, which at the same time, are affecting the microbial loop and their self auto-depurative capacity.

THE ORGANIC MATTER AND BENTHIC MICROBIAL LOOP IN RIVER ECOSYSTEMS

▪ ORGANIC MATTER IN RIVERS

The organic matter transported in rivers can vary largely in their content and composition. Some river waters are tea-coloured due to high concentrations of dissolved plant matter, while others have fewer chemical constituents and remain clear; these are known as blackwater and clearwater rivers, respectively (Fig. 1; Allan & Castillo, 2007).

Fig. 1. Fibyån River (Sweden) as an example of a blackwater river (A) and Fuirosos stream (Catalonia) as a clearwater river (B).

The amount (quantity) and origin (quality) of organic matter is one of the most important factors in determining the microbial metabolism and community structure (Bott et al., 1984, Judd et al., 2006) of a riverine ecosystem.

The total pool of organic matter in rivers can be divided in three categories separated in relation to the particle size: coarse particulate organic matter (CPOM), >1mm; fine particulate organic matter (FPOM), between 1mm and 0.5μm; and dissolved organic matter (DOM), <0.5μm.

The dissolved fraction of organic matter (DOM) has a key role in energy flow (Fischer et al., 2002) and is typically the largest pool of organic C in running waters (Fisher & Likens, 1973; Amon & Benner, 1996; Volk et al., 1997). It is also a vital resource affecting food webs either directly, by organisms' uptake, or indirectly by mechanisms such as turbidity, pH, metal chelation, and transport of contaminants (McDonald et al., 2004). The diversity of dissolved organic carbon (DOC) within a specific reach of a stream is dependent on the potential number of sources (Koetsier et al., 1997) and the availability of DOC to organisms. Sources of carbon can enter the river from a variety of sources, including autochthonous sources (carbon that derive from in-stream processes) and allochthonous sources (carbon that enter the stream from outside the channel, mostly from the hillslope).

Autochthonous sources are produced by the autotrophic organisms (algae, cyanobacteria, mosses) as a result of the photosynthetic processes. Primary producers can be important sources of DOM in streams and rivers, releasing mainly low molecular weight and labile matter as exudates and from cell lysis (Bott et al., 1984; Bertilsson & Jones, 2003). This organic C source may be available mostly during episodes of high primary production when exudates are produced, and within biofilms where exudates and products of cell lysis become concentrated. During springtime biofilm blooms, stream DOC concentrations increase as much as 37%, apparently due to extracellular release by algae (Kaplan & Bott, 1989).

Allochthonous sources are mainly composed of terrestrial plant materials (leachates from surroundings soils, grasses and inputs from riparian trees; Graça, 1993). This type of material enter to the stream mainly like CPOM but also as FPOM and DOM. This allochthonous material is considered to be more refractory and of lower quality (Kaplan & Newbold, 1995; Joffre et al., 2001). The overall POM (sum of CPOM and FPOM) may be seasonally pulsed, as occurs with autumn leaf fall into forested streams (Sabater et al., 2001; Artigas et al., 2004) or during storm flows. The proportion of allochthonous inputs can vary depending on the riparian vegetation type and on the stream size (order). In headwater streams the connection between the terrestrial and aquatic environments is tight (high terrestrial inputs), whereas in larger

rivers this relationship is weaker and the planktonic community prevail (Vannote et al., 1980).

Aquatic organisms use the organic matter entering the river as a C source (Whitton & Lucas, 1997). Macroinvertebrates (collectors, gatherers, shredders, grazers) feed principally on POM. Then, predators (e.g. fish and beetles larvae) feed on these macroinvertebrates. In contrast, bacteria and fungi mostly use DOM and FPOM (Fig. 2).

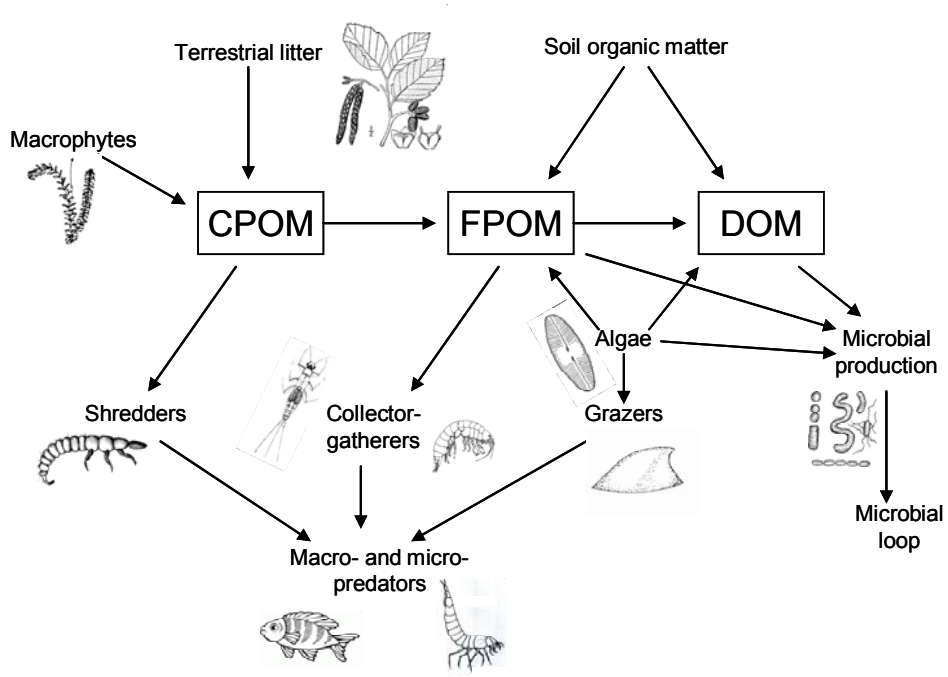


Fig. 2. Organic matter flux in a fluvial ecosystem. CPOM= coarse particulate organic matter; FPOM= fine particulate organic matter; DOM= dissolved organic matter. (Modified from Allan & Castillo, 2007).

The present thesis focuses on the benthic microbial loop, and therefore mostly in the fate of the DOM fraction. However, the input of POM in a river is obviously linked to the final DOM available for heterotrophs.

▪ DISSOLVED ORGANIC MATTER COMPOSITION AND BIOAVAILABILITY

DOM bulk in streams and rivers is highly refractory and of low bioavailability on timescales relevant to stream transport. However, river water also contains a smaller fraction of labile DOM, and this material constitutes a potentially important heterotrophic energy pathway.

The major classes of DOM in natural waters, including stream and river water, have been reviewed extensively (Thurman, 1985). The most common measure of organic matter is as organic carbon, and to facilitate a quantitative description of DOM we use units of carbon (DOC). From its composition the organic carbon in river water can be divided in two parts: a labile fraction of known biomolecular classes of compounds, including carboxylic acids (lipids), carbohydrates, amino acids and hydrophilic acids (Piccolo, 2001); and a humic fraction (fulvic and humic acids) of high molecular weight, which is refractory (McDonald et al., 2004; Fig. 3). Within these classes only the carbohydrate, amino acid and carboxylic acid categories contain a subset of monomers, of which the carboxylic acids perhaps constitute the largest though the less investigated pool (Fig. 4).

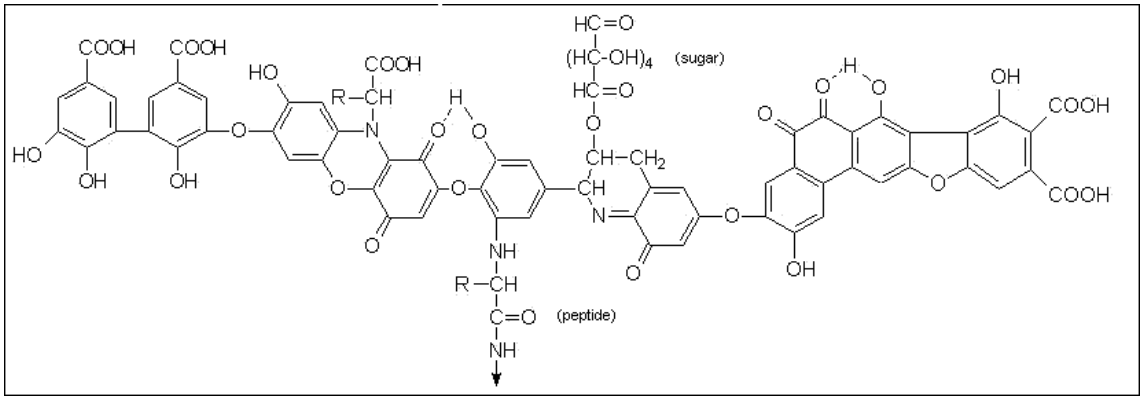


Fig. 3. Model structure of a humic acid (Stevenson, 1982). It is apparent that humic substances consist of a heterogeneous mixture of compounds for which no single structural formula will suffice. Nevertheless, humic acids are thought to be complex aromatic macromolecules with amino acids, amino sugars, peptides and aliphatic compounds involved in linkages between the aromatic groups. The hypothetical structure for humic acid, shown in figure, contains free and bound phenolic OH groups, quinone structures, nitrogen and oxygen as bridge units and carboxylic acid groups (COOH) variously placed on aromatic rings.

Biological, chemical and physical processes play important roles in modifying the chemical structure and composition of organic matter and transporting DOM within ecosystems. An important aspect of monomer sources is that not all monomers are the result of polymer degradation by either enzymatic hydrolysis or photolysis. A high number of monomers in streams derive from processes such as excretion by algae (Nalewajko, 1966), bacteria (Allen, 1976) and animals (Park et al., 1997), plus death and lysis of stream organisms. Among these, amino acids have received considerable attention because of their importance to protein synthesis and bacterial metabolism, providing a potential carbon and nitrogen source for heterotrophs.

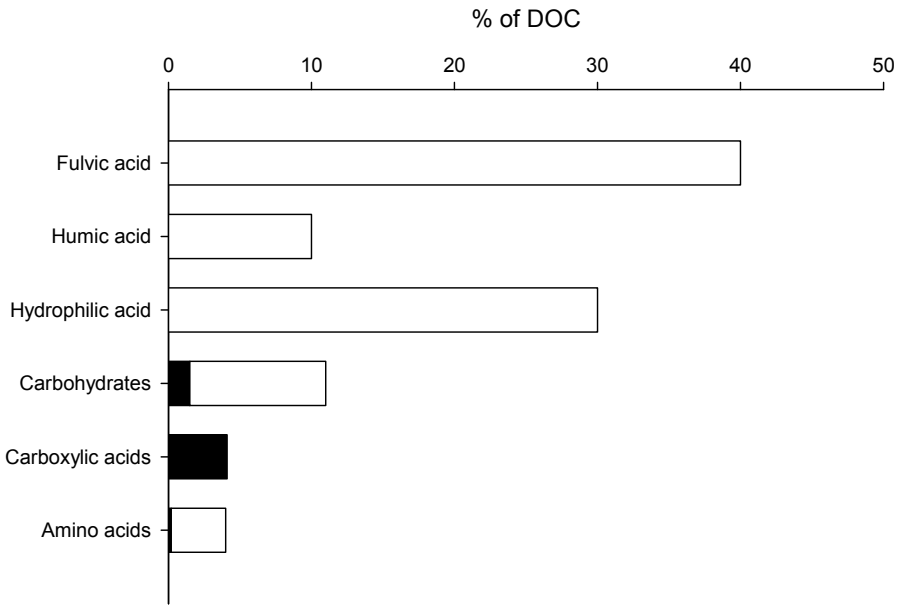


Fig. 4. Major classes of organic carbon in river water. Monomeric-C is represented by black shading within the histograms. From Kaplan & Newbold, 2003.

▪ BIOGEOCHEMICAL PROCESSES PATHWAYS (DOM ACQUISITION PATHWAYS)

At the ecosystem scale, carbon flow from DOM to bacteria moves along four major pathways: (1) direct uptake, (2) extracellular enzymes-mediated uptake, (3) photolysis-mediated uptake and (4) sorption-mediated uptake (Findlay & Sinsabaugh, 1999; Fig. 5).

Direct uptake is the assimilation of an organic molecule without mediation by an external process. Small monomeric subtrates (e.g. amino acids, fatty acids, saccharides) up to a few hundred daltons in size can be consumed. For extracellular enzymes-mediated uptake, the rate-defining step is the degradation of macromolecules into assimilable products by the action of extracellular enzymes (Chróst, 1990; Burns, 2001). These enzymes may be hydrolytic or oxidative, and reaction products may include sources of nitrogen, phosphorous, and other nutrients as well as carbon. In photolysis-mediated uptake, the rate-defining step is the generation of assimilable products from the photo oxidation of DOM. The absorption of solar radiation causes chemical oxidation reactions that can generate products for microbial assimilation (Moran & Zepp, 1997; Wetzel et al., 1995). Sorption-mediated uptake is probably the least quantified uptake pathway. The sorption of dissolved molecules to exopolymers, cell fragments, biofilms and particle surfaces concentrates carbon and other nutrients (Lock, 1994).

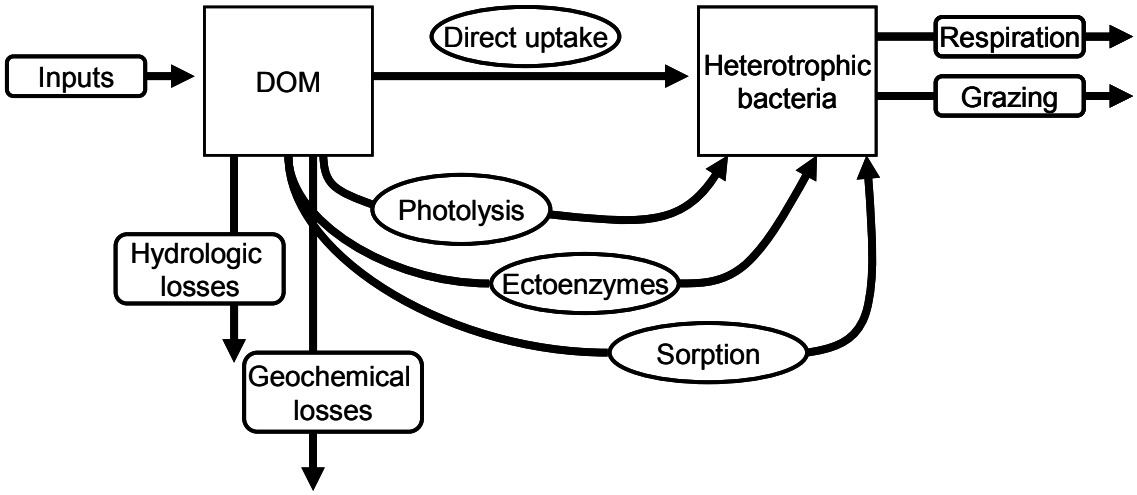


Fig. 5. Pathways for DOM consumption. There are four types of pathways processes that connect DOM to bacterial assimilation: (1) direct uptake, (2) extracellular enzymes-mediated uptake, (3) photolysis-mediated uptake and (4) sorption-mediated uptake. From Sinsabaugh & Foreman, 2003.

▪ WHAT ARE BIOFILMS? THE BENTHIC MICROBIAL COMMUNITY

Biofilms are abundant in a multitude of aquatic environments in which they cover inorganic and organic solid surfaces. Rock, cobbles, sand, and wood coexist in the stream reach, and all of these substrata host biofilms with differing structural and compositional characteristics (Lock, 1993). Biofilms are an assemblage of algae, fungi, protozoa, bacteria and unicellular animals, imbedded in a matrix of polysaccharide exudates and detritus (Wetzel, 1983; Fig. 6).

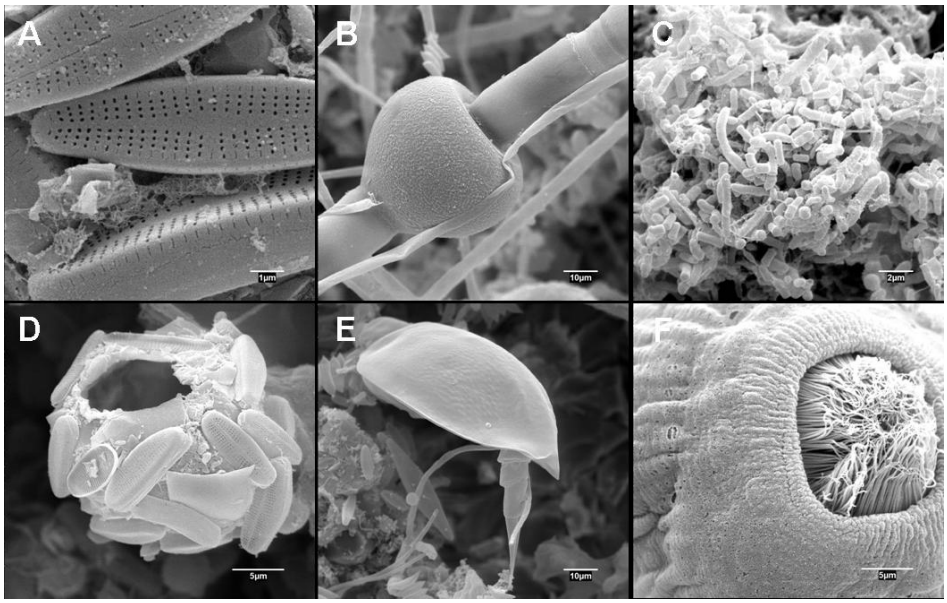


Fig. 6. Images of different organisms of the biofilm made with scanning electron microscopy (SEM). A) diatoms (*Achnanthidium minutissimum*), B) filamentous algae (*Oedogonium* sp.), C) bacterial aggregates, D) protozoa (Thecamoebians), E) rotifera (*Colurella* cf.), F) protozoa (*Vorticella* sp.).

Where light prevails, biofilms are dominated by photosynthetic organisms (autotrophs), the algae (Lock et al., 1984), particularly Chlorophyta (green algae), Bacillariophyta (diatoms) and Cyanobacteria (Peterson, 1996). Biofilms in low light environments are predominantly heterotrophic and dominated by bacteria (Blenkinsopp & Lock, 1994; Fig. 7). The balance between autotrophy and heterotrophy within the biofilm is not solely controlled by light availability, since the type of substrata (organic or inorganic), the nutrient availability and physical disturbances (Peterson, 1996) also play important roles.

Biofilms possess many attributes which make them useful ecological indicators of disturbances in riverine systems. They are sensitive to changes in environment conditions, are abundant and cosmopolitan in their distribution, have short generation time and sessile nature, can be sampled rapidly and have a wide range of attributes (structural and functional) which can be measured quantitatively (Steinman & McIntire, 1990). The measurement of functional biofilm attributes provides an insight into ecosystem processes fundamental to river health that is not available through structural attributes.

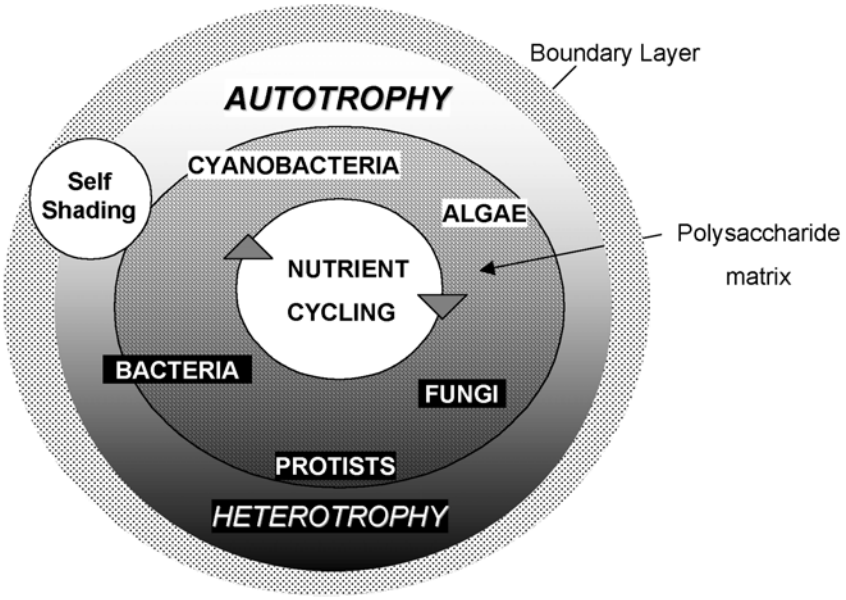


Fig. 7. Diagrammatic representation of a biofilm. Autotrophs (Cyanobacteria and algae) and heterotrophs (bacteria, fungi and protists) distribution within the polysaccharide matrix is shown. Cycles within the main circle illustrate that the biofilm can also control its own microenvironment: self-shading, and nutrient transfer between heterotrophic and autotrophic cells. The boundary layer of the microenvironment created by the biofilm is depicted by the grey border. From Burns & Ryder, 2001

▪ THE BENTHIC STREAM MICROBIAL LOOP

Benthic biofilms are the main responsible for the organic matter processing in streams and rivers (Bretschko, 1995; Romaní & Sabater, 2001). The organic matter derives from the water column and from material produced or entrapped within the biofilm matrix (including algal exudates and extracellular polymeric substances; Sabater & Admiraal, 2005). The trophic pathway in aquatic environments where DOC is reintroduced to the food web is named microbial loop (Azam et al., 1983, Tranvik, 1988). However, the organic matter processing by the benthic microbial populations is still poorly known in comparison with the planktonic microbial food web (Azam et al., 1983). This benthic microbial loop becomes the base of autodepurative fluvial processes (Fischer, 2003; Battin et al., 2003).

In this microbial loop, bacteria, fungi, and to some degree algae, transform carbon obtained from dissolved and fine particulate sources into microbial biomass. The interactions between these three compartments (algae, bacteria and fungi) are both positive and negative (see next section). Inside the biofilm, bacteria are consumed mostly by protists such as flagellates and ciliates (Bott & Kaplan, 1990). These protists, in turn, are consumed by larger aquatic organisms (micro-metazoans) like copepods, oligochaetes, rotifers, nematodes and insect larvae which are found interstitially as well as on the substrate surface. At the end, these micro-metazoans can be consumed by larger invertebrates and fish (Fig. 8).

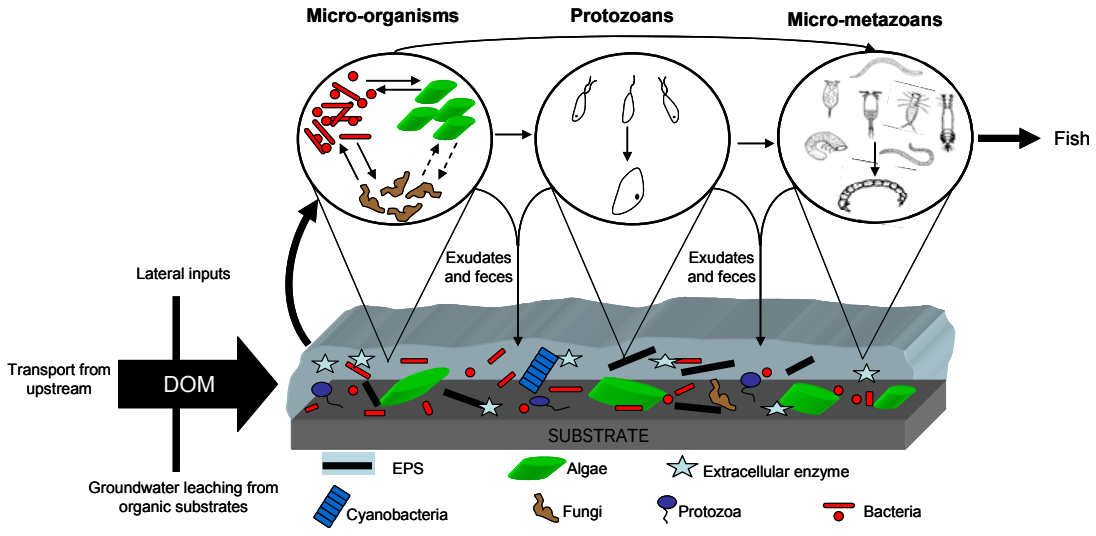


Fig. 8. Benthic stream microbial loop. Modified from Allan & Castillo, 2007.

Because microbes are the base of the food web in most aquatic environments, the trophic efficiency of the microbial loop has a profound impact on key aquatic processes (ultimately leading to the amount of carbon exported to the ocean floor).

Even this is of great importance, there is still a lack of knowledge about the flux of materials among the different benthic microorganisms (Bott & Borchardt, 1999; Parry, 2004). Many structural and functional studies of the biofilm point to the great significance of the organic matter use in the biofilm itself thanks to the observed trophic interactions among the biofilm organisms (Freeman & Lock, 1995), but usually in an indirect way, through, for instance, positive correlations between algal and bacterial growth or between algal biomass and certain enzymes expression.

The structural complexity of biofilms hampers the direct study of the relations between their components. The separation of the different trophic groups of microorganisms implies performing laboratory experiments to sort out the role of the different biofilm compartments through the utilization from pure microorganism's cultures grown in artificial substrata (Bruckner & Kroth, 2009). Also the utilization of restrictive conditions for one or another group (like using light to splitting out the auto-heterotrophic biofilms from those totally heterotrophic) is an alternative.

▪ EXTRACELLULAR ENZYMES AS INDICATORS OF ORGANIC MATTER USE

Low-molecular-weight substrates can be transported directly from the surrounding medium into the cell, but these substrates may be present only in low concentrations, and competition for such resources may be high (Arnosti, 2003). The bulk of organic substances in aquatic environments are macromolecules not ready for the direct incorporation into the bacterial cell (Hoppe, 1993). These materials have to be preconditioned by extracellular enzymes, so that they can be available for bacterial growth and nutrient cycles. The activity of these enzymes is in many cases a limiting factor for substrate decomposition and bacterial growth. These enzymes represent the "toolboxes", which enable heterotrophic bacteria to obtain suitable substrates from a diverse array of compounds (Arnosti, 2003).

It has been widely assumed in aquatic microbial ecology that most extracellular enzymatic activities except phosphatases are of bacterial and fungal origin (e.g. Hoppe et al., 1988; Chróst, 1989, 1990 and 1991; Baldy et al., 1995). However, to a minor extent, algae and protozoa may also express enzymatic activities (Vrba et al., 2004). Biofilm extracellular enzyme activities play a key role inside the microbial loop; they are the result of the internal recycling of organic matter and microbial interactions (competition/synergism) within the biofilm, such as algal-bacterial and fungal-bacterial interactions.

The expression of a certain set of enzymes is handy to "fingerprint" the composition of the organic matter pool in use (Hopkinson et al., 1998). The use of extracellular enzyme activity as fingerprints relies on the assumption that as the

composition of the bulk DOM pool fluctuates, bacteria have the capacity to express a different set of enzymes to gain carbon from the new mixture (Chróst, 1991). Thus, extracellular enzyme activities are good proxies to characterize the nature and quantity of available DOC and nutrients (N and P).

Extracellular enzymes are generally located outside the cytoplasmic membrane (Priest, 1992). In order to distinguish between enzymes which are still associated with their producers and those which can occur dissolved in the water or adsorbed to particles, Chróst (1991) suggested calling the former “ectoenzymes” and the latter “extracellular” enzymes. Substrates for hydrolysis are generally proteins, carbohydrates, fats and organic P- or S- compounds. To study the impact of bacterial extracellular enzyme activities on bacterial substrate uptake *in situ* biochemical methods can be used. These methods are based on the application of fluorogenic model substrates. These substrates have some characteristics in common: (1) they contain an artificial fluorescent molecule and one or more natural molecules (e.g. glucose, amino acids), linked by a specific binding (e.g. peptide binding, ester binding); fluorescence is observed after enzymatic splitting of the complex molecule (Fig. 9); (2) the hydrolysis of model substrates is competitively inhibited by a variety of natural compounds with the same structural characteristics; (3) hydrolysis of model substrates follows first order enzyme kinetics; and (4) application of those model substrates allows enzyme activity measurements under natural (in situ) conditions within short incubation periods (Hoppe, 1993).

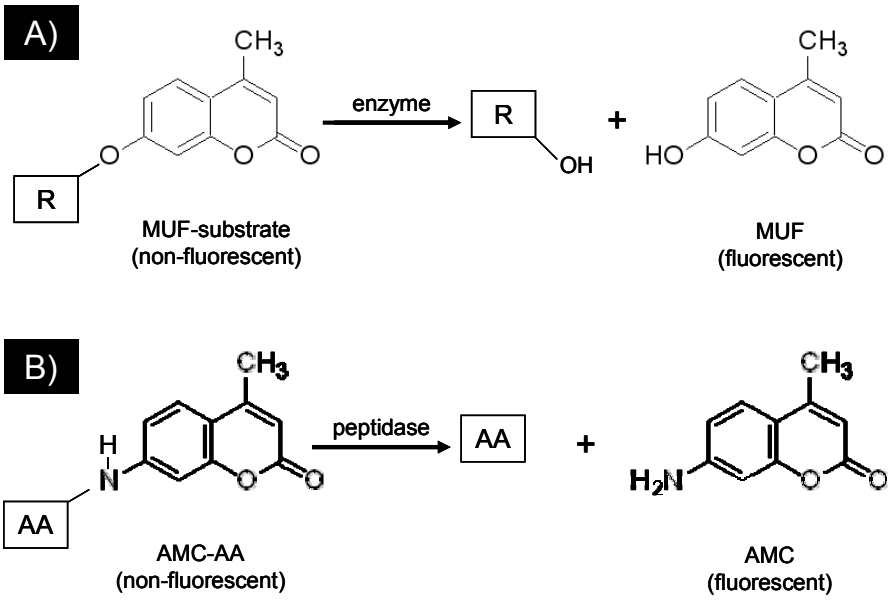


Fig. 9. Molecular structure and enzymatic hydrolysis products of 4-methylumbelliferyl (MUF) substrate (A) and 7-amino-4-methylcoumarin (AMC) substrate (B).

In this thesis, several hydrolytic and oxidative extracellular enzyme activities were assayed (Table 1) in microbial communities developed on distinct stream substrata. Measurements of the extracellular hydrolytic enzymes activities followed the methodology described by Romaní & Sabater (2001). A total of 4 different hydrolytic enzyme assays were performed in this study. Polysaccharide compounds degradation was determined by means of the β-glucosidase, β-xylosidase and cellobiohydrolase activity. Peptides decomposition was determined by means of the leucine-aminopeptidase activity. β-glucosidase, β-xylosidase and cellobiohydrolase activities were measured using MUF (4-methylumbelliferyl) fluorescent-linked substrates while leucine-aminopeptidase activity was measured using AMC (7-amino-4-methylcoumarin). These fluorescing agents (MUF and AMC; Fig. 9) are supplied to natural water or biofilm communities at substrate saturating conditions, which are determined from previously performed saturation curves (Romaní, 2000).

The phenol-oxidase activity was also measured. This oxidative enzyme activity is mainly produced by fungi and oxidizes lignin polymers. This assay involve a substrate (L-3,4-dihydroxyphenylalanine; L-DOPA) which was used as a electron donor, generating a product that can be quantified spectrophotometrically (Sinsabaugh & Linkins, 1990; Sinsabaugh et al., 1994).

Extracellular enzyme	Artificial substrate (from Sigma-Aldrich)	Involved in the metabolism of
Cellobiohydrolase (EC 3.2.1.91)	MUF-cellobioside	C (hydrolyses cellulose to cellobiose)
β-D-glucosidase (EC 3.2.1.21)	MUF-β-D-glucopyranoside	C (last step of cellulose decomposition, decompostion of cellobiose to glucose)
β-D-xylosidase (EC 3.2.1.37)	MUF-β-D-xylopyranoside	C (last step of hemicellulose decomposition, decomposition of xylobiose to xylose)
Lipase (EC 3.1.1.3)	MUF-palmitate	hydrolysis of ester bonds in water-insoluble lipid substrates
Leucine-aminopeptidase (EC 3.4.11.1)	Leu-AMC (L-leucine-4-methyl-7-coumarinylamide)	N, C (hydrolyses polypeptides to leucine and other hydrophobic amino acids)
Phenol oxidase (EC 1.14.18.1)	L-DOPA	Degradation of lignin

Table 1. Extracellular enzyme activities used in this study. The artificial substrate and the mode of action of each enzyme are shown. Between parentheses, the EC (Enzyme Commission) number is specified.

FACTORS AFFECTING BIOFILM ORGANIC MATTER USE IN STREAMS

The biofilm organic matter use in streams might be affected by factors such as organic matter bioavailability, bioreactivity and chemical composition. Other factors like discharge variability and stream water temperature (both enhanced by climate change), microorganism's interactions within the biofilm and microbial diversity may also play a significant role in this organic matter uptake.

▪ ORGANIC MATTER AVAILABILITY AND USE UNDER GLOBAL CHANGE PREDICITONS

Climate change models predict that higher water temperatures are likely to occur over rivers and streams in temperate regions (IPCC, 2007). Climate change is also expected to affect the severity and frequency of storm and drought events. Increasing water temperature and hydrological changes (drought and rewetting periods) are likely to lead to shifts in the quality and quantity of DOC export from terrestrial sources to rivers as well as changes in DOC processing rates (Porcal et al., 2009).

Mediterranean fluvial systems, especially in their headwaters and middle parts, are characterized by important discharge variability (Gasith & Resh, 1999). This variability is likely to increase determining the effects of extreme hydrologic episodes (droughts and floods) on the biochemistry of organic matter (quality and concentrations of DOC). In drought periods the hydrological connectivity between the superficial water and the hyporheic water is lost. The most superficial zones become small ponds where the sediments and the organic detritus accumulate and can not be exported (Lake, 2003). Severe drought periods followed by intense rainfall (as occurs frequently in autumn) supposes the transport of great amount of material (leaves and associated organisms) which accumulated in the river bed. During the dry-wet interphase an important mobilization of DOC, especially the most biodegradable fraction, and nitrates has been observed (Bernal et al., 2002; Romaní et al., 2006).

Warming could also have strong effects in the stream organic matter decomposition. It is hypothesized that higher stream water temperature may differentially affect the decomposition of labile and recalcitrant material by the microbial biofilm communities. Since the recalcitrance of a molecule is defined by the activation energy required to break the chemical bonds (Thornley & Cannell, 2001), higher temperature and activation energy are required to ensure degradation of resistant rather than easily degradable organic substrates. The temperature sensitivity of organic

carbon decomposition has been extensively studied in soils (Davidson & Janssens, 2006; Kirschbaum, 2006). Even though, relatively little is known about responses of benthic communities to stream organic matter decomposition under varying temperature (Sand-Jensen et al., 2007).

▪ EFFECTS OF BIOFILM ALGAL-BACTERIAL INTERACTIONS TO ORGANIC MATTER USE

The interactions between aquatic bacteria and algae have been extensively studied. In marine and freshwater plankton, these relationships have been widely described (Cole, 1982; Bird & Kalff, 1984; White et al., 1991) and it is generally accepted that heterotrophic bacteria directly use products excreted by planktonic algae (Brock & Clyne, 1984; Siuda et al., 1991; Goto et al., 2001; Descy et al., 2002). A similar relationship between algae and bacteria has been found in freshwater benthic systems (Fig. 10). An early study of Haack & McFeters (1982a, 1982b) on epilithic biofilms demonstrated that algae may supply bacteria with DOC resulting from excretion processes during photosynthesis. Other studies of epilithic biofilms also concluded that algal exudates are a major carbon source for bacteria (Geesey et al., 1978; Kaplan & Bott, 1989; Hepinstall & Fuller, 1994). Algae release extracellular organic carbon during active metabolism (enhanced during photosynthetic production) or following cell lysis and natural senescence. This extracellular organic carbon released consists largely of low molecular weight compounds (Sundh, 1992) which are rapidly used by bacteria and can result in increased bacterial productivity. In fact, up to 50% of algal primary production is released as DOC (Lyche et al., 1996) and algal-released DOC may support up to 95% of bacterial production (Coveney, 1982; Lyche et al., 1996).

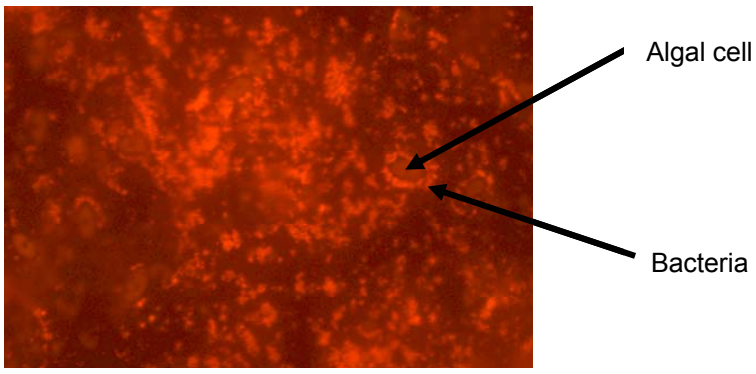


Fig. 10. In this epifluorescensce picture (after incubation with CTC, 5-Cyano-2,3-ditolyl tetrazolium chloride, 400x) of an epilithic intact biofilm, algal cells are surrounded by active respiring bacteria (red) evidencing the interaction between them.

Algal accumulation in the epilithic biofilm influences the use of organic matter by the heterotrophic community by increasing the amount of organic substrate available for bacteria (Wetzel, 1993; Romaní & Sabater, 1999 and 2000). This fact may be related to the physical proximity between the two, but also to the high proportion of polymeric carbohydrates and peptides included in algal exudates and lysis products, whose use is enzyme-mediated. The activity of leucine-aminopeptidase has been positively linked to the activity of photosynthetic primary producers (Francoeur & Wetzel, 2003), and to the use of algal-released proteinaceous compounds by microbial heterotrophs (Romaní et al., 2004). On the other hand, β-glucosidase activity has been correlated with the degradation of either organic compounds of algal origin (algal exudates and EPS) or polysaccharidic organic compounds dissolved in flowing water (Jones & Lock, 1993). In biofilm communities grown on artificial substrata, algal photosynthesis simultaneously increased the activities of extracellular α-glucosidase, β-glucosidase and β-xylosidase (Espeland et al., 2001). Furthermore, bacterial phosphatase activity is also stimulated by the amount of extracellular organic carbon accumulated within the biofilm matrix during photosynthesis (Espeland & Wetzel, 2001). Thus, photosynthetic activity of benthic algae has been recognized as a potentially important stimulator of extracellular enzyme activities within biofilms (Francoeur et al., 2006; Rier et al., 2007) which reinforces the existence of a link between autotrophic and heterotrophic organisms.

Although many studies have reported strong algal-bacterial coupling in lotic systems, some studies have found that algal-bacterial co-variation is weak or undetectable (Coffin & Sharp, 1987; Findlay et al., 1991 and 1993; Sobzack, 1996; Gao et al., 2004). Bacteria are less likely to utilize algal-generated DOC under specific conditions: 1) under extreme light limitation, 2) when a labile source of allochthonous DOC is available, or 3) under extremely oligotrophic conditions where algae are severely nutrient limited.

When light intensities are sufficient to stimulate benthic photosynthesis, bacterial production usually is coupled with algal production (Kaplan & Bott, 1989; Jones & Lock, 1993). Hence, biofilms growing under light reduce the uptake of dissolved organic compounds from the flowing water pool thanks to the use of algal released organic matter (Romaní et al., 2004). Nevertheless, streams with light limiting conditions (like forested streams), often have heterotrophic biofilms that rely on allochthonous organic matter rather than algal-derived extracellular organic compounds (Findlay et al., 1993). In this light-limited environments, some algae are capable of heterotrophically metabolize a diverse range of organic carbon sources. The proximal

relationship between heterotrophically metabolizing algae and bacteria may also lead to competition between those for organic substrates. However, Tuchman et al., 2006 found that bacteria were able to oxidize a more diverse array of organic substrates including carboxylic acids and large polymers, while diatoms appeared to more readily utilize the complex carbohydrates. By oxidizing different organic substrates than bacteria, heterotrophically metabolizing diatoms may be reducing direct competition and enhancing coexistence with bacteria. A mutualistic relation between heterotrophically algae and bacteria might exist in dark conditions.

The amendment of an external organic matter source such as a labile DOC (glucose) on biofilms grown under light conditions also weakened the algal–bacterial coupling. Sobczak (1996) observed that a labile DOC in water column may be controlling bacteria throughout biofilm colonization, with epilithic algae becoming increasingly important in late stages of biofilm development. A thin biofilm should rely primarily on nutrients from the water column (allochthonous DOC), whereas a thick, mature biofilm may rely on both allochthonous and autochthonous carbon.

The role of nutrients in the interplay between autotrophic and heterotrophic production in stream epilithon is under debate. Rier & Stevenson (2001) observed that there was only a positive statistical relationship between algae and bacteria when streams with periphyton chlorophyll *a* values greater than 5µg cm^{-2} were included in the analysis. Findlay et al. (1991) and Gao et al. (2004) found no significant correlation between bacterial abundance and chlorophyll concentration. These studied streams varied in their trophic status and correlation between benthic bacterial number and chlorophyll might be observed if streams with chlorophyll concentrations higher than 5µg cm^{-2} were included.

Low algal biomass occurred primarily in nutrient-poor streams, suggesting a potential competitive interaction between algae and bacteria for nutrients. In very low nutrient environments, heterotrophic bacteria may compete with algae for inorganic nutrients (Currie & Kalf, 1984a; Jansson et al., 1988; Grover, 2000). Since bacteria have higher growth rates than algae (Rhee, 1972) and a greater surface area to volume ratio (Lewis, 1979), they are considered superior competitors over algae for inorganic phosphorus and nitrogen (Currie & Kalf, 1984b). Even though, results from Rier and Stevenson (2002) and Carr et al., (2005) point that there was no evidence of algae being negatively affected by competition with bacteria for nutrients.

Rier and Stevenson (2002) went one step farther to suggest that nutrient-poor biofilms might exhibit stronger algal-bacterial coupling than nutrient-rich biofilms. Based on these results, Scott et al., (2006 and 2008) tested the hypothesis that algal-bacterial coupling decreases with increasing nutrient availability, but decoupling also occurs in

very-low-nutrients environments because of algal-bacterial competition for nutrients. The degree of coupling between autotrophs and heterotrophs in periphyton could therefore decrease along nutrient-enrichment gradient, algae becoming less reliant on bacterial mineralisation of nutrients and bacteria becoming less stressed for photosyntethically derived extracellular organic carbon (Scott et al., 2008). However, decoupling does not appear to occur as a result of competition for nutrients in nutrient-poor streams. Their results support the idea of mutual facilitation between photoautotrophs and heterotrophic bacteria in aquatic microbial communities.

The algal bacterial coupling mainly refers to the positive effect of algae on bacteria by providing algal exudates, while algae can also benefit from bacteria. This relationship can be therefore defined as mutualism or co-operation within the biofilm. As described before, bacteria may use algal exudates released during algal photosynthesis for energy source (Descy et al., 2002) as well as inorganic nutrients for growth (Hepinstall & Fuller, 1994). Heterotrophic bacteria may also rely on the use of O_2 released during algal photosynthesis (Kuhl et al., 1996). Algae require special vitamins (B vitamins) for their growth which are basically synthesized by heterotrophic bacteria (Cole, 1982). Thus, algae acquire vitamin B_{12} through a symbiotic relationship with bacteria (Croft, 2005). Autotrophic organisms are also favoured by the CO_2 released during heterotrophic respiration (Cole, 1982). Moreover, bacteria may also release extracellular enzymes that degrade complex organic molecules not transportable across cell membranes (Sinsabaugh & Linkins, 1988), making both organic and inorganic carbon (N and P) available to algae (Wetzel, 1993; Klug, 2005).

▪ LINKS BETWEEN MICROBIAL DIVERSITY AND FUNCTION. EFFECTS ON ORGANIC MATTER USE CAPACITY

The microbial diversity of the biofilm might further determine biofilm capacity for organic matter use. To understand the connections of biodiversity and ecosystem functioning has become one of ecology's primary goals in the last decade (Grime, 1998; Lawton et al., 1998; Loreau et al., 2001; Duffy, 2003; Luck et al., 2003; Loreau et al., 2006). Most of these publications focus on terrestrial ecosystems and much less in aquatic microbial systems (McGrady-Steed & Morin, 2000; Morin & McGrady-Steed, 2004; Petchey et al., 2004; Langenheder et al., 2005; Szabo et al., 2007). Considering that bacteria comprise the majority of diversity and at the same time drive the bulk of ecosystem processes (Ghilarov, 2000), it is pivotal to fill this knowledge gap.

Measuring biodiversity is not simple especially for microbial diversity. Most often biodiversity is measured in terms of species richness (e.g. the number of species at a

site). Other approaches focus on the distribution of individuals among species, referred to as evenness, and some measure phenotypic differences among species (Purvis & Hector, 2000). All of these techniques measure some aspect of diversity, but none of them include all aspects of biodiversity. Moreover, there are methodological constraints on the ability to measure biodiversity. For example, the widely used community fingerprinting techniques commonly detect only app. 10% of the most common bacterial species that coexist in freshwater habitats (Torsvik et al., 2002).

Ecosystem functioning can be defined as the processes that ecosystems perform to sustain ecological goods and services (Loreau et al., 2006). Walker (1992) and Lawton (1994) elaborated a range of hypotheses concerning species richness and ecosystem functioning. According to the *redundant species hypothesis* or *insurance hypothesis* several species in an ecosystem perform the same function; therefore the loss of one species will be compensated by another species. In contrast, the *rivet hypothesis* or *predictable change hypothesis* suggests a linear relationship as each time a species is lost a specific function is lost as well. The *idiosyncratic response hypothesis*, on the other hand, suggests a change of ecosystem function each time a species is lost, but magnitude and direction of change is unpredictable, as each species contributes in an indefinite way and to an unpredictable extent to the overall ecosystem function. The *null hypothesis* is that there is no coupling of diversity and functioning (Lawton, 1994).

Due to methodological difficulties related to the estimation of bacterial species richness, only very few studies addressed these hypotheses for microorganisms (McGrady-Steed et al., 1997; Naeem & Li, 1997; Naeem, 1998; Langenheder et al., 2005; Loreau et al., 2006).

OBJECTIVES OF THIS STUDY

The main goal of this thesis is to determine the use of dissolved organic matter (DOC) by the benthic stream microbial biofilms. It is aimed to determine the efficiency of the fluvial system in the use of the different materials that circulate in it. This efficiency will depend in one hand on the biofilm itself (e.g. the microorganisms involved, their function and interactions); and on the other hand on the available organic matter (quantity and quality).

Due to the fluvial benthic communities process a great amount of the organic mater reaching to the river, it is hypothesize that the inputs of material (autochthonous and allochthonous) as well as its nature (chemical composition) and intensity

(concentration in the river) will determine the structure and function of the autotrophic and heterotrophic benthic community, their possible interactions and their microbial composition.

The specific objectives of the thesis are:

1. To characterize the changes in organic matter (quantity and quality) at the different benthic stream compartments (cobbles, sand and leaves) and in the stream water (dissolved and particulate fractions) during a typical summer drought (transition from wet to dry) and the posterior water recovery (dry to wet conditions) in an intermittent Mediterranean stream. Specifically, to describe the changes in quality (polysaccharides, proteins and lipids content) and quantity of the dissolved, particulate and benthic organic matter pools particularly in these wet-drought-wet periods.

2. To study the stream amino acid composition in detail during the drought-rewetting processes in Mediterranean streams. In particular, to compare amino acid composition (quality) and abundance (quantity) in different organic matter pools in order to determine if changes in dissolved and benthic amino acid compositions are representative of the diagenetic state and quality of the organic matter during the pre- and post-drought phases.

3. To examine how the predicted stream water temperature increase in Mediterranean streams may differently affect DOC (labile and recalcitrant sources) biofilm degradation capacity.

4. To determine whether differences in organic matter processing and biofilm structure and composition exist between auto-heterotrophic (grown under light) and heterotrophic (grown under dark) biofilm communities, when an allochthonous labile organic matter is provided (glucose additions). At the same time, study the possible interactions between biofilm algae and bacteria in these circumstances.

5. To investigate the relationship between biofilm diversity and ecosystem functioning, by focusing on DOC degradation and use. Moreover, determine the effects of manipulated bacterial communities (with contrasting bacterial diversity levels) on the likelihood to sustain multifunctionality under different DOC quality sources (labile and recalcitrant).

STUDY SITE

In order to achieve the presented objectives, some experiments were developed in the field and others under laboratory conditions.

The field research was developed in a Mediterranean stream, the Fuirosos. Fuirosos is a third-order stream, with a catchment area of 15.6 km^2, located in the Montnegre-Corredor Natural Park, a forested range close to the Mediterranean Sea (50 km north of Barcelona, NE Spain; Fig. 11). The traditional land uses in the watershed are forestry (land clearing and riparian logging), cattle raising and agriculture. Agriculture was widespread in the past, but now occupies a small part of the lower basin. The stream has a Mediterranean flow regime, with usual summer drought (Sabater et al., 2001; Acuña et al., 2007; Fig. 12). Precipitation is distributed irregularly, and mostly falls in autumn and spring with occasional summer storms. The basal flow ranges from 5 to 20 L s^{-1} but can increase by more than 100 times during autumn and spring spates (Bernal et al., 2005; Butturini et al., 2008).

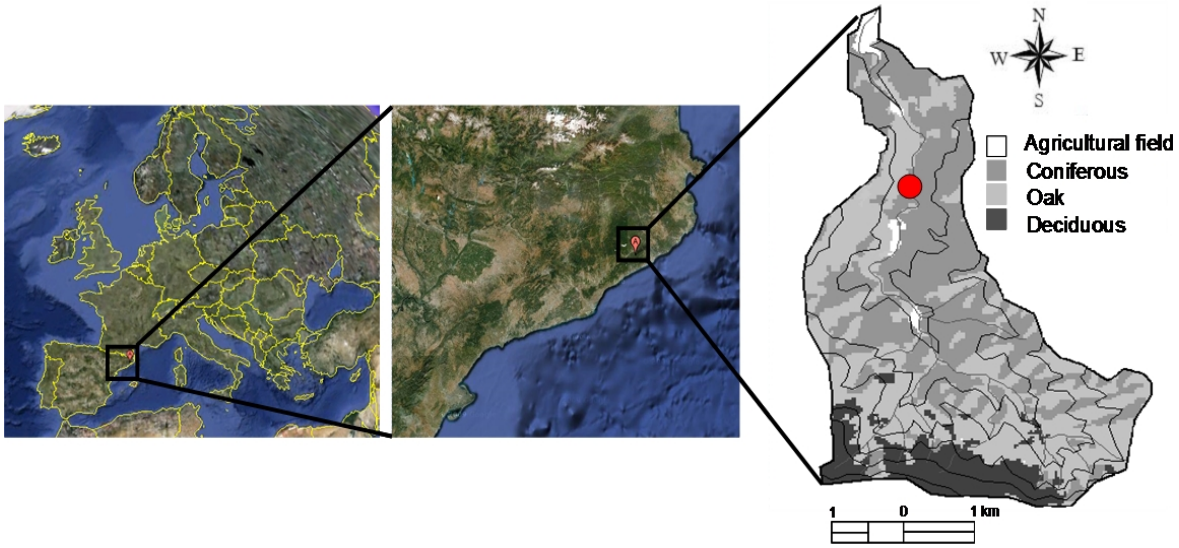


Fig. 11. Geographical location of the Fuirosos stream watershed. The Fuirosos is a tributary of *La Tordera* River which flows into the Mediterranean Sea. The red dot indicates the sampling site. Vegetation cover is shown by different shadings.

The studied stream reach is 3–5 m wide and 10 m in length. The riparian vegetation is made up of alder (*Alnus glutinosa*), hazelnut (*Corylus avellana*), poplar (*Populus nigra*) and plane trees (*Platanus acerifolia*) which form a closed canopy from May to October. Shrubs (especially *Rubus ulmifolia*) and lianas constituted the lower

vegetation layer. Most of the leaf input into the river channel occurs in summer due to hydric stress, and from autumn (especially *Alnus* leaves) to late winter (especially *Platanus* leaves). The streambed morphology is very similar throughout the study area, and is characterized by altering riffles (with boulders and cobbles) and pools (with sand and leaf material accumulated).

Fig. 12. Fuirosos stream reach during the wet phase (A) and during a summer drought (B).

This study site at the Fuirosos stream provides two relevant characteristics, apart from its Mediterranean hydrology: 1) it is a near-pristine Mediterranean stream watercourse, with no remarkable anthropogenic pressures, and 2) since 1998 numerous studies in our research group have been developed there. Thus, many papers have been published describing the processes occurring in the riparian zone (Butturini & Sabater, 2000; Sabater et al., 2001; Buttrini et al., 2002; Bernal et al., 2002) as well as those occurring instream (Sabater et al., 2005 and 2006). The structure and function of the Fuirosos stream biofilm has been well defined (Romaní et al., 2004) as well as its metabolism (Acuña et al., 2004 and 2005). Some studies also pointed out the relationships between biofilm algae and bacteria (Romaní & Sabater, 1999) and between biofilm bacteria and fungi (Artigas et al., 2009). Some studies also focused on the organic matter use by benthic microbial heterotrophs (mainly bacteria) in different stream substrata (Romaní et al., 2004; Sabater et al., 2005). In spite of all these investigations, there is still a lack of information concerning the availability (quantity), use, transformation and composition (quality) of the fluvial organic matter in the benthic compartment.

DEVELOPMENT OF THE PROJECT

The development of the thesis project includes both field research and sampling under specific and relevant periods and experimental research in the laboratory in order to control the most relevant factors affecting DOC heterotrophic use.

In order to evaluate the effects of drought and rewetting (effect of flow change) in relation to the availability of material (quantity and quality) for the microorganisms, a field monitoring during these periods was done. During the pre-drought process (wet conditions), drought (summer) and later during the process of basal flow recovery in autumn (post-drought), a sampling of the flowing water and the benthic accumulated material (epilithic and epipsammic biofilms as well as leaves) was made. This sampling was intensified in the relevant moments of change affecting the benthic community and the ecosystem functioning. This translated into a more intense analysis during the loss of flow during the drought and during the fast flow recovery after the late-summer rains. Analyses of polysaccharides, lipids and proteins content and DOC characterization (analyses of DOC, dissolved organic nitrogen (DON) and biodegradable DOC (BDOC)) were done. The availability of materials was investigated from measures of the enzymatic activities (β-glucosidase, leucine amino-peptidase and lipase) in the different habitats (cobbles, sand and leaves substrata) tied to the changes in organic matter content and concentration in the flowing water.

From these samplings a more detailed study of the amino acid composition was derived. The specific amino acid composition was analyzed as they can be used as good indicators of the organic matter diagenetic state, in order to detect the changes in the organic matter quality during these wet-drought-wet periods.

Besides, because of the predictions that the stream water temperature will increase by warming and due to climate change may drive shifts in DOC quality and concentration (e.g. accumulation of recalcitrant DOC during summer drought) a laboratory experiment considering two extreme DOC qualities (labile and recalcitrant) and two water temperatures (stream basal temperature and increasing 4ºC this basal temperature) was done. It was hypothesized that higher stream water temperature may differentially affect the decomposition of labile and recalcitrant material by the microbial biofilm communities. The increase of temperature might be of high importance for organic matter cycling and hence for the whole aquatic system.

A laboratory experiment was also designed to study the trophic relationships among stream algae and bacteria in the utilization of the organic matter. Light and dark conditions were used to differentiate an auto-heterotrophic biofilm from a heterotrophic

biofilm (without any inputs of good quality autochthonous material). Treatments with and without glucose were used to determine the effects of adding a labile DOC source on the biofilm degradation capacity. Saturation enzymatic curves were carried out to estimate the efficiency in the utilization of organic compounds (β-glucosidase and leucine- aminopeptidase activities) and the biofilm bacterial composition (Denaturing Gradient Gel Electrophoresis; DGGE) was described. This study added knowledge to the expected changes in organic matter processing by auto-heterotrophic or heterotrophic stream biofilms when labile organic matter was available and pointed to the algal-bacterial relationships.

Bacteria may also play a significant role in the organic matter use. However, basic understanding about the connections of biodiversity and ecosystem functioning of aquatic microbial communities is limited. A laboratory experiment was designed by using bioreactors to address the link of bacterial diversity and DOC degradation capacity as an ecosystem function. We worked with different treatments playing with the source of available organic matter (more labile or more recalcitrant) and with the dilution of the colonizing inoculum (higher dilution less diversity due to the lost of the less abundant species). Emphasis was done on the bacterial diversity (Terminal Restriction Fragment Length Polymorphism; TRFLP) and in the use of the amended organic material by means of enzymatic activities (leucine-aminopeptidase, β-glucosidase, β-xylosidase, cellobiohydrolase and phenol-oxidase). The ambition was to investigate the relationship between biofilm diversity and ecosystem functioning, by focusing on DOC degradation; prove if a loss of species was directly related to a loss of function and whether such a relationship would be different for overall functions (such as the activity of five enzymes). Moreover, determine the effects of bacterial diversity on the likelihood to sustain multifunctionality under different DOC quality sources (labile and recalcitrant). This experiment was done at the University of Uppsala (Sweden) in the department of Ecology and Evolution under the supervision of the Prof. Lars Tranvik.

METHODOLOGY USED

In order to achieve the planned objectives, several methodologies were used which are outlined below, and described in detail in the specific chapters:

- Biomass analysis of different microbial groups:
  - Analysis of the algal biomass through chlorophyll extraction and absorbance measurements (Jeffrey & Humphrey, 1975).
  - Analysis of bacterial biomass differentiating live and dead bacteria by using a double fluorescent stain (live/dead viability kit) with propidium iodide and Syto9 (Freese et al., 2006) and posterior counting by epifluorescence microscopy.
  - Measurement of the bacterial biomass in the flowing water by flow cytometry using Syto13 (Molecular Probes, Inc.) stained cells (Del Giorgio et al., 1996).

- Structure and composition of the biofilm microbial community:
  - Extracellular polysaccharidic content (EPS) extractions by using a cation-exchange resin (Dowex Marathon) following Romaní et al., (2008). Posterior polysaccharide content quantification using the phenol-sulphuric acid assay (Dubois et al., 1956).
  - Observation and microscopic determination of the different algal groups in order to determine and quantify the algal biodiversity.
  - Observation of the intact biofilm by the scanning electron microscope (SEM).
  - Analysis of the bacterial community through molecular techniques: PCR (Polymerase Chain Reaction), DGGE (Degrading Gradient Gel Electrophoresis) and TRFLP (Terminal Restriction Fragment Length Polymorphism).

- Biofilm metabolism:
  - Extracellular enzyme activities (leucine-aminopeptidase, β-glucosidase, β-xylosidase and cellobiohydrolase) through utilization of artificial substrata and posterior fluorescence (MUF and AMC) or absorbance (L-DOPA, Phenol-oxidase activity) detection. Potential measures and saturation curves in order to gauge V_{max} and K_m and to evaluate the efficiency in the utilization of organic compounds (Romaní, 2000; Romaní & Sabater, 2001).
  - Measures of photosynthetic efficiency (Schreiber et al., 2002) by using the PAM (Fluorimeter of Modular Amplitude).

- Net primary production by measuring differences in oxygen concentration (oxygen balance method).
- Biofilm respiratory activity, measured through the electron transport system (ETS) assay after the reduction of the electron transport acceptor INT (2-3 tetrazolium chloride from Sigma-Aldrich) into INT-formazan (Blenkinsopp & Lock, 1990).
- Respiration (oxygen consumption) in heterotrophic communities grown in bioreactors, measured as the difference in oxygen concentration between the inflow and the outflow. The medium passed through a flow-through oxygen probe (FTC, PreSens, Germany), connected via an optical fiber to a Fibox 3 oxygen meter (PreSens, Germany).

➢ Composition of the dissolved, particulate and benthic material:

- Amino acids. Determination of the amino acid content by using a HPLC chromatographic method (Waters AccQ-Tag® kit).
- Polysaccharides. Polysaccharidic content quantification through a colorimetric protocol (MBTH; Pakulski & Benner, 1992; Chanudet & Filella, 2006).
- Lipids. Lipidic content measurements by using a colorimetric technique (Zollner & Kirsch, 1962).

➢ DOC quality indices:

- Water absorbance measurements at 250 nm. Data absorbance was used to calculate the ratio Abs_{250}/ total DOC which indicates the proportion of humic material in total DOC (Morris & Hargreaves, 1997; Fischer et al., 2006).
- Water fluorescence measurements at 370 nm excitation and 450 and 500 nm emission wavelengths. Data was used to determine the Fluorescence index (FI; McKnight et al., 2001) which was calculated as the ratio between 450 and 500 nm of emission intensity (with excitation at 370 nm). This FI index measures the origin of the dissolved organic matter which can be more terrestrially or microbially derived.

➢ Physical and chemical water analyses:

- Dissolved oxygen concentration, pH and conductivity in the field, measured using probes (Hach meters).

- Quantification of DOC and DON in water samples using a Shimadzu TOC 5050A. Measurements of the BDOC following Servais et al., (1989).
- Nutrient content. Soluble reactive phosphorus (SRP) analysis following Murphy & Riley, (1962); nitrate analysis by ion chromatography and ammonia was determined following Hach, (1992).

CHAPTER I

ORGANIC MATTER AVAILABILITY DURING PRE AND POST-DROUGHT PERIODS IN A MEDITERRANEAN STREAM

Ylla, I., Sanpera-Calbet, I., Vázquez, E., Romaní, A. M., Muñoz, I., Butturini, A. & Sabater, S. 2010. Hydrobiologia 657:217-232

ABSTRACT

Mediterranean streams are characterized by water flow changes caused by floods and droughts. When intermittency occurs in river ecosystems, hydrological connectivity is interrupted and this affects benthic, hyporheic and flowing water compartments. Organic matter use and transport can be particularly affected during the transition from wet to dry and dry to wet conditions. To characterize the changes in benthic organic matter quantity and quality throughout a drying and rewetting process, organic matter and enzyme activities were analyzed in the benthic accumulated material (biofilms growing on rocks and cobbles, leaves, and sand) and in flowing water (dissolved and particulate fractions). The total polysaccharide, amino acid and lipid content in the benthic organic matter were on average higher in the drying period than in the rewetting period. However, during the drying period, peptide availability decreased, as indicated by decreases in leucine aminopeptidase activity as well as amino acid content in the water and benthic material, except leaves; while polysaccharides were actively used, as indicated by an increase in β-glucosidase activity in the benthic substrata and an increase in polysaccharide content of the particulate water fraction and in leaf material. During this process, microbial heterotrophs were constrained to use the organic matter source of the lowest quality (polysaccharides, providing only C), since peptides (providing N and C) were no longer available.

During the flow recovery phase, the microbial community rapidly recovered, suggesting the use of refuges and/or adaptation to desiccation during the previous drought period. The scouring during rewetting was responsible for the mobilization of the streambed and loss of benthic material, and the increase in high quality organic matter in transport (at that moment, polysaccharides and amino acids accounted for 30% of the total DOC). The dynamics of progressive and gradual drought effects, as well as the fast recover after rewetting, might be affected by the interaction of the individual dynamics of each benthic substratum: sand sediments and leaves providing refuge for microorganisms and organic matter storage, while on cobbles an active bacterial community is developed in the rewetting. Since global climate change may favour a higher intensity and frequency of droughts in streams, understanding the effects of these disturbances on the materials and biota could contribute to reliable resource management. The maintenance of benthic substrata heterogeneity within the stream may be important for stream recovery after droughts.

Keywords: Mediterranean stream, drought, organic matter, lipids, polysaccharides, amino acids, extracellular enzyme activities, benthic substrata.

INTRODUCTION

Both natural and anthropogenic factors regulate water intermittency (Lehner et al., 2006) and water flow alterations. Climate and global change will produce shifts in the precipitation and discharge patterns of rivers and streams in temperate regions (Schröter et al., 2005). These are expected to cause an increase in the frequency and intensity of floods and droughts (Arnell et al., 1996), and a substantial change in organic matter accumulation and processing (Lake, 2000; Acuña et al., 2007). Mediterranean stream ecosystems are characterized by a high hydrological variability (Acuña et al., 2005). This variability is likely to increase (Mariotti et al., 2002), determining the effects of extreme hydrological episodes on the biochemistry of organic matter (OM) and on carbon cycling in streams. This effect is expected to be increasingly relevant, not only in Mediterranean streams but also in many other climates (Sabater & Tockner, 2010).

The transport and recycling of OM are two major river ecosystem functions (Fisher & Likens, 1973; Cummins, 1974), in which the velocity and efficiency of OM processing, microbial use, and biogeochemical transformations are highly dependent on river hydrology (Butturini et al., 2003; Acuña et al., 2005). All these processes can be affected during the transitions from wet to dry (summer drought) and dry to wet (flood or rewetting events) conditions that occur under water intermittency. Mediterranean streams are physically, chemically and biologically shaped by predictable floods and droughts through the annual cycle (Gasith & Resh, 1999). Nowadays, the influence of floods and droughts on river and stream catchments have been relatively well studied (e.g. Fisher & Grimm, 1991; Poff et al., 1997; Caramujo et al., 2008), but little is known about their effects on the OM.

Drought severity determines the degree of loss of the hydrological connectivity, which is often sequential during the drought period (Butturini et al., 2003). In the final stages of the drying process, the fluvial network may be converted into a fragmented landscape of isolated water pools where sediments and organic detritus accumulate and cannot be exported (Lake, 2003). As a result, the drying process is gradual in time and heterogeneous in space. Carbon limitation for the microorganisms during these dry periods benefits autotrophic production (Humphries & Baldwin, 2003). The leaf fall dynamics are also affected by flow cessation. In dry years a longer leaf fall period is related to hydric stress, causing progressive accumulation of OM in the streambed (Sabater et al., 2001; Acuña et al., 2007). Plausibly, the quality of materials in transport after the first rains is affected by processes occurring in the OM accumulated in dry conditions (Langhans & Tockner, 2006). Photodegradation is one of these processes since solar radiation causes chemical oxidation reactions in the OM accumulated in the

streambed (Wetzel et al., 1995; Moran & Zepp, 1997). The importance of photochemical reactions to the microbial communities in aquatic systems will depend on the sources of the dissolved organic matter (DOM) and on its initial bioavailability (Howitt et al., 2008).

Severe drought periods can be followed by intense rainfall episodes (punctuated changes), often leading to floods, and therefore to the mobility of materials downstream and between compartments. Infiltration/exfiltration processes at the surface as well as in the groundwater affect the accumulation and biochemistry of OM (Dahm et al., 2003). Nitrate mobilization occurs in the transition from dry to wet interfaces (Butturini et al., 2003), and dissolved organic carbon (DOC) drastically changes in content and composition during rewetting (Vázquez et al., 2007). Since the mobilized DOM is a heterogeneous mixture of carbohydrates, proteins, lignins, organic acids, and humic substances (Thurman, 1985), the relative increase in biodegradable DOC (BDOC) increases microbial activity during rewetting (Romaní et al., 2006). A high amount of carbon is derived from floodplain sources in arid rivers after flooding, having a significant impact on river productivity (Burford et al., 2008). Microbial activity may positively correlate with sediment moisture content and respond to the existence of senescent algae, which can restrain water loss from surface sediments in the moist habitat (Claret & Boulton, 2003). Thus persistence of microbial communities in drought conditions may be dependent on available wet refuges in the ecosystem (Amalfitano et al., 2008).

This study aimed to characterize the changes in benthic OM (quantity and quality) during the transition from wet to dry and dry to wet conditions in an intermittent Mediterranean stream. We hypothesized that abrupt water flow alterations (water disappearance and return) in headwater streams will impact on the OM concentration and composition in the flowing water as well as in the benthic substrata. A different effect of drought and rewetting was expected to occur on the biofilm benthic substrata of the stream (cobbles and sand) and on leaf material due to their specific mobility and sensitivity to flow regime and specific autotrophic/heterotrophic microbial community associated with different substrata. Both rates and direction of temporal changes in OM content and composition in the flowing water should differ during drought, with gradual changes expected, versus during rewetting when pulsed changes are predicted; and temporal changes should vary among habitat types. Therefore, special attention has been given within and between habitat types during the pre- and post-drought periods.

METHODS

Study site

Fuirosos is a third-order stream located in Montnegre-Corredor Natural Park, a forested range close to the Mediterranean Sea (50 km north of Barcelona, NE Spain). The climate is typically Mediterranean; precipitation is distributed irregularly, and mostly falls in autumn and spring with occasional summer storms. A long dry period (2-3 months) in summer is followed by a short but intense (80L m^{-2} $month^{-1}$; Acuña et al., 2007) stream recharge period in late summer–early autumn.

The studied stream reach is 3–5 m wide and 10 m in length. The riparian vegetation is made up of alder (*Alnus glutinosa*), hazelnut (*Corylus avellana*), poplar (*Populus nigra*) and plane trees (*Platanus acerifolia*). Most of the leaf input into the river channel occurs in summer due to hydric stress, and from autumn (especially *Alnus* leaves) to late winter (especially *Platanus* leaves). The DOC concentrations in the stream water during basal and storm discharge conditions range between 2 and 4 mg L^{-1} and 5 and 10 mg L^{-1} respectively (Vázquez et al., 2007). The DOC concentration can increase up to 10–20 mg L^{-1} during the hydrological transition between the dry and wet periods (Butturini et al., 2008). The stream channel morphology of the studied reach included a riffle (with boulders and cobbles) and a large pool (with accumulated leaf material and sand). The superficial water flow was progressively interrupted and the hydrological connectivity between the stream habitats was lost since June 19, 2006 and remained completely dry until September 13, 2006 (Fig. 1). The pool (maximum depth of 45 cm) was the last stretch to dry out completely.

Sampling strategy

The first sampling period was before drought (pre-drought period) and the second one after drought (post-drought period). The pre-drought period (with a total of six sampling occasions) started on May 8, 2006, when the stream flow was at basal levels (7.7 L s^{-1}). During this period the stream discharge decreased progressively until June 19, 2006, when there was no surface water. At that time two samplings were done for benthic material. The post-drought period with six sampling occasions started on September 13, 2006 when water flow started after two months of drought. The post-drought period ended on December 4, 2006, when basal hydrological conditions were resumed (Fig.1). Four transects (3 m apart) were defined in the studied reach, and the relative cover (%) of each streambed substratum was identified every 20 cm in each sampling occasion. Dissolved oxygen, water temperature (Hach DO meters) and conductivity (WTW conductivity meter) were measured in the field.

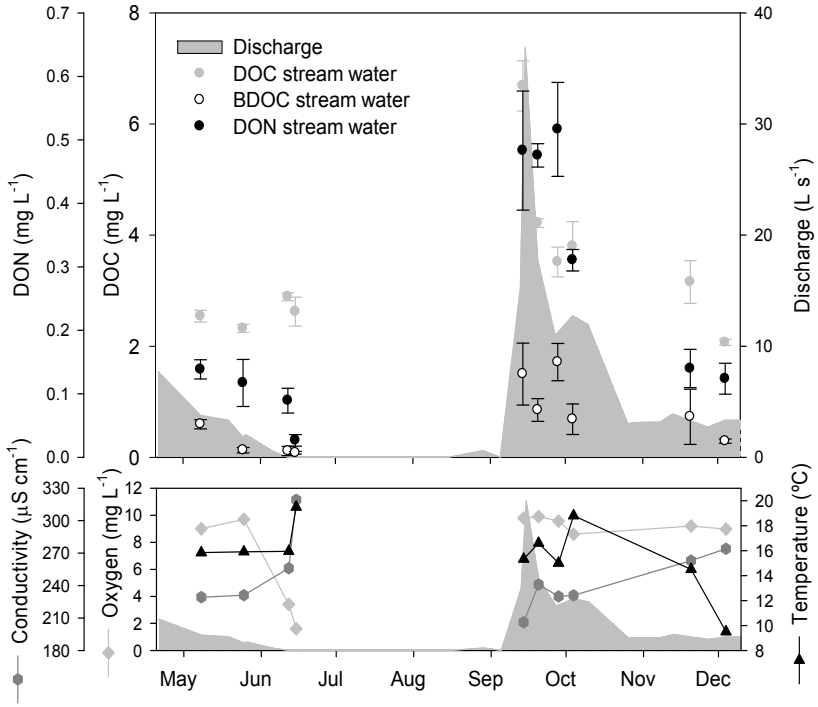


Fig.1. Temporal variations in discharge dissolved organic carbon (DOC) and dissolved organic nitrogen (DON) in the Fuirosos stream from May to December 2006. The lower graph corresponds to the temporal variation in temperature, conductivity and oxygen during the same period. Each sampling campaign is represented in a temporal scale; each dot corresponds to one sampling day (the 2 sampling campaigns done when there was no surface water were not represented).

Organic matter (quantity and quality) was analyzed in three types of benthic accumulated material, leaves and particulate material, biofilms growing on rocks and cobbles, and biofilm and fine material accumulated in sand, as well as in the flowing water (dissolved and particulate fractions). On each sampling occasion, one sample of each benthic substratum type was randomly collected in the same site of each of the four transects. Sand and leaf materials were sampled by coring an area (4.3 cm^2) between 5–10 cm in depth; cobbles were taken directly from the streambed. Stream water (approx. 8 L) was also collected for analysis. Moreover, the water discharge was measured in each sampling day, and water samples to analyze DOC, BDOC and dissolved organic nitrogen (DON) were collected. All the samples were refrigerated and transported to the laboratory (1hour travel time). Once in the laboratory, water was immediately filtered through precombusted GF/F filters in order to separate the DOM from the particulate organic matter (POM). The organic matter from the different benthic substrata was detached by immersing the sand samples in 120 ml of distilled water and then sonicating them (3 min, Selecta sonication bath at 40 W and 40 kHz). Leaf material was also immersed in 120 ml distilled water, sonicated (3 min, Selecta sonication bath at 40 W and 40 kHz) and then homogenized with a mixer (kitchen mixer). Leaf material, therefore, included both the leaves as well as their microbial colonizers. Cobbles were immersed in 60 ml of distilled water, scraped with a toothbrush and sonicated (3 min, Selecta sonication bath at 40 W and 40 kHz) to

obtain the epilithic material. The extracted and homogenized benthic material (which was separated into subsamples) and the particulate and dissolved water fractions, were analyzed for chlorophyll (excluding the water fractions) and bacterial biomass; polysaccharide, lipid and protein content; and extracellular enzyme activities.

DOC, BDOC and DON analysis

Water samples to determine DOC, BDOC and DON were filtered (precombusted GF/F glass-fibre filters; Whatman) before analysis. DOC and total nitrogen (TN) concentrations were determined using a Shimadzu TOC-VCS with a coupled TN analyzer unit. The BDOC was measured (Servais et al., 1989) in samples incubated for 28 days at room temperature in the dark. Glassware was previously heated at 450 ºC for 4 h to ensure complete organic carbon release. All DOC samples were acidified with 2M HCl (2%) and preserved at 4 ºC until analysis.

DON was determined as the difference between the total nitrogen and the total inorganic forms (the sum of nitrate, nitrite and ammonia). Inorganic forms were determined colorimetrically using a Technicon autoanalyzer. Nitrate was determined by the Griess-Ilosvay method (Keeney & Nelson, 1982) after reduction by percolation through a copperised cadmium column. Ammonia was determined after oxidation by salicylate using sodium nitroprusside as the catalyst (Hach, 1992).

Microbial biomass and extracellular enzyme activities in water and benthic material

Benthic chlorophyll concentration. Chlorophyll *a* was measured in sand and cobbles biofilms and in leaf material (four replicates). For each sample, between 5–10 ml of the extracted and homogenized benthic material were filtered (GF/C Whatman) and then chlorophyll was extracted in 90% acetone for 12 h in the dark at 4 ºC. Samples were further sonicated (2 min, Selecta sonication bath at 40 W and 40 kHz) to ensure complete chlorophyll extraction. After filtration (GF/C Whatman) of the extract, the chlorophyll concentration was determined spectrophotometrically (Lambda UV/VIS spectrophotometer, Hitachi) following Jeffrey and Humphrey (1975).

Bacterial density. Bacterial density was estimated in sand and cobbles biofilms, in leaf material and in water samples (four replicates per substratum). Live and dead bacteria were counted using the Live/Dead Baclight bacterial viability kit, which contains a mixture of SYTO® 9 and propidium iodide. The live cells (with intact cell membranes) appeared green after excitation with blue light, whereas dead cells (with damaged cell membranes) appeared red (Freese et al., 2006). Fifty microliters of the extracted

benthic material of the sand samples were diluted in 2 ml of sterilized river water. Aliquots of 200 µl of cobbles and leaves extracts were also diluted with 2 ml of sterilized river water. For water samples 2 ml were taken directly (no dilution). After appropriate dilution, a 1:1 mixture of SYTO® 9 and propidium iodide was added (3 µl), and samples were incubated for 15 min. Samples were then filtered through 0.2 µm black polycarbonate filters (Nucleopore, Whatman). The filters were dried, placed on a slide with mounting oil and examined by epifluorescence microscopy (Nikon E600). At least 20 random fields were examined on each slide for a minimum of 300 bacteria cells as a compromise between observational effort and reliability. The fraction of live bacteria was calculated as the abundance of live cells divided by the total count obtained with the Live/Dead method.

Extracellular enzyme activities. Sand and cobbles biofilms, leaf material and water samples (POM and DOM, four replicates) were analyzed to determine the activity of the enzymes β-D-1,4-glucosidase (EC 3.2.1.21), lipase (EC 3.1.1.3) and leucine-aminopeptidase (EC 3.4.11.1). Each sample from sand, cobbles and leaves consisted of 1 ml of the extracted and homogenized benthic material. Four ml of river water and GF/F filtered river water were considered respectively for the total and dissolved activity in water. Extracellular enzyme activities were determined spectrofluorometrically using the artificial substrates 4-methylumbelliferyl-β-D-glucopyranoside for β-glucosidase, 4-methylumbelliferyl palmitate for lipase activity and L-leucine-7-amido-4-methylcoumarin hydrochloride for peptidase activity (Sigma-Aldrich), as the respective substrate-analogues.

All the samples were incubated with 0.3 mM substrate (saturated conditions; Romaní & Sabater, 2001) in the dark under continuous shaking for 1 h at 18 ºC. Blanks and standards of MUF (methylumbelliferone) and AMC (aminomethylcoumarin) were also incubated. At the end of the incubation glycine buffer (pH 10.4) was added (1/1 vol/vol), and the fluorescence was measured at 365/455 nm excitation/emission for MUF and 364/445 nm excitation/emission for AMC.

Polysaccharide, protein and lipid content in water and benthic material

Polysaccharide, protein and lipid content was analyzed in water (dissolved and particulate fraction), in the biofilm benthic materials collected from sand and cobbles and in leaf material. Four replicates were considered for each sample type.

Polysaccharide content. Polysaccharide content was measured following the 3-methyl-2-benzothiazolinone hydrochloride (MBTH) method with modifications (Pakulski

& Benner, 1992; Chanudet & Filella, 2006). A total of 50 ml of DOM, filters for POM (Whatman GF/F), and 10 ml of the previously obtained extracted material from the samples (sand and cobbles biofilms; leaf material) were freeze-dried. The dried samples were then acidified with 1 ml of 12 M H_2SO_4 for 2 h at room temperature. Then, the samples were diluted with 4 ml Milli-Q water, sonicated (2 min) and hydrolyzed at 100 ºC for 3 h. After cooling, the pH of the hydrolysis solution was neutralized with NaOH. Next, monosaccharides were reduced to alditols by the addition of potassium borohydride. The reduction reaction was terminated by the addition of 2 M HCl. The samples were left overnight at 4 ºC. The following day, triplicate aliquots of hydrolysis products (and duplicate blanks) were placed in test tubes and were oxidized to formaldehyde by the addition of 0.025 M periodic acid. The oxidation reaction was terminated by the addition of 0.25 M sodium metaarsenite. After the addition of 2 M HCl, the aldehyde was reacted with MBTH reagent, ferric chloride solution and acetone. Absorbance was measured at 635 nm with a spectrophotometer (Spectronic® 20 Genesys). Absorbance of the blanks was subtracted from all samples. Glucose standard curves were generated concurrently.

Protein content. Amino acids were analyzed using High Performance Liquid Chromatography (HPLC). All samples were first freeze-dried (including 5 ml of DOM, filters for POM (Whatman GF/F), and 100 µl of the previously extracted material from sand and cobbles biofilms as well as from leaf material) and then hydrolyzed in sealed vials with 6 M HCl at 110 ºC for 20 h. After this step the remaining HCl was removed using nitrogen flushing steps. The residue was derivatized with a fluorescent reagent (AccQ Fluor reagent, Waters®) following the manufacturer's instructions, and then analyzed on a Waters HPLC amino acid analysis system. The HPLC system included a Waters AccQ Tag column for separation of amino acids, a Waters 2475 fluorescence detector, a 717plus auto-sampler and a 1525 Waters binary pump. An internal standard (α-aminobutyric acid) was added during the treatment of the samples and standards.

***Lipid content*.** Samples were first freeze-dried. Cobbles and sand biofilms as well as leaf material samples were weighed to the nearest 0.1 mg. Samples were homogenized with an ultrasonic homogenizer (200 W, 24 kHz; Hielscher Ultrasonics Gmbh, Teltow, Germany). The lipids were extracted with a mixture of chloroform and methanol (2:1) following Bligh & Dyer (1959). The total lipid content was analyzed by the colorimetric sulphophosphovanillin method (Zollner & Kirsch, 1962). The dissolved fraction could not be analyzed because the lipid content was below the detectable level (< 0.01 mgL^{-1}).

Statistical analyses

Differences in chlorophyll, bacterial density, enzyme activities and total polysaccharide, protein and lipid content within the pre-drought and the post-drought periods for the different substrata were analyzed using a 1-way repeated measures analysis of variance (RM-ANOVA). Probabilities within groups (Day and Day x S) were corrected for sphericity using the Greenhouse-Geisser correction. All probabilities were adjusted by the Dunn-Sidak correction. All variables included in the analyses were log(x+1) transformed in order to improve the homogeneity and heterogeneity of variance which was checked by visual inspection of the residues distribution.

Differences of bacterial density, total polysaccharide, protein and lipid content between the last day of the pre-drought period and the first day of the post-drought period were checked using multivariate analysis of variance (MANOVA).

Pearson correlation was performed to determine the potential relationships between the biogeochemical and biological variables studied. The relationships between the OM composition (proteins, polysaccharide and lipid content) of the benthic substrata and their enzyme activities were examined by regression analyses. All statistical analyses were carried out using the SPSS software package for Windows (Ver.14.0.1, SPSS Inc. 1989–2005).

RESULTS

Physical and chemical parameters

During the pre-drought period there was a progressive increase in the stream water conductivity (from 229 to 329 µS cm^{-1}). Dissolved oxygen decreased to very low values (1.6 mg O_2 L^{-1}). The maximum water temperature (19.5 ºC) was achieved in the pool immediately before stream water cessation (Fig.1).

The water flow peak on September 13^{th} (36.8 L s^{-1}; Fig. 1) was associated with an increase in DOC (6.7 mgL^{-1}), BDOC (1.5 mgL^{-1}) and DON (0.5 mgL^{-1}) in the stream water. DOC, BDOC and DON were positively correlated with water flow ($r=0.705$, $p<0.001$; $r=0.891$, $p<0.001$; $r=0.896$, $p<0.001$ respectively) throughout the studied period. During the high flow episode dissolved oxygen increased (9.8 mg O_2 L^{-1}) and conductivity returned to pre-drought basal values (206 µS cm^{-1}) (Fig. 1). Peak flow in September caused important changes in the structure of the stream bed. While the reach during the drought period consisted of a riffle (with cobbles and rocks covering 70 % of the streambed surface), a pool (with a large accumulation of sandy sediment, 80 %) and some patches of deposited leaves (5 %), the heavy rains homogenized the stream bed. After the high flow episode, the stream became dominated by cobbles and

rocks (70 %). Both the fine substrata (sand) and leaves were washed downstream after the episode.

Microbial biomass and metabolism

Benthic chlorophyll and organic matter. Benthic chlorophyll overall decreased significantly during the pre-drought period, but remained unchanged during the post-drought period (Table 1). During the pre-drought period, chlorophyll decreased in sand and cobbles biofilms, but remained steady on leaves (Table 1, Day x S interaction). Chlorophyll-*a* accounted for 182 ± 47.3 mg chl m^{-2} in sand and 23.5 ± 8.9 mg chl m^{-2} in cobbles during the pre-drought period. These values decreased respectively to 126 ± 39.7 and 5.8 ± 1.73 mg chl m^{-2} after drought. The chlorophyll content in leaves was most similar before and after the drought (1489 ± 825 and 1229 ± 388 mg chl m^{-2} respectively).

The dry weight per cm^2 of benthic materials was higher in the pre-drought than in the post-drought period. Sand had 1.27 ± 0.26 g cm^{-2} and cobbles had 0.03±0.008 g cm^{-2} in the pre-drought period, and 0.95 ± 0.26 and 0.01±0.008 g cm^{-2} respectively after drought. Similar dry weight values were obtained before and after drought (1.66 ± 1.16 and 1.69 ± 0.57 g cm^{-2} respectively) in leaves.

Source of variation	Chl	Total bacterial density	Life bacteria (%)	Polysaccharide content	Amino acid content	Lipid content	β-Glucosidase	Peptidase	Lipase
Pre-drought period									
Day	**0.032**	**<0.001**	0.134	**<0.001**	**<0.001**	0.594	**<0.001**	**<0.001**	**<0.001**
Substratum	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Day x S	**<0.001**	**<0.001**	**0.020**	**<0.001**	**<0.001**	**0.044**	**<0.001**	**<0.001**	0.430
Post-drought period									
Day	0.953	**0.017**	0.118	**0.002**	**<0.001**	0.527	**<0.001**	**0.002**	**<0.001**
Substratum	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.002**	**<0.001**	**<0.001**	0.359
Day x S	0.766	0.200	0.283	0.179	**0.047**	0.302	**<0.001**	**<0.001**	**0.005**

Table 1. Results of the repeated-measures analysis of variance considering one factor: Substratum type (cobbles and sand biofilms; and leaf material) for biofilm structure and composition, and activity variables. Probability within groups (Day and Day x S) are corrected for sphericity by the Greenhouse-Geisser correction. All probabilities are adjusted by the Dunn-Sidak correction. Values <0.05 are indicated in boldface type.

Bacterial density. Total bacteria and the percentage of live bacteria progressively decreased during the pre-drought period in the stream water (RM-ANOVA, Day effect, p=0.001) and in the cobble biofilms substrata, but increased in the sand biofilms up to the end of June (Table 1, Day x S interaction effects; Fig. 2). The first rains after the drought caused an instantaneous recovery of live bacterial density in water and cobbles (MANOVA, p<0.02).

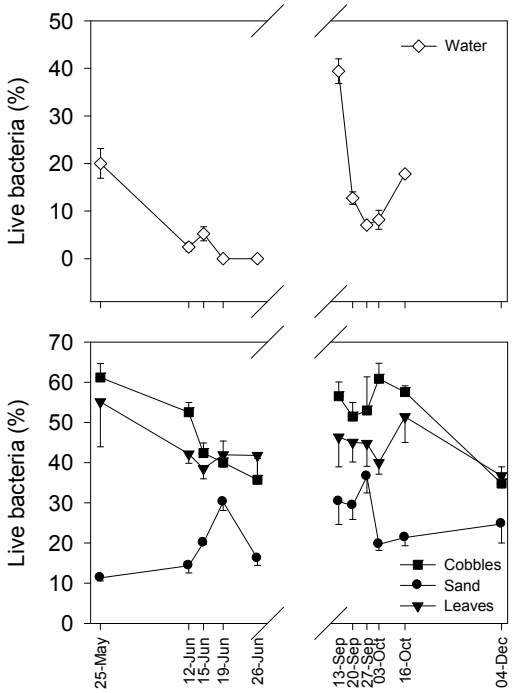


Fig. 2. Temporal dynamics of live bacteria (%) in water, cobbles and sand biofilms and leaf material in the Fuirosos stream, May 2006 to December 2006. On the x axis the exact sampling days are shown. Means ± standard errors (vertical bars), n=4.

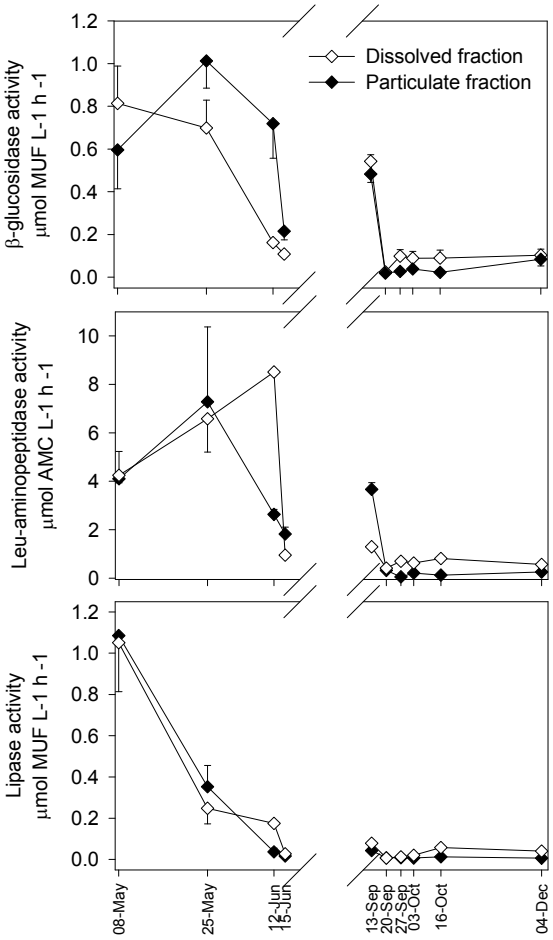


Fig. 3. Temporal changes in extracellular enzymatic activities in the stream water particulate fraction and dissolved fraction in the Fuirosos stream, May 2006 to December 2006. On the x axis the exact sampling days are shown. Means ± standard errors (vertical bars), n=4.

The total number of bacteria (live plus dead cells) was higher in leaf and sandy material ($3 \cdot 10^8$ bacteria cm^{-2}) than on cobbles biofilms ($5 \cdot 10^6$ bacteria cm^{-2}) both in the pre-drought and post-drought periods. However, the highest number of active bacteria was observed on cobbles biofilms, followed by leaf material, sand biofilms and finally in the stream water.

Extracellular enzyme activities. The extracellular enzyme activities measured in the dissolved water fraction followed a similar pattern than in the particulate fraction (Fig. 3). The extracellular enzyme activities progressively decreased when the stream dried

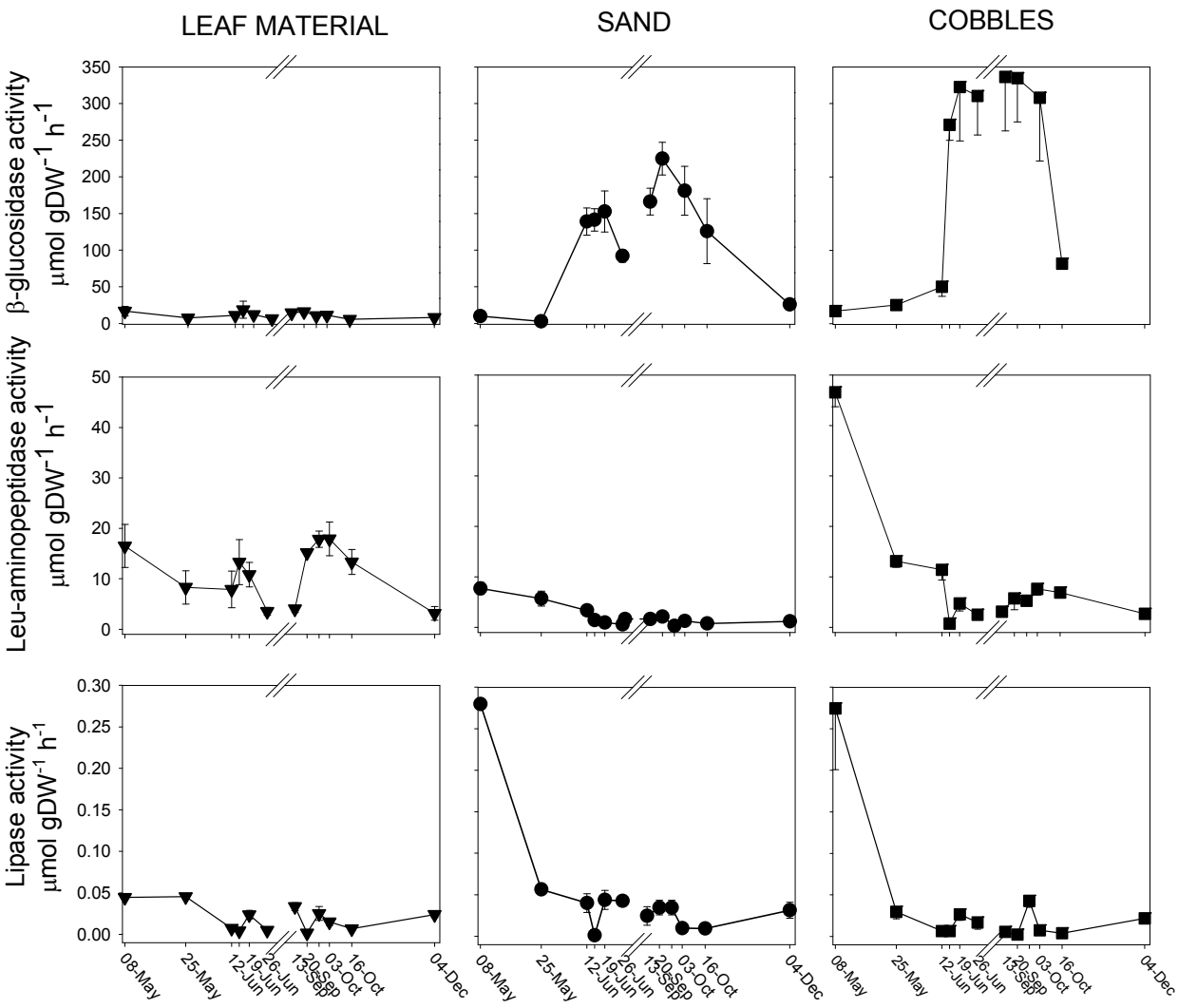


Fig. 4. Changes in extracellular enzyme activities (β-glucosidase, leucine amino peptidase and lipase) in leaf material, and in sand and cobble biofilms in the Fuirosos stream during the study period. On the x axis the exact sampling days are shown. Means ± standard errors (vertical bars), n=4.

out (RM-ANOVA, Day effect $p<0.001$). Enzymatic activities peaked after the rewetting, coinciding with that in the proportion of active bacteria (Fig. 2).

The enzyme activities in the benthic substrata (Fig. 4) followed similar patterns as those in the water except for β-glucosidase activity. Leucine aminopeptidase and lipase activities decreased during the drying process while β-glucosidase activity increased (Table 1, Day effect). After rewetting, enzyme activities recovered and were maintained at similar levels to those before drought. β-glucosidase activity before and after drought was the lowest in leaf material whereas peptidase activity had the lowest values in the sand biofilm (Table 1, Substratum effect; Fig. 4). Lipase activity behaviour was similar during the drought process for the three benthic substrata; however, after

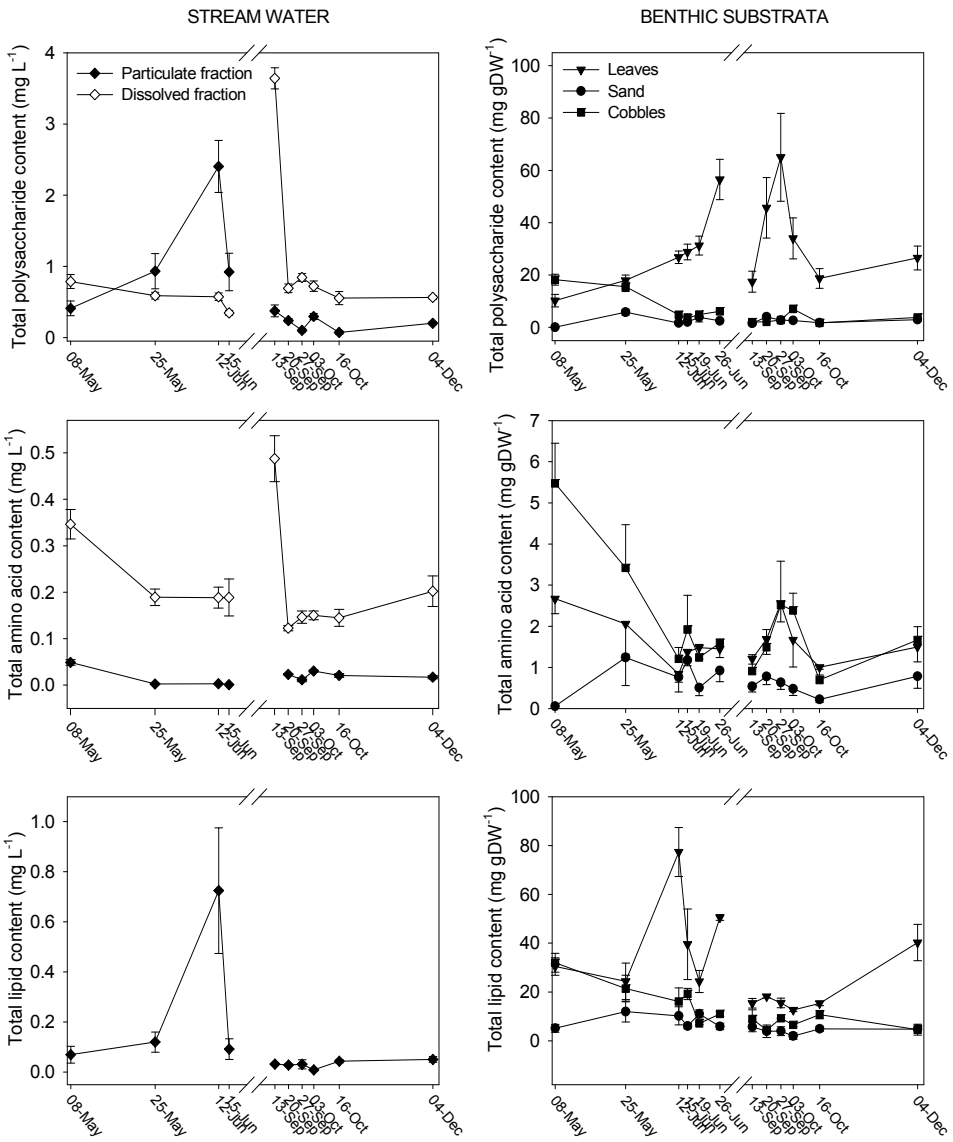


Fig. 5. Dynamics of the different components of organic matter (polysaccharides, amino acids and lipids) in the stream water (dissolved and particulate fraction) and in the different substrata (leaf material; sand and cobbles biofilms). Values are means of four replicates for each sampling date ± standard error. Results of polysaccharide content were expressed as glucose equivalents. In the x axis the exact sampling days are shown.

drought lipase activity recovered faster on leaves and sand biofilm (Table 1, Day x S effects; Fig. 4).

During the whole study period, β-glucosidase activity on the benthic substrata was the highest, followed by leucine aminopeptidase and then, with very low values by lipase. However, in the stream water leucine aminopeptidase activity was the highest followed by β-glucosidase and finally by lipase.

Organic matter composition

Water. Polysaccharides accumulated in the particulate water fraction while amino acids decreased (RM-ANOVA, Day effect, $p<0.005$; Fig. 5) in the pre-drought period. Total lipids in water also accumulated during the pre-drought period (RM-ANOVA, Day effect, $p<0.005$; Fig. 5). During the post-drought period, time differences in OM composition were not detected.

In the dissolved water fraction polysaccharide and amino acid content decreased (RM-ANOVA, Day effect, $p<0.005$; Fig. 5) during the pre-drought period. Dissolved polysaccharides reduced from 12% to a 5% of total DOC during the pre-drought period (Fig. 6). Similarly, total dissolved amino acids reduced from 5.6% to 3% of total DOC during the same period (Fig. 6).

A peak of polysaccharides and peptides in the dissolved water fraction occurred immediately after rewetting (Fig. 5). These peaks contributed to the immediate increases of DOC and DON (Fig. 1). At this rewetting moment, polysaccharides accounted for 20% of total DOC and amino acids were 3% of total DOC (Fig. 6). When basal water flow conditions were re-established (end of post-drought period), amino acids had increased up to 4% of total DOC (Fig.6).

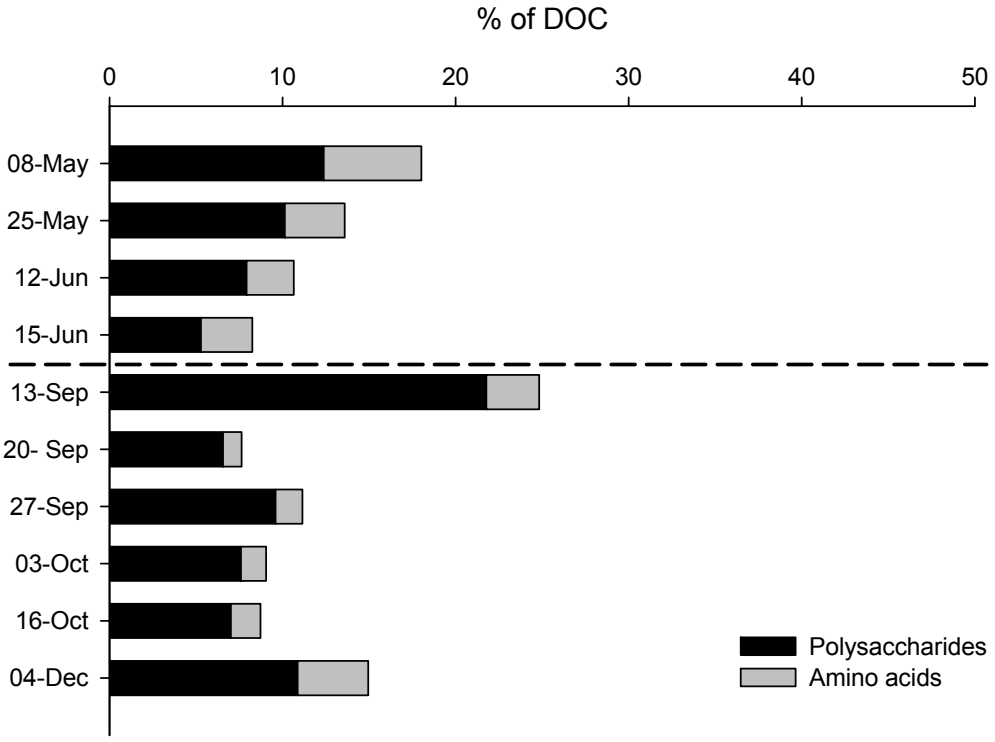


Fig. 6. Contribution of polysaccharides and amino acids (in units of C) to the total DOC. The dashed line divides the pre-drought period from the post-drought period.

Benthic substrata. Total polysaccharides, amino acid and lipid content per dry weight showed significant time differences as the stream dried out. The amino acid content (most notably) and the lipid and polysaccharide content decreased in sand and cobbles (Table 1, Day x S interaction; Fig. 5) in parallel with the progressive decrease in benthic chlorophyll content. However, polysaccharide and lipids increased on the leaf material.

In the post-drought period, differences between benthic substrata were evidenced by the highest values of polysaccharides (most remarkably) and lipids in leaves, as well as low amino acids in sand biofilms (Table 1, Substratum effect).

Total polysaccharides, amino acid and lipid content per dry weight on the benthic substrata were higher when the stream was dried out than at the rewetting (MANOVA, $p<0.05$).

Relationships between extracellular enzyme activities and organic matter

The extracellular enzyme activities in the stream water were higher when the available OM from the benthic substrata was also high. β-glucosidase in the stream water was correlated with the polysaccharide content in cobble biofilms ($r=0.674$, $p=0.033$), and lipase in the stream water was correlated with lipid content in cobble biofilms ($r=0.766$, $p=0.010$). Furthermore, in some moments, the available dissolved organic matter was also related to the water enzyme activities. In the post-drought period, β-glucosidase activity measured in the dissolved water fraction was correlated to the polysaccharide content in it ($r=0.978$, $p=0.001$), while peptidase activity in this same water fraction was associated with the amino acid availability in it ($r=0.847$, $p=0.033$).

The enzyme activities on the benthic substrata were significantly related to their OM composition. Peptidase activity on cobbles and sand biofilms and in leaf material were positively related with their amino acid content ($R^2=0.663$, $p<0.001$) according to a linear regression (Fig. 7). In contrast, β-glucosidase was negatively and exponentially related to substratum-associated polysaccharides ($R^2=0.147$, $p=0.021$; Fig.7), mainly showing that in the lower range of polysaccharide content (as it occurs in cobbles and sand), there can be high β-glucosidase activity which decreases rapidly with increasing polysaccharide content; and in the higher range (as it occurs in leaves) changes in polysaccharide content are not affecting the β-glucosidase activities which are very low (Fig. 7). No significant relationship was found between lipase and the respective lipid composition of benthos (Fig. 7).

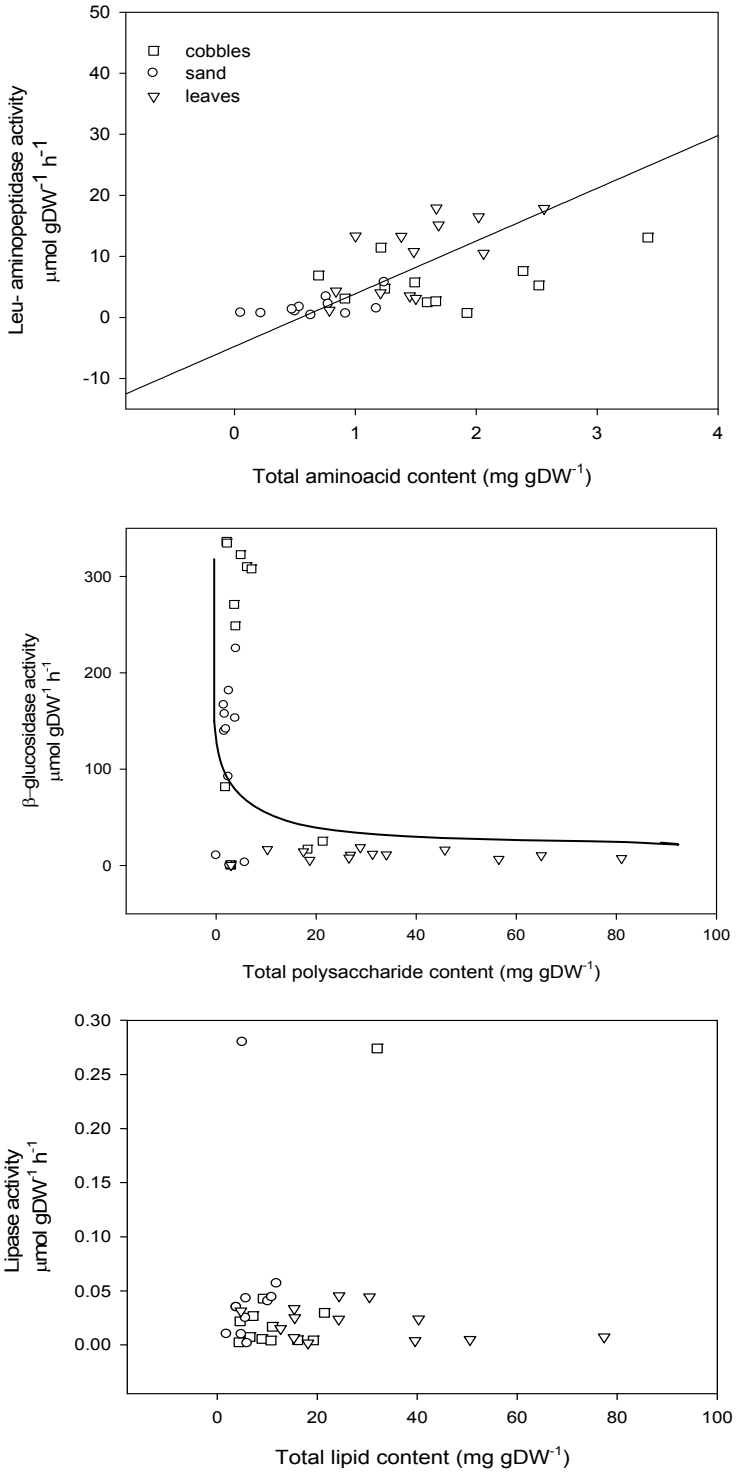


Fig. 7. Regression analyses between the organic matter composition (amino acid, polysaccharide and lipid content) of cobbles and sand biofilms as well as of leaf material and their enzyme activities (Leu-aminopeptidase, β-glucosidase and lipase).

DISCUSSION

Drought and rewetting caused mostly two linked effects in the Mediterranean forested stream: 1) changes in the microbial use of available organic matter, and 2) changes in downstream loss of organic matter dynamics.

Drought affected the availability of OM for microbial use as well as its food quality, in a differential way in the pre- and post-drought periods. Drought events in Mediterranean river ecosystems have a great effect not only on hydrological and biogeochemical conditions but also on OM availability, use and recycling (Sabater & Tockner, 2010). Droughts affect the biota and the stream ecosystem in a degree according to their duration and time of occurrence (Boulton, 2003; Lehner et al., 2006). As drying proceeds, shallow surface habitats such as riffles disappear first, and a series of fragmented pools remain together with a low water flow in the hyporheic zone (Lake, 2003). Increases in water flow may cause a complete resetting of the physical habitat, as well as the downstream drift of many individuals and materials. It has been observed that the biogeochemical response to water flow increases is closely linked to the previous hydrological "history" of the system (duration of drought) (Vázquez et al., 2007). Our observations during the drying and hydrological recovery of a Mediterranean stream indicate that the processes occurring during the post-drought period (the fast recovery of active bacteria and most extracellular enzyme activities; the transport of high quality OM) are related to those occurring during the pre-drought period.

In Mediterranean streams, the major drought period takes place in early to mid summer. Prior to this period, we expect the highest accumulation of benthic material derived from in-stream primary producers. In the Fuirosos, the optimal period for the development and growth of the biofilm communities is in the spring. The high availability of light and nutrients, as well as the steady hydrological conditions in that period, led to very high biofilm biomass as well as to high primary production and microbial metabolism (Ylla et al., 2007). High quality OM (mainly rich in peptides and polysaccharides) is therefore available to the stream food web during that period. The abundance of algal material, with equal contributions of amino acids and carbohydrates and lower amounts of lipids (Harvey & Mannino, 2001) probably explain the higher leucine aminopeptidase and β-glucosidase activity in the benthic substrata, as well as the lower lipase activity.

When discharge declines and the stream dries out, hydric stress on aquatic biota causes a gradual loss (faster in the last days with flowing water) of aquatic habitat, depletion of food resources and a decline in water quality. As the dry season progresses and water flow is even more reduced, habitat conditions become harsher (Gasith & Resh, 1999), transport of OM (detritus, leaves and plant material) and fine sediments declines and high quantities of OM are stored in pools (Cuffney & Wallace,

1989; Boulton & Lake, 1992; Wright & Symes, 1999). In addition, conductivity and water temperature rise, and low oxygen levels lead to facultative aerobic and anaerobic respiration.

In the Fuirosos these physical and biogeochemical changes were accompanied by variations in the quantity and quality of the OM and in its use by the microbial biota. During the drought process there was a progressive decrease of the polysaccharide, amino acid and lipid content in cobbles and sand biofilms. The loss of high quality OM during this period is striking, and is probably due to decaying organisms. In the final stages of the drought, when water ceased to flow, such effects were more substantial on the epilithic material than on the epipsammic and epixylic. At the same time, water was progressively enriched in its particulate fraction, with higher polysaccharide and lipid content, but lower peptide content. Besides, in the dissolved water fraction there was a reduction of polysaccharides and amino acid content. Altogether, the quality and biodegradability of materials in the dissolved fraction progressively decreased during the drought. Polysaccharide plus amino acid content decreased from 18% to 8% of total DOC throughout the drying process (Fig. 6). These changes in the quality of the OM were accompanied by changes in extracellular enzyme activities in the benthic substrata: leucine aminopeptidase and lipase activities decreased, and β-glucosidase activity increased during the drought period. Therefore, as drought progressed, stream microbial heterotrophs mostly used the lowest quality organic matter (polysaccharides, providing only C), since peptides (providing N and C) were no longer available. At the same time, the expression of extracellular enzyme activities was modulated by the nature of available OM in each substratum (Artigas et al., 2008). This was evidenced by the positive relationship between amino acid content of benthic substrata and leucine-aminopeptidase activity (Fig. 7). However, the opposite relationship was found for the β-glucosidase activity and polysaccharide content, eventually resulting in no relationship between high polysaccharide content and β-glucosidase. This might be related to the use of the available polysaccharides from the particulate water fraction during drought, when polysaccharides in cobbles and sand biofilms decrease.

The harsh conditions for the microbial community during the drying of the stream caused a reduction of both algae (reduction in chlorophyll) and active bacteria. Lower algal density during drought is related to cells breaking under desiccation (Usher & Blinn, 1990; Peterson et al., 2001; Stanley et al., 2004). Hydric stress is also lethal to bacterial cells by damaging membranes, proteins and nucleic acids (Billi & Potts, 2002). Further, the decrease in DOC inputs during drought may lead to C limitation, and consequently lower heterotrophic production (Humphries & Baldwin, 2003). Harsh

conditions were more evident on cobbles biofilms than on sand biofilms and leaf material, where moist conditions could be more easily maintained, providing a place in which aquatic bacterial communities could survive (Amalfitano et al., 2008).

The return of water to a previously dry streambed can be seen as a "hot biogeochemical moment" (McClain et al., 2003). Biogeochemical reactions restart or accelerate after long quiescent periods. Aerobic penetration increases in previously dry sediments as once anaerobic zones become aerobic (Baldwin & Mitchell, 2000). Re-wetted sediments liberate phosphorus and nitrogen, as a consequence of death-induced microbial cell lysis, which again may enhance instream primary productivity. Such enormous changes were recorded in the Fuirosos when the first rains occurred in autumn. The September flow peak basically had two effects: the cleaning of the streambed and the downstream transport of materials, and the simultaneous import of OM from upstream. This flow moved and redistributed streambed materials, including the OM accumulated on the streambed during drought. This OM was mostly made up of allochthonous (terrestrial) material coming from the decaying leaves and plant litter. This leaf material produce a fresh and biodegradable high quality lixiviates (Francis & Sheldon, 2002). There is also a noticeable fraction of autochthonous material derived from algal origin. The cleaning effect of the flow peak reduced the total amount of dry weight per cm^2 and reduced the polysaccharide, peptide and amino acid content of the benthic materials. There was a mobilization of OM downstream, and high polysaccharide and amino acid concentrations were detected, especially in the dissolved water fraction. At that moment, these high polysaccharides and amino acids concentrations accounted for 30% of the total DOC, indicating a high loading of high quality OM. The high DOC, BDOC and DON concentrations after rewetting revealed the remarkable transport of dissolved C and N compounds following the first important rains.

The high availability of OM during the peak flow was related to the high metabolic activities (extracellular enzyme activities and active bacteria) recorded in the stream water. This initial response after rewetting lasted for a week, and then values returned to basal levels. Bioavailability of materials returns to basal flow levels at the end of the flow peak (Stepanauskas et al., 2000). During the flow peak there is therefore fast and efficient microbial use of the available labile fresh material transported during the rewetting period (Romaní et al., 2006).

In spite of the substantial losses of benthic material during the peak flow, microbial activity recovered immediately after rewetting. The existence of refuges in the stream (for instance in the hyporheic), as well as the capacity of the biota to recover from droughts once these have finished, may explain the fast recovery. Robson (2000)

determined that the presence of dry residual biofilms on rocks enhanced recovery and strongly influenced community development. Cobbles, litter, and coarse woody debris in the dry streambed could be used as refuges for microbial organisms (Bond et al., 2008). The physiological plasticity of some taxa makes them able to withstand extreme desiccation and recover rapidly (Stanley et al., 2004). For example, desiccated cyanobacterial mats, started to photosynthesize in the laboratory within 2 h after rewetting (Romaní & Sabater, 1997). The excretion of extracellular polysaccharides by algae and cyanobacteria facilitates cellular water retention in the biofilm, and makes rapid rehydration possible. In our study, algae in sand and leaves were more resilient to the drought than algae on cobble.

In conclusion the dynamics of progressive and gradual drought effects, as well as the fast and punctuated recover after rewetting, might be affected by the interaction of the individual dynamics of each benthic substratum. As an example, while sediments provide refuge during desiccation, recovery of living bacteria and algal biomass is faster on cobbles; leaf material serves as a refuge and as a source of OM during the whole period. Since global climate change may favour a higher intensity and frequency of droughts in streams (Arnell et al., 1996), understanding the effects of these disturbances on the materials and biota will contribute to reliable resource management (Lake, 2003). The maintenance of benthic substrata heterogeneity within the stream may increase resilience of stream ecosystem processes with increasing frequency and duration of drought-rewetting disturbances.

ACKNOWLEDGEMENTS

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CHAPTER II

ORGANIC MATTER CHARACTERISTICS IN A MEDITERRANEAN STREAM THROUGH AMINO ACID COMPOSITION: CHANGES DRIVEN BY INTERMITTENCY

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Submitted to Aquatic Sciences

ABSTRACT

The amino acid composition (quality) and abundance (quantity) of organic matter (OM) in an intermittent Mediterranean stream were followed during transitions from wet to dry and dry to wet conditions. Amino acids were analyzed in benthic material (epilithic biofilms, sediments, leaf material) as well as in the flowing water (dissolved organic matter-DOM). A principal component analysis as well as the estimation of the amino acid degradation index (DI) allowed to differentiate patterns of amino acid composition and quality during the wet-drought-wet conditions. The amino acid content and composition analyzed were dependent on the source of OM as well as on its diagenetic state. The highest-quality OM (high DI and high N content) occurred on epilithic biofilms and the most degraded and lowest-quality OM occurred in sediments. Differences between the pre- and post-drought periods were mostly evident in DOM; autochthonous-derived material prevailed during the pre-drought, while allochthonous inputs dominated during the post-drought period. In contrast, the steadier composition of the benthic compartments indicates the in situ origin of OM, which quality progressively decreased throughout the drought period. While in the benthic material, drought-rewetting episodes determine subtle changes in OM quality, river DOM degradation is highly driven by water intermittency.

Keywords: amino acids, biofilms, intermittency, Mediterranean stream, degradation index, drought periods.

INTRODUCTION

Ubiquitous in natural bodies of water, organic matter (OM) is a main source of C and N in many freshwater ecosystems (Fellman et al. 2009) as well as a major component of global carbon cycles (Porcal et al. 2009). OM in aquatic systems plays a main role in food webs (Thingstad 2003), regulates the bioavailability of dissolved nutrients and metals (Dillon and Molot 2005; Shiller et al. 2006) and affects the optical properties of natural waters (Retamal et al. 2007). In streams, OM can accumulate in the benthic compartments (e.g., biofilms growing on cobbles, wood, leaves or detritus) or be transported by the flow of the stream. Biofilms play a key role in OM processing because they retain, decompose and transform OM from the water column while also producing additional OM. Bacteria are the main decomposers of both particulate and dissolved OM (POM and DOM, respectively; Meyer 1994; Amon and Benner 1996; Amon et al. 2001).

The OM that occurs in stream ecosystems is a heterogeneous complex mixture of carbohydrates, proteins, lipids, lignins, organic acids, and other less well-characterized compounds, such as humic substances (Thurman 1985). OM reactivity is largely dependent on its composition, which is controlled in part by source materials (Volk et al. 1997; Findlay and Sinsabaugh 1999). Local soil and plant litter inputs (France et al. 1996; Dalzell et al. 2005) as well as autochthonous material from instream primary producers (Webster and Meyer 1997) contribute to stream DOM. Its arrival to the stream and degradation dynamics is related to local rainstorm events in the watershed (Da Cunha et al. 2001), but also to other hydrological episodes such as droughts and water flow resumption. The source and diagenetic state of riverine OM are important factors determining its ultimate fate. Chemical biomarkers such as lignins, phenols, amino acids, sugars and fatty acids are clues for identifying the sources and diagenetic pathways of OM (Dauwe and Middelburg 1998; Amon and Benner 2003). Of the many potentially informative biomolecules, carbohydrates and amino acids are dominant components of cells. Since proteins are ubiquitous components of all source organisms and degradation mixtures (Cowie and Hedges 1992), characterizing the composition of amino acids provide valuable insight into the origins and reactivities of OM in complex mixtures (Dauwe and Middelbourg 1998; Dauwe et al. 1999; Chen et al. 1999; Wu et al. 2003). Amino acids, which are found in proteins, polypeptides, and combined or free amino acids, are a major form of organic nitrogen and amongst the most labile fractions of bulk OM (Spitzy and Ittekkot 1991). Their degradation supports microbial production and enlarges the pools of mineralized NO_3, NO_2 and NH_4 (Stepanauskas et al. 1999). The amino acid composition has been extensively studied in bulk DOM and POM in marine environments (Mannino and Harvey 2000; Amon et al. 2001; Davis and Benner 2005), but only a few studies have focused on comparisons between the particulate and dissolved fractions in fresh water (Hedges et al. 2000; Wu et al. 2003; Duan and Bianchi 2007; Tremblay and Benner 2009). The present study uses detailed data on amino acid composition in freshwater benthic organic matter and dissolved organic matter to comprehend the diagenetic processes occurring in the stream during intermittency.

Mediterranean stream ecosystems show high hydrological variability (Acuña et al. 2005) featuring extreme hydrological episodes (droughts and floods). Mediterranean streams are physically, chemically and biologically shaped by these episodes (Gasith and Resh 1999). In particular, the transport and degradation of OM in Mediterranean streams can be affected during the transitions from wet to dry (drought) and dry to wet (flood or rewetting events) conditions (Ylla et al. 2010). The quality of the materials transported after the first rains is affected by processes occurring in the accumulated

dry OM, and dissolved organic carbon (DOC) drastically changes in composition during rewetting (Butturini et al. 2003, Vázquez et al. 2007). The mobilized DOM increases biodegradable DOC (BDOC) content and microbial activity during rewetting (Romaní et al. 2006). On the other hand, OM degradation and microbial activity during drought is favoured by sediment moisture (Amalfitano et al. 2008) and responds to the existence of senescent algae that restrain water loss from surface sediments (Claret and Boulton 2003).

The amino acids' abundance and composition during these biogeochemically relevant periods ("hot moments"; McClain et al. 2003) could be helpful for understanding the sources and biogeochemical cycling of labile OM in rivers (Thomas 1997). In this study, we analyze the amino acid composition and degradation of all OM sources in a stream reach during a typical Mediterranean drought disturbance and the subsequent water recovery. The guiding hypothesis of this study is that intermittency is a driving factor of OM decomposition. Our prediction is that OM will be degraded during the water period (pre-drought) and after water resumption OM will mobilize providing high quality OM to the recovering benthic biofilms. The main objectives of the study were 1) to compare amino acid composition (quality) and abundance (quantity) in the stream during the drying and rewetting in the different OM pools and 2) to determine if changes in dissolved and benthic amino acid compositions are representative of the diagenetic state and quality of the OM during the pre- and post-drought phases.

METHODS

Stream water samples and materials from benthic habitats (e.g., cobbles, sand sediment, decaying leaf materials) were collected from the Fuirosos stream (third order). The Fuirosos is situated in the Montnegre-Corredor Natural Park (50 km north of Barcelona, NE Spain). The area has a Mediterranean climate, with precipitation mostly in autumn and spring and occasional summer storms. Most of the years, the superficial water flow progressively decreases during summer, and the hydrological connectivity between the stream habitats is eventually interrupted.

The sample collection took place during the drought and rewetting periods occurring in spring-summer in the stream. The physical and chemical characteristics of the stream water (i.e., dissolved oxygen, water temperature and conductivity), the DOC and dissolved organic nitrogen (DON), and the fraction of BDOC differed between the drought and rewetting periods (Ylla et al. 2010). DOC and total nitrogen concentrations were determined using a Shimadzu TOC-VCS with a coupled total nitrogen analyzer unit. DON was determined as the difference between the total nitrogen and the total inorganic forms (the sum of nitrate, nitrite, and ammonia).

The pre-drought period started on 8 May 2006, when the stream flow was at basal level (7.7 L s^{-1}), and finished on 26 June 2006, when the stream had dried up completely. The stream remained completely dry nearly three months until 13 September 2006, when the water flow resumed in the stream (rewetting). The considered post-drought period extended until 4 December 2006, when basal hydrological conditions after autumnal rains occurred. A total of six samplings were carried out during the pre-drought period. The last two were done just after the stream had dried out completely, when benthic material was only present (no flowing water, but the subsurface layers were still wet). Six more samplings were scheduled after the water flow resumed. The reach during the drought period consisted of a riffle (cobbles and rocks covering 70% of the streambed surface), a pool (with a large accumulation of sandy sediment, 80%) and some patches of deposited leaves (5%). The autumnal heavy rains homogenized the stream bed, and the stream became dominated by cobbles and rocks (70%); both the fine substrata (sand) and leaves were mostly washed downstream after the episode (Ylla et al. 2010).

The amino acids composition and abundance was analyzed both in the benthic material (leaf material, biofilms growing on rocks and cobbles, biofilm and fine sediment material), as well as in the flowing water (DOM). Four replicates were randomly collected at each sampling. Sand and leaf samples were obtained from the streambed by coring a relevant area (4.3 cm^2). Sediment samples were obtained from 0–5 cm depth. Cobbles were collected directly from the streambed. Stream water (approx. 8 L) was also collected for analysis. Water was immediately filtered once in the laboratory through precombusted GF/F filters (0.7 μm nominal porosity) to separate the DOM from the POM. The OM from the cobbles and sand was detached by immersion in distilled water and sonication (3 min, Selecta sonication bath at 40 W and 40 kHz). Leaf material was not separated from the biofilm growing on it, and it was homogenized with a mixer for analysis (Ylla et al., 2010).

Amino acid content

The collected benthic material and filtered water samples were freeze-dried and then hydrolyzed in sealed vials with 6 M HCl at 110ºC for 20 h. The samples analyzed were 5 ml for DOM and 100 μl of material extracted from sand, leaves and cobbles. The HCl remaining after hydrolysis was removed by a nitrogen flush. The residue was derivatized with a fluorescent reagent (AccQ Fluor reagent, Waters®) according to the manufacturer's instructions. After these preparation steps, all of the derivatized samples were filtered using centrifuge filters (micro-spins, 0.45 μm) to remove particles and prevent problems with the HPLC column. Afterwards, amino acids were analyzed

using High Performance Liquid Chromatography (HPLC, Waters). The HPLC system included a Waters AccQ Tag column for separation of amino acids, a Waters 2475 fluorescence detector (excitation wavelength 250 nm; emission wavelength 395 nm; gain 10), a 717 plus auto-sampler and a 1525 Waters binary pump. The eluents were acetate-phosphate buffer (Waters® AccQ·Tag eluent A concentrate) and 60% acetonitrile HPLC grade. The gradient profile was provided in the manufacturer's instructions. The run time for each sample was 50 minutes. The injection volumes were 5 µl for the benthic samples and 10 µl for the water samples. Content of each amino acid in the analyzed samples was calculated in moles (%), in µg g DW^{-1} and µg L^{-1} for amino acids in DOM.

The validity of the method was checked by the addition of an internal standard (50 pmol of α-aminobutyric acid), which was recovered nearly 100% (50 pmol ± 5 for DOM and 50 pmol ± 10 for benthic samples) during the treatment and analysis of the standards and samples. Amino acids were identified by retention times and quantified by comparison between standard and sample peaks. The amino acid standards came from a hydrolyzed protein that contained a mixture of 17 primary amino acids (Table 1). All of them could be quantified except cysteine, due to analytical problems.

Amino acid classification

Amino acids were classified as essential or non-essential, as well as in terms of their functionality. The amino acids classified as essential were isoleucine, leucine, lysine, methionine, phenylalanine, threonine, valine, arginine and histidine. Essential amino acids cannot be synthesized de novo by the organism and must be supplied by the diet. Amino acid classification into functional groups (Dauwe and Middelburg 1998; Mannino and Harvey 2000; Wu et al. 2003) separated basic (histidine, arginine, lysine), acidic (aspartic acid, glutamic acid), hydroxylic (serine, threonine), neutral (glycine, alanine, proline, valine, isoleucine, leucine), sulfuric (methionine) and aromatic (tyrosine, phenylalanine) amino acids. The amount of each functional group as well as the percentage of essential versus non-essential amino acids were calculated for all water and benthic samples analyzed.

Degradation index

The amino acid degradation index (DI; Dauwe et al. 1999) was developed in biogeochemistry studies in marine sediments to determine the diagenetic state of OM. This index links the amino acid composition to the OM diagenesis and is used to quantify the quality of the OM based solely on its chemical composition. The index considers the whole suite of amino acids in the calculation and allows different samples

to be directly and quantitatively compared. The DI of our samples was calculated following the empirical formula of Dauwe et al. (1999) and the molar percentage of amino acids in the samples. Highly negative DI values are indicative of severely degraded samples, whereas positive DI values are indicative of fresh materials.

Data analysis and statistics

Differences between the pre-drought and post-drought periods in DI, essential amino acids and amino acid functional groups were analyzed for benthic materials and DOM using a one-way repeated measures analysis of variance (RM-ANOVA). Probabilities within groups (Day and Day x Substrata) were corrected for sphericity using the Greenhouse-Geisser correction. All probabilities were adjusted using the Dunn-Sidak correction. A post hoc multiple comparison test (Tukey HSD) was used to examine the differences between substrata.

The percentage of DOC represented by amino acids in units of C (AA-C/DOC) and the percentage of DON represented by amino acids in units of N (AA-N/DON) were characterized. AA-C/DOC was calculated as the hydrolysable amino acids in units of C (AA-C) in total DOC. Similarly, AA-N/DON was calculated as the hydrolysable amino acids in units of N (AA-N) in total DON (Fig. 6).

A Pearson correlation analysis was performed to determine the potential relationship between the DI and the percentage of N in amino acids (% AA-N, calculated as the sum of the hydrolysable amino acids in units of N divided by the total amount of amino acids in each sample). The SPSS software package for Windows (Version 14.0.1, SPSS Inc., 1989–2005) was utilized.

The amino acid composition of the stream compartments during drought and water recovery were examined by means of multivariate analyses. Data were based on the relative abundances (mol %) of amino acids in the four analyzed compartments (three benthic compartments and DOM) for the twelve sampling campaigns. An arcsine square root transformation was applied to normalize the variables. Amino acid data were first analyzed with detrended correspondence analysis (DCA; Hill and Gauch 1980) to determine the length of the gradient for the first two axes. DCA indicated that the maximum gradient length was shorter than 3 SD units (0.548). Therefore, the use of linear ordination techniques was appropriate (ter Braak and Smilauer 2002). Accordingly, PCA was performed using CANOCO version 4.5.

RESULTS

Amino acid composition

The concentration of amino acids in epilithic biofilms and sand sediments (µg gDW^{-1} and µg L^{-1} summarized in Table 1) were higher in the pre-drought than in the post-drought period. However, amino acid concentrations were similar in the two periods in the leaf material. The concentrations of all amino acids decreased throughout the rewetting, especially in epilithic biofilms, and peaked in DOM.

	EPILITHIC BIOFILM (µg gDW^{-1})			SAND SEDIMENT (µg gDW^{-1})			LEAVES (µg gDW^{-1})			WATER DISSOLVED (µg L^{-1})		
	PRE	REW	POST	PRE	REW	POST	PRE	REW	POST	PRE	REW	POST
ASP	160 (40)	70	140 (30)	70 (20)	50	50 (10)	100 (10)	90	120 (40)	8.44 (2.3)	10.53	10.20 (0.74)
SER	170 (40)	70	130 (20)	60 (10)	50	50 (10)	110 (10)	90	120 (40)	12.19 (2.23)	37.59	13.21 (1.35)
GLU	230 (60)	60	140 (30)	60 (10)	50	50 (10)	110 (20)	80	140 (60)	26.92 (3.12)	68.16	15.58 (4.04)
GLY	140 (40)	70	100 (30)	80 (20)	40	40 (10)	120 (10)	100	120 (60)	9.41 (1.54)	24.49	21.50 (2.34)
HIS	190 (90)	20	100 (50)	20 (0.0)	40	60 (20)	40 (10)	30	40 (10)	31.99 (5.67)	80.45	9.90 (5.12)
ARG	300 (90)	80	160 (30)	80 (20)	40	50 (10)	110 (10)	70	90 (20)	11.94 (2.04)	20.09	6.55 (0.55)
THR	220 (60)	80	160 (30)	70 (10)	50	60 (10)	130 (20)	100	140 (50)	17.87 (2.89)	42.11	13.40 (0.45)
ALA	200 (70)	70	140 (30)	60 (10)	40	40 (10)	130 (10)	110	160 (60)	18.53 (3.54)	49.16	11.52 (1.48)
PRO	160 (50)	60	100 (20)	40 (10)	30	30 (0.0)	110 (20)	80	130 (50)	29.33 (13.43)	53.68	9.28 (1.73)
TYR	120 (30)	30	30 (10)	0.0 (0.0)	20	20 (10)	80 (10)	50	60 (10)	8.77 (1.99)	13.40	3.43 (0.57)
VAL	190 (60)	70	140 (30)	50 (10)	40	40 (10)	130 (20)	110	140 (50)	14.36 (1.97)	32.78	10.79 (0.35)
MET	20 (0.0)	10	10 (0.0)	0.0 (0.0)	10	0.0 (0.0)	10 (0.0)	10	10 (0.0)	8.97 (4.13)	0.84	7.54 (1.09)
LYS	100 (30)	20	60 (20)	20 (10)	10	10 (0.0)	60 (10)	40	60 (20)	8.54 (1.88)	12.82	2.06 (1.64)
ILE	140 (40)	60	100 (20)	40 (10)	20	20 (0.0)	110 (10)	80	110 (40)	6.41 (1.94)	13.30	4.68 (0.27)
LEU	170 (50)	70	130 (20)	60 (20)	40	40 (0.0)	130 (20)	100	140 (50)	11.12 (2.8)	21.62	6.89 (0.24)
PHE	190 (70)	70	100 (10)	30 (10)	30	20 (10)	100 (10)	70	90 (20)	11.63 (2.28)	19.85	6.64 (0.77)
Total aa	2710 (810)	910	1750 (330)	740 (170)	540	610 (90)	1600 (180)	1210	1660 (250)	236.43 (47.7)	500.87	153.17 (13.15)

Table 1. Amino acid concentrations in the pre-drought (PRE, *n*=6 for the benthic materials and *n*=4 for the water fraction), rewetting (REW, *n*=1) and post-drought (POST, *n*=5) periods. Amino acid abbreviations: aspartic acid (Asp), serine (Ser), glutamic acid (Glu), glycine (Gly), histidine (His), arginine (Arg), threonine (Thr), alanine (Ala), proline (Pro), cysteine (Cys), tyrosine (Tyr), valine (Val), methionine (Met), lysine (Lys), isoleucine (Ile), leucine (Leu) and phenylalanine (Phe). Reported values are the average and the standard error (in parentheses).

The composition spectra of individual amino acids (Fig. 1) differed between the benthic materials and the DOM. Glycine, alanine, serine, threonine and valine where the most abundant and accounted for over 50% of the total amino acids in the benthic

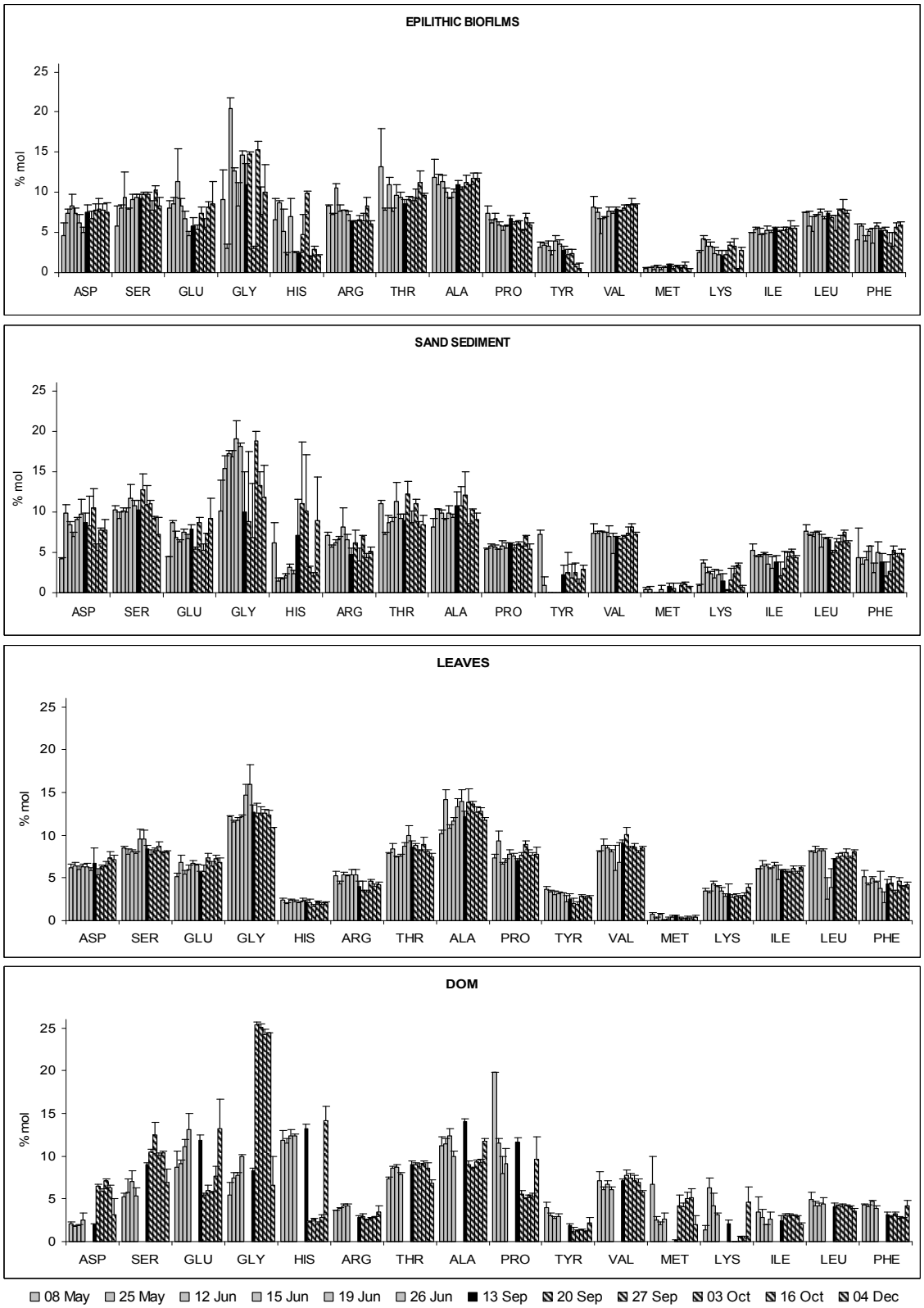

Fig. 1. Amino acid spectra (mole percentage) in epilithic biofilms, sand sediment, leaf material and in the dissolved water fraction throughout the wet-drought-wet process (12 campaigns). From 08 May 2006 to 26 June 2006 corresponds to the pre-drought period (grey bars), the 13 September 2006 corresponds to the rewetting day (black bar) and from the 20 September 2006 to 04 December 2006 corresponds to the post-drought period (diagonal pattern bar). Means and standard errors are shown (*n*=4). Amino acids abbreviations are given in Table 1.

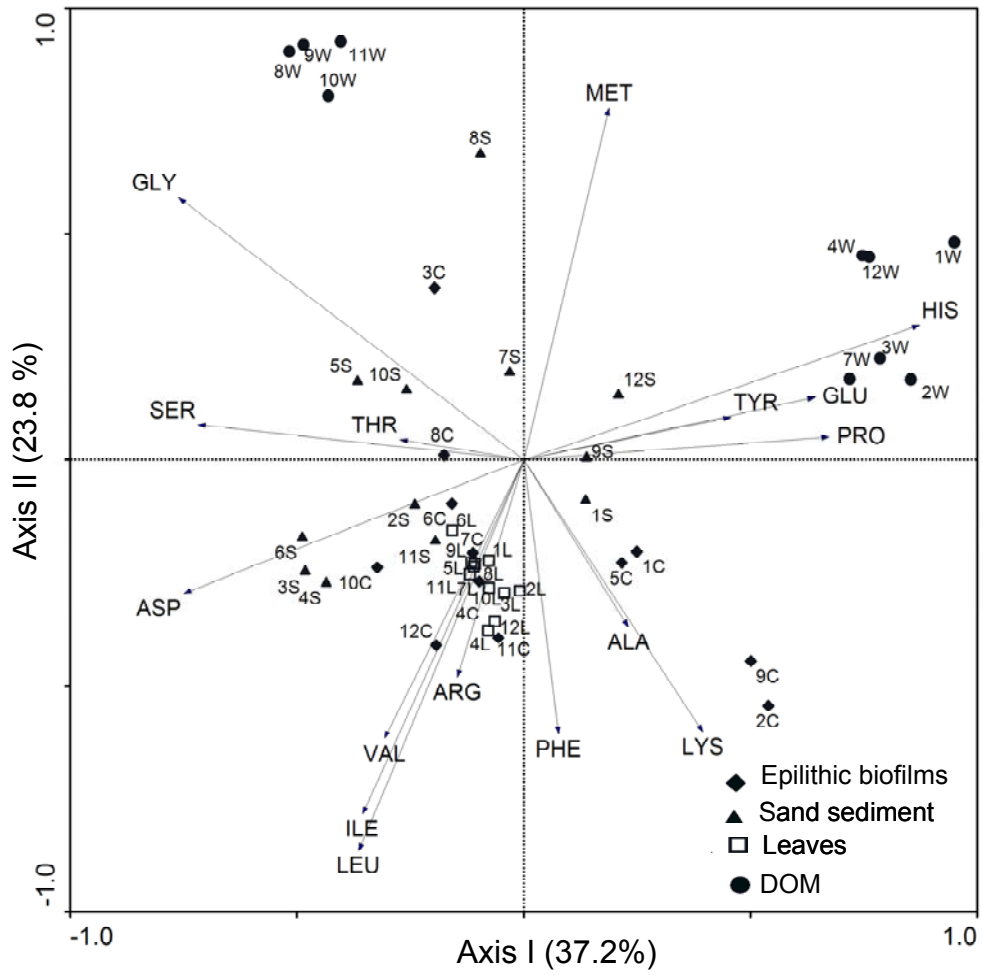

Fig. 2. Plot of the first and second axes of the principal components analysis (PCA) carried out with the amino acid compositions from epilithic biofilms (C), sand sediment (S), leaf material (L) and DOM (W) during the wet-drought-wet period (the numbers indicate the sampling campaign; from 1 to 6 corresponds to the pre-drought period, the 7 to the rewetting day and from the 8 to 12 to the post-drought period). The different sample groups are indicated by circles. Amino acid abbreviations are given in Table 1.

materials. DOM was dominated by glycine, alanine, glutamic acid and proline (accounting for 43.4% of the total amino acids). Methionine was nearly absent in the benthic substrata but relatively abundant (3.5% of the total amino acids) in the DOM. A huge peak of glycine (25% of total amino acids) was recorded in the DOM during the post-drought period.

The PCA yielded two significant ordination axes (Fig. 2, Table 2). The first axis (37.2% of the total variance) opposed the amino acids histidine, proline, glutamic acid and tyrosine to glycine, aspartic acid and serine. The former occurred in the DOM during the pre-drought period as well as in the rewetting (13th September) and at basal flow conditions (last sampling date). Glycine, aspartic acid and serine were characteristic of the post-drought water samples (except the initial day after the water

	First axis	Second axis
ASP	**-0.750**	-0.295
SER	**-0.719**	0.078
GLU	**0.643**	0.138
GLY	**-0.762**	**0.581**
HIS	**0.873**	0.299
ARG	-0.147	-0.481
THR	-0.273	0.045
ALA	0.231	-0.371
PRO	**0.674**	0.051
TYR	0.458	0.095
VAL	-0.308	**-0.617**
MET	0.188	**0.780**
LYS	0.395	**-0.603**
ILE	-0.356	**-0.784**
LEU	-0.364	**-0.865**
PHE	0.077	**-0.608**
Eigenvalue	0.372	0.238
Cumulative variance (%)	37.2	61.0

Table 2. Factor loadings, eigenvalues and cumulative variance of the principal components analysis performed with amino acid compositions from epilithic biofilms, sand sediment, leaf material and DOM during the wet-drought-wet period. The highest loading factors (larger than 0.5) at extremes of the axes (positive and negative) are marked in bold. Amino acid abbreviations are given in Table 1.

return). The amino acids composition of the benthic substrata did not change much during the pre- and post-drought periods. However, the epilithic biofilms had higher presences of histidine, proline, glutamic acid, tyrosine, leucine, isoleucine, valine, phenylalanine, lysine, arginine and alanine, while those from sand sediment had higher presence of the amino acids methionine, glycine, aspartic acid, serine, leucine, isoleucine, valine and threonine.

The second axis (23.8% of the total variance) separated the amino acids according to their occurrence either in DOM or in benthic materials. The amino acid composition of the DOM was mostly of methionine and glycine, whereas the benthic substrata were abundant in leucine, isoleucine, valine, phenylalanine, lysine, arginine and alanine.

Functional groups and essential amino acids

The relative abundances of amino acid functional groups in the benthic material did not change between the drought and rewetting periods (RM-ANOVA, Day effect, $p>0.05$). However, a progressive decline in essential amino acids occurred during the pre-drought period (RM-ANOVA, Day effect, $p=0.043$ $F_{2.7,\ 24.5}=4.4$). Significant

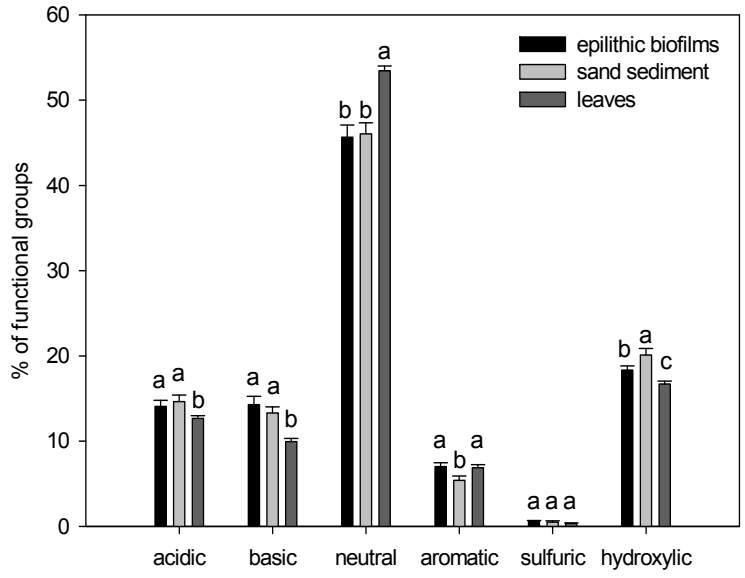

Fig. 3. Functional groups of protein amino acids (%). Acidic (aspartic acid, glutamic acid); Basic (histidine, arginine, lysine); Neutral (glycine, alanine, proline, valine, isoleucine, leucine); Aromatic (tyrosine, phenylalanine); Sulfuric (methionine) and Hydroxylic (serine, threonine). Means and standard errors are shown (*n*=12 corresponding to the 12 mean sampling values). Significantly different groups as determined by Tukey's test (a>b>c, α=0.05) are also shown.

differences were observed between benthic substrata. Epilithic biofilms had the highest percentage of essential amino acids (49%), followed by sand sediment and leaf material (44%) (Tukey's test, p<0.05). Sand sediments showed a lower proportion of aromatics and a higher proportion of hydroxylic amino acids than epilithic biofilms and leaf material (Fig. 3). Lower percentages of acidic, basic and hydroxylic amino acids and a higher percentage of neutral amino acids characterized the leaf material (RM-ANOVA, Substrata effect, p<0.05, Fig. 3).

Temporal changes in functional amino acid groups were particularly relevant in the DOM during the post-drought period (RM-ANOVA, Day effect, p<0.05, Fig. 4A). In the first day of the rewetting (13th September), the sulfuric group disappeared and the aromatics lowered off. The sulfuric group recovered during the following days (RM-ANOVA, Day effect, p<0.05 $F_{1.7,\ 5.3}$=6.2), whereas basic amino acids were strikingly reduced. The concentrations of functional groups when the basal flow was re-established (last sampling day), were similar to those observed during the drought (Fig. 4A). The proportion of essential amino acids in the DOM decreased during the pre-drought period and rose to 44% in the rewetting phase (Fig. 4B).

Organic matter diagenesis indicators

Sand sediment accumulated the most degraded benthic material during the pre-drought period, while the least degraded material was in epilithic biofilms (Tukey's test, p<0.001). The DI of amino acids decreased during the pre-drought period in the benthic materials (RM-ANOVA, Day effect, p=0.005 $F_{2.4,\ 21.4}$=9.5, Fig. 5), especially in sand and leaves. The DI in sand and leaf material increased immediately after

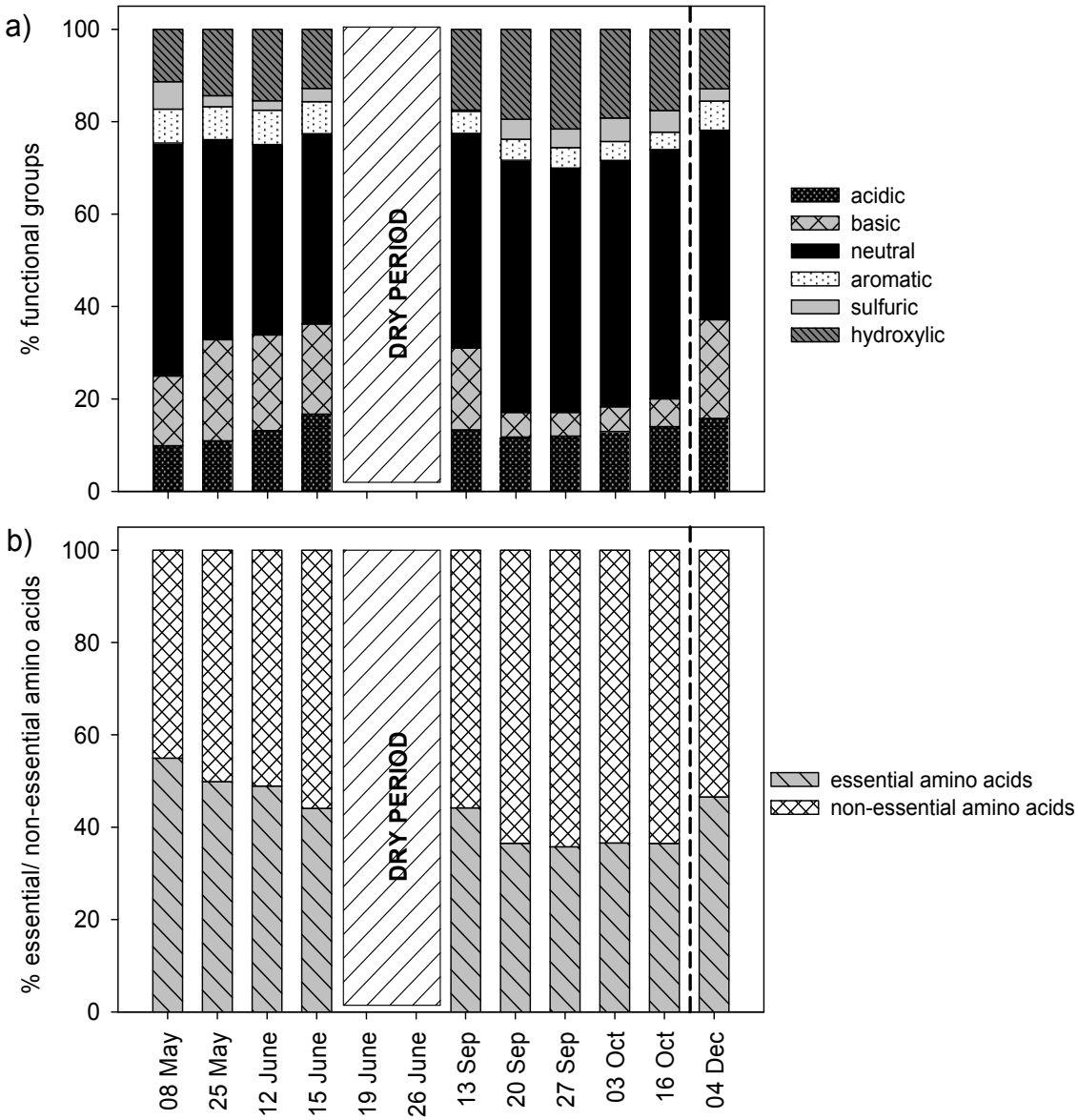

Fig. 4. In the upper graph (a) are represented the functional groups of protein amino acids in dissolved organic matter throughout the wet-drought-wet process. In the lower panel (b) is shown the evolution of the essential and non-essential amino acids during the same period. The dashed line indicates when the basal hydrological conditions were re-established.

rewetting. However, the DI after rewetting in the epilithic biofilm was similar to that in the pre-drought. The DI of DOM abruptly increased with the discharge peak (Fig. 5).

There was a positive relationship between the DI and the percentage of amino acids (AA-C/ DOC) in DOM (Fig. 6A). Higher DI values were related to higher percentages of AA-C/DOC during the pre-drought period. However, the DOM was more degraded during the post-drought period, presenting lower DI values and lower percentages of AA-C/DOC. During rewetting and when the stream's basal conditions

were re-established (4th December), the DI and the percentages of AA-C/DOC were close to the pre-drought values. Analogous relationships between the DI and the

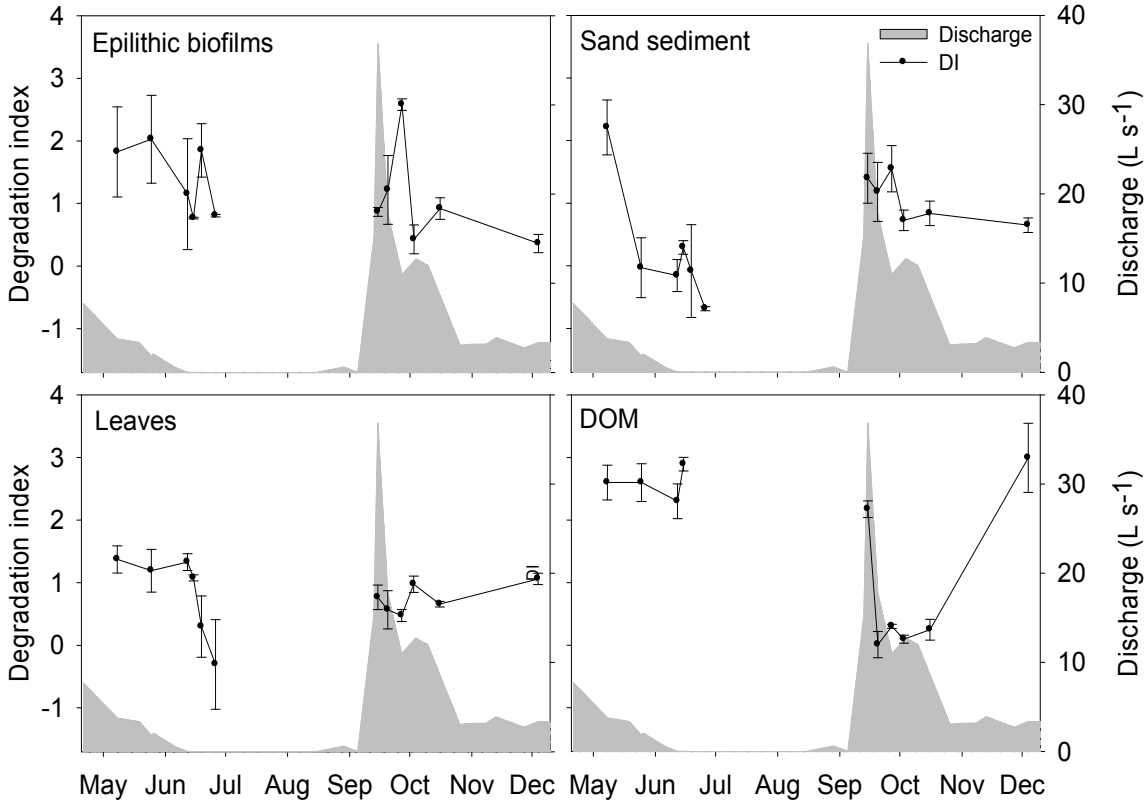

Fig. 5. Evolution of the degradation index and the water discharge throughout the wet-drought-wet period in epilithic biofilms, sand sediment, in leaf material and in dissolved organic matter. For the degradation index, means and standard error are shown (*n*=4).

percentage of DON represented by hydrolysable amino acids in units of N (AA-N/DON) occurred in the pre- and post-drought periods and in the rewetting (Fig. 6B).

The positive correlation (r=0.539; p<0.001) between the DI and the percentage of N in amino acids (% AA-N) suggests that higher DI values are linked to higher percentages of AA-N. The highest DI and % AA-N in DOM and epilithic biofilms were recorded during the pre-drought period and contrasted to values observed in the post-drought period. The lowest DI values occurred in sand biofilms during the pre-drought period, indicating that benthic organic matter rapidly degraded there. There was no difference between periods in the leaf material, which always had low percentages of AA-N. In the rewetting, DOM had high DI values and % AA-N while benthic substrata had the lowest percentage of AA-N (Fig. 7).

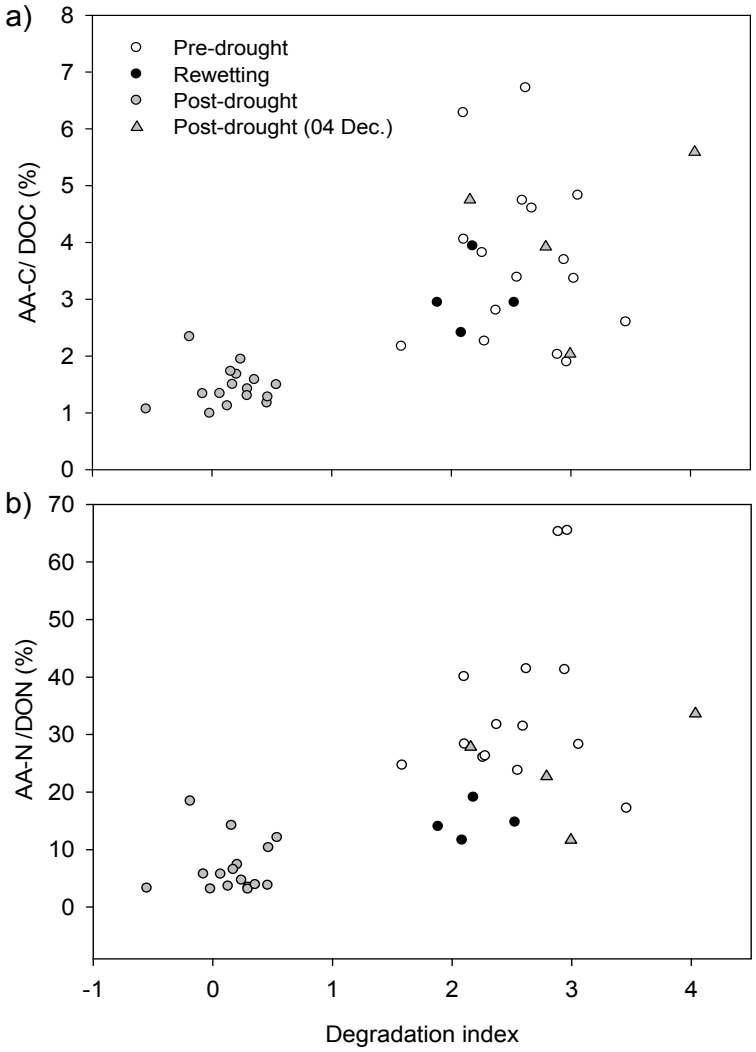

Fig. 6. The upper panel (a) corresponds to the relationship between the degradation index and the contribution of amino acid-carbon to DOC (AA-C/DOC) in the dissolved organic matter. The lower graph (b) corresponds to the relationship between the degradation index and the contribution of amino acid-nitrogen to DON (AA-N/DON) in dissolved organic matter. In total, ten sampling campaigns were carried out, and four replicates per sampling campaign are represented.

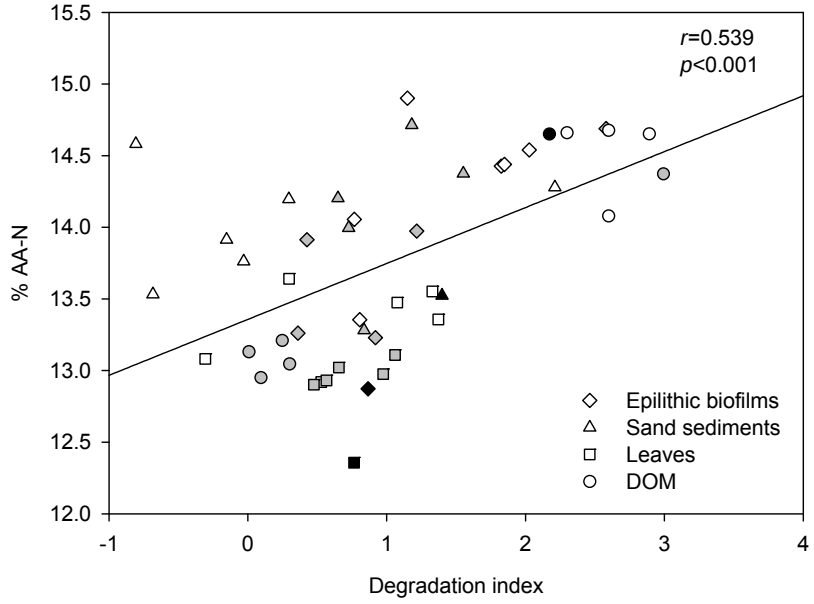

Fig. 7. Relationship between the degradation index and the %AA-N of epilithic biofilms, sand sediment, leaf material and dissolved organic matter (DOM) in the pre-drought (white dots), rewetting (black dots) and post-drought periods (grey dots).

DISCUSSION

Regardless of their abundance, amino acids are informative biomolecules by which we can measure OM diagenesis (Ittekkot et al. 1984; Cowie and Hedges 1994; Dauwe and Middelburg 1998). The composition of specific amino acids as well as differences in degradation indices were informative of the different natures and diagenetic states of the accumulated material in dissolved and benthic OM in a Mediterranean stream. Drought and rewetting episodes differentially affected the degradation of OM and this difference was more noticeable on flowing water than on benthic compartments.

The most abundant amino acids in Fuirosos were glycine and alanine in benthic and DOM. The predominance of these two amino acids has already been described in DOM and POM of aquatic systems (Mannino and Harvey 2000; Duan and Bianchi 2007) and in sediments (Keil et al. 1998). The relatively conservative behavior of glycine may be related to its abundance in the structural matrices of diatoms and bacteria as well as to its comparatively minor food value to micro- and macroconsumers because of its short chain length (Dauwe and Middelburg 1998).

The detailed analysis of amino acids composition show remarkable differences between the benthic substrata and the DOM, indicating that differential processes took place in the two compartments. The most degraded and lowest quality OM occurred in sand sediments. This compartment showed the highest concentration of hydroxylic acids (Ser and Thr) and the lowest of aromatics (Tyr and Phe). Amino acids such as glycine, serine and threonine preferentially accumulate during decomposition (Lee and Cronin 1984; Müller et al. 1986) and are considered to be highly recalcitrant and indicative of old OM (Chen et al. 2004). The epilithic biofilm showed instead high concentrations of histidine, alanine and glutamic acid, as well as tyrosine and phenylalanine. These amino acids are indicative of less degraded, fresher OM. Histidine and alanine usually occur in fresh materials (Jennerjahn and Itekkot 1997), and glutamic acid, tyrosine and phenylalanine are considered the most labile and easily degradable species (Cowie and Hedges 1992; Cowie et al. 1992). These last three amino acids (Glu, Tyr and Phe) are concentrated in cell plasma (Hecky et al. 1973) and are quickly consumed, justifying their low concentrations in decomposing material (Dauwe and Middelburg 1998; Lomstein et al. 2006). In epilithic biofilms is where we have found the highest amino acid concentrations, since the total concentration of amino acids is in relation to the abundance of autotrophic organisms. Algal biomass provides an important source of labile OM (Mannino and Harvey 2000) and epilithic biofilms have a higher presence of autotrophs than sediments (Romaní and Sabater 2001), though heterotrophic bacteria can also add significant N content to the OM pool.

In comparison with the other benthic substrata, the amino acids characteristic of leaf material were leucine, isoleucine, valine and phenylalanine, common in fresh material (Yamashita and Tanoue 2003), as well as structural amino acids (like glycine, serine and threonine; Hecky et al. 1973). Their mixed occurrence indicates that both benthic microorganisms and plant material coincide on leaf litter (Artigas et al. 2008).

Though amino acids in the benthic compartments were similar in the pre-drought and post-drought periods, there was a progressive degradation of benthic OM (progressive decrease in the DI) during the pre-drought, particularly in sand sediments and leaf material. That essential amino acids were preferentially consumed as degradation proceeded could have implications for consumers. Lower concentrations of essential amino acids (for instance Met) may limit macrofauna growth (Phillips 1984; Dauwe and Middelburg 1998). This might be particularly relevant in sand where the degradation index is low (DI= -0.7) indicating that the most degraded material accumulated there and in leaf material (DI= -0.3), but much less in epilithic biofilms (DI= 0.8). Sediments are generally more degraded than materials on superficial substrata (like epilithic biofilms; Dauwe et al. 1999). The very low DI in sand sediments is indicative of processes occurring in the epipsammic biofilms but also of those occurring in allochthonous OM sources (like terrestrial sources), which ultimately accumulate in the sediment.

While changes in amino acid composition were subtle in the benthic substrata throughout the wet-drought-wet period, they were much more remarkable in flowing water (DOM). The pre-drought DOM mostly is microbial-derived and shows a different composition than the post-drought DOM (which mostly is terrestrial-derived and more degraded). The water samples had higher content of amino acids within the total DOC and the total DON (AA-C/DOC, AA-N/DON) in the pre-drought period and showed higher DI values than those in the post-drought period. This difference indicated that OM was more labile during the pre-drought period and that diagenesis occurred in the temporal direction running from the pre-drought to the post-drought period. The temporal direction of this process is supported by the observed changes in the proportions of amino acid functional groups. Aromatic and basic amino acids in DOM were significantly reduced in the post-drought period, their decomposition being preferential relative to that of neutral amino acids during DOM degradation (Wu et al. 2003). The decrease in basic amino acids (positively charged) can be also related to their sorption to aluminosilicate clay minerals (negatively charged) (Meier et al. 1999; Aufdenkampe et al. 2001). This process can enrich the amino acid content in the POM and reduce them in DOM. When the reconnection between the flowing water and the benthic compartments recovered, high-quality OM accumulated in the streambed

mobilized and an ephemeral peak of labile DOM occurred (DI= 2.2). At that moment, resuspended material could rapidly be used by the organisms (Romaní et al. 2006; Ylla et al. 2010). After that pulse, only the resuspended older and highly-degraded sediment particles occurred in solution. The peak of glycine found in DOM during the post-drought period could be related to the mobility of older sand sediments, where glycine accounted for 20% of all amino acids.

Lower percentages of AA-N occurred in largely altered samples and vice-versa. The DOM in the pre-drought was more recent and of higher quality (higher N) than that of the benthic substrata. During the drought period decreased both the DI and the AA-N content, which rapidly recovered after water resumption. The low percentage of AA-N can consequently be diagnostic of the potential depletion of N as a key nutrient element (Cowie and Hedges 1994) which occurs during the pre-drought. The different source inputs and the specific stream channel dynamics between the pre- and post-drought periods were reflected in the temporal variation of DOM (both in terms of amino acid quantity and quality). However, the benthic compartments showed stable amino acids composition that reflected the respective nature of the OM sources, as well as their declining quality along the drought period. These slight changes in the benthic substrata were not greatly affected by the hydrological changes occurring in the stream. Therefore, the initial prediction that OM degradation was driven by water intermittency in Mediterranean streams is stronger to the dissolved organic matter, and weaker to the stream benthic substrata.

ACKNOWLEDGMENTS

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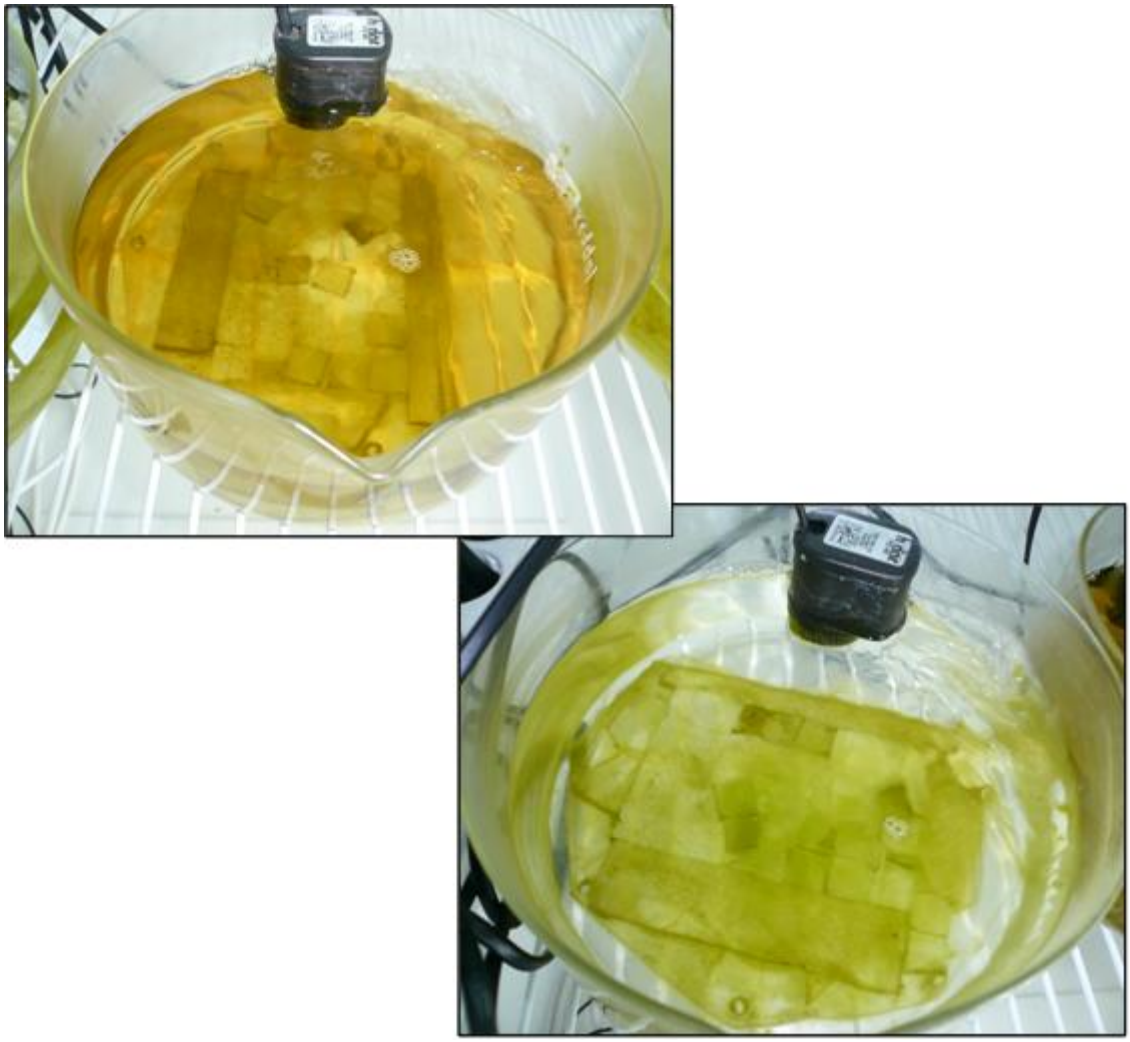


CHAPTER III

DIFFERENTIAL EFFECTS OF INCREASING WATER TEMPERATURE ON LABILE VERSUS RECALCITRANT BIOFILM DISSOLVED ORGANIC CARBON USE

Ylla, I., Romaní, A. M. & Sabater, S. 2010.
Submitted to Limnology and Oceanography

ABSTRACT

Dissolved organic carbon (DOC) inputs exhibit seasonal shifts in temperate streams, from high quality DOC when primary productivity is high to recalcitrant DOC after long periods of organic matter accumulation. Climate change may reinforce these shifts in DOC quality and concentration. A laboratory experiment was performed to determine the effect of higher water temperature on degradation of DOC by microbial biofilms and whether it diverged between labile and recalcitrant DOC. The microbial community was clearly affected by higher water temperatures; bacterial cell densities, respiratory activity (ETS) and bacterial metabolism (enzyme activity) were all higher. The addition of labile DOC (dipeptide plus cellobiose) caused a further augmentation of heterotrophic biomass and respiratory activity. The fluorescence index and the ratio Abs_{250}/total DOC indicated that recalcitrant DOC was degraded by higher temperatures. The experiment showed that naturally occurring pulses of highly available carbon, together with higher water temperature, might have consequences for the processing of flowing DOC. The more bioavailable organic matter will be rapidly cycled irrespective of higher temperature, while degradation of recalcitrant substances will be enhanced by warming.

Keywords: climate change, temperature, dissolved organic matter, labile organic matter, recalcitrant organic matter, enzyme activities, biofilms.

INTRODUCTION

Rivers act as pipes that transport dissolved organic matter (DOM), and biofilms covering the streambed are the main agent responsible for organic matter processing (Romaní et al., 2004). DOM quantity and quality depend on the hydrological phase of the stream (e.g., base runoff, floods or rewetting) and on its origin from allochthonous (terrestrial-derived) or autochthonous (derived from within the aquatic ecosystem) sources. DOM bioavailability varies with DOM source; while DOM released from autochthonous production is more labile and bioavailable, DOM that is terrestrially derived is more recalcitrant and more resistant to biological degradation (Bertilsson & Tranvik, 2000; Joffre, 2001; Bianchi et al., 2004). The composition of DOM can be viewed as a combination of a non-humic fraction (quickly degraded) and a humic fraction. The former consists of a large array of biomolecules, including lipids, carbohydrates, polysaccharides, amino acids, proteins, waxes and resins (Piccolo, 2001). The latter encompasses heterogeneous refractory organic substances, which are yellow to black in color and of high molecular weight (McDonald et al., 2004).

Therefore, DOM in rivers usually includes minor amounts of biodegradable material that are rapidly recycled and large amounts of refractory residues that are partially degraded (photochemically or microbiologically) (Cabaniss et al., 2005; Wiegner et al., 2006).

Current climate models predict that mean annual temperature will increase by 3.5 ºC in the air and 2.2-4.3 ºC in streams by 2100 (IPCC, 2007). It is widely accepted that global warming broadly affects ecosystems (Walther et al., 2002; Winder & Schindler, 2004; Kathol et al., 2009). For example, it influences both dissolved organic carbon (DOC) quality and quantity available to stream ecosystems and its microbial use and recycling (Porcal et al., 2009). The dependence of organic matter decomposition on temperature could lead to a substantial loss of carbon in stream ecosystems and further enhance global warming (Jenkinson et al., 1991). The hypothesis we aim to test in this study is that higher stream water temperatures during periods of moderate temperature (spring and autumn) may differentially affect the decomposition of labile and recalcitrant material by the microbial biofilm communities. Because the recalcitrance of a molecule is defined by the activation energy required to break its chemical bonds (Thornley & Cannell, 2001), higher temperature and activation energy are required to ensure degradation of resistant versus easily degradable organic substrates. The Arrhenius equation shows that the higher the activation energy for a reaction, the higher its temperature dependence. Thus, elevated temperature will theoretically accelerate the breakdown of recalcitrant compounds to a greater degree than the breakdown of labile compounds. The temperature sensitivity of organic carbon decomposition has been extensively studied in soils, and despite much research, the temperature sensitivity of soil organic carbon decomposition remains controversial (Reichstein et al., 2005; Davidson & Janssens, 2006; Kirschbaum, 2006). Some studies have indicated that the decomposition of older, more recalcitrant soil organic matter is less temperature sensitive (Giardina & Ryan, 2000), equally temperature sensitive (Fang et al., 2005a and 2005b; Conen et al., 2006), or more temperature sensitive (Knorr et al., 2005; Conant et al., 2008; Hartley & Ineson, 2008) than labile soil organic matter decomposition. These last three references were in accordance with kinetic theory, which predicts that the temperature sensitivity of decomposition should increase with substrate recalcitrance (Davidson & Janssens, 2006; Fierer et al., 2005).

In river ecosystems, microbial biofilms respond to a wide array of carbon manipulations, even those that might be considered recalcitrant. Microorganisms play a key role in the degradation of DOM and the release of its associated energy (Tranvik, 1988; Meyer, 1994). Bacterial extracellular enzymes convert high molecular weight

compounds to lower molecular weight compounds that are easily assimilated by heterotrophic microbes (Chróst, 1990). Because the synthesis of extracellular enzymes is regulated by the organic molecules available, they provide a powerful tool to understand the potential microbial use of DOC (Chróst, 1991). In this study, four enzyme activities were used that covered the degradation of labile compounds such as simple polysaccharides and peptides (β-glucosidase and leucine-aminopeptidase), complex polysaccharides such as hemicellulose (β-xylosidase) and recalcitrant compounds such as lignin (phenol-oxidase). The high population density within the biofilm, the expression of extracellular enzymes and even cell-to-cell signals contribute to the response of biofilms to pulsed inputs (Fischer, 2003). However, relatively little is known about the potential responses of the organic matter (OM) degradation mediated by biofilms at higher water temperatures. While Sand-Jensen et al., (2007) did not observe differences in DOC degradation related to temperature in a small stream, observations in the laboratory showed that changes in water temperature affected biofilm structure and determined its DOM microbial use (Díaz et al., in prep).

The potential combination of OM pulses and higher water temperatures may provide different outcomes depending on the source. A laboratory experiment mimicking these different OM sources was performed at two water temperatures (14 and 18 ºC). A temperature of 14 ºC was chosen to simulate the current mean water temperature measured in temperate systems in autumn and spring periods (Artigas et al., 2009), while 18 ºC correspond to a 4 ºC temperature increase that has been predicted by climate change models in temperate areas (scenario A2; IPCC, 2007). We aimed to determine whether a temperature increase could differentially affect the degradation of labile versus recalcitrant DOC by microbial biofilms. Our prediction was that the recalcitrant OM would be more intensively used at the higher water temperature, while the use of labile carbon would be barely affected by the temperature increase.

METHODS

Experimental procedure

A laboratory experiment was designed with two contrasting DOC qualities (labile and recalcitrant) plus a control (where no extra C had been added) and two water temperatures (14 ºC and 18 ºC). Six treatments were defined: High Temperature-Labile DOC (HT-L), High Temperature-Recalcitrant DOC (HT-R), High Temperature-Control (HT-C), Low Temperature-Labile DOC (LT-L), Low Temperature-Recalcitrant

DOC (LT-R) and Low Temperature-Control (LT-C). Three replicate microcosms were used per treatment.

Each microcosm consisted of a glass jar (19 cm in diameter, 9 cm high) with 40-45 glass tiles attached to the jar's bottom. The microcosms were filled with 2 L of simulated stream water that was recirculated by means of a submersible pump (Hydor, Pico 300, 230V 50 Hz, 4.5 W). The simulated stream water was obtained by dissolving pure salts (12 mg L^{-1} Na_2SO_4, 20 mg L^{-1} Na_2SiO_3, 30 mg L^{-1} $CaCl_2$, 1 mg L^{-1} KCl, 2 mg L^{-1} $MgSO_4$, 20 mg L^{-1} $NaHCO_3$) in MilliQ water to reproduce the chemical composition of a typical headwater forested stream, the Fuirosos (Vázquez et al., 2007). The glass tiles in the jars were colonized with biofilms collected from the Fuirosos. Aliquots (10 ml) containing 35 µg of chlorophyll were added to each microcosm once a week. Grown biofilms were kept inside two incubators (SCLAB-PGA500) with a constant day-night light cycle (12 h/12 h) and maintained at 13-15ºC. During this colonization period, water from the mesocosms was replaced every 3–4 days. The biofilms were three weeks old at the onset of the experiment when the experimental temperatures and DOC conditions were applied to the respective microcosms.

The two temperature treatments consisted of 18/15 ºC day/night temperatures, respectively, for the high temperature treatment and 14/11 ºC day/night temperatures, respectively, for the low temperature treatment. The different DOC treatments received daily pulses of 10 mg L^{-1} of labile DOC (labile treatment) or 10 mg L^{-1} of recalcitrant DOC (recalcitrant treatment) during three consecutive days. A total of 30 mg L^{-1} DOC was received by these microcosms. No extra DOC was added to the control treatment. The labile DOC (10 mg L^{-1}) was a mixture of cellobiose (5 mg L^{-1}, from Sigma-Aldrich) and the dipeptide leucine-proline (5 mg L^{-1}, from Sigma Aldrich). The recalcitrant DOC enrichment consisted of pure humic substances isolated from the Swannee River (from the International humic substance society, IHSS, ref. 2S101H). No further water replacements were done after the DOC pulses so that potential changes in DOC quality and quantity could be monitored after the pulses. Ammonium phosphate (30 µg L^{-1}) and ammonium nitrate (750 µg L^{-1}) were added to all treatments every 3-4 days during the whole experiment to prevent phosphorus and nitrogen depletion.

Water temperature in each microcosm was recorded every five minutes by a submerged temperature data logger (ACR SmartButton reader) to register all possible fluctuations. Water nutrient content and chemical parameters were monitored twice a week during biofilm colonization and at each sampling date during the experiment. Water aliquots from each microcosm were collected for analysis of inorganic nutrient and DOC after filtration (0.2 µm pore-diameter nylon filter, Whatman). Nitrate was

analyzed by ion chromatography (Metrohm 761 compact IC); ammonia was determined following Hach (1992) and phosphate was analyzed spectrophotometrically, as described by Murphy & Riley (1962). DOC was determined with a Shimadzu TOC 5050A. Oxygen concentration (Hach DO meter) and conductivity (conductivity meter, WTW) were also measured in all microcosms.

After the experimental temperature conditions and DOC pulses were applied, biofilms in the microcosms were sampled on day 1 (one day after the third DOC pulse) and later on days 3, 6, 10 and 17. Glass tiles were collected at random from each microcosm. Changes in DOC quality (by means of fluorescence index and absorbance ratio) as well as biofilm extracellular enzyme activities, primary production and respiration were analyzed. Biofilm chlorophyll and bacterial densities were also measured.

DOC quality

Fluorescence index (FI). Filtered water samples (GF/F; 5 ml) were collected from each microcosm on each sampling day and were adjusted to pH 2 with concentrated hydrochloric acid. Fluorescence was measured at 370 nm excitation and 450 and 500 nm emission and blank corrected (milli-Q water acidified with HCl to pH 2). FI was calculated as the ratio of emission intensity (450 nm/500 nm) at 370 nm excitation (McKnight et al., 2001).

DOC absorbance. Water absorbance at 250 nm (Shimadzu UV-1800) was used to calculate the ratio Abs_{250}/total DOC to indicate the proportion of humic material in the total DOC (Morris & Hargreaves, 1997; Fischer et al., 2006). Samples from the recalcitrant DOC treatments were diluted (1/2) with milli-Q water.

Extracellular enzyme activities

The extracellular enzyme activities of β-D-1,4-glucosidase (EC 3.2.1.21) and β-xylosidase (EC 3.2.1.37) were determined spectrofluorometrically using fluorescent (MUF, methylumbelliferone)-linked artificial substrates (4-MUF-β-D-glucopyranoside and 4-MUF-7-β-D-xyloside respectively). Leucine-aminopeptidase activity (EC 3.4.11.1) was analyzed by the fluorescent-linked artificial substrate L-leucine-7-amido-4-methylcoumarin hydrochloride (7-AMC-leucine from Sigma-Aldrich); and phenol-

oxidase activity (EC 1.14.18.1) was measured using L-3,4 dihydroxyphenylalanine (L-DOPA, from Sigma-Aldrich).

After the corresponding substrate addition (at saturating concentrations, 0.3 mmols L^{-1}) to the glass tiles (one for each microcosm and sampling date), samples were incubated in the dark at the respective treatment temperatures (14 and 18 ºC) and under continuous shaking for 1 h. Blanks and standards of MUF and AMC were also incubated. At the end of the incubation, glycine buffer (pH 10.4) was added (1/1 vol/vol), and fluorescence was measured at 365/445 nm excitation/emission for MUF and at 364/445 nm excitation/emission for AMC (spectrofluorimeter Kontron SEM 25). The intensity of fluorescence of the blanks was subtracted from all samples to correct for hydrolysis of the substrate or fluorescent substances in the water solution.

Phenol-oxidase activity was measured following the method outlined by Sinsabaugh et al., (1994). Incubations (1 h) were performed at 5 mM L-DOPA concentration. Samples were also incubated at the respective treatment temperatures (14 or 18 ºC) on agitation and in darkness. At the end of incubations, the absorbance of the liquid phase was measured at 460 nm (Shimadzu UV-1800).

Respiratory activity and primary production

Respiratory activity. The electron transport system (ETS) activity in each microcosm on each sampling day was assayed after the reduction of the electron transport acceptor INT (2-3 tetrazolium chloride from Sigma-Aldrich) into INT-formazan (iodonitrotetrazolium formazan) (Blenkinsopp & Lock, 1990). One tile for each microcosm plus 4 ml of water from each corresponding microcosm and two blanks were incubated in 0.02% INT solution. Incubations were performed in a shaker at the respective treatment temperatures (14 and 18 ºC) for 8 h in the dark. After this, INT-formazan was extracted with methanol for a minimum of 1 h at 4 ºC in the dark followed by sonication (2 min, Selecta, 40-W power). The extracts were filtered (GF/F filters, Whatman) and their absorbance measured spectrophotometrically at 480 nm (Shimadzu UV-1800). A stock solution of 30 µg ml^{-1} INT-formazan (Sigma-Aldrich) in methanol was used to prepare a standard curve.

Primary production. Net primary production and respiration were measured on the last sampling day of the experiment after the differences in oxygen concentration (oxygen balance method) were determined in each microcosm. Three glass tiles from each microcosm were removed and incubated in Winkler vials for 1.5 h. A first incubation in the early morning under dark conditions was performed to measure

respiration. Three consecutive incubations with the same glass tiles but under light conditions were performed to determine net primary production.

Biofilm structure

Bacterial density. Live and dead bacteria were counted using the Live/Dead Baclight bacterial viability kit (Invitrogen® Molecular probes) (Freese et al., 2006). Bacterial density was estimated after ultrasonication of each glass tile (one per microcosm on days 1 and 17) for 90 s using a sonication bath (Selecta) operating at 40 W and 40 kHz. After appropriate dilution (10 times) with sterile water, a 1:1 mixture of SYTO® 9 and propidium iodide was added to the samples and incubated for 15 min. Samples were then filtered (0.2 µm pore-diameter black polycarbonate filters, Nucleopore, Whatman) and at least 20 fields were randomly counted in each slide (Nikon E600 epifluorescence microscope). The fraction of live bacteria was calculated as the abundance of live cells divided by the total counts obtained with the Live/Dead method.

Chlorophyll-a. Chlorophyll *a* (Chl-*a*) concentration on the glass tiles (one replicate for each microcosm on each sampling date) was measured after extraction in 90% acetone for 12 h in the dark at 4 ºC. To ensure complete extraction of chlorophyll, samples were further sonicated for 2 min in a Selecta sonication bath operating at 40 W and 40 kHz and previously protected from light. Extracts were filtered through 1.45 µm mesh fiberglass filters (GF/C Whatman) and Chl *a* concentration was determined spectrophotometrically using a Lambda UV/VIS spectrophotometer (Hitachi), following Jeffrey & Humphrey (1975).

Algal composition. Glass substrata from the last sampling day (one tile for each microcosm) were preserved and stored with 4% formalin until analysis. Glass substrata were scraped with cell scrapers to achieve complete detachment of the microbial community. Three aliquots from each sample were observed under a light microscope (Nikon E600) at 400x to qualitatively determine the algal community composition at the genus level.

Scanning Electron Microscope (SEM) observations. Samples for SEM (one glass tile from each microcosm collected on the last sampling day) were fixed immediately after sampling with 2.5% glutaraldehyde in 0.1 mol L^{-1} cacodylate buffer (pH 7.2–7.4). Afterwards, a series of ethanol baths (65 to 100%) were used to dehydrate the samples, which were further dried by the critical point of CO_2 (CPD). Samples were finally coated with gold using a sputtering diode. Samples were viewed under a Zeiss DSM 960 scanning electron microscope.

Statistical Analyses

Differences in extracellular enzyme activity, fluorescence index, ETS and chlorophyll-*a* between the different treatments over the five sampling days were analyzed using a two-way repeated measures analysis of variance (RM-ANOVA). Probabilities within groups (Day and Day x S) were corrected for sphericity using the Greenhouse-Geisser correction. All probabilities were adjusted using the Dunn-Sidak correction. Chlorophyll-*a*, fluorescence index and ETS were log(x) transformed, while enzymes activities were log(x+1) transformed. A post hoc multiple comparison test (Tukey HSD) was used to examine the differences between the DOC treatments.

Variability in bacterial biomass (from days 1 and 17) and primary production (from day 17) were analyzed by single ANOVA's. This analysis was used to test for the single source effects of the studied factors and their interactions: DOC source (labile, recalcitrant and control) and temperature (low and high). In the case of the bacterial biomass the comparison between the two sampling dates was also included. Bacterial biomass was log (x) transformed, while primary production was log (x+1) transformed.

The SPSS software package for Windows (Ver. 14.0.1, SPSS Inc. 1989–2005) was used for all statistical analyses.

RESULTS

Chemical conditions at the microcosms

Conductivity increased in all microcosms (from 125 to 225 μS cm^{-1}) throughout the experiment, while dissolved oxygen remained steady (10.5 ± 0.9 mg L^{-1}). After the onset of the experimental temperature conditions, temperature remained steady in all microcosms (constant day-night temperature cycle; day high temperature of 18 ± 0.8 ºC and night high of 15 ± 1.1 ºC; day low temperature of 14 ± 0.8 ºC and night low of 11 ± 0.3 ºC; n=1836). The decrease in nutrient content achieved 5 ± 3 μgPO_4 L^{-1}, 11.2 ± 5 μgN-NH_4 L^{-1} and 360 ± 56 μgN-NO_3 L^{-1} in each 3-4 day period, but the periodic additions of N and P reestablished the initial nutrient conditions.

Changes in DOC content and quality

The DOC content remaining after the DOC pulse significantly decreased with higher temperature (Fig. 1, Table 1). The initial labile DOC pulse concentration (30 mg L^{-1}) rapidly decreased until day 6, while the microcosms amended with the recalcitrant DOC pulse showed a slight DOC increase throughout the experiment (Fig. 1). DOC

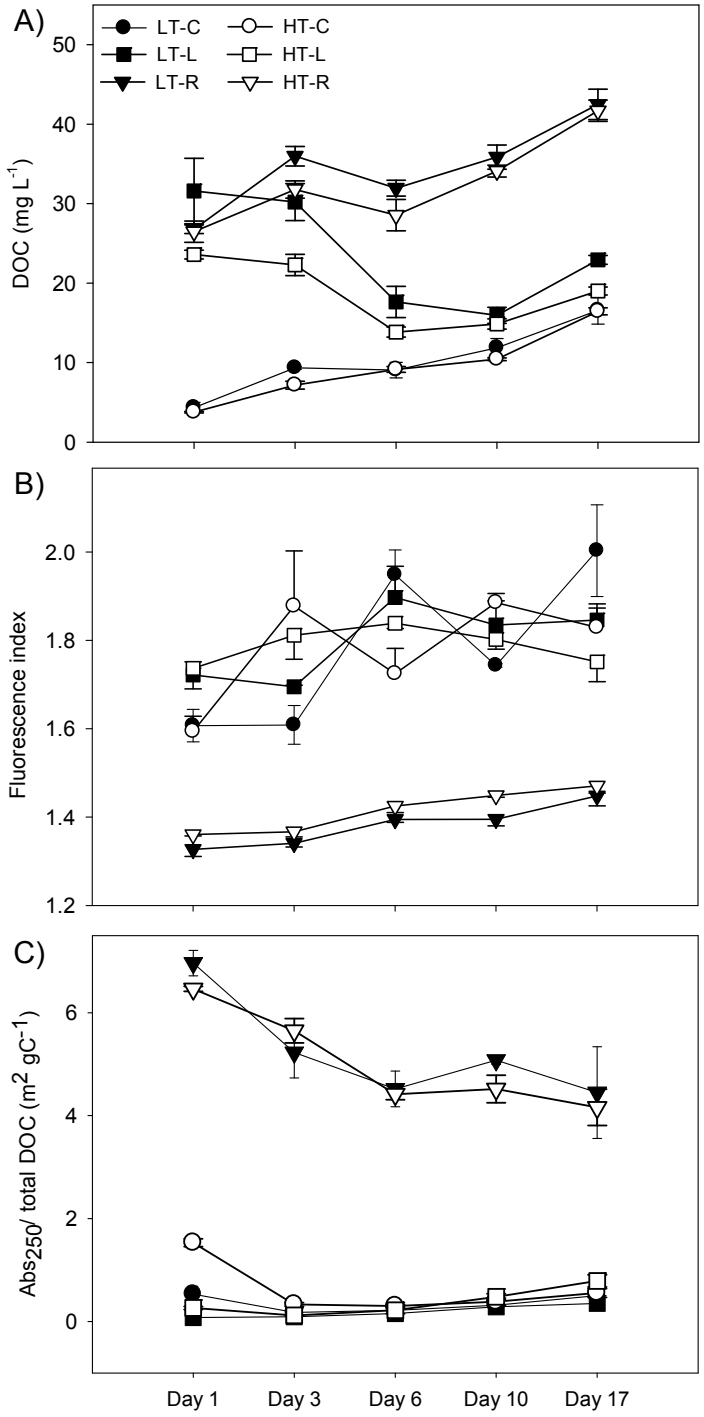


Fig. 1. (A) Amount of DOC, (B) Fluorescence index (FI) and (C) ratio Abs_{250}/total DOC under each set of conditions on the different sampling days. Values are means ± standard errors (vertical bars), *n* = 3

concentrations in the control treatment increased from 4 ± 0.4 to 16 ± 1.0 mg L^{-1} (Table 1; Fig. 1) with a similar trend to that of the recalcitrant treatment.

The fluorescence index ranged between 1.6-1.9 in the labile and control treatments and was significantly lower in the recalcitrant treatment (1.4-1.5; Fig. 1, Table 1). Temperature enhanced the index values in the recalcitrant treatment

throughout the experiment (Temperature × DOC × Day interactions, Table 1), but not in the other two treatments.

The Abs_{250}/total DOC index was much higher in the recalcitrant than in the labile and control treatments (Table 1). The absorbance index significantly decreased in the recalcitrant microcosms with higher temperature (Temperature × DOC interaction, Table 1).

Source of variation	DOC	Fluorescence index	Abs_{250}/ total DOC	β-glucosidase	Peptidase	β-xylosidase	Phenol-oxidase	ETS
Temperature	**<0.001**	0.838	0.564	**0.013**	**<0.001**	0.581	0.999	**0.05**
DOC	**<0.001**	**<0.001**	**<0.001**	0.726	**<0.001**	0.264	0.275	**0.020**
Day	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Temperature x DOC	0.192	0.584	**0.027**	0.936	0.442	0.998	0.997	0.848
Temperature x Day	0.341	**<0.001**	0.976	0.652	0.156	**<0.001**	0.994	0.883
DOC x Day	**<0.001**	**0.020**	**<0.001**	0.356	0.264	**0.006**	*0.067*	0.174
Temperature x DOC x Day	0.291	**0.006**	0.510	*0.054*	**0.013**	0.31	0.999	**0.013**

Table 1. Repeated measures ANOVA results considering the effects of temperature (high or low) and DOC source (control, labile or recalcitrant) on DOC quality as well as on biofilm metabolic parameters. Probability within groups was corrected for sphericity by the Greenhouse–Geisser correction and p-values were adjusted by the Dunn–Sidak correction. Values <0.05 are indicated in boldface type and values <0.1 are indicated in italics.

Biofilm extracellular enzyme activities, respiration and primary production

Temperature significantly increased β-glucosidase and peptidase activities (Temperature effects, Table 1, Fig. 2), as well as β-xylosidase activity over time (Temperature × Day interaction, Table 1), but did not affect phenol-oxidase activity (Table 1). An increasing pattern of phenol-oxidase activity was only observed under HT-R (Fig. 2).

DOC quality as a single factor only affected peptidase activity, which was significantly lower in the recalcitrant treatment (Table 1, Fig. 2; Tukey's test, $p<0.001$). Nevertheless, the addition of DOC affected the time pattern of β-xylosidase and phenol-oxidase activities. While β-xylosidase activity increased when both labile and recalcitrant material were added, phenol-oxidase activity increased when recalcitrant material was added (DOC × Day interaction, Table 1).

Respiratory activity (ETS) of the biofilms was enhanced with increased temperature and higher values were measured for the labile DOC treatments (Temperature and DOC effects, Table 1, Fig. 3). At the lower temperature, changes in

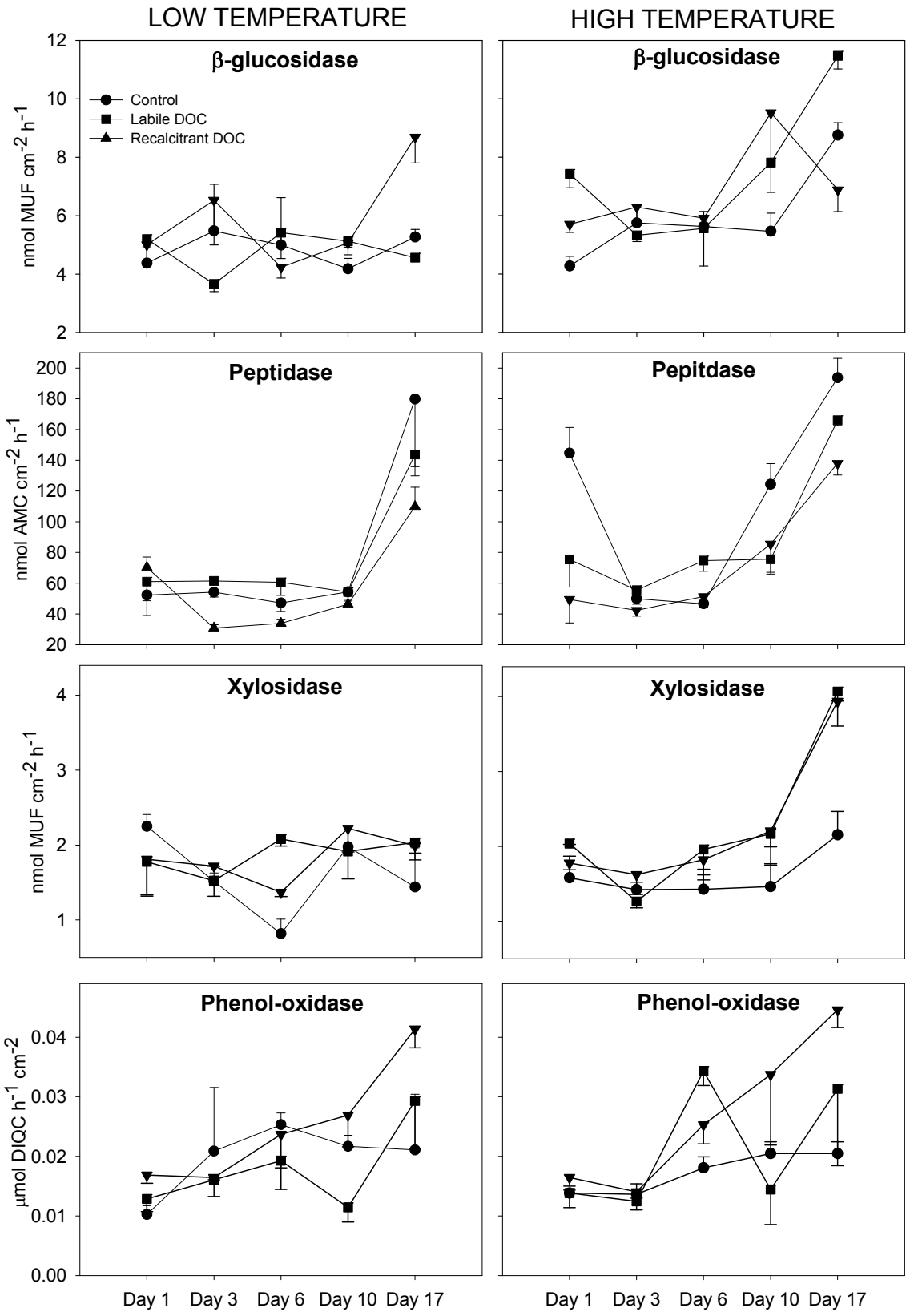


Fig. 2. Temporal changes in extracellular enzymatic activities (β-glucosidase, leucine amino peptidase, xylosidase and phenol-oxidase) at the colonized substrata of each treatment. Values are means ± standard errors (vertical bars), n = 3.

ETS over time were affected by DOC treatment, reaching the highest values in the labile DOC treatment, but less in the control treatment and even less in the recalcitrant DOC treatment. At the higher temperature, respiratory activity reached similar values in the three DOC treatments (Fig. 3). Community respiration measured at the end of the experiment with the oxygen balance method (Fig. 4) also showed the effect of temperature on increasing respiration in the control and recalcitrant treatments, while in the labile treatment ETS was already high at the lower temperature (ANOVA, Temperature effect, $p<0.001$ and DOC effect, $p<0.001$).

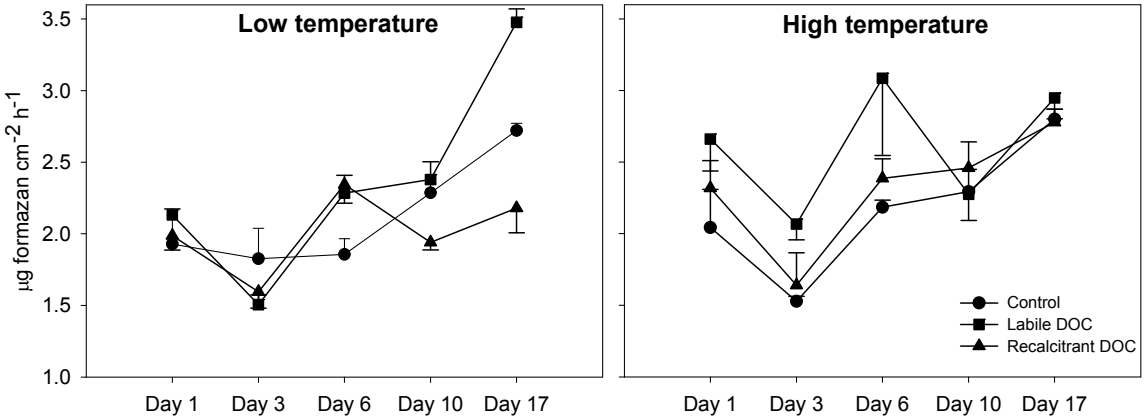


Fig. 3. Temporal variation of epilithic biofilms (growing on the artificial glass substrata) and respiratory activity (electron-transport-system [ETS]). Values are means ± standard errors (vertical bars), n = 3.

The net primary production (NPP) of the biofilm community (Fig. 4) decreased at the higher temperature, especially for DOC-amended treatments (labile and recalcitrant DOC additions, ANOVA, temperature effect $p<0.001$, DOC effect, $p=0.026$). NPP was lower in the recalcitrant than in the labile and control treatments (Tukey's test, $p=0.007$).

Biofilm structure

The total number of bacteria (live plus dead cells) increased during the experiment in all treatments (ANOVA, $p<0.001$, Table 2). The maximum bacterial concentration was achieved at day 17 in the HT-L treatment (Table 2), but the highest proportion of live bacteria occurred at the first sampling day in the HT-L (50 ± 3.6%) and LT-L (44 ± 1.4%) treatments. The percentage of living bacteria in the other treatments and days ranged from 36-40%.

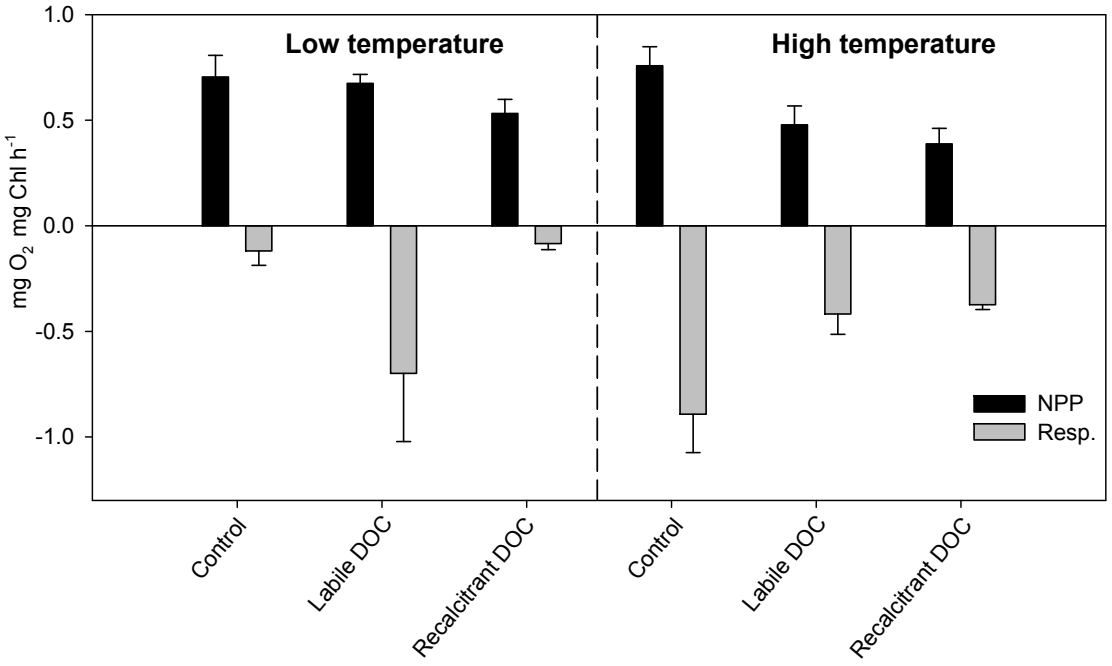


Fig. 4. Net primary production (NPP) and Respiration rate (Resp.) per unit of chlorophyll for each set of treatments. Each bar represents the mean (± SE) for three incubations performed during the day.

Chlorophyll *a* ranged between 3.5-4.8 µg cm^{-2} at the beginning of the experiment. The addition of a recalcitrant DOC source enhanced chlorophyll density to 7 µg cm^{-2}, but density remained similar in the control and labile treatments (RM-ANOVA, DOC interaction $p<0.001$; Table 2). Temperature effects on chlorophyll density were not significant.

		LT-C	LT-L	LT-R	HT-C	HT-L	HT-R
Total bacteria ($\times10^7$ bact. cm^{-2})	Day 1	1.32 ± 0.09	1.11 ± 0.1	1.07 ± 0.06	1.37 ± 0.09	1.16 ± 0.03	1.65 ± 0.2
	Day 17	1.74 ± 0.07	1.80 ± 0.2	1.85 ± 0.03	2.02 ± 0.3	2.36 ± 0.4	2.03 ± 0.2
Chl *a* (µg chl cm^{-2})	Day 1	3.43 ± 0.43	3.65 ± 0.2	3.62 ± 0.41	4.26 ± 0.29	4.29 ± 0.44	4.82 ± 0.13
	Day 17	3.69 ± 0.56	4.13 ± 0.5	6.86 ± 1.79	4.32 ± 0.86	4.07 ± 0.03	7.35 ± 0.5

Table 2. Total bacterial abundance ($\times10^7$ bacteria cm^{-2}) and chlorophyll *a* (µg chl cm^{-2}) concentration in the colonized glass substrata under each treatment. Values are means ± standard errors, n=3.

The algal community of the biofilms (observed under optical and SEM microscopy) was similar between treatments. It was composed of filamentous green algae (*Stigeoclonium tenue*, *Oedogonium* sp., *Mougeotia* sp.), non-filamentous green algae (*Scendesmus* sp., *Tetraspora* sp.), cyanobacteria (*Oscillatoria* sp., *Lyngbya* sp.)

and various diatom species. The higher temperature favored the presence of filamentous algae (basically *S. tenue* and *Oedogonium* sp.) as well as high densities of protozoa and rotifera (*Colurella* sp. and *Lecane* sp.). Large growths of *Stigeoclonium* appeared in the labile treatment, particularly in the higher temperature conditions.

DISCUSSION

It is well known that DOC degradation rates are temperature-dependent (Cabaniss et al., 2005) because increased temperatures leads to higher microbial activity and hence greater DOC decomposition (Baulch et al., 2005). However, the detailed response pattern of different organic substrates remains obscure. Fierer et al., (2005) suggested that the decomposition of lower quality substrates (more recalcitrant types or specific C compounds of lower lability) would be more sensitive to changes in temperature than that of higher quality C substrates.

In this experiment, the significant effect of temperature on decreasing total DOC content, as well as increasing most extracellular enzyme activities (glucosidase, peptidase and xylosidase) and bacterial density, indicates that DOC use is greater at higher water temperatures, thus promoting faster C cycling. The experiment also showed that the effects of temperature were specific for the different DOC sources. The decrease in DOC was mostly of labile carbon, while for the recalcitrant and control treatments total DOC was increasing. The dynamics of DOC changes responded both to uptake and release mechanisms within the biofilm (bacterial mineralization, algal release and cell lysis); thus, under the control and recalcitrant conditions a net release of DOC was measured while a net consumption of DOC was observed for the labile treatment. Furthermore, the overall increase in DOC in all microcosms' treatments in the last days of the experiment can be attributed to the solute concentration as a consequence of water evaporation (indicated by the increase in conductivity). However, changes in DOC content were accompanied by changes in DOC quality. The progressive increase of the fluorescence index and the decrease of the absorbance ratio (Abs_{250}/total DOC) respectively highlight that humic substances were losing aromaticity (McKnight et al., 2001), and the amount of phenolic compounds in total DOC was diminishing (De Hann, 1993; Fischer et al., 2006) in the recalcitrant treatments. The patterns of both indices indicated that transformation of material was occurring after the reception of the recalcitrant DOC. The two indices showed a more marked behavior (higher values of the fluorescence index, lower values of the absorbance ratio) at the higher temperature. These two DOC quality parameters did not show any change related to temperature in the labile treatment, therefore stressing the greater effect of temperature in the degradation of recalcitrant compounds.

The response of biofilm to the labile DOC pulse was a fast rate of uptake and remarkable changes in microbial biomass and metabolism. The relevance of the labile N and C inputs to the biofilm was expressed in the increase in active bacterial density. The biofilm experienced an increase in respiratory activity; however, the four degrees of temperature increase did not further enhance respiration rates that were already activated by the labile DOC availability at 14 ºC. Respiration rates of algae, bacteria and invertebrates increase with temperature (Baulch et al., 2005; Phinney & McIntire, 1965), but the quality of the DOC matters for this response. Claret (1998) showed that respiratory activity was enhanced by labile DOC and that refractory DOC limited the respiration activity in a hyporheic biofilm. Sand-Jensen et al., (2007) observed that the influence of temperature on respiration was not dependent on the quality of organic material. Our data indicated that labile DOC sources stimulated high respiratory activity, irrespective of the temperature contrast, and that biofilm respiratory activity with recalcitrant DOC increased with warming. However, the enzymes β-glucosidase and leucine-aminopeptidase did not increase their activity even when their respective substrates (cellobiose and a dipeptide) were added in the labile DOC treatment. The auto-heterotrophic biofilm communities were probably not limited by those substrates because the fresh algal exudates provide sufficient polysaccharides and proteinaceous substances to bacteria (Espeland et al., 2001; Francoeur & Wetzel, 2003). The high lability of the amended cellobiose and the dipeptide probably meant that they were preferentially and rapidly used and so their effects quickly vanished, but the substrate provided N and C for bacterial growth.

Recalcitrant DOC enhanced the enzymes closest to their degradation (β-xylosidase and phenol oxidase), and with time their activity increased under higher temperature. However, the expression of phenol-oxidase activity (lignin decomposition) and β-xylosidase activity (hemicellulose decomposition) was slow, indicating that the process of acquiring C from complex molecules needs a longer activation process carried out by more specific and probably less common enzyme activities.

Increased utilization of recalcitrant organic matter with higher temperature had been measured in soils and tied to microbial activation (Andrews et al., 2000). Waldrop & Firestone (2004) observed that increasing utilization of old carbon under high temperatures was associated with changes in microbial community composition and the related expression of enzyme activities. In our experiment, we cannot rule out that shifts in community composition (especially bacterial) could contribute to the responses in community metabolism (enzyme activities, respiration rates). Findlay et al., (2003) show that bacterial communities may diverge when exposed to labile versus recalcitrant DOM sources.

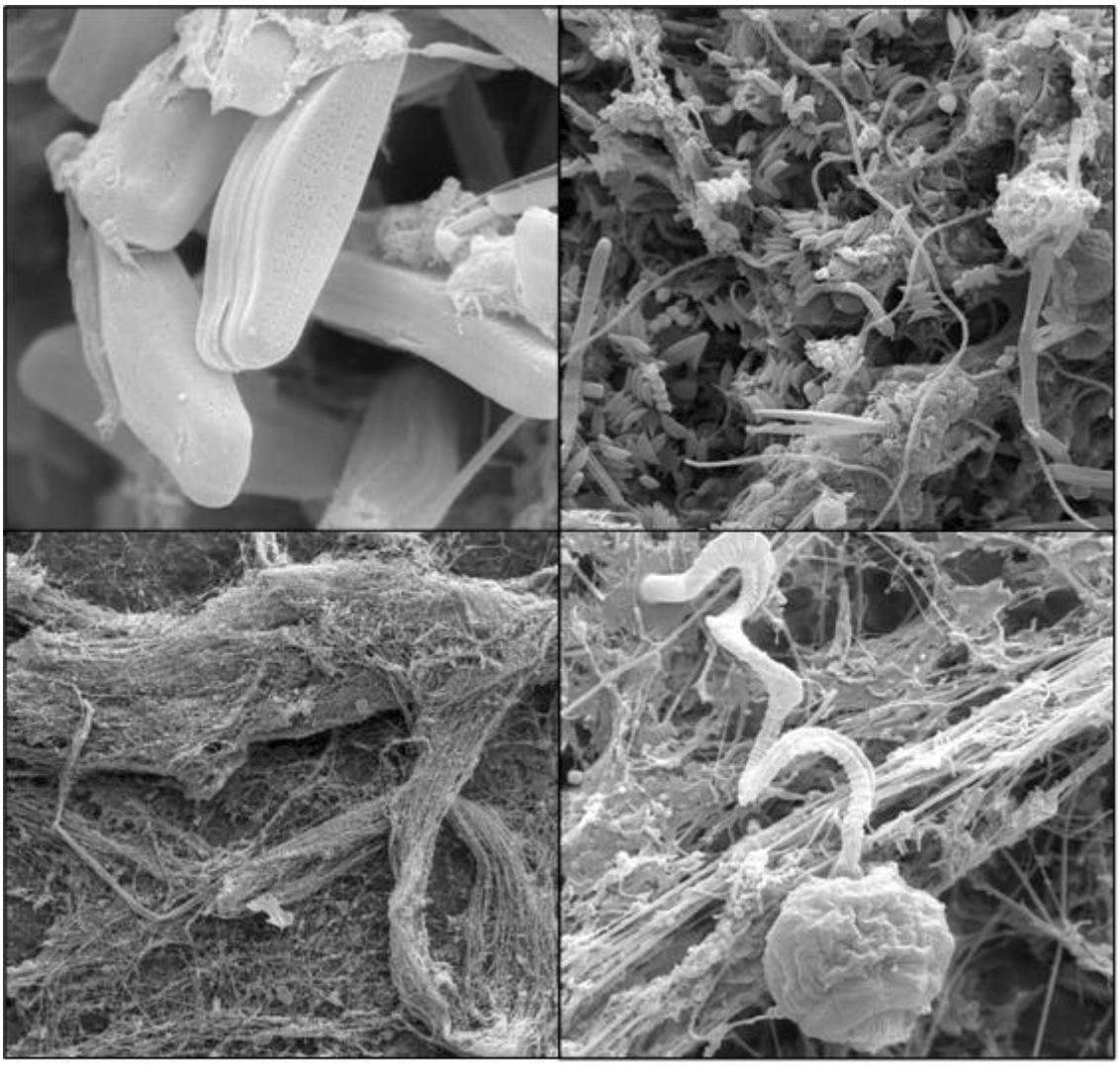
The addition of the humic material might have had indirect effects on the biofilm microbiota. Because dissolved recalcitrant substances alter water color (making it brownish-orange), attenuate both visible and UV light and reduce light extinction (Jansson et al., 1996), algal communities may respond with increased chlorophyll-*a* concentrations (Guasch & Sabater, 1995). Though the algal community grown on the glass tiles did not greatly differ between treatments, the higher chlorophyll-*a* content in recalcitrant treatment did not prevent the algal community from being the least productive, a potential depression effect of humic matter on primary productivity (Jackson & Heckey, 1980). The lowest peptidase activity level that was observed in the recalcitrant treatment could be related to the inhibitory effect of humic matter on certain metabolic processes by the complexation and inactivation of bacterial enzymes (Meyer et al., 1987; Freeman et al., 1990; Wetzel, 1992) or an indirect effect after photosynthesis inhibition (Francoeur & Wetzel, 2003; Ylla et al., 2009).

Our results hint that the composition of the carbon supply pulses and warmer water temperatures (4 ºC increase) influence how DOC materials are processed. Labile DOC pulses trigger the response of heterotrophic biofilms that rapidly (hours or days) decompose those substances, irrespective of higher temperature. Consequently, the impact of these bioavailable substances on the microbial community was punctual and short in time. Biofilms need much more time (weeks or longer) and higher temperatures to degrade recalcitrant DOC. The impact of the recalcitrant pulses was much more prolonged as more time was needed to trigger the biofilm response (expression of phenol-oxidase and xylosidase enzymes and reduction in photosynthetic activity).

In conclusion, the present results suggest that warming could differentially affect DOC degradation in stream ecosystems depending on the C source. Under warming conditions, recalcitrant materials will decompose faster, while labile sources will be less affected because they are so rapidly taken up by freshwater biofilms that temperature increase might be irrelevant. The findings of this paper indicate that pulses of terrestrial material, such as recalcitrant humic substances, in streams of temperate regions might be most affected by temperature increases that can accelerate their microbial use and decomposition. As a consequence, the predicted increase of 2-4ºC could determine lower inputs of humic material reaching the oceans.

ACKNOWLEDGEMENTS

This study was funded by projects CGL2007-65549/BOS, CGL2008-05618-C02/BOS of the Spanish Ministry of Science and Innovation and SCARCE (Consolider-Ingenio CSD2009-00065). We thank Juanita Mora for her help in the laboratory analysis and Helmut Fischer for his advice on DOC quality interpretation.



CHAPTER IV

AVAILABILITY OF GLUCOSE AND LIGHT MODULATES THE STRUCTURE AND FUNCTION OF A MICROBIAL BIOFILM

Ylla, I., Borrego, C., Romaní, A.M. & Sabater, S. 2009. FEMS-Microbiology Ecology 69:27- 42

Irene Ylla, Carles Borrego, Anna M. Romaní, Sergi Sabater. “Availability of glucose and light modulates the structure and function of a microbial biofilm”. FEMS microbiology ecology. Vol. 69, issue 1 (July 2009) : p. 27-42

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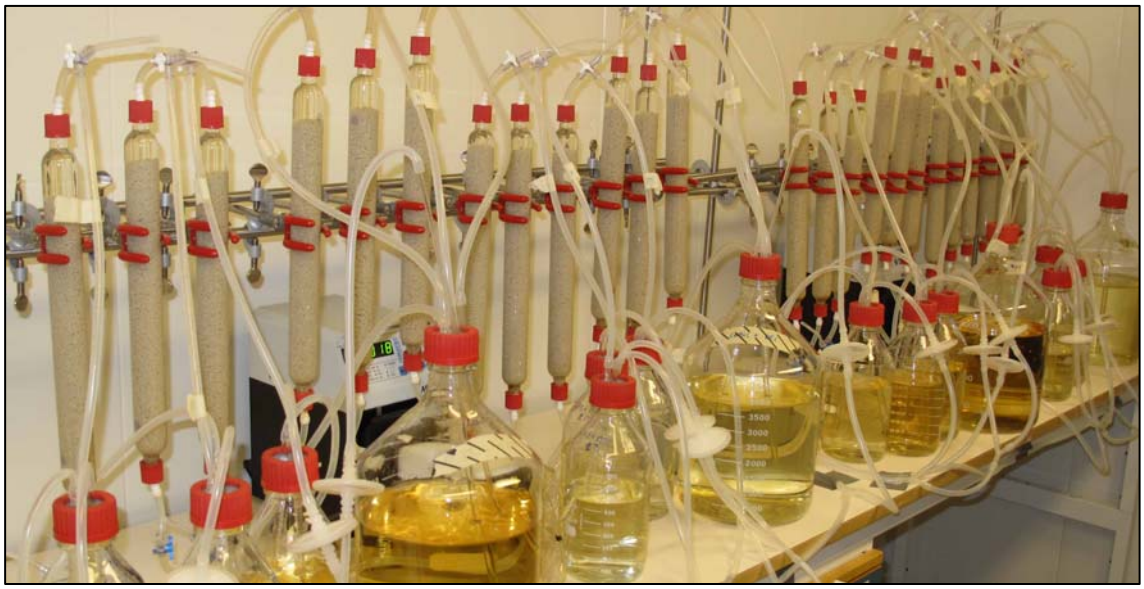
Abstract

We have studied the differences in the organic matter processing and biofilm composition and structure between autoheterotrophic and heterotrophic biofilm communities. Microbial communities grown on artificial biofilms were monitored, following incubation under light and dark conditions and with or without the addition of glucose as a labile organic compound. Glucose addition greatly affected the microbial biofilm composition as shown by differences in 16S rRNA gene fingerprints. A significant increase in β-glucosidase and peptidase enzyme activities were also observed in glucose-amended biofilms incubated in the dark, suggesting an active bacterial community. Light enhanced the algal and bacterial growth, as well as higher extracellular enzyme activity, thereby indicating a tight algal–bacterial coupling in biofilms incubated under illumination. In these biofilms, organic compounds excreted by photosynthetic microorganisms were readily available for bacterial heterotrophs. This algal–bacterial relationship weakened in glucose-amended biofilms grown in the light, probably because heterotrophic bacteria preferentially use external labile compounds. These results suggest that the availability of labile organic matter in the flowing water and the presence of light may alter the biofilm composition and function, therefore affecting the processing capacity of organic matter in the stream ecosystem.

Keywords

Microbial biofilm; Extracellular enzymes; Glucose; Light; Algae–bacteria coupling; Biofilm composition



CHAPTER V

MULTIFUNCTIONALITY IS AFFECTED BY LOSS OF DIVERSITY IN MICROBIAL BIOFILM COMMUNITIES

Peter, H., Ylla, I., Gudasz, C., Romaní, A. M., Sabater, S. & Tranvik, L. 2010. In prep.

ABSTRACT

Bacteria account for a significant fraction of biodiversity and intervene in most biogeochemical processes in aquatic ecosystems. However, the basic connections between biodiversity and aquatic ecosystem functioning remain obscure. The degradation and respiration of dissolved organic carbon (DOC) by bacterial biofilms is a key step of carbon cycling in streams and rivers. The effects of loss of bacterial species richness on respiration of labile and recalcitrant DOC were examined by means of a bioreactor experiment. DOC concentrations, oxygen consumption rates, bacterial abundances and activities of five extracellular enzymes were measured over 10 days in reactors colonized for 4 and 7 weeks, respectively. Gradients in bacterial community composition from the inflow to the outflow of the reactors were sampled for molecular fingerprinting by Terminal Restriction Fragment Length Polymorphism (T-RFLP). The bacterial communities rapidly degraded a substantial fraction of labile DOC, but DOC concentration remained unchanged and oxygen consumption rates were lower in the recalcitrant treatments. The intentional decrease in bacterial diversity was linked to a decrease in the enzyme multifunctionality. The multifunctionality decrease occurring at lower diversity was larger under recalcitrant DOC conditions and in older biofilms. The loss of functionality was not “equal” for all functions; those enzymes linked to the use of complex compounds were the first to be lost. The results show the implications of bacterial biodiversity in the maintenance of ecosystem functioning.

Keywords: biodiversity, biofilm, bioreactors, T-RFLP, extracellular enzyme activities, multifunctionality, labile DOC, recalcitrant DOC.

INTRODUCTION

Several recent reviews and meta-analyses indicate that ecosystem functioning is positively related to species richness (Covich et al., 2004; Hillebrand & Cardinale 2004; Balvanera et al., 2006; Cardinale et al., 2006). These relations have been reported with respect to biomass accrual (Downing, 2005), resource use efficiency (Cardinale et al., 2006) and temporal stability (Balvanera et al., 2006).

Microbes encompass the highest diversity of all life forms on Earth (Curtis et al., 2006). They drive the bulk of ecosystem functions and services and inhabit even the most hostile habitats. However, the roles microbial diversity play in controlling ecosystem functioning remain largely overlooked (Hillebrand & Matthiessen, 2009). Microorganisms are regarded as keyplayers in most biogeochemical cycles although their diversity is commonly treated as “black boxes” in biogeochemical models (Allison

& Martiny, 2008). Large-scale ecosystem functions are often regarded as independent from biodiversity; heterotrophic bacterial respiration may for example be independent from diversity because of the high number of similarly performing species (Langenheder et al., 2005).

Species assemblages both affect and are affected by properties of ecosystems in which they live. However, the interplay of environmental heterogeneity and species diversity on ecosystem functioning has only recently been addressed (e.g. Solan et al., 2004; Weis et al., 2008; Langenheder et al., 2010; Singer et al., 2010). Langenheder et al., (2010) hypothesised that ecosystem functioning increases with species and resource richness due to complementarity effects. Complementarity suggests that species discriminate between resources through niche diversification, allowing for facilitative interactions between species (Tilman et al., 1997). As a contrasting force, selection drives species differentiation with regard to their effects on processes, and species-rich communities are more likely to contain species with a large effect on ecosystem functioning than species-poor communities (Tilman et al., 1997).

Dissolved organic carbon (DOC) is an important component of the global carbon cycle in aquatic ecosystems (Battin et al., 2009; Porcal et al., 2009). In low-order streams, benthic microbial communities have a key role in organic matter degradation and in the associated energy release (Meyer, 1994; Battin et al., 2003). In most cases, those biofilms constitute a complex assemblage of bacteria, fungi, protozoa and algae, embedded in a polysaccharide matrix (Lock et al., 1984). Bacterial extracellular enzymes accumulate in these biofilms and are responsible for the conversion of high molecular weight compounds to low molecular weight compounds which subsequently can be assimilated by heterotrophic microbes (Chròst & Overbeck, 1990). Moreover, heterotrophic bacteria mediate trophic transfer of DOC and carbon fluxes at all scales (Azam et al., 1983; Tranvik, 1988) and their activity can be controlled by naturally occurring changes in the amount and origin of organic matter (Fischer et al., 2002). Available dissolved organic matter (DOM) for biofilms can be either autochthonous (produced by algae) or allochthonous from other sources in the catchment. These DOM types substantially differ in quality. Autochthonous DOM is more labile and easily degradable by bacteria compared to the more recalcitrant compounds of humic substances from allochthonous origin.

Findlay et al., (2003), performed a series of experiments using small scale perfusion cores and large mesocosms and added different sources of DOC. They found strong effects of different sources of DOC on bacterial metabolism (O_2 consumption, production, enzyme activity) as well as on bacterial community composition. However, they did not manipulate microbial diversity.

Potential relationships between DOC sources and bacterial community composition and metabolism have been recently explored. Early studies on biodiversity effects on ecosystem functioning mainly focused on single ecosystem processes (Reiss et al., 2009). Hector and Bagchi (2007) and Gamfeldt et al., (2008) highlighted the importance of simultaneous effects of biodiversity on ecosystem functions, i.e. that different species perform multiple functions at the same time. Their results were monoculture derived therefore an analysis of the joint effects of several functions across a diversity gradient in natural bacterial communities is still lacking.

In this study we hypothesized that the reduction of diversity will determine the likelihood to sustain bacterial functions. In order to test this hypothesis we manipulate biofilm bacterial diversity and investigate its effect on the degradation of DOC as an ecosystem function. We installed laboratory-based bioreactors and manipulated resource availability by supplying biofilms in the reactors with labile and recalcitrant DOC. We measured five specific enzyme activities which are related to the degradation of DOC in natural communities, the quality and total concentration of organic carbon over time and reactor-wide oxygen consumption. The main goal of the present study was to investigate whether the relationship between biofilm diversity and ecosystem functioning is affected, by focusing on DOC degradation and use. The probabilities of the manipulated bacterial communities (with contrasting bacterial diversity levels) to sustain multifunctionality are tested under different DOC quality sources.

METHODS

Bioreactor design and experiments

A set of 36 reactors were installed in a constant temperature room (20°C) and kept in the dark. The bioreactors were designed according the description of Kaplan and Newbold (1995). Glass columns (app. 200 mL volume) were filled with borosilicate glass beads to amplify the attachment area (90,000:1 surface to volume ratio). Three-way valves at the inflow and outflow of the reactors were used as sampling ports. Peristaltic pumps constantly supplied the reactors with liquid medium at a flow rate of 1 mL min^{-1}. Resource quality was manipulated by providing two types of liquid medium: a mixture of carbon-free artificial lake water medium with either 15 mg L^{-1} labile and recalcitrant C (called labile treatment) or 15 mg L^{-1} recalcitrant C only (called recalcitrant treatment). The artificial lake water medium was prepared as described in Bastviken et al., (2004) and in the labile treatments was amended with a mixture of 7.5 mg L^{-1} of recalcitrant aged river water and 7.5 mg L^{-1} of an artificial labile carbon source (including 3.75 mg L^{-1} of cellobiose and 3.75 mg L^{-1} dipeptide leucine-proline, respectively, both Sigma-Aldrich, St. Louise, MO, USA). In the recalcitrant treatment 15

mg L^{-1} of aged river water (recalcitrant) was added to the artificial lake water medium. The river water was aged for 2 month at 20 ºC and contained 110.8 mg L^{-1} of total organic carbon (TOC). The reactors were assembled sterilely; the beads were muffled at 450 ºC for 4 h, while the reactors and tubing were steam autoclaved (121 ºC for 20 min). Medium retention time was estimated in a separate reactor by changing from pure water to a saline solution in the inflow and measuring conductivity in the outflow. Retention time was estimated to be ca. 300 minutes.

Two 10 days-experiments were performed. The first experiment was performed after 4 weeks acclimation (referred to as "younger biofilms"), while the second experiment was performed after 7 weeks acclimation ("older biofilms") (see below). In each experiment, we had three diversity levels each with 6 bioreactors, 3 of them corresponding to the labile treatment and the other 3 to the recalcitrant treatment. We measure individually TOC concentration and quality (absorbance at 250 nm), respiration rates as the difference in oxygen concentration between the in- and the outflow for the whole reactors and bacterial abundance in the outflow. After 10 days, the corresponding reactors were opened (destructive sampling) and the glass beads were sampled to measure five different enzyme activities (β-glucosidase, β-xylosidase, cellobiohydrolase, leucine aminopeptidase, and phenol-oxidase). The molecular community composition was analysed using Terminal Restriction Fragment Length Polymorphism (T-RFLP) along a three-step gradient from the inflow to the outflow.

Preparation of the bacterial inoculum and acclimation

A dilution-to-extinction approach (e.g. Szabo et al., 2007) of a natural microbial community was used to establish a gradient in diversity in pre-cultures. The dilution approach results in a non-stochastic removal of rare organisms first (Franklin et al., 2001). Numerically rare species are more prone to extinction and therefore the elimination of species from natural ecosystems is most likely not a random process either (Giller et al., 2004). In each dilution step, the rare species were removed from the community. This means that the low diversity treatment represents a reduced set of species present at the highest diversity.

The bacterial community used was collected from a natural bacterial biofilm of a river (Fibyån, Sweden, N 59° 53′ 7″ E 17° 20′ 43″). Sediment and water were sampled, homogenized and filtered (GF/F, Whatman) to eliminate eukaryotes. Batch cultures with particle free (< 0.2 µm, Supor, PALL) sterilized river water (2 times autoclaved at 121°C for 20 min, with a 24h interval) were installed and inoculated. Cell number was adjusted to 10^7 cells and stepwise (1:10) diluted to 10^1 cells. This resulted in a total of 24 batch cultures; twenty-one were seven dilution steps and their three replicates, the

remaining three were sterile controls. The cultures were allowed to grow at 20°C in the dark and bacterial abundance was measured daily. After seven days of incubation, three of the pre-cultures were chosen to inoculate the bioreactors. One culture from the largest nominal inoculum size (10^7 cells, high richness), one culture from medium inoculum size (10^5 cells, medium richness) and one culture from a small inoculum (10^3 cells) were used to inoculate the bioreactors. At that time, the cultures had reached similar abundances, avoiding the hidden effects of differences in starting biomass/abundance.

After inoculating the reactors, the cells were allowed to colonize the surface of the glass beads for 4 and 7 weeks, respectively for young and older biofilms. Bacterial abundance was monitored in the medium as well as TOC and dissolved oxygen concentration (see below for a description of the methods). The medium was recycled during the acclimation phase and once a week we replaced the medium (4 L) with freshly prepared medium and inoculated it with ca. 500 mL of the old medium to avoid additional dilution effects. Replicates for the diversity and carbon source treatments were connected to the same medium flask, during this acclimation phase. However, during the 10-days experiments, each replicate were connected to and independent flask and the medium was not further replaced.

Bacterial abundance estimation

Bacterial abundance in the bioreactor medium was monitored by flow cytometry of Syto13 (Molecular Probes, Inc.) stained cells (del Giorgio et al., 1996). Cells were sampled daily, fixed with 3.7% final conc. formaldehyde and stored at 4ºC until processing. The samples were analysed using a Partec Cyflow cytometer equipped with a Robbywell 96 well-plate autosampler. For this purpose, 150 µL of samples were loaded into the 96 well-plate and stained for 10 minutes with a 1.25 µM Syto13 solution. The detector settings were optimized for the samples to 465 FS1 (fluorescence at 508 nm) and 245 FFC (forward scatter).

Total Organic Carbon (TOC)

Samples for the analysis of TOC content were taken daily during both experiments from the in- and outflow of the bioreactors into muffled 17 mL vials. TOC concentration was immediately analyzed using Shievers 900 TOC analyzer with an accuracy range of ± 0.5 ppb. At the beginning and the end of the two experiments absorbance at 250 nm was measured using a spectrophotometer (Lambda 40, Perkin Elmer) and a 1cm quartz cuvette. Data from the absorbance measurement and the

TOC analysis were used to calculate the ratio Abs_{250}:TOC, which indicates the proportion of humic material in the total organic carbon pool (Fischer et al., 2006).

Oxygen consumption

Oxygen consumption was measured as an estimate of total respiration on days 0, 1, 5 and 10 during the two experiments. The consumption was estimated as the difference in oxygen concentration between the inflow and the outflow. For this purpose, medium was withdrawn directly before and after the passage through the bioreactors using a three-way valve. For the measurement, the medium passed through a flow-through oxygen probe (FTC, PreSens, Germany), connected via an optical fibre to a Fibox 3 oxygen meter (PreSens, Germany). The measurements were temperature-compensated and excess medium was discarded.

DNA extraction and terminal restriction fragment length polymorphism (T-RFLP) analysis

Cells were harvested by filtering 50 mL of the bacterial community growing on the glass beads onto 0.2 µm membrane filters (Supor, Pall) and stored at -80 ºC. DNA extraction was carried out using the Ultraclean Soil DNA extraction kit (MoBio Laboratories). Frozen filters were cut into pieces, directly added to the bead tubes and then treated according to the manufacturer's instructions for maximum yield. DNA extracts were used as templates for PCR amplification of the 16S rRNA gene with the universal primers 27-forward, labelled with hexachlorofluorescein (HEX) and unlabelled 519-reverse. Thermocycling was carried out with a MyGene MG 96 Thermocycler (Longene Scientific Instruments, Hangzhou, China) using an initial 30 s denaturation at 98ºC, 28 cycles of 98ºC for 10 s, 50ºC for 30 s and 72ºC for 30 s followed by a final 7 min extension step at 72ºC. Pooled PCR products were purified using MultiScreen PCRµ96 plates (Millipore). Reactions of the PCR product with the restriction enzymes hae I and hinf I and corresponding buffer were incubated at 37º C for 16 h (Liu et al., 1997). Terminal fragments were sized by electrophoretic separation and detection on a capillary sequencer (ABI 96, Applied Biosystems). The size and quantity of terminal restriction fragments were analyzed using GeneMarker software.

Extracellular enzyme activities

We measured the activity of five extracellular enzymes produced by the biofilm communities: cellobiohydrolase and β-glucosidase, involved in cellulose degradation (cellobiohydrolase degrades the polymer cellulose to cellobiose, and β-glucosidase decomposes cellobiose to glucose; Deshpande & Eriksson, 1988); β-xylosidase, which

degrades xylobiosic molecules and is involved in degradation of hemicellulose (Lachke, 1988); leucine-aminopeptidase which drives peptides decomposition (Hoppe et al., 1988); and phenol-oxidase responsible for lignin degradation (Sinsabaugh et al., 1994). β-glucosidase (EC 3.2.1.21), β-xylosidase (EC 3.2.1.37) and cellobiohydrolase (EC 3.2.1.91) were determined spectrofluorometrically by using fluorescent (MUF, methylumbelliferone)-linked artificial substrates (4-MUF-β-D-glucopyranoside, 4-MUF-7-β-D-xyloside and 4-MUF- β-D- cellobioside, from Sigma-Aldrich). Leucine-aminopeptidase (EC 3.4.11.1) was analyzed by using the fluorescent-linked artificial substrate L-leucine-7-amido-4-methylcoumarin hydrochloride (7-AMC-leucine from Sigma-Aldrich). Phenol-oxidase activity (EC 1.14.18.1) was measured by using L-3,4 dihydroxyphenylalanine (L-DOPA, Sigma-Aldrich). In a pre-study, two bioreactors were colonized for one month in order to determine the saturation curves for each of the five enzymes. Based on these results we used 0.3 mmol L^{-1} substrate for β-glucosidase, β-xylosidase and leucine-aminopeptidase, 0.8 mmol L^{-1} substrate for cellobiohydrolase and 5 mmol L^{-1} for the measurement of phenol oxidase activity. During the experiment, substrates were added to 4 mL beads, which were kept for 2 h in the dark at 20ºC under continuous shaking. Cellobiohydrolase, however, was incubated for 5 h. Blanks and standards of MUF and AMC were included. At the end of the incubation period, glycine buffer (pH 10.4) was added (1/1 vol/vol), and fluorescence was measured at 360/465nm excitation/emission for MUF and AMC using a plate reader (Ultra 384, Tecan). Blanks were subtracted from the samples to correct for hydrolysis of the substrate or fluorescent substances in the water solution. Phenol oxidase activity was measured following the method outlined by Sinsabaugh et al., (1994). Incubations were performed for 2 h at 20ºC on agitation in darkness. At the end of incubations, the absorbance of the liquid phase was measured at 460 nm (Lambda 40, Perkin Elmer).

Multifunctionality

The logic outlined in Gamfeldt et al. (2008) was used in order to address multifunctional effects of diversity treatments. Overall functioning is understood as the joint effects of multiple constituent functions and cannot be expressed as the average of those functions because a decline in one function cannot be compensated by an increase in another function (Gamfeldt et al., 2008). Therefore a specific level must be defined for each functioning (enzyme activity) to be sufficient to sustain a function in the community. If any of the individual functions drop below the threshold, the specific function should be considered lost and multifunctionality impaired. Setting an initial threshold at 50% of the maximum enzyme activities for each enzyme meant that as long as the community was able to perform 50% of the maximum enzyme activity of all

samples, we would regard it as positive. However, when functioning of a specific enzyme dropped below 50% was considered a loss of function. We calculated the probability that at least one sample within the reactor would perform above the threshold level for all five enzyme activities. Subsequent steps were taken by increasing the threshold level to 0.75 and 0.9 of the maximum activity.

Statistical analysis

Non-metric Multidimensional Scaling (nMDS) using Bray-Curtis Similarities on the peak height (= relative abundance) data derived from the molecular fingerprinting of the 16S rRNA genes were calculated using PAST ver. 2.01 (Hammer et al., 2001). Variability in the enzymatic activity measurements (β-glucosidase, β-xylosidase, cellobiohydrolase, leucine aminopeptidase, and phenol-oxidase) were analyzed by multivariate analyses of variance (MANOVA). This analysis was used to test for the single-source effects and the interactions between three factors: diversity (high, medium and low), organic carbon source (labile or recalcitrant) and the position within the gradient from the in-to the outflow (inflow, middle and outflow). Differences of TOC content, oxygen consumption and Abs_{250}/TOC between the sampling days and treatments were checked by using ANOVA's. All variables included in the analyses were log(x+1) transformed in order to attain homogeneity of variances and normal distribution. All statistical analyses were performed using the SPSS software package for Windows (ver.14.0.1, SPSS Inc., 1989–2005).

RESULTS

Abundance

Cell numbers in the medium increased steeply during the acclimation phase and reached between 6×10^5 and 1.5×10^6 cells mL^{-1} in the outflow within the four weeks period. During the experiments, bacterial abundance in the outflows ranged between 1.5×10^5 and 1.8×10^6 cells mL^{-1}, irrespective of the diversity treatment. Bacterial counts in the outflow were slightly higher in the younger biofilms compared to older ones and culture growing on the labile carbon source reached slightly higher numbers compared to cultures growing on the recalcitrant carbon source.

Bacterial community composition

In total 77 different operational taxonomic units (OTUs) have been detected by Terminal Restriction Fragment Length Polymorphism, with 34 OTUs detected by the restriction enzyme hinf I and 43 by hae III. On average, 14 ± 2 different OTUs per sample were detected, with no difference between the diversity treatments (ANOVA,

p=0.12). Non-metric multidimensional scaling (nMDS) was used in order to graphically analyse community composition (Fig. 1). When all samples were included in the analysis, the high diverse treatments clustered closely together, while the medium and low diverse samples occupied more space in the bivariate plane (Fig. 1A). Separate analysis of the high, medium and low diverse treatments revealed a strong impact of age of the reactors and the carbon source, while the gradients in community composition from the inflow to the outflow were less pronounced. Under high diversity, the outlines of the treatments overlap considerably, although they appear clearly separated by age (Fig. 1B). This separation of community composition by carbon source and age increased in strength when diversity was reduced (Fig. 1C and 1D)

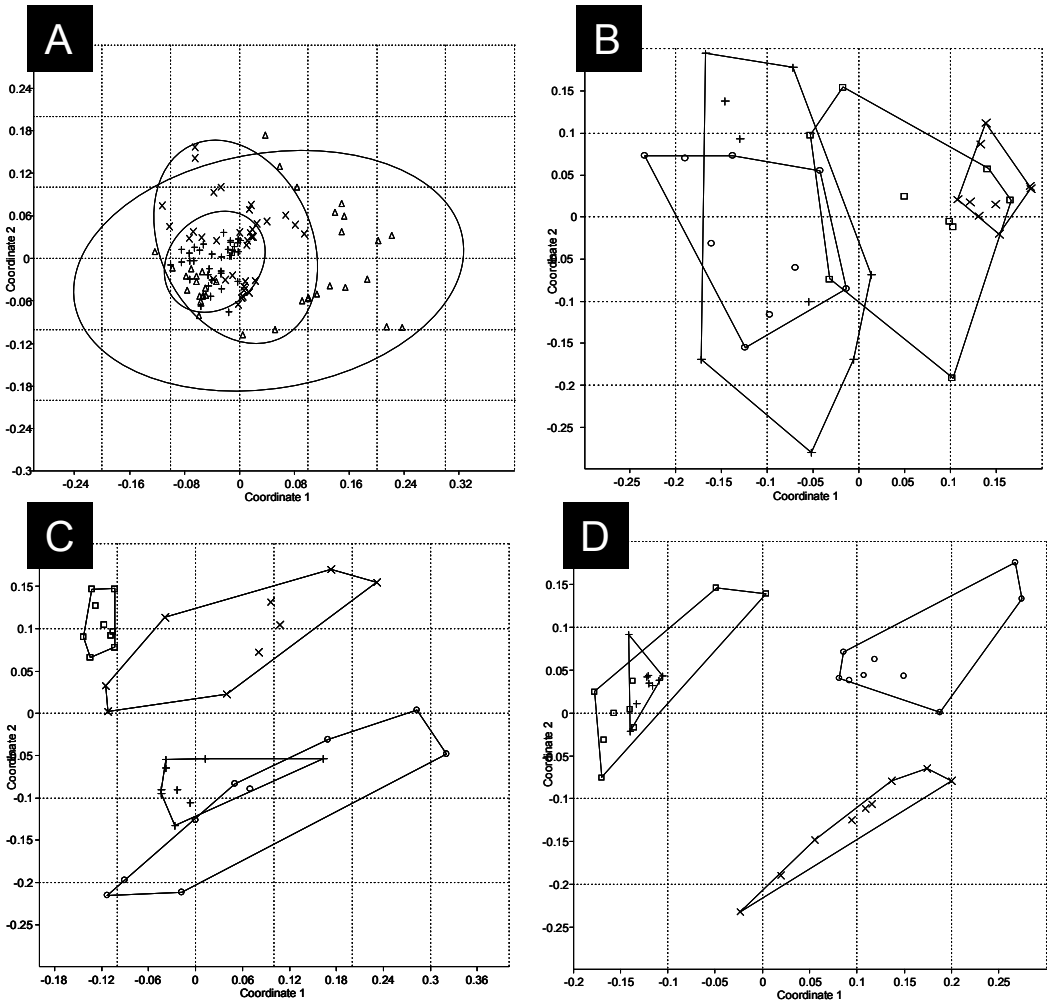


Fig. 1. Bacterial Community Composition. Non-metric multidimensional scaling (nMDS) of the 16 S rRNA community profiles of the whole dataset (A), only high (B), only medium (C) and only low (D) diversity treatments. In panel (A), circles indicated 95 % intervals (bootstrapped) for the diversity treatments (+ high diversity, × medium diversity and Δ low diversity). In panel B, C and D the outlines indicate the groups of □ recalcitrant-older, × labile-older, + labile-younger, ○ recalcitrant-younger communities.

Total organic carbon

The initial labile TOC pulse concentration was rapidly degraded until day 1 and increased slightly in the last sampling days (ANOVA, $p<0.001$) in both experiments (younger and older biofilms). Bioreactors amended with recalcitrant TOC showed no TOC degradation throughout both experiments but, similarly to the labile TOC treatments, a slight TOC increase was observed in the last days of the experiment (Fig. 2). TOC degradation at the bioreactors was not significantly affected by the diversity treatments (ANOVA, $p>0.05$); the TOC utilization pattern was the same for the high, medium and low diversity.

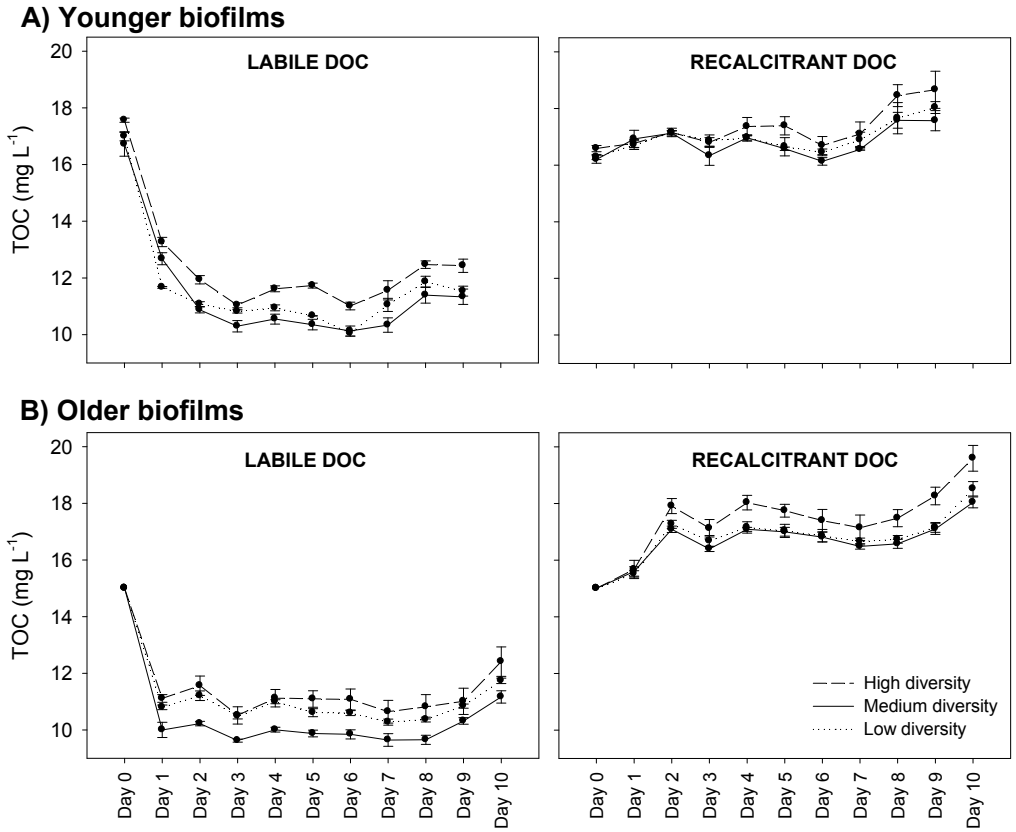


Fig. 2. TOC concentration during the 10 days of experiment in the younger biofilms (panel A) and older biofilms (panel B) for the labile or recalcitrant DOC and in three diversity levels (high, medium, low). Error bars indicate ± standard deviations, n=9.

The ratio Abs_{250nm}/TOC was used as a proxy for the quality of the organic substrate. In the recalcitrant treatments, a similar amount of humic substances was detected between day 0 and 10 in the younger biofilms (ANOVA, $p=0.999$). In the older biofilms, the recalcitrant humic substances were strikingly low on day 10 (ANOVA, $p<0.001$; Fig. 3). In the labile treatments, a significant increase of the contribution of humic substances on TOC was observed from day 0 to day 10, following a similar trend

in younger and older biofilms. The diversity treatments did not influence these patterns of DOC utilization.

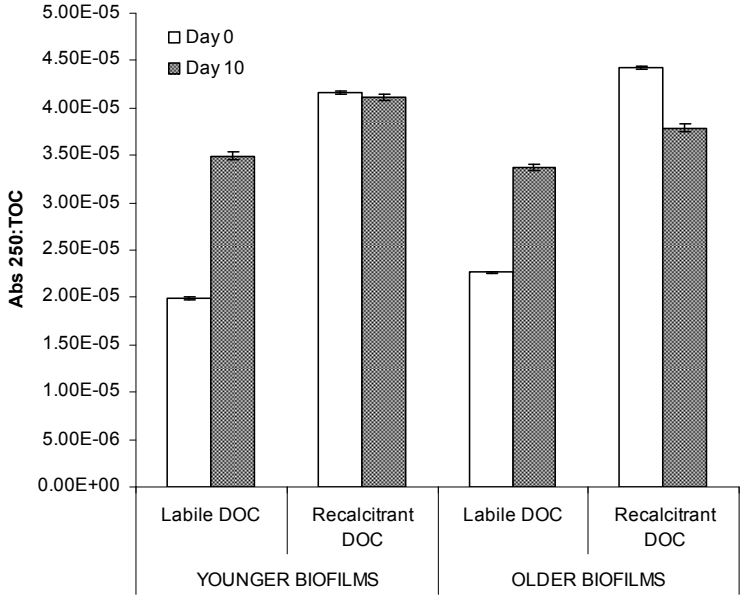


Fig. 3. Change in ratio of absorbance at 250 nm to TOC between the start (day 0) and the end (day 10) of the two experiments (younger and older biofilms) for reactors fed with labile and recalcitrant carbon, respectively. Error bars indicate ± standard deviations, n=9.

Oxygen consumption

Oxygen consumption was mainly driven by substrate availability (Fig. 4). In both experiments (older and younger biofilms), respiration was much higher in the labile treatments than in the recalcitrant treatments (ANOVA, $p<0.001$). However, a strong progressive decrease in oxygen consumption was detected in the labile treatments (ANOVA, $p<0.001$), reaching to similar values as in the recalcitrant treatments since day 5. No significant differences between the three levels of diversity were observed (ANOVA, $p=0.621$).

Extracellular enzyme activities

The enzyme activities were generally much higher in the labile treatments than in the recalcitrant treatments (ANOVA, $p<0.001$, Fig. 5). However, phenol-oxidase was very low and similar under labile and recalcitrant conditions. All enzyme activities were enhanced in the older biofilms with respect to the younger biofilms (ANOVA, $p<0.001$).

The enzyme activities followed the diversity gradient in the younger biofilms amended with labile compounds, with the highest values measured under high diversity, followed by medium and low diversity (Tukey's test, $p<0.001$). In the older

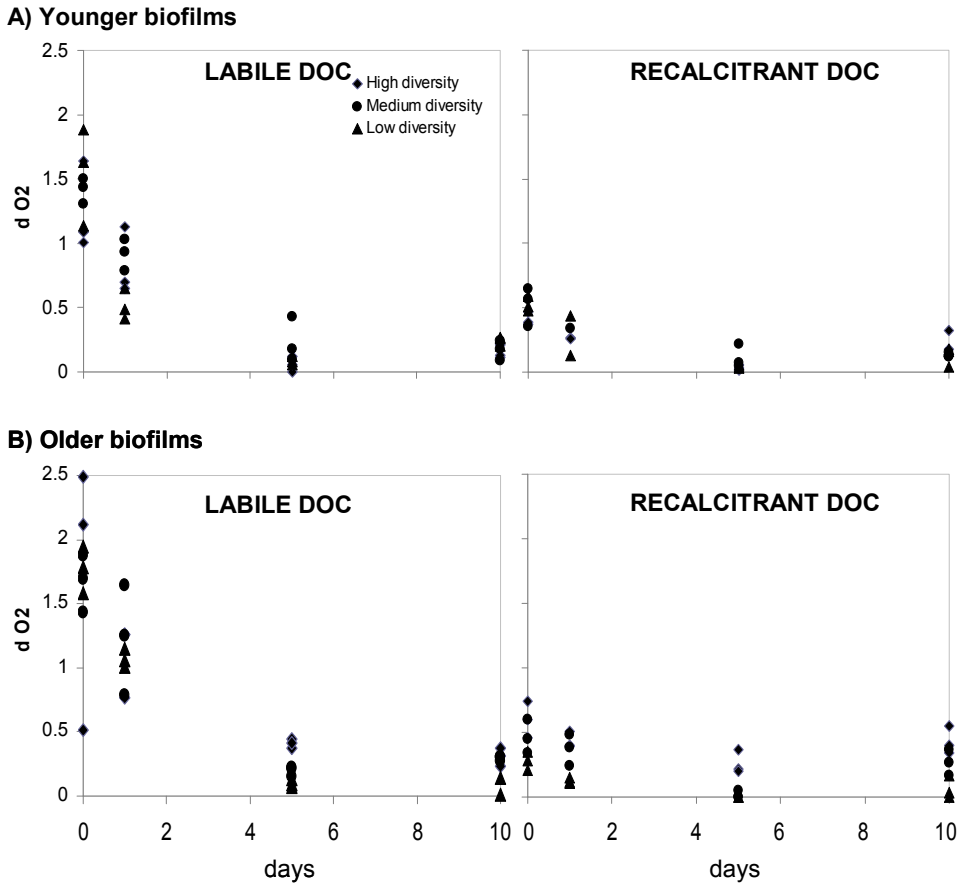


Fig. 4. Reactor-wide respiration in day 0, 1, 5 and 10 in the younger biofilms (panel A) and older biofilms (panel B) for the labile or recalcitrant DOC and in three diversity levels (high, medium, low).

biofilms, the effect of the addition of labile DOC on the extracellular enzyme activities was similar compared to the younger biofilms; higher enzyme values occurred in the high and medium diversity and decreased in the low diversity treatments (Tukey's test, $p<0.001$), mostly in the activities related to polysaccharides decomposition (cellobiohydrolase, β-glucosidase and β-xylosidase). Low diverse communities expressed ca. 65% of the β-glucosidase and β-xylosidase activity of the high diversity treatments. Cellobiohydrolase reduced to 33% of the activity in relation to the high diversity treatments, both in the younger and older biofilms. In contrast, the reduction of the peptidase activity following bacterial diversity reduction was much less evident. This reduction was not significant for the younger biofilms and for the older biofilms 88% and 84% of the maximum activity occurred in the medium and low diversity treatments respectively.

The decrease in enzyme activities with decreasing diversity was much more evident under the reception of recalcitrant DOC. Enzymes degrading polysaccharides (cellobiohydrolase, β-glucosidase and β-xylosidase) and peptides (peptidase activity)

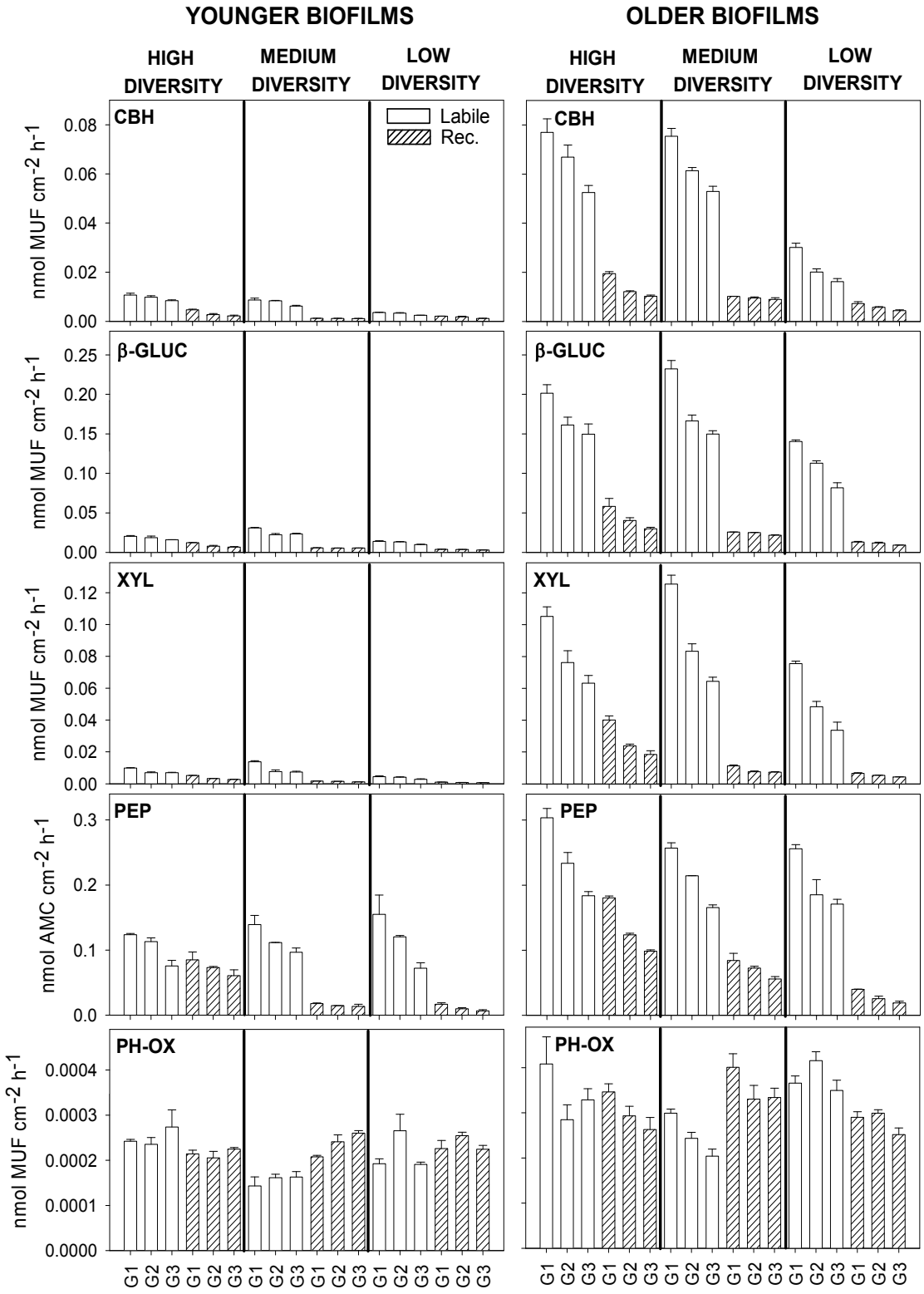


Fig. 5. Extracellular enzymatic activities (cellobiohydrolase, β-glucosidase, β-xylosidase, leucine-aminopeptidase and phenol-oxidase) in the three diversity treatments (High, Medium and Low diversity) in the two sampling occasions (younger and older biofilms). G1, G2 and G3 acronyms correspond to the three glass beads gradients (from the in- to the outflow). Mean ± standard error bars were shown, n=3.

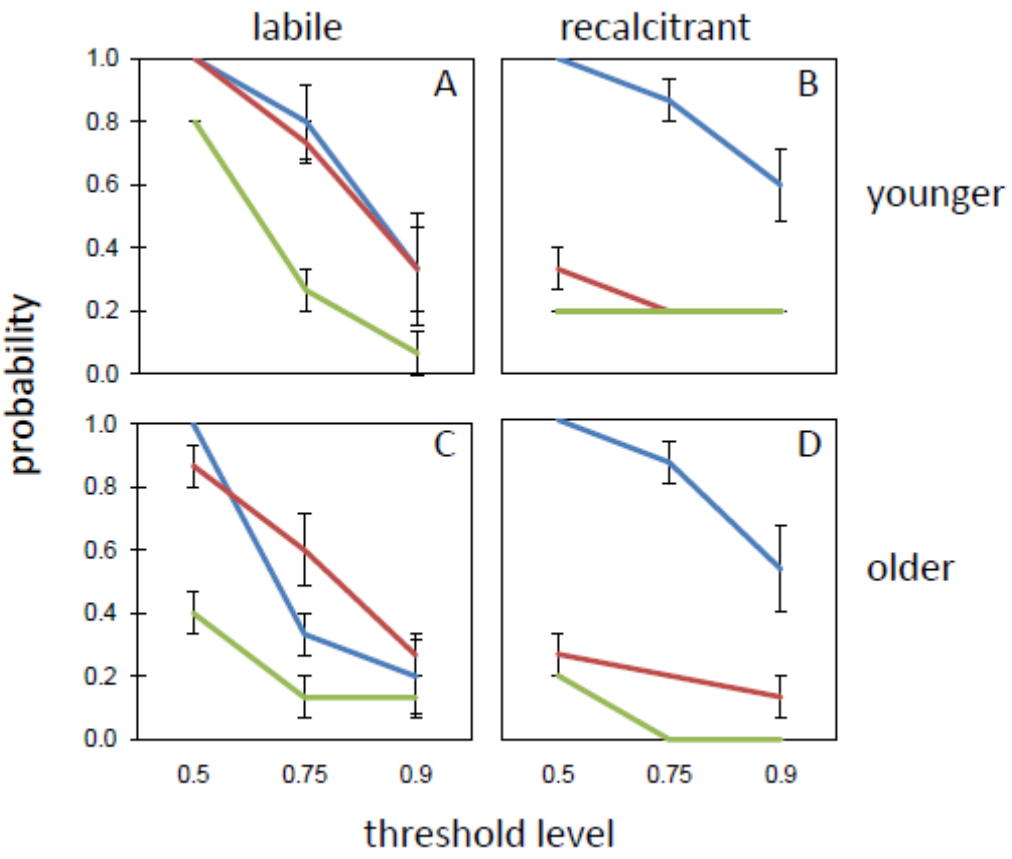


Fig. 6. Probabilities for all 5 enzymes to be represented above a threshold level from 0.5, 0.75 and 0.9 of the maximum activities among reactors fed with labile (A and C) and recalcitrant (B and D) organic carbon. Shown are differences in the likelihood to sustain joint functioning for younger (A and B) and older (C and D) biofilms. Blue lines represent the average high diversity treatment, red lines the medium diversity treatment and the green lines the low diversity treatment. Error bars indicate standard error estimates, n=3.

were higher under high diversity, followed by medium and lower diversity (Tukey's test, $p<0.001$). The values at low diversity decreased to 20-30% of those measured at high diversity. No differences between younger and older biofilms were measured.

The five enzymes clearly showed that there was a marked gradient from the inflow to the outflow with a progressive reduction of all the activities along the reactors (Tukey's test, $p<0.001$, Fig. 5).

Multifunctionality

The estimates of diversity loss on multifunctionality are shown in Figure 6. The analysis of the probability to find all five enzymes active above a certain threshold strongly depended on the source of organic carbon and on the age of the reactors. As expected, the consequences of species loss on joint ecosystem functioning were much

more dramatic when the threshold level stepped from 0.5 to 0.75 and 0.9, however there were also large differences between the diversity treatments. In general, at higher diversities the probabilities were much higher as compared to treatments where diversity was reduced. Reactors which were fed with a labile source of organic carbon, where able to compensate to a certain extend for the loss of diversity, however differences between diversity treatments were more pronounced when the biofilms were older. In reactors which were fed exclusively with recalcitrant carbon, the effects of diversity on multifunctionality were very strong, with a reduction of the likelihood to sustain all enzymes from 1 at highest diversity to 0.2 at lowest diversity (threshold 0.5). In this treatment, only the highest diversity could maintain a sustainable level of functioning considering the three thresholds levels. Again, younger biofilms were less affected by diversity loss than older biofilms.

DISCUSSION

How bacterial diversity and ecosystem function are related is an ongoing debate in ecology. Most research on the relationship between diversity and functioning has been done for short times and in uniform environments (Hillebrand & Matthiessen, 2009). Only recently the influence of resource complexity on microbial diversity has been brought into focus (Langenheder et al., 2010; Singer et al., 2010). The present results help to address the mechanistic controls of this relationship and suggest that the reduction of the microbial diversity leads to a loss of functions. The obtained data also highlight that maintaining high diversity is key to sustain multifunctionality.

The dilution of a natural occurring complex community to create a gradient in diversity seemed to be successful although the number of OTUs detected by the molecular fingerprinting technique is restricted in the ability to support this conclusion (Bent et al., 2007). The dilution approach results in removal of rare organisms first (Franklin et al., 2001) but TRFLP only shows community composition of the most abundant members, doesn't tell us about the diversity. Non-metric multidimensional scaling just showed that the communities were different; along the dilution gradient similarities between bacterial communities were reduced. This might be the effect of specific species being present in the cultures depending on the initial evenness of the communities. When diversity is high, the likelihood to include a specific species that would dominate the communities in the biofilms was high too. However, these specific species might be removed from some of the medium and low diversity treatments, where other species would then dominate the community. Consequently, under high diversity all species are present, while under low diversity only the initially most abundant species remained. The bacterial composition in the reactors also depends on

the age of the biofilm and the resources available. Those effects seem to be greater in the low diversity treatments, where the older and younger biofilms were more dissimilar if a labile or recalcitrant DOC was available. Variations in DOM supply may affect the abundance of the phylogenetic groups if there are systematic differences in how these groups use or respond to DOM or in the case that these groups are consistently dominated by a few species that differ in DOM utilization (Pinhassi et al., 1999; Cottrell & Kirchman, 2000; Findlay et al., 2003). Thus, the DOC quality exerts a greater effect on the lower diversity community composition than on the medium or high diversity. Biofilm age or carbon source affected the relative abundance of the heterotrophic bacterial groups which could have an impact on DOM hydrolysis (Olapade & Leff, 2004; Judd et al., 2006).

Differences in bacterial function due to DOC availability and biofilm age were measured. Respiration rates where much higher in the labile bioreactors than in the recalcitrant ones (Claret, 1998) until the labile DOC was depleted. Changes in humic content of the TOC might indicate that when labile compounds are available, the most biodegradable C is used first and later the most recalcitrant. In contrast, when a recalcitrant compound is available, this might be slowly degraded decreasing the proportion of humics (shown in our experiment by the decrease in Abs_{250}/TOC after 10 days).

The more specific microbial function of organic matter decomposition capacity (as shown by the extracellular enzyme activities) was also clearly affected by the quality of the DOC available as well as by the age of the biofilm (Battin et al., 2003). Almost certainly, the addition of the labile organic matter source provided N and C to the bacterial community which might lead to increased heterotrophic biomass (Olapade & Leff, 2006). Therefore, higher enzymatic activities were measured in those bioreactors (Ylla et al., 2009) than in those amended with humic substances. At the same time, humic substances might also cause the complexation and inactivation of bacterial enzymes (Freeman et al., 1990; Wetzel, 1992).

When integrating the biofilm functionality (as expressed by the five enzyme activities) at the different diversity levels, biofilm age and DOC availability, the results show a diversity-multifunctionality relationship. Many studies report that higher bacterial diversity is linked with higher bacterial functions (Langenheder et al., 2010; Tilman et al., 1997). Our results are in accordance with these studies, thus under the higher diversity level the expression of four out of the five enzymes analyzed (in exception of phenol-oxidase) was greater, decreasing at the medium and low diversity levels. The loss of enzyme activities was, however, not equal for all measured enzymes. Under the labile treatments, the activities related to degradation of complex polysaccharides such

as cellobiohydrolase (involved in cellulose degradation) showed a greater loss of activity than other more energetically relevant for bacteria such as β-glucosidase and β-xylosidase, (decomposing simple polysaccharides and providing monomeric sugars) and leucine aminopeptidase (decomposing simple peptides). Especially the peptidase activity showed the lowest loss degree with decreasing bacterial diversity. Therefore the loss of functionality is not “equal” for all functions. At the same time, the effect of decreasing diversity on the reduction of enzyme multifunctionality was much greater when the biofilms were submitted to a recalcitrant C source, where all functions were drastically reduced with diversity loss. The process of acquiring C from complex molecules such as humic substances needs activation of more specific and probably less common enzyme activities. It can be considered that leucine- aminopeptidase and β-glucosidase are enzyme activities commonly expressed by the whole bacterial community being present and active in all bacterial species. However, cellobiohydrolase and phenol-oxidase might be less abundant, not expressed by all bacterial species, and/or expressed when there is the need to degrade complex substances such as hemicellulose, cellulose and humic compounds in case other labile compounds (such as peptides and polysaccharides) are no longer available.

To our knowledge, this is the first study to manipulate microbial diversity in biofilms and investigate the effects on a wide range of functions from a multifunctional perspective. In natural systems the interplay of several enzymes might be of importance for ecosystem functioning, and a multifunctional perspective should allow further insight in those complex interactions. The results from the present paper on the effects of a loss of diversity on multifunctionality along a gradient in diversity shows that the consequences of species loss on joint ecosystem functioning were much more dramatic when the threshold level was increased from 0.5 to 0.75 and 0.9. However which threshold is the one required in natural systems to sustain ecosystem functioning will likely differ for different sets of functions as well as for different ecosystems. In microbial communities average diversity effect sizes in aquatic and terrestrial ecosystems correspond to a reduction of 40% in functioning (Cardinale et al., 2006, Gamfeldt et al., 2008). Our study also showed that multifunctionality was determined by the age of the biofilm community. Multifunctionality was less affected in younger compared to older biofilms, which might reflect the relevance of succession in the interactions between species. Biofilms differ in their abilities to utilize labile or recalcitrant sources of organic carbon depending on their molecular diversity and environmental heterogeneity (Singer et al., 2010), which are summarized by the successional stage. Biodiversity effects on functioning become more relevant with time

(Hillebrand & Matthiessen, 2009), and differences in biofilm age in the reactors extends this notion.

The substrate source also influenced the way multifunctionality was maintained. Our mixed medium of labile and recalcitrant sources was able to compensate to a certain extend for the reduction in diversity, however, if the substrate contained only the complex, recalcitrant carbon source, only the highest diversity could maintain a sustainable level of functioning. Hence, high species diversity increases the likelihood that the genetic capacity to decompose humic substances is present within the heterotrophic biofilm (Findlay et al., 2003). In the mixed medium, the resource diversity could lead to higher functioning due to a reduction in bacterial competition and enhancing complementarity among species due to species-specific trade-offs.

ACKNOWLEDGEMENTS

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GENERAL DISCUSSION

GENERAL DISCUSSION

ORGANIC MATTER AVAILABILITY AND USE UNDER GLOBAL CHANGE PREDICTIONS

Recycling of organic matter is one of main river ecosystem functions. The rates and efficiencies of organic matter accumulation, transport, biogeochemical transformation and microbial use are highly dependent on river hydrology. Global climate change may favor more intense and more frequent occurrence of floods and droughts, as well as increase the stream water temperature. Comprehending the effects of these disturbances on material processing and the potential role and implications of the biofilm community is highly relevant. The hypothesis of the present thesis is that abrupt water flow changes (water disappearance and return) in headwater streams will impact the organic matter concentration and composition in the flowing water as well as in the benthic substrata. The results obtained are in accordance with this hypothesis: the availability of OM (quantity) for microbial use, as well as its food quality is affected by the pre- and post-drought periods.

Prior to the drought period, a high accumulation of benthic material derived from in-stream primary producers (autochthonous OM) occurred, which provided high quality OM to the stream food web (high concentration of polysaccharides and peptides). During the drought process, polysaccharides, amino acids and lipids progressively decreased in the benthic substrata. In spite of this decrease the total polysaccharide, amino acid and lipid content in the benthic organic matter were on average higher during the drying than during the rewetting period. The water was progressively enriched in its particulate fraction, with higher polysaccharide and lipid content, and lower concentration of peptides. Besides, there was a reduction of polysaccharides and amino acid content in the dissolved water fraction. Altogether, the quality and biodegradability of materials in the dissolved fraction progressively decreased during the drought. These changes in the quality of the OM were accompanied by changes in extracellular enzyme activities in the benthic substrata. Leucine-aminopeptidase and lipase activities decreased and β-glucosidase activity increased during the drought period. As drought progressed, stream microbial heterotrophs mostly used a lower quality organic matter source (polysaccharides, providing only C). Peptides (providing N and C sources) were no longer available during that period. The detailed study of the amino acid composition also revealed that in spite of the similar occurrence of amino acids in the benthic materials throughout the wet-drought-wet period, there was a progressive OM degradation during the drought (reduction of essential amino acids and

progressive decrease of the degradation index), particularly in sand sediments and leaf material. The amino acids composition showed that the compartment with the most degraded and lowest quality OM was the sand biofilm, whereas the highest-quality organic matter (high degradation index and high N content) occurred on cobble biofilm substrata. In DOM, the amino acid composition differed notably from the benthic amino acids. Furthermore, the amino acid composition of DOM was highly different between the pre-drought and post-drought periods (low degradation index and low N content). The pre-drought DOM was mostly algal-derived, and had a different composition than the degraded post-drought DOM (which was mostly terrestrially derived).

The return of the water caused the fast recovery of the microbial activity (evidenced by the increase of active bacteria), which can come from external sources (with the water flow) and/or from the river bed (withstanding desiccation and using refuges). The September flow peak basically had two effects: the cleaning of the streambed and the downstream transport of these materials. This return flow moved and redistributed streambed materials, including the OM accumulated during summer drought. This OM was mostly made up of allochthonous (terrestrial) material coming from the decaying leaves and plant litter. This leaf material produce a fresh and biodegradable high quality lixiviates (Francis & Sheldon, 2002). There was also a noticeable fraction of autochthonous material derived from algal origin. The scouring during rewetting was responsible for the loss of benthic materials (decrease in polysaccharide, amino acid and fatty acid content), and the increase in high quality organic matter in transport (at that time polysaccharides and amino acids accounted for 30% of the total DOC). The amino acid degradation index as well as the DOC, DON, BDOC and most extracellular enzyme activities peaked, revealing the remarkable transport of dissolved high quality C and N. This first response after rewetting lasted for a week, and then returned to basal values. This response indicated a fast and efficient microbial use of available labile fresh material transported during rewetting (Fig. 1; Romaní et al., 2006).

The progressive and gradual drought effects, as well as the fast recovery after rewetting could be buffered by the interaction of the individual dynamics of each benthic substratum. Sand sediments and leaf material provided refuge for microorganisms and organic matter storage; rocks and cobbles hosted an active bacterial community in the rewetting. The composition of specific amino acids as well as differences in degradation indices was informative of the different natures and the diagenetic state of the accumulated material in dissolved and benthic OM. Amino acids could be used as a tool to detect changes in organic matter characteristics. The steadier amino acid composition in the benthic compartments despite the relevant

hydrological changes occurring in the stream, reflected the in situ OM sources (autochthonous), which declined in quality along the drought period. Instead, the amino acid composition of DOM reflected the heterogeneous mixture of source inputs: autochthonous inputs prevailed during the pre-drought while allochthonous inputs dominated during the post-drought.

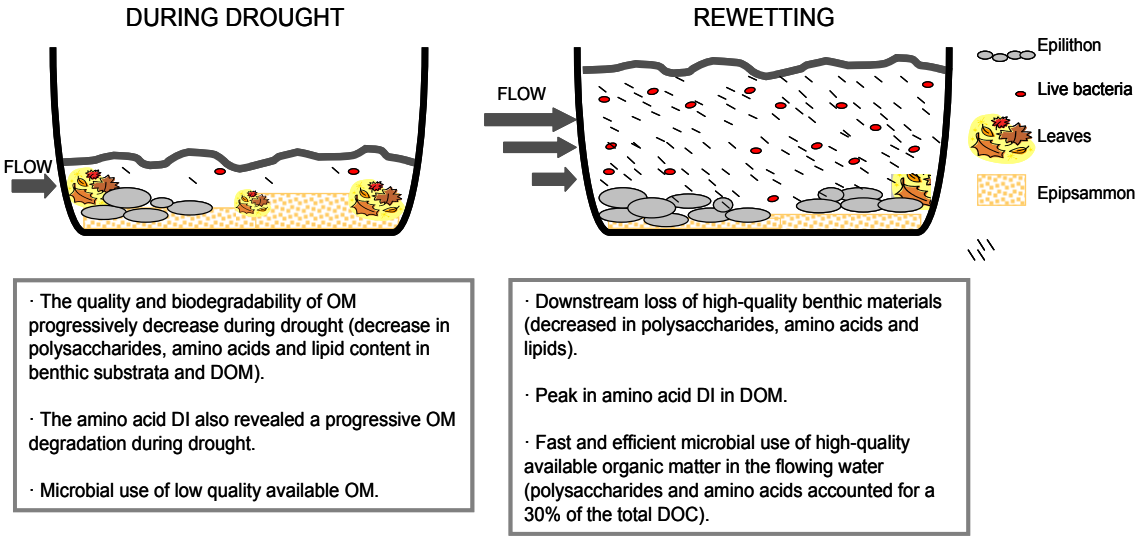


Fig 1. Schematic representation of the stream section during drought and during the rewetting. A differential distribution and transport of the organic matter between these two moments is represented.

Given that climate change will likely intensify the frequency of droughts in Mediterranean streams, the knowledge of the effects of these disturbances on the materials and biota could contribute to reliable resource management. For instance, it can be derived from our results, that the benthic substrata heterogeneity within the stream may be important for stream recovery after droughts, and that altering this distribution would hamper recovery. The results also show that depending on the hydrological river conditions (drought or wet periods), the differential changes in benthic organic matter quality and quantity might determine changes in the quality and quantity of organic matter loss which apart from affecting the microbial communities they will also affect the meio and macrofauna usually feeding on it (Phillips, 1984; Dauwe & Middelburg, 1998).

Climate change could also lead to increase stream water temperature, which could influence the OM microbial use and recycling (Porcal et al., 2009). Current climate models predict that mean annual stream water temperature will increase by

2.2-4.3 ºC by 2100 (IPCC, 2007). In this thesis, we test whether increasing stream water temperature may differentially affect the decomposition of labile and recalcitrant material by the microbial biofilm communities. The temperature sensitivity of organic carbon decomposition has been extensively studied in soils, but controversial conclusions were obtained (Davidson & Janssens, 2006; Kirschbaum, 2006). Our results highlighted that the recalcitrant OM would be more intensively used under higher water temperature, while the use of labile carbon would be barely affected by the temperature increase (Fig. 2).

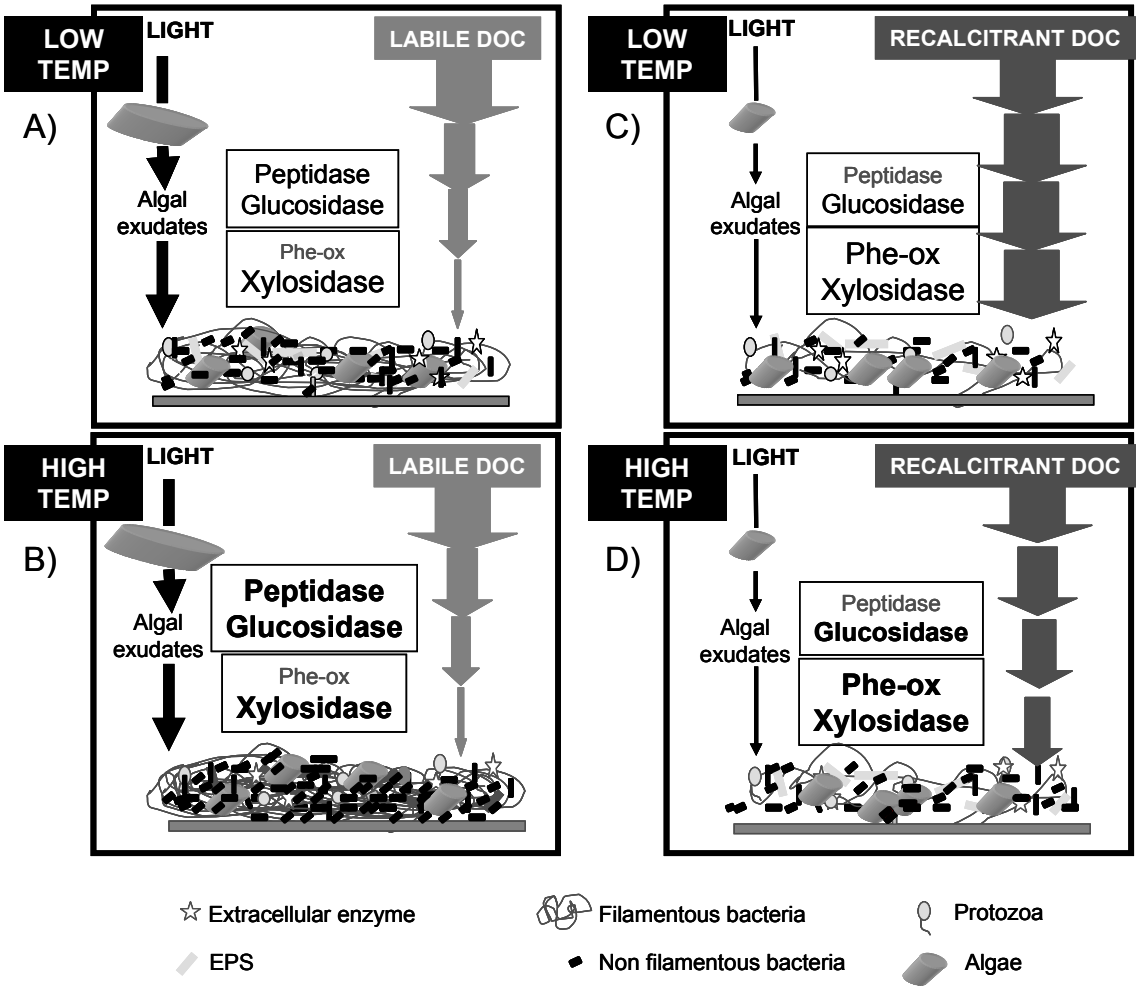


Fig. 2. Scheme of biofilm structure and function in relation to temperature and DOC availability (labile or recalcitrant). Under labile treatments (A and B) there was higher bacterial biomass and respiration. The labile DOC was rapidly taken up by freshwater biofilms equally under both temperatures (high and low temperatures). Under high temperature (B), enzymes activities were enhanced (higher peptidase, glucosidase and xylosidase). Under recalcitrant conditions (C and D), lower light arrives to the biofilm and stimulates the increase in chlorophyll-*a*. However, primary productivity was low due to a potential depression effect of humic matter. The lowest peptidase activity could be related with the inhibitory effect of that humic matter or an indirect effect after photosynthesis inhibition. Nevertheless, recalcitrant DOC triggered the expression of phenol-oxidase activity. The recalcitrant DOC was faster decomposed under higher temperature

treatments (D). A temperature increase also enhanced enzymes activities expression (higher glucosidase, phenol-oxidase and xylosidase).

DOC inputs shifts from high quality-labile DOC (e.g., in spring when primary productivity is high or during water flow peaks after summer drought) to recalcitrant DOC (e.g., after long periods of organic matter accumulation, occurring during summer drought or after leaf fall) in temperate streams. Warming will have a different effect on these DOC sources; recalcitrant materials will decompose faster, while labile sources will be less affected because they are so rapidly taken up by freshwater biofilms that temperature increase might be irrelevant. Therefore, pulses of terrestrial material, such as recalcitrant humic substances, in streams of temperate regions might be most affected by temperature increases that can accelerate their microbial use and decomposition. As a consequence, the predicted increase of 4ºC could determine lower inputs of humic material reaching downstream reaches.

EFFECTS OF BIOFILM ALGAL-BACTERIAL INTERACTIONS TO ORGANIC MATTER USE

It is hypothesized that the inputs of materials as well as its nature (chemical composition) and intensity (concentration in the river) will determine the structure and function of the autotrophic and heterotrophic benthic community, their possible interactions and their microbial composition. A laboratory experiment was designed in order to address the above hypothesis, and the results showed that the availability of labile organic matter in the flowing water and the presence or absence of light modified the biofilm composition and function, therefore affecting the processing capacity of organic matter in stream ecosystem. Glucose availability affects the microbial community composition considerably and favoured the heterotrophic biomass and activity, leading to a greater use of polysaccharides. Light conditions allows the development of a mixed, more diverse biofilm community comprising auto- and heterotrophic members and favors the heterotrophic use of peptides released by algae. The effects of readily available organic carbon were greater in dark-grown than in light-grown biofilms (Fig.3). Under the latter conditions, the release of organic molecules by phototrophs clearly benefits the activity and growth of heterotrophic microbiota by buffering their response to the artificial addition of organic matter. Consequently, the autoheterotrophic biofilm showed a tighter bacterial–algal link when not supplemented with readily available organic carbon.

Results could be extrapolated to the ecosystem (stream) scale of oligotrophic streams, where periods of higher availability of labile organic matter might occur. These periods have been described in intermittent streams during flood episodes after

drought periods (Romaní et al., 2006) as well as in streams suffering from anthropogenic urban contamination. The potential effect would therefore differ between those stream biofilms growing on illuminated surfaces (upper surfaces of rocks, cobbles and sand grains) than in those on dark surfaces (in sediments, under rocks and cobbles, and on leaves).

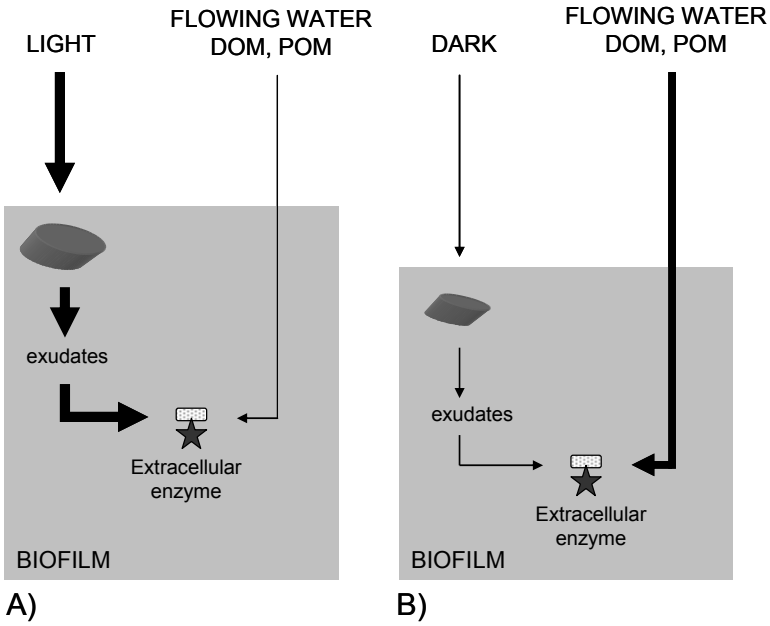


Fig. 3. Scheme of biofilm carbon cycling under light and dark conditions. In high light conditions (A), algal biomass was developed and thus photosynthetic activity. In this thicker and more structured biofilm, microbes were mainly using organic matter released by algae (high quality fresh molecules) instead of organic molecules from the flowing water. In dark conditions (B), a thinner biofilm was developed, characterized by lower algal biomass and lower available organic matter within the biofilm. Microbes in this situation were mainly using molecules from the dissolved organic matter (DOM) and particulate organic matter (POM) pool of the surrounding water. The addition of glucose under light availability weakened the algal-bacterial coupling probably because of heterotrophic bacteria preferentially use the external labile compounds (not represented in this model). Modified from Romaní, 2010.

LINKS BETWEEN MICROBIAL DIVERSITY AND FUNCTION. EFFECTS ON ORGANIC MATTER USE CAPACITY

Several recent reviews indicate that functioning is positively related to species richness (Covich et al., 2004; Hillebrand & Cardinale, 2004; Balvanera et al., 2006; Cardinale et al., 2006). However, the role that microbial diversity plays in controlling ecosystem functioning remain largely overlooked (Hillebrand & Matthiessen, 2009). One objective of the present thesis was to manipulate bacterial diversity of a natural bacterial biofilm community and investigate its effects on the degradation of DOC (labile-recalcitrant) as an expression of ecosystem function so the effects on the probabilities to sustain multifunctionality could be ascertained.

Many studies report that higher bacterial diversity is linked with higher bacterial functions (Langenheder et al., 2010; Tilman et al., 1997). Our results are in accordance with those studies, and showed that in both DOC amendments, and under high diversity level the expression of four out of the five enzymes analyzed (not in phenol-oxidase) was higher, decreasing at the medium and low diversity levels. Under the mixed DOC treatment (labile plus recalcitrant), the activities related to degradation of complex polysaccharides such as cellobiohydrolase (involved in cellulose degradation) showed a greater loss of activity when the bacterial diversity was reduced than other more energetically relevant for bacteria such as β-glucosidase and β-xylosidase (decomposing simple polysaccharides and providing monomeric sugars) and leucine aminopeptidase (providing amino acids). Especially, the leucine aminopeptidase activity showed the lowest loss degree with decreasing bacterial diversity. So, the loss of functionality is not “equal” for all functions. At the same time, under recalcitrant DOC availability all functions were drastically reduced with diversity loss.

In natural systems, the interplay of several enzymes might be of importance for ecosystem functioning, and a multifunctional perspective should allow further insight in those complex interactions. Our results on the effects of a loss of diversity on multifunctionality along a gradient in diversity shows that the consequences of species loss on ecosystem functioning was less affected in younger compared to older biofilms, which might reflect the importance of time for the establishment of interactions among species (Hillebrand & Matthiessen, 2009). The substrate source could also influence the way multifunctionality was maintained. The mixed medium of labile and recalcitrant sources could compensate for the reduction in diversity; however, if the substrate contained only the complex, recalcitrant carbon source, only the highest diversity could maintain a sustainable level of functioning. Hence, high species diversity increases the likelihood that the genetic capacity to decompose humic substances occurs within the heterotrophic biofilm (Findlay et al., 2003).

Overall, these data suggest the importance of the maintenance of bacterial diversity to sustain multifunctionality in river ecosystems.

GENERAL ECOLOGICAL IMPLICATIONS DERIVED FROM THE THESIS

Rivers and streams ecosystems include visible components (the stream channel, riparian trees, stream water, cobbles, fish, freshwater algae, etc.), and the microorganisms which are not apparent to our eyes, but that make up a large fraction of the system in terms of structure and function. Those microorganisms cover all the solid surfaces from the stream (in the channel and in the hyporheic zone) building a

structured microbial community known as biofilms. Those biofilms are the base of the autodepurative fluvial processes, through which flows an important quantity of the OM and energy processed by the river (benthic microbial loop).

Natural disturbances like those derived from the Mediterranean stream fluctuations (drought/rewetting periods, changes in stream water temperature, shifts in DOC quality and abundance) affect the fluvial ecosystems. In our times, Mediterranean streams are also highly threatened by human activities (e.g. dams construction, the inappropriate functioning of wastewater treatment plants leading to enormous amounts of labile OM reaching to the steams, riparian deforestation, water recreational uses, irrigation, etc) as well as by climate change (higher intensity and frequency of droughts and floods, plus a potential rise of stream water temperature). Understanding the effects of these disturbances on the biofilm community could significantly contribute to understanding the functioning of the biological fluvial systems in Mediterranean streams.

The overall thesis shows that the inputs of DOC material (autochthonous/ allochthonous, chemical composition and concentration) as well as the stream temperature and water intermittency (drought/ rewetting periods) will determine the structure and function of the autotrophic and heterotrophic benthic community, their possible interactions and their microbial composition (biodiversity) which at the end will determine the overall efficiency in the use of the flowing materials (OM processing; Fig.4).

In the present thesis, the integration of these three factors (water intermittency, temperature and DOC quality) has been done in Mediterranean streams ecosystems. These systems are used to summer droughts and flood events; nevertheless if the climate change intensifies their frequency and duration as well as the stream water temperature, a substantial change in OM accumulation and composition will take place. Throughout pre-drought periods jointly with an increase in the stream water temperature (by warming), will affect the usual accumulation of a low quality and recalcitrant OM; under warming conditions those recalcitrant materials will be faster decomposed. As a consequence, lower inputs of humic material will reach to the oceans and higher CO_2 to the atmosphere feeding further warming (positive feedback). On the other hand, during the rewetting phases (peak discharges or flood events) there is a high mobilization of a high quality OM (labile OM). Is derived from the results of the present thesis, that at this moment, an increase in the stream water temperature will not increase the degradation rates of this labile OM, due to labile materials were rapidly taken up by freshwater biofilms irrespective of higher temperature. Consequently, the impact of these labile substances on the microbial community would be punctual and

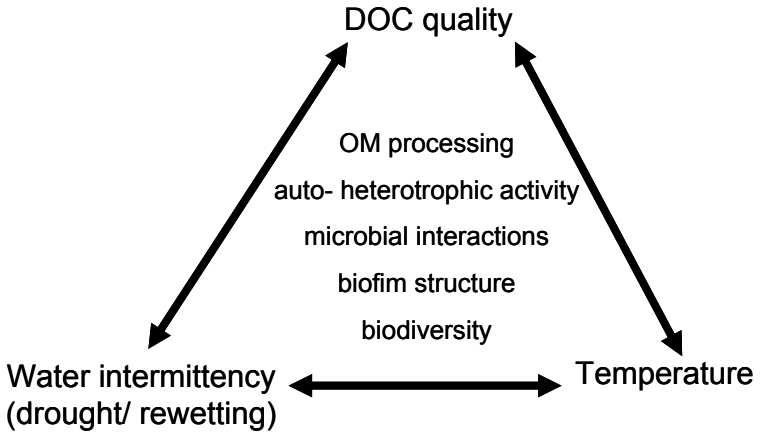


Fig. 4. Conceptual scheme linking the three most important factors studied in this thesis (DOC quality, water intermittency and temperature) and their effects on the biofilm structure and functioning.

short in time. However, during the peak of stream primary production as well as in streams suffering from anthropogenic urban contamination, when large and continuous inputs of highly available OM occur, a remarkable effect on the biofilm community composition and function could be present. The processing of this OM will favour the fluvial heterotrophic compartment and weaken the algal-bacterial relationships because bacteria preferentially use the external labile compounds. A subsequent result of these inputs will be a reduction of the algal biomass which will affect the micro- and macro-consumers usually feeding on them.

During both drought and water return episodes, there is a reduction of the microbial diversity. Severe drought periods will lead to the disappearance of those species not able to resist such dryness and flood episodes will lead to the cleaning of the streambed, thus a huge number of organisms is washed downstream. As a consequence of this reduction on the microbial diversity, the stream functions might also be reduced and so the availability to use and decompose a high array of OM compounds.

Whatever the future brings (naturally or anthropogenically disturbances), increasing temperature and hydrological changes are likely to lead to changes in DOC export and DOC processing rates (benthic microbial loop) in streams which will alter concentrations and composition of DOC and its constituents ultimately being transported to the ocean. At present, knowing how the fluvial systems could be pushed by Global Change (climate change and human being effects) is key to make a good management of resources and to preserve our ecosystems.

GENERAL CONCLUSIONS

GENERAL CONCLUSIONS

CHAPTER I: ORGANIC MATTER AVAILABILITY DURING PRE- AND POST-DROUGHT PERIODS IN A MEDITERRANEAN STREAM

1. Drought affected the availability of organic matter as well as its food quality differently in the pre- and post-drought periods. The total polysaccharide, amino acid and lipid content in the benthic organic matter were on average higher in the drying than in the rewetting periods.

2. During the wet-drought period a decreasing proportion of active bacteria were constrained to use the organic matter source of the lowest quality (polysaccharides, providing only C), since peptides (providing N and C) were no longer available.

3. The flow recovery after the drought caused the mobilization of the streambed and the loss of benthic material (decrease in the polysaccharide, amino acid and fatty acid content in the three benthic compartments), as well as the increase in high quality organic matter in transport which might be the responsible of the fast heterotrophic metabolism recovery.

4. The effects of gradual drought, as well as the fast recovery after rewetting, might be affected by the interaction of the individual dynamics of each benthic substratum: sand sediments and leaves provided refuge for microorganisms and favoured organic matter storage, while an active bacterial community developed on cobbles during the rewetting. The maintenance of benthic substrata heterogeneity within the stream may be important for stream recovery after droughts.

CHAPTER II: ORGANIC MATTER CHARACTERISTICS IN A MEDITERRANEAN STREAM THROUGH AMINO ACID COMPOSITION. CHANGES DRIVEN BY INTERMITTENCY

5. The amino acid content and composition analyzed at the different compartments were highly dependent on the organic matter source as well as on its diagenetic state. This indicated that amino acids were sensitive enough to detect changes in organic matter characteristics.

6. The most abundant amino acids in Fuirosos during both sampling periods were glycine and alanine in benthic and dissolved organic matter.

7. The compartment with the most degraded and lowest quality organic matter was the sand biofilm, whereas the highest-quality organic matter (high degradation index and high N content) occurred on cobble biofilm substrata.

8. Episodes of drought and rewetting differentially affected the organic matter compartments, with a stronger effect on DOM than on benthic compartments. The amino acid composition of DOM reflected the heterogeneous mixture of source inputs: autochthonous inputs prevailed during the pre-drought while allochthonous inputs dominated during the post-drought period. In contrast, the steadier composition of the benthic compartments indicated organic matter of in situ origin, whose quality progressively decreased throughout the drought period.

CHAPTER III: DIFFERENTIAL EFFECTS OF INCREASING WATER TEMPERATURE ON LABILE VERSUS RECALCITRANT BIOFILM DISSOLVED ORGANIC CARBON USE

9. The microbial community was clearly affected by higher water temperatures. Bacterial cell densities, respiratory activity (ETS) and bacterial metabolism (enzyme activities) were all higher. The addition of labile DOC (dipeptide plus cellobiose) caused a further augmentation of heterotrophic biomass and respiratory activity.

10. Higher temperature conditions differently affected DOC degradation; recalcitrant materials will decompose faster, while labile sources will be less affected. Labile materials were rapidly taken up by freshwater biofilms irrespective of higher temperature. Consequently, the impact of these labile substances on the microbial community was punctual and short in time.

11. Biofilms need much more time (weeks or longer) and higher temperatures to degrade recalcitrant DOC. The impact of the recalcitrant pulses was much more prolonged as more time was needed to trigger the biofilm response (expression of phenol-oxidase and xylosidase enzymes and reduction in photosynthetic activity).

12. Pulses of terrestrial origin, such as recalcitrant humic substances, in streams of temperate regions might be most affected by temperature increases that can accelerate their microbial use and decomposition. Potential lower inputs of humic material downstream can occur after the predicted increase of 4ºC.

CHAPTER IV: AVAILABILITY OF GLUCOSE AND LIGHT MODULATES THE STRUCTURE AND FUCNTION OF A MICROBIAL BIOFILM

13. Glucose addition affected the microbial community composition and favoured the heterotrophic biomass and activity, leading to a greater use of polysaccharides. Conversely chlorophyll density and photosynthetic activity declined. Light conditions allowed the development of auto-heterotrophic organisms enhancing the heterotrophic use of peptides released by algae. Thus, the effects of a readily available organic carbon are higher in biofilms grown in the dark than under the light.

14. Without glucose addition, algae and bacteria showed functional coupling probably due to a high dependency of bacterial activity to the use of algal exudates as a C source. When glucose was amended, the relationships between algal and bacterial weakened.

15. Availability of labile organic matter in the flowing water and the presence of light alter the biofilm composition, structure and function, therefore affecting the processing capacity of organic matter in the stream ecosystem.

CHAPTER V: FUNCTIONING IS AFFECTED BY A LOSS OF DIVERSITY IN MICROBIAL BIOFILM COMMUNITIES

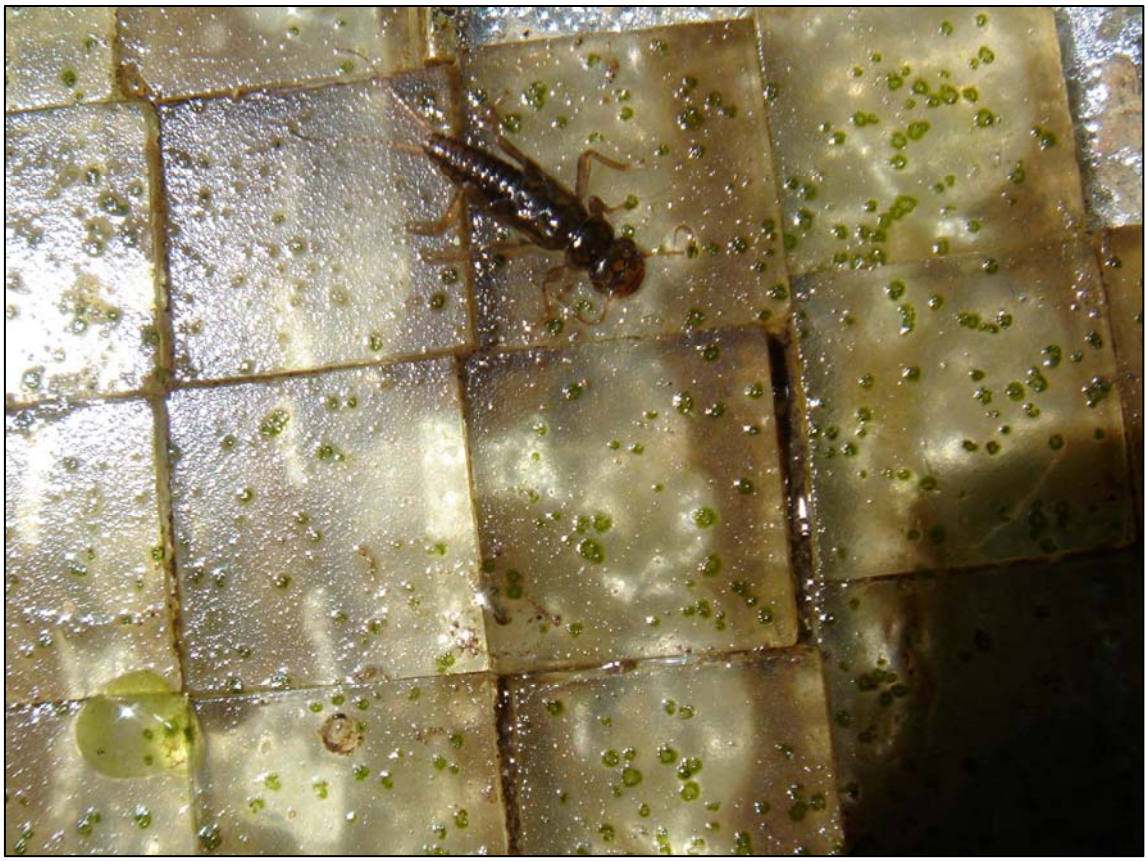
16. The reduction of the microbial diversity leads to a loss of functions.

17. The loss of functionality was not “equal” for all functions; those enzymes linked to the use of complex compounds were the first to be lost.

18. The effect of decreasing diversity on the reduction of enzyme multifunctionality was larger in older biofilms and under recalcitrant DOC conditions. The mixed medium of labile and recalcitrant sources was able to compensate to a certain extend for the reduction in diversity, however, if the substrate contained only the

complex, recalcitrant carbon source, only the highest diversity could maintain a sustainable level of functioning.

19. The maintenance of the bacterial diversity is key to sustain ecosystem functioning.



NEW TRENDS AND FUTURE PERSPECTIVES

NEW TRENDS AND FUTURE PERSPECTIVES

Organic matter (OM) use and decomposition by microorganisms in streams and rivers has been a relevant research subject in the last decades and techniques from both biogeochemical and microbiology expertise has been approached. The OM utilization by biofilms has been approached by means of extracellular enzyme activities (Sinsabaugh et al., 1994; Romaní & Marxsen 2002; Francoeur & Wetzel, 2003). However, more realistic and diverse methods are needed to measure the activities of extracellular enzymes on a wide range of substrates. Although much has been learned with the techniques at hand, little may be gained without methodological improvements. To develop more complex substrates (like in a real world), more specific information about the molecular structure and composition of DOC is required.

Our understanding of OM structure contains two persistent gaps: the twin problems of variability within a single OM sample and variability among different samples collected at different times or locations. Variability within a single sample, also referred to as structural heterogeneity, is well attested to by the complexity of OM behaviour and spectra (Saleh, 1989; Schmitt-Kopplin et al., 1998). We do not know how similar the OM molecules are to each other. Do most OM molecules have similar carbon 'skeletons', differing principally in specific functional groups and average size? Conversely, do the carbon 'skeletons' differ greatly according to the precursor material, with similar collections of functional groups imparting similar reactivity? (McDonald et al., 2004; Cabaniss et al., 2005). Without understanding the structural OM heterogeneity, the structural models developed are just hypothetic (Stevenson, 1982) hampering the development of new and realistic artificial OM substrates.

Recently, a new method called Biolog Ecoplates has been designed for scanning the biological properties of a microbial community to decompose a large number (95) of sole carbon sources. This Biolog technology is a rapid, economic and effective community-level approach. The method involves direct inoculation of environmental samples into Biolog microtiter plates, and uses colour formation from reduction of a tetrazolium dye to assess utilization of 95 separate sole carbon sources during a 2–7 day incubation period. Analyses of colour responses can be quantified from digitized images or from spectrophotometry readings and reveal distinctive degradation patterns among microbial communities (Garland & Mills, 1991; Garland, 1997). This approach, called community-level physiological profiling, has been effective at distinguishing spatial and temporal changes in microbial communities, but this technique also have some disadvantages, like is a culture-based method in which the

biases of enrichment culture may render the results unrepresentative of the native microbiota (Konopka et al., 1998). Another approach involves the inoculation of isolated environmental strains into these microtiter plates. The use of carbon use patterns obtained provided information on the cultivable fraction of the microbial community.

On the other hand, the conventional extracellular enzymes activities measured by fluorescent substrates (MUF and AMC substrates) in biofilms communities reflect the potential activity of the whole microbial community, making difficult to determine the degradation capacities among the different microbial groups forming the community (bacteria, fungi, cyanobacteria, protozoa). In order to solve this problem, new specific fluorescent reporters (e.g. ELF97 substrate for phosphatase) have been designed. This technique combined with confocal laser scanning microscopy (CLSM) permit to monitor which organisms are expressing this enzyme (Van Ommen Kloeke & Geesey, 1999). However, this technique has not been extensively used basically due to two reasons: 1) the CLSM use is expensive and 2) few ELF substrata have been designed as analogous of natural substrates. Further, quantifying the ELF signal and making it representative of a given stream site is difficult in biofilms, which are characterised by their high heterogeneity even at small scales.

The use of pure microorganism's cultures could be another solution to improve the knowledge about the organism's responsible of each enzyme activity expression. But, the achievement of pure cultures from natural samples is not easy. The structural complication of the biofilm hampers the separation of the different trophic groups of microorganisms. The utilization of pure microorganisms cultures grown in artificial substrata (Bruckner & Kroth, 2009) and/or the utilization of restrictive conditions for one or other groups like the light (splitting out the biofilms with an entry of photosynthetic material from those totally heterotrophic) supposes laboratory experiments attempting to sort out the different biofilm compartments. Inside the microbial loop, biofilm extracellular enzyme activities are the result of the internal recycling of organic matter and microbial interactions (competition/ synergism) such as algal-bacterial, fungal-bacterial and fungal-algal interactions. Thus, the success of these cultures could also be a hint for the comprehension of the microbial interactions and flux of materials within biofilms (Hopkinson et al., 1998; Bott & Borchardt, 1999; Parry, 2004). Furthermore, the comprehension of this energy fluxes could help to determine the trophic relationships established under different organic matter availability. From this thesis, is derived that in Mediterranean streams the high flow variability during the year, lead to changes in OM quantity and quality, which is reflected in changes in polysaccharides,

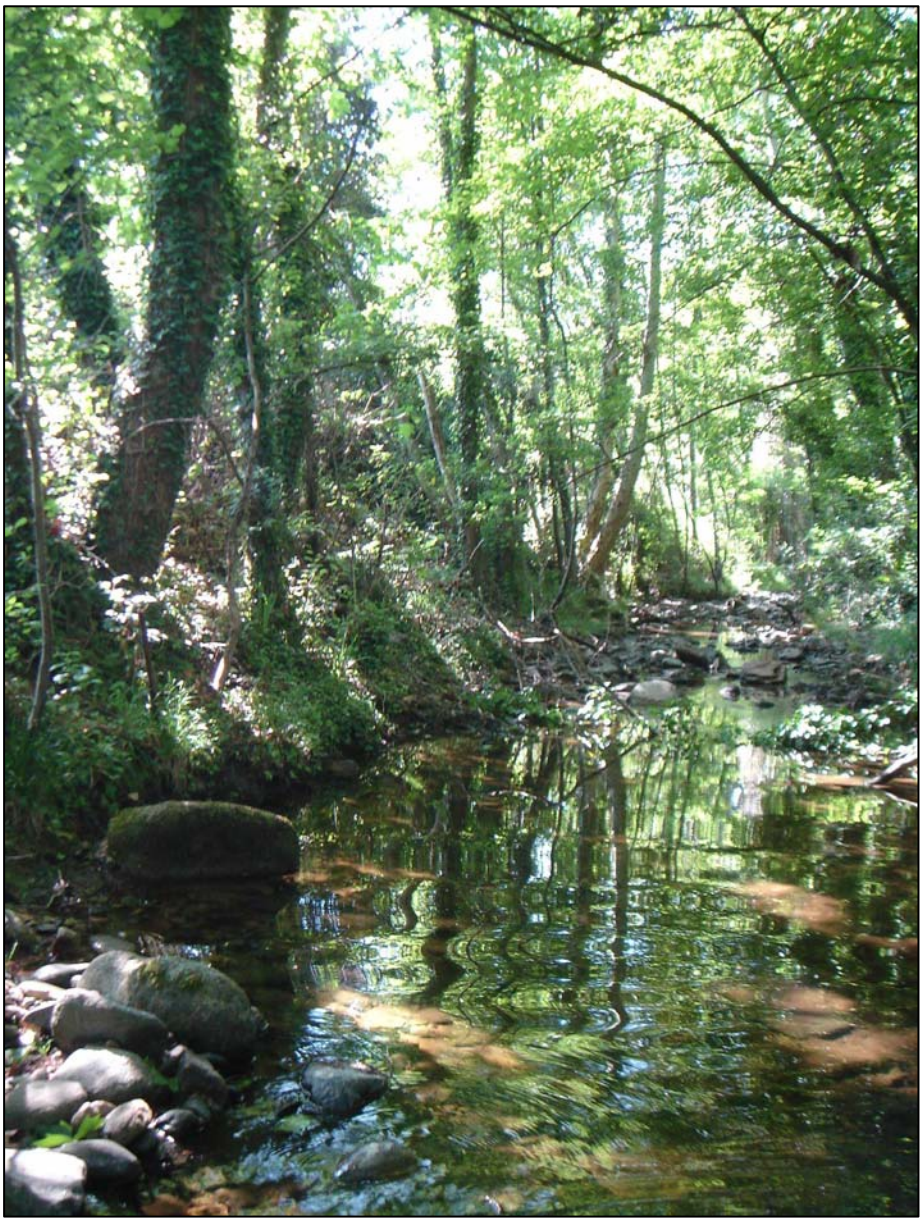
lipids an amino acids content, but we have no clues about the effects of this OM changes on higher trophic levels (meiofauna, macrofauna, fish). Nowadays, a new methodology involving the analysis of phospholipids fatty acids as trophic biomarkers (Torres-Ruiz et al., 2007; Arts et al., 2009) could help to understand these relationships.

Microbial communities are often regarded as “black boxes”. We know what goes into and comes out of the box, but which organisms are involved in actively facilitating the transfers is unknown (Foreman & Covert, 2003). It is time to begin linking both structural and functional studies to get a better understanding of ecosystems dynamics. With the booming of new molecular techniques, some studies focusing on identifying the bacteria that use different constituents of DOM are currently initiated. For instance the analysis of the functional gene expression may help us to charaterise what microbes are doing, what compounds they can use (e.g. if they have the gene of the chitinase they could decompose chitin).

The link between specific members of the bacterial community with specific aspects of DOM processing is highly relevant to the cycling of DOM in aquatic ecosystems, but also can have technological applications. For instance, in wastewater treatment plants, the use of certain bacterial strains which are known to have the capabilities to degrade highly complex macromolecules (lignin, chitin) would lead to obtain higher water quality more efficiently.

However, what becomes apparent when trying to understand microbial OM use is the lack of information that we have at present. Are more abundant groups necessarily for DOM processing? Or, is it only a reduced number of groups that have and advantage in metabolizing DOM? What is the level, if any, of functional redundancy in a bacterial community? How rapidly do shifts in bacterial community structure occur in response to changing environmental conditions? Martinus Beijerinck, one of the early pioneers of microbial ecology, stated, “everything is everywhere, the environment selects” (cited in Atlas and Barta, 1993). Our task is to determine what aspects of the particular environments are crucial to this selection process (Foreman & Covert, 2003).

The challenge now lies in increasing the studies on species identification and relationships between bacterial diversity and ecological processes to test the many unanswered questions of microbial ecology. We need to move from studies that focus on describing phylotypic diversity toward more integrated studies to explain these patterns of diversity. Now we have the capabilities to open the “black box”.



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