Universitat de Girona

THE USE OF PULSE AMPLITUDE MODULATED FLUORESCENCE TECHNIQUES FOR METAL TOXICITY ASSESSMENT IN FLUVIAL BIOFILMS

Natàlia CORCOLL CORNET

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Ph.D.THESIS

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Dirigida per:

Dra. Helena Guasch Padró
Institut d'Ecologia Aquàtica
Universitat de Girona

Dr. Manel Leira Campos
Facultat de Ciències
Universitat de Lisboa

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

Universitat de Girona

Institut d'Ecologia Aquàtica

La Dra. Helena Guasch i Padró, professora titular del departament de Ciències Ambientals de la Universitat de Girona i el Dr. Manel Leira Campos, investigador de la Facultat de Ciències de la Universitat de Lisboa,

CERTIFIQUEN:

Que aquest treball, titulat "The use of pulse amplitude modulated fluorescence techniques for metal toxicity assessment in fluvial biofilms", que presenta Natàlia Corcoll i Cornet, per a l'obtenció del títol de Doctora, ha estat realitzat sota la meva direcció.

Signatura,

Dra. Helena Guasch Padró

Dr. Manel Leira Campos

Girona, 2011

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Corcoll, N., Bonet, B., Leira, M., Guasch, H. 2011. Chl-a fluorescence parameters as biomarkers of metal toxicity in fluvial biofilms: an experimental study. *Hydrobiologia* 673: 119-136.

Corcoll, N., Bonet, B., Leira, M., Morin, S., Tlili, A., Guasch, H. The effect of metals on photosynthesis processes and diatoms metrics of biofilm from a metal contaminated river: a translocation experiment. (*Submitted).*

Corcoll, N., Bonet, B., Leira, M., Montuelle, B., Tlili, A., Guasch, H. Light history influences the response of fluvial biofilms to Zn exposure. (*Submitted).*

CONTENTS

SUMMARY

SUMMARY

Metal pollution is an issue of great concern in fluvial ecosystems affected mainly by mining and other industrial and urban activities occurring in its watershed, causing a loss of their integrity. Consequently, governments have developed different regulations to reduce pollution. In Europe, the implementation of the Water Framework Directive (WFD, Directive 2000/60/EC) aims to reduce chemical pollution of water bodies by 2015 and thus obtain a good ecological status of aquatic ecosystems (preserving the structure and function). In this context, phytobenthos (or phototrophic organisms of biofilms) have been defined by the WFD as a "biological quality element (BQE)" to assess the ecological status of fluvial systems. Biofilms are microphytobenthic communities made up of a consortium of organisms (algae, cyanobacteria, bacteria and protozoa among others) which live on submerged surfaces. Biofilms have an important role in many river ecosystems, being the main primary producers of these and therefore the base of the food chain. The relevance of the use of biofilms for the evaluation of chemical toxicity (e.g. metal toxicity) is attributed (1) to the fact that they offer the possibility to assess chemical toxicity at community level, (2) that they are sensitive to a wide range of toxic substances (including metals) and (3) to the fact that the observed effects at the level of biofilm can allow adverse effects on higher trophic levels to be predicted.

This thesis aims to evaluate the effects of metals on phytobenthic community of fluvial biofilms, refered in the text as biofilms, and the pertinence of the use of chl-a fluorescence parameters as biomarkers of metal toxicity. This study includes experiments with different types of time exposure (from hours to weeks) and with different scales of complexity (from microcosms to field conditions).

Photosynthetic processes play a key role in aquatic ecosystems. These processes are highly sensitive to the presence of toxicants, leading to an increase in

their use as ecotoxicological endpoints. Particularly, the use of chlorophyll-a fluorescence techniques to assess the impact of toxicants on the photosynthesis of the autotrophic component of fluvial biofilms has increased in the last decades. However, these photosynthetic endpoints are not currently used in water quality monitoring programs.

A review of the currently available literature -including studies dealing with toxicity assessment of metals and herbicides is presented in Chapter III. It allowed the discussion of the pros and cons of their use as metal pollution ecotoxicological endpoints in fluvial systems as well as their inclusion in regular monitoring programs.

Chlorophyll-a fluorescence measurements have the ability to respond to a large panel of chemical substances affecting fluvial biofilms, covering both functional and structural aspects of the photosynthetic apparatus of oxygenic photoautotrophs organisms found in the biofilm community. Moreover, they might provide early warning signals of toxic effects. The most relevant Chl-a fluorescence parameters used are: (1) the effective quantum yield (Φ'_{PSII}) which allows the assessment of the photosynthetic efficiency at photosystem II, (2) the optimal quantum yield (Φ_{PSII}) which allows determining the maximal photosynthetic capacity, (3) the minimal fluorescence (Fo) which is used as a surrogate of algal biomass and (4) the non-photochemical quenching (NPQ) used to evaluate the amount of light energy dissipated in non-photochemical reactions.

Thus, the application of the chlorophyll-a fluorescence measurement is recommended as a complementary measurement of metal stress in aquatic ecosystems (Chapter IV). Their application is of special interest in the context of the Water Framework Directive (WFD, Directive 2000/60/EC), which requires the development of new structural and functional endpoints of the biological quality elements (e.g. biofilms).

A study was carried out to evaluate the sensitivity of different chl-a fluorescence parameters measured in freshwater biofilms as metal pollution biomarkers of short- and long-term metal exposures at environmentally realistic concentrations (Chapter IV). A microcosm experiment was performed using indoor channels. Mature biofilms were exposed from hours to weeks to three different treatments: No-Metal, Zn (400 µg/L); and Zn plus Cd (400 µg/L and 20 µg/L, respectively). Metal concentration was based on a real case study: the Riou-Mort River (France) which presents a Zn and Cd metal pollution of 500-1000 µgZn/L and 12-15 µgCd/L measured in June 2008 Biofilms exposed to Zn bioaccumulated similar Zn contents per dry weight to those exposed to the mixture (Zn plus Cd) causing a similar inhibition of the effective quantum yield (Φ'_{PSII}) during the first hours of exposure. A reduction of the algal biomass, a shift in the community composition (a high reduction of diatoms), a reduction of the maximal quantum yield (Φ_{PSII}) and a strong reduction of non-photochemical quenching (NPQ) were observed from day 14 until the end of the experiment (35 days). The results indicate that the effects of the metal mixture present in the Riou-Mort on biofilms could be attributed to Zn toxicity. The use of a set of chl-a fluorescence measurements, including photochemical and NPQ parameters, are recommended as a reliable biomarker tool box to evaluate both short- and long-term effects of metals on biofilms containing oxygenic photoautotrophs, suggesting its use in field applications.

With the aim of evaluating cause-effect relationships between metal pollution and biofilm structure and function, a translocation study was performed in a metal polluted stream (la riera d'Osor, NE Spain). Biofilm responses were measured as temporal changes in chl-a fluorescence parameters, pigment composition and diatom species composition (Chapter V). Biofilms from a non polluted site were translocated to four downstream sites presenting a gradient of metal pollution, corresponding to scenarios of low and moderate metal pollution. Metal effects (Zn bioaccumulation, Fe bioaccumulation and Zn in water) were distinguished from other stressors (phosphate

concentration in water). Based on the results obtained, a fast bioaccumulation of Zn (after few hours of exposure) was linked to a decrease of photosynthetic efficiency and an enhancement of mechanisms of protection through the xanthophylls cycle. After longer exposure, differences in Zn and Fe bioaccumulation as well as Zn water concentration between sites were linked to diatom community changes; decrease of diatoms cell biovolume along with a decrease of the IPS index of diatoms. Based on our results, we recommend including Zn in the list of priority pollutants substances of the European Water Framework Directive (2000/60/EC) due to its occurrence and toxicity. In addition, our study supports the use of chl-a fluorescence parameters jointly with the analysis of photosynthetic pigmens (physiological markers) as suitable functional endpoints of early toxicity. Furthermore, diatom composition studies are recommended for their clear response after chronic exposure. However, new diatom indices beyond the use of the IPS are required to increase their sensitivity to metal pollution, e.g. identify the damage caused by moderate to low metal pollution.

Fluvial biofilms are exposed to multi-stress situations in natural ecosystems, such as the co-occurrence of light stress and metal toxicity. Many studies have examined these two factors separately, but studies simultaneously addressing both factors are rare. In Chapter VI, it was evaluated in microcosms the relationship between short-term light stress and Zn toxicity on the structure and function of fluvial biofilm communities photoadapted to different light conditions (shade and sun conditions). Biofilm communities photoadapted to low light (25 µmols photons /m^2/s, LL biofilms), to medium light (100 µmols photons/m^2/s, ML biofilms) and to high light (500 µmols photons/m^2/s, HL biofilms) were characterized by different attributes: chl-a, total biomass (AFDW), extracellular polymeric substances (EPS), algal groups, diatom taxonomy and photosynthesis-irradiances curves. HL biofilms showed a higher light saturation intensity and a higher production of xanthophylls than LL biofilms. In

contrast, LL biofilm communities had many structural differences, a higher proportion of diatoms in the community, lower AFDW and EPS contents than ML and HL biofilms.

A clear effect of light stress on Zn toxicity (1500 µgZn/L) was also demonstrated. Zn toxicity was enhanced when light stress (an increase or decrease of light intensity in comparison to growth light intensity) also occurred mainly to shade-adapted communities causing a higher inhibition of both the effective quantum yield (Φ'_{PSII}) and the maximal quantum yield (Φ_{PSII}). A decoupling of non-photochemical quenching (NPQ) versus de-epoxidation (DR) processes was also observed, indicating substantial damage on the photoprotective mechanisms of biofilms (i.e. xanthophylls cycle of diatoms) due to Zn toxicity.

Fluvial ecosystems are frequently exposed to strong spatial and temporal variations of light, from 20 µmols photons/m^2/s in shaded sites to full sunlight (1700 µmols photons/m^2/s) (Guasch and Sabater, 1998).This study highlights the need to account for environmental stress (e.g. light stress) caused by such variations in order to better assess the environmental risks of chemicals (e.g. metals)

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RESUM

RESUM

La contaminació per metalls en els ecosistemes fluvials és un problema ambiental de gran preocupació. Està lligada principalment a la mineria i a altres activitats industrials i urbanes que tenen lloc a la conca fluvial, causant una pèrdua de la seva integritat ecològica. En conseqüència, els governs han desenvolupat diverses normatives per tal de reduir la contaminació. A Europa, l'aplicació de la Directiva Marc de l'Aigua (DMA, Directiva 2000/60/CE) té per objectiu reduir la contaminació química de les masses d'aigua abans de 2015 i així, obtenir un bon estat ecològic dels ecosistemes aquàtics (conservant l'estructura i la funció). En aquest context, el fitobentos (o els organismos fototròfics del biofilm) s'han definit per la DMA com uns "elements de qualitat biològica (EQB)" per a l'avaluació de l'estat ecològic dels sistemes fluvials. Els biofilms són comunitats microfitobentòniques composades per diversos organismes (algues, cianobacteris, bacteris i protozous entre altres) que viuen unides a les superficies submergides. Els biofilms tenen un paper important en molts ecosistemes fluvials, sent els principals productors primaris d'aquests i per tant la base de la cadena tròfica.

La pertinència de l'ús dels biofilms per a l'avaluació de la toxicitat química (per exemple, la toxicitat dels metalls) s'atribueix a que els biofilms ofereixen (1) la possibilitat d'avaluar la toxicitat química a nivell de comunitat, (2) a que són sensibles a un ampli grup de substàncies tòxiques (entre elles els metalls) i (3) al fet que els efectes observats a nivell de biofilm permeten predir efectes adversos en els nivells tròfics superiors.

Aquesta tesi té com a objectiu avaluar els efectes dels metalls en els organismos fototròfics dels biofilms fluvials, i la pertinència de la utilització dels paràmetres de fluorescència de la chl-a com a biomarcadors de la toxicitat dels metalls. Aquest estudi inclou experiments realitzats amb diferents de temps d'exposició

(des d'hores fins a setmanes) i amb diferents escales de complexitat (des de condicions experimentals en microcosmos a condicions de camp).

Els processos fotosintètics juguen un paper clau en els ecosistemes aquàtics. Aquests processos són molt sensibles a la presència de substàncies tòxiques, el que porta a un augment del seu ús com a paràmetres de valoració ecotoxicològica. L'ús de les tècniques de fluorescència de la chl-a per avaluar l'impacte de les substàncies tòxiques en el processos fotosintètics del compartiment autotròfic del biofilm fluvial ha incrementat en les darreres dècades. No obstant, el seu ús no és aplicat de forma rutinària en els programes de seguiment de la qualitat de l'aigua.

En el Capítol III s'ha fet una revisió de la literatura actualment disponible sobre l'ús de la fluorimetria PAM en biofilms fluvials, incloent estudis que tracten tan de l'avaluació de la toxicitat dels metalls com dels herbicides. Aquesta revisió ha permès la discussió dels pros i contres del seu ús com a paràmetres ecotoxicològics en ecosistemes fluvials així com, la seva inclusió en programes de seguiment de la qualitat de l'aigua.

Els paràmetres de la fluorescència de la chl-a tenen la capacitat de respondre a un ampli grup de substàncies químiques que afecten el biofilm fluvial, que cobreixen tan aspectes funcionals com estructurals de l'aparell fototosintétic dels organismes fotòtrofs oxigènics que es troben en el biofilm. A més, poden proporcionar senyals d'alerta primerenca dels efectes de les substàncies tòxiques. Els paràmetres més utilizats de la fluorescencia de la chl-a són: (1) l'eficiència fotosintètica (Φ'_{PSII}) que permet avaluar l'activitat fotosintètica del PSII, (2) la capacitat fotosintètica (Φ_{PSII}) que permet determinar la capacitat fotosintètica màxima, (3) la fluoresència basal (Fo) que és utilitzada com un indicador de biomassa algal i (4) l'esmorteïment no fotoquímic (NPQ) que s'utilitza per avaluar la quantiat d'energia llumínica dissipada en reaccions no fotoquímiques.

L'ús dels paràmetres de fluorescència de la chl-a dels biofilms per avaluar la toxicitat dels metalls en els sistemes fluvials seria d'especial interès en el context de la Directiva Marc de l'Aigua (DMA, Directiva 2000/60/EC), en la qual es requereix el desenvolupament de nous paràmetres indicadors de l'estat estructural i funcional dels elements de qualitat biològics (EQB), com són els organismes fotosintètics del biofilm.

Es va portar a terme un estudi per avaluar la sensibilitat de diferents paràmetres de la fluorescència de la chl-a mesurats en biofilms fluvials com a biomarcadors d'exposició de curt i llarg termini a la contaminació per metalls a concentracions ambientalment realistes (Capítol IV). Es va realitzar un experiment en microcosmos mitjançant l'ús de canals artificials. Biofilms madurs van ser exposats des d'hores a setmanes a tres tractaments diferents: No-Metall, Zn (400 µgZn/L) i Zn més Cd (400 µgZn/L i 20 µgCd/L, respectivament). La concentració de metalls escollida estava basada en el cas d'estudi: el riu Riou-Mort (França) el qual presenta una contaminació per Zn i Cd de 500-1000 µgZn/L i 12-15 µgCd/L (Juny 2008). Els biofilms exposats a Zn van bioacumular un contingut de Zn per pes sec similar als biofilms exposats a la barreja (Zn+Cd), causant una inhibició semblant de l'eficiència fotosintètica (Φ'_{PSII}) durant les primeres hores d'exposició. Una reducció de la biomassa algal, un canvi de la composició de la comunitat (una elevada reducció de les diatomees), una reducció de la capacitat fotosintètica (Φ_{PSII}) i una forta reducció de l'esmorteïment no fotoquímic (NPQ) van ser observats des del dia 14 fins al final de l'experiment (35 dies). Els resultats indiquen que l'efecte de la barreja de metalls del riu Riou-Mort en els biofilms podria ser atribuït a la toxicitat del Zn. L'ús d'un conjunt de mesures de fluorescència de la chl-a, inclosos els paràmetres fotoquímics i no fotoquímics, es recomana com una caixa d'eines de biomarcadors fiables per avaluar els efectes a curt i llarg termini dels metalls en els biofilms que contenen organismes fotòtrofs oxigènics: el que suggereix el seu ús en aplicacions de camp.

Amb l'objectiu d'avaluar relacions causa-efecte entre la contaminació per metalls i l'estructura i la funció dels biofilms, es va dur a terme un estudi de translocació en una riera contaminada per metalls (la Riera d'Osor, NE, Catalunya). Les respostes dels biofilms als canvis temporals es van mesurar amb els paràmetres de fluorescència de la chl-a, la composició de pigmentes i la composició d'espècies de diatomees (Capítol V). Els biofilms d'un lloc no contaminat van ser traslladats a quatre llocs aigües avall del riu, els quals presentaven un gradient de contaminació per metalls: corresponent a escenaris de baixa i moderada contaminació per metalls. Els efectes dels metalls (la bioaccumulació del Zn, la bioacumulació del Fe i la concentració de Zn en l'aigua) es van distingir d'altres factors d'estrès (concentració de fosfat en l'aigua). Segons els resultats obtinguts, una ràpida bioacumulació de Zn (després de poques hores d'exposició) es va relacionar amb una disminució de l'eficiència fotosintètica del biofilm i un increment dels mecanismes de protecció d'estrès a través del cicle de les xantofil·les. Després d'una exposició més prolongada, les diferències en la bioaccumulació del Zn i del Fe, així com en la concentració de Zn en l'aigua entre els llocs de translocació es van vincular a canvis en la riquesa d'espècies de diatomees, a una disminució del biovolum de diatomees i amb una disminució de l'índex IPS (índex de sensibilitat de la contaminació basat en la comunitat de diatomees). D'acord amb els nostres resultats, es recomana incloure el Zn en la llista de substàncies contaminants prioritàries de la Directiva Marc de l'Aigua (2000/60/CE), per la seva presència i toxicitat. A més, el nostre estudi recolza l'ús dels paràmetres de fluorescència de la chl-a en forma conjunta amb l'anàlisi de pigments fotosintètics (indicadors fisiològics) dels biofilms com a eines adequades de valoració funcional de toxicitat primerenca. En canvi, els estudis de la composició de diatomees es recomanen per la seva resposta clara després de l'exposició crònica. No obstant això, nous índexs de diatomees, més enllà de l'ús de l'IPS, són necessaris per

augmentar la seva sensibilitat a la contaminació per metalls, per exemple, identificar els danys causats per escenaris de contaminació moderada i baixa per metalls.

Els biofilms fluvials estan exposats a múltiples situacions d'estrès en els ecosistemes naturals com és per exemple la co-ocurrència d'un estrès llum i l'exposició a metalls. Molts estudis han examinat com un d'aquests factors afecten els biofilms, però estudis que abordin ambdós factors al mateix temps són més rars. En el Capítol VI, es va realizar un estudi per tal d'avaluar en condicions experimentals (microcosmos) i a curt termini (hores), la relació entre l'estrès llum i la toxicitat del Zn en l'estructura i la funció en comunitats de biofilm fluvial fotoadaptades a diferents condicions de llum (condicions d'ombra i de llum). Les comunitats del biofilm fotoadaptades a poca llum (25 µmols fotons/m^2/s, LL biofilms), a mitjana llum (100 µmols fotons/m^2/s, ML biofilms) i a alta llum (500 µmols fotons/m^2/s, HL biofilms) es van caracteritzar mitjançant diferents atributs: la chl-a, la biomassa total (AFDW), substàncies polisacàrides extracel·lulars (EPS), els grups algals, la taxonomia de diatomees i les corbes d'irràdiancia-fotosíntesis. Els HL biofilms van mostrar una major intensitat de llum de saturació i una major producció de xantofil·les que els LL biofilms. Per contra, les comunitats de LL biofilms presentàven varies diferències estructurals; una major proporció de diatomees en la comunitat, menor contingut de AFDW i EPS que els ML and HL biofilms.

Es va demostrar un clar efecte de l'estrès de llum sobre la toxicitat del Zn (1500 µgZn/L). La toxicitat del Zn en el biofilms va ser major en condicions d'estrès de llum (un increment of disminució de la intensitat de llum en comparació a la intensitat de llum de creixement), i els efectes més grans es van veure en les comunitats fotoadaptades a l'ombra (LL biofilms) causant una major inhibició de l'eficiència fotosintètica (Φ'_{PSII}) i de la capacitat fotosintètica (Φ_{PSII}). També es va observar un desacoblament dels processos d'esmorteïment no fotoquímic (NPQ) versus la reacció

de de-epoxidació (DR), la qual cosa indica un dany substancial als mecanismes de fotoprotecció dels biofilms (en particular el cicle de xantofil.les de les diatomees) degut a la toxicitat del Zn.

Els ecosistemes fluvials estan sovint exposats a fortes variacions de la intensiat de llum des de 20 µmols photons/m^2/s en llocs d'ombra a 1700 µmols photons/m^2/s en llocs de plena llum del sol (Guasch and Sabater, 1998). Aquest estudi posa de manifest que cal tenir en compte l'estrès ambiental (per exemple, l'estrès de llum) per tal d'avaluar millor els riscos ambientals de les entrades de substàncies tòxiques (per exemple, els metalls) en el ecosistemes fluvials.

CHAPTER I

GENERAL INTRODUCTION

FLUVIAL ECOSYSTEMS AND THE OCCURRENCE OF METALS

In fluvial ecosystems there is a close relationship between water quality and landscape uses in the watershed. The anthropogenic activities occurring in their catchment will be relevant factors for understanding fluvial pollution. In the case of metals, their inputs to the fluvial ecosystems can take place mainly through two ways: point-sources, such as punctual spills or wastewater effluent discharges from urban or industrial activities, and diffuse-inputs from catchment drainage or mine run-off after rainfall events, among others. Consequently, metal inputs in fluvial ecosystems may produce adverse effects on biota and contribute to ecosystem integrity deterioration (Admiraal et al., 1999; Hill et al., 2000; Morin et al., 2008). Specifically, metal toxicity in aquatic organisms is influenced by metal speciation that may alter bioavailability, toxicity (Tessier et al., 1995; Morel and Hering 1993) and metal accumulation (Meylan et al., 2004).

In agreement with the well-recognized environmental risk of pollution, regulatory documents have been developed by management entities. For instance, in Europe the Water Framework Directive (Directive 2000/60/EC) aims to reduce chemical pollution in water bodies by 2015. This directive highlights the importance of evaluating the effects of priority pollutants (composed mainly by metals and organic substances with known mode of action) and emerging contaminants (compounds that are not currently covered by existing water-quality regulations and are thought to be potential threats to environmental ecosystems) on biota.

Metals in river ecosystems may be detected in different compartments: in the water body (e.g. metal concentration in surface water), in the sediment (e.g. metal accumulation in sediments) or accumulated in the biota (e.g. metal accumulation in biofilms). A list of different metallic elements are set out as priority pollutants in the EU according to the Water Framework Directive (WFD, Directive 2000/60/EC) and in the

USA according to the Environmental Pollution Agency (USEPA, 2006) legislations establishing the maximal and the available permissible concentrations of these metals in surface water (Table 1)

Table 1. Metals, which are set out as priority pollutants and their corresponding water quality criteria recommended in the EU by the WFD (Directive 2000/60/EC) and in the USA by the EPA (EPA legislation, 2006).

EU Surface water priority substances (2006)		MAC[a] (µg/L)	AA[a] (µg/L)	USA water priority substances	CMC[b] (µg/L)	CCC[b] (µg/L)
				Antimony (Sb)	-	-
				Arsenic (As)	340	150
Cadmium (Cd) and its compounds	(Class 1)*	≤ 0.45	≤ 0.08	Beryllium (Be)	-	-
	(Class 2)*	0.45	0.08	Cadmium (Cd)	2	0.25
	(Class 3)*	0.60	0.09	Chromium III (Cr)	570	74
	(Class 4)*	0.90	0.15	Chromium IV (Cr)	16	11
	(Class 5)*	1.50	0.25	Copper (Cu)	13	9
Lead (Pb) and its compounds		n.a.	7.2	Lead (Pb)	65	2.5
Mercury (Hg) and its compounds		0.07	0.05	Mercury (Hg)	1.4	0.77
Nickel (Ni) and its compounds		n.a.	20	Nickel (Ni)	470	52
				Selenium (Se)	-	5
				Silver (Ag)	3.2	-
				Thallium (Tl)	-	-
				Zinc (Zn)	120	120

[a] *MAC (maximal annual concentration) and AA (annual average) of priority substances in inland surface waters according to the EQS (Environmental Quality Standards) of the EU WFD (Directive, 2000/60/EC). n.a.: not applicable.*

** For cadmium and its compounds the EQS values vary depending on the hardness of the water as specified in five class categories (Class1: < 40 mg CaCO3/l, Class 2: 40 < 50 mg CaCO3/l, Class 3: 50 to < 100 mg CaCO3/l, Class 4: 100 to < 200 mg CaCO3/l and Class 5: ≥ 200 mg CaCO3/l).*

[b] *CMC (criteria for maximum concentration) and CCC (criteria for continuous concentration) of priority pollutants in freshwater systems according to the US EPA (2006).*

In this thesis, zinc was selected due to its widespead occurrence in fluvial ecosystems and the lack of investigations into its toxicity on biofilms at realistically low environmental concentrations. Zn is used in many industrial applications and is commonly used in a process called galvanization to protect other metals against rust and oxidation. It is also used in various inorganic zinc salts, which are used in numerous commercial applications such as batteries, chemical intermediates,

catalysts, pigments, vulcanization activators and accelerators in the rubber industry, UV stabilizers, and supplements in animal feeds and fertilizers. The anthropogenic sources of zinc in ecosystems are mining, steel production, combustion of coal and waste (ATSDR, 2005). In nature it is not found in its elemental form and is mostly present as zinc sulphides (ZnS) and smithsonite ($ZnCO_3$). It reacts relatively easily with weak acids and is of special concern in aquatic ecosystems, as it is more soluble than the majority of other metals (ATSDR 2005).

The Zn concentrations found in the study-cases of this thesis ranged between 400 and 900 µgZn/L in the Riou-Mort river (in France) and between 10 and 450 µgZn/L in the Riera d'Osor river (in Spain). In both cases metal pollution was linked to mining activities.

Cadmium was also investigated due to its common co-occurrence with Zn (Morin et al. 2007; Santos et al., 2010). Cd enters into the environment through mining, combustion of fossil fuels, incineration of household and industrial waste, and the disposal of metal containing waste and fertilizers. In water ecosystems Cd forms complexes with organics and is removed from the dissolved phase by sorption onto organics, clay minerals and hydrous Fe and Mn oxides (Burton and Pitt, 2002).

The Cd concentrations found in the case study of Riou-Mort river (France) ranged between 12 and 15 µgCd/L and its origin was linked to mining activities. In the Riera d'Osor river (Spain), no Cd pollution was detected.

BIOFILMS, A PERTINENT COMMUNITY ECOTOXICOLOGY APPROACH

Fluvial biofilms are benthic complex biological structures composed mainly by algae, cyanobacteria, bacteria, fungi and microfauna, located in close physical contact and embedded in a mucopolysacharide matrix that lives on stones and rocks (Lock et al., 1984) (Fig.1). In rivers and streams, biofilm has an important role in primary production (Guasch and Sabater, 1998) and in organic matter processing (Romaní and

Sabater, 2001). Biofilms, situated at the interface between the overlying water and the sediments, are suitable compartments for evaluating the effect of chemicals on river ecosystems. Their pertinence as bioindicators of water quality is based on their capacity to detect early and long-term effects produced by toxic substances (Sabater et al., 2007), providing a community ecotoxicology approach with higher ecological relevance than using single species (Clements and Newman, 2002; Guasch et al., 2010). Due to all these properties, phytobenthic community of biofilms (or phytobenthos) have been recognized by the WFD (Directive 2000/60/EC) as a "biological quality element" (BQE) for the assessment of the ecological status of water systems.

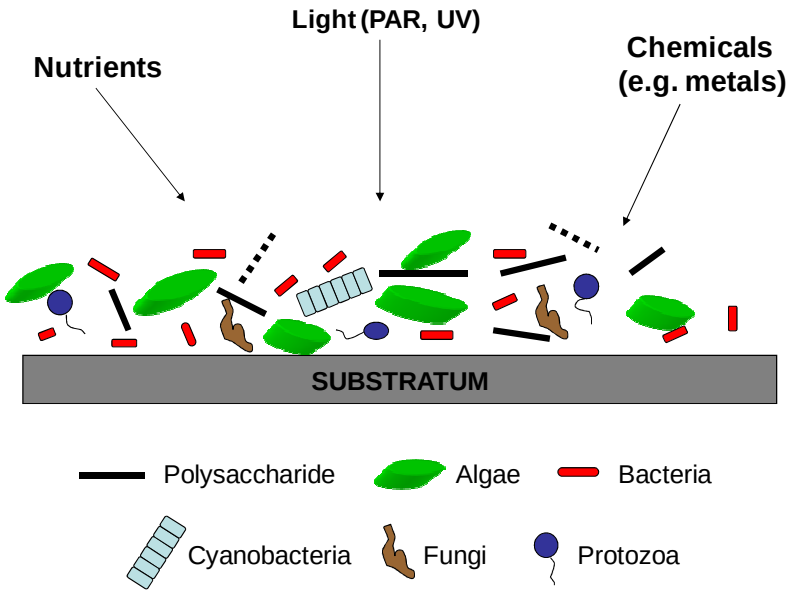


Figure 1. Scheme of fluvial biofilm structure. Adapted from Pusch et al. (1998).

- **Metal toxicity on fluvial biofilms**

Since the beginning of the ecotoxicology of communities in the late 1990s to date, different types of laboratory and field studies have been performed to assess the effects of metals on fluvial biofilms (Guasch et al., in press). These studies have contributed to generating a significant amount of information about the effects of metals on fluvial biofilms (Guasch et al., in press). Many structural, functional and metabolical

biofilm metrics have been applied to assess metal effects on biofilms. In general terms it has been reported that metals affect algal biomass (Paulsson et al., 2002; Navarro et al., 2002), the species composition and the structure of biofilm communities (Guasch et al., 2002). However, the co-occurrence of other environmental factors in field studies such as high nutrients and organic matter may mask metal effects on biofilms (Admiraal et al., 1999; Morin et al., 2007; Duong et al., 2008). Changes in diatom composition has widely been used to assess metal toxicity on biofilms in moderate and high-metal polluted mining areas contaminated by Cu, Zn, Fe, Cd, Pb and As (Hill et al., 2000; Holding et al., 2003; Sabater et al., 2000; Morin et al., 2007; Duong et al., 2008). Physiological biofilm parameters such as photosynthetic activities measured as chl-a fluorescence parameters, among others, have been applied to check photosynthesis-related effects of metals after a few hours (Barranguet et al., 2000; Serra et al., 2009) or several weeks of exposure (Barranguet et al., 2003), as well as in studies aiming to obtain the effective concentration of a compound that had the 50% of effect (EC_{50}) and/or assess pollution-induced community tolerance (PICT) (Guasch et al., 2003, Tlili et al., 2010). Within metal detoxification mechanisms, the production of extracellular polysaccharide substances (EPS) has been reported as an extracellular mechanism of metal protection by reducing metal penetration into cells (Admiraal et al., 1999; García-Meza et al., 2005). At intracellular level, the synthesis of phytochelatins to bind metals has been observed in biofilms exposed to metals, such as Cd (Le Faucheur et al., 2005). More recently, it has been demonstrated that biofilms also use different intracellular antioxidant enzymatic activities to cope with metal toxicity (Guasch et al., 2010a; Bonet et al., submitted).

In this thesis, the ecotoxicology of Zn, as well as its specific co-occurrence with Cd has been studied in fluvial biofilms. Zn is a trace element for photosynthetic organisms and therefore essential for life in low concentrations (0.78 to 65 ng/L) (Lane and Morel, 2000; Sunda and Huntsman, 2005). It is a coafactor of several enzymes

and a component of organic molecules such as carbonic anhydrase (Lane and Morel, 2000). Nevertheless, Zn toxicity is reported at very different concentrations depending on the species tested and the endpoint assessed: Chaloub et al. (2005) found Zn toxicity on cyanbacteria cultures between 30-332 mg/L and Posthuma et al. (2001) describes an EC_{50} of 15 µg/L in *Selenastrum capricornutum* algae. There is no regulation for the maximal concentration of Zn permissible in surface waters in the EU and, in the USA permissible maximal annual concentration in surface water is significantly higher for Zn than for most other metals (Table 1). The mode of action of Zn on algae is believed to be at the photosystem II (PSII) (Chaloub et al., 2005), like the majority of metals.

Cd and its compounds are not essential for photosynthetic organisms and are toxic to most species. They are classified as priority hazardous substances in the WFD (Directive 2000/60/EC). Water hardness also affects the toxicity of Cd (Table 1). In algae, PSII is believed to be the most sensitive target to Cd (Singh et al., 1993). Studies performed with *Selenastrum acutus* (Tupin) Kützing (Ilangovan et al. 1998) have shown Cd toxicity at high Cd concentration (EC_{50}= 500 µg/L). Zhou et al. (2006), observed Cd toxicity on cyanobacteria cultures of *Microcystis aeruginosa* at 448 µgCd/L.

Accordingly, in this thesis metal toxicity on fluvial biofilms has been assessed using several biofilm metrics focusing on chl-a fluorescence parameters as potential metal biomarkers, targeting mainly the autotrophic compartment of biofilms. To address this challenge, a multi-scale approach was followed, including different temporal and spatial scales.

- **Biofilm metrics**

Using a set of biofilm endpoints covering functional processes and structural changes, allows different degrees of effects/damage produced by metals on biofilms to

be evaluated. Biomarkers have been described as biochemical, physiological, or histological indicators of either exposure to or effects of xenobiotic chemicals (Huggertt et al. 2002). A broader application of the biomarker concept, defined as any quantitative measure of changes in the biological system that can be related to the exposure / toxic effects of environmental chemicals, has also been suggested and will be used in the this thesis. In biofilm communities, structural endpoints as total biomass, species abundances and biodiverstity will reflect more persistent and probably irreversible effects and metabolical or functional endpoints will inform about transitory effects on metabolism or community functioning (Geizinger et al. 2009).

The set of biofilm endpoints used in the thesis are: metal accumulation, biofilm structure and diatom composition, photosynthetic pigments and chl-a fluorescence parameters.

- **Metal accumulation in biofilms** was used as an indicator of metal exposure and potential toxicity, as weight of metal per biofilm dry weight (DW). Metal toxicity occurs once the toxic compound has entered into the cell (Escher and Hermens, 2002). The importance of biofilms in retaining metals from the water column has been extensively described (Newman and McIntosh, 1989; Farag et al., 1998; Behra et al., 2002; Meylan et al., 2003). Although biofilms could accumulate metals following abiotic factors (e.g. metal precipitation or sediment deposition), they can also follow different biotic mechanisms; absorption in extracellular polymeric substances, adsorption in cell surface or intracellular uptake of algae (Holding et al., 2003). Biotic accumulation often depends on time of exposure, species and kind of metal (Zbigniew et al., 2006). For biofilms, a high capacity to accumulate Zn, in comparison to other metals (Cu and Pb) has been reported (Ancion et al. 2010). To predict the toxicological effects of trace metals, several models have been developed that relate chemical speciation and bioavailability (e.g. free-ion activity model (FIAM); Morel and Hering (1983), biotic ligand model (BLM); Di Toro et al., 2001). However, these models may

fail in cases where intrinsic biological regulation is present (Hassler and Wilkinson, 2003). However, in this thesis these models were not applied. Aquatic organisms will accumulate less metal during a short exposure, causing a transient metabolic and physiological response than after a longer metal exposure, causing increases in toxicity and/or the development of mechanisms to regulate the accumulation of metals (Hassler and Wilkinson, 2003; Hassler et al., 2005). Thus, in this thesis, metal accumulation has been used as an indicator of metal exposure and potential indicator of metal toxicity on biofilms.

Metal accumulation and toxicity in biofilm may be influenced by different biotic and abiotic processes not addressed in this thesis. It is well reported that the pH has an influence on metal accumulation and toxicity (Sunda and Hunstsman, 1998). The presence of ligands in the media can also interact with metals, whether they are of mineral origin, such as chlorine and orthophosphates (Guasch et al., 2004; Rauch et al., 2004), or organics like fulvic acids (Ma et al., 2003). The structure of biofilms may also influence the bioavailabily of metals, and, therefore, their toxicity (Barranguet et al., 2000; Bradac et al., 2009).

- **Biofilm structure and diatom species composition.** Biofilm communities are characterized by a three-dimensional complex organization within a few millimetres thickness (cells are embedded in a polysaccharide matrix). This structure is susceptible to metal toxicity. To quantify algal biomass and total biomass the chl-a concentration and the ash-free dry weight (AFDW) respectively have commonly been measured. Both measurements may be used as indicators of metal toxicity (Navarro et al., 2002; Serra et al., 2009).

The quantity of extra polysaccharides (EPS) in which the algae are encased is influenced by the species composition, among other factors, and may play an important role in the biofilm structure and protection against metal toxicity (Admiraal et al., 1999;

Serra et al., 2009). Lower metal toxicity has been reported in biofilms presenting higher EPS content. Chronic metal exposure may cause an increase in the metal-binding sites of the biofilm linked to the enhancement of EPS production (García-Meza et al., 2005) as a detoxification mechanism.

Biofilm structure is also influenced by the algal assemblage composition presenting different sensitivity to metal exposure. Fluvial biofilms exposed during a long period of time to metals are characterized by presenting metal-resistant species. Tolerance of cyanobacteria and green algae to long-term exposures of Zn (Genter, 1987; Ivorra et al., 2000; Corcoll et al., 2011) and Zn mixtures (Takamura et al., 1989; Corcoll et al., 2011) has been reported in contrast to diatoms, which are more sensitive to long-term metal pollution (Guasch et al., 2002, Morin et al., 2008; Corcoll et al., 2011). Consequently, diatoms are widely used as biological indicators of water quality and a number of methods for monitoring European rivers based on diatoms have been already proposed (Kelly et al., 1998; Prygiel et al., 2002). They usually account for the highest number of species among the primary producers in aquatic systems (Pan et al., 1999) and often represent the major autotrophic proportion of biofilms (Navarro et al., 2002; McClellan et al., 2008; Morin et al. 2010). Several diatom metrics have been widely applied in ecotoxicology to assess metal pollution; not only at community level through shifts in dominant taxa and diversity patterns but also at an individual level with the appearance of teratological forms or size decrease (Gold et al., 2003; Morin et al., 2007; Falasco et al., 2009; Ferreira da Silva et al., 2009).

Figure 2. Detail of a diatom (*Gomphonema truncatum* Ehrenberg) observed by light microscope.

- **Photosynthetic pigments.**

Pigment composition can be used to assess the global taxonomic composition of the photoautotrophic community (a structural approach as mentioned above), based on pigments markers of algal classes. For instance, fucoxanthin used for diatoms, zeaxanthin for cyanobacteria and chl-b for gree algae (Wilhelm et al.,1991; Dorigo et al. 2007). Changes in the abundance of some photosynthetic pigments have also been used as markers of photosynthetic functional processes (Kana et al. 1997; Laviale et al. 2010). The occurrence of accessory pigments of the photosynthetic apparatus is indicative of its physiological status (Lohr and Wilhelm, 1999). Furthermore, carotenoids, especially xanthophylls, can help to dissipate the energy excess arriving at the PSII due to light or chemical stress (e.g. metals) throughout thermal radiation. Specifically, in diatoms, a way to dissipate this excess of energy that arrives at the PSII is by the de-epoxidation reaction (DR) that transforms diadionoxanthin into diatoxanthin pigments in the diatoxanthin cycle (Lohr and Wilhelm, 1999; Jin et al., 2003) (Fig. 3). This process, which is also named xanthophyll cycle is part of the so-called non-photochemical quenching (NPQ) (Müller et al., 2001), has been studied in diatoms of biofilm communities (Serôdio et al., 2005; Laviale et al., 2009; Chevalier et al., 2010); finding a strong correlation between NPQ and DR increases when biofilms are exposed

to increasing light irradiances. Moreover, a reduction of DR when the biofilm was exposed to isoproturon has also been observed (Laviale et al., 2010).

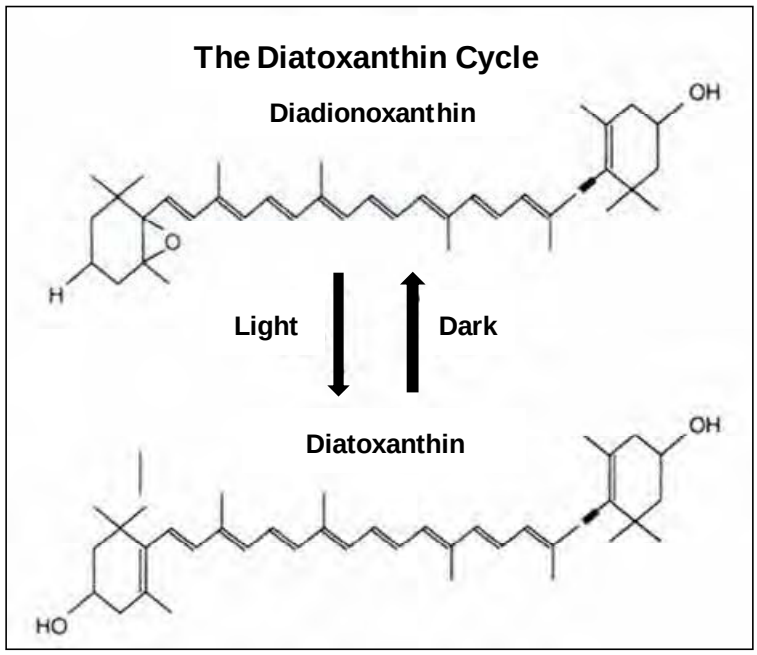


Figure 3. The xanthophyll cycle. In diatoms, the epoxidated xanthophylls (diadinoxanthin) is enzymatically de-epoxidated and converted to diatoxanthin in the light. The backreaction is enzymaticaly catalyzed in the dark. Diatoxanthin appears to be a quencher of excitation. Modified from Falkowsky and Raven (2007).

- **Chl-a fluorescence parameters** allow quantify the fluorescence emissions from Chl of photsystem II after excitation by light conditions. As fluvial biofilms are made up by a great variety of phototrophic organisms (aglae and cyanobacteria), the chl-a fluorescence parameters are presented as fast descriptors of photosynthetic processes (photochemical and non-photochemical) of algae and cyanobacteria composing biofilms, being sensitive to chemical compounds targeting photosynthesis, either directly (e.g. herbicides) or indirectly (e.g. metals). So, despite chl-a fluorescence parameters are not specific to metal toxicity, they are very sensitive to metal effects (Ralph et al., 2007). Chl-a fluorescence parameters are obtained after *in vivo* chl-a

fluorescence measurements by pulse amplitude modulated (PAM) fluorometers. It is based on the principle that light energy absorbed by PSII pigments of algae can either drive the photochemical energy conversion at PSII reaction centres (which allow photosynthesis activity), be dissipated into heat (non-photochemical energy) or be emitted in the form of chl-a fluorescence (Fig.4)**.** As these three pathways of energy conversion are complementary, the fluorescence yield may serve as a convenient indicator of time- and state-dependent changes in the relative rates of photosynthesis and heat dissipation (Maxwell and Johnson, 2000).

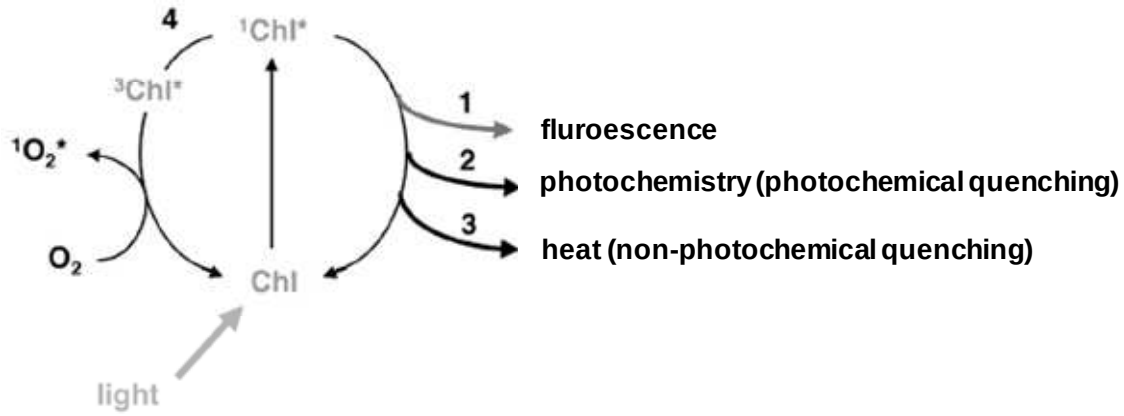


Figure 4. Possible fates of excited Chl. When Chl absorbs light it is excited from its ground state to its singlet excited stat, ^{1}Chl*. From there it has several ways to relax back to the ground state. It can relax by emitting light, as seen as fluorescence (1). Its excitation can be used to fuel photosynthetic reactions (2), or it can de-excite by dissipating heat (3); both of these mechanisms reduce the amount of fluorescence. They are therefore referred to as photochemical quenching and non-photochemical quenching of Chl fluorescence. Last, ^{1}Chl* can, by intersystem crossing, produce ^{3}Chl* (4), which in turn is able to produce $^1O_2$*, a very reactive oxygen species. Modified from Müller et al. (2001)

Several related chl-a fluorescence parameters have been applied in this t*hesis.*

The minimal fluorescence yield (Fo) reflects the chl-a fluorescence emission of all open reaction centres in an non-excited status. Fo can be used as a surrogate of algal biomass since chlorophyll fluorescence is proportional to total chlorophyll content (Serôdio et al., 1997; Rysgaard et al., 2001), but it has been shown that this method has its limitations at h*i*gh biomasses (Guasch et al., 2003; Schmitt-Jansen and Altenburger, 2008). It is expected that Fo will decrease if the toxicants cause a reduction in the number of cells due to cell death (structural damage) or the chlorophyll content of a sample.

The **maximal or optimal quantum yield (Φ_{PSII})** is a photochemical quenching parameter that reflects the quantum yield of PSII electron transport in a dark-adapted state. It has been shown in many studies that Φ_{PSII} can also be a measure of the quantum yield of photosynthesis (Schreiber et al., 1994; Genty et al., 1989). It is an estimate of the potential maximal photosynthetic activity and it is expected to be effected if a chemical produces alterations in the structure of the photosynthetic apparatus (i.e. shade-adapted chloroplasts). In general, this situation occurs when biofilms are exposed to chemicals at high concentrations or during long-term exposure (García-Meza et al., 2005; Schmitt-Jansen and Altenburger, 2005a). **Photochemical quenching (qP)**, represents the proportion of excitation energy "trapped" by opens PSII reaction centers used for electron transport (Horton et al., 1999). It has been applied in algal cultures to monitor the energy used by the electron transport chain which leads to carbon fixation. It presents a similar significance with Φ_{PSII}. An inhibition of qP has been observed in algal cultures exposed to Cu (Juneau et al., 2002) and Hg (Juneau and Popovic, 1999).

The **effective or operation quantum yield (Φ'_{PSII})** is also a photochemical quenching parameter that reflects the efficiency of excitation energy capture by the

open PSII reaction centres under light conditions (Schreiber et al., 1986; Genty et al., 1989). A reduction of the Φ'_{PSII} after toxic exposure indicates that the toxicant is reducing the electron flow in the PSII (Barranguet et al., 2000, 2003; Serra et al., 2009). This parameter is very sensitive to PSII-inhibiting compounds if they block the electron transport flow.

The **non-photochemical quenching (qN)** reflects the amount of light energy dissipation inducing fluorescence quenching that a non-radiative energy process involves (Horton et al., 1999). Algal cultures exposed for 5h to Cu showed an increase in qN (Juneau et al., 2002). Similar results were observed when algal cultures were exposed to Hg (Juneau and Popovic, 1999). Another way to evaluate the excess of light energy arriving to PSII and dissipated in non-radiative processes is **non-photochemical quenching (NPQ).** It is a simplified non-photochemical quenching value, which assumes that NPQ is caused only by one quenching factor (Buschmann, 1999), omitting the many energy-consuming processes, not directly involved in the PSII activity (Pospísil, 1997). Non-photochemical quenching parameters have been used less on biofilms than photochemical quenching parameters. An inhibition of the NPQ could be linked to damage in the pigments where the NPQ occurs and has been observed in biofilms exposed to toxic organic compounds (Laviale et al., 2010; Ricart et al., 2010b).

Measurements of chl-a fluorescence can also be applied to quantify the relative contribution from the different algal components of the whole biofilm community. This can be carried out by using a multi-wavelength PAM fluorometer (e.g. PhytoPAM flurometer, Walz, Germany). This instrument addresses this challenge by simultaneously using light at different wavelengths for excitation of the algal class-specific light harvesting complexes based on the internal ‘reference excitation spectra’ of a pure culture (Schreiber et al., 2002). So, the biofilm strucuture (in terms of algal

groups composition) using chl-a fluorescence parameters could also be addressed (see more details about biofilm structure in the previous section). In biofilms exposed chronically to Cu, Serra et al. (2009) an increase of the fluorescence linked to cyanobacteria was observed. In Boninneau et al. (2010), the toxicity of β-blockers to biofilm communities was assessed by analyzing the sensitivity of the different phototrophic groups through their specific photosynthetic efficiencies.

- **A multi-scale approach**

With the aim of evaluating cause-effect relationships between metal(s) exposure, confounding factors (i.e. nutrients, light …) and biological responses; in this thesis, metal toxicity on fluvial biofilms was addressed using experimental approaches performed at different temporal and spatial scales (Fig.3).

This thesis pesents several experiments addressing the role of time in toxic exposure by performing experiments at different temporal scales (Chapter IV and V). In this way it has been possible to evaluate functional alterations occurring after a few hours of exposure, as well as the structural changes caused after several days or weeks of exposure.

Experiments were performed at different spatial scales. Experimental testing in indoor artificial channels (Fig. 4) allowed us to identify the effects caused by specific stress factors (Chapter IV and VI), either alone or in combination, while field experiments (Chapters IV and V) were used to describe metal exposure effects under real multi-stress situations.

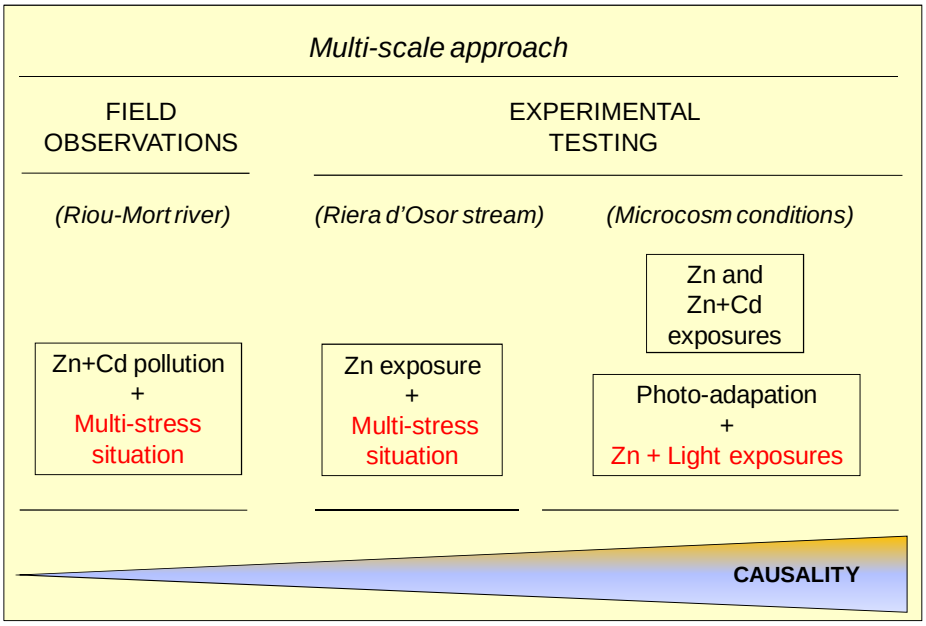


Figure 3. Graph illustrating the steps conducted in this thesis to derive causality (metal toxicity and biofilm responses). Hypothesis formulated on the basis of field observations should be experimentally tested for confirmation.

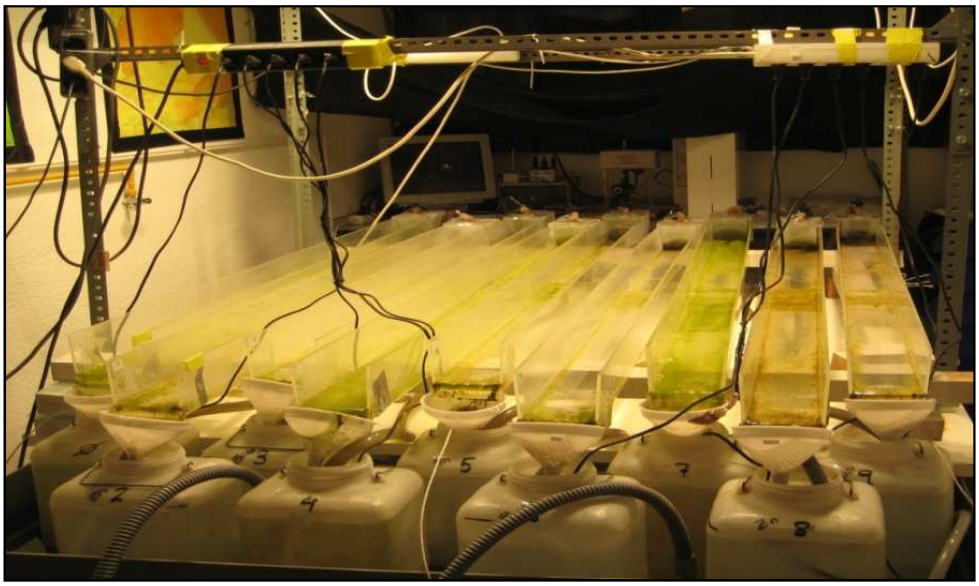


Figure 4. Artificial channels used to evaluate the toxicity of metals on biofilms in microcosm conditions.

OBJECTIVES OF THIS STUDY

The present thesis aims to investigate the potential use of chl-a fluorescence techniques on biofilms for the assessment of metal toxicity in fluvial ecosystems and to find cause-effect relationships between metal(s) exposure, other environmental factors (i.e. light) and biological responses.

This main objective was approached by the following specific objectives:

- To evaluate the sensitivity of different chl-a fluorescence parameters as metal pollution biomarkers of short- and long-term metal exposures under realistic Zn exposure concentrations applied either alone or in combination with Cd.
- To explore the convenience of using a set of biofilm metrics, including chl-a fluorescence parameters, for the assessment of metal effects under a real field exposure situation in fluvial systems.
- To explore the response of biofilms to a multiple-stress situation, i.e. environmental stress caused by light and Zn toxicity. And, also, to assess if adaptation to one of these stress factors (i.e. light) modifies the response.

HYPOTHESIS OF THIS STUDY

Based on the current knowledge of metal ecotoxicology in fluvial biofilms and chl-a fluorescence methods development, the following hypotheses have been formulated:

- The use of several chl-a fluorescence parameters measured in biofilms will allow the evaluation of the effects of metal pollution (Zn alone or Zn in combination with Cd) at realistic concentrations. Short-term exposure will mainly cause functional alterations, whereas structural changes will appear after long-term exposure. Shifts in algal composition will also occur due to selection

pressure according to differences in the metal tolerances or sensitivities of species (addressed in chapter IV).

- The use of chl-a fluorescence parameters will provide a pertinent set of biofilm metrics for the assessment of cause-effect relationships between Zn pollution and biological damage in fluvial systems (addressed in chapter V).

- Zn toxicity on photosynthesis of fluvial biofilms will be influenced by light conditions prevailing during biofilm growth before exposure as well as sudden changes in the light regime during Zn exposure (addressed in chapter VI).

PROJECT DEVELOPMENT

This manuscript thesis includes an introductory chapter, a methodological chapter, a review literature of investigations dealing about the application of chl-a fluorescence parameters on biofilms for metal toxicity assessment, experimental researches on field and microcosms conditions and a general discussion chapter.

In the introductory chapter it is presented the environmental problematic of metal pollution in rivers, focused on Zn and Cd pollution, the pertinence of use several biofilm metrics to assess chemical pollution and some concepts about cause-effect relationship. The objectives and hyphothesis of this study are also presented.

The methodological chapter describes the methods applied in this thesis for water and biological analysis.

Then a bibliographical chapter is presented, it reviews the most relevant literature about studies using chl-a fluorescence parameters to assess chemical pollution on fluvial biofilms. A publication was derived from this work;

- Corcoll, N., Ricart, M., Franz, S., Sans-Piché, F., Schmitt-Jansen, M., Guasch, H. The use of photosynthetic fluorescence parameters from autotrophic biofilms for monitoring the effect of chemicals in river ecosystems, In Guasch, H., Ginebreda, A. & Geizinger, A. [Eds].The Handbook of Environmental Chemistry. Spriner, (in press).

Based on experimental research, a field survey in a highly metal polluted area, Riou-Mort river (in France), was performed to assess metal toxicity on biofilms. The main remark of this field study was that metal pollution was linked to Zn and Cd pollution and caused a decrease of algal biomass. Then, a microcosms experiment was performed in controlled conditions to investigate the contribution of Zn, the metal found a higher concentration in the Riou-Mort river, on the the effects produced by Zn plus Cd. Also the role of time exposure, short vs. long-term exposures was addressed. The obtained experimental results showed that metal toxicity was attributed to Zn pollution. A publication was derived from the microcosm experiment:

-Corcoll, N., Bonet, B., Leira, M., Guasch, H. 2011. Chl-a fluorescence parameters as biomarkers of metal toxicity in fluvial biofilms: an experimental study. Hydrobiologia 673: 119-136.

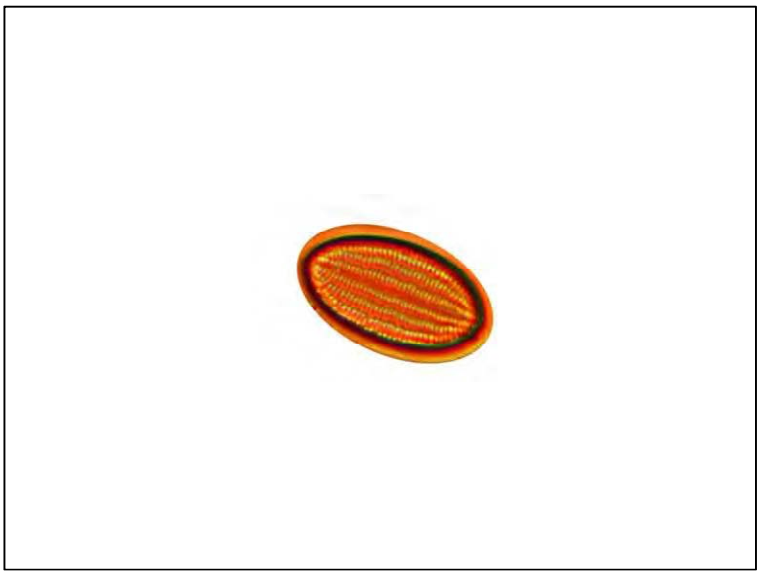
In another field study, in the Riera d'Osor stream (Calalunya, Spain), the convenience of using a set of biofilm endpoints, including chl-a fluorescence parameters, pigments composition and diatom metrics, as indicators of Zn pollution was evaluated in a biofilms translocation experiment. Functional biomarkers responded to short-term metal exposures (hours) meanwhile, structural ones were more sensitive to long-term (weeks) metal pollution. A scientific article will be published derived to this work:

-Corcoll, N., Bonet, B., Leira, M., Morin, S., Tlili, A., Guasch, H. The effect of metals on photosynthesis processes and diatoms metrics of biofilm from a metal contaminated river: a translocation experiment. (*Submitted).*

The following microcosm experiment deals to evaluate the biofilm response to a multiple-stress situation, i.e. environmental stress caused by light and Zn toxicity. The study also aimed to assess if adaptation to one of these stress factors (i.e. light) modifies this response. A clear effect of light stress on Zn toxicity was demonstrated. Zn toxicity was enhanced when light stress also occurred mainly to shade-adapted communities causing a higher inhibition of photosynthetic processes and a de-coupling between non-photochemcial quenching process and the de-expoxidation reaction of diatoms. A scientific article will be published derived to this work:

-Corcoll, N., Bonet, B., Leira, M., Montuelle, B., Tlili, A., Guasch, H. Light history influences the response of fluvial biofilms to Zn exposure. (*Submitted).*

The last part of this manuscript presents a general discussion focusing on the main results derived from this thesis.



CHAPTER II

METHODOLOGY USED

In order to achieve the planned objectives, several methodologies were used for water and biofilm analyses in the different studies presented (Chapters IV, V and VI).

WATER ANALYSIS

- **Temperature (ºC), pH, dissolved oxygen (mg/L) and conductivity (µS/cm)** were measured using multiparametric probes (WTW METERS, Weilheim, Germany). Applied in Chapters IV, V and VI.

- **Phosphate concentration** in terms of soluble reactive phosphorus was analysed using the Murphy and Riley (1992) molybdenum blue colorimetric method following APHA (1989). Applied in Chapters IV, V and VI.

- **Nitrate concentration and the major cations and anions concentrations** were analysed by ion chromatography (761 Compact IC, METROHM, Herisau, Switzerland) following standard methods (APHA, 1989). Applied in Chapters IV, V and VI.

- **Dissolved organic carbon (DOC)** was measured on a Shimadzu TOC 5000 following standard method (APHA, 1989). Applied in chapters IV and V.

- **Total dissolved metals in water** were analysed by ICP-MS (7500c Agilent Technologies, Inc. Wilmington, DE) according to Serra et al. (2009a). In Chapter IV (Al, Fe, Zn, Cd, Ni, Cu and Pb), in Chapter V (Al, Fe, Zn, Cd, Ni, Cu, Pb and Sb).

BIOFILM ANALYSIS

- **Bioaccumulation.** In order to measure the total amount of metals accumulated in the biofilm, dried biofilm samples were lyophylized and weighed (g) to determine the dry weight (DW) (Meylan et al., 2004). Then, *c.* 200 mg of DW were digested with 4 mL of concentrated HNO_3 (65%, suprapure) and 1 mL of H_2O_2 (31%, suprapure). After

dilution with MiliQ water, water samples were acidified (1% nitric acid suprapure) and stored at 4ºC. Digested samples were analyzed following the procedure described for dissolved metals in water. Metal bioaccumulation was expressed as dissolved metal contents per biofilm dry weight (µg/gDW). It was applied in Chapters IV and V.

- **Chlorophyll-a (Chl-a) concentration and the carotenoid ratio (430:665)** were obtained from biofilm samples after extraction with 90% acetone and measured spectrophotometrically (Jeffrey and Humphrey, 1975). Chl-a concentration was used as a measure of algal biomass. Carotenoid ratio was calculated as the quotient between optical densities at 430 and 665 nm (Margalef, 1983) and it was used as an indicator of the proportion of protection pigments such as carotenoids per unit of active chlorophyll. These techniques were applied in chapter IV. On the other hand, in Chapter VI, chl-a concentration was estimated from pigment analysis by high pressure liquid chromatography (HPLC) (see photosynthetic pigments analysis). Quantification of identified chl-a was made from external calibration on standard chl-a (C55H72MgN405, Carl Roth GmBH & Co). Final chl-a concentration was given as µgChl-a/cm^2.

- **Total biomass** content was measured by calculating the ash-free dry weight (AFDW). Biofilm was dried for 24h at 105 ºC in order to calculate dry matter. Afterwards, samples were combusted in an oven at 450 ºC (Obersal MOD MF12-124, Spain) for 4h, and then weighed again to calculate the mineral matter content. The AFDW was calculated by subtracting the mineral matter from the total dry matter. Results are expressed as g/cm^2. It was applied in Chapter VI.

- **Content of extracellular polymeric substances (EPS)** was extracted by using cation exchange resin (Dowex Marathon C, Na-form, strongly acid, Sigma-Aldrich) according to the procedure described in Romaní et al. (2008) and Ylla et al. (2009). Polysaccharide content was measured by the phenol–sulfuric acid assay (Dubois et al., 1956) after the extraction of EPS. Standards of glucose (0–200 mg/mL) were also

prepared. Results are expressed as µg glucose equivalents / cm^2. It was applied in Chapter VI.

- **Diatoms metrics.** Diatom species identification and counting was used to calculate several diatom metrics: species richness (S) in chapters V and VI, the Shannon index of diversity (H') in Chapter V and VI, relative abundance of each specie, cell biovolume, % teratological forms and the Index of Pollution Sensitivity-IPS (Indice de Polluosensibilité Spécifique, Coste in Cemagref, 1982) in Chapter V. IPS values were used to characterize water quality (Coste in Cemagref, 1982). Samples were digested with hydrochloric acid (37% HCl) and hydrogen peroxide (30% H_2O_2) according to Leira and Sabater (2005). Frustules were mounted on permanent slides using Naphrax (r.i. 1.74; Brunel Ltd, UK) and at least 400 valves were counted on each slide in random transects under light microscopy using Nomarski differential interference contrast optics at a magnification of (1000X). Krammer & Lange-Bertalot (1986-1991) and recent nomenclature updates were used for identification, and theoretical biovolumes of each species were also calculated following the geometric formulas of Hillebrand et al. (1999), and expressed as mean cell biovolume per sample.

- **Photosynthetic pigments** were analysed by high pressure liquid chromatography (HPLC) according to Tlili et al. (2008). Pigments were extracted with 4mL of methanol/0.5M ammonium acetate (98/2, v/v) solution as described in Dorigo et al. (2007). An injection volume of 100 µL of purified biofilm extract was used to determine the lipophilic pigment composition of the biofilm by HPLC. Pigments were separated on a 4.6mm×250mm column (Waters Spherisorb ODS5 25 µm). Solvents and elution gradient used for HPLC analysis are presented in Table. Standard pigments used for calibration were: chl-a, chl-b, zeaxanthin, lutein and fucoxanthin (Carl Roth GmBH & Co). Each pigment was identified from its retention time and absorption spectrum using a diode array detector (DAD) according to the scientific

committee for oceanic research (SCOR) (Jeffrey et al. 1997). Each pigment was expressed as the percentage of the sum of the areas for all the pigments in the sample. It was applied in Chapters V and VI. The percentage area obtained for diadinoxanthin and diatoxanthin pigments were used to calculate the de-epoxidation ration (DR): DR= diatoxanthin / (diadinoxanthin + diatoxanthin) in Chapter VI.

Table 1. Gradient of HPLC elution used for pigment analysis. Solvent A: 0.5 M ammonium acetate, Solvent B: 90% acetonitrile, Solvent C: ethyl acetate.

time (minutes)	Solvent A (%)	Solvent B (%)	Solvent C (%)	Event
0-4	100	0	0	injection
4-18	0	100	0	logarithmic gradient
18-21	0	20	80	linear gradient
21-24	0	100	0	linear gradient
24-32	100	0	0	linear gradient
32	100	0	0	return to equilibrium

- **In *vivo* chl-a fluorescence measurements** of biofilms were performed by using pulse amplitude modulated-PAM fluorometry based on the method described by Schreiber et al. (1986) by Mini-PAM fluorometer (Walz, Effeltrich, Germany) in chapter V and by Phyto-PAM fluorometer version EDF (Walz, Effeltrich, Germany) in chapters IV and VI. The difference between both flurometers is due to Mini-PAM only excites chlorophyll fluorescence at one measuring light wavelenght (665nm) unlike the Phyto-PAM employs an array of light-emitting diodes (LED) to excite chlorophyll fluorescence at different wavelengths (470, 520, 645 and 665 nm). Hence, using the Phyto-PAM instrument is possible to evaluate the fluorescence contribution of main algal group composing biofilm. This instrument addresses this challenge by simultaneously using light at different wavelengths for excitation of the algal class-specific light harvesting complexes based on the internal 'reference excitation spectra' of a pure culture (Schreiber et al., 2002). This application has been validated in fluvial biofilms by

Schmitt-Jansen and Altenburger (2008) and provides a relative measure of the fluorescence abundance of each phototrophic group composing biofilm (green algae, diatoms and cyanobacteria). For instance, Chl *b*, which characterises chlorophytes as a key pigment, shows absorption peaks at 470 nm and 645 nm. Diatoms which are characterized by the key pigments Chl *c* and carotenoids, especially fucoxanthin, can be studied by fluorescence excitation in the blue and green spectral range. On the other hand, in the case of cyanobacteria, fluorescence excitation in the blue and green spectral range is weak, while strong excitation is observed around 620-640 nm, in the absorption range of phycocyanin / allophycocyanin.

Accessory equipment was designed to adapt Phyto-PAM fluorescence measurements to biofilm samples (Fig.1). This material consisted in: 1) a set of transparent Perspex boxes (9 x 9 x 6 cm^3) used to incubate biofilm samples and measure fluorescence parameters avoiding the manipulation of the samples (Chapter IV), and 2) a micrometric device used for fixing the sensor of the Phyto-PAM under the transparent boxes which contained the biofilm samples (chapter IV, V and VI). The Phyto-PAM sensor was placed below the box at 6 mm of total distance from the biofilm samples. The measuring distance was kept constant for all PAM measurements.

PAM fluorometry is also so-called saturation pulse quenching analysis. The PAM-fluorescence method employs a combination of three different types of light (modulated or measuring= 0.05 µmols photons/m^2·s in µsec pulses; actinic= from 1 to 600 µmols photons/m^2·s and saturating= 8000 µmols photons/m^2·s) (Fig. 2), which allow the adequate analysis of the fluorescence-induction kinetics of photosynthetic organisms.

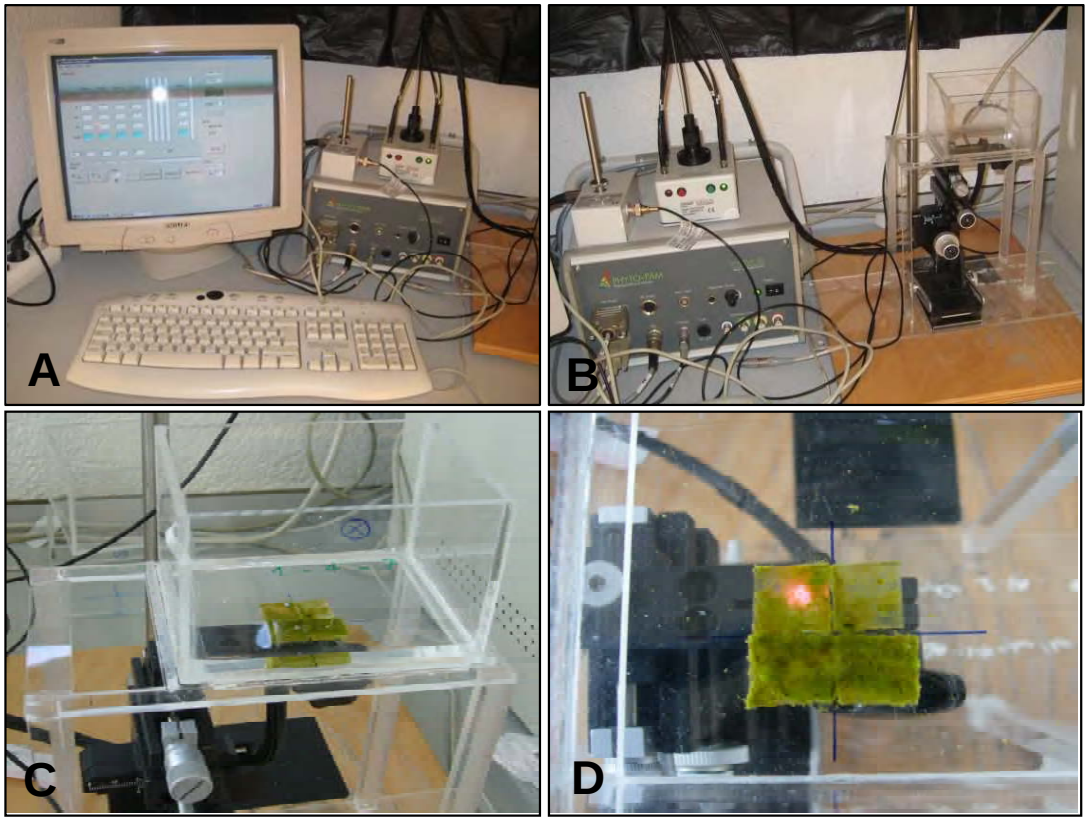


Figure 1. Details of the PhytoPAM instrument (A), the accessory equipment (B, C) and the measurements on biofilm (D).

Biofilm samples were incubated for 15 min in dark conditions in order to ensure that all reaction centres were open, and then a weak measuring light was applied in order to record the minimum fluorescence yield (Fo) (Fig. 2, Table 2). The Fo of dark-adapted cells was always recorded at 665 nm (chapter IV). When the de-convolution of the overall Fo fluorescence in the relative contribution of each class to the whole community was performed (by Phyto-PAM instrument), the fluorescence attributed to cyanobacteria, was refered to as Fo(Bl), the fluorescence attributed to green algae, referred to as Fo(Gr) and the fluorescence attributed to diatom, referred to as Fo(Br). The standards used for the deconvolution of fluorescence signals in algal class were based on Schmitt-Jansen and Altenburger (2008). It was applied in Chapters IV and VI.

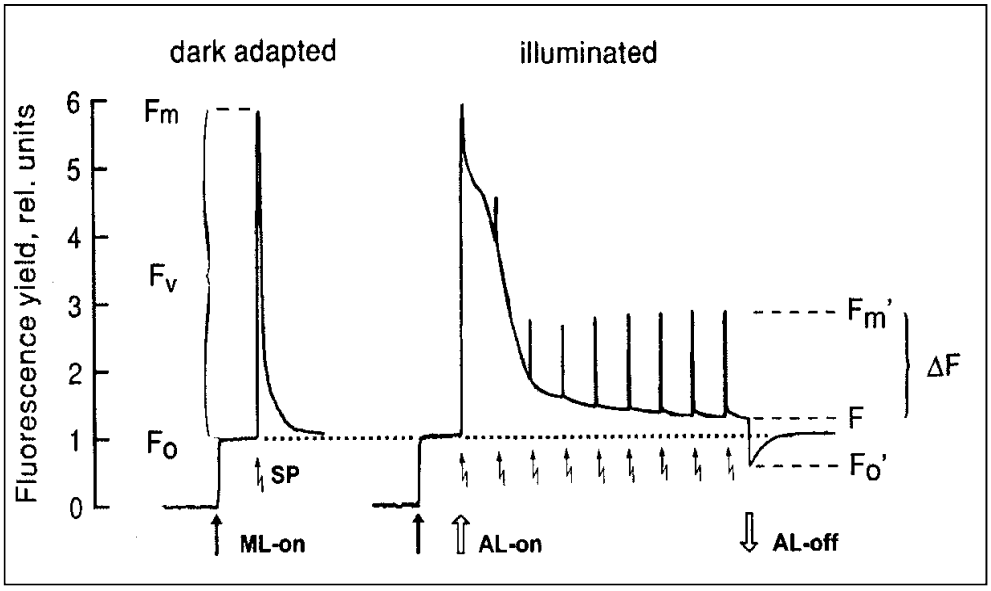


Figure 2. Schematic representation of the slow fluorescence kinetics analysis by using the PAM-fluorometry. The different types of light are indicated (ML = measuring light; AL = actinic light; SP = saturation pulse). Modified from Schreiber (2004).

Dark adapted samples used for the measurement of Fo, were thereafter exposed to a short SP of light of 8000 µmol photons/m^2·s to induce the Fm. Biofilm samples were then illuminated (120 µmol photons/m^2·s) by AL during 15 min to induce the typical Kautsky effect (Kautsky and Hirsch, 1931) and the change of the Fm' in light conditions was induced by five SPs at the end of this AL period. Afterwards, the AL was turned off and then the reoxidation of the photosynthetic pigments the Fo' was recorded (Fig. 2). Using both dark and light fluorescence measurements, several fluorescence parameters were calculated based on the fluorescence recorded at 665nm (Table 1).

Table 2. Fluorescence parameters obtained by using PAM fluorometry.

Parameter	*Name*	*Equation*	*Reference*
Fo	minimal fluorescence yield		Serôdio et al., 1997; Rysgaard et al., 2001
Φ_{PSII}	maximal quantum yield	$\Phi_{PSII} = (Fm - Fo) / Fm$	Schreiber et al., 1986; Genty et al., 1989
Φ'_{PSII}	effective quantum yield	$\Phi'_{PSII} = (Fm' - F) / Fm'$	Schreiber et al., 1986; Genty et al., 1989
qP	photochemical quenching	qP = (Fm' - F) / (Fm' – Fo')	Schreiber et al., 1986
qN	non-photochemical quenching	qN= 1 - [(Fm' - Fo') / (Fm - Fo)]	Schreiber et al., 1986
NPQ	non-photochemical quenching	NPQ = (Fm - Fm')/ F'm	Bilger and Björkman, 1990

CHAPTER III

REVIEW OF THE USE OF PAM FLUOROMETRY FOR MONITORING THE EFFECT OF CHEMICALS ON FLUVIAL BIOFILMS

INTRODUCTION

The use of pulse amplitude modulated (PAM) fluorometry techniques to assess photosynthesis performance under changing environmental conditions has widely been proved as a rapid, non-invasive, reliable method (Krause and Weis 1991; Schreiber et al., 1994). Since the first acknowledgement of the analytical potential of chl-a fluorescence techniques (Kautsky and Franck, 1943; Kautsky et al., 1960) until now, extensive research has been carried out to apply this technique in different research fields. In the case of ecotoxicology, chl-a fluorescence techniques have been used to evaluate the toxicity of different pollutants (mainly herbicides) and to locate their primary sites of damage in photosynthetic organisms.

This chapter focuses on the use of chl-a fluorescence parameters (measured by pulse amplitude modulated (PAM) fluorometry) to assess metal effects on the autotrophic component of fluvial biofilms, a non-target community present in aquatic systems. A detailed description of the basis, interpretation and measurement of chl-a fluorescence parameters are provided in Chapters I and II ("chl-a fluorescence parameters" pages 39-42 and "in *vivo* chl-a fluorescnece mesuraments" pages 54-58, respectively).

Fluvial biofilms are made up mainly of algae, bacteria, fungi and other micro- and meiofauna organisms, embedded in a matrix of extracellular polymeric substances (Sabater and Admiraal, 2005). In contrast to other autotrophic groups, such as phytoplankton, biofilms stand out for living immobile; they are therefore suitable for long-term monitoring and allow chemical toxicity to assessessed at community level. This community-approach is much closer to the processes of an ecosystem than the regularly monitoring of single species tests and a biological quality element (Clements and Newman, 2002; Guasch et al., 2010) for the assessment of the ecological status of water systems. Biofilms have the capacity to modify the transport and accumulation of

substances such as nutrients (Freeman et al., 1995) as well as organic toxicants and heavy metals (Gray and Hill, 1995). Several studies have highlighted the sensitivity of these communities to a large panel of toxicants such as herbicides (Leboulanger et al., 2001; Guasch et al., 2003; Schmitt-Jansen and Altenburger, 2005a, b; Pesce et al., 2006) and heavy metals (Guasch et al., 2002; Ivorra et al., 2002; Pinto et al., 2003). Due to this sensitivity, fluvial biofilms can be used as early warning systems for the detection of the effects of toxicants on aquatic systems (Sabater et al., 2007). The pertinence of the use of chl-a fluorescence techniques for toxicity assessment on fluvial biofilms is attributed to the basis that if a chemical produces effects on the photosynthesis processes (either directly or indirectly), the chl-a fluorescence parameters will reflect it. In the last decade several investigations have followed this approach (Barranguet et al., 2000; Navarro et al., 2002; Guasch et al., 2002, 2003, 2004; Schmitt-Jansen and Altenburger, 2005a; Schmitt-Jansen et al., 2007; Serra et al., 2009; Ricart et al., 2009, 2010a; Bonnineau et al., 2010; Corcoll et al. 2011).

Several PAM fluorescence instruments are provided to assess the photosynthetic performance in biofilms; each one presenting a specific characteristic: 1) Standard PAM fluorometers excite chlorophyll fluorescence at one wavelength (665 nm, excitation maximum of the chlorophyll a molecule) and have been applied on biofilms to evaluate the global photosynthetic response of the autotrophic compartment (e.g. Barranguet et al., 2000; Guasch et al., 2003). PAM fluorometers are available in different technical settings (Fa. Walz, Effeltrich, Germany). 2) Next to standard applications working with cuvettes, a microscopy PAM-fluorometer is available, suitable for the assessment of selected cells within a biofilm on a microscopic scale (Schmitt-Jansen and Altenburger, 2007). 3) The Maxi-Imaging PAM fluorometer was developed to assess the photosynthetic capacity of large surfaces, e.g. leaves or multiwell plates and is also suitable for measuring biofilms (McClellan et al., 2008; Schreiber et al., 2007).

4) Multi-wavelength excitation PAM fluorometers (e.g., Phyto-PAM) stand out for working with several excitation wavelengths, exciting pigments with different absorption spectra, e.g. carotenoids, which are characteristic for defined algal classes. After de-convolution of the fluorescent signals from mixed algal samples the multi-wavelength PAM-fluorometry has the potential to reveal the contribution of algal groups with different absorption spectra (Schreiber et al., 2002).

Different sources of chemical contamination (industrial, agricultural or urban activities) discharge a wide variety of compounds with different modes of action, toxic concentrations and persistence into the ecosystem (accumulation, degradation, etc.). The Water Framework Directive (2000/60/EC) defines a strategy for protecting and restoring clean waters across Europe. As a first step of this strategy, a list of priority substances was adopted in the Directive 2008/105/EC, in which 33 substances of priority concern were identified and regulated. The list includes mainly organic contaminants, such as pesticides and herbicides, and four toxic metals (Coquery et al., 2005). The WFD follows two different assessment strategies; chemical status evaluation and the ecological status. The “ecological status” represents the “quality of the structure and functioning of aquatic ecosystems associated with surface waters” (Directive 2000/60/EC). In order to establish the “ecological status”, the WFD requires the sampling and interpretation of data on a broad suite of “biological quality elements (BQEs)”. Observed metric values for BQEs in a water body undergoing monitoring are mathematically compared with the expected values of reference condition sites based on predictive modelling, hind-casting or expert judgment (Furse et al., 2006). Phytobenthic community of biofilms, referred to as phytobenthos in the WFD, have been included as one major BQE. Concerning this BQE, the WFD recognized the diatoms index within biofilms to evaluate structural effects, which has been widely applied for water managers. There is no recognized parameter to be evaluated for non-diatom species from biofilm.

This chapter summarizes studies which assess the toxicity of metals on autotrophic biofilms by using chl-a fluorescence techniques. Moreover, studies that evaluate the toxicity of herbicides (targeting PSII) on biofilms by using these same techniques are also reported because of their recognized sensitivity to PSII inhibitors (Juneau et al., 2007).

The aim of this chapter is to analyze the pros and cons of the use of chl-a fluorescence-techniques on biofilms for metal pollution quality monitoring programs in the context of the Water Framework Directive, based on the current bibliography.

THE USE OF FLUORESCENCE PARAMETERS TO ASSESS THE EFFECTS OF TOXICANTS ON BIOFILMS

PAM fluorometry gives the opportunity of evaluating both functional and structural alterations in oxygenic photosynthetic organisms. These alterations are related to the mode of action (MoA) of toxicants as well as the dose and time of exposure.

Several studies are presented in order to illustrate the pros and cons of the application of PAM fluorometry techniques on the growth of biofilm communities in the laboratory to assess the chemical toxicity of herbicides (compounds targeting PSII) and metals (not directly targeting PSII). The use of PAM fluorometry to assess chemical toxicity in field studies is also presented.

- **Herbicides, laboratory experiments**

Pollutants with MoA (mode of action) directly interfering with the PSII are mainly represented by organic herbicides (e.g. diuron, atrazine, isoproturon). Due to their specific MoA, studies dealing with their toxicity commonly use photosynthetic endpoints. The inhibition of the maximal and the effective quantum yield (Φ_{PSII} and Φ'_{PSII}, respectively) in biofilms exposed to PSII inhibitors for short periods of time

(hours) has already been demonstrated. McClellan et al. (2008) detected a reduction of the Φ_{PSII} in biofilms exposed to diuron. Similar results have been obtained with atrazine, prometryn and isoproturon (Schmitt-Jansen and Altenburger, 2005b, 2007, 2008). The sensitivity of biofilms to isoproturon under different light intensities was determined by Laviale et al. (2010), showing a clear reduction of photosynthesis (Φ'_{PSII} and Φ_{PSII}) and, under dynamic light conditions, a clear reduction of the non-photochemical quenching (NPQ) mechanisms. Effects of PSII inhibitors on the Φ_{PSII} and algal biomass measured by basal fluorescence (Fo) are less reported, especially in short-term toxicity assessment. Schmitt-Jansen and Altenburger (2005a) found a reduction on Φ_{PSII} of 90% after 1 hour of exposure to isoproturon. Focusing on long-term toxicity studies, Ricart et al. (2009) analyzed the long-term effect of the herbicide diuron on fluvial biofilms and detected a clear decrease in the Φ'_{PSII} with increasing concentrations of diuron, indicating a clear dose-dependent effect, probably attributable to the MoA of the herbicide. A reduction of the Φ_{PSII} of biofilms was also observed by Tlili et al. (2010) after 3 weeks of exposure to diuron. Schmitt-Jansen and Altenburger (2005a, 2008) used these techniques to evaluate the long-term effects of isoproturon to biofilm structure. They detected a reduction of Fo and a shift in the algal classes to a dominance of green algae. Effects of diuron on a simple food chain (biofilm and grazers (snail *Physella [Costatella] acuta*)) have also been evaluated using PAM fluorometry techniques. In a long-term experiment, López-Doval et al. (2010) used Φ'_{PSII} to monitor the physiological state of the biofilm, as well as to detect the effect of diuron, grazers and their interaction on the biofilm community. A significant reduction in Φ'_{PSII} was detected in both diuron and diuron+grazers microcosms.

Chl-a fluorescence parameters have been used as a physiological endpoint to investigate community adaptation following the Pollution Induced Community Tolerance (PICT) approach (Blanck et al., 1988). Briefly, the replacement of sensitive species by tolerant ones driven by the selection pressure from toxicants is expected to

increase the EC_{50} of the community measured in acute dose-response tests using physiological endpoints such as Φ'_{PSII}**.** PICT of periphyton to isoproturon (Schmitt-Jansen and Altenburger, 2005a, 2008), atrazine, prometryn (Schmitt-Jansen and Altenburger, 2005b) and diuron (Tlili et al., 2010) has been determined by testing inhibition of Φ'_{PSII} on pre-exposed and non pre-exposed biofilm communities.

- **Metals, laboratory experiments**

Chl-a fluorescence parameters have commonly been used to assess the toxicity of metals, chemicals not directly targeting PSII. Chemicals that damage membranes or proteins associated with photosynthetic electron transport or that inhibit any cellular process downstream of PSII, such as carbon assimilation or respiration, will lead to excitation pressure on PSII (Marwood et al., 2001). Therefore, chl-a fluorescence methods can also be used to assess the effects of chemicals affecting metabolic processes not directly linked to photosynthetic electron transport. In these cases, the obtained parameters may indicate secondary effects on the performance of the photosynthetic apparatus or on algal biomass. For instance, copper is a phytotoxic chemical affecting the activity of photosystem II and electron transfer rates (Mallick and Mohn, 2003). However, this metal is also known to cause the oxidation of sulphydryl groups of enzymes leading to their inhibition (Teisseire and Guy, 2000) or to generate reactive oxygen species like superoxide and hydroxyl radicals causing oxidative stress (for a review see Pinto et al. (2003)), reducing growth as well as photosynthetic and respiratory activities (Nalewajko and Olaveson, 1994). Several biofilm studies have used copper as a model toxic compound (Table 1), applying, in many cases chl-a fluorescence parameters to assess biological responses.

Serra et al. (2009) measured Φ'_{PSII} to check the photosynthetic performance of biofilms during the Cu retention experiment that lasted for a few hours. Transient

Table 1. Summary of studies applying chl-a fluorescence techniques to assess metal toxicity on fluvial biofilm communities. Significant responses observed are indicated by "-" for inhibition or by "+" for increase and its approximate magnitude by: "-" or "+" if it was slight (10-30% of control), by: "- -" or "+ +" if it was moderate (30-50% of control) and by: "- - -" or "+ ++" if it was high (>50% of control). n.s.: not significant (p>0.05).

Toxicant	Biofilm Community	Exposure	Conc.	Φ_{PSII}	Φ'_{PSII}	NPQ	Fo	Fo(Bl)	Fo(Gr)	Fo(Br)	Reference
Cu	Microcosms	24h; 3d	445-1905; 126 µg/L		- / - -		- - -				Barranguet et al., 2000; 2003
Zn	Microcosms	72 h	320 µg/L	n.s.	-	n.s	n.s.	n.s.	n.s.	n.s.	Corcoll et al., 2011
		5 wk	320 µg/L	-	n.s.	- - -	- - -	n.s.	n.s.	-	
Cu	Microcosms	6 d; 16d	0- 100 µg/L		-		- - -				Guasch et al., 2002; 2004
Cu, Zn, Cu+Zn	Microcosms	5 d	636; 6356 µg/L	-	n.s.			- - -	- - -		García-Meza et al., 2005
Cu	Microcosms	4 wk	30 µg/L					+++	n.s.	- - -	Serra et al., 2009
	no Cu *vs* Cu pre-exposure	6 h	100 µg/L		- / n.s.		n.s				
Cu	Microcosms	3 wk	30 µg/L	-							Tlili et al., 2010
			30 µg/L + PO_4^{3-}	n.s.							

inhibition of Φ'_{PSII} was also observed by Corcoll et al. (2011) after a few hours of Zn exposure; however, this parameter recovered after longer exposure (Table 1). In a study of Rotter et al. (2011), the capacity of biofilm to recover photosynthesis after long-term prometyn exposure was linked with a physiological adaptation and a selection of tolerant biofilm species. Physiological chl-a parameters such as Φ'_{PSII} may not consistently show toxic effects caused by metals after longer exposure due to community adaptation. Barranguet et al. (2003) observed that biofilms exposed during two weeks to Cu presented alterations in the Fo and that Φ'_{PSII} was less affected. In another study, Barranguet et al. (2000) have also observed that the main factor regulating the sensitivity of biofilms to Cu toxicity (based on the Φ'_{PSII} endpoint) during short-term exposures was the physical structure of the biofilm (package of cells and thickness), and not to the species composition. In this case, these endpoints were used to show differences in sensitivity between suspended cells and intact biofilm communities.

Fo and Φ'_{PSII} were also used by Guasch et al. (2002) and Garcia-Meza et al. (2005) to assess Cu and Cu plus Zn toxicity on biofilms for 6 and 5 days of exposure, respectively. In both cases, Fo appeared to be the most sensitive endpoint, whereas effective concentrations based on Φ'_{PSII} were always much higher (Table 1). In the second example, multi wave-length fluorometers allowed the effects on cyanobacteria and green-algae biomass to be followed as well as their respective Φ_{PSII}. Similarly, Serra et al. (2009a) demonstrated that community composition differed among biofilms with different Cu-exposure history. Community changes were identified using multi wave-length fluorimetry, whereas Cu adaptation was assessed by comparing the Φ'_{PSII} responses to short Cu exposure (Table 1). Tlili et al. (2010) used the Φ_{PSII} to evaluate the role of phosphorus on Cu toxicity on biofilms. They observed that biofilms pre-exposed to Cu presented a lower Φ_{PSII} than biofilms non pre-exposed to Cu or pre-exposed to Cu plus phosphorus (Table 1).

- **Field studies**

Pollution in fluvial ecosystems is related to the main activities that are carried out in their catchments. As a general rule, agricultural areas are more affected by organic chemicals (herbicides, insecticides or fungicides), industrial and mining areas by metals, and urban areas by personal care products and pharmaceuticals via sewage treatment plant effluents (Metcalfe et al., 2003; Halling-Sørensen et al., 1998). However, the predominant scenario found in most polluted rivers is a mixture of different chemicals. From an ecotoxicological point of view, mixture pollution implies that compounds with different chemical properties, modes of action and concentrations will coexist. Moreover, environmental factors such as light, nutrients or flow regime may also influence the fate and effects of chemicals on fluvial communities. Due to their complexity, the effects of toxicants on biofilm communities have been addressed by different methodological approaches including periodic monitoring sampling, translocation experiments or PICT approaches. In addition, different biofilm targeting endpoints have been used, including, in many cases, chl-a fluorescence parameters.

Ricart et al. (2010a) examined the presence of pesticides in the Llobregat river basin (Barcelona, Spain) and their effects on benthic communities (invertebrates and natural biofilms) (Table 2). Several biofilm metrics, including PAM parameters, were used as response variables to identify possible cause-effect relationships between pesticide pollution and biotic responses. Certain effects of organophosphates and phenylureas in both structural (chl-a content and Φ_{PSII}) and functional aspects (Φ'_{PSII}) of the biofilm community were suggested. The authors concluded that complemented with laboratory experiments, which are needed to confirm causality, this approach could be successfully incorporated into environmental risk assessments to better summarize biotic integrity and improve ecological management.

Table 2. Review of field studies where chl-a fluorescence techniques have been applied to assess the effects of toxicants on photosynthesis performance of fluvial biofilm communities. The significant responses observed are indicated by "-" for inhibition or by "+" for increase and its approximate magnitude by: "-" or "+" if it was slight (10-30% of control), by: "- -" or "+ +" if it was moderate (30-50% of control) and by "- - -" or "+ + +" if it was high (>50% of control). n.s.: not significant (p>0.05). The terminology used for mentioning the mode of action (MoA) of each compound was: "a" for PSII inhibitors and "b" for photosynthesis inhibitors not directly targeting PSII.

Toxicant	MoA	River	Growth substrate	Experimental Conditions	Φ_{PSII}	Φ'_{PSII}	NPQ	Fo	Fo(Bl)	Fo(Gr)	Fo(Br)	Reference
Cu	b	Ter River, Spain	artificial substrata	Biofilm from different polluted sites (spring and summer) was transported to the laboratory for Cu and atrazine short-term toxicity tests		- - -						Navarro et al. 2002
Atrazine	a					- - -						
22 pesticides (mixture)	a	Llobregat River, Spain	natural biofilm	Field monitoring of biofilm from different polluted sites during two years - natural exposure	-	-						Ricart et al. 2010a
Atrazine	a	7 streams from Catalonia Area, Spain	artificial substrata	Biofilm from different polluted sites and during different seasons was transported to the laboratory for atrazine and Zn short-term toxicity tests		- - -						Guasch et al. 2003
Zinc	b					- -						
Atrazine	a	Ozanne River, France	artificial substrata	Biofilm from different polluted sites and during different seasons was transported to the laboratory for atrazine and isoproturon toxicity tests - PICT concept	- - -							Dorigo et al. 2001, 2004
Isoproturon	a				- - -							

Zn, Cd, Ni, Fe (metal pollution)	b	Mort River, France	natural biofilm	Biofilm from different polluted sites was evaluated- natural exposure	-				Bonnineau et al. 2011
Diuron, DCPMU and 3,4-DCA	a	Morcille River, France	artificial substrata	Biofilm from different polluted sites, presenting a natural exposure, were sampled	-	- -			Pesce et al. 2010a, b
				Biofilm from different polluted sites was transported to the laboratory for short-term toxicity tests - PICT concept	- - -				
pesticides	a and b	Morcille River, France	artificial substrata	Biofilm from each sampling site was sampled before translocation to be structurally charachterized - natural exposure.		n.s.	n.s.	n.s.	Morin et al. 2010
prometryn	a	Elbe River, Germany	artificial substrata	Translocation experiment. Local and translocated biofilm was transported to the laboratory for short-term prometryn toxicity tests-PICT concept, structural characterization	- - -	+	+	- -	Rotter et al. 2011

In the Morcille River, located in a vineyard area of France, several studies have been performed to evaluate the effects of pesticides on biofilms (Morin et al., 2010; Pesce et al. 2010a, 2010b) (Table 2). Morin et al. (2010) studied the structural changes in biofilm assemblages induced by the transfer of biofilm communities from two contaminated sites to a reference site, expecting a recovery of the translocated communities, either in structure or in diversity. The proportions of the different algal groups in biofilms were estimated by *in vivo* chl-a fluorescence measurements, observing that biofilm was mainly composed by diatoms before and after translocation (Table 2). In the same river, Pesce et al. (2010a), used the Φ_{PSII} to evaluate individual and mixture effects of diuron and its main metabolites (DCPMU and 3,4-DCA) on biofilms. They observed that diuron was the most toxic of the evaluated compounds and that biofilms from contaminated sites presented a higher tolerance to diuron and DCPMU than biofilms from a reference site. In another study (Pesce et al., 2010b), diuron tolerance acquisition by photoautrophic biofilm communities due to their previous *in situ* exposure to this herbicide was investigated. For this purpose, the PICT approach (using the Φ_{PSII} as endpoint) together with multivariate statistical analyses, were combined. A spatio-temporal variation in diuron tolerance capacities of photoautotrophic communities was observed, the biofilm from the most polluted site being the most tolerant (Table 2). Similar tolerance results were found by Rotter et al. (2011) in a study in the Elbe River where prometryn toxicity on biofilm was evaluated by combining a translocation experiment and the PICT approach (Table 2). They observed that biofilms from a polluted site presented a higher EC_{50} (based on Φ'_{PSII} measures) than biofilms from a reference site, suggesting a prometryn tolerance induction. The proportion of each group (cyanobacteria, green algae and diatoms) in transferred and control biofilms was also measured by PAM fluorometry. In other studies, Dorigo et al. (2001, 2004) also used the PICT approach to investigate the toxicity of atrazine and isoproturon on biofilm from a herbicide-polluted river (Ozanne

River, France) (Table 2). Sampling was performed at different polluted sites and at different moments during the year. Using Φ_{PSII} as endpoint in short-term toxicity tests, differences in the EC_{50} between biofilms naturally exposed to different levels of atrazine and isoproturon could be observed.

A field study performed in the Ter River (Spain), which is affected by organic and metal pollution, applied short-term toxicity tests to evaluate biofilm sensitivity to toxic exposure (Navarro et al., 2002) (Table 2). It was concluded that the short-term toxicity tests using the Φ'_{PSII} as a physiological endpoint, may provide an early prospective quantification of the transient effects of a toxic compound on separate communities, predict which of these effects are not reversible and determine their intensity. Guasch et al. (2003) investigated the ecological and the structural parameters influencing Zn and atrazine toxicity on biofilms communities by testing the validity of this experimental approach (Table 2). They concluded that short-term toxicity tests seem to be pertinent to assess atrazine toxicity on biofilms. In contrast, the use of longer-term toxicity tests was proposed to assess Zn toxicity on biofilm to overcome the influence that biofilm thickness exerts on Zn diffusion and toxicity. Bonnineau et al. (2011), assessed in situ the effects of metal pollution on Φ'_{PSII} in natural communities from the Riou-Mort river (France) by comparing reference and polluted sites. Slight effects were observed based on Φ'_{PSII} and marked effects were observed based on antioxidant enzymatic activities, supporting the low sensitivity of Φ'_{PSII} for the assessment *in situ* of the chronic effects of metals.

Based on the field studies reported here, it can be concluded that the responses of the chl-a fluorescence parameter could serve as an early warning signal of biological effects after acute exposure to chemicals damaging either directly or indirectly PS II, but, in regular monitoring, PAM fluorometry may show low sensitivity or give false-negative signals if the community is already adapted to the prevailing toxic exposure

conditions. Tolerance induction evaluations and multi-biomarker approaches, together with the use of appropriate multivariate statistical analyses may partially overcome this limitation.

GENERAL DISCUSSION AND PROSPECTS

The main aim of this chapter was to analyse the pros and the cons of the use of chl-a fluorescence techniques for water metal pollution monitoring programs. While not being an exhaustive review, 19 different investigations have been analysed, including only field and laboratory investigations dealing about the application of chl-a fluorescence parameters on biofilm communities.

The use of chl-a fluorescence parameters as biomarkers of metal toxicity on biofilm communities has increased in the last few years, illustrating their applicability to show early and long-term effects of toxicants at community level using functional and structural descriptors.

Differences in sensitivity are reported between chl-a fluorescence parameters. Focusing on functional parameters, the effective quantum yield (Φ'_{PSII}) seems to be a very sensitive parameter to detect the effects on algae of toxic compounds targeting the PSII. Its use is well reported in both laboratory and field studies, supporting its use for these type of compounds. On the other hand, its sensitivity to chemicals with a mode of action different to the PSII, is highly influenced by the time of exposure of the toxicant (short-term *vs.* long-term exposure). In short-term studies, the Φ'_{PSII} seems to be a sensitive endpoint of toxicity but in long-term studies its sensitivity is often less obvious. This lack of sensitivity during long-term exposures could be related to a development of biofilm tolerance or recovery after longer exposures. It has been described that the exposure history of communities is defining the time-response of recovery and adaptation (Rotter et al. 2011, Morin et al. 2010). PICT-concept is shown

to be a suitable tool for analysis of recovery and adaptation processes of communities Rotter et al. (2011). Therefore, in long-term studies both in field and in laboratory conditions, supplementary methodological approaches, such as PICT tests based on Φ'_{PSII} may be used to show community adaptation.

The maximal photosynthetic capacity of biofilms (Φ_{PSII}) showed a similar sensitivity to chemicals as the Φ'_{PSII}. This parameter has been used more in ecotoxicological studies than the Φ'_{PSII}, probably due to its simplicity for being measured. The Φ_{PSII}, which is measured on dark-adapted samples, is independent of the light conditions prevailing during the incubation period. Therefore, it is more suitable than Φ'_{PSII} for comparing results of experiments using different light conditions (Laviale et al., 2010). In a recent study, Laviale et al. (2011) recomended the combined use of both photosynthetic parameters, the Φ_{PSII} and the Φ'_{PSII}, to get a better understanding of herbicides toxicity on biofilms. The use of the non-photochemical quenching parameter (NPQ) to assess chemical toxicity on biofilm is less documented than those reported above and applied only in microcosm studies. However, its use is promising due to its sensitivity to different types of toxicants, and also due to its complementarity to the more classical photosynthetic related parameters (Φ'_{PSII} and Φ_{PSII}) (Laviale et al., 2010; Ricart et al,. 2010b; Corcoll et al., 2011).

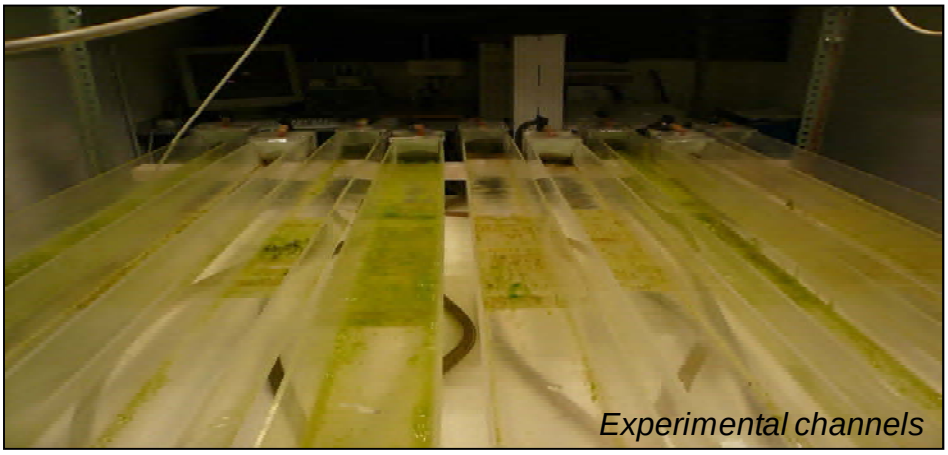
Concerning more structural chl-a fluorescence parameters; the minimal fluorescence parameter (Fo) has been applied for monitoring algal biomass and biofilm growth rate in microcosm studies (Serra et al., 2009; Corcoll et al., 2011). It has been successfully applied in ecotoxicological studies focusing on metals, compounds that damage photosynthetic processes without targeting the PSII. Since Fo is influenced by measuring conditions: instrument settings, distance between sensor and the biofilm sample or the light intensity measurement, absolute values are difficult to compare between different studies without using the same calibration. However, toxic effects on

Fo, in ecotoxicological studies, are usually reported in comparison to a non-exposed community (in relative terms) to avoid this limitation. The measurements of Fo may present other limitations not related to the algal biomass or biofilm thickness. Fo may increase if the addition of a herbicide causes a transfer of the electron on plastoquinone B (PQ_B) to the primary quinine acceptor, the plastoquinone A (PQ_A) and displacement of PQ_B by the herbicide; the reduced PQ_A leads to a higher Fo (Hiraki et al., 2003). Consequently, an overestimation of Fo could occur. Also, it is known that some chemicals emit fluorescence under excitation light (e.g. DCMU) that could not be distinguished from the fluorescence emitted from excited Chl-a (Fo measure). In these cases, the use of Fo to assess algal biomass is not recommended. In field applications, this phenomenon could play an important role, since pollution is often due to a mixture of compounds of unknown origin. On the other hand, the “greening effects”, in which Chl-a synthesis increases maintaining an efficient conversion of light energy to chemical energy (Waring et al. 2007), could also contribute to limit the use of Fo for measuring algal biomass or algal growth. This phenomenon may occur due to an increase in the number of chl-a molecules per unit of cell, without increasing the number of cells (Guasch et al., 1997; Ricart el al., 2009). This situation could produce an increase of Fo values with no increase in the number of cells.

A significant portion of the ecotoxicological studies included in this chapter has incorporated the deconvolution of the chl-a fluorescence signal into the contribution of the main autotrophic groups. These measurements allow the main autotrophic groups of biofilms (structural approach) to be characterized being in agreement with one of the most important assessment aims of the BQE. Nowadays, PAM fluorometers which incorporate a four-wavelength excitation method to deconvolute these photosynthetic parameters of microalgal communities (Fo(Bl), Fo(Gr) and Fo(Br)) by incorporating spectral groups based on a single species pigment spectra. This calibration could be improved in the future if spectres used for calibrating the fluorometer are based on a

mix of species from each group, based on the key species of each autotrophic group. This procedure could probably help to decrease uncertainty in these measurements. Accordingly, the use of Fo to assess algal classes as BQE , two main requeriments should be fulfilled: (1) fix a distance of measurement between sample and sensor to obtain comparable measurements between differents analysis, and (2) use standards calibrations for the deconvolution of main algal groups.

Riou Mort, June 2008



Experimental channels

CHAPTER IV

CHL-A FLURORESCENCE PARAMETERS AS BIOMARKERS OF METAL TOXICITY IN FLUVIAL BIOFILMS: AN EXPERIMENTAL APPROACH BASED ON THE RIOU-MORT RIVER CASE STUDY

INTRODUCTION

Among the great varieties of pollutants interfering with the metabolism of photosynthetic organisms at different stages, trace metals are one of the most common non-biodegradable pollutants reported at elevated concentrations in many parts of the world (Mallick and Mohn, 2003). Metal pollution is of great concern in freshwater systems affected by agricultural, mining and industrial activities. Recent studies performed in the Riou-Mort river, a river draining an active mining area in France, have demonstrated that the mixture of metals found is toxic to biofilms causing structural alterations such as growth inhibition and changes in the architecture and composition of the diatom community (Gold et al., 2003a; Duong et al., 2010; Bonnineau et al., 2011). Although this river presents high concentrations of Fe, metal pollution is attributed to Zn and Cd due to seepage from an active zinc factory (Morin et al., 2008). It is not clear, however, if the effects reported on biofilm were caused by Zn, Cd or mixture of both. Most of the previous experimental studies considered Cd alone as responsible of the toxicity observed, without taking into account either the role of Zn nor of the mixture (Cd + Zn) (Gold et al., 2003b; Morin et al., 2008; Duong et al., 2010). This study was focused on assessing the contribution of Zn to toxicity.

Zn ions at low concentrations are indispensable for algal growth but at high concentration they become toxic (Silver, 1998). Zinc toxicity on algae may affect the oxidizing side of PSII by noncompetitively inhibiting Ca^{2+} and Mn^{2+} binding at their native sites on the water oxidizing complex (Rashid et al., 1994). Based on algal monocultures, Zn toxicity is reported at very different concentrations depending on the species tested and the biomarker assessed: Chaloub et al. (2005) found Zn toxicity on cyanobacteria cultures between 30-332 mg/L and Posthuma et al. (2001) describe an EC_{50} of 15 µg/L in *Selenastrum capricornutum.* Cd is considered an important contaminant of natural waters due to its high toxicity at low concentrations (Deckert,

2005). Cd displaces metal cofactors (Zn^{2+} and Ca^{2+}) from undefined protein targets or directly binds amino acid residues, including cysteine, glutamate, aspartate, and histidine (Faller et al., 2005). However, Cd toxicity to autotrophic organisms at environmentally realistic concentrations is less documented. Studies performed with *Selenastrum acutus* (Turpin) Kützing (Ilangovan et al., 1998) have shown Cd toxicity at high Cd concentration (EC_{50}= 500 µgCd/L). Zhou et al. (2006), observed Cd toxicity on cyanobacteria cultures of *Microcystis aeruginosa* at 448 µgCd/L.

In our investigation, metal toxicity was assessed on biofilms because of its capacity to detect effects produced by toxic substances at the base of the trophic chain (Sabater et al., 2007) and to provide a community ecotoxicology approach with higher ecological relevance than using single species (Clements and Newman, 2002; Guasch et al., 2010a). Biofilms, also known as microphytobenthos or periphyton, are complex biological structures composed mainly by algae, cyanobacteria, bacteria, fungi and microfauna, located in close physical contact and embedded in a mucopolysaccharide matrix (Lock et al., 1984). In rivers and streams, the biofilm has an important role in the primary production (Guasch and Sabater, 1998) and in the organic matter processing (Romaní and Sabater, 2001).

Metal toxicity on biofilms differs from the results obtained with free-living organisms. Paulsson et al. (2000) reported an EC_{50} of 3640 µgZn/L based on the photosynthetic activity. In another study, this author (Paulsson et al., 2002) observed that Zn produced indirect effects on biofilms between 6-25 µgL. Guasch et al. (2003), reported that Zn toxicity on biofilms was highly related to algal biomass and for this reason the range of EC_{10} values based on photosynthesis tests was so broad (455 – 65,000 µgZn/L). In the case of Cd, previous studies (Gold et al., 2003b; Morin et al., 2008) showed slight effects at 10 µg/L and a marked reduction of the biofilm biomass

and diatom density, after chronic exposure to 100 µg/L (Gold et al., 2003b; Morin et al., 2008).

Based on the results reported above concerning fluvial biofilms, it is expected that the type of pollution present in the Riou-Mort river, Zn pollution ranges between 400 and 900 µgZn/L and Cd pollution ranges between 12 and 15 µgCd/L, may cause marked effects due to chronic Zn pollution and slight effects due to Cd. However, it is not clear whether the effects of the exposure to both metals will be similar, higher or lower than the exposure to Zn alone. While Morin et al. (2008) already investigated the response of biofilm to Cd exposure, showing slight effects, in the present investigation we focus on the difference between the effects of Zn alone and Zn with Cd. This approach will allow us to investigate the effects of the type of metal pollution found in the field and to elucidate the specific influence of Zn on freshwater biofilms. Zn and Cd pollution has been described in other metal polluted streams at higher concentrations: in the Dommel stream in Belgium (Ivorra et al., 1999) reached up to 282 µgCd/L and 3147 µgZn/L or the Guadiamar stream, in Spain, (Santos et al., 2010) presented up to 286 µgCd/L and 1001 µgZn/L. On the other hand, lower levels of Zn have been reported in other metal-polluted areas; i.e. around 600 µgZn/L in Osor River, Spain (ACA-Catalan Water Agency) or between 150 and 400 µgZn/L in Furtbach River, Switzerland (Le Faucheur et al., 2005).

Toxic concentrations of trace metals have been reported to act on the photosynthesis in higher plants (Sheoran and Singh, 1992), green algae (Samson et al., 1988), cyanobacteria and diatoms (Juneau et al., 2001). The pulse amplitude modulated (PAM) fluorimetry technique can be used to assess the direct and indirect effects of toxicants on photosynthetic processes (Ralph et al., 2007). In particular, the electron transport rate (Ralph and Burchet, 1998) or the water-splitting apparatus (Moustakas et al., 1994) can be inhibited. Within biofilms, PAM fluorimetry has been

used in several studies to evaluate metal toxicity based on the effective quantum yield (Φ'_{PSII}) and the maximal photosynthetic capacity (Φ_{PSII}) parameters (e.g. Guasch et al., 2002; Barranguet et al., 2003, Serra et al. 2009). However, the effects of metals in the use of the energy designated to radiative (qP) or non-radiative processes (qN and NPQ) is less documented in biofilm studies. These quenching parameters were incorporated in our study in order to widen the applications of the fluorescence parameters for the assessment of metal pollution effects on biofilm communities. Recent studies based on biofilms showed the potential use of the non-photochemical parameter (NPQ) as a pertinent fluorescence parameter to assess the effects of organic toxic substances (Laviale et al., 2010; Ricart et al., 2010a).

The duration of metal exposure is of great relevance for biofilms since metal bio-accumulation and toxicity increase with the time of exposure. Short-term metal toxicity on biofilms is usually found at concentrations higher than those described in free-living organisms (Guasch et al., 2010a,b). This marked difference is explained by the structure of biofilms, reducing metal penetration into the cells. On the other hand, longer metal exposures may allow the metals to reach the different types of organisms embedded in the biofilm (Guasch et al., 2003) increasing metal toxicity once the toxic compounds have entered into the cell (Escher et al., 2002). In field situations, point-sources of metal pollution might cause chronic and variable metal exposure depending on flow conditions (of lower concentration under high-flow conditions due to dilution) whereas diffuse sources such as the urban runoff would cause short-term metal exposures directly linked with rainfall episodes (Guasch et al. 2010a). Aquatic organisms will accumulate less metal during a short exposure causing a transient metabolic and physiological response. Internal concentration will increase after longer metal exposure causing increases in toxicity and/or the development of mechanisms to regulate the accumulation of metals (e.g. Hassler and Wilkinson, (2003); Hassler et al., (2005)).

Since the different species of algae and cyanobacteria found in a biofilm may differ in their metal sensitivity, it is also expected that long-term exposures to low metal concentrations may lead to changes in their competitive interactions producing community changes as it was observed by Serra et al. (2009a) in an experiment of chronic exposure of biofilms to Cu. In this study, cyanobacteria were the autotrophic group better adapted to Cu exposure. Thus, it is of great interest to investigate both short- and long-term effects of metal exposure providing the most appropriate endpoints for each temporal scale of exposure.

The main aim of this study was to evaluate the sensitivity of different chl-*a* fluorescence parameters as metal pollution biomarkers of short and long-term metal exposures under realistic metal exposure conditions by performing an experimental approach. Firstly, a field monitoring campaign was conducted in the Riou-Mort river to characterize water and biofilm from three different metal polluted sites (Bonnineau et al., 2011). Secondly, based on the Riou-Mort river case study (June 2008), a microcosm experiment was performed in which biofilm communities were chronically exposed to 400 µgZn/L and 20 µgCd/L. Moreover, the study also aimed to investigate the contribution of Zn, the metal found at higher concentration, on the effects produced by Zn plus Cd. With this aim, the effects of Zn alone were also investigated.

Our hypotheses are that: 1) The set of parameters studied will allow us to show the effects of metal pollution (Zn+Cd) at realistic concentrations. 2) Short-term exposure will mainly cause functional alterations, whereas structural changes will appear after long-term exposure.

Functional alterations will cause changes in qP and $\Phi'_{PSII,}$ closely related to the electron transport chain, and NPQ and qN activated to avoid stress. Structural damage of the photosynthetic apparatus such as the ultra-structure of the thylakoid membrane and the pigments composition, will lead, respectively, to a reduction of the Φ_{PSII} and the

NPQ. Shifts in algal composition will also occur due to selection pressure according to differences in the species metal tolerances or sensitivities.

FIELD SURVEY

- **Study site**

In order to evaluate *in sit*u metal effects on biofilms, a field sampling was carried out in the Riou-Mort river (SW Aveyron department, France), a small tributary of the Lot River located in an industrial basin. This stream is highly contaminated by different metals from its confluence with the Riou-Viou, a stream carrying seepage from a former active zinc factory (Morin et al., 2007). Three sites located on the main course of the Riou-Mort were chosen; one before the confluence with Riou-Viou (S1, Fig. 1) and two downstream (S2, S3, Fig. 1). Sampling was conducted in June 2008. S1 was considered a reference site. S2 was situated immediately after the confluence of Riou-Viou and Riou-Mort streams, so it was expected to receive point-source of metal pollution from zinc factory seepage (Fig.1). S3 was located further downstream and it was expected receiving lower metal loads due to upstream dilution and/or retention (Fig.1).

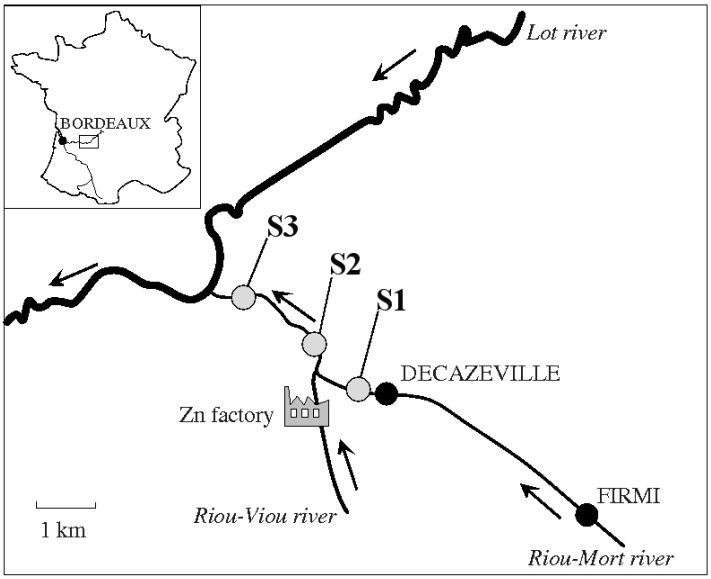


Figure1. Localization of sampling sites (S1, S2, S3) on the Riou-Mort river (adapted from Morin et al., 2007).

- **Field sampling**

Each site was sampled three times; in the morning at 8:00h, after 6h and after 24h. This strategy allowed accounting for diurnal variability in water chemistry and biofilm characteristics. Three replicates were collected at each sampling site. Temperature, conductivity, pH and dissolved oxygen were measured *in situ* by a multi-parametric probe (WTW Meters, Weilheim, Germany). Light was also measured *in situ* at each sampling site (LI-COR Inc., Lincoln, Nebraska,USA). Light measurements were performed on submerged biofilm in order to measure light irradiance arriving on biofilm communities. Water samples were taken for the analysis of phosphate, nitrate, dissolved organic carbon (DOC), alkalinity and dissolved metal concentrations. All water samples were immediately filtered (Whatman nylon filters 0.2 µM nylon). Samples for metal analysis were also acidified with 1% HNO_3 (65 % suprapure, Merck). All samples were transported to the laboratory and stored until analysis.

Biofilm sub-samples were obtained from different cobbles. A surface of 20 cm^2 of biofilm was scraped from each sample for metal bioaccumulation analysis and 1.5 cm^2 for chl-a analysis. For chl-a fluorescence analysis 1.5 cm^2 of biofilm was scarped and conserved in transparent glass vials filled with 10ml of water from the corresponding sampling site. Afterwards, chl-a fluorescence analysis were performed *in vivo* by Phyto-PAM instrument. All water and biofilm analysis followed the procedures described in Chapter II.

- **Field data analysis**

In order to integrate the mixture effects of metals present in the Riou-Mort river, the metal concentrations were used to calculate CCUs (Cumulative Concentration Units) and used as estimates of potential metal toxicity (Clements et al., 2000). The CCU is a score based on the sum of the ratios between metal concentrations measured in surface waters and the corresponding criterion value (US EPA's National Recommended Water Quality Criteria, http://www.epa.gov/waterscience/criteria/

wqctable/) used to investigate the responses of aquatic organisms to metals (Clements et al., 2000). Three categories of CCU were used; CCU below 1 corresponded to "background" category, CCU between 1 and 2 corresponded to "low" category indicating low potential toxicity, and CCU between 2 and 10 corresponded to "moderate" category indicating moderate metal potential toxicity. Above 10 CCU we added a further high metal category "high", compared to Guasch et al. (2009) and Morin et al. (in press) indicating high potential toxicity.

One-way ANOVA was performed to detect significant differences ($p<0.05$) in the light irradiance, water physical and chemical parameters, CCUs, metal accumulation in biofilm and biofilm metrics among sites. Data were transformed to achieve normality. Tukey's HSD test was applied to identify significant differences ($p<0.05$) between sites (S1, S2 and S3).

Principal Component Analysis (PCA) was employed to explore the ordination of sampling sites according to physical and chemical characteristics of water and according to the different biofilm metrics measured. The PCA analyses were done after standardization and normalization of variables (PRIMER, Clarke and Warwick, 2001).

MICROCOSM APPROACH

- **Experimental design**

The experiment was carried out indoors in recirculating channels. The microcosm system was made of nine independent Perspex channels (170 cm long, 9 cm wide and 9 cm high each) as those described in Serra et al. (2009a), except the light intensity that was between 100 and 120 µmolphotons/m^2s^1. In each channel, two sizes of glass substrates (8.5 x 12 cm and 8.5 x 2 cm) were placed horizontally to facilitate biofilm growth. Water was recirculated by means of water pumps and renewed every 2-3 days in order to minimize water chemistry changes.

The experiment lasted for ten weeks: the first five weeks were dedicated for colonization and the next five weeks for metal exposure. Sampling strategy was planned to monitor the colonization (referred to as colonization samples) and to compare mature biofilms before metal exposure (referred to as Pre-exposure) and during metal exposure (referred to as short-term and long-term exposures). Samples corresponding to the short-term exposure phase were taken after 6, 24 and 72h of exposure whereas those corresponding to the long-term exposure were taken at weeks 1, 2, 3, 4 and 5.

Pre-exposure samples were analysed at three different moments at the end of the colonization period: five and three days before exposure and the same day metal additions started. These samples are labelled as -5 d, -3 d and 0 d, respectively; and the moment when metal addition started is considered time zero (T0). From this moment on, the three channels with no metal addition will be referred to as "No-Metal treatment"; the three with Zn addition as: "Zn treatment" and the three channels with the addition of Zn and Cd as "Zn+Cd treatment".

- **Colonization**

In the channels system described above, inocula obtained from natural biofilms were used to colonize the surface of etched glass substrates during five weeks, after Serra et al. (2009a). Natural biofilms were sampled from the Llémena River, a small calcareous tributary of the Ter River (North-East Spain). This river is relatively unpolluted by metals (Catalan Water Agency) making it possible to avoid communities already pre-selected for zinc or cadmium tolerance. No metals were added during the whole colonization period (five weeks).

- **Metal exposure**

After the five weeks of biofilm colonization, the additions of the Zn and Zn+Cd started (T0). From T0 until week 5, three channels were exposed to a nominal concentration 400 µgZn/L (Zn treatment), another three to nominal concentrations of 400 µgZn/L plus 20 µgCd/L (Zn+Cd treatment), and the remaining three were not exposed to metals (No-Metal treatment). Zn and Cd metal solutions were prepared from $ZnCl_2$ and $CdCl_2$ from Tritisol (Merck, Darmstadt, Germany) with deionised water (18Ω Q-H2O grade Barnstead Nonopure). Equilibration of the metal with the water was done in carboys during a period of 24 hours at room temperature, before water renewals.

- **Water Sampling**

During the colonization period, water samples were taken to characterize the content in dissolved organic carbon (n=3), alkalinity (n= 15), anions (n=39) and cations (n=20). Physical and chemical parameters (temperature, pH, dissolved oxygen and conductivity) were measured before and after each water renewal (three times per week in three different channels each time) during the colonization period (n=114) and during metal exposure (n=57). Water samples (10 mL) were taken in triplicate to determine phosphate concentration during the colonization (n=54) and during the metal exposure (n=27). Water samples (5 mL) were also taken before and after each water renewal for Zn and Cd analysis during the colonization (n=18) and during the metal exposure (n=27). All water samples were immediately filtered through 0.2 µM nylon membrane filters (Whatman). Samples for metal analysis were also acidified with 1% HNO_3 (65% HNO_3, Suprapur, Merck, Germany). Samples were stored at 4 ºC.

For detailed information about methodologies applied for water analysis see Chapter II.

- **Biofilm sampling**

In order to monitor the changes in algal biomass during the colonization period and to know the growth phase of biofilm communities at the moment when metal additions began, biofilms were sampled twice a week (total n=49). Each sampling time, three colonized glass substrata (17cm^2) were randomly removed from each channel. These samples were used for Fo measurements using a Phyto-PAM fluorometer (version EDF, Walz, Effeltrich, Germany), see PAM measurements paragraph. Since Phyto-PAM measurement is a non-destructive technique, glass substrata were returned back to the channels after these fluorescence measurements.

During Pre-exposure (-5, -3 and 0d; n=9 for each treatment), Short-term exposure (6, 24 and 72h; n=9 for each treatment) and chronic exposure (wk1, wk2, wk3, wk4 and wk5; n=15 for each treatment) three glass substrates (17cm^2) were also removed at random from each treatment and fluorescence parameters measured. Specifically, following measurements were done: 1) the fluorescence of the different algal classes composing the biofilm communities, 2) the Fo as indirect measure of algal biomass and 3) $\Phi_{PSII,}$ Φ'_{PSII}, qP, qN and NPQ (see PAM measurements).

A total number of 81 biofilm samples were taken for chl-*a* concentration and also used to calculate the carotenoids ratio (430:665). During the Pre-exposure (at days -5,-3 and 0d and three samples per treatment), during Short-term exposure (at hours 6, 24 and 72h and the same number of samples per treatment) and during Long-term exposure (at weeks W1, W3, W5 and the same number of samples). Samples for chl-a analysis were obtained after scraping biofilms from a small substratum (17cm^2) and were kept in polypropylene vials at -4ºC until further analysis (see biofilm analysis).

A total of 72 samples were taken for bioaccumulation analyses (Cd.B: the Cd content per dry weight in µgCd/gDW, Zn.B: the Zn content per dry weight in µgZn/gDW). Each sample corresponded to 51 cm^2 of colonized substrata (half of a big

substratum). The biofilm was scraped and stored at -80ºC until their laboratory analysis (see biofilm analysis). Biofilms were taken in triplicate twice (at days -5 and 0) per treatment during the Pre-exposure; three times (at 6, 24 and 72h) per treatment during the Short-term exposure; and three more times (at wk1, wk3 and wk5) per treatment during Long-term exposure. Substrata were always randomly chosen.

For detailed information about methodologies applied for biofilm analysis see Chapter II.

- **Data analysis of microcosm experiment**

Changes in algal biomass (Fo) throughout the ten weeks of the experiment (from the beginning of colonization until the end of the metal exposure period) were adjusted to a logistic model in order to compare the growth curves between channels throughout the different stages of growth (including the periods of colonization and metal exposure). Data from the three channels assigned to each different treatment were used to fit the corresponding growth curve. Data were fitted to a logistic model using the SIGMAPLOT software (Version 8.0).

$$y = \frac{k}{\left(1 + \exp\left[-\frac{x - xo}{b}\right]\right)}$$

The *1/b* parameter represents the growth rate (*r*), the *xo* parameter represents the time when maximal community growth rate is achieved and the *k* parameter represents the carrying capacity. In our experiment the *r* parameter was used to compare the growth rates during the colonization period, the *xo* parameter to evaluate when biofilms reached the maximal growth rate and the *k* parameter to evaluate the carrying capacity (Romaní, 2010).

An ANOVA of repeated measures was applied to examine the temporal tendency of each biological parameter (chl-a, 430:665, Zn.B, Cd.B, Fo(Bl), Fo(Gr), Fo(Br), Φ_{PSII}, Φ'_{PSII}, qP, qN and NPQ) during the Pre exposure, the Short-term exposure and the Long-term exposure periods. The post-hoc Tukey's HSD test was applied to check if tendencies were different between treatments. ANOVA analyses were realized by SPSS software (Version 17.0).

Principal Component Analysis (PCA) was used to explore the ordination of samples according to the different biofilm variables measured. The data set included photosynthetic parameters, structural parameters (pigment analysis, Fo and the fluorescence of each algal class) and bio-accumulation values. A preliminary PCA was done using all sampling times in order to check the supposed difference in responses during short and long-term exposures.

PCA analyses were done after elimination of auto-correlated variables (R^2>0.8), and standardization and normalization of remaining variables (chl-a, 430:665, Zn.B, Cd.B, Fo(Bl), Fo(Gr), Fo(Br), Φ_{PSII}, Φ'_{PSII}, qP, qN and NPQ) (PRIMER, Clarke and Warwich, 2001).

RESULTS

- **Field sampling**

Physical and chemical parameters are summarized in ST1 (Supplementary Table 1). Detection limit for total metals dissolved in water (Al, Fe, Zn, Cd, Ni, Cu and Pb) was <0.001 µg/L.Cu and Pb concentrations were below detection limit in all cases. Accordingly, these metals were considered of low significance and were not included in the multivariate analysis. Based on the Concentation Cumulative Units (CCUs) differences were observed between sites. The highest values were obtained in S2 and S3 sites with a "high" potential toxicity level mainly attributed to Cd (50%), Zn (30%)

and Fe (14%). CCUs were lower in S1 with “moderate” potential toxicity mainly attributed to Fe (86%) and Al (17%). Light irradiance reaching biofilms at S1 was 615±684, while at S2 was 768±721 and at S3 was 1656±45 (AVG±SD; n=3), being comparable between sites ($p>0.05$; $F=1.747$).

PCA analysis of water physical and chemical parameters showed the ordination of sampling points within the first two axes (Fig. 2a). The first axis explained 78.9% of variance and separated the most polluted sites (S2 and S3) with highest Zn, Cd and NO_3^{2-} concentrations in water from S1 with a highest alkalinity, conductivity and Fe and SO_4^{2-} concentrations in water. The PCA second axis explained a smaller part of total variation (12.2%), and separated S3 from S1 and S2 sites (Fig. 2a). In fact, PO_4^{3-} and Cl^- concentrations were higher at S3 than at the other sites (Fig.2a).

Metal accumulation in biofilms and biofilm metrics (chl-a, carotenoids ratio-430:665, Φ_{PSII}, Φ'_{PSII} and NPQ) measured during biofilm field monitoring are shown in sumplementary tables 2 and 3 (Annex Chapter IV), respectively. PCA analysis of these data showed the ordination of sampling points within the first two axes (Fig. 2b). The first axis explained the 85.3% of the variance and separated the most polluted sites (S2 and S3) with highest metal accumulation (Zn, Cd, Pb, Ni and Cu) and a higher carotenoids ratio (430:665) from biofilms at S1 site. In contrast, biofilms from S1 had higher chl-a concentration and slightly higher Φ_{PSII} than biofilms form polluted sites (S2 and S3). The second axis accounted only for a 5.8% of total variance, showing that in occasional samplings at S1 and S2, biofilm accumulated higher amounts of Fe and Al (Fig.2) which was negatively correlated with chl-a fluorescence parameters (NPQ, Φ_{PSII} and Φ'_{PSII}).

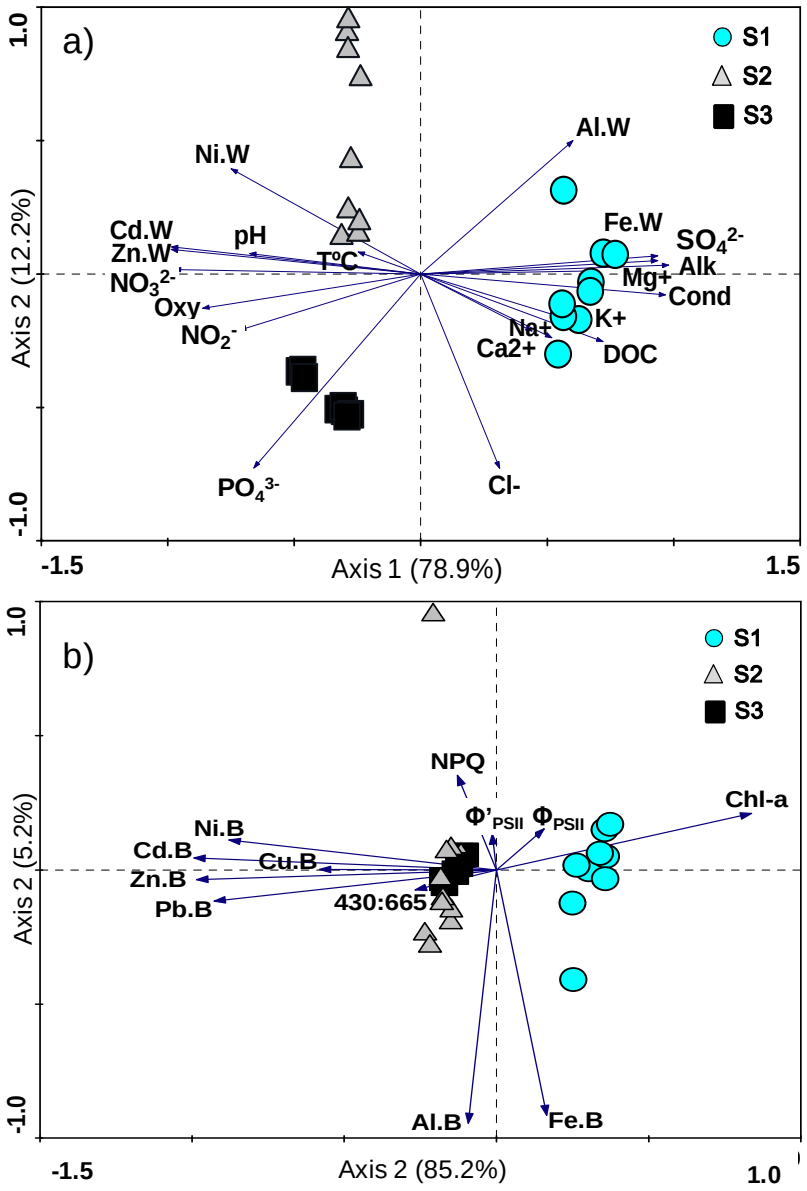


Figure 2. a) Principal Components Analysis (PCA) based on the physical and chemical parameters of Riou-Mort river water measured at each site (S1, S2 and S3) during field sampling. The acronym of each parameter is defined in ST1 (Annexe Chapter IV). b) Principal Components Analysis (PCA) based on biofilm metal accumulation and biofilm metrics measured at each site of Riou-Mort river (S1, S2 and S3) during field sampling. The acronym of each parameter is defined in ST2 and ST3 (Annexe Chapter IV).

➢ Microcosm study

➢ Physical and chemical characteristics of the water from the artificial channels system

Major anions and cations, as well alkalinity and DOC concentrations are summarized in Table 1. Physical and chemical conditions remained stable during the colonization and metal exposure periods and were similar between treatments (Table 2). Metal concentration in the No-Metal treatment was always <10 µg/L (Table 2). Zn concentration was similar in the Zn and Zn+Cd treatments. Zn concentration in the Zn treatment was 21 % below nominal concentration (400 µgZn/L), Zn concentration in the Zn+Cd treatment was 20 % below nominal concentration (400 µgZn/L) and Cd concentration in the Zn+Cd treatment was 28% below nominal concentration (20 µgCd/L).

Table 1. Chemical characteristics of water channels. Average (AVG) and standard error (SE) of water samples (n) taken throughout the duration of the microcosm experiment.

	SO_4^{2-} ($mg.L^{-1}$)	Cl^- ($mg.L^{-1}$)	K^+ ($mg.L^{-1}$)	Na^+ ($mg.L^{-1}$)	Ca^{2+} ($mg.L^{-1}$)	Mg^{2+} ($mg.L^{-1}$)	Alkalinity ($mEq.L^{-1}$)	NO_3^- ($mg.L^{-1}$)	NH_4^+ ($mg.L^{-1}$)	DOC ($mg.L^{-1}$)
AVG	73.33	86.13	3.34	24.88	41.4	8.06	1.47	4,353	0.05	4.92
SE	±8.75	±3.98	±0.11	±0.94	±1.75	±0.30	±0.03	±0.204	±0.03	±0.95
n	39	39	20	20	20	20	14	20	20	3

Table 2. Summary of dissolved oxygen (Oxy), water temperature (Temp); conductivity (Cond); phosphate (PO_4^{3-}); zinc (Zn) and cadmium (Cd) concentrations during the colonization and metal exposure periods for each treatment (No-Metal, Zn and Zn+Cd). Each value corresponds to the average (AVG) of before and after water renewals and ± standard error (SE). b.d.l.: below detection limit.

	Colonization			**Metal Exposure**								
				No-Metal Treatment			**Zn Treatment**			**Zn+Cd Treatment**		
	AVG	**SE**	**n**	**AVG**	**SE**	**n**	**AVG**	**SE**	**n**	**AVG**	**SE**	**n**
Oxy. ($mg{\cdot}L^{-1}$)	8.82	0.04	114	9.44	0.05	57	9.08	0.03	57	9.15	0.04	57
Temp. (ºC)	19.29	0.14	114	18.55	0.14	57	18.40	0.15	57	18.41	0.02	57
pH	8.36	0.02	114	8.61	0.03	57	8.52	0.02	57	8.53	0.02	57
Cond. ($\mu S{\cdot}cm^{-1}$)	326.72	1.40	114	310.72	3.62	57	341.72	2.58	57	341.51	2.66	57
PO_4^{-3}($\mu g{\cdot}L^{-1}$)	29.18	2.59	54	23.12	2.08	27	28.81	3.66	27	29.20	3.26	27
Zn ($\mu g{\cdot}L^{-1}$)	8.64	0.76	18	4.88	0.75	27	317.53	24.94	27	321.44	22.08	27
Cd ($\mu g{\cdot}L^{-1}$)	bdl		18	bdl		27	bdl		27	14.30	1.09	27

- **Biofilm growth curves**

The growth curves of the biofilm based on Fo showed significant fits to the logistic model (Fig.3). All growth curves had a similar growth rate (r=0.083 day^{-1}, p<0.0001 for No-metal; r= 0.085 day^{-1}, p<0.001 for Zn and r=0.092 day^{-1}, p<0.05 for Zn+Cd) indicating that biofilms presented comparable growth rates during the colonization (until day 36). However, the time to achieve the maximal community growth rate (*xo*) differed between treatments. In the No-Metal treatment it was 46 days (p<0.0001), slower than in the Zn treatment (xo=23 days; p<0.0001) or in the Zn+Cd treatment (xo=26 days, p<0.0001). The carrying capacity (k) of biofilm from No-Metal treatment was 300 units of Fo, 98 for the Zn treatment and 103 for the Zn+Cd treatment (67% and 65% lower than k in the No-Metal treatment, respectively) (Fig.3).

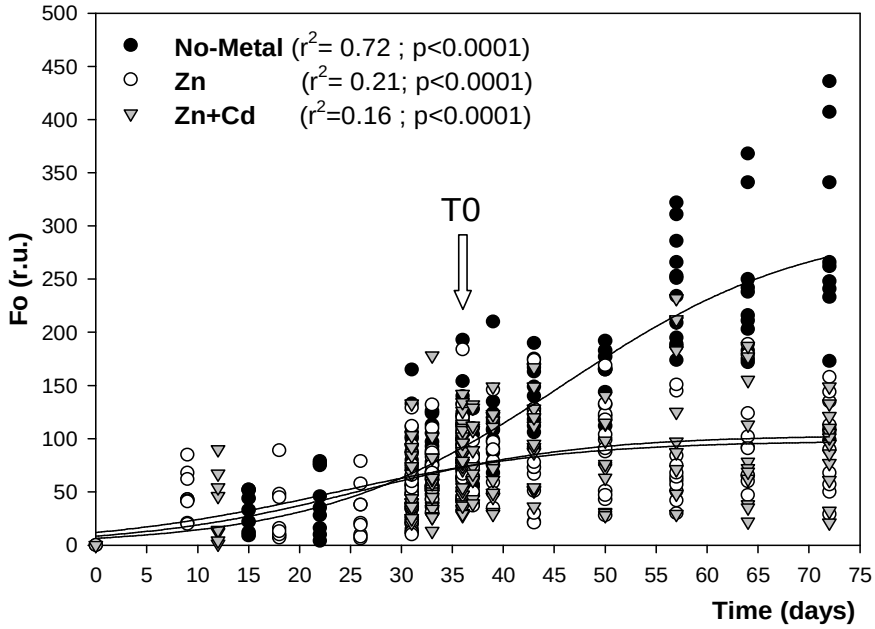


Figure 3. Growth curves of biofilms from the nine channels where the No-Metal, Zn and Zn+Cd treatments were applied. Fo measurements recorded throughout the 10 weeks that the experiment lasted. Lines represent curves fitted to the sigmoidal model. T0 corresponds to the time when metal exposure begun (day 36).

- **Biofilm characterization**

Chlorophyll-a concentration. During Pre-exposure and the first days of exposure (Short-term exposure period), chl-a concentration was low and remained stable in all treatments (Table 3). The carotenoids ratio (430:665) was also similar between treatments (Table 3). However, after longer exposure (Long-term exposure period), chl-a concentration and the carotenoids ratio showed significant differences between treatments (ANOVA of repeated measures; $F= 52.53$, $p<0.001$ and $F= 11.96$, $p<0.001$, respectively). Chl-a increased progressively over time in the No-Metal treatment, reaching values 3 times higher than those measured in the Zn and Zn+Cd treatments (Table 3). Ch-a was significantly correlated to Fo measurements during biofilm growth ($R^2=0.86$; $p<0.05$). The carotenoids ratio showed a trend to decrease in the No-Metal treatment, while it remained stable in the Zn+Cd treatment and increased in the Zn treatment as well, showing significant differences between the No-Metal and the Zn treatments (Table 3).

Bioaccumulation. During Pre-exposure, biofilm metal contents remained low and stable (Table 3). Metal bioaccumulation increased over time in the biofilms exposed to metals. A positive trend was observed for Zn during both Short and Long-term exposure periods in both metal treatments (ANOVA of repeated measures; $F=101.14$, $p<0.05$ and $F= 45.45$, $p<0.05$, respectively) reaching in both cases values between 1 and 2 orders of magnitude higher than controls (Table 3). Cd bioaccumulation was also observed in the Zn+Cd treatment, both during Short and Long-term exposures (ANOVA of repeated measures; $F=57.55$, $p<0.0001$ and $F= 36.88$, $p<0.0001$, respectively), reaching up to 74 µgCd/gDW (Table 3).

Table 3. Biofilm values throughout the three periods: Pre Exposure, Short-term and Long-term metal exposures in the three treatments: No-Metal, Zn and Zn+Cd. Each value corresponds to the AVG±S.E; n=3. For each parameter, different letters indicate significant differences ($p<0.01$) between treatments during the exposure period analyzed (Tukey's HSD test from ANOVA of repeated measures). b.d.l.: below detection limit; n, m.: not measured; n.s.: non significant differences between treatments.

	Treatments	Pre-exposure				Short-term exposure				Long-term exposure			
		-5d	-3d	0d	Tukey	6h	24h	72h	Tukey	wk1	wk3	wk5	Tukey
Chla (µg/cm^2)	No-Metal	1.16 ±0.37	1.02 ±0.05	0.88 ±0.13	n.s.	0.95 ±0.14	1.00 ±0.19	1.22 ±0.09	n.s.	1.50 ±0.20	2.51 ±0.49	3.59 ±1.19	a
	Zn	0.98 ±0.23	0.91 ±0.14	1.35 ±0.42		1.41 ±0.38	1.11 ±0.22	0.82 ±0.14		0.77 ±0.06	0.70 ±0.06	0.87 ±0.37	b
	Zn+Cd	1.49 ±0.33	0.82 ±0.12	0.68 ±0.01		0.81 ±0.10	0.89 ±0.20	1.05 ±0.22		0.73 ±0.15	1.09 ±0.38	0.99 ±0.36	b
430:665	No-Metal	3.04 ±0.19	3.40 ±0.09	2.90 ±0.10	n.s.	2.95 ±0.14	2.85 ±0.03	2.79 ±0.03	n.s.	2.84 ±0.09	2.57 ±0.13	2.48 ±0.02	a
	Zn	2.96 ±0.25	3.13 ±0.05	3.19 ±0.13		2.99 ±0.21	2.94 ±0.07	2.98 ±0.10		3.06 ±0.19	3.39 ±0.39	3.48 ±0.15	b
	Zn+Cd	2.82 ±0.00	2.99 ±0.15	3.08 ±0.33		2.99 ±0.11	2.92 ±0.32	3.00 ±0.15		3.08 ±0.21	2.90 ±0.22	3.02 ±0.20	ab
Zn.B (µgZn/gDW)	No-Metal	25.74 ±4.91	n.m.	17.87 ±4.19	n.s.	33.87 ±7.56	26.55 ±2.83	14.86 ±3.44	a	18.16 ±2.10	17.09 ±0.52	8.78 ±2.97	a
	Zn	23.3 ±3.06	n.m.	17.34 ±1.26		146.6 ±21.22	239.87 ±9.14	220.97 ±9.53	b	574.38 ±49.34	735.01 ±32.60	1571.24 ±186.53	b
	Zn+Cd	31.63 ±13.16	n.m.	20.91 ±10.94		205.84 ±42.10	183.44 ±7.19	224.31 ±8.64	b	371.38 ±16.59	1051.16 ±237.77	1570.91 ±136.04	b
Cd.B (µgCd/gDW)	No-Metal	bdl	n.m.	b.d.l.	n.s.	b.d.l.	b.d.l.	b.d.l.	a	b.d.l.	b.d.l.	b.d.l.	a
	Zn	1.96 ±0.62	n.m.	b.d.l.		b.d.l.	b.d.l.	b.d.l.	a	2.23 ±0.23	b.d.l.	b.d.l.	a
	Zn+Cd	2.34 ±1.01	n.m.	b.d.l.		6.3 ±3.26	9.84 ±1.22	10.62 ±0.41	b	25.47 ±1.44	57.73 ±9.31	74.35 ±16.87	b

The algal classes. Cyanobacteria, diatoms and green algae were detected with the Phyto-PAM. The percentage of each group was stable during Pre-exposure (data not shown) and Short-term exposure in all treatments (Fig.4). However, during Long-term exposure, a marked reduction in the percentage of diatoms was observed in both metal treatments, differing from the No-Metal treatment (ANOVA of repeated measures; $F=9.715$, $p<0.05$) (Fig.4). At week 5, the reduction in Fo(Br) reached 88% of the No-Metal values in the Zn treatment and a 99% in the Zn+Cd treatment (Fig.4).

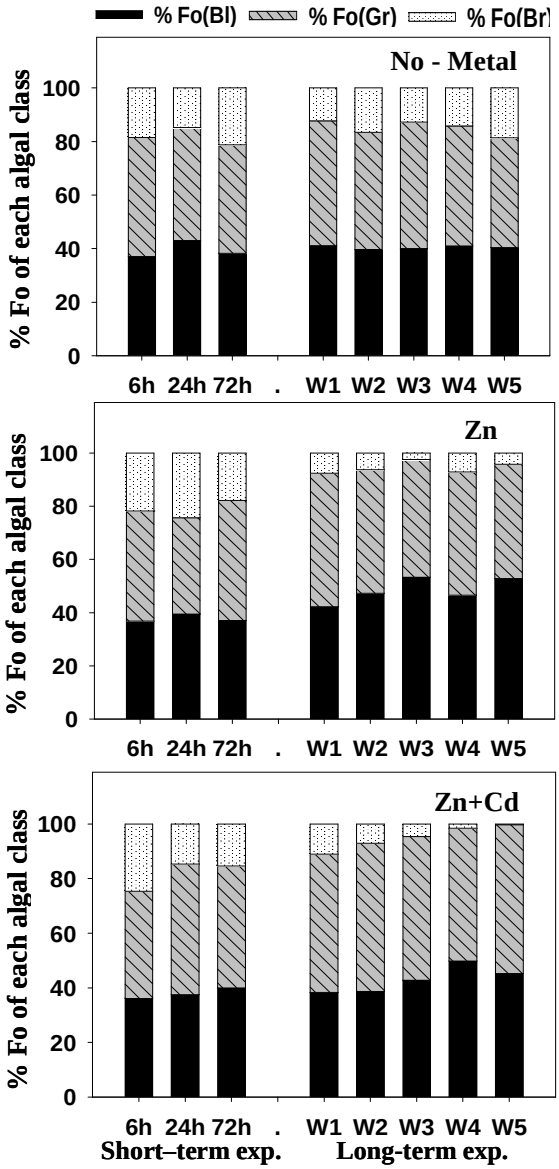


Figure 4. Percentage of fluorescence of each class: cyanobacteria (Fo(Bl)), green algae (Fo(Gr)) and diatoms (Fo(Br)) from biofilms of No-Metal, Zn and Zn+Cd treatments in each sampling time during Short and Long-term metal exposures (AVG; n=3).

Photosynthetic parameters. All photosynthetic parameters remained stable and were similar in the different channels before metal exposure (Pre-exposure). The qP and qN were stable over time showing similar values in all treatments. Φ'_{PSII} ranged between 0.1 ± 0.01 and 0.2 ± 0.02 (AVG±SE; n=3), Φ_{PSII} between 0.41 ± 0.02 and 0.54 ± 0.05 and NPQ between 0.53 ± 0.03 and 1.02 ± 0.13 in the No-Metal treatment, and were differently affected by metal exposure.

Short-term exposure. The Φ'_{PSII} parameter was inhibited in both metal treatments showing significant differences with the No-Metal treatment and also between them (ANOVA of repeated measures: $F=37.157$, $p<0.001$). It was 23% below controls in the Zn treatment and 8% below controls in the Zn+Cd treatment (Fig.5). Metal exposure effects on NPQ were less consistent, showing a significant difference between the No-Metal and the Zn+Cd treatments after 6h of exposure (ANOVA one way: $F=13.22$, $p<0.05$) as it is shown in (Fig.5) in percentage of controls.

Long-term exposure. Φ_{PSII} decreased in both metal treatments (ANOVA of repeated measures; $F= 22.35$, $p<0.01$) reaching values up to 38% below controls (Fig.5). Φ'_{PSII} slightly decreased over time in biofilms from No-Metal treatment and was not affected by metal exposure (Φ'_{PSII} from metal treatments was not significantly different from the No-Metal treatment). Effects of metal exposure on NPQ were significant (ANOVA of repeated measures; $F=26.57$, $p<0.01$), reaching, in both metal treatments, values up to 74% below controls (Fig.5).

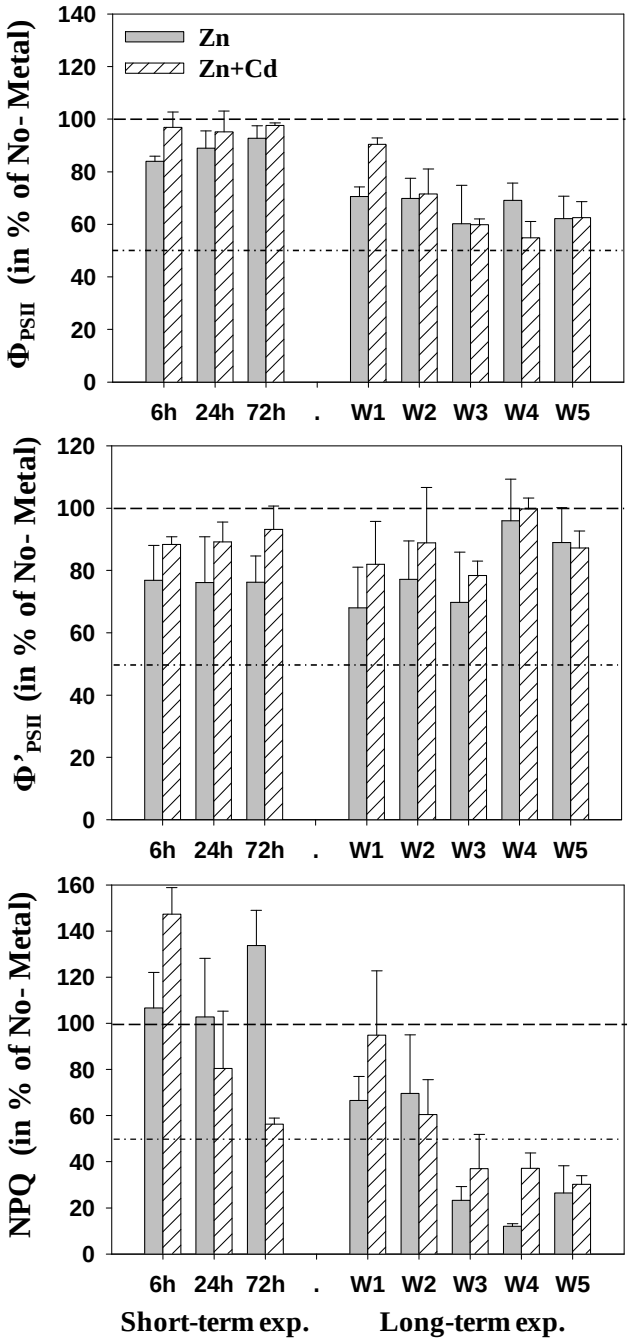


Figure 5. Maximal quantum yield (Φ_{PSII}), effective quantum yield (Φ'_{PSII}) and non-photochemical quenching (NPQ) of biofilms from the Zn treatment (grey bars) and the Zn+Cd treatment (dashed bars), in percentage of the No-Metal treatment, during the Short and the Long-term metal exposure periods (AVG+SE; n=3).

Multivariate analysis. The PCA carried out with all sampling times and variables separated data from T0 up to 72h (Short-term exposure) from data obtained after W1 (Long-term exposure), therefore these two periods were analysed separately supporting the initial separation between Short and Long-term exposure.

PCA first axis of the Pre-exposure and Short-term exposure periods explained 22.9% of the variance while the second axis explained 16.5 % (Fig.6a). Biofilm samples were separated along the first axis based on their metal bioaccumulation: Zn and Cd bioaccumulation samples were situated to the left, while NPQ, Φ_{PSII} and Φ'_{PSII} were grouped to the right (Table 4 and Fig. 6a). The second axis represented a gradient with positive values related to Φ'_{PSII} and negative scores associated with non-photochemical quenching parameters (qN and NPQ) and the cyanobacteria and green-algae groups (Table 4 and Fig. 6a).

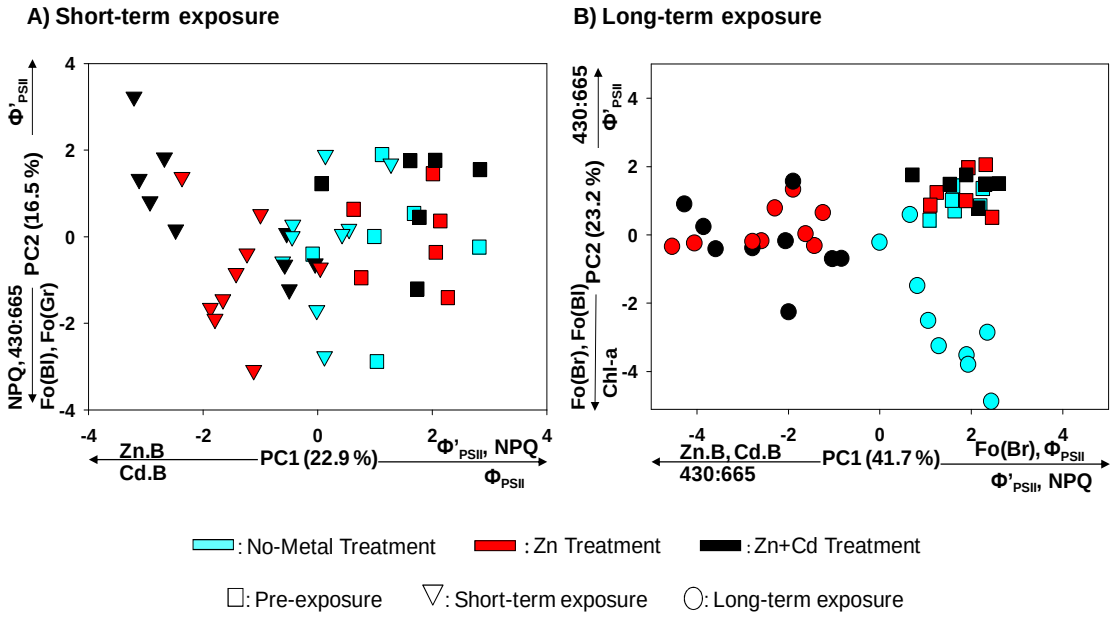


Figure 6. Principal Component Analysis (PCA) of biological variables from biofilms in the No-Metal, Zn and Zn+Cd treatments during the Pre-exposure and Short-term exposure (left graph-Fig.6a) and during the Pre-exposure and Long-term exposure (right graph-Fig.6b).

Table 4. Axis loadings of biological variables for principle components analysis (PCA) of the biological correlation matrix (Short and Long-term exposures). Zn.B: the Zn content per dry weight (µgZn/gDW), Cd.B: the Cd content per dry weight (µgCd/gDW).

	PCA loadings			
	PCA: Short-term exposure		**PCA: Long-term exposure**	
	First axis	Second axis	First axis	Second axis
Eigenvalues	2.74	1.99	5	2.78
% Variation	22.9	16.5	41.7	23.2
Biological variable	Eigenvectors PC1	Eigenvectors PC2	Eigenvectors PC1	Eigenvectors PC2
Chl-a	0.109	-0.034	0.201	-0.451
430:665	0.043	-0.338	-0.235	0.322
qP	0.128	-0.067	0.107	-0.132
qN	0.177	-0.534	0.236	-0.104
NPQ	0.306	-0.359	0.358	-0.176
Φ_{PSII}	0.461	-0.036	0.409	0.153
Φ'_{PSII}	0.376	0.424	0.331	0.266
Fo(Bl)	-0.262	-0.339	-0.165	-0.515
Fo(Gr)	-0.286	-0.301	-0.162	-0.513
Fo(Br)	0.183	-0.222	0.378	-0.09
Zn.B	-0.464	-0.014	-0.415	0.049
Cd.B	-0.302	0.172	-0.252	-0.016

The first axis of the PCA carried out with data from the Pre-exposure and Long-term exposure periods explained a 41.7% of the variance (Fig.6b). This first axis was related with metal toxicity. Samples with high Zn and Cd bioaccumulation values were palced on the left (see Table 4 and Fig. 6b) and those with low NPQ, Φ_{PSII} and Fo(Br) values on the right (see Table 4 and Fig. 6b). The second axis explained a 23.2% of the variance and was related with the maturity of the community, thus positively associated with the chl-a content and the relative abundance of green algae (Fo(Gr)) and cyanobacteria (Fo(Bl)), and negatively with the degradation state of the pigments (430:665) and the effective quantum yield (Φ'_{PSII}).

DISCUSSION

- **Field sampling**

The field sampling conducted in the Riou-Mort river allowed us to characterize the three sites in terms of water physical and chemical variables, metal accumulation and some biofilm metrics (e.g. chl-a concentration). According to CCUs results, S2 and S3 sites had a high metal pollution, mainly attributed to Zn and Cd. Metal pollution was comparable between S2 and S3 sites, probably due to groundwater inputs before S3 (Coynel et al. 2007). However, S1 had a moderated metal pollution, mainly attributed to Fe and Al probably due to the geochemical background of the stream (gres and lutite). S1, the reference site, had also a slightly higher concentration of DOC than downstream sites (S2 and S3), probably it could be explained due to the depuration effect of a water treatment plant (WWTP) between S1 and S2 sites. Accordingly, S1 was not a pertinent reference site, highlighting the difficulty in finding good reference sites in polluted areas (Guasch et al., in press; Hurlbert, 1984). Nevertheless, S1 differed from highly polluted sites (S2 and S3) which accumulated high amounts of Zn and Cd, had a lower algal biomass (chl-a) and a slightly lower photosynthetic capacity (Φ_{PSII}) than biofilms from S1, indicating that Zn and Cd accumulation caused structural and functional damaging effects on biofilms. A reduction of algal biomass due to chronically metal exposure has also been reported in Barranguet et al. (2002). On the other hand, Φ_{PSII} alterations may indicate that the structure of the PSII complex is indeed damaged by metals (Rosenqvist and van Kooten, 2003). The observed decrease in Φ_{PSII} could be linked with a reduction of antennae pigments (Lazar, 1999) due to the observed increase of degraded pigments (430:665). In contrast, no metal effects on NPQ and Φ'_{PSII} of biofilm from high polluted sites (S2 and S3) were detected. We hypothesize that these functional processes, which are crucial for biofilm fitness and survive, were maintained in biofilms from high metal polluted sites by two

processes: (1) a physiological acclimatation; e.g. by a reconstruction of the photosystem and (2) adaptative processes; e.g. by replacing sensitive species for tolerant ones (Foster, 1982; Deniseger et al., 1986; Lindstrom and Rorslett, 1991). In the case of diatoms, this adaptative process has been shown by previous studies along the Riou-Mort river (Gold et al. 2003b, Morin et al. 2008, Duong et al 2010): at least, strong community changes were demonstrated. We conclude that the observed metal effects in the Riou-Mort river could be mainly attributed to the co-occurrence of Zn+Cd pollution. However, experimental approaches in controlled conditions are needed to corroborated cause-effect relationship and evaluate separately the contribution of Zn, the metal bioaccumulated at higher proportions, to the observed Zn plus Cd toxicity on biofilms. These field observations also point out the relevance to evaluate the effects of Fe on biofilms due to its high occurrence in metal polluted rivers.

- **Microcosm study**

In agreement with our hypothesis, under controlled conditions metal pollution (Zn and Zn+Cd) at realistic concentrations and exposure time (hours to weeks) led to a variety of biofilm community alterations including changes in the kinetics of algal growth, metal bioaccumulation, chl-a concentration, community composition, and different PSII related fluorescence parameters(Φ_{PSII}, Φ'_{PSII} and NPQ) that are discussed below.

The kinetics of growth summarizes the integrated response of biofilms throughout the entire metal exposure period. The effect of metals on the growth curves was very marked and no differences were observed between metal treatments (Zn or Zn+Cd). In contrast to Non-exposed biofilms that reached the highest biomass at the end of the experiment, algal growth in metal-exposed biofilms was clearly reaching plateau values up to three times lower than non exposed biofilms (considered as controls). In comparison with controls, plateau values were around 66-67% lower in both metal treatments indicating a lower carrying capacity, which is in agreement with

the chl-a difference observed at the end of the experiment (63-65% below controls). Serra et al. (2009) also found that biofilms exposed to Cu reached a plateau state (in terms of Fo) before controls. The observed growth reduction may have marked effects on the fluvial ecosystem due to their role in the uptake or retention of inorganic and organic nutrients (Fischer et al., 2002; Romaní et al., 2004) affecting self-depuration processes (Cazelles et al., 1991), and transfer of energy to higher trophic levels (Hall and Meyer, 1998; Schmitt et al., 1995; Sheldon and Walker, 1997). On the other hand, biofilms exposed to Zn and Zn + Cd bioaccumulated these metals reaching values 1-2 orders of magnitude higher than the non metal-exposed communities. Metal bioaccumulation can have adverse effects for fish and invertebrates that prey on it due to biomagnification (Rhea et al., 2006; Farag et al., 2007; Hill et al., 2010).

- **Zn and Cd effects depending on the time of exposure**

After short-term metal exposure, the effects observed were of low magnitude, always below 30% in comparison to controls, and this was attributed to the relatively low metal exposure conditions simulated. NPQ, Φ_{PSII} and Φ'_{PSII}, decreased as metal bioaccumulation increased (Fig.7a and Table 6) but these changes explained a small portion of total variance based on the PCA analysis (22.9% of the variance). Zn bioaccumulation was 8 times higher in metal exposed biofilms than in the non-exposed ones, causing, in agreement with our hypothesis, mainly functional community responses. The Φ'_{PSII} decreased (23% and 8% in the Zn and Zn+Cd treatments, respectively) indicated an inhibition in the electron transport flow during the light-dependent photosynthesis activity (Juneau et al., 2007). This effect could also be attributed to the *in vivo* substitution of magnesium, the central atom of chlorophyll, by heavy metals leading to a breakdown in photosynthesis (Küpper et al., 1998, Zvezdanovic and Markovic, 2009). This damage mechanism, reported in heavy metal stressed plants, was not directly addressed in this investigation. NPQ response was

less consistent. Although a tendency to decrease with exposure is indicated by the PCA analyses, a significant increase was observed after 6h of exposure in the Zn+Cd treatment (Fig.3). Niyogi (2000) described an increase in the NPQ as a mechanism to avoid damage on the photosynthetic apparatus produced by an excess of light reaching the PSII which cannot be used in the photosynthetic process. Similar NPQ responses have been observed in bioassays with algal monocultures (Perales-Vela et al., 2007) and cyanobacteria (Juneau et al., 2001) exposed during few hours to metals. The maximal photosynthetic activity (Φ_{PSII}) showed a similar pattern than Φ'_{PSII} (Fig. 7), indicating that structural damage was already occurring during the first hours of metal exposure.

Other studies indicate that higher Zn concentrations (450 µgZn/L to 40 mgZn/L) are required to affect biofilm photosynthesis (50% inhibitions) after short-term exposure (Admiraal et al., 1999; Blanck et al., 2003; Guasch et al., 2003). The structure of biofilm could play an important protective role against Zn toxicity (Guasch et al., 2010a) since metal toxicity is linked to metal penetration into cells (Escher et al., 2002) and biofilm thickness reduces Zn and Cd diffusion (Ivorra et al., 2000; Duong et al. ,2010). Since our biofilm was exposed after 4 weeks of colonization, a slow rate of diffusion into the biofilm matrix was expected during the first hours.

During long-term metal exposures, clear effects were observed on metal exposed biofilms: algal growth was deeply inhibited (above 50% of controls), community composition was altered and most chl-a fluorescence parameters remained affected. In agreement with our expectations, these marked effects were linked to a higher metal bioaccumulation, result of a longer time of metal exposure. According to PCA analysis, metal exposure effects account for a high portion (around 42%) of the total variance (Fig.7b and Table 7). Zn bioaccumulation increased progressively over time, reaching values 1-2 orders of magnitude higher than the non metal-exposed

communities, within the range found in Zn polluted rivers (Behra et al., 2002; Morin et al., 2008, Hill et al., 2010). Biofilm endpoints linked to Zn and Cd bioaccumulation were NPQ, Fo(Br) and Φ_{PSII}. In NPQ inhibition was very marked (above 50% controls in both metal treatments), indicating that this non-photochemical quenching mechanism did not take place. It supports the hypothesis that metal toxicity caused structural effects, and these effects included the damage of pigments where NPQ processes occur. Ricart et al. (2010a) also found a drastic reduction of this parameter when biofilms were exposed during a long period of time to triclosan and they also attributed these findings to an alteration of the photosynthetic pigments. In our study the NPQ was even more sensitive than qN to metal stress, so it can, therefore be considered the most sensitive photosynthesis-related parameter evaluated. This high sensitivity of NPQ versus qN was reported recently in a review work about the use of PAM fluorometry to assess herbicides toxicity on monocultures (Juneau et al., 2007). In contrast, slight effects were observed in the Φ_{PSII}. The reduction of the Φ_{PSII} indicates that the structure of the PSII complex was indeed damaged by metals. The maximal photosynthetic capacity is a parameter extremely sensitive to alterations of the PSII/LHCII complex in the thylakoids (Rosenqvist and van Kooten, 2003). Lazar (1999) also suggested that a destruction of the antennae pigments may be partially responsible for the reduction of Φ_{PSII}. An increase in the carotenoids ratio (430:665) was also observed (mainly in the Zn treatment), supporting the occurrence of structural damage (Martínez-Abaigar and Núñez-Olivera, 1998). Photosynthesis efficiency was slightly affected after long-term metal exposures following the same pattern observed during the first hours of exposure. Φ'_{PSII} remained inhibited, indicating a persistent alteration on the electron transport chain when a metal exposure takes place over a long period. However, photosynthesis inhibition decreased over time reaching values similar to controls. This apparent lack of inhibition can be explained by the patterns observed in controls, decreasing their Φ'_{PSII} due to maturity.

The community composition of biofilm was highly altered due metal toxicity. Metal-exposed biofilms suffered a marked decrease of the relative abundance of diatoms (between an 80 and 90% in comparison to controls) while the relative abundance of cyanobacteria and green algae remained stable. These results indicate that cyanobacteria and green algae are more tolerant to Zn and Zn plus Cd exposures than diatoms. Other studies, based on biofilms, have already reported a metal resistance of green algae to Zn (Ivorra et al., 2000) as well as resistance of cyanobacteria to Zn (Genter et al., 1987) and Zn mixtures (Takamura et al., 1989). Serra et al. (2009) also observed a higher tolerance of cyanobacteria than green algae or diatoms, when biofilms were chronically exposed to Cu.

On the other hand, changes in the Φ'_{PSII} and the carotenoid ratio were mainly attributed to biofilm's maturity (23.3% of the total variance, Table 4). It is well reported that photosynthetic activity declines with biofilms age (Hill and Boston, 1991; Hawes, 1993) due to an increase of self-shading and a decrease of nutrient availability with biofilm thickness (Paul and Duthie, 1989; Dodds et al., 1999).

Our experimental results allowed to link short-term metal exposure (at the concentrations used) with functional alterations in biofilm: mainly the Φ'_{PSII} that may be therefore used as biomarkers of pollution. As such, they provide an early indicator of toxic exposure that can be later confirmed by even structural alterations.

- **Zn *vs* Zn+Cd effects on biofilm**

In agreement with our expectations, the effects caused by Zn+Cd could be attributed to Zn toxicity. The influence of Cd bioaccumulation during the short-term exposure was evident in the short-term exposure according to the multivariate analysis (Fig. 6a and Table 4), showing that biofims exposed to Zn+Cd presented higher photosynthetic efficiency that those exposed to Zn alone. However, Cd had a low weight in the long-term exposure, indicating a lack of difference between the two metal

treatments applied. Wang et al. (1995) reported in a study with marine algae, that when the Cd concentration was low and Zn concentration was high, the toxicity depends upon Zn concentration.

As it was pointed out before, the Zn and Cd concentrations were very different: Zn concentration was 22 times higher than Cd concentration, so no equal toxicity can be expected. The similarities observed between Zn and Zn+Cd treatments agree with the results obtain by Gold et al. (2003b), showing low effects of 10 µgCd/L in laboratory biofilms, but clear effects of Zn+Cd in the field (Gold et al., 2003a). Ivorra et al. (2000), based on different endpoints to the ones we used (chl-a concentration and diatoms taxonomy), also found that biofilm suffered similar effects when it was exposed to Zn+Cd or Zn alone.

- **The use of chl-a fluorescence parameters in biofilms as early warning systems for metal toxicity detection**

The set of chl-a fluorescence parameters included in this experimental study was very sensitive to metal exposure. However, its sensitivity to detect metal effects in field studies may be lower due to a lack of specificity and, for this reason, supplementary chemical analysis are needed. In our experiment, the effects observed after short-term exposure may be indicative of changes that will be detected after longer exposure. The selection towards a metal-adapted community had an energetic cost detected after short-term exposure as a physiological alteration leading to marked growth changes. Based on our results, the reduction of Φ'_{PSII} and the alterations on Φ_{PSII} and NPQ in biofilm communities could be used as biomarkers of short-term metal impact if they are compared to controls and complemented with metal exposure and accumulation data.

CONCLUSIONS

Our results suggest that chronic exposure of biofilms to the Zn concentrations found in several fluvial systems (Morin et al., 2007; Blanck et al., 2003), may have negative effects on biofilms. Our experimental approach demonstrates that in natural streams such as the Riou-Mort river, with a chronic pollution of Zn plus Cd, most of the metal toxicity reported on the autotrophic component of biofilms could be attributed to the metal found at higher concentration (in this case Zn). However, based on the high amounts of Fe detected in Riou-Mort river it is suggested the need of evaluate in controlled conditions the effects of Zn on biofilms when Fe pollution is also co-occurring. The use of chl-a fluorescence parameters seems to be a potential tool to assess metal pollution in natural ecosystems. Metals effects can be assessed *in vivo* at different temporal scales, following a community ecotoxicology approach. Its use can inform about: a) functional and quick alterations on photosynthesis processes (that could occur after a punctual spill during a short period of time) as well as, b) structural changes or functional alterations linked to changes in the community composition (that could occur in situations of chronic metal pollution). However, chemical analysis and comparisons between metal-impacted and non metal-impacted (reference) sites should also be performed because of its lack of specificity.

ANNEX CHAPTER IV

Supplementary table 1. Physical and chemical characteristics of stream water of Riou-Mort river determined at each sampling site. Each value corresponds to the AVG ±SD of n=3. Cond.: conductivity, Temp.: Temperature, Alk.: alkalinity, CCU: cumulative concentration unit. Tt: toxicity threshold according to EPA. n.d.: not detected.

	S1	S2	S3	Tt (µg/L)	ANOVA	
					F	p value
pH	7.84 ± 0.50	8.30 ±0.11	8.28 ± 0.15		2.11	n.s
Cond. (µS/cm)	1535 ± 35^{a}	1037 ± 14^{b}	1041 ± 36^{b}		266.9	<0.05
Temp. (ºC)	21.33 ± 2.57	22.70 ± 2.20	22.10 ± 1.73		0.29	n.s
O_2 (mg/L)	8.30 ± 1.26^{a}	10.08 ± 0.23^{b}	10.36 ± 0.23^{b}		6.64	<0.05
Alk.(meq/L)	4.65 ± 0.07^{a}	2.95 ± 0.07^{b}	2.80 ± 0.04^{b}		894.1	<0.05
Cl^- (mg/L)	17.35 ± 0.87^{a}	14.36 ± 0.65^{b}	17.53 ± 0.15^{b}		23.72	<0.05
NO_2^- (mg/L)	0.85 ± 0.35^{a}	1.92 ± 0.48^{ab}	2.21 ± 0.6^{b}		6.43	<0.05
NO_3^{2-} (mg/L)	0.39 ± 0.07^{a}	27.83 ± 5.56^{b}	29.43 ± 6.72^{b}		31.53	<0.05
SO_4^{2-} (mg/L)	110.9 ± 2.9	70.4 ± 3.7	64.9 ± 1.7		0.122	n.s
PO_4^{3-} (µg/L)	30.40 ± 15.02^{a}	42.97 ± 21.3^{a}	807.5 ± 383.6^{b}		12.06	<0.05
Na^+ (mg/L)	40.27 ± 7.10	29.56 ± 1.45	33.73 ± 1.34		4.83	n.s
NH_4^+ (mg/L)	0.52 ± 0.45	0.44 ± 0.18	1.05 ± 0.48		2.16	n.s
K^+ (mg/L)	18.7 ± 2.67	13.73 ± 0.84	15.04 ± 2.49		4.26	n.s
Ca^{2+} (mg/L)	161.6 ± 10.8	136.1 ± 16.4	142.7 ± 3.4		4.01	n.s
Mg^+ (mg/L)	175.1 ± 35.3^{a}	117.7 ± 9.8^{b}	113.5 ± 1.9^{b}		7.91	<0.05
DOC (mg/L)	6.96 ± 0.88^{a}	5.34 ± 0.34^{b}	5.88 ± 0.21^{b}		6.61	<0.05
Al (µg/L)	102.9 ± 30.1	75.55 ± 29.6	46.32 ± 11.30	150	3.77	n.s
Fe (µg/L)	4364 ± 276^{a}	3222 ± 117^{b}	2927 ± 93^{b}	1000	52.45	<0.05
Ni (µg/L)	3.90 ± 1.26	86.49 ± 110.8	60.02 ± 21.77	96	0.840	n.s
Zn (µg/L)	8.01 ± 4.16^{a}	753.1 ± 175.1^{b}	619.7 ± 191.6^{b}	106	21.08	<0.05
Cd (µg/L)	n.d. ^{a}	14.09 ± 0.92^{b}	12.84 ± 0.76^{b}	1.1	380.5	<0.05
CCU	5.17 ± 0.09^{a}	24.54 ± 1.74^{b}	21.38 ± 3.25^{b}	2	71.67	<0.05

Supplementary table 2. Metal accumulation in biofilm from each site (S1, S2 and S3). Each value correspond to AVG (±SD); n=3.

	S1	S2	S3	ANOVA	
				F	p value
Zn.B (µgZn/gDW)	724.2 ± 173.5[a]	5350 ± 1139[b]	4734 ± 235.3[b]	41.03	<0.05
Cd.B (µgCd/gDW)	1.17 ± 0.37[a]	76.94 ± 22.23[b]	67 ± 3.24[b]	30.25	<0.05
Fe.B (µgFe/gDW)	41484 ± 1573	41625 ± 9809	37304 ± 2508	0.52	n.s.
Al.B (µgAl/gDW)	26920 ± 6967	28802 ± 5308	26718 ± 1389	0.15	n.s.
Ni.B (µgAl/gDW)	31.89 ± 5.87[a]	84.34 ± 20.64[b]	78.7 ± 22.09[b]	7.87	<0.05
Cu.B (µgCu/gDW)	77.84 ± 49.77	124.58 ± 27.99	82.54 ± 3.44	1.82	n.s.
Pb.B (µgPb/gDW)	66.34 ± 11.56[a]	288.07 ± 40.97[b]	218.3 ± 29.69[b]	42.94	<0.05

Note: Zn.B; Zn bioaccumulation, Cd.B; Cd bioaccumulation, Fe.B; Fe bioaccumulation, Al.B; Al bioaccumulation, Ni.B; Ni bioaccumulation, Cu.B; Cu bioaccumulation, Pb.B; Pb bioaccumulation.

Supplementary table 3. Biofilm metrics of each site (S1, S2 and S3). Each value correspond to AVG (±SD); n=3.

	S1	S2	S3	ANOVA	
				F	p value
chl-a (µg/cm^2)	191.2 ± 11.13[a]	36.74 ± 9.13[b]	72.28 ± 9.54[c]	197.40	<0.05
430:665	1.49 ± 0.35	1.75 ± 0.08	1.91 ± 0.09	2.99	n.s.
NPQ	0.63 ± 0.07	0.64 ± 0.12	0.7 ± 0.1	0.41	n.s.
Φ_{PSII}	0.59 ± 0.05	0.57 ± 0.03	0.57 ± 0.05	0.11	n.s.
Φ'_{PSII}	0.30 ± 0.04	0.29 ± 0.02	0.32 ± 0.05	0.51	n.s.

Note: Chl-a; chlorophyll-a concentration, 430:665; carotenoid ratio, NPQ; non photochemical quenching, Φ_{PSII}; maximal quantum yield, Φ'_{PSII}; effective quantum yield.

Riera d'Osor, May 2009

CHAPTER V

METAL EFFECTS ON PHOTOSYNTHESIS AND DIATOM METRICS OF METAL CONTAMINATED FLUVIAL BIOFILMS. A TRANSLOCATION EXPERIMENT

INTRODUCTION

The occurrence of metal contamination in fluvial ecosystems is commonly due to urban and mining activities occurring in its watershed (Guasch et al., 2009; Ferreira da Silva et al., 2009; Sierra and Gómez, 2010). This is the case of the Osor stream (NE Spain), our case of study, which presents Zn pollution due to old mining activities in its watershed (Bruguera, 2004). Trace metals may produce toxic effects on aquatic organisms depending on metal speciation, which, in turn, determines bioavailability, toxicity (Tessier et al., 1995) and metal accumulation (Meylan et al., 2004). In accordance with this environmental problem, regulatory documents have been developed by management entities. In Europe, the Water Framework Directive (Directive 2000/60/EC) aimed at reducing chemical pollution in water bodies to reach a "good ecological status" (i.e. close to reference conditions) by 2015, highlighting the importance of evaluating the effects of chemical substances on the biota. To date, no water quality criterion has been recommended for Zn according to the WFD. However, its occurrence is still a matter of concern in Europe. For instance, in the Furtbach river, Switzerland, Zn concentrations ranged from 150 to 400 µgZn/L (Le Faucheur et al., 2005), in the Dommel river, Belgium, ranged from 76 to 2216 µgZn/L (Ivorra et al., 2002), in the Riou-Mort river, France, reached up to 1000µgZn/L and 3000µgZn/L (Bonnineau et al. 2011 and Morin et al. 2008, respectively) and/or in Osor stream, Spain, our case of study, ranged from 25 to 450 µgZn/L (ACA, Catalan Water Agency). According to the criteria proposed by the US Environmental Protection Agency (US EPA, 2006), Zn concentrations reported in these polluted cases are above the toxicity threshold (120 µgZn/L). In Osor stream, significant concentrations of Fe are also reported (60 - 500 µg/L) (ACA, 2009) but no toxicity on biota is expected according to the EPA's criteria, which sets the Fe toxicity threshold at 1000 µgFe/L.

Fluvial biofilms are microbenthic communities mainly composed by algae and cyanobacteria, as well as bacteria, protozoa and fungi, which live attached to submerged substrata (Boeije et al., 2000). They have been widely used for bio-monitoring in rivers due to their rapid response to environmental changes which makes them suitable as early warning systems for toxicity evaluation (Sabater et al., 2007). Phytobenthic community of biofilms (named also phytobenthos) are recognized for the WFD as a "biological quality element" (BQE) for the assessment of the ecological status of water systems. Biofilms constitute an integrative indicator of metal exposure occurring over a period of days or weeks (Ancion et al., 2010), supporting its use as metal pollution indicators (Mages et al., 2004; Ancion et al., 2010). However, the relationships between metal toxicity and biofilm responses may differ depending on the metal dose and exposure time. It is expected that metal exposure will produce firstly metabolic and functional alterations and secondly, after long-term exposures when much higher metals will be accumulated, it will cause structural changes (Serra et al., 2009; Guasch et al., 2010). Hence, the relevance of using a multi-biomarkers approach including functional and structural indicators (Bonnineau et al., 2010). Focusing on functional biofilm community endpoints, the use of chl-a fluorescence parameters will reflect direct or indirect damage caused by a toxicant (e.g. a metal) (Barranguet et al., 2000; Guasch et al., 2002; Serra et al., 2009). Changes in the composition of photosynthetic pigments have also been used as indicators of changes in the physiological processes in the photosynthetic apparatus of plants, aglae or phytobenthic communities as response mechanism to avoid environmental stress (Demming et al. 1998; Kana et al. 1997; Chevalier et al. 2010; Laviale et al., 2010). Accordingly, several accessory photosynthetic pigments of chl-a (diadinoxanthin, diatoxanthin, β-carotene, antheroxanthi or zeaxanthin, among others) were chosen in this study as functional markers of physiological processes of photosynthetic apparatus.

Focusing on structural biofilm endpoints, diatom metrics have been widely reported to respond to chemical stress not only at the community level through shifts in dominant taxa and diversity patterns but also at the individual level with increasing occurrence of frustules deformities or decrease in cell size (Morin et al., 2007; Falasco et al., 2009). Diatom indices have been considered in the WFD for water quality assessment. Although the WFD mentions the relevance of preserving the function and structure of aquatic ecosystems, no functional biofilm endpoint has to date been included. Hence, the interest in developing new structural and functional parameters as BQEs (Johnson et al., 2006) for the assessment of water quality

In field studies, finding causality between chemical toxicity and biofilm responses is not always an easy task because of the common occurrence of confounding factors (Guasch et al., 2010). Moreover, any disturbance in the river ecosystem might be buffered or enhanced by complex biological interactions (Geiszinger et al., 2009). To address this issue, in this study an experimental design based on biofilms translocation (active bio-monitoring; De Kock and Kramer, 1994) was applied and multivariate statistical techniques were used for data analysis. Active bio-monitoring is based on the translocation of organisms from one place to another and quantifying their biochemical, physiological and/or organismal responses for the purpose of water quality monitoring (De Kock and Kramer, 1994). Few hours and several weeks after starting translocations, biofilm metrics were measured to assess acute and chronic effects of metal exposure. This approach has been successfully applied for the assessment of stress responses (Ivorra et al., 1999; Sierra and Gómez; 2010) or recovery (Morin et al., 2010; Rotter et al., 2011) in polluted rivers. Multivariate techniques have been used to evaluate the combined effects of environmental factors and toxicants substances on biofilms (Guasch et al., 2009; Ricart et al., 2010a). In particular, variance partitioning (Borcard et al., 1992) was used in this study to

distinguish the effects caused by metal pollution from those caused by other chemical and physical factors.

The main aim of this study was to evaluate the convenience of using a set of biofilm endpoints, including chl-a fluorescence parameters, pigments composition and diatoms metrics as indicators of Zn pollution in a fluvial ecosystem.

STUDY SITE

The translocation experiment was conducted in the Osor stream “riera d’Osor”, a second order stream situated in Girona (NE, Spain) tributary of the River Ter (Fig.1). The climate of this region is Mediterranean. Annual average precipitation is 950 mm and mean temperature is 12ºC. The Osor stream is 23.5 km long and drains a catchment area of 8890 ha (mainly from Guilleries Mountains). Stream type is a siliceous Mediterranean mountain stream (Munné et al., 2004). It is relatively well preserved with low human pressure. Urban activities are limited, discharging low amounts of waste waters from the Osor village (354 inhabitants) and from a wastewater treatment plant located upstream (St. Hilari Sacalm with 5064 inhabitants).

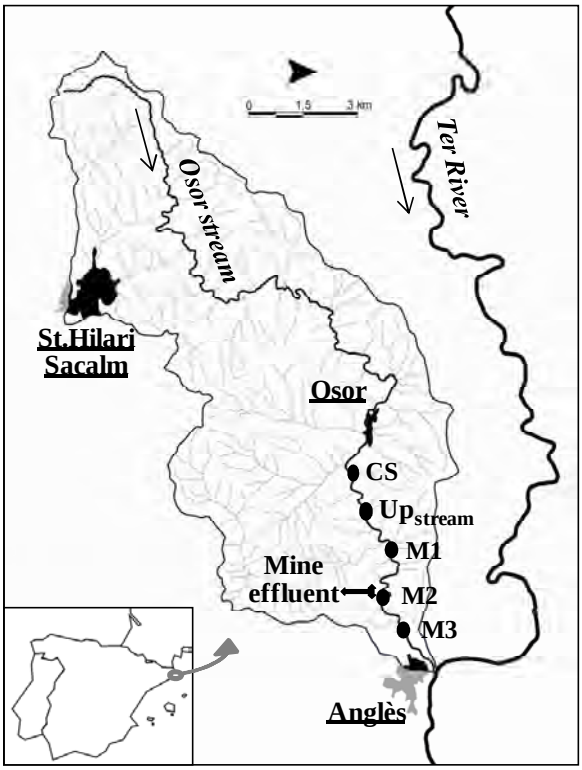


Figure 1. The watershed of Osor stream "la riera d'Osor" (NE Spain, Ter river Basin), showing the location of the sites involved in the biofilm translocation experiment (CS, Up_{stream}, M1, M2 and M3).

The stream is mainly affected by continuous (mine effluent) and diffuse metal inputs (mine run-off) remaining from former mining activities (finished in 1980). The mine effluent is a canal draining old underground galleries of the mine. The mine ore exploited were fluorite (CaF_2), sphalerite (ZnS) and galena (PbS) (Bruguera, 2004).

EXPERIMENTAL DESIGN

For the translocation study, 5 sites located on the main course of the Osor stream were chosen (Fig.1). The experiment was conducted from May to June 2009. Biofilms were grown on submerged artificial substrata. Each artificial substratum was made up of heavy stoneware (40x40cm) with attached sand-blasted glass substrata (70 units of 8.5x2cm and 80 units of 1.2x1.2cm) in horizontal position. All substrata were first placed in the same site, called colonization site (CS) (Fig.1), and left for 5

weeks to obtain mature biofilms. The CS, is located upstream of the mining area. Metal inputs are expected to be low, mainly coming from urban and agricultural activities.

Mature biofilms from CS were translocated to four sites (Fig.1): the upstream site (Up_{stream}), and three sites located within the mining area: mining 1 (M1), mining 2 (M2) and mining 3 (M3). In this way, all translocated biofilms had the same origin and maturity and suffered comparable stress during their transport. This moment was considered as T0 of the translocation. The Up_{stream} was located after the CS and before the mine effluent (Fig.1). It is considered a reference site, similar to the CS, with good ecological status and background metal pollution. The M1 is situated immediately before the main mine effluent and expected to receive diffuse metal pollution (Fig.1). The M2, is located after the mine effluent (Fig.1), receiving continuously metals. Further downstream, the M3 is expected to have lower metal concentrations than M2 due to metal retention in zones of sediment deposition and/or dilution (Fig.1).

SAMPLING

During the experiment water was sampled 9 times and biofilms 4 times. At the colonization site, water samples were taken at days 29 and 31 (6 and 4 days before T0, respectively) and at T0. In the translocation sites (Up_{stream}, M1, M2 and M3), water and biofilm samples were taken 6 and 24 hours (acute exposure) and 3 and 5 weeks (chronic exposure) after the translocation. During the course of the experiment, high precipitations were recorded between weeks 3 and 5, which potentially influenced the metal dilution factor of the continuous source as well as the diffuse input via run-off.

At each sampling time, temperature, conductivity, pH and dissolved oxygen were measured *in situ* with a multi-parametric probe (WTW Meters, Weilheim, Germany). Light was also measured *in situ* at each sampling site (LI-COR Inc., Lincoln,

Nebraska, USA). Light measurements were performed in the middle of the stream and at both banks in order to calculate the percentage cover (Cover %). Water samples were taken for the analysis of phosphate, nitrate, dissolved organic carbon (DOC) and dissolved metals concentrations. All water samples were taken in triplicate and immediately filtered through 0.2 µM nylon membrane filters (Whatman), while samples for metal analysis were also acidified with 1% HNO_3 (65 % suprapure, Merck). All samples were transported to the laboratory and stored until analyses. All biofilm samples were also taken in triplicate. Two big colonized substrata (8.5 x 2 cm) were scrapped for metal bioaccumulation, and used for each replicate. For chl-a fluorescence analysis a small colonized substrata (1.5 x 1.5 cm) was used for each replicate. Each colonized substrata was individually placed on the bottom of a transparent glass vial filled with 10ml of water from the corresponding sampling site and used for chl-a fluorescence measurements. As this measurement is non destructive; the same samples were fixed after measurements with formaldehyde 4% for further diatoms analysis. Several metrics from the diatom community were obtained: species richness (S), cell biovolume, % teratological forms and the Index of Pollution Sensitivity-IPS (Indice de Polluosensibilité Spécifique, Coste in Cemagref, 1982). S and IPS were calculated using Omnidia 4.1 software (Lecointe et al., 1993). IPS values were used to characterize water quality (Coste in Cemagref, 1982). The WFD recognize five categories of IPS to determine water river quality. Values of IPS higher than 16 correspond to “very good” quality, between 13 and 16 correspond to “good” quality, between 9 and 12 correspond to “moderate” quality, between 5 and 8 correspond to “poor” quality and below 5 correspond to “bad” quality. For pigment analysis, a small glass was used for each replicate. Samples were placed in individual centrifuge tubes (Corning) and kept at -25ºC until analysis. All biofilm samples were transported to the laboratory in a dark cool box. Samples for pigments and bioaccumulation analysis were kept frozen until analysis. Details about water and

biofilm analysis are provided in Chapter II focused on material and methods for water and biofilm analysis.

DATA ANALYSIS

In order to use an estimate of metal concentration and potential toxicity level, metal concentrations were used to calculate CCUs (Cumulative Concentration Units) and used as estimates of potential metal toxicity (Clements et al., 2000). The CCU is a score based on the sum of the ratios between metal concentrations measured in surface waters and the corresponding criterion value (US EPA's National Recommended Water Quality Criteria), http://www.epa.gov/waterscience/criteria/wqctable/) used to investigate the responses of aquatic organisms to metals (Clements et al., 2000). Three categories of CCU were used; CCU below 1 corresponded to "background" category, CCU between 1 and 2 corresponded to "low" category indicating low potential toxicity and CCU between 2 and 10 corresponded to "moderate" category indicating moderate metal potential toxicity.

One way ANOVA was performed to detect significant differences in the physical and chemical water characteristics among sites and between the biofilm metrics (chl-a fluorescence parameters, metal accumulation, photosynthetic pigments and diatoms metrics) in the translocated biofilms during acute and chronic exposures. Data were transformed to achieve normality. If metal concentrations were below detection limits, a value equal to half of the method detection limit was assigned to these data in the statistical analyses (Helsel, 1990). Tukey's HSD test was applied to identify significant differences between sites (Up_{stream}, M1, M2 and M3). ANOVA analyses were realized by SPSS software. A 5% significance level ($p<0.05$) was used for rejection of null hypothesis in all cases.

Multivariate analyses were performed using the CANOCO software version 4.5 (Ter Braak and Smilauer, 1998). Except pH, all variables were previously log-

transformed to reduce skewed distributions. Principal component analysis (PCA) was used to analyse the variability of water physical and chemical environment. Detrended correspondence analysis (DCA) was used to determine the maximum gradient length of biofilm metrics in order to decide between linear or unimodal methods. Relationships between biofilm metrics and metal concentrations, and water physical and chemical variables during acute and chronic exposures were assessed with redundancy detrended analysis (RDA). Two independent RDA analysis were performed; one using data from acute exposure (RDA acute exposure) and another including data from chronic exposure (RDA chronic exposure). Biofilm metrics included in the first RDA (acute exposure) were chl-a fluorescence parameters and photosynthetic pigments, while in the second RDA (chronic exposure) diatom metrics were also included. To avoid co-linearity, variables were selected based on inspection of variance inflation factors (VIF<20) (Ter Braak and Smilauer, 1998). Forward selection was used to select the environmental variables that significantly explained the distribution pattern of the biofilm metrics with a cut-off point of p=0.1. Significance of the RDA axes was assessed using the Monte Carlo permutation test (999 unrestricted permutations). Probabilities for multiple comparisons were corrected using the Bonferroni correction. The variance partitioning technique was applied to separate the effects of metal concentrations on biofilm metrics distribution from other chemical and physical variables, when pertinent (i.e. acute exposure) (Borcard et al., 1992). Accordingly, the explanatory variables were grouped into two subsets: (a) physical and chemical variables and (b) metals.

RESULTS

- **Physical and chemical conditions**

Physical and chemical conditions during the experiment are summarized in Table 1 covering the colonization period at the CS (May 2009) and the translocation

period at Up_{stream}, M1, M2 and M3 (June 2009). Percentage of cover (% cover) was similar between CS and all mining sites (M1, M2 and M3) but it was lower in the Up_{stream} (Table 1). Lower temperature was observed at CS in comparison to the other sites due to seasonality. Cu, Pb and Cd concentrations were in most cases (94%) below detection limit. Accordingly, these metals were considered of low significance and were not included in the multivariate analysis. Detection limit for Cu was 0.12 µg/L, for Zn was 2.82 µg/L, for Al was 0.72 µg/L, for Pb was 0.40 µg/L, for Ni was 2.44 µg/L, for Fe was 17.18 µg/L and for Cd was 0.32 µg/L. Based on the calculated Concentration Cumulative Units (CCU) the highest value was obtained in M2 with a "moderate" potential toxicity level. Up_{stream} and M1 sites had a "low" potential toxicity while M3 had a "background" level (Table 1).

Table 1. Average and standard errors (in parenthesis) of physical and chemical parameters: in the colonization site (CS) during the colonization period (n=9) and, in the translocation sites: upstream (Up_{stream}), mining 1 (M1), mining 2 (M2) and mining 3 (M3) during the translocation period (n= 12). For each parameter, different letters indicate significant differences ($p<0.05$) between sites after ANOVA and Tukey's HSD test. n.s.: non significant differences between sites. Tt: Toxicity threshold (µg/L) according to EPA.

Parameter	Colonization Site	up_{stream}	M1	M2	M3		ANOVA results	
	AVG (± SE)	AVG (± SE)	AVG (± SE)	AVG (± SE)	AVG (± SE)	Tt	$F_{(4,52)}$	p value
Cover (%)	37.53 (3.46)^{a}	4.48 (1.93)^{b}	34.28 (12.60)^{a}	59.81 (11.23)^{a}	38.00 (12.7)^{a}		12.37	< 0.001
pH	8.14 (0.02)^{a}	8.14 (0.04)^{a}	8.13 (0.07)^{a}	8.15 (0.04)^{a}	7.68 (0.07)^{b}		7.25	< 0.001
Cond.(µS/cm)	183.3 (6.60)^{a}	226.7 (5.17)^{abc}	370.1 (77.84)^{bc}	356.8 (29.80)^{c}	222.9 (4.03)^{ab}		5.64	< 0.001
Oxy. (mg/L)	9.40 (0.06)^{a}	9.12 (0.09)^{ab}	8.84 (0.18)^{b}	9.11 (0.07)^{ab}	9.23 (0.09)^{ab}		3.25	< 0.05
TºC	14.27 (0.43)^{a}	18.02 (0.27)^{b}	18.22 (0.23)^{b}	18.6 (0.2)^{b}	17.63 (0.19)^{b}		25.77	< 0.001
DOC (mg/L)	4.69 (0.92)^{a}	3.45 (0.23)^{ab}	3.59 (0.25)^{ab}	3.29 (0.28)^{b}	4.21 (0.39)^{ab}		3.06	n.s.
PO_4^{3-} (µg/L)	220.8(18.89)^{ab}	380.2 (33.60)^{a}	316.5 (20.75)^{ab}	218.4 (10.02)^{b}	298.6 (29.44)^{ab}		2.8	< 0.05
NO_3^{-} (mg/L)	3.04 (0.12)^{ab}	3.05 (0.21)^{a}	2.24 (0.14)^{bc}	1.87 (0.18)^{c}	2.89 (0.16)^{ab}		9.06	< 0.001
Cu (µg/L)	bdl	bdl	bdl	bdl	bdl	9		
Zn (µg/L)	bdl^{a}	19.19 (9.65)^{a}	31.11 (11.24)^{a}	289.9 (44.19)^{b}	21.04 (10.71)^{a}	120	16.14	< 0.001
Al (µg/L)	11.89 (3.32)^{a}	5.52 (1.43)^{b}	9.74 (1.95)^{a}	0.88 (0.29)^{b}	bdl^{b}	nd	16.1	< 0.001
Pb (µg/L)	bdl	bdl	bdl	bdl	bdl	2.5		
Ni (µg/L)	3.65 (0.42)^{ab}	3.26 (0.27)^{a}	9.53 (3.73)^{b}	bdl^{a}	bdl^{a}	52	6.29	< 0.001
Fe (µg/L)	280.4(13.45)^{a}	295.8 (4.8)^{a}	303.9 (8.74)^{a}	455.9 (40.80)^{a}	88.17 (27.42)^{b}	1000	13.32	< 0.001
Cd (µg/L)	bdl	bdl	bdl	bdl	bdl	0.25		
CCU	1.74 (0.17)^{a}	1.17 (0.06)^{ac}	1.36 (0.09)^{ac}	3.04 (0.33)^{b}	0.93 (0.09)^{c}	2	14.76	< 0.001

bdl: below detection limit

PCA analysis of water chemistry shows the ordination of sampling points within the first two axes (Fig. 2). The first axis explained the 58.8% of variance and separates the most polluted site (M2) with highest Zn concentration in water, highest conductivity and lowest nutrients (nitrate and phosphate concentrations) from Up_{stream}, M1 and M3 sites (Table 1). Nutrient (nitrate and phosphate) concentrations were lower and Zn concentrations higher at M2 site, due to the dilution (in the first case) or input (in the second case) coming from the groundwater mine effluent with high metal concentration but low nutrient levels (data not shown). The second axis of the PCA explained a 25.2 % of variance and separates CS, $Up_{stream,}$ and M1 from M3 (Fig.2). In fact Al, Ni and Fe water concentrations and pH were lower at M3 than at the other sites (Table 1). CS and Up_{stream} were plotted close together in the PCA, indicating that their physical and chemical characteristics were very similar.

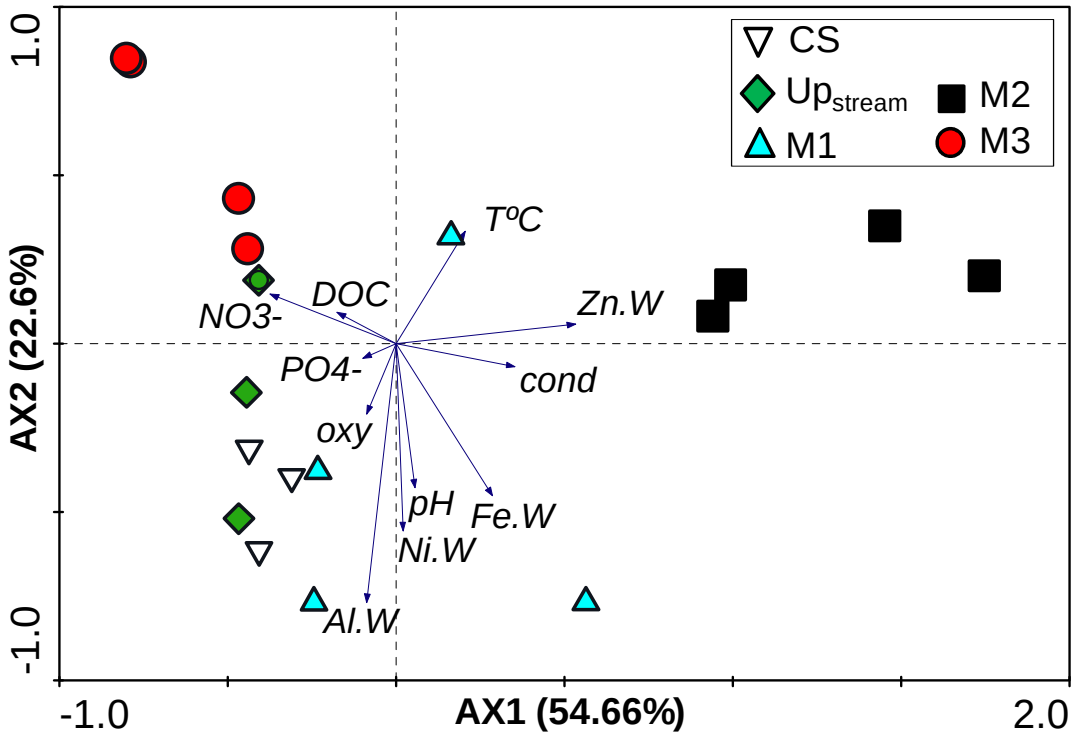


Figure 2. Principal Components Analysis (PCA) based on the physical and chemical parameters of stream water measured at each site during the experiment summarized in Table 1.

- **Metal bioaccumulation**

Translocated biofilms accumulated different amount of metals depending on the translocation site (Up_{stream}, M1, M2 or M3) and the time of exposure (acute vs. chronic) (Fig.3). Cd bioaccumulation was always below detection limit. Few hours after translocation (after 6 and 24h), Al and Fe bioaccumulation were similar in most sites (Table 1), except at M3, where Fe bioaccumulation was slightly lower (Fig. 3). Ni, Cu and Pb bioaccumulation were two orders of magnitude lower than Al and Fe and were comparable between sites (Fig.3). Zn bioaccumulation was 2.5 times higher at M1, M2 and M3 than at Up_{stream} (Fig.3).

After several weeks of exposure (after 3 and 5 weeks), Al and Fe remained lower or similar than during the first hours of exposure, whereas Pb and Zn showed a clear increase in the most metal polluted sites (M1, M2 and M3). Al and Cu were 1.5 and 2 times higher at M1 and M3 than Up_{stream} and M2, respectively. Ni bioaccumulation during chronic exposure was comparable to that one observed during acute exposure. Differences in Pb bioaccumulation between sites were similar to those observed for Zn, but with lower concentrations. Pb bioaccumulation was 9 times higher at M1, M2 and M3 than at Up_{stream}. Zn bioaccumulation was maximum at M2; 6 and 7 times higher than at M1 and M3, respectively, and 43 times higher than at Up_{stream} (Fig. 3).

- **Acute exposure**

Six hours after translocation, a decrease of the Φ'_{PSII} was observed in biofilms from mining sites (M1,M2 and M3) in comparison to biofilms from Up_{stream} (Fig. 4) ($p<0.05$; $F=43.29$). Later on, 24h after translocation, a reduction of the Φ_{PSII} was also observed in biofilms from M1 and M2 in comparison to Up_{stream} and M3 (Fig. 4) ($p<0.05$; $F=8.53$). A marked change in NPQ was observed between 6h ($p<0.05$; $F=78.26$) and

24h ($p<0.05$; $F=40.59$) after translocation (Fig.4), and this change was mainly observed at Up_{stream} and M1. After acute exposure, the relative abundance of dinoxanthin pigment was higher in mining sites (M1, M2 and M3) than in Up_{stream} (Table 2). Otherwise, violaxanthin decreased in mining sites (M1, M2 and M3) in comparison to Up_{stream} (Table 2).

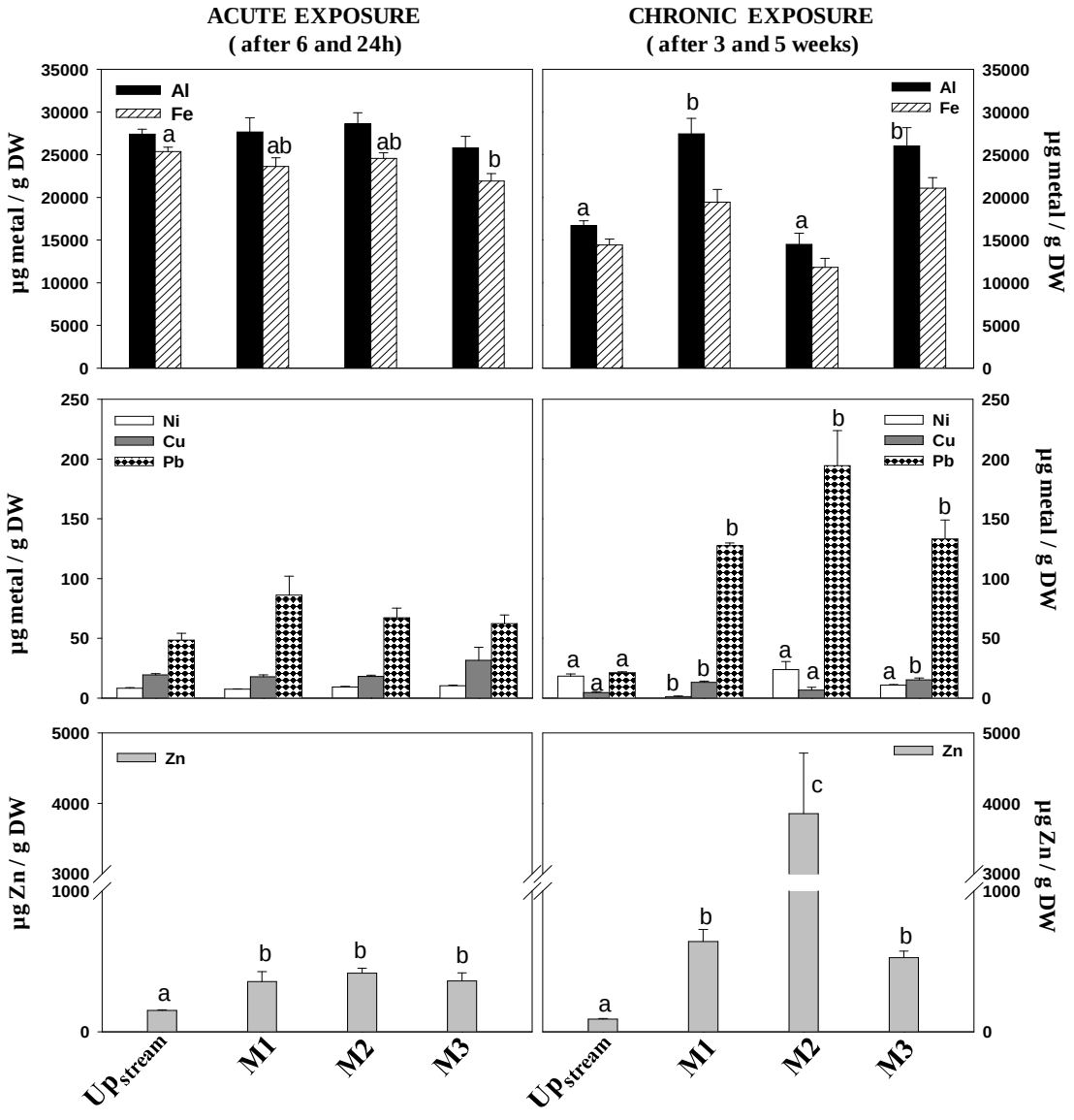


Figure 3. Metal bioaccumulation in biofilms translocated to Up_{stream}, M1, M2 and M3, after acute (6 and 24h) and chronic (3 and 5 weeks) exposures. Each column corresponds to the AVG±S.E; n=6. Different letters indicate significant differences ($p<0.05$) between sites after ANOVA and Tukey's HSD test.

Table 2. Relative abundance of pigments (%) from biofilm translocated after acute exposure (6 and 24h) and chronic exposure (3 and 5 weeks). Values are in average (± standard error); n=6. For each pigment, different letters indicate significant differences ($p<0.05$) between sites after ANOVA and Tukey's HSD test. n.s.: non significant differences between sites.

Acute exposure	Up_{stream}	M1	M2	M3	ANOVA	
					$F_{(3.20)}$	p value
Ch-la	48.90 (1.45)	43.40 (2.35)	41.88(1.32)	49.85 (3.42)	2.91	n.s.
Lutein	2.32 (0.14)	1.92 (0.28)	1.55 (0.18)	1.68 (0.36)	1.93	n.s.
Diatoxanthin	0.44 (0.2)	0.12 (0.12)	nd	nd	3.04	n.s.
Violaxanthin	2.81 (0.47)^{a}	0.59 (0.12)^{b}	0.29 (0.11)^{b}	0.25 (0.11)^{b}	27.94	<0.05
Diadinoxanthin	3.22 (1.0)	4.05 (0.72)	4.61 (0.19)	3.81 (0.29)	1.36	n.s.
Dinoxanthin	0.32 (0.16)^{a}	1.91 (0.46)^{b}	2.47 (0.14)^{b}	1.99 (0.43)^{b}	7.31	<0.05
Pheophytin-a	0.33 (0.16)	0.58 (0.25)	0.34 (0.13)	0.41 (0.02)	0.40	n.s.
β-Carotene	0.37 (0.24)	18.0 (0.18)	0.03 (0.01)	1.60 (0.89)	2.48	<0.05
Antheroxanthin	nd	nd	0.08 (0.04)	0.04 (0.02)	3.52	n.s.
Zeaxanthin	nd	nd	nd	nd		

Chronic exposure	Up_{stream}	M1	M2	M3	ANOVA	
					$F_{(3.20)}$	p value
Chl-a	68.26 (0.71)^{a}	72.1 (0.81)^{ab}	72.79 (1.76)^{ac}	74.17 (1.76)^{bc}	3.58	<0.05
Lutein	0.80 (0.45)^{ac}	0.26 (0.12)^{a}	3.07 (0.35)^{bd}	1.62 (0.15)^{cd}	15.33	<0.05
Diatoxanthin	0.05 (0.03)^{a}	0.42 (0.15)^{b}	0.13 (0.09)^{ab}	0.26 (0.04)^{ab}	3.66	<0.05
Violaxanthin	0.19 (0.03)	0.2 (0.03)	0.17 (0.04)	0.13 (0.01)	1.08	n.s.
Diadinoxanthin	0.91 (0.08)	1.02 (0.04)	1.21 (0.37)	0.83 (0.07)	0.52	n.s.
Dinoxanthin	1.10 (0.12)	1.05 (0.15)	0.82 (0.18)	0.67 (0.02)	2.48	n.s.
Pheophytin-a	0.59 (0.13)	0.47 (0.03)	0.35 (0.07)	0.44 (0.06)	1.54	n.s.
β-Carotene	0.27 (0.11)	0.11 (0.02)	0.14 (0.03)	0.34 (0.08)	2.45	n.s.
Antheroxanthin	nd^{a}	0.19 (0.09)^{b}	nd^{a}	0.01 (0.01)^{a}	4.46	<0.05
Zeaxanthin	nd^{a}	0.72 (0.05)^{b}	1.45 (0.26)^{c}	0.27 (0.13)^{a}	24.71	<0.05

nd: not detected

Since the maximum length of the gradient was 0.553 for chl-a fluorescence parameters and 1.212 for pigments data, DCA results indicated that linear methods would be appropriate (Ter Braak and Smilauer, 2002). RDA run on the acute exposure data arranged the cases maximizing the correlation with Zn bioaccumulation and phosphate concentration (Fig. 5). This analysis gathered a 53.7 % of the total variance. First axis (RDA1) explained 35.8% of variance and arranged biofilm cases following a gradient of Zn bioaccumulation. Second axis (RDA2) explained a 17.9 % of variance

and arranged biofilm communities maximizing the correlation with phosphate concentration. Biofilms sampled after 6h were separated to those sampled at 24h (Fig. 5) that accumulated higher amounts of Zn, had lower Φ'_{PSII}, lower relative abundance of violaxanthin and a higher relative abundance of dinoxanthin than biofilms from Up_{stream} (Fig.5 and Table 3). The second RDA axis was related to a gradient of phosphate (Fig.5) and focussing on biofilm metrics related to this axis it was observed, on the one hand, that biofilms had a higher NPQ activity after 24h of exposure than after 6h of exposure. On the other hand, biofilms had a higher Φ_{PSII} and a higher relative abundance of pheophytin-a at 6h than at 24h (Fig.5 and Table 3). Variance partitioning revealed that bioaccumulated Zn was the only metal (31.6%) and that phosphate concentration in water was the only physical or chemical variable presenting significant effects on biofilms (24%). The shared variance between metal and physicochemical variables accounted for a 1.9%. Six pigments and Φ'_{PSII} were the only biofilm metrics exclusively related to Zn bioaccumulation (Table 3).

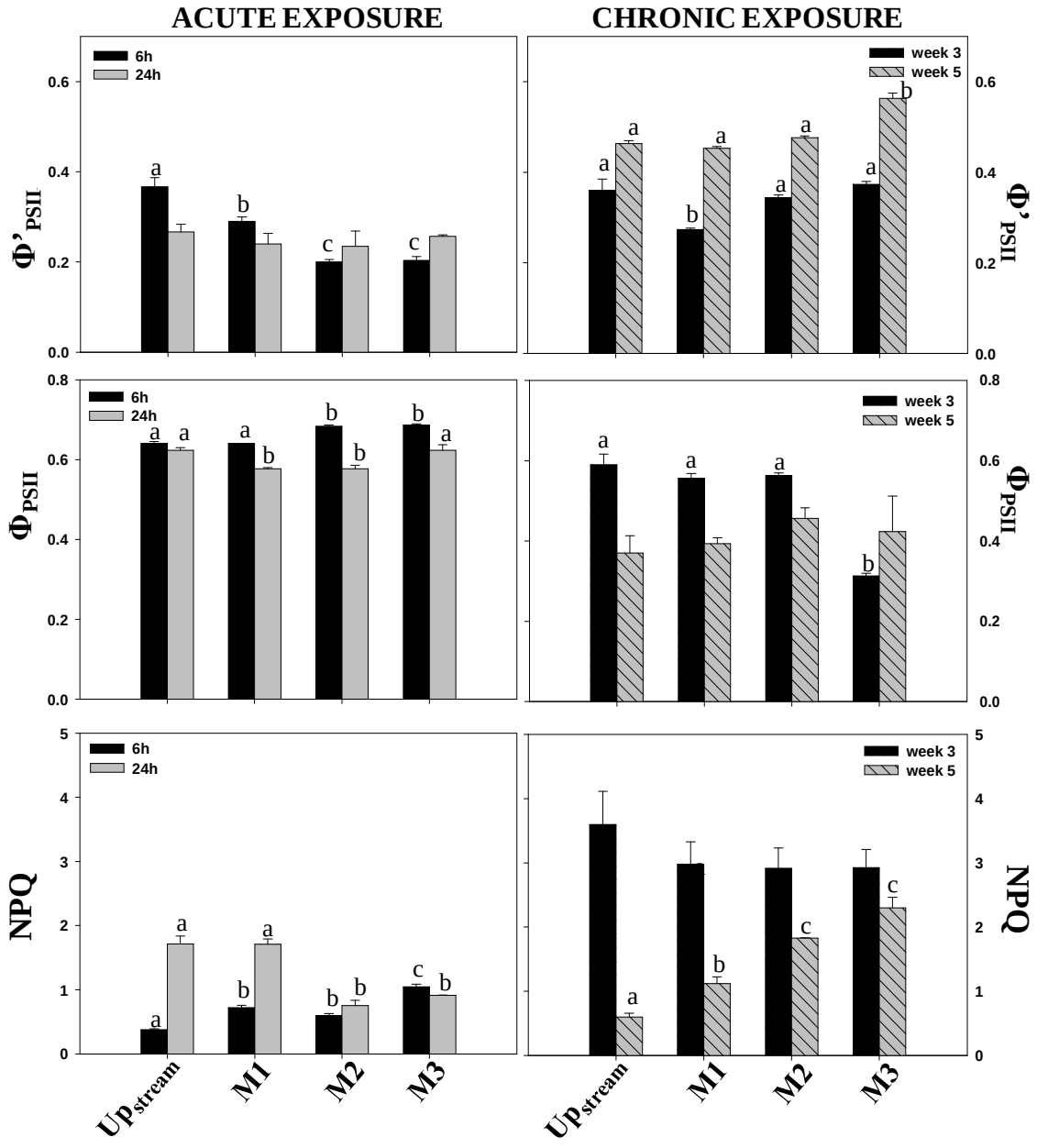


Figure 4. Φ'_{PSII}: effective quantum yield, Φ_{PSII}: maximal photosynthetic capacity and NPQ: non-photochemical quenching of biofilm translocated to Up_{stream}, M1, M2 and M3 after 6 and 24 hours (acute exposure) and after 3 and 5 weeks (chronic exposure) of exposure. Each value corresponds to the AVG±S.E; n=3. For each sampling time, different letters indicate significant differences ($p<0.05$) between sites after ANOVA and Tukey's HSD test.

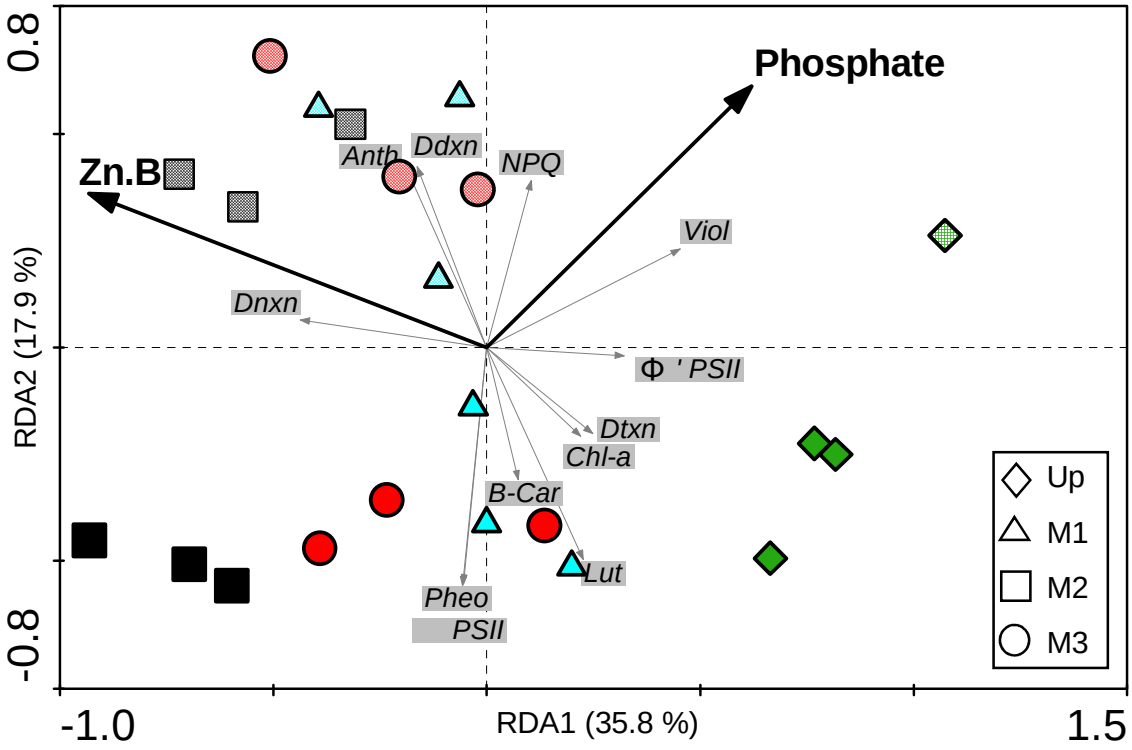


Figure 5. Triplot based on RDA acute exposure. Solid symbols correspond to biofilm sampled after 6h of exposure and dashed symbols correspond to biofilm sampled after 24h of exposure. Zn.B: Zn bioaccumulation. Phosphate: phosphate. The acronyms of pigments are defined in Table 3.

- **<u>Chronic exposure</u>**

Φ'_{PSII} did not show a consistent variation between sites after three and five weeks of translocation (Fig. 4). Similar results were observed based on Φ_{PSII} and NPQ (Fig.4). Four pigments, chl-a, lutein, diatoxanthin and antheroxanthin, showed differences between sites after chronic exposure (Table 2). Also, differences based on diatom metrics (species richness, teratoforms (%), biovolume and IPS) were observed between sites during chronic exposure (Table 4).

Table 3. Results of RDA acute exposure (Fig.5). On the left, the fraction of total variance is shown; bold is used to indicate the total variance higher than 10% for physicochemical variables or metals. On the right, the fraction of explained variance (%) is shown: bold and underlined values indicate biofilm metrics mainly related with physicochemical or metal variables (% of explained variance >60 % for one group of variables and <30% for the other group of variables).

Biofilm metrics	Fraction of total variance		Fraction of explained variance (%)	
	Physical and chemical variables	Metals	Physical and chemical variables	Metals
Φ_{PSII}	**44.83**	7.14	**86.26**	13.74
Φ'_{PSII}	11.31	**30.37**	27.14	**72.86**
NPQ	**29.66**	0.62	**97.95**	2.05
Chl-a	0.03	**25.34**	0.12	**99.88**
Lutein (Lut)	8.64	**49.11**	14.96	**85.04**
Diatoxanthin (Dtxn)	0.30	**29.82**	1.00	**99.00**
Violaxanthin (Viol)	57.39	37.03	60.78	39.22
Diadinoxanthin (Ddxn)	7.95	**29.98**	20.96	**79.04**
Dinoxanthin (Dnxn)	17.31	**59.84**	22.44	**77.56**
Pheophytin-a (Pheo)	**45.79**	7.11	**86.56**	13.44
β-Carotene (B-Car)	6.70	**10.52**	38.91	61.09
Antheroxanthin (Anth)	6.20	**34.04**	15.41	**84.59**

Table 4. Diatom metrics from biofilm translocated to Up_{stream}, M1, M2 and M3 after 3 and 5 weeks of exposure. Each value corresponds to the AVG± SE; n=6. For each parameter, different letters indicate significant differences ($p<0.05$) between sites after ANOVA and Tukey's HSD test.

Chronic exposure	up_{stream}	M1	M2	M3	ANOVA	
					$F_{(3,8)}$	p value
S	37.83 (1.27)^{a}	35.0 (1.93)^{ab}	29.17 (1.30)^{b}	39.17 (2.06)^{a}	7.173	<0.05
teratoforms (%)	0.54 (0.23)^{a}	2.39 (0.56)^{b}	3.35 (0.65)^{b}	2.52 (0.25)^{b}	10.33	<0.001
biovolume	1358 (49.5)^{a}	1830 (118.7)^{a}	225.3 (26.45)^{b}	1573 (83.6)^{a}	178.78	<0.001
IPS	15.22 (0.16)^{a}	14.25 (0.31)^{ac}	8.62 (0.23)^{b}	13.55 (0.21)^{c}	159.4	<0.001

The maximum length of the gradient (DCA) for the chronic exposure diatom metrics data was 0.556 for chl-a fluorescence parameters, 0.846 for pigments and 0.504 for diatoms indicating that again linear methods would be appropriate (Ter Braak

and Smilauer, 2002). RDA gathered a 68.6 % of the total variance and arranged the cases maximizing the correlation with Zn and Fe bioaccumulation and Zn in water (Fig.6). First axis explained 54.2 % of the variance and arranged biofilms along a joint gradient of Zn bioaccumulation and Zn in water and Fe bioaccumulation (Fig.6). Second axis explained 9.2 % of the total variance and showed mainly a gradient of Zn in water (Fig.6). A positive correlation between Zn in water and Zn bioaccumulation in biofilm was observed. Diatoms biovolume, IPS index, and zeaxanthin and β-carotene pigments relative abundance were the biofilm metrics better explained (>60% of the variance) by the model (Table 6). According to the first axis, biofilms from M2 were strongly separated of biofilms from the other sites (Up_{stream}, M1 and M3) because of the higher amounts of bioaccumulated Zn and water Zn, and lower amounts of bioaccumulated Fe than biofilms from Up_{stream}, M1 and M3. Biofilms from M2 were mainly characterized by a lower diatom biovolume, lower IPS value, and higher relative abundance of zeaxanthin and lutein pigments than biofilm communities from Up_{stream}, M1 and M3 (Fig. 6). The percentage of diatom teratological forms was higher in mining sites (M1, M2 and M3) than in Up_{stream} , reaching the maximum values at M2 (Table 4). The IPS values at Up_{stream} , M1 and M3 qualify their biological status as “good quality” whereas at M2 the IPS value corresponded to a “poor” biological status.

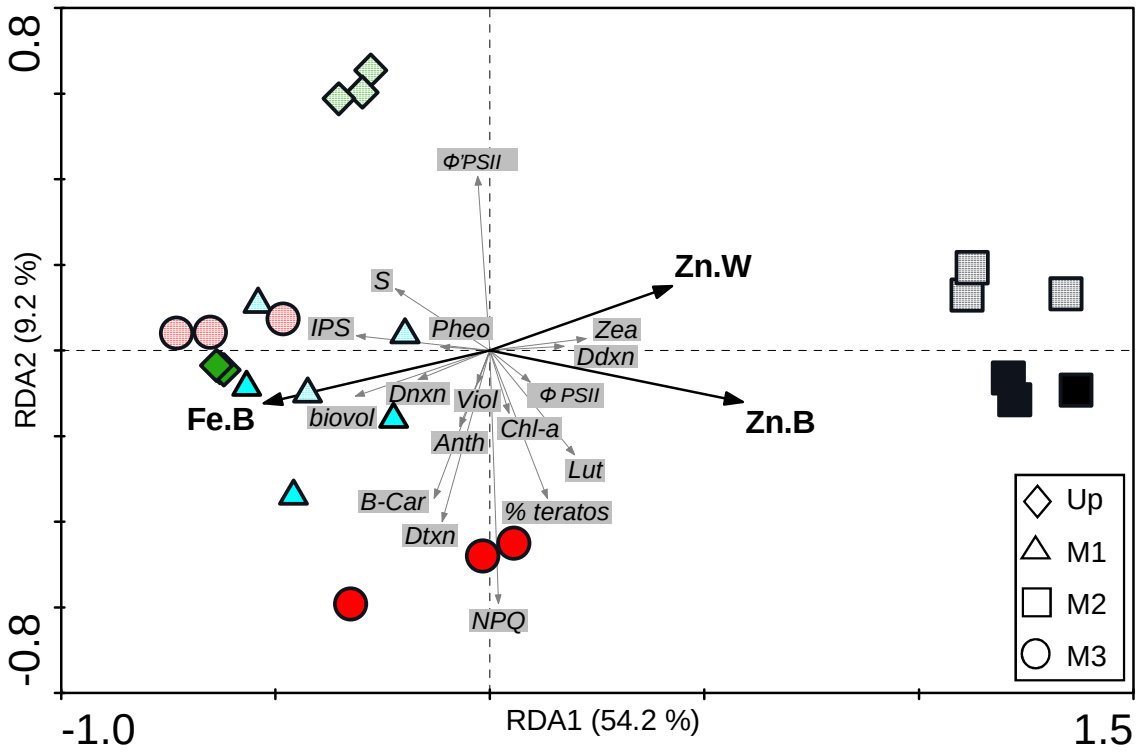


Figure 6. Triplot based on redundancy analysis of RDA chronic exposure. Solid symbols correspond to biofilm sampled after 3 weeks of exposure and dotted symbols correspond to biofilm sampled after 5 weeks of exposure. Zn.B: Zn bioaccumulation, Zn.W: Zn dissolved in water and Fe.B: Fe bioaccumulation. The acronyms of pigments are defined in table 3 and those from diatoms metrics in table 4.

DISCUSSION

The experimental setup allowed us to simulate two different metal pollution scenarios. First a low metal pollution scenario, characterized by elevated metal contents in biofilms but not a detectable metal concentration increase in the water phase (at sites M1 and M3). Secondly, a more clear case of metal pollution, characterized by elevated Zn concentration in the water phase (above toxicity thresholds) in addition to increased metals contents in biofilms that we define as a moderate M-pollution scenario (Table 5). Metal concentration in water is highly variable in time and space depending on the source of pollution (diffuse or point-source), the hydrological regime and the processes affecting their transfer from the water phase to

other compartments. However, metal content in biofilms, may better reflect the real metal exposure of biofilm organisms since metal content in biofilms integrate the different biotic and abiotic processes occurring in biofilms through time (Guasch et al., 2010).

In addition, it was also possible to simulate metal exposure scenarios of different duration: 1) within 24h: simulating a short-term episode of metal pollution and 2) after several weeks: simulating a situation of chronic metal pollution.

Table 5. Summary of significant biofilm metrics showing metal toxicity and their potential goodness associated as biological indicators for fluvial ecotoxicological studies. n.m.: not measured, n.s.: no significant.

		SCENARIOS				METRICS GOODNESS					
		exposure		exposure							
biofilm analysis	biofilm metrics	Low M-pollution	Moderate M-pollution	Low M-pollution	Moderate M-pollution	Sensitivity	Level exposure-dependency	Specificity	Fast effects detection	Chronic effects detection	Easy to measure
M-bioac	Zn-bioac	↑	↑	↑	↑↑	✓	✓	✓	✓	✓	
Chl-a fluor.	Φ'_{PSII}	↓	↓	n.s.	n.s.	✓			✓		✓
HPLC	Xantophylls cycle	↑	↑	n.s.	n.s.	✓			✓		
	Green algae & cyanobacteria	n.s.	n.s.	n.s.	↑		✓			✓	
Diatoms	Richness	n.m.	n.m.	↓	↓↓	✓	✓		n.m.	✓	
	Biovolume	n.m.	n.m.	n.s.	↓		✓		n.m.	✓	
	% teratoforms	n.m.	n.m.	↑	↑	✓		✓	n.m.	✓	
	IPS	n.m.	n.m.	↓	↓↓	✓	✓		n.m.	✓	

- **<u>Bilofilm metrics</u>**

- **Metal bioaccumulation**. The Zn content in biofilms from Up_{stream}, the reference site in Osor stream, ranged between 75 and 150 µgZn/gDW. Comparable values have been found in biofilms at non-contaminated river sites (Ivorra et al., 1999; Behra et al., 2002). Biofilms translocated to M1, M2 and M3 bioaccumulated enough Zn after few

hours of exposure (2.5 times more than biofilm translocated to Up_{stream}) to produce inhibitory effects on Φ'_{PSII} and a change on the photosynthetic pigments composition in spite of the low dissolved metal concentration found at M1 and M3. Our results are in agreement with other studies which also observed that metal toxicity is more related to metal bioaccumulation than to metal concentration in water (Bradac et al., 2009). Furthermore, several weeks after the biofilm translocations, Zn bioaccumulation continued increasing in the biofilms translocated to all metal-impacted sites but differed between them. Zn bioaccumulation was 6 and 7 times higher at M1 and M3, respectively, than in Up_{stream} site, and 43 times higher at M2 site than at Up_{stream} site. It is known that biosorption of trace metals by algal cells generally increase with time of exposure (Collard and Matagne, 1994). On the other hand, Ni, Cu, Al and Fe accumulation didn't increase with time of exposure, suggesting that the amounts observed could be considered the background contents for the biofilm of this stream. During this period, the RDA indicates that biofilm responses were mainly attributed to metals: to Zn bioaccumulation, followed by Fe bioaccumulation and Zn in water. According to the obtained results, metal -bioaccumulation (M-bioac.) is shown to be a biofilm metric sensitive to short and chronic exposure scenarios, level exposure-dependent and specific to metal pollution (Table 5).

- **Chl-a fluorescence parameters**. Φ'_{PSII} was inhibited at M1, M2 and M3 after few hours of exposure following the same pattern than Zn bioaccumulation (RDA analysis). This response could be explained by the mode of action of Zn targeting several photosynthetic processes of algae and cyanobacteria (Admiraal et al., 1999; Prasard et al., 1999). The observed decrease of Φ'_{PSII} shows that Zn exposure caused an inhibition of the electron transport flow during the light -dependent photosynthesis activity (Juneau et al., 2007). Under microcosm's conditions, where direct toxicity is easier to assess, Corcoll et al. (2011) observed similar results at a similar

concentration (400 µgZn/L) after few hours of exposure. Similar effects were also observed by Ivorra et al. (2000), two days after the addition of higher Zn concentrations (2216 µgZn/L) on young biofilms. In contrast to Φ'_{PSII} , Φ_{PSII} and NPQ did not show a response linked to metal exposure, indicating that these parameters are less sensitive to metal exposure. Under microcosm conditions, García-Meza et al. (2005) also observed a higher sensitivity of Φ'_{PSII} than Φ_{PSII} to Zn exposure (24h of exposure biofilms to high Zn concentrations: 6500 and 65000 µgZn/L). After chronic exposure (Table 5) biofilm communities from mining sites (M1, M2 and M3) did not show alterations either in Φ'_{PSII}, Φ_{PSII} or NPQ attributed to metals, suggesting a recovery after chronic exposure. The observed response could be attributed to an adaptation process, where sensitive species to metals were replaced by tolerant ones. Similar results were observed in biofilms exposed chronically to metals (García-Meza et al., 2005; Serra et al., 2009). However, some authors found in microcosms experiments a decrease of NPQ mechanisms after long term exposures of triclosan (Ricart et al. 2010b) or Zn (Corcoll et al. 2011). Temporal variability of Φ_{PSII} and NPQ (during acute and chronic-exposures) was higher at Up_{stream} and M1 than at M2 and M3, and was attributed to physical and chemical variations. Overall, we can conclude that among the chl-a fluorescence metrics investigated, which are relatively easy to measure, Φ'_{PSII} is the most sensitive metric that allow to detect quick effects of low and moderate M-pollution (Table 5).

- **Pigments analysis**. Focusing on photosynthetic pigments, concomitant effects to the inhibition of Φ'_{PSII} were observed during acute exposure. The RDA indicates that Zn toxicity may enhance the synthesis of dinoxanthin as well as the synthesis of antheroxanthin and diadinoxanthin, which could result from an activation of the xanthophylls cycle of brown and green algae as protective mechanism to avoid Zn toxicity (e.g. on Φ'_{PSII}). Xanthophylls play an important role in the use of the excess in

excited energy to avoid photoinhibition due to effects of high light irradiances or chemical substances (e.g. metals) that could potentially block light-dependent photosynthesis reactions (Armstrong and Hearst, 1996; Martínez-Abaigar and Núñez-Olivera, 1998). Specifically, a way to dissipate this excess of excited energy that arrives at the PSII is the xanthophylls cycle. In brown algae (dinoflagellates and diatoms) and in green algae (chlorophytes) it occurs by the de-expoxidation reaction. Diadinoxanthin is transformed into dinoxanthin / diatoxanthin (brown algae) and violaxanthin into antheroxanthin or zeaxanthin (green algae) (Lohr and Wilhelm, 1999; Jin et al., 2003). On the other hand, after chronic exposure biofilms from M2 presented a higher relative abundance of lutein and zeaxanthin in comparision to biofilm translocated to Up_{stream}. The occurrence of lutein and zeaxanthin could be linked either to the presence of green algae and cyanobacteria species, respectively, in biofilms (Wilhelm, 1991) or to the synthesis of accessory pigments composing the light harvesting complex as a mechanism to avoid a blockage of the light-dependent photosynthesis reactions (Armstrong and Hearst, 1996). A negative correlation between β-carotenes and violaxanthin with lutein and zeaxanthin, respectively, due to carotenoids transformation would confirm this last hypothesis, (Armstrong and Hearst, 1996). Since, this situation was not observed in our study we have attributed the increase of the abundance of lutein and zeaxanthin in M2 to a community composition change: an increase in the abundance of green algae and cyanobacteria. Changes in the community composition have been described previously in biofilms as a response to an induction of tolerance to metals. For example, Corcoll et al. (2011) showed that green algae and cyanobacteria were more selected after several weeks of Zn exposure (400 µgZn/L) in microcosm experiments. A metal resistance of green algae and cyanobacteria were also reported by Ivorra et al. (2000) and Genter et al. (1987) to Zn exposures. Thus, based on pigment analysis we can conclude that pigments linked to the xanthophylls cycle are suitable and sensitive biofilm metrics allowing the detection

of short-term effects caused by low and moderate M-pollution (Table 5). On the other hand, photosynthetic pigments linked to different groups (green algae and cyanobacteria) are less sensitive but dose-dependent biofilm metrics responding to chronic and moderate M-pollution (Table 5).

- **Diatoms metrics.** In agreement with our expectations, after a relatively long period of exposure, metal effects on biofilms were mainly structural (species composition). Diatoms, which presented a significant abundance (20-30%) in all biofilm communities (Tlili et al. 2011), were strongly affected. Based on diatoms metrics, biofilm communities from the highest Zn impacted site (M2) were largely different to those from Up_{stream}, M1 and M3. Among the 132 diatom taxa identified, the assemblages were characterised by an association of *Achnanthidium minutissimum* (Kützing) Czarnecki, *A. pyrenaicum* Hustedt, *Cocconeis placentula var. placentula* Ehrenberg, *C. placentula var. lineata* (Ehrenberg) Van Heurck, *C. placentula var. euglypta* (Ehrenberg) Grunow, *Eolimna minima* (Grunow) Lange-Bertalot and *Planothidium frequentissimum (Lange*-Bertalot) Round & L. Bukhtiyarova. Diatom communities of biofilm translocated to M2 were characterized by small-sized diatom species (low biovolume), either pioneer, substrate-adherent species which are more metal-tolerant (Medley and Clements, 1998), or species that are frequently reported in metal-contaminated environments (Morin et al., in press). The decrease in the diatom biovolume is associated with the predominance of smaller growth forms in the biofilms in impacted sites (Sabater and Admiraal, 2005). IPS values recorded at M2 indicate a "poor" biological quality of the water (according to the WFD) which supports its relevance to assess chronic point sources of Zn pollution. However, though significant differences between diatom richness (S) and IPS values were obtained between Up_{stream}, M1 and M3. Based on IPS, the water quality of these sites presented the same category ("good" biological quality) according to the WFD. According to the results, the

IPS is not sensitive enough to detect situations of diffuse pollution of Zn, given that Zn bioaccumulation in biofilms from M1 and M3 was significantly higher than Up_{stream} site and the IPS index did not differentiate these situations. The limitations of IPS for the assessment of metal pollutions have been recently pointed out (e.g. Morin et al. 2008) and are mainly due to the fact that this index was originally built to assess trophic pollution. The specific responses may however differ depending on the type of stressor (trophic vs. toxic). More specific endpoints are needed, such as cell size decrease or the occurrence of diatom deformities that was fairly high in M1, M2 and M3 (with values > 1%, proving metal stress; Morin et al. in press). Combining both data on cell size and abundances of deformities thus allows for the discrimination of different levels of metal stress, lower in M1 and M3 (only deformities) than in M2 (where deformities are associated to very low biovolumes). Thus, in chronic exposure scenarios the use of IPS and diatoms richness offers a similar goodness to assess the effects of low and moderate-M pollution in terms of sensitivity and level exposure-dependency. The percentage of teratoforms will also provide specific information on metal pollution (Table 5).

CONCLUSIONS

Our results indicate that the Zn concentrations found in Osor stream may produce different damaging effects on biofilms depending on the time and intensity of metal exposure. Early effects are mostly affecting biofilms function, supporting the use of chl-a fluorescence techniques in combination with the analysis of the photosynthetic pigments as signals of early toxicity. Longer-term exposure effects are better detected using diatom indices and traits such as cell biovolume or deformities of diatoms. Nevertheless, more research is needed to develop diatom indices that may allow identifying situations of low metal pollution.

Overall, our results provide information about the goodness of several biofilm metrics of interest to assess the effects of metal pollution. It also provides enough

evidences of occurrence and toxicity of Zn for recommending the inclusion of Zn in the list of priority pollutants substances of the European Water Framework Directive.

Sumplementary table 1. Diatom community composition (species relative abundances in %) in biofilms translocated to Up_{stream}, M1, M2 and M3, after 3 weeks of started translocation in riera d'Osor stream. a.f.: abnormal form.

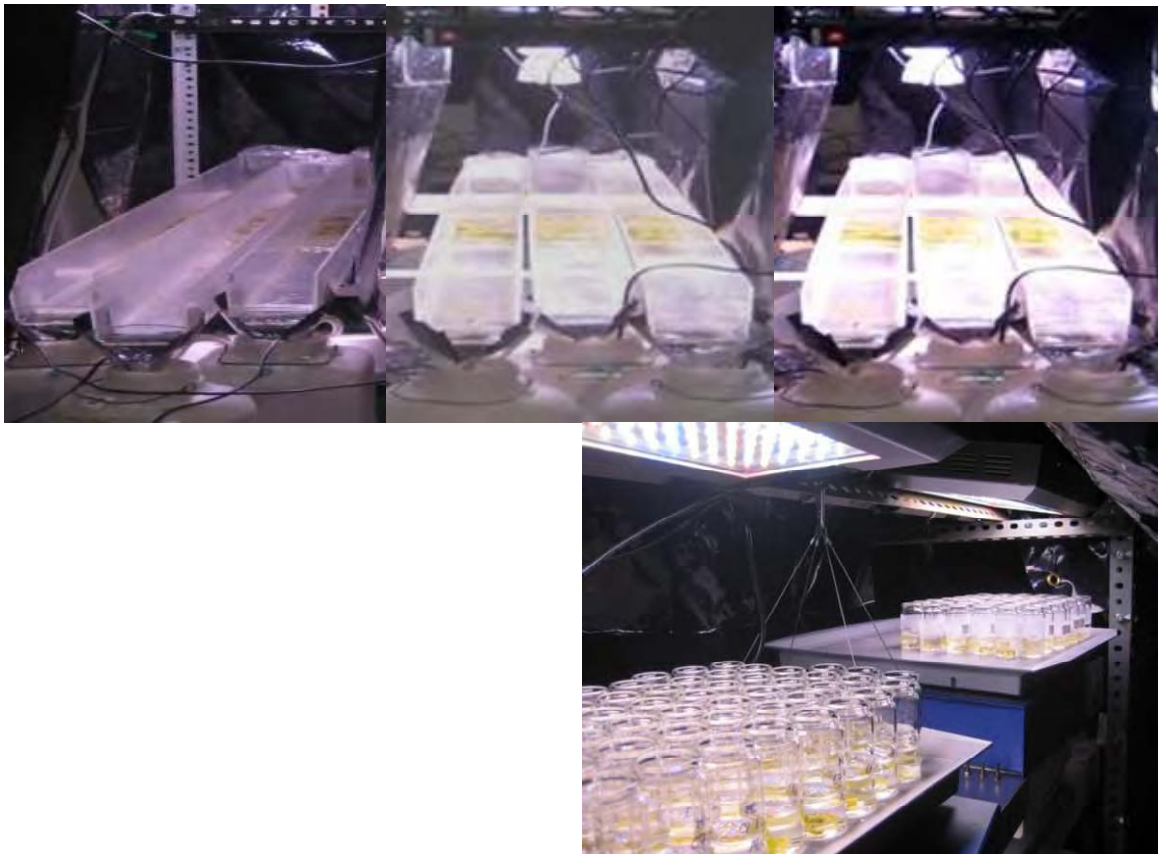
Week 3	Up_{stream}		M1		M2		M3	
	AVG	(±SE)	AVG	(±SE)	AVG	(±SE)	AVG	(±SE)
Achnanthidium affine (Grun) Czarnecki	0.00	(0.00)	0.00	(0.00)	0.58	(0.16)	0.27	(0.14)
Achnanthidium minutissimum (Kützing) Czarnecki a.f.	0.00	(0.00)	0.15	(0.15)	0.00	(0.00)	0.14	(0.14)
Achnanthidium minutissimum (Kützing) Czarnecki	7.42	(3.74)	4.95	(0.99)	0.56	(0.36)	6.40	(3.16)
Achnanthidium pyrenaicum (Hustedt) Kobayasi	2.39	(1.39)	3.74	(1.71)	1.93	(1.93)	2.81	(0.66)
Achnanthidium pyrenaicum (Hustedt) Kobayasi a.f.	0.16	(0.16)	0.29	(0.14)	0.30	(0.30)	0.27	(0.27)
Achnanthidium saprophilum (Kobayasi & Mayama) Round & Bukhtiyarova	0.00	(0.00)	0.53	(0.10)	0.00	(0.00)	0.00	(0.00)
Amphora inariensis Krammer	0.00	(0.00)	0.00	(0.00)	0.14	(0.14)	0.14	(0.14)
Amphora ovalis (Kützing) Kützing	0.42	(0.03)	0.00	(0.00)	0.00	(0.00)	0.00	(0.00)
Amphora pediculus (Kützing) Grunow	8.19	(0.90)	2.78	(0.41)	0.15	(0.15)	1.56	(0.40)
Aulacoseira lirata (Ehrenberg) Ross *in* Hartley	0.47	(0.47)	0.00	(0.00)	0.00	(0.00)	0.00	(0.00)
Caloneis amphisbaena (Bory) Cleve *f.amphisbaena*	0.00	(0.00)	0.14	(0.14)	0.00	(0.00)	0.00	(0.00)
Caloneis bacillum (Grunow) Cleve	0.42	(0.03)	0.12	(0.12)	0.85	(0.24)	0.25	(0.25)
Caloneis bacillum (*Grunow) Cleve a.f.*	0.00	(0.00)	0.00	(0.00)	0.14	(0.14)	0.00	(0.00)
Caloneis hyalina Hustedt	0.00	(0.00)	0.00	(0.00)	0.00	(0.00)	0.12	(0.12)
Cocconeis pediculus Ehrenberg	1.07	(0.60)	0.00	(0.00)	0.00	(0.00)	0.00	(0.00)
Cocconeis placentula var. euglypta a.f.	0.00	(0.00)	0.00	(0.00)	0.14	(0.14)	0.12	(0.12)
Cocconeis placentula Ehrenberg a.f.	0.16	(0.16)	0.42	(0.26)	0.14	(0.14)	0.81	(0.25)
Cocconeis placentula Ehrenberg var. placentula	30.77	(1.21)	33.67	(7.27)	1.60	(0.98)	34.16	(5.11)
Cocconeis placentula Ehrenberg var. euglypta (Ehrenberg) Grunow	2.51	(0.20)	7.21	(1.65)	0.58	(0.29)	9.30	(1.18)
Cocconeis placentula Ehrenberg var. lineata (Ehrenberg)Van Heurck	0.44	(0.27)	12.62	(3.81)	0.43	(0.01)	0.27	(0.14)
Craticula cf. accomoda (Hustedt) Mann	0.28	(0.14)	0.40	(0.24)	0.00	(0.00)	0.00	(0.00)
Craticula molestiformis (Hustedt) Lange-Bertalot	0.00	(0.00)	0.00	(0.00)	0.00	(0.00)	0.14	(0.14)
Cyclotella meneghiniana Kützing	0.79	(0.40)	0.43	(0.24)	0.14	(0.14)	0.26	(0.13)
Diadesmis gallica var. perpusilla (Grunow) Lange-Bertalot	0.00	(0.00)	0.12	(0.12)	0.00	(0.00)	0.00	(0.00)
Diploneis oblongella (Naegeli) Cleve-Euler	0.14	(0.14)	0.00	(0.00)	0.00	(0.00)	0.00	(0.00)
Encyonema minutum (Hilse *in* Rabhenhorst) D.G. Mann	0.42	(0.03)	0.00	(0.00)	0.28	(0.14)	0.00	(0.00)
Encyonopsis microcephala (Grunow) Krammer	0.00	(0.00)	0.00	(0.00)	0.00	(0.00)	0.12	(0.12)
Eolimna minima (Grunow) Lange-Bertalot	1.07	(0.60)	1.93	(00.97)	69.6	(3.45)	6.25	(4.72)
Eolimna minima (Grunow) Lange-Bertalot a.f.	0.00	(0.00)	0.00	(0.00)	0.56	(0.13)	0.80	(0.48)
Eolimna subminuscula (Manguin) Moser Lange-Bertalot & Metzeltin	0.53	(0.27)	0.26	(0.13)	1.44	(0.41)	1.33	(0.17)
Eucocconeis laevis (Oestrup) Lange-Bertalot	0.13	(0.13)	0.00	(0.00)	0.00	(0.00)	0.00	(0.00)
Fallacia lenzi (Hustedt) Lange-Bertalot	0.28	(0.28)	0.00	(0.00)	0.00	(0.00)	0.00	(0.00)
Fallacia subhamulata (Grunow *in* Van Heurck) D.G. Mann	0.00	(0.00)	0.15	(0.15)	0.14	(0.14)	0.14	(0.14)
*Fragilaria capucina Desmazièeres var.vaucheriae (*Kützing) Lange-Bertalot	0.47	(0.47)	0.00	(0.00)	0.41	(0.41)	0.92	(0.26)
Fragilaria capucina var. vaucheriae (Kützing) Lange-Bertalot a.f.	0.00	(0.00)	0.00	(0.00)	0.14	(0.14)	0.00	(0.00)
Gomphoneis minuta (Stone) Kociolek & Stoermer *var.minuta* a.f.	0.26	(0.13)	0.00	(0.00)	0.00	(0.00)	0.00	(0.00)
Gomphonema acuminatum Ehrenberg	0.28	(0.28)	0.00	(0.00)	0.00	(0.00)	0.00	(0.00)
Gomphonema minutum(Agardh) Agardh *f. minutum*	1.43	(0.56)	0.00	(0.00)	0.00	(0.00)	0.26	(0.13)
Gomphonema parvulum (Kützing) Kützing *var. parvulum f. parvulum*	0.41	(0.41)	0.12	(0.12)	0.14	(0.14)	0.26	(0.13)
Gomphonema parvulum Kützing *a.f.*	0.00	(0.00)	0.00	(0.00)	0.15	(0.15)	0.00	(0.00)
Gomphonema pumilum (Grunow) Reichardt & Lange-Bertalot	0.00	(0.00)	0.15	(0.15)	0.14	(0.14)	0.52	(0.11)
Gomphonema pumilum (Grunow) Reichardt & Lange-Bertalot a.f.	0.00	(0.00)	0.00	(0.00)	0.14	(0.14)	0.00	(0.00)

Gomphonema truncatum Ehrenberg	0.29	(0.15)	0.00	(0.00)	0.00	(0.00)	0.00	(0.00)
Hippodonta capitata (Ehrenberg) Lange-Bertalot Metzeltin & Witkowski	0.00	(0.00)	0.15	(0.15)	0.14	(0.14)	0.00	(0.00)
Karayevia clevei (Grunow) Bukhtiyarova	0.67	(0.23)	0.43	(0.24)	0.00	(0.00)	0.14	(0.14)
Karayevia ploenensis (Hustedt) Bukhtiyarova	0.56	(0.14)	0.00	(0.00)	0.00	(0.00)	0.00	(0.00)
Luticola cf. cohnii (Hilse) D.G. Mann	0.14	(0.14)	0.00	(0.00)	0.00	(0.00)	0.00	(0.00)
Mayamaea atomus var. permitis (Hustedt) Lange-Bertalot	0.00	(0.00)	0.41	(0.03)	0.43	(0.24)	0.52	(0.11)
Melosira varians Agardh	1.54	(1.15)	0.96	(0.16)	0.56	(0.13)	1.09	(0.89)
Meridion circulare (Greville) C.A. Agardh *var. circulare*	0.13	(0.13)	0.00	(0.00)	0.00	(0.00)	0.12	(0.12)
Navicula capitatoradiata Germain	2.60	(0.63)	0.24	(0.24)	0.00	(0.00)	0.00	(0.00)
Navicula catalanogermanica Lange-Bertalot & Hofmann	0.00	(0.00)	0.51	(0.31)	0.29	(0.14)	0.28	(0.28)
Navicula cincta (Ehrenberg) Ralfs *in* Pritchard	0.00	(0.00)	0.12	(0.12)	0.00	(0.00)	0.00	(0.00)
Navicula cryptotenella Lange-Bertalot	0.54	(0.33)	0.00	(0.00)	0.00	(0.00)	0.00	(0.00)
Navicula gregaria Donkin	0.16	(0.16)	3.40	(0.39)	1.14	(0.15)	2.22	(0.38)
Navicula gregaria Donkin a.f.	0.00	(0.00)	0.00	(0.00)	0.00	(0.00)	0.14	(0.14)
Navicula lanceolata (Agardh) Ehrenberg	5.88	(1.06)	3.34	(1.17)	0.98	(0.36)	3.57	(0.25)
Navicula oligotraphenta Lange-Bertalot & Hofmann	0.00	(0.00)	0.26	(1.13)	0.00	(0.00)	0.00	(0.00)
Navicula tripunctata (O.F.Müller) Bory	1.08	(0.31)	0.00	(0.00)	0.00	(0.00)	0.12	(0.12)
Nitzschia archibaldii Lange-Bertalot	0.86	(0.29)	0.42	(0.42)	0.73	(0.39)	0.38	(0.21)
Nitzschia dissipata (Kützing) Grunow a.f.	0.00	(0.00)	0.12	(0.12)	0.14	(0.14)	0.00	(0.00)
Nitzschia dissipata (Kützing) Grunow *var.dissipata*	0.96	(0.32)	3.57	(1.79)	0.43	(0.01)	1.34	(0.56)
Nitzschia fonticola Grunow *in* Cleve & Möller a.f.	0.00	(0.00)	0.00	(0.00)	0.14	(0.14)	0.14	(0.14)
Nitzschia fonticola Grunow *in* Cleve & Möller	0.81	(0.48)	2.96	(1.08)	0.99	(0.12)	2.41	(0.47)
Nitzschia frustulum (Kützing) Grunow *var.frustulum*	0.85	(0.43)	1.32	(0.26)	0.58	(0.16)	0.66	(0.36)
Nitzschia graciliformis Lange-Bertalot & Simonsen	0.14	(0.14)	0.00	(0.00)	0.00	(0.00)	0.00	(0.00)
Nitzschia inconspicua Grunow	2.54	(0.38)	2.40	(0.24)	1.16	(0.54)	2.50	(0.87)
Nitzschia inconspicua Grunow *a.f.*	0.13	(0.13)	0.00	(0.00)	0.00	(0.00)	0.00	(0.00)
Nitzschia linearis (Agardh) W.M.Smith *var.tenuis* (W.Smith) Grunow	0.57	(0.29)	0.41	(0.03)	0.14	(0.14)	0.14	(0.14)
Nitzschia palea (Kützing) W.Smith a.f.	0.00	(0.00)	0.14	(0.14)	0.00	(0.00)	0.14	(0.14)
Nitzschia palea (Kützing) W.Smith	0.00	(0.00)	1.83	(0.98)	0.15	(0.15)	1.71	(0.24)
Nitzschia recta Hantzsch *in* Rabenhorst	0.00	(0.00)	0.00	(0.00)	0.00	(0.00)	0.14	(0.14)
Nitzschia sigma (Kützing) W.M.Smith	0.00	(0.00)	0.14	(0.14)	0.00	(0.00)	0.00	(0.00)
Pinnularia microstauron (Ehrenberg) Cleve *var. microstauron*	0.00	(0.00)	0.00	(0.00)	0.15	(0.15)	0.00	(0.00)
Planothidium frequentissimum (Lange-Bertalot) Lange-Bertalot a.f.	0.00	(0.00)	0.00	(0.00)	0.14	(0.14)	0.00	(0.00)
*Planothidium frequentissimum (*Lange-Bertalot) Lange-Bertalot	0.26	(0.13)	1.18	(0.31)	6.68	(0.70)	1.18	(0.19)
Planothidium hauckianum (Grunow) Round & Bukhtiyarova	0.00	(0.00)	0.00	(0.00)	0.29	(0.14)	0.00	(0.00)
Planothidium lanceolatum (Brébisson ex Kützing) Lange-Bertalot a.f.	0.29	(0.15)	0.00	(0.00)	0.00	(0.00)	0.00	(0.00)
Planothidium lanceolatum (Brébisson ex Kützing) Lange-Bertalot	15.96	(1.72)	4.15	(0.79)	0.87	(0.45)	10.97	(0.88)
Planothidium rostratum (Oestrup) Lange-Bertalot	0.00	(0.00)	0.00	(0.00)	0.15	(0.15)	0.12	(0.12)
Reimeria sinuata (Gregory) Kociolek & Stoermer	0.28	(0.14)	0.50	(0.31)	0.43	(0.01)	0.38	(0.21)
Rhoicosphenia abbreviata (C. Agardh) Lange-Bertalot	0.54	(0.33)	0.12	(0.12)	0.00	(0.00)	0.94	(0.37)
Stauroneis thermicola (Petersen) Lund	0.26	(0.13)	0.12	(0.12)	0.00	(0.00)	0.12	(0.12)
Surirella angusta Kützing	0.00	(0.00)	0.12	(0.12)	0.14	(0.14)	0.12	(0.12)
Surirella brebissonii Krammer & Lange-Bertalot a.f.	0.00	(0.00)	0.15	(0.15)	0.00	(0.00)	0.00	(0.00)
Surirella brebissonii Krammer & Lange-Bertalot *var.brebissonii*	0.25	(0.25)	0.26	(0.13)	1.00	(0.37)	0.26	(0.13)
Surirella brebissonii var. kuetzingii Krammer & Lange-Bertalot	0.13	(0.13)	0.00	(0.00)	1.41	(0.50)	0.00	(0.00)
Ulnaria oxyrhynchus (Kützing) Aboal	0.70	(0.27)	0.00	(0.00)	0.00	(0.00)	0.26	(0.13)
Ulnaria ulna (Nitzsch) Compère	0.00	(0.00)	0.12	(0.12)	0.00	(0.00)	0.26	(0.13)

Sumplementary table 2. Diatom community composition (species relative abundances in %) in biofilms translocated to Up_{stream}, M1, M2 and M3, after 5 weeks of started translocation in riera d'Osor stream. a.f.: abnormal form.

Week 5	Up_{stream}		M1		M2		M3	
	AVG	(±SE)	AVG	(±SE)	AVG	(±SE)	AVG	(±SE)
Achnanthidium affine (Grunow) Czarnecki	0.73	(0.14)	0.00	(0.00)	0.00	(0.00)	0.00	(0.00)
Achnanthidium eutrophilum (Lange-Bertalot) Lange-Bertalot	0.08	(0.08)	0.00	(0.00)	0.00	(0.00)	0.00	(0.00)
Achnanthidium minutissimum (Kützing) Czarnecki a.f.	0.00	(0.00)	0.18	(0.18)	0.64	(0.29)	0.24	(0.15)
Achnanthidium minutissimum (Kützing) Czarnecki	3.00	(0.45)	0.86	(0.31)	1.16	(0.59)	3.99	(1.27)
Achnanthidium pyrenaicum (Hustedt) Kobayasi	1.38	(0.78)	1.17	(0.42)	0.47	(0.26)	2.59	(1.12)
Achnanthidium pyrenaicum (Hustedt) Kobayasi a.f.	0.00	(0.00)	0.00	(0.00)	0.00	(0.00)	0.32	(0.06)
Achnanthidium saprophilum (Kobayasi & Mayama) Round & Bukhtiyarova	0.00	(0.00)	0.00	(0.00)	0.23	(0.13)	1.24	(0.49)
Achnanthidium subatomus (Hustedt) Lange-Bertalot	0.24	(0.14)	0.09	(0.09)	0.00	(0.00)	0.00	(0.00)
Amphipleura cf. pellucida Kützing	0.00	(0.00)	0.00	(0.00)	0.00	(0.00)	0.08	(0.08)
Amphora copulata (Kützing) Schoeman & Archibald	0.08	(0.08)	0.08	(0.08)	0.00	(0.00)	0.00	(0.00)
Amphora pediculus (Kützing) Grunow	4.70	(0.34)	1.57	(0.66)	0.24	(0.14)	2.26	(0.73)
Caloneis bacillum (Grunow) Cleve	0.16	(0.08)	0.35	(0.18)	0.16	(0.08)	0.77	(0.30)
Cocconeis sp. a.f.	0.00	(0.00)	0.50	(0.28)	0.00	(0.00)	0.09	(0.09)
Cocconeis pediculus Ehrenberg	0.32	(0.08)	0.08	(0.08)	0.00	(0.00)	0.08	(0.08)
Cocconeis pediculus Ehrenberg a.f.	0.00	(0.00)	0.09	(0.09)	0.00	(0.00)	0.00	(0.00)
Cocconeis placentula Ehrenberg *var. euglypta* (Ehrenberg) Grunow a.f.	0.00	(0.00)	0.08	(0.08)	0.00	(0.00)	0.00	(0.00)
Cocconeis placentula Ehrenberg a.f.	0.00	(0.00)	1.12	(0.19)	0.00	(0.00)	0.67	(0.32)
Cocconeis placentula Ehrenberg *var. placentula*	18.36	(1.13)	31.77	(3.17)	0.47	(0.22)	35.29	(3.45)
Cocconeis placentula Ehrenberg *var.euglypta* (Ehrenberg) Grunow	0.41	(0.41)	6.53	(2.26)	0.24	(0.14)	12.37	(1.40)
Cocconeis placentula Ehrenberg *var. lineata* (Ehrenberg) Van Heurck	13.18	(1.32)	16.27	(2.20)	0.08	(0.08)	0.53	(0.28)
Cocconeis pseudolineata (Geitler) Lange-Bertalot	1.71	(1.00)	0.17	(0.08)	0.00	(0.00)	0.55	(0.18)
Cyclotella meneghiniana Kützing	0.32	(0.08)	0.34	(0.08)	0.00	(0.00)	0.16	(0.08)
Diadesmis biceps Arnott ex Grunow	0.08	(0.08)	0.00	(0.00)	0.00	(0.00)	0.09	(0.09)
Diadesmis gallica var. perpusilla (Grunow) Lange-Bertalot	0.41	(0.17)	0.00	(0.00)	0.00	(0.00)	0.00	(0.00)
Diatoma mesodon (Ehrenberg) Kützing	0.08	(0.08)	0.00	(0.00)	0.00	(0.00)	0.00	(0.00)
Diploneis oblongella (Naegeli) Cleve-Euler	0.00	(0.00)	0.00	(0.00)	0.00	(0.00)	0.07	(0.07)
Encyonema minutum (Hilse in Rabhenhorst) D.G. Mann	0.00	(0.00)	0.36	(0.24)	0.08	(0.08)	0.47	(0.24)
Encyonema prostratum (Berkeley) Kützing	0.00	(0.00)	0.08	(0.08)	0.00	(0.00)	0.00	(0.00)
Eolimna minima (Grunow) Lange-Bertalot	2.34	(0.44)	1.11	(0.08)	61.99	(4.44)	2.59	(0.46)
Eolimna minima (Grunow) Lange-Bertalot a.f.	0.00	(0.00)	0.17	(0.08)	3.38	(0.80)	0.17	(0.09)
Eolimna subminuscula (Manguin) Moser Lange-Bertalot & Metzeltin	1.13	(0.21)	0.18	(0.09)	0.49	(0.15)	0.59	(0.30)
Fallacia subhamulata (Grunow in Van Heurck) D.G. Mann	0.16	(0.16)	0.08	(0.08)	0.00	(0.00)	0.00	(0.00)
Fragilaria capucina Desmazières a.f.	0.00	(0.00)	0.00	(0.00)	0.08	(0.08)	0.07	(0.07)
Fragilaria capucina Desmazières *var.capucina*	0.00	(0.00)	0.00	(0.00)	0.08	(0.08)	0.00	(0.00)
Fragilaria capucina Desmazières *var.vaucheriae* (Kützing) Lange-Bertalot	0.73	(0.13)	0.50	(0.39)	0.25	(0.25)	0.16	(0.08)
Fragilaria capucina var. vaucheriae (Kützing) Lange-Bertalot a.f.	0.00	(0.00)	0.00	(0.00)	0.08	(0.08)	0.00	(0.00)
Gomphoneis minuta (Stone) Kociolek & Stoermer *var.minuta*	0.16	(0.08)	0.00	(0.00)	0.00	(0.00)	0.00	(0.00)
Gomphonema clavatum Ehrenberg	0.08	(0.08)	0.00	(0.00)	0.00	(0.00)	0.00	(0.00)
Gomphonema micropus Kützing *var. micropus*	0.08	(0.08)	0.17	(0.17)	0.00	(0.00)	0.00	(0.00)
Gomphonema minutum (Agardh) Agardh *f. minutum*	0.24	(0.14)	0.09	(0.09)	0.00	(0.00)	0.00	(0.00)
Gomphonema olivaceum var. olivaceum (Hornemann) Brebisson	0.16	(0.08)	0.00	(0.00)	0.00	(0.00)	0.08	(0.08)
Gomphonema parvulum (Kützing) Kützing var. parvulum f. parvulum	0.65	(0.09)	0.18	(0.18)	0.23	(0.13)	0.80	(0.16)

Gomphonema pumilum (Grunow) Reichardt & Lange-Bertalot	0.08	(0.08)	0.17	(0.08)	0.08	(0.08)	0.33	(0.09)
Halamphora montana (Krasske) Levkov	0.08	(0.08)	0.00	(0.00)	0.00	(0.00)	0.00	(0.00)
Karayevia clevei (Grunow) Bukhtiyarova	0.41	(0.29)	0.33	(0.17)	0.00	(0.00)	0.09	(0.09)
Karayevia ploenensis (Hustedt) Bukhtiyarova	0.64	(0.35)	0.27	(0.16)	0.00	(0.00)	0.07	(0.07)
Karayevia ploenensis (Hustedt) Bukhtiyarova a.f.	0.00	(0.00)	0.00	(0.00)	0.00	(0.00)	0.07	(0.07)
Luticola cf. cohnii (Hilse) D.G. Mann	0.00	(0.00)	0.00	(0.00)	0.00	(0.00)	0.09	(0.09)
Mayamaea atomus var. permitis (Hustedt) Lange-Bertalot	0.32	(0.21)	0.08	(0.08)	2.53	(0.67)	0.16	(0.08)
Mayamaea atomus var. permitis (Hustedt) Lange-Bertalot a.f.	0.00	(0.00)	0.00	(0.00)	0.08	(0.08)	0.00	(0.00)
Melosira varians Agardh	1.37	(0.28)	1.49	(0.65)	0.32	(0.09)	0.87	(0.32)
Meridion circulare (Greville) C.A. Agardh *var. circulare*	0.00	(0.00)	0.00	(0.00)	0.08	(0.08)	0.46	(0.21)
Navicula catalanogermanica Lange-Bertalot & Hofmann	0.16	(0.08)	0.08	(0.08)	0.08	(0.08)	0.16	(0.08)
Navicula cryptotenella Lange-Bertalot	0.73	(0.25)	0.27	(0.27)	0.00	(0.00)	0.00	(0.00)
Navicula gregaria Donkin	4.63	(0.82)	2.61	(0.68)	0.64	(0.09)	1.79	(0.37)
Navicula lanceolata (Agardh) Ehrenberg	2.28	(0.51)	1.98	(0.39)	0.16	(0.08)	1.86	(0.71)
Navicula tripunctata (O.F.Müller) Bory	0.32	(0.16)	0.00	(0.00)	0.00	(0.00)	0.16	(0.08)
Nitzschia acula Hantzsch	0.00	(0.00)	0.08	(0.08)	0.00	(0.00)	0.00	(0.00)
Nitzschia archibaldii Lange-Bertalot	2.98	(0.68)	0.00	(0.00)	0.00	(0.00)	0.00	(0.00)
Nitzschia dissipata (Kützing) Grunow a.f.	0.00	(0.00)	0.25	(0.15)	0.00	(0.00)	0.00	(0.00)
Nitzschia dissipata (Kützing) Grunow *var.dissipata*	2.10	(0.44)	1.20	(0.24)	0.48	(0.15)	1.38	(0.68)
Nitzschia fonticola Grunow in Cleve & Möller	0.25	(0.14)	2.07	(1.23)	0.24	(0.01)	3.92	(0.54)
Nitzschia frustulum (Kützing) Grunow a.f.	0.00	(0.00)	0.16	(0.16)	0.00	(0.00)	0.00	(0.00)
Nitzschia frustulum (Kützing) Grunow *var. frustulum*	0.97	(0.15)	3.35	(0.61)	0.48	(0.01)	2.16	(0.79)
Nitzschia inconspicua Grunow	0.88	(0.45)	1.44	(0.42)	0.39	(0.20)	1.57	(0.43)
Nitzschia inconspicua Grunow a.f.	0.00	(0.00)	0.17	(0.08)	0.00	(0.00)	0.08	(0.08)
Nitzschia linearis (Agardh) W.M.Smith *var.linearis*	0.00	(0.00)	0.00	(0.00)	0.16	(0.16)	0.09	(0.09)
Nitzschia linearis (Agardh) W.M.Smith *var.tenuis* (W.Smith) Grunow	0.33	(0.33)	0.00	(0.00)	0.00	(0.00)	0.30	(0.19)
Nitzschia palea (Kützing) W.Smith a.f.	0.00	(0.00)	0.27	(0.27)	0.00	(0.00)	0.24	(0.01)
Nitzschia palea (Kützing) W.Smith	1.53	(0.62)	1.74	(0.58)	0.16	(0.08)	2.32	(0.57)
Nitzschia solgensis Cleve-Euler	0.00	(0.00)	0.00	(0.00)	0.00	(0.00)	0.33	(0.09)
Nitzschia umbonata (Ehrenberg) Lange-Bertalot	0.08	(0.08)	0.00	(0.00)	0.00	(0.00)	0.00	(0.00)
Planothidium frequentissimum (Lange-Bertalot) Lange-Bertalot a.f.	0.00	(0.00)	0.25	(0.14)	0.32	(0.22)	0.41	(0.10)
Planothidium frequentissimum (Lange-Bertalot) Lange-Bertalot	5.52	(1.04)	5.58	(2.08)	22.23	(5.64)	4.49	(1.69)
Planothidium hauckianum (Grunow) Round & Bukhtiyarova	0.08	(0.08)	0.17	(0.08)	0.08	(0.08)	0.00	(0.00)
Planothidium lanceolatum (Brébisson ex Kützing) Lange-Bertalot a.f.	0.08	(0.08)	0.09	(0.09)	0.00	(0.00)	0.00	(0.00)
Planothidium lanceolatum (Brébisson ex Kützing) Lange-Bertalot	21.89	(1.82)	9.95	(3.56)	0.65	(0.23)	8.31	(0.82)
Reimeria sinuata (Gregory) Kociolek & Stoermer	0.32	(0.22)	1.16	(0.66)	0.08	(0.08)	0.70	(0.24)
Rhoicosphenia abbreviata (C.Agardh) Lange-Bertalot	0.57	(0.22)	0.00	(0.00)	0.00	(0.00)	0.56	(0.05)
Rhoicosphenia abbreviata (C.Agardh) Lange-Bertalot a.f.	0.00	(0.00)	0.09	(0.09)	0.00	(0.00)	0.00	(0.00)
Stauroneis thermicola (Petersen) Lund	0.00	(0.00)	0.09	(0.09)	0.08	(0.08)	0.00	(0.00)
Surirella angusta Kützing	0.25	(0.25)	0.08	(0.08)	0.16	(0.08)	0.15	(0.15)
Surirella brebissonii Krammer & Lange-Bertalot *var.brebissonii*	0.00	(0.00)	0.00	(0.00)	0.00	(0.00)	0.23	(0.13)
Surirella brebissonii var. kuetzingii Krammer & Lange-Bertalot	0.00	(0.00)	0.00	(0.00)	0.16	(0.08)	0.00	(0.00)
Ulnaria oxyrhynchus (Kützing) Aboal	0.08	(0.08)	0.08	(0.08)	0.00	(0.00)	0.00	(0.00)
Ulnaria ulna (Nitzsch) Compère	0.08	(0.08)	0.18	(0.09)	0.00	(0.00)	0.23	(0.13)
Ulnaria ulna (Nitzsch) Comère a.f.	0.00	(0.00)	0.08	(0.08)	0.00	(0.00)	0.00	(0.00)



CHAPTER VI

LIGHT HISTORY INFLUENCES THE RESPONSE OF FLUVIAL BIOFILMS TO ZN EXPOSURE

INTRODUCTION

Fluvial biofilms are phytobenthic communities composed mainly by algae, cyanobacteria, fungi, bacteria and protozoa embedded in a polysaccharide matrix on benthic substrata, and are responsible for most of the primary production in many fluvial ecosystems (Sabater et al., 2007; Romaní et al., 2010). Within biofilm processes, photosynthesis is critical for fitness and survival of algae and cyanobacteria. These communities are frequently exposed to strong spatial and temporal variations of light regime, from 20 µmols photons/m^2/s in shaded sites to full sunlight (1700 µmols photons/m^2/s) (Guasch and Sabater, 1998)., as well as other environmental stresses such as low temperature, drought or nutrient deficiency. Seasonal fluctuations in light intensity are related to the riparian vegetation canopy (open *versus* close canopy cover sites), and play an important role in the development of the structure and composition of biofilms. In headwaters and medium-sized rivers where riparian vegetation casts its shade on the benthic autotrophs, light may become a limiting factor below 25 µmols photons/m^2/s (Hill, 1996). Hence, biofilms photosynthetically adapted to shade or sun conditions will present a different efficiency in the use of light energy for photosynthetic processes; biofilms adapted to shade conditions are more efficient in the use of light energy than those that are adapted to sunlight (Guasch and Sabater, 1998; Roberts et al., 2004; Villeneuve et al., 2010). Furthermore, biofilms are also exposed to diurnal fluctuations in light intensity (occurring in a period of a few hours), which can result in the absorption of more light energy than can be utilized productively by photosynthesis. Under these conditions, safe dissipation of excess photons and electrons is necessary to protect the photosynthetic apparatus from light-induced damage (Niyogi, 2000). When considering short time scales, microalgae respond to light increase by a photoinhibition of photosynthesis efficiency while light decrease is expected to enhance photosynthesis efficiency (Laviale et al., 2009). Among the different photoprotective mechanisms, the

production of carotenoids, especially those of the xanthophyll cycle, can help to dissipate energy excess throughout thermal radiation. Specifically, in diatoms, a way to dissipate this excess of excited energy arriving at the PSII is the xanthophylls cycle through the de-epoxidation reaction (DR) that transforms diadinoxanthin into diatoxanthin pigments (Lohr and Wilhelm, 1999; Jin et al., 2003). This process, which is part of so-called non-photochemical quenching, NPQ, (Müller et al., 2001), has been recently studied in diatoms of biofilm communities (Serôdio et al., 2005; Laviale et al., 2009; Chevalier et al., 2010), finding a strong correlation between NPQ increase and DR activation when biofilms are exposed to increasing light irradiances.

Regarding metal pollution in freshwater ecosystems, Zn plays an important role due to its widespread occurrence and toxicity in fluvial biofilms. Zn pollution in fluvial ecosystems has been described from low concentrations, ranging between 150 and 600 µgZn/L (Le Faucheur et al., 2005; Tlili et al. 2011), to higher concentrations ranging between 1000 and 3000 µgZn/L (Ivorra et al., 1999,2000; Morin et al., 2007; Bonnineau et al., 2011). Damage caused on functional processes, such as photosynthetic activity or the synthesis of photosynthetic accessory pigments (Ivorra et al., 2000; Corcoll et al., submitted) and phytochelatins (Le Faucheur et al., 2005) has been atributed to Zn toxicity on biofilms. At structural level, several studies reported Zn effects on the diatom community, such as a decrease in the number of species (diatom richness) and/or the replacement of sensitive species by tolerant ones (Ivorra et al., 2000; Morin et al., 2007; Corcoll et al., submitted). In this study, Zn was selected to evaluate, in controlled conditions, the interaction between light stress and Zn toxicity due to their frequent co-occurrence in nature.

The influence of light history on biofilm sensitivity to chemical stress (i.e. atrazine herbicide) has already been demonstrated (Guasch and Sabater, 1998; Guasch et al. 2003); biofilms from open sites (with high light irradiation) were more sensitive to atrazine than those growing in low light. Moreover, effects of the co-

occurrence of chemical exposure (i.e. isoproturon herbicide) and light stress on biofilms were also investigated (Laviale et al., 2010), showing that a dynamic light regime increased periphyton sensitivity to isoproturon by increasing photosynthesis inhibition and challenging its photoprotective mechanisms, such as the xanthophyll cycle. Since chemical stress is expected to differ depending on light history; the different response of shade or sun-adapted communities to the co-occurrence of two different stress factors: chemical toxicity and light stress deserves further investigations.

The aim of this study was to evaluate the biofilm response to a multiple-stress situation, i.e. environmental stress caused by light and Zn toxicity. Also, the study aimed to assess if adaptation to one of these stress factors (i.e. light) modifies this response. In order to reach this goal, shade- and sun-adapted biofilm communities were first obtained. These communities were then exposed to different light intensities (10, 25, 50, 100, 250 and 500 µmols photons/m^2/s) and Zn (1500 µgZn/L). Chl-a fluorescence parameters and photosynthetic pigments were analyzed to characterise photosynthetic mechanisms of adaptation and to evaluate short-term responses.

We hypothesize that Zn will affect the phenotypic plasticity of biofilms to regulate light stress and avoid photodamage. In particular, it is expected that Zn will reduce the operation of photoprotective mechanisms, such as NPQ and the xanthophyll cycle (Serôdio et al., 2005; Laviale et al., 2009; Chevalier et al., 2010) of biofilms in a light stress situation. It is also expected that photoadaptation will influence their sensitivity to light stress and Zn toxicity due to differences in pigment composition, specifically those linked with the xanthophyll cycle (Guasch and Sabater, 1998; Laviale et al., 2009; Chevalier et al., 2010)

BIOFILM COLONIZATION

Fluvial biofilms were grown during four weeks (colonization period) in microcosm conditions using an experimental set-up of nine perspex channels (Serra et al., 2009). Carbon-dechlorinaded tap water supplied from 10 L carboys was located at the end of each channel and was recirculated at a rate of 1 L/min through centrifuge pumps. Water from channels was renewed three times a week. In each water renewal, 30 $\mu gPO_4^{3-}/L$ (nominal concentration) were added as KH_2PO_4 (Merck, Darmstadt, Germany) to avoid phosphate limitation. During the colonization period, water was sampled periodically to determine physical and chemical parameters (temperature, pH, dissolved oxygen and conductivity), phosphate concentration, and the major cations and anions. Glass substrata ($1x1cm^2$) were placed at the bottom of each channel to facilitate colonization. Biofilm communities were obtained from field inocula after scraping three cobbles of the Llémena River. This stream is a small calcareous tributary of the Ter River (NE Spain) and considered not polluted (ACA, Catalan Water Agency). During the four weeks of the biofilm colonization period, a new biofilm inocula was provided to each channel once a week. Throughout colonization, bioflms had three different light conditions. Three channels were used for biofilm colonization under a low light intensity of 25 µmols photons $/m^2$ /s, far from saturating (Hill, 1996). This community is referred to in the text as LL biofilm. Three other channels were used for biofilm colonization under a medium light intensity of 100 µmols photons $/m^2$ /s, within the range of light saturation conditions commonly reported for natural stream communities (Hill, 1996). This community is referred to as ML biofilm. The remaining three channels were used for the colonization of biofilm al high light intensity of 500 µmols photons $/m^2$ /s which is comparable to light inhibition levels (Hill, 1996). This community is referred to in the text as HL biofilm (Table 1). Light was provided by LEDs lamps (Lightech, Spain) and the light regime was 12:12h light:dark cycle. Light intensity

was checked twice a week with an underwater cell (LI-COR Inc., Lincoln, Nebraska, USA) placed above the colonized glass substrata.

BIOFILM SAMPLING

At the end of the colonization period, the different biofilm communities obtained were characterized (Table 1) in terms of chl-a concentration (Chl-a), ash free dry weight (AFDW), extracellular polymeric substances (EPS), algal groups, diatoms taxonomy, photosynthetic pigments and electron transport rate *vs*. irradiance (ETR-I) curves (see biofilm analysis section). For this purpose, one glass substrata from each channel was sampled at the end of the colonization period for Chl-a, AFDW, EPS, algal groups and photosynthetic pigment analysis, and three glass substrata for ETR-I curves. Algal groups and ETR-I curves were measured immediately by PhytoPAM instrument (see biofilm analysis section). As this technique is not destructive, the sample was fixed with formaldehyde (final concentration 4%) after the PAM measurements and used for further diatoms taxonomy analysis.

Samples for pigments were stored at -80ºC, and those for AFDW and EPS at -20ºC until analysis.

Table 1. Experimental design including the list of endpoints measured and the light and Zn conditions tested.

	Light colonization (µmols phot/m^2/s)	Light incubation (µmols phot/m^2/s)	Zn exposure (µg Zn/L)	biofilm endpoints
Biofilm colonization (lasted 4 weeks)	25-100-500			ETR-I curves, photosynthetic pigments, Chl-a, AFDW, EPS, algal groups, diatom taxonomy
Light and Zn toxicity tests (lasted 6h)		10-25-50-100-250-500	0-1500	Φ'_{PSII}, Φ_{PSII}, NPQ, DR

SHORT-TERM TOXICITY TESTS

After the biofilm colonization period, a short-term toxicity test (6h) was performed with LL, ML and HL biofilms exposed to different light intensities and Zn

concentrations to assess the effects of Zn when a sudden increase or decrease in light irradiance was also taking place (Table 1). This test is named in the text as Light and Zn toxicity test. It was performed in vials (10ml) containing a single colonized glass substrata. Biofilms were incubated for 6h at 10, 25, 50, 100, 250 and 500 µmols photons /m^2/s and exposed to 1500 µgZn/L (Zn treatment) or 0 µgZn/L (NoZn treatment). The concentration of 1500 µgZn/L was chosen because it was expected to cause effects on the biofilm after the short duration of exposure (Paulsson et al., 2000; Corcoll et al., 2011) and due to its realistic environmental concentration (Morin et al., 2008; Bonnineau et al., 2011).

During incubation, samples were constantly agitated using single-speed orbital mixers (KS260 Basic, IKA®). Light was provided by the same LEDs lamps (Lightech, Spain) used during colonization and Zn was added as zinc chloride (ZnCl2; Zn titrisol, Merck, Darmstadt, Germany). Biofilm samples were taken at the end of the exposure (6h) to measure photochemical and non-photochemical quenching parameters and analyze photosynthetic pigments (see biofilm analysis section for more details). A total of 3 toxicity tests were run: one for LL biofilms, one for ML biofilms and one for HL biofilms. 36 glass substrata were used for each test: 3 replicates (provided from each channel), six light levels (10, 25, 50, 100, 250 and 500 µmols photons /m^2/s) and two concentrations (0 and 1500 µgZn/L). Growth light conditions were always included and considered as controls in the tests performed.

ELECTRON TRANSPORT RATE – IRRADIANCE CURVES

At the end of the colonization period, three glass substrata were sampled from each channel to analyse electron transport rate-ETR *vs.* irradiances-I curves (ETR-I curves) by in *vivo* chl-a fluorescence measurements using the Phyto-PAM fluorometer (Heinz Walz, Germany). The PhytoPAM instrument offers the possibility to perform "rapid light curves-RLC" from 1 to 600 µmols photons/m^2/s light intensities with

illumination intervals at 10s. By using rapid light curves, relevant information on the saturation characteristics of electron transport can be obtained (Schreiber et al., 1997; Rascher et al., 2000). The relationship between the ETR and irradiances were fitted to the Eilers and Peeters curve-fitting model (1988). Photosynthetic parameters assessed with this model were: the initial slope of ETR (α) which expresses the efficiency of light use, the maximal electron transport rate (ETRm) and the light of the semi-saturation parameter (Ik). The use of RLCs to describe ETR-I relationships has been demonstrated successfully in other studies of benthic algal communities (Kühl et al., 2001; Roberts et al., 2004).

DATA ANALYSIS

- **ANOVA one-way.** To examine differences between light treatments (LL, ML and HL) in physical and chemical data of water (Table 2) and biofilm metrics (Chl-a, AFDW, EPS content, algal groups: Fo(Bl), Fo(Br), Fo(Gr), diatom richness (S), diatom diversity (H'), relative abundance of diatom species, α, ETRm and Ik parameters) after colonization period, a one-way ANOVA test followed by a Tukey post hoc test ($p<0.05$) was performed. In the first case (physical and chemical parameters), the analysis included data from 3 to 24 sampling times (n= 9 – 84) along the colonization period whereas biofilm metrics were compared at the end of the period (n=3). For light and Zn toxicity tests, one-way ANOVA test was also used to detect differences of Φ'_{PSII} and Φ_{PSII} parameters between biofilms exposed to Zn and those non-exposed. Before all ANOVA tests, data were transformed to achieve normality.

- **ANOVA three-ways.** To test for effects of light regime during colonization (Light colonization-L_{col}), irradiance of incubation (Light incubation-L_{inc}) and biofilm sensitivity to Zn (Zn exposure-Zn) a three-way ANOVA test was applied. The analysis included three factors: light conditions during colonization, with three levels (LL, ML and HL), incubation light- with six levels (10, 25, 50, 100, 250 and 500) and Zn exposure with

two levels (0, 1500). The interactions between these factors were also evaluated. The SPSS software was used for all ANOVA analyses.

- **Principal Component Analysis (PCA) and clustering analysis**. To explore the ordination of biofilm samples according to photosynthetic pigments, a principal component analysis (PCA) was done using CANOCO software version 4.5 (Ter Braak and Smilauer, 2002). Comparison of photosynthetic pigments and diatom communities was done with cluster analysis (average linkage method and Bray-Curtis dissimilarity coefficient) by means of PRIMER-6 software (Clarke and Gorley, 2005). Before analysis, all data were square root transformed. Only diatom species representing more than 1% relative abundance were included. The Simprof test was used to test significant differences ($p<0.05$) between cluster groups.

- **Linear regression analysis**. To test the relationship between DR and NPQ during light and Zn toxicity tests, data of DR and NPQ were adjusted to a linear regression model according to Chevalier et al. (2010) by SigmaPlot software. When significant correlations between DR and NPQ were found ($p<0.05$), the obtained slope parameter (b) was used for comparing kinetics between biofilm communities

RESULTS

- **Water physical and chemical characteristics**

Physical and chemical conditions, nutrient concentrations as well as the major anions and cations remained stable during the colonization period and were similar between light colonization treatments ($p>0.05$, Table 2). Light intensity of each light colonization treatment (25, 100 and 500 µmols photons /m^2/s, respectively) remained stable along biofilm colonization, varying in all cases <10 %. Measured Zn concentrations during light and Zn toxicity tests were 14.78±14.47 µg Zn/L in the NoZn treatment (AVG±SD; n=3) and 1400.67±99.22 µg Zn /L in the Zn treatment (AVG±SD; n=3).

Table 2. Summary of physical and chemical characterization of water during the biofilm colonization period. LL: corresponds to light treatment of 25 µmols photons /m^2/s; ML: corresponds to light treatment of 100 µmols photons /m^2/s and HL: corresponds to light treatment of 500 µmols photons /m^2/s.

	LL	ML	HL	
	AVG ± SD	**AVG ± SD**	**AVG ± SD**	**n**
pH	8.38 ± 0.17	8.35 ± 0.19	8.37 ± 0.20	87
Cond (µS/cm)	409.2 ± 25.3	402.6 ± 23.9	404.2 ± 26.9	87
Oxy (mg/L)	9.23 ± 0.22	9.31 ± 0.31	9.30 ± 0.35	87
Temp (ºC)	17.72 ± 1.19	17.53 ± 1.37	17.62 ± 1.50	87
K^+ (mg/L)	5.18 ± 0.97	5.1 ± 0.66	5.07 ± 0.55	9
Ca^{2+} (mg/L)	121.9 ± 4.6	119.6 ± 15.8	119.6 ± 8.2	9
Mg^{2+} (mg/L)	21.99 ± 2.32	23.09 ± 3.64	22.68 ± 1.88	9
Cl^- (mg/L)	30.99 ± 3.23	29.68 ± 1.40	31.79 ± 2.52	9
SO_4^{2-} (mg/L)	56.08 ± 7.39	69.40 ± 37.28	59.63 ± 10	9
NO_3^- (mg/L)	0.55 ± 0.07	0.51 ± 0.04	0.55 ± 0.06	9
NO_2^- (mg/L)	0.11 ± 0.01	0.09 ± 0.03	0.11 ± 0.02	9
PO_4^{3-} (µg/L)	28.07 ± 25.81	28.04 ± 23.76	28.18 ± 23.72	24

- **Biofilm communities after the colonization period**

Based on ETR-I curve parameters (Table 3), LL and ML biofilms had similar initial slopes (α) which were significantly higher than those from HL biofilms (Table 3). LL and ML biofilms had similar semi-saturation irradiances (Ik) which were lower than those from HL biofilms (Table 3). Chl-a concentration was higher for LL biofilms than for ML and HL biofilms ($F=61.57$; $p<0.001$) (Fig.1a). The content of extracellular polymeric substances (EPS) was lower in LL biofilms than for ML and HL biofilms ($F= 61.57$; $p<0.001$) (Fig.1a). Total biofilm biomass was lower for LL biofilms than for ML and HL biofilms ($F= 72.96$; $p<0.001$) (Fig.1a) leading to an autotrophic index (Chl-a/AFDW) 10 times higher in LL biofilms than for ML and HL biofilms ($F= 10.93$; $p<0.001$) (Fig.1a).

Table 3. Photosynthetic parameters derived from ETR-I curves; α: initial slope, ETRm: maximal electron transport rate and Ik: the light saturation parameter. Each parameter corresponds to the AVG±SD; n=3. For each parameter, different letters indicate significant differences (p <0.05) between light colonization treatments (Tukey's HSD test from one-way ANOVA).n.s.: non significant differences between light colonization treatments.

Photosynthetic parameters	LL	ML	HL	ANOVA	
				$F_{(2, 6)}$	p value
α	0.18 ± 0.02^{a}	0.16 ± 0.02^{a}	0.02 ± 0.02^{b}	14.43	p<0.001
ETRm	26.48 ± 2.96	32.01 ± 12.32	30.03 ± 2.19	0.39	n.s.
Ik	144.6 ± 13.2^{a}	204.6 ± 50.5^{a}	314.6 ± 56.6^{b}	13.22	p<0.05

The relative abundance of algal groups differed between LL, ML and HL biofilms (Fig.1b). Diatoms, Fo(Br), were more abundant in LL biofilms than in ML and HL biofilms (F=14.19; p= p <0.001, one-way ANOVA). Green algae, Fo(Gr) were not detected in LL biofilms, and had a similar abundance in ML and HL biofilms (F=996.78; p= p <0.001, ANOVA one-way) (Fig. 1b).

Similar diatom richness (S) was measured for LL, ML and HL biofilms (F=0.56; p>0.05) (Fig. 1c). Diatom diversity (measured using the Shannon index) was also similar (F=0.41; p>0.05) (Fig.1c). Significant differences were observed on the relative abundance of some species (Fig.3). *Achnanthidium minutissimum* (Kützing) Czarnecki taxon was more abundant in HL biofilms than in LL biofilms (F= 7.66; p<0.05) (Fig.3). *Eolimna minima* (Grunow) Lange-Bertalot and *Fragilaria radians* (Kützing) D.M.Willians & Round taxa were more abundant in LL biofilms than in ML and HL biofilms (F= 12.49; p<0.05 for *E. minima* and F= 7.8; p<0.05 for *F. radians*, respectively). *Nitzschia amphibia* Grunow was the dominant taxon in LL biofilms with a comparable abundance in ML biofilms being higher than in HL biofilms (F= 14.77; p<0.05). *Nitzschia palea var. debilis* (Kützing) Grunow was the dominant taxon in ML biofilms and had a similar abundance in HL biofilms being more abundant than in LL biofilms ((F= 17.49; p<0.05). Overall, diatom species composition was different between LL biofilms and the rest of the treatments with a similarity <50 % (Fig. 3).

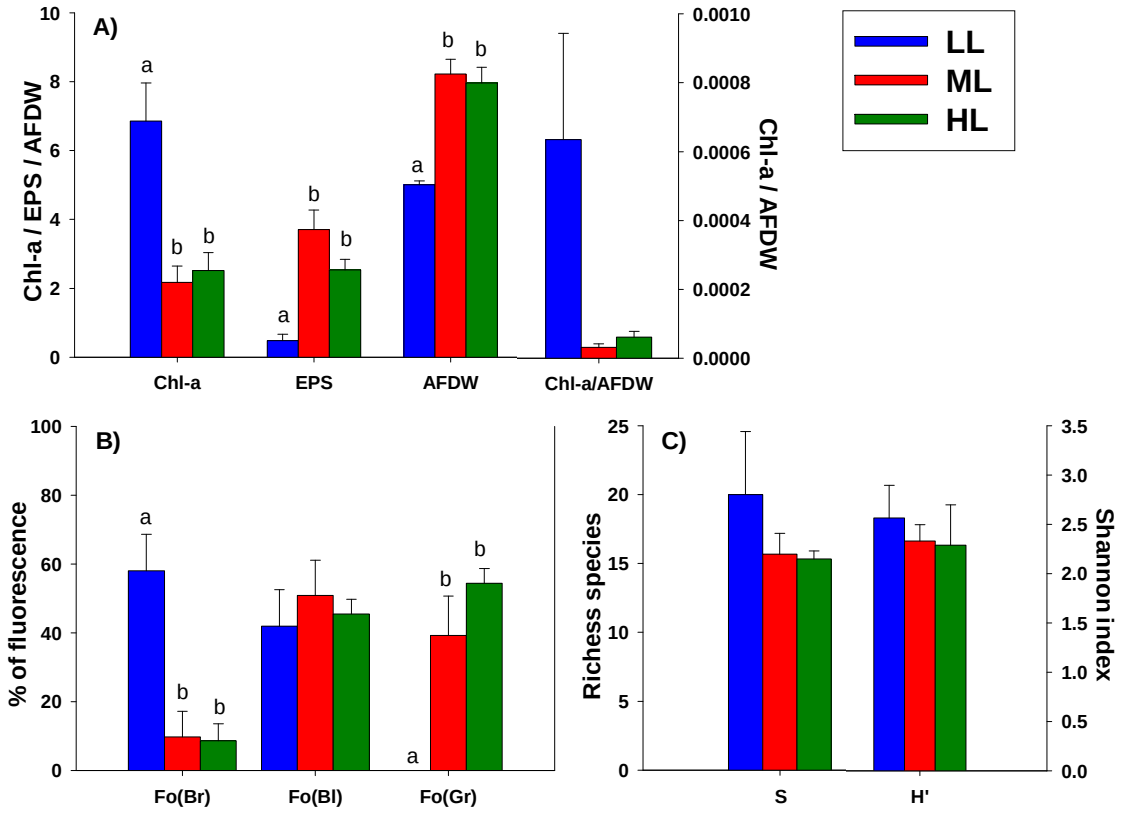


Figure 1. a) Chlorophyll-a concentration (Chl-a) in µgChl-a/cm^2, extracellular polymeric substances (EPS) in µgGlucosa eq./cm^2, ash free dry weight (AFDW) in gAFDW/cm^2 and chl-a/AFDW ratio in µgChl-a/µgAFDW cm^2 of LL, ML and HL biofilms. b) The relative abundance of each algal group based on Phyto-PAM fluorescence analysis. c) Shannon index (H') and richness species (S) based on diatom community. Each value corresponds to the AVG±SD; n=3. Different letters indicate significant differences ($p < 0.05$) between LL, ML and HL biofilms (Tukey's HSD test from ANOVA one way).

Photosynthetic pigments were also different between light colonization treatments (Fig.2). PCA first axis explained 57.3% of variance and separated LL biofilms with higher chl-a, chl-c1+c2, zeaxanthin and fucoxanthin pigments from ML and HL biofilms (presenting an intermediate and low relative abundance of these pigments, respectively). HL biofilms had higher relative abundance of pheophytin-a, diatoxanthin, diadinochrome, diadinoxanthin, violaxanthin and lutein pigments than ML and LL biofilms. The second axis of the PCA explained a 27.8 % of variance and separated ML from LL and HL biofilm communities (Fig. 2) with higher relative abundance of neoxanthin and unidentified pigments (others) (Fig.2). Cluster analysis

(Fig. 2) separated ($p < 0.05$) biofilm communities in two groups. LL biofilms (similarity <75 %) were different from ML and HL biofilms with a similarity between them >80% ($p<0.05$). In addition, a high similarity between replicates of each treatment was also obtained (above 88%) (Fig. 2).

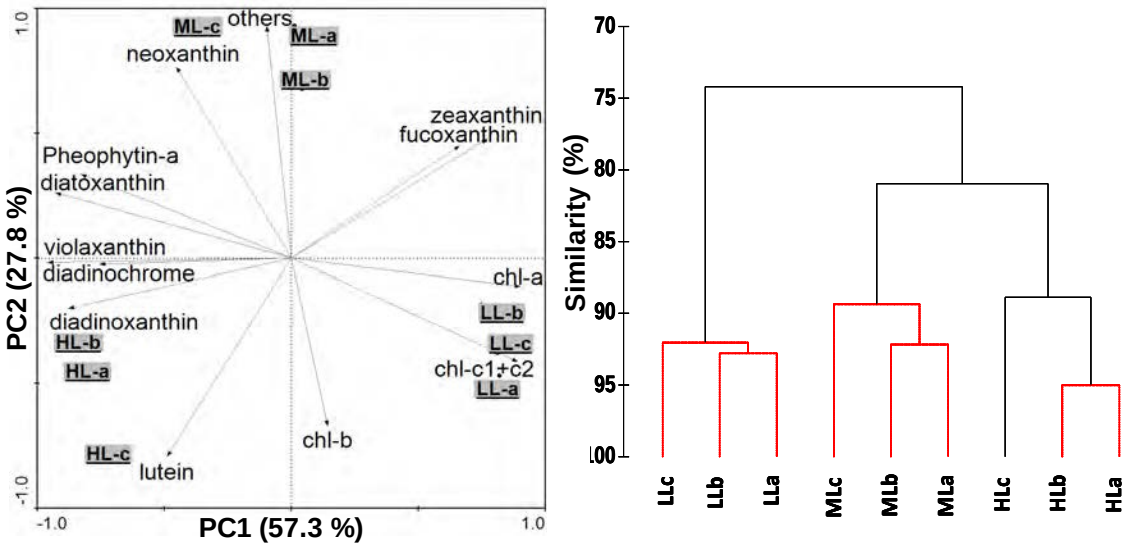


Figure 2. Left graph, PCA of photosynthetic pigments of biofilm samples from LL, ML and HL biofilms. Right graph, cluster of similarity distances between LL, ML and HL biofilm samples based on photosynthetic pigments. Red lines denote no significantly different groups and black lines indicate significantly different groups. Labels a, b and c indicate samples taken from three different channel belonging to the same light colonization treatment.

- **Light and Zn toxicity tests**

The results of the three-way ANOVA summarize the interdependence of the treatment factors (Light colonization-L_{col}, light incubation-L_{inc} and Zn exposure-Zn) (Table 4). LL, ML and HL biofilms had different Φ'_{PSII} , and Φ_{PSII} depending on the light of colonization, lower in LL biofilms (Fig. 4). Light of incubation affected Φ'_{PSII} and Φ_{PSII} of all biofilms (LL, ML and HL biofilms) but these effects were different depending on light colonization (L_{col}*L_{inc}; $p<0.05$). Clearly, LL and ML biofilms were more inhibited at high light (Table 4 and Fig. 4).

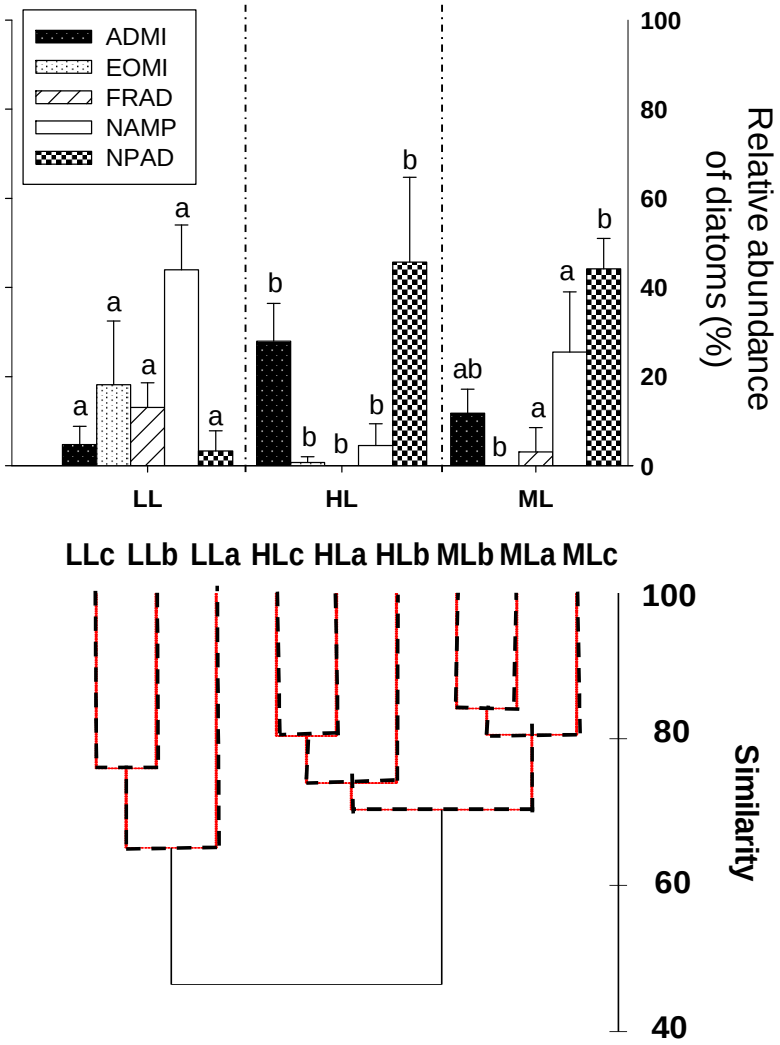


Figure 3. Upper panel, relative abundance of diatom taxa; *Achnanthidium minutissimum* (ADMI), *Eolimna minima* (EOMI), *Fragilaria radians* (FRAD), *Nitzschia amphibia* (NAMP) and *Nitzschia palea var. debilis* (NPAD). Each value corresponds to the AVG±SD; n=3. Different letters indicate significant differences ($p < 0.05$) between LL, ML and HL biofilms (Tukey's HSD test from ANOVA one way). Down panel, cluster of the corresponding similarity distances. Dashed lines denote not significantly different groups and solid lines denote significantly different groups. Note: a, b and c indicate samples taken from different channels belonging to the same treatment.

Table 4. Results of analysis of variance (ANOVA 3 ways) for Φ'_{PSII}: effective quantum yield, Φ_{PSII}: maximal quantum yield, NPQ: non-photochemical quenching and DR: de-epoxidation ratio of biofilm communities influenced by light during colonization -L_{col} (LL, ML and HL), by light irradiance of incubation - L_{inc} (10, 25, 50, 100, 250 and 500 µmols photons/m^2/s) and by zinc concentration-Zn (0 and 1500 µg/L). n.s.: non significant differences (p>0.05).

Source	Φ'_{PSII}		Φ_{PSII}		NPQ		DR	
	F_{value}	p_{value}	F_{value}	p_{value}	F_{value}	p_{value}	F_{value}	p_{value}
L_{col}	326.0	<0.001	33.01	<0.001	0.82	n.s.	2.14	n.s.
L_{inc}	899.9	<0.001	174.8	<0.001	187.1	<0.001	7.36	p<0.001
Zn	101.1	<0.001	18.19	<0.001	0.06	n.s.	18.28	p<0.001
L_{col} * L_{inc}	39.65	<0.001	13.06	<0.001	22.94	<0.001	1.51	n.s.
L_{col} * Zn	3.45	<0.05	13.48	<0.001	45.63	<0.001	18.28	p<0.001
L_{inc} * Zn	6.07	<0.001	0.49	n.s.	40.69	<0.001	9.78	p<0.001
L_{col} * L_{inc} * Zn	4.23	<0.001	4.72	<0.001	14.78	<0.001	5.31	p<0.001

Zn affected Φ'_{PSII} and Φ_{PSII} producing an inhibition which was higher on Φ'_{PSII} than on Φ_{PSII}. In addition, Zn inhibition differed depending on the light of colonization being of higher magnitude in LL biofilms in comparison to ML and HL biofilms. The Φ'_{PSII} of all Zn exposed biofilms (LL, ML and HL biofilms) was lower (23%, 10% and 6%, respectively) than in those non Zn exposed. Similar results were observed based on Φ_{PSII} but treatment effects were of lower magnitude. Zn effects also depended on the incubation light. This interaction was mainly observed at high light irradiances in LL biofilms, and at intermediate irradiances in ML and HL biofilms. The interaction between incubation light and Zn exposure was not significant (Linc*Zn; p>0.05) for Φ_{PSII} (Table 4 and Fig.4).

DR and NPQ had a positive relation in all non Zn-exposed biofilms (Fig.5). The slope was lower in HL biofilms, presenting lower values of NPQ than in LL and ML biofilms (Fig.5). This relationship was not observed in all Zn-exposed biofilms. It was due to a reduction of NPQ in LL biofilms and a reduction of DR in HL bioiflms (Fig. 5).

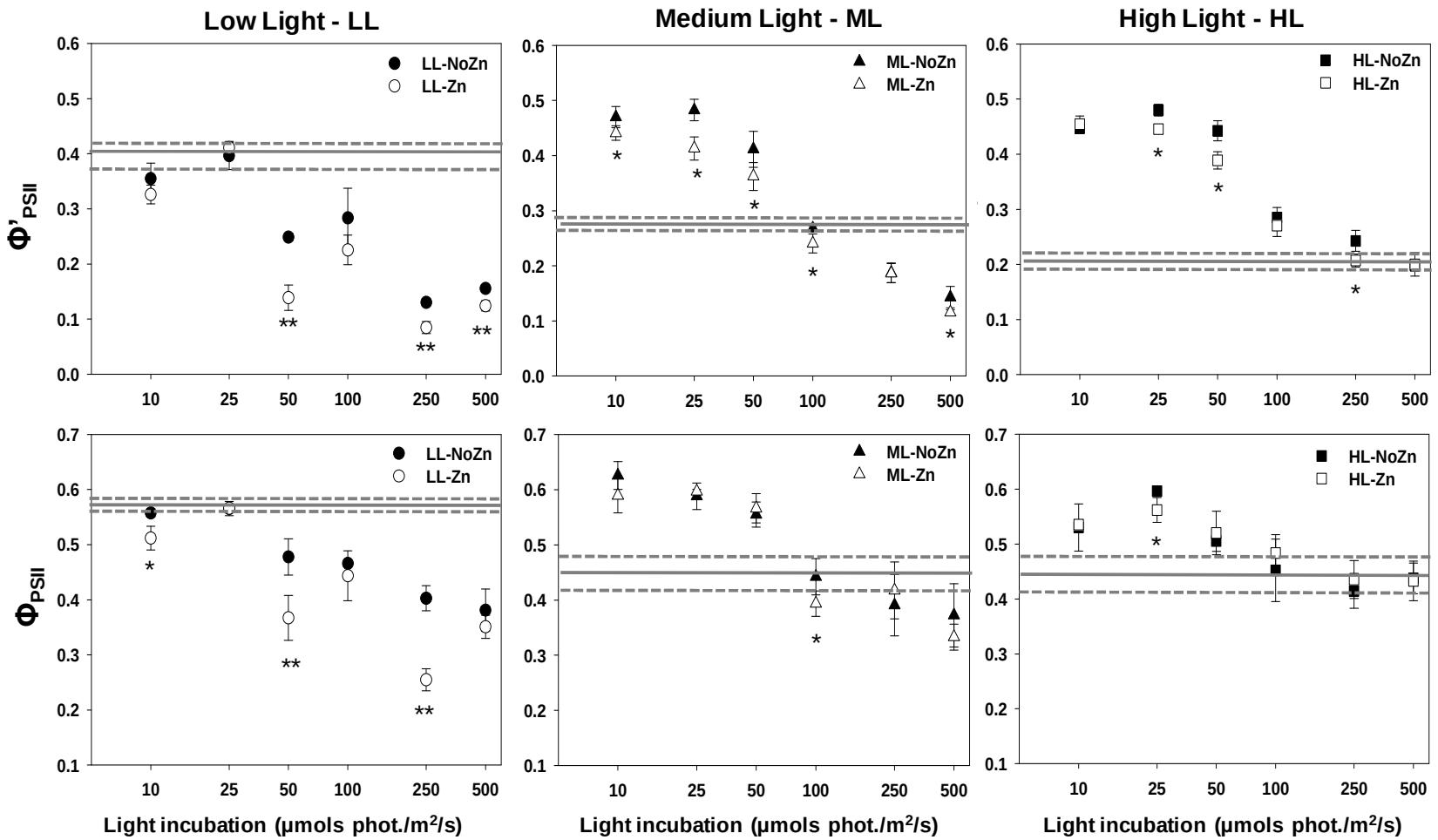


Figure 4. Φ'_{PSII}: effective quantum yield and Φ_{PSII}: maximal quantum yield after 6h of exposure of biofilm communities to different light intensities (10, 25, 50, 100, 200 and 500 µmols photons /m^2/s) without Zn addition (NoZn-black symbols) and with 1500 µgZn/L (Zn-white symbols). In all graphs, each value corresponds to the AVG±SD; n=3. A plain grey line indicates the control value and the dotted lines indicate their corresponding standard deviation. Significant differences between NoZn and Zn treatments for each light incubation are refered with *for $p<0.05$ and ** for $p<0.001$ of the one-way ANOVA.

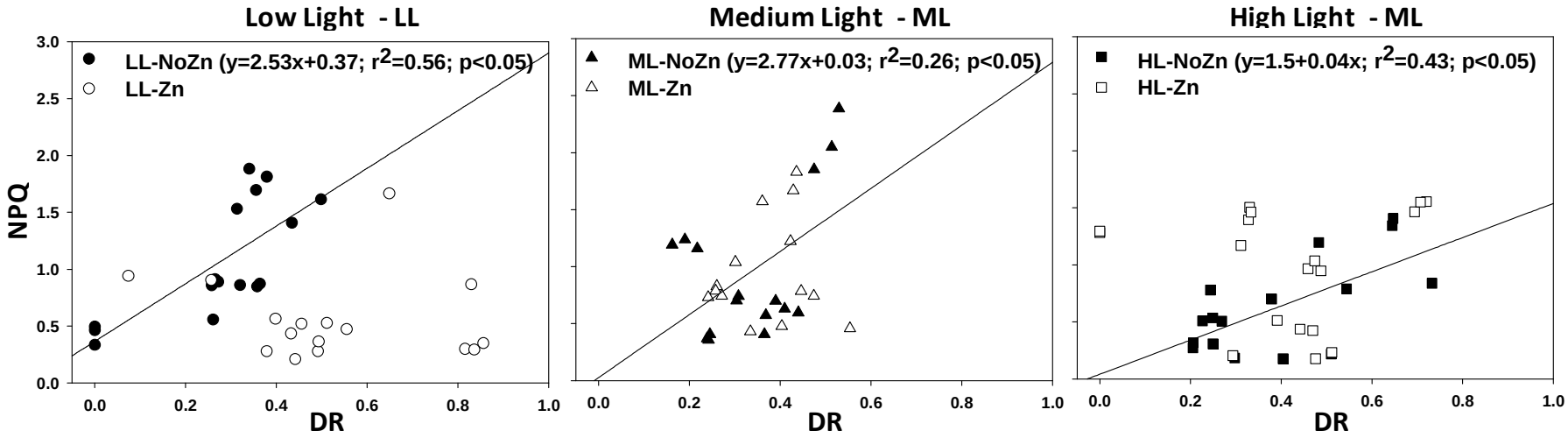


Figure 5. NPQ processes as a function of de-epoxidation reaction (DR) after 6h of exposure of biofilm communities (LL, ML and HL biofilms) to different light intensities without Zn addition (NoZn-black symbols) and with 1500 µgZn/L (Zn-white symbols). Black solid line denote significant linear regression ($p<0.05$) between NPQ vs DR for NoZn exposed communities.

DISCUSSION

The microcosm experiment performed allowed us to obtain three different biofilm communities adapted structurally and physiologically to different light conditions. A biofilm community adapted to shade conditions (a low light intensity of 25 µmols photons/m^2/s: LL biofilms); and two others adapted to sun conditions (a saturating light intensity of 100 µmols photons/m^2/s: ML biofilms and a high light intensity of 500 µmols photons/ m^2/s: HL biofilms). All of them reached comparable values of maximal ETR after four weeks of colonization, indicating that each community could achieve a similar efficiency to use of light energy growth but differing in their specific optimal light conditions.

The different physiological and structural attributes observed in LL, ML and HL biofilms allowed them to optimize the use of light. It is reported that organisms can acquire tolerance to light conditions mainly through two mechanisms: (1) physiological acclimation via existing cellular and enzymatic processes and (2) genetic adaptation via mutation and selection of genes that modify physiological functions (Klerks and Weis, 1987; Posthuma and van Straalen, 1993). Several generations of algae and cyanobacteria were expected to occur during biofilm growth period due to their fast division cycle. In fact, either physiological or structural attributes were affected, suggesting that eventually both photo-accclimation and photo-adaptation occurred within the community. Indeed, the light selection pressure caused a clear change in the species composition, with the removal of some species and the selection of others, actually changing the gene pool of the community. Therefore we can conclude that the communities were photodapted.

Biofilms adapted to shade conditions were dominated by diatoms, probably linked with their high efficiency in the use of low light (DeNicola, 1996; Hill, 1996). In

contrast, sun-adapted communities had more green algae, in agreement with previous investigations where their high efficiency in the use of high light intensities was demonstrated (DeNicola, 1996; Hill, 1996). The abundance of cyanobacteria was comparable between photo-adapted communities, suggesting that this phototrophic group is efficient in light utilization in a wide range of light intensities (from 25 to 500 µmols photons/ m^2/s). Similar differences in community composition were also reported in other studies based on natural biofilms (Guasch and Sabater, 1998; Barranguet et al., 2005). Diatom community structure changed considerably in response to light conditions during growth, in agreement with other studies performed under experimental conditions (Liess et al., 2009; Lange et al., 2011).

In agreement with our expectations, differences in the efficiency of light utilization at different light intensities were observed between these photoadapted communities. Algae photo-adapted to limiting and saturating light intensities were more efficient in light utilization than those adapted to high light irradiances, as shown with the initial slope of the P-I curve. In addition, higher light irradiance was needed to produce saturation (Ik) on algae adapted to high light than those adapted to saturating or limiting light conditions. These results are in agreement with other studies performed with natural biofilms (Guasch and Sabater, 1998; Roberts et al., 2004; Laviale et al., 2009), in which the differences in photosynthesis characteristics and/or sensitivity were attributed either to physiological differences of some particular species within the community or taxonomic differences in community composition. Furthermore, different mechanisms of photoadaptation between communities were observed based on biofilm structure and pigment composition (EPS, AFDW, chla/AFDW). Sun-adapted biofilms had higher EPS and total biomass indicating a higher proportion of non-photosynthetic structures. An increase in the proportion of EPS content under high light conditions was also described by Brouwer and Stal (2002), and attributed to a product of overflow

metabolism when algae and cyanobacteria grew under non limiting light irradiances. Focusing on pigment composition, shade adaptation was clearly shown as a higher amount of chl-a per unit of biomass. It is reported that shade-adapted plants generally have a higher content of photosynthetic pigments than light-adapted plants of the same species (Björkman, 1981), and the reaction centre of each PSII becomes associated with more light-harvesting chlorophyll-a which contribute to a higher susceptibility to photoinhibiton at high irradiances (Falkowski, 1981). Similar results were described for shade-adapted biofilms in the field (Roberts et al., 2004). In contrast, sun-adapted biofilms adjusted their intracellular photosynthetic pigments differently in response to light (Kana et al., 1997). ML and HL biofilms produced higher amounts of xanthophyll pigments (diatoxanthin, diadinoxanthin, violaxanthin, diadinochrome) as well as lutein and pheophytin-a. Xanthophyll and lutein pigments compose the main accessory pigments of the light harvesting complex (LHC) and their increase is used to avoid PSII damage and to generate the reactive oxygen species when the excited energy is too high (Demmig et al., 1987). An increase in xanthophylls has been observed in biofilms exposed to increasing light irradiances (Laviale et al., 2009) and in macroalgae exposed to high light irradiances (Nielsen et al., 2010).

As expected, a sudden increase or decrease in light (differing from light conditions prevailing during biofilm growth) produced a fast change in photosynthetic processes (Φ'_{PSII} and Φ'_{PSII}) and in photoprotective mechanisms (NPQ and DR) for all biofilm communities. These quick physiological responses were attributed to the phenotypic plasticity of algae and cyanobacteria composing biofilms to maximize photosynthesis but also to protect the photosynthetic apparatus from light stress. It has been extensively reported that NPQ processes play an important photoprotective role in dissipating the excess of energy caused by a sudden increase in light (Lavaud et al., 2004; Niyogi et al., 2005). Accordingly, we observed that all biofilm communities showed a dependence of DR and NPQ processes, suggesting that these physiological

mechanisms were relevant for oxygenic photosynthesis organisms composing biofilms (i.e. algae and cyanobacteria) to cope with the effects of light on Φ'_{PSII} and Φ_{PSII}. Biofilms photo-adapted to low and medium light intensities presented a higher slope derived from DR vs NPQ linear regressions than biofilms photo-adapted to high light irradiances (Fig. 4), suggesting that the resulting slope of the correlation between DR vs NPQ could be an indicator of biofilm photoadaptation to shade or sun conditions.

- **The role of light on Zn toxicity**

In agreement with our hypothesis, light history played an important role in the sensitivity of biofilms to Zn exposure. Zn toxicity on Φ'_{PSII} and Φ'_{PSII} was higher in biofilms photo-adapted to shade conditions (23 and 16%, respectively) than those adapted to higher light conditions (10% and 3%, respectively for ML biofilms and 6% and 1%, respectively for HL biofilms). This indicates that the structural and functional photoadaptation mechanisms developed for ML and HL biofilms contributed to reduce their sensitivity to Zn exposure. We hypothesize that some of the mechanisms developed for photo-adaptation to saturating and high light irradiance might be responsible for the observed Zn tolerance. An effect of photo-adaptation to sun conditions was the increase in biofilm thickness by developing more EPS and total biomass (EPS and AFDW were 3 and 1.6, respectively, times higher in ML and HL biofilms than in LL biofilms), which could play a protective role against Zn toxicity, reducing metal penetration into cells. EPS may reduce metal toxicity because they contain high amounts of negatively-charged functional groups such as carboxyl, phosphate, and sulphate groups, acting as metal-binding sites (Kaplan et al., 1987; Loaec et al., 1997; Admiraal et al., 1999; García-Meza et al., 2005; Serra et al., 2009b). Furthermore, it is also known that community composition plays a relevant role in the sensitivity of biofilm to metal exposure. Previous studies reported higher tolerance to metal exposure for green alga and cyanobacteria than for diatoms (Admiraal et al., 1999; Guasch et al., 2004). Hence, another factor that could explain why sun-adapted

biofilms (ML and HL biofilms), which were dominated by green algae and cyanobacteria, were less sensitive to Zn than shade-adapted biofilms (LL biofilms) which had more diatoms. Finally, according to our hypotheses another factor that could influence the observed lower sensitivity of sunadapted biofilms to Zn exposure in comparison to shade-adapted biofilms is the content of xanthophyll pigments. We suggest that the observed photoprotective role of xanthophyll pigments in sun-adapted biofilms (Guasch and Sabater, 1998; Laviale et al., 2009; Chevalier et al. 2010) may also provide a protection to Zn exposure by avoiding Zn photo-inhibitory effects by blocking light-dependent photosynthesis reactions, which are reported to occur under light or chemical stressors (Armstrong and Hearst, 1996; Martínez-Abaigar and Núñez-Olivera, 1998). Nielsen et al. (2010) observed that Cu inhibition on the PSII of the macroalgae *Fucus serratus* was lower in algae adapted to high light irradiances than in algae adapted to low light irradiances. They attributed these differences to a lower light-harvesting potential associated with each reaction centre in algae adapted to high light.

This study showed that Zn toxicity was enhanced in a light-stress situation. In fact, Zn exposure caused a magnification of light inhibitory effects on photosynthesis activity, suggesting that both stressors (light and Zn) target the PSII photochemical reactions. In algal cultures, the decrease of chlorophyll fluorescence in response to metal exposure has been correlated with chlorophyll damage (Küpper et al. 2002; Nielsen et al. 2010). Furthermore, in agreement to our hypothesis, at the level of photoprotective mechanisms, it was clearly shown that Zn produced a decoupling of DR vs NPQ at increasing light intensities indicating a loss of biofilm phenotypic plasticity to cope with light stress. It suggests that the xanthophyll cycle mediated NPQ (Demming-Adams, 1998; Li et al. 2000) is also a target for Zn toxicity. This decoupling could be linked with Zn damage effects on some factors influencing NPQ processes; (1) the formation of a proton gradient across the thylakoid membrane (ΔpH) and/or also

(2) the operation xanthophylls cycle (e.g. de-epoxidation reaction (DR) in diatoms) (Lavaud et al., 2004). In algal monocultures, Nielsen et al. (2005) also observed that Cu toxicity on the marine macroalgae *Fucus serratus* caused an un-coupling between NPQ and the total pool of xanthophylls.

The interaction between light stress and Zn exposure showed clear adverse effects on the photosynthetic processes of fluvial biofilms. Besides, biofilm sensitivity depended on its light history. These results suggest that in natural ecosystems where metal pollution occurs along with other environmental stressors (e.g. light irradiance), metal toxicity could be enhanced. Furthermore, it could be expected that in river sites where canopy vegetation is well developed, generally at headstreams, the co-occurrence of light stress and Zn exposure could cause more important damaging effects on biofilms than in open canopy sites, generally situated at the mid or lower sections. This study highlights the need to account for environmental stress caused by such variations (i.e. light regime and light stress) in order to better assess the environmental risk of chemicals.

CONCLUSIONS

Overall, this experimental study shows that the sensitivity of biofilms photoadapted to low light exposed to a multi-stress situation (i.e. light stress and Zn exposure) was higher than in biofilms photoadapted to saturating and high light conditions. Hence, it is suggested that in natural ecosystems it will be necessary to take into account the light history of biofilms to better understand the effects of multiple stressors (i.e. light stress and Zn toxicity). Therefore, the effects of a multistress situation (i.e. light stress and Zn toxicity) on upstream biofilms photo-adapted to a closed canopy cover will be higher than on downstream biofilms photo-adapted to an open canopy cover.

Suplementary table 1. Diatom community composition (species relative abundance in %) of biofilm communities colonized at different light conditions: at 25 µmols photons/m^2/s-LL biofilms, at 100 µmols photons/ m^2/s-ML biofilms and at 500 µmols photons/ m^2/s-HL biofilms.

	LL biofilms		ML biofilms		HL biofilms	
	AVG	(±SE)	AVG	(±SE)	AVG	(±SE)
Achnanthidium minutissimum (Kützing) Czarnecki	4.93	(2.20)	11.80	(3.13)	27.94	(4.91)
Achnanthidium catenatum (Bily & Marvan) Lange-Bertalot	0.10	(0.10)	0.00	(0.00)	0.00	(0.00)
Achnanthidium lanceolatum Grunow	0.21	(0.21)	0.00	(0.00)	0.11	(0.11)
Achnanthidium lineare Grunow	0.84	(0.84)	2.18	(0.79)	2.97	(0.18)
Achnanthidium straubianum Lange-Bertalot	1.60	(0.73)	2.72	(0.10)	2.13	(0.90)
Amphora indistincta Levkov	0.32	(0.19)	0.00	(0.00)	0.15	(0.15)
Amphora pediculus (Kützing) Grunow	0.74	(0.28)	0.44	(0.29)	0.00	(0.00)
Cocconeis pediculus Ehrenberg	0.10	(0.10)	0.00	(0.00)	0.15	(0.15)
Cocconeis placentula var. lineata Ehrenberg	0.00	(0.00)	0.00	(0.00)	0.15	(0.15)
Encyonema minutum (Hilse *in* Rabh.) D.G. Mann	0.00	(0.00)	0.22	(0.22)	0.00	(0.00)
Eolimna minima (Grunow) Lange-Bertalot	18.18	(8.26)	0.61	(0.31)	1.05	(0.59)
Fragilaria capucina Desmazières *var. capucina*	0.84	(0.84)	0.00	(0.00)	0.00	(0.00)
Fragilaria construens (Ehrenberg) Grunow *f.venter* (Ehrenberg) Hustedt	0.43	(0.21)	0.22	(0.22)	0.00	(0.00)
Fragilaria gracilis Østrup	0.00	(0.00)	0.11	(0.11)	0.64	(0.38)
Fragilaria pararumpens Lange-Bertalot, Hofmann & Werum	4.11	(1.90)	0.51	(0.18)	2.61	(0.81)
Fragilaria radians (Kützing) Williams & Round	13.04	(3.22)	3.35	(3.03)	0.39	(0.26)
Gomphonema exilissimum (Grunow) Lange-Bertalot & Reichardt	2.33	(0.93)	0.69	(0.16)	3.00	(1.21)
Gomphonema minutum (C.Agardh) C.Agardh	0.11	(0.11)	0.00	(0.00)	0.00	(0.00)
Gomphonema parvulum Kützing	0.52	(0.52)	4.32	(1.37)	5.78	(1.57)
Gomphonema parvulum var.saprophilum Lange-Bertalot & Reichardt	0.00	(0.00)	0.33	(0.33)	0.00	(0.00)
Gyrosigma aff. nodiferum (Grunow) Reimer	0.11	(0.11)	0.00	(0.00)	0.00	(0.00)
Halamphora veneta (Kützing) Levkov	0.10	(0.10)	0.15	(0.15)	0.00	(0.00)
Melosira varians Agardh	0.43	(0.11)	0.22	(0.22)	0.91	(0.44)
Navicula cryptotenella Lange-Bertalot	0.75	(0.22)	0.54	(0.29)	0.20	(0.20)
Navicula gregaria Donkin	0.00	(0.00)	0.00	(0.00)	0.00	(0.00)
Navicula reichardtiana Lange-Bertalot	0.43	(0.29)	0.00	(0.00)	0.00	(0.00)
Navicula tripunctata (O.F.Müller) Bory	0.00	(0.00)	0.11	(0.11)	0.00	(0.00)
Navicula veneta Kützing	0.64	(0.32)	0.00	(0.00)	0.00	(0.00)
Nitzschia amphibia Grunow	43.96	(5.82)	25.52	(7.77)	4.54	(2.82)
Nitzschia dissipata (Kützing) Grunow	0.53	(0.21)	0.11	(0.11)	0.53	(0.29)
Nitzschia fonticola Grunow	0.00	(0.00)	0.15	(0.15)	0.00	(0.00)
Nitzschia frustulum var. inconspicua Grunow	0.32	(0.18)	0.87	(0.22)	0.80	(0.25)
Nitzschia media Hantzsch	0.52	(0.38)	0.00	(0.00)	0.00	(0.00)
Nitzschia palea var. debilis (Kützing) Grunow	3.58	(2.47)	44.21	(3.92)	45.72	(10.96)
Nitzschia recta Hantzsch	0.00	(0.00)	0.00	(0.00)	0.11	(0.11)
Nitzschia sociabilis Pringsheim	0.10	(0.10)	0.00	(0.00)	0.00	(0.00)
Nitzschia umbonata Hustedt	0.00	(0.00)	0.15	(0.15)	0.00	(0.00)
Rhoicosphenia abbreviata (C.Agardh) Lange-Bertalot	0.10	(0.10)	0.00	(0.00)	0.11	(0.11)
Synedra ulna (Nitzsch) Ehrenberg	0.00	(0.00)	0.51	(0.26)	0.00	(0.00)

CHAPTER VII

GENERAL DISCUSSION

GENERAL DISCUSSION

With the aim of evaluating the potential use of the chl-a fluorescence parameters of fluvial biofilm for assessing metal toxicity in fluvial ecosystems and to find cause-effect relationships between metal(s) exposure, confounding factors (i.e. light) and biological responses, several experimental approaches have been performed at different spatial and temporal scales. Experiments conducted in microcosms (using a system of artificial channels) helped us to establish cause-effect relationships between metal exposure and biological responses under controlled conditions and foresee the effect of single stress factors in the environment (Chapter IV and Chapter VI) (Fig. 1). On the other hand, the field experiment in the riera d'Osor stream allowed us to test *in situ,* under multiple-stress situations, the cause-effect relationships previously described (Chapter V) (Fig. 1). The field sampling conducted in Riou-Mort river did not allow us to find cause-effect relationships between metal exposure and biofilm responses (Fig. 1) suggesting that it is not a good kind of approach to assess metal pollution *in situ*. Maybe the lack in cause-effects relationship in the Riou-Mort could be linked to other confounding factors as light irradiance or nutrient availability that may mitigate biofilms´ responses to Zn+Cd contamination. Causality, metal toxicity and biofilm responses, was higher in experimental testing studies than in field observation study (Fig. 1).

The duration of metal exposure was shown to be an important factor to explain metal toxicity. Within hours of exposure (referred to as short-term metal exposure) biofilms suffered mainly functional alterations (in the Φ'_{PSII} and in the xanthophyll cycle), indicating temporary alterations. Responses occurring after several weeks of exposure (referred to as long-term exposure) were linked, in most cases, with changes in the structure of the community (algal group composition, algal biomass, total biomass and/or diatom community) leading to more persistent alterations. Zn accumulation in

biofilm was shown to be a good indicator of metal exposure (level of exposure and time) and it was linked with Zn toxicity on biofilm. In addition, Zn toxicity was also demonstrated in environmental concentrations, as well as the influence of light history on the responses of fluvial biofilm to Zn exposure under a multi-stress situation (i.e. light stress and Zn exposure).

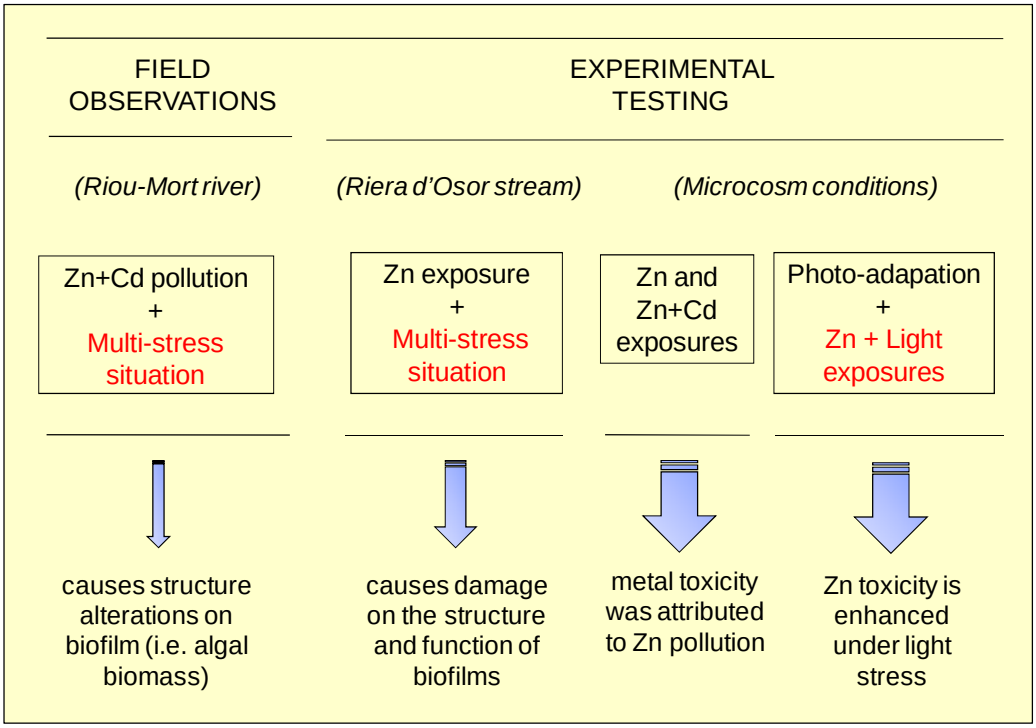


Figure 1. Graph illustrating the steps conducted in this thesis to derive causality and the main results obtained. The arrow width is proportional to the degree of causality.

- **<u>The sensitivity of chl-a fluorescence parameters of biofilm to metal toxicity</u>**

The experiments performed showed differences in metal toxicity sensitivity between chl-a fluorescence parameters and also between times of exposure, providing a set of biofilm biomarkers with different sensitivities and specificity. Focusing on the functional parameters investigated, the effective quantum yield (Φ'_{PSII}) was the most sensitive parameter for detecting the effects of Zn on algae and cyanobacteria composing biofilm after a short-term exposure (few hours). Its sensitivity was observed

both in microcosms and field experiments. In microcosms, Zn concentration in water ranged from 292 to 342 µgZn/L, similar to the one recorded at the highest Zn polluted site (M2) in the riera d'Osor stream (from 246 to 334 µgZn/L). Lower Zn concentrations in water were detected in the less polluted sites (M1 and M3) of the riera d'Osor stream, which ranged from 10 to 40 µgZn/L. In the microcosm experiments, Φ'_{PSII} was 23% below controls during the first 72h of Zn exposure. However, in the field, Φ'_{PSII} effects were only detected after 6h of the translocation. Φ'_{PSII} was 46% lower both in M2 and M3 than at the Up_{stream} site (reference site), while in M1 Φ'_{PSII} was 22% lower. Although, different magnitude of Zn effects on Φ'_{PSII} were observed between microcosm and field studies, all of them indicate negative effects of Zn on biofilm Φ'_{PSII} after short-term exposure at concentrations from 10 to 342 µgZn/L supporting its use for the assessment of short-term metal toxicity. A reduction of the Φ'_{PSII} indicates that the toxicant is reducing electron flow in the PSII (Barranguet et al., 2000, 2003; Juneau et al., 2002). This decrease is expected to lead to a reduction in the photosynthetic activity, one of the main functions of algae and cyanobacteria for growth.

The sensitivity of Φ'_{PSII} after long-term metal exposure (several weeks) was less obvious. This lack of sensitivity was attributed to biofilm tolerance acquisition. It may therefore be necessary in long-term studies, both in field as in laboratory conditions, use supplementary methodological approaches, such as Pollution Induced Community Tolerance (PICT) tests based on $\Phi'_{PSII,}$ to demonstrate community adaptation (Tlili et al. 2011) Briefly, the replacement of sensitive species by tolerant ones driven by the selection pressure of the toxicant is expected to increase the EC_{50} of the community measured in acute dose-response tests using physiological endpoints such as Φ'PSII (Blanck et al., 1988**).** This approach has been successfully applied to assess community adaptation to long-term chemical exposure in several studies (Schmitt-Jansen and Altenburger, 2005a, 2008; Tlili et al., 2010). In this investigation, community tolerance induction was addressed through changes in community

composition. A pollution-tolerant community is expected to have either a different genotype, strain and/or species composition compared to a sensitive community (Eriksson et al., 2009). Detailed results are fully explained in the section focused on algal group composition.

The maximal photosynthetic capacity of biofilms (Φ_{PSII}) was less sensitive to short-term metal exposure (hours, days) than the effective quantum yield (Φ'_{PSII}). It has been reported in monoculture bioassays that short-term metal exposures on algae cause firstly an inhibition of photosynthetic electron transport leading to a decrease in Φ'_{PSII}. And secondly, if the exposure persists throughout time, damage on the PSII structure occurs leading to a decrease in Φ_{PSII} (Juneau et al., 2002; Geoffroy et al., 2007). On the other hand, this parameter was more sensitive than Φ'_{PSII} after long-term metal exposure in microcosms showing the damaging effects on the structure of the photosynthetic apparatus. The Φ_{PSII} is an extremely sensitive parameter to alterations of PSII/LHCII complex in the thylakoids (Rosenqvist and Van Kooten, 2003). Other authors reported similar effects on Φ_{PSII} when biofilms were exposed to chemicals at high concentrations or during long-term exposure (García-Meza et al., 2005; Schmit-Jansen et al., 2005a). However, Φ_{PSII} changes were not attributed to long-term metal exposures (weeks) in the field, indicating that this parameter is not specific to metal toxicity, so the co-occurrence of other environmental factors (e.g. light stress, temperature, organic matter, and so on) could influence the Φ_{PSII} response, masking the effects caused by metal exposure. We may conclude that the use of both photosynthetic parameters, Φ'_{PSII} and $\Phi_{PSII,}$ allowed complementary information to be obtained, including structural and functional alterations related to photosynthetic processes.

The non-photochemical quenching parameter (NPQ) provided information about metal effects on photoprotective mechanisms of oxygenic photoautotrophic organisms composing biofilms. NPQ sensitivity was restricted to experiments performed in

controlled conditions (microcosm conditions). Furthermore, NPQ did not show the expected activation after short-term metal exposure in the microcosms. This unexpected result may probably be due to the fact that NPQ activation is restricted to a very specific period of time, from minutes to a few hours after the stress started (Müller et al., 2001), which has not been recorded in our experiments. Laviale et al. (2010) observed in microcosm experiments that the herbicide isoproturon caused a reduction of NPQ when biofilm was exposed during few hours to high light irradiances over a simulated daily cycle. The observed NPQ decrease was correlated with a decrease in de-expoxidation from diadinoxanthin to diatoxanthin, which was attributed to a state of relaxed proton gradient across the thylakoid membrane resulting from isoproturon inhibition on the electron transport (Laviale et al., 2010). On the other hand, in this thesis, NPQ showed clear effects after long-term metal exposure in the microcosms (Chapter IV). Metal exposure caused a marked reduction of NPQ suggesting damage on accessory pigments (xanthophylls) where NPQ processes occurred (Müller et al., 2001). Therefore, a decrease in NPQ was indicative of damaging effects on the pigmentary structure of the photosynthetic apparatus. Similar effects were observed in Ricart et al. (2010b). They also observed a marked reduction in NPQ when biofilm was exposed to high concentrations of the bactericide triclosan for 48h, damage being expected in the pigments where the NPQ takes place. In the field experiments, this parameter was more sensitive to physical and chemical variability (Chapter VI) than to metal toxicity, indicating a low specificity to metal exposure. Hence, the use of NPQ is mainly recommended for ecotoxicological experiments performed in laboratory conditions, providing complementary information about photosynthesis processes to the more classical photosynthetic related parameters (Φ'_{PSII} and Φ_{PSII}).

On the other hand, chl-a fluorescence parameters of biofilms were demonstrated to be useful tools for monitoring alterations in photosynthetic processes and photoprotective mechanisms under a multi-stress situation (i.e. light stress and Zn

exposure). Zn toxicity under light stress caused an inhibition of photosynthetic processes (Φ'_{PSII} and Φ_{PSII}) and an inhibition of NPQ linked with a decoupling with the xanthophyll cycle of diatoms. So, in this investigation the pertinence of measuring chl-a fluorescence parameters to assess effects of multiple-stress factors (i.e. light stress and Zn toxicity) was demonstrated (Chapter V).

The minimal fluorescence parameter (Fo) was applied for monitoring algal biomass changes over time and used to describe the kinetics of growth in biofilms under different metal exposure conditions (Chapter IV). The kinetics of growth summarizes the integrated response of biofilms throughout the entire metal exposure period. It can be considered as a global indicator, which integrates effects of metals on different metabolic processes causing pigment damage and/or cell death. The effect of metals on the growth curves was very marked. Biofilms exposed to metals reached plateau values up to three times lower than non metal-exposed biofilms (considered as controls). Serra et al. (2009a) also found that biofilms exposed to Cu reached a plateau state (in terms of Fo) earlier than controls.

The deconvolution of the Fo signal into the contribution of the main autotrophic groups - cyanobacteria Fo(Bl), green-algae Fo(Gr) and diatoms Fo(Br) - allowed the effects of metals on the algal groups composing biofilms to be easily evaluated (structural approach) (Chapter IV). As expected, metal effects on algal composition were only observed after long-term exposure. While diatoms were the most sensitive group, cyanobacteria and green algae showed a higher tolerance to metals. Other studies have already reported metal resistance of green algae to Zn (Ivorra et al., 2000) as well as resistance of cyanobacteria to Zn (Genter et al., 1987) and Zn mixtures (Takamura et al., 1989). Serra et al. (2009) also observed a higher tolerance of cyanobacteria than green algae or diatoms, when biofilms were chronically exposed to Cu.

- **Zn toxicity on biofilm communities and the occurrence of Zn pollution in fluvial ecosystems**

In this investigation the potential impact of an input of Zn at realistic concentrations (300 - 400 µgZn/L) on fluvial ecosystems was observed *in situ* in a Zn metal-polluted river (the riera d'Osor stream) (Chapter V) by performing a biofilm translocation experiment, and validated under controlled exposure conditions in microcosms (Chapter IV). The most challenging issue in field studies is the ability to discriminate biological responses caused by chemical pollution from those caused by environmental factors (also named confounding factors), which generally account for a higher significance and/or a higher explained variance (Leira and Sabater, 2005; Ricart et al., 2010a). The results obtained in the study performed in the riera d'Osor stream (Chapter V), pointed out the pertinence of using multivariate analysis techniques to indicate biological responses attributable to chemical toxicity or other environmental stress factors. Zn toxicity on biofilms was linked to Zn accumulation in biofilms. Changes in Zn accumulation were attributed to exposure level (Zn concentration in water) and time of Zn exposure (increasing over time). Zn toxicity on biofilms caused an inhibition of Φ'_{PSII} (either in the microcosms or in the field) and an activation of the xanthophyll cycle (reported in the field) after short-term exposure. When these functional damages took place in biofilms, Zn accumulation (both in microcosms and in field studies) ranged between 150 and 450 µgZn/gDW. Therefore, this range of Zn accumulation in biofilms could be considered high enough to cause negative effects in biofilm function (Fig.1). Accordingly, from the results obtained a toxicity threshold based of 150 µgZn/gDW (Fig.1) is suggested, based on Zn accumulation in biofilm. The concentration of Zn in water obtained in Zn-exposed channels during the microcosm experiment (Chapter IV) was comparable to that observed in the highest polluted site of the riera d'Osor stream (M2) (Chapter V), being in both cases around 300 µgZn/L. In contrast, Zn concentration in low metal polluted

sites of the riera d'Osor stream (M1 and M3) were one order of magnitude lower, between 10 and 40 µgZn/L. In spite of the fact that Zn concentrations in water differed between samplings sites, all biofilms accumulated comparable amounts of Zn and similar toxic effects were observed after short-term exposure. These results highlight that metal accumulation in biofilm may reflect real metal exposure and toxicity better than water concentrations. According to multivariate variance partitioning analysis performed with the field study data (Chapter V), Zn bioaccumulation was significantly discriminated by the Zn concentration in water, as well as by the other metals, and was the main chemical factor correlated with biological responses observed after short-term metal exposure. In agreement, Morin et al. (2008) demonstrated that Zn and Cd accumulation in biofilm caused damaging effects on the diatom community. However, Zn and Cd accumulation in biofilm were not significantly correlated with Zn and Cd concentrations in stream water. Bradac et al. (2010) showed that Cd accumulation in biofilm was pertinent for reflecting dynamic Cd concentrations in stream water, in this case due to the dilution factor of rain events.

Chronic inputs of metals in nature may happen in mining areas with continuous point-source metal pollution inputs into the river (e.g. the riera d'Osor stream, our case of study). In this investigation based on microcosm and field data, it was noticed that biofilms exposed for a long-term period (weeks) to Zn (from 20 to 300 µgZn/L) accumulated significant amounts of Zn (between 500 and 1700 µgZn/gDW) leading to a combination of functional and structural changes (Fig. 2). Thus, harmfull effects on the biota are expected above the 20 µgZn/L threshold of chronic exposure. A cause-effect relationship between Zn accumulation and alteration on the NPQ and Φ_{PSII} parameters was demonstrated in the microcosm experiment but not detected in the field. As reported above, this lack of agreement might be due to the fact that these chl-a fluorescence parameters have low metal specificity and high sensitivity to other environmental factors. Furthermore, it was also observed in the microcosms that Zn

toxicity occurred when Zn content in biofilms were between 500 and 1700 µgZn/gDW, causing a decrease in algal biomass (chl-a) and an increase in carotenoid pigments (430:665 index). In the riera d'Osor stream, Zn accumulation in the highest polluted site (M2) reached up to 4000 µgZn/gDW 5 weeks after translocation, much higher than the results obtained in the microcosms. Similar amounts of Zn accumulation have been reported in natural biofilms from a moderately metal-polluted river (Behra et al. 2002) and in biofilms translocated for two weeks in extremely Zn polluted river (Ivorra et al. 1999). Changes in algal group composition, either in the microcosms or in the field, were reported in biofilms that accumulated up to 500 µgZn/gDW (Fig.1).

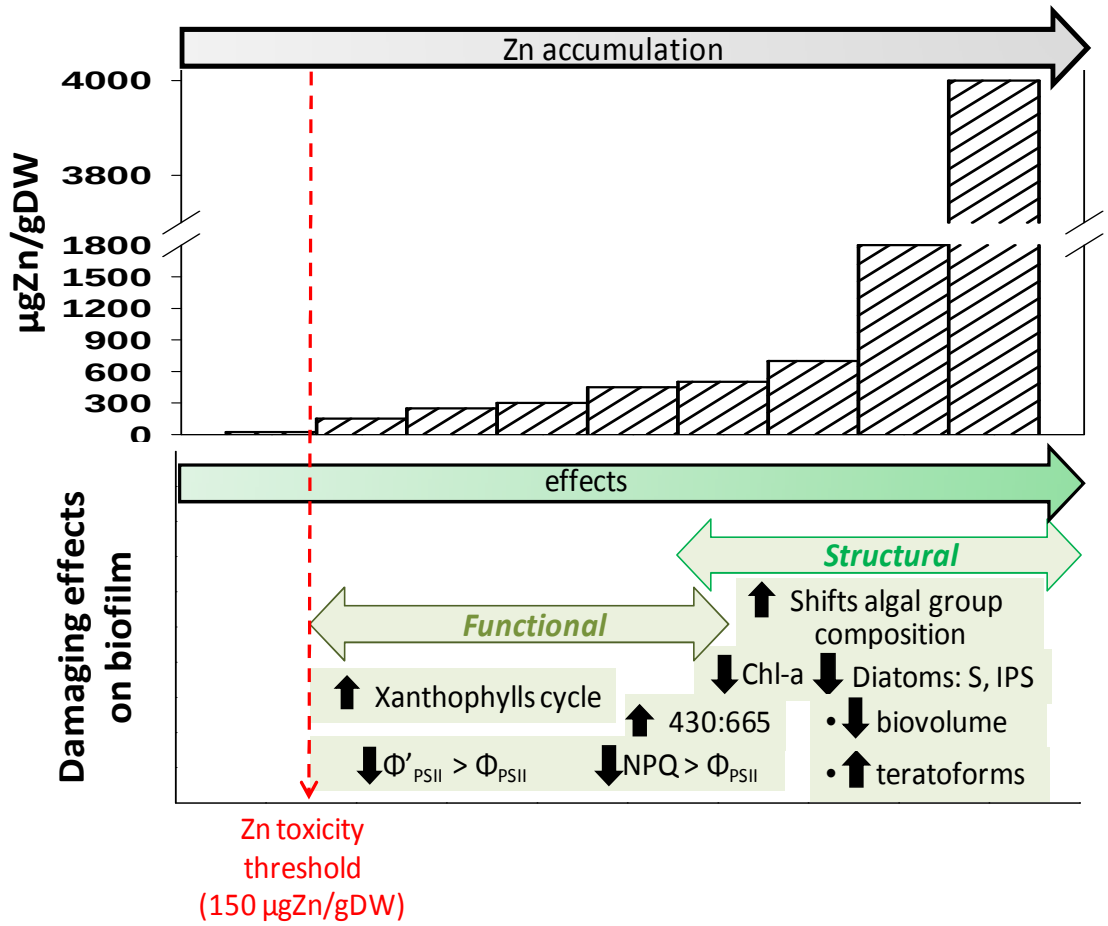


Figure 2. Zn accumulation in biofilms and its effects on functional and structural attributes of biofilm communities based on the results obtained in this investigation. The vertical dashed red line indicates the content of Zn accumulated in biofilm from which there are Zn toxic effects on biofilm communities.

While diatoms were strongly reduced by Zn accumulation, green-algae and cyanobacteria appear to be the most tolerant groups to Zn (either in the microcosms or in the field), suggesting an adaptative response to Zn pollution. In agreement, Tlili et al. (2011) reported in the same stream, the “riera d’Osor”, that Zn contamination exerted a selection pressure proportional both to exposure level and exposure time on the species composition of autotrophic and heterotrophic communities composing biofilms (PICT concept), as an indicator of contaminant effects. Within the autotrophic compartment, Zn pollution leads to a proliferation of cyanobacteria and metal-tolerant diatom species. Accordingly, a higher EC_{50} based on photosynthetic activity was reported in Zn-adapted communities than in reference communities. Within the heterotrophic compartment, although the bacterial community was shown to be more sensitive than fungi, Zn contamination exerted a selection pressure on the different heterotrophic communities by eliminating Zn-sensitive species. Our field results also showed that Zn toxicity on the diatom community caused both a loss of diatom biodiversity (detected by a decrease of S) and also alterations of diatoms morphology (increase of abnormal forms and decrease of cell mean biovolume). Other studies reported similar effects on the biofilm diatom community exposed to high amounts of metals (Gold et al., 2003; Morin et al., 2007; Ferreira da Silva et al., 2009). Although S and IPS indexes appear to be sensitive and metal exposure level-dependent , their potential goodness to distinguish scenarios of low and moderate metal pollution is unlcear. However, the occurrence of diatom teratologies showed to be a sensitive and specific metric to assess scenarios of low and moderate metal pollution. Our results are in agreement with other studies (Dickman, 1998, Morin et al., 2008). The occurrence of diatoms with small mean size showed to be a good metric to assess metal pollution due to its potential metal exposure level-dependency. Extrapolsaccharidic matrix of alga offers mechanical protection to metal toxicity by diminishing metal uptake (Vasconcelos et al. 2001). This mechanical protection can be

more effective for small-sized species, and thus this might be a mechanistic explanation for their positive selection under chronic metal pollution areas (Joux-Arab et al. 2000; Morin et al. 2007). Overall, according to diatom metrics applied in this thesis, it is recommended for the assessment of chronic metal pollution in local watersheds the relevance of comparing reference vs. polluted sites and use both taxonomic and morphologic diatom analysis.

- **Applied issues and ecological implications**

To preserve the ecological status of fluvial ecosystems, the conservation of structural traits (e.g. biodiversity) and functional traits (e.g. photosynthetic activity of primary producers) is requiered. In Europe, this goal is addressed by the WFD which aims to reduce chemical pollution in water bodies in order to reach a "good ecological status" (i.e. close to reference conditions) by preserving structural and functional traits of freshwater ecosystems. To date, 33 substances have been recognized as priority pollutants (PP's) according to the WFD with associate water quality criteria and limitation guidelines. Zn is not considered a priority pollutant in Europe, so neither water nor biota quality criterions are established for Zn in freshwater ecosystems. However, Zn occurrences in European fluvial ecosystems are well reported ranging from 20 up to 2500 µgZn/L (Ivorra et al., 2002; Le Faucheur et al., 2005; García-Meza et al., 2005; Morin et al., 2007; Gold et al., 2003; Bonnineau et al., 2011). In this context, based on the studies performed in this thesis which reveal Zn adverse effects on biofilms at range concentrations from 200 to 400 µgZn/L, we propose including Zn in the European list of priority pollutants and thereby establishing limitation guidelines. In this regard, Zn accumulation in biofilm reflected better the potential of Zn toxicity on biota than Zn concentration in water. Therefore, establishing a criterion of permissible Zn concentration in fluvial ecosystems based on a threshold of Zn accumulation in

biota is suggested (150 µgZn/gDW). This investigation demonstrated that from 150 µgZn/gDW accumulated in biofilm functional traits of biota are damaged (Fig.2).

Although, we focused on the effects of Zn on biofilms in this investigation, we do not forget that Zn accumulated in biofilms might cause adverse effects to other trophic levels like fish and invertebrates that prey on it due to biomagnification (Rhea et al., 2006; Farag et al., 2007; Hill et al., 2010).

In fluvial ecosystems, the quantity of light reaching the biofilm can vary over several orders of magnitude depending on the season and canopy vegetation (Guasch and Sabater, 1998), and on a time scale that ranges from minutes to hours due to the daily light cycle (Laviale et al., 2009). Based on the experimental results obtained, it might be expected that biofilms from a shaded river section would be more sensitive to a multi-stress situation (i.e. light stress and Zn exposure) than biofilms from an open section; photo-adapted to saturating and high light irradiances (Chapter VI). Furthermore, the expected stress caused by a sudden increase in light, i.e. caused by riparian vegetation, may also enhance Zn toxicity towards its shade-adapted community.

PROSPECTS

Although field and laboratory studies are both needed in ecotoxicology to establish cause-effect relationships, field investigations remain scarce. The experimental design applied *in situ* in a Zn-polluted river, in the riera d'Osor stream, was based on a biofilm translocation experiment (active monitoring). This type of approach proved to be an efficient tool for evaluating short- and long-term effects of Zn on biofilm in natural conditions. In addition, the simultaneous sampling of chemical and biological data and its later use in multivariate statistical analysis has been proved as an efficient tool for exploring the relationships between stressors and their effects.

Hence, this approach could be of special interest for biomonitoring programmes in fluvial systems.

The ecotoxicology of Zn on biofilm communities at environmental concentrations was selected due to lack of knowledge. This investigation demonstrated the toxicity of Zn at environmental concentrations on functional and structural biofilm attributes. In this regard, knowledge of the ecotoxicology of other metals currently detected in fluvial ecosystems (e.g. Cr, As, Se) still remains scarce, as well as their regulation in water legislations, especially in Europe. In this investigation, the toxicity of Zn plus Cd in comparison to Zn toxicity alone on fluvial biofilm was also addressed. It was observed that at the concentrations used (300µgZn/L and 20 µCd/L), metal toxicity on biofilms was attributed to Zn, the compound which was present at a higher concentration. Since the co-occurrence of a large number of compounds (metals and organic compounds) is common to fluvial ecosystems, addressing the toxicity of a mixture of chemicals on biofilm communities is a present-day challenge for ecotoxicology. Furthermore, in this investigation the influence of light on Zn toxicity was also investigated. Since fluvial ecosystems are exposed to many stressors, investigating the influence of other environmental variables like temperature, flow or nutrients on toxicity is also necessary.

We recommend the use of chl-a fluorescence parameters of biofilms as a complementary set of biofilm metal biomarkers to the more traditional ones applied so far (e.g. diatom indices) due to their capacity to provide different warning signals of early and late metal toxicity effects on fluvial biofilms. Their application is of special interest in the context of the WFD, where the development of new structural and functional parameters of the biological quality standards (e.g. biofilms) is required (Johnson et al., 2006). With regards to biomarkers, it could be of special interest to incorporate chl-a fluorescence parameters jointly with other biomarkers which have a

higher metal specificity and level of exposure-dependence. Phytochelatins are metal-binding polypeptides produced by algae under metal exposure and they have been described as pertinent indicators of metal toxicity in biofilms (Le Faucheur et al., 2005). In addition, the determination of biomarkers of metal toxicity at gene expression level could also offer a promising tool to evaluate low concentrations of metals affecting cellular and molecular level processes both in laboratory (Huthcins et al., 2010) and in field approaches (Simon et al., 2011). Nevertheless, more investigations are needed to enable the implementation of this genetic approach in biofilm ecotoxicology since the genome sequence of most algae and cyanobacteria species living in bioifilms is still unknown.

GENERAL CONCLUSIONS

UPDATE OF THE USE OF PAM FLUOROMETRY FOR MONITORING THE EFFECT OF CHEMICALS ON FLUVIAL BIOFILMS

- A significant portion of ecotoxicological studies based on biofilms have incorporated the use of chl-a fluorescence parameters to assess positively and easily the effects of herbicides on biofilms. Their application for metal toxicity assessment on biofilms is recommended due to their reported sensitivity for detecting direct and indirect effects of chemicals on photosynthetic processes.

CHL-a FLUORESCENCE PARAMETERS AS BIOFILM BIOMARKERS OF METAL TOXICITY

- Our results showed that the specific effects of metal toxicity on the chl-a fluorescence parameters of fluvial biofilms are better detected in experimental studies performed in microcosm than in field investigations due to their low metal specificity and their high sensitivity to other environmental factors.

- In microcosm studies, the use of chl-a fluorescence parameters for metal toxicity assessment on fluvial biofilms were pertinent to assess: a) functional and quick alterations in photosynthesis processes (that could occur after a punctual spill during a short period of time) as well as, b) structural changes or functional alterations linked to changes in the community composition (that could occur in situations of chronic metal pollution). In field studies, chl-a fluorescence parameters only responded to functional and quick alterations (early responses).

Zn TOXICITY ON FLUVIAL ECOSYSTEMS AND THE PERTINENCE OF USING DIFFERENT BIOFILM METRICS FOR ITS ASSESSMENT

- The hypothesis derived from the Riou-Mort river observations, which pointed out that metal toxicity was mainly attributed to Zn and Cd, was confirmed in the experimental study performed on microcosm. Furthermore, it was also demonstrated that the observed toxicity of Zn (320 µgZn/L) plus Cd (20 µgCd/L) on fluvial biofilms could be attributed to Zn. On the other hand, the translocation experiment performed in the "riera d'Osor" stream, confirmed that in nature Zn alone may cause damaging effects on biofilms at similar concentrations (between 200-400 µgZn/L).

- Zn accumulation in biofilms was shown to be a good indicator of metal exposure. Zn effects on biofilm communities were detected from 150 µgZn/gDW.

- The study performed in the riera d'Osor stream provided enough evidence of occurrence and toxicity of Zn to recommend its inclusion in the list of priority pollutant substances of the European Water Framework Directive. The experiment performed demonstrated that the use of a set of biofilm metrics which include: chl-a fluorescence parameters, pigment analysis, metal accumulation in biofilms and diatom taxonomic analysis, could be pertinent to assess acute and chronic Zn effects on natural biofilms. Short-term exposure mainly affected biofilm function, supporting the use of chl-a fluorescence techniques in combination with the analysis of photosynthetic pigments as signals of early toxicity. Longer-term exposure effects were better detected using diatom indices and traits such as cell biovolume or diatom deformities.

THE ROLE OF CONFOUNDING STRESSORS (i.e. LIGHT STRESS) ON THE TOXICITY OF Zn ON FLUVIAL BIOFILMS

- Algae and cyanobacteria that make up biofilms present several functional and structural mechanisms adapted to the light conditions prevailing during growth and optimizing the use of light. These mechanisms of light adaptation influence their sensitivity to Zn exposure.

- Shade-adapted biofilms are more sensitive to a multi-stress situation (i.e. light stress and Zn exposure) than sun-adapted biofilms, leading to a higher inhibition of maximal and effective quantum yield as well as a decoupling between de-epoxidation reactions and non-photochemical quenching processes.

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