FUNCTION AND STRUCTURE OF RIVER SEDIMENT BIOFILMS AND THEIR ROLE IN DISSOLVED ORGANIC MATTER UTILIZATION

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Universitat de Girona

Doctoral thesis

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Universitat de Girona

Dra. Anna M. Romaní i Cornet of Univeristat de Girona ,

I DECLARE:

That the thesis entitled "**Function and structure of river sediment biofilms and their role in dissolved organic matter utilization"** presented by **ANNA FREIXA CASALS** to obtain a doctoral degree, has been compled under my supervision and meets the requirements to opt for an International Doctorate.

For all intents and purposes, I hereby sign this document.

Dra. Anna M. Romaní Cornet

Girona, January 2016

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LIST OF PUBLICATIONS

List of publication derived from this doctoral thesis, two of them are published in two international journals.

- **Freixa A.** and Romaní A.M. (2014). *Shifts in carbon substrate utilization in sediment microbial communities along the Llobregat river*. Fundamental and Applied Limnology, 185: 247-262. DOI: 10.1127/fal/2014/0588

- **Freixa A.**, Rubol S., Carles-Brangarí A., Fernàndez-Garcia D., Butturini A., Sanchez-Vila X. and Romaní A.M. (2016). *The effects of sediment depth and oxygen concentration on the use of organic matter: An experimental study using an infiltration sediment tank*. Science of the Total Environment, 540: 20-41. DOI**:**10.1016/j.scitotenv.2015.04.007

- **Freixa A.**, Acuña V., Casellas M., Pecheva S. and Romaní A.M. *Warmer night-time temperature promotes microbial heterotrophic activity and clearly modifies river sediment community.* In prep**.**

- **Freixa A.**, Ejarque E., Crognale S., Amalfitano S., Fazi S., Butturini A. and Romaní A.M. *Microbial sediment communities rely on different dissolved organic matter sources along a Mediterranean river continuum*. Under revision in Limnology and Oceanography journal.

LIST OF ACRONYMS

AFDW: Ash-Free Dry Weight

AMC: Aminomethylcoumarin

ANOSIM: Analysis of Similarity

ANOVA: Analysis of variance

AWCD: Average Well Colour Development

BCC: Bacterial community composition

CARD-FISH: Catalysed Reported Deposition- Fluorescence in-situ Hybridization

Chl-a: Chlorophyll-a

CLPP: Community Level Physiological Profile

CNP: Carbon Nitrogen Phosphorous

CO_2: Carbon dioxide

DAPI: 4,6-diamidino-2-phenylindole

DIQC : 2,3-dihydroindole-5,6-quinone-2-carboxilate

DOC: Dissolved organic carbon

DOM: Dissolved organic matter

EEA: Extracellular enzyme activities

EPS: Extracellular Polymeric Substances

ETS: Electron Transport System

FI: Fluorescence index

GLU or BG: β-glucosidase activity

HIX: Humification Index

INT: 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride

INT-formazan: iodonitrotetrazolium-formazan

L-DOPA: L-3,4-dihydroxyphenylalanine

LEU: Leucine-aminopeptidase activity

LIP: Lipase activity

MUF: Methylumbelliferyl

NH_4: Ammonium

NMDS: Non-metric multi-dimensional scaling

NO_3: nitrate

NUSE: Nitrogen use index

O_2: Oxygen

OD: Optical Density

PCA: Principal component analysis

PHEN: Phenol oxidase activity

PHO: Phosphatase activity

PO_4: phosphate

POM: Particulate organic matter

RCC: River Continuum Concept

SUVA: Specific Ultra-Violet Absorbance

XYL: β-Xylosidase activity

WWTP: Waste Water Treatment Plant

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SUMMARY

Microbial communities in sediments play a key role in the degradation of organic matter in river ecosystems. These communities are directly influenced by environmental variables that fluctuate through time and space. In addition, in Mediterranean streams, object of study of this thesis, environmental variability is enhanced by the impact of human activities and further affected by global climate change. The main objectives of this thesis are to study the changes in the biofilm structure and dissolved organic matter utilization in river sediments at different spatial and temporal scales and, to detect main environmental drivers that modify these microbial communities. To meet these objectives, two field studies and two laboratory experiments were performed. First, at the Llobregat river, changes in the utilization of organic carbon compounds from mid to lower reaches in river sediments were analysed, using the Biolog EcoPlates technique (Chapter 2). Previously this technique was extensively reviewed in the first chapter of this thesis (Chapter 1). At the Tordera river, an intense longitudinal sampling of sediments and water were performed at two different hydrological periods, in order to study differences in bacterial community composition and microbial organic matter utilization (Chapter 5). In the laboratory, an infiltration sediment tank experiment was performed to study microbial organic matter utilization in sediment depth and at different oxygen concentrations (Chapter 3). Finally, artificial streams were used to study the warming effect and alteration of diel temperature pattern in carbon metabolism and biofilm composition (Chapter 4).

Results from this thesis suggest that **Biolog EcoPlates** are a practical and useful approach to study the spatial and temporal differences in carbon substrate utilization and functional diversity of fresh environmental sediment samples. In this way, results suggested that preserving frozen samples is not recommended, because significant biases can be observed such as a reduction of the overall functional diversity and richness of the microbial community inoculated in the plates.

Related to **spatial variability** in sediment biofilm microbial functioning and structure, results from this thesis showed that the terrestrial inputs of organic matter in headwaters dictated the longitudinal pattern of organic matter utilization along the Tordera river. In headwaters the potential degradation of cellulose and hemicellulose dominated and this was linked to higher input of allochthonous material from riparian forest, whereas accumulation and degradation of more recalcitrant compounds became relevant downstream. Otherwise, in the Llobregat river, the increase in water pollution downstream could be responsible for changes in structure and functioning of biofilm communities. In this river, the biofilm located downstream, in the most polluted sampling sites, was less structured (with lower content of extracellular polysaccharides) and was characterized by being able to degrade more complex carbon compounds such as polymers, when compared to upstream sites. On the other hand, in a smaller spatial scale, from the laboratory experiment using a sediment infiltration tank, evident vertical gradient were observed in microbial organic matter utilization between surface and depth sediments (50 cm) linked with the depletion of dissolved oxygen in depth. In this experiment, microbial communities in surface sediments were capable of using a wider range of organic substrates in contrast to deeper sediments where microbial organic matter degradation was clearly affected by the anoxic conditions.

In relation to **temporal variability**, the study in the Tordera river showed that seasonality (especially due to different hydrological conditions) modified river sediment functioning and bacterial composition. During the drought period**,** the disconnection of the river continuum, stimulated the formation of microhabitats where gamma and delta-Proteobacteria dominated and microbial community was potentially able to degrade more diverse organic compounds particularly in relation to nitrogen. In contrast, during base flow, higher use of carbon organic compounds and abundance of alpha-Proteobacteria were observed. On the other hand, in the laboratory experiment using artificial streams where sediment carbon metabolism was studied in a shorter temporal scale, a clear diel pattern was observed for β-Glucosidase activity peaking at night linked with dissolved organic carbon reduction. This result highlights the importance to taking into account the

exact sampling hour when measuring microbial activities, especially those linked to the use of labile and easy to decompose compounds. Moreover, from this experiment and in relation to **climate change**, results pointed out that an expected increase of diel temperature, and especially at night-time, may have large consequences in carbon metabolism, enhancing heterotrophic activities. At the same time, warming would also modify the interactions between the different biofilm groups and thus impact on the sediment food web.

The findings of this thesis show a large temporal and spatial variability of microbial dissolved organic matter utilization in Mediterranean river sediments. At the same time, environmental variables such as temperature, oxygen, dissolved organic matter quality clearly modify sediment biofilm. Finally, warming and pollution might also significantly affect the sediment biofilm activity and structure. Thus, river sediments, which play a key role for ecosystem functioning and carbon cycle, appear to be very vulnerable to global change.

RESUM

Les comunitats microbianes dels sediments juguen un paper clau en la degradació de la matèria orgànica en els ecosistemes fluvials. Aquestes comunitats estan directament influenciades per les variables ambientals que fluctuen a través del temps i l'espai. A més, en els rius Mediterranis, objectiu d'estudi d'aquesta tesi, aquesta variabilitat ambiental es veu reforçada per l'impacte de les activitats humanes i a la vegada pel canvi climàtic global. Els principals objectius d'aquesta tesis són estudiar els canvis en l'estructura del biofilm i en la utilització de la matèria orgànic dissolta en els sediments dels rius a diferents escales temporals i espacials, i detectar quines variables ambientals modifiquen principalment aquestes comunitats microbianes. Per complir aquests objectius es van realitzar dos estudis de camp i dos de laboratori. Primer, al riu Llobregat, es van estudiar els canvis en la utilització dels compostos orgànics de carboni dels sediments des del tram mig fins a la desembocadura, utilitzant la tècnica dels Biolog EcoPlates (Capítol 2). Prèviament aquesta tècnica es va revisar extensament al primer capítol d'aquesta tesi (Capítol 1). Al riu Tordera, es va portar a terme un mostreig intensiu longitudinal del sediment i l'aigua en dos períodes hidrològics diferents, per estudiar les diferències en la composició de la comunitat bacteriana i la utilització de la matèria orgànica (Capítol 5). Al laboratori, es va realitzar un experiment utilitzant un tanc d'infiltració per estudiar la utilització microbiana de la matèria orgànica al llarg d'un gradient de profunditat al sediment i a diferents concentracions d'oxigen (Capítol 3). Finalment, es van utilitzar rius artificials per estudiar l'efecte de l'escalfament i l'alteració dels patrons diaris de temperatura en el metabolisme del carboni i la composició del biofilm (Capítol 4).

Els resultats d'aquesta tesis mostren com els **Biolog EcoPlates** són una tècnica pràctica i útil per estudiar les diferències temporals i espacials en la utilització de substrats de carboni i de la diversitat funcional en mostres fresques de sediments. En aquest sentit, els resultats suggereixen que no és recomanable preservar les mostes congelades perquè es van observar biaixos significatius, com una reducció general de la diversitat i de la riquesa funcional de les comunitats microbianes inoculades a les plaques.

En relació a les **diferències espacials** en el funcionament i l'estructura del biofilm del sediment, els resultats d'aquesta tesis han mostrat com les entrades de matèria orgànica a la capçalera del riu dictaven el patró longitudinal d'utilització de la matèria orgànica al llarg del riu Tordera. A la capçalera dominava la degradació potencial de la cel·lulosa i hemicel·lulosa lligada a una entrada major de material al·lòcton procedent del bosc de ribera, mentre que els compostos més recalcitrants s'acumulaven i es degradaven riu avall. En canvi, al riu Llobregat, l'augment de la contaminació de l'aigua al llarg del riu podria ser la responsable dels canvis en l'estructura i el funcionament del biofilm. En aquest riu, el biofilm localitzat a la desembocadura, en els punts de mostreig més contaminats, era el menys estructurat (amb menys contingut de polisacàrids extracel·lulars) i era caracteritzat per ser capaç de degradar compostos de carboni complexes com els polímers, en comparació amb els punts de capçalera. D'altra banda, a una escala espacial més petita, a l'experiment de laboratori realitzat amb un tanc d'infiltració de sediment, es va observar un evident gradient vertical d'utilització microbiana de matèria orgànica entre la superfície i els sediments profunds (50 cm) lligat a un esgotament de l'oxigen dissolt en profunditat. En aquest experiment, la comunitat microbiana a la superfície era capaç de degradar una àmplia gamma de substrats, contràriament al que s'observava en profunditat, on la degradació microbiana de la matèria orgànica estava clarament afectada per les condicions anòxiques.

En relació a la **variabilitat temporal**, l'estudi al riu Tordera va mostrar com l'estacionalitat (principalment deguda a les diferents condicions hidrològiques) modificava el funcionament i la composició bacteriana del sediment. Durant el període de sequera, la fragmentació hídrica del continu del riu, va estimular la formació de micro-habitats on dominava gamma i delta-Proteobacteria i a on la comunitat microbiana era capaç de degradar més diversitat de compostos orgànics, especialment relacionats amb el nitrogen. En cas contrari, durant les condicions de cabal basal, es va observar una major utilització dels compostos orgànics de carboni i una abundància més alta d'alpha-Proteobacteria. Per altra banda, a l'experiment amb rius artificials on es va estudiar com variava el metabolisme del carboni en una escala temporal curta (hores), es va observar un patró diari clar de l'activitat β-glucosidasa que arribava el seu màxim durant la nit, lligat a una reducció del carboni orgànic dissolt. Aquest resultat ressalta la

importància de tenir en compte l'hora del mostreig quan es mesuren activitats microbianes, especialment aquelles relacionades amb la utilització dels compostos més làbils i fàcils de degradar. A més, en aquest experiment i en relació amb el **canvi climàtic,** els resultats indicaven que l'augment de la temperatura diària, especialment durant la nit, podia ocasionar importants conseqüències al metabolisme del carboni, incrementant les activitats heterotròfiques. Alhora, l'escalfament podria també modificar les interaccions entre els diferents grups del biofilm i per tant, impactar en la xarxa tròfica del sediment.

Aquesta tesis conclou que existeix una gran variabilitat espacial i temporal en la utilització microbiana de la matèria orgànic dissolta en els sediments dels rius Mediterranis. Alhora, les variables ambientals tal i com la temperatura, l'oxigen i la la qualitat del carboni orgànic dissolt clarament modifiquen el biofilm del sediment dels rius. Finalment l'escalfament i la pol·lució també poden significativament afectar l'activitat i l'estructura del biofilm. Per tant, el sediment dels rius, que juga un paper clau en el funcionament dels ecosistemes i del cicle del carboni, sembla ser molt vulnerable al canvi global.

RESUMEN

Las comunidades microbianas de los sedimentos juegan un papel clave en la degradación de la materia orgánica en los ecosistemas fluviales. Estas comunidades están directamente influenciadas por las variables ambientales que fluctúan en el tiempo y en el espacio. Además, en los ríos Mediterráneos, objetivo de estudio de esta tesis, esta variabilidad ambiental se ve reforzada por el impacto de las actividades humanas y a su vez, por el cambio climático global. Los principales objetivos de esta tesis son estudiar los cambios en la estructura del biofilm y en la utilización de la materia orgánica disuelta en los sedimentos de los ríos en diferentes escalas temporales y espaciales, y detectar qué variables ambientales modifican principalmente estas comunidades microbianas. Para cumplir estos objetivos se realizaron dos estudios de campo y dos en el laboratorio. Primero, en el río Llobregat se estudiaron los cambios en la utilización de los compuestos de carbono orgánico en los sedimentos desde el tramo medio del río hasta la desembocadura, utilizando la técnica de los Biolog EcoPlates (Capítulo 2). Previamente esta técnica se revisó extensamente en el primer capítulo de esta tesis (Capítulo 1). En el río Tordera se desarrolló un muestreo intensivo longitudinal del sedimento y el agua durante dos períodos hidrológicos diferentes, para estudiar las diferencias en la composición de la comunidad bacteriana y la utilización microbiana de la materia orgánica (Capítulo 5). En el laboratorio, se realizó un experimento utilizando un tanque de infiltración para estudiar la degradación de la materia orgánica disuelta a lo largo de un gradiente de profundidad del sedimento y a diferentes concentraciones de oxígeno (Capítulo 3). Finalmente, se utilizaron ríos artificiales para estudiar el efecto del calentamiento y de la alteración de los patrones diarios de temperatura en el metabolismo del carbono y en la composición del biofilm (Capítulo 4).

Los resultados de esta tesis muestran cómo los **Biolog EcoPlates** son una técnica práctica y útil para estudiar las diferencias temporales y espaciales en la utilización de los sustratos de carbono y de la diversidad funcional en muestras frescas de sedimentos. En este sentido, los resultados sugieren que no es recomendable preservar las muestras congeladas porque se observaron sesgos significativos

tales como una reducción general de la diversidad y la riqueza funcional de las comunidades microbianas inoculadas en las placas.

En relación a las **diferencias espaciales** en el funcionamiento y la estructura del biofilm del sedimento, los resultados de esta tesis muestran cómo las entradas de materia orgánica en la cabecera del río dictaron el patrón longitudinal de utilización del carbono orgánico disuelto a lo largo del río Tordera. En la cabecera dominaba la degradación potencial de la celulosa y la hemicelulosa ligada a una mayor entrada de material alóctono procedente del bosque ripario, mientras que los compuestos más recalcitrantes se acumularon y se degradaron aguas abajo. En cambio, en el río Llobregat, el aumento de la contaminación en el agua a lo largo del río podría ser el responsable de los cambios en la estructura y el funcionamiento del biofilm. En este río, el biofilm localizado en la desembocadura, en los puntos de muestreo más contaminados, era menos estructurado (con menor contenido de polisacáridos extracelulares) y estaba caracterizado por ser capaz de degradar compuestos de carbono complejos como los polímeros, en comparación con los puntos de cabecera. Por otro lado, a una escala espacial más pequeña, en el experimento de laboratorio realizado con un tanque de infiltración de sedimento, se observó un evidente gradiente vertical de degradación microbiana de materia orgánica entre la superficie y los sedimentos profundos (50 cm) ligado al agotamiento del oxígeno disuelto en profundidad. En este experimento, la comunidad microbiana de la superficie era capaz de degradar una amplia gama de sustratos de carbono contrariamente a lo que ocurría en los sedimentos profundos, donde la degradación de la materia orgánica estaba claramente afectada por las condiciones anóxicas.

En relación a la **variabilidad temporal**, el estudio del río Tordera mostró cómo la estacionalidad (especialmente debida a las diferentes condiciones hidrológicas) modificaba el funcionamiento y la composición bacteriana del sedimento. Durante el periodo de sequía, la fragmentación hídrica del continuo del río estimuló la formación de micro hábitats donde dominaban los gamma y delta- Proteobacteria y donde la comunidad microbiana era capaz de degradar más diversidad de compuestos orgánicos especialmente en relación al nitrógeno. En caso contrario, durante las condiciones de caudal basal, se observó una mayor utilización de

compuestos orgánicos del carbono y una abundancia más alta de alpha-Proteobacteria. Por otro lado, en el experimento en ríos artificiales donde se estudió cómo cambiaba el metabolismo del carbono en una escala temporal corta (horas), se observó un patrón claro para la actividad β –Glucosidasa, que llegaba a su máximo durante la noche ligado a una reducción del carbono orgánico disuelto. Este resultado resalta la importancia de tener en cuenta la hora del muestreo cuando se analizan actividades microbianas, especialmente aquellas relacionadas con la utilización de los compuestos más lábiles y fáciles de degradar. Además, en este experimento y en relación al **cambio climático**, los resultados indicaron que un incremento de la temperatura diaria, especialmente durante la noche, puede ocasionar importantes consecuencias en el metabolismo del carbono, aumentando las actividades heterotróficas. Al mismo tiempo, el calentamiento podría también modificar las interacciones entre los diferentes grupos del biofilm y por tanto impactar en la red trófica del sedimento.

Esta tesis concluye que existe una gran variabilidad espacial y temporal en la utilización microbiana del carbono orgánico disuelto en los sedimentos de los ríos Mediterráneos. Al mismo tiempo, las variables ambientales como la temperatura, el oxígeno y la calidad del carbono orgánico disuelto claramente modifican el biofilm de los sedimentos del río. Finalmente, el calentamiento y la polución también pueden afectar significativamente a la actividad y la estructura del biofilm. Por tanto, los sedimentos de los ríos, que juegan un papel clave en el funcionamiento de los ecosistemas y en el ciclo del carbono, parecen ser muy vulnerables al cambio global.

GENERAL INTRODUCTION

1. Function and structure of Mediterranean rivers ecosystems
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1. Function and structure of Mediterranean rivers ecosystems

1.1 Ecological concepts and models

Freshwater represents a 2.5 % of all water on the earth, and only a 0.006% of total freshwater reserves are in rivers. In spite of this small percentage, the important ecological relevancy of rivers is well known (Allan and Castillo 2007) and understanding the structure and functioning of river ecosystems continues to be a common goal of many aquatic ecologists. Rivers and streams transport transform and store organic material and nutrients along the flow path linking terrestrial to marine systems. Rivers are hierarchically organized and transport water and their components downstream. Due to this transport, a longitudinal transition of biological communities and patterns of river functioning from the headwaters to the river mouth is manifested. This concept was first conceptualized by the **River Continuum Concept (RCC)** (Vannote et al. 1980) with the identification of consistent patterns of loading, transport, utilization and storage of organic matter, as well as a turnover of the functional groups within biological communities along the length of the river. Soon after, other authors questioned the predictability and simplicity of the river continuum concept and incorporated the **spatial complexity** in new models (e.g. Statzner and Higler 1985; Sedell et al. 1989). For example, from a longitudinal perspective some authors suggested including the effect of tributaries confluences (Rice et al. 2001) or urban infrastructures (Kaushal and Belt 2012) in the RCC. In addition, later, some authors begun viewing rivers as discontinuous, suggesting that rivers, instead of continuum gradients, should be better conceptualised as a mosaic of patches (Thorp et al. 2006) where local environmental factors would be highly relevant to river function and structure (Poole 2002).

A part from the continuous or discontinuous gradient along the longitudinal dimensions of rivers, the **temporal variability** is highly relevant, especially in Mediterranean rivers. In this context, including the temporal variability of river ecosystems, the **flood pulse concept** arose from dissatisfaction with the generality of RCC, emphasizing more on lateral than longitudinal connectivity. The flood pulse concept reported the seasonal nature of the hydrological link between the river

channel and the floodplain as the primary source of material (Junk et al. 1989). At that time, Ward (1989) defined **the four dimension of the river,** not included as an ecosystem model but describing the connectivity inside of the rivers. In this sense, in addition to temporal and longitudinal variability, this author describes the connectivity of river including the lateral (interaction between river channel and riparian/floodplain systems) and vertical (interaction between surface water and groundwater) connectivity.

Later on, Thorp et al. (2006) merged the previous existing river ecosystem concepts in the **riverine ecosystem synthesis** defining rivers as downstream arrays of large hydro geomorphic patches formed by catchment geomorphology and climate. However, recently a new concept is reported, improving the existing one in a simple holistic model trying to unify river ecosystem concepts by the **river wave concept** (Humphries et al. 2014). This concept defines the source of organic matter, the storage, transformation and transport of material and energy in river systems as function of temporal and spatial position on the river wave, including climate, geology, geomorphology and anthropogenic regulation.

Despite this historical perspective, the ecological relevance of structure and functioning of river systems remains currently in discussion. In this sense, this thesis try to increase the knowledge about how structure and funcionining of microbial communities in Mediterranean rivers change at various temporal and spatial scales.

1.2 Mediterranean river ecosystems: key characteristics

Mediterranean climate regions include areas of the Mediterranean Sea basin, the north western pacific coast of America, the central Chilean coast, the Western Cape region of South Africa and some part of West and South Australia. These areas are subjected to rainfall seasonality due to both very low precipitation periods and, at the same time, high precipitation episodes. These contrasting precipitation events over an annual cycle cause typical episodes of floods and droughts in rivers (**Fig. 1**) that are the major forms of natural disturbance of river ecosystem structure and function (Lake 2000). Water stress deficit periods are determined by low or nul

precipitation also enhanced by high evapotranspiration and temperatures. These drough periods significantly affect benthic microoganisms which develop survival strategies (Romaní et al. 2013). Flood events alter the physical characteristics of sediments through erosion and transport, affecting the composition and function of attached biofilms (Biggs et al. 1999). These changes in flow have an influence on the quantity and quality of dissolved organic matter transported by river water, which may affect biofilm microbial metabolism (Ylla et al. 2010; Vázquez et al. 2011, 2015).

Flood Base Flow Drought

Figure 1. Pictures of different hydrological conditions (flood, base flow and drought)in the Tordera river. Images corresponds to March 2013 (flood), April 2012 (base flow) and July 2012 (drought).

1.3 Global climate change and anthropogenic pressures in Mediterranean rivers

The natural dynamics of most rivers is constantly impacted by multiple **human activities** and stressed by **climate change**. Human activities directly affect river ecosystems by physical (e.g. reservoir construction, water abstraction, and channelization) chemical (e.g. increase of water pollutants, pesticides and fertilizers) or biological stressors (e.g. introduction of invasive species). This multiple disturbances can alter the structure and functioning of river communities and highly contribute to the degradation of whole river ecosystem (Dudgeon 2010). Most Mediterranean rivers are located in heavy urbanized areas, receiving heavy contamination pressures from extensive urban, industrial and agricultural activities and are subjected to water abstraction that often implies an overpressure to river ecosystems

On the other hand, the IPCC predicts that the Mediterranean region will be particulary sensitive to global climate change. Models predict an increase of average temperature and a decrease of annual precipitation with a higher frequency of extreme events (IPCC 2013). Particulary in Mediterranean rivers, the expected reduction in precipitation would directly affect river discharge and water resources and management (García-Ruiz et al. 2011). These future scenarios could have large consequence in structure and functioning of microbial communities in river systems. In this sense, it is widely accepted that temperature increase broadly affects river ecosystems due to temperature regulation of all metabolic rates (Gillooly et al. 2001). For instance, it is reported that temperature dependence could enhance primary production and respiration in rivers (Acuña et al. 2008; Rosa et al. 2013), increase metabolic rates, and accelerate organic matter microbial degradation (Perkins et al. 2012; Ylla et al. 2014). In addition, the expected changes in frequency and duration of floods and droughts events could potentially affects on river ecosystem processes (Zoppini et al. 2010; Romaní et al. 2013).

2. River sediment biofilm communities

2.1 Sediment biofilm structure

River biofilms develop on different substrates such as sediments, rocks and cobbles, wood and leaves. The nature of the substratum determines the composition and structure of the biofilms and, in consequence, its metabolism (Romaní 2010). This thesis focuses on biofilm growing in river sediments.

River sediment is a hard, inert substrate, characterized by the smaller size of sand grains in comparison to other substrates (Mora-Gómez et al. 2015), and accumulate large amounts of detritic material. Microbial communities in river sediments are principally composed by bacteria, algae, fungi and cyanobacteria attached to sand grains and assembled in a polymeric matrix in a three-dimensional biofilm structure (Pusch et al. 1998) (**Fig. 2**). In addition, meiofauna, including mainly protozoa, coexist together inside of the biofilm matrix (Giere 2009).

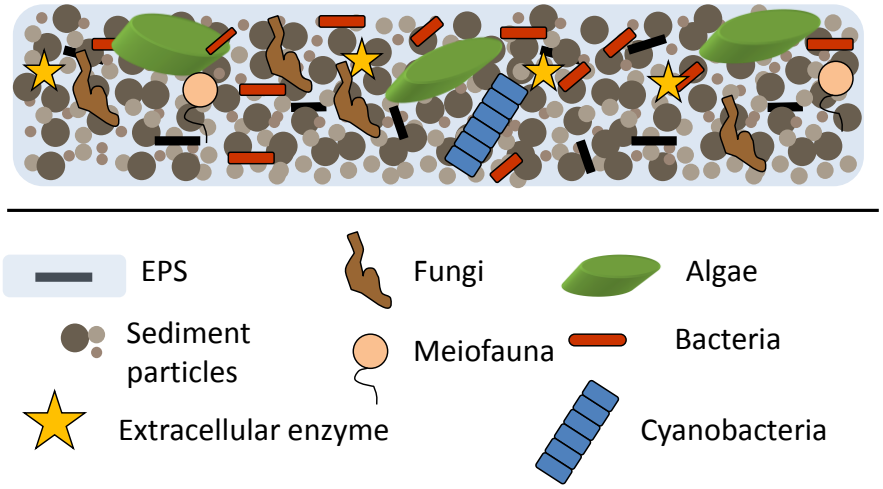


Figure 2. Sediment biofilm with their biotic compounds. Modified from Romaní 2010.

Sediment biofilms are in general more heterotrophic (with higher contributions of bacteria and fungi) than the biofilm developing on rocks (Romaní and Sabater 2001; Brablcová et al. 2013; Timoner et al. 2014). Trophic interactions occur within the biofilm, for instance, bacteria constitute a valuable and nutritious food resource for benthic consumers such as protozoa (Giere 2009). Studies found that biofilms typically host protozoa (ciliates and flagellates) and other meiofauna groups including copepods, nematodes and rotifers. These meiofauna communities have the potential to influence other organisms in stream biofilms directly (consumption and competition for food) and indirectly (influencing the microbial activity rates) (Hakenkamp and Morin 2000).

Moreover, the benefits of studying biofilms are many. Overall, biofilms are ubiquitous, have short generation times, are abundant and can be sampled rapidly; especially they are sensitive to environmental changing conditions responding quickly to these changes (Burns and Ryder 2001).

2.2 Functional relevance of surface and hyporheic sediment biofilms

Sediment biofilm communities dominate river ecosystem metabolism being a major component for the uptake, storage and cycling of carbon, nitrogen and phosphorous (Pusch et al. 1998). In this sense, heterotrophic bacteria are the main

DOM decomposers in rivers, responsible for a large proportion of ecosystem respiration. Actually, sediment bacteria account for 60 % of total community respiration in rivers (Marxsen 2006) and are responsible for most of the metabolic activity (Storey et al. 1999) including the microbial degradation of organic matter and the reduction of electron acceptors (e.g. oxygen, nitrate and sulfate) (Ghiorse and Wilson 1988; Hedin et al. 1998). Therefore, sediment biofilms play a key role in transfer carbon to higher trophic levels (Findlay 2010; Majdi et al. 2013). In this sense, as a result of recognizing the important role in the carbon cycle in river sediments, the study of functioning of this sediment biofilm has received an increasing interest.

As a consequence of biofilm metabolism, when river sediments are sufficiently thick, an oxygen profile zonation in depth is usually present. Sediment spatial zonation promotes an oxidized zone in the surface sediment and a reduced zone in deeper layers creating anoxic zones (Boulton et al. 1998). This oxygen gradient occurs together with water exchange processes at the hyporheic zone. The interface ecotone between river and groundwater ecosystems, defined as the **hyporheic zone** (Brunke and Gonser 1997) promotes the exchange of water, nutrients, organic matter and biota between alluvial groundwater and stream water (Boulton et al. 1998; Marmonier et al. 2012). This exchange, in turn, influences stream water quality (Sobczak and Findlay 2002) and organic matter degradation (Rulík et al. 2004). In addition, the hyporheic zone offers protection (as a temporary refuge) to the sediment biofilm communities against high discharge, desiccations, and extreme temperatures (Brunke and Gonser 1997).

3. Dissolved organic matter dynamics in rivers

3.1 Inputs and transformations of dissolved organic matter

Dissolved organic matter is the major form of organic matter in aquatic ecosystems (Wetzel 1992) and clearly influences in global carbon cycle and food webs. The term dissolved organic matter (DOM) refers to the pool of organic molecules that are dissolved in water (material that passes through a filter of 0.5 μm; McDonald et

al. 2004). DOM is commonly measured in terms of carbon (as DOC; dissolved organic carbon). Total dissolved organic carbon accounts for about 50% of total DOM (Findlay et al. 2003; Allan and Castillo 2007; Battin et al. 2008) while lower proportion is usually found for organic forms of nitrogen (DON) or phosphorous (DOP).

Rivers receive constant inputs of allochthonous and autochthonous DOM that can be processed, transported or stored through the river (**Fig. 3**). Although, as indicated above, DOM is the major pool, a significant percentage of organic matter enters the river as a particulate fraction (POM, particulate organic matter, >0.5μm) especially in headwaters, such as plant material (**Fig. 3**, allochthonous sources). Organic matter can be transported from upstream to downstream and lateral exchanges can be substantial wherever the river is connected to a floodplain. For instance, Findlay et al. (1997) reported that 65% of organic matter in a 2nd order river was exported downstream. Moreover organic matter can be immobilized by the polysaccharide matrix in sediment surfaces.

Allochthonous sources of organic matter are mainly composed by terrestrial plant materials (leaves, twigs and branches) contributing for instance between a 75% and 99% of inputs of organic matter in 2n order and sub-artic streams respectively (Findlay et al. 1997; Naiman and Link 1997). The most direct input into stream water is leaf litter leachate which directly provides of dissolved organic compounds to the river (McDowell and Fisher 1976). In this sense, leaves are usually a relevant contribution of allochthonous organic matter entering the river system. This type of material is usually humic material, more refractory and with lower quality than autochthonous material (Hein et al. 2003; Kaplan and Newbold 2003). Humic compounds are high -molecular-weight compounds resistant to microbial degradation, typically celluloses, tannins and lignins (McDonald et al. 2004). Moreover, other allochthonous organic matter sources, with less importance, are also contributing to the quantity of DOM in rivers such as precipitation, surface runoff, and water movement through soils and ground water (Findlay et al. 2001; Aikenhead-Peterson et al. 2003). In addition, in catchments with high human pressure, anthropogenic activities can alter the quantity and

quality of DOM. Typical sources of anthropogenic DOM are inputs of waste water treatment plants, industries and urban sewage.

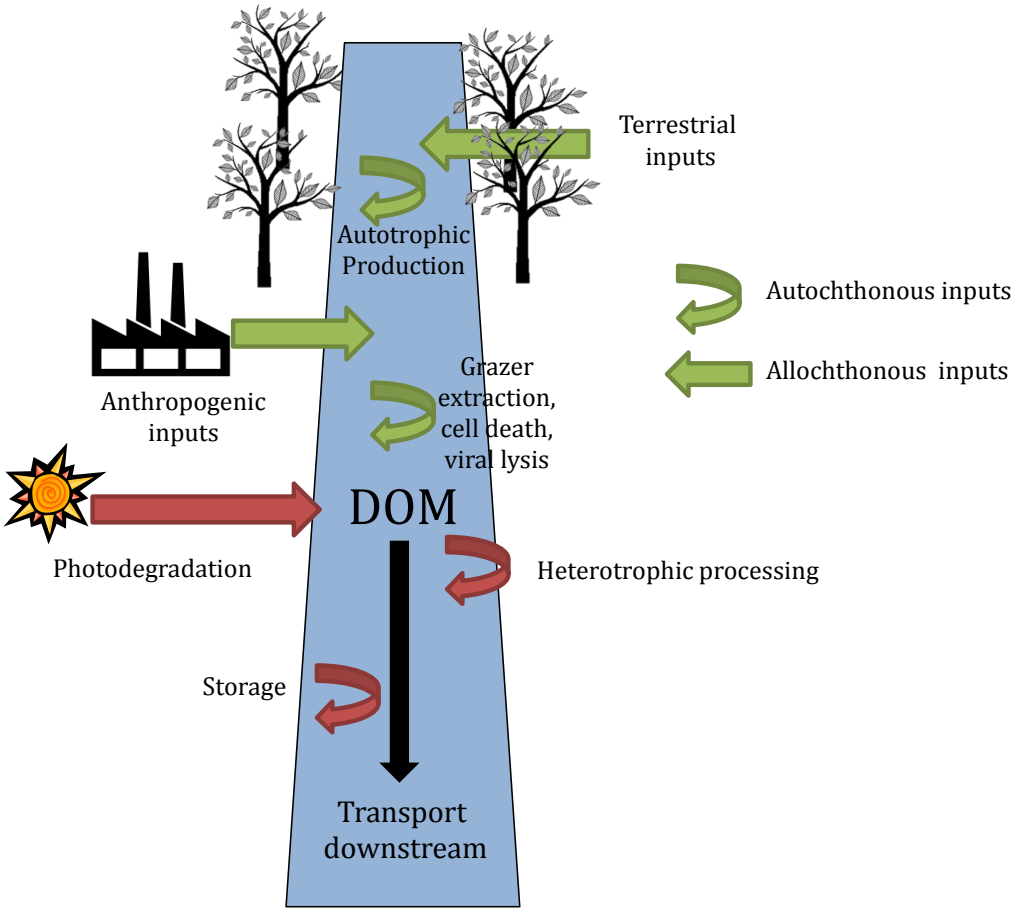


Figure 3. Conceptual diagram of main inputs (green arrow) and outputs/processing (red arrow) of organic matter in rivers systems. The black arrow represents the downstream transport of DOM.

On the other hand, **autochthonous inputs** are mainly the autotrophic production of DOC as a result of photosynthetic processes by autotrophic organisms (benthic algae, cyanobacteria, phytoplankton and macrophytes). Several studies showed that primary production can account for between 40 and 99% of total carbon inputs in rivers (Jones et al. 1997; Webster and Meyer 1997). In addition, the excretion of grazers, cell death and viral lysis of cells can also contribute to increase the amount of DOC in rivers (Bertilsson and Jones 2003). Autochthonous materials are mainly non-humic compounds such as carbohydrates, amino acids,

lipids with relatively low molecular weight (Wetzel 2001) usually considered labile and more susceptible to microbial processing (Thorp and Delong 2002). The photosynthetic production of DOC is the major source of metabolic substrates for heterotrophic microorganisms in aquatic ecosystems (Bertilsson and Jones 2003).

Internal microbial metabolism and photo degradation are the main **outputs** or decrease of DOM in rivers (Cole et al. 2007). DOM exposed to sunlight (photo degradation) results on the decrease in its average molecular weight (Bertilsson and Tranvik 2000) and its mineralisation to CO_2 (Miller and Zepp 1995) or assimilation by bacteria (Moran and Covert 2003). On the other hand, the rate of DOC utilization by microbial heterotrophy is difficult to estimate but the majority of studies point to bacterial assimilation as the principal removal process of DOC. For instance, in the Hudson river over ¼ of the organic carbon entering or produced within the river was either remineralized or converted into biomass by heterotrophic bacteria (Maranger et al. 2005). This internal microbial metabolism of DOC is presented in detail in the next section.

3.2 Microbial metabolism of dissolved organic matter

Dissolved organic carbon is removed from streams through the microbial assimilation of organic C into microbial biomass or released as CO_2 after being respired. In fact, a smaller fraction of DOC is considered labile and potentially important for heterotrophic communities. Bacteria play an important role in this pathway; greater to that of fungi (Meyer 1994). Actually fungi are considered to be responsible for the leaf litter decomposition whereas bateria mainly for the last stages of decomposition when organic matter is dissolved (Kuehn 2016). The assimilation of DOC by bacterial heterotrophic communities is a recycling pathway of organic carbon within the ecosystem due to carbon flow from DOC to bacteria to protozoa to metazoan or/and to higher trophic levels (Azam et al. 1983). This carbon pathway is known as a microbial loop, first described by Azam et al. (1983) in the marine water column, and was later on also described in river systems by Meyer (1994).

Incorporation of DOC into bacteria can follow 3 major pathways: 1) direct uptake, 2) extracellular enzyme uptake and 3) photo-degradation uptake (Sinsabaugh and Foreman 2003, **Fig. 4**)

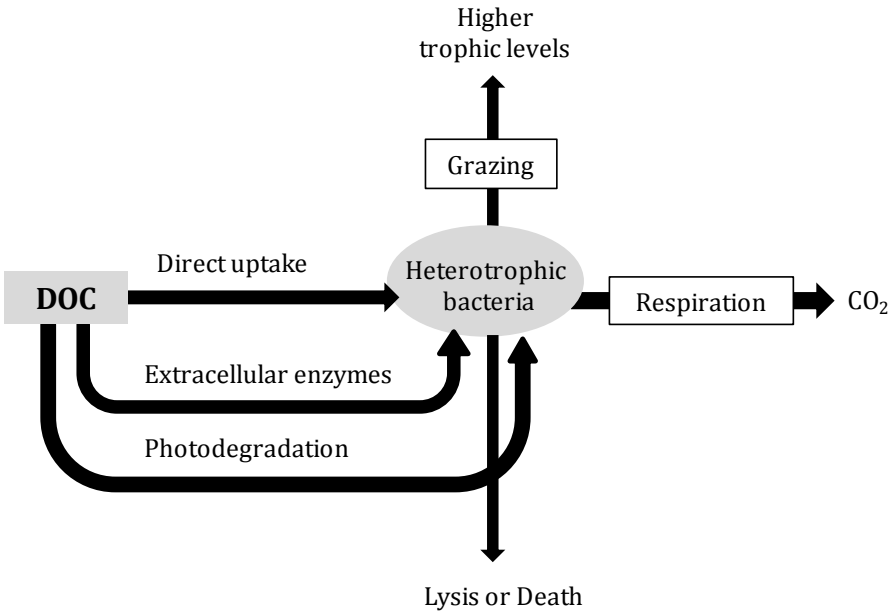


Figure 4. Pathways of DOC incorporation by heterotrophic bacteria. Modified from Sinsabaugh and Foreman (2003).

Direct uptake occurs when small monomeric substrates can be assimilated directly, without external processes. The extracellular enzyme uptake consists of the degradation of polymeric and macromolecular organic matter into assimilable products (low-molecular weight) thanks to the action of extracellular enzymes released by microorganism (Chrost 1991). Extracellular enzymes are synthesised at low basal rate in the absence of the appropriate substrate, whereas when the substrate becomes available in the environment, there is a stong increase in the production rate of this particular enzyme (Chrost 1991; Romaní et al. 2012). In this sense, enzymatic activities can inform about the availability of each substrate in the environment. Finally, the photo degradation uptake consists in the incorporation of organic products generated by the effect of solar radiation (chemical degradation) on degrading complex organic compounds and results in a decrease in their average molecular weight (Bertilsson and Tranvik 2000).

4. Links between function and structure of microbial communities

River microbial structure refers to the composition and abundances of microbial taxa whereas river microbial functioning refers to the main microbial metabolic activities relevant for ecosystem functioning such as microbial degradation of organic matter, primary production, nutrient cycling and others. Nowadays, there remains still limited information about the link between microbial community composition and functioning in aquatic ecosystems.

A general principle in microbial ecology is that community structure determines ecosystem functioning. However, some studies in aquatic ecosystems presented weak coupling or disconnected patterns between functioning and community composition(Langenheder et al. 2005; Frossard et al. 2012; Gibbons et al. 2014). These different responses suggest that both community composition and functioning can respond or remain unaltered after disturbances. Disturbances are discrete events in time that cause changes in resources or in the physical environment and, thus, influence community structure (Berga et al. 2012). For example, it has been observed that changes in organic matter quantity and quality can induce changes in bacterial community composition (Kirchman et al. 2004; Docherty et al. 2006).

It is reported that microbial community composition could be resistant or resilient to disturbances (Allison and Martiny 2008, **Fig. 5**) defining at the same time the stability of the community (Shade et al. 2012). Bacterial communities are resistant when they can withstand the disturbance without compositional changes. On the contrary, they are resilient when the community composition change but is able to quickly recover and return to its original state (Pimm 1984; Allison and Martiny 2008). On the other hand, some studies observed differences in bacterial community composition but not in functions suggesting a high functional redundancy of microbial freshwater communities (Frossard et al. 2012; Lear et al. 2014; Wagner et al. 2014). Functional redundant communities means that

different species have similar functional roles in the community. Alternatively, bacterial community could have high functional plasticity when only functions but no community composition change after disturbance (or having metabolic plasticity, Comte et al. 2013). In this case, bacterial communities usually change their morphological and physiological characteristics of cells, for example, modifying the size and/or activity (Schimel et al. 2007) suggesting that functional pasticity can lead to compositional resistance.

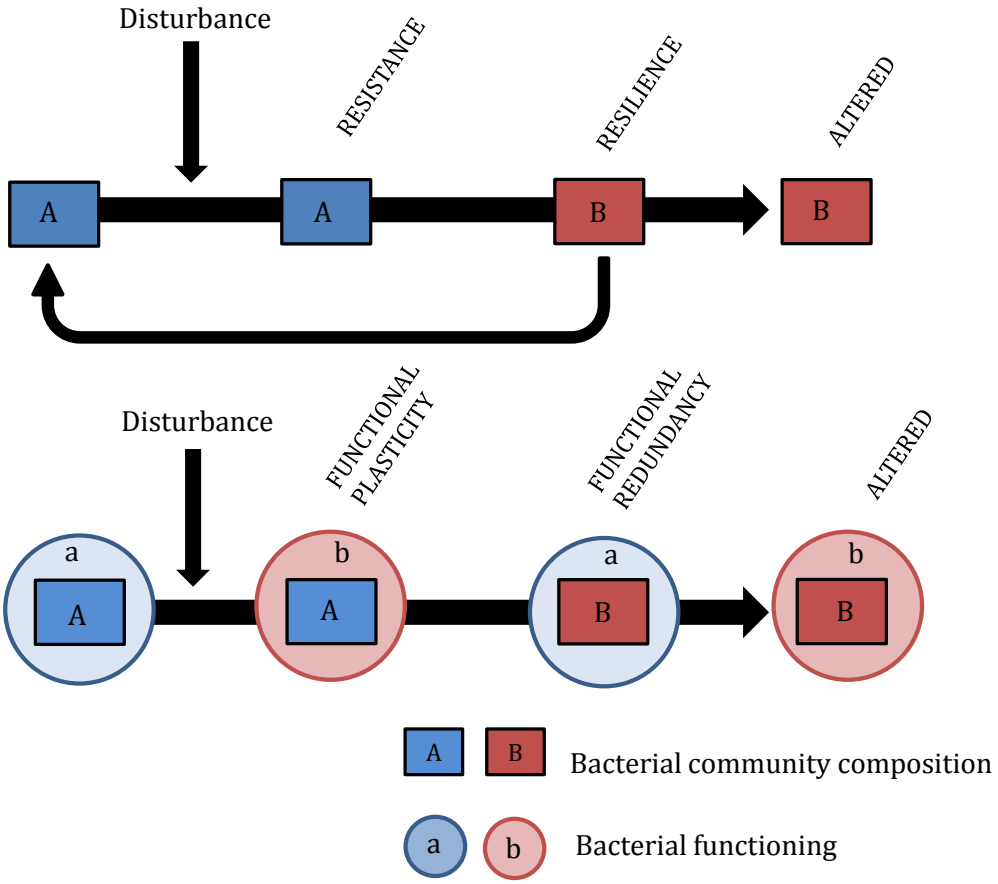


Figure 5. Diagram of how disturbance can affect microbial community composition and functioning. Modified from Allison and Martiny (2008).

GENERAL OBJECTIVES

GENERAL OBJECTIVES AND HYPOTHESIS

The main objective of this thesis is to analyse changes in the biofilm structure and dissolved organic matter utilization in river sediments at different spatial (reach, upstream-downstream, vertical and depth) and temporal scales (hydrological period and hours) and determine how they are affected by changes in environmental variables.

The specific objectives and hypothesis to reach this main goal are the following:

1. To review the methodological protocols, applicability and expected results from Biolog EcoPlates utilization with environmental samples for the measurement of carbon substrate degradation capabilities in river sediments (**Chapter 1**).

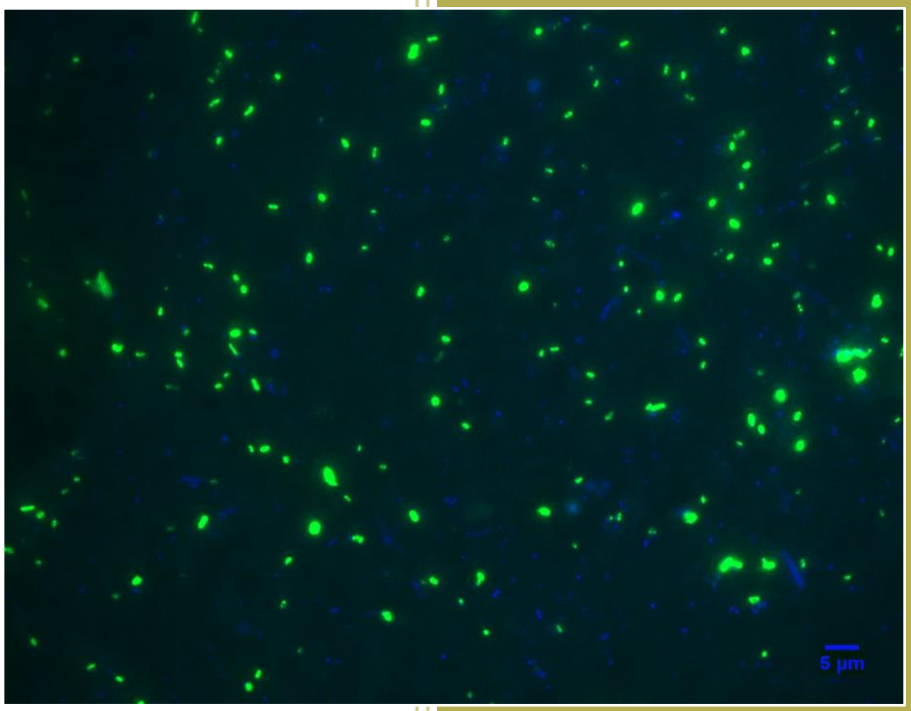
2. To investigate how carbon source utilization and functional diversity change in sediments along a polluted river system (**Chapter 2**). It is expected that differences in the metabolic fingerprint would occur between upstream and downstream samples as well as a reduction of functional diversity at the most polluted sites.

3. To analyse changes in microbial organic matter use at different sediment depths and oxygen concentrations under continuous infiltration experimental conditions (**Chapter 3**). It is hypothesized that microbial activity and biomass would be higher at the sediment surface and decline with depth. Gradients in depth would be more pronounced at the end of the experiment consistent with a vertical oxygen gradient.

4. To study the effect of warming and alteration of diel temperature patterns on the sediment biofilm community and functioning involved in carbon metabolism (**Chapter 4**). It is hypothesized that carbon metabolism would change over the diel period and that warming would raise the differences between day and night-time. Moreover, it is expected that warming would stimulate the growth rates of biofilm communities with subsequent effects on the biofilm food-webs.

5. To ascertain the longitudinal patterns in sediment bacterial community composition and organic matter utilization in response to environmental factors under base flow and drought conditions (**Chapter 5**). It is expected that the heterotrophic biofilm bacteria would adapt to availability and quality of organic matter from upstream to downstream. In addition, it is hypothesized to find greater differences in microbial functioning and bacterial community composition between sampling sites during drought than during base flow periods.

MATERIALS AND METHODS



In order to achieve the objectives of this thesis, different methods were used throughout the different thesis chapters. *Chapter 1* is a review of the Biolog Ecoplates technique and thus it is a methodological paper itself. Then, field studies were conducted at two Mediterranean rivers (Llobregat and Tordera rivers) (*Chapters 2 and 5)*. Two laboratory experiments were performed one using an infiltration sediment tank (*Chapter 3*) and the other using 12 artificial stream channels (*Chapter 4*). In this general Materials and Methods section, the main techniques used in the thesis are summarized but specific methodological details (specific protocols and sampling strategies) and experimental designs are described within each chapter.

STUDY SITES

The field work was developed at two different Mediterranean rivers located at the northeast of the Iberian Peninsula; the Llobregat and the Tordera rivers (**Fig. 1**), The Llobregat river was selected in order to represent a highly impacted river by anthropogenic pressures, and the selecting sampling sites included a downstream pollution gradient. On the other hand the Tordera river, lesser polluted than the Llobregat, is characterized by having a pristine upstream reach and without any reservoir along its main course. The Tordera river size (60 km long) made possible to perform a longitudinal sampling from headwater to the river mouth.

The **Llobregat river,** drains an area of 4948 km^2 and it is 157 km long. The geological substratum of this river is mainly calcareous. It is one of the most important water-courses in north-east Spain, because it supplies 35% of the drinking water to Barcelona city area. Together with its two main tributaries, the Cardener (1412 km^2) and the Anoia rivers (929 km^2), the Llobregat is a clear overexploited Mediterranean river (Muñoz et al. 2009). The Llobregat watershed, especially in the lower-medium courses, is densely inhabited and the river receives large inputs from industry and urban origin (WWTP) as well as surface runs off from agricultural areas (Kuster et al. 2008). Thus, an increased in the downstream content of nutrients, pesticides and pharmaceuticals compounds was reported in water and sediments along the Llobregat basin (Casas et al. 2003; Kuster et al. 2008; González et al. 2012). The main channel of the river is interrupted by a

series of dams and derivation channels. As a Mediterranean river, it is highly dependent on climatic conditions, mean annual precipitation is between 400–600 mm and mean annual discharge is around 8 m^3 s^{-1} although monthly mean values range between 2 – 416 m^3 s^{-1} (Fernández-Turiel et al. 2003). The five sampling sites selected for our study were located at the mid to lower part of the river after main dams (**Fig. 1**).

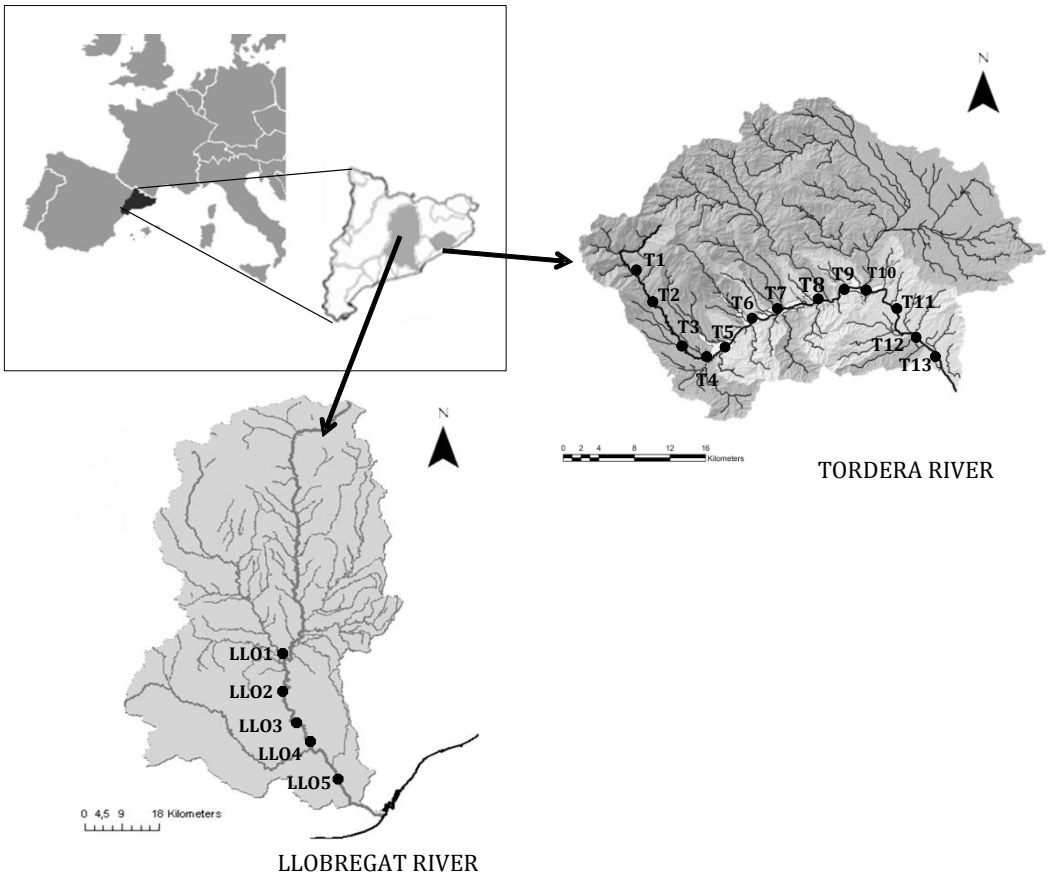


Figure 1. Map of the study sites with the location of the sampling sites for the Tordera and Llobregat rivers.

The **Tordera river**, drains an area of 865 km^2 and it is 60 km long. The geological substratum is mainly calcareous. 77% of the catchment is forested, 16% has crop coverage (in the alluvial deposits) and 7% is covered by urban and industrial areas specially located at the lowland of the river (Caille et al. 2007). Riparian vegetation shades the river bed during the first 25 km. The main tributaries are the Santa

Coloma (275 km^2) and Arbúcies (104 km^2) rivers. The river network receives the effluents from 13 waste water treatment plants and 10-15 industries. Mean annual rainfall ranges from 1000 mm at the mountains to 600 mm at the coast (Rovira and Batalla 2006). Mean annual discharge is around 4 $m^3\ s^{-1}$ (ACA 2002; Rovira and Batalla 2006) but as a Mediterranean river the annual hydrology is very variable. The 13 sampling sites selected for our study were located at the river main stem from headwaters to the mouth (**Fig. 1**).

METHODS

The different techniques used to study the structure and function of microbial communities in river sediments are listed in Table 1, also specifying the chapter/s were each technique has been applied. The techniques are briefly described below.

Physical and biogeochemical analysis in river water

- **Physicochemical parameters** (pH, conductivity, temperature and dissolved oxygen) in water were analysed in-situ using portable probes (WTW).
- **Inorganic nutrients.** Ammonium was measured using the Reardon et al. (1966) salicylate method. Nitrate and sulphate were analysed using an ion chromatograph following standard methods (APHA, 1989). Phosphate was analysed spectrophotometrically using the ammonium molybdate colorimetric method (Murphy and Riley 1962).
- **Dissolved organic carbon (DOC)** concentration was determined using a total organic carbon analyser (Shimadzu TOC-V CSH).
- **Dissolved organic matter (DOM) properties**: SUVA, HIX and FI indexes. SUVA, as an indicator of DOM aromaticity, was calculated by the absorbance at 254nm normalized by DOC concentration (Weishaar et al. 2003). HIX, as a measure of the humification degree of DOM, was calculated as the ratio between the area under excitation/emission 254/435-480nm and the area under 254/ 330-345nm (Zsolnay et al. 1999). Fluorescence Index (FI), indicator of DOM origin (terrestrial vs microbial), was measured as the fluorescence intensity at excitation/emission 370/470nm divided by fluorescence at 370/520nm (McKnight et al. 2001)

Table 1. Summary of the different methods used in each chapter (CHP) of this thesis. Methods used for water and sediment samples are distinguished.

		CHP2	CHP3	CHP4	CHP5
Water	Physicochemical parameters	■	■	■	■
	Inorganic Nutrients	■	■	■	■
	DOC	■	■	■	■
	DOM properties			■	■
Sediment	Bacterial density	■	■	■	■
	Bacterial viability (L/D)	■	■		
	Bacterial community composition				■
	Respiratory activity (ETS)	■			
	Extracellular enzyme activities		■	■	■
	Biolog Ecoplates	■	■		■
	Extracellular polymeric substances (EPS)	■			
	Organic matter	■	■	■	■
	Granulometry and C:N content				■
	Chorophyll-a			■	■
	Meiofauna			■	

Bacterial density, viability and community composition

- **Bacterial density** was estimated through fluorescent staining with DAPI (4,6-diamidino-2-phenylindole) (Porter and Feig 1980) and posterior counting by epifluorescence microscope at 1000x magnification. Moreover, bacterial density was also estimated by flow cytometer using Syto13 stained cells.
- **Bacterial viability**. Abundance of live and dead bacteria were estimated using the LIVE/DEAD double- stain kit with Syto 9 and propidium iodide (Freese et al. 2006) and posterior counting by epifluorescence microscope at 1000x magnification. Moreover the green fluorescence of each sample (excitation/emission being 480/500 nm for Syto 9) was measured as an indicator of live bacterial abundance (Roth et al. 1997).
- **Bacterial community composition** was assessed by performing the Catalysed Reported Deposition- Fluorescence in-situ Hybridization (CARD-FISH) following procedures described in Fazi et al. (2005). Eubacteria, Alpha-, Beta-, Gamma-, and Delta-Proteobacteria rRNA-target HRP-labeled

probes were hybridized and cells were counted using epifluorescence microscopy at 1000x magnification.

Biofilm functioning

- **Respiratory activity** was measured through the electron transport system **(ETS)** assay after reduction of electron transport acceptor INT (2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride) to INT-formazan (iodonitrotetrazolium-formazan) (Blenkinsopp and Lock 1990). INT-formazan was extracted with methanol and its absorbance was measured at 480nm.
- **Extracellular enzyme activities** were analysed through incubation of artificial substrates with samples and posterior fluorescence (for methylumbelliferyl (MUF) or aminomethylcoumarin (AMC)) or absorbance (for L-3,4-dihydroxyphenylalanine (L-DOPA)) incubations detection following procedures described in Romaní and Sabater 2001. The different enzymes measured in the thesis are summarized in Table 2.

Table 2. Summary of extracellular enzymes measured in each chapter of this thesis. The EC (enzyme commission) number for each enzyme is also indicated.

Enzyme	Artificial substrate	CHP2	CHP3	CHP4	CHP5
β-D-glucosidase (EC 3.2.1.21)	MUF-β-D-glucoside				
Alkaline phosphatase (EC 3.1.3.1-2)	MUF-phosphate				
Leucine-aminopeptidase (EC 3.4.11.1)	Leu-AMC				
β-D-xylosidase (EC 3.2.1.37)	MUF-β-D-xyloside				
Lipase (EC 3.1.1.3)	MUF-palmitate				
Phenol oxidase (EC 1.10.3)	L-DOPA				

- **Biolog EcoPlates**. Analysis of carbon substrate utilization profiles were analysed by incubation of sediment extracts inside of the Biolog EcoPlates at 20ºC in dark conditions for 6 days. Optical density in the plates was read every 24h at 590m using microplate reader. A deep description and discussion of the protocol for this technique is included in *Chapter 1*.

Biofilm structure

- **Extracellular polymeric substances (EPS)** from the sediments were extracted using cation exchange resin following the procedure described in Romaní et al. 2008. The polysaccharide content was measured by the phenol-sulphuric acid assay after the extraction of EPS (Dubois et al. 1956).
- **Organic matter** was measured as ash-free dry weight (AFDW). Sediment was first dried (placed at 70 ºC for 72 h) and then combusted (at 450 ºC for 4 h) using a muffle furnace.
- **Carbon and nitrogen content** was measured in dry sediments through a CN Elemental Analyser.
- **Chlorophyll-a** was used as a proxy for algal biomass. It was extracted with 90% acetone and concentration was determined using spectrophotometer following the protocol of Jeffrey and Humphrey 1975.
- **Meiofauna** organisms were counted using a microscope at 10x magnification and distinguished at the group of level for ciliates, rotifers nematodes, copepods, annelids and turbellaria.

Grain size distribution

- **Granulometry** was determined by sieving dry sediment through different sieves and calculating the percentage of gravel (2-4 mm), sand (0.2-2mm) and silt (<0.2mm).
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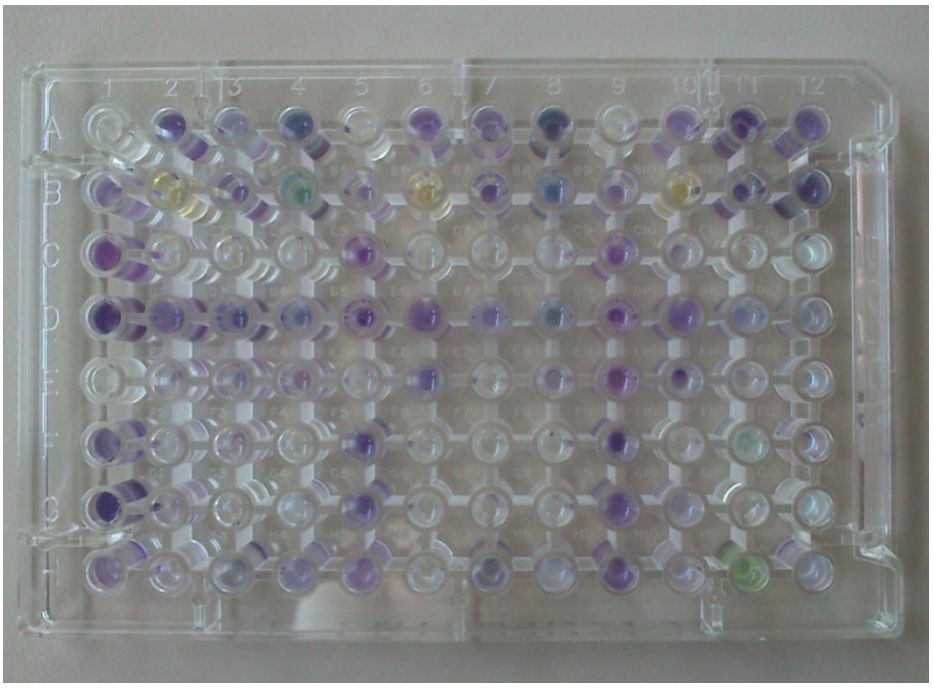
CHAPTER 1

REVIEW OF THE BIOLOG ECOPLATES TECNHIQUE AS A TOOL TO STUDY CARBON SUBSTRATE UTILIZATION IN ENVIRONMENTAL MICROBIAL COMMUNITIES



ABSTRACT

Biolog EcoPlates have been designed to provide a method for determining functional diversity and metabolic fingerprint based on carbon substrate utilization profiles in environmental samples and have been mainly used in soil and aquatic microbial communities. Biolog EcoPlates have well-known method limitations because of being a culture-based approach constrained by incubation conditions. Big amount of data is generated using this technique but not without problems in terms of interpretation due to different possibilities in data treatment. In this sense, the lack of standardization of this technique makes it hard to compare results across studies. Thus, this chapter tries to clarify and review the methodological variability of Biolog EcoPlates utilization in environmental microbial communities. Furthermore, finally this review discusses the interpretation of Biolog Ecoplate data and compares this technique with other functional and structural approaches.

BACKGROUND

Origin and uses of Biolog Ecoplates incubation method

The Biolog microplate incubation method was first utilized in soils to determine the metabolic fingerprint of heterotrophic microorganisms (Garland and Mills 1991). It is known that heterotrophic microorganisms are extremely diverse metabolizing different types of carbon sources. Researchers found that after inoculating microbial communities in a microplate containing different carbon substrates it was possible to define the community level physiological profile (CLPP) of the specific microbial community inoculated. This technique is recommended for comparing the functional diversity between samples and it has been successfully applied for monitoring temporal and spatial changes in bacterial and fungal communities (Garland 1997; Staddon et al. 1997; Dickerson and Williams 2014) as well as changes caused by environmental fluctuations, pollution or disturbance (e.g. Goberna et al. 2005; Tiquia 2010).

The first commercial microplate was the Biolog GN, composed by 95 different carbon sources. This microplate was created for a rapid identification of carbon source utilization of Gram-negative bacteria. After that, numerous studies used different commercialized microplates distinguished by the type of inoculum and number and type of substrates they contained (Preston-Mafham et al. 2002). Briefly, most common microplates are those designed for Gram-negative bacteria (GN plates), Gram-positive bacteria (GP plates), environmental bacterial community samples (EcoPlates), or fungal communities (SFN and SFP plates). Fungal microplates use dimethylthiazolyl-diphenyletrazolium bromide (MTT) as indicator of fungal growth while bacterial plates use tetrazolium violet (Dobranic and Zak 1999; Buyer et al. 2001). Particularly, Biolog EcoPlates were created specifically for bacterial analysis of environmental samples being that replicates of carbon sources and substrates with more ecological relevance were provided. Using replicates increases the probability that the obtained metabolic profile represents the microbial community studied (Stefanowicz 2006). Comte and del Giorgio (2009) observed that Biolog EcoPlates replicates results yield remarkably similar and consistent metabolic profiles, indicating that the final results were not

a consequence of random growth. The Biolog EcoPlates have been extensively used in soils (Goberna et al. 2005; Braun et al. 2010; Floch et al. 2011; Yu et al. 2012) and they have been also successfully applied in freshwater systems such river sediments (Tiquia 2010; Bushaw-Newton et al. 2012; Freixa and Romaní 2014), sediment wetlands (Salomo et al. 2009; Douterelo et al. 2010), sediment reservoirs (Christian and Lind 2007) and sewer sediments (Biggs et al. 2011). Moreover the Biolog EcoPlates have been also used for incubating environmental water samples such as marine bacterioplankton (Sala et al. 2008) or freshwater from rivers and lakes (Sinsabaugh and Foreman 2001; Dickerson and Williams 2014).

This technique is widely used because it is simple, inexpensive, fast, useful for replicated large–scale studies and because the procedure yields a great deal of information linked to organic matter use and thus to ecosystem functioning (Preston-Mafham et al. 2002). However, although a large amount of data is easily generated by this technique, their interpretation is complex since incubation conditions and data selection can significantly modify the obtained results. In this review we aim to contrast the different protocols reported to obtain data from Biolog EcoPlates to clarify its interpretation. Finally, the last section is focused on the ecological interpretation of this data in order to detect advantages and limitations of Biolog EcoPlates utilization for environmental samples in comparison to other functional and structural approaches.

Carbon sources

Each Biolog EcoPlate (a 96-well plate) provides 31 different carbon substrates in triplicate with a redox dye and a control well (A1) with no added carbon substrate. The Biolog EcoPlates are composed by substrates considered ecologically relevant (Derry et al. 1998) some of them are typically found in terrestrial environments and at least nine are considered as constituents of plant root exudates (Preston-Mafhan et al. 2002).

Individual wells within the plates can be grouped into different carbon sources categorized by substrate guilds such as amines, amino acids, carbohydrates, carboxylic acids, phenolic compounds and polymers (Insam 1997; Christian and

Lind 2006) (Table 1). All substrates contain carbon in their chemical structure, 10 substrates contain both C and N and 2 contain both C and P.

Table 1. List of 31 carbon substrates with their chemical formula grouped in 6 different groups, modified from Insam 1997.

Group of substrate	Substrate	Formula	Group of substrate	Substrate	Formula
Amino acids (n = 6)	Glycyl-L-Glutamic Acid	$C_7H_{12}N_2O_5$	**Carboxylic acids (n =7)**	γ-Hydroxybutyric acid	$C_4H_8O_3$
	L-Arginine	$C_6H_{14}N_4O_2$		α-Ketobutyric acid	$C_4H_6O_3$
	L-Asparagine	$C_4H_8N_2O_3$		D-Galacturonic acid	$C_6H_{10}O_7$
	L-Phenylalanine	$C_9H_{11}NO_2$		D-Glucosaminic acid	$C_6H_{13}NO_6$
	L-Serine	$C_3H_7NO_3$		Itaconic acid	$C_5H_6O_4$
	L-Threonine	$C_4H_9NO_3$		D-Malic acid	$C_4H_6O_5$
Carbohydrates (n = 10)	D-Cellobiose	$C_{12}H_{22}O_{11}$		Pyruvic acid methyl ester	$C_2H_6O_3$
	i-Erythritol	$C_4H_{10}O_4$	**Phenolic compounds (n=2)**	2-Hydroxybenzoic acid	$C_7H_6O_3$
	D-Galactonic acid c-lactone	$C_6H_{10}O_6$		4-Hydroxybenzoic acid	$C_7H_6O_3$
	N-Acetyl-D-glucosamine	$C_8H_{15}NO_6$	**Polymers (n = 4)**	α-Cyclodextrin	$C_{36}H_{60}O_{30}$
	Glucose-1-phosphate	$C_6H_{13}O_9P$		Glycogen	$(C_6H_{10}O_5)n$
	β-Methyl-D-glucoside	$C_7H_{14}O_6$		Tween 40	
	D,L-a-Glycerol phosphate	$C_3H_9O_6P$		Tween 80	
	α-D-Lactose	$C_{12}H_{22}O_{11}$	**Amines (n = 2)**	Phenylethylamine	$C_8H_{11}N$
	D-Mannitol	$C_6H_{14}O_6$		Putrescine	$C_4H_{12}N_2$
	D-Xylose	$C_5H_{10}O_5$			

Basis of Biolog Ecoplate measurements: Formazan formation

Plates are inoculated with a bacterial community and then incubated during a period of time. Over the time, each carbon source is degraded by bacteria and CO_2 is released through respiration. Respiration reduces the tetrazolium dye (present inside each well) which is transformed into the insoluble purple formazan salt that can be observed visually and quantified spectrophotometrically (Fig. 1). The intensity of purple colour in each well is proportional to the amount of carbon being respired by the bacterial community and thus informing about the capacity of the microbial community to use (i.e. degrade) each carbon source. It is important to know that the use of this dye can be a limitation in some cases since,

as it happens for fungi, some bacterial groups are not able to reduce the tetrazolium dye (Winding and Hendriksen 1997) due to toxic effects of the redox dye (Ullrich et al. 1996).

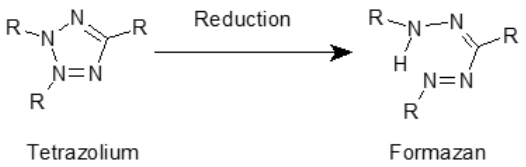


Figure 1. Reduction of tetrazolium to formazan.

PROTOCOL

The protocol to incubate Biolog EcoPlates using environmental samples requires several steps (summarized in Fig. 2). Each step requires attention and depending on research purposes and type of samples it is necessary to make decisions accordingly. The Biolog EcoPlates technique is a culture-based method which means that it is dependent on the bacterial inoculum and the incubation conditions. Results might be biased in favour of the bacterial species able to grow inside of the plates. Several authors indicated that only a cultivable fraction of fast growing aerobic and heterotrophic bacteria can grow inside the wells (Konopka et al. 1998; Campbell et al. 2003). In this sense, several limitations on the incubation conditions are already reported (see critical review by Preston-Mafham et al. 2002). In this section, the different specific protocols most frequently used are discussed step by step.

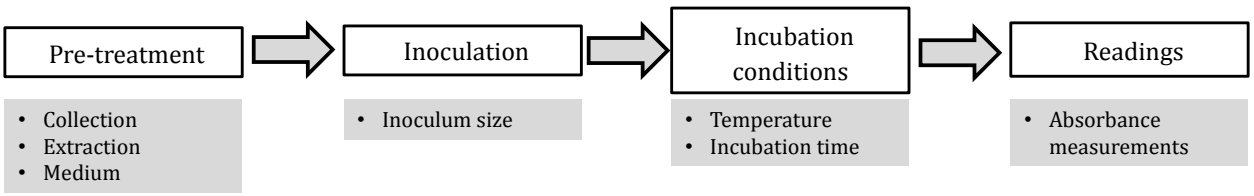


Figure 2. Scheme of the different steps for Biolog EcoPlates samples incubation. For each step, factors and variables to be considered are listed.

Pre-treatment

Samples are collected from the field, placed in a sterilized container and transported to the lab in fresh conditions (i.e. 4ºC). The time elapsing from sample collection to inoculation of plates has to be as short as possible (24h as maximum) in order to avoid changes in microbial community composition (Preston-Mafham et al. 2002). Storage as cold or frozen samples can clearly reduce and modify the capacity of a microbial community to degrade carbon sources (see Chapter 2 and Freixa and Romaní 2014).

In river studies, typically collected samples for Biolog EcoPlates incubations are river water or sediment samples. In the case of water, samples can be directly inoculated without previous treatments whereas sediment suspensions are used for sediment samples in order to extract and dilute bacteria. Overall for sediments, the dilutions procedures are carried out using an isotonic solution with no osmotic effects on the cells such as solutions of pyrophosphate or phosphate buffer (Preston-Mafham et al. 2002; Schmitt et al. 2006; Yu et al. 2012) or a solution of salts such as sterile NaCl or Ringer (Goberna et al. 2005; Douterelo et al. 2010; Floch et al. 2011). These solutions are the ones most often used to homogenize the effect of different water quality in each sampling sites. Moreover sterilized river water (for example filtered by 0.2 μm filters) can be also employed. Sometimes, in the case of sediment samples, turbidity (caused by suspended sediment particles) can interfere in the absorbance measurements, incrementing the final absorbance values. In this case, in order to reduce it, samples could be centrifuged and the supernatant is used to inoculate microplates. In this sense, it is recognized that some loss of bacteria may occur during the centrifugation process depending on centrifugation speed because some bacteria remain attached to sediment particles (Calbrix et al. 2005). In addition, the effects of turbidity (in case that it is not very high) can be corrected a posteriori, subtracting the initial well reading (background colour) at time zero from subsequent readings (Campbell et al. 2003) or subtracting the absorbance value at 750 nm.

In case of seawater and estuarine water samples, it is also important to control the calcium concentration because interferes with the micro plate chemistry. Someauthors found that Biolog EcoPlates could cause false positive readings when values greater than 100 ppm of calcium were detected in water (Pierce et al. 2014).

Inoculation

The plates are inoculated with 130-150 µl of sediment suspension inside of each well. The inoculum size (i.e. density of bacteria) influences in the colour development over the time. When the inoculum size is larger, higher average well colour development (AWCD) values are observed (Fig. 3) and more number of carbon sources (positive wells) are measured for the initial incubation days (up to 72 h in the example from Fig. 3). Therefore, when higher bacterial density is inoculated a shorter lag phase before formazan formation is observed (Haack et al. 1995; Konopka et al. 1998). In order to minimize the effect of having different bacterial density in samples, different options are proposed a posteriori during the data treatment (these are summarized in the next section, standardization of inoculum density, page 51). On the other hand, some authors propose to inoculate plates with approximately the same bacterial density between 1×10^6 and 1×10^8 cells ml^{-1} (Konopka et al. 1998; Insam and Goberna 2004) for sediment and soil suspensions. However, a minimum inoculum density about 10^8 cells ml^{-1} is required to produce an observable colour change (Konopka et al. 1998). In this sense, in case of a large field sampling it is practical to know beforehand the average or expected range of bacterial density in order to decide the optimal dilution factor.

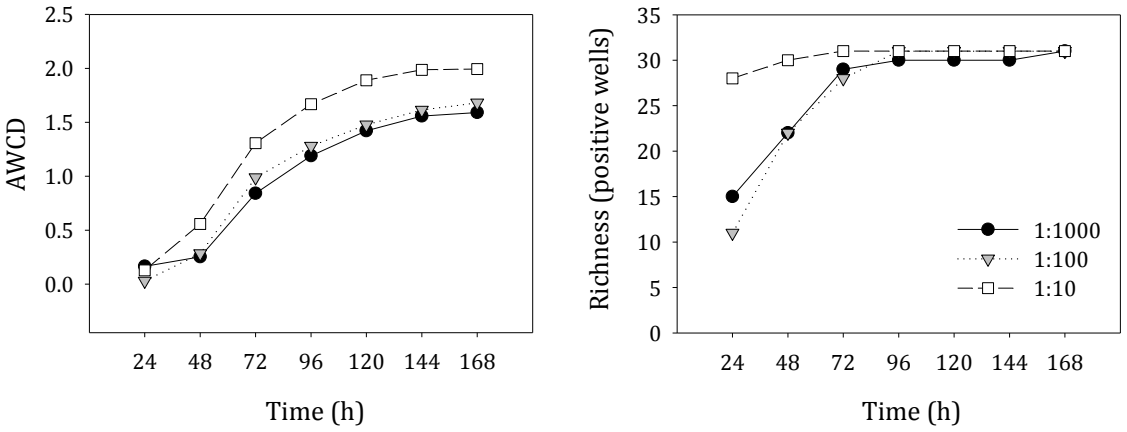


Figure 3. Results from AWCD and Richness (number of positive wells in the plate, see Data treatment section) after incubating river sediment sample extracts by using different inoculum sizes, diluting the initial extract by 1:10, 1:100, and 1:1000). Values were measured from 24 to 168 hours.

Incubation conditions

The different incubation conditions can modify the colour development inside of the plates and reduce or increase the differences between samples. The incubation conditions (such as temperature, oxygen and substrates selection) can favour the growth of some bacteria causing structural and functional changes with respect to the original inoculum (Konopka et al. 1998; Preston-Mafham et al. 2002; Christian and Lind 2006).

Incubation time and temperature are inversely linked, i.e. higher temperatures imply short incubation periods and vice versa. Temperature has an effect on the metabolic fingerprint and also on rates of colour development (Christian and Lind 2006; Biggs et al. 2011). Most studies use fixed incubation temperatures between 15 and 28ºC (Preston-Mafham et al. 2002; Calbrix et al. 2005) and 20ºC and incubation times over 2 to 7 days are the most commonly used (Garland 1997; Schmitt et al. 2006; Braun et al. 2010). Incubations at lower temperatures (< 10ºC) do not allow to observe a clear pattern of colour development within a few days of

incubation due to the reduced microbial activity. Therefore, when working at low temperatures incubation time need to be increase, which also means that bacterial communities are subjected for a longer time to cultivation effects. This could induce phenomena such as cell lysis and extracellular storage polymers (Haack et al. 1995). On the other hand, incubation at higher temperatures (> 30ºC) promotes evaporation and stimulates the microbial activity modifying the metabolic fingerprint. In this case, differences between samples, due to quickly depletion of carbon source, are sometimes not observed (Calbrix et al. 2005). In case of short-incubation time the colour development in some wells is limited because of some carbon substrates need more time to be degraded (Verschuere et al. 1997). Finally, another alternative is to incubate the microplates at in situ temperatures measured during sample collection in order to reproduce field conditions. However, in this case, the results obtained in different seasons would not be comparable (Christian and Lind 2006).

Readings

The optical density (OD) to detect the colour development inside of the wells is measured at 590, 592 or 595 nm using an absorbance plate reader. In addition, other studies also measured absorbance at 750 nm in order to detect turbidity of the samples and subtract it from the measurement at 590 nm (Yu et al. 2012). As explained before, the time of incubation is very variable and depends on the inoculum and incubation conditions. Mainly the absorbance readings are performed every 12 or 24 hours and for 2-10 days.

DATA TREATMENT

Biolog EcoPlates provide large amounts of data and a method to extract the information in a rapid and effective way is needed. Data treatment consumes most of the time in the utilization of Biolog EcoPlates technique due to the complexity of data analysis. One of the main limitations is that the rate of colour development during incubation time is non-linear and usually follows a sigmoid or logistic curve with time, thus making selection of data complicated. In this sense, after absorbance measurements, raw data is obtained and there are several steps to follow for a good data interpretation (Fig. 4).

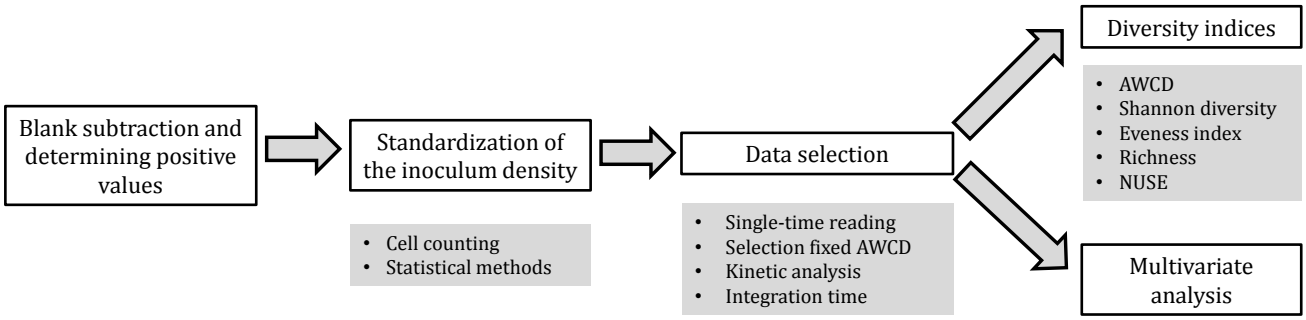


Figure 4. Data treatment process after Biolog EcoPlates absorbance measurement. Parameters to be considered at each step are listed.

Blank subtraction and determining positive values

The absorbance measured in control wells (no substrate) is first subtracted from the absorbance measured in each well and for each time reading in order to eliminate background colour and turbidity effects (water samples or sediment extracts). Then, negative values and values less than 0.03-0.25 (it depends on the author) are set to zero. For instance, some authors reported that the limit value selected to determine positive wells were OD values higher to 0.03, considering lower values as a noise and no significant to realize catabolic activity or OD values higuer of 0.06 because were the detection limit of the spectrophotometer (Guckert et al. 1996; Heuer and Smalla 1997; Classen et al. 2003).

Standardization of the inoculum density

As discussed above, it is reported that different inoculum bacterial density would affect the final absorbance value. In this sense, there are principally three options for standardizing potential differences in the inoculum size between samples.

a) The first option consists on cell counting of the sample before the inoculation in order to estimate bacterial density previously and inoculate the same inoculum size inside of the plates (Haack et al. 1995). This option requires time and usually the application of laborious methods for cell enumeration. When large field samplings are performed this option is not appropriate.
b) The second option is to diminish biases between samples by normalising the obtained data. The most common is dividing the optical density (OD) from each well by the AWCD for each time reading (Garland and Mills 1991; Stefanowicz 2006). This second approach has proven to be effective, but only if variation in AWCD is not higher than 30-50% (Garland 1996) and with samples with similar number of positives wells, since zero values cannot be effectively normalized (Garland 1997).
c) The third approach is also a statistical method that uses a fixed level of AWCD, explained in the next section ("Select a fixed AWCD").

Nevertheless, inoculum size standardization is not always necessary when the objective of the study is to observe particularly differences in general metabolic response for instance after exposure to contaminants or comparisons after environmental disturbance.

Data selection

As mentioned before, the colour development over time is a non-linear process which generates doubts about how to select the data for its analysis. The next four approaches propose different options for data selection:

a) Selecting a single-time reading. This consists of selecting the absorbance readings at one specific time point (e.g. after 24, 96 or 144 hours, or any time point of incubation). Selecting a single-time point is only feasible when the inoculum density are previously standardised by one of the methods

explained above. This is the most widely used strategy (Insam and Goberna 2004) however, different responses can be observed depending on the time reading selected. Selecting short incubation times (24h or less) minimizes the effects of the culture technique, but it does not allow evaluating slower metabolized substrates (Konopka et al. 1998). On the other hand, selecting long incubation times (such as 144h; usually when colour development is saturated) or when most of wells have an OD around 2 (Insam and Goberna 2004) implies greater cultivation biases, but results can be used as an indicator of the potential capacity of microbial community to degrade the present carbon substrates (Douterelo et al. 2010).

b) Selecting a fixed level of AWCD. This approach consists on selecting the absorbance readings at specific value of AWCD. For example, values of 0.25, 0.5, 0.75, or 1.0 for AWCD have been proposed as fixed levels for absorbance readings (Insam and Goberna 2004; Garland et al. 2001). In this case, it is necessary to monitor colour development over time and then to select time readings with AWCD as close as possible to the one chosen as a fixed level. In addition, some authors used the kinetic approach (see approach c) to calculate the precise OD values at selected fixed AWCD using a linear interpolation (Salomo et al. 2009). By using this option, Stefanowicz (2006) reported that choosing measurements of AWCD equal to 0.75 is one of the best solutions because most of wells have positive values and the wells with the most active microbial communities reach the asymptote of colour development. Other authors also choose the AWCD at the time when readings are in the exponential phase (see option c) kinetic approach; Dickerson and Williams 2014; Tiquia 2010). This option avoids the problems caused by differences in inoculum density.

c) Analyzing the kinetics of the color development. The kinetic parameters can be estimated by fitting the curve of OD versus time to a sigmoid or logistic curve (Lindstrom et al. 1998; Mondini and Insam 2003). The sigmoid equation is represented as:

$$y = \frac{a}{1 + e^{-(\frac{x - x_0)}{b})}}$$

In this equation the maximum absorbance of colour saturation is represented by the parameter *a*, the slope *(1/b)* represents the maximum rate of colour development per incubation time, and the time when maximum colour development rate is achieved is represented by the parameter x_0. With this three kinetic parameters (*a*, *1/b* and x_0) it is possible to evaluate how rapidly different carbon sources can be metabolised by the microbial community. Nevertheless, the kinetic parameters are usually correlated with the inoculum density (Garland et al. 2001) and need to be standardized prior to the statistical analysis (Stefanowicz 2006).

d) Selecting the **curve integration** data. By this approach, the total area under the curve (OD versus time) is calculated. The obtained value integrates the information of the entire incubation period (Guckert et al. 1996). The use of the integrated value is particularly useful when data do not fit the sigmoid curve, but results can be difficult to interpret. Some authors observed that differences in the metabolic fingerprint between using single-time reading and the integration values were few (Comte and del Giorgio 2009). For this reason they proposed to select the first option (single-time reading) because the curve integration require more time to analyse data and is more sensitive to inoculum size (Garland et al. 2001).

Data analysis

From the standardized data, diversity indices and multivariate analysis are frequently applied. Different functional diversity indices are usually applied using selected Biolog EcoPlates data (single-time reading, selected AWCD or integrated values) (Table 2).

Table 2. Different functional diversity indices used with Biolog EcoPlates data.

Index	Formulae	Definitions	Reference
AWCD	$\mathbf{AWCD = \sum(c-r)/n}$	c = the raw absorbance in each well r = the absorbance in the control well n = number of substrates inside the plate	Garland and Mills 1991
Shannon Index (functional diversity)	$\boldsymbol{H = -\sum p_i (ln\ p_i)}$	pi= the ratio of the corrected absorbance value of each well to the sum of the absorbance value of all the wells in each plate.	Shannon and Weaver 1963
Evenness index	**H/ log S**	S = number of positive wells H= Shannon index	
Substrate richness (functional richness)	**S**	S = number of positive wells	
NUSE Index (nitrogen use index)	**NUSE=**% (Abs N / Total Abs)	Summed absorbance of all N- containing substrates, over the total absorbance measured in each EcoPlate	Sala et al. 2006

For data representation, data can be analysed using multivariate statistical techniques to evaluate the relative similarity in metabolic fingerprint patterns among samples (Zak et al. 1994; Garland 1997). Principal component analysis (PCA) is the most used ordination procedure in order to reduce the data set into principal components, followed by clustering, canonical correspondence analysis (CCA) and multidimensional scaling (MDS).

INTERPRETATION AND ECOLOGICAL RELEVANCE

Interpretation of Biolog EcoPlates results

The analysis and interpretation of the Biolog EcoPlates data are often complicated. The results obtained from Biolog EcoPlates are expected to be related to the composition of the inoculated bacterial community (Rutgers et al. 2015) but the method should not be applied for bacterial community characterization (Stefanowicz 2006). Actually, the interpretation of the Biolog Ecoplates results as an indicator of "in situ" specific carbon use is erroneous, as well as the cause-effect relationships between substrates inside the plates and in the environment. Most of the organic substrates in the natural environment are more complex than those found in the plates and, at the same time, some substrates in the plate may not be found in the environment (Smalla et al. 1998). In this sense, a positive response to a carbon substrate does not mean that this substrate is available at the sampling site, but gives us an idea of the potential type of substrates that the community will be able to metabolize.

Further, the plates are selective and may not represent the activity of all community members (Haack et al. 1995). The bacterial community can change inside of the plates during the incubation time with respect to the initial inoculum as a result of the differential growth and interaction between species in the wells (cooperation and competition) (Preston-Mafham et al. 2002; Haack et al. 1995). As it is expected, the diversity of microorganisms growing in the wells is usually lower to that found in the original samples (Smalla et al. 1998). Even thought, some authors found that both non-culturable and culturable bacteria contributed to the formazan formation, showing that the viability inside the wells was higher than the culturability (Garland 1997; Winding and Hendriksen 1997).

Overall, the general utilization patterns as defined as the community level physiological profile (CLPP) or the metabolic fingerprint can be nicely interpreted. Particularly, Zak et al. (1994) found that analysis of substrate groups instead of individual substrates provided additional insight into microbial community differences among sites.

Comparison of Biolog EcoPlates to other functional and structural approaches

Many studies have been employed Biolog plates over the last 20 years in order to get information about microbial functional diversity in natural environments. However, other similar methodologies have been developed with the aim of estimating ecosystem metabolism and functional diversity in natural ecosystems. For instance, the Substrate Induced Respiration **(SIR)** and the micro respirometry system (**MicroResp)** are very useful to measure metabolic diversity and they do not require culturability of bacteria (Degens and Harris 1997; Campbell et al. 2003). Particularly, the SIR measures the amount of CO_2 before and after the addition of different substrates inside of a glass bottle. On the other hand, the MicroResp was designed to measure microbial respiration by capturing the CO_2 produced during a short period of time (6h) inside of microplates where different carbon substrates are placed before the addition of sample. Samples and carbon substrates are placed within a 96-well plate and a second plate, the detection plate, is used to detect the responses using a pH indicator dye that changes through CO_2 released from samples (Campbell et al. 2003). Both SIR and MicroResp use directly the sample (not an extract) and monitor the activity (i.e. microbial respiration) over a short period of time before any significant growth have occurred (Chapman et al. 2007). Nevertheless, generally less carbon substrates are used with these techniques compared to 31 present in Biolog EcoPlates. Therefore, the selection of each methodology depends on the particular study objectives and hypothesis (Chapman et al. 2007).

Other authors have also compared the Biolog EcoPlates results to those obtained from **extracellular enzyme activities** measurements. Extracellular enzyme activities provide information about microbial organic matter degradation capacity (Romaní et al. 2012). Previous studies have not found any direct correlation for both approaches for bacterioplankton (Sinsabaugh and Foreman 2001), salt marsh sediments (Costa et al. 2007b), freshwater sediments (Freixa et al. 2016, Chapter 3), or polluted sediments (Liu et al. 2015). However, weak correlations were observed in river biofilms where the capacity to use phenol oxidase activity was

correlated to the greater utilization of polymers (Ylla et al. 2014). Overall, these studies suggest that extracellular enzyme activities are more indicative of bacterial activity in the environment whereas Biolog EcoPlates provide assessments of potential functional diversity that microbial community could able to use. In this way, Floch et al. (2010) found that enzyme activities were more sensitive to detect pesticide contamination than Biolog EcoPlates in soils.

On the other hand, recently some authors studied the relationships between functional diversity (measured by Biolog EcoPlates) and bacterial community composition (taxonomic composition) (Maila et al. 2005; Leflaive et al. 2008; Xue et al. 2008; Comte and del Giorgio 2009; Yu et al. 2012; Severin et al. 2014; Romaní et al. 2014; Ruiz-González et al. 2015). Overall, results show very different responses for the relationships between CLPP from Biolog EcoPlates and results from molecular methods such as PCR-DGGE or 454 sequencing to detect taxonomic composition of bacterial communities. For instance, contrary patterns for taxonomic composition and functional diversity of microbial communities were observed in rivers and lakes (Ruiz-González et al. 2015) or weak associations between variation in bacterial community composition (from 454-sequencing) and variation in functional traits (Biolog EcoPlates) in freshwater communities (Severin et al. 2014). Interestingly, Ros et al. (2008) analysed community composition in different wells inside of Biolog plates by PCR-DGGE, and after some days of incubation in two clearly different soils. They found the same bacterial species (only 3–4 genera, detected after taxonomic assignments of DGGE bands) in the two soil samples but a clear different CLPP indicating that first, few species contribute to the colour development and second that the same bacterial species could perform different functions suggesting a high functional plasticity (Allison and Martiny 2008). In this sense, relationships between carbon substrate utilization using Biolog EcoPlates and bacterial community composition imply no direct relationships mainly due to high functional plasticity of environmental microbial communities. However, some specific relationships were observed by Romaní et al. (2014) between bacterial community composition (specific OTUs) and a group of substrates (i.e. carbohydrates) in a river biofilm experiment.

CONCLUSIONS

The main aspects to take care of when applying Biolog EcoPlates are related to the protocol definition and data treatment procedure. Many studies have used this technique but so far there is not a standard protocol. Using the same protocol (i.e. pre-treatment and incubation conditions) the standardization of the data treatment (i.e. inoculum density and data selection), and a correct interpretation of results (thanks to functional diversity indices and multivariate data treatment) are really important to properly use and compare Biolog EcoPlates results.

On the other hand, it is fundamental to know the limitations of the method when the results are interpreted. This technique may serve as a proxy for understanding the pattern of carbon substrate utilization (functional diversity) for a part of the bacterial community which is metabolically active and able to grow in plate conditions. Actually, the plates are selective and may not represent the activity of all community members confounding ecological interpretation. However, differences in the metabolic fingerprint imply differences in structure and/or function of this bacterial community, and spatial and temporal changes between samples can be effectively compared because the same community selection occurs among plates. Other techniques are more appropriate (such as MicroResp) when the objective of the study is to characterize the utilization of specific carbon sources from sampling sites. Biolog EcoPlates technique does not replace the typical functional and structural community approaches (such as enzyme activities or bacterial community composition) but they provide complementary knowledge about the pattern of carbon substrate utilization.

CHAPTER 2

SHIFTS IN CARBON SUBSTRATE UTILIZATION IN SEDIMENT MICROBIAL COMMUNITIES ALONG THE LLOBREGAT RIVER

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https://www.schweizerbart.de

Abstract

River sediment microorganisms have the capacity to metabolize, uptake and decompose flowing water organic materials, which strongly depend on their metabolic capabilities. The changes of microbial functional diversity in river sediments were analysed at five sites along the River Llobregat (NE Spain), using the Biolog EcoPlates incubation method. In parallel, we tested the potential application of the EcoPlates with preserved frozen samples (–80 °C). Although functional diversity (Shannon index) did not show significant differences, the specific carbon substrates that were used differed between sampling sites. The microbial community from the upstream site was the most active as shown by the highest respiratory activity and abundance of living bacteria, being able to metabolize more labile substrates. Downstream sediment communities showed a decrease in bacterial viability, respiration activity and EPS content, suggesting a less structured biofilm. The functional fingerprint analyses clearly distinguish the sediment microbial communities from the downstream most polluted sites, which showed a potential capacity to use more complex carbon substrates such as polymers. Freezing sediment samples is not recommended, since a significant reduction in the functional diversity and changes in the fingerprint patterns were observed together with a reduction of the number of substrates utilisation especially at two downstream sites. The Biolog EcoPlates appear to be useful for detecting changes in the microbial functional fingerprint in fresh sediments along a river pollution gradient

Keywords

biolog ecoplates; carbon substrate utilization pattern; freezing effect; functional diversity; mediterranean river

ABSTRACT

River sediment microorganisms have the capacity to metabolize, uptake and decompose flowing water organic materials, which strongly depend on their metabolic capabilities. The changes of microbial functional diversity in river sediments were analysed at five sites along the River Llobregat (NE Spain), using the Biolog EcoPlates incubation method. In parallel, we tested the potential application of the EcoPlates with preserved frozen samples (-80°C). Although functional diversity (Shannon index) did not show significant differences, the specific carbon substrates that were used differed between sampling sites. The microbial community from the upstream site was the most active as shown by the highest respiratory activity and abundance of living bacteria, being able to metabolize more labile substrates. Downstream sediment communities showed a decrease in bacterial viability, respiration activity and EPS content, suggesting a less structured biofilm. The functional fingerprint analyses clearly distinguish the sediment microbial communities from the downstream most polluted sites, which showed a potential capacity to use more complex carbon substrates such as polymers. Freezing sediment samples is not recommended, since a significant reduction in the functional diversity and changes in the fingerprint patterns were observed together with a reduction of the number of substrates utilisation especially at two downstream sites. The Biolog EcoPlates appear to be useful for detecting changes in the microbial functional fingerprint in fresh sediments along a river pollution gradient.

BACKGROUND AND AIMS

River sediments are generally colonised by microbial biofilms, which constitute a biological complex assemblage, mainly consisting of bacteria and fungi, responsible for most of the metabolic activity in streams (Findlay et al. 1993). Bacteria are generally three orders of magnitude more abundant in the surface layer of sediments than in the water column (Sala and Güde 2006), and play an important role in maintaining the ecological functions in aquatic ecosystems (Blume et al. 2002; Fierer et al. 2003). Also, a greater heterotrophic capacity for using and decomposing fluvial organic matter is measured in stream sediments when compared to the epilithic biofilm (Romani and Sabater 2001), sediment bacteria being responsible for over 90% of community respiration (Pusch and Schwoerbel 1994). Specifically, sediment bacteria are capable of uptaking, storing and transforming fluvial dissolved organic matter and nutrients (Pusch et al. 1998; Battin and Sengschmitt 1999), contributing to water purification processes (Cazelles et al. 1991). The high heterotrophic activity and decomposition of organic matter occurring in river sediments might be linked to the capacity of the microbial community to metabolize organic compounds. In this way, high extracellular enzyme activity has been measured in river sediments which showed increased capacity to decompose polysaccharides and recalcitrant compounds (high β-glucosidase and phenoloxidase activities)(Artigas et al. 2009). The presence of a wide range of enzyme capabilities and the capacity to metabolize a wide range of carbon substrates may help the sediment microbial community to grow and deal with all available organic molecules in the river, including complex organic compounds. In this sense, it has been described that higher functional diversity and functional richness may be related to higher microbial diversity (Cardinale et al. 2006) and environmental heterogeneity and, at the same time, affect ecosystem functioning (Langenheder et al. 2010).

A practical approach to measuring microbial functional diversity and define metabolic fingerprints is the incubation of the microbial communities with a wide range of substrates as obtained by Biolog EcoPlates (Salomo et al. 2009). The Biolog EcoPlates incubation method was first utilised in soils to determine the

metabolic fingerprint of heterotrophic microorganisms (Garland and Mills 1991). This method has several well-documented limitations, which we should be aware of. It is known that only a fraction of microbes are able to grow in plates (aerobic, heterotrophic and cultivable fraction) (Verschuere et al. 1997; Smalla et al. 1998). When increasing the incubation time, the differences between samples may become smaller and the incubation conditions (temperature, oxygen and inoculum density) could favour the growth of some bacteria causing structural and functional changes in the original inoculum (Konopka et al. 1998; Preston-Mafham et al. 2002). However, the use of normalized data and the standardization of the protocol (i.e. similar inoculum size and incubation conditions) provide robust results (Stefanowicz 2006). The Biolog EcoPlates has been successfully applied to plankton (Sala et al. 2008; Sinsabaugh and Foreman 2001), sediments (Salomo et al. 2009; Douterelo et al. 2010; Tiquia 2010), soils (Derry et al. 1998; Goberna et al. 2005; Braun et al. 2010; Floch et al. 2011) and sediment-water interface communities (Christian and Lind 2007).

Sediment heterotrophic metabolism and microbial utilization of organic compounds may be relevant in eutrophic and polluted rivers. However, in these systems, enhanced microbial biomass and metabolism can mask the detection of more detailed changes linked to the quality of organic matter being used, as suggested by Proia et al. (2013). Many studies have been conducted on river and microbial communities, but there are very few reports studying the metabolic diversity and their link to river pollution (Tiquia 2010). In this study we focus on the sediment metabolic fingerprint along the River Llobregat. The pollution of water and sediments in this river has been well characterized. An increase in the downstream content of pesticides, heavy metals, drugs, herbicides, surfactants, pharmaceuticals and personal care products has been reported (Casas et al. 2003; Kuster et al. 2008; González et al. 2012). Previous studies in the River Llobregat showed that changes in the species composition of bacteria, diatoms and macroinvertebrate communities occurred, and were linked to the increasing downstream levels of pesticides and pharmaceuticals(Muñoz et al. 2009; Ricart et al. 2010; González et al. 2012). It is known that a decrease in the water quality may further determine a reduction in the activity, structure and diversity of the

microbial community (Lehman et al. 1997), in part linked to the disappearance of the more sensitive species (Kandeler et al. 1996).

As a practical research question, this study also aims to investigate the potential effect of freezing environmental samples for performing metabolic measurements after storage. Environmental studies often involve extensive sample collection and storage before laboratory analyses. In general, it is well known that lengthy sample storage may be linked to significant changes in microbial activity, and short-term storage is better than long-term storage (Zelles et al. 1991). Freezing sediment samples in situ is a successful method for preserving samples and obtaining high-resolution results (Lotter et al. 1997). The quick freezing of samples in liquid nitrogen has been recommended in order to preserve microbial functions (Shikama and Yamazaki 1961). However, some studies have reported that rapid freezing fails to prevent some proteins from undergoing freezing denaturation (Cao et al. 2003). For the application of the Biolog EcoPlates incubation method in field environmental studies, it is useful to ascertain whether freezing the samples would change the organic carbon sources used.

Within this framework, the main objective of this study was to investigate how functional fingerprint obtained from carbon source utilization profiles change along the river sediment in a polluted system. Little is known about the potentiality and sensitivity of Biolog EcoPlates to detect metabolic diversity changes in river sediments affected by water pollution gradient. At the same time, we tested the potential effects of freezing the samples in the field and preserving them frozen prior to the EcoPlate incubation. We hypothesized that differences in the metabolic fingerprint would occur between upstream and downstream samples as well as a reduction of functional diversity at the most polluted sites. For this purpose, five replicates of sediment samples were collected from five different sites from the middle to the lower reaches of River Llobregat. The microbial functional diversity was measured using the Biolog EcoPlates incubation method with both fresh and frozen (-80ºC) samples. Complementary sediment biofilm structural and functional parameters, such as bacterial density and viability, polysaccharide content in EPS (Extracellular Polymeric Substances), organic matter content and respiratory activity were measured.

METHODS

Study area and sampling strategy

Five sampling sites from middle to lower reaches of Llobregat river were selected for this study (see map Fig. 1, page 32). Sampling was permormed in October 2010 during base flow conditions. Five surface sediment samples (2 cm depth) were collected at random with a methacrylate corer (4.5 cm in diameter) in order to encompass the heterogeneity of each sampling site. From each core, subsamples with a volume of 1 mL were collected using an uncapped syringe (1.2 cm in diameter) for each parameter. Sediment samples for Biolog EcoPlates incubations and bacterial density and viability analysis were collected in duplicate from each sediment core. One aliquot was frozen immediately in the field with liquid nitrogen and then stored at -80ºC and the other aliquot was processed while still fresh. Fresh sediment samples for bacterial viability, Biolog EcoPlates incubations and respiratory activity measurements were transported at 4 ºC and processed after 2-5 hours of field sampling. Samples for bacterial density were fixed with formalin (4%) and samples for polysaccharide content in EPS were frozen and preserved at -20ºC prior to analysis.

Physico-chemical parameters

Oxygen (WTW oxygen meter), conductivity (WTW conductivity meter), pH, temperature (WTW pH meter), and current velocity (Schiltknecht 43221; MiniAir2) were measured in the field along a cross-transect (minimum of 3 measurements for each site) placing the probes at 10 cm depth. River water samples were collected in triplicate and filtered (pre-combusted during 4 h at 450 ºC, 0.7 µm pore size filters, Whatman GF/F) for DOC (Dissolved Organic Carbon) and DIC (Dissolved Inorganic Carbon) analysis. DOC and DIC were measured using a total organic carbon analyser (Shimadzu TOC-V-CSH 230V). Water samples for nitrate, ammonium and phosphate were collected in triplicate filtered (0.2 µm pore size, nylon filters, Whatman) and analysed using automated analyser (WESTCO SmartChem 140). Discharge data were obtained from the Catalan water authorities.

Biolog EcoPlates incubations

Five EcoPlates were used for each treatment (fresh and frozen sediment samples) and for each sampling site (50 microplates in total, 25 for frozen and 25 for fresh samples). Sediment samples were placed in sterile vials with 15 mL of Ringer's solution (Scharlau S.L) and were sonicated for two times 60 s (Selecta, 40 W and 40 kHz). Sterilized Ringer solution instead of river water, was used in order to homogenize the effect of different water quality observed in each sampling site (Goberna et al. 2005, Douterelo et al. 2010). The sediment extracts were diluted 100 times before inoculation in the plates. Dilution factor was previously determined by testing different dilution factors with sediment samples from the same river, in order to obtain similar inoculum size and initial bacterial densities to those recommended by other authors (1.0 g wet weight of sand sediment L^{-1} or $>10^6$ cell ml^{-1}; Haack et al. 1995, Garland et al. 2001). EcoPlates were inoculated under sterile conditions with 130 µl of inoculum and incubated at 20 ºC in dark conditions. For the frozen samples the same incubation protocol was followed, except for a prior melting process. Samples stored at -80 ºC were thawed at 4 ºC (12 hours) and later at room temperature (3-4 hours) until completely thawed. Plates were read every 24 hours for 6 days at 590 nm using a microplate reader (BioTek, SynergyTM 4).

Bacterial density and viability

Bacterial density and viability were measured from the sediment extracts prepared for the EcoPlates for each sampling site. Both fresh and frozen sediment samples were considered in order to control the potential effects of freezing.

Bacterial density was estimated through fluorescent staining with DAPI (4,6-diamidino-2-phenylindole). Sodium pyrophosphate (final concentration 0.05 mmol L^{-1}) was added to the sediment extracts and agitated for 30 min to avoid aggregates. Then samples were stained with DAPI (final concentration 2 µg ml^{-1}) in the dark for 15 min and filtered through 0.2 µm black polycarbonate filters (Nuclepore) (Porter and Feig 1980). Bacteria were counted using an epifluorescence microscope (Nikon E600) at 1000x magnification. Twenty fields

per filter were counted. The results were expressed as cells per cm^2 of sediment projected area.

Abundance of live bacteria was estimated using the LIVE/DEAD double-stain kit (Molecular Probes, Inc.). Syto 9 is a cell-permeating nucleic acid stain having green fluorescence and labels all the cells, while propidium iodide only penetrates bacteria with altered membrane permeability, and cells appear fluorescent red. The combination of the double stain results in the appearance of live bacteria as the green ones and the appearance of dead (membrane damaged) bacteria as the red ones, since the combination of both stains on damaged cells gives a final red fluorescence. The green fluorescence of the suspension was used as a measure of bacterial viability (Roth et al. 1997). Bacterial viability was estimated with 2 ml of the same sample suspension used for the Biolog EcoPlates incubations. Solution was stained with a 1:1 mixture of Syto 9 and propidium iodide and incubated for 15 min in the dark. Fluorescence for SYTO 9 was measured at 480/500nm excitation/emission wavelengths (Yagüe et al. 2010) using a fluorimeter (Kontron SFM 25). The results were given in relative fluorescence units.

Respiratory activity

The electron transport system (ETS) activity of the microbial community in the sediment was measured as an indicator of respiratory activity (Trevors et al. 1982). Sediment samples were placed in sterile vials with 4 ml of filtered river water (0.2 μm, nylon filters, Whatman). In the laboratory, each sample was incubated in 0.02% INT solution (2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride, Sigma-Aldrich), in a shaker at 20 ºC for 12-14 h in the dark (Blenkinsopp and Lock 1990). INT-formazan (iodonitrotetrazolium-formazan) was then extracted with methanol for 1 h at 4ºC in the dark. Blanks without substrate additions were also incubated. The extracts were filtered (GF/F filters, Whatman) and their absorbance was measured at 480 nm (Shimadzu UV-1800). A stock solution of 60 μg ml^{-1} of INT-formazan (Sigma-Aldrich) in methanol was used to prepare a standard curve. The results were expressed as μg INT-formazan per cm^2 of sediment projected area per hour.

Polysaccharide content in Extracellular Polymeric Substances (EPS)

EPS from each sand sediment sample were extracted using cation-exchange resin (Dowex Marathon C, Na^+ form, strongly acidic, Sigma-Aldrich, Steinheim, Germany) following the procedure described in Romaní et al. (2008). The polysaccharide content was measured by the phenol-sulphuric acid assay (Dubois et al. 1956) after the extraction of EPS. Glucose standards (0–200 μg mL^{-1}) were also prepared. The results were given as glucose equivalents per cm^2 of sediment projected area.

Organic matter content

Samples of 1 mL of wet sediment were dried at 70 °C during 72 h, then burnt for 4 h at 550 °C in order to determine ash free dry mass (AFDM). The results were expressed as mg per cm^2 of sediment projected area.

Statistical Analyses

Biolog data processing

Raw absorbance data obtained from each EcoPlate were corrected by the mean absorbance of the control wells (without substrate) in each plate. Values < 0.05 were set to zero. The average well colour development (AWCD) was calculated from each plate at each reading time as AWCD = $\sum$ (c - r)/n, where c is the raw absorbance in each well, r is the absorbance of the control well, and n the number of wells in the plate (Garland and Mills 1991). As a measure of the diversity of substrate utilization, the Shannon diversity index was calculated (Staddon et al. 1997). AWCD follows a sigmoid curve with time and three kinetic parameters were estimated by fitting the sigmoid equation to the data (Lindstrom et al. 1998, *Chapter 1*, page 53). The three kinetic parameters (a , $1/b$ and x_o) were estimated using SigmaPlot 2000 (Systat Sofware, Inc., San Jose, CA, USA). These parameters were calculated for each plate and were then averaged to obtain mean values for each sampling site.

Differences between sampling sites

Extensive analysis of differences in sediment microbial degrading capabilities between sampling sites was performed with data obtained after 144 h of EcoPlates incubation (Insam and Goberna 2004). After this incubation time period most wells achieved their saturation of the sigmoidal colour development (optical density ~ 2) and the value of AWCD was similar to 0.75 (Stefanowicz 2006). The results were also grouped according to carbon source classification including polymers, carbohydrates, carboxylic acids, amino acids and amines (Choi and Dobbs 1999).

Differences between sampling sites for colour development and Shannon diversity index throughout Biolog EcoPlates incubation time (from 24 to 144 h) were analysed by one way repeated measures analysis of variance (rm-ANOVA). Probabilities within time and interaction site x time were corrected for sphericity using Greenhouse-Geisser correction. Moreover, differences between sampling sites for Biolog EcoPlates parameters (kinetic parameters of sigmoid curve, AWCD and Shannon index values after 24 h incubation) organic matter, respiratory activity, bacterial cell density and viability and polysaccharide content in EPS, were tested independently using analysis of variance (one-way, ANOVA). When significant differences ($p<0.05$) were obtained after ANOVA analysis, means were separated using a post-hoc Tukey-b test analysis to check for significantly different groups between sampling sites. Data were log transformed, except for AWCD and Shannon index to approach normality and homogeneity of variance and to comply with the assumptions of ANOVA analyses. Logarithmic regression analyses were performed for biological variables (ETS, EPS, bacterial density and live bacteria) with distance (km) from the first sampling site (LLO1) to downstream site (LLO5) as distance as an independent variable. These statistical analyses were performed using the SPSS programme v.15.0 (SPSS, Inc., Chicago, IL, USA).

Results from the 31 carbon sources for each EcoPlate were standardized (divided by AWCD) (Garland and Mills 1991) and a distance matrix was built with Bray-Curtis similarity. Based on the distance matrix, a one-way similarity percentages

(SIMPER) analysis was used to highlight and detect the characteristic substrates used at each site, ordering the substrates from more to less contribution to the total within sampling site similarity (cut-off at 50%, Clarke 1993). To visualize and test the spatial differences in the metabolic fingerprint between samples, MDS (Non-metric multi-dimensional scaling) followed by ANOSIM were performed based on the distance matrix. These analyses were performed using PRIMER v.6.0 for Windows (Primer-E Ltd., Plymouth, UK).

Finally, two principal component analyses (PCA) were performed to identify the environmental or biological variables most responsible for the spatial variation between river sites. The first PCA included environmental variables (physical and chemical parameters from Table 1, n=3), and the second PCA included biological variables (EPS, ETS, bacterial density, live bacteria and Biolog EcoPlates carbon groups, n=5). All variables were previously centred and scaled to homogenize the different units and scales. The analyses were performed by using the "prcomp" function of the R package "vegan".

Freezing effect

Paired t-test analyses were performed on logarithmically transformed data to test for differences between fresh and frozen samples for Biolog EcoPlates, bacterial density and viability. A two-way analysis of variance (two-way ANOVA) was also performed in order to detect potential interaction between freezing and site effects. The kinetic curve parameters from the EcoPlates results for fresh and frozen samples were also compared using one-way ANOVA analysis. These statistical analyses were performed using the SPSS programme v.15.0. In order to compare the fingerprint pattern between frozen and fresh samples from Biolog EcoPlates data, Mantel test (RELATE function integrated in the PRIMER 6 software) was performed (Legendre and Legendre 1998) using PRIMER v.6.0 for Windows. Similarity matrices were built with Bray-Curtis similarity and Spearman rank correlation method was selected.

RESULTS

Physico-chemical parameters

The average values of physico-chemical parameters at the five studied sites along the River Llobregat are shown in Table 1. The concentration of dissolved oxygen decreased downstream, while nutrient concentration and conductivity increased downstream. The pH remained stable at all sampling sites, except in LLO5 where it decreased to 5.9. This sampling site also showed the highest water temperature (28ºC) and the lowest oxygen concentration. A flow event occurred during the sampling period, particularly affecting the two middle sites where discharge increased (Table 1, sites LLO2 and LLO3).

Table 1. Physico-chemical parameters at the flowing water for all sampling sites of the river Llobregat. Mean value (n=3) and standard error (in parenthesis) for nutrients and flow velocity are shown. nd= no data.

Parameters	Units	**LLO1**	**LLO2**	**LLO3**	**LLO4**	**LLO5**
Tº	*ºC*	17.10	21.00	19.90	17.70	28.00
Oxygen	*mg/L*	9.80	9.48	7.35	7.87	5.90
pH		8.16	8.24	7.67	8.05	5.90
Conductivity	*µS/cm*	1003	1147	1414	1557	1560
Flow Velocity	*m/s*	0.20(0.08)	nd	0.83 (0.02)	0.50 (0.02)	0.54 (0.05)
Discharge	m^3/s	6.80	11.68	11.90	6.98	4.75
NO_3	*mg/L*	11.09(0.68)	8.17 (0.09)	7.60 (0.17)	10.96(1.05)	18.46(0.30)
NH_4	*mg/L*	0.31 (0.17)	0.22 (0.07)	0.40 (0.26)	0.50 (0.12)	1.59 (0.06)
PO_4	*mg/L*	0.19 (0.02)	0.22 (0.02)	0.18 (0.03)	0.43 (0.06)	2.46 (0.04)
DIC	*mg/L*	5.06 (0.93)	4.95 (0.21)	6.64 (0.43)	34.30(2.68)	29.71(1.19)
DOC	*mg/L*	6.66 (0.75)	5.47 (0.20)	6.47 (0.08)	10.24(1.35)	7.74 (0.24)

Respiratory activity, polysaccharide content, organic matter content, and bacterial density and viability in river sediments

The respiratory activity (ETS), polysaccharide content in the EPS and bacterial viability gradually decreased downstream following a significant negative logarithmic decay as increasing distance (Fig. 1a, b and d). Significant differences between sites were distinguished after the ANOVA for all variables ($p<0.05$).

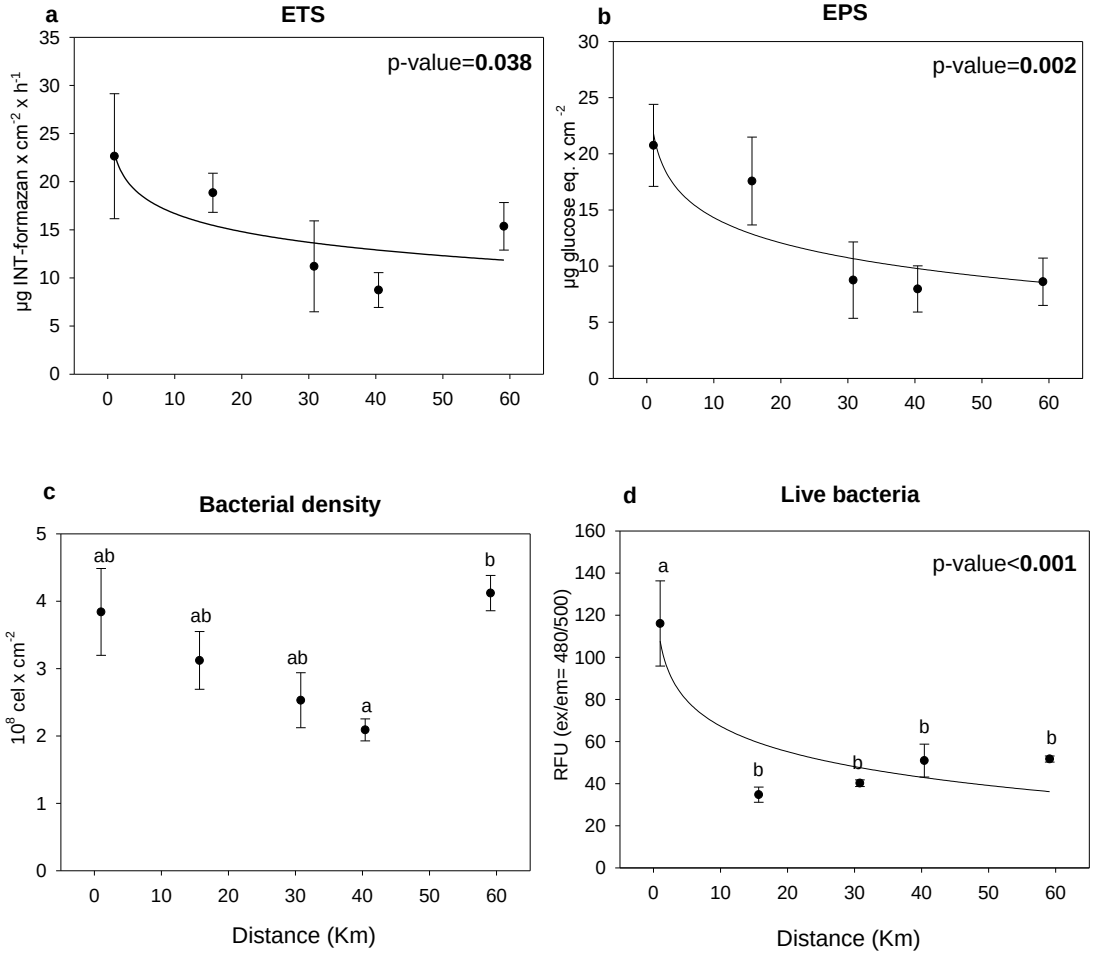


Figure 1. a) Respiratory (electron transport system, ETS) activity, b) Polysaccharide content in extracellular polymeric substances (EPS), c) Bacterial density and d) Live bacteria represented as green fluorescence at five sampling sites along the river length (km). Values are means with standard error (n=5). Solid line is the logarithmic regression of each variable vs. river km. Significance of the regression is also indicated. Letters indicate significantly different groups found by Tukey-b test after ANOVA analysis ($p<0.05$).

Bacterial density decreased downstream up to site LLO4 but peaked at the last sampling site. The highest values for bacterial viability (represented as live bacteria) occurred at LLO1, and values were significantly lower at the downstream sites (Fig. 1d). The mean value of sediment organic matter content (AFDM) was 12.6 ± 5.8 mg cm^{-2} and no significant differences between sampling sites were observed (ANOVA, p=0.062).

Microbial functional diversity in river sediments

The AWCD in the EcoPlates (from 24 to 144 hours) for each sampling site increased over time and fit to sigmoid curves (Fig. 2a). AWCD during incubation time were significantly different between sampling sites (rm-ANOVA, site and time x site effects, p<0.001). In particular, community colour development was significantly faster in LLO1 (highest *1/b* value, Tukey-b test, p<0.05) and the maximum metabolic capacity was significantly higher at sampling sites LLO1, LLO4 and LLO5 (Fig. 2a, Tukey-b test, p<0.05). However, after 24 h of incubation (initial or latent phase), the AWCD of the sediment samples was significantly lower at LLO5 (ANOVA, p=0.025, Fig. 2a). Shannon index increased over time until 72 h of incubation and did not show significant differences between sampling sites (rm-ANOVA, site and time x site effects, p=0.292, p=0.062, respectively, Fig. 2b), neither after 24 h of incubation (ANOVA, p=0.481). Although functional diversity at the downstream sites (LLO3, LLO4, and LLO5) were the lowest after 24 h, they became the highest after 48h of incubation (Fig. 2b).

After 144 h of incubation, saturation phase of sigmoid colour development was reached and the percentage of positive wells was 92% on average. The SIMPER analysis allowed us to identify the potential characteristic organic substrates being used by each microbial community, by analysing the contribution of each substrate to the similarity between replicates (Table 2). The average similarities were rather high, especially at sites LLO4 and LLO5 where percentages were >85% (Table 2). Some organic substrates were potentially degraded by all microbial communities, with in particular 3 substrates ranking top of the similarity contribution list; these were two polymers (*Tween 80* and *Tween 40*) and one carbohydrate (*D-Mannitol*). Communities from site LLO1 in particular were characterized by the use of the

carbohydrate *D-Cellobiose* and the amino acid *L- Asparagine*. Organic matter use capacities at the communities from sites LLO4 and LLO5 were clearly separated from those at LLO1 and characterized by the use of polymers and carbohydrates (Table 2 and Fig. 3). Particularly, some substrates were only commonly used by communities from sites LLO4 and LLO5, where the contribution to similarity was mainly represented by 3 polymers (*Tween 80, Glycogen and Tween 40*, Table 2). The sediment microbial community was not able to degrade the phenol substrate *2-hydroxybenzoic acid* anywhere along the river (0% of contribution in all sampling sites).

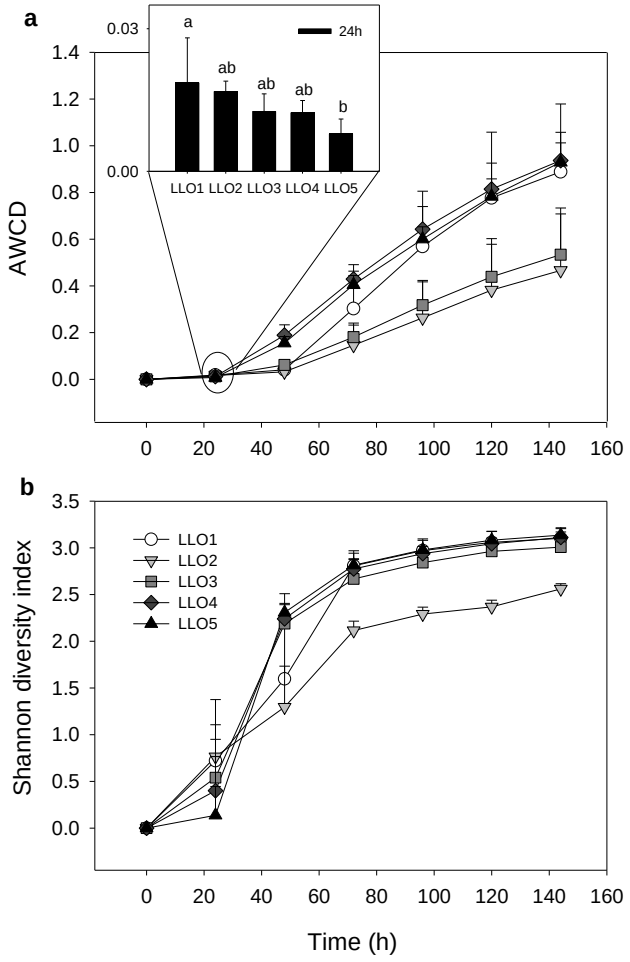


Figure 2. Temporal development of AWCD (a) and functional diversity (b) (Shannon index) during incubation time (from 24 to 144 hours) at different sampling sites. The supplementary plot in graph 2a) indicates the differences in AWCD between sites after 24 hours of incubation of the EcoPlates and letters indicate significantly different groups found by Tukey-b test ($p<0.05$). Values are means with standard error (n=5)

Table 2. SIMPER analysis results. The average similarities between the five replicates collected at each sampling site are shown. The organic substrates listed for each sampling site correspond to those mostly contributing to the site similarity up to ca. 50%. Sum of contribution to average similarity for each sampling site is shown

LLO1		**LLO2**		**LLO3**		**LLO4**		**LLO5**	
79.28% average similarity		75.46% average similarity		71.08% average similarity		87.64% average similarity		87.65% average similarity	
Substrates	**%**	**Substrates**	**%**	**Substrates**	**%**	**Substrates**	**%**	**Substrates**	**%**
D-Cellobiose	7.41	*D-Galacturonic acid*	7.69	*Tween 80*	8.90	*Tween 80*	9.76	*Tween 80*	9.79
L-Asparagine	7.30	*N-Acetyl-D-glucosamine*	7.39	*Tween 40*	7.55	*Glycogen*	8.89	*Glycogen*	8.92
Tween 80	7.17	*Tween 40*	6.98	*β-Methyl-D-glucoside*	7.27	*Tween 40*	7.58	*Tween 40*	7.26
D-Mannitol	6.98	*Tween 80*	6.88	*Glycogen*	7.09	*D-Cellobiose*	6.65	*D-Cellobiose*	6.44
Tween 40	6.27	*L-Serine*	6.18	*L-Serine*	7.03	*N-Acetyl-D-glucosamine*	6.56	*N-Acetyl-D-glucosamine*	6.28
Glycogen	5.78	*D-Mannitol*	6.07	*D-Mannitol*	6.86	*D-Mannitol*	6.39	*D-Mannitol*	6.25
L-Serine	5.74	*β-Methyl-D-glucoside*	5.62						
Total contribution to average similarity	**46.65**		**46.81**		**44.70**		**45.83**		**44.94**

Multivariate analysis of the Biolog EcoPlates data revealed significant differences in the fingerprint pattern between sampling sites (Fig. 3, ANOSIM p= 0.001) and specifically separating the sites LLO4 and LLO5 from the sites LLO1, LLO2, and LLO3 (p = 0.008 for all significant pairwise comparisons). Also, the two middle site replicates (LLO2 and LLO3) were more dispersed than LLO4 and LLO5 where the heterogeneity between replicates in each site was lower (Fig. 3).

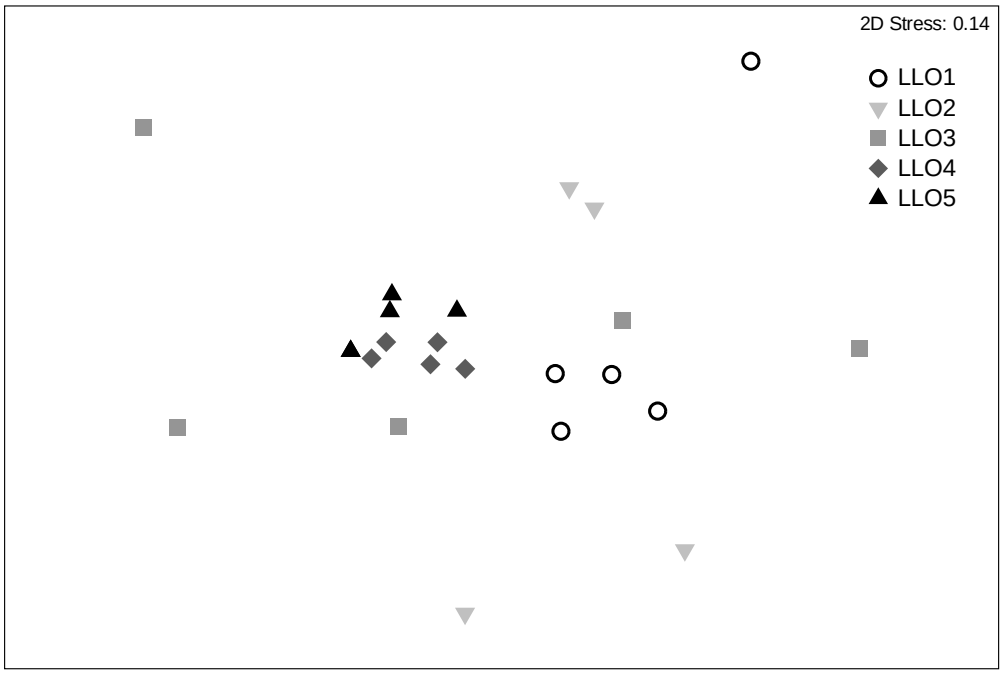


Figure 3. Non-metric multidimensional scaling (MDS) ordination plot of each sampling sites using Bray-Curtis similarities on Biolog EcoPlates data after 144 hours of incubation. Kruskal 2D stress is shown. The different sampling sites were significantly separated (ANOSIM analysis, global R=0.295, p=0.001).

Spatial differences in physico-chemical and biological parameters along the Llobregat river

Two principal component analyses (PCA) were used to visualize the differences between the five sampling sites and to look for the biological and environmental parameters most responsible for these differences (Fig. 4). The first axis of the environmental PCA (explaining 68.4% of the variance) was linked to the pollution gradient, with higher values of nutrient concentration (NH_4, PO_4, NO_3) and conductivity in LLO5. At the opposite side of this first axis, upstream sites were located and characterized by higher oxygen content, pH and flow (Fig. 4a). The second axis mainly distinguished site LLO4 due to its higher DOC content. On the other hand, the first axis of the biological PCA (explaining 36.5% of the variance) showed higher EPS, respiratory activity and use of amines in upstream sites, being opposite to downstream sites linked to the utilization of polymers (Fig. 4b).

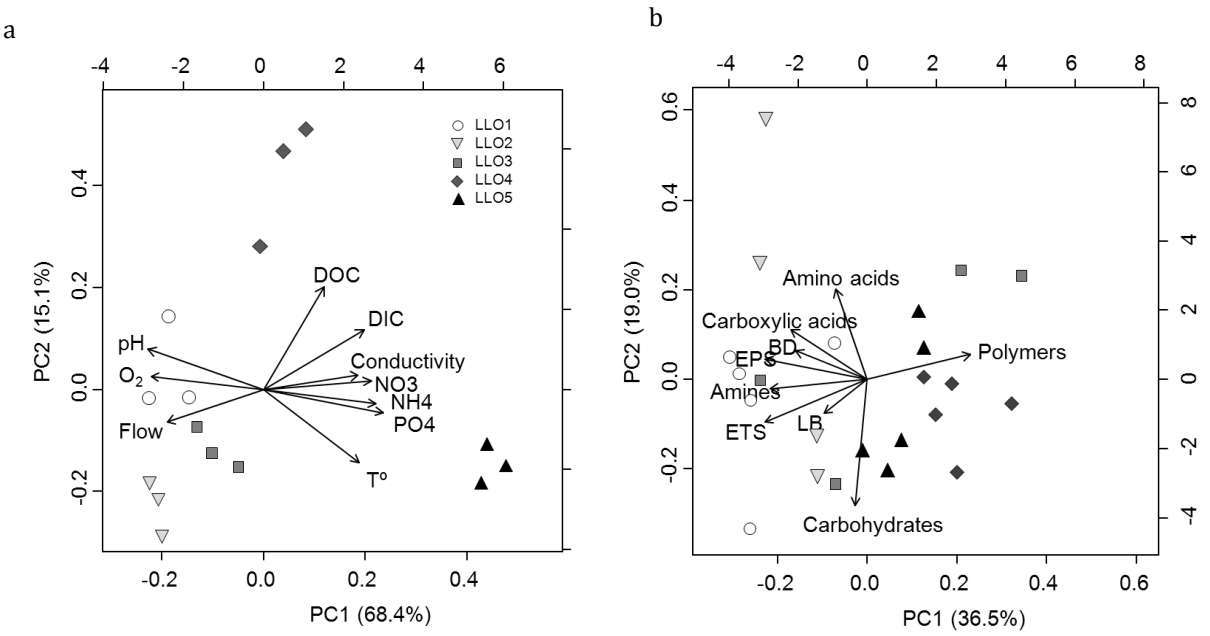
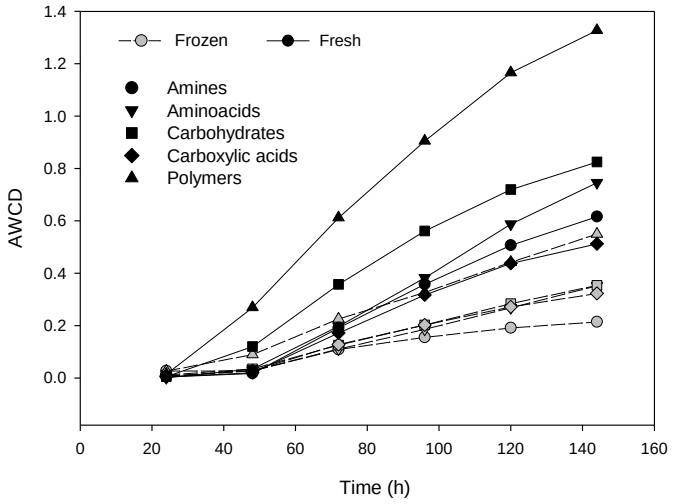


Figure 4. Principal Component Analysis (PCA) based on environmental data (a) and biological data (b). ETS (electron transport system), EPS (polysaccharide content in extracellular polymeric substances), BD (bacterial density) and LB (live bacteria). Legend symbols represent different sampling sites. Numbers in parenthesis represent the amount of total variance explained by each principal component.

Freezing effect

The kinetic analysis of the sigmoid curves showed significant maximum absorbance (parameter *a*) for the frozen sediment samples for all substrate categories ($p<0.05$) (Fig. 5). Moreover, slope (parameter 1/b) of the fitting sigmoid curve was significantly lower for the frozen sediment samples for all carbon groups excepting amines and carboxylic acids (Fig. 5). In the case of carbohydrates, the day when the maximum slope in colour development was reached (parameter x_0) was significantly earlier for fresh than for frozen sediment samples (Fig. 5b, p= 0.033).

a



b

Parameters	Amines		Amino acids		Carbohydrates		Carboxylicacids		Polymers	
	Fz	**Fh**	**Fz**	**Fh**	**Fz**	**Fh**	**Fz**	**Fh**	**Fz**	**Fh**
a (absorbance at 590nm)	0.25	**0.64**	0.49	**0.82**	0.50	**0.84**	0.38	**0.52**	0.75	**1.37**
1/b (h^{-1})	0.04	0.05	0.03	**0.05**	0.03	**0.05**	0.04	0.06	0.03	**0.05**
x_0 (h)	84.62	91.53	112.68	99.02	**110.48**	80.73	94.09	87.84	106.24	79.34

Figure 5. a) Mean values of AWCD for each metabolic group during incubation time for frozen (Fz) and fresh (Fh) samples (SE is not shown for clarity). For all sigmoid curves R^2 adjusted ranged 0.947-0.999, $p<0.001$. b) Mean results of kinetic parameters obtained after fitting to a sigmoid curve (*a, 1/b* and x_o) for each carbon source group are shown. Significant differences between Fh and Fz are highlighted in boldface and underlined (ANOVA, $p<0.05$).

When the EcoPlates results after 144 h incubation were compared, significant lower AWCD and Shannon metabolic diversity levels were measured after freezing samples (paired t-test $p<0.001$). Furthermore, the freezing effect on these two metabolic parameters was not equal for all sampling sites (ANOVA, site x freezing effect, Table 3) the greater decrease being measured downstream (LLO4 and LLO5).

Table 3. Results of ANOVA testing the differences between sampling sites (site effect), and the freezing effect (freezing) on bacterial density and viability based on sediment samples and AWCD and functional diversity (Shannon diversity index) from the Biolog EcoPlates. Mean values for each site (n=5) and standard error (in parenthesis) are shown. Significant probability values <0.05 are indicated in boldface type.

	Bacterial density *10^8 cell/cm^2*		**Live bacteria** *Green fluorescence units*		**AWCD** *OD at 590nm*		**Shannon diversity**	
	fresh	frozen	fresh	frozen	fresh	frozen	fresh	frozen
LLO1	3.84 (0.48)	3.66 (0.21)	116.05 (20.23)	57.54 (12.58)	0.88 (0.13)	0.58 (0.07)	3.10 (0.04)	3.02 (0.03)
LLO2	3.12 (0.42)	3.24 (0.32)	34.76 (3.61)	45.64 (3.65)	0.45 (0.13)	0.40 (0.08)	2.85 (0.19)	2.89 (0.06)
LLO3	2.53 (0.41)	3.40 (0.48)	40.20 (1.57)	38.00 (12.07)	0.53 (0.08)	0.34 (0.18)	3.00 (0.05)	2.55 (0.18)
LLO4	2.09 (0.16)	2.91 (0.25)	50.96 (7.81)	41.83 (5.43)	0.93 (0.05)	0.25 (0.09)	3.11 (0.02)	2.22 (0.27)
LLO5	4.12 (0.26)	4.17 (0.14)	51.68 (1.44)	32.98 (3.92)	0.91 (0.04)	0.23 (0.08)	3.12 (0.04)	2.41 (0.16)
Source of variation								
Site	**0.002**		**<0.001**		**0.027**		0.124	
Freezing	0.102		**0.014**		**<0.001**		**<0.001**	
Site x Freezing	0.455		**0.029**		**0.016**		**0.013**	

In particular, the activity lost due to freezing is highlighted in Figure 6, which shows that communities from sampling sites LLO4 and LLO5 lost the ability to utilise up to 15 and 14 substrates respectively. In contrast, the microbial communities at site LLO1 only lost 2 specific metabolic capacities after freezing. Two substrates were clearly affected by freezing: *D- Galactonic acid γ-lactone* (carbohydrate) and *γ- Hydroxybuturic acid* (carboxylic acid), which were degraded

everywhere for the fresh samples and were not degraded by any community for the frozen samples (Fig. 6). The similarity matrix based on the 31 carbon substrates of Biolog EcoPlates for each sample obtained with the frozen samples was not correlated with the matrix obtained with the fresh samples (Rho=0.099, p=0.23) as determined by Mantel test. A significant reduction in bacterial viability was measured for the frozen samples, whilst bacterial density was not affected by the effects of freezing (paired t-test, p=0.043, p=0.089 respectively).

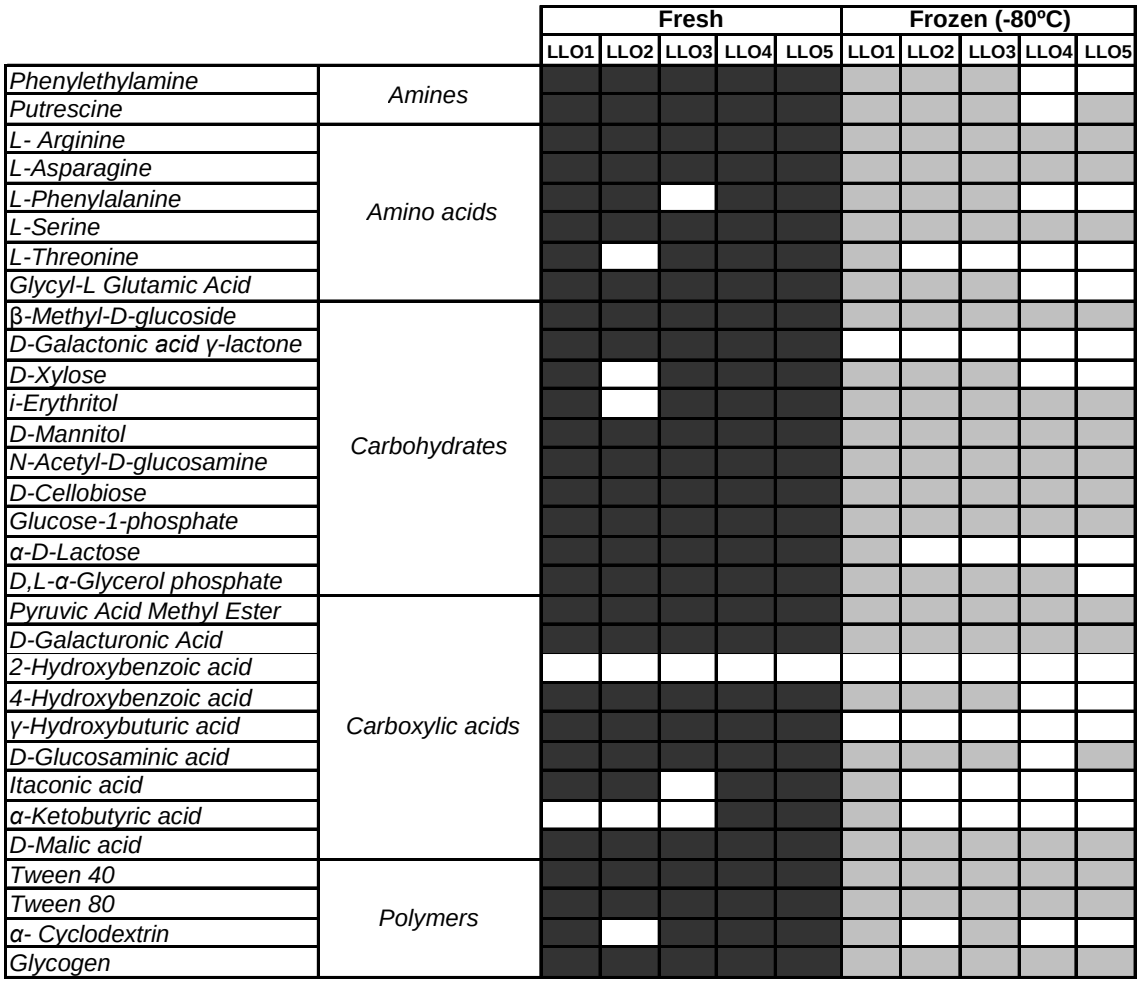


Figure 6. Pattern of utilization of 31 carbon substrates for frozen and fresh samples of five sampling sites studied (based on mean of AWCD, positive when mean value>0.05). Coloured cells (grey for frozen, black for fresh) represent positive metabolic activity for each substrate. White cells represent no substrate utilisation.

DISCUSSION

Microbial functional diversity in river sediments

River sediments play a key role in organic matter cycling and it is therefore relevant to discern potential shifts in the sediment microbial capabilities for using organic matter compounds along the river as they will affect the whole ecosystem functioning. A previous study in the River Llobregat showed not significant differences in the microbial heterotrophic metabolism of the benthic biofilm between sites in the middle and lower reaches, measured as the capacity to decompose carbon, nitrogen and phosphorus compounds (by measurements of extracellular enzymes β-glucosidase, leucine-aminopeptidase and phosphatase) (Proia et al. 2013). Similarly, in the present study, functional diversity results (Shannon index) from EcoPlates (Garland 1997) did not show significant differences between sites, thus indicating that the sediment microbial communities were potentially able to use a similar range of the available substrates (Fig. 2). However, although this diversity index did not appear to be sensitive to define functional differences between sites, when looking for all specific carbon sources used, differences in the functional fingerprint between sites arise (Table 2, Fig. 3).

Microbial communities of the Llobregat River sediments are potentially capable of using a wide range of compounds after 144h of incubation, however carbohydrates and polymers (with higher value of AWCD) were used by all sampling sites, as was also observed for aquatic aggregates communities (Lyons and Dobbs 2012). The higher use of these two groups of substrates might be related to the available carbon sources in flowing waters. In aquatic ecosystems, much of the biodegradable dissolved organic matter that is released into the system by natural processes (such as release from primary producers) are carbohydrates (Grover and Chrzanowski 2000; Tiquia 2010). Of the carbohydrates and polymers, 3 carbohydrates (D-*Cellobiose, N-Acetyl-D-glucosamine* and *D-Mannitol*) and 3 polymers (*Tween 80, Tween 40* and *Glycogen*) were the ones potentially most frequently used by all sediment biofilm communities in the Llobregat River. The carbohydrates *N-acetyl-D-glucosamine* and *D-Cellobiose* have been shown to be important sources for bacterial growth in seawater (Sala et al. 2005). From this

general pattern, specific metabolic fingerprints characterised the communities from the different sampling sites, especially the upstream site (LLO1) differed distinctly from the two downstream sites (LLO4 and LLO5, Fig. 3). At the upstream site the most potential substrate used by microbial communities was a carbohydrate, *D-Cellobiose* (Table 2), a disaccharide subunit of cellulose, which might be linked to the availability of similar substrates present at this site, such us compounds from terrestrial origin like leaves or wood. Also, a relevant use of amino acids and amines, and therefore of a high quality organic matter sources (providing C and N to the cells) was measured for these upstream communities. In contrast, the metabolic fingerprint of the two downstream communities is characterised by a greater potential capacity to use polymers as was similarly found in harbours where this group of compounds are used extensively (Sala and Güde 2006). Our investigation showed that the significant changes in the metabolic fingerprint at the two downstream sites might be related to increasing nutrient concentration, DOC and conductivity (Fig. 4). Furthermore, previous studies showed an increase of organic contaminants in the downstream reaches of the River Llobregat (coinciding with our LLO4 and LLO5 sites, Muñoz et al. 2009; Petrovic et al. 2011). Although the use of a specific carbon substrate from the EcoPlates does not mean that this substrate was available at the environment, the appearance of specific metabolic capabilities suggests that similar substrata may exist at the collecting sediment site. In our downstream communities, a higher use of *Tween 40* and *Tween 80* (a class of emulsifiers from synthetic origin used in some pharmaceuticals and food preparation) might be linked to the presence of pharmaceutical compounds specifically measured in other studies at these sites (Kuster et al. 2008; Muñoz et al. 2009).

Moreover, the similarity between LLO4 and LLO5 (small distances in MDS, Fig. 3) could be related to possible selection of a bacterial community (e.g. increase in Actinobacteria, Proia et al. 2013) adapted to the specific quality and high content of inorganic and organic compounds in water (Fig. 4a), decreasing the spatial heterogeneity in organic matter use capabilities in sediment samples. In contrast, the two middle sites (LLO2 and LLO3) were more difficult to characterise due to a larger heterogeneity between sediment replicate samples (Fig. 3 and Fig. 4). This

suggests a greater heterogeneity of the streambed physical characteristics at these sites in contrast to probably more homogeneous characteristics at downstream sites. Previous studies at these sites indicate the presence of cobbles, gravel and sand sediment at site LLO3 while at site LLO5 the streambed was all only covered by sediment (Ricart et al. 2010).

The results from AWCD obtained from the sediments of the River Llobregat after colour saturation inform us about the community's potential capacity to degrade the available substrates (Lindstrom et al. 1998), and thus define specific metabolic fingerprints. However, our results also show a different pattern when considering data after 24 hours of the EcoPlates incubation. The results obtained after 24 h represent the microbes present in initial inoculums (Konopka et al. 1998), especially those that are fast-growing and responsible for the utilization of more easily available substrates such as carbohydrates, amino acids and amines (Douterelo et al. 2010). Although after 144 h, LLO1, LLO4 and LLO5 showed the highest AWCD, after 24 h of incubation, there was a significant downstream decrease in AWCD. This result could be linked to the lower density of active bacteria at the downstream sites as similarly found by Proia et al. (2013). On the other hand, the microbial community from the upstream site appears to be the most active, showing the fasted colour development slope in the plates. The highly active characteristic of the upstream community is further evidenced by its highest respiratory activity (ETS) and live bacteria levels (Fig. 1). This short incubation time could minimize the effects of the culture technique but it only allows for the evaluation of some of the available compounds; no more than 6-7 different compounds (out of the 31) were detected. During incubation time, the differences between sites changed, and both functional diversity and AWCD recovered at two downstream sites. It could be that the microbial communities at these two sites had a greater capacity to degrade more complex substrates such as some polymers, which need more time for degradation, or that sediment samples had more spore-former that take an increased time to germinate.

Freezing effect

Significant differences in microbial functional diversity and bacterial viability were obtained after freezing (under -80ºC) the river sediment samples. Lower values of AWCD, slope and Shannon diversity were observed after frozen samples, which might be due to the loss of important functional groups of microorganisms. The death of bacteria because of the freezing was probably the main factor responsible for the general decrease in the functional diversity (Table 3). This result is consistent with other studies, which recommend that environmental samples that are to be used to analyse microbial functionality should not be frozen (Shishido and Chanway 1998).

This study suggests that storage frozen samples induces significant changes in sediment microbial communities. After freezing, samples showed a different fingerprint pattern (as shown by the not significant correlation between matrices obtained from fresh and frozen samples, mantel test). Moreover, we observed that the freezing effect affected the sampling sites differently. The downstream sites lost the capability to use 14 and 13 substrates respectively (Fig. 6), indicating a reduced recovery capacity of the microbial community after being kept frozen. It could be that the community from these sites was more fragile due to low bacterial population densities from the different species and/or had low levels of functional redundancy. It was also reported that freezing may have a selective deleterious effect on microorganisms by eliminating certain bacteria only (Shishido and Chanway 1998). The disappearance of species should determine a reduction in functions, although this might depend on functional redundancy in the specific community (Konopka et al. 1998; Leflaive et al. 2008). Hector and Bagchi (2007) concurred on the importance of biodiversity for maintaining ecosystem multifunctionality, and it has been shown that decreases in microbial diversity may reduce multifunctionality (Peter et al. 2011). Additionally, in the lower reaches of the river, the increase of sensitivity to freezing in sediment microbial community could be also linked to a less consistent biofilm structure, as shown by the lower EPS-polysaccharide content (Fig. 1). The EPS matrix contributes to the biofilm cohesions and stability (Gerbersdorf et al. 2008) and could favour the resistance of

the microbial sediment to the freezing affect, buffering the changes that occur during the freezing process such as the reduction in the water content and changes in conductivity (Or et al. 2007).

CONCLUSIONS

Differences in sediment microbial biofilm structure and metabolism downstream the Llobregat river were detected. Microbial community at the upstream site was the most active as shown by the highest respiratory activity and abundance of living bacteria. The greatest EPS content at this site may be responsible for a higher consistency of the biofilm structure. The functional fingerprints were different between sites. Specially, microbial communities at downstream sites showed a differential and homogenous functional fingerprint and the potential capacity to use more complex carbon substrates such as polymers which may be linked to changes in the water quality at these sites. Our results also concluded that the analyses using Biolog EcoPlates should be performed with fresh river sediment samples. Preserving frozen samples leads to significant biases in the results, reducing the functional diversity of the microbial community, and the magnitude of the effect might depend on the sediment biofilm structure thus modifying the fingerprint pattern.

CHAPTER 3

THE EFFECTS OF SEDIMENT DEPTH AND OXYGEN CONCENTRATION ON THE USE OF ORGANIC MATTER: AN EXPERIMENTAL STUDY USING AN INFILTRATION SEDIMENT TANK

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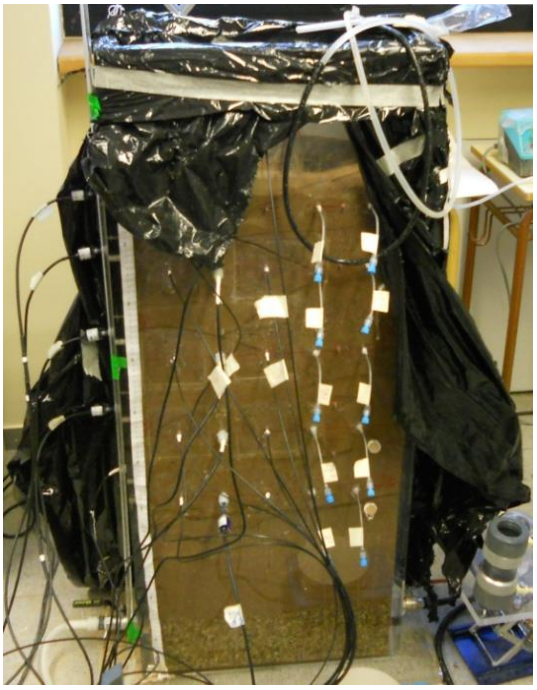

ABSTRACT

Water flowing through hyporheic river sediments or artificial recharge facilities promotes the development of microbial communities with sediment depth. We performed an 83-day mesocosm infiltration experiment, to study how microbial functions (e.g., extracellular enzyme activities and carbon substrate utilization) are affected by sediment depth (up to 50 cm) and different oxygen concentrations. Results indicated that surface sediment layers were mainly colonized by microorganisms capable of using a wide range of substrates (although they preferred to degrade carbon polymeric compounds, as indicated by the higher β-glucosidase activity). In contrast, at a depth of 50 cm, the microbial community became specialized in using fewer carbon substrates, showing decreased functional richness and diversity. At this depth, microorganisms picked nitrogenous compounds, including amino acids and carboxyl acids. After the 83-day experiment, the sediment at the bottom of the tank became anoxic, inhibiting phosphatase activity. Coexistence of aerobic and anaerobic communities, promoted by greater physicochemical heterogeneity, was also observed in deeper sediments. The presence of specific metabolic fingerprints under oxic and anoxic conditions indicated that the microbial community was adapted to use organic matter under different oxygen conditions. Overall the heterogeneity of oxygen concentrations with depth and in time would influence organic matter metabolism in the sediment tank.

BACKGROUND AND AIMS

The connection between surface water, groundwater and the processes occurring in this interface (i.e. the hyporheic sediment) are important for river ecosystem metabolism (Brunke and Gonser 1997; Nogaro et al. 2013). The hyporheic zone promotes the exchange of water, nutrients, and biota between alluvial groundwater and stream water (Boulton et al. 1998). This exchange, in turn, influences stream water quality (Sobczak and Findlay 2002). Microbial communities in sediments are principally composed of heterotrophic microorganisms including bacteria, fungi, and small metazoans which are attached to sand grains and assembled in a polymeric matrix (Pusch et al. 1998) that plays key roles in biogeochemical processes (Findlay et al. 1993; Mermillod-Blondin et al. 2005). Microbial communities are responsible for most of the metabolic activity in hyporheic sediments (Storey et al. 1999), including the degradation of organic matter and the reduction of electron acceptors (e.g. oxygen, nitrate and sulphate) (Ghiorse and Wilson 1988; Hedin et al. 1998). These processes act as water purification processes, ultimately impacting the water quality of river and aquifer systems. Similarly, when water flows through sediments in the vadose zone, microbial activity enhance the quality of surface water as in slow infiltration bed or in managed artificial recharge facilities (Greskowiak et al. 2005) removing organic carbon content, nutrients and trace organic chemicals (Li et al. 2012; Regnery et al. 2015).

Decomposition of organic matter is one of the main metabolic roles of microorganisms in soils and sediments. Extracellular enzymes released by microbes promote organic carbon cycling, by transforming polymeric material into soluble monomers that can be assimilated by microbes. These actions constitute a limiting step in the entrance of organic matter to the food web (Allison et al. 2007; Romaní et al. 2012). Although many studies have analyzed enzyme activities in surface sediments (Romaní and Sabater, 2001), much less is known about how these activities change according to depth. For instance, in the upper 12 cm of river sediment, extracellular enzyme activities involved in the degradation of cellulose, hemicellulose, and organic phosphorus compounds decreased together with

bacterial density (Romani et al. 1998). Changes in the utilization of organic matter at different sediment depths may be linked to microbial colonization. Indeed, microorganisms are found in largest quantities at the soil surface, and their abundance declines rapidly with increasing depth (Taylor et al. 2002). Microbially active zones are often limited to the top sediment layer (<60 cm) where bacterial biomass and exchange rates between the river and the hyporheic zone are the highest (Taylor et al. 2002). Bacteria in deeper sediments are more sensitive to physical and chemical changes compared to those in surface layers (Fierer et al. 2003) due to the relatively more stable conditions (Fischer et al. 2005). In deeper sediments, organic matter use may be further affected by physical and chemical changes in oxygen, pH, temperature and nutrient availability (Douterelo et al. 2011). Moreover, the sediment biofilm structure reduces the water infiltration capacity by pore clogging (e.g. Or et al. 2007), also decreasing the soil porosity, stream bed permeability, and thus the water exchange between river and vadose zone (Brunke and Gonser 1997; Descloux et al. 2010).

Physicochemical conditions appear to be highly heterogeneous at different sediment depths (Storey et al. 1999), and this heterogeneity promotes the coexistence of aerobic and anaerobic microbial communities in sediments (Harvey et al. 1995; Storey et al. 1999). Previous publications showed vertical oxygen consumption in sediments (Revsbech et al. 1986; Glud et al. 2005), but they did not focus on how to link this oxygen gradient to the decomposition of organic matter along the sediment's profile. This is in spite of oxygen and organic matter being known to play key roles in nutrient cycles (Hedin et al. 1998; Rubol et al. 2012; Nogaro et al. 2013). Low oxygen content and redox potential in deeper sediments may cause shifts in microbial metabolism. Indeed, decomposition of organic matter is more rapid and efficient in oxygenic conditions (Storey et al. 1999) and some extracellular enzymatic activities are inhibited in anoxic conditions (Goel et al. 1998).

The objective of this study was to analyze changes in microbial organic matter use at different sediment depths under continuous infiltration conditions. We hypothesized that microbial activity and biomass would be higher at the sediment

surface and decline with depth. At that deeper layer gradients would be more pronounced at the end of the experiment consistent with a vertical oxygen gradient. Specifically, the experiment aims at: i) analyzing organic matter decomposition capabilities and microbial functional diversity of the community developed in depth as a result of a colonization sequence; and ii) investigating the vertical changes of organic matter use due to different oxygenic conditions.

To reach these objectives, a 1- meter sediment tank with continuous infiltration of synthetic water was used to monitor several physical and chemical parameters, including oxygen, temperature, conductivity, inorganic nutrients, dissolved organic carbon, and microbial metabolism. Activities of β-glucosidase, leucine-aminopeptidase and phosphatase were assessed to monitor the hydrolysis of organic compounds containing carbon, nitrogen, and phosphorus (Romaní et al. 2012). Functional diversity and functional fingerprints of sediment microbial communities were analyzed on the community-level using Biolog Ecoplates (Salomo et al. 2009). A meso-scale was chosen to produce biogeochemical and microbial parameters under controlled interstitial flow conditions, similar to those experimental studies using sediment columns (Battin and Sengschmitt 1999; Mermillod-Blondin et al. 2005) or a sediment tank (Weber and Legge 2011).

METHODS

Experimental design

An infiltration (flow-through) experiment was conducted in a vertical intermediate-scale tank reconstructed with a heterogeneous sediment porous media. The dimensions of the sediment tank were 1.20 m high × 0.45 m long × 0.15 m wide. The base of the tank was filled with a 15 cm layer of silicic sand (0.7 to 1.8 mm diameter, supplied by Triturados Barcelona, Inc.) covered with a permeable geo-synthetic fabric membrane to prevent soil flowing through. Sediments were collected from a managed aquifer recharge facility site located in the Llobregat River near Barcelona (UTM coordinates 418446.63 N, 4581658.18 E). Dry sediments were sieved at 0.5 cm and packed in the tank by a repeating series of

wetting and drying cycles, see Rubol et al. (2014) for details. The top 20 cm of the tank were left free of sediment to allow ponding. A concentrated synthetic solution of 10 L mixture of inorganic and organic compounds was prepared in a carboy. This concentrated solution was diluted with deionized water prior to its injection into the infiltration pond of the tank. The carboy solution was continuously mixed with a magnetic stirrer (AREX 230v/50Hz, VELP Scientific) and supplied at the surface of the tank with no recirculation. The carboy was replaced every 4-7 days (depending on water consumption). The chemical composition of the mixture mimics the typical Llobregat River water reported by Fernández-Turiel et al. (2003) which is characterized by high nutrient content (NO_3 ~ 8 mg/L, NH_4~ 1.2 mg/L , PO_4~ 0.5 mg/L and DOC~ 7 mg/L, Table 1).

Table 1. Synthetic water composition used as input water to the flow-through system during the experiment

Synthetic water composition	
Compound	**mg/L**
$CHNaO_3$	179.9
KH_2PO_4	1.3
$MgCl_2\ 6H_2O$	238.2
$Mg(NO_3)_2\ 6H_2O$	22.5
KCl	67.7
$CaCl_2\ 2H_2O$	396.9
Na_2SO_4	269.7
NH_4Cl	4.5
Na_2SiO_3	18.0
Cellobiose	1.5
Leucine-proline	1.5
Humic acid	7

The upper layer in the tank was exposed to natural light (sun light reaching the tank from the laboratory windows), while the lateral walls were covered with dark plastic to prevent photoautotrophic activity. To stimulate biofilm colonization, an inoculum prepared from sediment collected at the pristine riverbed nearby the site, was added to the top of the tank at the beginning of the experiment. This inoculum contained 2.27 ± 0.41 x 10^6 bacterial cells mL^{-1} (mean value ± standard error).

The tank was equipped with duplicate liquid ports (located in the middle and on the left part of the sediment tank) at depths 5, 15, 30, 45 and 58 cm (all distances are measured from the surface of the sediment). Sediment sampling ports consisting of 1.5 cm horizontal holes tapped with cork caps located at 20 and 50 cm depth on the right part of sediment tank. Samples at 20 and 50 cm depth were collected with a methacrylate corer (1.5 cm in diameter, 12 cm long) displayed horizontally and samples from the surface were collected vertically. Despite sampling collection led to local changes in hydraulic conductivity right after sampling, the system used minimizes the overall impact as it readjusts quickly to fill the gap created. Then, three subsamples of 0.5 mL of sediment from the beginning, middle and edge of each core were collected in triplicate with an uncapped syringe for each analysis.

Physical and chemical analysis

Measured values of temperature and volumetric water content were recorded continuously by using capacitance sensors (5TE, Decagon Devices, Pullman, WA) placed at 3 different depths. A handheld multiparameter instrument (YSI Professional Plus) recorded temperature, electrical conductivity, dissolved oxygen and pH in continuous at the tank outlet. Dissolved oxygen concentrations were measured continuously with optical fibers (FiboxPresens, Germany) and corrected for temperature. The evolution of infiltration rate (R(t)) with time was determined from the water balance accounting for the infiltration rate and temporal changes in the ponding water level. Direct evaporation was estimated, and found negligible to the overall balance.

Inorganic nutrients were measured from the water samples collected at days 0, 3, 8, 13, 16, 20, 24, 28, 33, 36, 40, 43, 49, 53 and 83 at 5 depths (5, 15, 30, 45 and 58 cm measured from the surface). Water samples were collected in 9 mL vacuum vials and filtered at 0.2 μm (Whatman). Analysis for NO_3^-, NH_4^+ and Cl- were performed by High Performance Liquid Chromatography (HPLC). Measurements of dissolved organic carbon (DOC) were obtained at the same depths as those of nutrients from the water samples collected at days 13, 16, 20, 24, 28, 33, 36, 40, 43, 49, 53 and 83. Samples were filtered (Whatman GF/F), conditioned with 2M HCl

and stored at 5°C until analyses were performed. DOC was measured using a total organic carbon analyser (Shimadzu TOC-V-CSH 230V, Tokyo, Japan). Three replicates were used for each sample. Due to technical problems phosphate (PO_4) were analysed only at days 3, 13, 49 and 83 at 3 different depths (5, 15, and 45 cm). Phosphate was analysed spectrophotometrically as described by Murphy and Riley (1962).

Biological analysis

Microbial activity and bacterial abundance were analysed from sediment samples and were processed during the same sampling day. Samples for extracellular enzyme activities were collected on days 0, 3, 6, 9, 14, 21, 34, 50 and 83. Bacterial abundance and viability and sole- carbon-source utilization profiles (Biolog EcoPlates) were estimated on days 3, 14, 34, 50 and 83.

Bacterial abundance and viability

Live and dead bacteria in sediment were counted using Live/Dead bacterial viability kit (Invitrogen Molecular Probes, Inc.). On each sampling day, each collected sediment subsample (1 mL of sand volume) from each depth (3 replicates per depth) was placed in a sterile vial with 10 mL of Ringer solution (Scharlau S.L). Bacteria were detached from sediment after sonication for 1 min using an ultrasonic bath (Selecta, 40 W and 40 kHz). The extract was diluted (20 times at the beginning of the experiment, 50 times from day 14) with Ringer solution. The diluted sediment extract was then used for bacterial density and viability analysis and also as the inoculum for the Biolog EcoPlates incubations (see below). The extract dilution was determined in advance following the recommendations for EcoPlates incubations with bacterial density values around 10^6 cell mL^{-1} (Garland et al. 2001).

For each diluted sediment extract, 2 mL were stained by a 1:1 mixture of Syto9 and Propidium Iodide and incubated for 15 min in dark conditions. Samples were filtered through a 0.2 µm pore-size black polycarbonate filters (GE Water and Process Technologies) and then mounted on a microscope slide. Twenty randomly chosen fields were counted for each slide for live and dead bacteria (Nikon E600

epifluorescence microscope, 1000X, Nikon Corporation, Tokyo, Japan). Results are expressed as cells/ g DW (dry weight) of sediment.

Extracellular enzyme activities

Three extracellular enzyme activities were analysed in the sediment, linked to the capacity to decompose cellobiose (β-glucosidase activity, EC 3.2.1.21, BG), peptides (leucine-aminopeptidase activity, EC 3.4.11.1, LEU) and phosphomonoesters (phosphatase activity, EC 3.1.3.1, PHO).

Extracellular enzyme activities were determined with a spectrofluorometer using artificial fluorescent substrates 4-methylumbelliferone (MUF)-β-D-glucoside, MUF-phosphate, and L-leucine-4-7-methylcoumarylamide (AMC), for BG, PHO, and LEU, respectively in triplicate for each time and depth. Sediment samples were placed in vials filled with 4 mL of filtered water from the tank (0.2 μm nylon, Whatman). Samples were incubated at saturating conditions (final concentration of 300 μM) at 20ºC under continuous shaking (150 rpm) during 1 h in dark conditions. Blanks (with 0.2 μm filtered water from the tank) were also incubated to eliminate the background signals and water fluorescence. At the end of the incubation period, 4 mL of glycine buffer (pH 10.4) solution was added, and fluorescence was measured at 365/455 nm excitation/emission wave lengths for MUF and at 364/445 nm excitation/emission wave lengths for AMC (Kontron SFM 25, Munich, Germany). Standard curves (0-200 nmol/L) were prepared for MUF and AMC, separately. Activity values are expressed as nmol of AMC or MUF released per g DW of sediment per hour.

Carbon substrate utilization profiles

Biolog EcoPlates (Biolog Inc., Hayward, California, USA) were used in order to determine the differences in the metabolic fingerprint in time and depth of the sediment tank based on carbon source utilization.

Each sampling day, the diluted sediment extracts from each depth (3 replicates per depth, see extraction procedure in Bacterial abundance and viability section) were incubated in the EcoPlates immediately after sampling (maximum 5 h). EcoPlates

were inoculated with 130 µL of each sediment extract under sterile conditions and incubated at 20ºC in dark conditions for 6 days. Optical density (OD) in the plates was read every 24 h at 590 nm using a microplate reader (SynergyTM 4, BioTek, Winooski, VT, USA). After 6 days (144 h) most wells had achieved sigmoid color development saturation and the AWCD (Average Well Color Development) was close to 0.6 (Insam and Goberna 2004). Raw absorbance data obtained from Biolog Ecoplates were corrected by the mean absorbance of the control wells (3 wells with no substrate) in each plate. Values < 0.05 (or negative) were set to zero. Data from each EcoPlate were analysed by calculating the AWCD, Shannon diversity index (H') and substrate richness (S) to evaluate microbial community functional diversity and functional richness (Garland and Mills 1991). Substrate richness is the number of different substrates used by the community (counting all positive OD readings, i.e. positive wells). Moreover, kinetic analysis was carried out for AWCD for each time and depth. Three kinetic parameters (a, 1/b and x_0) were estimated by fitting the curve of color development on plates to a sigmoid equation (Freixa and Romaní 2014; Lindstrom et al. 1998) where a is the maximum absorbance in the event of color saturation, 1/b is the slope of the maximum rate of color development and x_0 is the time when maximum color development rate is achieved. The three kinetic parameters (a, 1/b and x_0) are invariant with respect to inoculums density (Lindstrom et al. 1998). To evaluate utilization of dissolved organic nitrogen compounds, the nitrogen use (NUSE) index was calculated as the proportion (expressed as percentage) of the summed absorbance of those substrates that have C and N over the total absorbance measured in each EcoPlate (Sala et al. 2006).

Extracellular enzymes and carbon substrate utilization profiles under anoxic conditions in the 50 cm depth sediment

Vertical variability in oxygen concentrations was observed during the experiment. For this reason, we performed a test to analyse possible differences in microbial functioning under oxic and anoxic conditions for samples collected at 50 cm depth.

To test the potential effect of oxygen conditions on sediment microbial metabolism, an extra set of samples from days 14, 34, 50 and 83 at 50 cm depth were collected for Biolog EcoPlates and extracellular enzyme activity measurements under anoxic conditions. The analytical protocols were the same as those described above, except that the incubations were performed under an anoxic atmosphere and the collected samples and sediment extracts were purged with nitrogen gas at the moment of collection. The incubations for Biolog EcoPlates and extracellular enzyme activities were performed within a hermetic bottle with anoxic conditions already created inside (AnaeroGen system, Oxoid, UK). For the Biolog EcoPlates incubations, plates were further covered with silicone sealing film (Sigma). Oxygen values were measured before and after incubations (WTW oxygen meter).

Data analysis

Differences among depths and days for temperature, oxygen, extracellular enzymes, bacterial density and viability, and parameters obtained from carbon substrate utilization profiles (AWCD, Shannon diversity index, Richness, NUSE index and kinetic parameters) were tested using repeated measures analysis of variance (rm-ANOVA, depth and days as factors). All variables were logarithmically transformed, except for AWCD and Shannon index and kinetic parameters to render symmetric variables. Differences between depths observed on day 83 were further analysed using a one-way analysis of variance (ANOVA, depth as a factor) between enzyme activities, Biolog parameters (Shannon diversity index, Richness, NUSE index) and live and bacterial density. Also, the differences between oxic and anoxic incubations for enzyme activities and Biolog EcoPlates were tested by analysis of variance (ANOVA, oxygen as a factor). Nutrients (NO_3, NH_4, PO_4, DOC and Cl) for each day and depth were analysed using a two-way analysis of variance (ANOVA, depth and time as factors). All data were previously logarithmically transformed. All of these statistical analyses were performed using the program SPSS v.15.0 (SPSS, Inc., Chicago, IL, USA) and differences were considered to be significant at $p < 0.05$.

The ratios between carbon, nitrogen and phosphorus degrading enzymes (BG: LEU, BG: PHO, and LEU: PHO, as indicators of C:N, C:P and N:P nutrient needs and nutrient acquisition capabilities relationships, respectively) obtained under oxic and anoxic conditions were calculated in order to estimate potential imbalances in nutrient needs and capabilities. These enzyme ratios were estimated based on linear regression analysis of the natural log transformed enzyme activities. Results were expressed in terms of the slope and 95% confidence interval (as proposed by Sinsabaugh et al. (2011, 2009)). This analysis was performed with Sigmaplot 11.0 (Systat software, Inc, CA, USA).

Non-metric multi-dimensional scaling (NMDS) ordination plots were performed to visualize the spatial distribution pattern of the metabolic profiles in time and depth obtained from the Biolog EcoPlates of the 31 carbon substrates as well as to distinguish between oxic and anoxic metabolic profiles obtained at 50 cm depth. A previous distance matrix with Bray-Curtis similarity was created. NMDS is based on the rank order relation of dissimilarities where the largest distance between samples denotes the most different microbial functional profile. In addition, as suggested by Choi and Dobbs (1999), the 31 carbon sources in the plate were grouped in six functional categories including polymers (n=4), carbohydrates (n=10), carboxylic acids (n=7), phenolic compounds (n=2), amines (n=2) and amino acids (n=6). Data for all substrates and group of substrates from Biolog were previously standardized by sampling dates and then were fitted to the ordination plot using the "envfit" function of the "vegan" package in R software. This function was used to identify the correlations ($p<0.05$) with the ordination space to identify the groups of substrates mostly responsible for the spatial distribution of the samples in the NMDS plot (Legendre and Legendre 1998; Blanchet et al. 2008). Based on these data, ANOSIM (analysis of similarity) (Clarke 1993) were performed using the "vegan" package in R software to test for differences between functional profiles in depth and time.

RESULTS

Physicochemical parameters

Dissolved oxygen concentrations decreased at all depths after the start of the experiment, approaching values below 2 mg/L after day 34. Significant differences were observed among depths indicating lower oxygen concentration at the bottom of the tank ($p < 0.01$, Fig. 1). Based on oxygen data, three time periods were used for analyses of nutrient content and enzyme ratios.

- **Period 1 (P1**): From day 1 to 28, defined by the development of a clear oxygen gradient ranging from 9.5 mg/L at the sediment surface to 4.5 mg/L at 50 cm depth.
- **Period 2 (P2)**: From day 33 to 53, defined by a reduction of the oxygen gradient, with small differences between the different depths and values close to 4 mg/L.
- **Period 3 (P3)**: From day 64 to 83, defined by a decrease in oxygen concentrations, ranged from 4 mg/L to 0.5 mg/L at the sediment surface and from 2.7 to 0.02 (anoxic conditions) at the bottom (50 cm in depth).

Water temperature increased from 18.14 ± 0.10 ºC to 25.18 ± 0.14 ºC (mean ± standard error) during the experiment, although no significant differences in temperature were observed among depths, indicating rapid re-equilibration with atmospheric conditions. The infiltration rate changed dynamically throughout the experiment, ranging from an initial value of 40 L/day to 15 L/day at day 83 (Fig. 1).

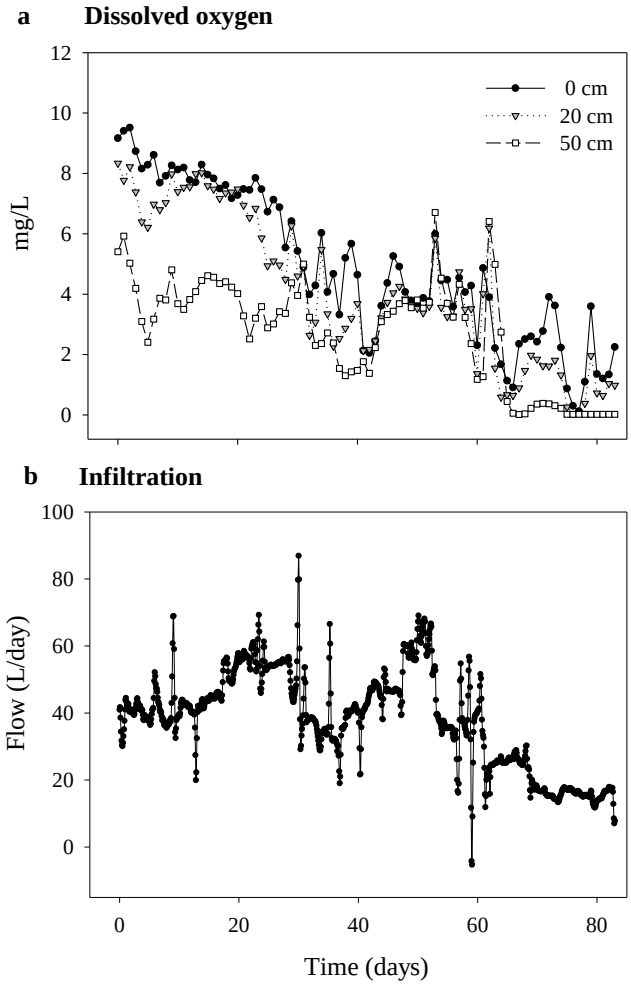

Figure 1. Temporal evolution of dissolved oxygen concentration at three different depths (a) and infiltration rates (b). a) Point values represent daily means and had been corrected for the drift of the instruments due to varied with temperature.

The chemical composition of the interstitial water varied according to time and depth (Fig. 2), whereas the pH values remained relatively stable throughout the experiment (pH 7.6–8). Dissolved NO_3^- varied from 3.5 to 28.2 mg/L over time ($p = 0.01$). NH_4^+ also varied according to depth ($p = 0.043$) and time ($p < 0.01$), peaking at 1.5 mg/L during P1 and remaining below 0.05 mg/L after day 33. Dissolved organic carbon (DOC) concentration_values diminished over time ($p < 0.01$), but did not differ by depth ($p > 0.05$). Inorganic phosphorous did not show any trend with depth, however a decrease of phosphate was observed at the end of the experiment. Chloride concentration remained stable over time and depth, ranging from 186 to 227 mg/L ($p > 0.05$; Fig. 2).

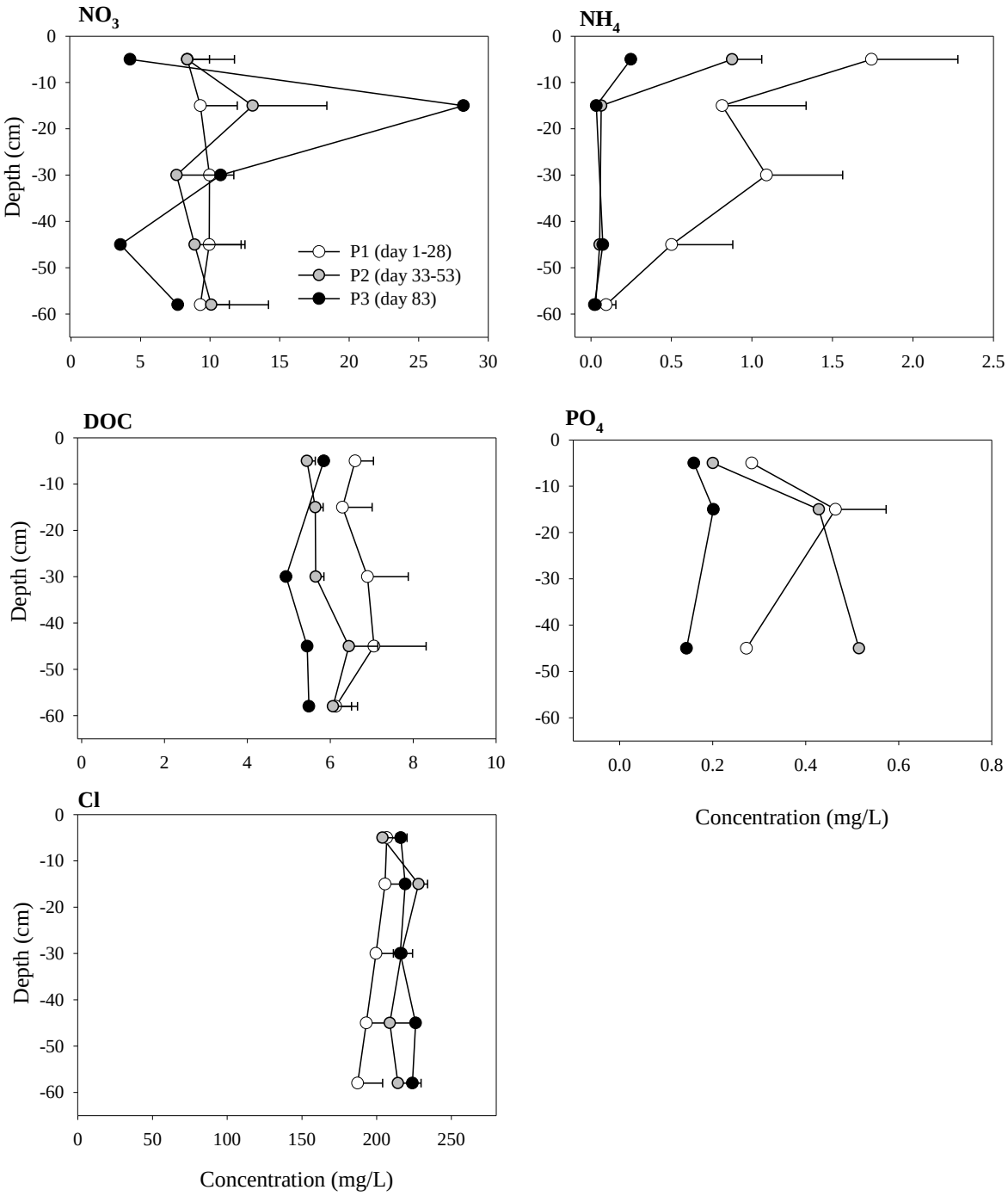

Figure 2. Mean values of nitrate (NO_3), ammonia (NH_4), dissolved organic carbon (DOC) phosphate (PO_4) and chloride (Cl) at the three selected periods. Periods are defined as P1 (days 1 to 28), P2 (days 33 to 53) and P3 (day 83) as a function of depth. PO4 data were only collected at days 3, 13 (P1), 49 (P2) and 83 (P3) at 3 different depths.

Biological parameters

Bacterial abundance and viability

Bacterial density increased rapidly during the colonization process, with a mean maximum of 1.20 × 10^9 cells/g dry weight on day 83. The maximum value was 1.96 × 10^9 cells/g dry weight obtained at day 34 in surface sediment (Table 2). Live bacteria accounted for 44.5% ± 7.1% of the average total bacteria for the whole experiment. No significant differences in bacterial density and viability at different depths were observed ($p > 0.05$, Table 3).

Table 2. Bacterial density and viability (live bacteria) at different sampling depths and dates. Values are means (n=3) ± standard error, except for day 3 (n=1). All data expressed as 10^9 cells/g of DW of sediment

	Bacterial density			Live bacteria		
Days	**0 cm**	**20 cm**	**50 cm**	**0 cm**	**20 cm**	**50 cm**
3	0.11	0.07	nd	0.03	0.03	nd
14	0.19±0.04	0.36±0.01	0.29±0.03	0.09±0.01	0.15±0.02	0.12±0.01
34	1.96±0.25	1.23±0.07	1.41±0.03	1.17±0.16	0.47±0.04	0.78±0.03
50	1.39±0.18	1.08±0.03	1.11±0.30	0.58±0.11	0.39±0.03	0.41±0.20
83	1.69±0.65	1.87±0.36	1.48±0.36	0.73±0.24	0.78±0.07	0.69±0.15

Extracellular enzyme activities

Leu-aminopeptidase (LEU) and phosphatase (PHO) activities increased significantly during the experiment (Fig. 3, Table 3). LEU activity increased from the beginning of the experiment, and the highest values were depicted on day 83. In contrast, PHO activity increased slowly until day 21 and was stable until the end of the experiment (Fig. 3). At the end of the experiment, PHO activity was the highest, followed by LEU and β-glucosidase (BG) activities. Significant increases in phosphatase activity were observed at day 83 at the bottom of the tank (Table 3). BG was significantly higher in surface sediment and decreased with increasing depth for the whole experiment (Table 3, Fig. 3).

Table 3. Repeated measures ANOVA results considering two factors (time and depth) for extracellular enzyme activities (BG: β-glucosidase, LEU:Leu-aminopeptidase and PHO: phosphatase), functional diversity indexes from Biolog EcoPlates (H': Shannon index, S: Richness and NUSE index) and live and total bacterial density (LIVE and TOTAL). Probabilities are corrected for sphericity by the Greenhouse–Geisser correction. Analysis of variance at day 83 (ANOVA, one factor) for each parameter is added. Significant values are indicated in boldface type. F-values and degrees of freedom are also indicated

	BG	**LEU**	**PHO**	**H'**	**S'**	**NUSE**	**LIVE**	**TOTAL**
Time	0.127	**0.005**	**0.001**	**0.025**	**0.013**	0.150	**0.005**	**0.010**
	$F_{1.0,2.1}$= 6.08	$F_{1.3,2.6}$ = 83.46	$F_{1.3,2.6}$ = 253.4	$F_{1.1,2.3}$ = 27.39	$F_{1.7,3.4}$ = 21.28	$F_{1.4,2.7}$ = 4.03	$F_{1.1,2.2}$ = 125.2	$F_{1.1,2.2}$ = 71.34
Depth	**0.012**	0.551	0.213	0.098	**0.007**	**0.019**	0.362	0.509
	$F_{1.0,2.0}$ = 73.81	$F_{1.1,2.2}$ = 0.54	$F_{1.0,2.0}$ = 3.21	$F_{1.0,2.0}$ = 8.53	$F_{1.5,2.9}$ = 39.33	$F_{1.3,2.7}$ = 25.76	$F_{1.3,2.5}$ = 1.36	$F_{1.2,2.4}$ = 0.68
Time x Depth	0.133	0.156	0.092	0.123	0.080	0.303	**0.043**	0.071
	$F_{1.8,\ 3.7}$ = 3.62	$F_{1.8,3.5}$ = 3.29	$F_{1.6,3.3}$ = 5.46	$F_{2.0,4.0}$ = 3.71	$F_{1.8,3.7}$ = 5.22	$F_{1.7,3.4}$ = 1.68	$F_{1.2,2.5}$ = 4.38	$F_{1.5,2.9}$ = 7.45
Day 83 Depth	0.317	0.230	**0.044**	0.057	**<0.001**	**0.035**	0.783	0.628
	$F_{2,6}$= 1.40	$F_{2,6}$= 5.50	$F_{2,6}$ = 1.85	$F_{2,6}$ = 5.35	$F_{2,6}$ = 49.49	$F_{2,6}$ = 7.04	$F_{2,6}$ = 0.25	$F_{2,6}$ = 0.50

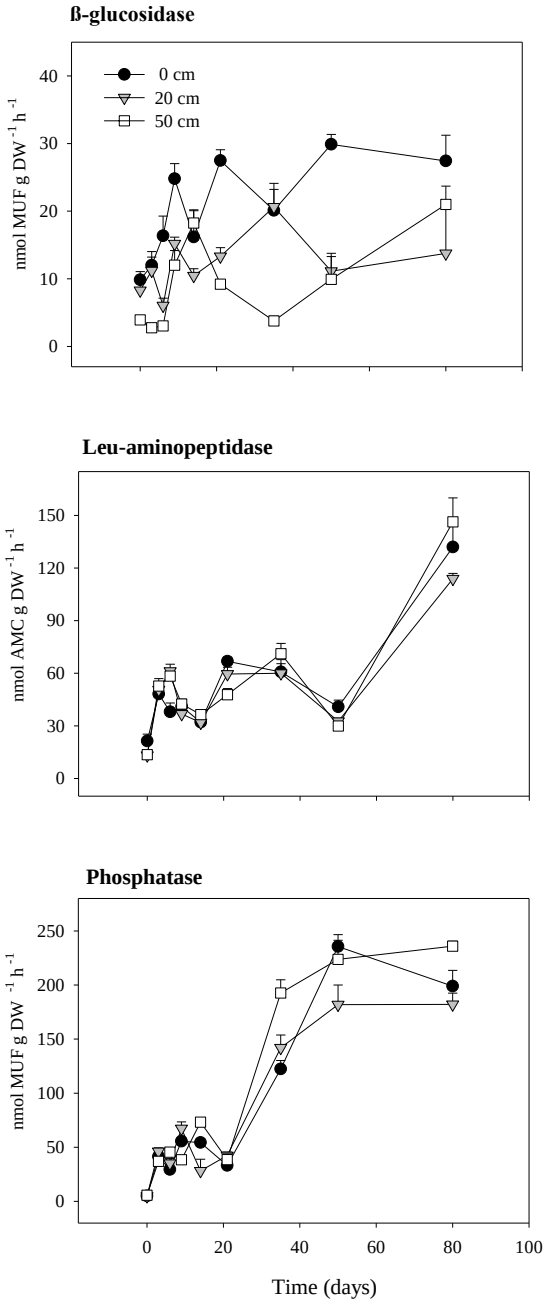

Figure 3. Temporal changes in extracellular enzyme activities at 3 different depths, after incubating samples under oxic conditions. Values are means ± standard error (from 3 replicates).

Differences in extracellular enzyme activities were observed under different oxygenic conditions (Fig. 4). PHO and LEU activities were significantly reduced in anoxic conditions, mainly PHO activity was reduced 82% compared to oxic conditions. In contrast, BG activity was not significantly affected by oxygen concentrations (Fig. 4).

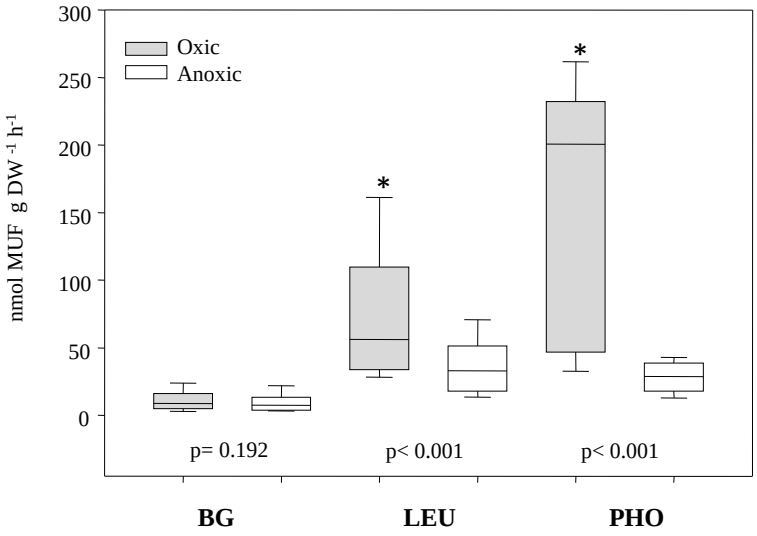

Figure 4. Box plot of extracellular enzymes (BG, LEU, PHO) comparing activities measured under oxic conditions versus activities measured under anoxic conditions for 50 cm depth sediment samples (n=12, including data from days 14, 34, 50 and 83). The asterisk indicates significant different values respect to anoxic data.

The ratio of Ln BG: Ln LEU activities (obtained as the slope of the linear regression between Ln LEU vs. Ln BG, Fig. 5) was used as an indicator of the greater or lower capacity of the microbial community to degrade organic matter, containing carbon in contrast to the its capacity to degrade organic matter containing both carbon and nitrogen. Results from these activities measured under oxic conditions showed a slope of 0.90, a value close to the equilibrium (1:1 ratio, 95% confidence interval was 0.71-1.10, Fig. 5). In contrast, slopes for Ln BG: Ln PHO and Ln LEU: Ln PHO ratios were 0.56 (95% confidence interval was 0.41-0.71) and 0.53 (95% confidence interval was 0.44-0.63) respectively, indicating enhanced ability to degrade organic compounds containing phosphorus to that containing carbon and/or nitrogen. The largest increase in PHO relative to BG and LEU was observed during P3, indicating that the microbial communities first acquired more carbon and nitrogen, while more phosphorous was assimilated during the third period of the experiment (Fig. 5). Slopes close to 1 were measured in anoxic conditions at a depth of 50 cm. No differences between depths were observed for enzyme activity ratios (data not shown).

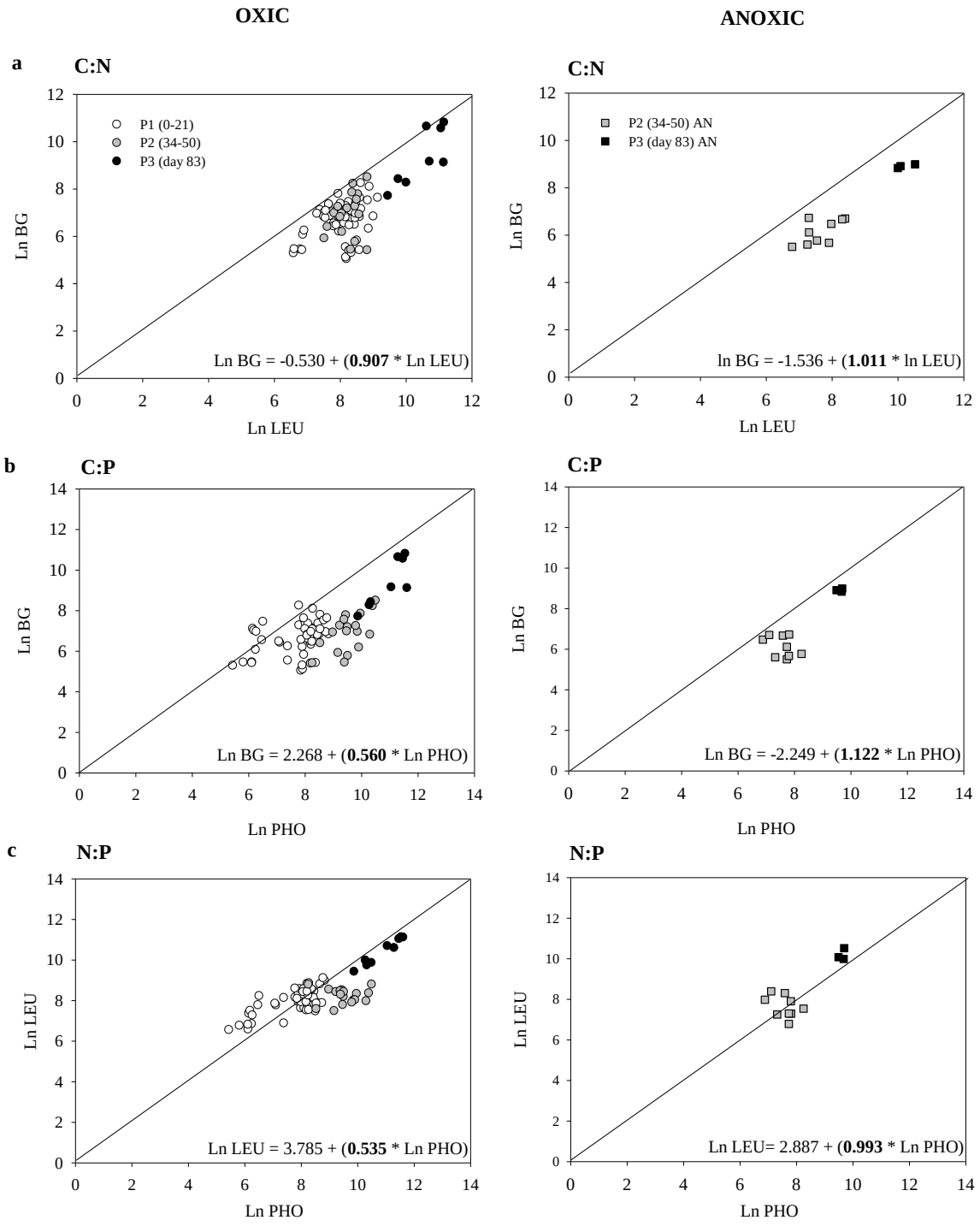

Figure 5. Relationships (in ln space) between BG:LEU (a) BG:PHO (b) LEU:PHO (c) organic matter degradation enzymes. Data are values from each sampling day grouped by periods (P1, P2 and P3). Relationships on the left are those from oxic measurements while those on the right are those from anoxic measurements. The solid line indicates a 1:1 relationship. Linear regressions are included for each chart and the slopes are highlighted in bold.

Carbon substrate utilization profiles

Biolog Ecoplates were used to characterize the functional diversity and metabolic fingerprint of the sediment tank communities according to depth and time (Fig. 6). The percentage of positive wells (richness) ranged between 65 - 100%, with lower values being measured at the end of the experiment. Consistently, functional diversity (Shannon index) and functional richness (positive wells) also decreased significantly through time (Table 3, Fig. 6). Significant differences were found at different sediment depths; functional richness was highest at the surface and decreased with depth over time (Fig. 6, Table 3). However, at 50 cm, measurements under oxic and anoxic conditions were not significantly different ($p = 0.92$, Shannon index; $p = 0.59$, functional richness). Moreover, differences in the use of nitrogen compounds (NUSE index) at different depths were detected. High NUSE index values were found at 50 cm on day 83 (Fig. 6, Table 3). The NUSE index at 50 cm was not significantly different between oxic and anoxic conditions ($p = 0.54$).

The change in the metabolic fingerprint with depth was also remarkable. The community present at depth 50 cm was clearly distinct from that of the surface and the first 20 cm, as shown in the NMDS plot (Fig. 7a) and in the ANOSIM analysis (depth factor, $R= 0.228$, $p=0.001$ between surface - 50cm: $R= 0.114$, $p=0.009$ between 20cm -50 cm). At 50 cm, microbial communities were able to degrade amino acids and carboxylic acids, including L-asparagine and pyruvic acid, whereas surface and 20 cm communities principally degraded polymers (Tween 80) and carbohydrates (α-D-lactose and D-xylose) (Fig. 7a). At 50 cm, high dispersion in the ordination analysis (NMDS) was found, indicating larger heterogeneity between samples; this finding was especially relevant on day 83. Metabolic fingerprints of oxic and anoxic communities at 50 cm were different (ANOSIM analysis, Global $R= 0.232$, $p = 0.007$, Fig. 7b). However, anoxic samples from day 14 were similar to oxic samples from days 14, 34, and 50, whereas clear differences were observed between oxic and anoxic conditions for other sampling dates (Fig. 7b). Under anoxic conditions, decomposition of carboxylic acids and amino acids were enhanced, whereas phenolic compounds and amines were degraded in the presence of oxygen

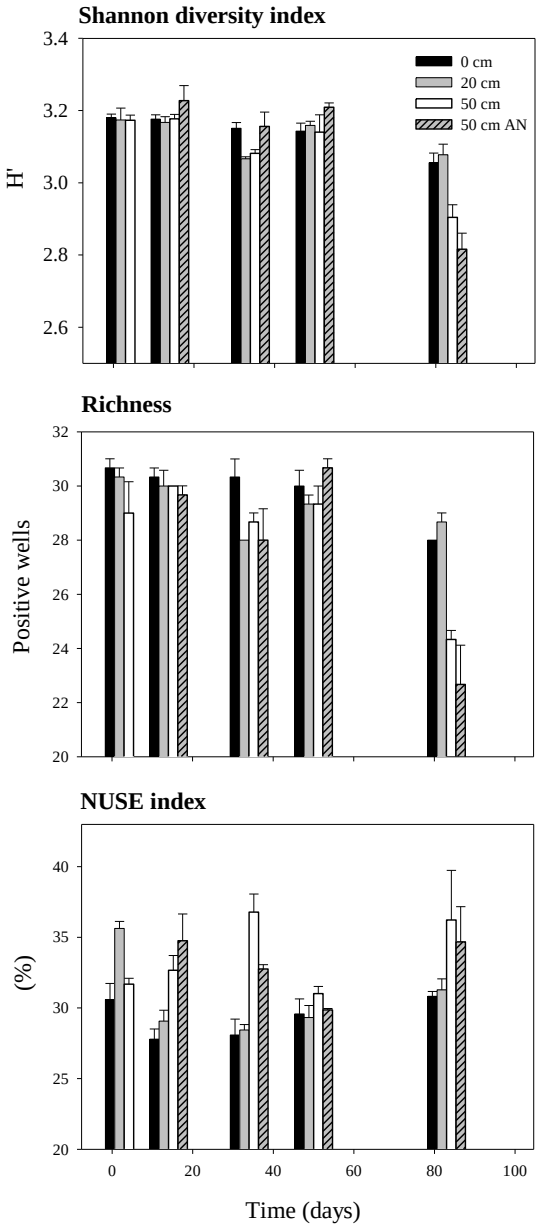

Figure 6. Shannon diversity index, Richness (positive wells) and NUSE index at different depth (0, 20 and 50 cm) for 5 different sampling dates. Values at 50 cm in anaerobic incubations are also included after day 14. Values are means and standard deviation (from 3 replicates).

Average well color development (AWCD) values revealed significant differences in kinetic parameters a and x_0 between oxic and anoxic incubation conditions at 50 cm ($p < 0.001$, a; $p = 0.038$, x_0). Under aerobic conditions, metabolic activity took longer (higher x_0 values) to achieve maximum color development and higher maximum metabolic capacity (a) was observed on all sampling dates. In contrast, no differences were observed between oxic and anoxic conditions at day 83 ($p > 0.05$ for a, $1/b$, and x_0).

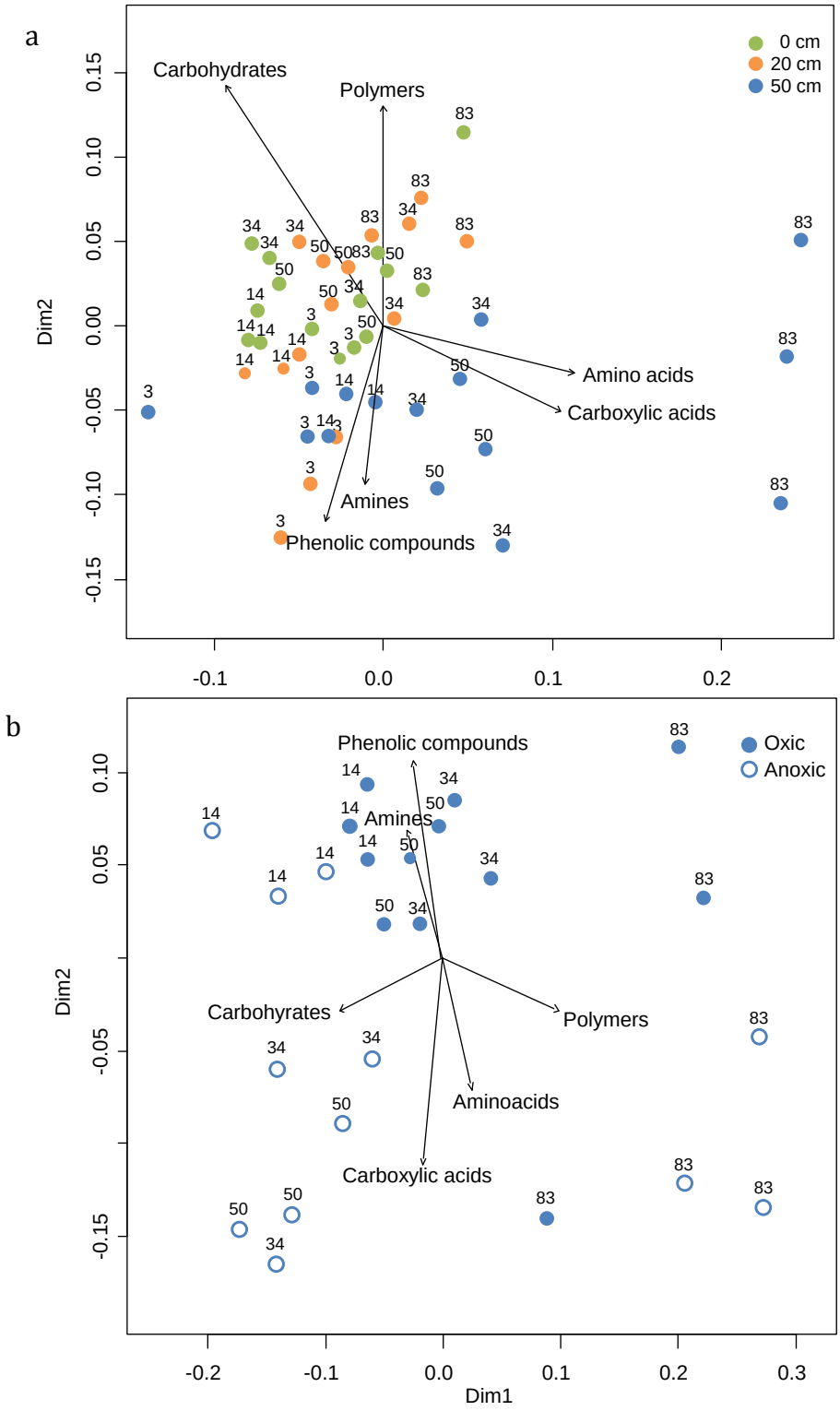

Figure 7. NMDS ordination plots based on Bray-Curtis distances according to 31 substrates of Biolog Ecoplates after 144 hours of incubation. a) Data include all depths. Color indicates different depths and numbers the sampling date. b) Data for 50 cm depth after incubation at different oxygen conditions. Color indicates oxic / anoxic incubated samples, and numbers the sampling date. The six groups of carbon substrates are fitted on the ordination plot $p<0.05$. Kruskal 2D stress is equal to 0.15 and 0.11, respectively.

DISCUSSION

Changes in microbial community metabolism and functional diversity at selected depths were found to occur in a controlled porous media subjected to continuous infiltration. Previous studies described the structure and activity of microbial communities with soils depth being driven by physicochemical factors (e.g., grain size, oxygen, pH, temperature, and redox potential (Bundt et al. 2001; Fierer et al. 2003). Here we complement this knowledge by reporting changes in microbial metabolism as a function of sediment depth and oxygen conditions.

Our findings indicate that bacteria colonizing the sediment tank had different capacities to decompose organic compounds depending on depth. At the surface, bacteria used simple polysaccharides through β-glucosidase activity, and this activity decreased with depth. Previous studies documented the decrease of β-glucosidase activity in sand filters (Hendel et al. 2001) and deep-sea sediments (Boetius et al. 2000) as the result of reduced availability of simple polysaccharides and low bacterial densities in deeper sediments (Romani et al. 1998; Fischer et al. 2002). In our study, no significant differences in alive or total bacteria density were found. The latter is consistent with the work of Franken et al. (2001), that also reported no differences in bacterial densities until 60 cm in a hyporheic zone temperate stream. Additional studies linked the decrease values of temperature, pH and organic matter content to the reduction in enzyme activities with increasing depth (Douterelo et al. 2011). However, in our experimental conditions, no significant differences in temperature or pH were observed. Although significant reduction of oxygen content was measured, the decrease in β-glucosidase activity along the vertical profile could not be explained by low oxygen content, as BG activity was not affected by incubation under different oxygen conditions (Fig. 4). According to Kristensen et al. (1995), the availability of labile organic matter limits bacterial heterotrophic activity in various aquatic ecosystems, regardless of oxygen concentration. Therefore, the decrease in simple polysaccharide use with depth may be explained by the presence of easy-to-decompose material (more labile) and first degraded compounds at the tank

surface. This first degradation in the surface sediment might determine a reduction of labile organic matter in depth, and more resistant material were accumulated in deeper sediments (Costa et al. 2007a). Moreover, our results indicated that metabolic changes should be mainly linked to DOC quality than to DOC quantity, since we did not observe differences in DOC with depth. Nevertheless, high DOC values at depth (50 cm), as well as constant pH and temperature were likely promoted by the high infiltration rates measured during the experiment, which were in the upper range of the ones measured in the hyporheic zone, but similar to those observed in artificial recharge areas (Pedretti et al. 2011).

Microbial functional diversity was also depth dependent, and differences were more evident by the end of the experiment. Biolog EcoPlates incubations were used to characterize carbon source utilization in the sediment tank. Despite the limitations associated to this method (Verschuere et al. 1997; Konopka et al. 1998; Smalla et al. 1998), robust information to describe microbial functional diversity can be obtained after the data normalization and protocol standardization (i.e., use of similar inoculum size and incubation conditions) (Stefanowicz 2006). At the end of the experiment, the microbial community became more specialized and used a narrower range of carbon substrates, as indicated by the lower Shannon diversity and richness scores. These data suggested that the microorganisms had assembled to those better adapted to the environmental conditions of the sediment tank. This ecological specialization was defined by Devictor et al. (2010) as an adaptation process to the diversity of resources used by a species in different environments. Decreased use of available substrates was also observed at 50 cm, similar to results reported by Griffiths et al. (2003), that in substrate utilization decreased at 20 cm compared to surface samples. Likewise, the microbial community showed different functional fingerprints depending on depth (Fig. 7). Carbohydrates and polymers were used readily at the surface (0 and 20 cm depth, Fig. 7a). Similarly, concentrated use of carbohydrates and polymers was observed at the seawater surface (Sala et al. 2008). These compounds are considered to be the largest bioavailable source of carbon in sediments (Oliveira et al. 2010) and greater use of them at the surface is consistent with high surface labile compounds as β-glucosidase activity. On the other hand, the metabolic fingerprint at 50 cm was

distinct from fingerprints at 0 and 20 cm (Fig. 7a). The former was mainly characterized by the use of nitrogen compounds, as shown by higher NUSE index values. Until day 50, microbial communities used amines and phenolic compounds and from day 50 until the end of the experiment, amino acids and carboxylic acids were used (Fig. 7a). These results indicate the significant use of nitrogen-containing organic compounds at the bottom of the tank, consistent with the maintenance of leu-aminopeptidase activity.

Differences in microbial metabolism with depth may have been affected by oxygen availability, most significantly by the end of the experiment. Bacterial colonization and biofilm formation may have contributed to pore clogging, providing a substantial decrease in permeability and infiltration flow rate, and an increase in anoxia with increasing depth (Rubol et al. 2014). In our experiment, decrease in dissolved oxygen and infiltration rate followed a similar pattern during the experiment, but temperature increased. Although a temperature increase determines a lower viscosity of water and thus would increase water infiltration, our results suggest that microbial colonization and metabolism are the main responsible for changes in water infiltration, determining its decrease in time. In river ecosystems, decreases in dissolved oxygen along the vertical profile correlates with microbial respiration, interstitial flow, and water residence time (Brunke and Gonser 1997; Fischer et al. 2005). Indeed, oxygen plays an important role in microbial metabolism and diversity (Brune et al. 2000; Rubol et al. 2013). In our study, a significant reduction in the degradation of organic nitrogen and phosphorus compounds was found under anoxic conditions, whereas no polysaccharide degradation reduction was detected (Fig. 4). This result suggests that inactivation rates of the hydrolytic enzymes vary for different enzymes (Goel et al. 1998; Freeman et al. 2004). Christy et al. (2014) reported that during anaerobic and aerobic decomposition, polysaccharides are hydrolysed by secreted enzymes, such as cellulase and cellobiase, and cellulose-hydrolysing enzymes, including β-glucosidase, can be released under different oxygen conditions. In contrast, hydrolysis of organic phosphorus compounds was inhibited by anoxic conditions. The differential effects of anoxia on extracellular enzyme activities at different depths affected the balance between carbon, nitrogen, and phosphorus

acquisition. Sinsabaugh et al. (2009) suggested that C:N:P activity ratios of 1:1:1 indicate equilibrium between organic matter composition, nutrient availability, and microbial metabolism. Specifically, extracellular enzyme activities and carbon, nitrogen, and phosphorus acquisition might be correlated with water and sediment chemistries (Hill et al. 2012). In our study, oxic conditions led to greater degradation of phosphorus compounds compared to carbon and nitrogen over time. Although the extracellular enzymes measured do not account for the degradation of all C, N and P available organic compounds, these enzymes describe the last steps of the decomposition process for a broad range of organic compounds. This wide-ranging justify the comparison of different ratios to the potential nutrient imbalances and needs of microbial community present inside the tank. In these sense, equilibrium was observed between C:N acquiring enzymes, but it remained imbalanced for C:P and N:P acquiring enzymes (Fig. 5). These data suggest that the sediment community inside the tank was phosphorus limited, especially at the end of the experiment, as we observed a reduction of available inorganic phosphorus in interstitial water. Phosphorus limitation in sediment may affect bacterial growth rates and microbial nutrient assimilation (Sinsabaugh et al. 2011); similar imbalances in sediments were recently reported by Hill et al. (2012) and Romaní et al. (2013). However, due to inhibition of phosphatase activity under anoxic conditions, the equilibrium between phosphorus-acquiring enzymes and carbon- and nitrogen-acquiring enzymes was re-established (Fig. 5). Reduction of phosphatase activity in deep anoxic sediment was also reported by Steenbergh et al. (2011), who suggested the presence of lower biological phosphorus retention efficiency under anoxic conditions in Baltic Sea sediments.

In our study, a different functional fingerprint was obtained for communities incubated in oxic and anoxic conditions at 50 cm (Fig. 7b) where carboxylic acids and amino acids were used preferentially under anoxic conditions. Tiquia (2011) reported that low oxygen conditions promoted the use of carboxylic acids and amino acids in an urbanized river. In our sediment tank, distinct metabolic fingerprints were observed due to depth and anoxia occurred gradually over time; e.g., results for day 14 in anoxia were still similar to those found under oxic

conditions (Fig. 7b). The gradual change in oxygen conditions suggests that both aerobic and anaerobic processes may have taken place simultaneously. Indeed, nitrification and denitrification processes might also have occurred with time, as shown by NH_4^+ consumption and NO_3^- production in the first 20 cm of the sediment tank. Toward the bottom of the tank, NO_3^- was consumed and no ammonium was present, suggesting that nitrogen had to be acquired from complex nitrogen compounds. These data hints the spatially coexistence of nitrification and denitrification in the sediment profile, already reported in marine sediments (Bonin et al. 1998). The gradual change in the metabolic fingerprint with increasing depth may be related to changes in the quality of the available organic matter and changes in metabolic processes that occurred due to depleted oxygen concentrations. However, although not measured in this study, changes in the composition of bacterial communities through the sediment tank may also have occurred. Adaptation of the communities to anoxic conditions was shown by the presence of active bacteria at all depths, including the transition zone from oxic to anoxic conditions. For instance facultative bacteria, capable to live in sediments with fluctuating oxygen concentrations, may have colonized the tank. Indeed, microorganisms responsible for oxidation of organic matter are not only aerobic bacteria (Brune et al. 2000). In areas of low oxygen, components of anaerobic respiration (e.g., nitrifiers, sulphate reducers and methanogenic bacteria) can metabolize organic carbon (Kristensen et al. 1995; Storey et al. 1999). Maintenance of live bacteria at monitored depths was shown by similarities in kinetic parameters (a, b, and x_0) under oxic and anoxic conditions. These data indicate that aerobic and anaerobic communities metabolized substrates in the plate with similar velocities, suggesting that the microbial communities adapted to the environmental conditions after the lag phase (Kristensen et al. 1995). In this context, it is known that oxic and anoxic bacteria can hydrolyse particulate material or to mineralize dissolved organic matter equally fast in sea sediments (Hulthe et al. 1998).

Our experiments revealed higher heterogeneity between replicates at greater depths and under anoxic conditions, especially at the end of the experiment,

indicating larger spatial heterogeneity combined with lower functional richness and diversity. Functional heterogeneity may be linked to physicochemical conditions in sediments, which also appear to have high spatial and temporal heterogeneity at greater depths (Storey et al. 1999).

Functional approaches, including measurements of extracellular enzyme activities and Biolog EcoPlate incubations, provided complementary information on the microbial community in the sediment tank. Previous authors analysed results from extracellular enzymes and Biolog EcoPlates, but found no direct correlations for bacterioplankton (Sinsabaugh and Foreman 2001) and salt marsh sediments (Costa et al. 2007b), and slight correlations in river biofilms (Ylla et al. 2014). These studies suggest that extracellular enzyme activities reflect the inherent activity of the resident community, whereas Biolog EcoPlates assess the potential functional diversity of microbial communities. In our study, extracellular enzyme activities showed larger differences over time compared to Biolog EcoPlates, which were more sensitive to spatial differences. Altogether, these data indicate that while biogeochemical processes changed over time, the functional diversity characteristics changed with depth.

The laboratory experiment used allowed the simulation and monitoring of sediment conditions subject to continuous infiltration at the meso-scale study. Although extrapolation of these results to natural aquatic ecosystems must be done with caution, meso-scale studies can be seen as a first step to better understand the biogeochemical processes occurring at the hyporheic zone and artificial recharge facilities. The transferability of these results at larger scales should be through a directly field experiment, as a solution to validate the laboratory experiment, simulating the same conditions in the field, by using fine scale hydrology tools (such as the monitoring of hydraulic conductivity by temperature sensors) and intensive and extensive core sampling and interstitial water sampling for microbiological and biogeochemical analysis.

CONCLUSIONS

We conclude that the microbial community showed different abilities to degrade organic matter at different sediment depths. Greater decomposition of carbon compounds occurred in surface sediments, and greater use of nitrogen compounds occurred at greater depths. Under anoxic conditions at increased depths, phosphatase activity was inhibited, limiting phosphorus availability. Milder effects of anoxia were found for peptidase activity, and glucosidase activity was not affected. Coexistence of aerobic and anaerobic communities, promoted by greater physicochemical heterogeneity, was also observed in deeper sediments. Bacteria (including living bacteria) occurred at all sediment depths and were able to adapt to different oxygen concentrations. These factors may affect the biogeochemical potential of deep sediment tanks for water purification processes.

CHAPTER 4

WARMER NIGHT-TIME TEMPERATURE PROMOTES MICROBIAL HETEROTROPHIC ACTIVITY AND MODIFIES STREAM SEDIMENT COMMUNITY

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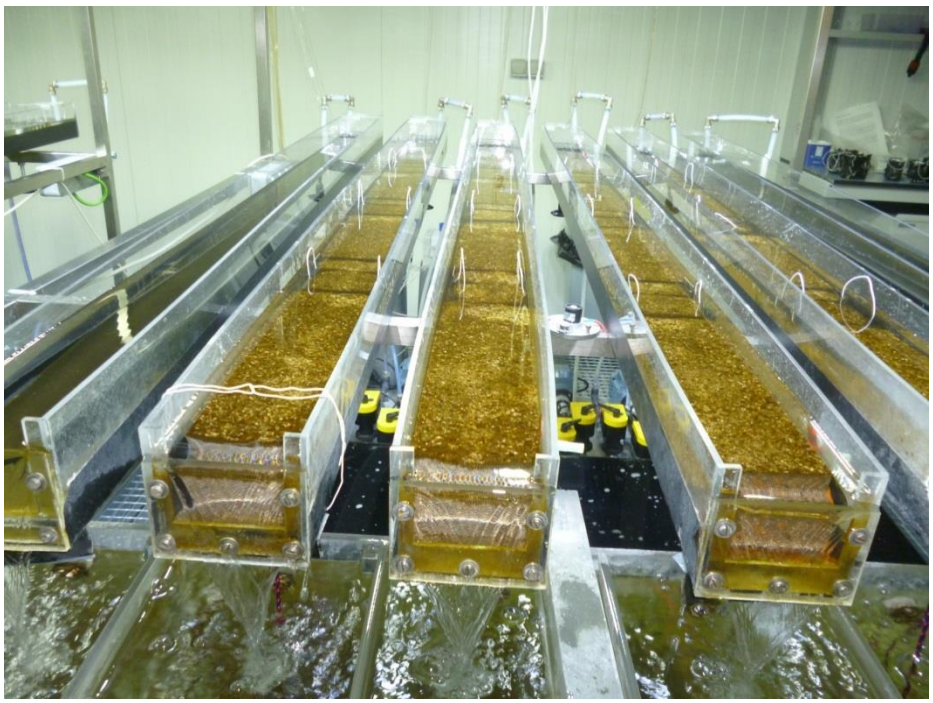
In prep.



ABSTRACT

The diel temperature patterns are changing because of global warming, with an overall increase of temperature, which is expected to be more pronounced during the night. Because of the temperature dependence of biological reactions, global warming might alter biofilm structure and functioning. Our objective was to study the effect of warming and alteration of the diel temperature patterns on the river sediment biofilm structure and functioning tied to carbon metabolism. We performed a 5 weeks experiment with 12 artificial streams submitted to three different diel temperature patterns: warm-days, warm-nights, and control.

Regarding warming treatments, our results showed that neither biofilm function nor biofilm structure were affected by warming on the short term (~24 h), but on the long term (2-5 weeks). Biofilm functioning was affected by warming mainly for the warm-nights treatment which stimulated β-Glucosidase activity and reduced phosphatase activity. A clear opposite diel pattern was observed for DOC and β-Glucosidase activity, suggesting that sediment bacteria may quickly consume during night-time the input of photosynthetic DOM labile compounds created during light-time. Furthermore, the biofilm structure was also altered by warming, as both warm-days and warm-nights treatments enhanced rotifer and copepod abundances, which in turn controlled the bacterial, algal and ciliate communities. Overall we conclude that warming river water temperature might drastically change river sediment biofilm structure and functioning which might consequently affect in higher trophic levels. However the alteration of the diel temperature patterns (mainly due to warm-night temperatures) alters only the functioning of river sediment communities, enhancing the heterotrophic activities.

CHAPTER 5

MICROBIAL SEDIMENT COMMUNITIES RELY ON DIFFERENT DISSOLVED ORGANIC MATTER SOURCES ALONG A MEDITERRANEAN RIVER CONTINUUM

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Abstract

Heterotrophic bacteria play a key role in the degradation of organic matter and carbon cycling in river sediments. These bacterial communities are directly influenced by environmental factors that differ spatially and temporally in rivers. Here we studied the longitudinal patterns in sediment bacterial community composition and dissolved organic matter utilization under base flow and drought conditions in a Mediterranean river. Our results indicated that sediment microbial communities are affected by dissolved organic matter quality and origin along the river continuum. In headwaters the potential degradation of cellulose and hemicellulose dominated (i.e. higher β-glucosidase and β-xylosidase), suggesting higher microbial utilization of allochthonous detritus from terrestrial origin. On the other hand, the accumulation and transport of recalcitrant compounds (i.e. decrease on the recalcitrant index) became potentially relevant downstream. Furthermore, the seasonal variability had clear effects on bacterial community composition and function. The hydrological fragmentation of the river continuum during drought period generated sediment microhabitats dominated by gamma and delta-Proteobacteria, with a high potential capacity to degrade a wide range of compounds, particularly nitrogen containing moieties. During the base flow conditions, a higher occurrence of alpha-Proteobacteria and a higher potential use of allochthonous and more recalcitrant organic carbon compounds were observed. Overall, our findings highlight an upstream-downstream longitudinal transition of microbial river sediment communities that rely on allochthonous to autochthonous dissolved organic matter; and a shift towards autochthonous dissolved organic matter reliance during drought.

GENERAL DISCUSSION

1. Function and structure of river sediment microbial communities clearly change at different temporal and spatial scales
   - 1.1 Spatial variability
   - 1.2 Temporal variability
2. The impact of climate change on structure and functioning of river sediment biofilm communities.
3. Experimental and field observations: learning from this thesis
4. Methodological limitations and trends

One of the greatest challenges faced by aquatic ecologist researchers is the ability to detect the key environmental drivers that modify the structure and functioning of river ecosystems. Particularly, many studies observed that changes in water temperature, nutrient concentration, dissolved organic matter quantity and composition or flow influenced on the functioning and composition of river biofilm (Kirchman et al. 2004; Olapade and Leff 2005; Zoppini et al. 2010; Romaní et al. 2013; Ylla et al. 2014). These environmental factors vary spatially and temporally in river systems naturally or due to anthropogenic activities. Actually, Mediterranean streams are highly affected by human activities (e.g. increase in pollutants concentration in water) as well as by climate change (e.g. increase in water temperature). Understanding how the function and structure of biofilm communities linked to environmental drivers change temporally and spatially could significantly contribute to our knowledge on Mediterranean rivers functioning.

The main objectives of this thesis were to study the changes in the biofilm structure and dissolved organic matter utilization in river sediments at different spatial and temporal scales, and to determine main environmental drivers that modify these microbial communities.

Results from this thesis have shown different temporal and spatial responses for each parameter analysed in river sediment biofilm. Overall, a clear **longitudinal pattern** (upstream to downstream of the river) in microbial organic matter degradation and biofilm structure was observed in river sediments linked to the quality of organic matter inputs (*Chapter 5*) or due to a pollution gradient (*Chapter 2*). In addition, differences in microbial organic matter degradation were observed between surface sediments and deep sediments (50 cm) in a laboratory experiment and this was linked to depletion of oxygen availability in depth (**vertical-scale**, river sediment depth) (*Chapter 3*). On the other hand, **seasonality** (especially due to differences in flow conditions) modified river sediment functioning and bacterial communities were distinguished between base flow and drought conditions (*Chapter 5*). Also, at the short temporal scale, clear **daily patterns** were observed mainly for the decomposition of labile compounds due to rapid carbon microbial degradation which determined clear differences between

day-time and night-time carbon metabolism (*Chapter 4*). This chapter also showed that in a context of climate change, a small increase of the temperature regime could dramatically affect biofilm community composition and carbon metabolism. In this general discussion the results obtained from the different chapters are combined and the variability of several parameters at different temporal and spatial scales are compared. This scaling comparison allows going a step further on the knowledge of temporal and spatial variability in river sediment microbial communities.

1. Function and structure of river sediment microbial communities clearly change at different temporal and spatial scales

Many researchers have identified ecological scale as a principal concern in aquatic ecology research (Habersack 2000; Schneider 2001). The study of temporal and spatial variability in river ecosystems has a great importance for understanding the heterogeneity and complexity of microbial communities. The view of river systems as a hierarchically organized system, requires considering different **spatial scales** to examine the patterns and processes in rivers (Frissell et al. 1986; Boulton et al. 1998). Biotic and abiotic processes and patterns in rivers can be studied at a large scale including the whole river basin (i.e. longitudinal pattern from headwaters to the mouth of the river) at the reach scale (typically extending between upstream and downstream tributary juncture) or at the habitat or microhabitat scale (e.g. including the biofilm community composition developing at different substrata or differences between surface, subsurface or depth sediments). For instance, differences in flow velocity are observed at a small spatial scale (cm and m) whereas differences in water temperature generally occur at a larger scale (km) (Dorigo et al. 2009). Changes at the microhabitat scale could have great impact at the ecosystem level (Frissell et al. 1986).

On the other hand, the relevance of **temporal variability** in microbial communities is well known. Microorganisms have a rapid growth and short generation times that implies rapid changes after environmental disturbances

(Habersack 2000). For instance, the accumulation of bacterial cells or induction of extracellular enzymes could take hours to days and slowest shifts in bacterial community composition can range between 10's of hours to many days (Findlay et al. 2003). The temporal dimension can be studied at diel or seasonal cycles (which includes hydrological variability, very relevant in Mediterranean rivers).

Here, results of spatial and temporal variability observed from the thesis are discussed independently, although the interaction between space and time are also considered.

1.1 Spatial variability

Rivers transport, storage and utilize dissolved organic carbon (DOC) from terrestrial and freshwater ecosystems to the marine systems. During the transport, DOC can be assimilated by bacteria (assisted by extracellular enzymes) and, depending on changes in DOM quantity and quality, this microbial metabolism may be modified along the river length (Romaní et al. 2012). The results from *Chapter 2 and 5* of this thesis showed a clear **longitudinal pattern** for microbial organic matter decomposition separating mainly headwaters and lower river reaches. In this way, previous studies showed how metabolic processes (P:R, Production: Respiration) vary along the river where heterotrophic metabolism was dominating in upstream sites while metabolism became autotrophic downstream (McTammany et al. 2003; Maranger et al. 2005) but very few studies revealed differences in biofilm organic matter degradation along the flow path. In this thesis, potentially higher utilization of cellulose and hemicelluloses (β-Glucosidase and β-Xylosidase activities) were observed in headwaters might be related to the high DOM inputs of allochthonous origin (terrestrial inputs) in the Tordera river. Similarly at the upstream site of the Llobregat river, D-Cellobiose, carbohydrate subunit of cellulose, was the substrate most used by microbial communities incubated in Biolog Ecoplates, which might be linked to the availability of similar substrates (such as leaves or wood) present at this site. Related to biofilm structure, bacterial density were higher in upper reaches than in lower reaches in Llobregat river and this was also occurring in la Tordera river during base flow conditions. This might be because bacterial abundances are higher in biofilms

grown under higher flow velocities (typical in upstream sites) in comparison to biofilms grown under slower velocities located in downstream sites (Battin et al. 2003). Actually, water flow variability modifies bacterial abundances and selects the bacteria species adapted to these fluctuating hydrological condicions (Artigas et al. 2012; Fazi et al. 2013). In addition, a more stable and cohesive biofilm (observed by higher extracellular polymeric substances (EPS)) were observed upstream of Llobregat river. Biofilms have a large content of EPS produced by bacteria, algae and meiofauna. It is reported that the EPS matrix contributes to the river sediment stabilization and cohesion that could favour the resistance of these communities after disturbances (Gerbersdorf et al. 2008). In contrast, at the downstream sites of Llobregat river the biofilm EPS matrix was thinner, suggesting a microbial community more susceptible to possible disturbances. At these lower reaches, higher potential use of more recalcitrant and not easy to microbial degradation compounds such as lignin (as shown by higher phenoloxidase activity) was detected in la Tordera River (Sinsabaugh et al. 1994). Similarly, at the downstream sites of the Llobregat river, a higher utilization of complex compounds such as polymers (from synthetic origin used in pharmaceutics) were also observed which might be linked to the presence of pharmaceutical compounds detected at these sites (Muñoz et al. 2009). These findings show a longitudinal gradient of microbial organic matter utilization from headwater to lower reaches where downstream sites communities might be more sensitive and fragile to disturbances due to being more affected by human activities.

Nevertheless, these results are from a **steady point of view** (snapshot) without considering the temporal variability in river sediments along the year. Microbial communities in rivers are dynamic in time due to rapid life cycles and temporal changes of environmental variables. In this sense, differences in river longitudinal patterns were observed depending on the sampling time as it was observed for different hydrological periods (base flow, drought or flood conditions) (Fig. 1). β-Glucosidase activity (GLU) showed higher variability along the flow path during base flow and drought conditions whereas few differences were observed during flood period. These results indicated large upstream-downstream differences in microbial activity during drought and base flow periods but a drastic reduction in spatial variability and homogenization through the flow path during flood.

Interestingly, opposite results were observed for DOC where higher spatial variability along the main stem was observed during the flood period. At high flow events, the transport of solutes and particulate organic matter clearly increases downstream (Artigas et al. 2009) and a positive correlation between DOC and discharge have been widely reported (Butturini et al. 2008). DOC concentration rose during flooding due to the flushing and runoff in the watershed and the riparian zone that increases the terrestrial material (particulate and dissolved compounds from leaves and wood) that enters in the river system. In addition, DOC composition during flood events is characterized by an increase in high molecular weight compounds containing a large fraction of aromatics and lignin (Vidon et al. 2008) which are compounds difficult to microbial degradation. This could be the reason for the low mean GLU activity observed for this sampling period in comparison to other periods. On the other hand, when comparing the different studied river systems, DOC concentration was higher in Llobregat than Tordera river at base flow, which might be because several inputs of industrial, agricultural and urban wastewaters were entering from middle-lower part of the Llobregat river constantly increasing the DOC concentration in river waters (González et al. 2012). For bacterial density in river sediments, similar to GLU, the lowest spatial variability and lowest value was observed for the flood period (Fig. 1). Probably the flood removed the bacterial cells and caused a sustainable biofilm abrasion through sediment movement (Biggs et al. 1999; Augspurger and Küsel 2010).

On smaller spatial scales, **vertical differences** in sediment microbial organic matter degradation capacity were also observed (Fig. 1). These differences were linked to the depletion of oxygen availability in deep sediments, comparable with other observations in marine sediments (Glud et al. 2005). Results from the sediment tank experiment showed that surface sediments were colonized by bacteria capable of using a wider range of substrates mainly related to the decomposition of carbon compounds while at depth of 50 cm microbial community was specialized in using fewer carbon substrates, reducing their functional diversity (*Chapter 3*). Results from day 50 and day 80 from the sediment tank experiment showed that higher bacterial density was observed at surface sediment

whereas it decreased with depth, similar to the findings from other studies (Franken et al. 2001; Taylor et al. 2002) where the decrease in bacterial abundance was correlated with the decrease in organic matter concentration (Fischer et al. 2002).

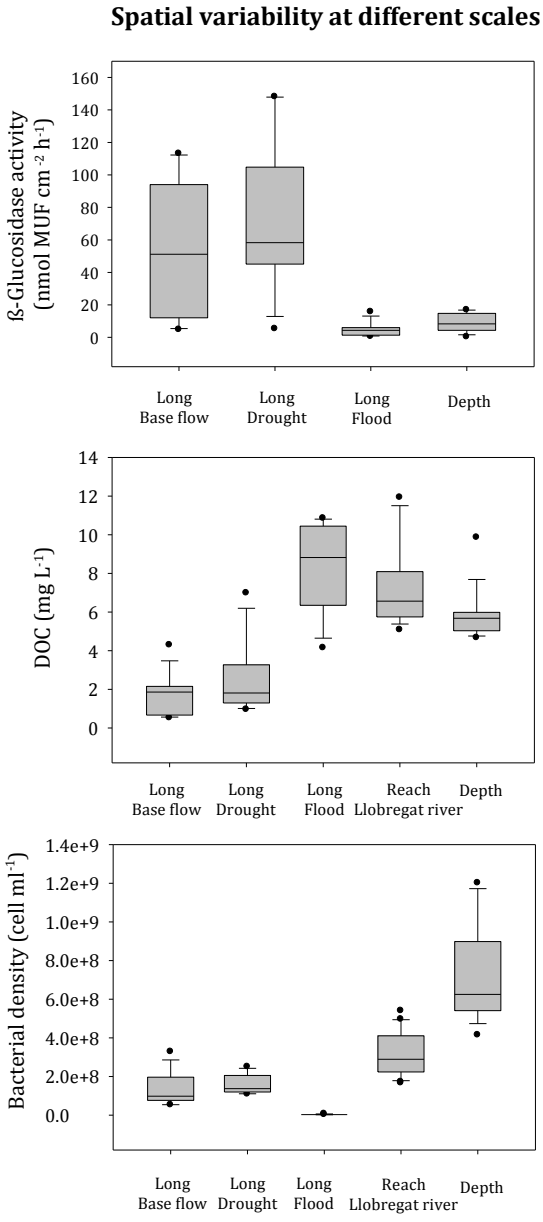

Figure 1. Box plot of spatial variablity at different scales of β-Glucosidase activity, dissolved organic carbon (DOC) and bacterial density from different chapters data. Longitudinal (**Long**) data are mean values from Tordera river including base flow, drought and flood (data from flood were not included in the chapter), n=45 (*Chapter 5*). **Reach** data are from the 5 sampling sites of Llobregat river, n=25 (*Chapter 2*). **Depth** data are mean values from day 50 and 80 of laboratory experiment including 3 different depths (0, 20 and 50cm) n = 18 (*Chapter 3*).

β-glucosidase and DOC did not show much variability at the vertical-scale (Depth data) in comparison to the observed longitudinal variability (Long and Reach data) that clearly modifies their activity and concentration along the Tordera river main stem (Fig. 1). Interestingly, variability in bacterial density was stronger at the vertical-scale (Depth) than longitudinal-scale along the river (Fig. 1).

1.2 Temporal variability

In Mediterranean rivers, water discharge has large fluctuations from basal conditions to drought and floods. These hydrologic oscillations have an influence on the quantity and quality of dissolved organic matter transported by river water, which may affect biofilm microbial metabolism (Ylla et al. 2010; Vazquez et al. 2011) and bacterial composition (Marxsen et al. 2010). Results from this thesis show that during drought periods the fragmentation of river water continuum stimulated the formation of microhabitats with higher bacterial functional diversity and heterogeneity between sites. In addition, changes in the main subclasses of bacteria were observed, where higher gamma- and delta-Proteobacteria abundances were reported during drought conditions whereas alpha-Proteobacteria were dominating in base flow conditions (*Chapter 5*).

On the other hand, results from *Chapter 4* showed a clear diel pattern for β-Glucosidase activity peaking at night linked with DOC reduction. The daily-time scale variability in microbial activity particularly affected the easy and faster to degrade compounds, increasing the relevance for any experimental design for the control of the sampling hour when microbial activity patterns are analysed. Interestingly, daily changes in GLU activity were around 27% of increase from day-time to night-time, and this was similar to the observed variability between hydrological periods at downstream sites (Fig. 2). However, at headwater sites, greater fluctuation between hydrological periods was observed. As noted in the previous subsection, GLU activity might be related to the availability of allochthonous inputs linked to higher presence of riparian forest at headwaters, that are in fact, subjected to temporal variability. For instance, in autumn increase of plant material inputs (due to leaf litter fall) in streams stimulating the

productivity of GLU activity (Romaní et al. 2013). In case of DOC, low variability changes in daily time-scale was observed in comparison to that observed for the different hydrological periods (Fig. 2). DOC fluctuation depends on the allochthonous and autochthonous material inputs throughout the year, but it is mainly during flood events when large quantities of organic material are transported downstream (Fig. 1).

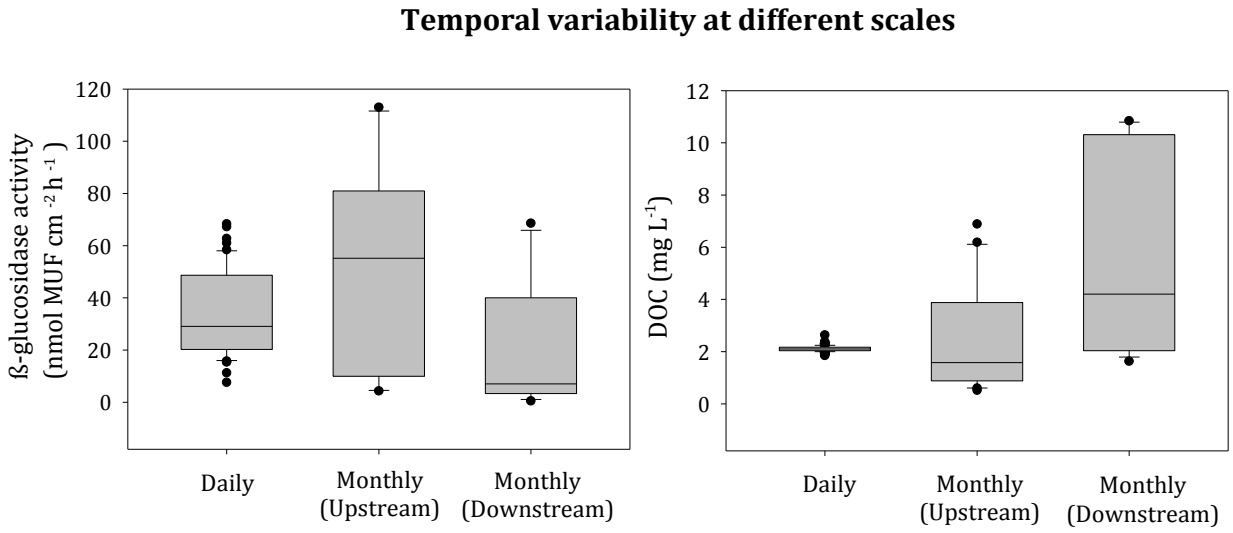

Figure 2. Box plot of temporal variablity at different time scales of β-Glucosidase activity and dissolved organic carbon (DOC) with data from different chapters. Monthly data refers to different hydrological periods. Data are mean values from Tordera river including base flow, drought and flood conditions (data for flood were not included in chapter 5) from sites T1-T5 (Upstream sites) and T12-T15 (Dowstream sites) n=18 (*Chapter 5*). Daily data are from artificial streams including data for all sampling hours at day 14 and 35 for control treatment, n= 64 (*Chapter 4*).

Traditional sampling approaches implicitly assume that biological processes occur in a steady state or that changes are minimum in short-time scales. Nevertheless, results from this thesis highlight the importance to take into account the hour of sampling for microbial activities, especially for those involved in the degradation of labile compounds, while other parameters variation such as DOC show largest variations at the larger time scales (such as season/flow periods). Thus, depending on the objective of the study, the different sampling strategies (spatial or temporal scale) have to be properly designed.

2. The impact of climate change on structure and functioning of river sediment biofilm communities

Global climate change scenarios predict a higher frequency and intensity of occurrence of floods and droughts in Mediterranean rivers, as well as an increase in the stream water temperature of 2.2 - 4.3 ºC by 2100 (IPCC, 2013). Numerous studies have been published on the effects of warming on structure and functioning of microbial communities in freshwater ecosystems (reviewed in Romaní et al. 2015).

Results from *Chapter 4* of this thesis showed that microbial organic matter degradation of sediment biofilm was affected by warming, promoting β-Glucosidase activity and reducing phosphatase activity. The different sensitivity to temperature of extracellular enzyme activities has been demonstrated also in other studies (Wallenstein et al. 2009; Ylla et al. 2014). On the other hand, warming considerably changed the structure of river sediment biofilm communities modifying trophic interaction between biotic groups inside of the biofilm. Particularly, warming stimulated the abundance of rotifers and copepods that at the same time may regulate the development of ciliates, bacteria and algae, creating a clear distinct biofilm community respect to the control treatment. These results were not expected because most of studies conclude that warming stimulates biofilm biomass, which was not very clear in our study, however very few of them were focusing on the biofilm trophic interactions. Thus, the abundance of different groups of microbes and meiofauna are controlled by interactions between the different species through consumption and competition for food (Hakenkamp and Morin 2000). These results point out that expected increases of temperature due to climate change could have large consequences not only on carbon metabolism but also on interactions in the biofilm communities affecting the whole food web.

3. Experimental and field observations: learnings from the thesis

In this thesis, in order to study de complexity of microbial communities in river sediments, field observations and laboratory experiments have been combined. Laboratory experiments are very popular, in part due to the degree of experimental control that they offer. In *Chapter 3* a meso-scale experiment was chosen to study biogeochemical and microbial parameters under controlled flow conditions using a sediment tank, similar to those experimental studies using sediment columns (Battin et al. 1999; Mermillod-Blondin et al. 2005) or also a sediment tank (Weber and Legge 2011). On the other hand, in *Chapter 4*, artificial streams were used, to study carbon metabolism and biofilm community structure after warming in a controlled temperature system. The advantages of using artificial channels are the replicability and the ability to control and simulate the conditions of the river systems. Actually, the purpose of laboratory experiments is to simplify nature in order to more easily understand ecological processes and patterns (Jessup et al. 2004). Contrary, the disadvantages of the laboratory experiments are to be overly simplified, contrived and too small and short in spatial and temporal scales and fundamentally different from natural systems (Table 1).

Table 1. Summary of the main advantages and disadvantages of using field observations or laboratory experiments.

	Advantages	**Disadvantages**
Field observations	Reproduce the natural complexity	Difficult to explore ecological patterns Difficult to answer ecological questions
Laboratory experiments	Experimental control, Simplified to better understand, Answer ecological questions Replicability	Simplified, Contrived, Too small spatial scale, Too short temporal scale, Not reproduce natural systems

However, the laboratory experiments are useful to answer ecological questions that can be difficult to address using field observations. Particularly, laboratory experiments are orders of magnitude larger than the microorganisms they contain, making it possible to explore ecological patterns occurring at several spatial scales (Jessup et al. 2004). In this sense, as in *Chapter 4*, laboratory experiments are largely used to investigate the effects of simulated climate change (temperature increased) on biofilm communities (Petchey et al. 1999; Romaní et al. 2014).

Despite this, the extrapolation and interpretation of experimental results to natural aquatic ecosystems must be done with caution. Meso-scale studies are the first step to better understand the ecological processes occurring at river systems, but also a field experiment should be necessary as a solution to validate the laboratory experiment. On the contrary, the large scale field studies (with a lot of data) provide real results from river ecosystem, as shown from the Tordera river study. From field studies it is probably more difficult to obtain clear unidirectional conclusions due to large interactions between factors. However, after proper data analysis, conclusions from these studies include the real environmental variability in contrast to those obtained in the laboratory experiments.

In this way, results from the different chapters of this thesis of functional diversity measured by the Biolog EcoPlates, obtained from the field and laboratory experiments were analysed together for comparison (Fig. 3). Field observations (Llobregat and Tordera rivers) were compared to the laboratory experiment performed in a sediment tank. Results showed a clear different fingerprint in carbon substrates utilization, showing clear spatial and temporal differences between studies. First, significant differences between rivers was observed (ANOSIM pairwaise-test, Llobregat versus Tordera, $p<0.01$). Llobregat river, the most polluted river, showed higher heterogeneity between sampling sites whereas Tordera river exhibited clear different responses between hydrological periods. Second, in case of laboratory experiment, almost no heterogeneity between samples was observed, although samples represented the 80 days of experiment. Thus, environmental heterogeneity among samples was stronger in the two field studies compared to the laboratory experiment. This interesting result suggested

that the experiments represent only a fraction of the variability of microbial communities in the field, showing a more similar functional diversity between samples, whereas results from field observations probably reflect far more the complexity and diversity of carbon substrate utilization of these microbial communities in the environment.

Finally, significant differences between the laboratory experiment and Tordera river samples were shown (ANOSIM pairwaise-test, Lab versus Tordera, $p<0.01$) whereas no significant differences was observed with Llobregat. In addition, these results from Biolog EcoPlates strengthen the conclusions of *Chapter 1* defining the technique as a good tool to analyse functional diversity for carbon substrate utilization in river sediment samples, allowing the comparison between spatial and temporal scales and field and laboratory experiments.

Figure 3. NMDS plot using normalized data from Biolog EcoPlates at 96 h after Bray Curtis dissimilarity. Data from la Tordera river integrates data from drought and base flow events for all sampling sites (n=78). Llobregat river data are from all sampling sites (n=25). Laboratory experiment are oxygenic data from surface sediment (0 cm) for all the sampling days (n=15).

4. Methodological limitations and trends

The main limitations of the methods used in the different chapters of this thesis are discussed below. In the case of biofilm functioning, the measurement of extracellular enzymes and Biolog plates are a result of incubations under substrate saturation and enrichments, respectively. For this reason the results obtained are the potential measurements of these activities or carbon substrates use which can be far from the direct activity observed in the ecosystem. Thus, the ecological interpretation of obtained results has to be meticulous. In the case of the Biolog Ecoplate method, the method limitations and ecological interpretation of data were extensively discussed in *Chapter 1*. In the case of extracellular enzyme activities the results of this thesis were obtained through the utilization of artificial fluoresencent substrates. The measurements were based on an incubation of the natural sample with an artificial fluorescent substrate (e.g., methylumbelliferone, MUF, or aminomehtyl coumarin, AMC) that was linked to the substrate that the enzyme was able to hydrolyse. It is reported that the fluorescence measurement after a short incubation is directly related to the proportion of artificial substrate molecules cleaved by the natural enzyme activity (Romaní et al. 2012). Using this approach, the main limitation is that we are detecting the potential enzyme activity of that microbial community (because of the artificial substrate is added at saturating concentration). In many cases obtained values are an overestimation of the real activity since available natural substrate in the environment exists in lower concentrations. However, since all enzymes are potentially measured, it is then feasible to compare the distinct capacities of microbial communities in relation to their potential capability to degrade compounds from different origins or sources. An advantage of extracellular enzyme activities measurements is that they are measured with the fresh samples and incubations are short, so, better reflecting potential degrading capacities and potential key role of the community on biogeochemical cycles than other longer incubation techniques.

On the other hand, in case of biofilm structure, in this thesis the abundance of different biofilm groups have been analysed (bacteria, algae and meiofauna) as well as the major phylogenetic groups of bacterial community composition using CARD-FISH technique. In case of measurement of bacterial density different

approaches were used such as the utilization of epifluorescence microscopy (*Chapters 2-3*) and flow cytometer (*Chapters 4-5*) using fluorescence stains. Although the two methods are somewhat similar in the results they yield, there are differences. The traditional microscopic techniques consist on a direct counting that requires time that do not lend themselves to large-scale studies (Gasol and Del Giorgio 2000). On the other hand, the use of flow cytometer significantly reduces the time employed with similar level of resolution and precision than epifluorescence microscopy, and allows to quantify physical and biochemical aspects of individuals cells (such as cell size)(Monfort and Baleux 1992). However, in the case of river sediment samples an efficient detachment and previous purification process is required in order to quantify bacterial cell abundances (Amalfitano and Fazi 2008). However, using an appropriate protocol, the results of this thesis have proved that the flow cytometer is more useful than epifluorescence microscopy to analyse bacterial abundances in river sediments.

On the other hand by using fluorescence in situ hybridization techniques (e.g. CARD– FISH), the bacterial community composition can be identified without requiring culture methods. Nevertheless, there are some problems in the application of this technique (Kubota 2013). One of the main limitations is that CARD-FISH uses group-specific probes to hybridize cells that could underestimate the final results because of no complete coverage of all species within a specific group (Amann and Fuchs 2008). Particularly, the probe mixture Eubateria (EUB338 I–III) currently covers 94% of bacterial entries, but misses a few deep-branching groups, such as the Aquificales (Pernthaler et al. 2002; Amann and Fuchs 2008). Furthermore, in this thesis bacterial structure is not exhaustively described since no sequencing data is provided. However CARD-FISH approach detects large taxonomic groups (such as classes of proteobacteria) that facilitates and initial assessments of the most abundant groups of bacteria and allow to be studied in their natural environment (Amann and Fuchs 2008).

Finally, nowadays the trends for studying bacterial communities in greater detail are the use of next-generation sequencing techniques. The extremely high diversity of microbial communities in rivers is less commonly studied than in marine and

lake ecosystem (Zinger et al. 2012) and usually is treated as a black box in many models. However, recently **the black box has been opened** due to the rapidly accessibility of molecular tools. Currenlty, the application of next-generation sequencing techniques, or high throughput sequencing (e.g. 454 pyrosequencing, Ilumina MiSeq) at higher level of detail, have increased the rate of data generation in comparison to cloning or sequencing techniques largely used in last decades (such as ARISA, T-RFLP, DGGE, CARD-FISH) (Maclean et al. 2009). Furthermore recently many studies are addressed to study the metagenomics (MacLean et al. 2009), that sequenced total DNA from a microbial population. However, the development of these techniques requires constant improvements of bioinformatics due to the enormous datasets and continuously advances in microbial diversity detection. Overall, these revolutionary new techniques are a great opportunity for aquatic ecologists to better understand the microbial diversity and its link to microbial activities and ecosystem processes.

GENERAL CONCLUSIONS

Chapter 1. Review of Biolog EcoPlates technique as a tool to study carbon substrate utilization in environmental microbial communities

1. When studying microbial functional diversity using Biolog Ecoplates technique, the utilization of the same protocol, the standardization of the data treatment, and a correct interpretation of results is required.
2. Biolog EcoPlates are useful for studying carbon substrate utilization fingerprints but represent the part of the bacterial community which is metabolically active and able to grow in plate conditions.
3. Biolog EcoPlates are a good tool to compare the carbon substrate utilization profiles at different spatial and temporal scales as well as field and laboratory experiments using environmental samples.
4. This technique is not appropriate to characterize in-situ sampling sites by they own and need to be complemented by other functional and structural approaches.

Chapter 2. Shifts in carbon substrate utilization in sediment microbial communities along the Llobregat river

1. Differences in sediment microbial biofilm structure and metabolism along the Llobregat river were observed linked to a pollution gradient.
2. The biofilm community at the **upstream** site was the most active (highest respiratory activity and abundance of living bacteria) and thickest (highest EPS content). On the contrary, biofilm at **downstream** sites showed a homogenous functional fingerprint and the potential capacity to use more complex carbon substrates such as polymers.
3. **Biolog EcoPlates** analysis should be performed with fresh river sediment samples. Preserving frozen samples reduce the functional diversity (Shannon diversity) and richness (number of positive substrates) showing higher sensitivity to frozen in most polluted sites.

Chapter 3. The effects of sediment depth and oxygen concentration on the use of organic matter: An experimental study using an infiltration sediment tank

1. Differences in microbial organic matter degradation were observed with depth. **Surface** sediments were colonized by bacteria capable of using a wide range of substrates, mainly related to decomposition of carbon compounds. **At a depth of 50 cm,** the community was specialized in using fewer carbon substrates and greater use of nitrogen compounds.
2. **Bacteria** (including live bacteria) occurred at all sediment depths and were adapted to fluctuations in oxygen concentrations.
3. Under **anoxic conditions** phosphatase activity was inhibited, limiting phosphorus availability. Milder effects of anoxia were found for peptidase activity, and β-Glucosidase activity was not affected.
4. Microbial communities were adapted to use organic matter under different oxygenic conditions at different depths.
5. The heterogeneity of oxygen concentrations with depth and over time clearly influence the degradation of organic matter in the sediment tank.

Chapter 4. Warmer night-time temperature promotes microbial heterotrophic activity and clearly modified river sediment community

1. Neither biofilm function nor biofilm structure were affected by warming on the short term (~24 h), but effects were detected on the long term (2-5 weeks).
2. Biofilm functioning was affected by warming mainly in the warming-night treatment which stimulated microbial heterotrophic community promoting degradation of labile compounds (such as β-Glucosidase activity).
3. A clear diel pattern was observed for β-Glucosidase activity peaking at night linked to the consumption of DOC by microbial communities.
4. The biofilm structure was altered by warming, as both warm-days and warm-nights treatments, enhancing rotifer and copepod abundances, which in turn control the bacterial, algal and ciliate communities.

5. Warming highly altered both the structure and functioning of river sediments which might affect carbon metabolism and higher trophic levels.

Chapter 5. Microbial sediment communities rely on different dissolved organic matter sources along a Mediterranean river continuum

1. DOM quality and origin varied at spatial and temporal scales, modifying bacterial community composition and functioning in la Tordera river sediments.
2. The terrestrial inputs in headwaters dictated the longitudinal pattern of microbial organic matter degradation along the river continuum. **In headwaters**, degradation of cellulose and hemicellulose dominated, linked to a higher input of allochthonous detritus from plant origin, whereas accumulation and degradation of recalcitrant compounds became significant **downstream**.
3. Different hydrological conditions clearly altered community composition and functioning. During **drought**, the fragmentation of the fluvial continuum stimulated the formation of microhabitats dominated by gamma and delta-Proteobacteria with higher functional heterogeneity between sites. In contrast, during **base flow**, greater use of carbon compounds and a higher relative abundance of alpha-proteobacteria were observed.

General Conclusions

1. We conclude that functiona and structure of river sediment biofilm clearly change at different temporal and spatial scales. And the sampling strategies need to be designed specifically for each type of variable.
2. Laboratory experiments represent only a fraction of the variability of microbial communities in the field whereas field observations show higher heterogeneity among samples, reflecting far more the complexity and diversity of microbial communities in the environment.

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