BIOFILM RESPONSES TO MULTIPLE STRESSORS ASSOCIATED TO GLOBAL CHANGE IN RIVER ECOSYSTEMS

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Dipòsit legal: Gl. 1869-2012
http://hdl.handle.net/10803/96523

Universitat de Girona

PhD THESIS

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PhD Thesis

Biofilm responses to multiple stressors associated to global change in river ecosystems

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2012

PROGRAMA DE DOCTORAT EN CIENCIES EXPERIMENTALS I SOSTENIBILITAT

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

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CERTIFIQUEN:

Que aquest treball titulat "Biofilm responses to multiple stressors associated to global change in river ecosystems", que presenta Lorenzo Proia, per a l'obtenció del títol de Doctor, ha estat realitzat sota la nostra direcció i que compleix els requeriments per poder optar a Menció Europea.

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El doctorand:
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Girona, març 2012

A Vicky, Enrica, Roberto e Alessandro

ACKNOWLEDGEMENTS

This PhD thesis was developed within the Marie Curie Research Network Training KEYBIOEFFECTS (MRTN-CT-2006-035695).

Financial support was also provided by the EU projects MODELKEY (SSPI-CT-2003-511237-2), and the Spanish projects SCARCE (Consolider-Ingenio CSD2009-00065), WARMTEMP (CGL2008-05618-C02-01/BOS), VIECO (009/RN08/011), and FLUVIALMULTISTRESS (CTM2009- 14111-CO2-01).

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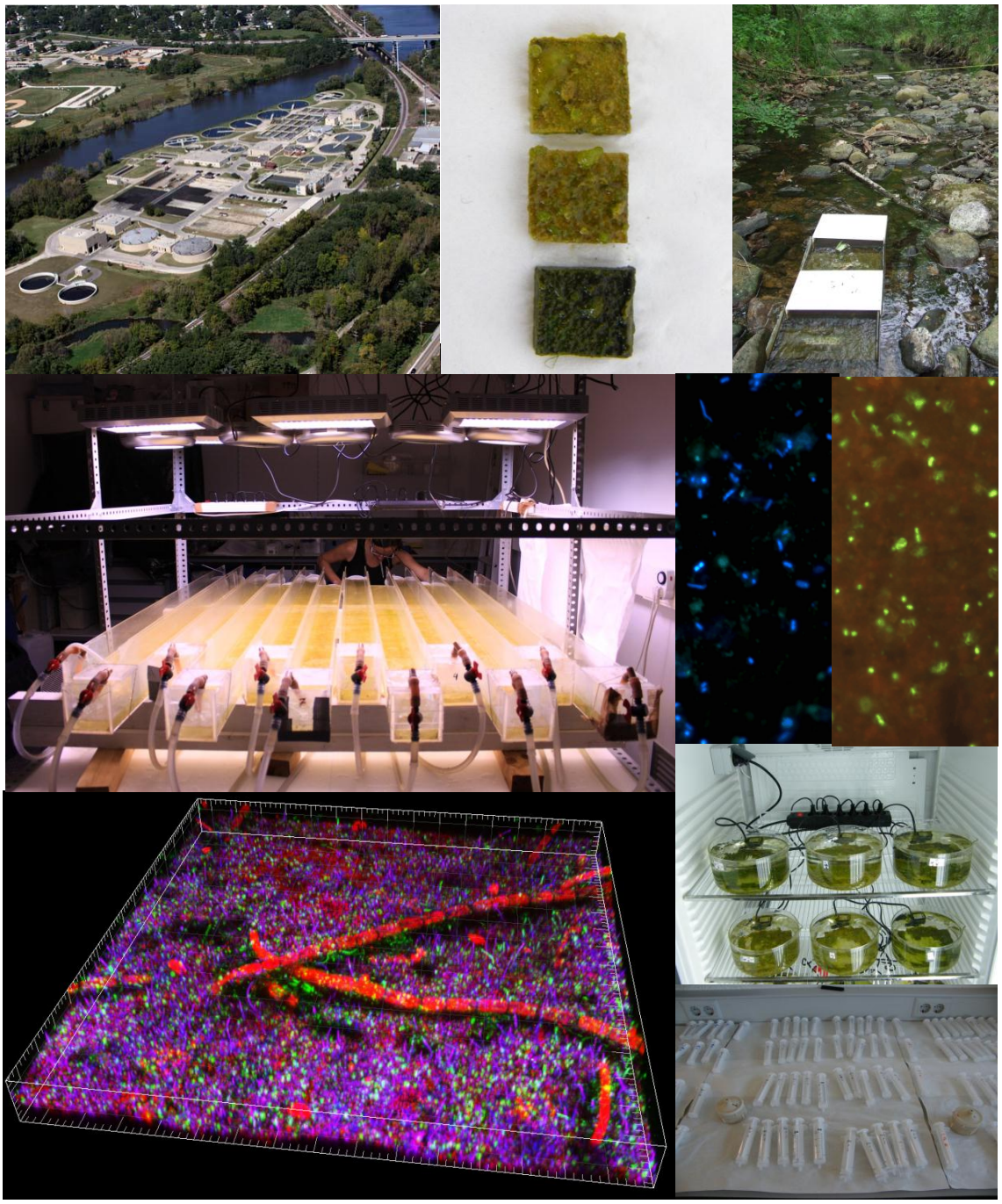

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SUMMARY (English)

Water from rivers, streams and lakes is the most important source for human use (drinking water, industry, agriculture etc) even if less than 1% of the total is stored in freshwater systems. At present, most freshwater bodies are affected by consequences of global change resulting from both climatic change and human activities. Consequences of these changes include alterations of flow regime and temperature, increasing chemical pollution and eutrophication, riparian and river network simplifications, geomorphologic and hydrologic alterations. All these factors affect the functional organization of streams and rivers, and lead to a simplification and impoverishment of the biota within these ecosystems, reducing the capacity of fluvial systems to recover from natural disturbances. The co-occurrence of climate change and pollution pressures can be envisaged in systems receiving waste waters from industrial, agricultural and urban areas located in regions affected by increasing water scarcity problems. Because there are thousands of different contaminants and their potential toxicity may vary in response to environmental changes, there is an increasing need of developing reliable bioindicators and early warning biomarkers of stress. Attached microbial communities respond rapidly, justifying the use of biofilms as good early warning indicators of disturbances related with global change consequences. These communities are in general structured by autotrophs (diatoms, green algae and cyanobacteria) and heterotrophs (bacteria, fungi, protozoa) embedded in extracellular polymeric matrix adhered on substrata conferring a biofilm structure. Biofilms integrate the effects of environmental conditions over extended periods of time, mainly because of their small size and rapid growth, species richness, and the physiological variety of the organisms of which they are formed. Moreover biofilms are ubiquitous, have short generation times, and are easy to manipulate under controlled conditions, constituting ideal systems for assessing the impacts of stressors that are not feasible to manipulate in the field. As attached communities are micro ecosystems where complex interactions conflict, responses to multiple stressors can be different depending on target organisms and thus both direct

and indirect effects can be inferred. The main goal of this thesis is to investigate the effects of global change, specifically water flow decrease, nutrient excess and pollutants occurrence, on the structure and function of fluvial microbial biofilms. To achieve the main objective a multi-marker approach has been used at different experimental scales, which have ranged from field studies to mesocosms and laboratory experiments. The methodologies used to reach the planned objectives are described in detail in Chapter 2 of this thesis. Multiple metrics related with structure and function of both autotrophic and heterotrophic biofilm compartments were used to describe direct and indirect effects and to investigate the ecological interactions occurring at the microbial scale. In particular, the structure of autotrophic community was investigated by measuring chlorophyll-*a* densities, community composition as the auto-fluorescence signal of the main photosynthetic groups and biomass of diatoms while the function of autotrophs in biofilms was studied mainly by measuring the photosynthetic parameters. Heterotrophs structure was investigated as community composition (molecular techniques) as well as by counting the live/dead bacteria ratio, while function was mainly studied by measuring extracellular enzymatic activities. Moreover microscopies were used in some chapter to complement information about superficial and tri-dimensional structure of biofilms. Finally, the measure of phosphorus uptake capacity of river biofilm was investigated as whole community function related whit ecological services provided by river biofilms.

Chapter 3: NUTRIENTS AND LIGHT EFFECTS ON STREAM BIOFILMS: A COMBINED ASSESSMENT WITH CLSM, STRUCTURAL AND FUNCTIONAL PARAMETERS

This chapter aims to investigate the individual and combined effects of nutrients and light on river biofilm development in oligotrophic forested streams. To this end a field experiment was designed in a Mediterranean forested stream (Fuirosos, Spain). Biofilms grew on artificial

substrata in both enriched and unenriched reaches where shade conditions were simulated. Four different treatments were generated: Higher Light Unenriched (HL-U), Lower Light Unenriched (LL-U), Higher Light Enriched (HL-E) and Lower Light Enriched (LL-E). Several structural and functional descriptors of both autotrophic and heterotrophic biofilm compartments were analyzed during the colonization at days 4, 9, 16, 22 and 52. Confocal Laser Scanning Microscopy (CLSM) was used to determine differences in biofilm architecture at day 52. CLSM evidenced differences in thickness and structural complexity of biofilms grown in different conditions. Biofilms in HL-E were the thickest and had the most complex structure. The CLSM highlighted that the extracellular polymeric substances were agglomerate in the upper layer of enriched-grown biofilms, but evenly distributed through the biofilm in unenriched communities. CLSM 3-d images suggested that cyanobacteria increased under higher nutrient conditions. Nutrient enrichment caused the decrease of extracellular phosphatase activity. Interaction between the two factors affected extracellular peptidase activity. HL-E had the highest peptidase and the lowest phosphatase activities, an indication that biofilm responses to nutrients mostly occurred under high light availability conditions. Our results revealed that the conjoint availability of light and nutrients caused the highest changes in biofilm spatial organisation, microbial structure and functioning in oligotrophic forested streams.

Chapter 4: BIOFILM RESPONSE TO TRANSLOCATION ALONG A POLLUTION GRADIENT IN A HIGHLY IMPACTED RIVER: THE EFFECTS OF PESTICIDES AND PHARMACEUTICALS

A huge number of chemical compounds reach running waters as a result of agricultural, industrial and urban activities. Amongst these, pesticides and pharmaceuticals are the most commonly detected. The Llobregat is a highly impacted Mediterranean river affected by

strong urban, industrial and agricultural activities. In this chapter we investigate the effects of pharmaceuticals and pesticides detected in Llobregat waters on the structure and function of river biofilms by means of translocation experiments performed under controlled conditions. Three sampling points were selected: Castellbell (CB) and Mina de Terrassa (MT) as less polluted sites and Sant Joan Despí (SJD) as hotspot. River water was collected from each site and used as inoculums and medium for biofilm growing in independent mesocosms. After 25 days three biofilm translocations were performed: from MT to SJD, from CB to MT and from CB to SJD. Several structural and functional biofilm descriptors were measured four times during the experiment: one before and three after translocations. Fifty-seven pharmaceutical compounds from 14 different therapeutic groups and sixteen compounds from 5 different pesticide families were detected in river waters. The differences between biofilms before translocation mostly concerned the autotrophic compartment. The biofilms responses were different depending on the translocation. The translocation of biofilms from CB to MT was the least responsive while the translocation from MT to SJD caused important structural and functional responses. The translocation from CB to SJD was the most effective in terms of the biofilm responses. The redundancy analysis showed that conductivity, analgesics and barbiturics groups were the variables that significantly influenced the response of biofilms to translocation and pollution gradient of Llobregat River. Moreover, the partitioning variance technique revealed that analgesics and antinflamatories significantly affected biofilm responses to translocations, particularly the commonly used Ibuprofen and Acetaminophen (Paracetamol) resulted related with negative effects on autotrophic compartment while Diclofenac was more related with responses of heterotrophs. This study evidenced how non-regulated emerging compounds, reaching continuously river systems, may cause biological responses at the base of river food web with still unknown consequences for the whole freshwater ecosystem, especially in Mediterranean rivers.

Chapter 5: RESPONSES OF BIOFILM BACTERIAL COMMUNITIES TO ANTIBIOTICS IN RIVER WATERS: A TRANSLOCATION STUDY

Anthropogenic activities are increasing the levels of priority and emerging contaminants derived from pollution point and diffuse sources, which reach freshwater ecosystems. Antibiotics are bioactive compounds against bacteria in natural microbial communities, and their presence in aquatic environment may lead both to short-term physiological alterations, as well as to long-term changes in the microbial biomass or shifts in community composition. In this chapter we investigated the structural and functional responses of biofilm bacterial community grown along an antibiotic pollution gradient in the River Llobregat, to translocation in river waters differently contaminated by antibiotics. Three sampling points were selected: Castellbell (CB) and Mina de Terrassa (MT) as less polluted sites and Sant Joan Despí (SJD) as hotspot. River water was collected from each site and used as inoculum and medium for biofilm growing in independent mesocosms. After 25 days three biofilm translocations were performed: from MT to SJD, from CB to MT and from CB to SJD. Biofilm bacterial community composition, bacterial live/dead ratio and extracellular enzymes activities were measured twice during the experiment: one before and one after translocations. A total of sixteen antibiotic compounds were detected in river water from the three sampling sites. The family of sulfonamide antibiotics was the most concentrated at each sampling site followed by quinolones and macrolids families. The bacterial communities showed structural but not functional differences between sampling sites before translocation. Bacterial community structure changed after nine days of translocation. In particular an increase of Actinobacteria (HGC) was measured in all translocated biofilms. The Canonical Correspondence Analysis confirmed that communities were separated by sampling site/translocation and that Actinobacteria were associated to increasing antibiotics concentration. Biofilm communities translocated to water with higher antibiotics concentrations increased bacteria mortality and modulate heterotrophic metabolism, particularly increasing Leucine-aminopeptidase and

decreasing Alkaline phosphatase activities. Mantel test confirmed significant correlation between antibiotics concentrations and structural-functional biofilm responses. Our study showed that continuous entrance of antibiotics in freshwater systems may lead to structural and functional changes in microbial attached communities.

Chapter 6: RESISTANCE AND RECOVERY OF RIVER BIOFILMS RECEIVING SHORT PULSES OF TRICLOSAN AND DIURON

This chapter aim to investigate the resistance and recovery of river biofilms receiving short pulses of Triclosan and Diuron. To this end, was designed a system of mesocoms where biofilms were exposed during 48-hours to short pulses of either DIU or TCS under controlled conditions. The direct and indirect effects of each toxicant on the biofilms, and the subsequent recovery of the biofilms, were evaluated according to structural and functional biomarkers. These parameters were analyzed immediately before exposure, immediately after exposure, and 9 and 16 days post-exposure. DIU caused an increase in diatom mortality (+79%), which persisted until the end of the experiment. TCS also affected diatom mortality (+41%), although the effect did not appear until one week post-exposure. TCS caused an increase in bacterial mortality (+45%); however, this parameter returned to normal values 1 week post-exposure. TCS compromised the cellular integrity of the green alga *Spirogyra* sp., whereas DIU did not. TCS also strongly inhibited phosphate uptake capacity (-71%), which did not return to normal values until 2 weeks post-exposure. DIU directly affected algae, but barely affected the heterotrophs, whereas TCS seriously impaired bacteria (direct effect) as well as autotrophs (indirect effect). However, the biofilms recovered their normal structure and function within only a few days to a few weeks. These findings demonstrate the capacity of biofilms to cope with periodic inputs of toxicants, but also the risks associated to repeated exposure or multi-contamination in aquatic ecosystems.

Chapter 7: DROUGHT EPISODE MODULATES THE RESPONSE OF RIVER BIOFILMS TO TRICLOSAN

This chapter aims to investigate how an episode of drought might influence the response of river biofilms to pulses of Triclosan (TCS). The objectives were to assess the separate and combined effects of simulated drought (achieved through drastic flow alteration) and of TCS exposure on biofilms growing in artificial channels. Thus, three-week old biofilms were studied under four conditions: Control (normal water flow); Simulated Drought (1 week reduced flow + 2 days interrupted flow); TCS only (normal water flow plus a 48-hour pulse of TCS); and Simulated Drought + TCS. All channels were then left for 2 weeks under steady flow conditions, and their responses and recovery were studied. Several descriptors of biofilms were analyzed before and after each step. Flow reduction and subsequent interruption were found to provoke an increase in extracellular phosphatase activity, bacterial mortality and green algae biomass. The TCS pulses severely affected biofilms: they drastically reduced photosynthetic efficiency, the viability of bacteria and diatoms, and phosphate uptake capacity. Latent consequences evidenced significant combined effects caused by the two stressors. The biofilms exposed only to TCS recovered far better than those subjected to both altered flow and subsequent TCS exposure: the latter suffered more persistent consequences, indicating that simulated drought amplified the toxicity of this compound. This finding has implications for river ecosystems, as it suggests that the toxicity of pollutants to biofilms may be exacerbated following a drought episode.

Chapter 8: USING BIOFILM PHOSPHORUS UPTAKE EFFICIENCY AS A TOOL FOR THE ASSESSMENT OF THE EFFECTS OF POLLUTANTS ON RIVERS SELF-DEPURATION CAPACITY.

Biofilm communities are key elements in river self-depuration processes since they can retain nutrients trough different mechanisms. Autotrophs and heterotrophs in the biofilm use nutrients from the river water to build up their growing cells. Uptake of phosphorus (P) is mainly a biotic process that depends from the affinity of organisms for P. Microbial biofilms active role in P interception suggests that careful examination of their potential role will be beneficial to both managers and scientists. Several physical and biological factors affect the efficiency of biofilms to retain nutrients. The input of pollutants in the flowing water altering the biofilm structure and functioning might determine changes in the P-uptake capacity and ultimately in the river self-depuration. This chapter aims to analyze the effect of pollution on the biofilm P-uptake capacity by comparing the specific effect of the bactericide triclosan in different experimental conditions with the more general effect of a pollution gradient. To this aim the response of biofilm P-uptake measured in four different experiments was analyzed and compared. Particularly, three experiments were performed testing triclosan (TCS) toxicity on biofilm in different conditions: TCS alone, with grazers, and after simulated drought episode. The fourth experiment was performed with biofilm exposed to river polluted water. This comparison evidenced that 48 hours of exposure to concentrations of TCS higher than 15 $\mu g\ L^{-1}$ significantly reduce the capacity of biofilm to uptake phosphorus while exposure to lower concentration affects phosphorus uptake capacity only under grazing pressure. Moreover, the simulated drought episode also induces a decrease of biofilm phosphorus uptake capacity and worsened triclosan negative effects. Results of the fourth experiment showed biofilm uptake capacity decrease along a pollution gradient. Our study validates the use of biofilm phosphorus uptake capacity as a powerful tool for studies aiming the understanding of ecological consequences of pollution and environmental variation on river self-depuration capacity.

RESUMEN (Castellano)

A pesar de que menos del 1% del agua total del planeta esta almacenada en ecosistemas de aguas dulces, el agua de ríos, arroyos y lagos es el recurso mas importante por el uso humano (agua potable, industria, agricultura etc.). Actualmente, la mayoría de los sistemas acuáticos continentales resultan afectados por las consecuencias del cambio climático y las actividades humanas. Las consecuencias de estos cambios incluyen la alteración del régimen de caudal y temperatura, el incremento de contaminación química y eutrofización y la simplificación de la red fluvial y de su vegetación ribereña. Todos estos factores afectan a la organización funcional de arroyos y ríos, y llevan a la simplificación y el empobrecimiento de la biota dentro de estos ecosistemas, reduciendo la capacidad de los sistemas fluviales de recuperar de perturbaciones naturales. Los sistemas receptores de aguas residuales de origen industrial, agrícola y urbano situados en regiones afectadas por problemas crecientes de escasez de agua se ven afectados por la coocurrencia del cabio climático y la contaminación. Debido a la presencia de miles de contaminantes diferentes cuya toxicidad potencial puede variar como consecuencia de cambios ambientales, existe una creciente necesidad de desarrollar bioindicadores seguros y biomarcadores de estrés rápidos y sensibles. Las comunidades bentónicas microbianas responden rápidamente, justificando su uso como buenos indicadores de perturbaciones relacionados con las consecuencias del cambio global. Estas comunidades están básicamente formadas por autótrofos (diatomeas, algas verdes y cianobacterias) y heterótrofos (bacterias, hongos y protozoos) incrustados en una matriz polimérica extracelular adherida a substratos que le confiere una estructura denominada biofilm. Los biofilms integran los efectos de las condiciones ambientales durante amplios periodos de tiempo, sobre todo gracias a su tamaño pequeño, rápida capacidad de crecimiento, riqueza de especies y la variedad fisiológica de los organismos que los componen. Además, los biofilms son ubicuos tienen tiempo de generación cortos y son fáciles de manipular en condiciones controladas. Por todas estas razones los biofilms constituyen un sistema ideal para la evaluación del impacto de

los estresores que no se pueden manipular fácilmente en el campo. A pesar de que las comunidades bentónicas son micro-ecosistemas donde se dan interacciones complejas, su respuesta a múltiples estresores puede ser diferente en función del organismo afectado y de este modo ambos efectos directos e indirectos pueden ser inferidos. El principal objetivo de esta tesis es investigar los efectos del cambio global, concretamente de la disminución de caudal, del exceso de nutrientes y contaminantes sobre la estructura-función de los biofilms microbianos fluviales. Para este fin, un enfoque de multi-marcadores ha sido utilizado a diferentes escalas experimentales, de estudios de campos hasta experimentos de laboratorio en mesocosmos. Las metodologías utilizadas para alcanzar los objetivos están descritas con detalle en el Capitulo 2 de la presente tesis. Múltiples métricas relacionadas con la estructura y función de los compartimentos autotróficos e heterotróficos del biofilm han sido utilizadas para describir los efectos directos e indirectos y para investigar las interacciones ecológicas que ocurren a escala microbiana. En particular la estructura de la comunidad autotrófica ha sido investigada midiendo las densidades de clorofila-*a*, la composición de comunidad mediante la señal de auto-fluorescencia de los principales grupos fotosintéticos y la biomasa de las diatomeas mientras que la función de los autótrofos ha sido estudiada principalmente midiendo los parámetros fotosintéticos. La estructura de los heterótrofos ha sido investigada como composición de comunidad (mediante técnicas moleculares) así como mediante el contaje de la ratio entre bacteria vivas y muertas, mientras que la función se ha estudiado principalmente midiendo las actividades enzimática extracelulares. Además, microscopios has sido utilizado para complementar la información sobre la estructura superficial y tridimensional de los biofilms. Finalmente, la medida de la capacidad de retención de fósforo ha sido investigada como una función global de la comunidad relacionada con los servicios ecológicos proporcionados por los biofilms fluviales.

CAPITULO 3. EFECTO DE LUZ Y NUTRIENTES SOBRE BIOFILMS FLUVIALES: UNA EVALUACÍON COMBINADA ENTRE PARÁMETROS ESTRUCTURALES Y FUNCIONALES Y CLSM.

El objetivo principal de este capítulo es la investigación de los efectos individuales y combinados de la luz y los nutrientes sobre el desarrollo de un biofilm fluvial en un río forestado oligotrófico. Con esta finalidad, se ha llevado a cabo un experimento de campo en un río Mediterráneo (Fuirosos, España). Los biofilms crecieron adheridos a substratos artificiales en dos tramos (enriquecido y no enriquecido en nutrientes) en donde se generaron artificialmente condiciones de sombra. Se aplicaron cuatro tratamientos diferentes: Alta luz-No enriquecido (HL-U), Baja luz-No enriquecido (LL-U), Alta luz-Enriquecido (HL-E) y Baja luz-Enriquecido (LL-E). Se analizaron diferentes parámetros estructurales y funcionales de los compartimentos autotrófico y heterotrófico de los biofilms durante el proceso de colonización en los días 4, 9, 16, 22 y 52. Se utilizó un Microscopio Confocal de Escaneo Láser (CLSM) para determinar diferencias en la arquitectura de los biofilms al día 52. El CLSM evidenció diferencias de grosos y complejidad estructural en biofilms crecidos en condiciones diferentes. Los biofilms HL-E resultaron los más gruesos y estructuralmente complejos. . El CLSM evidenció que la matriz extracelular polimérica resultaba más concentrada en las capas más superficiales de los biofilms crecidos en el tramo enriquecido, mientras que se encontraba uniformemente distribuido en las comunidades del tramo no enriquecido. Las imágenes tridimensionales del CLSM sugirieron un incremento de las cianobacterias en condiciones de mayor disponibilidad de nutrientes. El enriquecimiento causó una bajada de actividad de la fosfatasa extracelular. La interacción entre los dos factores, afectó la actividad de la peptidasa extracelular. Los biofilms HL-E tuvieron la actividad de peptidasa más alta y la de fosfatasa más baja, lo cual indica que la respuesta de los biofilms más importantes a la variación de concentración de los nutrientes ocurre principalmente en condiciones de luz más elevada. Los resultados revelaron que la disponibilidad conjunta de luz y nutrientes causó los mayores cambios en la organización espacial, la estructura microbiana y el funcionamiento de los biofilms en un río forestado oligotrófico.

Capitulo 4. RESPUESTA DEL BIOFILM A LA TRANSLOCACION A LO LARGO DE UN GRADIENTE DE CONTAMINACION DE UN RIO SUMAMENTE IMPACTADO: LOS EFECTOS DE FARMACOS Y PESTICIDAS.

Actualmente, una elevada y variada cantidad de compuestos químicos se encuentra presente en los ecosistemas acuáticos como consecuencia de la influencia directa de las actividades agrícola, industrial e urbana. Entre estos, pesticidas y fármacos son los más frecuentemente detectados. El Llobregat es un río Mediterráneo sumamente impactado por una intensa actividad urbana, industrial y agrícola. En este capítulo hemos investigado los efectos de los fármacos y pesticidas detectados en las aguas del Llobregat sobre la estructura y función de los biofilms fluviales mediante experimentos de translocación realizados bajo condiciones controladas. Se seleccionaron tres puntos de muestreo: Castellbell (CB) y Mina de Terrassa (MT) como puntos menos contaminados y Sant Joan Despí (SJD) como punto muy contaminado. El agua superficial procedente de cada punto de muestreo se utilizó como inóculo y medio para el crecimiento del biofilm en mesocosmos independientes. Transcurridos 25 días, se realizaron tres translocaciones: de MT a SJD, de CB a MT y de CB a SJD. Se midieron varios descriptores estructurales y funcionales de biofilm cuatro veces durante el experimento: uno antes y tres después de la translocaciones. 57 fármacos de 14 grupos terapéuticos distintos y 16 compuestos de 5 familias de pesticidas diferentes fueron detectados en las aguas superficiales del río. Las diferencias entre biofilms antes de la translocación concernieron en su mayor parte el compartimiento de los autótrofos. Las respuestas de los biofilms fueron diferentes dependiendo de la translocación. La translocación de CB a MT fue la menos sensible mientras que la translocación de MT a SJD causó respuestas estructurales y funcionales importantes. La translocación de CB a SJD fue la más efectiva en cuanto a respuesta del biofilm. El análisis de la redundancia mostró que la conductividad, los analgésicos y los barbitúricos fueron las variables que influyeron significativamente en la respuesta de biofilms a las translocaciones y la contaminación del Río de Llobregat. Además, la técnica de partición de la varianza, reveló

que los analgésicos y antinflamatorios afectaron significativamente las respuestas del biofilm a las translocaciones y el gradiente de contaminación. Los fármacos comúnmente utilizados Ibuprofeno y Paracetamol, resultaron relacionados con efectos negativos sobre el compartimiento autotrófico, mientras que el Diclofenac fue más relacionado con las respuestas de los heterótrofos. Este estudio evidenció cómo los compuestos emergentes no regulados, que alcanzan continuamente los sistemas fluviales, pueden causar respuestas biológicas en la base de la cadena trófica del río con consecuencias aun desconocidas para el ecosistema, especialmente en ríos Mediterráneos.

CAPITULO 5. RESPUESTAS DE LA COMUNIDAD BACTERIANA DEL BIOFILM A LOS ANTIBIOTICOS EN AGUAS DE RIOS: UN ESTUDIO DE TRANSLOCACION.

Las actividades antrópicas están incrementando los niveles de contaminantes prioritarios y emergentes, derivados de fuentes difusas y puntuales, que alcanzan los ecosistemas acuáticos continentales. Los antibióticos, son compuestos bioactivos que actúan contra bacteria de comunidades microbianas naturales y su presencia en el ambiente acuático puede dirigir modificaciones fisiológicas a corto plazo, así como cambios a largo plazo en la biomasa o cambios en la composición de la comunidad. En este capítulo, hemos investigado las respuestas estructurales y funcionales de la comunidad bacteriana de biofilms crecidos a lo largo del gradiente de contaminación por antibióticos del Rio Llobregat, a la translocación en aguas con diferente grado de contaminación por antibióticos. Se seleccionaron tres puntos de muestreo: Castellbell (CB) y Mina de Terrassa (MT) como puntos menos contaminados y Sant Joan Despí (SJD) como punto muy contaminado. El agua superficial procedente de cada punto de muestreo se utilizó como inóculo y medio para el crecimiento del biofilm en mesocosmos independientes. Transcurridos 25 días, se realizaron tres translocaciones: de MT a SJD, de CB to MT y de CB a SJD. La composición de la comunidad bacteriana, la proporción entre bacterias vivas y muertas y las actividades enzimáticas extracelulares fueron medidas dos veces durante el experimento: uno

antes y otro después de la translocación. Un total de 16 compuestos antibióticos fueron detectados en las aguas del río de los tres puntos de muestreo. La familia de las sulfonamidas resultó ser la más concentrada en cada punto de muestreo, seguida por las familias de las quinolonas y los macrólidos. La comunidad bacteriana mostró diferencias estructurales pero no funcionales entre puntos de muestreo antes de la translocación. La estructura de la comunidad bacteriana cambió nueve días después de la translocación. En particular, el incremento de las Actinobacteria (HCG) fue medido en todos los biofilms translocados. El análisis de Correspondencias Canónicas (CCA) confirmó que las comunidades se separaron por punto de muestreo y translocación, y que las Actinobacteria resultaron asociadas al incremento de concentraciones de antibióticos. Las comunidades de biofilms translocadas en aguas con concentraciones de antibióticos más elevadas vieron incrementada la mortalidad bacteriana y modificaron su metabolismo heterotrófico, en particular incrementando la actividad de la leucina-aminopeptidasa y bajando la actividad de la fosfatasa alcalina. El test de Mantel confirmó la correlación significativa entre la concentración de antibióticos y las respuestas estructurales y funcionales del biofilm. El estudio mostró que la entrada continua de antibióticos en sistemas acuáticos continentales puede provocar cambios estructurales y funcionales en las comunidades microbianas bentónicas.

CHAPTER 6. RESISTENCIA Y RECUPERACION DE BIOFILMS FLUVIALES QUE RECIBIERON CORTOS PULSOS DE TRICLOSAN Y DIURON.

Este capitulo tiene como objetivo el estudio de la resistencia y recuperación de biofilm fluviales que recibieron pulsos cortos de Triclosan (TCS) y Diuron (DIU). Con este fin, se diseñó un sistema de mesocosmos donde los biofilms fueron expuestos durante 48 horas a pulsos cortos de DIU y TCS bajo condiciones controladas. Los efectos directos e indirectos de cada tóxico sobre el biofilm y la posterior recuperación, fueron evaluados mediante el uso de bio-marcadores estructurales y funcionales. Estos parámetros fueron analizados inmediatamente antes y después de

la exposición, 9 y 16 días después de la exposición. El DIU causó un incremento de la mortalidad de las diatomeas (+79%), el cual persistió hasta el final del experimento. El TCS también afectó la mortalidad de las diatomeas (+41%), aunque el efecto no apareció hasta transcurrido una semana del final de la exposición. El TCS causó un incremento de la mortalidad bacteriana (+45%); aunque este parámetro recuperó valores normales una semana después de finalizar la exposición. El TCS comprometió la integridad celular del alga verde Spyrogira sp., mientras que el DIU no. El TCS inhibió fuertemente la capacidad de captación de fósforo del biofilm (-71%), que no recuperó valores normales hasta pasadas dos semanas de finalizar la exposición. El DIU afectó directamente las algas, pero apenas afectó los heterótrofos, mientras el TCS dañó gravemente las bacterias (efectos directos) así como los autótrofos (efectos indirectos). Sin embargo, el biofilm recuperó su estructura y función normales dentro de unos días o unas semanas. Estas conclusiones demuestran la capacidad de los biofilms para enfrentarse a entradas periódicas de tóxicos, pero también los riesgos asociados a la exposición repetida o a la multi-contaminación en ecosistemas acuáticos.

CAPITULO 7. UN EPISODIO DE SEQUIA MODULA LA RESPUESTA DEL BIOFILM FLUVIAL AL TRICLOSAN.

Este capítulo tiene como objetivo el estudio de como un episodio de sequía puede influenciar la respuesta del biofilm fluvial a pulsos de triclosan (TCS). Los objetivos fueron la evaluación de los efectos independientes y combinados de un episodio de sequía simulado (logrado con una drástica alteración del caudal) y de la exposición al TCS de biofilms crecidos en canales artificiales. Con este fin, biofilms de tres semanas de edad fueron estudiados en cuatro condiciones diferentes: control (caudal normal); sequía simulada (una semana de caudal reducido seguida de dos días de caudal interrumpido); TCS solo (caudal normal y 48 horas de pulsos de TCS); sequia simulada + TCS. Transcurrido ese tiempo, el sistema se llevó a condiciones de caudal estable durante dos

semanas, y se realizó el estudio de las respuestas y la recuperación del biofilm fueron estudiadas. Varios descriptores del biofilm se analizaron antes y después de cada paso. La reducción de caudal y la posterior interrupción, causaron un incremento de la actividad de la fosfatasa alcalina, de la mortalidad bacteriana y de la biomasa de las algas verdes. Los pulsos de TCS, dañaron gravemente el biofilm: causaron una drástica reducción de la eficiencia fotosintética, la capacidad de captación de fósforo y la viabilidad de diatomeas y bacterias. Las consecuencias tardías evidenciaron efectos significativos y combinados causados por los dos estresores. Los biofilms expuestos sólo al TCS se recuperaron mejor que los que estuvieron sujetos a caudal alterado y posterior exposición al TCS. Estos últimos sufrieron consecuencias más persistentes, indicando que la simulación de sequía, amplificó la toxicidad de este compuesto. Este hallazgo tiene implicaciones para los ecosistemas fluviales, como sugiere el hecho que la toxicidad de contaminantes para los biofilms puede ser acentuada después de un episodio de sequía.

CAPITULO 8. UTILIZAR LA EFICIENCIA DE CAPTACION DE FOSFORO DEL BIOFILM COMO UNA HERRAMIENTA PARA LA EVALUACION DE LOS EFECTOS DE CONTAMINANTES SOBRE LA CAPACIDAD DE AUTO-DEPURATION DE LOS RIOS.

Las comunidades de biofilm son elementos clave en los procesos de auto-depuración de los ríos dado que pueden retener nutrientes mediante mecanismos diferentes. Los organismos autótrofos y heterótrofos del biofilm utilizan los nutrientes disueltos en el agua del río para para desarrollar sus células crecientes. La captación de fosforo (P) es principalmente un proceso biótico, que depende de la afinidad de los organismos por el este elemento. El papel activo de los biofilms microbianos en la captación de fósforo sugiere que un atento examen de su papel potencial, beneficiaria gestores y científicos. Varios factores físicos y biológicos afectan la eficiencia de los biofilms en la captación de nutrientes. La entrada de contaminantes a las aguas superficiales altera

la estructura y función del biofilm y puede determinar cambios en la capacidad de captación de fósforo y finalmente en la capacidad de auto depuración de los ríos. Este capítulo tiene como objetivo analizar los efectos de la contaminación sobre la capacidad de captación de fósforo de los biofilms, mediante la comparación de los efectos específicos del bactericida triclosan (TCS) bajo diferentes condiciones experimentales con el efecto mas general de un gradiente de contaminación. Con este fin, se analizó la respuesta de la capacidad de captación de fósforo del biofilm medida en cuatro experimentos diferentes concretamente se realizaron tres experimentos evaluando la toxicidad del TCS sobre el biofilm en condiciones diferentes: el TCS sólo, en presencia de caracoles, y después de un episodio de sequia simulado. Esta comparación evidenció que 48 horas de exposición a concentraciones de TCS speriores a 15 $\mu g\ L^{-1}$ redujeron significativamente la capacidad del biofilm de captar fósforo, mientras la exposición a concentraciones más bajas afectó la capacidad de captación de fosforo solo en presencia de los caracoles. Además, la simulación de un episodio de sequía también indujo una disminucion de la capacidad de captación de fósforo del biofilm y empeoró los efectos negativos del triclosan. Los resultados del cuarto experimento mostraron una bajada de la capacidad de captación del biofilm a lo largo del gradiente de contaminación. El estudio, validó el uso de la capacidad de captación de fósforo del biofilm como una herramienta poderosa para su utilización en estudios enfocados en la comprensión de las consecuencias ecológicas de la contaminación y la variación ambiental sobre la capacidad de auto-depuración de los ríos.

RESUM (Català)

L'aigua dels rius, rieres i llacs és el recurs més important per a l'ús humà (aigua potable, indústria, agricultura etc.) tot i que menys de l'1% de l'aigua total del planeta està emmagatzemada en ecosistemes d'aigües dolces. Actualment, la majoria dels sistemes aquàtics continentals es veuen afectats per les conseqüències del canvi climàtic i les activitats humanes. Les conseqüències d'aquests canvis inclouen l'alteració del règim de cabal i temperatura, l'increment de la contaminació química i eutrofització, i la simplificació de la xarxa fluvial i de la seva vegetació ripària. Tots aquests factors afecten l'organització funcional del rius i rieres, i porten a una simplificació i empobriment de la biota reduint la capacitat dels sistemes fluvials de recuperar-se de pertorbacions naturals. La concurrència de canvi climàtic i contaminació pot ser especialment rellevant en sistemes que reben aigües residuals d'origen industrial, agrícola i urbà situats en regions afectades per problemes creixents d'escassetat d'aigua. Degut a la presència de milers de contaminants diferents que poden variar la seva potencial toxicitat en resposta a canvis ambientals, hi ha una creixent necessitat de desenvolupar bioindicadors segurs i biomarcadors d'estrès ràpids i sensibles. Les comunitats bentòniques microbianes responen ràpidament, justificant el seu ús com a bones indicadores de pertorbacions relacionades amb les conseqüències del canvi global. Aquestes comunitats estan formades en general per autòtrofs (diatomees, algues verdes, i cianobacteris) i heteròtrofs (bacteris, fongs i protozous) englobats en una matriu polimèrica extracel·lular adherida a substrats que li confereixen una estructura anomenada biofilm. Els biofilms integren els efectes de les condicions ambientals durant períodes de temps més o menys llargs (en funció de l'estabilitat del biofilm), sobretot gràcies a la seva mida i a la seva ràpida capacitat de creixement, riquesa d'espècies, i varietat fisiològica del organismes que els componen. A més a més, els biofilms són ubiqüitaris, tenen un temps de generació curt i són fàcils de manipular en condicions controlades. Per totes aquestes raons, són un sistema ideal per a l'avaluació dels impactes d'estressos que no es poden manipular fàcilment al camp. Considerant que les comunitats bentòniques són micro-

ecosistemes en els quals es donen interaccions complexes, les respostes a estressos múltiples poden ser diferents en funció de l'organisme afectat i per tant poden tenir lloc dins el biofilm efectes tan directes com indirectes. L'objectiu principal d'aquesta tesi és el d'investigar els efectes del canvi global, específicament del decrement de cabal, l'excés de nutrients i contaminants, sobre l'estructura i funció dels biofilms fluvials microbians. Amb aquesta finalitat s'ha utilitzat un enfoc de multi-marcadors a diferents escales experimentals, des d'estudis de camp fins a experiments de laboratori en mesocosms. Les metodologies utilitzades per arribar a complir amb els objectius planejats estan descrites en detall en el capítol 2 de la present tesi. Múltiples mètriques relacionades amb la estructura i funció del compartiments autotròfic i heterotròfic del biofilm es van fer servir per descriure els efectes directes i indirectes i per investigar les interaccions ecològiques que es donen a escala microbiana. En particular la estructura de la comunitat autotròfica es va investigar mesurant les densitats de clorofil·la *a,* la composició de comunitat mitjanament la senyal de auto-fluorescència dels principals grups fotosintètics i la biomassa de les diatomees mentre que la funció dels autòtrofs es va estudiar principalment mesurant els paràmetres fotosintètics. L' estructura dels heteròtrofs es va estudiar com composició de comunitat (mitjanament tècniques moleculars) tan com mitjanament el comptatge de la relació entre bacteris vius i morts mentre que la funció es va estudiar principalment mesurant les activitats enzimàtiques extra cel·lulars. A mes a mes els microscopis es van fer servir per complementar la informació sobre l'estructura superficial i tridimensional dels biofilms. Finalment, la mesura de la capacitat de retenció de fòsfor es va investigar com una funció global de la comunitat relacionada amb els serveis ecològics proporcionats pels biofilms fluvials.

CAPÍTOL 3. EFECTE DE LES CONDICIONES DE LLUM I NUTRIENTS SOBRE ELS BIOFILMS FLUVIALS: UNA AVALUACIÓ COMBINADA ENTRE PARÀMETRES ESTRUCTURALS I FUNCIONALS I MICROSCOPIA CONFOCAL

L'objectiu principal d'aquest capítol és la investigació dels efectes individuals i combinats de llum i nutrients sobre el desenvolupament d'un biofilm fluvial en un riu forestal oligotròfic. Amb aquesta finalitat es va realitzar un experiment de camp en un riu Mediterrani (Furiosos, Espanya). Els biofilms van créixer adherits a substrats artificials en dos trams (enriquit i no enriquit) on es van generar artificialment condicions d'ombra. Es van generar quatre tractaments diferents: Alta llum-No enriquit (HL-U), Baixa llum-No enriquit (LL-U), Alta llum-Enriquit (HL-E) i Baixa llum-Enriquit (LL-E). Durant el procés de colonització, als dies 4, 9, 16, 22 i 52, es van analitzar diferents paràmetres estructurals i funcionals dels compartiments autotròfic i heterotròfic dels biofilms. El Microscopi confocal Làser d'Escaneig (CLSM) es va utilitzar per determinar diferències en l'arquitectura el dia 52 de colonització. Les observacions amb el CLSM van evidenciar diferències de gruix i complexitat estructural en biofilms crescuts en condicions diferents. Els biofilms HL-E van resultar els més gruixuts i estructuralment complexos. El CLSM també va evidenciar que la matriu extracel·lular polimèrica es trobava concentrada en les capes més superficials dels biofilms crescuts en el tram enriquit, mentre que es trobava uniformement distribuïda en les comunitats del tram no enriquit. Les imatges tridimensionals del CLSM van suggerir un increment dels cianobacteris en condicions de més disponibilitat de nutrients. L'enriquiment va causar una baixada d'activitat de la fosfatasa extracel·lular. La interacció entre els dos factors (llum i nutrients) va afectar l'activitat de la peptidasa extracel·lular. Els biofilms del tractament HL-E van mostrar l'activitat peptidasa més alta i la de fosfatasa més baixa, una indicació que les respostes dels biofilms més importants a la variació de concentració dels nutrients es donen principalment en condicions de llum més alta. Els nostres resultats van revelar que la disponibilitat conjunta de llum i nutrients causava els majors canvis en l'organització espacial, l'estructura microbiana i el funcionament dels biofilm en un riu forestal oligotròfic.

CAPÍTOL 4. RESPOSTA DEL BIOFILM A LA TRANSLOCACIÓ AL LLARG D'UN GRADIENT DE CONTAMINACIÓ D'UN RIU IMPACTAT: ELS EFECTES DE FÀRMACS I PESTICIDES.

Un gran nombre de compostos químics entra a les aigües corrents en conseqüència de les activitats agrícola, industrial i urbana. Entre aquests, pesticides i fàrmacs són dels més freqüentment detectats. El Llobregat és un riu Mediterrani summament impactat per una intensa activitat urbana, industrial i agrícola. En aquest capítol hem investigat els efectes del fàrmacs i pesticides detectats en les aigües del Llobregat sobre l'estructura i funció dels biofilms fluvials mitjançant experiments de translocació realitzats en condicions controlades. Es van seleccionar tres punts de mostreig: Castellbell (CB) i Mina de Terrassa (MT) com a punts menys contaminats i Sant Joan Despí (SJD) com a punt molt contaminat. L'aigua del riu es va agafar de cada punt de mostreig i va ser utilitzada com a inòcul pel creixement del biofilm en mesocosms independents. Als 25 dies, es van realitzar tres translocacions: de MT a SJD, de CB a MT i de CB a SJD. Varis descriptors estructurals i funcionals del biofilm es van mesurar quatre vegades durant l'experiment: una abans i tres després de les translocacions. A les aigües del riu es van detectar 57 fàrmacs de 14 grups terapèutics i 16 compostos de 5 famílies de pesticides diferents. Les diferències entre els biofilms abans de la translocació tenien a veure majoritàriament amb el compartiment autotròfic. Les respostes dels biofilms van ser diferents depenent de la translocació. La translocació de CB a MT va ser la menys sensible mentre que la translocació de MT a SJD va causar importants respostes estructurals i funcionals. La translocació de CB a SJD va ser la més efectiva en quan a resposta del biofilm. L'anàlisi de redundància va mostrar que la conductivitat, els analgèsics i els barbitúrics van ser les variables que van influir significativament en la resposta dels biofilms a les translocacions i al gradient de contaminació del riu Llobregat. A més a més, la tècnica de partició de la variància va revelar que els analgèsics i antiinflamatoris van afectar significativament les respostes del biofilm a les translocacions i al gradient de contaminació. Concretament, els

comunament utilitzats Ibuprofè i Paracetamol van resultar relacionats amb efectes negatius sobre el compartiment autotròfic mentre que el Diclofenac va ser més relacionat amb les respostes dels heteròtrofs. Aquest estudi va evidenciar com els compostos emergents no regulats, que entren contínuament als sistemes fluvials, poden causar respostes biològiques a la base de la xarxa tròfica del riu amb conseqüències encara desconegudes per l'ecosistema sencer, especialment en rius mediterranis.

CAPÍTOL 5. RESPOSTES DE LA COMUNITAT BACTERIANA DEL BIOFILM ALS ANTIBIOTICS EN AIGÜES DE RIU: UN ESTUDI DE TRANSLOCACIÓ.

Les activitats antròpiques estan incrementant els nivells de contaminants prioritaris i emergents derivats de fonts difoses i puntuals que entren als ecosistemes aquàtics continentals. Els antibiòtics són compostos bioactius contra els bacteris i la seva presència a l'ambient aquàtic pot determinar modificacions fisiològiques a curt termini així com canvis a llarg termini tan en la biomassa com en la composició de la comunitat. En aquest capítol hem investigat les respostes estructurals i funcionals de la comunitat bacteriana de biofilms crescuts al llarg d'un gradient de contaminació per antibiòtics del Riu Llobregat, mitjançant la translocació de biofilms en aigües amb diferent grau de contaminació per antibiòtics. Es van seleccionar tres punts de mostreig: Castellbell (CB) i Mina de Terrassa (MT) com a punts menys contaminats i Sant Joan Despí (SJD) com a punt molt contaminat. L'aigua del riu es va agafar de cada punt de mostreig i va ser utilitzada com a inòcul pel creixement del biofilm en mesocosms independents. Als 25 dies, es van realitzar tres translocacions: de MT a SJD, de CB a MT i de CB a SJD. La composició de la comunitat bacteriana, el quocient entre bacteris vius i morts i les activitats enzimàtiques extracel·lulars van ser mesurades dues vegades durant l'experiment: una abans i una després de la translocació. Es van detectar un total de 16 compostos antibiòtics a les aigües del riu dels tres

punts de mostreig. Les concentracions més altes van correspondre a la família de les sulfonamides seguida per les famílies de les quinolones i dels macròlids. La comunitat bacteriana va mostrar diferències estructurals però no funcionals entre punts de mostreig abans de la translocació. L'estructura de la comunitat bacteriana va canviar nou dies després de la translocació. En particular, l'increment d'Actinobacteri (HCG) va ser mesurada en tots els biofilms translocats. Les anàlisis de Correspondències Canòniques (CCA) van confirmar que les comunitats se separaven per punt de mostreig i translocació i que els Actinobacteris van resultar associats amb l'increment de concentracions d'antibiòtics. Les comunitats de biofilms translocades cap a aigües amb concentracions d'antibiòtics més elevades van incrementar la mortalitat bacteriana i van modular el seu metabolisme heterotròfic, en particular augmentant l'activitat de leucina-aminopeptidasa i reduint l'activitat de fosfatasa alcalina extracel·lulars. El Mantel test va confirmar la correlació significativa entre la concentració d'antibiòtics i les respostes estructurals i funcionals del biofilm. El nostre estudi va mostrar que l'entrada contínua d'antibiòtics en sistemes aquàtics continentals pot provocar canvis estructurals i funcionals en les comunitats microbianes bentòniques.

CAPÍTOL 6. RESISTÈNCIA I RECUPERACIÓ DELS BIOFILMS FLUVIALS A ENTRADES CURTES I PUNTUALS DE TRICLOSAN I DIURÓ.

Aquest capítol té com a objectiu l'estudi de la resistència i recuperació de biofilms fluvials en rebre entrades curtes i puntuals de Triclosan (TCS) i Diuró (DIU). Amb aquesta finalitat, es va dissenyar un sistema de mesocosms on els biofilms van ser exposats durant 48 hores a entrades de DIU i TCS en condicions controlades. Els efectes directes i indirectes de cada tòxic sobre el biofilm i la següent recuperació, van ser avaluats mitjanament l'ús de bio-marcadors estructurals i funcionals. Aquests paràmetres van ser analitzats immediatament abans i després de l'exposició, i 9 i 16 dies després de l'exposició. El Diu va causar un increment de la mortalitat de les diatomees

(+79%) que va persistir fins al final de l'experiment. El TCS també va afectar la mortalitat de les diatomees (+41%), tot i que l'efecte no va aparèixer fins passada una setmana després del final de l'exposició. El TCS va causar un augment de la mortalitat bacteriana (+45%), tot i que aquest paràmetre va recuperar els valors normals una setmana després de finalitzar l'exposició. El TCS va afectar la integritat cel·lular de l'alga verda *Spyrogira sp.*, mentre que el DIU no. El TCS va inhibir fortament la capacitat de captació de fòsfor del biofilm (-71%), que no es va recuperar fins passades dues setmanes de finalitzar l'exposició. El DIU va afectar directament les algues, però va afectar molt poc els heteròtrofs. El TCS en canvi va danyar greument els bacteris (efectes directes) així com els autòtrofs (efectes indirectes). En tot cas, el biofilm va recuperar la seva estructura i funció normals en uns dies o unes setmanes. Aquestes conclusions demostren la capacitat dels biofilms de suportar entrades periòdiques de tòxics, però també els riscos associats a una exposició repetida o a la contaminació múltiple en ecosistemes aquàtics.

CAPÍTOL 7. UN EPISODI DE SEQUERA MODULA LA RESPOSTA DEL BIOFILM FLUVIAL AL TRICLOSAN.

Aquest capítol té com a objectiu l'estudi de com un episodi de sequera pot influenciar la resposta del biofilm fluvial a entrades puntuals i curtes de triclosan (TCS). Els objectius concrets van ser l'avaluació dels efectes independents i combinats d'un episodi de sequera simulat (obtingut amb una dràstica alteració del cabal) i de l'exposició al TCS de biofilms crescuts en canals artificials. Amb aquesta finalitat, es van estudiar biofilms de tres setmanes en quatre condicions diferents: control (cabal normal); sequera simulada (una setmana de cabal reduït + dos dies de cabal interromput); TCS sol (cabal normal i 48 hores d'exposició al TCS); sequera simulada + TCS. Tots els canals van ser llavors deixats en condicions de cabal estable durant dues setmanes per tal d'estudiar les respostes i recuperació del biofilm. Abans i després de cada canvi de condicions,

es van analitzar diferents descriptors del biofilm. La reducció de cabal i la següent interrupció van causar un augment de l'activitat de la fosfatasa alcalina, de la mortalitat bacteriana i de la biomassa de les algues verdes. L'exposició al TCS va danyar greument el biofilm: va causar una dràstica reducció de l'eficiència fotosintètica, de la capacitat de captació del fòsfor i de la viabilitat de diatomees i bacteris. Les conseqüències retardades van evidenciar els efectes significatius i combinats causats pels dos estressos. Els biofilms exposats només al TCS es van recuperar millor dels que van ser subjectes a cabal alterat i següent exposició al TCS. Aquests últims van sofrir conseqüències més persistents, indicant que la simulació de sequera va amplificar la toxicitat del TCS. Aquestes evidències tenen implicacions pels ecosistemes fluvials, com suggereix el fet que la toxicitat de contaminants pels biofilms pot ser exacerbada després d'un episodi de sequera.

CAPÍTOL 8. UTILITZACIÓ DE L'EFICIÈNCIA DE CAPTACIÓ DE FÒSFOR DEL BIOFILM COM UNA EINA PER A L'AVALUACIÓ DELS EFECTES DELS CONTAMINANTS SOBRE LA CAPACITAT D'AUTO-DEPURACIÓ DELS RIUS.

Les comunitats de biofilms són elements clau en els processos d'auto-depuració dels rius ja que poden retenir nutrients mitjançant diferents mecanismes. Els autòtrofs i heteròtrofs del biofilm utilitzen els nutrients dissolts a l'aigua del riu pel seu creixement. La captació de fòsfor (P) és principalment un procés biòtic que depèn de l'afinitat dels organismes pel P. El rol actiu dels biofilms microbians en la intercepció de fòsfor suggereix que un examen detallat del seu rol potencial beneficiaria gestors i científics. Varis factors físics i biològics afecten l'eficiència dels biofilms en captar nutrients. L'entrada de contaminants en les aigües corrents altera l'estructura i funció del biofilm i pot determinar canvis en la capacitat de captació del P i finalment en la capacitat d'auto-depuració dels rius. Aquest capítol té l'objectiu d'analitzar els efectes de la contaminació sobre la capacitat de captació de fòsfor dels biofilm mitjançant la comparació dels efectes específics del

bactericida triclosan (TCS) en diferents condicions experimentals amb l'efecte més general d'un gradient de contaminació. Amb aquesta finalitat, la resposta de la capacitat de captació de fòsfor del biofilm mesurada en quatre experiments diferents va ser analitzada i comparada. En particular, tres experiments van ser realitzats testant la toxicitat del TCS sobre el biofilm en condicions diferents. El TCS sol, en presència de cargols, i després d'un episodi de sequera simulat. Aquesta comparació va evidenciar que 48 hores d'exposició a concentracions de TCS més altes de 15 $\mu g\ L^{-1}$ reduïen significativament la capacitat del biofilm de captar fòsfor mentre l'exposició a concentracions més baixes afectava la capacitat de captació de fòsfor només en presència dels cargols. A més a més, la simulació d'un episodi de sequera també va induir a una disminució de la capacitat de captació de fòsfor del biofilm i van empitjorar els efectes negatius del triclosan. Els resultats del quart experiment van mostrar una disminució de la capacitat de captació de fòsfor del biofilm al llarg del gradient de contaminació. Aquest estudi comparatiu va validar l'ús de la capacitat de captació de fòsfor del biofilm com una eina útil per a estudis que tenen per objectiu la comprensió de les conseqüències ecològiques de la contaminació i la variació ambiental sobre la capacitat d'auto-depuració dels rius.

CHAPTER 1:

GENERAL INTRODUCTION

GENERAL INTRODUCTION

River structure and function dynamics: from the watershed to the microbial biofilm.

River and stream ecosystems are heterogeneous and dynamic environments submitted to several physical and biological processes. Physical processes are mainly driven by geomorphology and hydrology characteristics, determining the quantity and quality inputs of organic and inorganic materials in the flowing ecosystem as well as the arrangement of habitat patchiness (hyporheic zones, wetlands, rapid-pools, floodplains; Allan and Castillo 2007). At the catchment scale, climate largely determines these processes. The biological processes, interacting with the river physical dynamics, are the main responsible for the organic matter cycling –release, uptake and transformations- throughout the river system.

Within the large complexity intrinsic of river watersheds key biogeochemical and biological processes occur at the microbial scale, and might be relevant at the ecosystem scale (Zehr, 2010). The study of microbial ecology of river ecosystems is a relatively recent discipline that has grown in the last decades. From the first description of the microbial loop by Azam et al. (1983) in marine systems several studies have been performed in marine, lentic and lotic environments (i.e. Descy et al. 2002; Hart et al., 2000; Fenchel 2008), highlighting relevant differences in the structure of the microbial communities. The importance of the food webs for organic matter re-mineralization and carbon fluxes has been widely described in the plankton of lakes and marine systems (Pomeroy and Wiebe 1988; Edwards et al. 1990). However the crucial role of the benthic microbial community in river energy flow (Battin et al. 1999) is largely unknown.

Attached microbial communities are in general structured by autotrophs (diatoms, green algae and cyanobacteria) and heterotrophs (bacteria, fungi, protozoa) embedded in extracellular polymeric matrix adhered on substrata (Figure 1) conferring a biofilm structure (Lock 1993). Attached bacteria start their colonization process by covering mineral surfaces with polysaccharide

glycocalix (Barlocher and Murdoch 1989). This extracellular matrix facilitates the adherence of micro autotrophs and traps organic matter particles permitting the development of more complex biofilms (Pusch et al. 1998). In spite of their mobility, micro-metazoans can also be found interstitially as well as on the substratum surface and they must be considered as part of the attached community because of their trophic relationship with biofilm microorganisms (Lamberti 1996). This close spatial relationship between different life-strategy organisms results in important interactions (i.e. bacterial utilization of algal exudates, Murray et al. 1986), that allow us to consider attached communities as micro ecosystems with a complex structure where important processes take place (Figure 1).

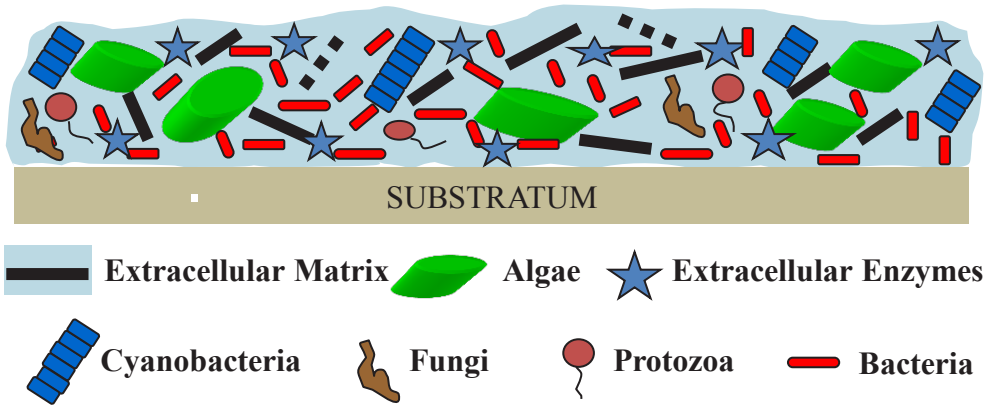


Figure 1. Schematic representation of epilithic river biofilm.

The role of microbial biofilms in running water ecosystems

River biofilms play an important role in the organic matter re-mineralization and inorganic nutrient fluxes all over the river network. The epilithic biofilms (developing on rocks and cobbles) have been described as the most relevant sites for organic and inorganic nutrients uptake and retention in most small and medium-sized rivers (Sabater et al. 2007). One of the key roles of bacteria and fungi within biofilm communities is their capability to decompose and transform organic molecules to finally mineralize them, thanks to their extracellular enzyme capabilities. The size of molecules limits its transport across biological membranes, making the activity of

extracellular enzymes the first biotic step of organic matter turnover in ecosystems (Pusch et al. 1998). Extracellular enzymes produced by fungi and bacteria exhibit substrate specificity. In general, fungal communities produce enzymes capable to degrade complex and recalcitrant compounds such as cellulose, hemicelluloses and lignin (by means of β-xylosidase, β-glucosidase, peroxidase and phenoloxidase enzyme activities), while bacteria mostly synthesize enzymes involved in degrading more simple polymers such as polysaccharides, peptides or organic phosphorus compounds (β-glucosidase, peptidase, and phosphatase; Romaní 2010). However, these enzyme capabilities could be modulated by microbial interactions within the attached community, such as the positive interaction between algae and bacteria in epilithic biofilms (Rier and Stevenson 2001; Francoeur and Wetzel 2003) or the synergistic/competitive relationship between fungi and bacteria in leaf litter breakdown (Bengtsson 1992, Romaní et al. 2006). Overall, microbial attached communities dominate the ecosystem metabolism in many aquatic systems and are major components for the uptake, storage and cycling of carbon, nitrogen and phosphorus (Pusch et al. 1998, Battin et al. 1999). Their relevance extends to the water purification processes (Cazelles et al. 1991) that occurs in rivers and streams, where biofilms are the first to interact with dissolved substances such as nutrients, organic matter, and toxicants, and can therefore be affected and later used to detect the early effects disturbances might cause on the ecosystem.

The consequences of global change in river ecosystems

Water from rivers, streams and lakes is the most important source for human use (drinking water, industry, agriculture etc) even if less than 1% of the total is stored in freshwater systems (Sabater and Elosegi, 2009). At present, most rivers and streams are affected by consequences of global change resulting from both climatic change and human activities. Consequences of these changes include alterations of flow regime and temperature, increasing chemical pollution

and eutrophication, riparian and river network simplifications, geomorphologic and hydrologic alterations. All these factors affect the functional organization of streams and rivers, and lead to a simplification and impoverishment of the biota within these ecosystems, reducing the capacity of fluvial systems to recover from natural disturbances (Sabater and Tockner, 2010). The co-occurrence of climate change and pollution pressures can be envisaged in systems receiving waste waters from industrial, agricultural and urban areas located in regions affected by increasing water scarcity problems. Mixtures of pollutants from agricultural, industrial and domestic origins enter the watercourses either continuously (producing potentially chronic effects) or in pulses (causing potentially acute effects). These inputs occur in function of flow episodes, crop treatments and/or industrial release (Ellis, 2006). Both chronic and periodic inputs under drought conditions may have larger effects on stream biota than when occurring under normal flow conditions. Environmental stressors account for extreme flow fluctuations and chemical pollution may cause a variety of responses at different biological levels (from molecules and cells to communities and ecosystems) that can provide measurable endpoints and are useful in ecological risk assessment and environmental management. Because there are thousands of different contaminants and their potential toxicity may vary in response to environmental changes (hydrodynamics, water temperature, etc), there is an increasing need of developing reliable bioindicators and early warning biomarkers of stress (Sabater et al., 2007). Over the last few years, the use of microbial communities as model systems in ecology and ecotoxicology has been increasing. Particularly, attached microbial communities respond rapidly, justifying the use of biofilms as good early warning indicators of disturbances related with global change consequences.

Responses of river biofilms to stressors: direct vs indirect effects.

Biofilms integrate the effects of environmental conditions, mainly because of their small size and rapid growth, species richness, and the physiological variety of the organisms of which they are formed (Sabater et al., 2007). Moreover biofilms are ubiquitous, have short generation times, and are easy to manipulate under controlled conditions, constituting ideal systems for assessing the impacts of stressors that are not feasible to manipulate in the field (Jessup et al. 2004; Duarte et al. 2008, 2009). As attached communities are micro ecosystems where complex interactions conflict (Freeman and Lock 1995, Wetzel 1993), responses to multiple stressors can be different depending on target organisms and thus both direct and indirect effects can be inferred (Figure 2). Probably the most important interactions occurring in attached microbial communities are between autotrophs and heterotrophs (Rier el al. 2007).

Interception by biofilms of toxicants in the water phase may result in two biofilm responses, which may differ in their temporal pattern—short-term physiological alterations and, long-term changes in community structure. Both types of change can be transient or irreversible, but the responses occur rapidly, justifying the use of biofilms as good early warning indicators in aquatic ecosystems (Navarro et al., 2002). For these reasons biofilms are amongst the biological compartments recognised by the Water Framework Directive (WFD, Directive 2000/60/EC) as a necessary target (Sabater et al., 2007).

When biofilms are exposed to pollutants we therefore might expect both direct and indirect effects on the autotrophic and/or heterotrophic compartments. This might depend on the mode of action of the compound and on the target organisms. Effects can be acute or chronic depending on the time of exposure and on the concentration levels of the pollutant (Figure 2).

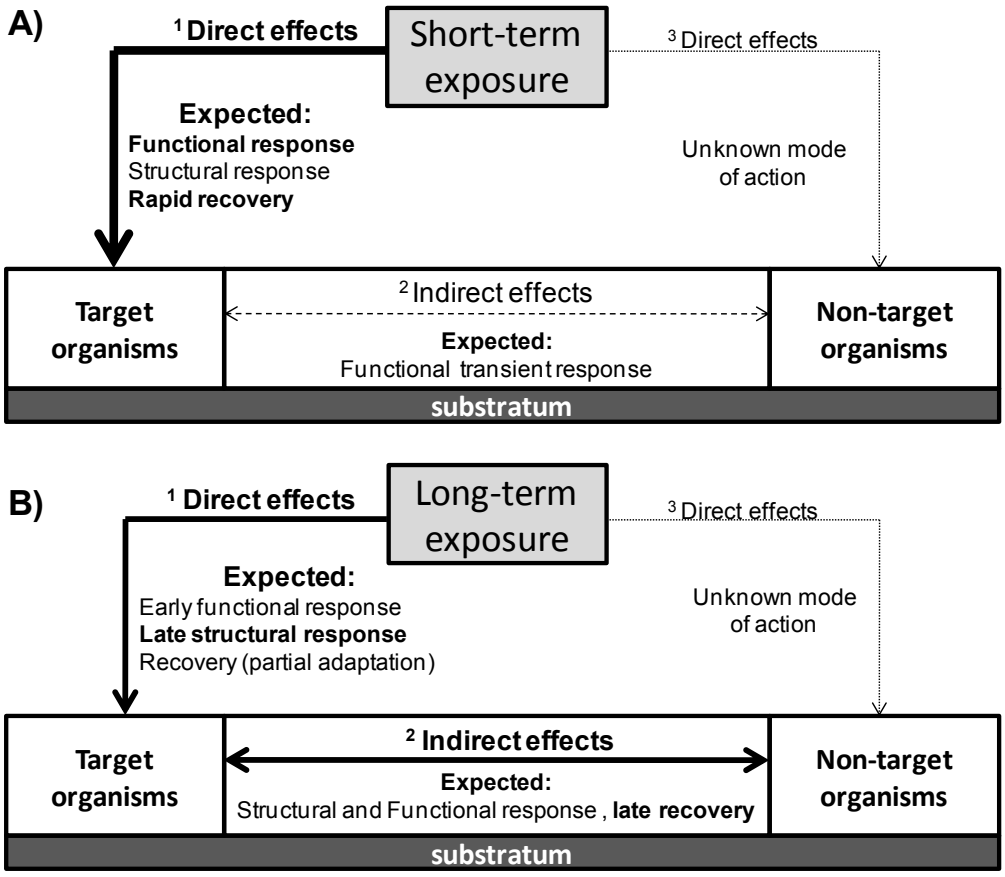


Figure 2. Schematic representation of the expected direct and indirect effects of sub-lethal concentrations of toxic compound/s on target and non-target organisms of freshwater microbial attached communities in case of **A)** short-term and **B)** long-term exposure. In the case of short-term exposure to toxic compound/s **(A)** direct effects of target organisms ([1]) are expected to be the most important ones. In particular, rapid effect and recovery of functional response would be expected. Some structural response could also occur as well as some direct effect on non-target organisms ([3]) due to unknown mode-of-action. These effects can also generate and indirect effect on target organisms. The magnitude of these effects is expected to be less relevant than direct ones. Indirect effects ([2]) are expected to be transient and mainly on function. In the case of long-term exposure to toxic compound/s **(B)** direct ([1]) and indirect ([2]) effects are expected to occur. In particular target organisms would respond quickly in terms of function and later at structural level ([1]). Recovery of these effects is expected to be partial depending on the magnitude of the response. For example, the exposure to some bactericide could result in an initial negative effect on some function sustained by bacteria (i.e. extracellular enzymatic activity). Nevertheless if exposure persisted, some resistant species are expected to be selected. This selection will results in shift of community composition (structural response). The structural response may therefore restore previous functional levels resulting in a general recovery of functional parameters. Nevertheless the structural response (shift in community composition) could be not being considered recovered until the original community will not be restored or even an adapted community will establish. Occurrence of indirect effects ([2]) is expected to be delayed respect to direct ones. Thus, the structural and functional response of non-target organisms will occur after the target organisms responded. The magnitude and delay of indirect effects depends on the interaction with the target organisms directly affected and on physiology, metabolism and life cycle of the non-target organism. Recovery of these indirect effects mainly depends on: resilience potential of non-target organisms, the magnitude and the duration of the direct effect observed that generated it. Direct effects on non-target organisms ([3]) could also occur in consequence of some unknown mode-of-action. These effects can also generate and indirect effect on target organisms. The magnitude of these effects is expected to be less relevant than direct ones.

In general, target organisms are affected directly and the effects can be exerted after relative short-time exposure to the toxic compound (this of course also depending on the toxicity of the pollutant). Direct effects are normally dose-dependent and can be recovered if the toxic concentration is sub-lethal, the exposure does not persist in time and the organism has a good resilience potential. Thus, indirect effects on non-target organisms are less probable in short-time exposure (Figure 2). However, chronic long-term exposure of biofilm to pollutants, in spite of being at sub-lethal concentrations, could result in persistence of direct effects and in the appearance of indirect effects due to interactions between target and non-target organisms within the microbial biofilm.

Non-target organisms may be affected both directly (unknown mode of action) or indirectly because of interaction with target organisms. Indirect effects are strictly dependent on the magnitude and timing of direct effects observed. Obviously they appear after the direct response occurred and the delay depends on the interaction with the target organism directly affected and on physiology, metabolism and life cycle of the non target organism affected. Recovery of indirect effects is therefore a more complex process with high ecological concern. Indirect effects recovery is also dependent on resilience potential of affected organisms, time of exposure and toxicant concentration but is moreover mainly dependent on the nature of the interaction with target organisms that generated it. In particular, it strictly depends on the behavior and the duration of the direct effect observed.

Taking the perspective of the complex communities is essential in order to evaluate possible effects of pollutants on freshwater ecosystem. As stated by Barranguet et al. (2003) "…*the effects of toxicants on each biofilm compartment studied separately will not give an accurate picture of its sensitivity under natural conditions*…".

Analyzing the biofilm responses to pollutants: the relevance of a multi-biomarker approach.

The complexity of biofilm communities requires of a large panel of markers that could be used to describe the effect of stressors and/or environmental factors on community structure-function. An appropriate set of endpoints should be selected in order to detect the wide range of possible responses of complex microbial communities to pollutants. As a general rule, the more functional and physiological descriptors should be used when acute responses are expected because of short-time exposure or transient perturbation in natural systems while the more structural descriptors should be selected as endpoints in studies aiming the investigation of potential chronic effects (Figure 2). However, due to the potential interaction between the biofilm compartments, both functional and structural parameters might be analyzed, enhancing the relevance of a multi-biomarker approach in ecotoxicological studies (Boninneau et al., 2010).

Functional descriptors may be specific or unspecific and are useful to detect acute effects of transient stressor(s) after short-term periods. Specific markers (e.g. those related with photosynthesis) mainly highlight direct effects, while unspecific ones (e.g. extracellular enzymatic activities) better describe indirect effects due to interactions occurring in biofilm communities.

Structural descriptors are useful to detect chronic effects of persistent stressors and normally respond to variations during longer time periods. Among these, community composition should best reflect long-term effects of the stressor(s), because it may cause a shift from a sensitive to progressively tolerant community. Nevertheless, structural-based approaches may do not adequately reflect cause effect relationships. Thus, considering the strong relation between structure and function into such complex microbial community an approach complementing the use of structural and functional descriptors is required to assess potential effects of stressors on fluvial ecosystem (Sabater et al., 2007). In fact, obtaining realistic results of the effects of stressor(s) on the fluvial ecosystem requires scaling up from physiological to structural responses. For this

reason, use of biofilms for detection of the effects of stressors cannot be reduced to simple tests, but implies the consideration of effects, and their interactions, in different biofilm compartments (Ricart et al., 2010a). In conclusion, this combination of approaches might enable detection of factors affecting the "good ecological status" of fluvial systems. A multi-descriptor approach has been used in each chapter of this Thesis, covering both functional and structural aspects of the biofilm communities.

Analyzing the biofilm responses to pollutants: the relevance of experimental and field approaches.

The use of biological communities such as biofilms is not standardized in the way that single specific tests species are defined, for which international standards and guidelines are provided (i.e. OECD guidelines). Nevertheless the complexity of the interactions occurring within these micro ecosystems allow more realistic scaling up to the whole ecosystem level of the responses observed. In order to evaluate the potential impact of pollutants on the biota, the use of controlled exposures provides an excellent basis (Graney et al., 1994) for the understanding of ecological risks associated to the presence of pollutants in river waters. The degree of experimental organization and biological complexity investigated determines the level of ecological realism and causality that can be reached (Figure 3).

Small scale laboratory experiments where mono specific cultures of the target organism are exposed in microcosms for short-time to toxicants have high experimental control and replication but lack of sufficient ecological realism. Larger scale field studies and experiments have greater ecological relevance but as the system increase in complexity and dimension, the experimental control and replication capacity decreases (Clements and Newman, 2002). The use of experimental mesocosms (artificial channels, glass jars etc) in which natural biofilm communities can develop

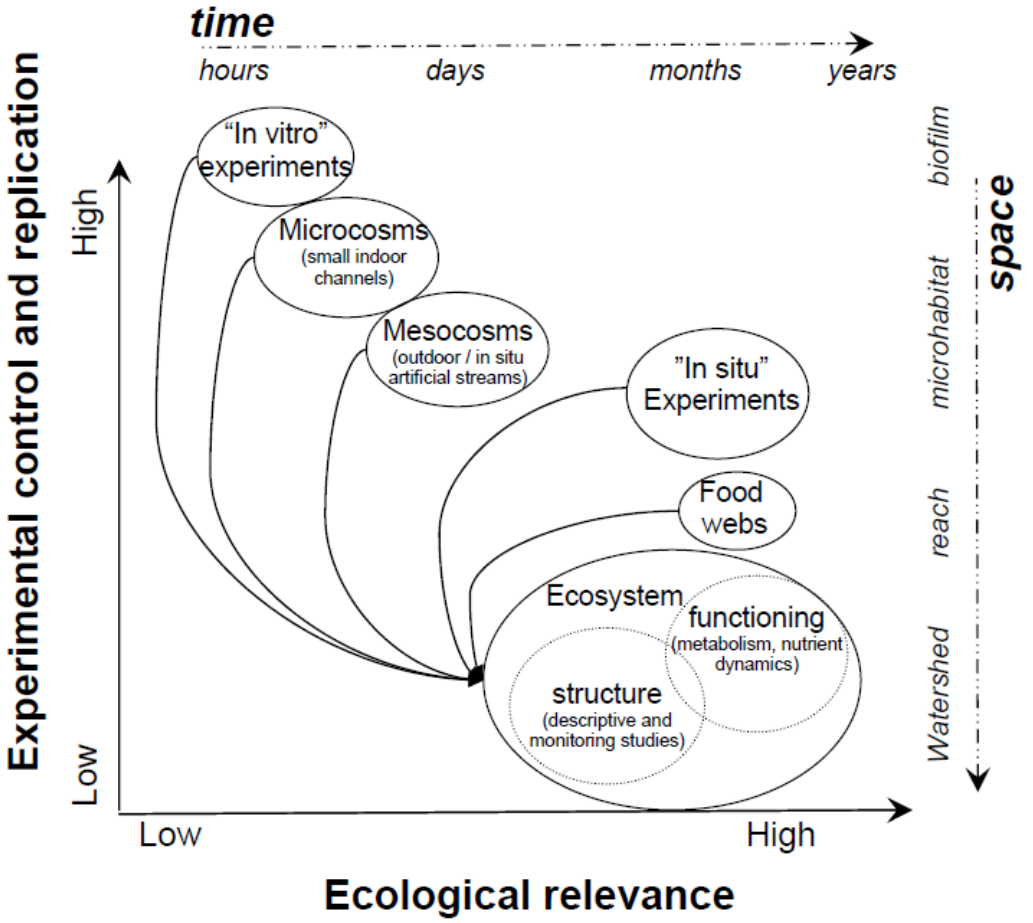


Figure 3. Relationship between ecological relevance, experimental control and replication in fluvial ecology experimental approaches. Modified from Clements and Newman, 2002.

may achieve the necessary compromise between simplification and standardization of the natural system and the required experimental replicability and repeatability (Ricart et al., 2010a).

It is true that mesocosms are less complex than real-world ecosystems; nevertheless they provide replicated and controlled test systems to perform ecosystem-level research in both manageable costs and logistics (Roussel et al., 2007). Experiments with biofilms in experimental channels (Navarro et al., 2008; Serra et al., 2009; Ricart et al., 2009; Ricart et al., 2010b) and glass jars (Diaz et al., 2011; Ylla et al., 2009) have been used to investigate responses of communities to some of the global change consequences, as well as effects of the interaction between toxicants and grazers (Muñoz et al., 2001; Real et al., 2003; López-Doval et al., 2010). Nevertheless, the use of such systems obviously represents a simplification of the complex interactions occurring in real systems therefore limiting the possibility to upscale the observed results to real ecosystems.

On the other hand, field studies can provide support for a causal relationship between stressors and community responses; however, field studies alone cannot be used to show causality (Clements and Newman, 2002). Compared with laboratory experiments, they are more realistic, although have less control (Ricart et al., 2010a). The combination of different approaches has been used in this work to reach the planned objectives.

Objectives and hypotheses of the present thesis

The main goal of this thesis is to investigate the effects of global change, specifically water flow decrease, nutrient excess and pollutants occurrence, on the structure and function of fluvial microbial biofilms. To achieve the main objective a multi-marker approach has been used at different experimental scales, which have ranged from field studies to mesocosms and laboratory experiments.

The specific objectives are:

- To determine the biofilm responses to independent and combined effects of different nutrients and light availabilities throughout their colonisation (Chapter 3).
- To investigate the effects of pharmaceuticals and pesticides detected in Llobregat waters on structural and functional responses of river biofilms (Chapter 4).
- To investigate the effects of antibiotics detected in several polluted sites of the Llobregat River on the biofilms bacterial community structure and function (Chapter 5).
- To investigate the relevance of direct and indirect effects of priority (Diuron) and emerging (Triclosan) compounds on both target and non-target organisms of fluvial biofilms, including recovery process after exposure (Chapter 6).
- To determine the structural and functional short- and long- term responses of fluvial biofilms to the separate and combined effects of drought and exposure to triclosan, including recovery process after disturbances (Chapter 7).

- To investigate the phosphorus uptake capacity of fluvial biofilm and its response to changing environmental conditions and chemical pollution (Chapter 8).

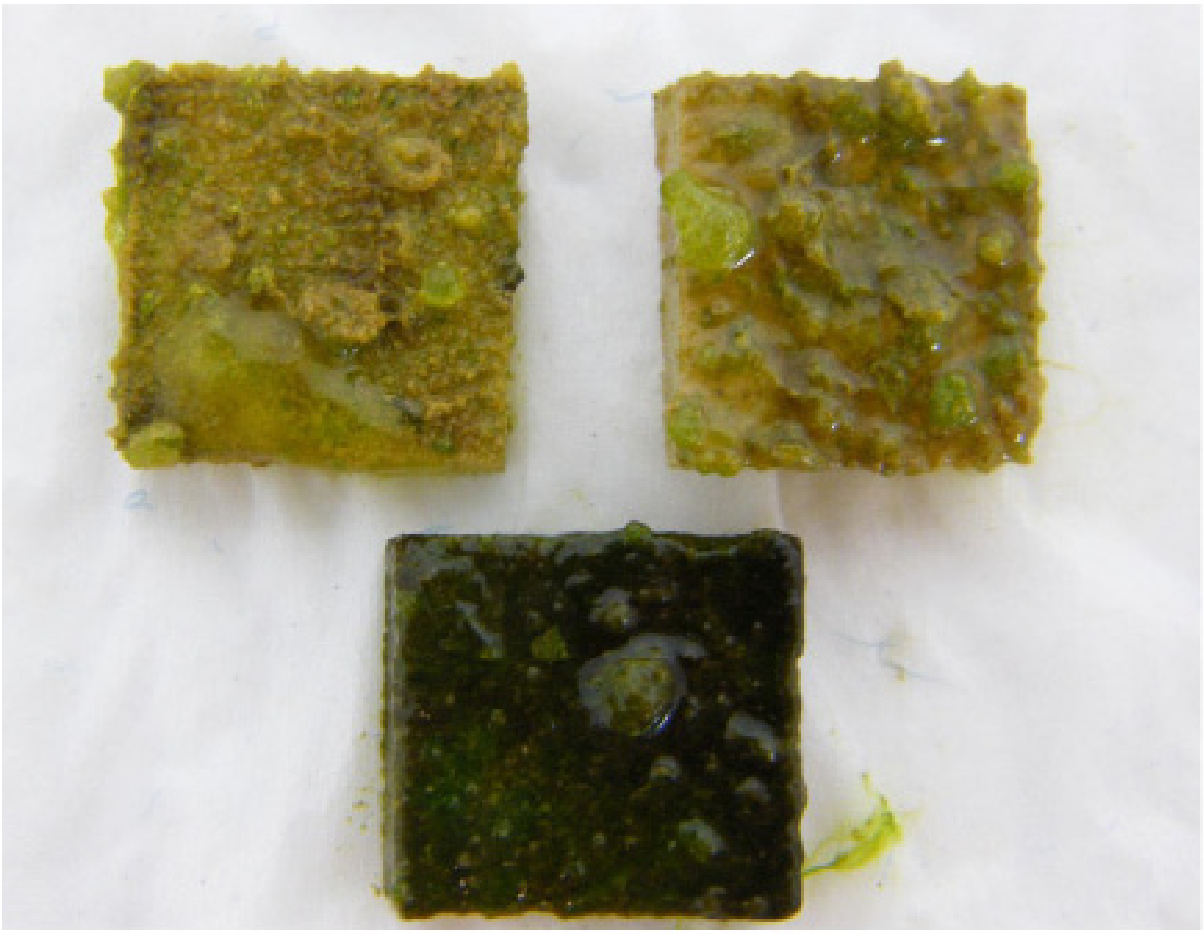
The described objectives aim to test the following hypotheses:

- Light and nutrient availability would mostly affect the biofilm structure and metabolism under higher light conditions and under the combined occurrence of higher light and nutrients.
- Bioactive compounds (pesticides and pharmaceuticals) detected in river waters will influence the responses of biofilms communities depending on their previous exposure to the toxicants. The magnitude of the responses would be related with the concentration of organic pollutants as well as on the origin of the biological community.
- Biofilms grown at different sites would present different bacterial assemblages but similar functioning; bacterial community would respond to physical changes (translocation) in terms of structure and function. Antibiotics detected in river waters would influence assemblages and responses of bacterial community of river biofilms.
- Diuron and Triclosan might provoke direct and indirect effects deriving from ecological interactions at the microbial scale. The direct effects were expected to occur immediately upon toxicant exposure, whereas the indirect ones were expected to appear later on. Moreover, it was predicted that the time required for the biofilms to recover from the pollutant pulse could be correlated to the presence of target organisms (direct effects) and non-target organisms (indirect effects) in the biofilms.
- Altered water flow would affect the structure and function of biofilms, consequently making them more sensitive to short-term TCS pulses; direct and indirect effects of TCS on biofilms would differ depending on flow history; recovery after short-term perturbations would differ according to whether the observed consequences were direct or indirect, and would be influenced by any combined effects from the two stressors.

- The capacity of biofilms in the uptake of phosphorus could be used as an endpoint to detect effects of stressors on self-depuration capacity of rivers.

Experimental approaches

In this Thesis several factors related with global change consequences for river ecosystems have been investigated at different levels of experimental organization (from lab to field) using microbial biofilm communities as a target. A field experiment assessing responses of biofilms to independent and combined effects of different nutrients and light availabilities has been performed to investigate the consequences of both eutrophication and riparian simplification for microbial benthic communities structure and function in Mediterranean stream (Chapter 3). A field-lab translocation experiment along the pollution gradient of the highly polluted river Llobregat has been performed to investigate: i) the effects of mixtures of pharmaceuticals and pesticides detected in river water on structural and functional responses of river biofilms (Chapter 4) and ii) the effects of mixtures of antibiotics detected in river water on structural and functional responses of heterotrophic compartment of river biofilms (Chapter 5). Mesocosms experiment assessing resistance and recovery potential of biofilms after exposure to pulses of one priority (Diuron) and one emerging (Triclosan) compounds has been performed to investigate direct and indirect effects on both target and non-target organisms (Chapter 6). Mesocosms experiment in artificial channels assessing responses of biofilms to independent and combined effects of drought and Triclosan has been performed to investigate how droughts episodes may modulate Triclosan toxicity for river biofilm (Chapter 7). In different laboratory experiments testing the effects of Triclosan on river biofilms in different conditions, the capacity of the communities to uptake phosphorus has been measured to investigate the use of this ecosystem-related endpoint in ecotoxicology studies with biofilms (Chapter 8).



CHAPTER 2:

MATERIAL AND METHODS

MATERIAL AND METHODS

The planned objectives required the use of several methodologies that are described in this chapter. Some of the methodologies used were common for almost each chapter while others were specific. The most common methods are those described in detail in this chapter, while the most specific are specifically addressed.

Biofilm parameters

Chlorophyll-a content

Analysis of autotrophic biomass was performed through chlorophyll-a extraction and quantification by absorbance measurements (Jeffrey and Humphery, 1975). Chlorophyll-content has commonly been used as a surrogate of autotrophic biomass in the assessment of the effects of herbicides (Guasch et al., 2007), heavy metals (Navarro et al., 2002) and new emerging compounds (White et al., 2005) on natural biofilm communities. In this study, samples for chlorophyll-a analysis were collected and stored in the dark at -20 °C until analysis. Chlorophyll-a in the biofilms was quantified after extraction in 90% acetone in the dark at 4 °C for 12/24 h. Concentration was determined spectrophotometrically after filtration (GF/F Whatman) of the extract, following the procedure of Jeffrey & Humphrey (1975).

In vivo chlorophyll-a fluorescence

The measurement of in vivo chlorophyll-a fluorescence of different autotrophic groups of biofilm has been found to be one of the most sensitive tools for the rapid detection of compounds and environmental factors that exhibit effects on photosynthesis (Brack and Frank, 1998; Fai et al., 2007, Corcoll et al., 2012). Light energy absorbed by chlorophyll molecules in photosynthetic organisms can be used to drive photosynthesis (photochemistry), can be dissipated as heat (i.e.

excess energy), or it can be reemitted as light (chlorophyll fluorescence). These three processes occur in competition. Therefore, by measuring the yield of chlorophyll fluorescence, information about changes in the efficiency of photochemistry and heat dissipation can be obtained (Maxwell and Johnson, 2000). These endpoints based on photosynthetic activity are adequate to describe direct and indirect effects of chemicals on photosynthetic performance. Several studies assessed the toxicity of different priority and emerging compounds to biofilm communities by analysing the sensitivity of the different groups of primary producers through their specific photosynthetic efficiencies (Bonnineau et al., 2010, Ricart et al., 2009; Ricart et al., 2010). The in vivo chlorophyll-*a* fluorescence parameters have been used in Chapters 4, 6 and 7. The chlorophyll-a fluorescence (F0), the photosynthetic capacity (Ymax) and the photosynthetic efficiency (Yeff) have been measured by the PhytoPAM (Pulse Amplitud Modulated) fluorometer (Heinz Walz GmbH) on biofilm grown on artificial substrata (glass slides). The PhytoPAM uses a set of light-emitting diodes (LED) that excite chlorophyll fluorescence using four different wavelengths (470, 520, 645, and 665 nm). For each glass slide sampled three fluorescence measurements were performed in order to represent small scale heterogeneity of autotrophs in biofilm. The measurements were based on the procedure described by Serra et al. (2009). The photosynthetic efficiency of photosystem II (PSII) (referred to as Yeff) and the photosynthetic capacity of PSII (referred to as Ymax) were estimated based on the fluorescence signal recorded at 665 nm and given as relative units of fluorescence. The minimum fluorescence level of the darkadapted samples was used as an estimation of algal biomass. This estimation was based on the fluorescence recorded at the four different excitation wavelengths (F1 at 470 nm, F2 at 520 nm, F3 at 645 nm, and F4 at 665 nm). F1 is linked to the chlorophyll of green algae, whereas F2 is mostly related to that of diatoms. The F3 signal is related to cyanobacteria chlorophyll and the F4 signal is related to the chlorophyll of the whole autotrophic community (Ricart et al., 2010). The ratio between F1 and F3 (green algae versus cyanobacteria) was calculated per each replicate.

Extracellular Enzymatic activities

Extracellular enzymatic activities are potentially useful for detecting the effects of environmental factors and toxicants on biofilm communities function. Biofilm extracellular enzyme activities play a key role in the microbial loop; they are the result of the internal recycling of organic matter and microbial interactions (competition/synergisms) within the biofilm, such as algal-bacterial interactions (Ylla et al., 2010), and, at the same time, they are reflecting the potential use and transformation of organic matter in flowing water (Romaní et al., 2011). The expression of a certain set of enzymes is handy to "fingerprint" the composition of the organic matter pool in use (Hopkinson et al., 1998). Sensitivity and a direct relationship with organic matter make them relevant tools for assessing the toxicity of specific compounds. Exposure of a microbial community to a toxic compound may induce either an increase or a decrease in enzyme activity, depending on the possible relevance of carbon, nitrogen, or phosphorus compounds as sources for microbial growth, and depending on eventual toxic effects on both target and non-target organisms. In this study, the extracellular activity of β-Glucosidase has been used in chapters 4, 5 and 7 while Leucine-amnopeptidase and Alkaline-phosphatase were used in all experimental chapters (from 3 to 7). Extracellular enzyme activities involved in the use and decomposition of organic matter were measured, specifically the β-glucosidase activity (involved in the decomposition of simple polysaccharides to obtain C), leucine-aminopeptidase (involved in the decomposition of peptides to obtain C and N), and alkaline phosphatase (involved in the decomposition of organic phosphorus compounds in order to obtain inorganic phosphorus), were measured by using fluorescent artificial substrate (Methyl-umbeliferone – MUF- β-glucoside, Leucine- Aminomethyl coumarin –Leu-AMC, and MUF-phosphate, respectively). Colonized glass were incubated with the artificial substrate at substrate saturation conditions (0.3 mM, Romaní et al., 2004), at controlled temperature (18ºC), in the dark and in a shaker. After one hour incubation, glycine buffer (pH 10.4,

1/1 v/v) was added to each sample and fluorescence was measured at 365/455 for MUF, and at 364/445 for AMC. Blanks and standards of MUF and AMC (0-100 μM) were also incubated and later measured for their fluorescence (SFM25 Kontron). Extracellular enzyme activities in the biofilm are expressed as nmol of MUF or AMC released per cm^2 and hour.

Bacterial density and viability

Analysis of bacterial biomass differentiating live and dead bacterial cells by using live/dead viability kit is potentially useful for detecting the effects of environmental factors and toxicants on bacteria compartment of biofilm communities. Live and dead bacteria were counted with epifluorescence microscopy using the LIVE/DEAD® Bacteria Viability Kit L7012 (*Bac*Light™, Molecular Probes, Invitrogen). The LIVE/DEAD BacLight Bacterial Viability Kits is a mixture of SYTO® 9, a green-fluorescent nucleic acid stain, and the red-fluorescent nucleic acid stain, propidium iodide. These stains differ both in their spectral characteristics and in their ability to penetrate healthy bacterial cells. When used alone, the SYTO 9 stain generally labels all bacteria in a population — those with intact membranes and those with damaged membranes. In contrast, propidium iodide penetrates only bacteria with damaged membranes, causing a reduction in the SYTO 9 stain fluorescence when both dyes are present. Thus, with an appropriate mixture of the SYTO 9 and propidium iodide stains, bacteria with intact cell membranes stain fluorescent green, whereas bacteria with damaged membranes stain fluorescent red. The excitation/emission maxima for these dyes are about 480/500 nm for SYTO 9 stain and 490/635 nm for propidium iodide. The background remains virtually nonfluorescent. Biofilm samples were sonicated (< 1 min, sonication bath at 40 W and 40 kHz, Selecta) and scraped (Nunc sterile silicone cell scraper) to obtain a biofilm suspension. Samples were then diluted with pre-filtered sterilized water from the mesocosms, and 2 mL subsamples were stained with 3 μL of a 1:1 mixture of SYTO 9 and

propidium iodide for 15-30 minutes in the dark. After incubation, samples were filtered through a 0.2 μm black polycarbonate filters (Nuclepore, Whatman). Filters were then dried, placed on a slide with mounting oil (Molecular Probes) and counted by epifluorescence microscopy (Nikon E600, 100x in immersion oil). Green (live) and red (dead) bacterial cells were counted in 20 random fields per filter. The method has been used in all experimental chapters except in chapter 4.

Diatoms density and viability

Analysis of diatoms density differentiating live and dead cells is potentially useful for detecting the effects of environmental factors and toxicants on diatom biofilm communities. This tool was used in chapters 6 and 7 following the protocol optimized by Morin et al. (2010).

The glass slides were scraped using polyethylene cell lifters (Corning Inc., NY, USA), and cells preserved with a drop of formalin solution and diluted to a final volume of 5 mL. Samples were ultrasonicated for 7 minutes to separate the aggregated cells without destroying the frustules. 125 μL of each sample were then pipetted onto a Nageotte counting chamber to count the total number of diatom cells in 10 microscope fields (1.25 μL each, 0.5 mm depth) selected at random, using light microscopy at a 10x magnification (photomicroscope Nikon Eclipse 80i, Nikon Co., Tokyo, Japan). Data were recorded as cells per unit area of sampled substrate (number of cells/ cm^2). Counting were separated into 2 types: empty cells that were considered as 'dead', and cells occupied by chloroplasts that, whatever their color (from pale yellow to green or brown), shape and number, were considered as 'alive' (Cox 1996). From live diatom counts, growth rates (as expressed in cell divisions/day) of the diatom community were calculated according to Guillard (1973). The optimal use of the live/dead cell indicator (L/D ratio) required:1) The observations/ counting to be carried out within 1 month after sampling. Chlorophyll content has been shown to decrease very quickly in formalin-preserved samples (Dell'Anno et al., 1999), and a decrease in pigment content would make it harder to distinguish between live and dead cells. Storing the

formalin-fixed samples in the dark will however delay chlorophyll degradation (Morin et al., 2010). Additionally, we used the same dilution per unit substratum surface area, in order to maximize comparability between data. Moreover, as the difference between live and dead cells may be subjective in some cases (e.g., very slight coloration of the cell content), all observations were performed by a single operator; 2) The proportion of live vs. dead diatoms was based on counting entire cells, i.e., those that exhibit an entire frustule. This required the ultrasonication to be limited to a maximum of 7 minutes, in order to minimize frustules damage.

Confocal Laser Scanning Microscopy (CLSM)

CLSM observations were performed in chapter 1 to establish possible differences in the spatial organisation, architecture and depth profiles of biofilms between treatments at the end of colonization process. The samples collected from the field were directly transported to the CLSM to be analysed *in vivo*. A Leica TCS-SP5 (Leica Microsystems Heidelberg GmbH, Mannheim, Germany) was used for the CLSM observations. Auto fluorescence of photosynthetic pigments was detected with the 561- (phycobiliproteins) and 633- (Chlorophylls) nm lines of an Ar/HeNe laser (excitation) and observed in the red and far-red channels at 590 to 800 nm (emission). The location of the EPS in the biofilms was preceded by staining with the carbohydrate-recognizing lectin concanavalin A (ConA)-Alexa Fluor 488 (Molecular Probes, Inc., Eugene, OR). This fluorochrome is excited with the 488-nm line of an Ar laser, and observed in the green channel from 490 to 530 nm. Resulting colours in images were blue for algae, pink for cyanobacteria and green for EPS.

Scanning Electron Microscopy observation

Observation of biofilms by Scanning Electron Microcopy (SEM) was used in chapter 6 to highlight eventual effects of potentially toxic compounds on community structure. Glass tiles were collected from mesocosms for SEM observation. Samples were fixed immediately with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2–7.4. Samples were dehydrated in graded ethanol (65–100%) and dried at the critical point of CO_2. Finally, samples were sputter coated with gold, and then observed by SEM (Zeiss DSM 960).

Polysaccharide content in Extracellular Polymeric Substances (EPS)

The analysis of polysaccharide content in EPS has been performed in chapter 1 to establish possible differences between biofilms grown under different light and nutrient availability conditions. Glass tiles for polysaccharide content in EPS (4 replicates per treatment and date) were collected and preserved at -20ºC until analysis. EPS were extracted using cation-exchange resin (Dowex Marathon C, Na^+ form, strongly acidic, Sigma-Aldrich, Steinheim, Germany), following the procedure described in Romaní et al. (2008). Polysaccharide content was measured by the phenol-sulphuric acid assay (Dubois et al. 1956) after the extraction of EPS. Standards of glucose (0–200 μg ml^{-1}) were also prepared. Results are given as glucose equivalents per cm^2 of biofilm surface area.

Catalized Reported Deposition-Fluorescence In Situ Hybridization (CARD-FISH)

Biofilms bacterial community composition has been analyzed by CARD-FISH in chapter 5 to investigate the effects of antibiotics mixtures detected in highly polluted river. Biofilms were scraped and treated optimizing the ultracentrifugation method described by Amalfitano and Fazi (2008) in order to isolate bacterial cells from extracellular matrix, particles and the other organisms of the biofilm consortia. Previously, bacterial recovery after optimized treatment was verified.

After treatment, bacterial cells were fixed on polycarbonate filters (Whatman). Subsequently, CARD-FISH was performed according to the protocols described by Pernthaler et al. (2002, 2004). Cells were immobilized on filter sections by embedding with agarose (Invitrogen Life Technologies) 0.2% for 10 min to avoid cell detachment, dried at 37°C for 10 minutes and then dehydrated in ethanol 96%. Filters were permeabilized (37°C for 1h) with lysozyme (20 mg ml-1 , Fluka, Steinheim, Germany) and dissolved in a buffer contained 0.5M EDTA pH 8, and 1M Tris-HCl - pH 7.4 for 1 h at 37 °C followed by a second permeabilization with Proteinase K (0.034 U µl-1 - SIGMA-ALDRICH, Steinheim, Germany, in Tris-EDTA buffer) for 25 minutes at 37° C. After permeabilization, filters were incubated in 0.01 M HCl at room temperature for 10 minutes in order to inactivate the proteinase K and intracellular peroxidases and then were washed in ultrapure water and 96% ethanol. We used horseradish peroxidase (HRP) labeled oligonucleotidic probes to target Bacteria (Eub I-III), Actinobacteria (HGC69a), Alpha-proteobacteria (ALF968), Cytophaga-Flavobacteria (CF319a) and Gamma-proteobacteria (GAM42a) with a competitor (Manz et al., 1992), (Biomers, Germany). Probes (50 ng µl-1) were added to the hybridization buffer (5M NaCl, 1M Tris/HCl - pH 7.4, 10% w/v dextran sulfate, 0.02% w/v sodium dodecyl sulphate, 10% w/v blocking reagent - Roche Diagnostic GmbH, Germany) containing the following concentration of formamide: 20% v/v for Alpha-proteobacteria, 55% v/v for Bacteria, CF and Gamma-proteobacteria (Amann et al., 1990; Daims et al., 1999) and 30% v/v for Actinobacteria (Fazi et al., 2007) and hybridization was performed overnight at 35°C. After hybridization, the samples were placed in 50 ml of pre-warmed washing buffer (5M NaCl, 1M Tris/HCl - pH 7.4, 0.5M EDTA - pH 8, 0.01% w/v sodium dodecyl sulphate in ultrapure water) at 48°C for five minutes. After washing, filters were placed in an amplification buffer (2 µl fluorescein-labelled tyramide - SIGMA-ALDRICH, Germany - in 30% H_2O_2, PBS - pH 7.4, 5M NaCl, 10% w/v blocking reagent, 10% w/v dextran sulphate) and incubated at 37°C for 10 minutes in the dark. Then filters were washed in PBS solution at room temperature in the dark for 25 minutes and counterstained

with 4'-6-diamino-2-phenylindole (DAPI, 1 µg ml-1 - Vector Laboratories, USA). DAPI stained and probe hybridized cells were observed and quantified by epifluorescence microscopy (EM) (Leica DM LB 30, at 1000X magnification).

Denaturing Gradient Gel Electrophoresis analysis

Biofilms bacterial community composition has been analyzed by CARD-FISH in chapter 5 to investigate the effects of antibiotics mixtures detected in highly polluted river. Samples (one glass from each mesocosm) were scraped in 1 ml of MilliQ water, and samples were frozen at -80°C until analysed. DNA extraction was performed by thermo shock from pellets of scraped biofilm obtained after centrifugation of samples at 13 400 g for 30 min in a 5415D centrifuge (Eppendorf, Germany) after sample collection. Between 6 and 8 cycles of freeze (13-15 minutes at -80 °C) and defrost (7 minutes at 60 °C) were performed in order to obtain nucleic acids extract from samples. DNA concentration and purity were determined spectrophotometrically from extracts using a Nanodrop ND-1000 UV-Vis spectrophotometer (Nanodrop, DE). Bacteria community structure and composition were determined by denaturing gradient gel electrophoresis (DGGE). The universal primers 27F (5'-AGA GTT TGA TC(AC) TGG CTC AG-3') with a degenerate base pair at one position with a GCclamp (5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G-5') spanning *Escherichia coli* positions 8–27 and 517R (5'-ATT ACC GCG GCT GCT GG-3') spanning *E. coli* positions 518–534 were used for amplification of a 566-bp long fragment of the 16S rDNA of *Eubacteria*. PCR was performed as follows: an activation step for the polymerase for 15 min at 95°C, 35 cycles with initial denaturation for 30 s at 94°C, annealing for 30 s at 54°C and elongation for 1.5 min at 72°C, followed by a final elongation step for 7 min at 72°C. The PCR mix contained 2 ml of the template, 1.25 U of the HotStar DNA Taq polymerase (PeqLab, Erlangen, Germany), 1 ml of dNTPs (0.2 mM final concentration per vial

and dNTP), 1.5 ml of each primer (20 pM final concentration per vial), and 5 ml of reaction buffer (10X), with the total volume being 50 ml. DGGE analysis of the PCR products (15–25 ml) was performed by means of the D-Code-System BioRadLaboratories GmbH, Munich, Germany) using polyacrylamide gels containing a 40–70% urea gradient. DGGE gels were run in 1 x TAE buffer (40 mmol l-1Tris, 20 mmol l-1 acetate, 1 mmol l-1 EDTA) at 70 V and 60°C for 16 h. The gels were stained with SYBR®Gold (Invitrogen, Karlsruhe, Germany). The stained gels were immediately analyzed using the Lumi-Imager Working Station (Roche Diagnostics, Mannheim, Germany). DGGE fingerprints were scored by the presence or absence of DNA bands. Intensively stained bands were excised from the DGGE gel, and the gel slices were equilibrated in 15 ml of sterile water overnight at room temperature. The DNA extract was re-amplified by PCR and subjected to DGGE again to verify the purity of the PCR re-amplification product. PCR products were purified using a ExoSap kit (usb,Staufen, Germany). The sequencing reaction was carried out using the BigDye ® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems), and sequence detection was accomplished using the ABI Prism 310 genetic analyzer (Applied Biosystems) according to the manufacturer's protocol. DNA identification was achieved by comparing the nucleic acid sequences with GenBank sequences using the BLAST program (http:// www.ncbi.nlm.nih.gov).

Phosphorus uptake capacity

Phosphorus (soluble reactive phosphorus, SRP) uptake capacity of biofilms was calculated by measuring the temporal decay of SRP after spiking in sterile glass jars (19 cm in diameter, 9 cm high; each jar corresponded to one channel) filled with 1.5 L of dechlorinated tap water recirculated using a submersible pump (Hydor, Pico 300, 230 V 50 Hz, 4.5W). The bottom of each mesocoms was occupied by a known area of colonized tiles. The starting SRP concentration in each mesocoms was previously analyzed. Subsequently, to quadruple the background concentration of phosphorus, each jar was spiked with Na_2PO_4 (10 mM). The biofilms were then incubated for 120

minutes under controlled temperature (21 ºC) and light (130-150 µmol photons m^{-2} s^{-1}) conditions (Radiber AGP-570). Aliquots (10 mL) for SRP concentration measurements were then taken at 1, 5, 10, 15, 30, 45, 60, 90 and 120 minutes after spiking, and immediately filtered through 0.2 µm nylon membrane filters (Whatman). The phosphate uptake rate coefficient (K, min^{-1}) was calculated as the coefficient of the negative exponential model represented by the decay of SRP concentration over time (Figure 1). The phosphate uptake rate (U) was calculated as the mass of phosphorous removed from the water column per unit area per unit time (µg P cm^{-2} min^{-1}). Particularly, the removed mass was calculated as the difference between intial and final masses obtained from the multiplication of the concentrations for the volumes. This mass was standardized for the area colonized by the biofilms and for the time required to remove it. Abiotic controls were performed under the same experimental conditions and for the same time: they did not exhibit any SRP decay.

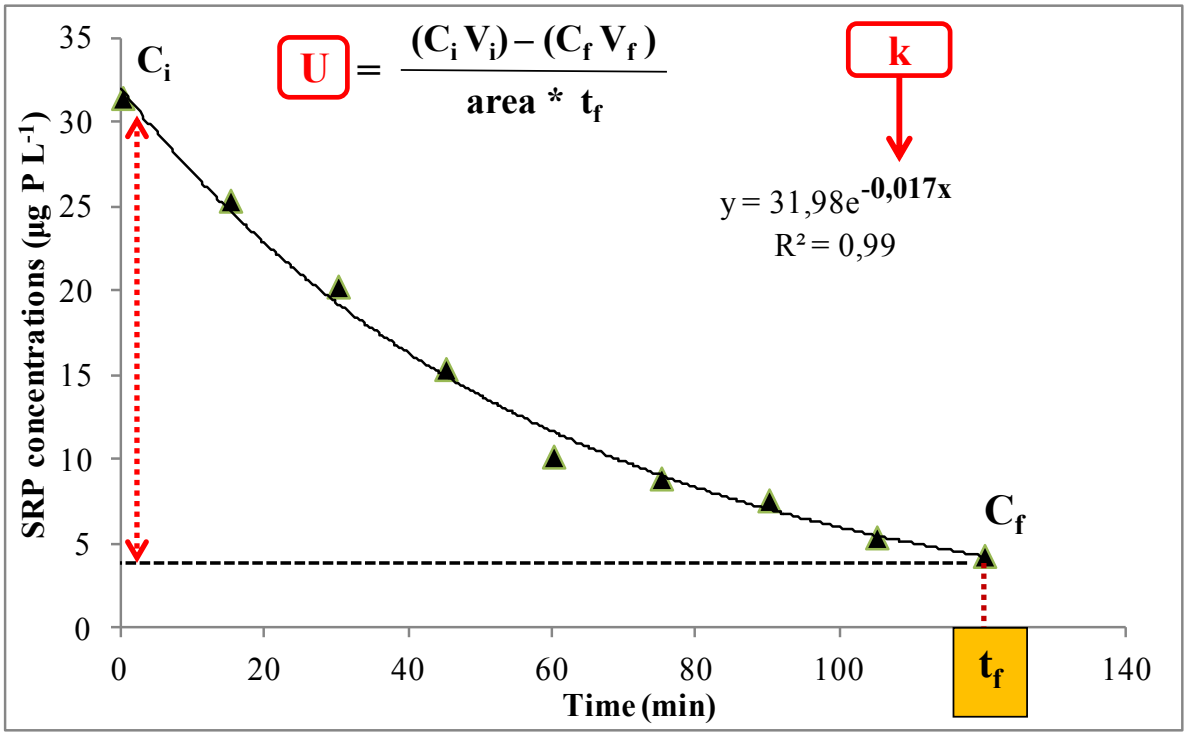
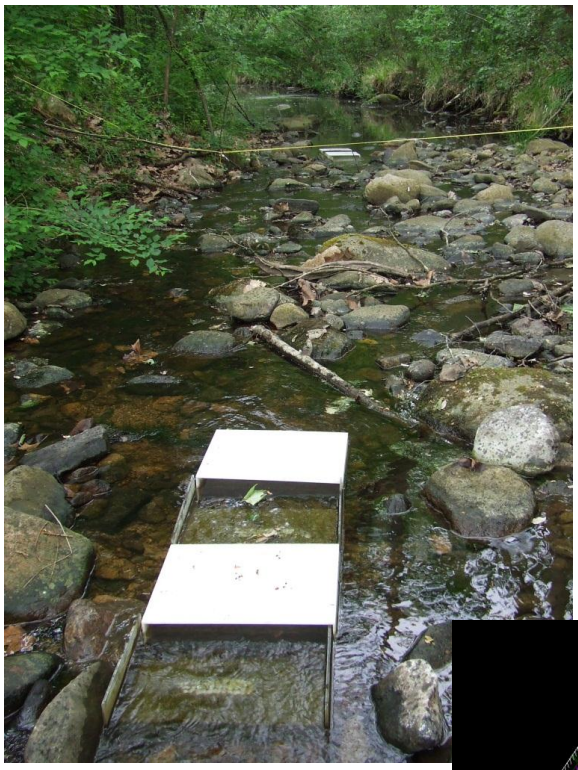
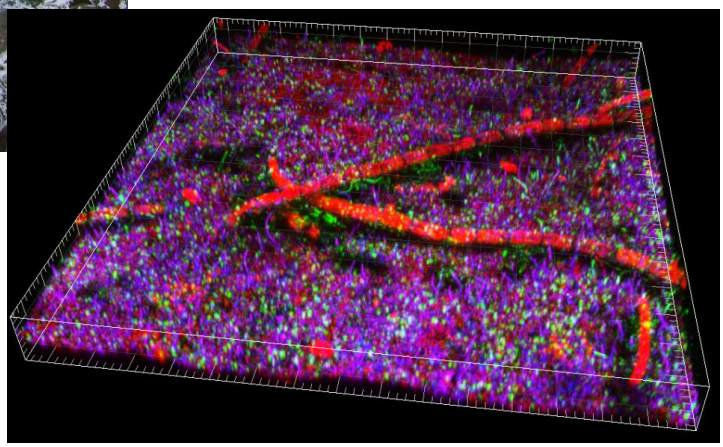


Figure 1. Example of dissolved inorganic phosphorus decay in time after controlled spike in response to biotic uptake by river biofilms. Uptake rate coefficient (K, min^{-1}) is calculated by fitting negative exponential curve to the data. The phosphate uptake rate (U) was calculated as the mass of phosphorous removed from the water column per unit area per unit time (µg P cm^{-2} min^{-1}).





CHAPTER 3:

NUTRIENTS AND LIGHT EFFECTS ON STREAM BIOFILMS: A COMBINED ASSESSMENT WITH CLSM, STRUCTURAL AND FUNCTIONAL PARAMETERS

NUTRIENTS AND LIGHT EFFECTS ON STREAM BIOFILMS: A COMBINED ASSESSMENT WITH CLSM, STRUCTURAL AND FUNCTIONAL PARAMETERS

Abstract

Nutrients and light are the most determinant factors for microbial benthic assemblages in oligotrophic forested streams. We investigated the importance of nutrients and light availability on the structure and the-function of epilithic biofilms in a Mediterranean forested stream (Fuirosos, Spain). Biofilms grew on artificial substrata in both enriched and unenriched reaches where shade conditions were simulated. Four different treatments were generated: Higher Light Unenriched (HL-U), Lower Light Unenriched (LL-U), Higher Light Enriched (HL-E) and Lower Light Enriched (LL-E). Chlorophyll-a, bacterial density, Extracellular Polymeric Substances (EPS), extracellular Leucine Aminopeptidase (LAmP) and Alkaline Phosphatase (APase) activities were analysed during the colonisation at days 4, 9, 16, 22 and 52. At day 52, Confocal Laser Scanning Microscopy (CLSM) was used to determine differences in biofilm architecture. CLSM evidenced differences in thickness and structural complexity of biofilms grown in different conditions. Biofilms in HL-E were the thickest and had the most complex structure. The CLSM highlighted that the EPS was agglomerate in the upper layer of enriched-grown biofilms, but evenly distributed through the biofilm in unenriched biofilms. CLSM 3-d images suggested that cyanobacteria increased under higher nutrient conditions. Nutrient enrichment caused the decrease of APase activity. Interaction between the two factors affected LAmP activity. HL-E had the highest LAmP and the lowest APase activities, an indication that biofilm responses to nutrients mostly occurred with high light availability. Our results revealed that the conjoint availability of light and nutrients caused the highest changes in biofilm spatial organisation, microbial structure and functioning in oligotrophic forested streams.

Keywords: Epilithic Biofilm, Confocal Laser Scanner Microscopy, Nutrients, Light, forested stream, extracellular enzyme activities

INTRODUCTION

Biofilms are structured communities of bacteria, algae, cyanobacteria, fungi and protozoa embedded in a polymeric matrix. Biofilms can be considered as micro ecosystems in which heterotrophic and autotrophic organisms are in close spatial relation, causing complex interactions to take place. All solid surfaces (cobbles, leaves etc.) in streams are colonised by biofilms, which are communities effective in organic matter transformation (Ainsworth & Goulder, 2000), playing a key role in nutrient cycling uptake and remineralisation (House, 2003; VonSchiller et al., 2007) and are energy and organic matter transducers to higher trophic levels (Lamberti, 1996). The colonisation of streambed substrata by biofilms has been described as a sequence of overlapping stages (Stock & Ward, 1989). This colonisation process results from progressive responses of involved organisms to resource availability as well as other abiotic factors (i.e., current velocity, temperature and pH). Nutrients and light are essential resources, and their availability in oligotrophic forested streams could determine changes in the structure and the function of microbial benthic communities. In terms of structure, autotrophs and heterotrophs in benthic communities could respond differently to nutrient supply. In heterotrophic functioning, nutrient availability can either directly (by influencing metabolic activities) or indirectly (by effect on primary producers) affect biofilm communities (Romaní et al., 2004). This response could change depending on light availability conditions. Several studies have focused on the effects of nutrient enrichment on the biofilm structure and function (Romaní et al. 2004; Sabater et al., 2005b). In small streams, riparian vegetation is the main factor determining light availability (Roberts et al., 2004; Acuña et al., 2005), which limits algal growth (Hill & Knight, 1988; Winterbourn, 1990). Though light is the primary limiting resource, especially in forested conditions (Mosisch et al., 2001), nutrients are often limiting (Taulbee et al., 2005). The higher availability of nutrients can effectively enhance algal biomass and productivity when light is present (Ylla et al., 2007).

While many of these studies have investigated the role of light and nutrients on the growth and community composition of biofilms in oligotrophic forested streams, much less is known about the combined effects of light and nutrients on spatial organisation, microbial biomass and biofilm functioning. Moreover, investigation of the colonisation process by both structural and metabolic parameters under different nutrients and light conditions may be a powerful tool to study ecological interactions within biofilm communities.

The aim of this study was to determine the relative importance of nutrients and light availability on the structure and the function of epilithic biofilms throughout their colonisation. The response of biofilms to different light and nutrient availability conditions was analysed in terms of biofilm metabolism (extracellular enzyme activities), structure (bacterial and algal density, EPS) and spatial architecture (CLSM). We hypothesised that light and nutrient availability would mostly affect the biofilm structure and metabolism under (1) higher light conditions and, (2) under the combined occurrence of higher light and nutrients. These hypotheses were tested in an oligotrophic forested Mediterranean stream under natural and artificially-enhanced nutrient concentrations in shaded and unshaded conditions for 52 days. The differences in the metabolism and structure were compared at the end of the experiment to the differences in biofilm architecture. The experimental observations in this paper can provide arguments for the consideration of light conditions during routine algal monitoring in nutrient-rich rivers.

MATERIAL AND METHODS

Study Area

The field experiment was conducted at the Fuirosos, a third order stream, with a catchment area of 15.6 km^2, located in the Natural Area of Montnegre-Corredor, a forested range close to the Mediterranean Sea (50 km N of Barcelona, NE Spain). The stream has a Mediterranean flow regime characterised by an extended summer drought (Sabater et al., 2001). Basal flow ranges

from 5 to 20 L s^{-1}. The stream is flanked by 20-m wide bands of riparian vegetation, dominated by alder (*Alnus glutinosa*), hazel-nut (*Corylus avellana*), sycamore (*Platanus hispanica)*, and cottonwood (*Populus* sp.) (Romaní et al. 2004). The streambed morphology is characterised by alternating riffles and pools. Boulders and cobbles dominate in riffles while sand accumulates in pools. Branches and leaves are scattered throughout the streambed, particularly in small debris dams in riffle areas (Sabater et al., 2005b).

Experimental design

Artificial glass substrata (1 cm^2 glass tiles) were attached with silicone to flagstones (25 x 75 cm) and left on the streambed to colonise in two analogous 15-m reaches. The downstream reach was enriched over 52 days, starting at 17 March 2008. A continuous flux of an enriched nutrient solution was contributed in this reach from a 100-L reservoir placed on the stream bank. The reservoir was connected by a dark silicone tube to a tap placed ca. 50 cm on the running water in correspondence of small waterfall in order to reach good mixing of added solution. The reservoir contained a concentrated solution of ammonium nitrate and ammonium phosphate designed to increase 10 times the basal concentrations of ammonium (NH_4) and soluble reactive phosphorus (SRP). The nutrient solution was adjusted and added weekly to respond to the changes in stream flow and basal nutrient concentrations. At both enriched and unenriched reaches, eight flagstones were placed considering two light conditions: higher light conditions (HL) and lower light conditions (LL). Lower light conditions were simulated by intercepting light by means of methacrylate pieces placed on top of the substrata, which caused a ca. 80% reduction of incident light. Higher light conditions were those naturally received by uncovered rocks in open reaches. In summary, four conditions were generated: Higher light Unenriched (HL-U), Lower light Unenriched (LL-U), Higher light Enriched (HL-E) and Lower light Enriched (LL-E).

Glass tiles were randomly collected from the streambed at days 4, 9, 16, 22, and 52 after

immersion for the analysis of several structural and functional descriptors of the biofilms. Analyses included chlorophyll-a, Extracellular Polymeric Substances (EPS), bacterial density, extracellular enzyme activities and Confocal Laser Scanning Microscopy (CLSM) observations.

Physical and chemical parameters

Dissolved oxygen, pH, conductivity, temperature, and light irradiance (quantum sensor Li-192SB; Li-Cor) were measured once per week in the field at the enriched and unenriched reaches. Current velocity (Schiltknecht 43221; MiniAir2) was also measured in a location nearby the artificial substrata at the two reaches. Water flow was measured using the pulse addition method (Stream Solute Workshop, 1990) with sodium chloride as conservative tracer. Water samples for nutrient content were collected in triplicate at the unenriched and enriched reaches one to two times per week. Water samples for chemical analyses were filtered (0.2 μm Nylon Membrane filters, Whatman) and stored at 4°C with analyses being performed no longer than 4 hours after collection. Ammonium and dissolved phosphorus were analysed according to standard methods (American Public Health Association, 1989).

Biofilm structure and function

Several biofilm metrics were measured in order to describe structural and functional responses of autotrophic and heterotrophic biofilm compartments to different nutrients and light availability conditions. In particular the structure of biofilm community was investigated by measuring the Chlorophyll-*a* and bacterial density. The heterotrophic capacity to degrade organic matter was investigated by measuring the extracellular enzymatic activities Leucine-aminopeptidase and Alkaline-phosphatase. Moreover, the polysaccharide content in extracellular polymeric substances (EPS) of biofilms was also measured. Finally, biofilms were observed by Confocal Laser Scanning Microscopy (CSLM) on day 52 to establish possible differences

in the spatial organisation, architecture and depth profiles of biofilms between treatments. The methodology used to analyze the biofilm descriptors measured is described in detail in chapter 2.

Statistical analysis

Biofilm biomass, EPS and extracellular enzyme activities were analysed by using a 2-way repeated-measures analysis of variance (ANOVA) to test for the differences between light (higher light and lower light), nutrient enrichment (enriched and unenriched) and the interaction between light and enrichment. Probabilities within groups (day and interactions) were corrected for sphericity using the Greenhouse–Geisser correction. All probabilities were adjusted by the Dunn-Sidak correction. Because bacterial abundances were measured only at days 4 and 52, we tested differences between the 4 treatments using a two-way ANOVA.

RESULTS

Physical and chemical parameters

The physical and chemical conditions at the enriched and unenriched reaches of the Fuirosos stream during the experiment (March – May 2008) are summarised in Table 1. This study was performed in a period without light limitation (>100 μmol photons m^{-2} s^{-1}, Table 1). Light reduction in the shaded substrata (lower light treatments) was 80% on average (79.4 ± 12.2 % and 81.2 ± 7.7 % in the enriched and unenriched reach respectively). Nutrient addition caused a 10.3 times increase of SRP and 10.8 times increase of NH_4 during the 52 days of colonisation process in the enriched reach. Water flow varied between 11.5 and 46.0 L s^{-1} during the study period, the peak (46 L s^{-1}) occurring on day 16. The water velocity was in average of 0.08± 0.02 m s^{-1}, and velocity was not significantly different between reaches.

		Enriched reach (E)	Unenriched reach (U)
P-PO4 (µg L^{-1})		277.78 (152.06)	24.73 (18.75)
N-NH4 (µg L^{-1})		454.35 (225.25)	50.79 (47.62)
pH		7.37 (0.09)	7.38 (0.11)
Temperature (C°)		11.23 (2.12)	11.43 (2.33)
O_2 (mg L^{-1})		10.21 (0.66)	10.30 (0.81)
Conductivity (µS cm^{-1})		215.5 (11.7)	211.1 (10.9)
Light (µmol m^{-2} s^{-1})	Lower-light (LL)	45.99 (28.10)	66.53 (27.31)
	Higher-light (HL)	253.05 (172.67)	400.41 (220.20)

Table 1. Physical and chemical parameters at the two Fuirosos stream reaches during the colonisation experiment (March-May 2007). Values are expressed as mean values and SD in parenthesis (n= 8).

Biofilm structure

Chlorophyll-a density

Chlorophyll-*a* density followed a sigmoid pattern of increase during colonisation in each treatment (Figure 1a). The most important increase in chlorophyll-*a* occurred in most treatments between days 16 and 22. An earlier chlorophyll increase occurred in the HL-U treatment. At the end of the colonisation period (day 52), chlorophyll-a density was slightly higher for LL-grown biofilms when compared with HL-grown biofilms (Figure 1a). There were not significant effects of the light and enrichment on chlorophyll density. However, the interaction between time and these factors indicate a different temporal pattern of chlorophyll density under the different light and enrichment conditions (Table 2).

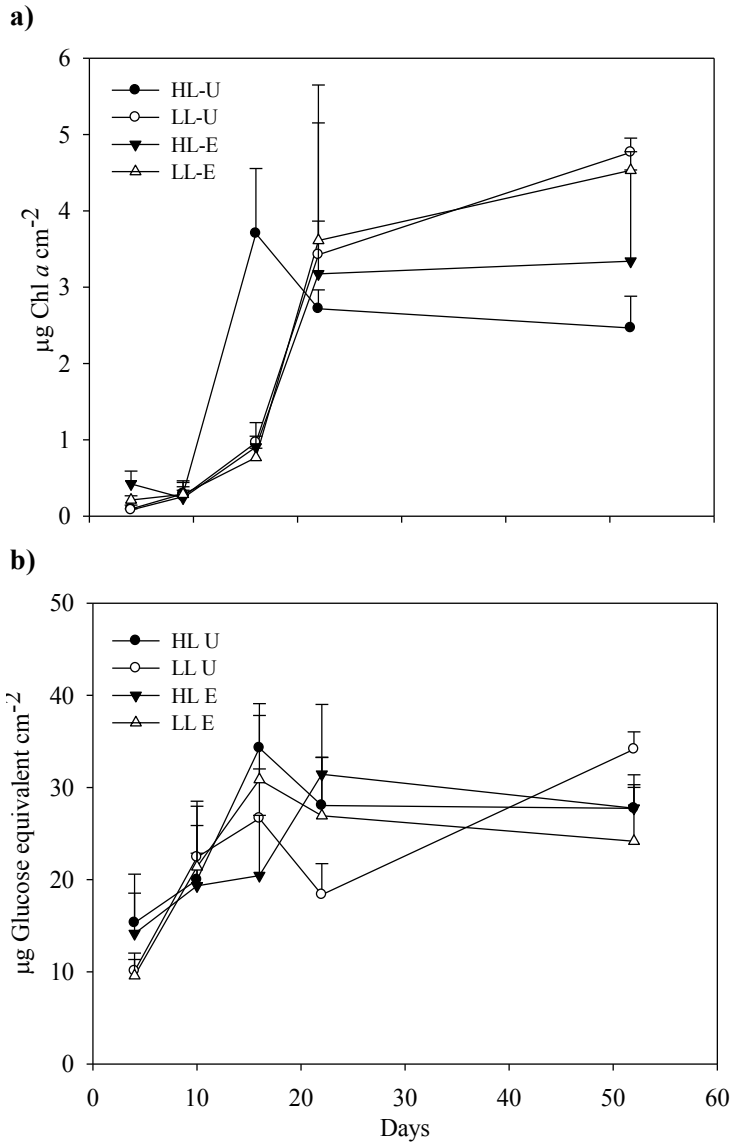


Figure 1. Chlorophyll *a* density (a) and polysaccharide content in EPS (b) patterns during colonization proccess. HL = higher light; LL = lower light; U = unenriched; E = enriched. Values are means ± SD (n = 4).

Polysaccharide content in Extracellular Polymeric Substances (EPS)

The polysaccharide content in biofilm EPS increased during the first two weeks (up to day 16) and then stabilised (Figure 1b). Biofilm polysaccharide content was slightly lower in the enriched than in unenriched reach. EPS polysaccharide content was not significantly affected by light and enrichment conditions throughout the biofilm formation (Table 2).

Source of variation	Chl	EPS	Peptidase	Phosphatase
Day	**0.007**	**0.007**	**0.007**	**0.007**
	F = 181.4	F = 25.5	F = 137.0	F = 7.0
Light	0.801	0.629	0.518	*0.088*
	F = 1.8	F = 2.6	F = 3.2	F = 8.6
Enrichment	0.914	0.831	*0.100*	**0.007**
	F = 1.2	F = 1.6	F = 8.1	F = 17.1
Day x L	**0.007**	0.421	0.731	**0.007**
	F = 22.2	F = 2.5	F = 2.0	F = 6.6
Day x E	**0.007**	0.356	1	0.598
	F = 10.6	F = 2.7	F = 0.3	F = 2.1
L x E	0.956	0.655	**0.041**	*0.061*
	F = 0.9	F = 2.5	F = 10.8	F = 9.7
Day x L x E	**0.007**	0.162	0.113	*0.055*
	F = 8.2	F = 3.6	F = 5.7	F = 4.7

Table 2. Results of a repeated-measures analysis of variance considering two factors: light (L) (high light and low light) and enrichment (E) (unenriched and enriched) availability for biofilm structural and functional descriptors. Probabilities within groups (day and interactions Day x L, day x E and day x L x E) are corrected for sphericity by the Greenhouse-Geisser correction. All probabilities are adjusted by the Dunn-Sidak correction. Values ≤ 0.05 are indicated in bold face type and those ≤ 0.1 are indicated in italic type. F-ratios are also indicated.

Bacterial density

Bacterial counting was performed in days 4 and 52 (Figure 2). Bacterial density was analysed as total bacteria (live plus dead). No significant differences of total bacterial density among treatments were found after 4 days. Instead, biofilms in high light conditions had the highest bacterial density ($p = 0.014$, two-way ANOVA) at day 52. Percentage of dead bacteria showed no significant differences between treatments and increased from 20.0 ± 7.0 (day 4) to 65.5 ± 12.8 % (day 52).

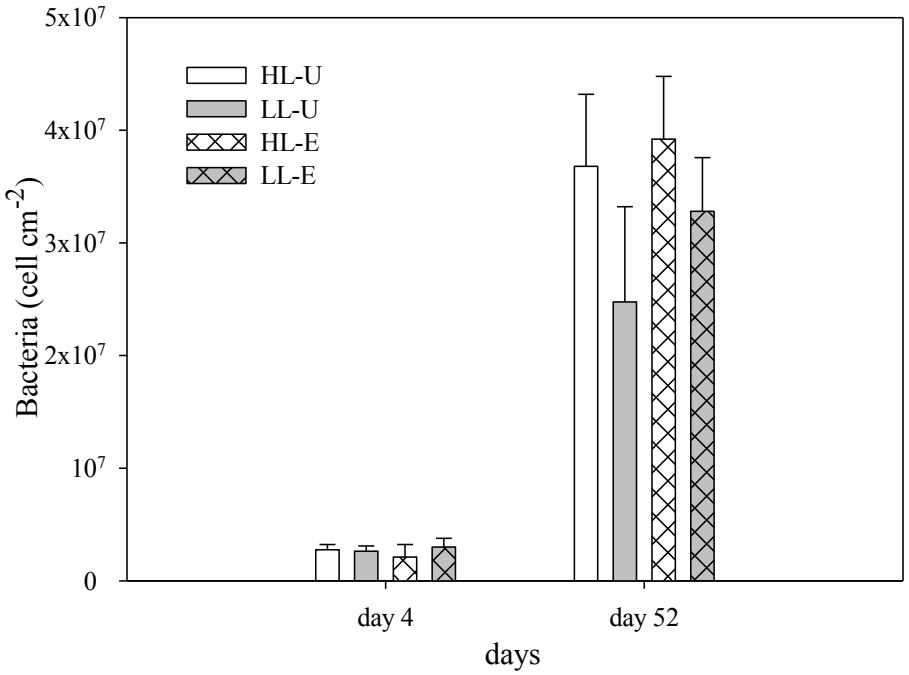


Figure 2. Bacterial density at days 4 and 52. HL = higher light; LL = lower light; U = unenriched; E = enriched. Values are means ± SD (n = 4).

Confocal Laser Scanning Microscopy (CLSM)

CLSM 3D images showed differences in biofilm thickness and structural profiles, as well as in group dominance, between treatments (Figure 3). HL-E produced the thickest biofilm of the different treatments. The thinnest biofilm occurred at LL- unenriched conditions (Figure 3). Maximum projection overlay and 3D images highlighted the differences in the biofilm architecture regarding light availability, especially under unenriched conditions. The green algae and diatoms autofluorescence signal (blue) was the most widespread in HL-U, while the autofluorescence signal of cyanobacteria (pink) dominated the image of biofilm in LL-U unenriched conditions (Figure 3c, 3d). This difference was not as evident in the enriched treatments, where both HL- and LL- treatments had a similar proportion of algae (greens and diatoms) and cyanobacteria (Figure 3a, 3b). The 3D image also revealed that the EPS (green) was concentrating agglomerates in the upper layer of the biofilm under enriched conditions, while under the unenriched conditions agglomerates were evenly distributed throughout the biofilm layers (Figure 3).

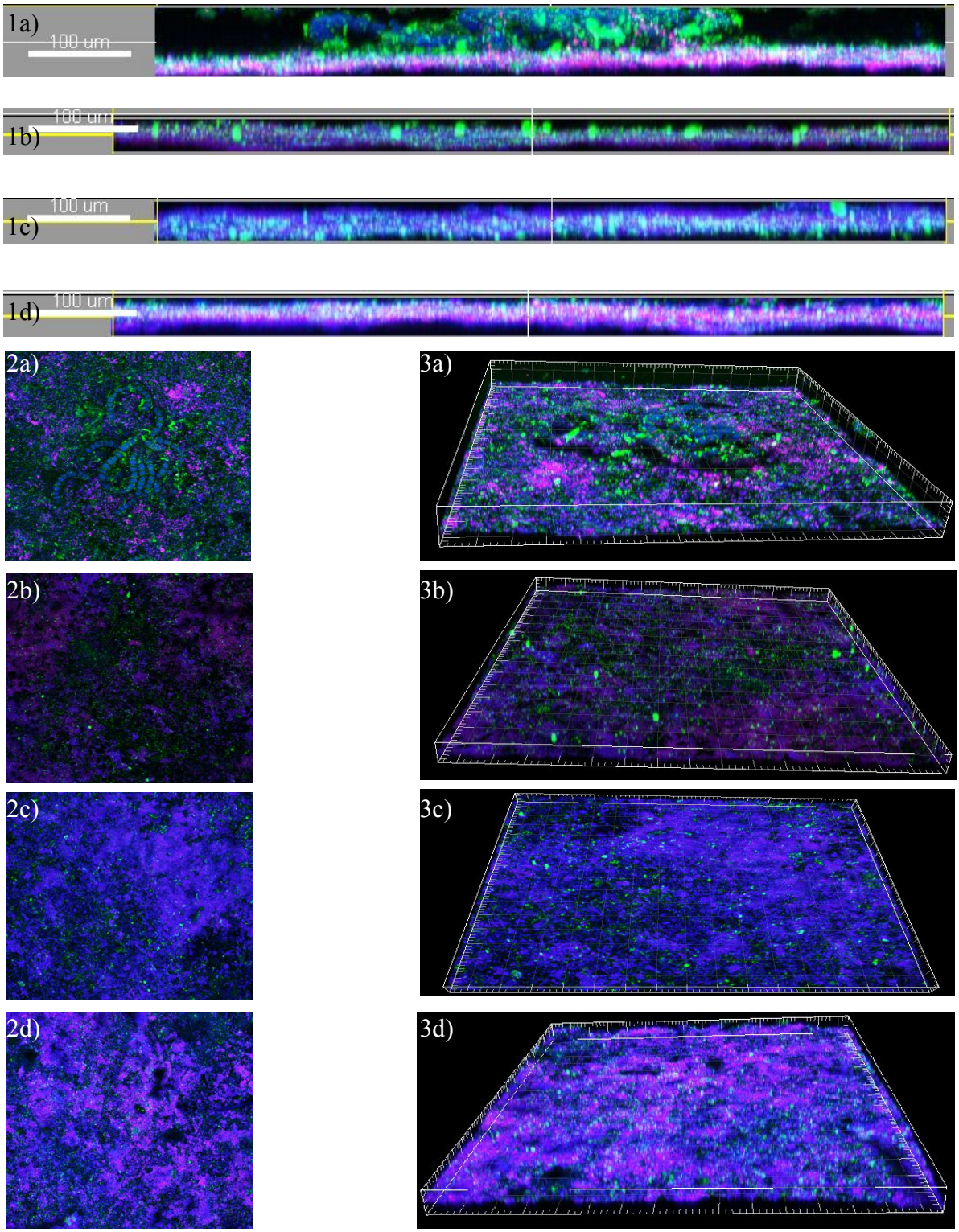


Figure 3. Depth profiles (1); Maximum overlay projection (2); and IMARIS 3d pictures (3) of biofilms growth in the four different conditions (day 52). a) Higher Light Enriched (HL-E, ≈ 70 µm); b) Lower Light Enriched (LL-E, ≈ 35 µm); c) Higher light Unenriched (HL-U, ≈ 40 µm; d) Lower Light Unenriched (LL-U, ≈ 27 µm). Blue colour = algae; Pink colour = Cyanobacteria; green colour = Extracellular Polymeric Substances.

Extracellular enzyme activities

Leucine aminopeptidase activity (LAmP) increased throughout the biofilm formation (Figure 4a). This enzyme activity was generally higher in enriched than unenriched conditions (Figure 4a, Table 2). However, the enrichment caused higher LAmP increases under higher than lower light conditions. This is confirmed by the significant effect of interaction between light and nutrients reported in table 2. Alkaline phosphatase activity was highly variable throughout the biofilm formation (Figure 4b). This enzyme activity was significantly affected by enrichment, decreasing under the enriched conditions (Table 2). The lowest alkaline phosphatase values were measured at higher light enriched conditions (except at day 4), indicating an interaction (though slight) between light and enrichment on this activity (Figure 4b, Table 2).

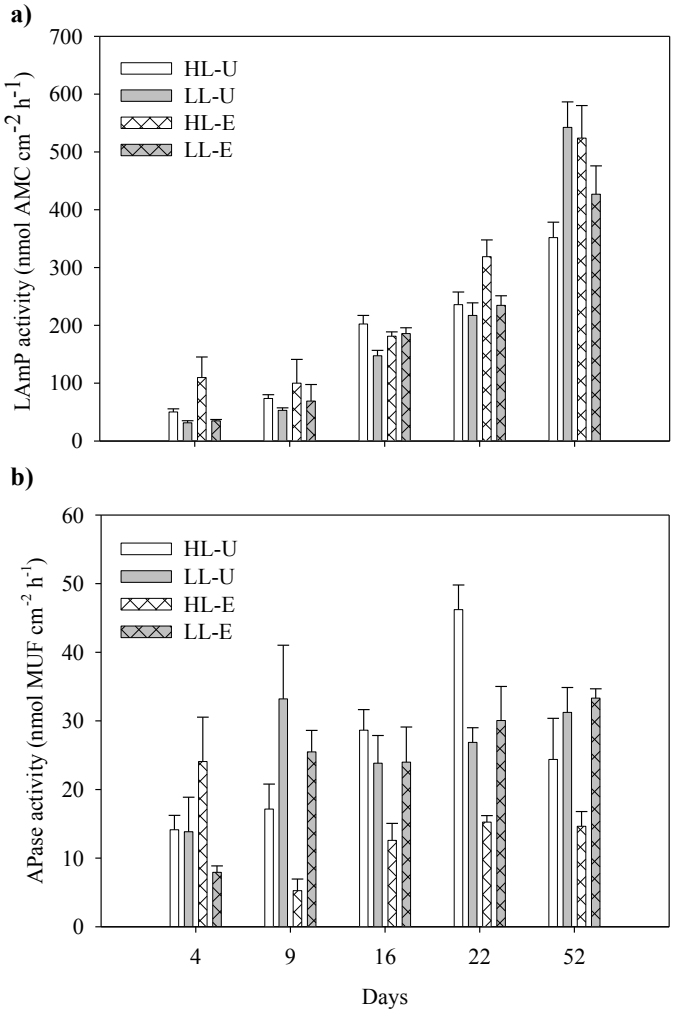


Figure 4. Leucine Aminopeptidase (LAmP) (a) and Alkaline Phosphatase (APase) (b) activities during colonisation process. HL = higher light; LL = lower light; U = unenriched; E = enriched. Values are means ± SD (n = 4).

DISCUSSION

High light and nutrient availability affected several structural and functional characteristics of biofilms during colonisation process in the light-limited stream. These single and interactive effects provided evidence for a synergic effect of light and nutrients on biofilms that have been already described to be highly relevant for the autotrophic component of biofilms (Rier & Stevenson, 2002; Sabater et al. 2005b; Ylla et al. 2007). While nutrient addition have been mostly described to affect biofilm autotrophs, the present experiment highlights that single an interactive effects of light and nutrients affect both heterotrophic and autotrophic compartments of biofilms.

The effects of nutrient enrichment and light availability on the stream biofilm did not show any significant pattern of algal biomass increase when compared to Ylla et al. (2007). Flow variability during the colonisation sequence probably interfered in the biomass accrual pattern (Sabater et al., 2008), making differences less consistent. Although increased light and nutrients did not significantly affect chlorophyll density and polysaccharide content in the EPS, there was a slightly higher chlorophyll density at lower light conditions. This might be an adaptation of photosynthetic apparatus of the algal community to the lower light availability (Guasch & Sabater, 1995). The bacterial density was higher in biofilms grown in high-light and enriched conditions at the end of the colonization (day 52). Though this result cannot be considered a systematic effect of light and nutrients on bacteria, the more complex autotrophic structure in the HL-E biofilm (shown by CLSM) can reinforce the positive relationships between algae and bacteria (Rier and Stevenson, 2002; Rier et al. 2007; Romaní & Sabater, 2000), and helps to explain the final higher bacterial density. The interaction of the two factors (light and nutrients) caused relevant effects on the biofilm structure (algal composition, 3D structure). These more subtle but important effects have been detected because of the coupled use of non-disruptive (CLSM) and disruptive techniques (chlorophyll and EPS extraction procedures). Differences detected in the algal component are underlined by the confocal microscope observations. There is a larger proportion of cyanobacteria

in biofilms grown under lower light, while cyanobacteria were accompanied by diatoms and green algae in high light grown biofilms, especially at unenriched conditions. Nutrient conditions also affected 3D biofilm structure. Biofilms grown under high light and enriched conditions were the thickest and most complex, with green algae dominating the upper biofilm layers and cyanobacterial aggregates mainly adhered to the substratum. The CLSM also showed that the EPS was concentrated in agglomerates in the upper layer of enriched-grown biofilms, but evenly distributed through the biofilm layers in unenriched biofilms (Figure 3). Thus, a similar amount of EPS might be produced by different biofilms (as shown by the extraction and analysis of extracellular polysaccharides) but this is differentially distributed within the biofilm 3D structure. The differential distribution of EPS within the biofilm might indicate a different mechanism for EPS production at both enriched and unenriched conditions. With low nutrient availability, EPS production throughout the biofilm is a response of possible nutrient limitation. In this situation, EPS is released as a quorum-sensing molecule to increase nutrient uptake capacity (Lazazzera, 2000; Artigas J., pers.com.). In contrast, under high nutrient availability, the development of EPS agglomerates might be specifically linked to the growth of certain algal groups under these conditions.

The previous results illustrate the relevance of using 3D non-disruptive techniques for biofilm studies as a complementary tool to the "classical" extraction procedures (Barranguet et al., 2004). In the past, CLSM has been used to determine the effects of nutrient addition and other factors on biofilms composition and structure in microcosms and mesocosms (Battin et al., 2003; Lawrence et al., 2004; Neu et al., 2005). The CLSM provides simultaneous information about the 3 dimensional structure of the biofilms and the identification of different components by both autofluorescence (algae and cyanobacteria) and fluorescent dyes for DNA (bacteria) and EPS (Neu et al., 2001; Battin et al., 2003). Even though quantification of these components is possible by means of image analysis, the potential limitation of this quantification is the fluorescence attenuation related to biofilm thickness (Barranguet et al., 2004). These authors identified the 35

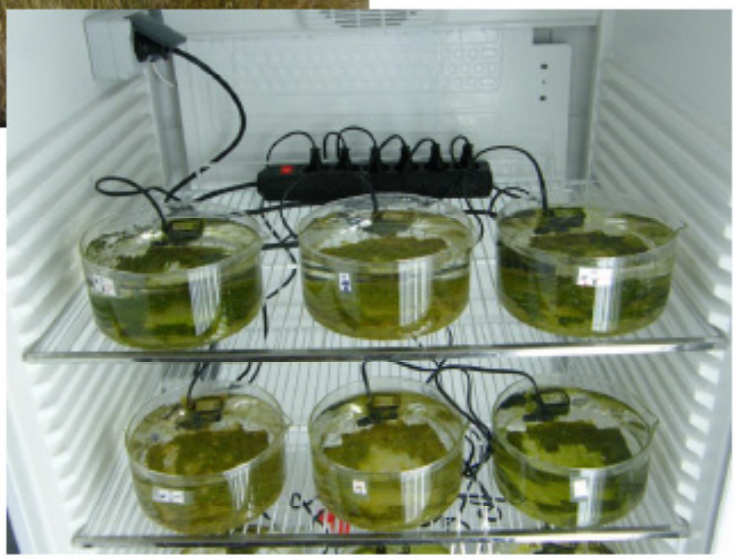
μm depth as the threshold for the optimal quantification of the biofilm components biomass by CLSM even if biofilm components could be visualised up to 200 μm depth. The average biofilm thickness in our samples (48 ± 16.9 μm) restrained us from attempting to quantify the fluorescence signal. Despite this, the qualitative results of this study reliably describe the spatial organisation and structural changes among treatments.

Our study also revealed that the structural changes in the biofilm due to light and nutrient availability were associated with effects on the heterotrophic extracellular enzyme activities. There was a significant decrease of phosphatase activity due to enrichment. The significant (though slight) interaction between light and nutrients revealed that the negative effect of enrichment on phosphatase activity mainly occurred under higher light conditions. Extracellular enzyme production is induced by the presence of substrates, and inhibited by the presence of end products and catabolic repression (Chróst,1990; Chróst,1991). Specifically, alkaline phosphatase catalyses the hydrolysis of phosphate esters, liberating inorganic phosphorus available for microbial uptake. The synthesis of alkaline phosphatase increases when inorganic phosphate is limited (Berman 1970; Siuda & Chrost, 1987) and, at the same time, abundance of available inorganic phosphorus inhibits the alkaline phosphatase activity of algae and bacteria (Chrost & Overbeck, 1987). This effect was previously observed in other experiments in Fuirosos (Romaní et al., 2004; Sabater et al., 2005b). Phosphatase activity in our study was significantly higher in the unenriched than in the enriched reach, mostly under high light conditions.

There was a significant effect of the interaction between light and nutrients on the leucine-aminopeptidase activity. In particular our results revealed that the increase of peptidase activity due to the enrichment occurred in higher light conditions. This result might be the consequence of interactions within the biofilm. Leu-aminopeptidase activity has been associated to heterotrophic bacteria that degrade high molecular weight molecules (substrate) present as dissolved organic matter (DOM), which cannot directly cross the cell membrane, to obtain small peptides and amino

acids (products) (Rosso & Azam 1987; Chrost 1991). Thereafter, the products can be uptaken by bacterial cells and be used for protein synthesis as well as for C and N sources. The higher LAmP activity of biofilm grown in enriched and high light conditions can be a result of higher biofilm biomass and higher biofilm complexity (Romani et al. 2004) and therefore a higher coupling between algae and bacteria within the biofilm (Romaní & Sabater, 2000; Rier et al. 2007). Francoeur & Wetzel (2003) suggested that area-specific enzyme activity may be altered in 3 ways: (1) changes in the abundance of enzyme producing organisms, (2) changes in the amount of enzyme produced per organism, and (3) changes in the activity of individual enzyme molecules. Our results support that higher LAmP activity in these biofilms could be related to the higher abundance of enzyme-producing organisms (bacteria) associated with higher biofilm complexity as well as to the occurrence of higher weight molecules (substrate) present in algal exudates and EPS. Biofilm thickness can be also related to the enzyme dynamics. An increased thickness of the biofilm can result in decreased diffusion (Dodds et al., 1999), which causes the lower arrival of fresh nutrients and the enhancement of internal biofilm recycling (Paul & Duthie, 1989). EPS may enhance storage of organic material susceptible of being used in the absence of external inputs (Freeman & Lock, 1995; Romaní & Sabater, 2000), and may favour their enzymatic activities.

In conclusion, light and nutrient availability may enhance biofilm thickness, architecture, internal recycling mechanisms and the interactions between the algal and bacterial component. Our results confirm that light and nutrient availability are key environmental factors in the structure biofilm characteristics (in terms of thickness, autotrophic composition, heterotrophic abundance), and that this structure leads to specific functioning (specific enzyme activity). Our work shows the importance of the use of CLSM as non-destructive technique able to highlight differences not shown by the other structural descriptors. Though mostly not considered during algal monitoring, the relevant interactions within the biofilm affect their functioning and architecture, and can heavily modify the expected effects of environmental parameters.



CHAPTER 4:

BIOFILM RESPONSE TO TRANSLOCATION ALONG A POLLUTION GRADIENT IN A HIGHLY IMPACTED RIVER: THE EFFECTS OF PESTICIDES AND PHARMACEUTICALS

BIOFILM RESPONSE TO TRANSLOCATION ALONG A POLLUTION GRADIENT IN A HIGHLY IMPACTED RIVER: THE EFFECTS OF PESTICIDES AND PHARMACEUTICALS

Abstract

A huge number of chemical compounds reach running waters as a result of agricultural, industrial and urban activities. Amongst these, pesticides and pharmaceuticals are the most commonly detected. The Llobregat is a highly impacted Mediterranean river affected by strong urban, industrial and agricultural activities. We investigate the effects of pharmaceuticals and pesticides detected in Llobregat waters on the structure and function of river biofilms by means of translocation experiments performed under controlled conditions. Three sampling points were selected: Castellbell (CB) and Mina de Terrassa (MT) as less polluted sites and Sant Joan Despí (SJD) as hotspot. River water was collected from each site and used as inoculums and medium for biofilm growing in independent mesocosms. After 25 days three biofilm translocations were performed: from MT to SJD, from CB to MT and from CB to SJD. Several structural and functional biofilm descriptors were measured four times during the experiment: one before and three after translocations. Fifty-seven pharmaceutical compounds from 14 different therapeutic groups and sixteen compounds from 5 different pesticide families were detected in river waters. The differences between biofilms before translocation mostly concerned the autotrophic compartment. The biofilms responses were different depending on the translocation. The translocation of biofilms from CB to MT was the least responsive while the translocation from MT to SJD caused important structural and functional responses. The translocation from CB to SJD was the most effective in terms of the biofilm responses. The redundancy analysis showed that conductivity, analgesics and barbiturics groups were the variables that significantly influenced the response of biofilms to translocation. Moreover, the partitioning variance technique revealed that analgesics

and antinflamatories significantly affected biofilm responses to translocations, particularly the commonly used Ibuprofen and Acetaminophen (Paracetamol) resulted related with negative effects on autotrophic compartment while Diclofenac was more related with responses of heterotrophs. This study evidenced how non-regulated emerging compounds, reaching continuously river systems, may cause biological responses at the base of river food web with still unknown consequences for the whole freshwater ecosystem, especially in Mediterranean rivers.

Keywords: Biofilm, Pharmaceuticals, Pesticides, Llobregat River, Translocation

INTRODUCTION

Chemical organic compounds have increased in the last decades in running waters. These substances mostly enter as a result of agricultural (via runoff), industrial and urban activities (via wastewaters and sewage treatment systems). A huge number of these compounds are detected in surface waters and their long term effect on biological communities, and therefore on ecosystems, is a challenging task.

Amongst the organic compounds reaching freshwaters pesticides and pharmaceuticals are the most commonly detected (Azevedo et al, 2000; Daughton and Ternes, 1999). Pesticides are mainly used in agricultural activity and enter aquatic environment via runoff after being sprayed in agricultural fields. Their concentration normally peak after rainfall events following spraying campaigns (Rabiet el al., 2010). Various pesticides are actually included in the list of priority substances (Decision 2455/2001/EC) within the Water Framework Directive (WFD, Directive 2000/60/EC), a regulatory document requiring the achievement of the good ecological status of European rivers by 2015. Pharmaceuticals by human and veterinary uses enter aquatic environment mainly via wastewaters and their concentrations in rivers are normally low (Petrovic et al., 2005). However, their entrance is generally chronic and their relevance may increase as a consequence of water scarcity situations (Kuster et al., 2008). Pharmaceuticals are intrinsically bioactive compounds and the knowledge about the effects on the aquatic ecosystems resulting from long-term low-dose exposure to pharmaceuticals is almost none (Ginebreda et al., 2010). Nevertheless, pharmaceuticals have been considered as new emerging pollutants by the EC but have not been included in monitoring programs or regulatory policy within the WFD. Environmental risk assessment procedures (ERA) have been developed for both pesticides and pharmaceuticals, but they are based on short-term single-species lab tests that could not reflect the real ecosystem situation (Ginebreda et al., 2010). To the date, few studies have investigated the effects of priority

and non-priority pollutants on real ecosystems (Hernando et al., 2006; Crane et al., 2006; Sanderson et al., 2004; Fent et al., 2006; Nunes et al., 2005; Pascoe et al., 2003).

River biofilms are complex microbial benthic communities composed by autotrophic and heterotrophic organisms embedded in extracellular polymeric matrix (Romaní, 2010). As an interface, they interact and respond rapidly to changes of environmental conditions and are therefore able to act as warning systems after disturbances (Sabater et al., 2007). Moreover they play a fundamental role in the trophic web and in the geochemical cycles within aquatic ecosystems (Battin et al., 2003; Lock et al., 1993). The short life cycle of biofilm microorganisms as well as the trophic interactions between the biofilm microbiota (algae, bacteria, fungi, protozoa) allows the detection of both short and long term, direct and indirect effects on the biofilm consortia (Proia et al., 2012a). River biofilms can be therefore useful to determine the effects of pollutants on freshwater ecosystems (Sabater et al., 2007).

This study aims to investigate the effects of pharmaceuticals and pesticides detected in Llobregat waters on the structure and function of river biofilms. The Llobregat River is the most important drinking water source for the city of Barcelona (Catalonia, NE Spain). The middle-low part of the river is densely populated and affected by strong industrial and agricultural activities. As consequence of these anthropogenic pressures, priority and emerging compounds in both water and sediments occur in relevant concentrations (Casas et al, 2003; Guerra et al., 2009; Kuster et al., 2008). The environmental risk assessment of the pharmaceuticals detected in the Llobregat River waters (Ginebreda et al., 2010), as well as the relationships between occurrence of pharmaceuticals (Muñoz et al., 2009) and pesticides (Ricart et al., 2010) with the structural composition of benthic communities (macroinvertebrates and diatoms) have been already approached in the Llobregat. The effect of polluted waters on less-tolerant communities can be a complementary tool to understand the effect of contaminants in the ecosystem. With this in mind, we performed a translocation experiment of biofilms from less to more polluted sites and we linked the responses of the biofilm

communities to pollutant concentrations and to the environmental factors. Three sampling sites were used to define a pollution gradient, and translocations from less to more polluted site were performed to determine the effects on biofilms. We hypothesized that bioactive compounds would influence the responses of biofilms to translocation, and that the magnitude of the responses should be related with the incoming higher concentration of organic pollutants. To test our hypothesis we identified the most effective translocation effects in terms of biofilm response, and related them to the differing environmental factors and chemical conditions in the sites.

METHODS

Study Site

The Llobregat is a typical Mediterranean river flowing through north-east of the Iberian Peninsula to south of the city of Barcelona (Catalonia, Spain). It is 165 km long and drains a catchment area of 4948 km^2 (Marcé et al. 2012). The water flow is characterized by a high variability with periodic flood and drought events modulated by the seasonal rainfall regime and the length of dry periods (Ricart et al., 2010). The mean annual precipitation is 3330 Hm^3 and it has an annual average discharge of 693 Hm^3 (Ginebreda et al., 2010). Nearly 30% of discharge flowing in Llobregat basin is used for drinking water (Muñoz et al., 2009). Together with its two main tributaries, the River Cardener and the River Anoia, the Llobregat River is a paradigm of overexploited Mediterranean rivers (Muñoz et al. 2009, Marcé et al. 2012). The middle-low part of the river is densely populated and interested by strong industrial, agricultural and urban activities. In fact its watershed is located into densely inhabited area (more than 3 million inhabitants) and receives significant inputs of industrial and urban wastewaters (~ 137 Hm^3 per year, Ginebreda et al., 2010) as well as surface runoff from agricultural areas (Kuster et al., 2008). Moreover, salt inputs deriving from the ancient salt mines of the Cardener watershed have caused an increase in

water salinity downstream, worsening the poor conditions of the low part of the river. In the present study, three sampling points were selected in the middle-lower part of the Llobregat River (Figure 1): two upstream the input of Anoia River (Castelbell, Mina de Terrassa) and one downstream (Sant Joan Despí).

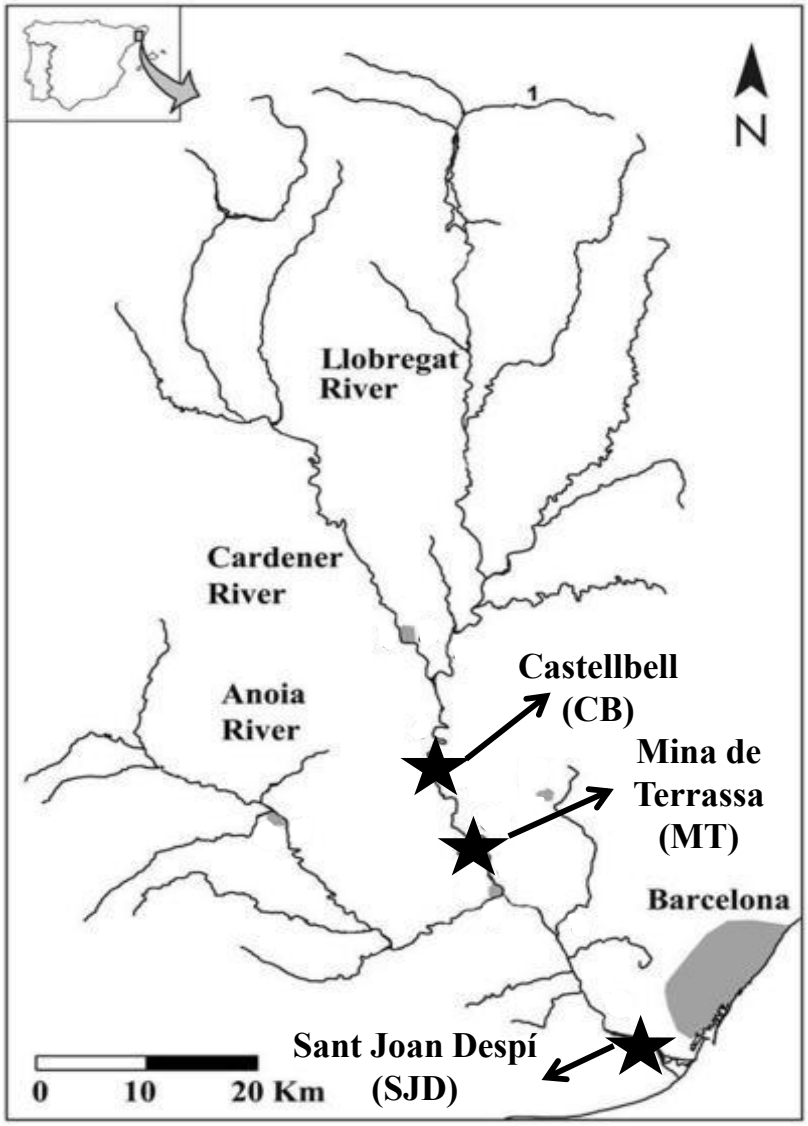


Figure 1. Study site with selected sampling sites (represented by stars) in the Llobregat River.

Experimental design

The biofilm responses to increasing pollutant concentrations in the Llobregat were investigated by means of translocation experiments performed under controlled conditions. The two upstream sampling sites Castellbell (CB) and Mina de Terrassa (MT) were selected as less polluted sites and Sant Joan Despí (SJD) as hotspot. River water was collected between two and three times per week from these three sites and was used as inoculums for biofilm growing in 18 independent mesocosms. Biofilms were colonized on glass slides (1 cm^2 each) placed at the bottom of each mesocosms (35 slides per mesocosm). The mesocosms consisted of sterile glass jars (19 cm in diameter, 9 cm high), filled with 1.5 L of river water which was re-circulated using a submersible pump (Hydor, Pico 300, 230 V 50 Hz, 4.5W). The water in each mesocosm was changed with respective river waters three times per week. All mesocosms were maintained in an incubator (SCLAB) under controlled temperature (18 °C) and light irradiance (150-180 μmol photons m^{-2} s^{-1}; darkness/light cycle of 12 h/12 h). After 25 days of colonization the translocation of the biofilms to a higher pollution site was performed. Three translocations (three replicate jars each) were performed: from Mina de Terrassa to Sant Joan Despí (MT→SJD), from Castellbell to Mina de Terrassa (CB→MT) and from Castellbell to Sant Joan Despí (CB→SJD). Further three replicate glass jars previously incubated with Castellbell, Mina de Terrassa and Sant Joan Despí waters were maintained with the same conditions after the translocation, as controls.

The biofilms were sampled four times during the experiments, one before (day 0) and three after translocation (day 2, day 9 and day 16). During all the experiment, the mesocosms water was replaced three times per week with freshly collected river water from the respective sites. Glass tiles from each mesocosm were randomly sampled.

Control of the physical and chemical parameters

Conductivity, temperature, pH and dissolved oxygen were measured with appropriate sensor probes (HACH LANGE GMBH, Germany) in the field and in the glass jars both before and after each water change. Water samples were collected for nutrient content from the glass jars before and after water renewals. All the water samples were filtered (Nylon Membrane Filters 0.2 μm, WHATMAN, Maidstone, UK) prior to their analysis. Soluble reactive phosphorus was measured following Murphy and Riley (1962). Samples for anions and cations analysis were preserved frozen until analysis by ion-chromatography (761 Compact IC, METROHM, Herisau, Switzerland).

Contaminants in water

The concentrations of 66 pharmaceuticals, belonging to different therapeutic groups, were analyzed in surface waters using the multiresidue analytical method based on LC-MS/MS after solid-phase extraction described by Osorio et al. (2011). The concentrations of 16 pesticides, belonging to different families, were analyzed in water following the method based on on-line SPE–LC–MS/MS described by Köck-Schulmeyer et al. (2012).

Biofilm metrics

The biofilm metrics analyzed were: chlorophyll in vivo fluorescence measurements (Photosynthetic efficiency, Photosynthetic capacity, Fluorescence signal of different autotrophic groups), chlorophylla density, extracellular enzyme activities (Leucine-aminopeptidase, Alkaline phosphatase and β-Glucosidase) and phosphorus uptake capacity. The methodology used for each biofilm metric analyzed is described in detail in chapter 2.

Data Analysis

Biofilm responses to translocations

Responses of each biofilm metric to translocations were analyzed independently for each translocation at each sampling day by one way ANOVA with treatment (control, translocated, and fate) as fixed factor. Effects were analyzed by *post hoc* with Tukey's b test. Statistical significance was set at $p = 0.05$. Analysis was performed using SPSS Version 15.0.

Relationship between biofilm responses and environmental conditions

Redundancy analysis (RDA) was used to determine the respective influence of pharmaceuticals, pesticides and other environmental factors on biofilm response to translocation The biological data included responses of each biofilm metric analyzed (Chlorophyll-a density, photosynthetic capacity and efficiency, F0, F1/F3, extracellular peptidase, phosphatase and β-glucosidase activities, phosphorus uptake rate) at the three samplings after translocation (day 2, 9 and 16). Biological data were square-root transformed. The environmental dataset was composed by thirty-one variables, including pH, conductivity, dissolved oxygen, NO_3, SO_4, SRP, K, Na, Mg, Cl, the concentrations of five pesticides classes (Triazines, Organophosphates, Phenylureas, Choroacetanilides and Thiocarbamates) and the concentration of fifteen pharmaceutical families (Analgesics and Anti inflammatory, β-Blockers, Lipid regulators Psychiatric drugs, Antibiotics macrolids, Antibiotics Fluoroquinolones, Antibiotics sulfonamides, Other Antibiotics, Tetracycline, Stomach treatment, Barbiturics, Blood pressure regulators, Fungicides, Anti-Cancer and Anti-diabetes). All environmental data were transformed by $\log(x + 1)$ to reduce skewed distributions. The maximum gradient length for biofilm metrics dataset was determined using detrended correspondence analysis (DCA). The maximum amount of variation was 0.866, indicating that linear methods would be appropriate (Ter Braak and Smilauer, 2002). To avoid

co-linearity, the variables were selected based on the inspection of variance inflation factors (VIF < 20) (Ter Braak and Smilauer, 1998). Forward selection was used to reduce the environmental variables that significantly explained the response of the biofilm to translocation at a cut-off point of $p = 0.05$. The significance of the RDA axes was assessed using the Monte Carlo permutation test (999 unrestricted permutations). Probabilities for multiple comparisons were corrected by applying the Bonferroni correction. To separate the effects of contaminants (pharmaceuticals and pesticides) from those of other chemical and physical variables on biofilm responses, the variance partitioning technique was applied following Ricart et al. (2010). This technique enabled us to assess the fractions of the explained variance that are shared by two predictor variables, and to determine which of them could be uniquely attributed to each of them (Borcard et al., 1992). The explanatory variables were therefore grouped into two subsets: (a) physical and chemical variables and (b) contaminants. The following sequence of RDAs was performed: (a) RDA of the biofilm metrics matrix constrained by physical and chemical variables, (b) RDA of the biofilm metrics matrix constrained by contaminants, (c) partial RDA of the biofilm metrics matrix constrained by physical and chemical variables using the contaminants as covariables and (d) partial RDA of the biofilm metrics matrix constrained by contaminants using the physical and chemical variables as covariables.

Based on the results of the first set of analyses, the contaminants dataset was reduced, and the new dataset included concentrations of each single compound of the families of contaminants significantly explaining the biofilm response in the first sequence of RDAs. With this new dataset of contaminants an additional sequence of RDAs was performed: (a) RDA of the biofilm metrics matrix constrained by the selected compounds, (b) partial RDA of the biofilm metrics matrix constrained by physical and chemical variables using the selected compounds as covariables and (c) partial RDA of the biofilm metrics matrix constrained by the selected compounds using the

physical and chemical variables as covariables.

RESULTS

Environmental conditions

Physical and Chemical parameters

The dissolved oxygen (DO) and pH did not differ among sites, but conductivity (Table 1) increased significantly from CB to SJD ($p < 0.05$). Concentration of soluble reactive phosphorus (SRP) gradually increased downstream, while nitrates (NO_3) concentration was similar in CB and

	Flow (m^3 sec^{-1})	**pH**	**Cond** (μS cm^{-1})	**DO** (mg L^{-1})	**SRP** (μgP L^{-1})	**NO_3** (mg L^{-1})
CB	6.93 *(1.73)*	8.43 *(0.17)*	1560.0 *(195.0)*	10.42 *(1.04)*	44.80 *(57.18)*	6.48 *(1.58)*
MT	6.16 *(2.43)*	8.40 *(0.15)*	1645.0 *(274.3)*	10.37 *(0.95)*	70.85 *(50.73)*	7.32 *(1.97)*
SJD	7.01 *(12.53)*	8.41 *(0.18)*	1819.0 *(330.2)*	10.49 *(1.00)*	104.63 *(38.04)*	10.84 *(2.62)*

Table 1. Results of environmental variables measured at each sampling site during the experiment. Values are expressed as mean values and SD in parenthesis (n= 16).

MT and increased significantly in SJD ($p < 0.05$, Table 1).

Contaminants in water

Fifty-seven pharmaceutical compounds from 14 different therapeutic groups were detected in the three sites (Table 2). SJD resulted the most polluted site while concentrations in CB and MT were lower and within similar range. Analgesics and anti-inflammatories had the highest concentrations at each sampling site. Ibuprofen was on average, the most concentrated compound of this group (Table 2). Psychiatric drugs and Sulfonamide antibiotics could also achieve concentrations higher than 100 ng L^{-1} at each sampling site. Blood pressure regulators had concentrations of 1928.2 ± 561.4 ng L^{-1} in SJD, and around 200 ng L^{-1} at CB and MT. The blood pressure regulator Hydrochlorothiazide, a diuretic drug of the thiazide class, reached a maximum concentration of 2930.9 ng L^{-1} at SJD (Table 2).

Sixteen compounds from 5 different pesticide families were detected in river water (Table 3). Concentrations were in general low (< 100 ng L^{-1}), the highest at SJD, and lower in CB and MT. The families accounting for higher concentrations were Triazines, Phenylurea and Organophosphate. Those having the highest concentrations in SJD were Terbuthylazine, Diuron and Diazinon

		CB			MT			SJD		
		min	**max**	**average**	**min**	**max**	**average**	**min**	**max**	**average**
Analgesics and Anti-inflammatory	**Ketoprofen**	14.9	106.3	48.8	0.5	86.3	31.6	5.2	292.6	96.4
	Naproxen	68.9	198.9	127.7	85.0	165.4	111.8	126.8	258.5	185.5
	Ibuprofen	128.0	523.9	270.4	94.8	404.9	251.3	200.3	642.0	391.1
	Indometacine	12.7	26.6	20.1	9.8	109.0	25.1	17.2	123.8	48.8
	Diclofenac	86.5	202.9	142.5	83.7	184.0	125.5	128.6	445.2	311.0
	Acetaminophen	86.6	824.3	348.1	10.9	586.2	225.8	77.3	421.4	225.6
	Propiphenazone	2.2	4.6	3.6	2.2	4.8	3.7	6.0	36.3	17.4
	Phenazone	7.9	18.6	11.8	8.3	12.7	10.6	12.6	94.0	45.9
	Phenybutazone	2.3	6.5	4.9	1.0	6.6	3.6	4.0	50.5	18.2
	Codeine	7.0	30.6	15.9	5.5	26.8	14.4	3.8	122.7	55.7
β-Blockers	**Atenolol**	21.2	47.8	37.4	17.2	45.1	33.3	62.5	251.2	153.8
	Sotalol	13.5	22.9	18.4	10.0	22.5	17.0	24.6	164.7	89.6
	Metoprolol	7.4	16.4	12.9	7.2	17.9	12.2	32.4	535.0	104.2
	Pindolol	0.0	0.2	0.1	0.1	0.3	0.1	0.2	0.4	0.3
	Carazolol	0.0	0.3	0.2	0.1	0.3	0.2	0.2	1.0	0.6
	Propanolol	8.8	25.8	14.3	11.9	22.5	16.2	10.3	70.4	37.8
	Timolol	1.2	153.5	14.7	1.0	2.9	1.8	2.2	10.3	6.8
	Nadolol	0.2	0.6	0.4	0.1	0.5	0.3	0.4	1.1	0.7
Lipid regulators	**Clorifibic acid**	1.3	18.7	3.4	1.1	2.4	1.8	6.8	40.1	20.8
	Gemfrobizil	12.0	25.4	18.3	9.0	24.3	16.2	21.2	152.0	85.5
	Benzafibrate	13.7	54.1	27.9	13.2	47.5	24.8	21.2	217.1	94.0
	Fenofibrate	24.8	97.6	47.1	19.3	56.6	34.9	35.1	277.6	148.8
	Atorvastatine	0.5	1.2	0.7	0.3	1.3	0.7	1.0	3.2	2.0
	Mevastatine	1.2	15.8	4.9	1.8	7.9	4.0	2.7	7.5	5.4
Psychiatric drugs	**Lorazepam**	87.1	204.2	163.5	102.6	196.5	158.9	113.8	705.5	391.6
	Carbamazepine	35.3	59.2	51.0	36.1	62.8	51.9	53.2	278.2	173.6
	Diazepam	2.2	3.9	2.9	1.6	3.8	2.8	3.1	32.0	16.5
	Fluoxetine	12.8	29.0	16.1	4.3	34.1	14.2	10.3	53.5	33.1
	Paroxetine	1.3	4.0	2.1	1.1	9.6	4.4	3.8	145.1	24.4
Antibiotics Macrolids	**Erytromicin**	1.9	7.4	4.8	1.3	12.8	4.1	0.1	45.2	15.9
	Azythromicin	3.5	7.1	6.7	6.9	7.1	7.0	3.6	7.2	6.4
	Roxythromycin	0.4	1.1	0.7	0.2	0.9	0.5	0.7	8.1	3.6
	Clarithromicin	13.5	51.9	38.4	13.8	52.9	34.2	21.2	232.1	115.1
	Tylosin	1.8	4.3	2.8	1.0	4.7	2.7	2.2	30.3	8.1
	Josamycin	0.3	0.7	0.5	0.3	0.7	0.5	0.8	3.6	2.2
	Spyramicin	4.4	16.2	8.1	4.8	15.2	7.4	6.9	52.8	28.5
	Tilmicosin	0.3	370.5	31.8	0.8	95.8	8.8	1.9	96.6	11.8

Table 2.Results of maximum, minimum and average pharmaceuticals concentrations measured at each sampling site during the experiment (n = 18). Values are expressed in ng L^{-1}.

		CB			MT			SJD		
		min	**max**	**average**	**min**	**max**	**average**	**min**	**max**	**average**
Antibiotics Fluoroquinolons	**Ofloxacine**	11.6	87.5	29.9	11.9	32.2	20.8	24.1	337.8	156.4
	Ciprofloxacine	16.9	56.4	27.7	18.5	36.6	25.3	27.7	164.6	80.3
	Enoxacine	6.1	14.8	9.3	6.5	11.6	8.9	0.7	36.4	18.4
	Enrofloxacine	2.3	45.8	7.0	2.1	7.6	4.0	6.0	303.7	113.7
	Flumequine	0.2	0.9	0.4	0.2	0.8	0.4	0.3	0.9	0.5
Antibiotics Sulfonamidas	**Sulfamethoxazole**	91.2	256.8	200.8	132.0	298.4	210.3	201.2	1576.0	717.2
	Sulfadiazine	2.7	17.8	6.5	2.6	45.7	12.4	7.1	43.4	30.1
Others antibiotics	**Trimethoprim**	3.5	8.6	6.3	2.9	7.6	5.7	6.8	37.4	22.3
	Tetracycicline	3.5	12.4	7.3	2.8	35.1	14.7	25.6	788.8	247.1
Stomach treatment	**Famotidine**	0.6	1.0	0.9	0.7	1.0	0.9	0.2	7.5	3.0
	Ranitidine	1.1	3.3	2.3	0.7	3.4	2.2	0.1	115.8	15.1
	Cimetidine	1.1	3.6	2.3	0.3	3.9	2.3	0.2	42.3	9.7
Barbiturics	**Butalbial**	1.9	14.6	7.0	1.8	27.3	11.5	2.2	4.3	3.0
	Pentobarbital	8.1	47.1	19.9	10.2	68.6	21.5	9.6	17.6	12.1
	Phenobarbital	2.3	25.4	9.2	2.6	22.1	11.0	2.3	12,1	5.8
Blood pressure regulators	**Enalapril**	2.4	12.0	6.4	1.8	11.8	6.0	6.2	32.0	11.8
	Hydrochlorothiazide	140.7	286.2	219.2	100.8	236.7	185.5	272.8	2930.9	1283.1
Fungicides	**Metronidazole**	0.2	0.6	0.3	0.1	0.6	0.3	0.6	4.5	2.7
Anti-cancer	**Tamoxifen**	0.3	1.5	0.6	0.2	1.7	0.5	0.2	1.5	0.6
Anti-diabetic	**Glibenclamide**	0.4	2.1	1.2	0.4	2.0	0.9	1.6	12.6	7.2

Table 2.Results of maximum, minimum and average pharmaceuticals concentrations measured at each sampling site during the experiment (n = 18). Values are expressed in ng L^{-1}.

		CB			MT			SJD		
		min	**max**	**average**	**min**	**max**	**average**	**min**	**max**	**average**
Triazines	**Atrazine**	0,02	3,12	0,27	0,02	0,46	0,19	0,04	0,43	0,16
	Cyanacine	0,20	2,06	0,34	0,19	0,19	0,19	0,25	0,25	0,25
	Deisopropilatrazine	3,57	9,58	3,90	4,20	4,20	4,20	7,33	36,89	8,97
	Desetilatrazine	0,19	2,73	0,38	0,17	0,17	0,17	0,40	1,33	0,48
	Simazine	0,25	4,07	0,73	0,04	1,05	0,60	0,86	3,40	1,94
	Terbuthylazine	0,04	50,08	30,97	0,04	65,74	33,88	0,05	82,84	34,78
Phenylurea	**Diuron**	1,12	5,04	2,60	1,38	5,09	3,04	10,53	31,13	22,65
	Isoproturon	0,01	0,71	0,20	0,01	0,60	0,14	0,02	0,81	0,28
	Linuron	1,02	1,02	1,02	0,92	0,92	0,92	1,17	1,17	1,17
	Clortoluron	0,03	3,09	0,30	0,03	0,59	0,09	0,06	6,20	1,29
Organophosphate	**Malation**	0,22	4,63	0,48	0,20	0,43	0,21	0,24	0,79	0,27
	Diazinon	0,82	9,12	3,56	0,78	5,72	3,08	7,12	79,03	24,72
	Dimetoate	0,50	2,37	0,70	0,49	1,06	0,52	0,78	3,52	1,08
Chloroacetanilide	**Alaclor**	0,68	0,68	0,68	0,66	0,66	0,66	0,90	0,90	0,90
	Metolaclor	0,13	1,31	0,31	0,13	0,13	0,13	0,17	1,30	0,23
Tiocarbamate	**Molinate**	0,52	4,22	0,72	0,50	0,50	0,50	0,59	0,59	0,59

Table 3..Results of maximum, minimum and average concentrations of pesticides measured at each sampling site during the experiment (n = 18). Values are expressed in ng L^{-1}.

respectively (Table 3).

Biofilm responses

The differences between biofilms before translocation (day 0) mostly concerned the autotrophic compartment. Biofilm grown with SJD water had significantly higher chlorophyll *a* density and fluorescence signal of all autotrophic groups (green algae, diatoms and cyanobacteria) than those grown with CB and MT waters (Figure 2, $p < 0.05$). Chlorophyll *a* density of biofilms in SJD was

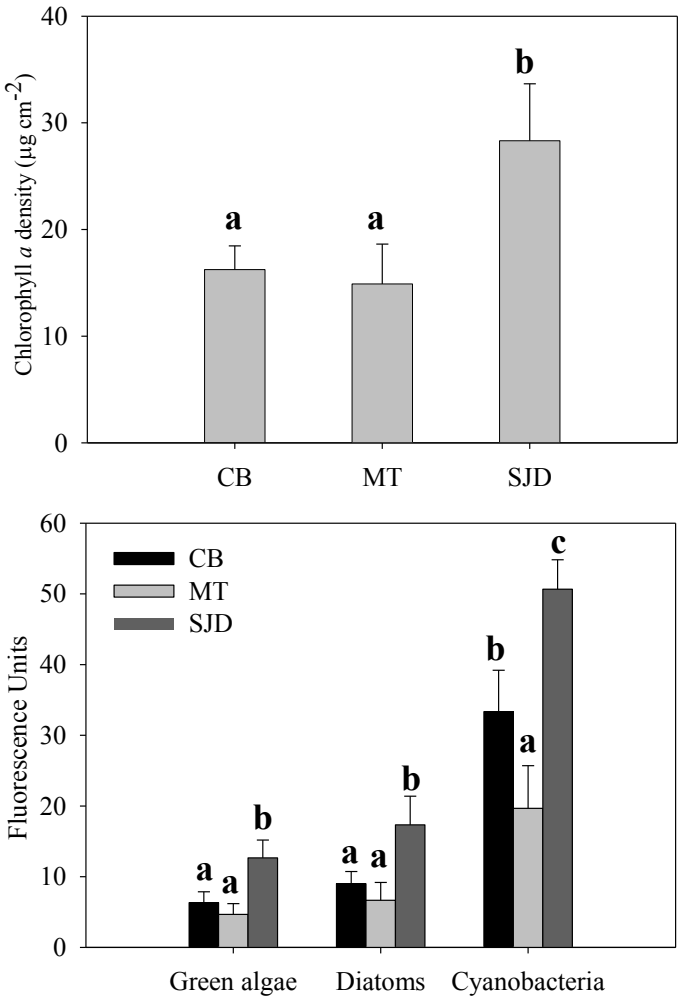


Figure 2. Variation of biofilm metrics before translocation. Values are means and standard deviation (n = 3). Letters represents results of the Tukey-b post-hoc test performed when differences resulted significant. Statistical significance was set at $p \leq 0.05$ (one-way ANOVA).

28.3 ± 5.3 μgChl cm^{-2} , 1.9 and 1.7 times higher than those measured in MT and CB respectively.

The biofilms responses to translocation were different depending on the magnitude of the translocation. The translocation of biofilms from CB to MT was the least responsive. CB→MT biofilms exhibited a significant decrease of extracellular peptidase activity and Fo at day 2 (Figure 3), but from day 9 these two metrics recovered to values similar to CB biofilms. Further, the photosynthetic efficiency of biofilms translocated between CB→MT decreased significantly on day 9, and recovered one week later (day 16, Figure 3).

In contrast, the translocation from MT to SJD caused structural and functional responses in the biofilm. Extracellular phosphatase activity decreased to values significantly lower than MT and SJD at day 2, and the extracellular peptidase activity increased at day 2 and 16 (Figure 3). Moreover, the phosphate uptake capacity of SJD biofilms was lower than that of MT biofilms. At day 16, the phosphate uptake rate of MT→SJD biofilm decreased to values closer to SJD biofilms (Figure 3). Regarding chlorophyll-*a* density, those of translocated biofilms increased to values closer to SJD, particularly on day 16. In contrast, Fo of translocated biofilms decrease significantly at day 2 and recovered later on (from day 9, Figure 3).

The translocation from CB to SJD was the most effective on the biofilm. CB→SJD biofilms exhibited significant increase in extracellular peptidase activity on day 9 and significant decrease of extracellular phosphatase activity on days 9 and 16 (Figure 3). The phosphate uptake capacity of SJD biofilms was lower than CB. The phosphate uptake rate of CB→SJD biofilm decreased to resemble SJD values (Figure 3). The photosynthetic efficiency of translocated biofilms significantly decreased on day 9 and recovered to CB values on day 16. The chlorophyll *a* density of SJD biofilms was significantly higher than CB and in translocated biofilms it tends to increase to values closer to SJD from day 9 until the end of the experiment (Figure 3). The Fo values of CB→SJD biofilms decreased significantly at day 2 and increased to values higher than CB and SJD on day

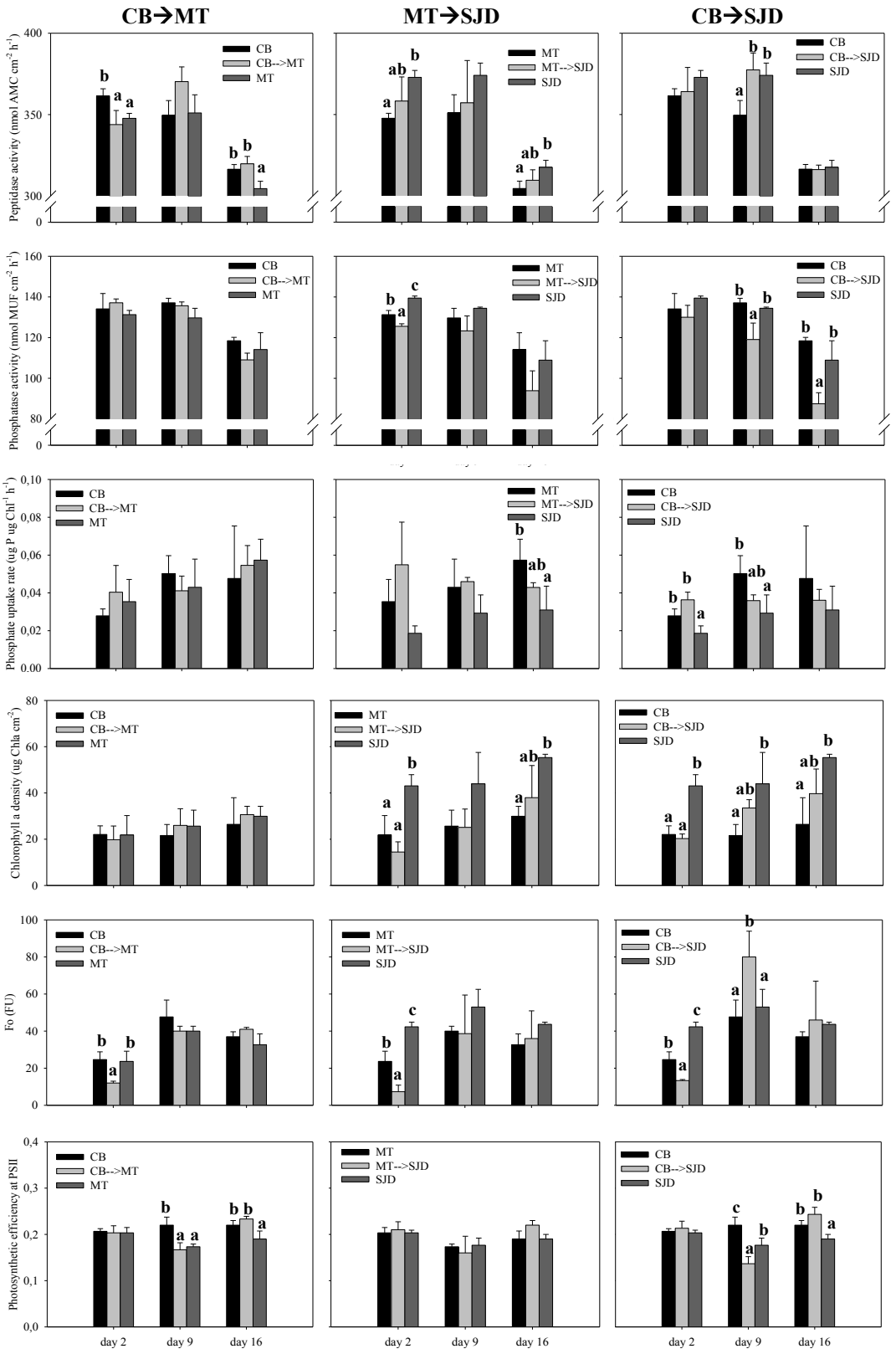


Figure 3. Variation of biofilm metrics in response of translocation. Values are means and standard deviation (n = 3). Letters represents results of the Tukey-b post-hoc test performed when differences resulted significant per each samplig date. Statistical significance was set at p≤0.05 (one-way ANOVA).

9 (Figure 3).

Relationships between biofilm responses and environmental conditions

The RDA analysis showed that conductivity, analgesics and barbiturics groups were the variables that most significantly influenced the response of biofilms to translocation. This RDA accounted for the 57.3 % of the variance.

The potential contribution of the two sets of variables (contaminants and physical and chemical variables) was estimated in the second redundancy analysis. Amongst the physical and chemical variables, both conductivity and SRP concentrations significantly influenced biofilm response accounting for the 24.4 % of the total variance. Amongst the contaminants the groups most significantly influencing the biofilm responses were Analgesics, Triazines, Barbiturics and Organophosphates, which accounted for the 60.4 % of the total variance. The partial redundancy analysis in order to evaluate the covariance explained by the six significant variables of the two sets of parameters evidenced that of the total variance (62.3 %) the physical-chemical variables (conductivity and SRP) account only for the 1.9%, while Analgesics, Triazines, Barbiturics and Organophosphates account for the 37.9%. The shared variance explained represented the 22.5 %. SRP increase was related with biofilm chlorophyll-*a* and photosynthetic capacity increases and with the decrease of phosphate upake rate (Figure 4a). Moreover, conductivity increase was related with decrease of photosynthetic efficiency, phosphatase and β- Glucosidase activities (Figure 4a). Analgesics, triazines and barbiturics affected most of the biofilm metrics; especially those of the autotrophs (Figure 4b). In particular, increase of analgesics was related with decrease of the F1/F3 ratio and photosynthetic capacity while the increase of triazines and barbiturics was related with chlorophyll*a,* Fo and phosphatase activity decreases (Figure 4b). The last iteration with multivariate analysis evidenced that amongst the compounds that most significantly influenced the

biofilm responses were the analgesics Diclofenac, Ibuprofen and Acetaminophen (Paracetamol), altogether accounting for the 25.4 % of the explained variance. Ibuprofen and Acetaminophen affected chlorophyll*a* density, peptidase activity, Fo, photosynthetic capacity and F1/F3 ratio (Figure 4c). In particular, Acetaminophen increase was related with the F1/F3 decrease, and the Ibuprofen increase was related with photosynthetic capacity, and that of diclofenac was associated to higher phosphatase activity.

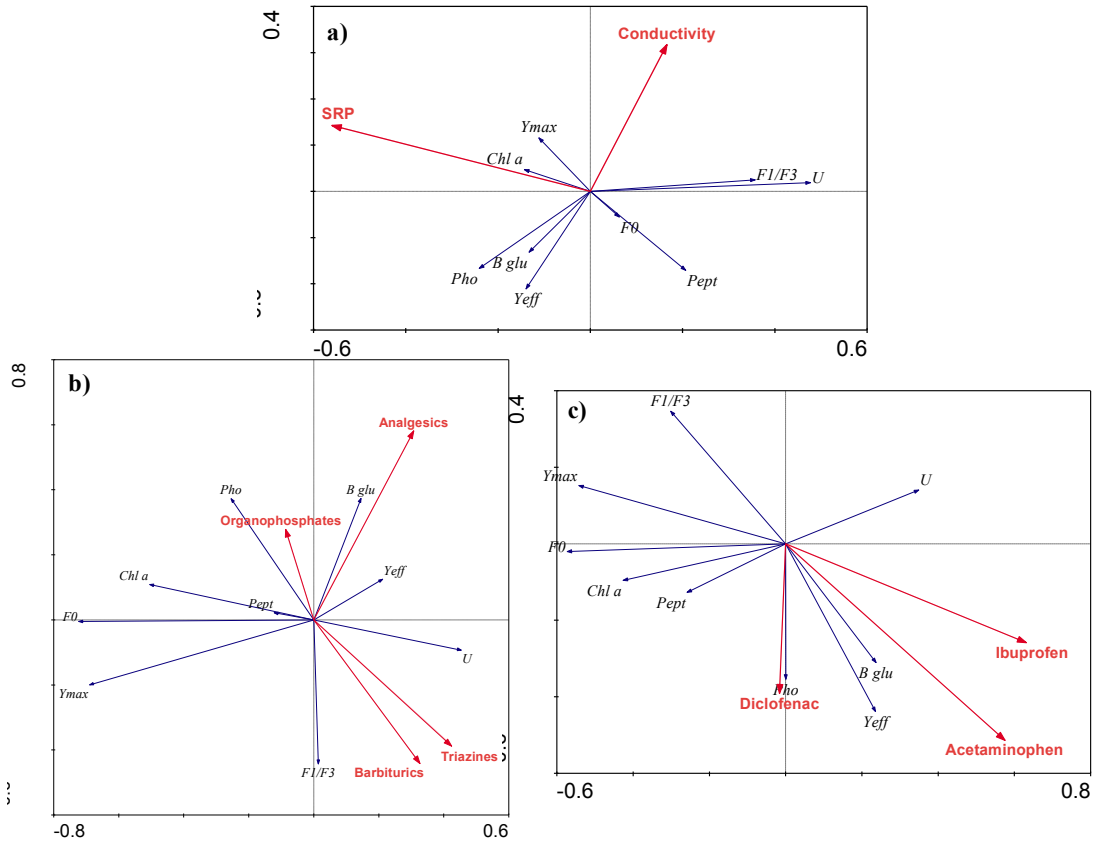


Figure 4. Sequence of RDAs performed. a) Partial RDA of the biofilm metrics matrix constrained by physical and chemical variables using the contaminants as covariables. b) Partial RDA of the biofilm metrics matrix constrained by families of contaminants using the physical and chemical variables as covariables. c) Partial RDA of the biofilm metrics matrix constrained by compounds using the physical and chemical variables as covariables. Variables that significantly explained the response of the biofilm to translocation are showed. Significance was set at cut off point p = 0.05. The significance of the RDA axes was assessed using the Monte Carlo permutation test (999 unrestricted permutations). Probabilities for multiple comparisons were corrected by applying the Bonferroni correction.

DISCUSSION

A total of 16 pesticides and 57 pharmaceutical compounds were detected in all sampling sites at concentrations ranging from < 1 ng L^{-1} to ca. 3 μg L^{-1}. These values were comparable to concentrations measured in other studies performed on the Llobregat (Kuster et al., 2008; Quintana et al., 2001). Concentrations of pharmaceuticals were higher than those of pesticides. These results could be explained by the hydrological features corresponding to the study period. Pesticides mostly enter river waters from diffuse source after rain fall events and peaks during floods in agricultural areas (Rabiet et al., 2010), and the period of our study was during low flow conditions (< than 10 m^3 sec^{-1}) favoring the concentration of products entering continuously from WWTP effluents (Kuster et al., 2008). The occurrence of pharmaceuticals in Llobregat river water matched with those most consumed in Spain (Spanish Public health Ministry; http://www.aemps.gob.es). The most abundant therapeutic group was the analgesics and anti-inflamatories, where Ibuprofen, Diclofenac and Acetaminophen (paracetamol) had large concentrations. This was also in accordance with the results of previous studies in the Llobregat river (Kuster et al., 2008; Muñoz et al., 2009; Ginebreda et al, 2010). The most abundant pharmaceutical product detected during our study was the blood pressure regulator Hydrochlorothiazide (Table 2), a diuretic compound which has shown cytotoxicity on fish cells (Caminada et al., 2006).

A few studies have investigated the occurrence and potential effects of pesticides (Ricart et al., 2009) and pharmaceuticals (Muñoz et al., 2009) on biological communities in field conditions. In this study we used translocations as experimental tool in order to define the response of natural biological communities to different mixtures of pesticides and pharmaceuticals. Translocation experiments have been successfully used to describe the responses of biofilms to various environmental and anthropogenic stressors (Victoria and Gómez, 2010; Ivorra et al., 1999; Tlili et al., 2011). Our experiments show that the mixtures of chemicals occurring in the Llobregat River affect the responses of structure and function of biofilms to simulated translocation, and that

this response is higher when there is higher concentration. Particularly, pharmaceuticals of the most abundant therapeutic groups significantly explained the variance of structural and functional responses of biofilms to changes in water quality. The different biofilm biological descriptors showed different responses in function of time and magnitude of translocation. In particular, our results revealed that most of the measured biofilm metrics responded to translocation from less (CB) to more (SJD) polluted site more than in the other translocations (Figure 3). In particular the responses of structural and functional parameters of both autotrophic and heterotrophic compartments of biofilm translocated from CB to SJD were related with strong differences in the water quality of the studied sites. In fact SJD water resulted the most polluted in terms of nutrients (mainly phosphates and nitrates) and contaminants (pesticides and pharmaceuticals) concentrations.

Multivariate statistical techniques are useful to establish spatial and temporal relationships between different types of stressors and their effects on biological responses (Muñoz et al., 2009). The redundancy analysis used in this study revealed that biofilm responses to translocation and to pollution gradient along Llobregat River were affected by both physicochemical and organic contamination factors. In particular, conductivity and SRP were the physico-chemical variables significantly explaining the variance of biofilm responses, while Triazines and Analgesics and anti-inflamatories were the most important groups of contaminants accounted for almost 61% of explained variance. These results were in accordance with previous studies performed in Llobregat River investigating the relationships between the structure of invertebrate and diatom communities and chemical pollution (Muñoz et al., 2009; Ricart et al., 2010). Significant relationships between SRP and Triazines concentrations and diatom community composition have been observed in epilithic and epipsammic biofilms (Ricart et al. 2010). The composition of the invertebrate community in the Llobregat River is significantly related with conductivity (Ricart et al. 2010), but also with inflammatory concentrations (Muñoz et al. 2009). The used biofilm metrics includes

both functional and structural biological attributes, and this allowed us to detect a wide range of responses. In particular we identified three analgesics and anti inflammatories compounds who explained an important portion of the variance of biofilm responses to translocation. Those products were the Diclofenac, Acetaminophen (paracetamol) and Ibuprofen. These three compounds are widely used and their presence has been described in aquatic ecosystems (Daughton and Ternes,1999; Ellis, 2006; Fent et al., 2006; Heberer et al., 2002). Their acute toxicity has been tested in several studies on numerous aquatic organisms (Cleuvers et al., 2003; David et al., 2009; Ferrari et al., 2004). In our study Acetaminophen (paracetamol) and Ibuprofen affected mostly autotrophic descriptors (Figure 4c). In particular acetaminophen was related to a decrease of the F1/F3 ratio of biofilms, suggesting a negative effect on green algae, and positive on cyanobacteria. Although no data are available about direct effects of paracetamol on algae and/or biofilms, indeed exists for other aquatic organisms (Brain et al., 2004; David et al., 2009; Kim et al., 2007; Kim et al., 2010). Brain and colleagues (2004) showed the acetaminophen toxicity on two species of aquatic macrophytes when mixed with other 7 pharmaceuticals. David et al. (2009) reported acute toxicity of acetaminophen on the embryonic development of zebrafish at concentrations in the range of those measured in Llobregat River. Furthermore, acetaminophen toxicity was higher with higher water temperature, and resulted as the most toxic tested pharmaceutical in a study performed on *D. magna* (Kim et al., 2010).

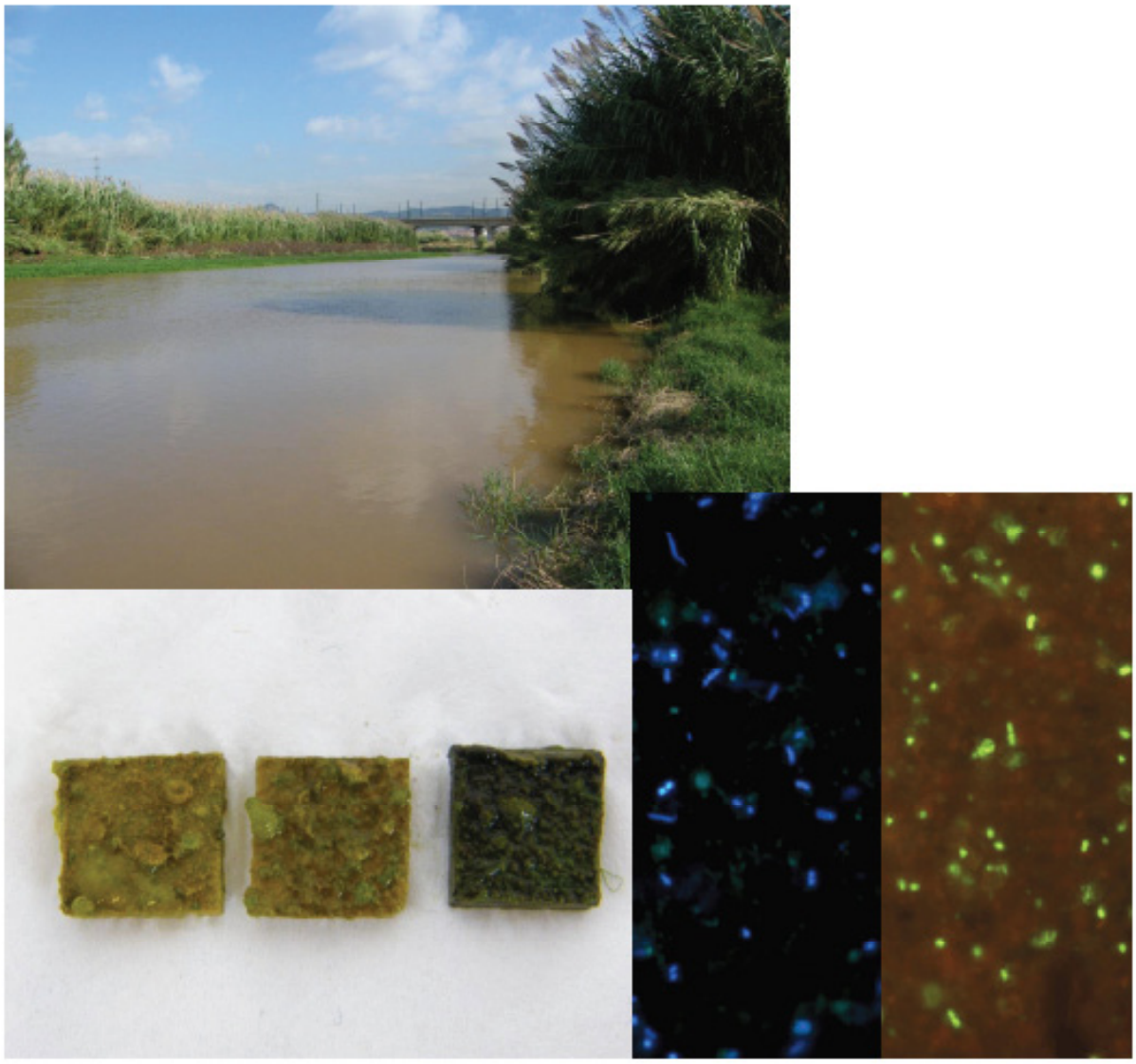
Our study evidenced how Ibuprofen may affect biomass of photosynthetic organisms of biofilm and their photosynthetic capacity as showed by Figure 3c. Moreover, Ibuprofen also was related to decrease of F1/F3 ratio as acetaminophen. This result could be explained by the stimulatory effect of ibuprofen on cyanobacteria growth at concentrations in the range of those measured in our study (Pomati et al., 2004).

Negative effects of ibuprofen on photosynthetic biomass of biofilm have been described

(Lawrence et al. 2005), and it results in both direct and indirect effects of ibuprofen on autotrophic organisms. Furthermore, ibuprofen resulted as one of the most effective compounds affecting invertebrate community structure of Llobregat River (Muñoz et al., 2009) and significantly affected food web in a long term microcosm experiment (Richards et al., 2004). The concentration addition approach justified that acute toxicity of ibuprofen and diclofenac mixture on algal growth can occur (Cleuvers, 2003). The same study concluded that these compounds act unspecifically by nonpolar narcosis on *Desmodesmus* and *Daphnia*. Our study did not show relationship between diclofenac and biofilm autotrophic metrics. In fact the increase of biofilm phosphatase activity was the only response clearly associated with diclofenac concentration increase. Diclofenac has been widely described as one of the more toxic compound group for aquatic organisms at multiple trophic levels (Cleuvers, 2003; Fent et al., 2006; Ginebreda et al., 2010). Our result suggested some direct effect of diclofenac on heterotrophic compartment of biofilms or indirect effects as a consequence of strong structural-functional relationships between autotrophs and heterotrophs within biofilm communities described in several studies (Bonnineau et al., 2010; Proia et al., 2011; Proia et al., 2012b; Ricart et al., 2011).

The present work confirmed that the measurement of the response of fluvial biofilms to translocations along pollution gradient is a sensitive tool to investigate effects of real mixtures of contaminants found in freshwater systems on biota. This tool showed clearly that the biological responses of biofilm community reflected water quality status of the sampled sites. The mixture of low concentrations of bioactive organic compounds that continuously reach the river systems may lead to chronic effects on biological communities. Using multivariate tools the most important compounds involved in the responses of fluvial biofilm were identified. Mediterranean rivers are extremely variable systems characterized by periodic floods and droughts that may modulate the relative importance of waste water treatment plants inputs as well as the capacity of communities to respond to chronic contamination. This study evidenced how non-regulated emerging compounds,

reaching continuously river systems, may become relevant during low flow periods, causing biological responses at the base of river food web with still unknown consequences for the whole freshwater ecosystem.



CHAPTER 5:

RESPONSES OF BIOFILM BACTERIAL COMMUNITIES TO ANTIBIOTICS IN RIVER WATERS: A TRANSLOCATION STUDY

RESPONSES OF BIOFILM BACTERIAL COMMUNITIES TO ANTIBIOTICS IN RIVER WATERS: A TRANSLOCATION STUDY

Abstract

Anthropogenic activities are increasing the levels of priority and emerging contaminants derived from pollution point and diffuse sources, which reach freshwater ecosystems. Antibiotics are bioactive compounds against bacteria in natural microbial communities, and their presence in aquatic environments may lead both to short-term physiological alterations, as well as to long-term changes in the microbial biomass or shifts in community composition. We investigated the structural and functional responses of biofilm bacterial community grown along an antibiotic pollution gradient in the River Llobregat, to translocation in river waters differently contaminated by antibiotics. Three sampling sites were selected: Castellbell (CB) and Mina de Terrassa (MT) as less polluted sites and Sant Joan Despí (SJD) as hotspot. River water was collected from each site and used as inoculum and medium for biofilm growing in independent mesocosms. After 25 days three biofilm translocations were performed: from MT to SJD, from CB to MT and from CB to SJD. Biofilm bacterial community composition, bacterial live/dead ratio and extracellular enzymes activities were measured twice during the experiment: one before and one after translocations. A total of sixteen antibiotic compounds were detected in river water from the three sampling sites. The family of sulfonamide antibiotics was the most concentrated at each sampling site followed by quinolones and macrolids families. The bacterial communities showed structural but not functional differences between sampling sites before translocation. Bacterial community structure changed after nine days of translocation. In particular an increase of Actinobacteria (HGC) was measured in all translocated biofilms. The Canonical Correspondence Analysis confirmed that communities were separated by sampling site/translocation and that Actinobacteria were associated to increasing antibiotics concentration. Biofilm communities translocated to water with higher antibiotics concentrations increased bacteria mortality and modulate heterotrophic metabolism, particularly

increasing Leucine-aminopeptidase and decreasing Alkaline phosphatase activities. Mantel test confirmed significant correlation between antibiotics concentrations and structural-functional biofilm responses. Our study showed that continuous entrance of antibiotics in freshwater systems may lead to structural and functional changes in microbial attached communities.

Keywords: Antibiotics, Biofilms, Llobregat River, Bacteria, Translocation

INTRODUCTION

Anthropogenic activities are increasing the levels of priority and emerging contaminants derived from pollution point and diffuse sources, which reach freshwater ecosystems. A large number of these compounds are detectable in river waters and their effects on biological communities are currently a matter of active research (Ginebreda et al., 2010).

In particular, antibiotics released into aquatic environments (e.g. ß-lactams, quinolones, tetracyclines, macrolides, sulphonamides; Kümmerer et al., 2009a) are a cause of concern, either as single compounds or in mixture, because of (i) the direct toxic effects at low concentrations on the aquatic microbes (Hernando et al., 2006) (ii) the potential to accelerate the acquisition of antibiotic resistance in several bacterial strains, including pathogens (Kümmerer and Henninger, 2003; Obst et al., 2006) (iii) the widespread and persistent contamination of water resources, since their mineralization is essentially due to the presence of microorganisms with specific catabolic activities (Brain et al., 2004; Costanzo et al., 2005; Pomati et al., 2006).

Between 30% and 90% of any administered dose of most antibiotics to humans and animals are excreted as active substance (Rang et al., 1999), and their occurrence is therefore common in aquatic environments (Gros et al., 2007, Luo et al., 2011; Managaki et al., 2007). Hirsch et al. (1998) detected the presence of 18 compounds from four classes of antibiotics in German surface water samples, and Watkinson et al. (2009) detected antibiotics in the 90% of freshwater, estuarine and marine samples in six river catchments.

Antibiotics are bioactive compounds against bacteria in natural microbial communities, and their presence may lead both to short-term physiological alterations, including cell death and altered metabolic functions (biomass production, respiration, and synthesis of extracellular enzyme activities), as well as to long-term changes in the microbial biomass or shifts in community composition (Bonnineau et al. 2010, Tlili et al. 2010). Most studies concerning antibiotic effects

in the aquatic environments have focused so far on planktonic microbial communities, and have disregarded the response of river biofilms. However microbial attached communities constitute the major component for the uptake, storage and cycling of carbon, nutrients and anthropogenic contaminants in many river sections (Pusch et al., 1998, Battin et al., 1999). Bacteria in biofilms occur together with other heterotrophs (fungi, protozoa), and autotrophs (diatoms, green algae and cyanobacteria), all of them embedded in an extracellular polymeric matrix (Lock, 1993). The mutual benefits and the close spatial relations between different life-strategy organisms strictly reflects the quality of the surrounding flowing waters, generating a complex micro-ecosystem in which specific cross-kingdom metabolic processes and interactions may take place (i.e. bacterial utilization of algal exudates, Murray et al., 1986). One of the key processes of biofilm bacteria is their capability to mineralize organic molecules, where extracellular enzymes have a major role (Pusch et al. 1998; Romaní, 2010; Proia et al., 2012), also influenced by the microbial interactions within the biofilm (Rier and Stevenson 2002; Francoeur and Wetzel, 2003). Keeping in mind this complexity, it is unknown whether or not the presence of antibiotics in natural waters modifies the structure and function of microbial communities in riverine biofilms.

The objectives of this study were (i) to investigate the structural and functional modifications of biofilm bacterial community along an antibiotic pollution gradient in the River Llobregat (Spain), (ii) to evaluate the bacterial responses to biofilm translocation in river waters differently contaminated by antibiotics. It was hypothesized that the biofilm communities inhabiting environments with lower antibiotic presence would be the most affected when translocated to more polluted waters and that this effect could be visible in the bacterial community composition of the biofilm. Testing this hypothesis in a field situation has been performed by means of translocation experiments. This methodology has been previously used for assessing the effects of metal pollution and industrial discharge in river biofilms (Ivorra et al., 1999; Victoria and Gomez, 2010; Tlili et al., 2011). The present study has analyzed the response of the biofilms after reallocating them in waters with progressively higher antibiotics contamination.

MATERIAL AND METHODS

Study Site and experimental design

The study site, the selected sampling points and the experimental design were already described in detail in the previous chapter (Chapter 4). In this experiment the biofilms were sampled two times during the experiment: before and 9 days after translocation. Glass tiles from each mesocosm were randomly sampled.

Physicochemical parameters

Conductivity, temperature, pH and dissolved oxygen were measured with appropriate multiparameter sensor probes (HACH LANGE GMBH, Germany) in the field and in the jars before and after each water change. Water samples were collected for nutrient content from the glass jars before and after water renewals. All the water samples were filtered (Nylon Membrane Filters 0.2 μm, WHATMAN, Maidstone, UK) prior to their analysis. Soluble reactive phosphorus was measured following Murphy and Riley (1992). Samples for anions and cations analysis were preserved frozen until analysis by ion-chromatography (761 Compact IC, METROHM, Herisau, Switzerland).

The concentrations of 16 antibiotics, belonging to different families, were analyzed in surface waters using the multiresidue analytical method based on LC-MS/MS after solid-phase extraction described by Osorio et al. (2011).

Biofilm metrics

The biofilm community structure was analyzed by Catalyzed Reported Deposition-Fluorescence In Situ Hybridization (CARD-FISH) and Denaturing Gradient Gel Electrophoresis

analysis (DGGE). Moreover, the live/dead bacterial ratio and the extracellular enzymatic activities β-Glucosidase, Leucine-aminopeptidase and Alkaline phosphatase were also measured at each sampling day as functional descriptors of biofilms bacterial compartment. The methodology used for each biofilm metric analyzed is described in detail in chapter 2.

Statistical analysis

Differences among biofilms before translocation and responses of biofilm metrics to each translocation were analyzed by ANOVA with sampling site and treatment as fixed factors. Effects were analyzed post hoc with Tukey's b test. Analysis was performed using SPSS Version 15.0. Cluster analyses of bacterial community composition determined by DGGE were performed with PRIMER 6.0 using Bray-Curtis similarity matrix. The relation between biofilm bacterial metrics and antibiotics concentrations was analyzed using Spearman correlation tests. Data were log-transformed before the analyses.

The multi-group SIMilarity PERcentage test (SIMPER) using the Bray-Curtis similarity measure was run on antibiotics dataset to assess which compounds were primarily responsible for the observed difference between all samples before and after the translocation. In the output table, antibiotics were sorted in descending order of contribution to group difference.

The Canonical Correspondence Analysis (CCA) was used to test how the environmental gradient of antibiotic concentrations affects the distribution patterns of the major bacterial groups in the analyzed samples (CARD-FISH analysis), before and after the translocation experiments.

The Mantel test was applied to identify the degree of correlation among five different Bray-Curtis similarity matrices (5000 randomized runs), computed by the combination of 1) the antibiotic concentrations, 2) the bacterial structural parameters (CARD-FISH and DGGE bands)

and 3) the bacterial functional parameters (Live/Dead ratio and extracellular enzymatic activities). For the multivariate analyses, all data were log-transformed and elaborated by the PAST software package (PAlaeontological STatistics, ver. 1.80).

RESULTS

Physicochemical water characterization

Dissolved oxygen (DO) and pH did not differ among sites, conductivity was relatively high in all the sampling sites (Table 1) and increase significantly in SJD ($p < 0.05$). Concentration of soluble reactive phosphorus (SRP) increased gradually downstream while nitrates (NO_3) concentration was similar in CB and MT and increased significantly in SJD ($p < 0.05$, Table 1). Discharge during the study period was in general low (< 7m^3 sec^{-1}) with significant lower flow in SJD respect to CB and MT (Table 1).

	Discharge (m^3sec^{-1})	**Conductivity (µS cm^{-1})**	**pH**	**Oxygen (mg L^{-1})**	**SRP (µgP L^{-1})**	**NH_4 (mg L^{-1})**	**NO_3 (mg L^{-1})**
CB	6.06	1649.5	8.52	10.89	44.0	0.09	7.40
	(0.50)	*(22.6)*	*(0.11)*	*(1.37)*	*(57.18)*	*(0.10)*	*(0.87)*
MT	4.85	1695.8	8.43	9.77	70.85	0.13	7.10
	(0.72)	*(38.7)*	*(0.06)*	*(1.09)*	*(50.73)*	*(0.03)*	*(0.83)*
SJD	2.91	2045.0	8.44	10.29	104.63	0.07	10.84
	(1.04)	*(67.4)*	*(0.13)*	*(1.38)*	*(38.04)*	*(0.04)*	*(2.62)*

Table 1. Results of physical-chemical variables measured at each sampling site. Values are expressed as mean values and SD in parenthesis (n= 16).

Antibiotics in water

A total of sixteen antibiotic compounds were detected in river water from the three sampling sites. Fifteen compounds from 4 different families of antibiotics (7 Macrolids, 5 Quinolones, 2 Sulfonamides, and 1 Tetracycline) were detected in river water (Figure 1). Moreover, the bacteriostatic antibiotic Trimethoprim (belonging to the class of chemotherapeutic agents) was detected at low concentrations in river water from each sampling site. Concentrations in Sant Joan Despí (SJD) were on average 6.1 times higher than in Castellbell (CB) and Mina de Terrassa (MT). The family of sulfonamide antibiotics was the most concentrated at each sampling site followed by quinolones and macrolids families. The most concentrated compound at each sampling site resulted Sulfamethoxazole that showed a peak of 1576 ng L^{-1} in SJD during the studied period. The most concentrated compound of the Macrolids family was Clarithromicin while among quinolones family Ofloxacine resulted the most concentrated one (Figure 1). The broad-spectrum antibiotic Tetracycline was detected at high concentration in SJD (average concentration = 336.61 ng L^{-1}) while concentrations in CB and MT were below 10 ng L^{-1}. SIMPER test indicated that 4 out of the 16 measured antibiotics had the major contributions to the overall average dissimilarity (19.9), respectively Enrofloxacine (2.7), Tetracycline (2.1), Roxythromycin (1.7), Ofloxacine (1.6).

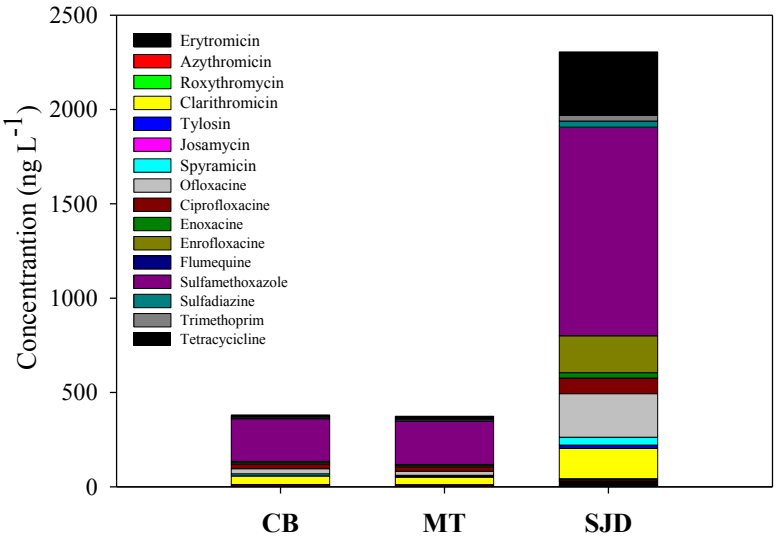


Figure 1. Average concentrations of antibiotics (n=9) measured at each sampling site.

Responses of biofilm bacterial community structure to translocation

The bacterial communities showed differences between sampling sites before translocation. The bacterial abundance increase significantly from CB to SJD ($p< 0.05$). In particular, CB biofilms resulted in bacterial density of 3.28 ± 0.81 x10^7 cells cm^{-2}, MT biofilms resulted 4.45 ± 1.48 x10^7 cells cm^{-2} and SJD biofilms resulted 5.70 ± 2.28 x10^7 cells cm^{-2}. The community composition analyzed by CARD-FISH highlighted that before translocation (Figure 2a), *Alpha-* proteobacteria abundance resulted significantly higher in SJD than in CB and MT, while *Beta* and *Gamma-* proteobacteria abundances were significantly lower in CB biofilms respect to MT and SJD ($p< 0.05$, Figure 2a). The Canonical Correspondence Analysis (CCA) of the bacterial community composition studied by CARD-FISH evidenced clear separation of communities grown in the three sampling sites and highlights the different pressure of antibiotic in the sites (Figure 3a). In particular, the Actinobacteria group (HCG) was highly associated to the 16 antibiotics compounds, and were important in SJD biofilms (Figure 3a). Differences in bacterial community composition of biofilms before translocation were confirmed by cluster analysis of DGGE gels (Figure 4a). The sequencing of selected bands highlight that the Betaproteobacteria *Variovorax paradoxus* (Burkholderiales; Comamonadaceae) was common in biofilms from the three sampling sites. The Alfaproteobacteria *Roseomonas lacus* (Rhodospirillales; Acetobacteraceae) and the Gammaproteobacteria *Legionella pneumophila* (Legionellales; Legionellaceae) were the two species only identified in biofilms from CB. The Alphaproteobacteria *Rhodobacter* sp. (Rhodobacterales; Rhodobacteraceae) and the Betaproteobacteria *Limnobacter* sp. (Burkholderiales; Burkholderiaceae) were detected in biofilms from MT. Finally, the Cyanobacteria *Cyanobium* sp. (Chroococcales) and the Betaproteobacteria *Acidovorax* sp. (Burkholderiales; Comamonadaceae) were two species only identified in biofilm from site SJD. The two complementary bacterial community structure analyses confirmed differences between communities and separated particularly SJD from CB and MT.

Bacterial community structure changed after nine days of translocation. Biofilms translocated from CB to MT showed a significant increase of Betaproteobacteria, Cytophaga-Flavobacteria and Actinobacteria ($p < 0.05$, Figure 2b), while only Actinobacteria significantly increased in MT→SJD biofilms ($p = 0.001$, Figure 2b). Gammaproteobacteria, Cytophaga-Flavobacteria and Actinobacteria increased significantly in the biofilm translocated from CB to SJD ($p < 0.05$, Figure 2b). The Mantel test confirmed the significant correlation between antibiotic concentrations and bacterial community composition analyzed by CARD-FISH (Table 2). The CCA of bacterial community composition studied by CARD-FISH evidenced clear separation of translocated biofilm communities and highlights the role of antibiotics (Figure 3b). In particular, Actinobacteria (HCG) increase observed in all translocated biofilms resulted associated to increasing antibiotics concentrations. The Spearman test confirmed the positive correlation between Actinobacteria abundances and concentrations of all antibiotics families ($R \geq 0.63$, $p \leq 0.005$).

The similarity analysis with the DGGE results showed that the translocated communities tend to shift to communities at the more polluted site where samples were translocated (Figure 4b), i.e. bacterial community of MT→SJD biofilm tends to shift to assemblage more similar to SJD

	Antibiotics	Structure	Function
Antibiotics	-	R= 0.003 p= 0.388	R= 0.403 **p= 0.013**
Structure	R= 0.108 **p= 0.041**	-	R= 0.015 p= 0.354
Function	R= 0.274 **p= 0.023**	R= 0.578 **p= 0.0002**	-

Table 2. Mantel tests between four different Bray-Curtis similarity matrices 1) the antibiotic concentrations, 2) the bacterial community structure (CARD-FISH and DGGE), and 3) function (live/dead ratio and enzyme activities) of biofilms, before and after the translocation experiments (respectively the upper and the lower part in the table). The Pearson's correlation coefficient (R) were calculated between all the entries in the two matrices and compared in 5000 random permutations. The reported p-values are one-tailed.

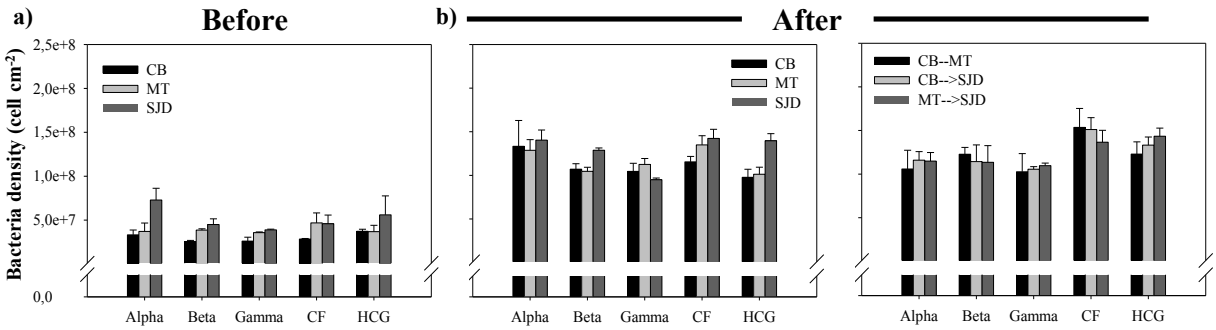


Figure 2. Abundances of the bacterial groups analysed by CARD-FISH before (a) and after (b) the translocation experiments. Values are means and standard deviations (n = 3).

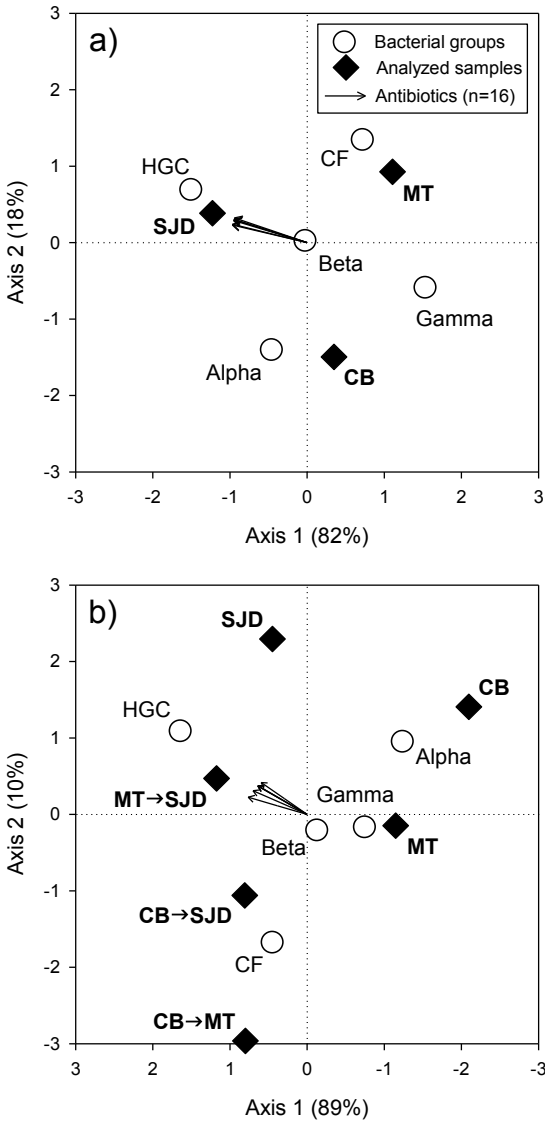


Figure 3. Responses of the biofilm bacterial groups to antibiotic contamination in river water before (a) and after (b) the translocation experiments as expressed by the Canonical Correspondence Analysis (CCA).

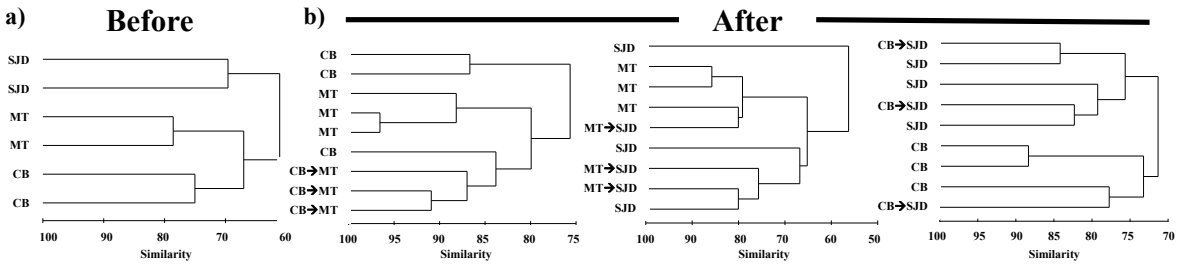


Figure 4. Cluster analysis based on Bray-Curtis similarity of biofilm bacterial community analyzed by DGGE: (a) before and (b) after translocation.

biofilms (Figure 4b).

Functional biofilm responses to translocation

Biofilm communities from the different sampling sites showed differences in bacteria viability. Particularly, biofilms in SJD before translocation had significantly lower proportion of live bacteria than in sites CB and MT ($p < 0.001$, Figure 5a). No significant differences in extracellular enzymatic activities were observed among sampling sites before translocation (Figure 5a). Mantel test analysis evidenced slight significant correlation between antibiotics and functional parameters analyzed before translocation (Table 2).

Bacterial community viability and functioning were affected after translocation. Significant increase of bacterial mortality was observed in the translocated biofilm of each translocation ($p < 0.001$, Figure 5b). Particularly, the most pronounced decrease of live/dead bacteria ratio was observed in CB→SJD biofilms (Figure 5b). The Spearman test revealed significant negative correlation between the live/dead bacteria ratio and tetracycline concentrations ($R = -0.551$ $p = 0.018$). Translocated biofilms showed a significant decrease in the extracellular peptidase activity, particularly when transferred to SJD ($p < 0.05$, Figure 6b). A significant decrease of extracellular phosphatase activity was measured in CB→SJD biofilms ($p = 0.008$, Figure 5b); however, β-Glucosidase activity did not change after translocation. The Mantel test confirmed the

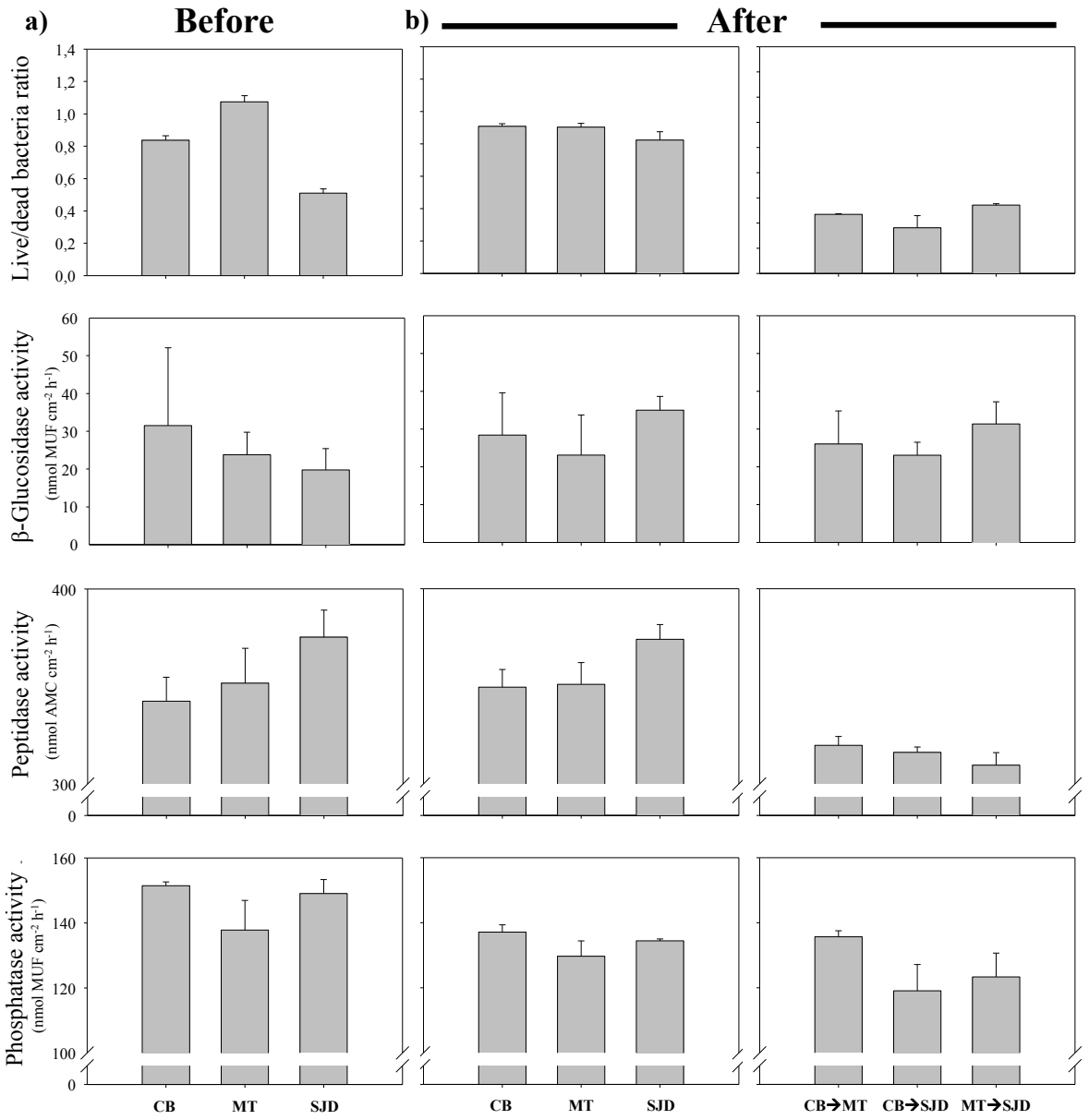


Figure 5. Functional responses of biofilm before (a) and after (b) the translocation experiments. Values are means and standard deviation (n = 3).

significant correlation between functional biofilms metrics and antibiotics concentrations after translocation (Table 2).

DISCUSSION

Antibiotics were detected in more than 90% of the water samples analyzed in the Llobregat, and concentrations were comparable (ng L^{-1}) to those observed in other impacted areas (Costanzo et al., 2005; Luo et al., 2011; Managaki et al., 2007; Watkinson et al., 2009). The entrances of antibiotics in running waters may occur either via point sources (PS) or non-point sources (NPS) (Watkinson et al., 2009). A continuous release of human used antibiotics in the aquatic environment is produced by PS waste waters, as well as NPS veterinary and agricultural used entering mainly after rainfall events. As a consequence, higher concentrations of antibiotics entering from PS should occur in densely populated areas during low flow dry periods (because of reduced dilution capacity) while peaks of compounds entering from NPS are expected in more rural zones during floods after rainfalls episodes. The higher concentrations of antibiotics in Sant Joan Despí are related both to the low flow recorded during the sampling (Table 1) as well as to the reception of a densely populated area. The most concentrated compounds were the sulfonamide Sulfamethoxazole, the macrolide Clarithromicin and the quinolone Ofloxacine, all of them used in human medicine. Thus, the input in the lower Llobregat was mostly via urban WWTP effluents.

The pollution gradient and differences in physical and chemical conditions might be responsible for the distinct bacterial community structure at the three selected sites. The higher proportion of dead bacteria in SJD biofilms could be explained by the influence of multiple pollutants on biofilms (Ricart et al. 2010, Muñoz et al. 2009, Proia et al. 2012). Furthermore, the higher autotrophic biomass observed in biofilm grown with Sant Joan Despí water (Chapter 4), may be another explanation for the higher proportion of death bacteria in these communities. An increased competition for resources as well as limited exchange with flowing water column

because of biofilm thickness could generate unfavorable conditions for bacteria resulting in increased proportion of death cells. Moreover, the direct effect of antibiotics on non-resistant bacteria could be an extremely relevant factor for the outcome of the bacterial community within the biofilm. The live/dead bacteria ratio was negatively correlated with concentrations of quinolones and sulfonamides ($R < -0.75$; $p < 0.05$). In particular, Sulfamethoxazole is an anti-bacterial sulphonamide that inhibits the synthesis of dihydrofolic acid, a compound which bacteria must be able to produce to survive (Isidori et al., 2005). At the same time, the patterns of bacterial diversity obtained by molecular fingerprinting under the different conditions showed that the bacterial communities were structured mainly by sampling site (Figure 4).

The capacity of bacteria to develop resistance to antibiotics has been widely described (Luo et al., 2010, Manivasagan et al., 2011, Storteboom et al., 2010, Schwartz et al., 2003). Moreover, antibiotics in water could represent a selective pressure during the first phases of biofilm development, when bacteria are early colonizers and cover the mineral surfaces with their polysaccharide glycocalix (Barlocher and Murdoch, 1989). The selection of different bacterial strains may occur in function of their resistance to antibiotics and other environmental stressors resulting in different assemblages at the end of the biofilm colonization process. The selective role of other chemical stressors (not as specific against bacteria as antibiotics) on bacterial community structure of biofilms has been demonstrated (Tlili et al., 2010). Our results showed the significant increase of the main bacterial groups abundances (*Alpha-, Beta and Gamma* - proteobacteria) in SJD. Nevertheless, these increases seem to be associated to the general increase of bacteria density along the pollution gradient that could be also explained by water trophic state. In fact, the multivariate analysis (CCA) revealed that despite the increase of all the proteobacteria groups in Sant Joan Despí the HCG (Actinobacteria) group was associated to antibiotics gradient. Antibiotics, which act directly on the bacteria, will cause rapid elimination of the most sensitive bacteria and consequently promote the development of other taxa not subjected to a strong competition thus

stimulating cell density increase (Fleeger et al., 2003; Le Jeune et al., 2007). Significant positive correlations between some antibiotics and abundances of some bacterial groups were observed in this work and confirm this hypothesis. Our study also revealed that the structural changes in the biofilm bacteria community before translocation were not associated with differences on the heterotrophic extracellular enzyme activities. This result confirms that the communities adapt to different physico-chemical conditions and are able to maintain similar functional levels.

Bacterial communities responded to translocation in terms of structure and function. The magnitude of the responses was associated with the magnitude of differences in antibiotics concentrations at sampling points. Particularly the most important responses were observed in communities transferred from less (Castellbell) to more (Sant Joan Despí) polluted site (CB→SJD). The significant increase of bacterial mortality when biofilms were translocated downstream (Figure 4a) could be associated to the presence of antibiotics in water. Most of the antibiotics detected in river water are able to differently induce the bacterial cell death; even though the concentrations are very low, their reactivity as well as the continuous arrival of these products can affect bacterial viability and community composition. The increase of antibiotics concentration of 5.5 times in Sant Joan Despí could explain this increase of mortality when communities were transferred at this point. Moreover, this increase was even higher in the case of the protein synthesis inhibitor Tetracycline (36.8 times higher in SJD than in CB and MT). The negative correlation between live/dead ratio and tetracycline concentration ($R = -0.551$ $p = 0.018$) confirms the significant role of this antibiotic in the induction of bacterial mortality in biofilm translocated to Sant Joan Despí. Nevertheless, bacterial mortality increase in biofilms translocated from Castellbell to Mina de Terrassa (CB→MT) suggests that some other factor could directly or indirectly induce bacteria mortality. In fact similar antibiotics concentrations at these two sampling points were detected (Figure 1). Indirect negative effects on bacteria of chemicals affecting authotrops of river biofilms have been already described (Bonninneau et al., 2010; Proia et al., 2012) and need to be also

considered. For example, the effect of Diuron on bacterial communities of treated biofilms has been observed and attributed to the indirect action of the herbicide on heterotrophs (Ricart et al., 2009; Lopez-Doval et al. 2009; Pesce et al 2006; Tlili et al. 2008). The complex trophic and metabolic interactions between autotrophs and heterotrophs within biofilm microbial community (Freeman and Lock, 1995, Wetzel, 1993, Rier el al., 2007) lies behind these observed indirect effects. The occurrence of many other priority and non-priority pollutants along the Llobregat River gradient described in other studies (Gros et al., 2007; Ricart et al., 2010) may be an additional source of direct and indirect stress for bacteria translocated to more polluted sites.

The translocation caused the increase of bacteria mortality, as well as the decrease of extracellular peptidase and phosphatase activities. These activities have been associated to heterotrophic bacteria that degrade high molecular weight molecules to obtain small peptides, amino acids and inorganic nutrients (Rosso & Azam 1987; Chrost 1991). Francoeur & Wetzel (2003) suggested that area-specific enzyme activity may be altered in 3 ways: (1) changes in the abundance of enzyme producing organisms, (2) changes in the amount of enzyme produced per organism, and (3) changes in the activity of individual enzyme molecules. Two of the antibiotics with higher concentrations in the Llobregat (Tetracycline, Clarithromycin) are protein synthesis inhibitors and may therefore affect the amount of enzyme per cell produced, such as peptidase and phosphatase; indication of this possibility is that phosphatase negatively correlated with tetracycline after translocation ($R= -0.621$; $p= 0.006$). However, the significant decrease of extracellular phosphatase activity measured in CB→SJD biofilms (Figure 4b) could also be related with the 2.3 times increase of inorganic phosphorus availability from CB to SJD (table 1), that could inhibit the phosphatase expression (Berman, 1970; Chrost and Overvebck 1987). Finally, as bacteria are the enzyme producing organisms, the increase of bacterial mortality in response to translocation could be another reason for the extracellular enzymatic activity decrease in translocated communities. The conjoint of these results highlight how mature communities

developed under different conditions maintain similar functional level while respond rapidly to changing environmental conditions.

Important changes in bacterial composition occurred in biofilms when transferred from less to more polluted sites. Particularly, the CCA evidences that HCG and CF were the groups associated with translocated communities and antibiotics gradient (Figure 3b). In fact, both groups increased in response to translocations (Figure 2b). High CG-content bacteria are Gram-positive bacteria of the class of Actinobacteria, a group that include some of the most common soil, freshwater and marine life microbes. The increase of this group could be directly related with the selective pressure of the antibiotics; this is confirmed by the positive correlation with all the families of antibiotics analyzed ($R > 0.63$; $p \leq 0.005$). Species of this bacterial group (and especially from the genus Streptomyces) are producers of hundreds of naturally occurring antibiotics (ex. Tetracycline, Streptomycin) (Watve et al., 2001). Antibiotics cause the rapid elimination of the most sensitive bacteria species and consequently promote the development of other more resistant taxa not subjected to a strong competition thus stimulating cell density increase (Fleeger et al., 2003; Le Jeune et al., 2007) as in the case of HCG in translocated biofilms. Similar selective pressure of antibiotics may account for the increase of Cytophaga Flavobacterium in translocated biofilms. CF is one of the most abundant bacteria group within aquatic environments (Kirchman et al., 2002). The positive correlation with tetracycline ($R = 0.491$ $p = 0.038$) suggests that resistant strains of this group of bacteria may be favored by high concentration of this antibiotic. Olapade and Leff (2004) demonstrated that this bacterial group clustered with conductivity, nitrates and soluble reactive phosphorus SRP in a study performed on epilithic biofilms of a US stream. Thus, the increasing conductivity, nitrates and SRP concentrations observed downstream in our study may be an additional co-factor explaining the CF behavior in translocated biofilm

Our study aimed to determine if the concentrations of antibiotics occurring in river waters can affect biofilm bacterial community structure and function. We showed that continuous entrance

of antibiotics in freshwater systems may lead to structural and functional changes in microbial attached communities. Even though other factors may interfere with the effects of antibiotics on microbial communities, their presence in urban areas needs to be considered a risk factor for aquatic ecosystems.

CHAPTER 6:

RESISTANCE AND RECOVERY OF RIVER BIOFILMS RECEIVING SHORT PULSES OF TRICLOSAN AND DIURON

RESISTANCE AND RECOVERY OF RIVER BIOFILMS RECEIVING SHORT PULSES OF TRICLOSAN AND DIURON

Abstract

The effects of the herbicide Diuron (DIU) and the bactericide Triclosan (TCS) were assessed on laboratory-grown stream biofilms. Four week-old biofilms were exposed in mesocosms to 48-hours of short pulses of either DIU or TCS. The direct and indirect effects of each toxicant on the biofilms, and the subsequent recovery of the biofilms, were evaluated according to structural and functional biomarkers. These parameters were analyzed immediately before exposure, immediately after exposure, and 9 and 16 days post-exposure. DIU caused an increase in diatom mortality (+79%), which persisted until the end of the experiment. TCS also affected diatom mortality (+41%), although the effect did not appear until one week post-exposure. TCS caused an increase in bacterial mortality (+45%); however, this parameter returned to normal values 1 week post-exposure. TCS compromised the cellular integrity of the green alga *Spirogyra* sp., whereas DIU did not. TCS also strongly inhibited phosphate uptake (-71%), which did not return to normal values until 2 weeks post-exposure. DIU directly affected algae, but barely affected the heterotrophs, whereas TCS seriously impaired bacteria (direct effect) as well as autotrophs (indirect effect). However, the biofilms recovered their normal structure and function within only a few days to a few weeks. These findings demonstrate the capacity of biofilms to cope with periodic inputs of toxicants, but also the risks associated to repeated exposure or multi-contamination in aquatic ecosystems.

Keywords:

Biofilms, Resistance, Recovery, Triclosan, Diuron, Pulses.

INTRODUCTION

Pollutants from agricultural, industrial and domestic activities enter watercourses either continuously (producing potentially chronic effects) or in pulses (causing potentially acute effects), in function of flow episodes, crop treatments and/or industrial release (Ellis, 2006). These chronic and periodic inputs are likely to have unexpected effects on the organisms living in aquatic environments. River ecosystems feature various ecological services (*e.g.* self-depuration and organic matter mineralization) directly related to processes driven by complex microbial communities (Mathuriau and Chauvet, 2002; Findlay et al., 1993; Sabater et al., 2007). These communities include benthic biofilms, which play a fundamental role in the trophic web and in the geochemical cycles within aquatic ecosystems (Battin et al., 2003; Lock, 1993). As interfaces between the water column and the substrata, biofilms are the first communities to suffer the consequences of pollutants (Sabater et al., 2007). Thus, understanding the resistance and resilience of biofilm communities to pollutants is crucial for ecological risk assessment of priority and emerging compounds.

This study analyzes the effects of two compounds on stream biofilms: the herbicide Diuron (DIU; 3-(3,4-dichlorophenyl)-1,1-dimethylurea), and the broad-spectrum bactericide Triclosan (TCS; 5-chloro-2-(2,4-dichlorophenoxy)phenol), which operate by different modes of action.

DIU is a photosynthesis inhibitor included on the list of priority pollutants of the EU Water Framework Directive (European Commission, 2000). As herbicide, DIU is active against phototrophic microorganisms and higher plants by blocking the chloroplast electron transport chain in Photosystem-II (Moreland, 1967). It has been used to control various annual and perennial broadleaf and grassy weeds, and is applied for vineyard protection. It has been also used on non-crop areas such as roads, garden paths and railway lines, and on many agricultural crops such as fruit, cotton, sugar cane, alfalfa and wheat (Giacomazzi and Cochet, 2004). Several studies have

reported the presence of DIU in surface waters (Azevedo et al., 2000; Blanchoud et al. 2004; Rodriguez-Mozaz et al., 2004).

TCS is active against both gram-positive and gram-negative bacteria. It is an inhibitor of the enzyme enoyl–acyl carrier protein reductase (ENR), which is involved in bacterial lipid biosynthesis (Adolfsson-Erici et al., 2002). For over 30 years TCS has been used in products such as anti-bacterial hand soaps, deodorants, household cleaners, dental hygiene products, and textiles (Singer et al., 2002). This emerging compound has been reported in sewage wastewater and sludge at significant concentrations (Halden and Paull, 2005; Samsøe-Petersen et al., 2003). Although wastewater treatment plants (WWTPs) are rather effective at removing TCS (Samsøe-Petersen et al., 2003; McAvoy et al., 2002), this compound still reaches freshwater systems, and has been reported in various aquatic habitats, including rivers, streams (Ellis, 2006; Kuster et al., 2008; Morral et al., 2004), lakes (Loos et al., 2007; Singer et al., 2002) and the sea (Xie et al., 2008). Both DIU and TCS have been widely tested for toxicity to myriad cultured aquatic organisms (Canesi et al., 2007; Capdevielle et al., 2008; De Lorenzo et al., 2007; Farré et al., 2008; Flaherty and Dodson, 2005; Giacomazzi and Cochet, 2004; Orvos et al., 2002; Wilson et al., 2003); however, they have not been extensively studied for toxicity to natural complex communities (Franz et al., 2008; Lawrence et al., 2009; Morin et al., 2010a; Pesce et al., 2006, 2008; Ricart et al., 2009).

DIU reaches running waters primarily via pulses from diffuse source, whereas TCS enters them periodically from fixed sources (chiefly, WWTPs). DIU pulses of up to 134.0 μg L^{-1} have been described during flooding events in vineyard catchments. These chronically affected environments show baseline concentrations of about 1 μg DIU L^{-1} between flood events (Rabiet et al., 2010). In contrast, TCS enters running waters chronically at low concentrations via urban sewage effluents, and its removal during wastewater treatment is variable (Ellis, 2006; Ricart et al., 2010). Measured TCS concentrations can be reduced to 80% on average trough waste water treatment plants (Kantiani et al., 2008), but still can reach river waters, where observed concentration range between 0.027

and 2.7µg L^{-1} (Ricart et al., 2009). In spite of these low concentrations, brief spikes of compounds such as TCS could occur during dry periods, and might result in transient perturbations of river ecosystems, with unknown long-term implications. These inputs may have specific or non-specific effects on both target and non-target organisms. Studying how biological communities are affected from these events is an ecological priority. Studying how communities recover from transient perturbations is important for assessing the risks associated with chronic contaminations

This study gauged the ability of biofilms to cope with short pulses of either DIU or TCS, assessing their initial responses and their subsequent recoveries. Considering the intrinsic complexity of biofilms, it was hypothesized that in addition to the specific effects of DIU on autotrophs, and of TCS on bacteria, these toxicants could provoke indirect effects deriving from ecological interactions at the microbial scale. The direct effects were expected to occur immediately upon toxicant exposure, whereas the indirect ones were expected to appear later on. Moreover, it was predicted that the time required for the biofilms to recover from the pollutant pulse could be correlated to the presence of target organisms (direct effects) and non-target organisms (indirect effects) in the biofilms. Given that indirect effects can involve much more complex mechanisms (Ricart et al., 2009), they can imply longer recovery times. Thus, the core hypothesis in this work was that early recovery of biofilm endpoints could be related to direct effects of pollutants, whereas late recovery could be related to indirect ones. In order to verify our hypothesis toxicants concentrations were selected after considering ecotoxicological data available (i.e. EC50, NOEC) both on single cultured species and on natural biofilm communities. It has been demonstrated how complex biofilm communities can result more resistant to toxicants than single species composing it (Franz et al., 2008). Several studies demonstrated the protective function of extracellular polymeric substances produced by biofilm organisms (Admiraal et al., 1999; Samrakandi et al., 1997). These evidences were considered for the selection of toxicants concentrations as well as for

consequent comparison of results with other studies.

In this work, 4-week old biofilms were subjected to 48-hours of short pulses of either DIU or TCS. Their responses to these compounds were measured just after exposure, and then once weekly for 2 weeks post-exposure. Each toxicant's effects on the biomass and survival of algae and bacteria were used to gauge their respective effects on biofilm structure. Furthermore, their effects on extracellular enzymatic activity and phosphate uptake were used to assess the ecological implications of their entry into running water—namely, in the context of nutrient retention and river self-depuration.

MATERIAL AND METHODS

Experimental design

Biofilms were scraped from rocks of the Fuirosos Stream, a third order pristine stream located in the Natural Park of Montnegre-Corredor, (50 km N of Barcelona, NE Spain), and then inoculated and colonized in twelve independent mesocosms. They were colonized on glass slides (1 cm^2 each) placed at the bottom of each mesocosm (35-40 slides per mesocosm).The mesocosms comprised sterile glass jars (19 cm in diameter, 9 cm high), filled with 1.5 L of artificial stream water which was recirculated using a submersible pump (Hydor, Pico 300, 230 V 50 Hz, 4.5W). Artificial stream water was produced adding pure salts to MilliQ water (Millipore) as described in Ylla et al. (2009). To avoid nutrient depletion the water in each mesocosm was changed twice weekly. All mesocosms were maintained in an incubator (Radiber AGP-570) under controlled temperature (17.5 ± 1.1 °C) and light irradiance (160-180 µmol photons m^{-2} s^{-1}; darkness/light cycle of 12 h/12 h). After 4 weeks colonization, four mesocosms were treated with TCS (IRGASAN,

Sigma Aldrich, >97% , CAS: 3380-34-5) up to nominal concentrations of 60 mg/L, and another four, with DIU (Sigma Aldrich, minimum 98%, CAS: 330-54-1) up to nominal concentrations of 15 mg/L. The last four mesocosms were left untreated and used as control. To minimize photodegradation of the toxicants and ionization of TCS (pKa = 8.1), water and toxicants were renewed every 3 h (during the light cycle) for 48 hours. The pH was monitored between water changes during toxicant exposure. After the exposure period, the mesocosms were refilled with unpolluted artificial river water (as described above) that was changed twice per week during the following 2 weeks. The biofilm was sampled four times: before contamination (day 0), after the 48-hour exposure (day 2), and 1 and 2 weeks post-exposure (days 9 and 16).

Glass tiles from each mesocosm were randomly sampled. Extracellular enzyme activities, photosynthetic parameters (F_0, Y_{eff} and Y_{max}), and bacterial densities were immediately measured after collection. Diatom samples for enumeration and taxonomical identification were preserved in formalin before being processed. Samples for chlorophyll determination were frozen (-20 °C) until analysis. Samples for Scanning Electron Microscopy (SEM) observation were collected at day 16. The phosphorus uptake (P-uptake) of the biofilm in each mesocosm was experimentally determined on each sampling day.

Water analysis

Concentrations of TCS, methyl-Triclosan (Me-TCS) and DIU were determined using high performance liquid chromatography (HPLC). Stock solutions (1 mg/mL) were prepared by dissolving pure standards of the highest purity available (HPLC grade, Sigma Aldrich) in methanol. An external calibration curve was then built for each compound by injecting different concentrations of individual standards prepared by different dilutions of the stock solution.

Water samples were collected once from each mesocosm. Samples were filtered through 0.45mm

nylon membrane filters (Whatman) and immediately loaded onto C18 SPE cartridges (Sep-Pak® Vac 3 cc tC18, Waters, Ireland) previously conditioned with 5 mL of HPLC water and methanol at a flow rate of 1 mL min^{-1}. Samples (500 mL) were loaded at a flow rate of 5 mL min^{-1}. After pre-concentration, the cartridges were completely dried *in vacuo* for 20 minutes to avoid hydrolysis and kept frozen until analysis. Thereafter, cartridges were eluted with 4 mL of methanol. Eluted samples were partially evaporated under a gentle nitrogen stream and reconstituted in a final volume of 1 mL methanol. Samples were then analyzed by liquid chromatography. The HPLC system comprised a binary HPLC Pump (Waters 1525), an auto sampler (Waters 717 Plus) and a UV-detector (Waters 2487 Dual λ Abs. Detector). The HPLC separation entailed use of a µm C_{18} reverse-phase column (Sunfire 4.6x150 mm). For DIU analysis, the injection volume was set at 20 mL and separation was performed using an isocratic gradient of 45% methanol/55% water at 0.8 mL min^{-1}. The DIU peak was detected at 251 nm. For TCS and Me-TCS analysis the injection volume was set at 50 mL and the flow rate was 1 mL min^{-1} of 90% methanol with isocratic flow. The TCS and Me-TCS peaks were detected at 280 nm.

Biofilm structure and function

Several biofilm endpoints were measured in order to describe structural and functional responses of autotrophic and heterotrophic biofilm compartments of biofilms to toxicants pulses. In particular the structure of the autotrophic community was investigated by measuring the Chlorophyll-*a* density (as surrogate of autotrophic biomass) as well as the growth rate, mortality and composition of diatom community. The structural response of heterotrophs was investigated by counting the live and dead bacterial cells. This counting allowed the calculation of live/dead ratio and therefore provides information about bacterial mortality. Scanning Electron Microscopy (SEM) observations were useful to compare the structure of treated and non-treated biofilm at the

end of the experiment. The *in vivo* fluorescence measurements described the functional response of phototrophic organisms (green algae, diatoms and cyanobacteria). The extracellular enzymatic activities described the heterotrophic capacity to degrade organic matter. Finally, the phosphorus uptake rate measurement was used to describe the capacity of the whole biofilm community to remove phosphate from the water column. The methodology used to analyze the biofilm descriptors measured is described in detail in chapter 2.

Statistical tests

Differences in the biofilm endpoints were tested daily using one-way analysis of variance (ANOVA), in which treatment (DIU or TCS) was set as the fixed factor. Effects were analyzed *post hoc* with Tukey's b test. Statistical significance was set at $p = 0.05$. Analysis was performed using SPSS Version 15.0. Growth rates were calculated from the slope of the linear portion of a curve showing the log of the cell number as a function of time, as described by Morin et al. (2008), and tested with ANOVA.

RESULTS

Physical and chemical conditions in the mesocosms

Conductivity, pH and dissolved oxygen in the mesocosms remained steady during the experiment: their mean values were 143.4 (± 34.1) µS cm^{-1}, 7.9 (± 0.5), and 9.2 (± 0.2) mg L^{-1} (n= 48), respectively. SRP concentration ranged from 16.9 (± 2.1) to 4.5 (±1.9) µg L^{-1} between water replacements. pH was monitored between water changes and ranged from 7.45 (± 0.02) to 7.62 (±0.04) (n = 8), never reaching the TCS pKa value of 8.1.

During the 48 hours of toxicant exposure, the DIU-contaminated mesocosms had 13.4 ±

1.3 µg DIU L^{-1} (n = 4), and the TCS-contaminated mesocosms, 60.8 ± 30.1 µg TCS L^{-1} (n = 4). The level of Me-TCS in the TCS-contaminated mesocosms was consistently below the detection limit. The water of control mesocoms resulted in no trace of both DIU and TCS (n = 8).

Biofilm structure: microbial biomass, diatom community composition, and SEM observations

Before toxicant exposure, the biofilms had chlorophyll-a concentration of 3.04 ± 0.72 µg cm^{-2} and a mean bacterial density of 3.06 ± 1.14 x 10^7 cells cm^{-2}. The diatom community was dominated by *Achnanthidium minutissimum* (Kützing), and also contained *Achnanthidium biasolettianum* (Grunow), *Ulnaria ulna* (Nitzsch) and *Gomphonema*. Live diatom density was 11.3 ± 3.7 x 10^4 cells cm^{-2}, and the diatom live/dead ratio was 10.9 ± 0.9. The bacteria live/dead ratio was 1.48 ± 0.58.

Chlorophyll-a density increased in all the treatments from day 0 to day 16 (Fig. 1a); however, that of the DIU-treated biofilms was not significantly different than that of the control biofilms. Chlorophyll-a density in TCS-treated biofilms decreased relative to that of the control by day 2, and subsequently increased significantly ($p = 0.026$ and $p = 0.007$, for days 9 and 16, respectively) (Fig. 1a).

Live diatom density increased exponentially (Fig. 2) from day 0 to day 16 in all treatments. Diatoms growth rates (Fig. 2) were higher (0.14 divisions day^{-1}) in DIU-treated biofilms than in either control or TCS-treated biofilms (Fig. 2; $p = 0.001$). Diatom composition did not significantly change in either DIU or control biofilms, but *Achnanthidium minutissimum* became dominant in the TCS-treated biofilms. The diatom live/dead ratio significantly decreased in DIU-treated biofilms starting from day 2 and remained lower than that of the control biofilms until the end of experiment (Fig. 1b). Diatoms of TCS-treated biofilms responded late to exposure (Fig. 1b): the live/dead ratio significantly decreased by day 9 (7.4 ± 0.7; 69% of the control value; p =

0.001), recovering moderately by day 16 (Fig. 1b). DIU exposure did not affect bacterial mortality. However, TCS did cause a significant but temporary surge in bacterial mortality (145% of the control value; $p < 0.001$; Fig. 1c).

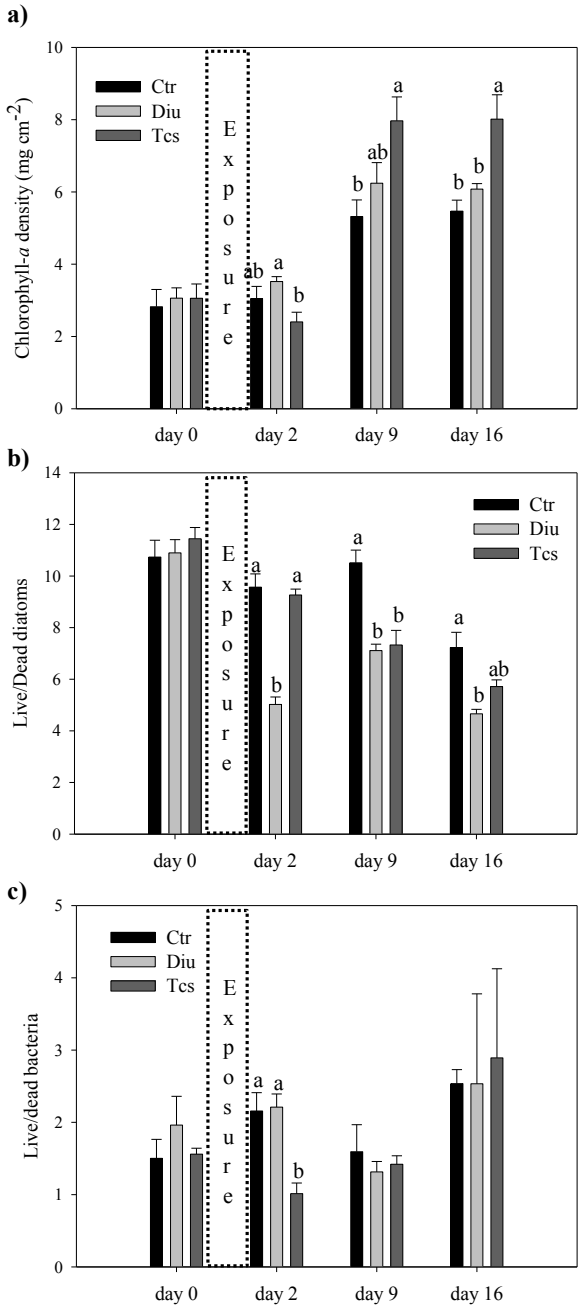


Figure 1. Changes of control (black) and treated (greys) biofilms in following endpoints: (a) Chla density; (b) live/dead diatom ratio and (c) live/dead bacteria ratio in each sampling date. Values are means and standard deviation (n = 4). Post-hoc Tukey-b analysis results are showed when treatment effect resulted significant. Statistical signicance was set at $p \leq 0.05$ (one-way ANOVA).

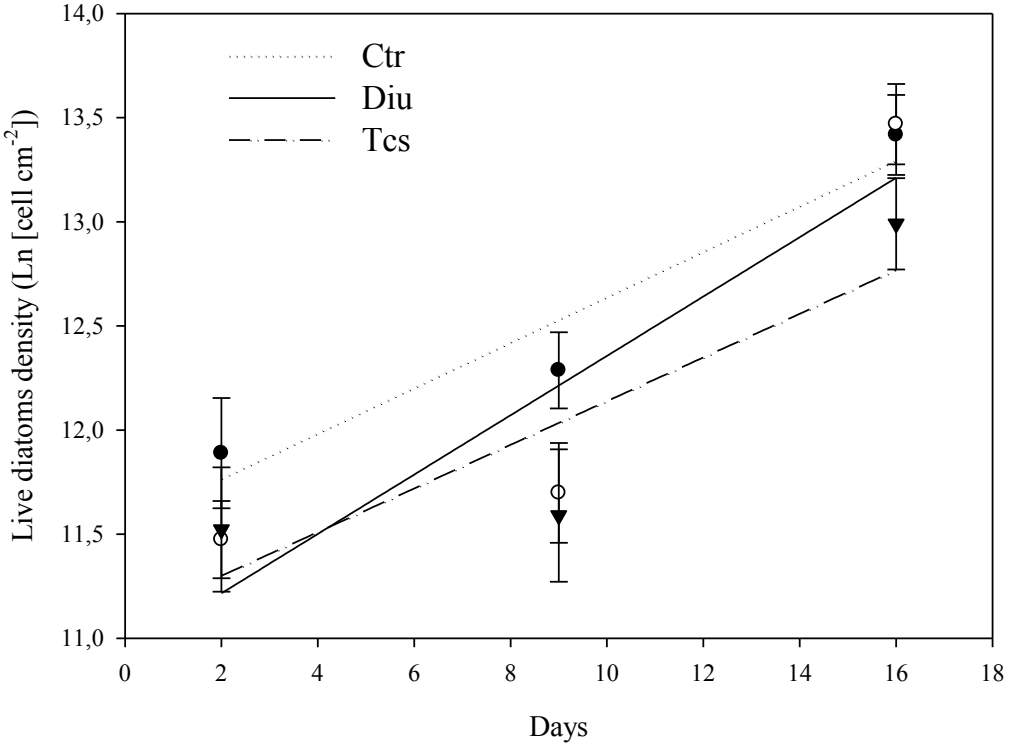


Figure 2. Diatom community evolution during experiment. a) Increase of live cell density from day 2 to day 16. Slope of linear curve is the grow rate (div day^{-1}) of diatoms.

The SEM showed that the filamentous green alga *Spirogyra* sp. thrived with unharmed filaments both in control and DIU-contaminated mesocosms (Fig. 3, day 16). However, the *Spirogyra* filaments were less abundant and visually damaged, hosting abundant epibionts (*Achnanthidium* species in Fig. 3b and c) in TCS-treated mesocosms. The green algae contribution to the total chlorophyll *a* content of the TCS-treated samples was less than 0.5% (data derived from fluorescence, not shown).

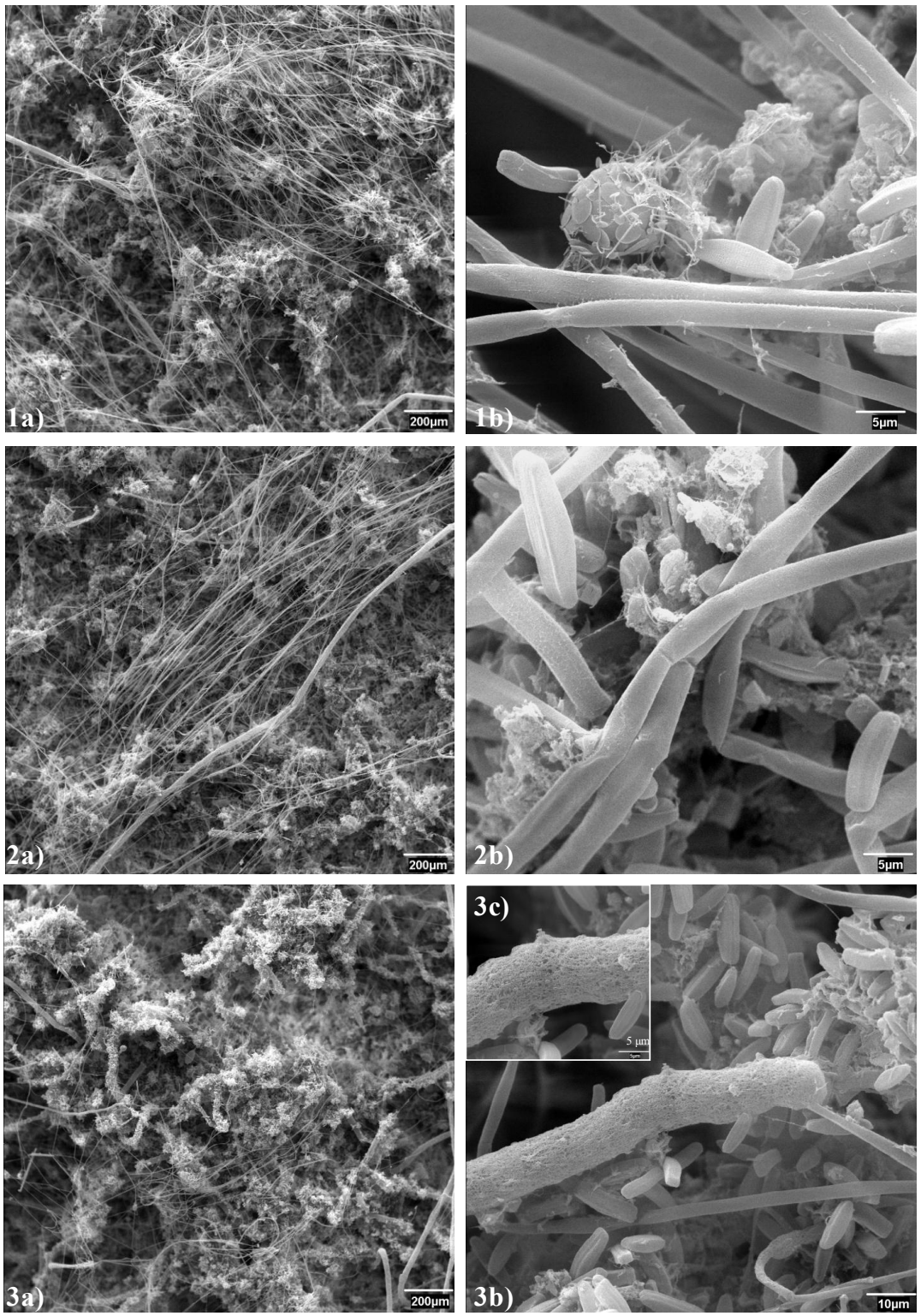


Figure 3. SEM pictures of Control (1), DIU-treated (2) and TCS-treated (3) biofilms at day 16.

Biofilm function: photosynthesis, extracellular enzyme activities, and phosphorus uptake

The results from photosynthetic capacity are included in Figure 4 while results from extracellularar enzymes are reported in this section. The extracellular enzymatic activity and the physiological parameters of the biofilms were similar among mesocosms before toxicant exposure. Leucine-aminopeptidase activity was 419 ± 78 nmol AMC cm^{-2} h^{-1} and alkaline phosphatase activity was 136 ± 21 nmol MUF cm^{-2} h^{-1}. Photosynthetic capacity was 0.47 ± 0.06 photon yield and the photosynthetic efficiency was 0.4 ± 0.05 photon yield. The P-uptake before exposure was 2.1 ± 0.3 µg P cm^{-2} h^{-1}.

After 48 hours treatment, DIU significantly affected both photosynthetic efficiency ($p < 0.001$) and capacity ($p = 0.004$), whereas TSC did not significantly affect either one (Fig. 4). However, in the DIU-treated biofilms, both photosynthetic parameters returned to normal levels by 1 week post-exposure.

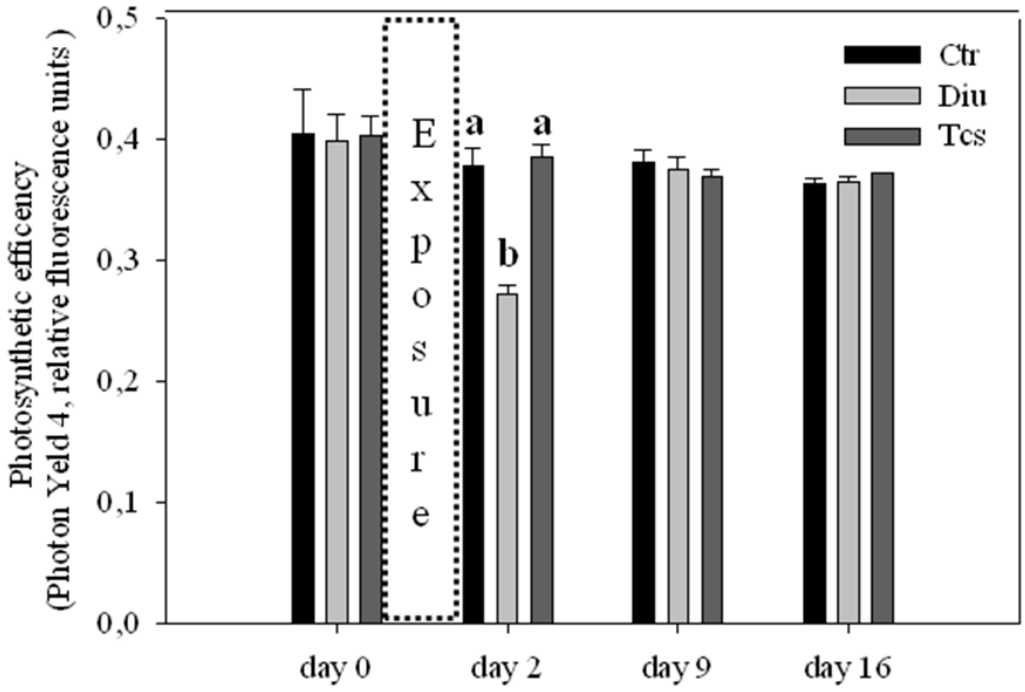


Figure 4. Changes of control (black) and treated (greys) biofilms in community photosynthetic efficency; in each sampling date. Values are means and standard deviation (n = 4). Post-hoc Tukey-b analysis results are showed when treatment effect resulted significant. Statistical signicance was set at $p \leq 0.05$ (one-way ANOVA).

Extracellular enzyme activity did not differ among the different biofilms and generally increased with time. Phosphatase activity increased up to 266 ± 28 nmol MUF cm^{-2} h^{-1} (day 9), and leucine-aminopeptidase increased up to 544 ± 58 nmol MUF cm^{-2} h^{-1} (day 9). These temporary changes were not associated to the effects of DIU or TCS. In contrast, DIU had enhanced specific extracellular enzyme activity per cell by day 2: the DIU-treated biofilms exhibited an increase in specific alkaline phosphatase and leucine aminopeptidase activity per cell, up to 250 % of the values in the control biofilms (data not shown). Both specific activities returned to normal levels by 1 week post-exposure (day 9).

P-uptake was not affected in the DIU-treated biofilms, but decreased significantly in the TCS-treated biofilms (Fig. 5): at day 2 its value was 1.1 ± 0.3 µg P cm^{-2} h^{-1} (29.3 % of the control value; $p < 0.001$). The TCS-induced effects persisted until 1 week post-exposure: at day 9 P-uptake was 2.1 ± 0.1 µg P cm^{-2} h^{-1} (77.4 % of the control value; $p = 0.001$), although by day 16, it had returned to normal levels (Fig. 5).

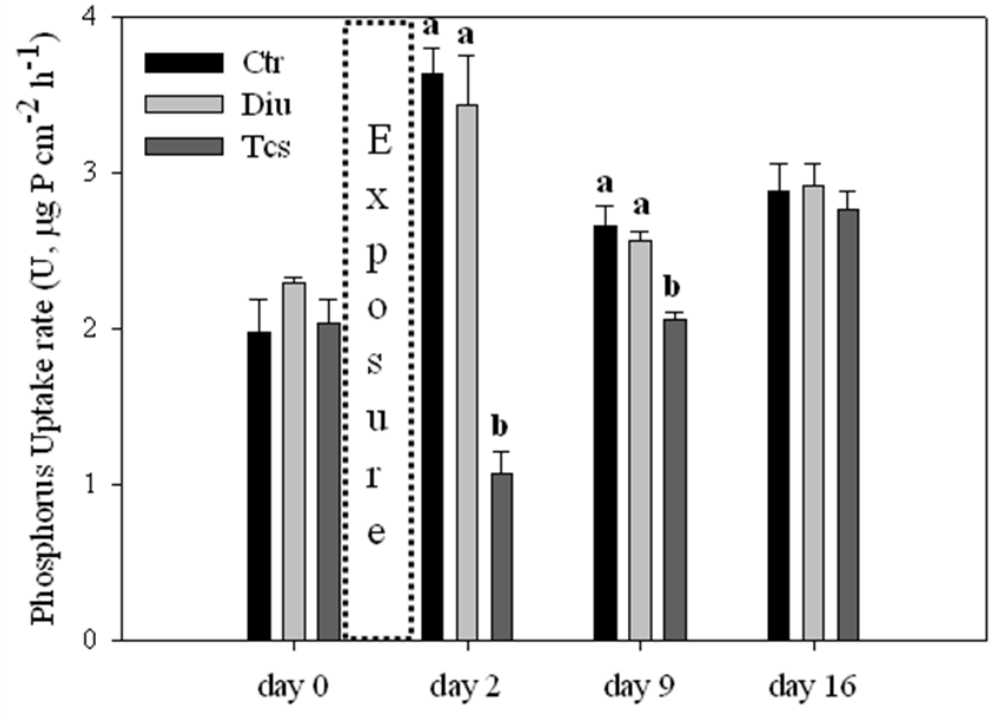


Figure 5. Changes in Phosphate Uptake rates (U) of control (black) and treated (greys) biofilms in each sampling date. Values are means and standard deviation (n = 4). Post-hoc Tukey-b analysis results are showed when treatment effect resulted significant. Statistical signicance was set at $p \leq 0.05$ (one-way ANOVA).

DISCUSSION

Several studies have investigated the effects of DIU (Pesce et al., 2006; Ricart et al., 2009; Tlili et al., 2008) and TCS on river biofilms (Franz et al., 2008; Lawrence et al., 2009; Ricart et al., 2010). Nevertheless, these studies mainly used dose-response designs, and exposure of biofilms to the toxicants, to describe the effects of chronic contamination. The results reported in the present work show that short pulses of either compound also affect natural biofilm communities, and that post-pulse behavior depends on the toxicant used and on the endpoint considered. Due to the different target of the two toxicants and the interactions between autotrophs and heterotrophs within the biofilm, direct and indirect effects are highlighted. Moreover, the study of the post-pulse behavior shows the timing of these effects (either rapid or delayed) as well as potential recovery (return to values not significantly different respect to the control). The multi-biomarker approach (Boninneau et al., 2010) employed in this study enabled description of direct and indirect effects, as well as their recovery, associated with short pulses of either toxicant. Short-term pulses can be considered as transitory disturbances, which can generate responses in the structure and function of fluvial biofilms. Whether the responses after these short-term stressors are immediate or delayed in time, responses depend on the organisms directly or indirectly targeted by the stressor, as well as on the mechanism associated to the measured parameter. Once a significant response occurred (i.e. increase or decrease of some activity; increase or decrease of mortality; shift in community composition etc.), the effects can persist in time, or instead recover to the original status. We considered that parameters recovered when values after the disturbance were close to values in the control after the disturbance. However, the significance of disturbances and the recovery at the ecosystem scale is a more complex subject that the one being dealt in the paper at a mesocosms scale, and therefore cannot be directly extrapolated to real systems..

The short pulses of DIU inhibited photosynthetic efficiency and capacity and increased

diatom mortality. These effects are related to its inhibition of photosynthesis via blockage of electron transport in photosystem II (Van Rensen, 1989). Several studies have confirmed this effect on natural epipelic and epilithic biofilm communities (Legrand et al., 2006; López-Doval et al., 2010). However, the short-term pulses had transient functional effects on the autotrophs and the photosynthetic parameters had rapidly recovered (returned to control values) by 1 week post-exposure. On other hand, recovery of functional parameters may hide specific effects on community (i.e. species replacement, composition shift). Ricart et al. (2009) evidenced how chronic DIU exposure induced shift in diatoms community composition and decrease in diatoms biovolume. Nevertheless the same study also evidenced that photosynthetic parameters did not recover despite the shift of community composition. The recovery of photosynthetic parameters in our study occurred despite the low resistance of diatoms and their slow recovery after 48 hours of short pulses (10 μg L^{-1}) of DIU. The significant increase in diatom growth rate (Fig. 2) and the absence of shift in community composition after the DIU pulses might indicate that the diatoms recovery was occurring at the end of the experiment. These results could be explained by the short time of exposure leading to transient direct effects on photosynthesis and on diatoms viability. A relevant side-effect was that algal biomass (chlorophyll-*a*) was moderately enhanced by DIU exposure. Other authors have also observed this increase in chlorophyll density and have related it to the interruption of electron flow in PSII provoked by DIU (Ricart et al., 2009; Tlili et al., 2008), as well as to the induction of shade-type chloroplasts with a higher concentration of photosynthetic pigments (Chesworth et al., 2004). On the other hand any significant effect of DIU on bacterial viability has been observed. In other studies, DIU did indirectly affect bacteria mortality and extracellular enzyme activity, but these studies involved long-term exposure (Ricart et al., 2009). Moreover, chronic exposure has been demonstrated to induce shift in bacterial community composition of biofilms in the case of DIU (Pesce et al., 2006; Tlili et al., 2008)

The moderate effects on periphyton structure and function after the DIU pulses showed in this study are consistent with the three-stage model proposed by Mølander and Blanck (1992). This model joins various effects of DIU on periphyton structure and function. In the first stage no long-term effects can be detected in spite of short-term effects, such as inhibition of photosynthesis. The second stage would be characterized by slight long-term effects such as the increase of chlorophyll-*a*. The final stage would occur when the sensitive species would be eliminated resulting in restructured community and increased community tolerance. Achieving this last stage should imply that the diuron stress would be sufficiently severe to cause cell mortality. The recovery of photosynthetic parameters and the increase of chlorophyll-*a* density evidenced in this study suggest that 48 hours exposure to 13µg L^{-1} of DIU can be considered a threshold between first and second stage impact (Mølander and Blanck, 1992) in biofilm communities.

In contrast to DIU, the mode of action of the bactericide TCS leads to a strong direct effect on bacterial viability in the biofilm. TCS might be inhibiting fatty acid synthesis and bacterial growth (Escalada et al., 2005). However, one week after the end of exposure, live/dead bacteria ratio values were similar than controls indicating a recovery of the bacterial community. Nevertheless, considering the biology and the short life cycle of bacteria, selection of resistant species and consequent shift in community composition could occur, although this would be most probably in response to longer exposure time. However, to our knowledge, no experimental data are available about chronic contamination effects of TCS on biofilm bacteria.

Although TCS has chiefly been described as a bactericide, it also significantly affected autotrophs (non-target organisms). Diatom mortality increased for 1 week post-exposure (day 9), and TCS exposure retarded development of the diatom community relative to the control. These results may reflect a delayed direct effect of TCS on diatoms or an indirect effect of bacteria mortality as a result of the tight interaction between these two biofilms components. The difficulty

of growing axenic cultures of benthic diatoms (Bruckner and Kroth, 2009) demonstrates that diatoms require bacteria for proper development (*e.g.* bacteria vitamin production for algae; Croft et al., 2005). The delayed effect on diatoms (day 9) could have been a late indirect response to the increase in bacterial mortality that had occurred on day 2. This scenario is corroborated by the fact that the diatoms recovered within 1 week after the bacteria had recovered. Chlorophyll concentration had decreased after 48 hours of TCS exposure, but eventually returned to normal levels. The negative effect of TCS on chlorophyll-a, described elsewhere (White et al., 2005), has been associated to modifications of biofilm architecture (Lawrence et al., 2009). In the present work, SEM images (Fig. 3) showed that TCS had damaged *Spirogyra* sp. filaments and reduced chlorophyll density. The effects of TCS on the cell walls of *Spirogyra* sp. can be related to its blocking of fatty acid synthesis. This has been described in bacteria (McMurry et al., 1998), and has been reported to compromise permeability-barrier functions (Phan and Marquis, 2006) and to destabilize cell membranes (Villalaín et al., 2001). Although no specific mode-of-action for TCS has yet been established for algae, in some aspects this contaminant could affect algae similarly to the way it affects bacteria (Lawrence et al., 2009; Ricart et al., 2010; Morin et al., 2010b). Moreover, some studies have described that algae are more sensitive to TCS than are bacteria (Tatarakazo et al., 2004).

Neither DIU nor TCS affected the extracellular activity of either phosphatase or leucine-aminopeptidase in any of the biofilms. This indicates that the toxicant pulses did not compromise the ability of the biofilms to process organic matter (proteins) or organic phosphorus, despite the increased microbial mortality. Thus, one could infer that the biofilm maintained certain major functions even when its constituent organisms were directly or indirectly affected. This could be explained by either a change in the bacterial community or by a relatively higher specific activity per live cell (Francoeur and Wetzel, 2003). In fact, normalization of extracellular activities per live

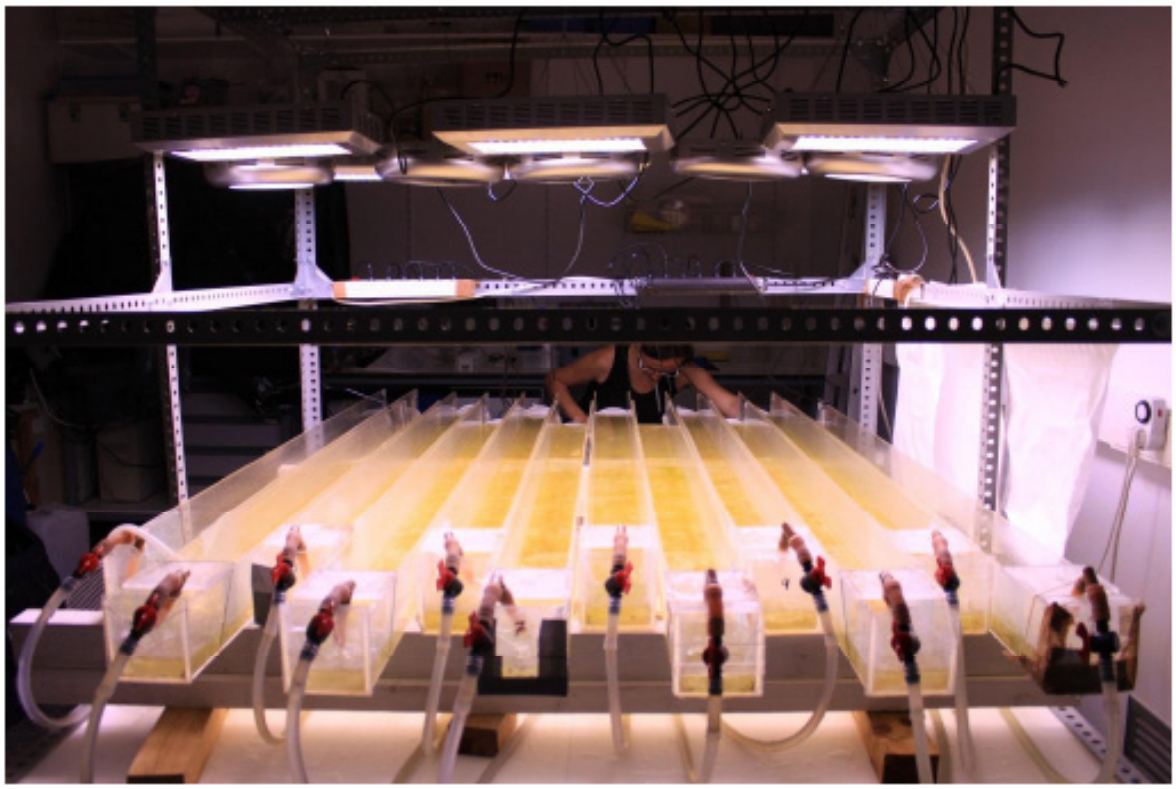
bacteria cell revealed that each toxicant had a distinct effect. In the TCS-treated biofilms, specific activity per cell was not affected, suggesting that an alternative mechanism dictates extracellular enzymatic activity in the biofilms. In contrast, the DIU-treated biofilms exhibited significant increases in both specific phosphatase and specific peptidase activity per live cell by day 2. Ricart et al. (2009) reported a similar indirect effect of DIU on the metabolism of live bacteria in the long-term. They concluded that the increase in extracellular leucine-aminopeptidase activity per live cell was a response to the release of proteinaceous material from DIU-induced lysis of algae cells.

DIU did not affect P-uptake, whereas TCS did. The bacterial death caused by TCS, together with its delayed indirect effect on diatoms and its toxicity to *Spirogyra* sp., could have caused the reduction in P-uptake. The fact that damage to heterotrophs and autotrophs generally had negative consequences for P-uptake highlights the utility of this endpoint as a descriptor of total biofilm function. Given that biofilms are the most important compartment in the biotic removal of inorganic dissolved nutrients from water columns (Sabater et al., 2007), TCS-induced loss in P-uptake is a clear threat to this ecological service of river ecosystems, which purifies water.

The results obtained with the large set of biomarkers confirm the central hypothesis of this work: that direct effects on target organisms would occur earlier and are recovered in a short period (especially if only physiological mechanisms were affected). The strong resistance of bacteria to DIU, and the rapid recovery of photosynthetic parameters following exposure to DIU, are consistent with the toxicant's specific mode-of-action, and support the aforementioned hypothesis. Nevertheless, slow recovery of diatoms suggests that a combination of direct and indirect effects could be at play. The hypothesis on delayed effects from indirect interactions was confirmed by the results from the TCS exposure experiments. These effects on diatom mortality appeared one week post-exposure and delayed biofilm recovery. Biofilms were more resistant and

resilient after the DIU pulses than after the TCS pulses. The fact that DIU did not affect P-uptake, and that TCS did, agrees with the more complex behavior of direct and indirect effects associated to this community function.

To conclude, the present study has confirmed the existence of direct effects of DIU and of TCS on specific biofilm components, as well as indirect effects of each toxicant due to ecological interactions within biofilms, as consequences of short-term pulses. Biofilms have shown their recovery capacity (by 2 weeks post-exposure they had recovered from nearly all the effects), but also that even very short pulses of toxicants can have relevant consequences for biofilm structure and function. It is reasonable to establish that short pulses can be seen as initial phases for effects on biofilms, that longer toxicant pulses could imply more persistent effects and finally that chronic concentrations of these toxicants could represent the most severe threat to biofilm diversity and function.



CHAPTER 7:

DROUGHT EPISODE MODULATES THE RESPONSE OF RIVER BIOFILMS TO TRICLOSAN

DROUGHT EPISODE MODULATES THE RESPONSE OF RIVER BIOFILMS TO TRICLOSAN

Abstract

The consequences of global change on rivers include altered flow regime, and entrance of compounds that may be toxic to biota. When water is scarce, a reduced dilution capacity may amplify the effects of chemical pollution. Therefore, studying the response of natural communities to compromised water flow and to toxicants is critical for assessing how global change may affects river ecosystems. This work aims to investigate how an episode of drought might influence the response of river biofilms to pulses of Triclosan (TCS). The objectives were to assess the separate and combined effects of simulated drought (achieved through drastic flow alteration) and of TCS exposure on biofilms growing in artificial channels. Thus, three-week old biofilms were studied under four conditions: Control (normal water flow); Simulated Drought (1 week reduced flow + 2 days interrupted flow); TCS only (normal water flow plus a 48-hour pulse of TCS); and Simulated Drought + TCS. All channels were then left for 2 weeks under steady flow conditions, and their responses and recovery were studied. Several descriptors of biofilms were analyzed before and after each step. Flow reduction and subsequent interruption were found to provoke an increase in extracellular phosphatase activity, bacterial mortality and green algae biomass. The TCS pulses severely affected biofilms: they drastically reduced photosynthetic efficiency, the viability of bacteria and diatoms, and phosphate uptake. Latent consequences evidenced significant combined effects caused by the two stressors. The biofilms exposed only to TCS recovered far better than those subjected to both altered flow and subsequent TCS exposure: the latter suffered more persistent consequences, indicating that simulated drought amplified the toxicity of this compound. This finding has implications for river ecosystems, as it suggests that the toxicity of pollutants to biofilms may be exacerbated following a drought.

Keywords: biofilms, drought, triclosan, recovery, river, toxicity.

INTRODUCTION

The consequences of global change on rivers include altered flow regime, and entrance of compounds that may be toxic to biota. Based on climate change scenarios, researchers have predicted substantial increases in both the frequency and magnitude of fluctuations in many ecosystems (Acuña, 2010). Alterations in flow regime, resulting from fewer precipitation days and more heavy rain events (Hyrabayashi et al., 2008; Sillmann and Roeckner, 2008), are expected to severely affect certain regions (*e.g.* the Mediterranean), chiefly through more frequent and more intense floods and droughts. Although flooding may strongly affect biogeochemical processes, drought implies more persistent consequences (Sabater and Tockner, 2010) namely, by disrupting hydrological connectivity and consequently favoring the extension of lentic habitats. During drought periods, shallow sections disappear and the stream becomes a series of fragmented, short-lived pools (Lake, 2003). Flow cessation triggers a cascade of effects on community structure and ecosystem function (Lake, 2003; Sabater and Tockner, 2010). Specifically, the benthic microbial community may respond to changes in water quality during drought (*e.g.* increased temperature, or altered quality of organic matter; Ylla et al., 2010), since it serves as an interface between the water column and the substrata (Sabater et al., 2007). Benthic biofilms are complex microbial communities adhered to solid surfaces (Mathuriau and Chauvet, 2002; Findlay et al., 1993; Sabater et al., 2007). They are fundamental in the trophic web and in the geochemical cycles within aquatic ecosystems (Battin et al., 2003; Lock, 1993). Field studies have shown the consequences of drought and intermittency on biofilm primary production and algal recolonization (Robson et al., 2004; Robson et al., 2008; Stanley et al., 2004; Ryder, 2004). Some benthic autotrophic groups and species are better adapted to drought than others, owing to structural traits (*e.g.* a higher content of extracellular polymeric substances) and/or to physiological properties (Ledger et al., 2008). The effects of drought on benthic heterotrophic microbial communities have been described mainly for sediment. They include changes in bacterial diversity, microbial activity and

biomass due to water stress (Amalfitano et al., 2008, Fierer et al., 2003).

In watersheds strongly influenced by human activity, the principal consequences of drastic flow reduction or interruption during drought periods include compromised dilution capacity, which may exacerbate chemical pollution (Guasch et al., 2010). Climate change and pollution pressures may simultaneously affect systems that receive wastewaters from industrial, agricultural and urban areas and that are located in regions affected by increasing water scarcity. Pollutants of agricultural, industrial or domestic origin enter watercourses either continuously (producing potentially chronic effects) or in pulses (causing potentially acute effects). These inputs occur in function of flow episodes, crop treatments and/or industrial release (Ellis, 2006). Both chronic and periodic inputs may affect stream biota more severely during drought periods than under normal flow conditions.

In this study, we investigated the combined effects of drought and pollution on river biofilms by following the respective impacts of drastic flow alteration (as a simulation of drought), and of exposure to the antibacterial agent triclosan (TCS), on biofilm community structure and function.

The broad-spectrum bactericide TCS (5-chloro-2-(2,4-dichlorophenoxy)phenol) is active against both gram-positive and gram-negative bacteria. It is an inhibitor of the enzyme enoyl–acyl carrier protein reductase (ENR), which is involved in bacterial lipid biosynthesis (Adolfsson-Erici et al., 2002). For over 30 years TCS has been used in products such as anti-bacterial hand soaps, deodorants, household cleaners, dental hygiene products, and textiles (Singer et al., 2002). It has been reported in sewage wastewater at concentrations between 6.1 and 16.6 μg L^{-1} (Halden and Paull, 2005; Samsøe-Petersen et al., 2003). Removal of TCS by wastewater treatment plants varies (Ellis, 2006; Kantiani et al., 2008; McAvoy et al., 2002); consequently, this compound can reach freshwater systems by entering running waters chronically at low concentrations via urban sewage effluents. Triclosan has been reported in rivers and streams (Kuster et al., 2008; Morral et al., 2004),

lakes (Loos et al., 2007; Singer et al., 2002) and the sea (Xie et al., 2008). Dry periods may cause drastic flow reduction and may entail brief spikes of compounds from sewage effluents, where TCS is commonly found. These spikes can provoke transient perturbation of river ecosystems with unknown long-term implications. The toxicity of TCS on aquatic organisms has been assessed by laboratory experiments (Capdeville et al., 2008). Exposure to TCS has significant consequences for biofilms, including increased bacterial mortality, as one would expect, and general effects on biofilm structure and function. These effects reach the autotrophic compartment, as reflected in biofilm responses to increasing concentrations (Franz et al., 2008; Ricart et al., 2010) or to short-term pulses (Morin et al., 2010; Proia et al., 2011) of TCS.

Our main objective in this study was to determine the structural and functional responses of fluvial biofilms to the separate and the combined effects of simulated drought (altered water flow) and of TCS exposure. Although several studies have revealed the independent effects of drought or TCS on river biofilm structure and function, the toxicity of TCS to biofilms experiencing multiple stressors (*i.e.* altered temperature, pH, oxygen levels, etc.) has not been assessed to date. Studying the combined effects of drought and of TCS exposure on river biological communities will facilitate risk assessment for this compound in freshwater ecosystems under stress. Thus, our specific objectives were firstly, to assess the individual effects of altered low and of TCS pulses on biofilm structure and function; secondly, to determine any combined action between these two stressors; and lastly, to evaluate the recovery potential of biofilms after these perturbations. We hypothesized that: i) altered water flow, as a simulation of drought, would affect the structure and function of biofilms, consequently making them more sensitive to short-term TCS pulses; ii) TCS would have direct and indirect effects on autotrophs; and iii) recovery after short-term perturbations would differ according to whether the observed consequences were direct or indirect, and would be influenced by any combined effects from the two stressors.

We tested our hypotheses using a multi-biomarker approach. We studied 3-week old biofilms under four conditions: Control (normal water flow); Simulated Drought (1 week reduced flow + 2 days interrupted flow); TCS only (normal water flow plus a 48-hour pulse of TCS); and Simulated Drought + TCS.

MATERIAL AND METHODS

Experimental setup

The experiment was conducted using twelve recirculating Perspex channels (170 cm long x 10 cm wide). Each channel unit was affixed with a Perspex piece to keep the water column height at 1.5 cm. Water input at the head of the first channel unit was provided by a 10 L carboy using a pump connected with silicone tubes (Pico Evolution1000, Hydor). The system was supplied with dechlorinated tap water filtered through an active carbon filter. All the carboys were placed in a water bath for water temperature control (21 ºC). To avoid nutrient depletion, the water in the carboys was renewed twice weekly. Phosphate supply was provided after each water change to reach a concentration of 30 μg L^{-1}. Light-emitting diodes (LEDs; Lightech, Spain) were used to provide natural light between 450 and 660 nm, at an intensity of 136.7 ± 8.0 μmol photons m^{-2} s^{-1} (n = 72), following a 12 h/12 h light and dark cycle. The bottom of each channel was covered with a sandblasted glass substratum (1 x 1 cm and 8.5 x 2 cm). Biofilm colonization was achieved by introducing aliquots of a natural microbenthic community obtained from the Llémena stream (NE Spain; Serra et al., 2009) weekly during the first 2 weeks. The biofilms colonized the artificial substrata under controlled flow conditions (3.5 L min^{-1}) for 21 days (Figure 1).

After colonization, four different conditions were assessed for their respective effects on biofilm structure and function: Control (normal water flow); Simulated Drought (1 week reduced

flow + 2 days interrupted flow); TCS only (normal water flow plus a 48-hour pulse of TCS); and Simulated Drought + TCS (Figure 1). First, six channels were maintained under Control, and the other six were subjected to Simulated Drought, whereby the flow was gradually decreased to one-third of the control value, over the course of 1 week (days 21 to 28), and then ultimately stopped, for 2 days (days 28 to 30). The physico-chemical parameters of all the channels were measured twice daily during the zero-flow period. The flow conditions were restored overnight in the Simulated Drought channels. Six channels (three Control and three Simulated Drought) were then treated with TCS (IRGASAN, Sigma Aldrich, > 97%, CAS: 3380-34-5) for 48 hours, and the remaining six channels (three Control and three Simulated Drought) were left untreated. Thus, each of the four established conditions (Control, Simulated Drought, TCS, and Simulated Drought + TCS) was tested in triplicate (three independent channels per treatment) (Fig. 1).

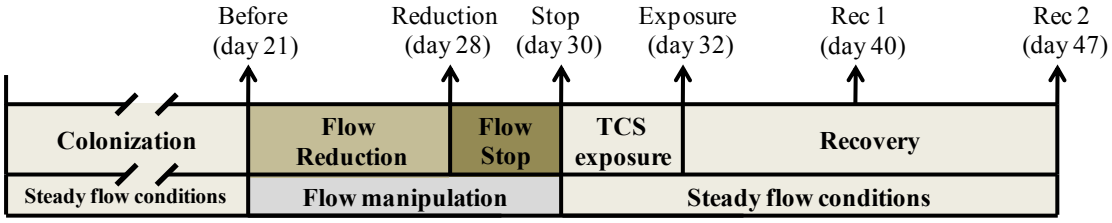


Figure 1. Schematic figure of the experimental design.

Triclosan can be photo-degraded in dioxins, especially at high pH values (Mezcua et al., 2004). Therefore, to maintain the TCS concentration levels, the water in the system was renewed every 3 hours during the light period of the light/dark cycle during TCS exposure. The water was sampled twice daily during TCS exposure, and to avoid TCS ionization (TCS pKa = 8.1, Tixier et al., 2002), the pH was monitored, and adjusted with diluted hydrochloric acid drops (as needed), to maintain values below 8.0. The biofilms were sampled after establishment of each condition to ascertain their structural and functional responses to each one as well as to any possible combined effects. Thus, the biofilms were sampled six times: before any manipulation (day 21, *Before*); after

1 week of flow reduction (day 28, *Reduction*); after 48 hours of interrupted flow (day 30, *Stop*); after 48 h of TCS exposure (day 32, *Exposure*); 1 week after the end of TCS exposure (day 40, *Recovery 1*); and 2 weeks after the end of TCS exposure (day 47, *Recovery 2*) (Fig. 1). On each sampling day, tiles of the colonized glass substrata were randomly collected from each channel and analyzed for extracellular-enzyme activity (EEA); photosynthetic parameters (F_0, Y_{eff} and Y_{opt}); phosphate uptake capacity (U); ratio of live-to-dead bacteria; ratio of live-to-dead diatoms; and biofilm biomass (ash-free dry mass [AFDM]).

Water analysis

The TCS concentrations were determined by a magnetic particle-based immunoassay (Kantiani et al., 2008), using the TCS IA kit (Abraxis LLC, Warminster, PA, USA). All contaminated channels were sampled twice daily after TCS addition and before water changes (n = 48). Furthermore, one contaminated channel was intensively sampled (n = 10) during the second day of exposure to determine the decay kinetics of the dissolved TCS. For this assay, the first five samples were collected every 5 minutes within the first 20 minutes (starting at time 0) after TCS addition; the sixth sample was taken 10 minutes later (30 min after addition), and the remaining samples, every 30 minutes until the next water change (3 hours after addition). Finally, samples were collected once a day in the Control channels (n = 12). The samples were immediately filtered through 0.2 μm nylon membrane filters (Whatman) and stored at -20 °C in the dark until analysis.

Biofilm sampling

Different sampling strategies were used depending on the endpoint. Biofilm functional measurements were performed on the day of sampling. One tile of the colonized glass substratum

(1 cm^2 per activity) was collected from each channel for EEA measurements. To measure the phosphate uptake capacity of the biofilms, five large tiles of the glass substratum (8.5 x 2 cm) were collected per channel. For the photosynthetic parameters analyzed by Phyto-PAM, three tiles of colonized glass substrata (17 cm^2) were used. Sample staining and preparation for microscopy (to count the live and dead bacteria) were also immediately performed after collection of one tile of glass substratum per channel. Biofilm biomass (AFDM) was measured in samples (one glass tile per channel) that were collected and immediately stored in vials at 50 °C. Since Phyto-PAM is a non-destructive, the three tiles of colonized glass substrata (17 cm^2) used for the fluorescence measurements were later fixed and used to determine the ratio of live to dead diatoms. For the latter task, the biofilms were scraped from the glass substrata using polyethylene cell lifters (Corning Inc., NY, USA), preserved with a drop of formalin solution, and diluted to a final volume of 5 mL. The methodology used to analyze the biofilm descriptors measured is described in detail in chapter 2.

Data analysis

To study the effects of drought and of TCS exposure on biofilms, the means of each biological variable measured per each channel at each sampling date were used as independent replicates. This dataset comprised the values for: extracellular enzymatic activities (phosphatase, peptidase and β-glucosidase); photosynthetic parameters (Y_{eff}, Y_{opt}, non-photochemical quenching [NPQ], total F_0, and F_0[Br], F_0[Gr], F_0[Bl]); biofilm phosphate uptake capacity (U and |K|); the ratio of live-to-dead bacteria; the ratio of live-to-dead diatoms; and the biomass (AFDM). All variables were log-transformed. Firstly, a principal component analysis (PCA) was performed to study the data ordination. Then, the effect of time was removed from the two sets of variables by performing a within PCA on each matrix, whereby for each variable, the mean value for the samples is

subtracted from the value for each sample in the group. All the group centers are, therefore, at the origin of the factorial map and samples are represented with the maximal variance around this origin. This enables comparison of variation patterns. The percentage of variance explained by the within PCA, *the intra-group variance*, corresponds to the variance due to factors other than time; it was calculated as the ratio between the sum of the Eigenvalues of the within PCA and the sum of the Eigenvalues of the simple PCA (Dray and Dufour, 2007). Statistical analysis was performed on the program R (R development Core Team, 2008; Ihaka and Gentleman, 1996) and on the software packages ade4 (Dray and Dufour, 2007) and Hmisc (Harrell, 2007). Statistical significance was set at $p < 0.05$.

The effect of the simulated drought episode on each parameter was also tested daily (sampling after flow reduction and after flow interruption) by one-way analysis of variance (ANOVA) in which Simulated Drought (F) was set as the fixed factor. The responses of the biofilms after TCS exposure were also analyzed daily (*Exposure, Recovery 1* and *Recovery 2* samplings) by two-way analysis of variance (ANOVA) to test for the effect of Simulated Drought (F), TCS treatment (T) and any combined effects of the two factors (F+T). Statistical significance was set at $p < 0.05$. Both analyses were performed using SPSS software, v. 15.0.

RESULTS

Experimental conditions

No significant differences in the physico-chemical parameter values among the channels were observed during the colonization period ($p > 0.05$). The conditions were set as follows: water flow, 3.5 ± 0.2 L min^{-1}; temperature, 21.7 ± 0.2 °C; pH, 7.7 ± 0.3; conductivity, 407.8 ± 6 µS cm^{-1}; dissolved oxygen, 8.5 ± 0.3 mg L^{-1} (n = 72); and phosphorus concentration, 33.7 ± 3.8 µg P L^{-1} (n = 18). During the flow reduction period, the water flow of the manipulated channels was 1.2 ± 0.6 L

min^{-1}, significantly lower than in the control channels ($p < 0.001$). The remaining parameter values during this period did not differ significantly between the Control channels and the Simulated Drought channels ($p > 0.05$). When the flow was stopped, the water temperature, conductivity and dissolved oxygen all increased significantly ($n = 26$): the temperature rose to 25.6 ± 0.8°C (2.4 °C higher than in the control; $p < 0.001$); conductivity, to 432.8 ± 20.4 µS cm^{-1} ($p = 0.001$); and dissolved oxygen, to 10.7 mg L^{-1} ($p = 0.031$). However, after the flow was restored, the values of the physico-chemical variables no longer differed significantly among the channels ($p > 0.05$).

After TCS addition, the measured TCS concentration in the treated channels was 87.2 ± 7.8 µg TCS L^{-1} ($n = 24$), 12.2% below the nominal concentration. The TCS concentration followed exponential decay kinetics, with a rate (K) of 0.018 min^{-1} ($r = 0.90$, $p < 0.05$): by 3 hours after addition, the concentration had dropped to 4.6 ± 0.3 µg TCS L^{-1} ($n = 24$) (5.2% of the initial concentration). The TCS concentration in the Control channels was always below the detection limit.

Biofilm responses to drought and to TCS exposure

Temporal variation

The biofilm changes over throughout the course of the experiment are described by the PCA (Figure 2). The first two axes of the PCA explain 53.5 % of the variance. Axis 1 clearly arranges samples by their sampling date (40.7 %, Figure 2). The live-to-dead ratios of bacteria and of diatoms, the phosphatase activity, and the photosynthetic efficiency and capacity all decreased over time. Contrariwise, the activity of β-glucosidase and of peptidase, the AFDM, the algal biomass (F_0), the fluorescence of cyanobacteria (F_0[Bl]) and of green algae (F_0[Gr]), and the phosphate uptake rate (U) all increased during the experiment (Figure 2, Table A.1).

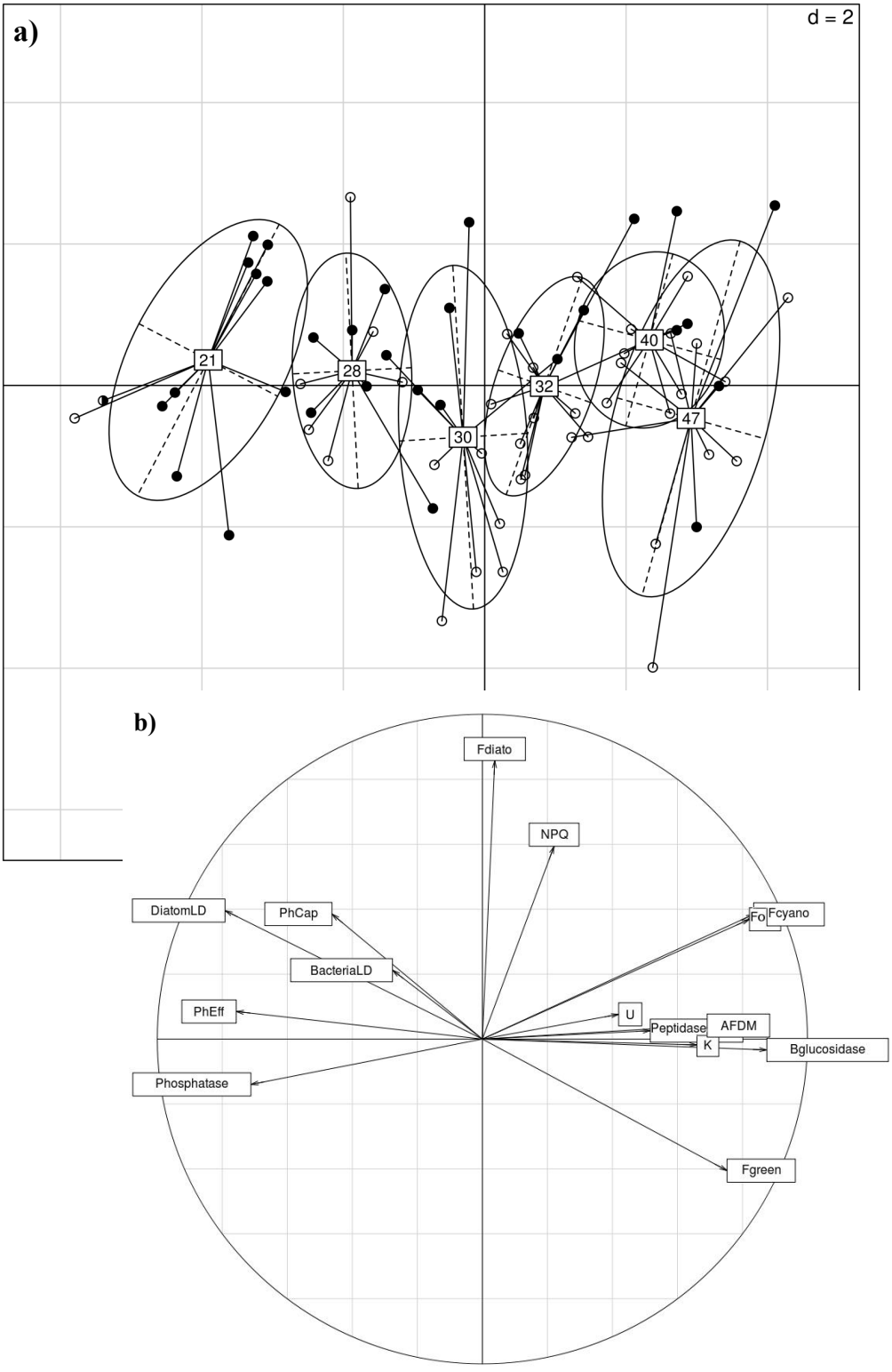


Figure 2. a) Factorial map of the sample ordination by the PCA. The samples are grouped by sampling day (indicated by the label). The solid black dots correspond to the Control samples, and the outlined dots correspond to the TCS-exposed samples. b) Normalized coefficients of the different variables on the first two axes of the PCA.

Effects of drought and of TCS exposure

The within PCA showed that 47.8 % of the variance (intra-group variance) was explained by factors other than time. Both flow alteration and exposure to triclosan affected the different biofilm structural and functional parameters, especially on days 30, 32 and 40 (Figure 3). The first two axes of the within PCA explain 40.7 % of variance (wPCA1: 25.0 %, wPCA2: 15.8 %) and are linked to flow- alteration effects and to TCS effects, respectively. The specific effects of flow alteration and of TCS exposure on the measured biofilm variables were further evidenced by the ANOVA analyses (Table 1).

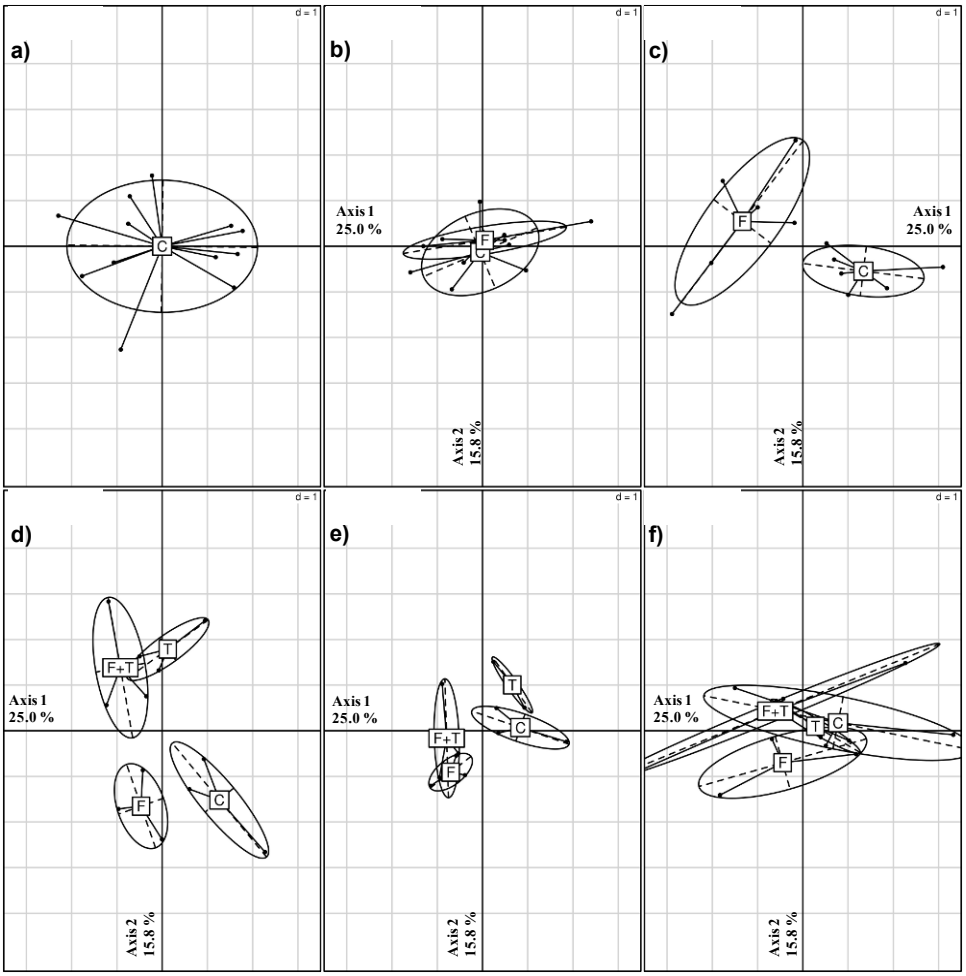


Figure3. Factorial map of the sample ordination by the within PCA, shown for each sampling day: a) day 21, Before; b) day 28, Reduction; c) day 30, Stop; d) day 32, Exposure; e) day 40, Recovery 1; and f) day 47, Recovery 2. Samples are grouped by treatments: C = Controls; F = Simulated Drought (altered flow); T = TCS exposure; and F+T = Simulated Drought + TCS exposure.

Table 1. Results of F and p values of the one-way ANOVA and two-ways ANOVA test performed. The most responsive endpoints are reported. p values below 0.01 are represented in bold.

	One-way ANOVA		MANOVA								
	Day 26 (low)	**Day 29** (Stop)	**Day 32** (Effect)			**Day 40** (Rec 1)			**Day 47** (Rec 2)		
	Flow (F)	Flow (F)	Flow (F)	Triclosan (T)	Interaction (FxT)	Flow (F)	Triclosan (T)	Interaction (FxT)	Flow (F)	Triclosan (T)	Interaction (FxT)
Phosphate Uptake Rate (U)	n.s.	n.s.	n.s	0.013 F = 10.2	n.s.	n.s	**0.008 F = 12.2**	0.036 F = 6.3	n.s	0.019 F = 8.5	n.s
Alkaline phosphatase activity	n.s.	**< 0.001 F = 61.6**	n.s	0.029 F = 7.0	n.s	n.s	n.s	*0.084 F = 3.9*	n.s	n.s	n.s
L/D bacteria	0.024 F = 7.1	**< 0.001 F = 31.3**	n.s	**0.009 F = 11.8**	n.s.	n.s	**0.001 F = 23.4**	*0.056 F = 5.0*	*0.067 F = 4.5*	0.012 F = 10.6	n.s
Ph.eff.	n.s.	n.s.	0.011 F = 10.9	**0.002 F = 19.6**	n.s.	0.014 F = 9.7	n.s	n.s	n.s	0.014 F = 9.9	0.047 F = 5.5
Ph.cap.	n.s.	**< 0.001 F = 32.1**	n.s.	**0.003 F = 18.3**	n.s.	n.s	n.s	n.s	n.s	n.s	n.s
Fgreen	n.s.	**0.003 F = 14.8**	**0.002 F = 18.8**	n.s.	n.s.	0.044 F = 5.7	n.s	0.023 F = 7.9	n.s	n.s	n.s
Fdiatoms	n.s.	**0.009 F = 10.2**	**0.001 F = 26.5**	n.s.	n.s.	**0.004 F = 16.3**	n.s	0.030 F = 6.9	n.s	n.s	n.s
L/D diatoms	n.s.	n.s.	n.s.	*0.053 F = 5.1*	**0.001 F = 28.8**	0.041 F = 5.9	n.s	n.s	**< 0.001 F = 128.9**	**< 0.001 F = 25.4**	0.044 F = 5.7

Effects of drought

At 2 days after flow interruption, the Control biofilms (C) and the Simulated Drought (F) biofilms are clearly separated along axis 1 of the within PCA (Figure 3c). Although the gap between them decreases during the recovery period, they remain somewhat separated until the end of the experiment (Figure 3). Flow reduction and further flow interruption caused a significant decrease in the number of live bacteria and of live diatoms (Figures 4a and b, respectively, and Table 1), in F_0(Br) (Figure 6 and Table 1) and in photosynthetic capacity (Figure 5b and Table 1), but it caused a significant increase in phosphatase activity (Figure 5a and Table 1) and in F_0(Gr) (Figure 6 and Table A.1). The differences in the proportion of algal groups remained until the end of the experiment (Figure 6). A lower proportion of live bacteria and of diatoms (*i.e.* a lower live-to-dead ratio for each) was still observed at day 47 in the Simulated Drought samples, relative to the Control samples (Figure 4, Table 1).

Effects of TCS

At 48 hours after TCS exposure (day 32), the exposed biofilms appear separate from the control biofilms along axis 2 of the within PCA (Figure 3d). These effects persisted for 1 week after the end of exposure (day 40, Figure 3e). However, 2 weeks after the end of exposure (day 47), exposed and non-exposed biofilms appear together (Figure 3f); nonetheless, some parameter values of the TCS-exposed biofilms (TCS, and Simulated Drought + TCS) were still significantly different from the non-exposed communities (Controls, and Simulated Drought) (Table 1).

In the TCS-treated biofilms, the peptidase activity and the F_0(Br) increased, whereas the F_0(Gr) and the AFDM decreased. The phosphatase activity in these biofilms increased significantly compared to controls on day 32, but recovered to control levels within 1 week after the end

of exposure (Figure 5a and Table 1). The photosynthetic parameters (Ph. capacity) were also significantly affected by TCS, but the effect was transient: the values had recovered by day 40 (Figure 5b and Table 1). Contrariwise, the negative effects of TCS exposure on the ratio of live-to-dead bacteria (Figure 4a) and on phosphate uptake rate (Figure 5c) persisted until the end of the experiment (Table 1).

Combined effects of drought and TCS exposure

The within PCA indicates that the short-term effects of TCS were not influenced by flow variations (days 32 and 40). However, the biofilms exposed to TCS only (T) recovered more quickly (Figure 3), and at day 47, the biofilms exposed to both Simulated Drought and TCS (F+T) appear separate from those exposed only to Simulated Drought (F) (Figure 3f). The delayed combined effects of Simulated Drought + TCS were observed in several structural and functional descriptors of biofilms (Table 1). For example, F_0(Gr) decreased in the TCS biofilms, but was not affected in the Simulated Drought + TCS biofilms (Figure 6). The ratio of live-to-dead bacteria and the phosphate uptake capacity remained lower in the Simulated Drought + TCS biofilms. However, the TCS communities recovered to normal values by day 47 (Figures 4a and 5c). In the F and the Simulated Drought + TCS biofilms, F_0(Br) decreased; the decline was more severe in the latter (Figure 6 and Table A.1). In the TCS-treated biofilms, the ratio of live-to-dead diatoms decreased after TCS exposure, but ultimately recovered to values higher than those of the control biofilms; however, in the Simulated Drought + TCS biofilms, it never recovered (Figure 4b).

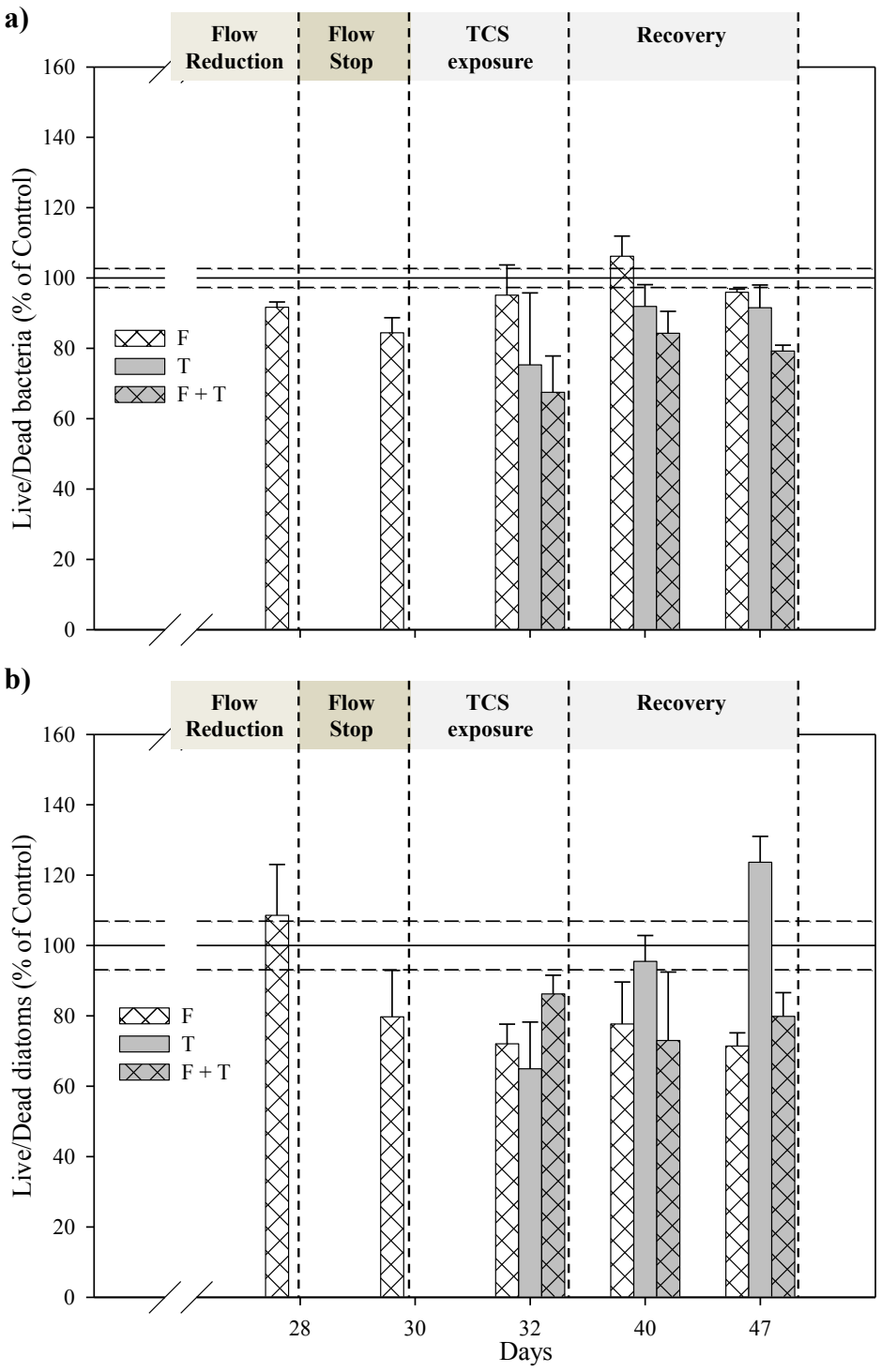


Figure 4. Altered composition in the treated biofilms. Changes in a) the live-to-dead bacteria ratio and b) the live-to-dead diatoms ratio, on each sampling date. The values are means with standard errors (n = 3). The solid horizontal line represents controls at ± 95% CI (dashed horizontal lines).

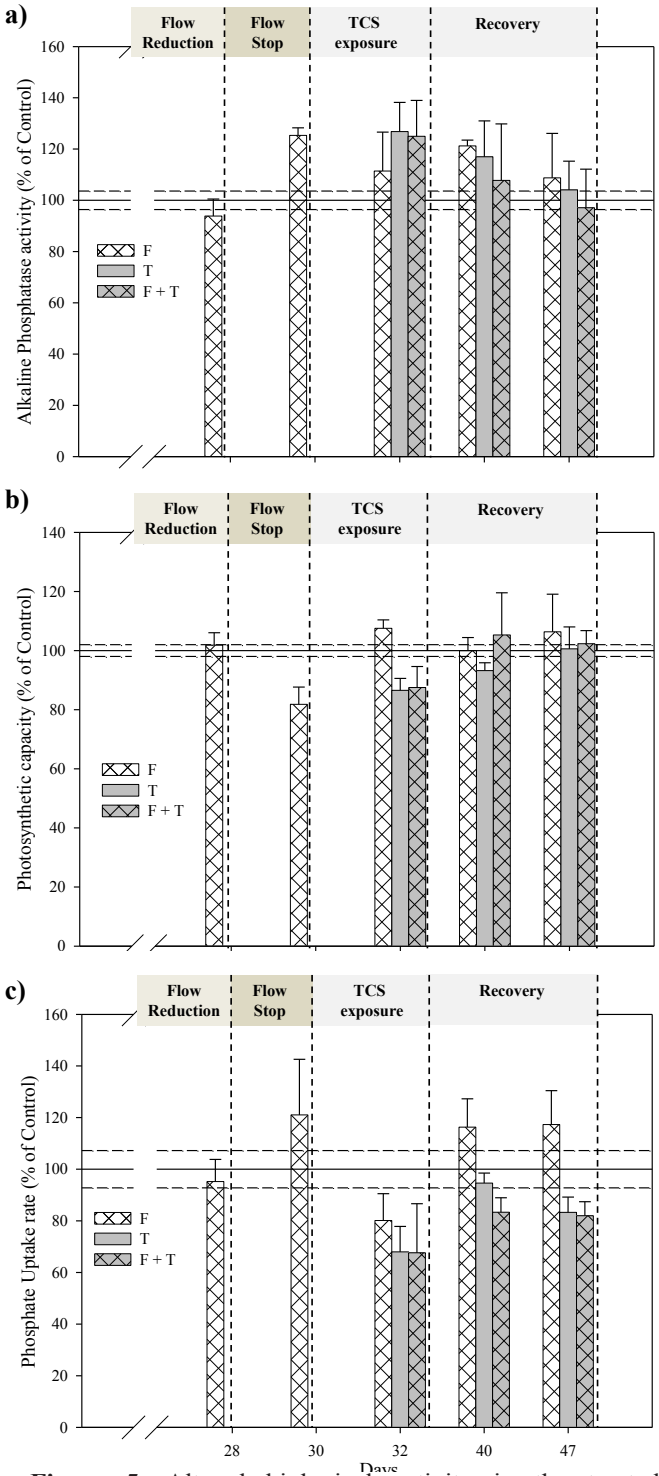


Figure 5. Altered biological activity in the treated biofilms. Changes in a) alkaline phosphatase activity, b) photosynthetic capacity, and c) phosphate uptake rate, on each sampling date. The values are means with standard errors (n = 3). The solid horizontal line represents controls at ± 95% CI (dashed horizontal lines).

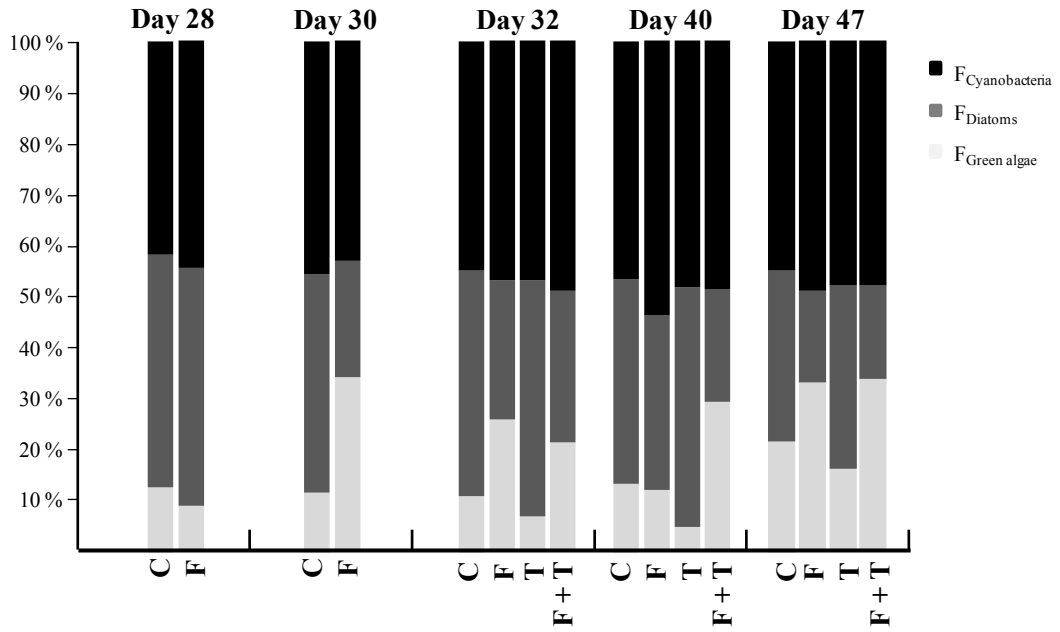


Figure 6. Altered fluorescence in the treated biofilms. Changes in the relative percentage of the fluorescence signal corresponding to each of the three autotrophic groups on each sampling date. The values are means (n = 3). Samples are grouped by treatments: C = Controls; F = Simulated Drought (altered flow); T = TCS exposure; and F+T = Simulated Drought + TCS exposure.

DISCUSSION

In this study, we investigated how simulated drought and TCS exposure can affect natural river biofilm communities, both separately and when combined. Our results confirmed our hypothesis that altered water flow, as a simulation of drought, can modulate the responses of biofilms to chemical exposure, even long after the flow is re-established.

Effects of drought

Our study revealed that the simulated drought episode (1 week of drastically reduced flow, followed by 2 days of totally blocked flow) was enough to cause structural and functional changes in biofilm communities. It induced changes in the structure and photosynthetic capacity of the autotrophic compartment, and provoked increased bacterial mortality and extracellular phosphatase activity.

Drought episodes in running waters produce drastic flow reduction and the subsequent disruption of longitudinal hydrological connectivity, with altered physico-chemical conditions and specific biotic responses (Lake, 2003; Sabater and Tockner, 2010). After two days of interrupted flow, we observed increases in temperature, conductivity and dissolved oxygen, all which have been described in disconnected systems (Stanley et al., 1997; Caruso et al., 2002). Researchers have shown that in unshaded pools, the higher temperatures and the photosynthesis-activating radiation can trigger algal blooms (Freeman et al., 1994). Indeed, in our lentic channels, the temperature increase associated with the steady photosynthetic radiation fostered an initial phase of such a bloom. Green algal biomass—particularly, of filamentous forms—increased significantly, which could be associated to the decrease in inorganic phosphorus (Dahm et al., 2003). The subsequent increase in the activity of extracellular alkaline phosphatase that we observed after the 2 days of

flow interruption could be associated to this apparent algal growth. Indeed, alkaline phosphatase catalyzes the hydrolysis of phosphate esters, liberating inorganic phosphorus available for microbial uptake, particularly when inorganic phosphate is limiting (Berman, 1970; Siuda and Chrost, 1987; Allison and Vitousek, 2005; Romaní et al., 2004). The reduced flow was also detrimental to diatoms, either because of direct effects, or because the diatoms had to compete with autotrophic groups that are more prone to lentic conditions (*e.g.* green algae). The observed changes in the algal compartment (increase in green algae and decrease in diatoms) associated to a slight decrease in biofilm photosynthetic capacity, but not to any changes in photosynthetic efficiency, indicating that the treatment affected the structure of the photosynthetic apparatuses without comprising their photosynthetic performance. The photosynthetic efficiency increased after day 32, supporting the existence of a selection pressure that favors the growth of green algae (Corcoll et al., in press). The significant decrease in the ratio of live-to-dead bacteria observed after the 2 days of flow interruption could be also associated to a described coupling between diatoms and bacteria (Croft et al., 2005). Indeed, indirect effects on bacteria have been induced by direct effects on autotrophs (López-Doval et al., 2010; Ricart et al., 2009; Pesce et al., 2006). Nevertheless, bacterial mortality was the only parameter that significantly increased after 1 week of flow reduction (day 22; Tables 1 and 2, and Figure 4a), confirming the high sensitivity of bacteria to water flow and suggesting that other mechanisms (direct and/or indirect) could have affected the biofilm heterotrophs during the first phases of the simulated drought episode. In particular, flow reduction may provoke changes in the internal water (and consequently, in the nutrients) that cycle into biofilms (Battin et al., 2003), which can directly affect bacteria in the first phases of a drought episode. Subsequent effects of interrupted flow on autotrophic community composition, as evidenced in our study, may indirectly contribute to maintaining and even reinforcing this negative direct effect.

TCS toxicity

Our results confirm that TCS may directly and/or indirectly affect fluvial biofilm structure and function as indicated in several studies (Franz et al., 2008; Lawrence et al., 2009; Morin et al., 2010; Proia et al., 2011; Ricart et al., 2010). The TCS pulses in our experiments provoked increased mortality among bacteria and diatoms, and led to reduced phosphate uptake, photosynthetic capacity and green algae biomass.

The TCS concentrations in water after the pulse exposure followed exponential decay kinetics: within 3 hours after addition, nearly 94% of the TCS disappeared from the water. Nevertheless, organic compounds such as TCS can be degraded to several bioactive sub-products, and can be adsorbed and trapped in biofilms by both extracellular and intracellular pathways.

The TCS exposure markedly influenced the biofilm communities, affecting both target (bacteria) and non-target (autotrophs) organisms. The mechanisms of TCS toxicity to bacteria are well documented: these include blocking of fatty acid synthesis (McMurry et al., 1998), compromising permeability-barrier functions (Phan and Marquis, 2006) and destabilizing cell membranes (Villalaín et al., 2001). Although no specific mode-of-action of TCS in algae has yet been determined, this agent could affect algae similarly as it does on bacteria (Lawrence et al., 2009; Ricart et al., 2010; Morin et al., 2010). The negative impact of TCS on green algal biomass, diatom viability and photosynthetic parameters reinforced our hypothesis that this bactericide would have direct and indirect effects on autotrophs. Similar negative effects of TCS on autotrophs in biofilms have been found in several studies (Franz et al., 2008; Morin et al., 2010; Proia et al., 2011; Ricart et al., 2010). Some researchers have described algae to be even more sensitive to TCS than bacteria (Tatarakazo et al., 2004), whereas others, having evaluating axenic algal cultures, have confirmed the existence of some direct—albeit unexplainable—effect of TCS on algae (Capdeville et al., 2008). The significant reduction in biofilm phosphate uptake induced by TCS could derive from its negative effects on algae and bacteria (Proia et al., 2011).

Influence of flow reduction history on TCS toxicity

The persistent effects of TCS on biofilm structure and function differed in relation to their water flow history (at day 32, the four treatment groups appear at distinct locations along the two axes of the within PCA analysis). The Simulated Drought + TCS biofilms were more sensitive to TCS exposure than were the TCS only biofilms: the former suffered more persistent effects and exhibited lower recovery potential than did the latter. Among the biofilms subjected to both Simulated Drought and TCS, the persistently higher mortality of bacteria and of diatoms (until the end of the experiment) correlated to the reduced phosphorus uptake, thereby confirming the aforementioned effects. Since biofilms are the most important biotic compartment in rivers, in terms of retaining inorganic nutrients from the water column (Sabater et al., 2007), any reduction in their phosphate uptake induced by organic compounds such as TCS, and worsened by previous drought episodes, can severely compromise the health of river ecosystems.

Based on our findings, we conclude that the flow history of rivers may alter the response of biofilms to pollutant exposure. In our experiments, the biofilms from channels that had experienced water flow reduction and interruption were more sensitive to subsequent TCS pulses than were those from channels that had experienced constant flow, confirming our hypothesis that altered water flow, as a simulation of drought, would modulate the response of biofilms to chemical exposure. Proia et al. (in press) has theorized that in rivers that have experienced a sequence of perturbations, the recovery process will dependent on: the duration and magnitude of disturbances (short-term and/or long-term); the observed effects (direct and/or indirect); and the resilience potential of the affected organisms (target and/or non-target).

In the present study, we investigated the acute response and recovery potential of biofilm communities after transient perturbations (simulated short-term drought followed by a highly-concentrated pulse of TCS). Given our findings, we conclude that the possible implications of chronic entrance of low concentrations of TCS into running water systems facing increasing water

scarcity must be investigated in work that accounts for the predicted effects of global climate change scenarios. Sequential exposure of biofilms to drought and TCS pulses may lead to early and to late effects, from which biofilm communities may only partially recover. Evidence that the toxicity of pollutants to biofilms may be augmented following a drought period should be further investigated for compounds and compound mixtures found in field conditions, especially in regions affected by water scarcity, taking into account the effects of global climate change.

CHAPTER 8:

USING BIOFILM PHOSPHORUS UPTAKE EFFICIENCY AS A TOOL FOR THE ASSESSMENT OF THE EFFECTS OF POLLUTANTS ON RIVERS SELF-DEPURATION CAPACITY

USING BIOFILM PHOSPHORUS UPTAKE EFFICIENCY AS A TOOL FOR THE ASSESSMENT OF THE EFFECTS OF POLLUTANTS ON RIVERS SELF-DEPURATION CAPACITY.

Abstract

Biofilm communities are key elements in river self-depuration processes since they can retain nutrients through different mechanisms. Autotrophs and heterotrophs in the biofilm use nutrients from the river water to build up their growing cells. Uptake of phosphorus (P) is mainly a biotic process that depends from the affinity of organisms for P. Microbial biofilms active role in P interception suggests that careful examination of their potential role will be beneficial to both managers and scientists. Several physical and biological factors affect the efficiency of biofilms to retain nutrients. The input of pollutants in the flowing water altering the biofilm structure and functioning might determine changes in the P-uptake capacity and ultimately in the river self-depuration. This study aims to analyze the effect of pollution on the biofilm P-uptake capacity by comparing the specific effect of the bactericide triclosan in different experimental conditions with the more general effect of a pollution gradient. To this aim the response of biofilm P-uptake measured in four different experiments was analyzed and compared. Particularly, three experiments were performed testing triclosan (TCS) toxicity on biofilm in different conditions: TCS alone, with grazers, and after simulated drought episode. The fourth experiment was performed with biofilm exposed to river polluted water. This comparison evidenced that 48 hours of exposure to concentrations of TCS higher than 15 $\mu g\ L^{-1}$ significantly reduce the capacity of biofilm to uptake phosphorus while exposure to lower concentration affects phosphorus uptake capacity only under grazing pressure. Moreover, the simulated drought episode also induces a decrease of biofilm phosphorus uptake capacity and worsened triclosan negative effects. Results of the

fourth experiment showed biofilm uptake capacity decrease along a pollution gradient. Our study highlights the use of biofilm phosphorus uptake capacity as a promising tool for studies aiming the understanding of ecological consequences of pollution and environmental variation on river self-depuration capacity.

Keywords: Phosphorus, Uptake, Biofilm, River, Self-depuration, Pollution

INTRODUCTION

Biofilm communities are key elements in river self-depuration processes (Pusch et al., 1998) since they are complex entities made up of living (algae, bacteria, fungi, protozoa) and non-living components (extracellular matrix) essential in the uptake or retention of inorganic and organic nutrients (Burkholder et al., 1990; Flemming, 1995). Dodds et al (2003) described several mechanisms that lead to increased retention of phosphorus by biofilm assemblages: a) direct removal from water column; b) reduction of water exchange across the substrata/water column boundary thus decreasing advective transport of P; c) interception of nutrients diffusing from the benthic sediments; d) alteration of local biochemical conditions that favor P deposition (i.e. increase of pH in consequence of phothosynthetic activity favor precipitation of calcium phosphate) and e) trap of particulate material from the water column (Adey et al. 1993). Autotrophs and heterotrophs in the biofilm use nitrogen and phosphorus from the river water to build up their growing cells (Bothwell, 1988). Uptake of phosphorus is mainly a biotic process, and depends in part upon the affinity (described with a Michaelis-Menten relationship) of organisms for P (Dodds et al., 2003). Algae and bacteria in the biofilm compete for inorganic nutrients (Cole, 1982), and this competition results in a higher efficiency of the biofilm to retain nutrients (Sabater et al., 2002). Microbial biofilms perform an active role in P interception, and the fact that major P fluxes can be altered by algal biofilms suggests that careful examination of their potential role will be beneficial to both managers interested in P control and scientists interested in basic ecosystem function (Dodds et al., 2003). Thus, among all biofilm retention mechanisms, phosphorus retention might be a specifically relevant ecosystem service to investigate.

Several factors affect the function and subsequent efficiency of biofilms to retain nutrients (Sabater et al., 2002). Of these factors, some are physical (e.g. water current, temperature, light penetration), and chemical (pH, nutrient availability) while others are biological (relative contribution of autotrophs and heterotrophs, community composition, biomass, thickness,

grazing) (Stevenson, 1996). Particularly, phosphorus uptake rates may be influenced by biofilm biomass, metabolic activity, and advective transport of P to biofilm assemblages (Dodds et al., 2003). Moreover, the uptake of P by algae can be time dependent and may reflects the present P-nutritional state. At the same time, the input of pollutants in the flowing water altering the biofilm structure and functioning might determine changes in the P-uptake capacity and ultimately in the river self-depuration. As rivers are one of the main sources of drinking water (Pimentel et al., 1997), the increasing number of substances entering freshwater systems complicates the provision of high quality drinking water. In countries with limited water resources this concern is even higher and river water purification becomes progressively a more sophisticated and costly procedure (Sabater et al., 2002). In flowing waters several processes occur naturally and lead to an important, cost- effective amelioration of water quality (Sabater et al., 1991).

Our aim was to analyze the effect of pollution on the biofilm P-uptake capacity by comparing the specific effect of the bactericide triclosan (considering different experimental conditions such as drought and grazing) with the more general effect of a pollution gradient. The specific experiments with triclosan effects on biofilms showed a clear decrease of P uptake capacity by the biofilm when exposed to the toxicant but this response is modulated either by the drought conditions or the presence of grazers (Proia et al., 2011; Proia et al., 2012; Ricart et al., *submitted*). In the present chapter, the degree of biofilm response in terms of P-uptake is analyzed and compared from data collected from four different experiments, including three experiments specifically with biofilm exposed to triclosan (TCS) and one experiment with biofilm exposed to river water from polluted sites. The study adds evidences for the potential use of biofilm phosphorus uptake capacity as endpoint for studies aiming the understanding of ecological consequences of pollution for river functioning.

MATERIAL AND METHODS

Data collection

For the comparison of the response of phosphorus uptake capacity of the biofilm submitted to different pollution conditions data from four experiments were assembled. In all experiments the same method for the measurement of P uptake was applied (as described below). Colonized biofilms on glass tiles submerged in mesocosms (glass jars, 1.5 L water volume and water pump) or in artificial channels (1.5 mt long) were used in all experiments.

Three of the experiments analyzed the effect of Triclosan on the biofilm by pulse exposure to the pollutant (48h). Biofilm colonization was achieved by introducing aliquots of a natural microbenthic community obtained from the Llémena stream (NE Spain) and the glass tiles were colonized during 3 weeks before TCS exposure. During triclosan exposure water was renewed every 2/3 hours during illuminated period in order to avoid triclosan photo degradation, and pH was monitored and maintained below 8.0 in order to avoid triclosan ionization (pKa = 8.1, Tixier et al., 2002). The first experiment included the effect of TCS up to nominal concentrations of 60 μg L^{-1} by using four mesocosms which were treated with TCS and four mesocosms which were left untreated and used as control (see chapter 6). In the second experiment, the potential effect of a grazer on biofilm response was included, considering four different conditions: Control, without triclosan and free from snails(Ctr); Grazing, without Triclosan and with snails (G); Triclosan, with 15 μg TCS L^{-1} and without snails(TCS(15)); and Triclosan + Grazing, with triclosan (15 μg TCS L^{-1}) and with snails (TCS(15)+G) (Ricart et al. *submitted*). The third experiment included the effect of drought on biofilm responses to triclosan exposure. In this case, artificial channels were used and the effect of drought was applied previously to the effect of triclosan. Half of the channels (6) were submitted to a 1 week of flow reduction + 2 days of flow interruption. Afterwards, TCS was applied to half of the channels (3 control and 3 previously submitted to the drought). Thus,

the following four treatment were obtained: Control (Ctr), with normal water flow; Simulated Drought (D), 1 week reduced flow + 2 days interrupted flow; TCS only (TCS(80)), normal water flow plus a pulse of 80 μg TCS L^{-1}; and Simulated Drought + TCS (TCS(80)+D) (see chapter 7).

The fourth experiment include the results from analyzing the development of natural biofilms using water from a polluted river as inoculum (Chapter 4). In this case, biofilms were grown under controlled conditions with water collected from three different sites along a pollution gradient of a highly impacted river. The three sampling sites were selected in the middle-low part of the Llobregat River following a pollution gradient: Castellbell (CB) and Mina de Terrassa (MT) as less polluted sites and Sant Joan Despí (SJD) as hotspot (see Chapter 4).

P uptake measurement

The capacity of biofilms to uptake phosphorus was measured in the four described experiments. For the three triclosan exposure experiments, biofilm P uptake was measured for all treatments after 48 hours of exposure to triclosan. In the fourth experiment, biofilm P uptake was measured after 34 days of biofilm growth. The methodology used for the biofilm phosphorus uptake measurements is described in detail in chapter 2.

Data analysis

For the comparison of the different biofilm responses to triclosan (first three experiments) the uptake rate coefficients have been calculated as percentage of control and tested by one-way analysis of variance (ANOVA) in which treatment was set as fixed factor. Effects were analyzed post hoc with Tukey's b test after passing the homogeneity variance test. Statistical significance was set at $p=0.05$. For the comparison of the biofilm grown under increasing pollution (fourth

experiment) uptake rate coefficients have been tested by one-way analysis of variance (ANOVA) in which sampling site was set as fixed factor. Differences were analyzed post hoc with Tukey's b test after passing the homogeneity variance test. Statistical significance was set at p=0.05. All data were previously tested for normal distribution with Kolmogorov-Smirnov test. Analyses were performed using SPSS Version 15.0.

RESULTS

Detailed results of the kinetics of phosphorus removal from water column as a consequence of biofilm uptake in the first three experiments are shown in figure 1. In particular in the experiment 1, the exposure to 60 μg L^{-1} of TCS during 48 hours provoked a clear reduction of the biofilms capacity to uptake phosphorus (Figure 1a). In the experiment 2, the biofilms with snails (G) slightly increase their capacity to uptake phosphorus while the combined effects of grazers and TCS clearly affect the P removal (Figure 1b). In contrast, TCS alone did not affect the kinetic of phosphorus uptake (Figure 1b). The experiment 3 highlighted how Drought and TCS reduce P uptake capacity of biofilms both independently and combined (Figure 1c). To compare results of the different experiments, the uptake rate coefficients have been expressed in percentage of control and plotted all together (Figure 2). This comparison confirms that 48 hours of exposure to concentrations of TCS higher than 15 μg L^{-1} significantly reduce the capacity of biofilm to uptake phosphorus. Moreover, the simulated drought episode also provokes a significant decrease of phosphorus uptake rate coefficient. Finally, exposure to lower concentration of TCS (15 μg L^{-1}) affects phosphorus uptake capacity of biofilms only under grazing pressure.

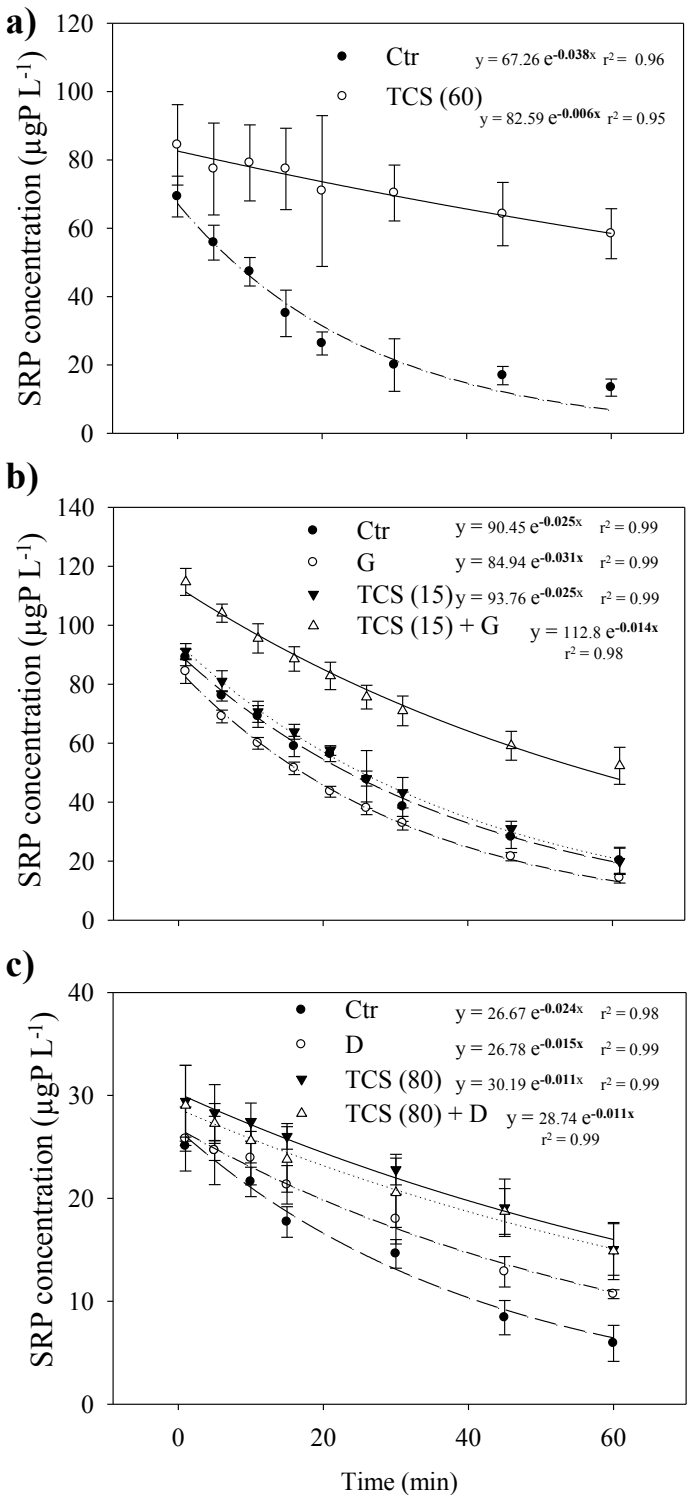


Figure 1. Variation of dissolved inorganic phosphorus in time after controlled spike of the three different experiments testing toxicity of the bactericide Triclosan (TCS) under different conditions: **a)** Experiment 1. Comparison between control (Ctr) and triclosan exposed (TCS(60)) biofilms; **b)** Experiment 2. Comparison among Control (Ctr), Grazed (G), triclosan exposed (TCS(15)) and Grazed + triclosan exposed (TCS(15)+G) biofilms and ; **c)** Experiment 3. Comparison among Control (Ctr), Simulated Drought (D) exposed, triclosan exposed (TCS(15)) and Simulated Drought + triclosan exposed (TCS(80)+D) biofilms. Values are mean ± standard deviation (n = 3 or 4).

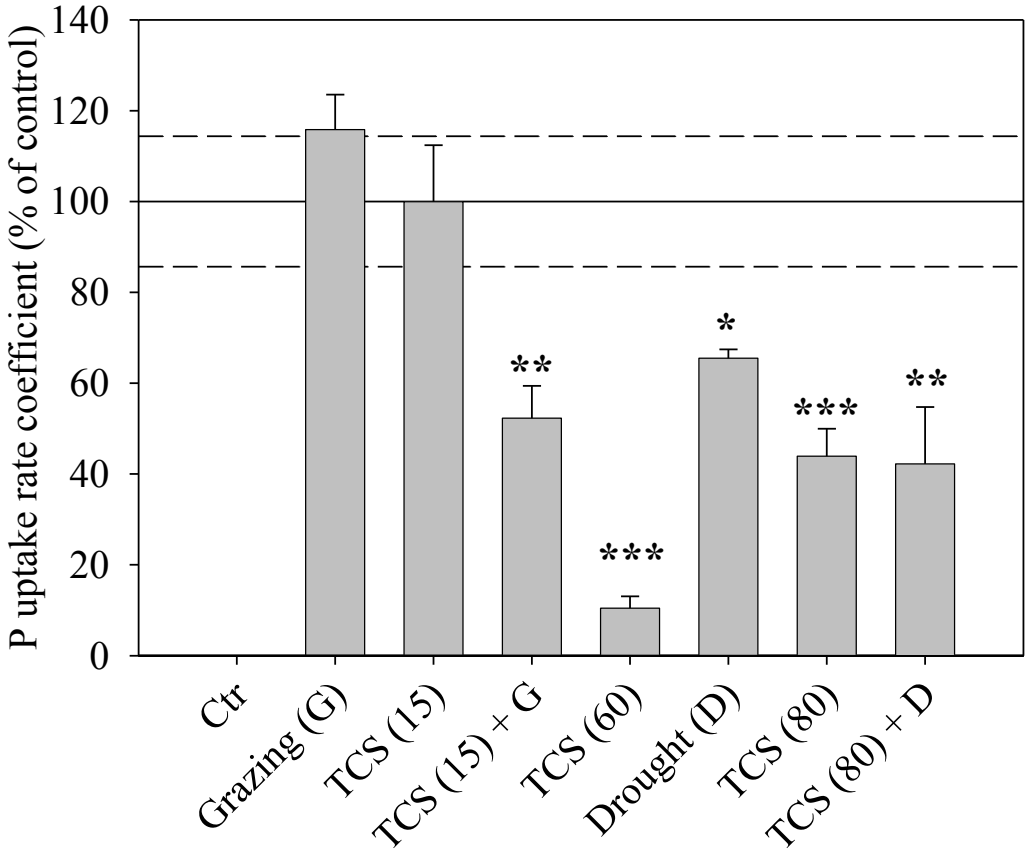


Figure 2. Comparison among the uptake rate coefficients (expressed as percentage of controls) of the treated biofilms in the three experiments testing Triclosan (TCS) toxicity under different conditions. Values are mean ± standard deviation (n = 3 or 4). The horizontal lines represents controls ± 95% confidence intervals.

Results of the fourth experiment are shown in Figure 4. Exponential decay of the phosphorus concentration in time was lower in biofilms grown with SJD water (Figure 3a). Comparison between phosphorus uptake rate coefficients (Figure 3b) showed how biofilm uptake capacity of biofilms decreases significantly from CB to SJD. In fact, more negative coefficient corresponds to higher phosphorus uptake efficiency and SJD resulted significantly the less efficient biofilms (Figure 3b).

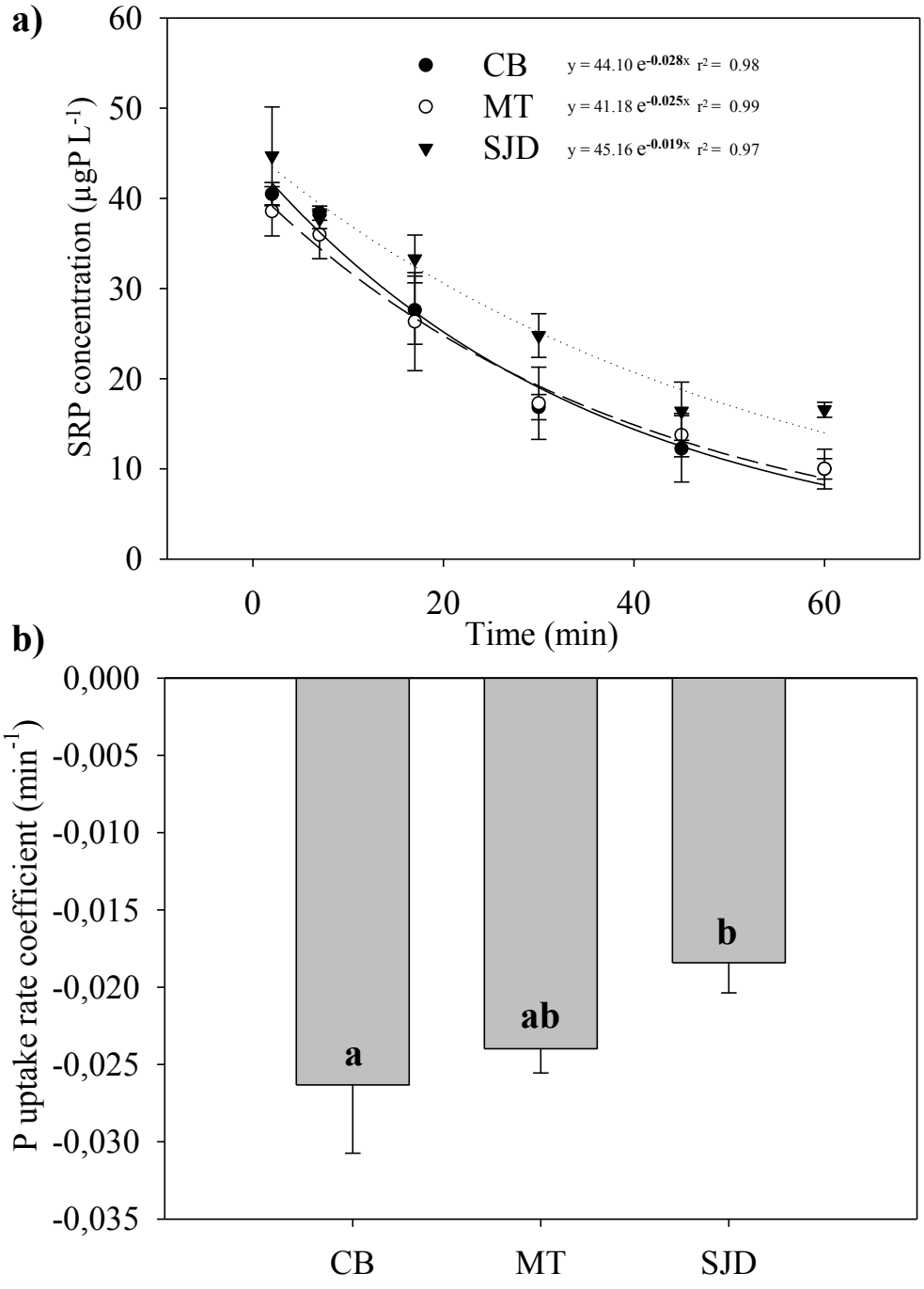


Figure 3. Comparison of phosphorus uptake capacities of biofilms grown with water from the three selected sampling sites along the pollution gradient of the LLobregat river (Castellbell = CB; Mina de Terrassa = MT; Sant Joan Despí = SJD). a) Variation of dissolved inorganic phosphorus in time after controlled spike. Values are mean ± standard deviation (n = 3). b) Uptake rate coefficients of the different biofilms. Letters represent results of the post hoc Tuckey b test when one-way analysis of variance (ANOVA) resulted significant ($p < 0.05$).

DISCUSSION

Our study confirmed the sensitivity of phosphorus uptake capacity as descriptor of the whole biofilm community function related with river self-depuration capacity. Our results demonstrated that biofilm phosphorus uptake efficiency responds to environmental and chemical factors. The use of this tool was particularly successfull evidencing biofilm responses to controlled exposures to toxicants, as well as decribing the effects driven by real mixtures of chemicals at low concentrations. The decrease in the capacity of biofilms to uptake phosphorus has been demonstrated in response to exposure to the bactericide triclosan as well as along the pollution gradient of the Llobregat River. This decrease could be explained by increasing pollution occurring downstream as a consequence of human activities. Urban and industrial sewage waters entering the river gradually increase the concentrations of both inorganic nutrients (phosphate and nitrates particularly) and chemical compounds potentially toxics (i.e. pharmaceuticals, personal care products). Is it known that biofilms growing under higher phosphorus concentration could be less effective in removing P from water column as a consequence of lowering competition for a limiting resource. Our results also demonstrated that biofilm capacity to uptake phosphorus could be reduced by chemicals with toxic effect for biota reaching freshwaters continuously. In chapters 4 and 5 we showed how pesticides, pharmaceuticals and antibiotics increased downstream in the Llobregat River and may have negative effects on structure and function of both autotrophic and heterotrophic biofilm compartments. In this case, the most polluted site (SJD) corresponds with the site with the highest P concentrations and where biofilm showed the lowest phosphorus uptake rate coefficient. More specifically, the toxicity of TCS for biofilm communities has been demonstrated in previous chapters (Chapter 7 and 8). Although in our study triclosan concentrations have not been measured, the downstream increase of other bioactive compounds, also entering from WWTP effluents (pharmaceuticals, antibiotics), allowed us to hypothesize that the same behavior of triclosan would occur in the Llobregat river for other compounds. In fact, the removal of triclosan

during sewage waters treatment is widely variable and its presence has been previously described in Llobregat river waters (Kantiani et al., 2008; Kuster et al., 2008). Nevertheless, the range of biofilm phosphorus uptake, along the Llobregat pollution gradient, is much less pronounced than in biofilms directly exposed to higher concentration of triclosan. This difference could be explained by the buffering effect of high nutrient availability on chemicals' toxicity as well as by adaptation of biofilms to not limiting conditions. Thus, is not possible to establish causality between one single factor and P uptake decrease in SJD, where is of its maximum the multiple stressors context of our study. The reduction of P uptake capacity observed in the biofilm downstream could be explained by the combined effects of eutrophication and chemical pollution. All these evidences confirm the sensitivity of this tool and highlight its usefulness in both controlled exposures and more realistic approaches in the investigation of the effects of pollutants on river ecosystems.

Comparing the results of the three different experiments allowed us to conclude on the risk associated with Triclosan presence in running waters for river self-depuration capacity. The independent effect of TCS on biofilms phosphorus uptake efficiency has been attributed to cumulative effects of the bactericide on both autotrophs and hetherotrophs (Chapters 7). This result cis in agreement with observations of direct effects on target organisms that may provoke indirect effects on non-target organisms. The interactions within biofilm communities result in a loss of whole community function as phosphorus uptake efficiency. As was shown in Chapter 8, environmental events linked with climate variation, such as drought episode, may provoke negative response of biofilms in terms of their capacity to remove phosphorus from water column. Our results showed that the toxic effects of TCS on P uptake capacity were worsened after the drought episode occurred. This result evidenced the importance to study the toxicity of chemicals, and the relative ecological risk associated to their presence in freshwaters, considering the natural environmental variation and the possible scenarios predicted by climate change models.

In this context of natural factors influencing the response of communities to potential toxic compounds the second experiment of this chapter has been performed adding the grazing on biofilm as a modulating factor on triclosan toxicity effects. In this case, grazers alone provoke a slight increase of the capacity of biofilms to uptake phosphorus while TCS only affect this function in presence of snails. This opposite behavior could be explained by the mode-of-action of grazers and TCS. Is it well known that grazers consume epilithic biofilm biomass, "cleaning" substrata that remain free for a new colonization process. Biofilm biomass submitted to grazing pressure resulted significantly lower than biofilms not grazed (data not shown). The colonization process of river biofilms can be resumed by sigmoidal curve, with the first phases characterized by exponential growth until reaching a plateau (Romaní et al., 2004). Exponential growth requires complete resources availability, and inorganic phosphorus is one of the most limiting resources. Thus, the increase of phosphorus uptake capacity of biofilm submitted to grazing pressure could be explained by the restart of colonization process after the snails' activity. Colonization is started by bacteria that attach by covering mineral surfaces with polysaccharide glycocalix (Barlocher and Murdoch 1989). This extracellular matrix facilitates the adherence of micro autotrophs and traps organic matter particles permitting the development of more complex biofilms (Pusch et al. 1998). Thus, the role of bacteria after the grazers' "reset" provoked is crucial to restart biofilm development and bacteria remain as the most important compartment taking up phosphorus during this phase. The anti-bacterial activity of TCS (previously described and discussed in this thesis' Chapters 7 and 8) and the significant decrease of P uptake efficiency of biofilms submitted to both grazing pressure and TCS exposure, can be therefore explained by the combination of direct actions of both factors on the biofilm: grazers clean substrata and cause the restart of colonization process in which bacteria are the most important compartment involved, and the bactericidal action of TCS during this phase induce a decrease of the whole community capacity to remove phosphorus from water column.

It should be also stressed that direct negative effects on both target and non-target organisms of river biofilms are dose dependent. Assembling, results from different experiments do not allows to build dose-response curves, but our observations evidenced that the highest concentrations of TCS (60 and 80 μgTCS L^{-1} respectively) caused significant decrease of phosphorus uptake capacity because of co-occurrence of direct and indirect effects on both autotrophic and heterotrophic biofilm compartments (Chapters 6 and 7). In contrast, the lowest concentration of TCS (15 μgTCS L^{-1}) did not induce P uptake inhibition alone, but resulted effective in the presence of grazers. Is also true that our studies assessed effects of exposure to relevant concentrations of TCS during only 48 hours. Thus, considering that the presence of grazers is the most realistic scenario in real ecosystems, and that TCS normally reach river waters at lower concentrations but in a continuous pase after waste water treatment plants effluents, our work conclude that the ecological risk associated to the presence of TCS (or other potential toxic new-emerging compounds) in river waters should be assessed taking into account complexity of relationships between communities in ecosystems.

To conclude, our study validates the use of biofilm phosphorus uptake capacity as a tool to understand the ecological consequences of pollution and environmental variation on river self-depuration capacity. However, the measure of biofilm P uptake capacity fits in the context of the multi biomarker approach used in this thesis, and enhance the possibility to understand interactions within complex microbial assemblages. In this sense the use of P uptake capacity as an endpoint in ecotoxicological studies increases the ecological relevance of conclusions that can be derived from other parameters with less obvious immediate impact.

CHAPTER 9:

GENERAL DISCUSSION

GENERAL DISCUSSION

The main assumption of this thesis was that river biofilms could be used as reliable tools to determine some of the consequences of global change in river ecosystems. The factors considered in this thesis vary from the increase of nutrients availability resulting from human activities to the increases of light incidence on the riverbed in response to riparian vegetation simplification. The presence of chemical compounds in river waters derived from agriculture and urban sources from both point and non-point sources is another factor that has been assessed in this thesis. Besides, the influence of drought episodes on the response of biofilms when faced to a key non-priority pollutant was used to approach the consequences of flow change predictions (in Mediterranean rivers) on the behavior of biological communities' responses to potential toxicant compounds.

The combination of field and laboratory experimental studies allowed to determining the effect of independent and combined perturbations on fluvial biofilm communities. Multiple metrics related with structure and function of both autotrophic and heterotrophic biofilm compartments were successfully used to describe direct and indirect effects and to investigate the ecological interactions occurring at the microbial scale. The observation of late effects after transient acute perturbations was used as a tool to study the recovery potential of biofilms and therefore to upscale the results to the overall ecological implications for river systems. The determination of biofilm phosphorus uptake capacity has been proposed in this thesis as a biofilm functional descriptor related to the river auto-depuration capacity, and ultimately with one of the most relevant ecosystem services provided by running water ecosystems. Multivariate analyses have been successfully used to elucidate the complexity and abundance of stressors that co-occur in the highly impacted Llobregat River.

NUTRIENTS AND LIGHT AVAILABILITY INFLUENCE ON BIOFILM STRUCTURE AND FUNCTION

Nutrients and light are essential resources, and their availability could determine changes in the structure and function of microbial benthic communities. They are environmental factors whose variability could be associated to both natural events and human activities. In fact biogeochemical cycles at the watershed scale may influence the availability of inorganic nutrients, as well as daily and seasonal fluctuations determine natural light availability. Nevertheless, despite the improvement of waste water treatments in last decades, agricultural land use and urban sewage effluents are still important punctual and diffuse sources of nutrient inputs to freshwater ecosystems. Human activities as urbanization and river canalization frequently result in riparian simplification with a consequent alteration of incident light regime on riverbed that could affect structure and function of microbenthic biota. Our study (Chapter 3) demonstrated that the co-occurrence of both increases in light and nutrient availabilities provokes the most important structural and metabolic responses of stream biofilm. In terms of structure, autotrophs and heterotrophs in benthic communities could respond differently to nutrient supply. The shifts in the relative abundances and in the spatial organization of autotrophic groups observed in our study may be reflected at functional level. The changes observed at relative short-term time scale in our experiment suggest that the increase in nutrient and light availability, both as consequence of human activities, may reflect changes in metabolism and functioning at the ecosystem scale. In fact, key biological processes occur at the microbial scale and the biogeochemical reactions driven by micro biota might be relevant at the ecosystem scale (Zehr 2010). For example, nutrient availability can either directly (by influencing metabolic activities) or indirectly (by effecting primary producers) affect heterotrophic functioning of biofilm communities (Romaní et al., 2004). Biofilms are the main responsible for the matter cycling –release, uptake and transformations- in most sections of the river system. This response

could change depending on light availability conditions as demonstrated by our study. Several studies have focused on the effects of nutrient enrichment on the biofilm structure and function (Romaní et al., 2004; Sabater et al., 2005). While many of these studies have investigated the role of light and nutrients on the growth and community composition of biofilms in oligotrophic forested streams, in this thesis the combined effects of light and nutrients on spatial organisation, microbial biomass and biofilm functioning were evidenced. Finally, investigation of the colonisation process by both structural and metabolic parameters under different nutrients and light conditions has been demonstrated as a useful tool to study ecological interactions within biofilm communities.

BIOFILM RESPONSES TO STRESSORS

Different experiments of this thesis were performed in mesocosms under controlled conditions in order to investigate biofilms responses to different types of stressors: from potential toxic compounds to simulated drought episodes. The exposure to real mixtures of compounds detected in river water (Chapter 4 and 5) allowed to approach realistic scenarios of multi-stressor situations, while the use of controlled exposures (Chapter 6 and 7) allowed the investigation of causality between the stressor and the response that would not be possible to establish in field conditions.

The use of river water from the pollution gradient of the highly impacted Llobregat River combined with the translocations experimental approach (chapter 4 and 5) resulted in a useful tool to investigate ecological relevance of real mixtures of priority and emerging compounds and to elucidate which compounds (or class of compounds) may represent a risk for river ecosystems. By selecting metrics that respond to different classes of stressors, results of multivariate analyses may be useful for identifying specific stressors in a multi-perturbed system (Clements and Newman, 2002). Results of our studies elucidated relationships between biological responses and water

quality. Focusing on the role of non-regulated emerging compound, we demonstrated that mixture of low concentrations of bioactive organic compounds that reach continuously river systems may lead to chronic effects on biological communities. The inclusion of several biofilm metrics provided interesting results showing structural and functional changes in the different biofilms compartments. Particularly, the commonly used analgesics and anti-inflammatories, still considered new-emerging compounds and frequently detected in freshwaters wherever, significantly affected biofilm responses, mainly the autotrophic compartment (Chapter 4). Antibiotics of human use were those determining changes in heterotrophic community structure and functioning of biofilm (Chapter 5).

In spite of the advance represented by the combination of field and controlled conditions in the laboratory in the test of real mixtures of contaminants on biofilm responses, this approach cannot be used to demonstrate causation between single stressor and specific response. As mentioned above, multivariate analysis is considered a useful tool to identify specific stressors among multiple perturbations (Clements and Newman, 2002) but not to establish causality. Therefore, controlled exposures have been used in chapter 6 and 7 to investigate causality between exposure to key compounds and response of biological communities that would not be possible to establish in field conditions.

The first experiment (Chapter 6) was performed testing the resistance and the recovery of biofilms after pulses of the herbicide Diuron and the bactericide Triclosan. Diuron is an herbicide included by the EU in the list of priority compounds with a well-known mode-of –action against autotrophs while Triclosan is a bactericide with unknown mode-of-action against autotrophs still considered as new-emerging compound by water managers. This study evidenced direct and indirect effects of TCS on biofilm autotrophs (non-target organisms) that added to direct effects on bacteria (target organisms), and that resulted in a loss of the whole community capacity to uptake phosphorus from water column (Figure 1).

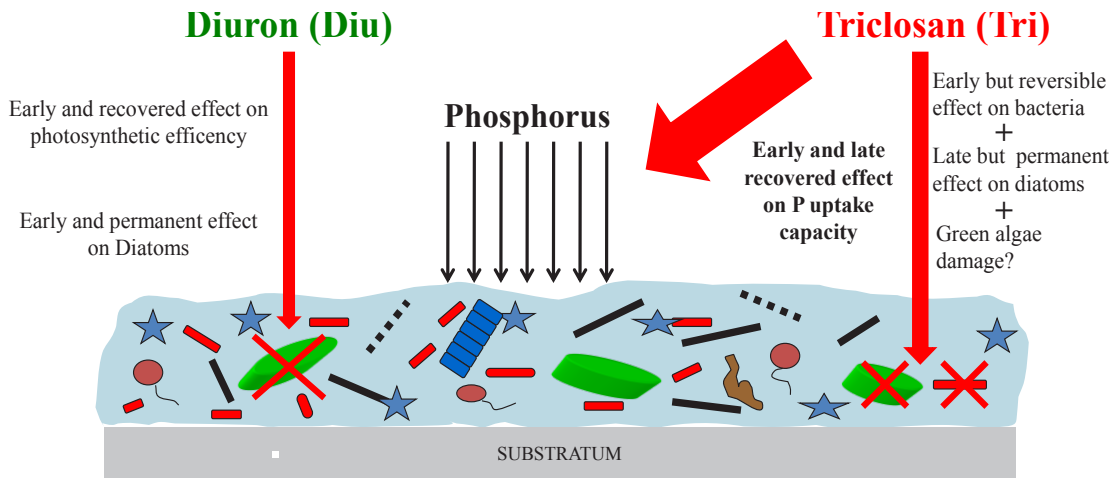


Figure 1. Schematic representation of the effects of triclosan and diuron pulses on river biofilms structure and function.

These results evidenced the risk of the presence of TCS for river self-depuration capacity. Moreover, the study of recovery process highlighted how effects after short-term exposure could persist in time suggesting a still higher risk associated to continuous entrance of TCS from waste water treatment plants effluents. In particular, the direct effect on bacteria viability would provoke later and persistent increase of diatoms mortality because of well-known positive interactions between these two groups. Similar results were observed in Chapter 7 were TCS toxicity under assumptions of climate change scenario were investigated. The effects of short-term simulated drought episode modulated toxicity of TCS to biofilm structure and function. The velocity in the recovery process revealed that persistent effects were produced on bacteria and diatom viability as well as on the biofilm phosphorus uptake capacity (Figure 2). Therefore, these two studies lead to conclude that there is a high toxicity risk of TCS for river biofilms. Moreover the evidences showed by these studies highlighted the relevance to investigate the recovery process after exposure to better assess ecological implications of acute toxicity and the importance to investigate toxicity of compounds considering interactions with environmental factors naturally variable.

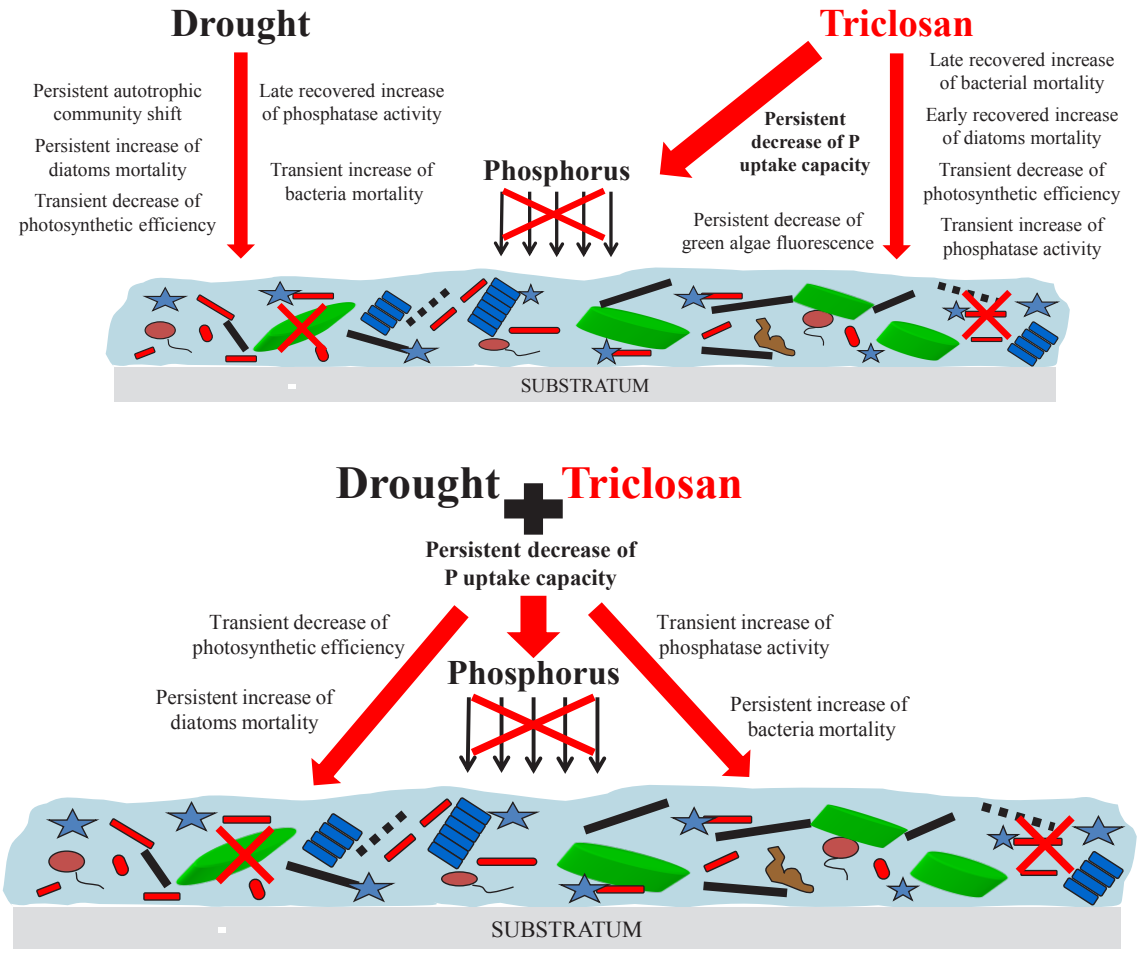


Figure 2. Schematic representation of the independent and combined effects of drought and triclosan on river biofilms structure and function.

THE MULTI-BIOMARKER APPROACH RELEVANCE IN THE ANALYSIS OF BIOFILM RESPONSES

The multi-descriptor approach used in this Thesis, has covered both functional and structural aspects of the biofilm communities. This approach allowed us to describe the effect of stressors and/or environmental factors on biofilm structure-function considering the complexity of such diverse communities. In this section the comparison among the responses of descriptors analyzed in all the experiments is presented and discussed. To compare results from experiments

performed at different spatial scales (from field to mesocosms) we calculated the percentage of control (Table 1). The first evidence highlighted by this comparison was the different sensitivity to different kind of stressors showed by biofilm bacterial compartment compared to the autotrophic.

	Chl-*a*	**Ymax**	**Yeff**	**Bacteria**	**APase**	**LamP**	**U**
Light	+37.4%	n.m.	n.m.	+9% (density)	-6.3%	+38.7%	n.m
Nutrients	+43.1%	n.m.	n.m.	+6% (density)	-21.3%	+31.5%	n.m.
Pollution gradient							
CB→MT	-10.2%	-10.1%	-1.6%	-11.6% (L/D)	+2.2%	-4.9%	+44.8%
MT→SJD	-33.7%	-11.7%	+3.3%	-11.2% (L/D)	-4.4%	+3.1%	+55.1%
CB→SJD	-8.0%	-6.7	+3.2	-22.6% (L/D)	-3.1%	+0.7%	+30.3%
Diuron	+15.6%	-10.5%	-20.8%	+2.4% (L/D)	+0.3%	+2.5%	-5.6%
Triclosan (60 µg TCS L^{-1})	-21.1%	-5.5%	+12.6%	-46.6% (L/D)	-14.5%	-5.6%	-70.7%
Drought	n.m.	+7.5%	+8.0%	-15.6% (L/D)	+25.3%	-0.0%	+21.0%
Triclosan (TCS) (80 µg TCS L^{-1})	n.m	-13.4%	-17.4%	-24.7% (L/D)	+26.8%	-11.6%	-32.0%
TCS + Drought	n.m	-12.5%	-3.7%	-32.5% (L/D)	+25.0%	+5.7%	-32.4%

Table 1. Variation of biofilm structural and functional parameters when submitted to different stressors. Values are percentages related to the respective control for all the experiments performed. Chl-*a* = Chlorophyll-a content; Ymax = Photosynthetic capacity; Yeff = Photosynthetic efficiency; Bacteria = bacterial density or live/dead ratio (L/D); LamP = leucine-aminopeptidase activity; APase = alkaline-phosphatase activity; U = phosphorus uptake rate

Despite the slight increase of bacterial density observed in biofilm grown under higher light and nutrients availability, bacteria live/dead ratio in general decreased in response to chemical substances, and the degree of decrease was proportional to the degree of perturbation. The direct effects of the exposure to the bactericide triclosan resulted in the highest decrease of the live/dead bacteria ratio. The real mixture of new-emerging chemicals (particularly antibiotics) detected in river waters also provoke significant bacteria responses in terms of mortality (Table 1) and community composition shifts (see Chapter 5). Despite light epilithic biofilms are fairly considered primary producers of freshwater systems because of dominance of autotrophic biomass, this thesis highlights the relevant role of hetherotrophs, particularly bacteria, in the assessment of global change consequences on running water ecosystems. The relevance of bacteria within biofilm communities has been demonstrated by several studies (Proia et al., 2012; Romaní et al, 2004; Ylla et al., 2010) and can be confirmed by comparing the complex pattern of extracellular enzymatic activities (EEAs) responses observed in our experiments (Table 1). The luecine-aminopeptidase (LamP) activity increased due to light and nutrient availbility increase and decreased due to the single effect of triclosan. However, LamP did not respond clearly to translocation to more polluted sites (Table 1). In contrast, alkaline phosphatase (APase) decreased in response to increasing nutrient concentrations in water and showed complex pattern in response to triclosan (Table 1). Responses of APase activity to translocation were also not relevant (Table 1). These results indicate that the EEAs are highly controlled by physical and chemical parameters as was also described when comparing pesticides and environmental factors effects on several biofilm metrics (Ricart et al., 2010). On the other hand, specific compounds such as triclosan, targeting directly on bacteria, showed a clear effect on enzymes, especially on leucine-aminopeptidase. Moreover, comparison of responses of LamP in the different experiments highlights the capacity of bacteria to modulate their functional levels in different conditions. In fact, the behaviour of bacterial density and viability is not always associated to responses of EEAs. Particularly, the decrease of

the live/dead ratio of the EEAs producers' organisms (bacteria) in response to translocations do not correspond to a decrease of the activities (Table 1). The specific interpretation of the EEAs behavior suggests that autotrophs-heterotrophs interactions within biofilms communities are often the main important explanation of the observed EEAs responses to changed conditions. In conclusion, all these evidences suggest that EEAs may be useful tools in the description of biofilm responses to global change related factors only in the multi-biomarker approach used in this thesis.

Furthermore the autotrophic biomass measured as chlorophyll-*a* density responded more clearly to environmental factors as nutrients and light availabilities than to exposure to chemicals. In fact, chlorophyll*a* density increased under higher nutrients and light availability conditions and in general decreased in response to chemical stress (Table 1). Nevertheless, the decrease of chlorophyll*a* in biofilms translocated from less to more impacted sites was transient and these communities finally tend to increase autotrophic biomass (see Chapter 4). This behavior may be explained by different factors acting at different time scale. The initial decrease may be a response to increasing concentrations of chemicals probably affecting autotrophs as confirmed by photosynthetic capacity (but not efficiency) decrease (Table 1), later on, when the communities adapted to the new conditions, the increased availabilities of inorganic nutrients (mainly phosphorus) may be the driven factor for the final increase of chlorophyll density of translocated biofilms. Nevertheless this behavior has been observed also after transient stress. The same pattern of chlorophyll*a* density and photosynthetic parameters was observed in biofilm exposed during 48 hours to the bactericide triclosan. In particular, autotrophic parameters significantly decreased just after exposure while recovered to values higher than control during the subsequent two weeks (see chapter 6). Moreover, in the experiments where chlorophyll fluorescence measurement were performed also by phyto-PAM, the information about responses of autotrophic compartment was complemented and completed allowing a deeper investigation of the effects of studied factors. For example the decrease of photosynthetic capacity (but not efficiency) informs about possible structural damages of autotrophs while decrease of both parameters concern basal photosynthetic

function and therefore may inform about whole community metabolism.

Finally the comparison of phosphorus uptake measurements performed in mesocoms experiments demonstrated how this whole community function is an integrative measure that cannot be explained only by the "sum" of the observed effects on each compartment. For example, biofilm transferred from less to more impacted sites showed a general increase of phosphorus uptake values despite a decrease of live/dead bacteria ratio and chlorophyll density two days after translocation (Table 1). On the other hand, the slight positive effect of simulated drought on this function is not reflected when flow treated biofilms were exposed to triclosan (Table 1) confirming that this function in the multi-descriptor context proposed in this study is a powerful and not redundant tool to investigate biofilm processes related to river self-depuration capacity.

APPROACHING HIGHER ECOLOGICAL RELEVANCE IN ECOTOXICOLOGICAL STUDIES.

The study of the recovery process in complex microbial communities after short-term perturbations provides new insights on the role of organisms to physical and chemical disturbances. In general, most of the ecotoxicological studies are focused on acute or chronic toxicity of compounds for single specie described by one or more descriptor/s, named endpoint/s. These approaches consist in the exposure of cultured organisms to the tested compound following standard procedures established by international protocols. Data from these studies are then used for ecological risk assessment (ERA) and subsequent management procedures. In particular, acute toxicity studies analyse direct effect on the target organism after controlled exposure to increasing concentrations of the tested compound in order to obtain a dose-response curve from which extrapolate values useful for ERA procedures (EC50, NOEC, etc). Although the procedures based on these approaches are necessary to advance in the management of policy, ecological relevance of results obtained from such simplified experiments is normally low. The use of

complex communities (i.e. river biofilms) combined with multi biomarker approaches in acute ecotoxicological tests has been used by several authors to increase the ecological relevance of observed results (Bonninneau et al., 2010; Ricart et al., 2010). Moreover, biofilms were also used to assess effects of long-term exposures to toxicant compounds in chronic ecotoxicological tests (Ricart et al., 2009; Tlili et al., 2010). Using the same long-term exposure approach, the pollution induced community tolerance (PICT) has been introduced as concept to describe chronic effects of contaminants on biological communities (Blanck et al., 1988) and has been applied with river biofilms (Tlili et al., 2011). This concept is based on the assumption that a complex community chronically exposed to toxic compound would shift its composition by replacing sensitive species to tolerant ones. This shift would be reflected in acquisition of tolerance relatively easy to measure by acute tests. All these approaches sensibly increased the capacity to upscale results obtained in laboratory to ecosystems, although limitations are still present. This thesis proposed further steps in the direction of increase the ecological relevance of ecotoxicological studies: the use of biofilm phosphorus uptake efficiency as endpoint related with river self-depuration capacity and the study of recovery process to investigate ecological implications of transient perturbations.

The use of phosphorus uptake efficiency as a general functional descriptor in ecotoxicological studies has been validated in this thesis and its advantages and limitations have been already discussed (Chapter 8). Instead, the discussion about the importance to study of recovery process after perturbations deserves more accurate attention in this section. Assessment of ecological consequences of disturbances, either natural or human-induced, confronts a fundamental problem. Ecosystems are complex, variable, and diverse in nature; consequently, simplification to essential features that would characterize ecosystems adequately is needed. Yet there is no firm prescription for what to measure in order to describe the resistance of ecosystems to stress and their resilience (Kelly and Harvell, 1990). In experimental ecotoxicological studies the recovery could be simplified as the return to the control conditions after a significant deviation in response to the

stressor. With this purpose, in the experimental designs of the studies performed in this thesis on TCS toxicity, two more weeks after the end of the two days exposure, were included in the sampling strategy. During these two weeks the conditions maintained during the colonization period were restored for all biofilms and two more samplings were performed in order to study recovery process. The study of the behaviour of biofilms after the removal of the stressor was also fundamental to detect eventual indirect effects due to interactions occurring within community. For example in the experiment of chapter 6 during the first week after the end of exposure diatoms mortality increased significantly in indirect response to direct bacterial mortality induced by TCS. Without including the observation of the behaviour of biofilms after the removal of the stressor in the experimental design, this indirect effect with important micro-ecological implications would not be described and assessment of TCS toxicity on biofilm would not be as complete as was. As another example, in the experiment reported in chapter 7, the study of recovery highlighted that previous drought episode worsened the negative effects of TCS on biofilm. In fact, some of the biofilms descriptors that recovered in biofilm only exposed to TCS did not in biofilms previously submitted to simulated drought. These differences did not appear just after the perturbations occurred, but required two weeks to be evidenced confirming the importance to study post perturbation period in order to approach higher ecological relevance of exotoxicological studies. In conclusion, the study of recovery period after short-term acute perturbation could also provide important information to formulate hypothesis about effects of longer or chronic perturbations more difficult to reproduce in laboratory conditions. For example in both studies assessing toxicity of TCS in this thesis, the not complete recovery of all biofilm descriptors two weeks after the end of exposure allow to conclude that the continuous entrance of the bactericide from waste water plants effluents may have important consequences for aquatic ecosystems, particularly for river self-depuration capacity. Finally, the evidences reported in this thesis validate the use of biofilm phosphorus uptake efficiency and the study of recovery process after exposures as useful tools in order to reach higher ecological relevance of ecotoxicological studies.

GENERAL CONCLUSIONS

CONCLUSIONS

1. Light and nutrient availability affects thickness and structural complexity of biofilm. Particularly, the biofilms grown under higher light and nutrient availability conditions were the thickest and showed the most complex structure as evidenced by Confocal Laser Scanning Microscopy (CLSM).

2. The CLSM highlighted that the extracellular polymeric substances were agglomerate in the upper layer of biofilms grown under nutrient-enriched conditions while were evenly distributed through the biofilm in communities grown in unenriched reach.

3. Biofilm function is affected by both nutrients and light. Particularly, nutrient concentrations increase caused the decrease of extracellular phosphatase activity while interaction between the two factors affected extracellular peptidase activity. Biofilms grown under higher light and nutrients conditions showed the highest peptidase and the lowest phosphatase activity. The conjoint availability of light and nutrients caused the highest changes in biofilm spatial organization, microbial structure and functioning in oligotrophic forested stream.

4. The lowest part of the Llobregat River was highly polluted by pharmaceuticals and pesticides. Pharmaceuticals concentrations were in general higher than pesticides. Analgesics and anti-inflammatories were the most concentrated at each sampling site with Ibuprofen as the most concentrated compound. Sant Joan Despí was the most polluted site while concentrations in Castellbell and Mina de Terrassa were lower.

5. The differences between biofilms before translocation mostly concerned the autotrophic compartment. Biofilm grown in Sant Joan Despì water had higher chlorophyll *a* density and fluorescence signal of all autotrophic groups (green algae, diatoms and cyanobacteria) than those grown with Castellbell and Mina de Terrassa waters.

6. The biofilms responses were different depending on the translocation. The translocation of biofilms from CB to MT was the least responsive while the translocation from MT to SJD caused important structural and functional responses. The translocation from CB to SJD was the most effective in terms of the biofilm responses. The multivariate analysis showed that conductivity, analgesics and barbiturics groups were the variables that significantly influenced the response of biofilms to translocation and pollution gradient of Llobregat River. Further, the partitioning variance technique revealed that analgesics and antinflamatories significantly affected biofilm responses to translocations and pollution gradient, particularly the commonly used Ibuprofen and Acetaminophen (Paracetamol) resulted related with negative effects on autotrophic compartment while Diclofenac was more related with responses of heterotrophs.

7. A total of sixteen antibiotic compounds were detected in Llobregat River waters. The sulfonamide antibiotics were the most concentrated at each sampling site followed by quinolones and macrolids. Concentrations in Sant Joan Despí were higher than in Castellbell and Mina de Terrassa.

8. The bacterial communities showed structural but not functional differences between sampling sites before translocation. The bacterial abundance increase from Castellbell to Sant Joan Despì, particularly *Alpha- Beta* and *Gamma-* proteobacteria. The Canonical Correspondence Analysis (CCA) of the bacterial community composition evidenced clear separation of communities grown in the three sampling sites. Differences in bacterial community composition of biofilms before translocation were confirmed by cluster analysis of DGGE gels.

9. Biofilm communities translocated to waters with higher antibiotics concentrations increased bacteria mortality and modulate heterotrophic metabolism, particularly increasing leucine-aminopeptidase and decreasing alkaline phosphatase activities. A CCA performed with

the bacterial community composition evidenced clear separation of translocated biofilm communities and highlights the role of antibiotics. In particular, Actinobacteria (HCG) increase observed in all translocated biofilms resulted associated to increasing antibiotics concentrations. The similarity analysis with the DGGE results showed that the translocated communities tend to shift to communities at the more polluted site where samples were translocated.

10. Short pulses of the bactericide Triclosan (TCS) and the herbicide Diuron (DIU) caused both direct and indirect effects on fluvial biofilms that may persist after the end of exposure. DIU directly affected algae, but rarely affected the heterotrophs, whereas TCS seriously impaired bacteria (direct effect) as well as autotrophs (indirect effect). DIU caused an increase of diatom mortality which persisted until the end of the experiment. TCS also affected persistently diatom mortality, although the effect did not appear until one week post-exposure. TCS caused an increase of bacterial mortality that recovered to normal values one week after the end of exposure. TCS strongly inhibited phosphate uptake, which did not return to normal values until 2 weeks post-exposure. TCS also compromised the cellular integrity of the green alga *Spirogyra* sp., whereas DIU did not.

11. Simulated drought episode, consisting in flow reduction and subsequent interruption, induced biofilms responses at structural and functional levels. Particularly, drought caused an increase of extracellular phosphatase activity, bacterial mortality and green algae biomass. The triclosan (TCS) pulses severely affected biofilms by reducing photosynthetic efficiency, bacteria and diatoms viability, and phosphate uptake capacity. The biofilms exposed only to TCS recovered far better than those subjected to both altered flow and subsequent TCS exposure: the latter suffered more persistent consequences, indicating that simulated drought amplified the toxicity of this compound.

12. The highest concentrations of TCS caused significant decrease of phosphorus (P) uptake capacity because of co-occurrence of direct and indirect effects on both autotrophic and heterotrophic biofilm compartments. Lower concentration of TCS did not induce P uptake inhibition alone while resulted effective in presence of grazers. Thus, the ecological risk associated to presence of TCS (or other potential toxic new-emerging compounds) in river waters should be assessed taking into account complexity of relationships between communities in ecosystems.

13. Biofilm P uptake capacity decrease along the pollution gradient of the Llobregat River. This decrease could be explained by increasing concentrations of both inorganic nutrients (phosphate and nitrates particularly) and chemical compounds potentially toxics (i.e. pharmaceuticals, personal care products).

14. The use of P uptake capacity as endpoint in ecotoxicological studies increases the ecological relevance of the conclusions that can be argued from the observed results.

15. The multiple endpoint criteria used along this research allowed the detection of both direct and indirect effects on river biofilms increasing the ecological relevance of the conclusions argued from the observed results

Publications derived from this thesis:

Proia L., Morin S., Peipoch M., Romaní A.M., Sabater S., 2011. Resistance and recovery of river biofilms receiving short pulses of Triclosan and Diuron. **Science of the Total Environment. 409: 3129–3137.**

Proia L., Vilches, C., Boninneau, C, Kantiani, L., Farré, M., Romaní, AM., Sabater, S., Guasch, H., 2012. Drought episode modulates the response of river biofilm to triclosan. **Aquatic Toxicology. doi:10.1016/j.aquatox.2012.01.006**.

Proia L., Romaní A.M., Sabater S., 2012. Nutrients and light effects on stream biofilms: a combined assessment with CLSM, structural and functional parameters. **Hydrobiologia.** DOI 10.1007/s10750-012-1117-x.

Proia L., Cassió F., Pascoal C., Tlili A., Romaní A.M., 2012. The Use of Attached Microbial Communities to Assess Ecological Risks of Pollutants in River Ecosystems: The Role of Heterotrophs. In Guasch H., Ginebreda A., Geiszinger A. (eds.), **Emerging and Priority Pollutants in Rivers, Hdb Env Chem** (2012) 19: 55–84, DOI 10.1007/978-3-642-25722-3_3. Springer-Verlag Berlin Heidelberg 2012.

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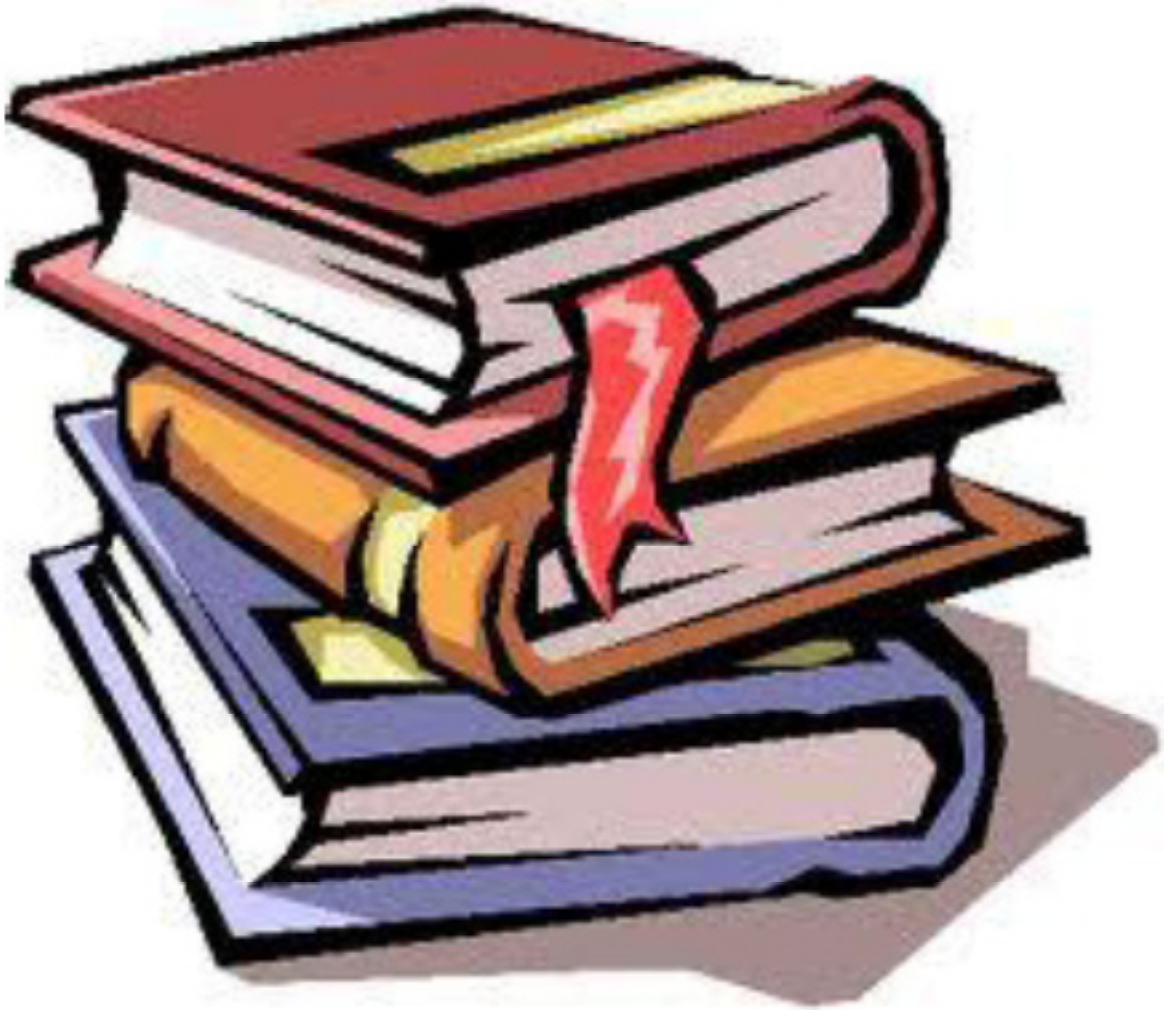

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