Universitat de Girona

STREAM BIOFILM RESPONSES TO FLOW INTERMITTENCY

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Dipòsit legal: Gi. 1799-2014
http://hdl.handle.net/10803/283569

PhD THESIS

STREAM BIOFILM RESPONSES TO FLOW INTERMITTENCY

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2014

Universitat de Girona
Institut d'Ecologia Aquàtica

TESI DOCTORAL

STREAM BIOFILM RESPONSES TO FLOW INTERMITTENCY

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2014

PROGRAMA DE DOCTRORAT
EN CIÈNCIES EXPERIMENTALS I SOSTENIBILITAT

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

Universitat de Girona
Institut d'Ecologia Aquàtica

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CERTIFIQUEN:

Que aquest treball titulat "Stream biofilm responses to flow intermittency", que presenta Xisca Timoner Amer, per a l'obtenció del títol de Doctora, ha estat realitzat sota la seva direcció i que compleix els requisits per poder optar a Menció Internacional.

Dr. Sergi Sabater i Cortés

Dr. Vicenç Acuña i Salazar

La doctoranda,
Xisca Timoner Amer

Girona, març 2014

A mumpare, mumare, es meu germà i en Jaume,

ACKNOWLEDGEMENTS

I was recipient of a Doctoral fellowship from the Spanish Ministry of science and technology (AP-2007-01945). Additional funds were also provided by the Spanish projects SCARCE (CONSOLIDER-INGENIO CDS2009-00065).

Sou molts/es els que d'alguna manera o un altra m'heu ajudat a arribar fins aquí! La llista s'ha anat fent llarga durant aquest camí, espero no deixar-me a ningú... Sort n'he tingut de tots vosaltres!

Primer de tot, Sergi i Vicenç us vull donar les gràcies per tot el que m'heu ensenyat, pel suport, la confiança i la feina feta durant tots aquests anys. La il·lusió amb què viviu la ciència m'ha transmès sempre curiositat i ganes de continuar!

Teresa, Carles i Dani, m'heu ajudat moltíssim i n'he après molt de vosaltres! Sense vosaltres aquesta Tesi no hauria sigut possible! Moltíssimes gràcies!

Gràcies també a tots els companys i amics de la Universitat i de l'ICRA... us hauria d'escriure milions de vegades gràcies!... Anna, Irene, Elisabet i Albert des del principi que m'heu donat suport, ensenyat i ajudat en tot, GRÀCIES! Carmen ja saps, optimitzar és la clau! ELA, ELA.... gràcies pel teu suport! Lorenzo, Juan, Susanna, Gemma, Natàlia, Berta, Chloé, Helena, Anna, Juanita, Gerard, Rosana, Roberto, Eli, Marta, Lydia, Maria, Rafa... tots m'heu fet costat! Olga, Marta, Sara i Àlex gràcies i no només pel vostre suport tècnic. Sara, Núri i Laura gràcies per ajudar-me amb el món de l'HPLC. Pau amb tu vam començar ben decidits del que volíem eh? sempre m'has fet veure les coses de manera positiva i amb entusiasme, tal com ets! I la resta de gent que sou a l'ICRA o n'heu format part durant tot aquest temps gràcies pel dia a dia que no és poc!

Javier! Te agradezco muchísimo tu ayuda en el trabajo de campo durante el tiempo que estuviste aquí con nosotros, fue intenso, pero nos lo pasmos bien!

Thanks Stuart to give me the opportunity to become one member of your department during three months and for all your support .Thanks Alisha, John, Laurisse, Peter, Carolyn, Ann, Iris, Lars, Alex, Simone, Virgilio and Migue for your help and specially to make me feel like home in the Down Under.

Carlos i Laura durant aquest temps m'heu fet costat, us aprecio moltíssim com a amics i com a científics! Carlos, les meves primeres passes en la recerca i en la docència van ser gràcies a tu, a més em vas ajudar a continuar i a decidir-me a fer el que més m'agrada!

Nines! Gràcies per ser-hi SEMPRE! Amb vosaltres no hi ha res millor com venir a Mallorca a carregar piles!

Lidia i Jordi gràcies pel vostre suport i ajuda.

Mumpare, mumare Miquel i Angelina, gràcies pel vostre suport incondicional, la vostra confiança i tranquil·litat. I sobretot per ser l'empenta i la força. No hi hauria pogut arribar sense vosaltres!

Jaume, gràcies per ser al meu costat, les teves ganes de créixer, la dedicació i l'entusiasme que hi poses a les coses enforteixen qui tens al costat. Sempre m'ajudes a aconseguir el que vull simplement amb el teu suport.

SCIENTIFIC PUBLICATIONS DERIVED FROM THIS THESIS

In this Thesis I present four scientific papers from which I am the first author. Two has been already published and the other two are under review. References are cited below:

En aquesta Tesi presento quatre articles dels quals en sóc la primera autora. Dos articles ja han estat publicat i els dos restants es troben en procés de revisió. Les referències dels articles són les següents:

- Timoner, X., Acuña, V., von Schiller, D. and Sabater, S. (2012) Functional responses of stream biofilms to flow cessation, desiccation and rewetting. *Freshwater Biology* **57**: 1565-1578.

- Timoner, X., Buchaca, T., Acuña, V. and Sabater, S. Do streambeds change in color when they dry? Photosynthetic pigment changes in biofilms during flow intermittency. *Submitted to Aquatic Sciences.*

- Timoner, X., Borrego, C., Acuña, V. and Sabater, S. The dynamics of biofilm bacterial communities is driven by flow wax and wane in intermittent streams. *Submitted to Limnology and Oceanography.*

- Timoner, X., Acuña, V., Frampton, L., Pollard, P., Sabater, S., Bunn, S. E. (2014) Biofilm functional responses to the rehydration of a dry intermittent stream. *Hydrobiologia.* **727**: 185-195.

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SUMMARY

Streams experiencing a recurrent non-flow phase (*i.e.*, flow intermittency) are characteristic of world regions with arid and semi-arid climates, where Mediterranean regions are part of. The temporal and spatial extent of flow intermittency is however increasing globally due to climate change, water abstraction and direct human uses. In temporary rivers, particularly during the dry season, water flow is interrupted due to the high temperatures and the decrease in precipitation, consequently the aquatic habitat is constrained to some pools or wetted areas, and desiccation of the streambed sediments generally occurs. Organisms in temporary rivers are directly affected by flow intermittency, however some can move for better conditions or have evolved some physiological adaptations, such as macroinvertebrates or fishes. The case of microorganisms, such as algae and bacteria, require specific structural and physiological adaptations to withstand the harsh conditions prevailing during the non-flow phase. Microorganisms assembled in biofilms lead a substantial part of the ecosystem processes. One of those is their involvement in organic matter processing, where they recycle the materials in the carbon and nutrient cycles. Biofilms are also on the base of the food web fueling energy to the higher trophic levels through the primary production. Therefore, understanding how biofilms respond to flow intermittency is a primary step in order to unravel the consequences of the increasing flow intermittency affecting stream ecosystems.

The main objective of this Thesis is to investigate the biofilm responses to flow intermittency, paying special attention to drying, non-flow, and rewetting phases in order to understand and predict how global change is affecting temporary rivers. Field studies were conducted in a Mediterranean headwater stream and in a subtropical Australian stream. In particular, different sampling dates were done during a hydrological cycle in the Mediterranean stream, with special emphasis to the drying, non-flow and rewetting phases. Physicochemical parameters and biofilm samples growing on the main streambed compartments (cobbles, superficial sand and subsuperficial sand) were collected at each sampling date. Three of the four chapters of this Thesis derive from this field study, where structural and functional biofilm responses were analyzed at the cellular level (algae and

bacteria), as well as at the whole biofilm responses (autotrophic *vs* heterotrophic processes). The last chapter is based on the subtropical Australian stream, where flow intermittency is a common phenomenon of the landscape. In this study the biofilm responses to a rehydration event (unpredictable rainfall) that occurred during the non-flow phase, were assessed by a community level physiological profile technique that allowed to simultaneously test the performance of different biofilm functions.

The main results of the Thesis pointed out that flow intermittency clearly affects the biofilm structure and function, being the biofilm growing on cobbles and the autotrophic processes the most affected by flow intermittency. On cobbles, both autotrophic and heterotrophic processes sharply decreased during non-flow, concentrations of chlorophyll-a become extremmely low, and the degradation capacity (measured by extracellular enzyme activities) also decreased. However, the two experienced a fast recovery when flow resumed, but heterotrophic processes were favoured during the rewetting. In sandy compartments chlorophyll-a also decreased, but this pigment remained largely unaltered during non-flow. Sandy compartments maintained their degradation capacities (heterotrophic processes) during non flow conditions, specifically for polysaccharides (β-glucosidase activity) and organic phosphorus (phosphatase activity) compounds. Nitrogen acquisition (leucine-aminopeptidase activity) was limited under non-flow conditions whereas carbon and phosphorus compounds were largely used throughout the entire hydrological cycle. Biofilm nutrient molar ratios suggested a change in the organic matter acquisition by the biofilm under increasing flow intermittency, towards an increasing utilization of carbon compounds.

The higher capacity of the autotrophic community to react immediately after flow resumption was evidenced by the physiological changes occurring in the algal cells during non-flow phase. Despite the high degradation rates of chlorophyll-a in the epilithic biofilm (up to 90 %), the accumulation of protective pigments, such as scytonemin and canthaxanthin, and the formation of cell resistant structures, such as enlarged membrane thickness or chloroplast reduction, played a vital role in protecting the photosynthetic apparatus and the remaining chlorophyll-a that reactivated immediately after flow resumption. Although chlorophyll-a was more preserved in sandy compartments than in cobbles (chlorophyll degradation in superficial sands was arround 70 %), the substratum

characteristics and the environmental conditions that prevailed during the rewetting phase (low water temperature and low light availability) were less suitable for algal growth. The maintenance of the heterotrophic processes during non-flow, mainly in sandy compartments, was explained by a change in the bacterial community through a selection of the most resistant species able to thrive under non-flow conditions. On cobbles, the occurrence of new bacterial species during the non-flow phase suggested the colonization of the dry biofilm by terrestrial species coming from elsewhere. The non-flow phase supposed significant changes for the bacterial communities in all compartments (diversity decreased), however bacterial diversity rapidly recovered once flow recovered, to similar diversity values than those observed before flow intermittency.

The rehydration of the streambed sediments due to unpredictable rainfall events stimulated bacterial functioning, and biofilm used a broad range of carbon sources when rehydrated, similar to submerged biofilms (no exposed to desiccation). However after the rainfall, desiccation affected again the biofilm capacity to degrade different carbon sources, evidencing that the rehydration of streambed sediments during the non-flow phase produced a 'reset' effect to flow intermittency.

Overall, the Thesis results highlight that biofilms in temporary rivers are structurally and functionally adapted to flow intermittency. During the non-flow phase biofilms show low metabolic capacities, however biofilms show a high capacity to react when favourable conditions occur (rehydration event and/or flow resumption). These adaptations might play a crucial role in recovering the ecosystem functions once the flow recovers.

RESUM

Els rius que experimenten períodes sense cabal, coneguts com a rius temporals, són característics de les regions àrides i semiàrides, de les quals en formen part les regions de la Mediterrània. L'extensió temporal i espacial dels períodes sense cabal o períodes secs, està augmentant a nivell mundial a causa del canvi climàtic i la sobreexplotació dels recursos hídrics. En rius temporals, sobretot durant la fase seca, el flux d'aigua s'interromp a causa de les altes temperatures i la disminució de les precipitacions, conseqüentment l'hàbitat aquàtic es veu primerament reduït en forma d'algunes basses o zones humides al llarg del curs fluvial, fins que posteriorment es pot produir la total dessecació de la llera del riu.

Per tant, els organismes en rius temporals es veuen directament afectats quan el cabal s'interromp, però alguns poden moure's per trobar millors condicions o fins hi tot han desenvolupat algunes adaptacions fisiològiques, com ara els macroinvertebrats o peixos. Els microorganismes, com ara algues i bacteris, requereixen adaptacions estructurals i fisiològiques específiques per suportar les dures condicions que es donen durant la fase seca. El conjunt de microorganismes que creix sobra la llera fluvial es coneix com a biofilm i té una gran importància en els processos biogeoquímics que es donen a l'ecosistema. El biofilm juga un paper clau en els cicles del carboni i nutrients, gràcies a la seva capacitat de processar la matèria orgànica. A més, els biofilms són a la base de la xarxa tròfica, aportant energia als nivells tròfics superiors. Per tant, entendre el funcionament del biofilm quan es dona la fase seca, és un pas clau per entendre i determinar les conseqüències del canvi global en l'ecosistema fluvial, tan en rius temporals com en rius que estan experimentant períodes sense cabal en les zones més temprades del planeta.

L'objectiu principal d'aquesta Tesi és investigar les respostes del biofilm a períodes sense cabal, posant especial atenció a la fase de reducció del cabal, la seca i la de recuperació el cabal, per tal d'entendre i predir com el canvi global està afectant l'ecosistema fluvial. Amb aquest finalitat es van realitzar dos estudis, un en un riu de capçalera de la regió Mediterrània i un altre en un riu de la zona subtropical d'Austràlia. En el riu Mediterrani, es va dur a terme un estudi durant un cicle hidrològic, amb campanyes de mostreig

repartides al llarg de l'any i on, per cada campanya de mostreig es van mesurar varis paràmetres fisicoquímics i es van recollir mostres de biofilm dels principals compartiments de la llera (pedres, sorres superficials i sorres subsuperficials). Tres dels quatre capítols d'aquesta Tesi provenen d'aquest estudi de camp, on es van analitzar les respostes estructurals i funcionals del biofilm des d'un punt de vista cel·lular (algues i bacteris), així com en el conjunt del biofilm (processos autotròfics i heterotròfics). L'últim capítol es basa en l'estudi realitzat en un riu en la zona subtropical d'Austràlia, on la interrupció del cabal és un fenomen comú del paisatge fluvial. En aquest estudi es va avaluar la resposta funcional del biofilm a un esdeveniment de rehidratació durant la fase seca (pluges impredictibles) mitjançant una tècnica que permet analitzar simultàniament diferents funcions, obtenint així una idea del rendiment global del biofilm.

Els principals resultats d'aquesta Tesi mostren que la interrupció del cabal afecta clarament a l'estructura i el funcionament del biofilm i que el biofilm que creix sobre les pedres i els processos autotròfics són els més afectats. En el biofilm que creix sobre les pedres, tant els processos autotròfics com heterotròfics van disminuir considerablement durant la fase seca, es van observar concentracions de clorofil·la-a molt baixes i una gran disminució en la seva capacitat de degradar la matèria orgànica (mesurada per diferents activitats enzimàtiques extracel·lulars). No obstant això, ambdós processos (autotròfics i heterotròfics) es van recuperar de manera molt ràpida un cop es va recuperar es cabal, tot i que els processos autotròfics disminuïren de seguida (es van observar valors més baixos en comparació a abans de la desaparició del cabal), mentre que els heterotrófics es mantingueren. Durant la fase seca, en el biofilm que creix en els compartiments sorrencs també es va observar una gran disminució de l'activitat fotosintètica, però es va observar una major concentració de clorofil·la-a, en comparació en el biofilm que creix sobre les pedres. Durant la fase seca, la capacitat del biofilm de degradar la matèria orgànica (processos heterotròfics) es va veure poc afectada en el biofilm dels compartiments sorrencs, concretament per als polisacàrids (activitat β-glucosidasa) i els compostos de fòsfor orgànic (activitat fosfatasa). Però l'adquisició de nitrogen (activitat leucina-aminopeptidasa) va disminuir considerablement, mentre que els compostos de carboni i fòsfor es van utilitzar en gran mesura al llarg de tot el cicle hidrològic. A més els canvis observats en la proporció molecular de carboni, nitrogen i fòsfor del biofilm, deguts a la fase seca, reflectiren un canvi en l'adquisició de la matèria orgànica per part del biofilm, sent els compostos de carboni els més usats.

La gran capacitat de la comunitat autotròfica a reaccionar immediatament després de la recuperació del cabal es va explicar mitjançant els canvis fisiològics observats a nivell cel·lular durant la fase seca. Malgrat les altes taxes de degradació de la clorofil la-a en el biofilm que creix sobre les pedres (a voltant del 90 %), l'acumulació de pigments protectors, com ara escitonemina i cantaxantina, i la formació d'estructures cel·lulars de resistència, com un increment en el gruix de la membrana cel·lular o la reducció del cloroplast, jugaren un paper vital en la protecció de la aparell fotosintètic i la poca clorofil·la restant durant la fase seca, que es va reactivar immediatament després de la recuperació del cabal. Encara que es va observar una major preservació de la clorofil·la-a en el biofilm de compartiments sorrencs (amb un índex de degradació del 70 %), les característiques del substrat i les condicions ambientals un cop el flux es va reprendre (baixa temperatura de l'aigua i la baixa disponibilitat de llum) van limitar el creixement de les algues. El manteniment dels processos heterotròfics durant la fase seca , principalment en els compartiments de sorra, s'explica per un canvi en la comunitat bacteriana a través d'una selecció de les espècies més resistents capaces de prosperar sota condicions de dessecació. En el biofilm sobre les pedres es va observar l'aparició de noves espècies de bacteris durant la fase seca, espècies terrestres procedents d'altres llocs capaces d'aprofitar els recursos existents en el biofilm sec. La desaparició del cabal va provocar canvis significatius en les comunitats bacterianes en tots els compartiments (disminució de la diversitat), però durant la recuperació del cabal tots es van recuperar ràpidament amb valors de diversitat similars als observats abans de la fase seca.

Finalment es va observar un augment en la capacitat del biofilm de degradar diferents fonts de carboni quan el biofilm es rehidrata causa d'un esdeveniment de precipitació durant la fase seca, amb valors similars als observats en biofilms no exposats a la dessecació. No obstant això després de la precipitació, la dessecació va afectar de nou la capacitat del biofilm per degradar diferents fonts de carboni, disminuint tant la seva capacitat com la quantitat de fonts de carboni que el biofilm és capaç de processar. Aquests resultats van posar de manifest la importància de la capacitat del biofilm a reaccionar quan es rehidrata i que les precipitacions que poden ocórrer durant la fase seca suposen una oportunitat per al funcionament del biofilm, reiniciant els efectes de la interrupció del cabal.

Així doncs, els biofilms en rius temporals tenen una sèrie d'adaptacions tant estructurals, com funcionals a la interrupció del cabal. Encara que durant la fase seca el biofilm mostra unes baixes capacitats metabòliques, aquest posseeix una gran capacitat de reaccionar quan es produeixen condicions favorables (o bé rehidratació per precipitacions o la represa de flux). Aquestes adaptacions juguen un paper crucial en la recuperació de les funcions de l'ecosistema després de la represa del flux.

RESUMEN

Los ríos que experimentan una fase de interrupción del flujo o fase seca, son los llamados ríos temporales, característicos de las regiones áridas i semiáridas, como las regiones Mediterráneas. Hoy en día la extensión temporal y espacial de la interrupción del flujo en los ríos está aumentando a nivel mundial, debido al cambio climático y a la sobreexplotación de los recursos hídricos. En los ríos temporales, normalmente durante la estación seca, el flujo es interrumpido por las altas temperaturas y la disminución de las precipitaciones. En consecuencia el hábitat acuático se ve reducido a pequeñas pozas o zonas más húmedas produciéndose la desecación del lecho fluvial.

Los organismos en ríos temporales están directamente afectados por la desaparición del flujo, sin embargo algunos pueden migrar río abajo y permanecer en pozas o en el curso principal si éste no se ve afectado, también han desarrollado algunas adaptaciones fisiológicas para evitar la desecación, como es el caso de los macroinvertebrados o los peces. Microorganismos, como algas y bacterias, requieren adaptaciones estructurales y fisiológicas específicas para soportar las duras condiciones que se dan durante la fase seca. Al conjunto de estos microorganismos que crecen en el lecho fluvial se les conoce como biofilm y tiene un papel fundamental en el funcionamiento del ecosistema ya que participa activamente en el procesamiento de la materia orgánica, reciclando los materiales que entran en el sistema y así contribuyendo en los ciclos del carbono y nutrientes. Además se encuentran en la base de la red trófica, aportando energía a los niveles tróficos superiores a través de la producción primaria. Por tanto, es importante entender como los biofilms responden a la interrupción del flujo para poder determinar como el cambio global afecta a los ecosistemas fluviales tanto en ríos temporales, como en ríos de zonas templadas que también están experimentando la interrupción del flujo.

El objetivo principal de esta Tesis es investigar las respuestas del biofilm a la interrupción del flujo en ríos temporales, prestando especial atención a la fase de reducción del flujo, la fase seca y a la reanudación del flujo, con el fin de entender y predecir cómo el cambio global está afectando los ríos temporales. Para este fin se llevaron a cabo dos estudios de

campo, uno en un río de cabecera mediterráneo y otro en un río de la zona subtropical de Australia. En el río de cabecera mediterráneo se tomaron muestras durante un ciclo hidrológico, se realizaron diferentes campañas de muestreo a lo largo de un año. En cada muestreo se midieron diferentes parámetros fisicoquímicos y se tomaron muestras del biofilm sobre los tres compartimentos fluviales principales (piedras, arena superficial y arena subsuperficial). Tres de los cuatro capítulos de esta Tesis derivan de este estudio de campo, donde se analizaron las respuestas estructurales y funcionales del biofilm a nivel celular (algas y bacterias), así como en su conjunto (procesos autotróficos y heterotróficos). El último capítulo se basa en el estudio realizado en el río de Australia subtropical, donde la interrupción del flujo es un fenómeno común del paisaje. En este estudio se determinaron las respuestas funcionales del biofilm en substratos arenosos a un evento de rehidratación (lluvias impredecibles) durante la fase seca. Las respuestas del biofilm se evaluaron mediante una técnica que permite analizar simultáneamente diferentes funciones, obteniendo así una idea del rendimiento global del biofilm.

Los principales resultados de esta Tesis muestran que la interrupción del flujo afecta claramente la estructura y el funcionamiento del biofilm, siendo el biofilm que crece sobre las piedras y los procesos autotróficos los más afectados. En el biofilm sobre piedras, ambos procesos (autotróficos y heterotróficos) disminuyeron considerablemente durante la fase seca, se observaron concentraciones de clorofila-a extremadamente bajas y una gran disminución de su capacidad de degradación de la materia orgánica (medida por actividades enzimáticas extracelulares). Sin embargo, ambos procesos experimentaron una rápida recuperación cuando se reanudó el flujo, aunque los procesos heterotróficos dominaron sobre los autotróficos (se observaron valores más bajos en comparación a los observados antes de la desaparición del flujo). Durante la fase seca, en el biofilm que crece en los compartimentos arenosos también se observó una disminución de la actividad fotosintética, no obstante se observó una mayor concentración de clorofila-a. El biofilm en los compartimentos arenosos mantuvo sus capacidades de degradación de la materia orgánica (procesos heterotróficos) durante la fase seca, específicamente para los polisacáridos (actividad β-glucosidasa) y los compuestos de fósforo orgánico (actividad fosfatasa). La adquisición de nitrógeno (actividad leucina-aminopeptidasa) en cambio disminuyó considerablemente, mientras que los compuestos de carbono y fósforo se utilizaron en gran medida a lo largo de las diferentes fases hidrológicas. Además los

cambios observados en la proporción molecular de carbono, nitrógeno y fósforo del biofilm, debidos a la interrupción del flujo, apuntaron a un cambio en la adquisición de la materia orgánica por parte del biofilm, siendo los compuestos del carbono los más usados.

La gran capacidad de la comunidad autotrófica (algas y cianobacterias) a reaccionar inmediatamente después de la reanudación del flujo se explica mediante los cambios fisiológicos que se observaron a nivel celular durante la fase seca. A pesar de las altas tasas de degradación de la clorofila-a en el biofilm sobre las piedras (alrededor de un 90 %), la acumulación de pigmentos protectores, tales como escitonemina y cantaxantina, y la formación de estructuras celulares de resistencia, como un incremento en el grosor de la membrana celular o la reducción del cloroplasto, jugaron un papel vital en la protección de la aparato fotosintético y la reactivación de la poca clorofila-a, restante durante la fase seca, inmediatamente después de la reanudación del flujo. Aunque se observó una mayor preservación de la clorofila-a en el biofilm de compartimentos arenosos (siendo la tasa de degradación de un 70 % en la arean superficial), las características del sustrato y las condiciones ambientales que prevalecieron una vez el flujo se reanudó (baja temperatura del agua y baja disponibilidad de luz) limitaron el crecimiento de algas en este compartimento. El mantenimiento de los procesos heterotróficos durante la fase seca, principalmente en los compartimientos de arena, se explica por un cambio en la comunidad bacteriana a través de una selección de las especies más resistentes capaces de prosperar bajo condiciones de desecación. En el biofilm sobre piedras se observó la proliferación de nuevas especies de bacterias durante la fase seca, especies terrestres procedentes de otros lugares capaces de aprovechar los recursos existentes en éste. La desaparición de flujo provocó cambios significativos en las comunidades bacterianas en todos los compartimentos (disminución de la diversidad), sin embargo durante la reanudación del flujo, la diversidad se recuperó con valores similares a los observados antes de la fase seca.

Finalmente se observó un aumento en la capacidad de degradación del biofilm de diferentes fuentes de carbono cuando el biofilm se rehidrata debido a un evento de precipitación durante la fase seca, similar a la observada en biofilms no expuestos a la desecación. Sin embargo después de la precipitación, la desecación afectó de nuevo la capacidad del biofilm de degradar diferentes fuentes de carbono, disminuyendo tanto su

capacidad como la cantidad de fuentes de carbono que el biofilm es capaz de procesar. Estos resultados ponen de manifiesto la importancia de la capacidad del biofilm de reaccionar cuando simplemente se rehidrata, sin necesidad de la recuperación del flujo. Las precipitaciones que pueden ocurrir durante la fase seca suponen una oportunidad para el funcionamiento del biofilm, reiniciando los efectos de la interrupción del flujo.

Así pues, los biofilms en ríos temporales poseen una serie de adaptaciones tanto estructurales, como funcionales a la interrupción del flujo. Aunque durante la fase seca el biofilm muestra unas bajas capacidades metabólicas, éste posee una gran capacidad de reaccionar cuando se producen condiciones favorables (rehidratación por precipitaciones o la reanudación de flujo). Estas adaptaciones pueden desempeñar un papel crucial en la recuperación de las funciones de los ecosistemas después de la reanudación del flujo.

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Flow is the master variable controlling physical, chemical, and biological processes in streams. But what happens if flow vanishes for a period of time? Some scientists consider that when a channel dries the ecosystem disappears, whereas others see that channel as a corridor for fauna or as a new habitat for terrestrial biota. Less clear even is the phase when drying is approaching. During this, the aquatic biota can seek for better conditions, such as a remaining pool or the perennial main stream, but this is difficult for settled organisms, such as the biofilms. The aquatic microorganisms that cannot move to a safer place; how do they perform, and which are the strategies during the periods of drying, non-flow (when desiccated), and rewetting?

STREAM FLOW INTERMITTENCY

Rivers and streams that experience flow intermittency (*i.e.*, a recurrent non-flow phase) are known as temporary. Several authors have classified them depending on the surface water permanence (Boulton and Brock, 1999; Williams, 2006; Morais *et al.*, 2009; Lake, 2012), and although a strict classification does not exist, most agree classifying them as ephemeral or intermittent. Ephemeral streams are those that flow briefly in response to precipitation, thus water flows occasionally and unpredictably. The groundwater table is situated below the streambed throughout the year, therefore the channel does no receive groundwater discharge. Intermittent streams have the groundwater table above the streambed during some periods of the year and below it during others. Intermittent streams receive water either unpredictably or predictably (Boulton and Brock, 1999; Williams, 2006). In those that receive flow predictably groundwater table remains above the streambed during some periods (*e.g.*, when receive water from a spring, from groundwater or melting snow), but during dry seasons the groundwater table drops below the streambed, and surface water ceases to flow. Other intermittent systems experience these periods not according to the seasons, and therefore flow is unpredictable. On the contrary, perennial streams in humid parts of the world, where precipitation exceeds evapotranspiration, have flow all year-round and base flow is maintained by ground water discharge (Fig.1).

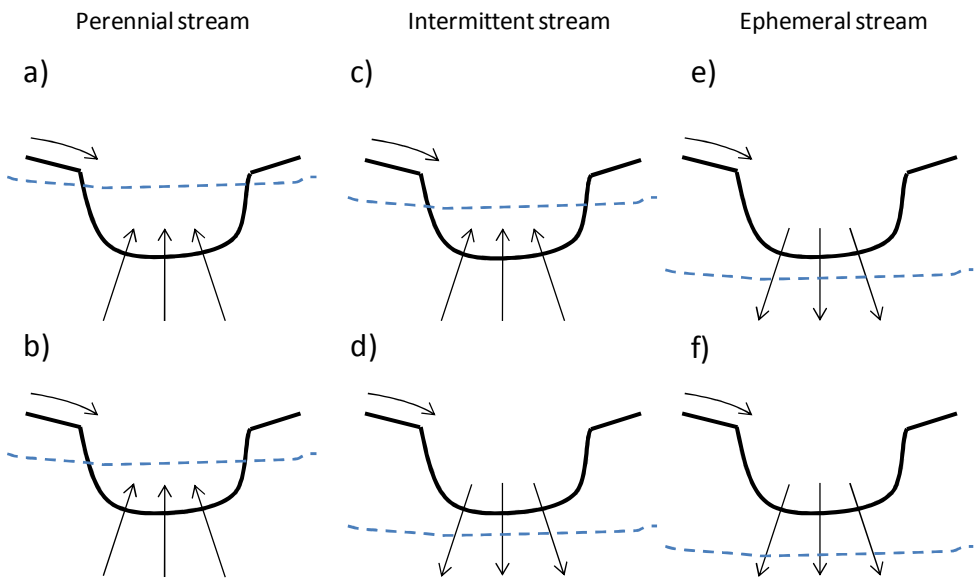

Figure 1. Cross channel sections showing perennial, intermittent and ephemeral streams under high (above) and low (below) groundwater table conditions. Dashed line indicates groundwater table elevation. Arrows indicate surface water and groundwater flow paths. a) and b) Perennial stream during high and low groundwater table. c) and d) Intermittent stream during high and low groundwater table. e) and f) Ephemeral stream during high and low groundwater table. Adapted from McDonough *et al.*, 2011.

Rivers that experience some flow intermittency have been described as being representative of the world's river systems (Dodds, 1997; Tooth, 2000; Larned *et al.*, 2010), even more than those with perennial flows (Williams, 1998). Estimates of the total length and discharge of temporary rivers are very rough, since they have been ignored for years, and few surveys of these ecosystems have been done. However they can be found in every continent, ranging in size from the smallest ephemeral streams with small drainage basins, to seasonally flowing headwaters, or to higher order river reaches that are spatially disconnected during some periods of the year. Intermittent streams mainly occur in arid and semiarid regions, occupying approximately 41 % of the Earth's land and affecting more than 30 % of the population (Whitford, 2002). These streams experience flow intermittency due to severe declines in rainfall, together with high temperatures and high evaporation rates. Similarly occurs in the regions subjected to a Mediterranean climate, where most of the tributaries of the main rivers are intermittent (Fig. 2).

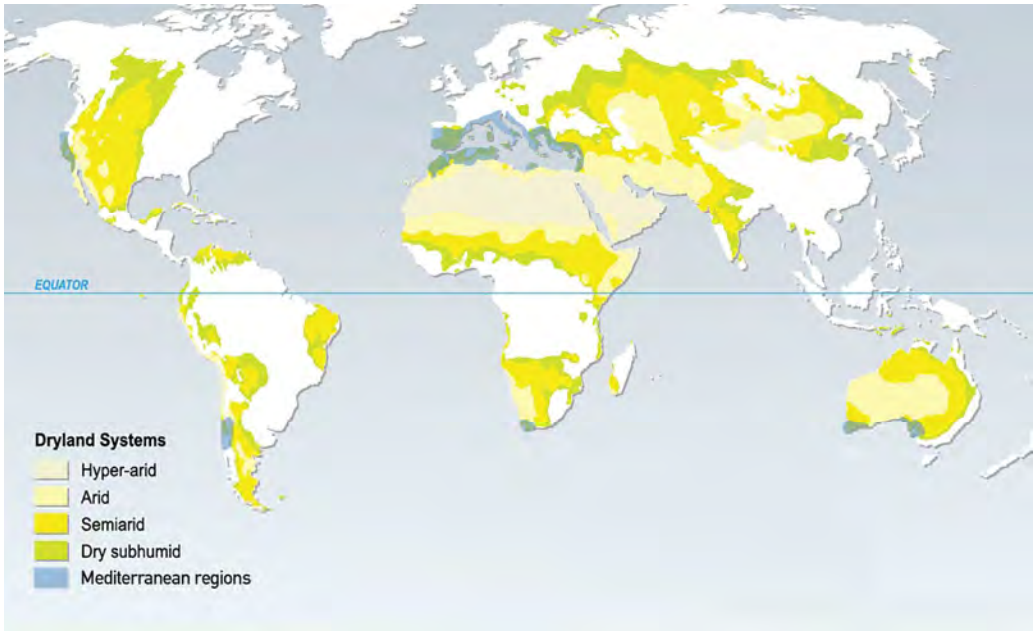

Figure 2. Location of arid and semiarid regions, and the Mediterranean regions (Adapted from: Millennium Ecosystem Assessment, 2005a).

In all these regions the duration and spatial extent of the non-flow phase has a climatic origin (climatic-droughts) and is highly dependent on the magnitude of the precipitation events (Williams, 2006). Furthermore, flow intermittency also occurs in Polar regions due to ice the formation (Stanish *et al.*, 2011), where water only flows during summer when higher temperatures provoke ice melting into the streams. Although in all these regions flow intermittency has a climatic origin, climate and global change severely affect these fluvial ecosystems (Fig. 3).

Since the last century there is detailed evidence that flow intermittency is increasing in these regions, and that will continue in the near future. Stream flow has declined for several decades in regions of Africa, Europe, North and South America, and Australia (Milly *et al.*, 2005; Xenopoulos *et al.*, 2005). Climate predictions indicate that flow intermittency will expand, whereas intermittency in Polar regions will probably reverse by the near future (due to ice melting) (Hughes, 2003; Christensen *et al.*, 2004; Milly *et al.*, 2005). A global-scaled runoff analysis predicts a decrease in annual average flows in 25–45 % of all river catchments by 2050, and a 2–3 fold increase in the frequency of severe low flows in southern Africa, Europe, the Amazon and western North America (Arnell, 2003).

Anthropogenic pressures in the form of construction works (*e.g.*, dikes, dams, or large reservoirs) or over-abstraction (to meet agricultural, urban and industrial demand), will further change the river hydrology and some river sections could shift from intermittent to permanent of from permanent to intermittent, severly affecting the residing biota in temporary rivers (Vörösmarty and Sahagian, 2000; Palmer *et al.*, 2008; Sabater and Tockner, 2010). These effects will also extend to many perennial streams that are currently experiencing stream flow intermittency due to the occurrence of exceptional drought events that are affecting temperate regions (Wilby *et al.*, 2006; Sutherland *et al.*, 2008).

Figure 3. Examples of temporary rivers: a) The Fuirosos an intermittent Mediterranean stream, b) The Negev, an intermittent stream in Israel, c) an ephemeral gully in South-East Queensland and d) The Lawson creek in the McMurdo Dry Valleys.

Temporary rivers are ecologically vulnerable since they are not recognized in most river management policies and neither considered in river health monitoring and assessment programs (Acuña *et al.*, 2014). For example, the European Union legislation ignores temporary rivers and extensively focuses on water quality (Water Framework Directive, WFD, EC 2000). In Australia, management policies failed on monitoring temporary

rivers using aquatic macroinvertebrates as biological indicators of river health, consequently a vast area of the river network could not be sampled owing to flow intermittency, and results were not representative enough (Ambient Biological monitoring and Assessment Program, ABMAP 2005, Steward, 2007; Sheldon *et al.*, 2005). Similarly occurs in the United States, where legislation failed to protect small temporary rivers, excluding them from the Clean Water Act (Environmental Protection Agency, EPA 2011). Furthermore river managers generally apply the same principles as for perennial rivers to manage temporary rivers, when making decisions about water allocation, river engineering, effluent discharge and restoration. For example, flow augmentation has been used to improve intermittent rivers by creating perennial flow (Wolff *et al.*, 1989; Henszey*et al.*, 1991; Cluett, 2005), resulting in invasions of non-native species (Howell and Benson, 2000), loss of native species and loss of refugia (Labbe and Fausch, 2000; Reich *et al.*, 2009). Fortunately, nowadays the role of global change and flow intermittency is receiving more attention by scientists and water resources managers (Garrote *et al.*, 2007; Morais *et al.*, 2009), but questions remain to be answered in order to provide water managers with tools and models to effectively manage temporary rivers. It is particularly important to expand the knowledge on the biological and biogeochemical responses to flow intermittency, in order to better understand and disclose the ecological values of temporary rivers and predict the consequences of global and climate change. Streams that naturally experience a non-flow period could serve as a template to understand and predict how much the increasing flow intermittency might affect them, and in which way will permanent streams respond to flow intermittency.

BETWEEN AQUATIC AND TERRESTRIAL ECOSYSTEMS

Temporary streams exist at the interface between aquatic and terrestrial ecosystems, the hydrological connections (when flowing) and disconnections (flow intermittency) create aquatic-terrestrial habitat mosaics that occur at multiple spatial and temporal scales (Stanley *et al.*, 1997; Malard *et al.*, 2006; Sheldon and Thoms 2006; Sponseller *et al.*, 2013). At the reach scale aquatic patches range in size from small pools to long reaches with flow permanence, and terrestrial patches range from emerged boulder tops to long reaches completely desiccated. At the catchment scale entire channels disconnect during non-flow phase and reconnect again when flow recovers, restoring hydrological

connections between pools, reaches, river channels, the floodplain and the riparian habitat (Tockner *et al.*, 1999; Jenkins and Boulton, 2003). The frequencies at which patches shift from terrestrial to aquatic and from aquatic to terrestrial range from days to years, depending on the rainfall pattern. The transition periods (flow recovery and drying) occur more or less rapidly, within minutes or hours, and days or weeks, respectively. Thus, the patches within this mosaic can be considered as aquatic, terrestrial or transitional (Fig. 4).

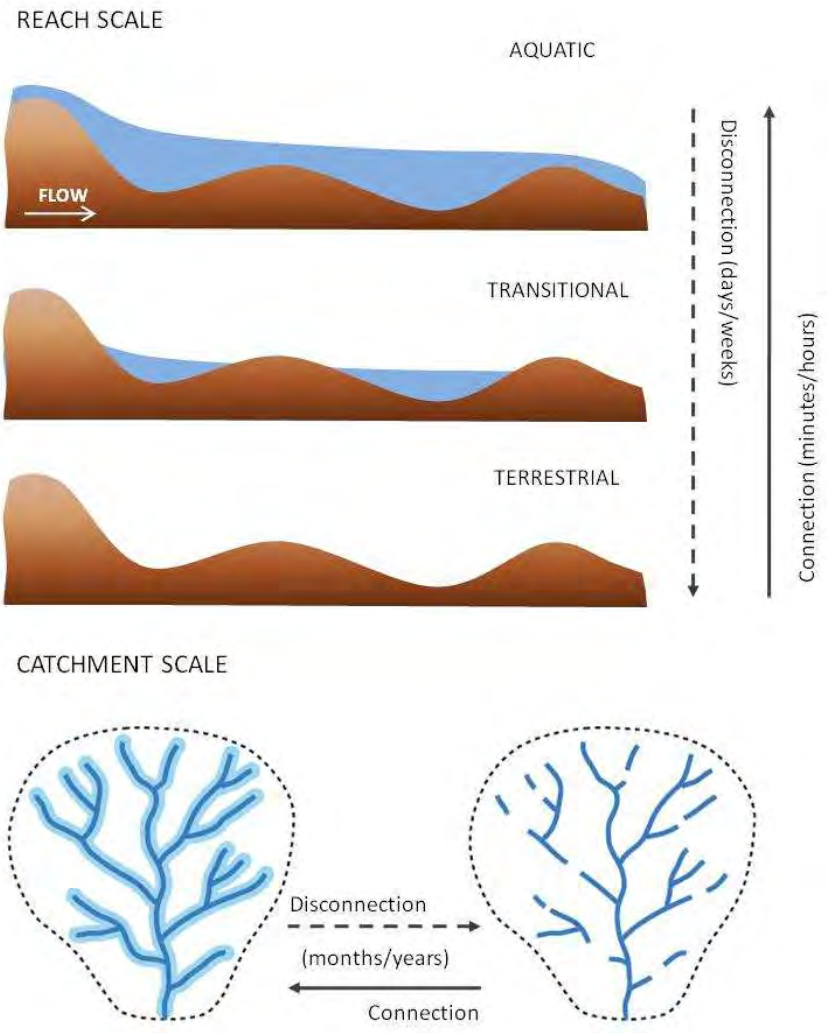

Figure 4. Temporal and spatial hydrological connection and disconnections in temporary streams. Adapted from McDonough et al., 2011 and Bernal *et al.*, 2013.

The presence or absence of water flow and the manner that drying and flow recovery occur represents a main constraint for biological communities (Poole *et al.*, 2006), as well as for nutrients and organic matter transport and processing (Stanley *et al.*, 1994; Lake, 2003; Boulton, 2003) affecting both the biogeochemical cycles (Baldwin and Mitchell, 2000) and ecosystem functioning (Fig. 5). Drying and flow recovery affect on the way that nutrients and energy are transformed in streams (Dahm *et al.*, 2003; von Schiller *et al.*, 2011; Ylla *et al.*, 2011). During drying (flow recession) the aquatic habitat reduces and disconnects (Boulton, 2003; Boulton and Lake, 2008), water temperature increases, and so do nutrients and dissolved substances. Oxygen depletes, and pH shows larger shifts. The overall water quality deteriorates and stressful situations for aquatic biota occur (Mundahl, 1990; Tramer, 1977). When flow recovers the terrestrial habitat is reduced and the aquatic one is reconnected again. Immediately after flow recovery large quantities of organic matter and nutrients are released and transported downstream, due to the accumulation during the terrestrial phase, and also from the flood plain and the riparian area, washing out them into the stream (Acuña *et al.*, 2004; Romaní *et al.*, 2006). Both drying and flow recovery are considered 'hot moments' (McClain *et al.*, 2003) for nutrients and organic matter processing in streams, since microbial respiration and denitrification-nitrification rates are enhanced (Baldwin and Mitchell, 2000; Artigas *et al.*, 2009; Ylla *et al.*, 2010; von Schiller *et al.*, 2011).

The desiccation of streambed sediments causes changes in their chemistry, mineralogy and microbiology (Mitchell and Baldwin, 1999; Baldwin and Mitchell, 2000), since nutrients and many other chemicals may be further transformed and stored as precipitated solutes through evaporation (McLaughlin, 2008). Once flow recovers the dissolution of these chemicals can produce stressful events. For example, lakes store sulfur in anoxic sediments as FeS, and the oxygenation of these sediments when desiccate), provoke the sulphur re-oxidation to sulphate leading to acid conditions once sediments are submerged in water affecting the biota, as well as a decline in dissolved organic carbon (DOC) concentration (Yan *et al.*, 1996; Schindler and Curtis, 1997). The drying velocity also matters for the biota, since slow drying allow longer permanence of refuges, such as remaining pools or wetted areas and also provide the necessary delay for structural and physiological adaptations to produce the regrowth once the flow recovers (Magoulick and Kobza, 2003; Robson *et al.*, 2008). Fish and macroinvertebrates have developed traits that increase

reproductive plasticity and reduce time constraints in life-history stages, such as rapid development, asexual reproduction and facultative dormancy (Nylin and Gotthard, 1998; Williams, 1998), together with physiological adaptations (*e.g.*, aestivation, and encystment) (Eldon, 1979; Kikawada *et al.*, 2005) or behavioral responses (burrowing and rheotaxis) (Del Rosario and Resh, 2000; Davey *et al.*, 2006; Lytle *et al.*, 2008; Stubbington *et al.*, 2011). Similarly, the permanence of refuges affects algal recolonization when the flow recovers (Robson and Matthews, 2004).

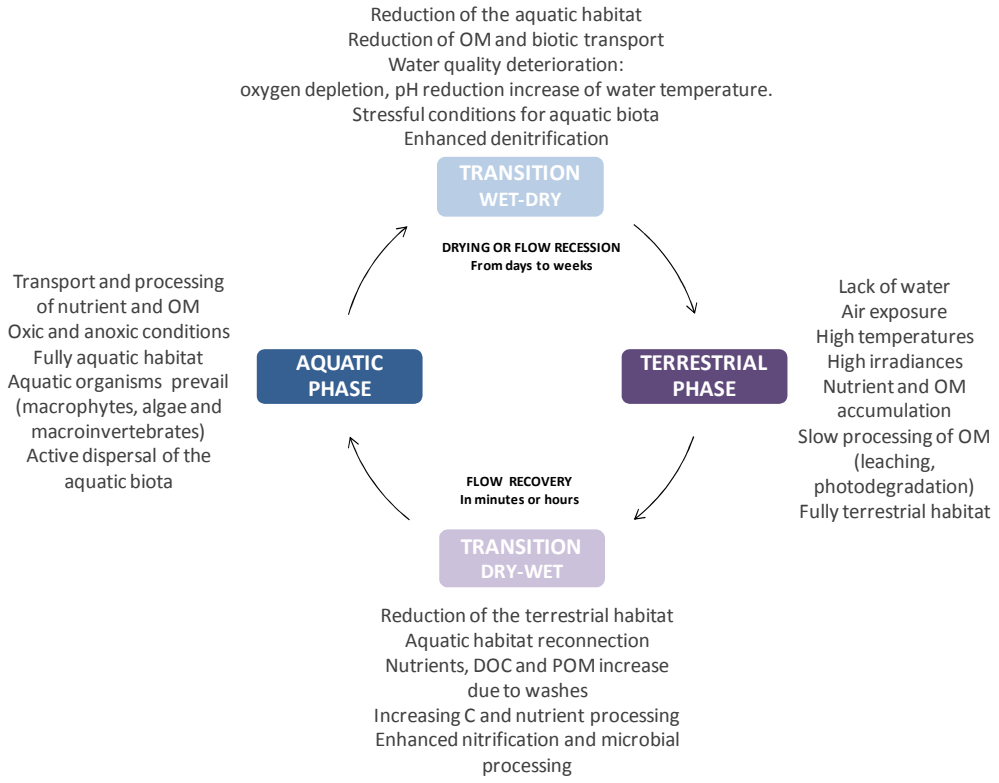

Figure 5. Physicochemical and biotic processes occurring in each of the hydrological phases in temporary rivers.

The terrestrial phase has been less studied by aquatic ecologists since it is often considered harsh and quiescent. Terrestrial ecologists have neither paid attention to the terrestrial phase, probably because they perceive the terrestrial phase outside of their respective disciplines. During the terrestrial phase streambed sediments desiccate and remain exposed to intense solar radiation (if riparian cover is scarce, as in arid regions), together with extreme temperatures and wind (Fig. 6).

Figure 6. Dry streambed in the Fuirosos stream during summer 2013, receiving high temperatures and intense solar radiation.

Recent studies have pointed out the importance and the ecological values of the terrestrial phase, or terrestrial patches (dry streambeds) (Steward *et al*., 2012). Marshall *et al*. (2006) considered that dry riverbeds serve as habitats and connectivity corridors for terrestrial organisms. Accordingly Steward *et al*. (2011) showed that dry riverbeds provide habitat for an unique set of terrestrial invertebrates. During the terrestrial phase dry riverbeds receive inputs of organic matter and nutrients mainly from the adjacent terrestrial ecosystems. However few studies have considered the importance of organic matter and nutrient processing that occurs during the terrestrial phase. As streambed dries up, microbial activity is reduced due to the low water availability (Amalfitano *et al*., 2008), and mineralization rates decrease. The aerobic environment expands and therefore aerobic processes are favored (nitrification and phosphorus transformations) (Baldwin and Mitchell, 2000; Austin and Strauss, 2011), and as a consequence, large amounts of organic matter and nutrients accumulate. This organic matter is slowly decomposed by physical (leaching and photodegradation) and microbial processes (Dieter *et al*., 2011; Corti *et al*., 2011). However streambed sediments can rehydrate for a short period of time due to unpredictable rainfalls, and these episodes probably contribute to the maintenance of the

microbial activity. The precondition of these accumulated organic matter during the terrestrial phase play an important role fuelling ecosystem metabolism during flow resumption (McClain *et al.*, 2003; Acuña *et al.*, 2004).

BIOFILM AND FLOW INTERMITTENCY

Biofilms are complex assemblages of microorganism, such as algae, bacteria, fungi and protozoa, embedded in a matrix of polysaccharides, exudates and detritus (Wetzel, 1983; Lock *et al.*, 1984). They are abundant in a multitude of natural environments (Wimpenny *et al.*, 2000) conditioning the bare surfaces for life (Miralles *et al.*, 2012). In streams, biofilms colonize rocks, cobbles, sand, aquatic plants, submerged leaves and wood in a variety of structural and compositional configurations (Lock, 1993). Previous studies have reported that fungi mainly colonize leaves and wood (Diez *et al.*, 2002, Hieber and Gessner, 2002; Gulis and Suberkroop, 2003), while bacteria prevail on fine mineral surfaces (sand) and detritic materials (Push *et al.*, 1998; Findlay *et al.*, 2002). Contrarily, algae prefer coarse and hard substrata (rocks and cobbles) where light is more available, and for that reason also grow on aquatic plants and on larger algae (Stevenson, 1996). For the development of this Thesis the biofilm growing on cobbles, sand and subsuperficial sand have been considered, and from now on we define the *epilithic* as the biofilm growing on rocks or cobbles, the *epipsammic* as the biofilm growing within superficial sand grains and the *hyporheic* as the biofilm growing within the sediments of the hyporheos, the section hydrologically linked to the open stream channel but just above the groundwater. These biofilms colonize the streambed compartment, and are responsive to environmental conditions indicating ecosystem changes (Steinman and McIntre, 1990; Burns and Ryder, 2001). Light availability is also an important factor when defining the biofilm structure and functioning. Where light prevails biofilms are dominated by photosynthetic organisms (autotrophs) (Lock *et al.*, 1984), particularly: Cyanobacteria, Chlorophyta (green algae), Bacillariophyta (diatoms) and some Rhodophyta (red algae) (Peterson, 1996). Low-light environments have biofilms predominantly heterotrophic (dominated by bacteria) (Blenkinsopp and Lock, 1994). The dominance of algae over bacteria may also be influenced by the nutrient availability, for instance cyanobacteria dominate under low nitrogen concentrations owing to their capacity to convert the atmospheric N_2 to ammonia and amino acids, enhancing the biofilm productivity (Peterson and Grimm,

1992). Organic enrichment (inputs of dissolved organic carbon, DOC) may decrease the richness of the algal community, favoring filamentous algal species when light is available and enhancing heterotrophy (Steinman and McIntre, 1990; Rosemound, 1993; Ylla *et al.*, 2009). Biofilm growth and metabolism is enhanced with water temperature (Brown *et al.*, 2004; Baulch *et al.*, 2005). Also water pH, rarely separated from other factors such as heavy metal abundances (low pH) or high salt and nutrient levels (high pH), determine the composition of the biofilm community since only specialized taxa can thrive under these extreme conditions. As an example, diatoms are commonly the dominant group of algae in very low pH environments (DeNicola, 2000; Sabater *et al.*, 2003). Flow variations determine the biofilm growth form and age. Low flow variability allows the development of thick biofilm mats with weak attachment to the substrata because of the basal cell senescence, thus small floods may be sufficient to remove these mats (Power, 1990; Peterson, 1996). Contrarily, in environments with high flow variability, such as the Mediterranean streams subjected to seasonal floods, biofilm grows tightly adhered to the substrata (Sabater, 1989). Turbulent flows occurring during floods clear out the substrata through scouring and abrasion (Grimm and Fisher, 1989; Uehlinger, 1991; Biggs *et al.*, 1999). Grazing also influences the biofilm thickness and productivity (Hart, 1992; Wellnitz *et al.*, 1996) by their direct consumption as well as by the remineralization of organic matter. Nutrient diffusion decreases in thick biofilms and O_2 gradients occur with oxic and anoxic microenvironments (Kühl and Jørgensen, 1992; Ramsing *et al.*, 1993) affecting the distribution of the microorganism within the biofilm. However, within the biofilm internal nutrients and organic substances entrapped within the polymeric matrix are recycled (Decho, 2000; Battin *et al.*, 2003), decoupling the biofilm functioning from the nutrient concentration in water (Mulholland *et al.*, 1994) when biofilms are sufficiently thick.

Biofilms are at the base of the food chain of the stream, fueling energy to the upper trophic levels (Lamberti, 1996) and driving carbon and nutrient cycles (Battin *et al.*, 2008). Streambed biofilms have been described as the most relevant sites for organic and inorganic nutrient uptake and retention in most small and medium-sized rivers (Sabater *et al.*, 2007). Since biofilms are key in recycling particulate (POM) and dissolve organic matter (DOM) in-stream (excreted by in-stream organisms) or terrestrially derived (from upstream or lateral inputs) (Bretschko, 1995; Romaní and Sabater, 2001). Biofilms also fix

or recycle organic nitrogen, and fix energy and carbon by photosynthesis and chemosynthesis into microbial biomass. Inside the biofilm, bacteria are consumed mostly by protozoa such as flagellates and ciliates (Bott and Kaplan, 1990). Biofilms, including bacteria, can also be used as food resources by macrozoobenthic organisms (grazers) (Gaudes *et al.*, 2009; Meyer, 1994) that, at their turn, are consumed by larger invertebrates and fish (Fig. 7).

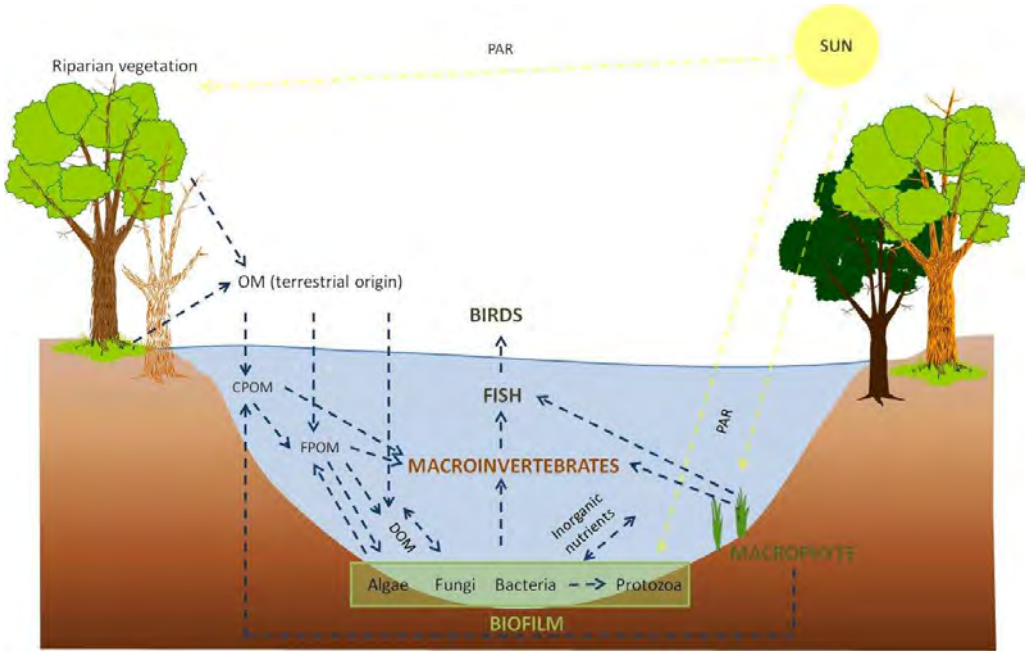

Figure 7. Stream food web interactions.
(PAR: photosyntetic active radiation, OM: organic matter, CPOM: coarse particulate organic matter, FPOM: fine particulate organic matter and DOM: dissolved organic matter).

In temporary rivers unpredictable drying and rewetting episodes exert an additional and critical abiotic control on biofilms (Fig. 8), and consequently on the processing and transformation rates of organic matter, with implications for the ecosystem functioning. The biofilm structure and activity in temporary rivers, as well as its metabolic processes (autotrophic and heterotrophic processes) have been investigated during flow recession and after flow recovery, but most often without considering the terrestrial phase. Changes in the bacterial activity and biomass occur in response to drying (Tzoraky *et al.*, 2007; Amalfitano *et al.*, 2008; McIntyre *et al.*, 2009). However a high resilience of the heterotrophic processes has been observed in intermittent Mediterranean streams, assessed

by a rapid recovery of the extracellular enzyme activities after flow resumption (Romaní and Sabater, 1997; Romaní *et al.*, 2006; Artigas *et al.*. 2009; Ylla *et al.*, 2010; Zoppini and Marxsen, 2011). These results suggest the importance of extracellular enzymes in the maintenance of biofilms during the terrestrial phase (Zoppini and Marxsen, 2011; Burns *et al.*, 2013). Furthermore, the responses of the bacterial community composition to drying and rewetting have been mainly studied in microcosm experiments (Amalfitano *et al.*, 2008; Marxsen *et al.*, 2010; Pohlon *et al.*, 2013) attributing the observed compositional changes to the water stress (Rees *et al.*, 2006). Similarly, in soils, Fierer *et al.* (2003) reported that drying and rewetting cycles affected the microbial community structure, but the major effects were observed on soils that underwent infrequent water stress. Thus, investigations suggest that bacterial communities in streams subjected to flow intermittency might be composed by a set of species capable to resist and thrive under extreme conditions (Febria *et al.*, 2011; Zeglin *et al.*, 2011). During the terrestrial phase unpredictable rainfall events, not enough to restore the flow, can rehydrate biofilms for some hours and affect the biofilm functioning. While rewetting processes in soils have been largely investigated, information is scarce for stream biofilms (Mamilov and Dily, 2002; William and Rice, 2007; Ioveno and Baath, 2008; Braun *et al.*, 2010). For instance, William and Rice (2007) found that soil bacteria exposed to rewetting during a dry season performed better than bacteria in soils not receiving water during the dry season, these results might indicate that unpredictable rehydration events will help to maintain biofilm functioning during the terrestrial phase.

Several studies also pointed out the resistance and resilience of the autotrophic community to water flow intermittency (Romaní and Sabater, 1997; Robson and Matthews, 2004; Robson *et al.*, 2008). These investigations pointed out the biofilm capacity to recover their photosynthetic efficiency when rewetted, and the importance of humid zones and refuges for the algal colonization after flow recovery. However structural and physiological adaptations of algae to flow intermittency are still unresolved. Based on structural properties (species composition and succession, and the formation of resistance structures), there are some groups and species better adapted than others to survive or thrive under desiccation (Kawecka, 2003; Robson and Matthews, 2004; Ledger *et al.*, 2008). For example, Cyanobacteria and green algae are better adapted to withstand flow intermittency than diatoms. Some Cyanobacteria in calcareous environments deposit

carbonates and produce stromatolitic-like mats, which provide protection to the cells living inside, as well as a high porosity prone to rewet soon after some rains (Sabater *et al.*, 2000). These structures do show quick autotrophic (and heterotrophic) response after rewetting (Romaní and Sabater, 1997). Algal communities experience succession during drying, in a reach scale there is transition from diatom mats to encrusting forms as dryness progresses (Romaní and Sabater*,* 1998; Ledger *et al.*, 2008). Diatoms are less resistant than other groups to desiccation, however some produce thick mucilaginous masses that may act as protectors of the living cells, others build inner plates to resist desiccation and osmotic variations, and some subsist in subaerial environments (Sabater, 2009). Other algae also possess thick cell walls that protect the cells from desiccation, for example some red algae and cyanobacteria produce permanent crusts that resist long desiccation and return to activity when flow recovers. Some filamentous green algae (*Spirogyra, Zygnema*, and other Zygnematales and Oedogoniales) produce zygospores through sexual reproduction, which are thick-walled and remain dormant to survive adverse conditions such as desiccation. There is a reduction in chlorophyll-a with the flow drawback (Ylla *et al.*, 2010), since streambed materials remain exposed to high temperatures and high incident light conditions particularly in open areas, which affect the autotrophic machinery. Thus, physiological adaptations (*e.g.*, changes in pigment composition) together with the structural changes mentioned above, might explain the capacity of some groups, such as cyanobacteria, to recover their photosynthetic activity quickly after rewetting (Romaní and Sabater, 1997). Similarly, terrestrial cyanobacteria recover extremely fast (from minutes to hours) their photosynthetic efficiencies when water is available (Scherer *et al.,* 1984). However, the photosynthetic recovery of epilithic diatoms or green algae is unknown. Accordingly, lower photosynthetic efficiencies during low flow conditions in a Mediterranean stream were associated to the carotenoid accumulation in sun-adapted biofilms (Guasch and Sabater, 1995) providing photooxidative protection to photoinhibition at summer ambient irradiances (Scherer *et al.*, 1988; Quesada *et al.*, 1995; Dieser*et al.*, 2010). UV-protective pigments such as carotenoids, xanthophylls and scytonemin accumulate in microbial mats (Fernández-Valiente *et al.*, 2007) and soil crust biofilms (Belnap *et al.*, 2007), and analogous processes might occur in fluvial biofilms as a response to flow intermittency.

Overall, the pattern of flow intermittency (*i.e.*, timing, duration and frequency) is likely to govern many in-stream processes, particularly biogeochemical rates which are governed by biofilm communities. However, despite their importance, information about their responses to flow intermittency as well as about their dynamic during the terrestrial phase is sparse.

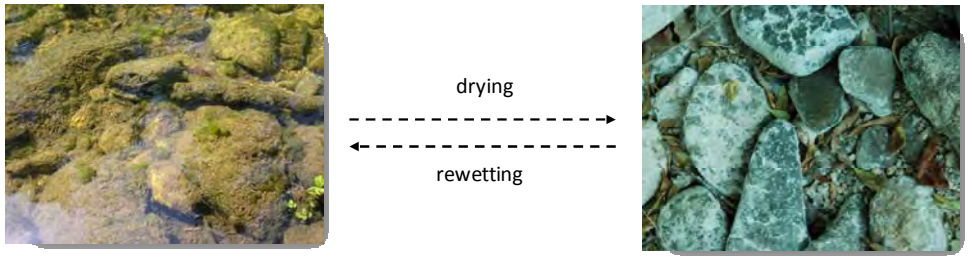

Figure 8. View of anepilithic biofilm when wet (left) and when dry (right).

OBJECTIVES OF THE THESIS

Flow intermittency produces unpredictable effects for biofilms and their associated ecosystem processes. Thus, the main objective of this Thesis is to investigate the biofilm responses to flow intermittency, paying special attention to the drying, non-flow and rewetting phases, in order to understand and predict how global change is affecting temporary rivers in the Mediterranean, as a template of rivers elsewhere in temperate regions that are currently experiencing flow intermittency.

Specific objectives are:

1. To determine the effects of flow intermittency on the biofilm structure and function, considering both autotrophic (algae and cyanobacteria) and heterotrophic (bacteria) components, and with particular interest in the three main streambed compartments where the biofilm grows (epilithic, epipsammic and hyporheic).

2. To investigate the role that algae and bacteria have in maintaining the ecosystem functions in temporary rivers, as well as the role of the biofilm on each streambed compartment.

3. To investigate the physiological adaptations of the biofilm to flow intermittency.

4. To study the effects of desiccation (and the associated environmental factors such as temperature and solar radiation) and rehydration on the biofilm structure and function

I expect, as the main hypothesis of this Thesis that biofilms in temporary rivers exhibit adaptation to flow intermittency and therefore show structural and functional characteristics that allow them to regain function immediately after rewetting.

The specific objectives aim to test the following specific hypotheses:

1. The epilithic will be more affected by flow intermittency than the epipsammic and hyporheic. Since the physical configuration of sandy compartments will probably confer higher protection to the biofilm to the changes associated to drying and rewetting, than the physical configuration of cobbles. This hypothesis is based on the evidence that cobbles are rapidly exposed to desiccation, whereas sand may show higher inertia to desiccation, since sand grains favour water retention interstitially.

2. The autotrophic community, which is more abundant in the epilithic, will show lower resistance to flow intermittency, since the lack of water will severely affect the photosynthetic processes. However during rewetting algae in this compartment will rapidly regain activity due to some cell physiological adaptations occurring during the non-flow phase.

3. The heterotrophic community will be more resistant to flow intermittency than the autotrophic community, and this resistance will be more obvious in sandy compartments (epipsammic and hyporheic biofilms), because some bacterial taxa will be able to thrive during the non-flow phase, and that will favour the maintenance of some ecosystem processes.

4. Bacteria will be able to take advantage of small rehydration events (*e.g.*, unexpected rainfall) that will favour the maintenance of heterotrophic processes during the non-flow phase.

METHODOLOGY

METHODOLOGY

Biofilms from two intermittent streams have been studied in this Thesis, one in the Mediterranean basin (Chapter 1-3), and the other in eastern Australia (Chapter 4).

STUDY SITES

Fuirosos stream

The first three chapters of this Thesis derive from the field monitoring study in the Fuirosos stream during 2009. Fuirosos is a Mediterranean third-order intermittent stream that drains a small forested granitic catchment of 15.2 km^2. It is located in the Montnegre-Corredor Natural Park, a forested range close to the Mediterranean Sea (50 km north of Barcelona, NE Spain) (Fig. 9).

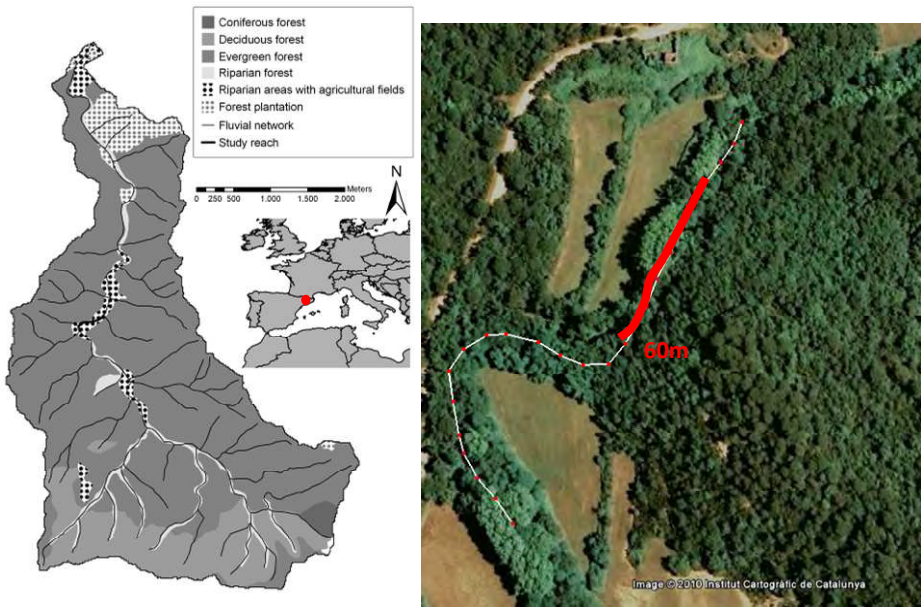

Figure 9. Fuirosos catchment and sky view of the study reach.

The traditional land uses in the watershed are forestry (land clearing and riparian logging), cattle and agriculture. Monthly mean air temperatures range from 5 ^{0}C in January to 24 ^{0}C in August. Mean annual precipitation is 677 mm (years 2000-2010; Dosrius meteorological station, Catalan Meteorological Service) with high inter-annual variability. Precipitation is distributed irregularly, mostly falling in autumn and spring, with occasional summer storms. Mean flow ranges 7-20 L s^{-1} and discharge is intermittent. Summer droughts usually evolve in a non-flow phase (variable in duration), followed by a short but intense stream recharge phase in late summer-early fall that usually last until late spring (Bernal *et al.*, 2005; Butturini *et al.*, 2008). Streambed morphology is very similar throughout the study area and consists of alternating riffles and pools. Boulders and cobbles are the dominant substrata in riffles, whereas sand accumulates in pools. Branches and leaves are scattered on the streambed and accumulate mainly in riffle areas during low water flow. The stream banks are steep and covered by dense riparian vegetation that forms a closed canopy. The riparian vegetation consists of alder (*Alnus glutinosa*), hazelnut (*Corylus avellana*), poplar (*Populus nigra*) and plane trees (*Platanus acerifolia*) which form a closed canopy from May to October. Leaf inputs into the river channel also occur in autumn (especially Alnus leaves) and late winter (Sabater *et al.*, 2001). The riparian tree community suffers intense hydric stress in summer, when flow declines the groundwater level. This stress causes an increased leaf input of up to 30 g C m^{-2}, which accumulates on the stream bed and stream margins (Acuña *et al.*, 2004). Because of the vegetation and the steep banks, light intensity in the river is moderate and heterogeneous throughout the year. Day light average PAR is usually <20 $\mu E \cdot m^{-2} \cdot s^{-1}$ for most of the year, except for a short period of time (from March to the end of May), when this parameter can reach values up to 70 $\mu E \cdot m^{-2} \cdot s^{-1}$ (Acuña *et al.*, 2004). Nutrient concentrations are low, ranging from 17 to 29 µg L^{-1} $P\text{-}PO_4$, 40 to 93 µg L^{-1} $N\text{-}NH_4$ and 260 to 390 µg L^{-1} $N\text{-}NO_3$ (Sabater *et al.*, 2005). Dissolved nitrogen (N) and phosphorous (P) peak in winter and summer, respectively (Bernal *et al.*, 2005; von Schiller *et al.*, 2008; Medici *et al.*, 2010), though the stream is considered P-limited for most of the hydrologic period (von Schiller *et al.*, 2008; Sabater *et al.*, 2011). DOC concentration in stream water ranges from 2 to 3 mg L^{-1} under basal discharge conditions. During the stream recharge period (September-October), however, stream DOC concentrations increase to 10-15 mg L^{-1}, occasionally reaching 20 mg L^{-1} (Bernal *et al.*, 2002). Abrupt changes in the physicochemical

characteristics of stream water occur before and after the dry phase (Vázquez *et al.*, 2007, Ylla *et al.*, 2011, von Schiller *et al.*, 2011).

Dam creek

Chapter 4 derives from another field study in the sub-tropics of South-East Queensland (Australia), where flow intermittency is a natural phenomenon of the landscape (Kennard *et al.*, 2010). Dam creek is subject to Subtropical climate and during the non-flow phase unpredictable summer storms can rehydrate the streambed sediments for some hours, even days. Dam Creek is an intermittent first-order headwater stream located in the upper Brisbane catchment in the Benarkin State Forest, located 6 Km from Moore (Fig. 10).

Figure 10. Dam creek catchment and sky view of the study reach.

Since European settlement in the catchment during the mid-19th century, the area has been extensively cleared with major land uses including forestry and pasture land for cattle farming. Dam Creek usually begins to dry during the austral winter until the austral summer, when unpredictable heavy rainfalls occur. Dam Creek has a poor canopy (*Casuarina* sp.) with only a few trees lining the bank due to extensive clearing. The materials forming the streambed are mainly silt/clay and some cobbles and boulders.

FIELD SAMPLING

Field sampling in the Fuirosos included specific and relevant moments of the hydrologic cycle, paying special attention to the non-flow phase. The sampling period was from May to December 2009 and a total of twenty seven sampling dates were realized. Sampling intensity was different depending on relevant moments of change affecting the biofilm and accordingly to the water permanence. Different hydrological phases were defined (Fig. 11): *i*) during the drying phase (*D*) that was from May to mid June, sampling was biweekly, at that time stream flow progressively declined. *ii*) Sampling was weekly during the fragmented phase (*F*) that occurred from the end of June to mid July, at this time stream flow was disconnected and some pools remained. *iii*) Then, during the terrestrial phase (*T*) sampling was biweekly, the stream run dry from mid July to mid October, for a total of 112 days. Finally *iv*) the rewetting phase (*R*) was sampled from the first day of the flow resumption, then every three days until one week, and then biweekly again until December. These four hydrological phases are used in chapter 1, whereas in chapter 2 and 3, although the data come from the same study period, only three hydrological phases were defined: *i*) the drying phase (*D*) which included the samplings from the fragmented phase. *ii*) the non-flow phase (*NF*) equivalent to the terrestrial phase in chapter 1 and *iii*) the rewetting phase (*R*), equivalent to the one in chapter 1.

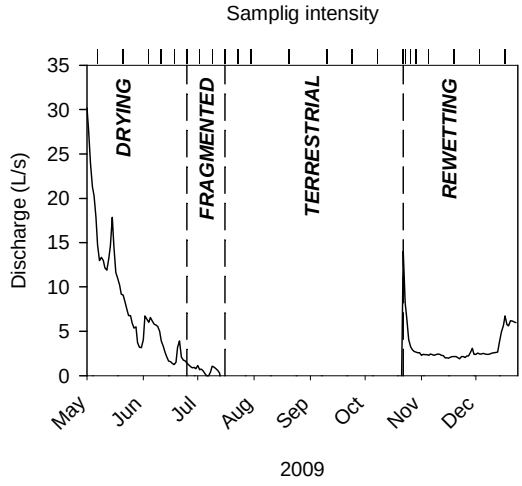

Figure 11. Sampling intensity in the Fuirosos stream.

A study reach of 60 m long, 3-4 m wide and ~ 10-50 cm deep was selected, from where physicochemical parameters and biofilm samples were collected at each sampling date (Fig. 9) Different physicochemical parameters were continuously monitored, such as discharge, water temperature and light (Table 1). While for other physicochemical parameters and biofilm samples, the sampling intensity followed the one described before.

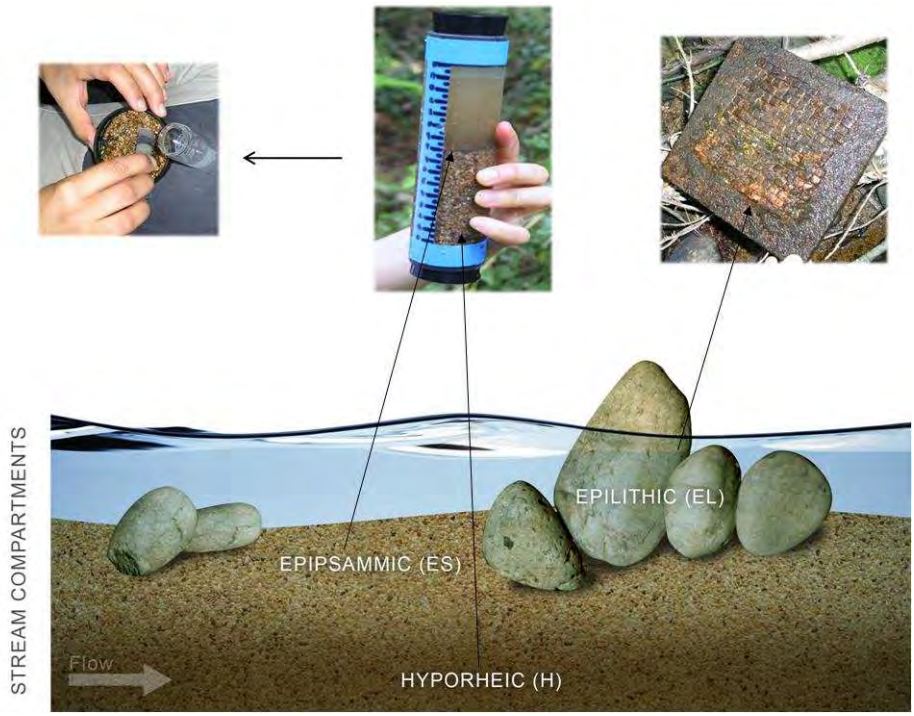

Figure 12. Stream biofilm compartments and the methodology used for sampling.

Biofilm sample collection was done following a randomized block design in order to include the habitat heterogeneity in the study reach. The biofilm from the three main streambed compartments was collected on each sampling occasion (Fig. 12). Unglazed ceramic tiles (1 cm^2) were used as surrogate substrata for epilithic biofilm (EL) (Lamberti and Resh, 1985). These substrata were glued onto flat bricks (100-150 tiles per brick), and randomly deployed along the study reach (20 bricks in total) 40 days previously to the beginning of the study, to allow biofilm colonization. Five bricks were sampled at random on each sampling day. Epipsammic and hyporheic biofilms were simultaneously collected from the surroundings of each sampled brick using a sample corer (2.6 cm in diameter).

The first 2 cm of sediment were considered epipsammic (ES), the next 3 to 6 cm were discarded in order to avoid edge effects, and from 7 to 10 cm were considered hyporheic (H) (Findlay, 1995; Romaní *et al.,* 1998). Subsamples of 1 mL were recovered from the corer using a plastic cylinder (1.2 cm in diameter). Some biofilm analyses were done under field conditions, other analyses were done in the laboratory, for which biofilm samples were preserved at 4 ^{0}C in the field. Once in the laboratory, the measurements that required in vivo analysis were immediately performed, while the rest of samples were frozen until analysis (Table 1).

Field sampling in the Dam creek lasted two months, from the end of October to the middle of December of 2011. Biofilms on the streambed sediments were sampled fortnightly at four consecutive sampling dates (T1, T2, T3, and T4) and in three different pools that formed in the same study reach once flow had ceased. The study reach was ca. 80 m long. Pools had similar morphometry and dimensions, and mainly differed in the length of time that they could remain dry. The downstream pool was permanently inundated (P) during all the study period, whereas the two upstream pools dried up and differed in the time remaining dry before the beginning of the study. Previous field observations allowed us to, respectively, define them as short-term dry pool (S; dried up 4-5 days before the first sampling date, T1) and long-term dry pool (L; dried since 1 month before T1). The S pool dimensions before drying were 7 m long and between 30-40 cm deep, and those of the L pool were of 4.5 m long and 30-40 cm deep. The P pool was 8 m long and 86-54 cm deep throughout the study period. A rainfall event occurred just before the second sampling date (T2) and this event rehydrated streambed sediments but did not suppose flow recovery. After the rainfall event, short and long term dry pools dried up again, since no significant rainfall occurred (Fig. 13). Physicochemical parameters and biofilm samples (three replicates) (Table 1) were collected in each stream pool at each sampling date. For this study only the biofilm on the superficial sand was investigated and it was collected as described before for the epipsammic.

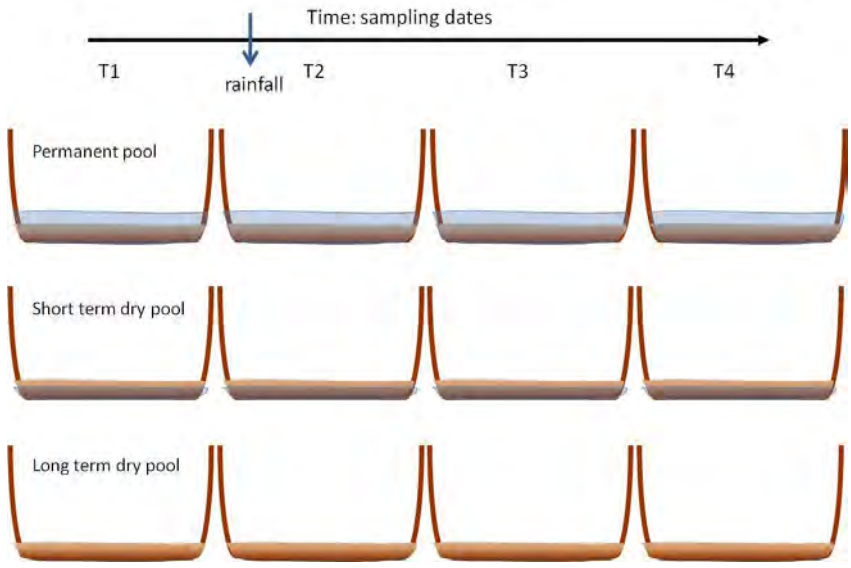

Figure 13. Dam Creek sampling dates in relation to rainfall, the blue arrow indicates the rainfall event.

LABORATORY METHODS AND ANALYSES

Biofilm responses are investigated from multiple perspectives and using a wide variety of techniques. Processes are analyzed at the cellular level (algae and bacteria), as well as at the whole biofilm responses (autotrophic *vs* heterotrophic processes). Temporal and spatial scales include the different hydrological phases (drying, non-flow and rewetting phases) and the main streambed compartments where the biofilm grows (epilithic, epipsammic and hyporheic), in order to better understand and predict the ecosystem responses to flow intermittency.

In order to determine the biofilm responses to flow intermittency structural and functional biofilm parameters were assessed in this Thesis. In chapter 1 the responses of the autotrophic community were assessed by means of the algal biomass and the photosynthetic efficiency, and the responses for the heterotrophic community were determined by the percentage of live bacteria and the extracellular enzyme activities. The whole biofilm responses were determined by the biofilm nutrient molar ratios (carbon, nitrogen and phosphorus (C, N and P) content of the biofilm, stoichiometry) to determine nutrient constrains. In chapter 2 pigment analysis were used to determine the

resistance and resilience of algae and cyanobacteria, as well as the physiological changes that the community experienced due to flow intermittency. Together with microscopic observations, in order to observe cell physiological adjustments, such as change in colour, enlarged membrane thickness and cell resistant structures. In chapter 3 the effects of flow intermittency on the bacterial community composition were assessed using massive parallel sequencing. The utilization of this molecular technique allowed to deeply investigate the changes observed in bacterial communities associated to flow intermittency, as well as their resistance and resilience by the applicability of some community analysis that have not been extensively applied for bacterial communities (nestedness and species turnover rates, here referred as OTUs turnover). And finally in chapter 4, the responses of the biofilm to a rehydration event during the non-flow phase, and to different flow intermittency extent (short and long term dry pools) were assessed by a community level physiological profile technique (BiologEcoPlates™) that allowed to test simultaneously the performance of different biofilm functions that is, the biofilm functional diversity.

Biofilm structural parameters

Algal biomass. Chlorophyll-a concentration per unit area of substrata, is commonly used to estimate algal biomass. Chlorophyll-a can be determined spectrophotometrically (Jeffrey and Humphrey, 1975). And from this measure the ratio of carotenoids or chlorophyll degradation products to active chlorophyll can be estimated as a rough index of the physiological state of algae (Margalef, 1983).

Bacterial cell density. DNA fluorescent stains allow bacterial cells counts under the epifluorescence microcope. Two different DNA stains were used in this Thesis. The LIVE/DEAD *Bac*light Bacterial Viability kit that allows to differentiate between live and dead cells. And the Acridine Orange stain that is used to determine the total bacterial density, all the bacterial cells are stained (Freese *et al.*, 2006).

Biofilm stoichiometry. C, N and P biofilm content and the elemental ratios (C:N, C:P and N:P) determine the biofilm stoichiometry. These ratios integrate biofilm and ecosystem functioning (Frost *et al.*, 2005; Sinsabaugh *et al.*, 2009).

Pigment analysis. Chlorophyll-a occurs in all photosynthetic organisms as the primary photosynthetic pigment, since the photosynthetic process does not occur without Chlorophyll-a. The other pigments (chlorophylls and carotenoids) function as accessory pigments, meaning that they absorb the light energy from those wavelength that the Chlorophyll-a cannot absorb and enable algae and cyanobacteria to utilize a much wider range of light. These accessory pigments are distributed distinctively within the different algal groups, thus the different pigments contained in a biofilm sample give clues to the contribution of green algae, diatoms, cyanobacteria and red algae in the biofilm (Rowan, 1989; Descy and Metens, 1996; Pinckney *et al.*, 1995). These set of pigments in a sample can be determined by High Performance Liquid Chromatography (HPLC) analysis (Jeffrey *et al.*, 1997; Schlüter *et al.*, 2006; Brotas and Plante-Cuny, 2003). Pigment analysis also gives valuable information about the physiological state of the algal community, since living algae contain mainly undegraded chlorophyll, however, during cell senescence chlorophyll is highly susceptible to destruction by its absorbing wavelengths, since some protection is lost because algae stop to synthesizing accessory pigments (Jen and Mackinney, 1970). Accessory pigments also play a key role against photooxidation protecting the algal cell (Porra *et al.*, 1997; Schubert, 2008). Therefore, the occurrence of chlorophyll degradation products and accessory pigments gives an idea about the physiological state of algae.

Algal community composition and cell resistant structures. Pigment analyses coupled with light microscopic observations provide the most accurate characterization of algal communities (Soma *et al.*, 1993). Light microscope observations allow the determination of physiological cell changes and cell resistant structures.

Bacterial community structure. Bacterial DNA sequencing is used to determine the bacterial community composition. In this Thesis sequencing was performed by Massive parallel sequencing. The main advantage of this technique is the accuracy and that allows the applicability of richness, diversity and relative abundance measures, as well as the other community analysis, such as OTUs turnover (Ronaghi, 2001).

Biofilm functional parameters

Photosynthetic efficiency. The photosynthetic efficiency can be estimated as chlorophyll fluorescence, which reflects the efficiency of energy conversion at the Photosystem II reaction centres (Schreiber *et al.*, 2002).

Extracellular enzyme activities Within the biofilms the organic matter decomposition is mediated by a wide range of extracellular enzymes produced by the microorganism (Hoppe *et al.*, 1988), these enzymes depolymerize the dissolved organic matter and detritus releasing soluble low molecular weight compounds that can be easily assimilated for the cellular metabolism (Chróst, 1990; Arnosti, 2003). Three extracellular enzyme activities related with the degradation of carbon (β-glucosidase activity), nitrogen (leucine-aminopeptidase activity) and phosphorous (phosphatase activity) compounds were analyzed in this Thesis.

BiologEcoPlates™. The Biolog technique allows the simultaneous determination of the biofilm capacity to degrade different carbon sources. Thus, different biofilm functions are measured simultaneously giving an idea of the biofilm functional diversity (Preston-Mafham *et al.*, 2002). Besides, the carbon degradation capacity gives information about the metabolic state of the biofilm.

The following table summarizes the physicochemical and laboratory techniques used in each chapter. Each method is well explained in the corresponding chapter.

Table 1. Summary of the methods used in each chapter of the Thesis.

Physicochemical parameters		
Discharge	continuously monitored, YSI probes	chapter 1-3
Temperature	continuously monitored, VEMCO data loggers and i-buttons	chapter 1-4
Photosynthetic active radiation (PAR)	continuously monitored, ODYSSEY data loggers	chapter 1-3
pH	Thermo Fisher TPS WP81 probe	chapter 4
Conductivity (EC)	YSI probe and Thermo Fisher TPS WP81 probe	chapter 4
Dissolved oxygen (DO)	TPS WP 82 probe	chapter 4
Benthic organic matter (fine and coarse)	Ash free dry weight (AFDW)	chapter 1-3
% Water content on cobbles and sand	Dry weight	chapter 1-4
Biofilm parameters		
Algae biomass	Chlorophyll-a concentration, spectrophotometric determination	chapter 1
Chlorophyll degradation and carotenoids accumulation	OD_{430}/OD_{665} spectrophotometric determination	chapter 1
Photosynthetic yield	Pulse amplitude modulation (PAM) fluorometer	chapter 1
Algal pigments	High Performance Liquid Chromatography (HPLC)	chapter 2
Algal cell resistant structures and community composition	Light microscope observations	chapter 2
Bacterial density	DNA stains: LIVE/ DEAD Baclight Bacterial Viability Kit and Acridine Orange. Epifluorescence microscope	chapter 1
Extracellular enzyme activities	Labeled substrates, related to the degradation of C, N and P compounds	chapter 1
Biofilm CNP content	CN analyzer and P basic digestion	chapter 1
Bacterial community structure	DNA analysis, massive pararell sequencing	chapter 3
Degradation of different C sources	BiologEcoPlates™	chapter 4

Chapter 1.

Functional responses of stream biofilms to flow cessation, desiccation and rewetting.

INTRODUCTION

Biofilms occur in a multitude of natural environments that provide sufficient nutrients and water for their development (Wimpenny *et al.*, 2000). In streams, biofilms colonize rocks, cobbles, sand, aquatic plants and submerged wood in a variety of structural and compositional configurations (Lock, 1993). Under light-limited conditions biofilms are dominated by bacteria and fungi (heterotrophs), whereas autotrophs (algae and cyanobacteria) can prevail when light is available (Sigee, 2005). Other physical factors (*e.g.*, temperature and flow velocity) as well as the type of colonized substrata (*e.g.*, organic or inorganic) further influence the relative relevance of heterotrophs or autotrophs in stream biofilms (Peterson, 1996).

Mediterranean headwater streams are subjected to high variability of hydrological and physicochemical characteristics, which can strongly affect biofilm composition and functioning, and may determine changes in the biogeochemical cycles and energy fluxes (Acuña *et al.*, 2005; Romaní *et al.*, 2006; Artigas *et al.*, 2009). Biogeochemical processes are strongly modified during droughts (Ylla *et al.*, 2010, 2011; Vázquez *et al.*, 2011; von Schiller *et al.*, 2011), which in the Mediterranean region mostly occur in summer, and usually extend for several weeks or months (Gasith and Resh, 1999; Butturini *et al.*, 2008). During droughts groundwater inflow is reduced, superficial water progressively disappears and the stream becomes intermittent, separated first in isolated pools, and finally completely dries up (Sabater and Tockner, 2010).

Drought progression affects biofilm structure and functioning, since the streambed loses water cover and humidity (Amalfitano *et al.*, 2008). Biofilms play a key role in the processing of organic matter and nutrients in streams (Pusch *et al.*, 1998; Romaní and Sabater, 2001; Battin *et al.*, 2003), therefore understanding the response and contribution of dry biofilms is essential to comprehend the effect of flow intermittency on stream ecosystem functioning. Several studies have revealed that desiccation and rewetting events represent physiological stress for heterotrophs, affecting their structure and functioning (Zoppini and Marxsen, 2011; Amalfitano *et al.*, 2008). A more extensive literature has

reported the capacity of autotrophs to cope with desiccation (Davis, 1972; Peterson, 1996; Ledger *et al.*, 2008) as well as their capacity to recover when the flow resumes (Benenati *et al.*, 1998; Robson and Matthews, 2004; Robson *et al.*, 2008). However, the joint response of autotrophs and heterotrophs within the biofilms, especially in the absence of surface water, is poorly understood. Stoichiometry represents an useful approach to examine ecosystem function and ecological processes since nutrient molar ratios (C:N:P) of the benthic microbial communities link element imbalances to ecosystem processes (Cross *et al.,* 2005; Frost *et al.*, 2005, Sinsabaugh *et al.*, 2009). Several studies have analyzed the response of biofilm stoichiometry to changes in the availability of resources, such as light and nutrients (Frost and Elser, 2002; Bowman, Chambers and Schindler, 2005); however none has yet examined the stoichiometric response of biofilms to desiccation.

Changes in climate and land use result in higher variability in runoff (Arnell, 1999, Huntington, 2006, IPCC, 2007), which may result in flow intermittency (New *et al.*, 2001; Huntington, 2006; Hirabayashi *et al.*, 2008). Hence, intermittency affects not only Mediterranean and other arid or semiarid streams, but also streams and rivers in more temperate regions where decreasing runoff and extended periods of desiccation have been observed, especially in their headwaters (Wildby *et al.*, 2006; Sutherland *et al.*, 2008). It might therefore occur that the seasonal flow intermittency, commonly found in Mediterranean systems, extends to other regions of the world. Mediterranean streams that naturally cease to flow and dry up could therefore serve as a template to better understand the biogeochemical and ecological implications of flow intermittency in more temperate regions.

This study aimed to determine how flow intermittency affects the structure and functioning of stream biofilms. Our first hypothesis was that some heterotrophic processes would maintain its activity during flow intermittency, while autotrophic processes would become dormant. The major relative decrease in autotrophic versus heterotrophic processes would be related to differences in humidity conditions among the different streambed compartments where biofilm grows. Our second hypothesis was that the use of carbon (C), nitrogen (N) and phosphorus (P) by biofilms would significantly change due to water stress conditions during the non-flow period, potentially leading to changes in the stream biofilms stoichiometry. To test these hypotheses, the functional response of the

main stream biofilms (epilithic, epipsammic and hyporheic) was analyzed in a Mediterranean stream during flow cessation, desiccation and rewetting.

METHODS

Study site and field sampling

This chapter derives from the field monitoring study in the Fuirosos that was performed from May to December 2009 (see more details of the study site and sampling procedures in the Methodology section).

Hydrology, temperature and light. Discharge was continuously estimated from water column depth measurements at the bottom of the study reach at 10 min intervals using YSI 600 OMS V2 multiparameter sondes (YSI Inc., Yellow Springs, Ohio). An empirical depth-discharge relationship was constructed based on additions of a conservative tracer. Daily mean temperature was recorded at 30-min intervals using three temperature recorders (12-bitMinilog Temperature logger, VEMCO, Canada) installed along the reach (at 0 m, 300 m and 1000 m upstream). Daily mean light reaching the streambed was measured at 10-min intervals by means of 2 Odyssey photosynthetic irradiance loggers (ODYSSEY, New Zealand) placed in the stream channel, calibrated according to manufacturer specifications.

Organic matter and water content of stream biofilm compartments. Five replicates of coarse and fine particulate benthic organic matter (CBOM and FBOM) were randomly collected with a core sampler (20 cm diameter) from near the bricks where the biofilm samples were collected on each sampling date. The FBOM was not collected during the terrestrial phase due to absence of water. Five replicates of the 3 different stream biofilm compartments that is, the biofilm itself and its substratum, were collected during the late drying, fragmented, terrestrial and early rewetting phases in each sampling date, to analyze their water content. Organic matter and samples for the water content analysis were transported to the laboratory for analysis.

Biofilm sampling. Biofilm from three different streambed compartments (epilithic, epipsammic and hyporheic) was collected at each sampling date (see more details in the Methodology section).

Laboratory analyses

Organic matter and water content of stream biofilm compartments. FBOM samples were passed through a net of 0.05 mm mesh size, and CBOM and FBOM samples were dried at 105 ^{0}C to constant weight and combusted at 450 ^{0}C for 4 h to estimate the ash free dry weight (AFDW). The water content of cobbles and sand was calculated as the difference between wet and dry weight of the sample after 24 h at 110 ^{0}C. Water content was expressed as the percentage of wet weight.

Autotrophic biomass. Chlorophyll-a (Chl-a) concentration for each biofilm was measured after extraction in 90 % acetone for 12 h in the dark at 4 ^{0}C (Steinman, Lamberti and Leavitt, 2006). Complete extraction of chlorophyll was ensured with sonication (1 min, Selecta, JP Selecta SA, Barcelona, Spain 360 W power, 50/60 Hz frequency). After filtration (Whatman GF/C, Whatman International Ltd., Maidstone, England) of the extract, the Chl-a concentration was determined spectrophotometrically using a Lambda UV/VIS spectrophotometer (U-2000 Spectrophotometer; Hitachi, Tokyo, Japan) and following Jeffrey and Humphrey (1975). The ratio of carotenoids and/or chlorophyll degradation products to active chlorophyll was estimated by comparing the optical densities at $OD_{430\ nm}$ and $OD_{665\ nm}$ (Margalef, 1983).

Photosynthetic efficiency. The photosynthetic efficiency of autotrophs was determined as chlorophyll fluorescence, which reflects the efficiency of energy conversion at Photosystem II (PS II) reaction centres (Schreiber *et al.*, 2002). Five replicates per biofilm type were used to determine the photosynthetic yield, which was measured in the field with a pulse amplitude modulation (PAM) fluorometer (MINI-PAM, WALZ, Effeltrich, Germany). The samples were set during 10 min under pre-determined low light conditions and a saturation pulse was done in order to estimate the photosynthetic efficiency.

Bacterial density. Live and dead bacteria were counted using the LIVE/DEAD *Bac*light Bacterial Viability Kit (Invitrogen Molecular probes Inc., Oslo, Norway) (Freese *et al.*,

2006). The bacterial density was estimated after ultrasonication (five replicates per biofilm) for 30 s using a sonication bath (Selecta, JP Selecta SA, Barcelona, Spain 360 W power, 50/60 Hz frequency). After appropriate dilution with sterilized stream water, a 1:1 mixture of SYTO 9 and propidium iodide was added to the sample under analysis and incubated for 15 min. Samples were then filtered (Whatman Nucleopore, Whatman International Ltd., Maidstone, England) and 20 randomly chosen fields were counted for each slide (Nikon E600 epifluorescence microscope, Tokyo, Japan). The fraction of live bacteria was calculated as the abundance of live cells divided by the total counts obtained with the live/dead method. Some cyanobacterial cells which could interfere in our analysis were present in the Fuirosos, mostly in the epilithic. However, the size and morphology of these forms (most of them are filamentous or colonial; Tornés and Sabater, 2010) was unmistakable at the epifluorescence microscope. In addition, sonication was carefully done, in order to avoid disaggregation of cyanobacterial structures as well as cell damages which could interfere with our results. Therefore, cyanobacteria were identified and excluded during bacterial countings and only heterotrophic bacteria were considered. Unfortunately, we cannot certainly discard that the smallest photoautotrophic cyanobacterial cells were not included in our epifluorescence countings, as it is practically impossible to differentiate between photoautotrophic and heterotrophic bacteria with this technique.

Extracellular enzyme activities. Three different extracellular enzyme activities were analyzed in the biofilms. The activities were related to the degradation capacity of C (β-glucosidase activity, BG), N (leucine-aminopeptidase, LAP) and P compounds (alkaline phosphatase, AP). The extracellular enzyme activities were measured by means of fluorescent-linked substrates (methylumbelliferyl [MUF] for BG and AP; aminomethyl-coumarin [AMC] for LAP). Colonized tiles (epilithic) and sand samples (epipsammic and hyporheic) were incubated for 1 h in the dark at 20 ^{0}C immediately after each sampling. Incubations were performed at a final concentration of 300 μmol L^{-1} (saturation concentration for these communities determined by Romaní *et al.*, 2004). Blanks and standards of MUF and AMC (0-100 μmol L^{-1}) were also incubated. At the end of the incubation, glycine buffer (pH 10.4) was added (1/1 vol/vol), and the fluorescence was measured at 365/455 nm excitation/emission for MUF, and at 364/445 nm excitation/emission for AMC. Values were expressed as nmoles MUF/AMC. The ratios

ln(BG): ln(LAP) and ln(BG): ln(AP) were calculated to compare the relative nutrient demand (organic P and organic N relative to organic C) among biofilms and hydrological phases (Sinsabaugh *et al.*, 2009).

All biofilm measurements (autotrophic biomass, bacterial density and extracellular enzyme activities) were related to the substrata surface area (cm^2). The surface area of sediments was obtained after granulometric analysis and the application of a conversion factor between sediment dry mass and surface area (1.003 g DW cm^{-2}; Romani and Sabater, 2001).

Biofilm C, N and P content. Biofilm detachment from sand (epipsammic and hyporheic) and tiles (epilithic) was achieved by ultrasonic bath (Selecta, Barcelona, Spain 360 W power, 50/60 Hz frequency) followed by successive cleanings using 10 mL of purified water for sand and 4 mL for tiles. The resultant suspensions were freeze dried during 72 h (Telstar freeze dryer, Lyoalfa 6-80, Telstar, Barcelona, Spain). Total C and N concentrations were determined with a CN analyzer (Thermo Finnigan Flash EA 1112, Waltham, MA, USA). Total P concentrations were analyzed after basic digestion (NaOH) at 110 ^{0}C for 90 min (Grasshoff *et al.*, 1983), and subsequent analysis of phosphate concentration following Murphy and Riley (1962). The total C, N and P concentrations of each biofilm were expressed as nutrient molar ratios (C:N and C:P).

Data analysis

Differences in extracellular enzyme activities, photon yield and Chl-a concentration among the different biofilms and during the different hydrological phases, were examined using multivariate analysis of variance (MANOVA). The differences in the percentage of live bacteria were examined by means of univariate analysis of variance (ANOVA). The Tukey HSD multiple comparison test was used to examine the differences between biofilms and phases. The percent water content of each stream biofilm compartment was related to the biofilm structure and functioning by means of Pearson correlation analysis. Correlations between the CBOM, extracellular enzyme activities, nutrient molar ratios, percentage of live bacteria and autotrophic biomass were also performed. The significance level was set at $P < 0.05$ for all analyses. Data were log transformed (x = log (x+1)) when

necessary to meet assumptions of parametric tests. The statistical analyses were carried out using SPSS for Windows (Version 17.0, SPSS, Chicago, IL, USA).

RESULTS

Physicochemical parameters

Hydrology, temperature and light. Discharge steadily decreased from late spring to the beginning of summer (end of June), when flow became disconnected and only isolated pools remained. At the end of July the streambed dried out completely, and this period lasted for 112 days. Flow resumed at the end of October. Mean daily water temperatures ranged between 3 and 19 ºC, but the streambed sediments during the terrestrial phase were exposed to a maximal mean daily temperature of 25-32 ºC (Fig. 1 a and b). Light reaching the streambed decreased (~ 70 %) from early May to early June, due to leaf emergence. The onset of leaf abscission in early August allowed higher light inputs (70 µE m^{-2} s^{-1}) (Fig. 1 b). PAR decreased in mid autumn due to the lower sun angle, and usually was < 20 µE m^{-2} s^{-1}.

Benthic organic matter. The amount of CBOM steadily increased during the terrestrial phase (from 199 ± 45 g AFDW m^{-2} to 695 ± 11 g AFDW m^{-2}; Fig. 1 a), and sharply decreased with flow onset at the beginning of the rewetting phase. The streambed was completely leaf-covered by the end of the terrestrial phase, when maximum values were attained. The FBOM values ranged from 0.51 ± 0.1 g AFDW m^{-2} to 88 ± 10 g AFDW m^{-2}, reaching maximum values during the rewetting phase (Fig. 1 a).

Water content of stream biofilm compartments. The lowest water content was recorded from the epilithic biofilm, and ranged from 1.1 ± 0.2 % during the drying and rewetting phases to 0.1 ± 0.1 % during the terrestrial phase. Water content in the epipsammic (0.2 ± 0.04 % to 23 ± 1.2 %) and hyporheic (0.6 ± 0.4 % to 22 ± 2 %) biofilms were similar (Fig. 1 c). The temporal pattern of water content also differed among the different biofilms. The reduction was relatively sharp in the epilithic biofilm but not so much in the epipsammic or hyporheic biofilms, where some moisture remained until mid August.

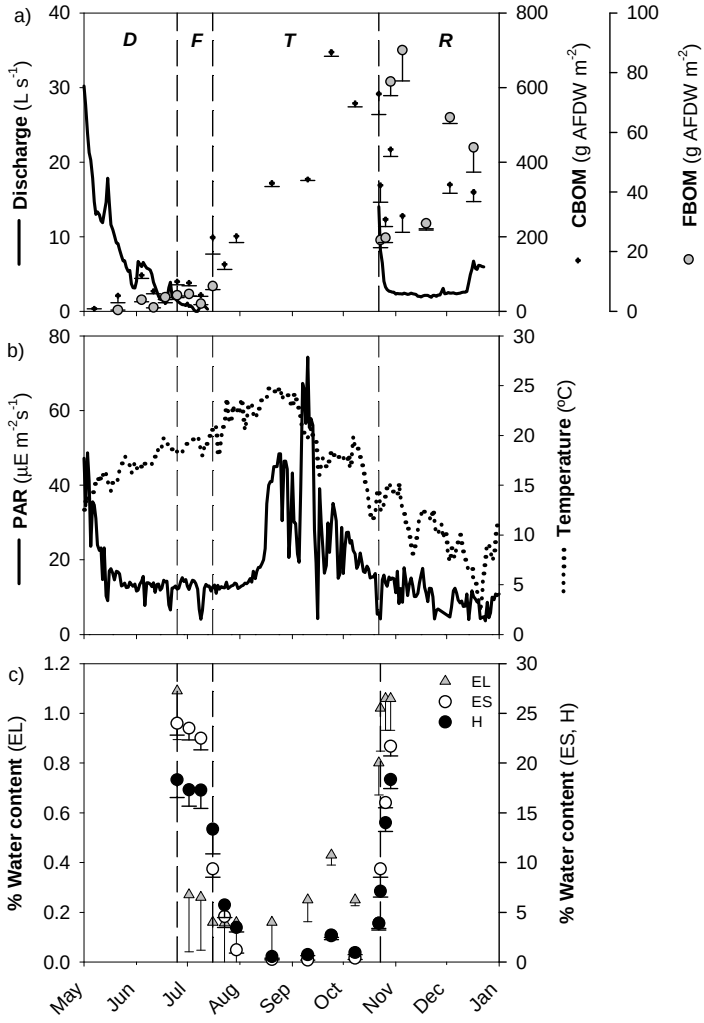

Figure 1. a) Discharge, coarse benthic organic matter (CBOM) and fine benthic organic matter (FBOM); b) Mean daily (from sunrise to sunset) photosynthetically active radiation (PAR) reaching the streambed and mean daily temperature (for water, except during the terrestrial phase); and c) Percentage water content of each biofilm compartment (EL-epilithic, ES-epipsammic, H-hyporheic). Error bars indicate standard errors. Hydrological phases (*D*-drying, *F*-fragmented, *T*-terrestrial, and *R*-rewetting) are differentiated with vertical dashed bars.

Biofilm structure and functioning

Autotroph structure and function. Autotrophic biomass, expressed as Chl-a concentration, ranged from 0.1 to 7.4 µg cm^{-2} (Fig. 2 a). Chl-a values were similar in the epilithic and epipsammic biofilms during the drying and rewetting phases, but diverged during the

terrestrial phase. The values in the hyporheic biofilm were the lowest amongst all biofilms except during the terrestrial phase, when the lowest values occurred in the epilithic biofilm. Chl-a concentration was not related to the percent water content of the different stream biofilm compartments, but negatively related to the CBOM (r = -0.396, P = 0.001). The OD_{430}/OD_{665} ratio ranged from 1.7 to 7.8 (Fig. 2 b), and higher values were recorded in epilithic and hyporheic biofilms. This ratio increased in the epilithic biofilm during the terrestrial phase, whereas it increased in the hyporheic during the rewetting phase. The epipsammic biofilm had consistently lower OD_{430}/OD_{665} values (Tukey's test, $P < 0.05$).

The epilithic biofilm had higher photosynthetic efficiencies than the epipsammic and hyporheic biofilms (Tukey's test, $P < 0.05$). The photosynthetic yield was always close to zero in the hyporheic biofilm, and also approached zero values in all biofilms during the terrestrial phase. These low values remained until flow resumption, when the epilithic biofilm showed the fastest recovery, the epipsammic biofilm had a slower recovery and the hyporheic remained extremely low (Fig. 2 c). The photosynthetic efficiency was negatively related to the percentage of water content in all streambed compartments (r = -0.350, P = 0.003).

Heterotrophs structure and function. Live bacteria ranged between 21.8 and 77.2 % of total bacterial density (Fig. 3), with the highest proportion occurring in epipsammic and hyporheic biofilms (Tukey's test, $P < 0.05$). The proportion of live bacteria decreased during the fragmented phase, and showed the highest values during the rewetting phase (Tukey's test, $P < 0.05$). Streambed desiccation involved a decrease in the proportion of live bacteria in all biofilms. However, the proportion of live bacteria steadily recovered during the terrestrial phase, and stopped with the onset of flow. The proportion of live bacteria was not related to the percent water content of the stream biofilm compartments but it was related to CBOM (r = -0.368, P = 0.001).

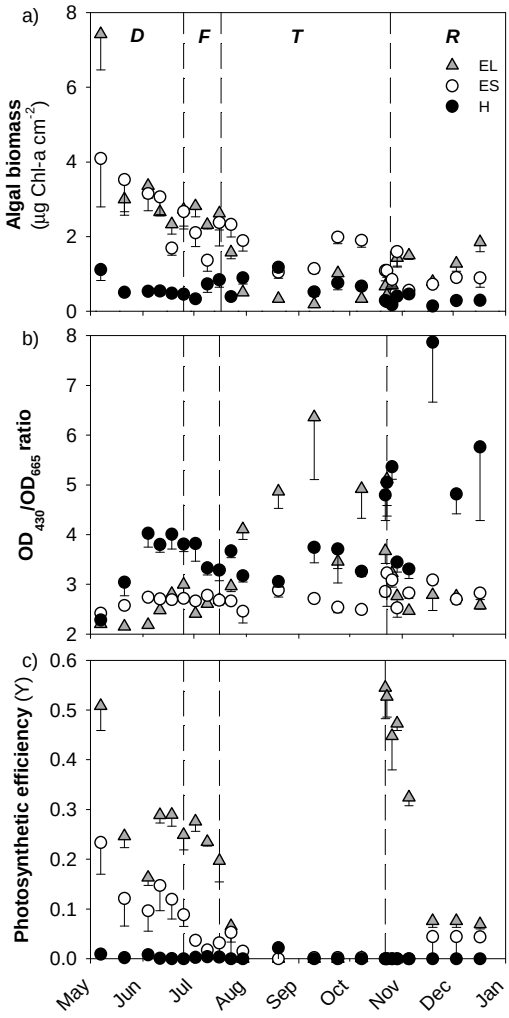

Figure 2. a) Chlorophyll-*a* content, b) Optical ratio; and c) Photosynthetic efficiency of each biofilm (EL-epilithic, ES-epipsammic, H-hyporheic). Values are the mean and standard error for each sampling date. Hydrological phases (*D*-drying, *F*-fragmented, *T*-terrestrial, and *R*-rewetting) are differentiated with vertical dashed bars.

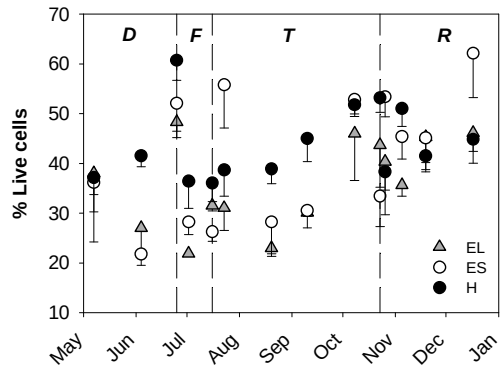

Figure 3. Percentage of live bacteria of each biofilm (EL-epilithic, ES-epipsammic, H-hyporheic). Values are the mean and standard error for each sampling date. Hydrological phases (*D*-drying, *F*-fragmented, *T*-terrestrial, and *R*-rewetting) are differentiated with vertical dashed bars.

The BG activity showed significant differences among biofilms and hydrologic phases (Table 1), with the highest values found in the epipsammic biofilm (Tukey's test, $P < 0.05$). High BG activity values in epipsammic and hyporheic biofilms during the terrestrial phase and at the beginning of the rewetting phase (Tukey's test, $P < 0.05$) were related to the CBOM accumulated on the streambed ($r = 0.523$, $P < 0.01$). The epilithic biofilm showed the lowest BG activity values, particularly at the beginning of the terrestrial phase, though at the end of this phase it showed large fluctuations (Fig. 4 a). The LAP activity was significantly higher in epilithic biofilms (Tukey's test, $P < 0.05$). The highest LAP activity occurred during the drying phase (mostly in epilithic; Tukey's test, $P < 0.05$, Table 1) and the lowest during the terrestrial phase. With flow resumption, the LAP activity of the epilithic biofilm returned rapidly to values similar to the pre-drought period, but the recovery of epipsammic and hyporheic biofilms was slower. Overall, the LAP activity was negatively related to the percentage of water content of the stream biofilm compartments ($r = -0.398$, $P = 0.001$) and positively with chlorophyll-*a* ($r = 0.489$, $P < 0.01$). Therefore, the highest LAP activity values occurred during the drying and fragmented phases (Fig. 4 b), when autotrophic abundance was higher. In addition, the LAP activity was positively related to the proportion of N in biofilms ($r = -0.655$, $P < 0.001$ with C:N and $r = 0.369$, $P = 0.001$ with N:P). The epilithic biofilm had the highest AP activity values of all the biofilms (Tukey's test, $P < 0.05$). The AP activity showed higher values during the fragmented and drying phases, but decreased during the terrestrial

phase (Tukey's test, $P < 0.05$), and again immediately after flow resumption (Fig. 4 c). The recovery of the AP activity after flow resumption took 4 weeks. The AP activity was negatively related to the percentage water content of the stream biofilm compartments (r = - 0.280, $P = 0.017$) and with the C:N and N:P nutrient molar ratios (r = -0.462, $P < 0.001$ and r = -0.350, $P = 0.003$).

Table 1. ANOVA results for each of the variables tested after the MANOVA analysis, considering single source effects and interactions of the two factors, biofilm type (B) and hydrological phase (P). *P*-values < 0.05 are indicated in boldface type. Y= photosynthetic efficiency, BG = β-Glucosidase activity; LAP = Leucine-aminopeptidase activity and AP= Phosphatase activity.

Sources	Chl-a	OD_{430nm}/OD_{665nm}	Y	BG	LAP
Biofilm	**$P < 0.001$** $F_{2,343} = 188.240$	**$P < 0.001$** $F_{2,343} = 33.322$	**$P < 0.001$** $F_{2,343} = 138.551$	**$P < 0.001$** $F_{2,343} = 36.200$	**$P < 0.001$** $F_{2,343} = 179.305$
Phase	**$P < 0.001$** $F_{4,343} = 66.284$	**$P < 0.001$** $F_{4,343} = 19.055$	**$P < 0.001$** $F_{4,343} = 12.695$	**$P < 0.001$** $F_{4,343} = 17.079$	**$P < 0.001$** $F_{4,343} = 41.186$
Biofilm*Phase	**$P < 0.001$** $F_{8,343} = 20.988$	**$P < 0.001$** $F_{8,343} = 11.104$	**$P < 0.001$** $F_{8,343} = 6.888$	**$P < 0.001$** $F_{8,343} = 4.282$	$P = 0.381$ $F_{8,343} = 1.073$

Sources	AP	C:N	C:P	BG:LAP	BG:AP
Biofilm	**$P < 0.001$** $F_{2,343} = 18.980$	**$P < 0.001$** $F_{2,343} = 27.342$	**$P < 0.001$** $F_{2,343} = 18.798$	**$P < 0.001$** $F_{2,343} = 19.361$	**$P < 0.001$** $F_{2,343} = 55.298$
Phase	**$P < 0.001$** $F_{4,343} = 17.021$	**$P < 0.001$** $F_{4,343} = 9.739$	**$P < 0.001$** $F_{4,343} = 21.261$	**$P < 0.001$** $F_{4,343} = 5.640$	**$P < 0.001$** $F_{4,343} = 27.897$
Biofilm*Phase	**$P < 0.001$** $F_{8,343} = 4.387$	$P = 0.425$ $F_{8,343} = 1.014$	$P = 0.052$ $F_{8,343} = 1.948$	$P = 0.776$ $F_{8,343} = 0.602$	$P = 0.001$ $F_{8,343} = 3.414$

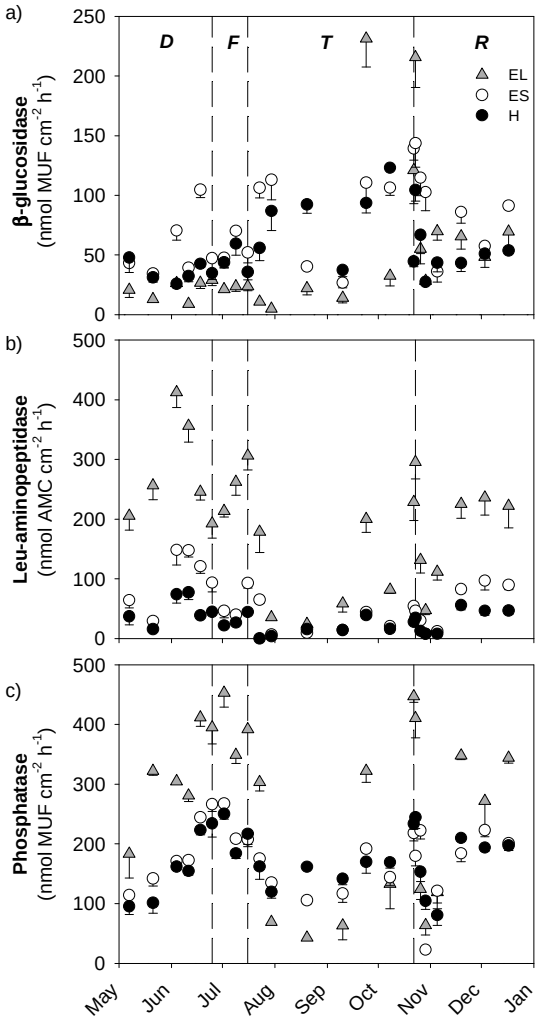

Figure 4. a) β-Glucosidase activity; b) Leucine-aminopeptidase activity; c) Phosphatase activity of each biofilm (EL-epilithic, ES-epipsammic, H-hyporheic). Values are the mean and standard error for each sampling date. Hydrological phases (*D*-drying, *F*-fragmented, *T*-terrestrial, and *R*-rewetting) are differentiated with vertical dashed bars.

Nutrient acquisition ratios varied significantly between phases and biofilms (Table 1). The ratio ln(BG):ln(LAP) ranged from 0.4 and 0.9 in epilithic, and from 0.6 and 1.7 in epipsammic and hyporheic biofilms (Fig. 5 b, Tukey's test, $P < 0.05$). The highest values occurred in sandy biofilms (epipsammic and hyporheic) mostly during the terrestrial and rewetting phases, and decreased (Fig. 5 a, Tukey's test, $P < 0.05$) in the drying and

fragmented phases. The ratio ln(BG):ln(AP) ranged between 0.3 and 1.0 in the epilithic and between 0.4 and 0.9 in the epipsammic and hyporheic biofilms (Fig. 4 b, Tukey's test, $P < 0.05$). The highest values occurred during the terrestrial and rewetting phases (Fig. 5 a, Tukey's test, $P < 0.05$).

C, N and P content of the biofilm. The nutrient molar ratios varied among biofilms and hydrologic phases (Table 1). The C:N and C:P ratios were significantly higher in sandy biofilms (epipsammic and hyporheic) than in the epilithic biofilm (Tukey's test, $P < 0.05$, Fig. 5 d), and higher during the terrestrial phase than during the other hydrologic phases (Tukey's test, $P < 0.05$, Fig. 5, c). The C:N ratio was related to the percentage water content of the stream biofilm compartments (r = 0.270, $P = 0.022$).

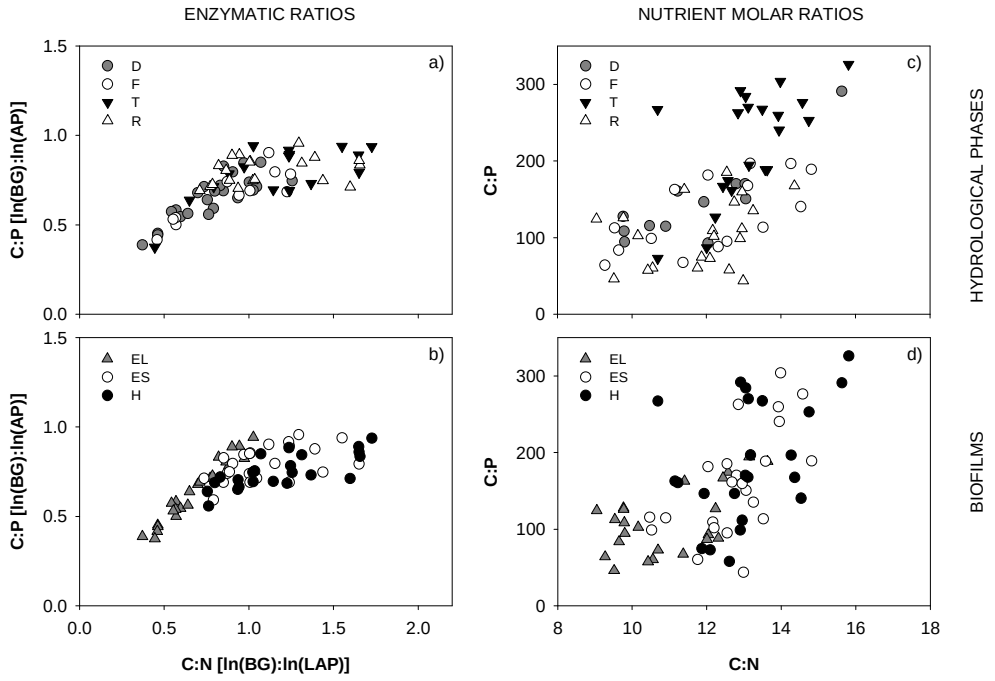

Figure 5. Enzymatic ratios (a and b) and nutrient molar ratios (c and d). In a) and b): ratios of ln(BG):ln(LAP), as an indicator of potential C:N acquisition activity, in relation to the ratio ln(BG):ln(AP), as an indicator of potential C:P acquisition activity. In c) and d): C:N nutrient molar ratio in relation to C:P nutrient molar ratio. Values in a) and c) are represented by biofilm (EL-epilithic, ES-epipsammic, H-hyporheic). Values in b) and d) are represented by hydrological phases (D-drying, F-fragmented, T-terrestrial, and R-rewetting). BG = β-Glucosidase activity; LAP = Leucine-aminopeptidase activity and AP= Phosphatase activity.

DISCUSSION

Flow intermittency had clear effects on the functioning of stream biofilms. According to our first hypothesis, heterotrophic activity remained during streambed desiccation, while stream drying caused a major effect on autotrophic activity. Autotrophs in the epilithic biofilm were the most affected, but showed a high capacity to recover after desiccation: autotrophic biomass in cobbles decreased by 80% with streambed desiccation, but recovered the fastest with flow resumption. Heterotrophs withstood flow intermittency, especially the biofilms growing in sandy substrata (epipsammon and hyporheos), where bacterial cell density decreased only by 20 %. The differential response of autotrophs *versus* heterotrophs was at least partially attributable to the physical conditions of the different stream biofilm compartments. According to our second hypothesis, the C, N and P use by the biofilm changed in response to streambed desiccation. While C and P breakdown capacities were maintained during dry conditions, especially in the epipsammic and hyporheic biofilms, the N breakdown capacity sharply decreased. In parallel, the stoichiometric C:N and C:P ratios of epipsammic and hyporheic biofilms increased with streambed desiccation.

Autotrophic versus heterotrophic functional responses

Intermittency had clear and specific effects on autotrophs (algae and cyanobacteria) and heterotrophs (bacteria). Autotrophs were more affected by streambed desiccation than heterotrophs. Autotrophic biomass (as Chl-a concentration) progressively decreased in the epilithic biofilm during the drying phase and abruptly decreased with streambed desiccation (terrestrial phase), reaching residual values. Differences between epilithic and epipsammic autotrophs were reflected in the much higher $OD_{430\ nm}/OD_{665\ nm}$ ratio in the former, as well as in its lower photosynthetic efficiency during the terrestrial phase. During this period, only a low proportion of active Chl-a remained. Values higher than 2 for the $OD_{430\ nm}/OD_{665\ nm}$ ratio (specially for the epilithic) and close to zero for the photosynthetic efficiency were observed, meaning that a large Chl-a fraction degraded during the stressing summer conditions (Guasch and Sabater, 1995; Romaní and Sabater, 1997). The hyporheic biofilm showed a slight increase of Chl-a during the terrestrial phase, suggesting that this compartment could act as a refuge for the autotrophic community under harsh conditions. In fact, diatoms are capable of migrate from superficial to deeper sediments as

a survival strategy (Mckew *et al.*, 2010). The gradual drying occurring in Fuirosos probably allowed structural and physiological adjustments in the stream autotrophs, as was reported in early studies on the effect of desiccation on algal communities (Davis, 1972; Peterson, 1996; Steinman and McIntire, 1990). Though not specifically observed in this study, algae and cyanobacteria are able to produce resistant structures, as well as extracellular mucilages to facilitate cellular water retention, or intracellular osmotic adjustments to increase resistance during the dry period (Hostetter and Hoshaw, 1970). At least some of this set of adaptations operated in Fuirosos, where the autotrophs remained dormant during the terrestrial phase but showed a rapid recovery after rewetting likely related to the physiological recovery of the dry algae and cyanobacteria that remained on the surface of the cobbles and sand (Dodds *et al.*, 1996; Robson, 2000; Robson *et al.,* 2008). The epilithic biofilm showed a slight increase in autotrophic biomass after the return of flow (reaching values common in analogous periods in the stream; Tornés and Sabater, 2010), and photosynthetic efficiency was remarkably higher than during drying and fragmented phases. This higher photosynthetic response occurred in parallel to the low values of the $OD_{430\ nm}/OD_{665\ nm}$ ratio that expressed the higher proportion of active chlorophyll in the rewetting phase. The recovery ability of the epilithic autotrophs was even more remarkable since limiting light conditions (low light irradiance, large CBOM accumulations) existed during the rewetting phase. The recovery of photosynthetic efficiencies was much slower in the epipsammic biofilm. Sandy biofilms showed lower and steadier values than those in the epilithic biofilm throughout the study period, this difference was probably related to the more stable humidity in sandy biofilms and that sandy compartments are less exposed than cobbles (Romaní *et al.*, 1998). There was no significant photosynthetic activity in any of the hydrological phases in the hyporheic biofilm, where light was not available. Though Chl-a is a surrogate of algal and cyanobaterial biomass, and as such can deviate from direct estimations of biomass (Stevenson, 1996), it was a practical estimate of the variations produced during the different phases in Fuirosos.

The heterotrophic component of the biofilm showed a higher capacity to withstand desiccation than the autotrophic component, especially in the epipsammic and hyporheic biofilms. Sandy substrata have higher capacity to preserve humid conditions and were less exposed to desiccation than cobbles, and most likely allowed for the better development

and survival of microorganisms (McKew *et al.*, 2010). Regardless of this capacity, both epipsammic and hyporheic biofilms showed higher percentages of live bacteria than the epilithic biofilm, indicating more favorable conditions for bacterial development (Romaní and Sabater, 2001). Streambed desiccation caused a decrease in the percentage of live cells in all biofilms because of almost complete dehydration, particularly in the epilithic biofilm which is the most exposed. However, this decrease was not consistent over time, as the percentage of live cells increased progressively during the terrestrial phase until reaching similar values to those observed before desiccation. This increase could be either caused by acclimation mechanisms to water stress or by a change in the microbial community composition in the stream biofilms during the terrestrial phase. Acclimation mechanisms could be both related to its capacity to withstand desiccation through the adoption of survival strategies, spores and dormant cells (Bär *et al.*, 2002; Schimel *et al.*, 2007), whereas changes in the community composition could be the result of the selection of the most resistant to water stress conditions among the existing species pool, or by the colonization of the dry streambed by terrestrial species (Fazi *et al.*, 2008). The sharp but short decrease of the percentage of live cells occurring with flow onset seems to indicate the latest option, but further analysis of the microbial community composition over the whole cycle of intermittency are needed to shed light on this issue.

Elemental and enzymatic stoichiometric responses

The physiological state of the stream microbial community is directly related to water availability (Fierer *et al.*, 2003; Rees *et al.*, 2006, Amalfitano *et al.*, 2008). As in upland soils, desiccation of stream and riparian sediments may cause physical disruption as well as changes in redox conditions and hydrophicity (Borken and Matzer, 2009), leading to changes in the microbial activity. The enzymatic assays testing the potential degradation of organic C, N and P compounds by the microorganisms (including those during the terrestrial phase, assimilated to those in moist and dry soils; Burns and Dicks, 2002) showed variable dynamics. The extracellular enzyme activities generally decreased during the terrestrial phase, but the potential capacity of the bacterial community to degrade C (BG) and P (AP) compounds remained, especially in the epipsammic and hyporheic biofilms. This did not stand for the N-related enzyme activity (LAP). The very low LAP values during this phase in the three biofilms indicated a potential restriction in the

biofilm capacity to degrade peptides. During the terrestrial phase, bacteria preferentially used C and P most likely aimed for energy use rather than N for growth (Schimel *et al.*, 2007).

The AP activity throughout the terrestrial phase was high in epipsammic and hyporheic biofilms, and remained relatively low in the epilithic biofilms. These differences were related to the specific impact of desiccation on the different stream biofilm compartments, the cobbles being more severely affected than the sandy compartments. While the primary producers greatly suffered during the dry conditions in the epilithic biofilm, the microorganisms in the sandy compartments (epipsammic and hyporheic) were predominantly heterotrophic and remained active during these harsh conditions. Maintaining AP activity reflected the importance of P as a key element for microbial metabolism (Healey and Hendzel, 1979; Mohamed *et al.*, 1998; Dodds, 2003). Carbon acquisition activity (expressed through BG) remained during the terrestrial phase, possibly due to the abundance of CBOM which provided substrate availability. Finally, the LAP activity was mostly depressed during the terrestrial phase, but showed a slight recovery at the end of this period with the first rain fall events that moistened cobbles and sand. The existence of enzyme activities during the terrestrial phase could be both related to the existence of free enzymes onto the surfaces that could be activated by the existing humidity, but also with the incoming terrestrial bacteria that occupied the dry streambed, and therefore provided new capacities for organic matter transformation. The differences between enzyme activities could be related to their substrate specificity. Most extracellular phosphatases hydrolyze phosphate from a wide range of substrates, therefore facilitating P acquisition from organic matter, but N acquisition is more complex because N is distributed among several classes of polymers as well as humic molecules (McGill and Cole, 1981; Manzoni *et al.*, 2008).

Extracellular enzyme activities reflect the interactions between microbial metabolism and resource availability (Sinsabaugh *et al.*, 2009). Therefore, the ratios of enzymes related to C, N and P degradation can be used to resolve energetic and nutrient constraints on microbial community metabolism. We applied this principle to the ratios between BG, LAP and AP activities, used as surrogates for the assimilation of organic C, N and P substrates by the biofilm during the drying-rewetting cycle. We observed an increase from

drying to rewetting in the BG:LAP and BG:AP ratios, related to the preferential use of C as energy source. The sequence was however mixed between the terrestrial and rewetting phases, where C use reached a plateau due to the limiting P assimilation. The stoichiometry (nutrient molar ratios) of the different biofilms indicated that the terrestrial phase was the most P limited, as well as richer in C than N.

An analogous pattern in the preferential use of C could be uncovered between the different biofilms. The initial values of the sequence corresponded to the epilithic, while the plateau values corresponded to the epipsammic and hyporheic biofilms. The epilithic biofilm, therefore, presented the lowest BG:LAP and BG:AP ratios, which indicated its major demand for N (LAP) and P (AP). This observation coincided with the lower C:N and C:P nutrient molar ratios in the epilithic biofilm. In this biofilm, algae and fine particles are abundant (Artigas *et al.,* 2008), and therefore these biofilms have the highest quality materials. Epipsammic and hyporheic biofilms showed similar patterns of higher C content and demand, especially during the terrestrial and rewetting phases when the availability of organic matter was high. Sandy biofilms are able to process large amounts of polysaccharides, related both to the high accumulation of organic matter on sand as well as to the structural simplicity of their biofilms (Marxsen and Witzel, 1991; Romaní and Sabater, 2001). These biofilms require less high-quality organic matter than the epilithic biofilm to maintain their own structure and functioning. The presence of algae, as well as the higher structural complexity of the biofilm growing on cobbles demands higher nutrient requirements (higher LAP and AP demand). These requirements could not be fulfilled during the terrestrial phase because of the loss of structural complexity with desiccation, but also because of the limited N and P breakdown capacities of the epilithic biofilm under dry conditions.

CONCLUSIONS

In conclusion, our findings of contrasting responses of autotrophic and heterotrophic processes in the different biofilms, suggest that increased flow intermittency as a consequence of climate and land use change is likely to increase the relative importance of heterotrophic processes in stream networks, as well as the relative contribution of the hyporheic biofilm to the C-N-P use. An increase of the non-flow period extent will impact

most on autotrophs recovery (loss of refuges and increased cell damage). Furthermore, the patterns observed during the terrestrial phase in Fuirosos suggest that the longer streams remain dry, the more the biofilm stoichiometry will differ, potentially leading to changes in the biogeochemical cycles at larger scales. Experimental manipulations of the extent of flow intermittency are needed to overcome the limitations of a field study like ours in which the streambed remained dry for a certain time period (112 days in our case). Moreover, further investigations should aim to characterize the microbial community composition in stream biofilms during the whole hydrological cycle, including the terrestrial phase, to determine whether acclimation mechanisms or substitution processes prevail during that phase.

Chapter 2.

Do streambeds change in colour when they dry? Photosynthetic pigment changes in biofilms during flow intermittency.

INTRODUCTION

Algae and cyanobacteria are the most important group of primary producers in streams and mid-sized rivers (Stevenson *et al.,* 1996). They develop on the different substrata occurring in the stream, and live in association with other microorganisms (bacteria, fungi and protozoa) within a matrix of polysaccharide exudates and detritus (Wetzel, 1983). These biofilms developing on the upper side of rocks, cobbles and sand are therefore the main source of autochthonous energy for higher trophic levels in the stream (Mayer and Likens, 1987). Substratum type, water chemistry, light availability, water temperature and flow are the main environmental factors that determine the algal assemblage structure and functioning (Burkholder, 1996; Bergey, 2005; Murdock and Dodds, 2007). Among these factors the one often determining the structure and functioning of biofilms is flow intermittency (Sabater *et al.,* 2000). Temporary streams are characterized by the occurrence of non-flow periods, and organisms are therefore periodically exposed to desiccation. Thus, flow intermittency may be viewed as a disturbance that affects the organisms dwelling in the stream (Lake, 2003). Unraveling the mechanisms behind the resistance and resilience to desiccation of algae and cyanobacteria in streams may help to understand and predict the ecosystem responses to flow intermittency. Lake (2013) defined resistance as the capacity of the biota to withstand a disturbance (*e.g.*, desiccation) without significant losses, whereas resilience is the capacity to recover from a disturbance (*e.g.*, when flow resumes) after incurring in losses. Climate change and its associated rising unpredictability and severity of drought events are increasing the duration and spatial extent of non-flow periods in streams and rivers worldwide (Milly *et al.,* 2005; Hirabayashi *et al.,* 2008; Döll and Schmied, 2012). Furthermore, fluvial ecosystems affected by flow intermittency comprise a substantial proportion of the total number, length and discharge of fluvial networks globally (Larned *et al.,* 2010). Within this scenario, understanding the response of primary producers in temporary streams, including the non-flow period, is relevant to predict the potential biogeochemical and ecological implications of flow intermittency in systems that are either intermittent and become ephemeral, or that are permanent and become intermittent.

An extensive classical literature about tolerance and recovery to desiccation already exists. There is good knowledge of the resistant structures such as zygotes, akinetes, or cysts that algae produce under unfavourable conditions and that allow the cells to be still viable upon rewetting even after months, years and decades of desiccation (Davis, 1972). These structures are widespread among algal classes and cyanobacteria, and in spite of their facultative formation, are rather specific (Evans, 1958, 1959). The absence of water or its episodic occurrence also induces structural changes in algal cells to promote resistance (Morison and Sheath, 1985). These include mucilage production, or wall thickening, but also the production and accumulation of internal low molecular weight substances that protect the cell against osmotic changes (Collyer and Fogg, 1955; Evans, 1958, 1959). The photosynthetic apparatus can become damaged when deprived of water and cells are exposed to extreme solar radiation (Billi and Potts, 2002). A decrease in Chlorophyll-a (Chl-a) and changes in pigment composition can be seen both as a consequence as well as a response to protect the cells (Angradi and Kubly, 1993; Fleming and Castenholz, 2007). These structural changes can have considerable adaptive benefits to algal resistance and recovery. It has been shown that cyanobacterial mats in Antarctic environments recover photosynthetic activity similar to rates before desiccation, in few minutes (Vicent and Howard-Williams, 1986; Hawes *et al.,* 1992; McKnight *et al.,* 2007). Romaní and Sabater (1997) also observed the high resilience of stromatolite-like structures to quickly return to the photosynthetic activity they had before its complete desiccation. The frequency of exposure to desiccation influenced the rate of recovery in a regulated river, where algae required between 1 to 4 months to achieve Chl-a values similar to pre-desiccation (Blinn *et al.,* 1995). It is therefore obvious that the rate and frequency of desiccation affects the permanence of refuges in the stream (Stanley *et al.,* 2004; Ledger *et al.,* 2008), and that algae and cyanobacteria survive slow desiccation better than rapid desiccation (Evans, 1959; Hostetter and Hoshaw, 1970). Also the type of substratum where algae and cyanobacteria grow influences the potential to preserve humidity during desiccation and host recolonization when rewetting (Romaní and Sabater, 1997; Sabater, 2000; Robson *et al.,* 2008).

Algae and cyanobacteria contain characteristic pigments and combination of pigments (marker pigments) depending on the Division or Class (Millie *et al.,* 1993; Jeffrey *et al.,* 1997). Carotenoids are important in all photosynthetic membranes because of their ability

to prevent photooxidative damage and to harvest light. Pigment composition has been successfully used to determine the response of algae and cyanobacteria to stressful situations such as high irradiances, or exposure to heavy metal and xenobiotic compounds (Dorigo *et al.,* 2004; Dieser *et al.,* 2010; Corcoll *et al.,* 2012). Algae and cyanobacteria synthesize accessory pigments such as carotenoids to adaptively protect the cell against oxidative damage (Armstrong and Hearstt, 1996). It might be expected that similar responses in the dynamics of pigments composition of benthic algae and cyanobacteria would also occur in temporary ecosystems (Caramujo *et al.,* 2008). Davis (1972) suggested that algae produce carotenoids as a photoprotection mechanism to desiccation (which is usually associated with high irradiances). Carotenoid synthesis would therefore be a mechanism to reduce the radiation impinging on the sensitive structures within the cell when they are dry. Garcia-Pichel and Castenholz (1991) showed that the formation of scytonemin (an UV-absorbing pigment) was a physiological adaptation of cyanobacteria to high irradiances and desiccation processes (Hills *et al.,* 1994; Fleming and Castenholz, 2007). Also, an increase in chlorophyll degradation products may result from physiological stress during desiccation and rewetting processes in intermittent streams (Daley, 1973). Therefore the analysis of pigment composition of algae and cyanobacteria could provide valuable information to better understand the mechanisms behind their resistance and resilience to flow intermittency.

In this study we characterized the changes occurring in the algae and cyanobacteria colonizing three different streambed compartments (the epilithic is the biofilm growing on cobbles, the epipsammic is the biofilm growing within the sand grains and the hyporheic is the biofilm growing within subsurface sediments) in a Mediterranean intermittent stream. We carefully examined the biofilm responses during the three hydrological phases encompassing intermittency in the stream (drying, non-flow and rewetting phases) by means of pigment composition and microscope observations. We hypothesize that *i*) algae will have a higher resistance to desiccation (in terms of Chl-a) in sand than on cobbles, because of the higher sand ability to retain humidity and the lower exposure of this compartment to desiccation and its associated environmental changes. *ii*) The non-flow phase will result in the accumulation of some protective carotenoids as a physiological adaptation to desiccation, as well as the formation of resistance structures that protect the cells during unfavourable growth conditions. These physiological and structural

adaptations will confer resistance to the biofilm, favouring its recovery when rewetting. *iii*) Changes in marker pigment composition will differ between biofilm types owing to the corresponding differences in algal assemblage structure. *iv*) An encrusting algal growth-form will confer protection to the assemblage and would determine the faster or slower recovery of their functionality after flow resumption.

METHODS

Study site and field sampling

This study derives from the field monitoring study in the Fuirosos that was performed from May to December 2009. Three hydrological phases with different survey intensity were defined: *i*) drying phase (*D*; biweekly and weekly sampling at the end of this phase); *ii*) non-flow (*NF*) which last 112 days (biweekly sampling); and *iii*) rewetting phase (*R*; firstly sampled every three days from the flow resumption until one week, then biweekly again).

Biofilm from the three different streambed compartments (EL-epilithic, ES-epipsammic and H-hyporheic) were collected on each sampling occasion in order to analyze benthic algal changes occurring during the different phases. Samples for pigment analysis were kept dark and cool before freezing (-20 ^{0}C) within 3-6 h of sampling. Samples were preserved frozen until extraction and HPLC analysis. Samples for algae identification were placed separately in 20 mL glass vials with 5 mL of stream water, and preserved with 4 % formaldehyde.

Light availability at the study reach was relatively low due to a closed canopy, with maximum values of 70 µE m^{-2} s^{-1} during the non-flow period. Complementary pigment analyses were performed during the non-flow phase in a downstream reach receiving higher irradiances throughout the year that reached ca. 100 µE m^{-2} s^{-1} during the flow periods and with maximum values of 260 µE m^{-2} s^{-1} during the non-flow phase. Both reaches also differed in that flow resumption occurred 10 days later in the downstream reach. The main conclusions of this study are based on the results from the study reach, where desiccation was not tightly associated to excess light exposure.

Detailed study site, sampling strategy and environmental parameters are explained in the Methodology section and in Chapter 1.

Laboratory analyses

The frozen biofilm samples were freeze-dried over 12 h (Telstar freeze dryer, Lyoalfa 6-80; Telstar, Barcelona, Spain) and pigments were extracted in 4 mL 90% acetone for 24 h in the dark at 0 ^{0}C to minimize pigment degradation (Bidigare *et al.,* 2005). Complete extraction of pigments was ensured with sonication (1 min, 360 W power, 50 ⁄60 Hz frequency, Selecta, JP Selecta SA, Barcelona, Spain). The extract was filtered through Whatman Anotop filters (0.1 µm pore size, 25 mm diameter) (Whatman International Ltd., Maidstone, England) and blown to dryness under a stream of N_2. The concentrated residue was re-dissolved in 500 µL of methanol and analyzed by HPLC. Samples were analyzed with an Agilent HPLC G1315D Photodiode array detector (Agilent Technologies, Darmstadt, Germany) on a C_{18} column (5 µm, 250 mm x 4.6 mm, Spherisorb-ODS1 Waters, Milford, USA) using the gradient flow described by Buchaca and Catalan (2007). The detector was set at 440 and 660 nm for carotenoid and chloropigment peak integration, respectively. Pigment standards (β-carotene, chlorophyll-a, chlorophyll-b, chlorophyllide-a, diatoxanthin, fucoxanthin, lutein, myxoxanthophyll, phaeophorbide-a1, phaeophytin-a1 and zeaxanthin; DHI Water and Environment, Denmark) were run individually and as a mixed standard to determine retention times and spectra. Pigment identification was performed by comparison of retention times and absorption spectra. Peaks not corresponding with pigment standards (Car-11, Car-8, canthaxanthin, chlorophyll-a allomer 1, chlorophyll-c1, chlorophyll-c2, diadinoxanthin, echinenone, neoxanthin, phaeophorbide-b1, phaeophythin-a2, -b1, -b2, scytonemin and violaxanthin) were identified by comparison with a library of pigment spectra obtained from Buchaca (2009). Extinction coefficients used for calculations were obtained from the literature (Davies, 1976; Rowan, 1989; Jeffrey *et al.,* 1997). In the case of unknown peaks we used an assumed extinction coefficient of 2000 for carotenoids (Mantoura and Llewellyn, 1983) and the extinction coefficient value of the closest peak in terms of spectral characteristics in the case of phorbins. Peak areas were converted to concentrations and then pigment measurements were related to the substratum surface area (cm^2). The surface area of sediments was obtained after granulometric analysis and the application of a

conversion factor between sediment dry mass and surface area (1.003 g DW cm^{-2}; Romani and Sabater, 2001), and then the pigment concentration was expressed as ng cm^{-2}.

Analysis, identification, and observation of resistance structures of the algal and cyanobacterial community were done by microscopic examinations at 600x (Nikon CS1, Tokyo, Japan). Tiles and sand were sonicated twice for 1min to separate algae gently (1 min, 360 W power, 50⁄60 Hz frequency, Selecta, JP Selecta SA, Barcelona, Spain). Algae and cyanobacteria were estimated by their qualitative abundances based on cell numbers and ranked on the following scale: 1 (very rare, ≤ 5 %), 2 (> 5 to ≤ 20 %), 3 (> 20 to ≤ 40 %), 4 (> 40 to ≤ 60 %), 5 (> 60 to ≤ 80 %) and 6 (very abundant, > 80 %) at genera level.

Both for pigment analysis and algae identification a total of 15 samples were collected in each sampling date (5 replicates of each biofilm type, see the Methodology section), the five replicates of each biofilm type were pooled together in order to account for the within-site variability and a composite sample from each biofilm type was analyzed.

Data analysis

Physiological changes during the study period were assessed by means of the Chl-a degradation index and the occurrence of carotenoids with a role in the protection against photo-oxidative damage (protective carotenoids) during the non-flow phase. The Chl-a degradation index (CD/a-phorb) was used to evaluate the chlorophyll degradation process over the study period. This index is the ratio between the Chl-a derivatives (CD), which are the Chl-a degradation products (chlorophyll-a allomer 1, chlorophyllide-a, phaeophorbide-a and phaeophythin-a1, -a2,) and the a-phorbins (a-phorb), which are the sum of the Chl-a and CD. For this ratio, a value of 1 or around 1 means a complete degradation of the Chl-a (adapted from Moss, 1968). Carotenoids were assumed to appear in biofilms to protect cells against photo-oxidative damage, in response to excess irradiance or water stress during desiccation. The relative importance of these environmental factors on the carotenoids occurrence was obtained by comparing the carotenoids occurrence of the study reach with the ones occurring in a nearby reach with similar flow regime but higher irradiances. It was assumed that carotenoid occurrence in both reaches would indicate water stress as a main cause of carotenoids occurrence, whereas the only

occurrence in the downstream reach might indicate that irradiance rather than water stress could be affecting the carotenoids presence.

Differences between biofilms and hydrological phases in terms of Chl-a concentration and Chl-a degradation index were examined using two-way analysis of variance (ANOVA) using SPSS for Windows (version 17.0; SPSS, Chicago, U.S.A.). Tukey's HSD multiple comparison test was used to examine the differences between biofilms and hydrological phases, the significance level was set at $P < 0.05$ for all analyses. Chl-a concentration was log-transformed (x = log (100x+1) to meet the assumptions of parametric tests. Overall pigment composition on the three biofilms during the different hydrological phases and their relation to environmental variables were examined by multivariate analyses. Data were based on the pigment concentration in ng cm^{-2} of the three analyzed biofilms for the 22 sampling dates; log transformation (x = log (100x+1)) was applied to normalize the variables. A detrended correspondence analysis (DCA, Hill and Gauch, 1980) was used to determine the length of the gradient of the data. DCA indicated that the maximum gradient length was shorter than 3 SD units (2.147). Therefore, the use of linear ordination techniques was appropriate (ter Braak and Smilauer, 2002). Accordingly, principal component analysis (PCA) was performed using CANOCO for Windows (Version 4.5, Microcomputer Power, Ithaca, USA). Shifts in the pigment composition observed in the PCA representation were assessed using a PERMANOVA of two factors using Euclidean distance (biofilm type and hydrological phase), performed with PRIMER-E v.6.1.11 & PERMANOVA + v.1.0.1 (PRIMER-E Ltd., Ivybridge, UK). In addition, the pigment ordination was related to several key environmental variables (percentage of water content, temperature and PAR) by correlating the first two PCA axes (Pearson coefficient) at a significance level of $P < 0.05$. Differences in the algal composition (microscope observations) between biofilms and hydrological phases were assessed using a PERMANOVA of two factors (biofilm type and hydrological phase) based on the ranked scores, which were square root transformed, Bray–Curtis was selected as a dissimilarity distance.

RESULTS

A total of 14 carotenoids and 12 chloropigments were identified in the three different biofilms (Supplementary Table S1) during the study period. Chl-a concentration differed significantly among biofilms and hydrological phases (Table 1). Chl-a concentration ranged from 19 to 951 ng cm^{-2} in the epilithic, from 14 to 814 ng cm^{-2} in the epipsammic and from 6 to 122 ng cm^{-2} in the hyporheic. During the non-flow phase the lowest values of Chl-a were observed in the epilithic, when a 95 % decrease of Chl-a concentration occurred. However nearly a 90 % of the Chl-a in the epilithic was recovered after 3 days of rewetting. In the epipsammic, a 75 % decrease of Chl-a concentration occurred during non-flow, but a slight increase was observed by the end of this phase. A recovery of 49 % occurred after 3 days of rewetting, but values remained low during the rewetting phase. The hyporheic biofilm showed low and relatively steady values of Chl-a during the study period, however Chl-a decreased by 65% during non-flow, and values did not recover during the rewetting phase (Fig. 1 a).

The degree of Chl-a degradation was assessed by means of the index which ranged from 0.23 to 0.92 during the study period (Fig. 1 b). Significant differences in this index occurred between hydrological phases, but not between biofilms (Table 1). The lowest values of this index (meaning higher proportion of active Chl-a) were observed in the epilithic during drying and rewetting phases. The maximum values of Chl-degradation occurred in the epilithic and hyporheic (0.92) during the non-flow phase, when the epipsammic showed lower values (0.71). During the rewetting phase the Chl-a degradation index increased in the epipsammic but decreased in the epilithic (that had the highest proportion of active Chl-a), and in the hyporheic remained high.

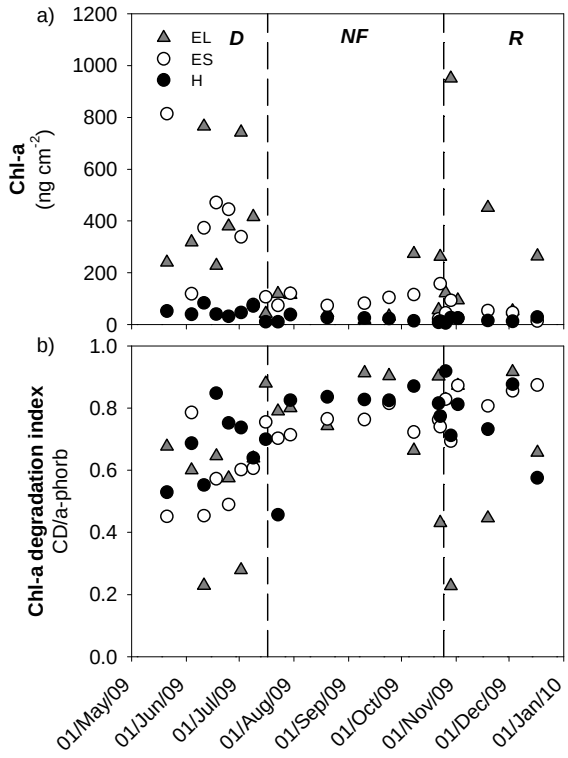

Figure 1. a) Temporal changes in Chl-a and b) Chl-a degradation index for each biofilm (EL-epilithic, ES-epipsammic and H-hyporheic). Hydrological phases (*D*-drying, *NF*-non-flow and *R*-rewetting) are differentiated with vertical dashed bars.

Table 1. Two-way ANOVA results for the Chl-a and the Chl-a degradation index, considering single source effects and interactions of the two factors, biofilm type and hydrological phase. *P*-values < 0.05 are indicated in boldface type.

Sources	Chl-a	Chl-a degradation index
Biofilm	***P* < 0.001**	*P* = 0.241
	$F_{2,57}$ = 37.169	$F_{2,57}$ = 1.457
Phase	***P* < 0.001**	***P* = 0.001**
	$F_{2,57}$ = 15.386	$F_{2,57}$ = 8.213
Biofilm*Phase	***P* = 0.043**	*P* = 0.171
	$F_{4,57}$ = 2.632	$F_{4,57}$ = 1.665

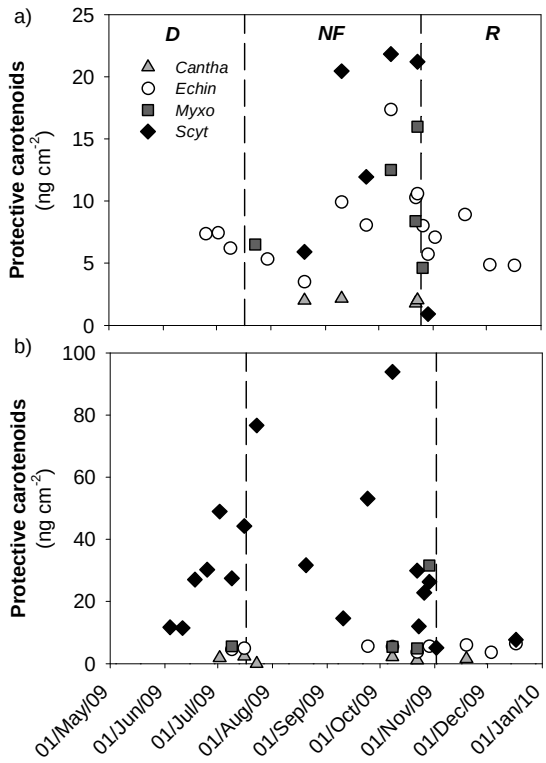

Figure 2. a) Protective carotenoids occurring on the epilithic biofilm in the study reach and b) in the downstream reach. Canthaxanthin (*Cantha*), Echinenone (*Echin*), Myxoxanthophyll (*Myxo*), Scytonemin (*Scyt*). Hydrological phases (*D*-drying, *NF*-non-flow and *R*-rewetting) are differentiated with vertical dashed bars.

Several protective carotenoids were measured in the epilithic (Fig. 2). Canthaxanthin, echinenone, myxoxanthophyll and scytonemin characterized the non-flow phase. Echinenone was evident in the study reach (3.5 to 17.4 ng cm^{-2}; Fig. 2 a) during non-flow phase, by it was only observed in the downstream reach by the end of the non-flow phase; echinenone remained during the rewetting phase. Canthaxanthin, myxoxanthophyll and scytonemin were detected during non-flow and persisted during the early rewetting phase (Fig. 2 a and b). Scytonemin occurred much earlier, during the drying phase, and achieved higher concentrations (up to 94 ng cm^{-2}) in the complementary reach. In the epipsammic and hyporheic the unidentified carotenoid car-11 and lutein were measured during the non-flow phase.

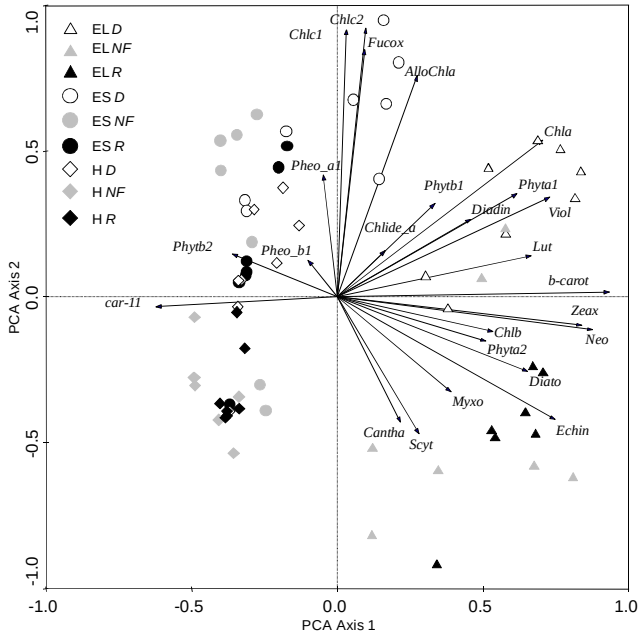

Figure 3. Principal component analysis (PCA) with the pigments analyzed on each biofilm compartment (EL-epilithic, ES-epipsammic and H-hyporheic) during the different hydrological phases (*D*-drying, *NF*-non-flow and *R*-rewetting). β-carotene (*b-carot*), canthaxanthin (*Cantha*), diadinoxanthin (*Diadin*), diatoxanthin (*Diato*), echinenone (*Echin*), fucoxanthin (*Fucox*), lutein (*Lut*), myxoxanthophyll (*Myxo*), neoxanthin (*Neo*), scytonemin (*Scyt*), violaxanthin (*Viol*), zeaxanthin (*Zeax*), unidentified carotenoid (*car-11*), chlorophyll-a (*Chla*), chlorophyll-b (*Chlb*),chlorophyll-c1 and c2 (*Chlc1* and *Chlc2*), chlorophyllide-a (*Chlide_a*), phaeophorbide-a1 and b1 (*Pheo_a1* and *Pheo_b1*)and phaeophytin-a1, a2, b1 and b2 (*Phyta1*, *Phyta2*, *Phytb1* and *Phytb2*).

The temporal changes in pigment composition were summarized by means of a PCA (Fig. 3). Pigment composition differentiated the epilithic from the epipsammic and hyporheic assemblages along the first axis (34.3 % of the total variance). The second axis (24.7 % of the total variance) separated many of the drying phase assemblages from the non-flow and rewetting assemblages based on differences in carotenoid composition. Accordingly, the PCA scores of the second axis were negatively correlated with the percentage of water content and temperature (r = -0.297, P = 0.015; r = -0.290, P = 0.018 respectively), but not with PAR values (P = 0.711). Furthermore, the results of the PERMANOVA confirmed the separation in pigment composition among the different biofilms (*pseudo-*$F_{2,57}$ = 23.766, P = 0.001) and hydrological phases (*pseudo-*$F_{2,57}$ = 7.887, P = 0.001) as well as among the levels of each factor (biofilm type*hydrological phase *pseudo-*$F_{8,57}$ = 9.049, P = 0.001). The epilithon had the largest and most variable number of pigments. β-carotene, lutein, violaxanthin, diadinoxanthin, zeaxanthin, neoxanthin and fucoxanthin prevailed

during drying, while canthaxanthin, diatoxanthin, echinenone, lutein, myxoxanthophyll, neoxanthin, scytonemin, and zeaxanthin were characteristic of the epilithic assemblages during the non-flow and rewetting phases. Epipsammic and hyporheic assemblages had a more reduced number of pigments. Fucoxanthin, chlorophyll-c1 and -c2 and in lower proportion lutein, chlorophyll-b and for β-carotene were observed during the drying phase in the epipsammic, and their presence decreased during the non-flow and rewetting phases. These pigments were also observed in lesser proportion in the hyporheic during the entire study period.

Microscope observations revealed differences in the algae and cyanobacteria composition among the different biofilms (*pseudo-*$F_{2,57}$ = 47.858, P = 0.001). However the community composition did not experience significant changes throughout the different hydrological phases (*pseudo-*$F_{2,57}$ = 1.5974, P = 0.215). Results from the interaction between biofilm type and hydrological phase pointed out that differences among the different biofilm compartments existed throughout the study period (*pseudo-*$F_{8,57}$ = 12.728, P = 0.001) (Supplementary Table S2). The epilithon was mainly dominated by Bacillariophyta (45 %), Cyanobacteria (25 %), Chlorophyta (20 %), and Rhodophyta (9 %) (Fig. 4 a). Epilithic diatoms such as *Achnanthidium minutissimum* and *Cocconeis placentula* coexisted with encrusting forms *Gongrosira* spp, and *Pleurocapsa* spp. during the entire study period, but were more abundant during the non-flow phase. The encrusting red alga *Hildenbrandia rivularis* was abundant during the non-flow phase, but declined following rewetting. Bacillariophyta were the main algal group (70 %) in the epipsammic, while Cyanobacteria (18 %), Chlorophyta (8 %) and Rhodophyta (2 %) were also present (Fig. 4 b). *Chlorococcus* spp. occupied the spaces between the sand grains during the entire study period, coexisting with diatoms, *Achnanthidium minutissimum*, *Planothidium lanceolatum*, *Cocconeis placentula*, *Gomphonema* spp. and *Meridion* spp. were the most common taxa. In the hyporheic Bacillariophyta was also the main algal group (85 %), and *Achnanthidium minutissimum* the most abundant taxa during all the study period. Cyanobacteria (6 %) and Chlorophyta (8 %) were present in minor proportions (Fig. 4 c). Some Euglenophyta (*Trachelomonas* spp.) were occasionally observed in all the biofilms (1 %) during drying and non-flow. Microscopic observations also revealed the occurrence of structural cell adaptations in the biofilm during desiccation, particularly during the non-flow phase (Fig. 5). Cells in the epilithic were organized into a dry tightly adhering layer similar to a crust

(Fig. 5 b). The epilithic developed a superficial protective layer rich in mucilage, and wall thickening was rather common together with the occurrence of resting spores and cyst formation (Fig. 5 c, d and e). Biofilm bleaching was also observed in the epilithic, where gray-white patches identified as filamentous green algae (*Oedogonium* sp.) remained on the cobbles and its resistant spores occurred beneath the bleached filamentous mat (Fig. 5 a and f). In the epipsammic and hyporheic cells only some spores were observed, but the reduction or/and colour change (from green to yellow and orange-red) of the chloroplast of many diatoms cells was observed (Fig. 5 g and h).

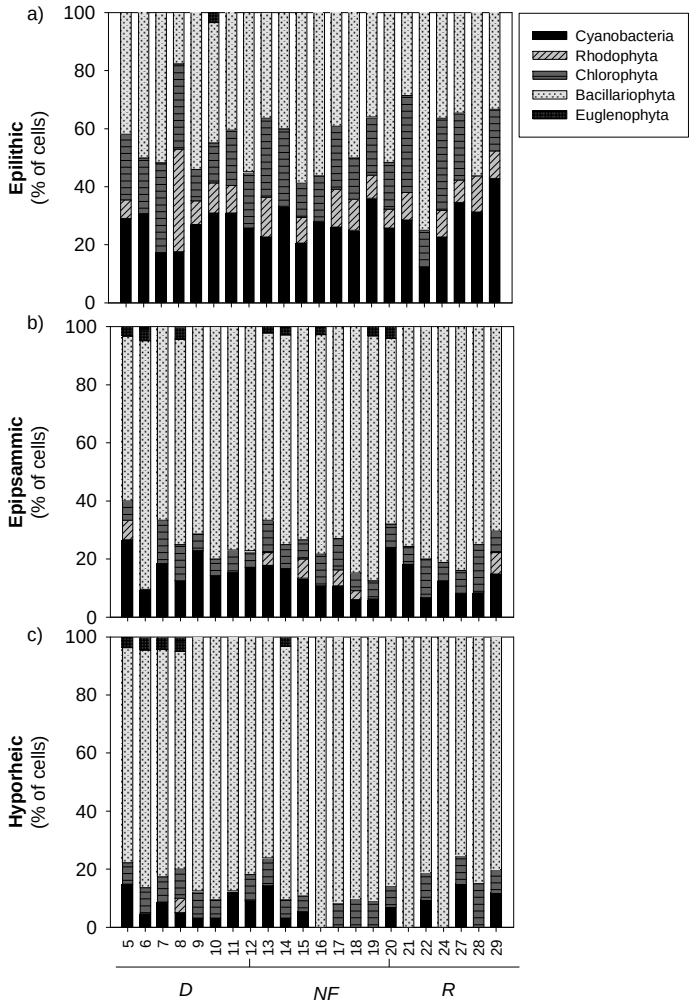

Figure 4. Temporal changes in a) epilithic, b) epipsammic and c) hyporheic biofilm of each algal classes observed by microscopy in Fuirosos throughout the different hydrological phases (*D*-drying, *NF*-non-flow and *R*-rewetting).

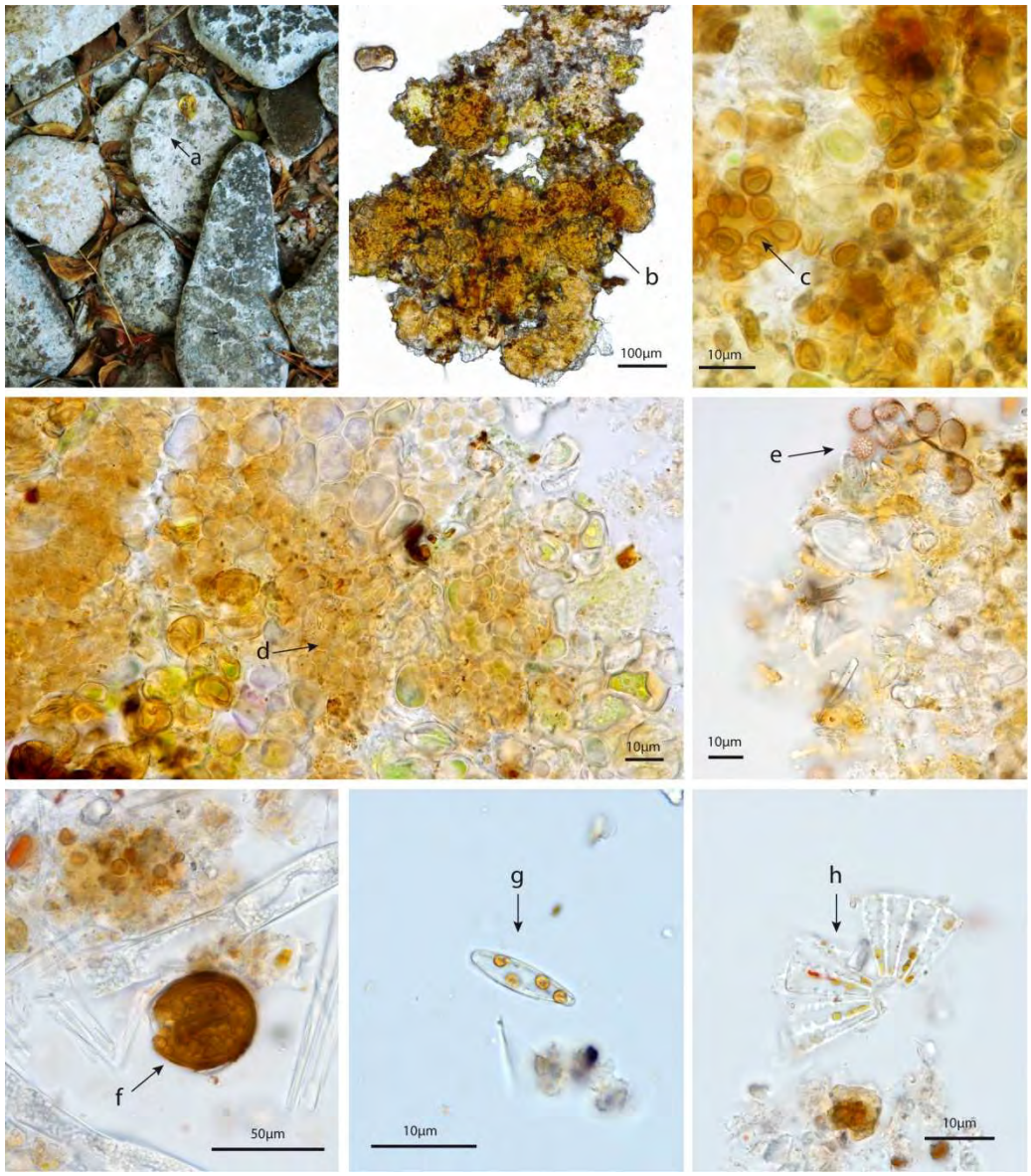

Figure 5. Cell resistance structures observed in the algal community during the non-flow phase. In the epilithic: bleached dry biofilm (a), general view of the crust formation (b), enlarged membrane thickness (c), change in colouration (d), spores (e) and a resistant spore of *Oedogonium sp*. (f). In the epipsammic: chloroplast reduction (g). In the hyporheic: chloroplast reduction (h).

DISCUSSION

Algae and cyanobacteria in the Fuirosos stream showed low resistance to flow intermittency, as it might be inferred from the 60 to 90 % reduction in active chlorophyll-a (Chl-a) during the non-flow phase. In contrast, fast recoveries of Chl-a when flow resumed indicated high resilience. Pigment composition revealed that the algal community in the epilithic was considerably different than the ones in the epipsammic and hyporheic. These differences were accordingly to the physical configuration of each biofilm that resulted in differences on the resistance and resilience to flow intermittency. The synthesis of protective carotenoids as well as the occurrence of cell resistance structures, observed in Fuirosos during the non-flow phase could be interpreted as mechanisms against desiccation stress to ensure survival (Davis, 1972; Peterson, 1996). Therefore, these mechanisms are likely important in enhancing resistance and resilience of algae and cyanobacteria in intermittent streams.

Photosynthetic pigment adaptations to flow intermittency

The low values of Chl-a occurring in Fuirosos during the non-flow phase suggested that algae and cyanobacteria likely remained inactive. Measurements of photosynthetic efficiency values, measured in a separated study (chapter 1), were nearly zero until the end of the non-flow phase indicating that many algal and cyanobacterial cells die and/or become dormant when desiccation occurs (Timoner *et al.,* 2012). Maximum values of Chl-a were observed in the epilithic, however during the non-flow phase the lowest values of Chl-a occurred in the epilithic indicating low resistance of algae and cyanobacteria growing on this compartment. Chl-a in the epipsammic also decreased due to desiccation, whereas steady low values of Chl-a were observed in the hyporheic throughout the study period, most likely due to the light limitation in this compartment. By the end of the non-flow phase Chl-a slightly increased in the superficial biofilms (epilithic and epipsammic) as a result of small rainfall events that enhanced the streambed humidity. Thus, algae and cyanobacteria showed the capacity to respond to these small rehydration events that might contribute in the recovery of biofilm activity immediately after flow resumption. Algae and cyanobacteria on the epilithic recovered immediately after flow resumption showing a high resilience, whereas Chl-a concentration in the epipsammic remained low during the rewetting phase. The Chl-a degradation index reached its maximum values in the epilithic

(near 1) confirming the physiological stress that algae experienced during the non-flow phase. Chl-a degradation index in the epipsammic during the non-flow phase was lower than in the epilithic and hyporheic, where the low concentration of Chl-a also experienced degradation due to flow intermittency. The rewetting phase, however, showed divergent responses in the different compartments: while the Chl-a degradation index decreased considerably in the epilithon, it remained high in the epipsammon and hyporheos. This difference suggests that the low concentration of Chl-a, which remained in the epilithon after the non-flow phase, could be rapidly activated during the flow resumption contrary to algae in the epipsammon that did not show the same capacity to recover. Overall, differences observed in the resistance and resilience of Chl-a between the different biofilms are especially informative of the role of the substratum where the biofilm grows in determining the biofilm responses to flow intermittency.

In various groups of microalgae environmental stresses such as high PAR irradiance and/or nitrogen deficiency bring about profound changes in photosynthetic apparatus structure and function as well as in lipid and pigment metabolism (Torzillo *et al.,* 2003; Solovchenko *et al.,* 2008). Light availability during the non-flow phase in the Fuirosos was relatively low (70 µE m^{-2} s^{-1}) due to the thick riparian canopy (Sabater *et al.,* 2008; Timoner *et al.,* 2012), and is well below the range that has been shown experimentally to induce carotenoid synthesis (Rabbani *et al.,* 1998). We suggest therefore that water stress concomitant to high summer air temperatures may have been important factors affecting chlorophyll degradation and carotenoid synthesis in the present study. Two basic types of pigments are produced to protect algae tissues from UV degradation that have been also observed when other stressor such as desiccation occur. The first one is the scytonemin, a coloured pigment that occurs in the extracellular polysaccharide sheaths of cyanobacteria, stopping incoming radiation (Garcia-Pichel and Castenholz, 1991). The synthesis of scytonemin has been observed in microbial mats (Fernández-Valiente *et al.,* 2007) and in soil crust biofilms exposed to desiccation and high solar radiation (Belnap *et al.,* 2007). A second group of pigments captures free radicals generated by UV penetration within the cell (Adams *et al.,* 1993); this includes echinenone, canthaxanthin, β-carotene, lutein, zeaxanthin, and myxoxanthophyll. These protect cells from lethal singlet oxygen generated by UV or other stress situations that produce cell oxidative damages (Karsten *et al.,* 1998). Some of these protective carotenoids occurred in dry biofilms in Fuirosos, especially more

in the epilithic than in the epipsammic and the hyporheic assemblages, probably because it was the most exposed to desiccation and the associated environmental changes. Echinenone was the first carotene detected by the end of the drying phase that consistently increased in concentration over the non-flow phase and persisted during the rewetting phase. This was followed by scytonemin accumulation during the non-flow phase, which later decreased when the flow resumed. Canthaxanthin and myxoxanthophyll was also produced after desiccation during the non-flow phase. The occurrence of these protective carotenoids due to flow intermittency was evident under low light availability. Thus, the present data suggest that the synthesis of these protective carotenoids occurred as a response to desiccation stress, more than as a photoprotective mechanism. Early synthesis of scytonemin occurred in the downstream reach (in early spring), where light availability was higher, during the non-flow phase scytonemin increased 50 % associated to desiccation and high irradiances. Similarly, Fleming and Castenholz (2007) found that desiccation-tolerant cyanobacteria concentrated more scytonemin when algae where exposed to desiccation and high light irradiances (~ 250 µE m^{-2} s^{-1}), than when algae were hydrated at the same light conditions. Accordingly, Bergey *et al.* (2010) showed that the riparian canopy shade minimizes harsh conditions in freshwater habitats by reducing the effects of desiccation on algal assemblages. Echinenone, canthaxanthin and myxoxanthophyll may all have been also produced in response to desiccation. Overall, the occurrence of protective carotenoids under stressful conditions may reflect an adaptive strategy to reduce long-term energy costs associated to cell repair (Fleming and Castenholz, 2007). In the Fuirosos, these physiological adjustments were evident in the epilithic biofilm, the most exposed to desiccation but the one that invested more in protection against desiccation by the synthesis of carotenoids. Thus, the accumulation of these protective carotenoids might help in the preservation of the photosynthetic machinery and the remaining Chl-a during the non-flow phase, facilitating its physiological recovery.

Changes in community composition and physiological cell adaptations due to flow intermittency

Both pigment composition and microscope observations revealed differences in terms of community composition and adaption to desiccation. A greater diversity of algal classes coexisted in the epilithic than in the epipsammic and hyporheic assemblages. In addition,

taxonomic structure was likely simpler in the latter habitats because of substratum instability and lower light availability. Bacillariophyta preferentially colonized the epipsammic and hyporheic biofilm, and were the most sensitive to desiccation, thus their participation in the recovery was minor. This was evidenced by the considerable decrease in the main marker pigment of this group (fucoxanthin) during flow recovery. The remaining diatom cells during the non-flow phase showed reduction (or even loss) of their chloroplasts. Instead, Cyanobacteria were apparently the most resistant to desiccation and were provided with protective carotenoids such as the scytonemin, echinenone and canthaxanthin, which are commonly synthesized under stressful environments (Takaichi, 2011). Canthaxanthin, lutein and chlorophyll-b (marker pigments of Chlorophytes) also decreased during the non-flow phase, but the presence of canthaxanthin could be indicative of the adaptative response of chlorophytes to flow intermittency (Takaichi, 2011), which were observed on the microscope examinations. Results of the current study suggest that prolonged intermittency may result in alterations to biofilm assemblage structure, particularly shifts towards cyanobacteria, which has also been observed elsewhere (Robson *et al.*, 2013).

The persistence of algal cells observed in the epilithic may also be related with the tightly adhered growth-form of algae on this compartment. The dry epilithic biofilm formed a protective crust and beneath it mucilages further protected the cells. Cell wall thickening was also a common response during desiccation, together with the change in algal colour (from green to yellow-orange) and the formation of spores. Therefore, crust formation may have helped to preserve and capture humidity, and conferred a refuge to the other biofilm cells living underneath. Crust formation is common in many semi-arid and arid ecosystems around the world (Belnap and Lange, 2003), and allows cells to become metabolically active only when wet. Thus if longer non-flow periods in the future occur, the recovery of the autotrophic processes is likely to come from dry biofilm on stones rather than from other refuges following the dry phase (Robson *et al.*, 2000).

CONCLUSIONS

Flow intermittency resulted in differential effects on the different biofilms where algae and cyanobacteria were present. The epilithic biofilm experienced the largest changes in

pigment composition but was most resilient to flow intermittency. This more complex structure showed crusts analogous to those in arid and semiarid regions, exhibiting a greater resilience to flow intermittency and ability to re-grow after flow resumption. Algae cells on the epilithic biofilm, the most sensitive to desiccation were the ones that most invested in cell protection against desiccation. The synthesis protective carotenoids during the non-flow phase prevented damage in the photosynthetic apparatus and accounted for the rapid reactivation of the Chl-a remaining in the cells. These cells might have a high capacity to synthesize Chl-a using the photosynthetic capacity offered by the Chl-a that persisted the desiccation, and resume their functioning in favourable conditions. Flow intermittency caused instead fewer changes in the epipsammic and hyporheic biofilms, most likely due to the simpler structure of these biofilms and the protective effect of the substratum in which they grow, which is less affected by desiccation and air exposure. Biofilm growing in these compartments was more resistant and therefore need less cell adaptations, but also less resilient to flow intermittency, presumably due to the environmental conditions during the flow resumption, that did not allow algae to grow. Overall, results from this study shed some light on the resistance and resilience capacity of algae and cyanobacteria to flow intermittency and suggest that the persisting desiccated biofilm is structurally and functionally adapted to intermittency, especially on cobbles and rocks, and might play a crucial role in recovering the ecosystem functions after rewetting in streams that periodically experience non-flow periods. This study also highlighted the effects of light irradiances and high air temperatures that might occur together with desiccation, associated to global change, and that the resistance and resilience capacity of algae and cyanobacteria are essential to the ecosystem.

SUPPLEMENTARY TABLES

Supplementary Table S1. Pigment composition on epilithic (EL), epipsammic (ES) and hyporheic (H) biofilms for each hydrological phase (numbers indicate the different samplig date).Values are pigment concentration in ng cm-^{2}.

PIGMENT	*Drying*							
	5 EL	6 EL	7 EL	8 EL	9 EL	10 EL	11 EL	12 EL
β,β-carotene	22.2	14.8	25.0	13.9	24.4	25.0	26.8	8.7
car-11	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
car-8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Canthaxanthin	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Chlorophyll-a	240.1	318.6	765.5	227.6	380.3	742.7	416.1	42.5
Chlorophyll-a alomer 1	0.0	5.1	18.7	9.1	6.8	13.3	11.3	0.0
Chlorophyll-b	162.2	293.4	119.3	129.7	150.0	149.4	177.1	51.9
Chlorophyll-c1	5.4	0.0	12.3	6.8	6.7	12.5	26.1	3.1
Chlorophyll-c2R	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Chlorophyll-c2	3.1	0.0	8.8	4.8	5.3	8.8	16.0	2.6
Chlorophyllide-a	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Diadinoxanthin	0.0	0.0	7.1	0.0	3.3	7.1	6.1	0.0
Diatoxanthin	0.0	0.0	1.6	0.0	1.6	2.3	0.0	0.0
Echinenone	0.0	0.0	0.0	0.0	7.4	7.4	6.2	0.0
Fucoxanthin	57.1	11.9	120.7	69.4	76.1	121.6	205.6	48.9
Lutein	59.2	61.8	36.6	27.7	44.8	38.6	33.1	10.0
Mixoxanthophyll	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Neoxanthin	19.8	9.5	4.9	5.7	9.4	8.7	11.2	8.7
Phaeophorbide-a1	50.6	25.3	14.3	10.8	26.1	16.3	15.9	9.7
Phaeophorbide-b1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Phaeophytin-a1	425.4	423.3	173.3	363.1	447.8	242.4	664.1	279.0
Phaeophythin-a2	27.2	25.3	21.6	31.3	34.2	16.5	45.7	22.8
Phaeophytin-b1	27.2	25.3	21.6	31.3	34.2	16.5	45.7	22.8
Phaeophythin-b2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Scytonemin	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Violaxanthin	10.7	9.5	4.3	2.5	2.8	6.9	2.0	0.0
Zeaxanthin	34.8	0.0	28.1	7.9	28.6	25.0	20.4	5.3

PIGMENT	*Non-flow*						
	13 EL	14 EL	15 EL	16 EL	17 EL	18 EL	19 EL
β,β-carotene	14.7	9.8	3.5	7.1	12.7	26.1	19.7
car-11	0.0	0.0	0.0	0.0	0.0	0.0	0.0
car-8	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Canthaxanthin	0.0	0.0	2.0	2.2	0.0	0.0	1.8
Chlorophyll-a	119.3	115.8	44.6	19.2	31.7	273.3	56.7
Chlorophyll-a alomer 1	2.1	0.0	0.0	0.0	0.0	0.0	0.0
Chlorophyll-b	131.8	79.9	29.0	27.2	25.7	173.3	86.5
Chlorophyll-c1	5.2	10.1	0.0	0.0	0.0	0.0	0.0
Chlorophyll-c2R	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Chlorophyll-c2	3.1	3.8	0.0	0.0	0.0	0.0	0.0
Chlorophyllide-a	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Diadinoxanthin	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Diatoxanthin	0.0	0.0	0.0	0.0	0.0	3.5	5.3
Echinenone	0.0	5.3	3.5	9.9	8.1	17.4	10.3
Fucoxanthin	98.1	106.4	9.0	0.0	11.9	7.4	4.1
Lutein	33.2	31.6	7.7	10.7	8.7	58.9	24.0
Mixoxanthophyll	6.5	0.0	0.0	0.0	0.0	12.5	8.4
Neoxanthin	20.9	13.4	2.2	0.0	0.0	12.4	5.1
Phaeophorbide-a1	0.0	19.1	12.4	0.0	14.2	16.3	11.3
Phaeophorbide-b1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Phaeophytin-a1	422.6	418.9	104.2	171.6	265.7	482.8	486.9
Phaeophythin-a2	24.5	26.3	12.1	30.5	18.5	41.0	23.4
Phaeophytin-b1	24.5	26.3	12.1	30.5	18.5	41.0	23.4
Phaeophythin-b2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Scytonemin	0.0	0.0	5.9	20.5	12.0	21.8	0.0
Violaxanthin	0.9	0.0	0.0	0.0	0.0	1.7	1.1
Zeaxanthin	14.1	16.6	4.3	0.0	0.0	21.8	7.8

PIGMENT	*Rewetting*						
	20 EL	21 EL	22 EL	24 EL	27 EL	28 EL	29 EL
β,β-carotene	26.6	22.5	34.8	24.9	28.0	12.8	19.5
car-11	0.0	0.0	0.0	0.0	0.0	0.0	0.0
car-8	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Canthaxanthin	2.0	0.0	0.0	0.0	0.0	0.0	0.0
Chlorophyll-a	262.9	122.3	951.1	94.7	451.0	52.0	264.2
Chlorophyll-a alomer 1	0.0	0.0	3.8	0.0	3.3	0.0	0.0
Chlorophyll-b	44.5	145.2	351.2	147.3	88.6	81.1	264.1
Chlorophyll-c1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Chlorophyll-c2R	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Chlorophyll-c2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Chlorophyllide-a	0.0	0.0	18.9	0.0	0.0	0.0	0.0
Diadinoxanthin	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Diatoxanthin	7.7	2.3	2.8	2.4	2.7	0.0	0.0
Echinenone	10.6	8.0	5.7	7.1	8.9	4.9	4.8
Fucoxanthin	0.0	5.7	3.3	4.6	3.1	2.5	4.0
Lutein	12.7	56.9	77.4	40.1	24.8	32.4	54.3
Mixoxanthophyll	16.0	4.6	0.0	0.0	0.0	0.0	0.0
Neoxanthin	0.0	8.6	17.5	8.3	3.6	5.3	10.5
Phaeophorbide-a1	0.0	10.5	42.4	18.7	91.2	35.8	32.0
Phaeophorbide-b1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Phaeophytin-a1	199.1	522.5	180.3	585.9	249.4	488.5	449.8
Phaeophythin-a2	0.0	51.0	36.3	42.2	19.2	49.9	25.3
Phaeophytin-b1	0.0	51.0	36.3	42.2	19.2	49.9	25.3
Phaeophythin-b2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Scytonemin	21.2	0.0	0.9	0.0	0.0	0.0	0.0
Violaxanthin	0.0	2.3	18.7	1.8	2.9	0.4	10.2
Zeaxanthin	6.3	0.0	0.0	22.7	20.6	18.0	24.9

PIGMENT	*Drying*							
	5 ES	6 ES	7 ES	8 ES	9 ES	10 ES	11 ES	12 ES
β,β-carotene	15.4	4.0	4.3	13.0	7.2	9.8	0.0	0.0
car-11	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
car-8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Canthaxanthin	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Chlorophyll-a	814.0	119.1	373.6	471.0	445.2	338.9	77.1	107.2
Chlorophyll-a alomer 1	37.6	6.8	20.0	29.2	12.7	0.0	2.0	0.0
Chlorophyll-b	42.1	0.0	0.0	50.7	23.1	61.6	10.1	0.0
Chlorophyll-c1	49.0	12.7	24.0	29.1	21.9	19.5	4.2	11.6
Chlorophyll-c2R	7.9	0.0	0.0	5.8	0.0	0.0	0.0	0.0
Chlorophyll-c2	38.2	12.9	11.4	21.3	13.7	11.2	2.4	11.2
Chlorophyllide-a	45.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Diadinoxanthin	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Diatoxanthin	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Echinenone	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Fucoxanthin	582.1	204.7	197.1	393.0	274.0	218.1	43.8	129.4
Lutein	18.9	4.1	6.2	16.9	6.9	12.7	2.2	4.9
Mixoxanthophyll	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Neoxanthin	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Phaeophorbide-a1	45.6	20.6	35.5	27.6	22.3	32.2	4.4	24.1
Phaeophorbide-b1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Phaeophytin-a1	540.6	409.6	243.2	561.3	384.9	452.7	112.5	288.6
Phaeophythin-a2	0.0	0.0	11.5	12.9	7.2	27.7	0.0	18.0
Phaeophytin-b1	22.6	8.1	8.8	23.0	17.1	30.9	10.6	28.3
Phaeophythin-b2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Scytonemin	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Violaxanthin	17.9	0.0	4.5	9.8	9.4	6.1	0.0	0.0
Zeaxanthin	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

PIGMENT	*Non-flow*						
	13 ES	14 ES	15 ES	16 ES	17 ES	18 ES	19 ES
β,β-carotene	0.0	0.0	0.0	0.0	0.0	0.0	0.0
car-11	0.0	1.5	1.4	0.0	1.4	1.3	1.0
car-8	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Canthaxanthin	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Chlorophyll-a	74.4	120.3	73.6	82.1	104.5	115.3	22.0
Chlorophyll-a alomer 1	0.0	0.0	11.4	11.7	3.9	5.0	0.0
Chlorophyll-b	26.9	20.7	16.0	11.7	22.2	28.4	17.6
Chlorophyll-c1	0.0	7.1	4.1	5.8	6.8	6.4	0.0
Chlorophyll-c2R	0.0	0.0	0.0	2.7	2.2	2.7	0.0
Chlorophyll-c2	0.0	3.7	1.9	3.3	3.4	3.6	0.0
Chlorophyllide-a	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Diadinoxanthin	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Diatoxanthin	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Echinenone	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Fucoxanthin	30.0	59.7	55.0	77.6	68.9	82.5	5.1
Lutein	11.7	11.5	9.9	7.2	22.3	13.6	5.1
Mixoxanthophyll	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Neoxanthin	0.0	0.0	0.0	0.0	0.0	0.0	2.0
Phaeophorbide-a1	20.4	39.1	17.3	30.8	120.0	41.8	11.6
Phaeophorbide-b1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Phaeophytin-a1	146.1	238.4	210.9	222.3	338.2	253.7	56.7
Phaeophythin-a2	9.8	22.3	0.0	0.0	0.0	0.0	2.2
Phaeophytin-b1	10.6	12.0	19.6	24.5	37.0	12.8	5.5
Phaeophythin-b2	0.0	0.0	13.1	10.2	20.8	14.3	0.0
Scytonemin	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Violaxanthin	0.0	0.0	0.0	0.0	6.7	0.0	0.0
Zeaxanthin	0.0	0.0	0.0	0.0	0.0	0.0	0.0

PIGMENT	*Rewetting*						
	20 ES	21 ES	22 ES	24 ES	27 ES	28 ES	29 ES
β,β-carotene	0.0	0.0	0.0	0.0	0.0	0.0	0.0
car-11	0.0	1.1	0.0	0.7	1.0	2.0	0.7
car-8	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Canthaxanthin	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Chlorophyll-a	157.7	45.0	93.6	24.3	53.9	45.8	13.7
Chlorophyll-a alomer 1	12.7	0.0	9.7	0.0	0.0	0.0	0.0
Chlorophyll-b	40.8	23.2	23.8	16.0	20.7	33.8	12.1
Chlorophyll-c1	5.5	2.0	4.5	1.5	2.0	3.3	0.0
Chlorophyll-c2R	1.9	0.0	2.1	0.0	0.0	0.0	0.0
Chlorophyll-c2	2.9	1.4	2.3	0.8	0.8	2.2	0.0
Chlorophyllide-a	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Diadinoxanthin	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Diatoxanthin	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Echinenone	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Fucoxanthin	71.8	46.9	78.5	22.3	40.5	46.5	6.6
Lutein	27.3	7.0	6.9	6.5	9.3	10.4	3.8
Mixoxanthophyll	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Neoxanthin	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Phaeophorbide-a1	159.6	27.3	26.1	36.8	30.5	24.3	11.2
Phaeophorbide-b1	32.4	0.0	0.0	5.8	0.0	0.0	0.0
Phaeophytin-a1	262.1	177.6	166.2	122.3	186.3	235.7	78.3
Phaeophythin-a2	15.2	12.2	10.1	7.9	8.8	10.6	5.5
Phaeophytin-b1	91.4	13.6	8.1	9.6	21.7	13.6	4.4
Phaeophythin-b2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Scytonemin	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Violaxanthin	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Zeaxanthin	0.0	0.0	0.0	0.0	0.0	0.0	0.0

PIGMENT	*Drying*							
	5 H	6 H	7 H	8 H	9 H	10 H	11 H	12 H
β,β-carotene	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
car-11	0.0	0.0	0.2	1.4	0.6	1.2	1.1	0.0
car-8	0.0	0.0	0.0	0.9	0.0	0.5	1.2	1.2
Canthaxanthin	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Chlorophyll-a	52.0	39.7	83.4	40.3	31.6	46.8	71.9	10.9
Chlorophyll-a alomer 1	4.3	0.0	3.0	0.0	0.0	0.0	2.5	0.0
Chlorophyll-b	8.3	14.3	11.0	15.1	12.4	13.9	17.6	5.0
Chlorophyll-c1	1.1	1.8	3.4	2.3	1.0	2.4	5.0	0.0
Chlorophyll-c2R	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Chlorophyll-c2	0.4	1.4	1.9	0.9	0.4	1.0	1.7	0.0
Chlorophyllide-a	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Diadinoxanthin	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Diatoxanthin	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Echinenone	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Fucoxanthin	14.2	31.2	37.1	22.4	9.8	21.6	32.5	3.2
Lutein	2.3	5.5	4.4	9.3	4.0	6.3	7.9	2.0
Mixoxanthophyll	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Neoxanthin	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Phaeophorbide-a1	4.6	8.9	22.1	24.6	11.7	12.8	22.4	5.8
Phaeophorbide-b1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Phaeophytin-a1	40.6	74.4	76.4	186.7	78.5	110.2	94.1	18.2
Phaeophythin-a2	8.9	3.7	1.6	13.4	5.7	8.5	8.8	1.4
Phaeophytin-b1	3.4	5.9	7.8	13.9	8.9	9.3	6.8	1.3
Phaeophythin-b2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Scytonemin	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Violaxanthin	3.3	0.5	1.6	0.0	0.0	0.0	0.0	0.0
Zeaxanthin	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

PIGMENT	*Non-flow*						
	13 H	14 H	15 H	16 H	17 H	18 H	19 H
β,β-carotene	0.0	0.0	0.0	0.0	0.0	0.0	0.0
car-11	0.0	2.7	1.5	1.2	1.3	0.7	0.0
car-8	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Canthaxanthin	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Chlorophyll-a	10.6	38.5	26.8	25.8	23.6	14.6	8.6
Chlorophyll-a alomer 1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Chlorophyll-b	4.6	29.4	17.4	12.7	17.0	8.9	11.3
Chlorophyll-c1	0.0	0.0	0.0	0.0	0.0	3.2	0.0
Chlorophyll-c2R	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Chlorophyll-c2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Chlorophyllide-a	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Diadinoxanthin	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Diatoxanthin	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Echinenone	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Fucoxanthin	1.7	9.0	8.0	8.6	3.8	7.3	0.0
Lutein	0.0	13.8	10.9	7.5	9.6	5.9	1.6
Mixoxanthophyll	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Neoxanthin	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Phaeophorbide-a1	5.3	16.6	23.3	18.9	11.4	14.4	4.9
Phaeophorbide-b1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Phaeophytin-a1	1.8	151.9	113.4	105.0	99.9	83.5	31.0
Phaeophythin-a2	1.8	13.1	0.0	0.0	0.0	0.0	2.0
Phaeophytin-b1	14.0	16.5	10.8	11.8	12.5	8.6	4.3
Phaeophythin-b2	0.0	0.0	9.0	8.5	5.4	5.6	0.0
Scytonemin	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Violaxanthin	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Zeaxanthin	0.0	0.0	0.0	0.0	0.0	0.0	0.0

PIGMENT	*Rewetting*						
	20 H	21 H	22 H	24 H	27 H	28 H	29 H
β,β-carotene	0.0	0.0	0.0	0.0	0.0	0.0	0.0
car-11	0.0	0.7	0.0	1.2	0.0	1.5	0.8
car-8	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Canthaxanthin	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Chlorophyll-a	13.4	6.4	26.6	25.3	15.6	12.6	29.1
Chlorophyll-a alomer 1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Chlorophyll-b	0.0	7.2	7.6	18.3	6.9	16.9	20.2
Chlorophyll-c1	0.0	0.0	0.9	0.7	0.0	0.0	0.0
Chlorophyll-c2R	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Chlorophyll-c2	0.0	0.0	0.0	0.3	0.0	0.0	0.0
Chlorophyllide-a	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Diadinoxanthin	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Diatoxanthin	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Echinenone	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Fucoxanthin	4.8	1.3	9.0	11.2	4.0	1.3	2.4
Lutein	1.5	3.6	2.5	5.7	2.1	5.6	6.4
Mixoxanthophyll	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Neoxanthin	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Phaeophorbide-a1	3.0	6.0	9.4	14.0	5.5	6.1	3.8
Phaeophorbide-b1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Phaeophytin-a1	38.0	61.4	52.1	89.7	34.1	80.0	34.2
Phaeophythin-a2	5.0	5.2	4.3	5.6	3.1	3.3	1.5
Phaeophytin-b1	4.1	9.4	3.6	12.2	3.4	9.3	4.2
Phaeophythin-b2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Scytonemin	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Violaxanthin	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Zeaxanthin	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Supplementary Table S2. Algal taxonomic composition on epilithic (EL), epipsammic (ES) and hyporheic (H) biofilms for each hydrological phase (numbers indicate the different sampling date). Qualitative abundances were recorded and ranked on the following scale: 1 (very rare, ≤ 5 %), 2 (> 5 to ≤ 20 %), 3 (> 20 to ≤ 40 %), 4 (> 40 to ≤ 60 %), 5 (> 60 to ≤ 80 %) and 6 (very abundant, > 80 %).

Class	Genera	*Drying*							
		5 EL	6 EL	7 EL	8 EL	9 EL	10 EL	11 EL	12 EL
CYANOBACTERIA	*Pleurocapsa* sp.	3	4	3	3	3	5	5	5
	Gleocapsa sp.		2					2	
	Homoeothrix janthina		2	2		2	2	2	
	Lyngbya sp.	2							
	Phormidium sp.	2				3		2	
	Calothrix sp.	2				2	2	2	3
RHODOPHYTA	Unidentified chantransia stage	2			2	3	3		
	Hildenbrandia rivularis				4			4	
CHLOROPHYTA	*Gongrosira* spp.	5	3	4	3	4	4	3	4
	Chlorococcal spp.	2	2	2	2				2
	Scenedesmus sp.							1	
	Oedogonium sp.			3				4	
BACILLARIOPHYTA	*Achnanthidium minutissimum*	3	3	3	2	4	3	4	4
	Planothidium lanceolatum	2	2	2	1	3	2	3	
	Gomphonema spp.			2		3	2		2
	Meridion sp.	2	2			3			
	Navicula spp.	2	2			2		2	2
	Cymbella spp.	2	2	2				2	2
	Amphora sp.								
	Fragilaria spp.			2				3	2
	Nitzschia spp.								2
	Eunotia sp.								
	Cocconeis placentula	2	2	4		5	5	3	3
	Pinnularia sp.								
	Surirella sp.								
	Cyclotella sp.								
EUGLENOPHYTA	*Trachelomonas* sp.						1		

Class	Genera	Non-flow						
		13 EL	14 EL	15 EL	16 EL	17 EL	18 EL	19 EL
CYANOBACTERIA	*Pleurocapsa* sp.	5	5	5	5	4	5	4
	Gleocapsa sp.							
	Homoeothrix janthina							2
	Lyngbya sp.							
	Phormidium sp.						2	
	Calothrix sp.			2	4	2		3
RHODOPHYTA	Unidentified chantransia stage							2
	Hildenbrandia rivularis	3		3		3	3	
CHLOROPHYTA	*Gongrosira* spp.	4	4	4	5	3	4	3
	Chlorococcal spp.	2						
	Scenedesmus sp.							
	Oedogonium sp.					2		2
BACILLARIOPHYTA	*Achnanthidium minutissimum*	3	2	5	4	5	3	
	Planothidium lanceolatum	2		3	3		2	
	Gomphonema spp.			2	2		2	2
	Meridion sp.			2				
	Navicula spp.			2	2		2	
	Cymbella spp.			2				2
	Amphora sp.							
	Fragilaria spp.				2		2	2
	Nitzschia spp.							
	Eunotia sp.							
	Cocconeis placentula	3	4	4	5	4	3	3
	Pinnularia sp.							
	Surirella sp.							
	Cyclotella sp.							
EUGLENOPHYTA	*Trachelomonas* sp.							

Class	Genera	*Rewetting*						
		20 EL	21 EL	22 EL	24 EL	27 EL	28 EL	29 EL
CYANOBACTERIA	*Pleurocapsa* sp.	4	4		3	5	5	4
	Gleocapsa sp.					2		
	Homoeothrix janthina	2	2	2	2	2		2
	Lyngbya sp.							
	Phormidium sp.							3
	Calothrix sp.	2		2				
RHODOPHYTA	Unidentified chantransia stage	2	2		2	2	2	2
	Hildenbrandia rivularis							
CHLOROPHYTA	*Gongrosira* spp.	3	5		4	4		3
	Chlorococcal spp.		2	2				
	Scenedesmus sp.							
	Oedogonium sp.	2		2	3	2		
BACILLARIOPHYTA	*Achnanthidium minutissimum*	5		4	3	3	3	2
	Planothidium lanceolatum		2	2	2			
	Gomphonema spp.	2		2		2	2	
	Meridion sp.			2		2		
	Navicula spp.			2				
	Cymbella spp.			2			2	
	Amphora sp.							
	Fragilaria spp.			2				
	Nitzschia spp.	2		2	1			2
	Eunotia sp.	2		2				
	Cocconeis placentula	5	4	4	2	2	2	3
	Pinnularia sp.							
	Surirella sp.							
	Cyclotella sp.							
EUGLENOPHYTA	*Trachelomonas* sp.							

Class	Genera	*Drying*							
		5 ES	6 ES	7 ES	8 ES	9 ES	10 ES	11 ES	12 ES
CYANOBACTERIA	*Pleurocapsa* sp.	4		3	3	2			2
	Gleocapsa sp.	2				2	2	2	2
	Homoeothrix janthina	2	2	2		2	2	2	2
	Lyngbya sp.								
	Phormidium sp.					1	1		
	Calothrix sp.					1			
RHODOPHYTA	Unidentified chantransia stage	2							
	Hildenbrandia rivularis								
CHLOROPHYTA	*Gongrosira* spp.			2					
	Chlorococcal spp.	2		2	2	2	2	2	2
	Scenedesmus sp.								
	Oedogonium sp.				1				
BACILLARIOPHYTA	*Achnanthidium minutissimum*	4	4	4	4	5	5	4	5
	Planothidium lanceolatum	2	2	2	3	3	3	2	4
	Gomphonema spp.	2	2		2	3	4	2	3
	Meridion sp.		2	2	2	2	2	2	2
	Navicula spp.	2		2	2	2	2	2	2
	Cymbella spp.		2	2		2	2	2	2
	Amphora sp.								
	Fragilaria spp.	2	2	2		2	2	2	2
	Nitzschia spp.		2	2		2	2	2	2
	Eunotia sp.	2					2		
	Cocconeis placentula		2	2	4	4	3	2	3
	Pinnularia sp.	2							
	Surirella sp.								1
	Cyclotella sp.	1					1		1
EUGLENOPHYTA	*Trachelomonas* sp.	1	1		1				

Class	Genera	*Non-flow*						
		13 ES	14 ES	15 ES	16 ES	17 ES	18 ES	19 ES
CYANOBACTERIA	*Pleurocapsa* sp.	2	2	2		2		2
	Gleocapsa sp.	2	2	2	2	2	2	
	Homoeothrix janthina	2			2			
	Lyngbya sp.							
	Phormidium sp.							
	Calothrix sp.	2	2					
RHODOPHYTA	Unidentified chantransia stage	1						
	Hildenbrandia rivularis	1		2		2	1	
CHLOROPHYTA	*Gongrosira* spp.	2			2	2		
	Chlorococcal spp.	3	3	2	2	2	2	2
	Scenedesmus sp.							
	Oedogonium sp.							
BACILLARIOPHYTA	*Achnanthidium minutissimum*	5	4	5	5	5	5	5
	Planothidium lanceolatum	4	2	2	3	3	3	3
	Gomphonema spp.	3	4	3	3	4	3	3
	Meridion sp.	4	3	2	3	3	3	3
	Navicula spp.	2	2	2	2	2	2	2
	Cymbella spp.	2	2	2	3	3	2	2
	Amphora sp.						1	
	Fragilaria spp.	2	2	2	2	2	3	2
	Nitzschia spp.	2	2	2	2		2	2
	Eunotia sp.	2	2		2	2	2	2
	Cocconeis placentula	3	3	2	3	3	2	3
	Pinnularia sp.							
	Surirella sp.							
	Cyclotella sp.							
EUGLENOPHYTA	*Trachelomonas* sp.	1	1		1			1

Class	Genera	Rewetting						
		20 ES	21 ES	22 ES	24 ES	27 ES	28 ES	29 ES
CYANOBACTERIA	*Pleurocapsa* sp.	2	2					2
	Gleocapsa sp.	2	2		2			
	Homoeothrix janthina	2	2	2	2	2	2	2
	Lyngbya sp.							
	Phormidium sp.							
	Calothrix sp.							
RHODOPHYTA	Unidentified chantransia stage							
	Hildenbrandia rivularis							2
CHLOROPHYTA	*Gongrosira* spp.						2	
	Chlorococcal spp.	2	2	2	2	2	2	2
	Scenedesmus sp.							
	Oedogonium sp.			2				
BACILLARIOPHYTA	*Achnanthidium minutissimum*	4	5	4	5	5	5	5
	Planothidium lanceolatum	2	2	2	3	2	2	2
	Gomphonema spp.	2	3	2	3	2	2	2
	Meridion sp.	2	2	2	2	2	2	2
	Navicula spp.		2	2				
	Cymbella spp.	2	2	2	3	2	2	2
	Amphora sp.							
	Fragilaria spp.		2	2	2	2	2	2
	Nitzschia spp.		2	2	2			
	Eunotia sp.	2	2	2	2	2		
	Cocconeis placentula	2	3	4	4	4	3	4
	Pinnularia sp.							
	Surirella sp.							
	Cyclotella sp.							
EUGLENOPHYTA	*Trachelomonas* sp.	1						

Class	Genera	*Drying*							
		5 H	6 H	7 H	8 H	9 H	10 H	11 H	12 H
CYANOBACTERIA	*Pleurocapsa* sp.	2	1					2	
	Gleocapsa sp.				1	1			
	Homoeothrix janthina	2		2					2
	Lyngbya sp.								
	Phormidium sp.								
	Calothrix sp.						1		
RHODOPHYTA	Unidentified chantransia stage				1				
	Hildenbrandia rivularis								
CHLOROPHYTA	*Gongrosira* spp.								
	Chlorococcal spp.	2	2	2	2	2	2		2
	Scenedesmus sp.					1			
	Oedogonium sp.								
BACILLARIOPHYTA	*Achnanthidium minutissimum*	4	4	4	4	5	5	4	4
	Planothidium lanceolatum	2	3	2		3	3		2
	Gomphonema spp.	2	2	2	3	4	4	2	2
	Meridion sp.	2	2	2	2	3	3		
	Navicula spp.	2					1		2
	Cymbella spp.	2	2	2	2	2	3	2	2
	Amphora sp.								
	Fragilaria spp.	1	2	2	2	3	3	2	2
	Nitzschia spp.			2		2	2	2	
	Eunotia sp.	2				2	1		2
	Cocconeis placentula	2	2	2	2	2	2	2	2
	Pinnularia sp.								
	Surirella sp.								
	Cyclotella sp.	1	1			2	2		
EUGLENOPHYTA	*Trachelomonas* sp.	1	1	1	1				

Class	Genera	Non-flow						
		13 H	14 H	15 H	16 H	17 H	18 H	19 H
CYANOBACTERIA	*Pleurocapsa* sp.	2	1					
	Gleocapsa sp.	1		2				
	Homoeothrix janthina							
	Lyngbya sp.							
	Phormidium sp.							
	Calothrix sp.							
RHODOPHYTA	Unidentified chantransia stage							
	Hildenbrandia rivularis							
CHLOROPHYTA	*Gongrosira* spp.							
	Chlorococcal spp.	2	2	2		2	2	2
	Scenedesmus sp.							
	Oedogonium sp.							
BACILLARIOPHYTA	*Achnanthidium minutissimum*	4	5	4	5	4	4	4
	Planothidium lanceolatum		2	2	2	2	2	2
	Gomphonema spp.	3	4	4	3	3	3	3
	Meridion sp.	2	3	2	2	2	2	2
	Navicula spp.		2	2	2			
	Cymbella spp.	2	2	2	2	2	2	2
	Amphora sp.		1	2				
	Fragilaria spp.	2	2	2	2	2	2	3
	Nitzschia spp.		2	2	1	2		2
	Eunotia sp.		2	2		1	1	
	Cocconeis placentula	3	3	4	2	2	2	2
	Pinnularia sp.			1				
	Surirella sp.			2				
	Cyclotella sp.			2	1	1		
EUGLENOPHYTA	*Trachelomonas* sp.		1					

Class	Genera	*Rewetting*						
		20 H	21 H	22 H	24 H	27 H	28 H	29 H
CYANOBACTERIA	*Pleurocapsa* sp.	1						2
	Gleocapsa sp.	1				1		1
	Homoeothrix janthina			2		2		
	Lyngbya sp.							
	Phormidium sp.							
	Calothrix sp.							
RHODOPHYTA	Unidentified chantransia stage							
	Hildenbrandia rivularis							
CHLOROPHYTA	*Gongrosira* spp.							
	Chlorococcal spp.	2		2		2	2	2
	Scenedesmus sp.							
	Oedogonium sp.							
BACILLARIOPHYTA	*Achnanthidium minutissimum*	4	4	4	4	4	4	4
	Planothidium lanceolatum	3	2			3		2
	Gomphonema spp.	3	3	4	3			3
	Meridion sp.	2	2			3		2
	Navicula spp.	2		2	2			
	Cymbella spp.	3	2	2				2
	Amphora sp.							
	Fragilaria spp.	2	2	2	3	2	2	2
	Nitzschia spp.			1				2
	Eunotia sp.	2	1				2	
	Cocconeis placentula	3	2	2		3	3	3
	Pinnularia sp.							
	Surirella sp.							
	Cyclotella sp.							
EUGLENOPHYTA	*Trachelomonas* sp.							

Chapter 3.

The dynamics of biofilm bacterial communities is driven by flow wax and wane in temporary streams.

Timoner, X., Borrego, C., Acuña, V. and Sabater, S. “The dynamics of biofilm bacterial communities is driven by flow wax and wane in a temporary stream”. Limnology and Oceanography. Vol. 59, issue 6 (2014) : 2057-2067

http://dx.doi.org/10.4319/lo.2014.59.6.2057

http://aslo.org/lo/toc/vol_59/issue_6/2057.html

INTRODUCTION

Drought climatic events are severely increasing in temperate regions worldwide (Wilby *et al.*, 2006; Parry *et al.*, 2007), and are usually associated to a temporal and spatial increase of non-flow periods in streams and rivers (Milly *et al.*, 2005; Hirabayashi *et al.*, 2008; Döll and Schmied, 2012). As a result many systems become temporary, with unknown biogeochemical and ecological consequences for biodiversity and ecosystem functioning (Acuña *et al.,* 2014). Particularly important are the non-flow and the flow recovery periods, when the system experiences desiccation and rewetting respectively. These periods affect the survival and functioning of the biota in the stream and select well-adapted communities (Lake, 2011).

In streambed sediments, microbial communities are directly affected by desiccation and rewetting (Amalfitano *et al.,* 2008; Zoppini and Marxsen, 2011; Timoner *et al.,* 2012). Prokaryotes inhabiting these sediments play a pivotal role in the production and degradation of organic matter and nutrient cycling (Meyer, 1994; Battin *et al.,* 2003). Bacteria colonize all streambed substrata (mainly cobbles or sand sediments), and together with other microorganisms (archaea, algae, fungi and protozoa) make part of biofilms (Wetzel, 1983; Lear *et al.,* 2012). The bacterial community structure and functioning is related to environmental factors such as resource availability and temperature, but also to the water content in the sediments (Zeglin *et al.,* 2011). Flow intermittency may cause changes in the bacterial community composition of biofilms (Rees *et al.,* 2006; Amalfitano *et al.,* 2008; Febria *et al.,* 2011), indicating that these communities respond to desiccation. Studies in soil bacterial communities and tidal sediments also show prominent changes subjected to desiccation and rewetting (Fierer and Schimel, 2002; Mckew *et al.,* 2011). During the non-flow phase, bacterial richness and diversity decrease, and only a few bacterial taxa thriving under desiccation dominate (Rees *et al.,* 2006; Febria *et al.,* 2011). Changes in the community composition associated to desiccation could result from a selection of the most resistant taxa (Evans and Wallenstein, 2014), or from the colonization by immigrant and tolerant taxa (Fazi *et al.,* 2008). Biofilm functioning, assessed through the ability of organic matter degradation (extracellular enzyme activities)

decreases due to flow intermittency (Zoppini and Marxsen, 2011) but does not disappear (Timoner *et al.,* 2012). Further, both the biofilm community structure and function rapidly recover after flow resumption (Rees *et al.,* 2006; Zoppini and Marxsen, 2011; Timoner *et al.,* 2012). These are evidences that bacterial communities in temporary streams are able to thrive and function according to the desiccation and rewetting cycles (Evans and Wallenstein, 2014).

The present study aims to understand the mechanisms associated to the dynamics of the bacterial communities during flow intermittency, by analyzing the specific responses of bacterial communities in the epilithic, epipsammic and hyporheic streambed compartments.We conducted a detailed profiling of these biofilm bacterial communities through pyrotag sequencing of the SSU rRNAgene. We hypothesized that: *i)* the effects of flow intermittency would be more pronounced and long-lasting on the epilithic bacterial community, because of the harsher environmental conditions (water content, light irradiances, air temperature) occurring during the non-flow phase. *ii)* Water flow wane would drive a selection towards desiccation-resistant bacteria from the taxa already present during the flowing period. Accordingly, we would also expect that flow resumption would restore bacterial species prevalent before desiccation. *iii)* Bacterial taxa from the nearby terrestrial environment could also be active colonizers in the more superficial streambed substrata. The resulting bacterial community would therefore exhibit high resilience, expressed as ecological strategies directed to thrive or persist under desiccation and rewetting cycles.

METHODS

Study site and field sampling

Results from this chapter are based on biofilm samples collected between May and December 2009 in the Fuirosos. Three hydrological phases were defined in terms of the hydrological condition of the stream during the study period: *i*) the drying phase prior to flow cessation (*D*), where the stream flow was relatively low and extended from May until mid-July; *ii*) the non-flow phase that lasted for 112 days (*NF*); and *iii*) rewetting (*R*), which started by the end of October and included the first day after flow recovered. From the 23 sampling dates performed, 17 were included in this study trying to represent the

hot moments for bacterial change, including all samples from the *NF* phase (7, weeks 13, 14, 15, 16, 17, 18 and 19) and samples obtained just prior to flow cessation (6, weeks 4, 6, 8, 9, 11 and 12) and after flow resumption (4, weeks 20, 22, 27 and 29). Details concerning about the study site, sampling strategy and environmental parameters are described in the Methodology section and in Chapter 1.

Laboratory analyses

DNA was extracted from biofilms using the Fast DNA Spin kit for soil (MP Biomedicals, Solon, OH, USA) according to the manufacturer's instructions. The extracted DNA was quantified using a Qubit® 2.0 Fluorometer (Invitrogen Molecular probes Inc., Oslo, Norway). The samples for 454 pyrosequencing were obtained after pooling equal amounts of DNA extracts from the five replicates of each biofilm compartment and processed as composite samples. Three composite samples corresponding to each biofilm compartment for each sampling date (a total of 51 samples) were subsequently analyzed through tag-encoded FLX-Titanium amplicon pyrosequencing at the Research and Testing Laboratory (RTL, Lubbock, TX, USA). Briefly, genomic DNA from the biofilm communities was used as a template in PCR reactions using primers 28F/519R targeting the V1-3 region of the bacterial 16S rRNA gene complemented with 454-adapters and sample-specific barcodes. Raw sequence dataset was pre-processed at RTL facilities to reduce noise and sequencing artifacts as previously described (Dowd et al. 2008). Demultiplexing according to sample barcodes, sequence quality assessments, chimera detection and downstream phylogenetic analyses were conducted in QIIME (Caporaso *et al.,* 2010a, see below). The denoised, quality-filtered and chimera-free sequence dataset (51 samples) consisted of 142,590 reads distributed in 2,796 sequences per sample (min = 920, max = 6,976, SD = 1,376.9), with an average length of 383 nt (min = 223, max = 534). The pyrosequencing data obtained from this study have been deposited in the NCBI database via the Biosample Submission Portal (http://www.ncbi.nlm.nih.gov/biosample/) under accession number SAMN02388893.

Phylogenetic analysis

The relative abundance of sequences associated with different bacterial Phyla across samples was corrected for the average number of rRNA operons present in their genomes

to avoid interpretation biases. The average values used for this calculation were obtained from the Ribosomal RNA Database (http://rrndb.umms.med.umich.edu/, Klappenbach *et al.,* 2001; *Lee et al.,* 2009) and are compiled in Table S1. The clustering of the sequences into OTUs (Operational taxonomic unit) was performed in QIIME using USEARCH (Edgar, 2010) using a cutoff value of 97 %. OTUs with less than 4 members were removed from downstream analysis to discard spurious OTUs generated by pyrosequencing errors. Representative sequences from each OTU were aligned to the Greengenes imputed core reference alignment (DeSantis *et al.,* 2006) using PyNAST (Caporaso *et al.,* 2010b). Taxonomical assignments for each OTU were performed using the RDP classifier (Wang *et al.,* 2007) retrained using the October 2012 Greengenes taxonomy database (http://greengenes.secondgenome.com). Most of the final 3,314 OTUs delineated across samples were classified at the class and family levels at a confidence threshold > 90 %.

For community analysis, the number of sequences in each sample was normalized using a randomly selected subset of 900 sequences from each sample to standardize the sequencing effort across samples and minimize any bias due to a different number of total sequences. QIIME was used to calculate β-diversity indicators of richness (Chao1), diversity (Shannon-Wiener index) and phylogenetic diversity (PD, Faith, 1992) for each biofilm type and sampling date and to calculate the similarity between bacterial communities (β-diversity) using UniFrac distances (Lozupone and Knight, 2005). The relative abundance of the most populated OTUs (> 100 members) for each biofilm type was calculated using the absolute abundance of each OTU across the samples of each biofilm type (OTU Heatmap in QIIME). Shared OTUs between biofilms and hydrological phases were graphically visualized in Venn diagrams constructed in Mothur (Schloss *et al.,* 2009) using the corresponding OTU tables exported from QIIME. To determine whether bacterial species occurring during non-flow were derived from aquatic environments subjected to desiccation or alternatively from terrestrial environments, a representative sequence of the most populated OTUs (> 100 members) was compared with the reference sequences of cultured microorganisms using BLAST (Altschul *et al.,* 1990).

Ecological analysis

We performed a nestedness analysis to determine whether the bacterial communities observed during the non-flow phase were or not subsets of those during the drying phase. The overall nestedness temperature (Atmar and Patterson, 1993) was calculated for each biofilm type and compared with the nestedness temperatures occurring during each sampling date. The nestedness temperature (*T*) is ananalog of the community entropy, and provides an idea about the magnitude of "disorder" in which species are gained or lost in communities. Within this context, low *T* (*i.e.,* low disorder in species gain and loss) indicate nested patterns (*i.e.,* subset of species from the preceding phase); and high *T* (i.e., high disorder in species gain and loss) indicate non-nested patterns (*i.e.,* random occurrence). The statistical significance of *T* was calculated based on the means of fixed-fixed null model, comparing the observed nestedness temperature with that of 1,000 simulated matrices (predicted nestedness temperature) using the Nestedness Temperature Calculator (Atmar and Patterson, 1995). In the fixed-fixed null model, both column (OTUs) and row (sampling dates) sums are fixed. This null model is used due to its restrictiveness and lower incidence of type I errors (Almeida-Neto *et al.,* 2008). Non-nested patterns occurring during sampling dates were attributed when the nestedness temperature value observed at a sampling date was higher than the overall nestedness temperature obtained for each biofilm type. Contrarily, nested patterns occurred when the nestedness temperature value observed at a sampling date was lower than the overall nestedness temperature obtained for each biofilm type. Moreover, the OTUs turnover between sampling dates during the non-flow phase was estimated using the Armstrong index. This index calculates the percentage of OTUs appearing and disappearing and considering the time elapsed between two consecutive sampling dates (Armstrong, 1969; Ruhí *et al.,* 2012).

Biofilm bacterial communities were classified into ecological strategies using an approach similar to the one developed by Grime (1977) for plants under hydric stress. A similar approach has recently been applied to soil bacterial communities subjected to drying and rewetting (Evans and Wallenstein, 2014). We calculated beforehand the relative abundances of the most populated OTUs (> 100 members) for each biofilm type. An OTU was considered to be present during a hydrological phase when its relative

abundance was greater than 0 over at least half of the sampling dates. Based on that, the relative abundance of each OTU was averaged for each hydrological phase and the percentage of change from drying to non-flow and from non-flow to rewetting was calculated. If an OTU increased more than 50 % from one phase to another it was considered opportunistic, if it decreased more than 50 % was considered sensitive, and if changes were less than 50 % it was considered tolerant.

Statistical analysis

Differences in the bacterial community composition between biofilm types and throughout the different hydrological phases were outlined by means of a principal coordinate analysis (PCO). This analysis was performed using the weighted UniFrac distance matrix (Lozupone *et al.,* 2006) obtained in QIIME. The shifts observed in the PCO representation were assessed using the PERMANOVA of two factors ("biofilm" and "phase") (Anderson, 2001).The RELATE function (a Mantel-type test) was used to determine if there were significant correlations between the bacterial community structure (based on the weighted UniFrac distance matrix) and the measured environmental variables (percentage of water content, temperature, photosynthetic active radiation-PAR and coarse benthic organic matter-CBOM). A similarity matrice for the environmental variables was calculated from normalized Euclidean distances (Clarke and Warwick, 2001). Variables were log-transformed (log (x+1)) when was necessary. The distance-based multivariate linear model (DistLM routine, McArdle and Anderson, 2001) selected (forward selection) the best combination of environmental variables to account for changes observed in the bacterial community composition. This method predicts the relative influence of each variable in the differences of the bacterial community structure. The significance level for all the analyses was $P < 0.05$. Statistical tests were performed using PRIMER-E &PERMANOVA software (PRIMER-E Ltd., Ivybridge, UK).

RESULTS

Intermittency effects on the bacterial community composition

The biofilm bacterial communities changed their composition between stream compartments as well as along the study period (Fig. 1). Sequences affiliated to

Cyanobacteria and *Firmicutes* dominated in the epilithic biofilm, while *Actinobacteria* and *Proteobacteria* were more common in the epipsammic and hyporheic. Particularly, cyanobacterial sequences prevalent during the drying phase in the epilithic biofilm were replaced by Firmicutes during the non-flow phase, but recovered two weeks after flow resumption (Fig. 1 a). Epipsammic and hyporheic biofilms showed minor variations in the relative abundance of sequences affiliated to *Actinobacteria*, *Alphaproteobacteria* and *Firmicutes* during non-flow. *Actinobacteria* moderately increased during rewetting in the epipsammic and hyporheic (Fig.1 b and c).

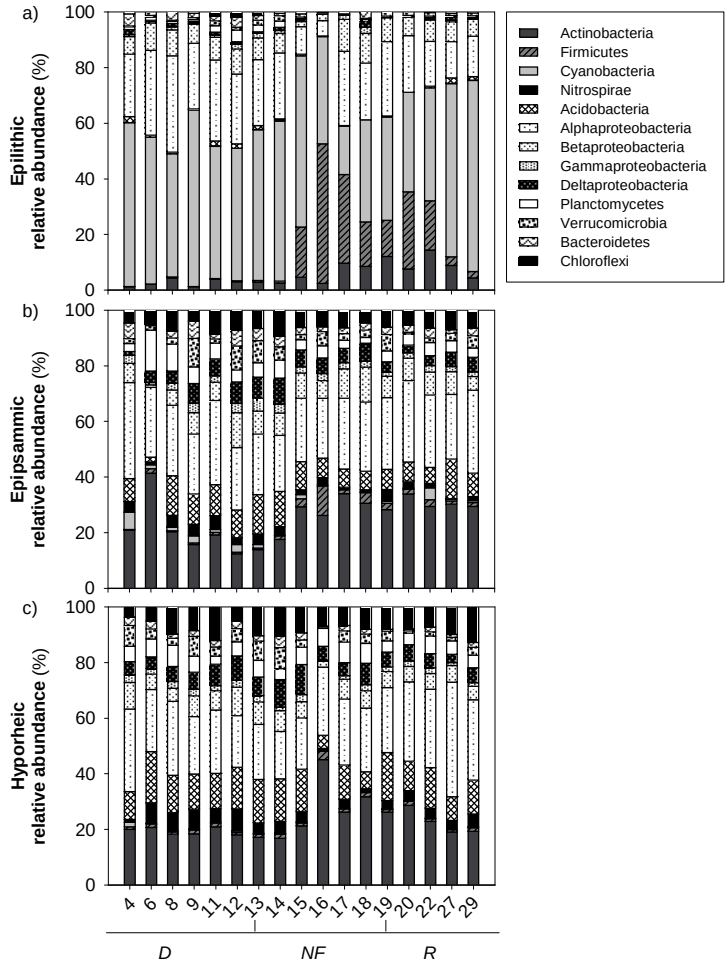

Figure 1. Relative abundance of 16S rRNA gene sequences affiliated with the bacterial phyla (class for *Proteobacteria*) in each biofilm compartment after correction for the average rRNA operon copies in their genomes (see main text for details). a) Epilithic, b) Epipsammic, and c) Hyporheic compartments.The labels at the top indicate the different hydrological phases (*D*-drying, *NF*-non-flow and *R*-rewetting).

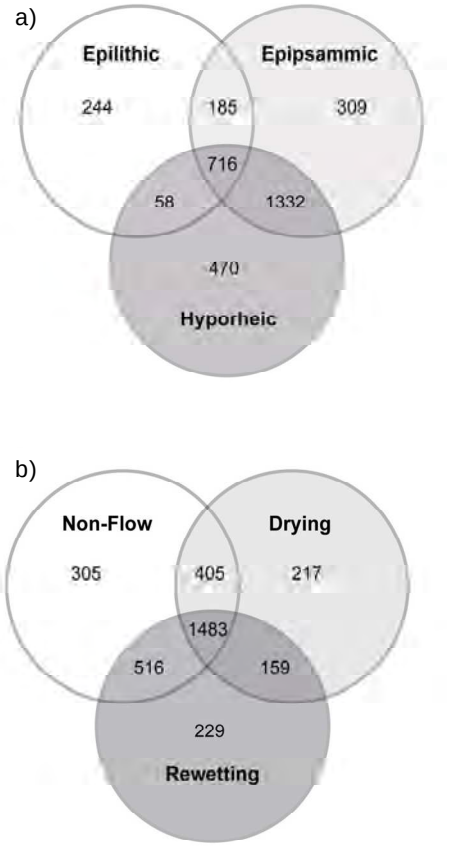

Figure 2. Venn diagrams showing shared OTUs among biofilm types (a) and hydrological phases (b).

Epipsammic and hyporheic biofilms shared 2,048 OTUs, 62 % of the total. The epilithic biofilm (Fig. 2 a) shared a similar number of OTUs with the epipsammic (901 shared OTUs, 21 %) and hyporheic biofilms (774 shared OTUs, 23 %). Approximately 22 % of the total OTUs (716 out of 3,314) were shared between the three studied biofilm types (Fig. 2 a). Up to 305 OTUs were characteristic of the non-flow phase, and the number of specific OTUs during drying and rewetting was lower (217 and 229 OTUs, respectively) (Fig. 2 b). The number of shared OTUs across all hydrological phases during the entire studied period was 1,483 (~45 %).

Prevailing OTUs in the epilithic during non-flow were affiliated to the genus *Exiguobacterium* (99 % identity to *Exiguobacterium acetylicum* and *E. undae*) and *Chryseomicrobium* (98 % identity to *Chryseomicrobium amylolyticum*). During the same period OTUs affiliated with *Actinobacteria* (*Nocardioides jensenii*, 96 % identity and *N. terrigena*, 94 % identity) and *Alphaproteobacteria* (*Nitrobacter vulgaris,* 99 % identity and *Sphingomonas suberifaciens*, 97 % identity) prevailed in the epipsammic and hyporheic.

Intermittency effects on α and β diversity

Epipsammic and hyporheic biofilms had higher richness (Chao1) and diversity (Shannon-Wiener diversity index and Phylogenetic diversity, PD) than the epilithic biofilm (Fig. 3). The non-flow phase was associated to a decrease in the richness and diversity of all bacterial biofilm communities. The diversity decrease was more pronounced in the epilithic, and non-flow samples were segregated after clustering according to weighted UniFrac distances using Principal Coordinate Analysis (PCO) (Fig. 4). This analysis separated the epilithic samples from epipsammic and hyporheic ones along the first horizontal axis (51.3 % of the variation), whereas the second vertical axis (20.4 % of total variation) segregated the epilithic samples according to the different hydrological phases. The epipsammic and hyporheic samples overlapped in the first axis. The first epilithic samples after flow cessation (weeks 13 and 14) clustered with those during the drying phase (from week 4 to 12); the first samples after flow recovery (weeks 20 and 22) grouped with those from the non-flow phase (weeks 12 to 19); and those collected two months after flow recovery (weeks 27 and 29) grouped with those in the drying phase. These differences between biofilm communities were further identified through PERMANOVA analysis (biofilm: *pseudo* $F_{2,42}$ = 30.758; P < 0.001 and phase: *pseudo* $F_{2,42}$ = 5.075; P < 0.001).

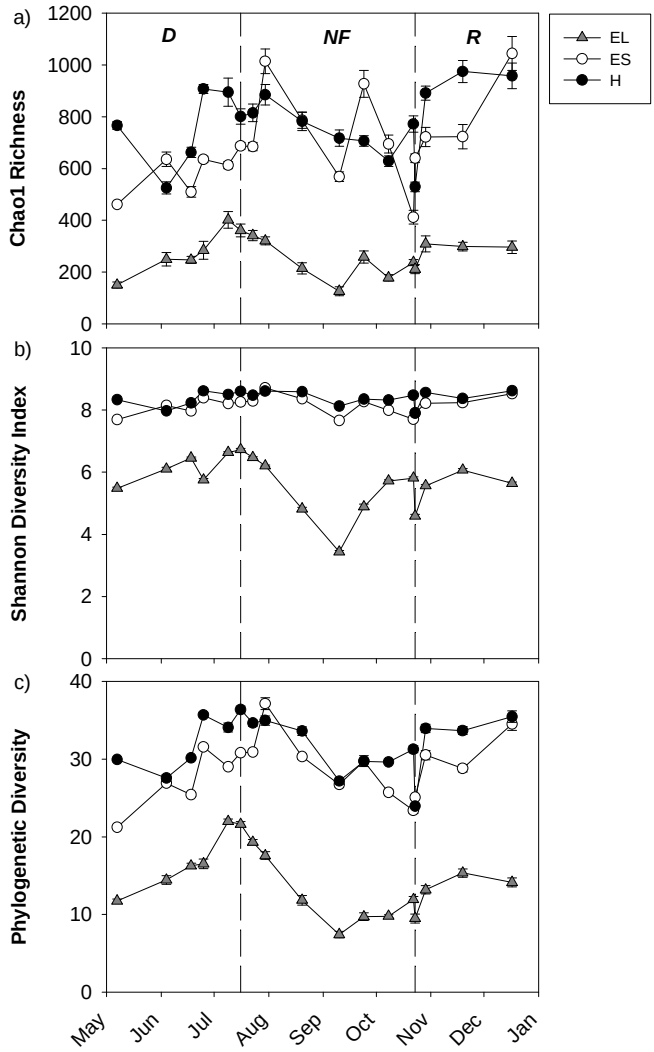

Figure 3. Dynamics of a) Chao 1 richness, b) Shannon diversity index, and c) Phylogenetic Diversityfor the three biofilm compartments during the study period. The symbols represent the different biofilmcompartments: epilithic (EL), epipsammic (ES), and hyporheic (H). The vertical dashed bars indicate the different hydrological phases (*D*-drying, *NF*-non-flow and *R*-rewetting).

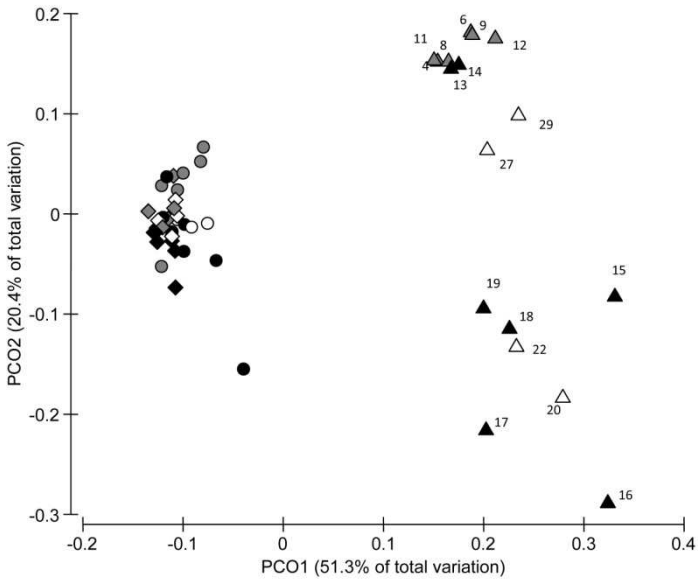

Figure 4. Principal coordinates analysis (PCO) of the UniFrac pairwise dissimilarity of the bacterial communities forall three biofilm compartments and during the study period. The numbers indicate sampling weeks, the symbols indicate the different compartments: epilithic (triangles), epipsammic (circles) and hyporheic (diamonds), and the colors indicate the different hydrological phases: drying (gray), non-flow (black) and rewetting (white).

Nestedness and OTUs turnover

Bacterial communities in the three different biofilms were highly structured, as indicated by the observed significant degree of nestedness. The overall observed nestedness temperature value for the epilithic (T_{obs} = 34.85 ºC [P (T< 34.85) = 1.09 × 10^{-64}]) was significantly lower than the predicted value for randomly generated communities (Atmar and Paterson, 1995) (T_{pred} = 56.10 ºC). Epipsammic and hyporheic biofilms also exhibited lower nestedness temperature*s* (T_{obs} = 35.45 ºC [P (T< 35.45) = 2.63 × 10^{-57}] and T_{obs} = 42.01 ºC [P (T< 42.01) = 1.86 × 10^{-107}], respectively) than the randomly predicted values (T_{pred} = 62.07 ºC, T_{pred} = 65.10 ºC, respectively). T_{obs} values of the epilithic biofilm during the non-flow phase were relatively similar or even higher (week 16) than the overall observed nestedness temperature for the epilithic (Fig. 5 a). However, the T_{obs} in the epipsammic and hyporheic during the non-flow phase were lower than their respective overall observed nestedness temperatures (Fig. 5 b and c). This suggests that sandy

biofilms during the non-flow phase were a subset of the taxa already present during the preceding drying phase. Precisely, the high T_{obs} of the epipsammic and hyporheic biofilms during the drying phase indicate the occurrence of random taxa. Also high T_{obs} characterized the flow resumption phase in all biofilms. The low T_{obs} during the rewetting phase indicated that the bacterial community was a subset of the preceding non-flow phase.

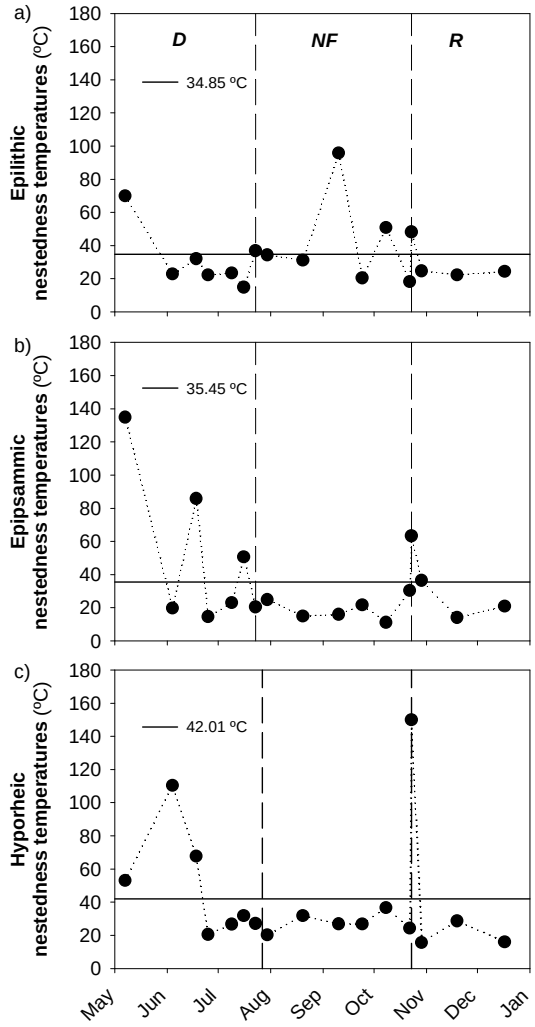

Figure 5. Nestedness temperatures calculated at each sampling date for the a) Epilithic, b) Epipsammic, and c) Hyporheic compartments. The vertical dashed bars indicate the different hydrological phases (*D*-drying, *NF*-non-flow and *R*-rewetting). The observed nestedness temperature for each biofilm compartmentsis indicated with a horizontal solid line.

OTUs turnover increased during the transition from drying to non-flow and, particularly, from non-flow to rewetting phases (from week 19 to week 20) (Table 1). A different trend occurred between biofilm compartments during non-flow, where OTUs turnover increased in the epilithic and decreased in the epipsammic and hyporheic (Table 1).

Table 1. OUTs turnover assessed using the Armstrong index between consecutive sampling dates at each biofilm compartment.

Sampling date (weeks)		Epilithic	Epipsammic	Hyporheic
Drying	04-Jun	0.000021	0.000006	0.000004
	06-Aug	0.000004	0.000008	0.000006
	08-Sep	0.000027	0.000019	0.000014
	09-Nov	0.000011	0.000008	0.000005
	11-Dec	0.000016	0.000016	0.000009
Non-Flow	Dec-13	0.000024	0.000015	0.000009
	13-14	0.000017	0.000013	0.000009
	14-15	0.000017	0.000003	0.000003
	15-16	0.000019	0.000005	0.000004
	16-17	0.000032	0.000007	0.000007
	17-18	0.000023	0.000005	0.000007
	18-19	0.000023	0.000008	0.000007
Rewetting	19-20	0.00019	0.000128	0.000137
	20-22	0.000035	0.000017	0.000023
	22-27	0.00001	0.000003	0.000002
	27-29	0.000005	0.000003	0.000001

Ecological strategies of bacterial communities to desiccation and rewetting

The ecological strategies to desiccation and rewetting varied among dominant phyla and between the different biofilms (Table S2 a and b). The epipsammic and hyporheic biofilms had a higher number of OTUs tolerant to desiccation (47 % and 86 % respectively) than the epilithic (27 %) (Fig. 6 a - c). OTUs sensitive to desiccation were abundant in epilithic biofilm (35 %) and much lower in the epipsammic and hyporheic

biofilms (15 % and 2 %, respectively). Opportunistic OTUs were higher during non-flow in the epilithic and epipsammic biofilms (~35 %) than in the hyporheic (12 %).

The number of OTUs tolerant to rewetting was higher in the epipsammic (60 %) and hyporheic biofilms (72 %) than in the epilithic biofilm (44 %) (Fig. 6 d - f). A major proportion of OTUs were sensitive to rewetting in the epilithic and the epipsammic (23 % and 19 %, respectively) than in the hyporheic (12 %). The opportunistic OTUs reached 33 % in the epilithic, but only 21 % in the epipsammic and 16 % in the hyporheic).

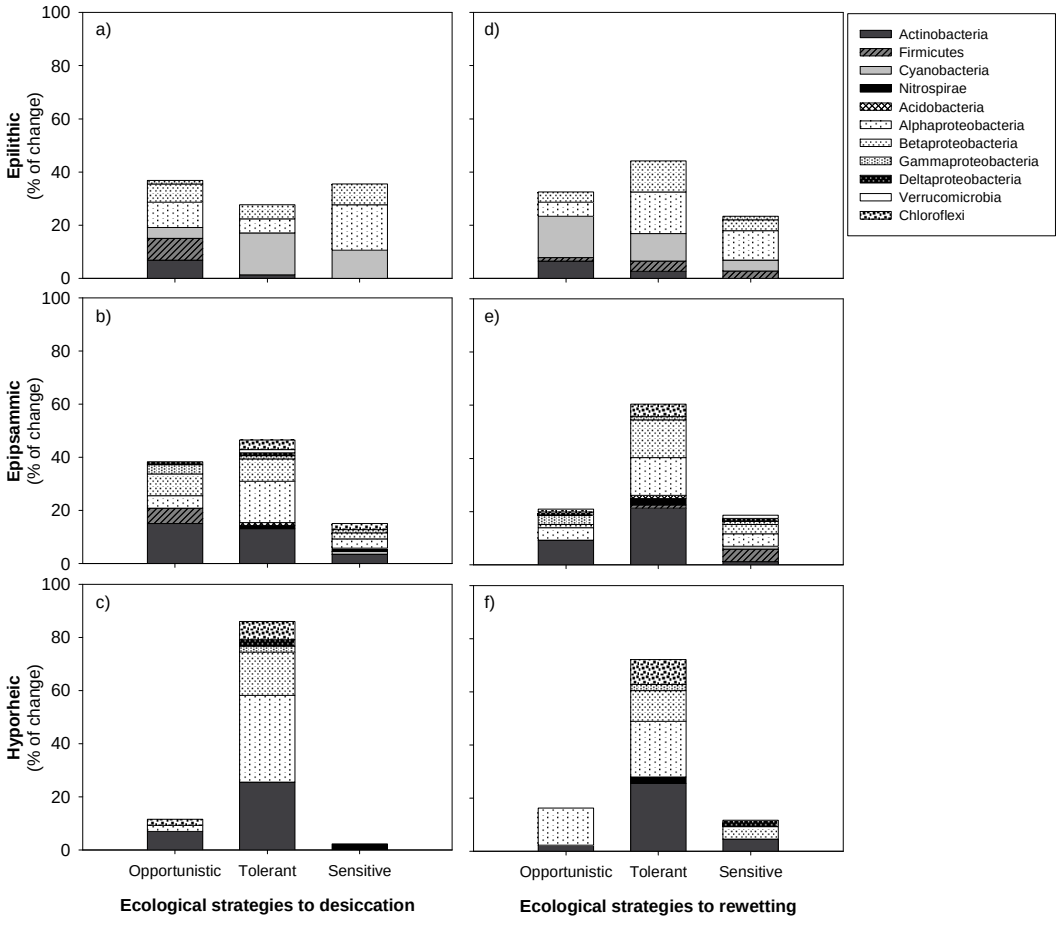

Figure 6. Ecological strategies of biofilm bacterial communities to desiccation on the epilithic (a), epipsammic (b) and hyporheic (c). Ecological strategies of biofilm bacterial communities to rewetting on the epilithic (d), epipsammic (e) and hyporheic (f).

Links between bacterial community composition and environmental variables

Several environmental variables (Mantel tests, rho = 0.229, P = .001) accounted for the bacterial community structure in the different biofilms and hydrological phases. Water content, CBOM and temperature accounted for 37.5 % of the total variation in the bacterial community composition (Table 2). Water content explained the greatest proportion of variation (21.9 %), followed by CBOM and temperature, which accounted for 9.2 % and 6.4 %, respectively.

Table 2. Relationship of the bacterial community composition (based on weighted UniFrac distances) between environmental variables, analyzed using a forward selection procedure in DistLM. The *P*-values were obtained using 9,999 permutations of residuals under a reduced model.

Variable	R^2 (cumulative)	SS(trace)	*pseudo*-F	*P*
% Water content	0.219	0.52	13.728	**0.001**
CBOM	0.311	0.22	6.4506	**0.001**
Temperature	0.375	0.15	4.7431	**0.005**
PAR	0.4	0.06	1.964	0.1

DISCUSSION

The community composition of biofilm bacteria was tightly associated to the hydrological phases in the temporary stream, but changes were not uniform for all stream compartments colonized by biofilms. The bacterial community in epilithic biofilms suffered the most abrupt composition changes during the non-flow phase, probably as a result of their major exposure to desiccation, higher air temperatures and major influence of terrestrial microbes reaching the streambed. The bacterial communities on sand compartments (epipsammic and hyporheic) showed smaller changes, likely because their physical configuration provided higher protection from desiccation and temperature variations (Tonolla *et al.,* 2010). The non-flow period generally reduced the richness and diversity of bacterial communities, and more importantly when the streambed desiccation

was the highest. This period also provided new opportunities for bacterial taxa involved in ecosystem functions such as dry organic matter mineralization.

Epilithic bacterial communities were the most singular in the stream (less than 25% of shared OTUs with other biofilm communities), while those on epipsammic and hyporheic were similar to each other (62 % of shared OTUs). These two biofilms had OTUs affiliated to *Actinobacteria* and *Proteobacteria* throughout the study period. These are taxa common in soils and river sediments (Gao *et al.,* 2005; Tamames *et al.,* 2010) and their members play an important role in the decomposition of organic matter (Santmire and Leff, 2006; Zoppini *et al.,* 2010). Sand sediments retain particulate and dissolved organic materials and their associated bacterial communities are responsible for up to 80 % of the carbon mineralization in streams (Marxsen, 2001; Romaní and Sabater, 2001). Epilithic biofilms had *Cyanobacteria* and *Firmicutes* as the most abundant bacterial groups. Cyanobacteria were predominantly observed during the drying and rewetting phases, when these phototrophic organisms have suitable water availability and light conditions, whereas members of *Firmicutes* were prevalent during the non-flow phase. The bacterial richness (Chao1) and diversity (Shannon-Wiener and Phylogenetic Diversity) were higher in the epipsammic and hyporheic biofilms, likely because of the high number of niches provided by sand particles (Sigee, 2005). The bacterial communities colonizing rocks and cobbles are generally limited by the availability of dissolved carbon in the flowing water and by that of the endogenous carbon derived from the biofilm activity (Freeman and Lock, 1995). However, the bacterial communities in sand sediments can obtain additional resources from entrained particulate and dissolved carbon (Kaplan and Bott, 1989; Battin *et al.,* 2003). Thus, lower diversity and richness in the epilithic biofilm might result from the dominance of phototrophic organisms, the lower number of niches, and the lower resource availability (Romaní and Sabater, 2001).

A major change in the composition of the bacterial community of epilithic biofilms occurred 14 days after flow cessation. This apparent resistance to change might be associated with the protective effect of the growth-form of epilithic biofilms, which are tightly attached to the substratum, resembling a 'crust' (Sabater, 2000; Belnap *et al.,* 2004). The proliferation of Firmicutes during the non-flow phase is probably associated to the characteristics of this group, which includes fast growing species in favorable

conditions (Stott *et al.,* 2008). Members of this group are adapted to thrive under extreme conditions such as desiccation (Vishnivetskaya *et al.,* 2009). Besides, the multiplicity of rRNA operons in the genomes of Firmicutes (7 on average) can also be considered key to the competitive success of these bacteria (Klappenbach *et al.,* 2000). Firmicutes were also observed in the epipsammic during the period of maximum desiccation (weeks 16 to 18). Representative sequences of the two dominant OTUs affiliated with this phylum were closely related to extremophilic members of the *Exiguobacterium and Chryseomicrobium* genera (OTU-0, -1, -1003 and -86) (Chatuverdi and Shivaji, 2006; Vishnivetskaya *et al.,* 2009; Raj *et al.,* 2013). OTUs affiliated to *Firmicutes* were classified as tolerant and opportunistic regarding to desiccation. During the non-flow phase, the most populated OTUs in the epipsammic and the hyporheic were affiliated to *Solirubrobacter* and *Nocardioides* (*Actinobacteria*, OTU-9, -10, -16, and -21), and to *Nitrobacter and Sphingomonas* (*Alfaproteobacteria*, OTU-3 and OTU- 29), common genera from soils showing tolerance to desiccation and in some cases capacity to grow under non flow conditions (opportunistic, OTU -16, OTU -21, and OTU -29). Some members of these phyla could also be considered as opportunistic, *i.e.*, able to growth under non-flow conditions. In fact, the number of living bacterial cells increased during the non-flow phase in all streambed compartments (Timoner *et al.,* 2012). *Firmicutes* and *Actinobacteria* have Gram-positive cell wall type (Fierer *et al.,* 2003; Schimel *et al.,* 2007), and this probably offers them a high resistance to desiccation and rewetting in temporary streams (Marxsen *et al.,* 2010) as well as in soils frequently exposed to hydrological variability (Cruz-Martínez *et al.,* 2009; Fierer *et al.,* 2007; Schimel *et al.,* 2007). There was therefore a wide group of bacterial taxa adapted to flow intermittency (45 % of OTUs shared between the three hydrological phases) in the sandy compartments in Fuirosos.

The observed decrease in richness and diversity during the non-flow phase was probably a reflection of the replacement of many sensitive species by a few other dominant species favored by the new environmental conditions. This decrease in richness and phylogenetic diversity occurred mostly by species sorting through the selection of species tolerant to desiccation (Webb *et al.,* 2002). The bacterial community shifted to a narrower phylogenetic diversity, where only specialized taxa survived (Febria *et al.,* 2011). The low nestedness temperatures in the epipsammic and hyporheic biofilms during the non-flow phase indicated that bacterial communities derived from the species pool of the preceding

wetted phase (the drying phase) accordingly, Zeglin *et al.* (2001) suggested that dormancy plays a key role in the recovery of bacterial diversity. However, the high nested temperatures in the epilithic during the same phase were indicative of a non-nested pattern, and suggested a poor adaptation of indigenous members of the community to desiccation. This was confirmed by the high occurring proportion of sensitive taxa to desiccation in that biofilm. The occurrence of immigrant bacteria (~ 36 % were opportunistic to desiccation) in the epilithic biofilm probably derived from terrestrial sources (*e.g.*, dust deposition and leaf accumulation). The Armstrong index also shows an increase in the OTUs turnover in epilithic biofilms associated to the occurrence of new taxa. The arrival of immigrant microorganisms from external sources is an expression of the biological link between terrestrial and aquatic ecosystems during the non-flow phase, and that has been proven very effective with other organisms (Steward *et al.,* 2012). The high nestedness temperatures also occurring during the drying phase might be associated to the patchiness during streambed contraction, which provides a wide range of habitats and conditions for versatile bacteria to thrive (Febria *et al.,* 2011).

Flow recovery promoted changes in the bacterial community composition, primarily in the epilithic compartment. The nestedness temperatures and the Armstrong index indicated that the transition from the non-flow phase to the rewetting phase severely impacted biofilm bacterial communities. While desiccation is a lengthy process that facilitates a gradual adaptation (Robson *et al.,* 2008), water flow returns occurs in few hours and drastically affects those taxa unable to resist inundation (sensitive taxa). Our results show, however, that epilithic and epipsammic bacterial communities tolerated better the rewetting than the desiccation. The increase in the nestedness temperature immediately after flow recovery might be due to the occurrence of opportunistic taxa in the stream after washout (Fazi *et al.,* 2008). The epilithic and epipsammic biofilms showed the highest proportion of sensitive and opportunistic OTUs to rewetting and indicated their tight association to flow intermittency. Those bacterial communities in the hyporheic had a more stable composition through the study period, with a major number of tolerant OTUs to both desiccation and rewetting (~ 80 %). The epilithic biofilm required more than two weeks to recover to a composition similar to that before desiccation, and this recovery was shorter in the sandy compartments. One week after flow resumption bacterial richness and diversity in all biofilms recovered to values similar to

those before desiccation. This certainly stresses the high resilience of bacterial communities (and especially of those in subsuperficial substrata) to cope with flow discontinuities in temporary streams. This recovery in community composition coincided with that in the biofilm activity. The β-glucosidase and Alkaline phosphatase enzymatic activities were higher during the rewetting phase than before flow cessation (Timoner *et al.,* 2012), and outlined the prevalence of the heterotrophic processes during that period (Romani *et al.,* 2013). When flow returns the high availability of good quality organic matter accumulated during the terrestrial phase (Ylla *et al.,* 2011; Timoner *et al.,* 2012) triggers the bacterial activity in a well-adapted community to the new situation.

CONCLUSIONS

Streams in temperate regions are under risk of recurrent flow intermittency, implying extended interactions between terrestrial and aquatic ecosystems, and potential effects on biogeochemical cycles and ecosystem functioning. Global change is accelerating the rate at which such changes occur, and potentially impair the capacity of microbial communities to cope with drastic fluctuations of environmental conditions. The resilience of biofilm bacterial communities in temporary streams might mitigate to some extent these effects, specifically when these are compared to other systems with lower intra-annual water flow variability. Assuming the crucial role of streambed microbial communities in biogeochemical cycles and ecosystem functioning, the understanding of how these communities respond and adapt to such environmental changes may help to anticipate potential consequences at local and global scales.

SUPPLEMENTARY TABLES

Supplementary Table S1. Average number of 16S rRNA operons in genomes of listed bacterial Phyla (class for *Proteobacteria*) according to the last version of the ribosomal RNA Database (http://rrndb.umms.med.umich.edu/, Klappenbach et al., 2001; Lee et al., 2009).

Phyla/Class	Average number of 16S rRNA operons
Acidobacteria	1.5
Actinobacteria	3
Bacteroidetes	3.3
Chloroflexi	1.7
Cyanobacteria	2.3
Firmicutes	7
Nitrospira	1
Planctomycetes	1.75
Proteobacteria	
Alphaproteobacteria	2.4
Betaproteobacteria	3.7
Gammaproteobacteria	5.7
Deltaproteobacteria	2.8
Verrucomicrobia	1.6

Supplementary Table S2 a. Ecological strategies to desiccation among the different bacterial Phyla (Class for Proteobacteria) for each biofilm type and the corresponding OTUs.

Biofilm type	Ecological strategy to desiccation	Phyla/Class	#OTU
Epilithic	Opportunistic	*Actinobacteria*	36, 39, 57, 76, 113
	Opportunistic	*Cyanobacteria*	18, 47, 70
	Opportunistic	*Firmicutes*	0, 1, 62, 73, 86, 1003
	Opportunistic	*Proteobacteria*	
	Opportunistic	*Alphaproteobacteria*	22, 77, 81, 112, 133, 160, 2138
	Opportunistic	*Betaproteobacteria*	11, 61, 94, 109, 1571
	Opportunistic	*Gammaproteobacteria*	56
	Tolerant	*Actinobacteria*	43
	Tolerant	*Cyanobacteria*	2, 4, 6, 7, 13, 15, 25, 63, 216, 416, 557, 902
	Tolerant	*Proteobacteria*	
	Tolerant	*Alphaproteobacteria*	3, 14, 74, 110
	Tolerant	*Betaproteobacteria*	26, 54, 68, 328
	Sensitive	*Cyanobacteria*	5, 28, 34, 45, 83, 154, 856, 1093
	Sensitive	*Proteobacteria*	
	Sensitive	*Alphaproteobacteria*	12, 19, 20, 23, 35, 40, 64, 72, 75, 80, 131, 134, 219
	Sensitive	*Betaproteobacteria*	8, 48, 103, 118, 148, 162
Epipsammic	Opportunistic	*Actinobacteria*	10, 16, 21, 39, 57, 76, 84, 92, 95, 101, 105, 117, 130
	Opportunistic	*Firmicutes*	0, 1, 99, 127, 1003
	Opportunistic	*Proteobacteria*	
	Opportunistic	*Alphaproteobacteria*	55, 81, 97, 115
	Opportunistic	*Betaproteobacteria*	11, 30, 44, 61, 94, 108, 1571
	Opportunistic	*Deltaproteobacteria*	150
	Opportunistic	*Gammaproteobacteria*	56, 60, 111
	Tolerant	*Acidobacteria*	58
	Tolerant	*Actinobacteria*	9, 17, 36, 38, 41, 43, 51, 65, 69, 102, 183
	Tolerant	*Chloroflexi*	52, 157, 209
	Tolerant	*Nitrospirae*	31
	Tolerant	*Proteobacteria*	
	Tolerant	*Alphaproteobacteria*	3, 12, 24, 29, 37, 42, 49, 50, 53, 71, 89, 106, 120
	Tolerant	*Betaproteobacteria*	8, 48, 68, 90, 103, 185, 1653
	Tolerant	*Deltaproteobacteria*	143
	Tolerant	*Gammaproteobacteria*	93
	Tolerant	*Verrucomicrobia*	66
	Tolerant		104
	Sensitive	*Actinobacteria*	79, 85, 87
	Sensitive	*Chloroflexi*	46, 151
	Sensitive	*Cyanobacteria*	34
	Sensitive	*Nitrospirae*	98
	Sensitive	*Proteobacteria*	
	Sensitive	*Alphaproteobacteria*	27, 32, 250
	Sensitive	*Betaproteobacteria*	67, 82
	Sensitive	*Gammaproteobacteria*	33
Hyporheic	Opportunistic	*Actinobacteria*	10, 16, 128
	Opportunistic	*Chloroflexi*	88
	Opportunistic	*Proteobacteria*	
	Opportunistic	*Alphaproteobacteria*	29
	Sensitive	*Nitrospirae*	31
	Tolerant	*Actinobacteria*	9, 21, 38, 41, 51, 65, 69, 87, 130, 183, 188
	Tolerant	*Chloroflexi*	46, 52, 151
	Tolerant	*Proteobacteria*	
	Tolerant	*Alphaproteobacteria*	3, 12, 24, 27, 32, 37, 42, 49, 50, 53, 55, 59, 106, 114
	Tolerant	*Betaproteobacteria*	30, 48, 68, 82, 91, 185, 1653
	Tolerant	*Deltaproteobacteria*	191
	Tolerant	*Gammaproteobacteria*	33

Supplementary Table S2 b. Ecological strategies to rewetting among the different bacterial Phyla (Class for Proteobacteria) for each biofilm type and the corresponding OTUs.

Biofilm type	Ecological strategy to rewetting	Phyla/Class	#OTU
Epilithic	Opportunistic	*Actinobacteria*	39, 57, 76, 113, 171
	Opportunistic	*Cyanobacteria*	2, 6, 13, 15, 18, 28, 34, 45, 154, 216, 416, 856
	Opportunistic	*Firmicutes*	86
	Opportunistic	*Proteobacteria*	
	Opportunistic	*Alphaproteobacteria*	74, 75, 134, 219
	Opportunistic	*Betaproteobacteria*	54, 94, 1571
	Tolerant	*Actinobacteria*	36, 43
	Tolerant	*Cyanobacteria*	4, 5, 7, 25, 63, 557, 902, 1093
	Tolerant	*Firmicutes*	0, 1, 1003
	Tolerant	*Proteobacteria*	
	Tolerant	*Alphaproteobacteria*	14, 20, 22, 35, 40, 72, 77, 81, 110, 112, 131, 2138
	Tolerant	*Betaproteobacteria*	8, 11, 26, 48, 61, 68, 109, 118, 328
	Sensitive	*Cyanobacteria*	47, 70, 83
	Sensitive	*Firmicutes*	62, 73
	Sensitive	*Proteobacteria*	
	Sensitive	*Alphaproteobacteria*	3, 12, 19, 23, 64, 80, 133, 160
	Sensitive	*Betaproteobacteria*	103, 148, 162
	Sensitive	*Gammaproteobacteria*	56
	Sensitive		107
Epipsammic	Opportunistic	*Actinobacteria*	16, 17, 38, 43, 57, 95, 101, 117
	Opportunistic	*Chloroflexi*	46
	Opportunistic	*Proteobacteria*	
	Opportunistic	*Alphaproteobacteria*	29, 32, 55, 81
	Opportunistic	*Betaproteobacteria*	8
	Opportunistic	*Deltaproteobacteria*	143
	Opportunistic	*Gammaproteobacteria*	56, 60, 111
	Tolerant	*Acidobacteria*	58
	Tolerant	*Actinobacteria*	9, 10, 21, 36, 39, 41, 65, 69, 76, 79, 84, 85, 87, 92, 102, 1(
	Tolerant	*Chloroflexi*	52, 151, 157, 209
	Tolerant	*Firmicutes*	127
	Tolerant	*Nitrospirae*	31, 98
	Tolerant	*Proteobacteria*	
	Tolerant	*Alphaproteobacteria*	3, 12, 24, 27, 37, 42, 49, 53, 97, 115, 120, 250
	Tolerant	*Betaproteobacteria*	11, 30 , 44, 61, 82, 90, 94, 103, 108, 185, 1571, 1653
	Tolerant	*Gammaproteobacteria*	33
	Tolerant		104
	Sensitive	*Actinobacteria*	51
	Sensitive	*Cyanobacteria*	34
	Sensitive	*Firmicutes*	0, 1, 99, 1003
	Sensitive	*Proteobacteria*	
	Sensitive	*Alphaproteobacteria*	50, 71, 89, 106
	Sensitive	*Betaproteobacteria*	48, 67, 68
	Sensitive	*Deltaproteobacteria*	150
	Sensitive	*Gammaproteobacteria*	93
	Sensitive	*Verrucomicrobia*	66
Hyporheic	Opportunistic	*Actinobacteria*	21
	Opportunistic	*Proteobacteria*	
	Opportunistic	*Alphaproteobacteria*	12, 29, 32, 42, 49, 55
	Tolerant	*Actinobacteria*	10, 16, 38, 41, 51, 65, 69, 87, 128, 183, 188
	Tolerant	*Chloroflexi*	46, 52, 88, 151
	Tolerant	*Nitrospirae*	31
	Tolerant	*Proteobacteria*	
	Tolerant	*Alphaproteobacteria*	3, 24, 27, 37, 50, 53, 59, 106, 1147
	Tolerant	*Betaproteobacteria*	48, 68, 91, 185, 1653
	Tolerant	*Gammaproteobacteria*	33
	Sensitive	*Actinobacteria*	9, 130
	Sensitive	*Proteobacteria*	
	Sensitive	*Betaproteobacteria*	30, 82
	Sensitive	*Deltaproteobacteria*	191

Chapter 4.

Biofilm functional responses to the rehydration of a dry intermittent stream.

INTRODUCTION

Rivers and streams that experience a recurrent non-flow phase are considered to be intermittent (Williams, 2006). Worldwide these ecosystems comprise a substantial proportion of the total number, length and discharge of fluvial networks (Tooth, 2000), forming more than half of the total river length in Australia (Davies *et al.*, 1994), South Africa (Uys and O'Keefe, 1997), United States (Nadeau and Rains, 2007), and SE Europe (Tzoraki *et al.*, 2007). In addition, virtually all running waters in the Arctic and Antarctic regions are intermittent owing to ice formation (Howard-Williams *et al.*, 1986). In arid or semiarid regions, the non-flow phase usually occurs during the dry season (Gasith and Resh, 1999) that fragment the stream into scattered pools or other wetted areas. The streams sometimes completely dry up (Gasith and Resh, 1999; Lake, 2003) and the associated biota of streambed sediments experience the effect of desiccation accompanied by high temperatures and intense solar radiation.

Flow intermittency has several effects on stream biota and ecosystem processes (Boulton and Lake, 1992; Bunn and Arthington, 2002; Dahm *et al.*, 2003). A substantial part of the carbon and nutrient cycling in streams is mediated by biofilms (Buesing and Gessner, 2003; Rees *et al.*, 2006), which are directly affected by desiccation. Biofilms are complex communities including bacteria, algae, fungi and protozoa within a protective matrix of extracellular polymeric substances (Wetzel, 1983; Lear *et al.*, 2012). There is evidence of changes in biofilm structure and function in response to flow intermittency (Amalfitano *et al.*, 2008; Febria *et al.*, 2011; Timoner *et al.*, 2012) that in turn affect food quality, nitrification, and production of organic materials (Ylla *et al.*, 2010; von Schiller *et al.*, 2011; Romaní *et al.*, 2012).

While research on the flow intermittency effects on biofilms has mostly focused on biodiversity and functional indicators such as enzymatic activities (Amalfitano *et al.*, 2008; Zoppini *et al.*, 2010; Romaní *et al.*, 2012), the effects of flow intermittency on the functional diversity of biofilms are largely unknown. It is true that the exclusion or addition of different bacterial types does not necessarily change the resultant community

function (Allison and Martiny, 2008), but the change associated with desiccation is not defined. Williams and Rice (2007) analyzed the functional diversity of soil microorganisms affected by dry periods and observed that their capacity to degrade different organic carbon sources increased in soils sporadically exposed to wet conditions compared with soils permanently exposed to dry conditions (Mamilov and Dilly, 2002; Iovieno and Bååth, 2008; Braun *et al.*, 2010). Similarly, intermittent streams in arid or semiarid regions might occasionally experience summer storms that rehydrate the streambed sediments during the non-flow phase, even for a short period of time. Since biofilm functioning rapidly recovers after flow resumption (Romaní and Sabater, 1997; Marxsen *et al.*, 2010; Timoner *et al.*, 2012), effects on the stream biofilm structure and functioning should also occur after these sequential events.

The duration and spatial extent of flow intermittency is increasing globally because of increasing unpredictability and severity of drought events due to climate change and excessive water abstraction (Milly *et al.*, 2005; Hirabayashi *et al.*, 2008; Döll and Schmied, 2012). Therefore, understanding how biofilm communities respond to desiccation and rehydration events during non-flow periods will help to formulate predictions on the biogeochemical and ecological implications of increasing flow intermittency.

The main objective of this study was to determine the effect of rehydration events on the biofilm functional diversity in an intermittent stream. We also wanted to determine the interaction between the length of the dry phase and the occurrence of the rehydration event on the biofilm response. Hence, we studied three different pools in the same study reach that differed in the length of time that they remained dry. While one of these pools was permanently inundated, a second dried up one week before the beginning of the study and the third dried up one month before. We hypothesized that *i*) biofilm functional diversity would decrease during the dry phase, and *ii*) that the functional diversity would recover to values similar to the permanently inundated biofilms after the rehydration events.

METHODS

Study site and field sampling

This chapter derives from the field monitoring study in the Dam Creek that was performed during the austral summer 2011 (see more details about the study site and field sampling in the Methodology section).

Temperature (T), conductivity (EC), pH (TPS WP81 pH, Total Dissolved Salts, Temperature and Conductivity Meter, Thermo Fisher, Scoresby, Australia) and oxygen saturation (DO) (TPS WP 82 Dissolved Oxygen and Temperature Meter, Thermo Fisher, Scoresby, Australia) were measured before the first sampling date for 5 months at the 3 stream pools, however, during the experiment, these parameters were measured only at the permanent pool due to the water permanence. Three temperature and humidity data loggers (Hydrochron iButtons DS1923, iButtonLink, USA) collected data every 5 min and were deployed in each pool for a period of 0.5 h to characterize streambed temperatures (between 10 to 12 pm). Dam Creek is not a gauged stream but rainfall data were available from three nearby meteorological stations (143010B-Emu Creek at Boat Mountain; 143016A-Maronghi Creek at Glendale and 143007A-Brisbane River at Linville, http://watermonitoring.derm.qld.gov.au/host.htm, DERM Water monitoring data portal). The rainfall data were expressed as the accumulated rainfall between sampling dates that is, between 11-13 days before each sampling date.

Biofilms on the superficial streambed sediments (0-2 cm in depth) were sampled in triplicate, with three different cores collected in each pool and at each sampling date using an untapped syringe of 60 mL (2.6 cm in diameter). Samples were sealed in sterilized plastic bags and stored at 4ºC until they were processed at the laboratory. Another set of samples (three replicates) from each pool and at each sampling date was collected to determine the percentage of water content of the streambed sediments.

Laboratory analyses

For each replicate, a subsample (1 mL) was recovered using a 5 mL syringe cut at the edge (1.2 cm in diameter), and each subsample was placed in sterilized vials with 15 mL of a sterile solution (Ringer solution, Scharlab, S.L., Barcelona, Spain). Biofilms were detached

from the sediments by two 1 min sonication steps using an ultrasonic bath (40 W and 40 kHz, Selecta, JP Selecta SA). Gentle sonication during moderate time avoids lysing bacterial cells (Romaní *et al.*, 2009). Samples were allowed to settle for 1 min after sonication in order to reduce the masking effects because of large particles (Kuwae and Hosokawa, 1999; Griebler *et al.*, 2001), and 1mL of the supernatant from the suspension was added to 49 mL of a sterile dilutant (Ringer solution) in a sterilized flask and the solution was swirled to ensure that it was thoroughly mixed. This biofilm suspension was used for bacterial density counts and BiologEcoPlates™ analysis, which were performed as described below. For the samples from the short and long term dry pools, the biofilm suspension was prepared right before the extraction of the biofilm for bacterial density and BiologEcoPlates™ analysis in order to avoid community changes due to rehydration before the analysis. All solutions, transfer equipment and glassware were sterilized in an autoclave prior to use.

Water content of the streambed sediments was calculated as the difference between wet and dry weight of the samples after 24 h at 110 ^{0}C. Water content was expressed as the percentage of wet weight.

Total bacterial cell densities were determined using a DNA stain, Acridine Orange (Sigma-Aldrich Co. LLC., St. Louis, USA). The biofilm suspension (1 mL), previously fixed with 0.5 % glutaraldehyde, was stained for 15 min at a final Acridine Orange concentration of 125 µg L^{-1}. Samples were then filtered through a 0.2 µm black polycarbonate filter (WhatmanNucleopore, Whatman International Ltd., Maidstone, England) and mounted on a microscope slide with non-fluorescing immersion oil. Total bacterial cells were counted on 15randomly chosen fields per slide using a fluorescence microscope (Leica DM 4000B, Wetzlar, Germany).

BiologEcoPlates™ (BIOLOG, Hayward, USA) were used to determine changes in the biofilm functional diversity based on the biofilm ability to degrade different carbon sources. The BiologEcoPlates™ were created specifically for community analysis and microbial ecological studies, since they contain environmentally applicable carbon sources (Garland, 1997; Insman, 1997). A Biolog plate contains three replicate sets of 31 different carbon sources and one control well (96 wells). If the microorganisms inoculated into each

well are able to oxidize the carbon source within the well, the colourless tretrazolium dye is reduced to violet formazan as a function of the degree of use of this carbon source. The colour development in each well is measured spectrophotometrically and over time. Thus, the number of used carbon sources, their colour intensity, as well as their identity give valuable information about functioning of the microbial community (Stefanowicz, 2006). Each well on the plate was inoculated with 150 µL of the previously diluted biofilm suspension and contained about 10^5 cells mL^{-1} based on epifluorescence microscope counts that allowed a proper sigmoidal colour development. This was tested before the beginning of the study with separate biofilm samples from the same study reach. In total, 36 plates were analyzed (using one plate per sample; that is, three plates per stream pool at each sampling date). Plates were incubated in a temperature-controlled laboratory at 23 ^{0}C in the dark and the optical density at 590 nm (OD_{590}) in each well was recorded after 24, 48, 72, 144 and 168 hours using a microplate reader (Microstation B070 Microplate Reader, BIOLOG, Hayward, USA).

Data analysis

For the Biolog data, 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 substrates in the plate (Garland and Mills, 1991). AWCD follows an asymptotic sigmoidal curve with time and the kinetic parameters of the curves were determined by fitting a density dependent logistic growth equation using Sigma Plot 11.0 (Systat Software, Inc., San Jose, USA):

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

where a is the asymptote, b is the maximum rate of colour development, being $1/b$ the velocity at which the colour development in the plate occurs and X_0 is the lag time passed until the first colour development in the plate, interpreted as the time that the community needs to begin degrading a carbon source (Lindstrom *et al.*, 1998). The two kinetic parameters $1/b$ and X_0 were used to analyze the biofilm functioning in each pool and at each sampling date.

Limits of the Biolog technique (Preston-Mafham *et al.*, 2002) require consideration during field and laboratory procedures, but also during data analysis. Biolog is a culture-based approach and suffers from the same inherent biases as a selective culturing technique. During the incubation the bacterial community in each well changes due to the selective forces of the incubation media, and measurements after a certain incubation time do not necessarily reflect the original community structure and functioning (Smalla *et al.*, 1998). Regarding the incubation time (48 or 72 h), 48 h allowed colour development in all the plates and allowed comparing the actual responses of the inoculated biofilm community rather than community changes.

Shannon diversity index was calculated based on the number of carbon sources used after 48 h of incubation, as described by Insam and Goberna (2004) to evaluate the functional diversity (Zak *et al.*, 1994). Differences in the patterns of carbon use between stream pools and sampling dates were standardized by the AWCD (Garland, 1996). Moreover, differences in the carbon source use pattern were calculated for the 31 carbon sources as the percentage of colour of each well with respect to the total colour in the plate (Sala *et al.*, 2006; Tiquia, 2010) in order to have a semi-quantitative idea of the number and identity of the carbon sources used by the biofilm at each pool and sampling date.

Differences in functional diversity (H'), maximum slope ($1/b$) and lag time (X_0) among the different pools and sampling dates were examined using analysis of variance (two-way ANOVA). Tukey's HSD multiple comparison test was used to examine the differences between pools and samplings dates. Percentage of water content, temperature and accumulated rainfall were related to the microbial functioning by means of Pearson correlation analysis. The significance level was set at $P < 0.05$ for all analyses. Data were log-transformed ($x = \log (x + 1)$) when necessary to meet the assumptions of parametric tests. Analyses were carried out using SPSS 17.0 for Windows (SPSS, Chicago, USA). Differences in the carbon use pattern for the biofilm in each pool and at each sampling date were graphically represented in a non-parametric multi-dimensional scaling ordination (NMDS), Bray–Curtis was selected as a dissimilarity distance. Shifts observed in the NMDS representation were assessed using a PERMANOVA of two factors (pool and sampling date) (Anderson, 2001). NMDS and PERMANOVA were performed with PRIMER-E v.6.1.11 and PERMANOVA + v.1.0.1 (PRIMER-E Ltd., Ivybridge, UK).

RESULTS

Physicochemical parameters

The three different stream pools (permanent, short term dry and long term dry) showed similar values in terms of pH, conductivity, and temperature before the streambed drying (Table 1), except for the highest oxygen saturation in the permanent pool. The main rainfall event occurred between T1 and T2 with an accumulated rainfall of 19 mm. The percentage of water content of the streambed sediments, at the beginning of the study was 21.2 ± 1.6 % in the permanent pool, 12.7 ± 0.6 % in the short term dry pool and 5.5 ± 0.4 % in the long term dry pool. The rainfall before T2 caused the rehydration of the dry streambed sediments in the short term dry to 16.1 ± 0.5 % and to 6.8 ± 1 % in the long term dry. Afterwards, the absence of rain resulted in extreme drying of the sediment in short and long term dry pools, which reached a water content of 2.1 ± 0.2 % and 1.6 ± 0.6 %, respectively at T3. The water content remained low at T4 in in short and long term dry pools, 2.1 ± 0.1 % and 4.32 ± 0.1 % respectively (Table 2). The maximum streambed temperature was reached during T3, with values of 45 ºC, coinciding with the minimum water content in the dry pools. Owing to water permanence, the permanent pool showed a relatively steady temperature of ~ 25 ºC and water content (21 %) during all sampling dates.

Table 1. Mean values and standard deviations of pH, conductivity (EC), dissolved oxygen (DO) and temperature (T) for each stream pool before the beginning of the study. Also the averaged values of the same parameters during the study period for the permanent pool are shown.

	pH	EC (µS cm^{-1})	% DO	T (ºC)
Permanent pool (n=3)	7.8±0.1	1255.3±39.5	98.2±4.5	17.3±1.1
Short term dry pool (n=3)	7.6±0.2	1257.3±32.3	67.2±15.8	15.9±1.9
Long term dry pool (n=3)	7.7±0.2	1248.7±31.2	81.1±21.1	16.7±0.9
Permanent pool (T1, T2, T3, T4, n=4)	7.7±0.2	1251.6±107.2	120.5±63.2	25.4±4.7

Table 2. Percentage water content of the streambed sediments (mean values and standard deviations) for each stream pool at each sampling date.

% Watercontent	T1 26/10/2011	T2 07/11/2011	T3 21/11/2011	T4 05/12/2011
Permanent pool (P) (n=3)	21.2±1.6	27.2±1.7	20.9±0.8	22.5±0.3
Short term dry pool (S) (n=3)	12.7±0.6	16.1±0.5	2.1±0.2	2.1±0.1
Long term dry pool (L) (n=3)	5.5±0.4	6.8±1	1.6±0.6	4.3±0.1

Biofilm functioning

Differences in bacterial cell density for each pool and sampling date where observed (Table 3), though values at the permanent pool were more consistent over time than at the short and long term dry pools. Bacterial density increased in short and long term dry pools at T2, after the wet pulse (Tukey's test, P <0.05) (Fig. 1). This rainfall event provided a slight increase of sediment water content but the biofilm reacted more at the long term dry pool tha at the short term dry, according to the time exposed to desiccation (r = -0.547, P < 0.05).

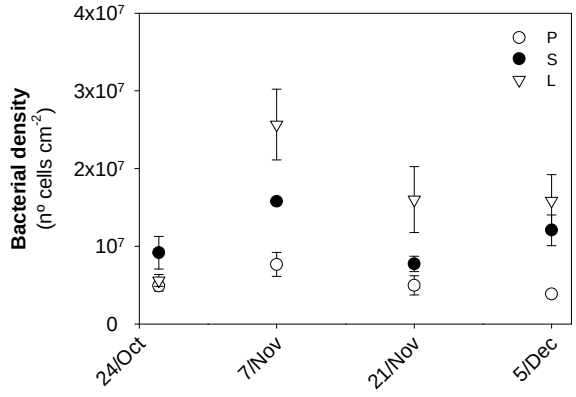

Figure 1. Bacterial density for each stream pool (P-permanent, S-short term dry and L-long term dry) and sampling date (sampling dates are refereed in the text as: T1= 27/Oct, T2= 7/Nov, T3= 21/Nov and T4= 5/Dec). The vertical arrow indicates when the rainfall event occurred.

Functional diversity (H'), based on the number of different carbon sources used by biofilm communities for each pool and at each sampling date, showed significant differences between sampling dates (Tukey's test, $P < 0.05$), but not between pools (Table 2) (Fig. 2 a). Biofilms from the short term dry pool showed the highest capacity to degrade different carbon sources at T1. After streambed rehydration (T2) H' increased in all pools (Fig. 2 a) ($r = 0.382$, $P < 0.05$). At subsequent sampling dates, functional diversity decreased, especially in biofilms from short and long term dry pools compared with biofilms in the permanent pool. Biofilms in short and long term dry pools were exposed to desiccation and high temperatures. The functional diversity of biofilms in the permanent pool was higher at T2.

Table 3.Two-way ANOVA results considering single source effects and interactions of the two factors, sampling date and stream pool. *P*-value < 0.05 was considered as significant. nº cells cm^{-2} =bacterial density; H' = Functional diversity, $1/b$= maximum slope and X_0 = lag time.

Sources	nº cells cm^{-2}	H'	$1/b$	X_0
Sampling date	***P* < 0.000**	***P* < 0.000**	***P* < 0.000**	***P* < 0.000**
	$F_{3,24} = 15.361$	$F_{3,24} = 8.843$	$F_{3,24} = 13.523$	$F_{3,24} = 12.141$
Pool	***P* < 0.000**	$P = 0.170$	***P* = 0.029**	***P* = 0.020**
	$F_{2,24} = 42.095$	$F_{2,24} = 1.907$	$F_{2,24} = 4.108$	$F_{2,24} = 4.618$
Sampling date *Pool	***P* = 0.002**	***P* = 0.004**	$P = 0.120$	***P* = 0.004**
	$F_{6,24} = 5.109$	$F_{6,24} = 4.432$	$F_{6,24} = 1.914$	$F_{6,24} = 4.449$

At T1, the biofilm from the short term dry pool took less time (low X_0 values) to degrade carbon sources in the plate, and accordingly kinetic parameter $1/b$ values were high. However, the biofilm from the long term dry pool showed a low metabolic capacity (low $1/b$ values) and took more time (high X_0 values) to degrade carbon sources. Once the biofilm was rehydrated at T2, $1/b$ was the highest for the biofilsm in the short term dry pool (Tukey's test, $P < 0.05$) (Fig. 2 b). Biofilms from short and long term dry pools showed the lowest values of X_0 at T2 (Tukey's test, $P < 0.05$) (Fig. 2 c). After the rainfall event, X_0 values decreased, specifically for biofilm samples from short and long term dry pools, and $1/b$ values increased for biofilm samples from short and long term dry pools, implying that the rehydration event stimulated the rapid biofilm response to carbon source

degradation (r = 0.389, $P < 0.05$). X_0 increased in biofilm samples from short and long term dry pools at T3 and T4, when desiccation of the streambed sediments occurred again. X_0 was also correlated with the amount of accumulated rainfall (r = -0.374, $P < 0.05$). Biofilm in the permanent pool showed an intermediate response, and the rainfall event produced $1/b$ and X_0 changes.

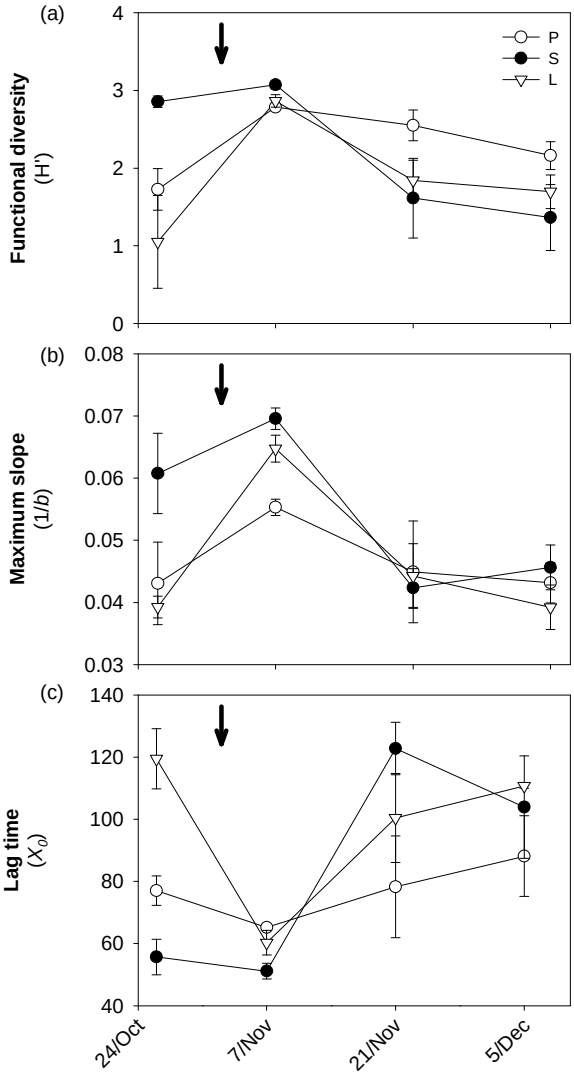

Figure 2. a) Functional diversity (H') after 48h incubation and sigmoid kinetic parameters: b) maximum slope ($1/b$) and c) lag time in hours (X_0) for each stream pool (P-permanent, S-short term dry and L-long term dry) and sampling date (referred in the text as T1= 27/Oct, T2= 7/Nov, T3= 21/Nov and T4= 5/Dec). The vertical arrow indicates when the rainfall event occurred.

The carbon use patterns of biofilms differed with time (Fig. 3). The samples from the permanent pool were grouped close together in the ordination analysis (NMDS) meaning that no marked changes occurred, while samples from the short and long term dry pools showed higher dispersion. Samples from all pools grouped together at T2, when the rehydration of streambed sediments occurred, suggesting that the carbon use pattern at that time was similar (Fig. 3). After the rehydration event, the capacity to degrade different carbon sources decreased for biofilm communities from short and long term dry pools, while communities from the permanent pool maintained the same capacity. The PERMANOVA analysis detected significant differences in the carbon use between stream pools (pool: *pseudo-*$F_{2,23}$ = 1.888, P = 0.004) and sampling dates (time: *pseudo-*$F_{3,23}$ = 3.245, P = 0.001).

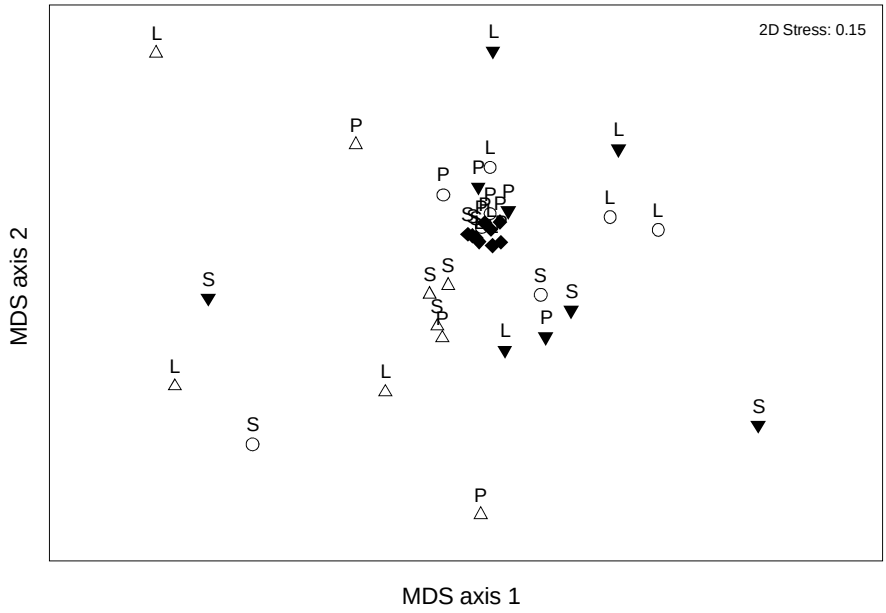

Figure 3. Non-parametric multidimensional scaling (MDS) representing samples over time and between stream pools. Symbols represent the different sampling dates: T1, up triangle; T2, black diamond; T3, white circle; and T4, black circle. And the letters the different pools: permanent (P), short term dry (S) and long term dry (L).

These differences showed the carbon use profile for the biofilm in each pool and sampling time (Fig. 4). A large number of carbon sources were used by biofilm communities from the permanent pool over the study period, whereas the number of used carbon sources varied considerably over the study period in biofilms communities from short and long term dry pools, decreasing at T3 and T4 (12 to 17) compared to T2 (27 to 29). The

capacity to degrade amino acids of biofilms from short and long term dry pools was low at T3 and T4, and amines were not used for the biofilm communities in the long term dry pool. Biofilms from the long term dry pool used a broader range of carbohydrates than those from the short term dry pool.

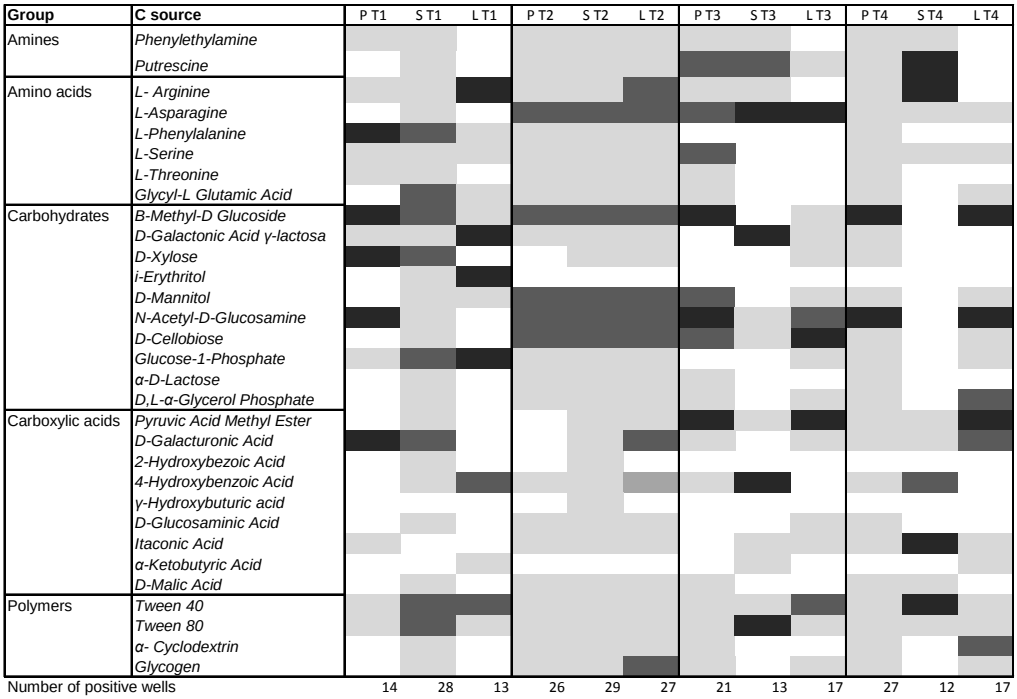

Figure 4. Pattern of carbon use based on the AWCD 48h of the 31 carbon sources for biofilms from permanent-P, short term dry-S and long term dry-L pools at each sampling date (T1, T2, T3 and T4). Shading in the boxes indicates the range of colour development in the plate, indicating the carbon source use. White colour indicates no use of the carbon source; light grey, < 6 %; dark grey 6-10 % and black > 10 %. At the bottom the number of used carbon sources for each pool and at each sampling date is shown.

DISCUSSION

The intermittent drying of streambed sediments caused changes in biofilm functioning, and the magnitude of those changes depended on the length of time that biofilm remained dry. Biofilms in short and long term dry pools differed in the velocity at which colour development in the plate occurred ($1/b$). The biofilm in the long term dry pool was exposed longer to desiccation (a month) and the decrease of $1/b$ may be due to the death or dormancy of the cells after desiccation (Schimel *et al.*, 2007; Placella *et al.*, 2012).

Biofilms in the short term dry pool, that experienced a shorter desiccation time, showed a hotspot of activity at the beginning of the non-flow phase (T1), when substrates and extracellular enzymes became more concentrated (Burns *et al.*, 2013). The physicochemical changes in streambed sediments at the beginning of the desiccation could stimulate the microbial activity.

These results indicate that changes occurring along the non-flow phase were not linear. Timoner *et al.* (2012) observed an increase of extracellular enzyme activity related with the degradation of carbon compounds at the beginning of the non-flow phase, and also noted that this activity decreased when streambed sediments became completely desiccated. The initial fostering effect and the progressive reduction were reflected in the time needed to degrade a carbon source (lag time, X_0). The long time exposure to desiccation also decreased the biofilm functional diversity (H'), particularly in the long term dry pool, where amines were not used under the long desiccation. Instead, biofilm functional diversity was stimulated in the short term dry pool where desiccation was recent, and where carbon sources were more extensive. Biofilm from the permanent pool showed intermediate functional diversity values and a broader use of different carbon sources during all the study period, specifically for amines, amino acids and polymers. Those biofilm communities were selective in terms of carbohydrates, for which B-Methyl-D-Glucoside and N-Acetyl-D-Glucosamine represented more than a 10 % of the total C used. The enhancement of microbial functioning in biofilms from the short term dry pool compared to those from the permanent pool (not desiccated) could be seen as a physiological mechanism for cells adaptation to harsher environment confronting desiccation (Chowdhury *et al.*, 2011; Wallenstein and Hall, 2012).

Rehydration of the streambed sediments produced a positive response on biofilm communities. A rapid response was evident on the increase of maximum slope ($1/b$) and on the reduction of the lag time (X_0). The sudden increase in water availability experienced by dry biofilms alleviated the drought-induced stasis and dormant organisms regained activity (Schimel *et al.*, 2007; Placella *et al.*, 2012). Kennedy (1993) and Belnap *et al.* (2004) also reported that an increase in water availability in soils stimulated microbial activity owing to an increase of nutrient availability and organic carbon for energy (Yancey *et al.*, 1982). These rapid responses may be related both to physiological adaptations as

well as to community changes to withstand desiccation (Bär *et al.*, 2002; Fierer and Schimel, 2002; Schimel *et al.*, 2007). Biofilm communities exposed to desiccation showed low metabolic capacities (Schimel *et al.*, 2007), and the enhancement after rehydration may be related to the release of nutrients from streambed sediments. Biofilms from the permaent pool also experienced an increase of the microbial functioning related to the rainfall event, but values were lower than the ones observed for biofilms in short and long term dry pools. In the permanent pool, though water availability was not the driver variable of change, water temperature might have influenced biofilm functioning. Sediment rehydration in short and long term dry pools implied similar patterns in biofilm carbon use with the permanently inundated biofilms, but is remarkable that biofilms exposed to desiccation are able to use of a higher number of carbon sources after rehydration. This shift can probably be related to community changes where the presence of fast growing species and or dormant species able to take advantage of the new environmental conditions can occur.

When streambed desiccation occurred again, bacterial cell density as well as the maximum slope ($1/b$) decreased, and differences no longer exist between the biofilms from short and long term dry pools, which differed in their original exposure to desiccation. Desiccation implied an increase of the lag time (X_0), and a reduction of bacterial growth and functioning (Timoner *et al.*, 2012). Functional diversity decreased again, specifically for biofilms exposed to desiccation. The number of carbon sources used by the biofilms from short and long term dry pools decreased. In particular biofilms from the short pool lost their capacity to use different amino acids and polymers, and only some of them available on the plate were used. Carbohydrate degradation capacity also decreased, and only a few substrates were used. Amines were not used for biofilms from the long term dry pool when desiccation occurred again, a signal of changes in the bacterial community potentially related to a terrestrialization of the microbial community, since this is the only one able to operate under low water availability (Timoner *et al.*, 2012). Biofilms from the permanent pool instead used similar carbon sources during all sampling dates. This could be a normal behavior in stream sediments of arid systems, since water permanency prevailed to changes in temperature and substrate mobilization after the rainfall event.

CONCLUSIONS

Overall, biofilm responses to desiccation differed as a function of the length of time that biofilms remained dry. Rehydration of these biofilms significantly increased biofilm functional diversity, and the dissimilarities between the biofilms decreased, irrespectively of the time length of desiccation. The rehydration pulse was sufficient to stimulate biofilm functioning, and produced a 'reset' effect on the desiccation exposure as the new desiccation caused the process to start again. Desiccation and rewetting might result in microbial selection, and community sorting based on drought tolerance strategies that can be associated to different biogeochemical capabilities (Allison and Martiny, 2008). Effects on organic matter decomposition, nutrient cycling and food webs interactions, mediated by biofilms, can therefore be expected. As a consequence of the altered precipitation patterns, unpredictable drought and rehydration pulses may complicate the potential predictions on biofilm functioning. The threshold at which biofilm communities can withstand desiccation needs to take into account the increasing frequency of extreme climatic events that acts as drivers of change in ecosystem functioning (Parry *et al*., 2007).

GENERAL DISCUSSION

GENERAL DISCUSSION

In temporary rivers, such as those in Mediterranean regions, floods and dry periods are part of the natural hydrological cycle, as well as of the biotic and abiotic components of the fluvial ecosystem, and therefore of the ecosystem functioning. Temporary systems in the Mediterranean can serve as a natural laboratory where to gain knowledge on how biofilms respond to desiccation and rewetting episodes, and to better understand and predict the biogeochemical and ecological implications of the increasing flow intermittency associated to global change. Mediterranean regions have characteristics that fall between, and include aspects of, both temperate and arid regions (Dallman, 1998; Bernal *et al.*, 2013). During the last decades flow intermittency has been more often associated with global change in temperate regions, (Wilby *et al.*, 2006, Sutherland *et al.*, 2008), therefore comprehending the biofilm responses to flow intermittency and the role that these responses play on the ecosystem functioning is highly relevant. The research that I present here aims to contribute to solving the puzzle of the biofilm responses to flow intermittency and how these responses affect to the ecosystem functioning, since biofilms orchestrate a substantial part of the ecosystem processes, and what occurs at the biofilm scale is reflected at the ecosystem scale. Biofilms may be considered as a whole entity when studying ecosystem processes (*e.g.,* ecosystem metabolism), or may be also used by taking into account either autotrophic (*e.g.*, primary production) or heterotrophic processes (*e.g.*, organic matter degradation capcity). But it is also important to understand the changes observed on the different biofilm components (*e.g.,* algae and bacteria) in order to better understand and predict the whole biofilm functioning.

Results from this Thesis show that both autotrophic and heterotrophic processes were favored during the drying phase, because of the low flow conditions, the increasing water temperature, and the high light availability that occurred at that time. Maximum values of Chl-a where observed during the drying phase that in turn favored the bacterial community, showing maximum values of phosphatase and leucine-amino peptidase activities, and a high capacity to degrade different carbon sources (as observed in chapter 4). Positive interactions occurred between algae and bacteria during the drying phase,

since the photosynthetic organisms release peptides favoring the bacterial activity with high quality carbon sources (peptides provide both C and N) (Rier and Stevenson, 2002; Francoeur and Wetzel, 2003; Ylla *et al.*, 2010). However, autotrophic processes (photosynthetic efficiency) dropped close to zero values when the streambed dried up, whereas heterotrophic processes (extracellular enzyme activities) remained mainly in sandy compartments. The leucine-amino peptidase activity decreased to very low values during the non-flow phase, this was due to the negligible photosynthetic activity that released N-rich compounds. However phosphatase and glucosidase activities remained, even though it was observed that the capacity to degrade different carbon sources was higher when biofilms were water covered (as observed in chapter 4 for the permanently inundated biofilms). During the non-flow phase, between 30-40 % of the total bacterial cells remained alive in the sandy compartments that might have contributed in maintaining the heterotrophic activity during that period. In fact, in all the streambed compartments live cells increased, suggesting that the bacterial community was able to adapt to the new environmental conditions during non-flow, mainly in sandy compartments where a high proportion of tolerant taxa persisted as observed in chapter 3. Differences however existed between streambed compartments, being the biofilm on cobbles (epilithic) the most affected by flow intermittency and the one that showed the lowest resistance. The epilithic biofilm, however, showed the highest responses immediately after flow recovery with photosynthetic and enzymatic values higher than those before flow cessation, and it was after a week that values return to normal conditions. Glucosidase activity in the epilithic biofilm was higher during the rewetting phase than before flow cessation, indicating the prevalence of the heterotrophic processes during that period (Romaní *et al.*, 2013). The high heterotrophic activity observed during the rewetting phase was favoured by the high availability of good quality organic matter that was accumulated during the terrestrial phase (Ylla *et al.*, 2011) and was further processed by the bacterial community that thrived under non-flow conditions. However, the low water temperatures and the low light availability during the rewetting phase limited algal growth (Tornés *et al.*, 2010). Thus, flow intermittency cannot be defined as a closed-loop process in natural conditions, since the conditions before flow cessation and after flow recovery differ, and so do the determinant factors of the biofilm structure and function.

As observed in this Thesis, the autotrophic processes dropped rapidly in response to flow intermittency, reaching residual values of Chl-a and photosynthetic efficiency due to desiccation. Immediately after flow resumption both Chl-a and photosynthetic efficiency rose quickly, however flow resumption was not enough to maintain autotrophic processes at the level observed during the drying phase, and fell to a lower stable state than the one observed during the drying phase and according with the environmental conditions occurring during the rewetting phase. Thus, desiccation and rewetting (immediately after flow recovery) brings the autotrophic processes to the lower and upper branches of the stables states (drying and rewetting) (Fig. 1). This pattern, in which forward and backward switches occur at different critical conditions, resembles a hysteresis (Scheffer *et al.*, 2001). A smooth transition was observed for the heterotrophic processes, probably due to the major resistance and adaptation of the bacterial communities and the persistence of extracellular enzymes during non-flow conditions. How forward and backward switches occur might play a crucial role on the resilience of the ecosystem, for instance if longer non-flow periods occur affecting forward switches (to a new lower branche, *e.g.,* impinging severe damages on algae and cyanobacteria, reducing even more the amount of Chl-a persinsting during non-flow) then the capacity of the autotrophic processes to recover (backward switches) could be affected too and the ecosystem capacity to return to the lower stable state. Results highlight the importance to define the thresholds at which both autotrophic and heterotrophic processes will be affected if longer and harsher non-flow periods occur.

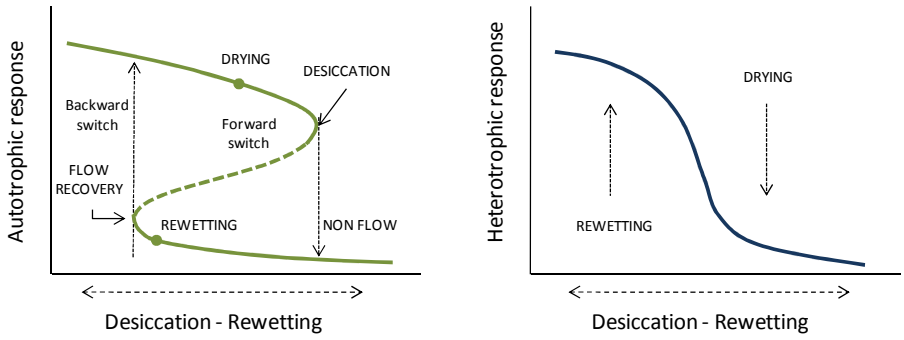

Figure 1. Changes in autotrophic (left plot, green line) and heterotrophic processes (right plot, blue line) due to flow intermittency (desiccation - rewetting). Dots on the left plot indicate the stable states at drying and rewetting. Forward and backward switches are indicated by dashed arrows. Note that the bifurcation points occur when streambed sediments desiccate (desiccation) and when flow recovers (flow recovery) bringing the autotrophic processes to the upper and lower branches of the two stable states.

Both autotrophic and heterotrophic responses were investigated at the cellular level, in order to determine which are the mechanisms that the biofilm has to resist flow intermittency. As observed, results pointed out that the autotrophic component of the biofilm (algae and cyanobacteria) was less resistant than the heterotrophic (bacteria) to flow intermittency. Chl-a dropped to extremely low values during the terrestrial phase, being in epilithic biofilms where the lowest values of Chl-a occurred. However in the epilithic, the synthesis of protective carotenoids and the formation of resistant cell structures played a crucial role in protecting the cell against desiccation and the high temperatures occurring during non-flow, conferring a high resilience to the algal community when flow recovered. During the terrestrial phase, algae and cyanobacteria in sandy compartments also showed low values of Chl-a, but the Chl-a degradation index was lower than in the epilithic. Major differences were also observed in the algal community composition between the three different biofilms. The high amount of pigments in the epilithic, as well as the microscope observations, indicated that a major number of algal classes prevailed and that cobbles are a suitable habitat for algae to grow. Diatoms were the dominant algal class in sand sediments, however the instability of sand grains as well as the low light availability (compared with the upper face of a cobble) made the epipsammic a less suitable habitat for algae to grow. In a laboratory experiment Acuña *et al*. (*unpublished data*) also observed that diatoms were the algal group most sensitive to desiccation, while cyanobacteria and green algae rapidly reacted once flow resumed. In streams subjected to high flow variability, algae grow tightly attached to the cobbles substrata (Sabater, 1989), and when dry up resemble a crust such as that occurring in arid zones (Belnap *et al*., 2007). Biofilms in arid zones take advantage of water pulses (short rain events), and rapidly become active when water is available. In temporary rivers a similar strategy to avoid flooding effects may be used to withstand desiccation. Results also pointed out to the importance of the maintenance of the riparian habitat, since the shade produced during the non-flow phase allowed algae to invest less energy in cell protection, since they had lower amount of accessory pigments than those observed in biofilm samples from a reach with an open canopy cover. Overall, the high resilience observed of the dry biofilm remaining on cobbles when rewet plays a crucial role in maintaining autotrophic processes in temporary streams.

Similar responses to flow intermittency were observed in biofilm bacterial communities. Major changes were also attributed to the differences between compartments, since the epilithic showed a singular bacterial community, where Cyanobacteria and Firmicutes prevailed, and was highly sensitive to flow intermittency probably because of the low resistance of Cyanobacteria and the high proportion of sensitive taxa. Epipsammic and hyporheic bacterial communities were similar one to the other, but richer and more diverse than the ones in the epilithic. This probably conferred resilience to the assemblages, since several core species persisted through the whole hydrological cycle, as observed in macroinvertebrate communities (Rieradevall *et al.*, 1999). During the non-flow phase, bacterial communities in sandy compartments were, a subset of species coming from the drying phase, meaning that there was a selection towards the ones that can thrive under low water conditions. In fact, in the subsuperficial compartment, the hyporheic a high proportion of taxa was tolerant to desiccation.In the epilithic new bacterial species (airborne or associated to the leaf inputs) proliferated on the dry biofilm remaining on cobbles. The crust formation could also provide some king of protection to the bacterial community. Moreover the occurrence of new bacterial species during the non-flow period highlighted the biological link between the dry streambed and the nearby terrestrial ecosystem, as well as the importance of the dry epilithic biofilm as a rich nutrient resource (richer in N and P than biofilms on sand) Flow recovery triggered bacterial community changes in all biofilm compartments, affecting species unable to resist flooding and favoring the occurrence of random taxa after washout (Fazi *et al.*, 2008). However bacterial communities showed a major tolerance to rewetting than to desiccation in all biofilms. Richness and diversity indicators recovered one week after flow recovery to similar values before non-flow in all biofilm compartments, pointing out again the high resilience of bacterial communities in temporary streams to flow intermittency.

During the non-flow phase, the occurrence of rainfall events not enough to restore the flow can rehydrate streambed sediments during some hours or days without flow resumption. But the high temperatures and high irradiances prevailing during the non-flow phase produce again the streambed desiccation, until the final resumption of water flow, when the drought season is over. The study conducted in the Dam Creek (chapter 4), where the rehydration of the streambed sediments occurred, revealed a high capacity of the biofilm to react after rehydration. During the non-flow phase differences in the

biofilm capacity to degrade different carbon sources (functional diversity) were related to the time that biofilms were exposed to desiccation. Short-term dry biofilm showed a higher functional diversity than permanently inundated biofilms, whereas long-term dry biofilms had lower functional diversity and as a consequence a low metabolic activity. The metabolic enhancement observed in the short-term dry biofilm during the non-flow phase was also observed in the Fuirosos stream at the beginning of the non-flow phase. This response can be considered a strategy to cope with desiccation, since taking up a resource in excess of requirements (luxury consumption) may serve as energy or nutrient reservoir to be used when resources become limiting (*i.e.*, during the non-flow phase) (Chapin *et al.*, 1987). The biofilm capacity to degrade different carbon sources was higher in rehydrated biofilms (both short and long-term dry pools) than in permanently inundated biofilms. Sediment rehydration implied similar patterns in biofilm carbon use with permanently inundated biofilms, but biofilms exposed to desiccation were able to use a higher number of carbon sources after rehydration. Afterwards the high temperatures and intense solar radiation prevailed and streambed sediments desiccated again affecting the biofilm capacity to degrade carbon sources and differences no longer existed between short and long term dry biofilms. Thus, the rehydration of streambed sediments during the non-flow phase produced a 'reset' effect to flow intermittency and that might play a crucial role in sustaining biofilm functional diversity, which in turn is important to maintain the ecosystem processes in temporary streams. To which extent enzyme activities measured during the non-flow phase are linked to the cell could reveal the importance of the maintenance of free enzymes for the biofilm recovery when favourable conditions occur (rainfall rehydration and flow resumption) in temporary rivers. The release of these enzymes before desiccation can be related with the cell lysis due to water stress or the enhancement of activity observed just before desiccation (Burns *et al.*, 2013). Both the capacity of the cells that are able to thrive and reduce their metabolism during the non-flow phase, and the enzymes freed on the environment play a crucial role in the maintenance and recovery of heterotrophic processes.

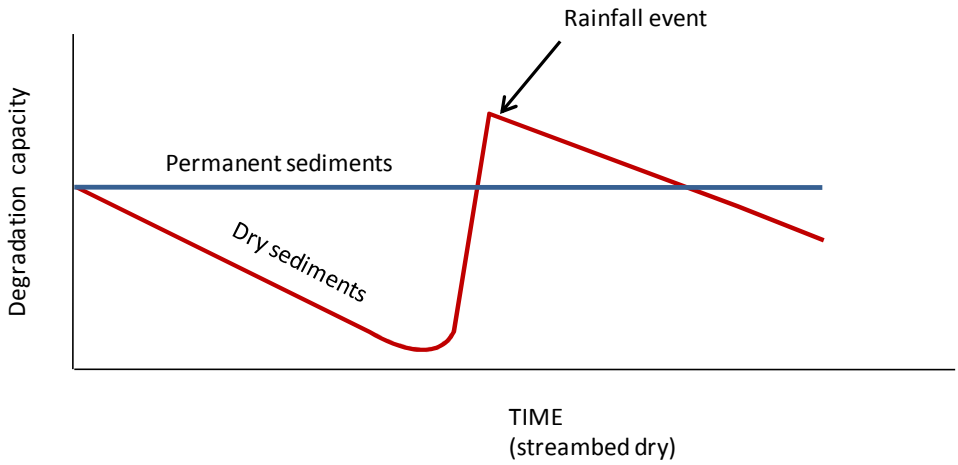

Figure 2: Schematic plot of the effects of rehydration on the biofilm capacity to degrade different carbon sources.

Overall, results highlight that biofilms in temporary rivers are structurally and functionally adapted to flow intermittency, and that during the non-flow phase biofilms have low metabolic capacities, but rapidly react when favourable conditions occur (rainfall rehydration or flow resumption). Biofilms have many strategies that allow resistance (community changes and physiological adaptations), facilitating further resilience (Bonada *et al.*, 2007; Hershkovitz and Gasith, 2013) and these might play a crucial role in recovering the ecosystem functions after rewetting.

GLOBAL CHANGE AND FLOW INTERMITTENCY: FROM THE BIOFILM TO THE ECOSYSTEM

Many ecosystem processes are simply the consequence of biofilm growth and activity, since biofilm interacts with and responses to the physical and chemical environment. In this Thesis biofilm nutrient molar ratios, together with ratios of extracellular enzymes, related with the degradation of C (β-glucosidase), N (leucine-amino peptidase) and P (phosphatase) compounds, are used to resolve biogeochemical constraints (Sinsabaugh *et al.*, 2009; Sinsabaugh and Follstad, 2012) since biofilm responses to flow intermittency may affect carbon and nutrient cycles. Extracellular enzyme activities reflect the microbial nutrient demand in relation to supply, and thus changes in organic matter availability, caused by flow intermittency, may affect the stoichiometry of extracellular enzymes (Allison *et al.*, 2010). Sinsabaugh *et al.* (2009) suggested that an equilibrium between C:N

and C:P ratios of extracellular enzymes of 1:1 indicates an equilibrium between the elemental composition of available organic matter and microbial nutrient assimilation in river sediments and soils. Biofilm nutrient molar ratios revealed that during the non-flow phase the biofilm become C and N rich and P poor, and that during the rewetting phase P in biofilms increased. Biofilms on sand were always richer in C than the epilithic biofilm. Accordingly, extracellular enzyme ratios revealed that there was a preferential C demand during non-flow and rewetting, while a preferential N demand occurred during drying and the fragmented phase (the superficial flow was already interrupted, but the streambed was water covered) (fig. 3 a). There was a clear preference for N demand vs C on the epilithic, while epipsammic and hyporheic had a major C demand (fig. 3 b). P demand prevailed vs C between hydrological phases (fig. 3 c) and biofilms (fig. 3 d) indicating that P was always limiting. These results highlight the role that the biofilm on each streambed compartment plays on biogeochemical cycles, being the biofilm on sandy compartments an important site for C cycle, whereas biofilm on cobbles had a high N and P demand which were not always fulfilled during the hydrological cycle. Thus, resulst suggest that in temporary rivers the use of organic compounds is unbalanced by the greater use of phosphorus and peptides (mainly in the epilithic), indicating N limitation during some periods of the year and P limitations during all the year, at least in the Fuirosos (Romaní *et al.*, 2013). The biofilm nutrient molar ratios also show that the biofilm degradation capacity change between hydrological phases and among the different biofilms, for instance a higher use of polysaccharides (β-glucosidase) than peptides (leucine-amino peptidase) was observed on the epilithic biofilm during the rewetting phase, pointing out the adaptation of the microbial biofilm communities to the available organic matter sources through the different hydrological phases in temporary rivers.

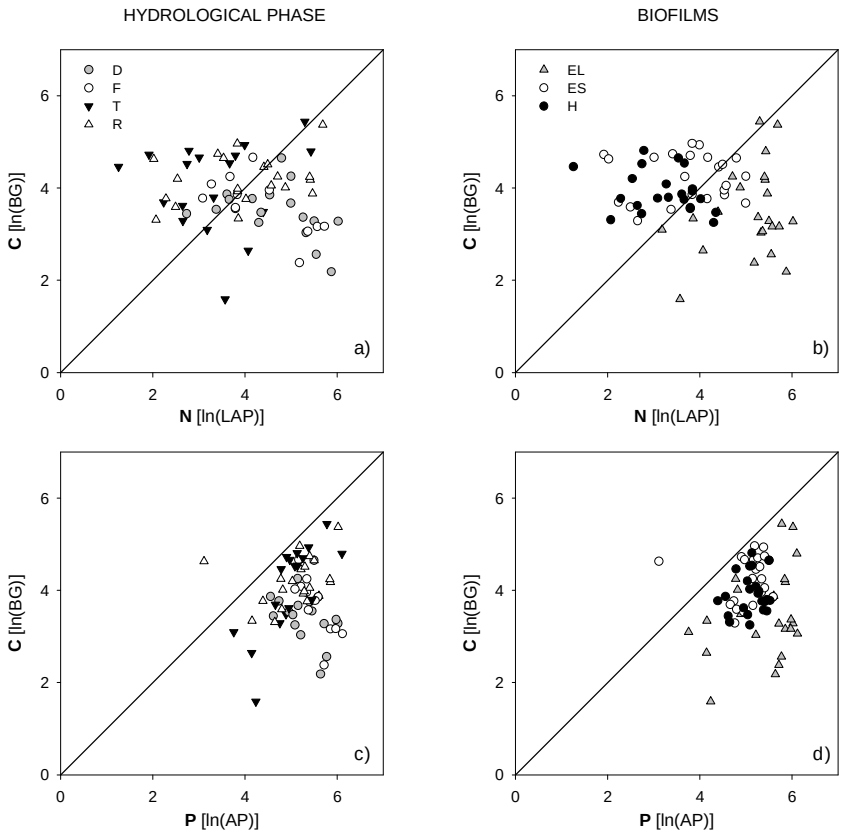

Figure 3. Extracellular enzyme ratios showing the differences in the stoichiometry of N or P demand relative to C. The reference line indicates balanced acquisition rates for the plotted elements, C vs N (a and b) and C vs P (c and d). The departure from this line indicates a preferential demand for one of the elements plotted. Extracellular enzymes were ln transformed. BG (β-glucosidase activity); LAP (leucine-amino peptidase activity) and AP (phosphatase activity). Symbols on the left indicate the different hydrological phases (*D*-drying, *NF*-non-flow and *R*-rewetting) and symbols on the right the different biofilms (EL-epilithic, ES-epipsammic and H-hyporheic).

Results from this Thesis highlight the importance of the biofilm on each streambed compartment in maintaining the ecosystem function during each hydrological phase, as well as the role of algal and bacterial communities (autotrophic processes vs heterotrophic ones). During the drying phase there was an accumulation of autochthonous organic matter due to the prevalence of autotrophic processes that in turn favored the heterotrophic ones; therefore a high recycling of in-stream material provided high quality sources downstream and for higher trophic levels (Romaní *et al.*, 2012). The maintenance of heterotrophic processes during the terrestrial phase enabled high quality allocthonous organic matter available during the rewetting phase, enhancing the overall stream heterotrophy (Romaní *et al.*, 2006, 2012). Thus, increasing flow intermittency is likely to

increase the relative importance of heterotrophic processes in streams, and the relative contribution of sandy biofilms (mainly the hyporheic) to organic matter processing during the terrestrial phase increasing C retention. Longer periods of flow intermittency could also affect the capacity of algae to recover by the loss of refuges that serve as inocula when flow recovers (permanence of pools and humid zones) (Robson and Matthews, 2004), furthermore longer desiccation might produce severe damages on algal cells that may hinder their recovery. Longer non flow periods could change the composition of the algal assemblage, favoring Cyanobacteria on cobbles, since is the algal group less sensitive to flow intermittency, thus the recovery of the autotrophs will depend on dry biofilms on cobbles prevailing Cyanobacteria, which are less palatable for stream grazers and that will reduce the algae base for the food webs (Robson *et al.*, 2013). A decrease in stream autotrophy (primary production) may limit the supply of autochthonous carbon downstream (Dodds *et al.*, 1996), and therefore the biofilm adaptation to windows of opportunity is essential; since production pulses (during the drying phase) may supply food webs with high quality carbon (Artigas *et al.*, 2009).

Understanding how biofilm communities adapt and how can they cope with flow intermittency provide the basis for predictions of which changes in biofilm structure and function will affect ecosystem responses to climate change. In temporary rivers some of the adaptations observed in biofilms (already adapted to flow intermittency) might mitigate the effects of global change, but streams in temperate regions with a low intra-annual water flow variability, are more likely to experience large biogeochemical shifts due to a lower adaptation of their biofilm communities. Prior hydrologic conditions over evolutionary timescales in temporary streams may have generated a species pool able to take advantage or at least accommodate such changes much better than species in permanent streams (used to steadier flow and temperature conditions). On the other hand, the increasing extent of flow intermittency might involve the local emergence of species that historically have been non-existed or extremely reduced in spatial extent (abnormal situation), and the potential responses of the biological communities may be limited by geographical constrains and life history traits of the available species pool. There are still few studies comparing biofilm communities in permanent and temporary streams. Tornés and Ruhí (2013) found that diatoms communities in temporary rivers had a higher niche breadth and were less diverse than the ones in permanent streams concluding that

increasing flow intermittency might lead to a loss of diversity and ecosystem functions. There is a great diversity in microbial natural populations, but the significance of this diversity with respect to microbial community function and ecosystem function is still an open question. Many studies report that higher bacterial diversity is linked with higher bacterial function (Langenheder *et al.*, 2010; Tilman *et al.*, 1997). Non-flow conditions in the studied streams produced a decrease in richness and diversity, however rapidly recovered after flow resumption (mainly in sandy compartments). The loss of functional diversity was also associated to the low biofilm metabolic capacities under harsh conditions (non-flow) however biofilm showed a high capacity to increase functional diversity when conditions become favorable. Shifts in community structure is an ubiquitous bacterial response to environmental change, a selection of the constituent populations that fits more to the new environment occurs and these changes can in turn affect the ecosystem functioning (Wallenstain and Hall, 2012). However, not necessarily a community change has to be accompanied by a loss of ecosystem function, since the new community can perform similar to the previous one, showing functional redundancy (Allison and Martiny, 2012). But global change is accelerating the velocity at which ecosystem changes and that could decrease the capacity of microorganism to adapt affecting the ecosystem function.

Furthermore, flow intermittency is accompanied by increasing water temperature that influence the biofilm use and recycling of organic matter. In a laboratory study Ylla *et al.*, (2012) observed that the use of labile carbon would be rarely affected by increasing water temperature, however recalcitrant carbon would be more intensively used, thus increasing water temperature together with extended periods of flow intermittency could determine lower inputs of carbon reaching downstream. Temporary streams are also threatened by human activities that reinforce flow intermittency, the interaction between flow intermittency, increased temperature and the entrance of pollutants is still a novel research. Proia *et al.*, (2013) found that biofilms exposed to Triclosan and flow intermittency had a lower capacity to recover after flow resumption than the ones only exposed to flow intermittency. The knowledge of the effects of these interactions on biofilms will contribute to a reliable resource management for temporary rivers. The study of this interaction is challenging because of the difficulty to mimic the conditions that prevail during the non-flow phase (high temperatures and high light availability) in laboratory conditions, as well as the assessment of relative long non-flow phases. Thus, it is

necessary to balance the simplicity of the biofilm communities growing under laboratory conditions and flow intermittency.

FUTURE PERSPECTIVES

The recent acceleration of temporary river research is promising, and represents an opportunity for rapid advances through collaborations between aquatic and terrestrial ecologists and hydrologists. Emerging ecological concepts may provide some insight for the management of temporary rivers. Aquatic microbial ecology has and always will be need to integrate across physics, chemistry and engineering to understand and manage water resources. But to understand the management of water resources in temporary streams the challenge is to cross aquatic and terrestrial microbial interactions to be able to understand how the biofilm scale control the global terrestrial-aquatic habitat mosaic tha occur in temporary rivers. A multiple scale perspective from the biofilm scale to the watershed scale is promising.

The multiple interactions between increasing water temperature, emergent pollution, and flow intermittency need to be addressed. As well as the interaction between other anthropogenic pressures, such as nutrient loads due to land use changes or waste water treatment plants, and dam construction. The increasing extent of the non-flow phase makes necessary to investigate the threshold at which biofilm communities can withstand desiccation in order to define the thresholds at the ecosystem level. To determine these thresholds it is necessary to take into account the multiple interactions that occur during the non-flow phase (air exposure, high temperatures, intense solar radiation and the possibility of rehydration) and the ones with global change. Thus, it will be important to design laboratory and field studies or a combination taking into account all these interactions. For example, the fast or slower desiccation of streambed sediments may affect on the biofilm capacity to recover, since physiological adaptations will depend on that, but sometimes it is difficult to assess in field conditions. How biofilms in temporary rivers under different Climatic zones respond to flow intermittency could address some of this topics. For instance, the Mediterranean summer is dryer than the Austral summer in the subtropics. Furthermore these comparisons might help to go further and define which are the ecological traits that characterize biofilms in temporary rivers worldwide.

The concept of the biofilm functional redundancy is another exciting topic of research in temporary rivers. In a natural systems the interplay of several enzymes (functions) might be of importance for the ecosystem functioning and a multifunctional perspective combined with the community structure should allow further insight in this topic. Recent studies pointed out the importance that fungi may play during the non-flow phase (Zoppini and Marxsen 2011), and another emerging and exciting topic is the role that archaea have on biofilms and in biogeochemical cycles, since the effects of flow intermittency on archaeal communities remain poorly understood.

GENERAL CONCLUSIONS

GENERAL CONCLUSIONS

1. The relative importance of heterotrophic processes in streams is enhanced by flow intermittency, and the relative contribution of the biofilm to the organic matter breakdown mostly occurs in sandy compartments (superficial and hyporheic).

2. Autotrophic processes show lower resistance to flow intermittency than the heterotrophic ones, but they show a higher resilience immediately after flow resumption.

3. Algae in the epilithic biofilm experience higher changes than those in the epipsammic and hyporheic, due to flow intermittency. However the complex structures resembling a "crust", and the physiological adaptations (protective pigments and cell resistant structures) confer high resilience to flow intermittency to the epilithic biofilm, facilitating algal reactivation and regrowth after flow resumption.

4. Algae in sand sediments are more resistant but less resilient to flow intermittency, because of the the protective effect of the sand grains and the simpler structure of these biofilms. Less physiological adaptations to flow intermittency are observed in algae in sand. The environmental conditions prevailing after flow resumption do not allow algae to grow in these compartments.

5. The remaining dry biofilm on cobbles and rocks is structurally and functionally adapted to flow intermittency, and play a crucial role in the recovery of the ecosystem functions after rewetting in streams that periodically experience non-flow periods.

6. Bacterial communities in the epilithic biofilm are more affected by flow intermittency than those in the epipsammic and hyporheic. Epipsammic and

hyporheic bacterial communities are made up of versatile bacterial groups that adapt to either inundation or desiccation conditions, and this is evidenced by the persistence of these bacteria along all hydrological phases.

7. The terrestrialization of the streambed during the non-flow phase is confirmed by the presence of bacterial taxa from terrestrial environments (mainly on cobbles) that colonize the new habitat using the available nutrient resources from dry epilithic biofilms.

8. Biofilm responses to desiccation differed as a function of the length of time that biofilms remained dry.

9. Rehydration of streambed sediments because of unpredictable rainfall events significantly increase biofilm functional diversity, and the initial dissimilarities between the biofilms decrease irrespectively of the time length of desiccation, entailing a 'reset' effect on the desiccation exposure as the new desiccation caused the process to start again.

10. An increase in the non-flow period extent will preferentially impact on the recovery of autotrophic than heterotrophic processes

11. As observed during the terrestrial phase, the longer the streams remain dry the most the biofilm stoichiometry will differ, potentially leading to changes in the biogeochemical cycles at larger scales.
12. Desiccation and rewetting result in microbial selection, with strategies that can be associated to different biogeochemical capabilities. The increase flow intermittency and recurrence can alter these microbial adaptations, favoring the colonization of terrestrial taxa in the epilithic compartment, and therefore effects on organic matter decomposition, nutrient cycling and food webs interactions, mediated by biofilms, can be expected.

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