Universitat de Girona

ANTIOXIDANT ENZYME ACTIVITIES IN FLUVIAL BIOFILMS AS BIOMARKERS OF METAL POLLUTION

Berta BONET SÁNCHEZ

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Ph.D. Thesis

ANTIOXIDANT ENZYME ACTIVITES IN FLUVIAL BIOFILMS AS BIOMARKERS OF METAL POLLUTION

Berta Bonet Sánchez

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Dirigida per:

Dra. Helena Guasch Padró

Institut d'Ecologia Aquàtica

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La Dra. Helena Guasch Padró, professora titular del departament de Ciències Ambientals de la Universitat de Girona

CERTIFICA:

Que aquest treball, titulat "*Antioxidant enzyme activities in fluvial biofilms as biomarkers of metal pollution*", que presenta Berta Bonet Sánchez per a l'obtenció del títol de doctor/a, ha estat realitzat sota la meva direcció i que compleix els requeriments per poder optar a Menció Internacional.

Signatura

Dra. Helena Guasch Padró

Girona, 2013

A la mare i en Sergi

AGRAÏMENTS

A qui ha cregut i confiat amb mi,

a qui m'ha ensenyat amb paciència,

a qui m'ha animat i escoltat

tant a nivell professional com personal,

MOLTES GRÀCIES!

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For those who have believed in and trusted me,

who have taught me with patience,

who have encouraged and listened to me

at both a professional and personal level,

MANY THANKS!

La tesi presentada a continuació té un format de tesi com a compendi d'articles ja que compleix els requisits establerts per la Comissió d'Autorització de Defensa de Tesis Doctorals en la sessió de 26 d'abril de 2012. En base a les condicions establertes en l'esmentada secció, tot seguit es mostra que es compleixen els mínims de qualitat i quantitat d'articles sol·licitats per presentar la tesi com a compendi de publicacions. La tesi està formada per dos articles publicats, un enviat i un article en preparació. Les referències i les característiques de les revistes segons l'ISI web of Knowledge són les següents:

1. Bonet, B., Corcoll, N., Acuña, V., Sigg, L., Behra, R., Guasch, H., 2012. Seasonal changes in antioxidant enzyme activities of freshwater biofilms in a metal polluted Mediterranean stream. Science of the Total Environment 444, 60-72.
Àrea temàtica: Environmental Science
Factor d'impacte: 3.286 (Any 2011) / Rang dins l'àrea temàtica: 29/205 (Q1)

2. Bonet, B., Corcoll, N., Tlili, A., Morin, S., Guasch, H., 2012. Antioxidant enzyme activities responses as biomarker of Zn pollution in a natural system: an active bio-monitoring study. Submitted to Ecotoxicology.

3. Bonet, B., Corcoll, N., Tlili, A., Morin, S., Leira, M., Guasch, H., 2012. Antioxidant enzyme activities responses in two Zn polluted streams: an active bio-monitoring. Submitted to Aquatic Toxicology.

4. Bonet, B., Corcoll, N., Guasch, H., 2012. Antioxidant enzyme activities as biomarkers of Zn pollution in fluvial biofilms. Ecotoxicology and Environmental Safety 80, 172–178.
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CONTENTS

RESUM

Els sistemes fluvials són dinàmics i complexos. Aquest dinamisme i complexitat es veu reflectit amb l'àmplia varietat de comunitats biològiques amb diferents estructures i funcions segons les característiques del sistema fluvial. És important ressaltar que la contaminació també contribueix a la modificació de la comunitats biològiques dels sistemes fluvials. Els metalls són uns dels tòxics més comuns i àmpliament distribuïts als sistemes fluvials que no poden ser degradats ni destruïts. Els seus efectes han estat vastament estudiats. Els experiments ecotoxicològics que utilitzen comunitats microfitobentòniques, conegudes també com a biofilms, són molt útils per estudiar el efectes dels metalls en els sistemes fluvials. Els biofilms juguen un paper clau en els ecosistemes fluvials i han estat definits, per les seves característiques, com a poderosos indicadors de l'estat de salut dels ecosistemes. En aquesta tesi, concretament, s'ha estudiat la resposta de les activitats enzimàtiques antioxidants (AEA) per la seva capacitat de resposta davant l'augment de les espècies reactives a l'oxigen (ROS) a causa de l'efecte tòxic dels metalls. No obstant, les AEA també es veuen afectades per processos metabòlics com la respiració o la fotosíntesi i també responen als canvis ambientals (ex. llum).

L'objectiu d'aquesta tesi és avaluar l'ús de les AEA dels biofilms com a biomarcadors de contaminació metàl·lica (principalment per zinc (Zn)) en els ecosistemes fluvials. Per tal d'estudiar la resposta de les AEA a la contaminació metàl·lica, així com també la resposta a altres canvis ambientals, s'han realitzat diversos experiments ecotoxicològics. S'ha fet un zoom des d'estudis de camp (amb un elevat realisme ecològic) fins a un estudi de laboratori utilitzant microcosmos (amb condicions controlades) per tal d'omplir el buit entre les dues aproximacions ecotoxicològiques i entendre la resposta de les AEA a situacions d'estrès múltiple (ambiental i metàl·lic).

Capítol 1: Canvis estacionals de les activitats enzimàtiques antioxidants dels biofilms en una riera Mediterrània contaminada amb metalls. Aquest capítol té per

objectiu descriure la variabilitat de les activitats enzimàtiques antioxidants (AEA) dels biofilms fluvials al llarg d'un any, en un escenari d'estrès múltiple degut a la variabilitat ambiental (ex. intensitat de la llum, cabal, temperatura) i a la contaminació metàl·lica (Zn, Mn i Fe). El monitoratge anual es va dur a terme en tres punts d'una riera amb diferents concentracions de metalls a l'aigua i al biofilm. La concentració de metalls es va veure modificada pel cabal. Amb cabals baixos la concentració de Zn dissolt era més elevada, així com també l'acumulació de Zn al biofilm i viceversa. La temperatura de l'aigua, la intensitat de la llum i la concentració de fosfats van ser els factors ambientals que van determinar la resposta estacional del biofilm, mentre que el Zn dissolt i acumulat en el biofilm van ser els paràmetres que van marcar la respostes biològiques en els punts i els períodes de l'any amb més contaminació metàl·lica. La successió natural de les comunitats algals, de diatomees en condicions fredes a algues verdes en condicions càlides, es va observar clarament en el punt sense contaminació metàl·lica però no en els contaminats, probablement degut a la selecció de comunitats més tolerants produïda pels metalls. La majoria de les AEA van mostrar una relació amb l'estacionalitat ambiental als punts amb poca o sense contaminació, excepte la glutatió-S-transferasa (GST) relacionada amb la contaminació de Zn (dissolt i acumulat al biofilm) que es donava al punt més contaminat. Podem concloure que la successió de les comunitats algals del biofilm i les seves funcions queden emmascarades per la contaminació metàl·lica. A partir dels resultats d'aquest estudi es suggereix l'ús de multiples biomarcadors, que incloguin les AEA i altres paràmetres biològics i fisicoquímics, com a eines per avaluar la contaminació metàl·lica en els sistemes fluvials.

Capítol 2. Resposta de les activitats enzimàtiques antioxidants com a biomarcadors de contaminació de Zn en sistemes naturals: estudi aplicant tècniques de monitoratge actiu. L'objectiu d'aquest estudi era explorar l'ús de les activitats enzimàtiques antioxidants (AEA) i la capacitat d'acumulació metàl·lica dels

biofilms en sistemes naturals com a indicadors de resposta a l'exposició metàl·lica en sistemes naturals. Per tal d'assolir aquest objectiu, es va fer un estudi aplicant tècniques de biomonitoratge actiu utilitzant biofilms al llarg de 5 setmanes. Després de la colonització en substrats artificials en un punt no contaminat, els biofilms es van translocar a 4 punts amb diferents concentracions de metalls situats aigües avall. Es va analitzar l'evolució temporal dels paràmetres ambientals i la resposta del biofilm (AEA, biomassa algal, la ràtio de les densitats òptiques 430/665 i l'acumulació de metalls al biofilm). Els paràmetres ambientals van ser diferents entre els mostrejos. També van diferir entre el punt més contaminat i la resta, bàsicament degut a la contaminació per Zn. Per altra banda, l'anàlisi de les AEA i l'acumulació de metalls en el biofilm va permetre diferenciar el punt amb una contaminació elevada del punt amb una contaminació moderada i la resta (amb poca o sense contaminació). El Zn, el metall amb més contribució a la toxicitat potencial, va presentar una ràpida i elevada capacitat d'acumulació en els biofilms.

En base els resultats obtingut de l'anàlisi multivariada, les AEA van presentar diferents respostes. Mentre la variabilitat de la catalasa (CAT) i l'ascorbat peroxidasa (APX) eren atribuides majoritàriament a l'estrès ambiental (pH, temperatura i la concentració de fosfats), la resposta de la glutatió-S-transferasa (GST) va ser deguda als metalls (Zn, Fe i Al). La glutatió reductasa (GR) i la superòxid dismutasa (SOD) estaven relacionades ambdós estressos, l'ambiental i el metàl·lic. Les AEA i l'acumulació de metalls es proposen com a eines sensibles a l'avaluació dels efectes provocats per l'entrada subtada de metalls (ex. un vessament accidental) en les comunitats naturals (biofilms fluvials) per la seva capacitat de respondre després de poques hores, però també en seguiments rutinaris per la seva persistència després d'algunes setmanes d'exposició. Aquestes eines podrien ajudar a la millorar de l'Estratègia d'Implantació Comuna (CIS) de la Directiva Marc de l'Aigua (DMA) com demanen els experts.

Capítol 3. La resposta de les activitats enzimàtiques antioxidants en dues rieres amb contaminació de Zn. En aquest capítol es van investigar els efectes de l'exposició de metalls (principalment el zinc (Zn)) en els biofilms en dues conques mineres: la Riera d'Osor (NE Catalunya, Espanya) i el Riou Mort (SO Bordeus, França). Ambdues rieres oferien les condicions òptimes per l'estudi dels efectes de la contaminació metàl·lica en comunitats naturals mitjançant l'ús de tècniques de biomonitoratge actiu (ex. experiments de translocació). El Riou Mort, més mineralitzat que la Riera d'Osor, presentava una conductivitat més elevada. En tots dos casos, els biofilms es van translocar de punts amb un nivells basal o moderats de contaminació metàl·lica a punts amb concentracions més elevades. Això va generar tres classes de contaminació metàl·lica: baixa, moderada i elevada. Al cap de 6 i 24h es van analitzar paràmetres físics, químics i biològics, incloent les activitats enzimàtiques antioxidants (AEA) (catalasa (CAT), ascorbat peroxidasa (APX), glutatió reductasa (GR), glutatió-S-transferasa (GST) i la superòxid dismutasa (SOD)) i altres paràmetres clàssics com l'eficiència fotosintètica (Φ'_M) i la biomassa algal (chl-a).

Al Riou Mort, amb més contaminació de Zn (de 10 a 776 µg Zn L^{-1} dissolt a l'aigua i de 757 a 5953 µg Zn g DW^{-1} acumulat al biofilm), es va observar una reducció general de les AEA (ex. CAT, GR i GST). Només l'APX va augmentar en el punt amb poc increment de concentració de Zn. En aquest mateix punt, també va augmentar la chl-a mentre que la Φ'_M va disminuir. A la Riera d'Osor amb menys contaminació de Zn (115 µg Zn L^{-1} dissolt a l'aigua i de 151 a 416 µg Zn g DW^{-1} acumulat al biofilm), l'APX, la GR, la SOD i Φ'_M van disminuir mentre que la CAT i la GST van augmentar.

Aquests resultats mostren que les AEA són capaces de mostrar una ràpida resposta als de efectes de toxicitat del Zn després de poques hores d'exposició (de 6 a 24h), i que aquesta resposta varia en funció de la magnitud de la contaminació. Per tant, l'ús

de les AEA del biofilm es proposen com a biomarcador sensible a la toxicitat dels metalls en els sistemes fluvials.

Capítol 4. Les activitats enzimàtiques antioxidants com a biomarcadors de contaminació de Zn en biofilms fluvials. En aquest estudi es va explorar el potencial de l'activitat enzimàtica catalasa (CAT) i l'ascorbat peroxidasa (APX) com a biomarcadors moleculars de toxicitat de Zn en biofilms fluvials, juntament amb altres paràmetres clàssics funcionals i estructurals (paràmetres fotosintètics, composició algal i bioacumulació). Els biofilms van creixer en un sistema de canals (microcosmos) durant 5 setmanes i després es van exposar a Zn (320 µg Zn L^{-1}) durant 5 setmanes més. Per avaluar els efectes del Zn, els biofilms es van mostrejar 5 i 3 dies abans de l'exposició, just abans de començar (0h) i després de 6 hores, 1, 3, 7, 21 i 35 dies de ser exposats. La majoria de paràmetres mesurats van respondre a la exposició del Zn al llarg de l'experiment. L'APX va ser l'únic paràmetre funcional que va respondre després de poques hores d'exposició, destacant el seu ús com a biomarcador de toxicitat aguda. Al cap de 3 dies d'exposició, la biomassa algal va disminuir mentre que la ràtio de les densitats òptiques 430:665 va incrementar. Es van observar canvis estructurals en les comunitats del biofilm es van observar al cap d'una setmana, passant d'una comunitat de diatomees a una comunitat dominada per cianobacteris i algues verdes. En aquest fase de l'experiment (al cap de 3 setmanes d'exposició) la CAT va augmentar degut a l'efecte directe de l'acumulació de Zn però també per l'efecte indirecte del canvi de les comunitats algals provocat per l'exposició crònica. Es pot concloure que les activitats enzimàtiques antioxidants poden evidenciar un estrès agut causat per l'exposició metàl·lica però també proporcionen informació dobre els mecanismes d'adaptació de la comunitat.

Finalment, es discuteix l'ús general de les AEA com a biomarcadors de contaminació metàl·lica (Zn) en biofilms fluvials. Les AEA van mostrar resultats més consistents en

els estudis d'exposició aguda que crònica, i diferents en funció del nivell de contaminació. L'acumulació de metalls també va ser molt sensible, havent estat detectats després de poc temps d'exposició. Es va observar que s'acumulaven ràpidament fins i tot quan els metalls a l'aigua eren inferiors als límits de detecció, i que en tots els casos desencadenaven efectes biològics a nivell funcional. A nivell crònic les AEA es van mostrar més sensibles davant l'estrès ambiental que a la toxicitat metàl·lica. Per tant, l'ús d'un conjunt de AEA seria útil per avaluar altres impactes als que estan sotmesos els sistemes fluvials com els produïts pel canvi climàtic (ex. els efectes biològics causats per períodes de sequera/inundacions, l'augment de la radiació dels UV o la temperatura). És important remarcar que l'estacionalitat de les AEA es va veure emmascarada pels efectes dels metalls en els punts contaminats.

SUMMARY

Fluvial ecosystems are dynamic and complex. This is demonstrated by the wide variety of biological communities with different structures and functions according to the characteristics of the fluvial system. Besides the complexity, it is important to highlight the effects of pollution which also contribute to modifying fluvial biological communities. Metals are common and widely distributed toxicants in aquatic systems which cannot be degraded or destroyed. Indeed, their toxic effects have been widely reported. Ecotoxicological experiments using microphytobenthic communities, also called biofilms, are useful to study the effects of metals in fluvial systems. Biofilms play a key role in fluvial ecosystems and due to their characteristics have been defined as powerful indicators for ecosystem health. Specifically, antioxidant enzyme activities (AEA) responses of biofilm to metal pollution have been studied due to their capacity to respond to the increase of reactive oxygen species (ROS) caused by metals. However AEA are also metabolic responses and respond to environmental parameters (e.g. light) too.

The present Thesis aims to evaluate the use of antioxidant enzyme activities (AEA) of biofilm communities as biomarkers of metal pollution (mainly Zn) in fluvial ecosystems. In order to test AEA as biomarkers of metal pollution as well as their responses to environmental variables, several ecotoxicological experiments have been performed. A zoom from field studies (with high ecological realism) to microcosm experiments (under controlled conditions) has been done to fill the gap between both approaches and understand AEA responses under a multiple-stress (environmental and metallic) scenario.

Chapter 1: Seasonal changes in antioxidant enzyme activities of freshwater biofilms in a metal polluted Mediterranean stream. This chapter aimed to describe antioxidant enzyme activity (AEA) variability in fluvial biofilms over an annual cycle, under multiple-stress scenarios due to environmental variability (e.g., light intensity,

water flow, and temperature) and metal pollution (Zn, Mn and Fe). The annual monitoring study was performed at three sites according to their water and biofilm metal concentrations. Metal concentration was affected by water flow due to dilution. Low flow led to higher dissolved Zn concentrations, and thus to higher Zn accumulation in the biofilm. Water temperature, light intensity and phosphate concentration were the environmental factors which determined the seasonality of biofilm responses, whereas dissolved Zn and Zn accumulation in biofilms were the parameters linked to sites and periods of highest metal pollution. Community algal succession, from diatoms in cold conditions to green algae in warm conditions, was clearer in the non metal-polluted site than in those metal-polluted, presumably due to the selection pressure exerted by metals. Most AEA were related with seasonal environmental variability at the sites with low or no-metal pollution, except glutathione-S-transferase (GST) which was related with Zn (dissolved and accumulated in biofilm) pollution occurring at the most polluted site. The main conclusions from this study are that seasonal variations of community composition and function are masked by metal pollution. From this study the use of a multi-biomarker approach, including AEA and a set of biological and physicochemical parameters is proposed as an effect-based field tool to assess metal pollution.

Chapter 2. Antioxidant enzyme activities responses as biomarker of Zn pollution in a natural system: an active bio-monitoring study. This study aimed to explore the use of antioxidant enzyme activities (AEA) and biofilm metal accumulation capacity in natural communities as effect-based indicator of metal exposure in fluvial systems. To reach this, an active bio-monitoring using biofilm communities was performed during 5 weeks. After colonization over artificial substrata in a non-polluted site, biofilms were translocated to 4 different sites with different metal pollution in the same stream. The evolution of environmental parameters as well as biofilm responses (AEA, algal biomass, optical densities 430:665 ratio and metal accumulation in biofilm) were analysed over time. Physicochemical parameters were different between sampling

times as well as between the most polluted site and the less polluted ones, mainly due to Zn pollution. In contrast, AEA and metal accumulation in biofilms allowed us to discriminate the high and moderate metal pollution sites from the rest (low and non-metal polluted sites). Zn, the metal with the highest contribution to potential toxicity, presented a fast and high accumulation capacity in biofilms.

According to the multivariate analysis, AEA showed different responses. While catalase (CAT) and ascorbate peroxidase (APX) variability was mainly attributed to environmental stress (pH, temperature and phosphate concentration), glutathione-S-transferase (GST) changes were related to metal pollution and reductase (GR) and superoxide dismutase (SOD) to both stress factors. AEA and metal accumulation are proposed as sensitive effect-based field methods to evaluate biofilm responses after acute metal exposure (e.g. an accidental spill) due to their capacity to respond after few hours, but also in routinely monitoring due to their persistent changes after some weeks of exposure. These tools could improve the Common Implementation Strategy (CIS) of the Water Framework Directive (WFD) as expert group request.

Chapter 3. Antioxidant enzyme activities responses in two Zn polluted streams: an active bio-monitoring. In this chapter the effects of metal exposure (mainly zinc (Zn)) in biofilm communities were investigated in two mine basins: Riera d'Osor (NE Catalonia, Spain) and Riou Mort (SW Bordeaux, France). Both streams offered the opportunity to study the effects of metal pollution on natural communities by means of active monitoring (i.e. translocation experiments). Riou Mort was more mineralized and metal polluted than Riera d'Osor, and thus presented higher conductivity. In both cases, biofilms were transferred from sites having either background or moderate levels of metal pollution to sites with higher pollution levels. This generated three classes of metal concentration: low, moderate and high. Physical, chemical and

biological parameters, including antioxidant enzyme activities (AEA) (i.e. catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), glutathione-S-transferase (GST) and superoxide dismutase (SOD)) and other classical endpoints like effective photosynthetic efficiency (Φ'_M) and algal biomass (chl-a), were analysed 6h and 24h after translocation.

In the Riou Mort, with higher Zn pollution (from 10 to 776 µg Zn L^{-1} dissolved in water and from 757 to 5954 µg Zn g DW^{-1} accumulated in biofilm), a general reduction of AEA (i.e. CAT, GR and GST) was observed. Only APX was enhanced under the lowest metal concentration change investigated. At this study-site, the highest Zn increase caused the enhancement of APX activity and chl-a, whereas photosynthetic efficiency decreased under the lowest Zn increase conditions. In Riera d'Osor with lower Zn pollution (115 µg Zn L^{-1} dissolved in water and 151 to 416 µg Zn g DW^{-1} accumulated in biofilm) a decrease in APX, GR, SOD and Φ'_M was observed whereas CAT and GST were enhanced.

The results of this study showed that AEA are able to reflect fast (from 6 to 24h) responses to Zn differing depending on the magnitude of pollution. Thus, the use of AEA in biofilms is proposed as a sensitive effect-based biomarker of metal toxicity in fluvial systems.

Chapter 4. Antioxidant enzyme activities as biomarkers of Zn pollution in fluvial biofilms. The potential of the antioxidant enzyme catalase (CAT) and ascorbate peroxidase (APX) as molecular biomarkers of Zn toxicity in freshwater biofilms has been explored in this study jointly with other classical functional and structural endpoints (photosynthetic parameters, algal group composition and bioaccumulation). Biofilms were colonized in an indoor microcosm system for 5 weeks and then exposed to Zn for 5 weeks. To evaluate Zn effects, biofilms were sampled 5 and 3 days before

exposure, just before exposure (time 0), and after 6 hours, 1, 3, 7, 21 and 35 days of metal exposure. Most endpoints measured were affected by Zn exposure (320 µgZn L^{-1}) during both periods of exposure. APX was the only functional parameter responding after a few hours of Zn exposure, highlighting its use as an early toxicity biomarker. Structural changes began after 3 days of exposure, starting with a decrease in algal biomass and an increase in the OD 430:665 ratio. Structural changes in biofilm communities were observed after 1 week, leading to a shift from diatoms to cyanobacteria and green algae-dominated communities. CAT activity was thereafter enhanced (after three weeks of exposure) and attributed not only to a direct effect of Zn bioaccumulation but also to an indirect effect of the community composition changes driven by chronic metal exposure. It can be concluded that biofilm antioxidant enzyme activities may provide evidence of early stress caused by metal exposure and also provide information about the mechanism of community adaptation.

Finally, the general applicability of AEA in fluvial biofilms as biomarkers of metal (Zn) pollution is discussed. AEA showed more consistent results in acute than in chronic studies, responding according to the metal pollution level. Metal accumulation was also very sensitive even after a short period of time and revealed that even when metals in water were below detection limits they can be accumulated in biofilms quickly and trigger biological effects at functional level. Furthermore, AEA at chronic level was more sensitive to environmental stress than metal toxicity. Thus a set of AEA could be useful to asses other impacts in aquatic systems such those produced by climate change (e.g. biological effects of droughts/floods events, increase of UV and temperature). It is important to highlight that AEA seasonality was masked in the metal polluted sites.

GENERAL INTRODUCTION

FLUVIAL ECOSYSTEMS AND METALS

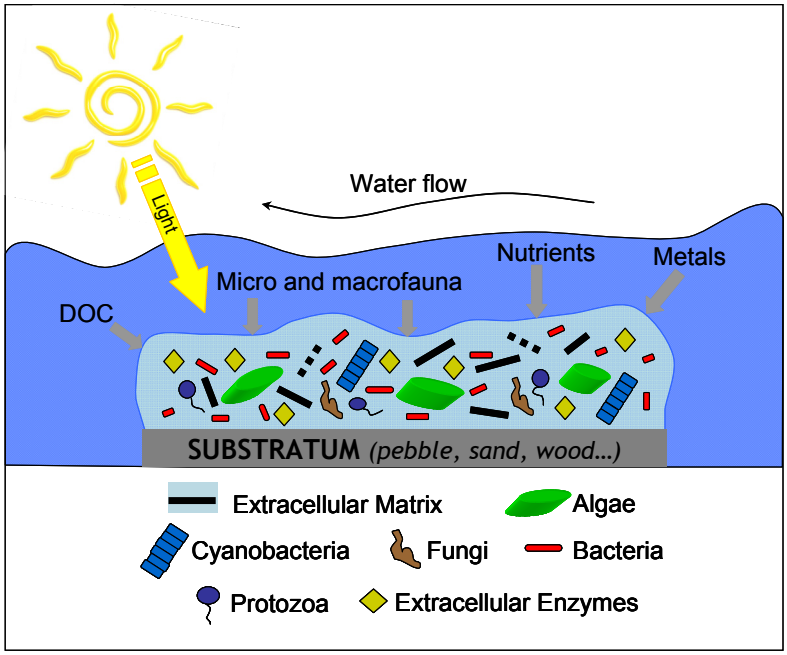
Fluvial ecosystems are dynamic and have high spatial and temporal variability. They are complex systems, with a high hydrological variability, heterogeneity of habitats (riparian cover, pools and riffles) and substrata (rocks, pebbles, sediments). This is demonstrated by the wide range of different biological communities with different structures and functions according to the characteristics of the fluvial system.

Light, temperature, discharge and nutrients are the main physicochemical factors that influence biological communities in fluvial systems. Biological communities can also modify water chemistry due to their biological activities (Allan 1995). For example, light availability determines the potential effect of nutrients in primary producers and, at the same time, primary producers can modify CO_2 concentration, and therefore, the water pH, due to photosynthesis (Sabater et al. 2006).

Microphytobenthic communities, also called biofilms, play a key role in fluvial ecosystems. Biofilms are defined as biological layers attached to different solid substrates (e.g. pebbles, sand, wood, etc) in contact with water. They are located at the base of the food chain and are primary producers (Vannote et al. 1980). Biofilms are made up of either autotrophic (green algae, diatoms and cyanobacteria) and heterotrophic (bacteria, fungi and protozoa) organisms forming a well-structured, complex community. All these organisms are located in close physical contact and embedded in a matrix composed of extracellular polymeric substances (EPS) and interact among one another (Fig. 1) (Romaní 2010). Biofilms have a 3D architecture which is strongly modified by the environment (e.g. water flow and light). Apart from being primary producers, biofilm communities carry out other important tasks, such as nutrient cycling or the interception of dissolved and particulate matter due to their physical and biological properties. By physical adsorption, biofilms remove substances (e.g. nutrients) from flowing water which can be assimilated later by organisms

(Kaplan, et al. 1987). In fact, EPS matrix offers potential binding sites for a variety of colloidal, organic (e.g. dissolved organic carbon (DOC)) and inorganic substances (e.g. nutrients) (Sabater et al. 2007).

Figure 1. Sketch of freshwater biofilm and the elements than affect this microbiological community. Adapted from Romaní et al. 2010



Besides the relationship between environmental parameters and biological communities, it is important to highlight the effects of humans which also contribute to modifying the dynamics and functions of fluvial ecosystems, and consequently, their biological communities (Allan 2004). Constructions of dams, derivation of water for human activities or pollution are some examples of how human activities can alter fluvial systems.

Metals are common elements present in the earth which cannot be degraded or destroyed (Mallick and Mohn, 2003). Humans have been using metals since ancient times (e.g. by Mesopotamians, Egyptians, Greeks and Romans) and have caused metal pollution in aquatic ecosystems basically due to metal manipulation and their wastes. In fact, several environmental disasters related with heavy metals have

occurred. Minamata Bay (Japan) in 1932, whose effects appeared after 20 years; the Sandoz chemical accident in the upper Rhine River (Switzerland) in the 80's, or the Summitville mine accident in the River Alamosa (Colorado). In 1998, another disaster occurred in the Spanish nature reserve Doñana. More recently in 2010 in Hungary, tons of alumina sludge were accidentally spilled. These accidental spills to aquatic systems produce serious environmental consequences and people hear about them in the media. However, every day there are metal spills and/or wastes in aquatic ecosystems which come from industry, agriculture and home waste which go unnoticed. All of them will seldom result in rapid and catastrophic change. Rather, the impact will be gradual, subtle and frequently difficult to disentangle from the process and effects of natural environmental change (Moore et al. 2004). Thus, to understand and solve ecosystem damage caused by pollution, it is crucial to evaluate the effects of pollution within the framework of ecological studies.

METAL TOXICITY

It is well known that metal concentrations vary widely over time and space due to differences in geology, hydrology and natural and anthropogenic loadings (Peijnenburg and Vijver, 2007). Moreover, the alteration of elemental stoichiometry by human activity is also another important way by which trace elements acquire toxic properties (Shanker 2008).

Metals enter into the organism due to their distinct chemical properties such as reduction and oxidation reactions under specific physiological conditions. These reactions, which are essential for life, are also the primary cause of their toxicity when present in surfeit (Peijnenburg and Vijver, 2007; Shanker 2008). The toxicity of most trace metals depends principally on the absorption, concentration and persistence of the eventual toxicant at its location of action (target molecule and/or organelle). In general, metals exist in strongly hydrated species and are unable to traverse biological

membranes by simple diffusion. Thus, essential as well as non-essential metals are transported from the media to cells by carriers or pores specific to this metal or with similar physicochemical characteristics, as occurs with Zn and Cd, which compete for binding sites (Peijnenburg and Vijver, 2007; Töpperwiewn et al. 2007).

In this Thesis, Zn has been the common factor among all performed studies. The calculation of cumulative criterion units (CCU, Clements et al. 2000) scoring as a measure of potential metal toxicity, revealed the important contribution of Zn in the most polluted sites. In fact, Zn (i) is one of the most widely-spread metals in aquatic ecosystems due to both natural and anthropogenic causes, (ii) it is present in a wide range of concentrations, (iii) it is an essential metal as well as toxic in surfeit (iv) its toxic effects have been widely reported (Admiraal et al. 1999; Behra et al. 2002; Besser et al. 2007; Morin et al. 2007; Tlili et al. 2011; Corcoll et al. 2012) and once in the cell (v) it is considered a borderline metal because it can be bound to many structurally diverse ligands/target molecules (cysteinyl residues in proteins, thiols, thiolates, purines and pyrimidines in nucleic acids, with nitrogen amino groups in purine bases) (Peijnenburg and Vijver, 2007; Shanker 2008).

These target molecules have regulation, maintenance and signalling functions. Thus, when attacked by metals, a cascade of effects is started. These effects include: (i) changes in gene expression, (ii) impaired protein synthesis, (iii) changes in internal and external maintenance causing impaired ATP synthesis, and (iv) altered membrane function leading to cell injury. All these molecular changes produce structural and functional cell changes (Shanker 2008). If these alterations cannot be repaired, toxic effects will affect cells, organisms, populations as well as communities.

Metal pollution commonly occurs as a mixture of metals (e.g. Zn, Fe, Al, Cu, Ni, Cd). This is the case in mine basins but also in rivers receiving urban wastes. It is well

known that different metals have different affinities for specific binding groups and do not develop the same toxicity. In addition, metal mixtures can have a synergistic, antagonistic or null interaction. The presence or deficiency of some substances (e.g. metals, nutrients) can also influence metal toxicity. For example, Zn, Cu and Ni toxicity has been attributed to Fe deficiency, or an increase in Pb, Zn and Cu toxicity has been related to phosphate deficiency (Peijnenburg and Vijver, 2007; Serra et al. 2009). In this Thesis, CCU scores have been used as a general estimate of the potential toxicity of metals (Chapter 2).

Another important step in metal toxicity is their speciation. Free metal ions are the most toxic metal species because they are bioavailable (Meylan et al. 2004; Luoma and Rainbow 2005; Sigg and Behra 2005). Different models like free ionic activity model (FIAM) or its derivative, the biotic ligand model (BLM), predict the influence of metallic compounds on the biota due to the activity of the free metal ion. However, some limitations and exceptions have been, and are still being, reported (Errécalde and Campbell, 2000; Heijerick et al. 2002; Meylan et al. 2003; Töpperwien et al. 2007). In fluvial systems, labile metal form in water, weak metal complexes which can be discomposed easily, as well as dissolved metals in water, can also contribute to metal toxicity because they are potentially bioavailable. For example, labile species can contribute towards the free metal concentration because their stability as a complex is so weak (Van Leeuwen et al. 2005; Bradac et al. 2010). Metal speciation was addressed in this Thesis in order to study if there were differences in toxicity due to its form (dissolved, free and labile). Metal speciation is given in molar concentration to make the comparison between different metal species easier. However, most results of metal concentration are presented in μg L^{-1}, the most common unit reported in the literature. Molar units are used in chapters where metal speciation has been done (Chapter 1), and in μg L^{-1} in others, where only total dissolved metals have been analysed (Chapter 2, 3 and 4).

ECOTOXICOLOGY AND BIOFILMS: A GOOD RELATIONSHIP

As a definition, 'Ecotoxicology is concerned with the toxic effects of chemical and physical agents on living organisms, especially on populations and communities within defined ecosystems. It includes the transfer pathways and their interactions with the environment' (Rand et al. 1995). Thus, ecotoxicology is a multidisciplinary field, which integrates toxicology and ecology to evaluate toxic effects in organisms, populations and biological communities to finally evaluate ecosystem responses.

Fluvial biofilms are aquatic communities commonly used in ecotoxicology. Biofilm 3D architecture might be strongly modified by toxicant exposure (e.g. metals). Their properties to remove substances from flowing water give these microbiological communities not only the great capacity to integrate the effects of environmental conditions but also the opportunity to evaluate effects of toxic exposure over extended periods of time (Guasch et al. 2003; Sabater et al. 2007). Thus, biofilms have been defined as powerful indicators for ecosystem health (Larson and Passy, 2005). Ecotoxicological experiments with biofilms have been used to investigate direct and/or indirect effect of metals in microcosms or mesocosms (Gold, et al. 2003; Morin, et al., 2008; Bonnineau et al. 2011; Bonet et al. 2012; Corcoll et al. 2012) as well as in field experiments (Guasch et al. 2003; Tlili et al. 2011; Bonet et al. 2013; Corcoll et al. 2012).

Acute Zn effects on biofilms have been reported at concentrations ranging from 400 µg Zn L^{-1} to 195 mg Zn L^{-1} (Admiraal et al. 1999; Blanck et al 2003; Tlili el al 2011; Corcoll et al. 2012), while chronic effects have been reported at lower concentrations ranging from 50 µg Zn L^{-1} to 2.5 mg Zn L^{-1} (Genter et al. 1987). However, indirect effects, such as the reduction of nutrient uptake in biofilms, have also been found at much lower concentrations (6-25 µg Zn L^{-1}) under oligotrophic conditions (Paulsson et al. 2002). Shehata et al. (1999) reported that Zn has a higher uptake in algal biomass in

comparison to other tested metals. Biofilms accumulate metals following three main mechanisms: (i) absorption in EPS, (ii) mainly cell surface adsorption and (iii) intracellular uptake. These mechanisms can be either passive or active (Garcia-Meza et al. 2005; Morin et al. 2008). Time will magnify metal accumulation in biofilms.

Several physicochemical and biological factors may affect the ecotoxicological response of biofilms. For example, high primary production changes pH gradients in biofilms and may change metal speciation, and therefore, metal toxicity. Different sensitivity of biofilms to toxicants have also been found to be dependent on the age and succession status of the community (Ivorra et al. 2000) as well as the protective role of nutrients (Serra et al. 2009). In this thesis, the complex nature of metal toxicity in nature has been addressed by the assessment, at different temporal scales, of biofilm responses to metal exposure under a large range of field conditions and also biofilm responses to Zn exposure in a laboratory study.

In ecotoxicology the term biomarker usually refers to biochemical, physiological or histological indicators and toxicant effects (Huggett et al. 1992; Forbes et al. 2006). A biomarker as a definition, is a biological element belonging to a wider system whose observation is expected to give information on the wider system based on the “a priori” knowledge of the links and interactions existing between the biomarker and this system (Bonnineau et al. 2012). In aquatic ecosystems, several attempts have been made to develop pollution biomarkers. Bonnineau et al. (2012) described three classes of biomarkers based on the fact that biomarkers can provide information on (1) the exposure to (2) the effects of or (3) the susceptibility to a perturbation. The first two classes (exposure and effects) are commonly used in ecotoxicology, whereas the last one, the biomarker of susceptibility, is rarely used. Some authors have suggested the use of antioxidant enzyme activities (AEA) as biomarkers. The main AEA function is to reduce reactive oxygen species (ROS) resulting from the metabolism or due to metal

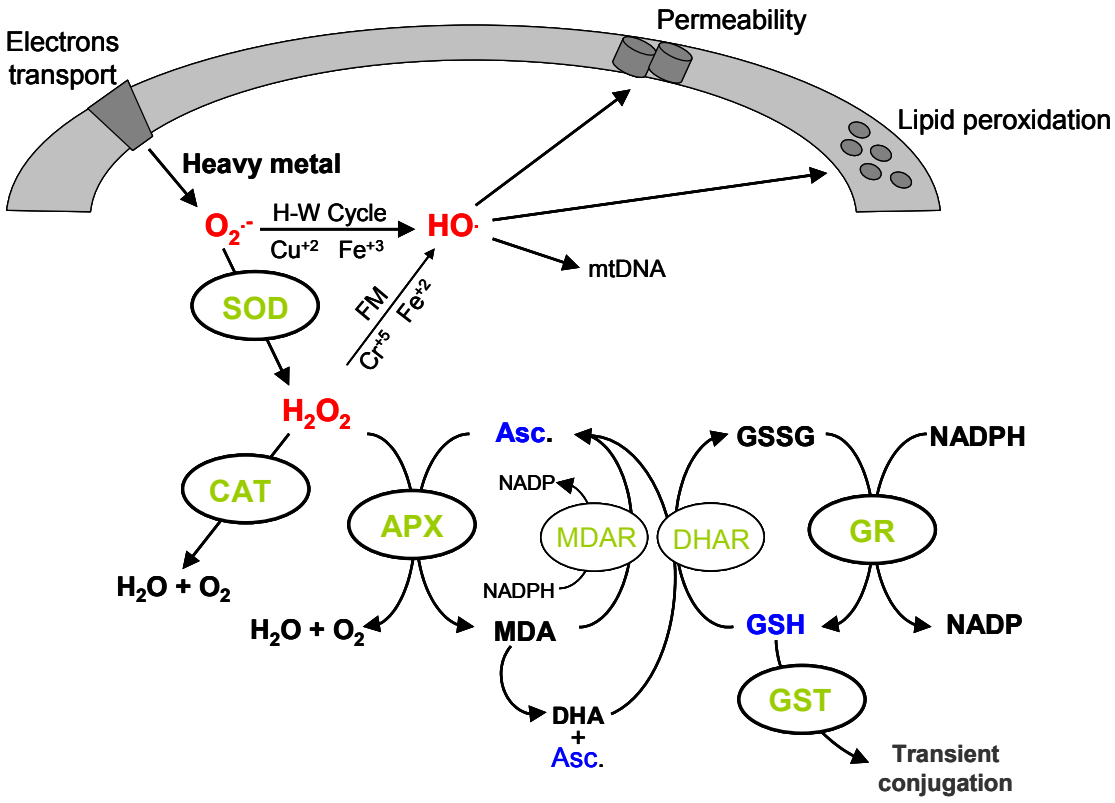
effects. AEA have been proposed as biomarkers of exposure and effects (1 and 2) due to their capacity to respond after short (a few hours) and long (several weeks) periods under pollution exposure (Valavanidis et al. 2006; Guasch et al. 2010; Maharana et al. 2010; Bonnineau et al. 2011a).

Virtually all ROS found in aquatic environments are produced by organisms through redox reactions with O_2 and they result in a common form of stress, oxidative stress. In fact, ROS are results of metabolism (e.g. photosynthesis or respiration) and can be found in several cell places like chloroplasts in autotrophic organisms (Edreva 2005; Asada 2006), in mitochondrion (Mittler 2002) as well as in endoplasmic reticulum and microbodies like peroxisomes and glyoxysomes (Lesser 2006). Some of the most notable culprits within the ROS family are the superoxide anion radical ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($HO^{\cdot -}$) resulting from the transfer of one, two or three electrons, respectively, to oxygen (Scandalios, 1993, Mittler 2002; Edreva 2005; Wolfe-Simon et al. 2005) (Fig. 2). These ROS molecules attack lipids, proteins, nucleic acids and damage most cellular machinery which often leads to alterations in cell structures and mutagenesis (Scandalios 1993; Stohs et al. 1995; Nagalakshmi and Prasad 1998; Mallik 2004; Wolfe-Simon et al. 2005; Lesser 2006) (Fig. 2). Thus, despite the energetic advantage gained from using O_2 as a terminal electron acceptor, cells are required to maintain an efficient defence system against its by-products, like AEA and other antioxidant mechanisms.

ROS, apart from being the result of a metabolic process, can be enhanced by toxicants like metals (Sauser et al. 1997; Nagalakshmi and Prasad 2001; Okamoto et al. 2001; Mallick 2004; Contreras et al. 2005; Zbigniew and Wojciech 2006; Shanker 2008). Metal exposure induces oxidative stress because the metals are involved in different types of ROS-generating mechanisms (Weckx and Clijsters 1997; Collén et al. 2003; Pinto et al., 2003; Zbigniew and Wojciech 2006). Although ROS are potentially toxic,

aquatic organisms may have capitalized the chemistry associated with ROS production to increase their fitness. Organisms have non-enzymatic mechanisms to cope with ROS by low molecular weight antioxidants, such as glutathione (GSH), carotenoids and phenolics (Okamoto et al. 2001). GSH is considered an important cellular antioxidant (Noctor and Foyer 1998; Nagalaskshmi and Prasad, 2001). However, the main response of the organism to metals is by induction of antioxidant enzyme activities (AEA). Among these AEA, this Thesis focuses on: superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR) and glutathione-S-transferase (GST) (Fig. 2).

Figure 2. Effects of heavy metals on cellular generation of ROS in algae and antioxidant enzyme activities (AEA). ROS are in red, AEA in green and antioxidant molecules in blue. Abbreviations: superoxide anion radical ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\cdot -}$), Haber-Weiss Cycle (H-W Cycle), Fenton Mechanism (FM), superoxid dismutase (SOD), catalse (CAT), ascorbate peroxidase (APX), monodehydroascorbate (MDA), MDA reductase (MDAR), ascorbate (Asc.), glutathione reductase (GR), glutathione disulphide (GSSG), glutathione (GSH), glutathione-S-transferase (GST), dehydroascorbate (DHA), DHA reductase (DHAR), glutathione-S-transferase (GST), nicotinamide adenine dinucleotide phosphate (NADP) and reduced nicotinamide adenine dinucleotide phosphate (NADPH). Adapted Noctor and Foyer 1998 and Mittler 2002.



Superoxide dismutase (SOD, EC 1.15.1.1), has been called the cell's first line of defence against ROS (Scandalios 1993). SODs constitute a family of metalloenzymes that catalyzes the dismutation of superoxide anion ($O_2^{\cdot-}$) into oxygen (O_2) and hydrogen peroxide (H_2O_2). SOD is typically localized in the chloroplasts, cytoplasm, mitochondria, peroxisomes and as an extracellular enzyme (Okamoto et al. 2001; Mittler 2002; Lesser 2006; Zbigniew and Wojciech 2006). The dismutation of $O_2^{\cdot-}$ radicals by SOD results in H_2O_2, which can be scavenged by CAT, APX and glutathione peroxidase (GPX) (Mittler 2002; Li et al. 2006). **Catalase** (CAT, EC 1.11.1.6) catalyzes the production of H_2O mainly from the degradation of H_2O_2 (Chelikani et al. 2004). It is mainly localized in peroxisomes and does not require co-factors (Mittler 2002). In contrast, **ascorbate peroxidase** (APX, EC 1.11.1.11) needs ascorbate (Asc.) as the principal electron donor to reduce H_2O_2 into H_2O (Sauser et al. 1997; Nagalakshmi and Prasad, 1998; Mallick 2004; Wolfe-Simon et al. 2005; Lesser 2006). APX can be found in the same cell places like SOD. H_2O_2 decomposed by APX forms H_2O and O_2 but also monohydroascorbate (MDA) and this, in addition to ascorbate, forms dehydroascorbate (DHA). Both molecules (MDA and DHA) are converted to ascorbate by monodehydroascorbate reductase (MDAR) and dehydroascorbate reductase (DHAR), respectively (Fig. 2). **Glutathione reductase** (GR, EC 1.8.1.7) reduces glutathione disulfide (GSSG) to the sulfhydryl form (GSH) using reduced nicotinamide adenine dinucleotide phosphate (NADPH). Furthermore, GR participates in the generation of the ascorbate needed by APX. Thus, GR and APX act in conjunction to metabolise H_2O_2 to H_2O through a widely known metabolic cycle: the ascorbate-glutathione cycle (Mittler 2002; Mallick 2004). The last AEA studied in this Thesis is **glutatione-S-transferase** (GST, EC 2.5.1.18), which catalyses the conjugation of pollutants using GSH (produced by GR) to inactivate oxidative compounds (Noctor et Foyer 1998). GST can be found in the cytosol, mitochondria and in the membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG) (Oliver de Franco et al. 2009). GST activities are an important group of

enzymes involved in the detoxification of both ROS and endogenous substances such as metals (Nagalaskshmi and Prasad, 2001) (Fig. 2).

RELATIONSHIPS WITHIN AEA, ENVIRONMENTAL PARAMETERS, METALS AND TIME OF EXPOSURE

Many antioxidant enzymes are metabolically linked and this has to be taken into consideration when interpreting ecotoxicological results. Superoxide radicals ($O_2^{\cdot-}$) and SOD can inactive CAT and hence the disproportionation of H_2O_2 is mainly done by APX in algae (Nagalakshmi and Prasad 1998). However, other studies suggest that higher concentrations of H_2O_2 would be more efficiently eliminated by CAT rather than APX due to their different affinity for H_2O_2 (APX at μM and CAT at mM order). Thus, APX is more effective for decomposing smaller concentrations of this ROS than CAT. As CAT does not require any co-factor for its function, it may be insensitive to the redox status of cells and its function might not be affected during stress (Mittler 2002; Barros et al. 2003). Furthermore, even though CAT is only present in peroxisoms, oxidative stress causes the proliferation of them (Mittler 2002).

The enzymes APX, GR and GST are very close in the ascorbate-glutathione cycle and thus are dependent. The maintenance of highly reduced per oxidized ratio of ascorbate and GSH is essential for the proper scavenging of ROS in cells. This ratio is maintained by GR, MDAR and DHAR enzymes using NADPH as reducing power (Fig. 2) (Mittler 2002). Thus, the variability of some of these AEA could produce a cascade of effects. For example, if GR activity decreases (e.g. due to the reaction of –SH groups with free ion metals), it could lead to a depletion in cellular GSH poll and GSH is used to (i) recycle the APX cofactor (Asc.), (ii) is conjugated by GST to inactivate oxidative compounds and (iii) is a precursor of other antioxidant mechanisms (e.g. phytochelatins biosynthesis) (Noctor and Foyer 1998; Nagalakshmi and Prasad, 2001, Le Faucheur, et al., 2005). The ascorbate-glutathione cycle and the SOD/CAT way

have to be considered as two complementary ROS-scavenging systems (Foyer et al. 1994; Prasad 1998). Mittler (2002) supported partly this “two-way defence system” for the differences in affinities for H_2O_2 between APX and CAT commented above.

There are other factors, external factors, which may influence AEA: environmental parameters and toxicants (e.g. metals). Environmental parameters such as light intensity can influence AEA due to the link of ROS with photosynthesis (Butow et al. 1997; Aguilera et al. 2002; Li et al. 2010; Bonnineau, 2011). High radiation enhanced CAT and SOD in some green and red algae while GR was reduced (Aguilera et al. 2002). In contrast, Lesser (2006) reported a reduction of CAT activity due to light as well as other factors like osmotic, heat or cold stress. Salinity also affects AEA. CAT and APX as well as non-enzymatic antioxidants (e.g. Asc. and carotenoids) increased against salt stress (Chakraborty et al., 2010) as hypothesized by Cairrão et al. (2004).

Environmental factors (e.g. temperature, discharge, light intensity or nutrients) can influence the sensitivity of biofilm faced by toxicants (Guasch and Sabater 1998; Lesser 2006; Navarro et al. 2008; Villeneuve et al. 2010; Tlili et al. 2010). Thus, environmental parameters play a double role regarding biofilms: as stressors and as modifiers of toxic effects. Bonnineau et al. (2011) found that biofilm AEA responded to organic (glyphosate) and inorganic (Cu) pollutants and also to light stress, and observed how light stress modified the biofilm sensitivity constraining its capacity to cope with further stress factors.

It has been shown that the slow increase in intracellular metal concentration may be accompanied by generalized metabolism changes inducing alternative metabolic pathways (Bozhkov et al., 2010). Different AEA responses due to metal exposure have been largely reported in cultures. SOD and APX activities increased while CAT did not in *Selenastrum sp.* after Cu exposure (Sauser et al. 1997). Using *Scenedesmus sp.* SOD, APX and CAT increased after Cu exposure (Nagalakshmi and Prasad, 1998 and

2001). Li et al. (2006) reported that Cu enhanced SOD and GPX while Zn reduced these activities in *Pavlova viridis*. CAT and GSH were enhanced by both metals. In spite of the fact that these cited studies used the same metals, AEA responses were different. Thus, it could be said that AEA responses differ in function of the tested organism/species as well as in function of the metal.

Exposure time is another factor that can affect AEA. In general AEA responses are proportional to the duration and severity of the stress applied to the biofilm (metal concentration and acute or chronic exposure). For example, an abrupt generation of high levels of ROS caused by a short period of metal exposure will usually exceed the total antioxidant capacity, whereas over time the enhancing of cellular antioxidants could allow cells to acclimatize to increasing stress (Okamoto et al., 2001). A unimodal temporal response to stress has been described in biofilms changing after chronic exposure due to adaptation (Bonnineau thesis, 2011).

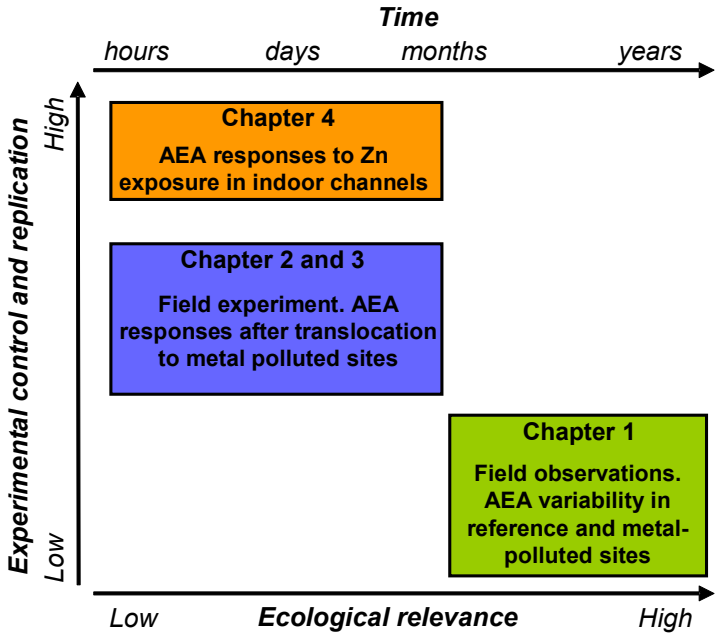
In culture studies, *Scenedesmus sp*. exposed to Cu showed an increase in SOD activity and was particularly high after a long-term rather than short-term exposure while CAT and APX were enhanced similarly in both periods of time. These results suggested that SOD activity alone could not alleviate the burden of ROS (Tripathi et al. 2006). Furthermore, AEA responses can be proportional to the maturity of the culture (Sauser et al. 1997). However, there are exceptions. FeSOD and MnSOD were stimulated by Cd regardless of the time of exposure (Zbigniew et al. 2006).

Until now, AEA responses to metals have been widely reported in cultures and also in the field, but mainly at the species level, showing their sensitivity to a large number of compounds and exposure conditions (Sauser et al. 1997; Li et al. 2006; Tripathi et al. 2006; Pereira et al. 2009). These studies have a low ecological realism (Depledge et al. 1995; Clements et al. 2002). Increasing the ecological relevance, microcosm studies have been done using periphyton communities (Guasch et al. 2010; Bonnineau et al.

2010; Bonnineau et al. 2011a; Bonnineau 2011b). Both experimental approaches show that AEA responses are dependent on time, dose, metal, as well as on organism (from single species to complex communities). Bonnineau's Thesis (2011) highlighted the use of biofilms as early warning systems rather than biomarkers of chronic exposure. She noted the high integrative capacity of AEA to reflect biofilm history in terms of oxidative stress. Furthermore, although AEA were not specific of a toxicant (organic or inorganic), they were defined as indicators of biofilm status, because the AEA measurements at community level, in biofilms, reflect the tendency (activation or inhibition) observed in the majority of individuals and species within the community. Thus, it was concluded that AEA could be considered a global indicator of biofilm health, more sensitive than traditional biomarkers (e.g. photosynthetic parameters), as reported in other studies (Dewez et al. 2005; Guasch et al 2010). Apart from these affirmations, Bonnineau (2011) recommended the analysis of other AEA as glutathione-S-transferase (GST) as well as further investigations to better understand AEA variations in multiple stress scenarios found in the field, to provide an insight into their variation *in situ*. These recommendations have been taken into account in this Thesis.

In order to assess the effect of metal pollution as well as environmental variables to AEA responses as biomarkers, several ecotoxicological experiments have been performed in this Thesis. A zoom from field studies (with high ecological realism) to microcosm experiment (high control conditions) has been done to fill the gap between both approaches and understand AEA responses (Fig. 3).

Figure 3. Experimental approach of the Thesis. Modified from Clements and Newman 2002



ZN WATER POLICIES

As commented before, Zn is one of the most widely-spread metals in aquatic ecosystems and often exceeds concentration thresholds marked by European and American legislation (Table 1). For example, the River Dommel, in Belgium, presents maximum Zn levels around 6600 µg L^{-1} (Admiraal et al. 1999; Ivorra et al. 2002); the River Boulder in Montana 1800 µg L^{-1} (Farag et al. 2007); the Riou Mort in France around 3000 µg L^{-1} (Morin et al. 2008) and after restoration management around 1200 µg L^{-1} (Arini et al. 2012). In the Riera d'Osor in Catalonia (Spain), maximum values of Zn are around 620 µg L^{-1} (Tlili et al. 2011).

In water management documents (EPA 2007), Zn is defined as a chemical with moderate toxicity, moderate natural occurrence and a high probability of being found in mixtures with other metals. Its environmental risk varies according to the metal form and the organism's ability to regulate and/or store it. Zn pollution has high relevance in ecological systems, requiring case-specific information for metal risk assessment (Menzie et al. 2009).

American and European legislation have developed laws that define guidelines and strategies against pollution of water in aquatic systems, the Clean Water Act (CWA, 1972) and the Water Frame Directive (WFD, 2000/60/EC), respectively. Organisms which safeguard these laws are the U.S. Environmental Protection Agency (EPA) and the European Commission (EC). The CWA define the National Recommended Water Quality Criteria (published pursuant to section 304(a)), which presents a summary table with approximately 150 (priority and non-priority) pollutants. Zn is in this list since 1995 as a priority pollutant. The WFD and its daughter, the Directive on Environmental Quality Standards (EQS, 2008/105/EC), defined a set of environmental quality standards (EQS) and elaborated a list of 33 priority pollutants and 8 priority hazardous substances. Zn is not in these lists. However, Zn is included in the list of "specific pollutants" in the Directive about water pollution by discharge of certain dangerous substances (76/464/EEC codified as 2006/11/EC). WFD requires countries to establish their own list of specific pollutants that are in significant quantities and EQS for them. The European list of priority pollutants is revised every 4 years. The European Commission has recently published the report of the revision (COM/2011/875), which includes new substances like aclonifen, bifenox, diclofenac or dioxins, but the presence of metals is still residual.

Both legislations define their own criteria about Zn concentration in aquatic systems (Table 1). The American aquatic life criterion (EPA 2006) distinguishes between the Criteria Maximum Concentration (CMC) which is an estimate of the highest concentration of a material in surface water to which an aquatic community can be exposed briefly without resulting in an unacceptable effect (acute effects) and Criterion Continuous Concentration (CCC), which is an estimate of the highest concentration of a material in surface water to which an aquatic community can be exposed indefinitely without resulting in an unacceptable effect (chronic effects). Both criteria (CMC and CCC) are close, ranging from 59 to 210 µg Zn L^{-1} in function of water hardness.

In Europe the environmental quality standards (EQS) define the Maximum Allowable Concentrations (MAC) for short-term, direct and acute toxic effects, and the Annual Average Concentrations (AAC) for protection against long-term and chronic effects. In this case, there is no European Zn threshold. In Spain, the EQS-AAC for Zn range from 30 to 500 μg Zn L^{-1} in function of water hardness too.

Table 1. Summary of American and European water polices.

	United States	European Community
Water laws	Clean Water Agency (CWA, 1972)	Water Framework Directive (WFD, 2000) and Environmental Quality Standards (EQS, 2008)
Organism	Environmental Protective Agency (EPA)	European Commission (EC)
Priority list	150 priority and non-priority pollutants (include Zn as priority pollutant since 1995)	41 priority pollutants and hazardous substances (Zn is not included)
Water criteria	Criteria Maximum Concentration (CMC, acute effects) Criterion Continuous Concentration (CCC, chronic effects)	Maximum Allowable Concentrations (MAC, acute effects) Annual Average Concentrations (AAC, chronic effects)
Zn Thresholds*	From 59 to 210 μg Zn L^{-1}	From 30 μg Zn L^{-1} to 500 μg Zn L^{-1} (in Spain)

* in function of water hardness, from soft to hard, respectively.

It is known that chemical analyses are not enough to understand the ecological status of aquatic systems. Thus, the Common Implementation Strategy (CIS) of the WFD has developed specific guidelines to provide an overall methodology approach for chemical monitoring, also including sediment and biota (EC. Guidance document n.25, 2010). The document highlights:

- The use of biota and/or sediment together with the water matrix to provide a coherent and comprehensive status of water bodies.
- The efficacy of biofilms to monitor metals due to their physicochemical preference for this matrix.

- The advantages of active biomonitoring rather than passive due to: (i) the possibility to choose the monitoring station, (ii) the knowledge of exposure duration, and (iii) the reduction of biological variability.
- The usefulness of bioassays, biomarkers and other ecotoxicological tests in base-effect monitoring methods to evaluate the real state of sediments/biota in which known and unknown contaminants are present at concentrations sufficient to cause toxicity in the organism.
- The great advantage of combining chemical, bioassays and ecological methods because they can give an answer that cannot be given by any of the individual methods by itself. The combination of these three methods is also known as the Triad approach, described in detail by Chapman (1990).

According to these remarks, active biomonitoring has been performed in this Thesis using the basis of the Triad approach to study the acute and chronic biological responses of biofilms exposed to different levels of metal pollution.

OBJECTIVES

The main objective of this Thesis is to evaluate the use of antioxidant enzyme activities (AEA) of biofilm communities as biomarkers of metal pollution (Zn) in fluvial ecosystems.

The specific objectives of this study are:

1. To explore, in a fluvial system, if the AEA of biofilm follow a seasonal pattern like other functional and structural biofilm parameters.
2. To distinguish between natural AEA variability of fluvial biofilms and AEA changes caused by metal pollution (multiple-stress situation).
3. To test the potential of AEA as a biomarker of short and long-term Zn pollution in fluvial biofilms.
4. To validate the use of AEA as biomarkers of metal pollution using active biomonitoring (effect-based) methods.

HYPOTHESIS OF THIS STUDY

Based on the current knowledge of metal ecotoxicology in fluvial biofilms and AEA responses, the following hypothesis have been formulated:

1. The AEA of biofilms are biomarkers of oxidative stress induced by metals.
2. The AEA of biofilms are influenced by environmental factors such as light or temperature.
3. The AEA of biofilms will show different magnitude and type of responses to different levels of metal exposure.

The AEA of biofilms will respond to short periods of metal exposure (~24h) but also to long-term exposure due to AEA changes linked to community adaptation

CHAPTER 1

Seasonal changes in antioxidant enzyme activities of freshwater biofilms in a metal polluted Mediterranean stream

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ABSTRACT

While seasonal variations in fluvial communities have been extensively investigated, effects of seasonality on community responses to environmental and/or chemical stress are poorly documented. The aim of this study was to describe antioxidant enzyme activity (AEA) variability in fluvial biofilms over an annual cycle, under multiple-stress scenarios due to environmental variability (e.g., light intensity, water flow, and temperature) and metal pollution (Zn, Mn and Fe). The annual monitoring study was performed at three sites according to their water and biofilm metal concentrations. Metal concentration was affected by water flow due to dilution. Low flow led to higher dissolved Zn concentrations, and thus to higher Zn accumulation in the biofilm. Water temperature, light intensity and phosphate concentration were the environmental factors which determined the seasonality of biofilm responses, whereas dissolved Zn and Zn accumulation in biofilms were the parameters linked to sites and periods of highest metal pollution. Community algal succession, from diatoms in cold conditions to green algae in warm conditions, was clearer in the non metal-polluted site than in those metal-polluted, presumably due to the selection pressure exerted by metals. Most AEA were related with seasonal environmental variability at the sites with low or no-metal pollution, except glutathione-S-transferase (GST) which was related with Zn (dissolved and accumulated in biofilm) pollution occurring at the most polluted site. We can conclude that seasonal variations of community composition and function are masked by metal pollution. From this study we suggest the use of a multi-biomarker approach, including AEA and a set of biological and physicochemical parameters as an effect-based field tool to assess metal pollution.

1. INTRODUCTION

The Water Framework Directive (WFD) (Directive 2000/60/EC), by means of the Directive on Environmental Quality Standards (EQS) (Directive 2008/105/EC), allows member states to include new EQS for their surface water bodies based on their actual state. For instance in Spain, Zn occurrence led to the establishment of Zn EQS (annual average values) of 0.5 μmol Zn L^{-1} for soft water and 7.65 μmol Zn L^{-1} for hard water (RD 60/2011), within the range of the North-American water maximum concentration quality criteria of 1.83 μmol Zn L^{-1} (EPA, 2006).

Many metal pollution cases are the result of chronic contamination mainly due to anthropogenic activities. This is the situation of our case study (Osor river, NE Spain), a small stream which presents minimum and maximum concentration values of Zn in water of approximately 5 and 9.5 μmol L^{-1}, respectively (Tlili et al., 2011; Corcoll et al., 2012). Chronic metal pollution may lead to gradual effects on the fluvial ecosystem, which are difficult to differentiate from those of natural environmental variations (Moore, 2002). Thus an important question is what tools can be used to evaluate the effects of chronic metal pollution in fluvial ecosystems and differentiate them from other environmental changes.

In fluvial ecosystems, biofilms have been used as effective bioindicators due to their characteristics: i) they have a complex community with functional and structural complexity (Sabater, 2007; Romaní, 2010); ii) they are the first step of the food chain in fluvial ecosystems and thus the first to be affected by metal pollution, which can rapidly affect their structure and function (Soldo and Behra, 2000; Morin et al., 2007; Dorigo et al., 2010; Corcoll et al., 2011; Bonet et al., 2012); iii) they integrate the effects of environmental conditions over extended periods (Dorigo et al., 2004; Sabater et al., 2007) and iv) they make it possible to show toxicological effects associated with metals using their metrics (Griffith et al., 2002). These characteristics justify their use as early-

warning indicators of toxicant exposure in aquatic ecosystems (Dorigo et al., 2004; Sabater et al., 2007; Romaní et al., 2010; Bonet et al., 2012) and also as biomarkers of chronic pollution (Corcoll et al., 2011; Tlili et al., 2011; Bonet et al., 2012). Recently there have been several attempts to develop pollution biomarkers. Some authors have suggested the use of antioxidant enzyme activities (AEA) as early warning systems and also as biomarkers of chronic pollution due to their capacity to respond after short (a few hours) and long (several weeks) periods of exposure (Valavanidis et al., 2006; Guasch et al., 2010; Maharana et al., 2010; Bonnineau et al., 2011; Bonet et al., 2012). AEA contribute to reduce reactive oxygen species (ROS), which are present in organisms due to metabolic processes such as photosynthesis and respiration, and also as a result of toxicant exposure. Until now, AEA have been widely reported in cultures and also in the field, but mainly at the species level, showing their sensitivity to a large number of compounds and exposure conditions (Sauser et al., 1997; Geoffroy et al., 2004; Li et al., 2006; Tripathi et al., 2006). However, most investigations have been performed in the laboratory, with low ecological realism (Clements and Newman, 2002). Microcosm studies have also been done using periphyton communities, increasing the realism of the approach (Guasch et al., 2010; Bonnineau et al., 2010, 2011; Bonnineau, 2011; Bonet et al., 2012). Both experimental approximations show that AEA responses are time, dose, toxicant (organic or inorganic) as well as organism (from single species to complex communities) dependent. However, several studies have also highlighted their limitations, since AEA response to stress is usually not linear and may also change after chronic exposure due to adaptation (Bonet et al., 2012).

Environmental variables such as temperature, discharge, light intensity, or nutrients can influence biofilm community sensitivity to toxicants (Guasch and Sabater, 1998; Lesser, 2006; Villeneuve et al., 2010; Tlili et al., 2010). For example, Bonnineau (2011) found that biofilm AEA may respond to organic and inorganic pollutants and also to

environmental factors such as light stress. In this case, what would be the response of AEA in the field under a multiple-stress situation including environmental and metal stress factors? Studies focussing on AEA responses in natural biofilms, under a multiple-stress situation (environmental and toxicant), are lacking. Thus, the present investigation aimed to distinguish, in the field, between natural AEA variability of fluvial biofilms (under background metal conditions) and AEA responses due to metal pollution, and also to explore if biofilm AEA followed a seasonal pattern like other functional and structural biofilm parameters.

To reach this goal, an annual monitoring was performed in the Osor stream, a fluvial stream affected by former mining activities. Three sites, characterized by having increasing values of water metal concentrations and biofilm metal contents, were selected. Several physical, chemical and biological parameters were determined to describe the system, including a set of biofilm antioxidant enzyme activities (AEA), described as a good mechanism to cope with ROS. Superoxide dismutase (SOD), which is the first line of defense, detoxifies superoxide to H_2O_2; catalase (CAT) and ascorbate peroxidase (APX) convert H_2O_2 to other non-damaging substances; furthermore APX jointly with glutathione reductase (GR) and glutathione-S-transferase (GST) is involved in the ascorbate–glutathione cycle (Mittler, 2002) which can directly reduce ROS (e.g. H_2O_2 can be reduced by APX) as well as conjugate metabolites to cope with oxidative stress (e.g. precursor of phytochelatins).

We expected to observe differences in the magnitude and type of AEA responses as well as other biological responses (functional and structural) between sites over the year due to their different metal concentrations. On the other hand, temporal changes in the biological responses were also expected in the unpolluted site due to environmental variability. Comparing these patterns with those occurring at the two polluted sites might allow us to elucidate possible interactions between biological

responses driven by environmental variability and those caused by Zn. Furthermore we expected that environmental changes over the year, like water flow, would affect metal concentration in the water and also in the biofilm. Moreover, differences in toxicity between metal species were also expected.

2. MATERIAL AND METHODS

2.1. Study site

The annual monitoring was conducted from July 2009 to July 2010 in the Osor stream, located in the north-east of Catalonia (NE Spain) (Fig. 1A). The Osor stream is a second-order stream. It is 23.5 km long and drains a catchment area (from the Guilleries Mountains) of 8890 ha. The stream's stone-bedded geological substratum is mainly siliceous, with moderate mineralization (173 mg L 162 $^{-1}$ $CaCO_3$, ACA, 2009). This stream is relatively well preserved and has well developed riparian vegetation. Urban pressures are low, with small amounts of residual sewage from the Osor village (354 inhabitants) and from a wastewater treatment plant located upstream (St. Hilari Sacalm, with 5064 inhabitants). The hydrology of the river is heavily altered due to the diversion of part of the stream's water flow for electric power production (Fig. 1B). The stream is also affected by effluents and runoff from a former mine that extracted sphalerite ((Zn,Fe)S) and galena (PbS). Although mining activities finished in 1980, no environmental rehabilitation was carried out and the stream is still receiving the input of a continuous mine effluent (referred to in the text as mine source (MS)) and also of diffuse metal inputs from mine run-over of metal-polluted landfills. This area is subject to the influence of the Mediterranean climate with an average temperature of around 12 °C and average rainfall of around 800–900 mm per year, with the maximum in the summer and autumn, respectively.

A 5 km stretch was selected starting after the Osor village and ending before Anglès town, including the mining area (Fig. 1A). Along this stretch, 3 reaches with different

levels of metal concentration were selected according to Tlili et al. (2011) and Corcoll et al.'s (2012) previous studies. From upstream to downstream, the first site was located before the mine source, with background metal concentration (referred to as B), thus metal contents were within the range found in non-metal-polluted streams. The second site, placed downstream from the mine source, had moderate metal pollution mainly due to Zn (referred to as M). Finally, the third site was placed farther downstream with low metal pollution in the water column (referred to as L) but with increased metal contents in biofilms (Fig. 1B).

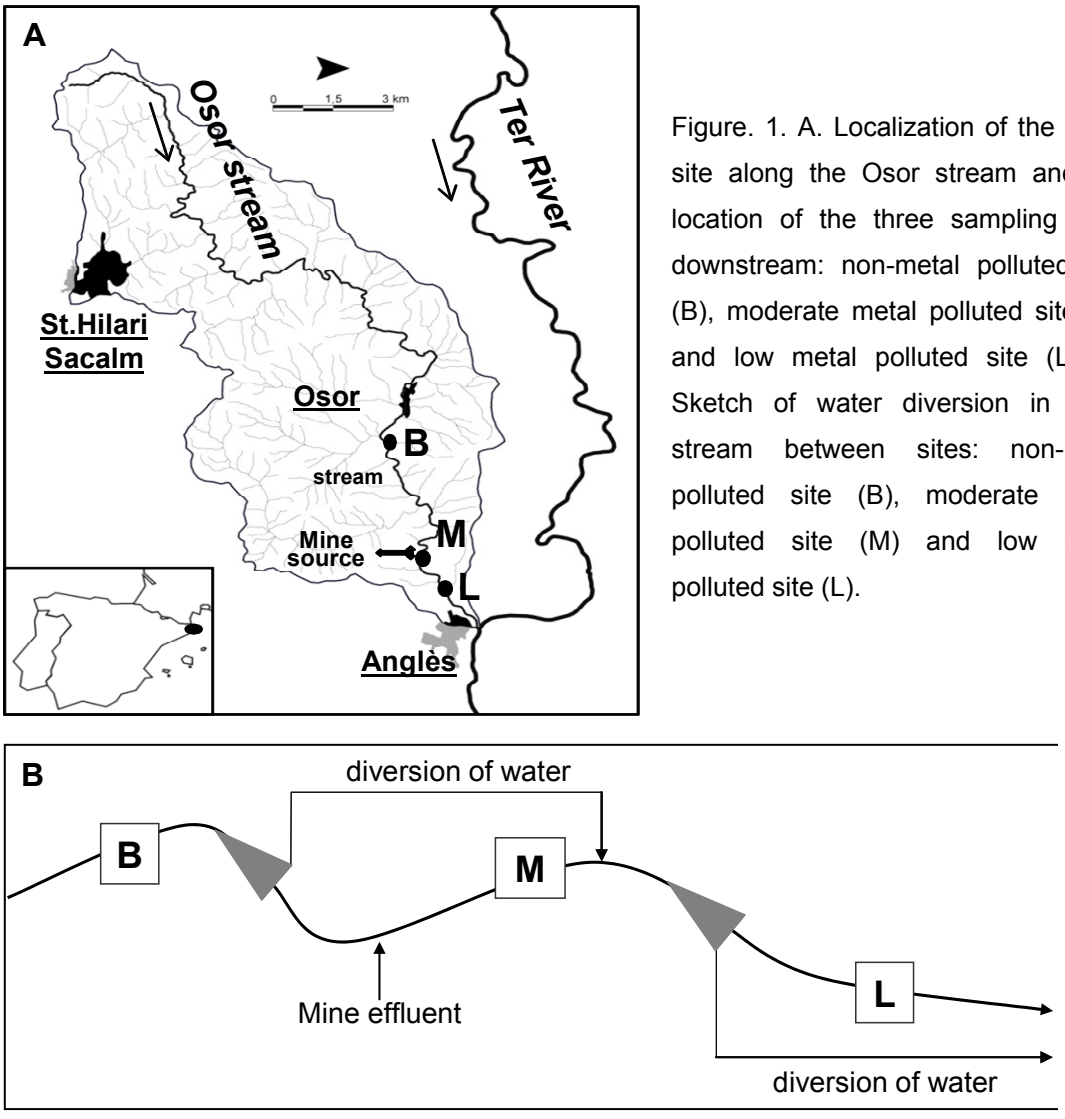


Figure. 1. A. Localization of the study site along the Osor stream and the location of the three sampling sites downstream: non-metal polluted site (B), moderate metal polluted site (M) and low metal polluted site (L). B. Sketch of water diversion in Osor stream between sites: non-metal polluted site (B), moderate metal polluted site (M) and low metal polluted site (L).

2.2. Monitoring design and sample collection

2.2.1. Physicochemical parameters

Temperature, dissolved oxygen, pH and electrical conductivity (k) were measured monthly in situ (WTW METERS, Weilheim, Germany). Water samples (10 mL) were filtered (0.2 µm nylon membrane filters, Whatman) to analyze monthly per triplicate PO_4^{3-} (following the Murphy and Riley (1962) method), anions (Cl^-, NO_2^-, NO_3^{2-}, CO_3^{2-} and SO_4^-) and cations (K^+, Na^+, NH_4^+, Ca^{2+} and Mg^{2+}) using ion-chromatography (761 Compact IC, Metrohm, Herisau, Switzerland) (Hach, 1992). Ion strength (IS) of water was calculated according to the following formula: $Im=1/2\Sigma m_B z_B^2$ where m_B was the molar concentration of ion and z_B, the charge number of that ion. The sum was taken over the major ions analyzed in the water which are mentioned above. Dissolved organic carbon (DOC) measurements were done once every two months using 20 mL of filtered water (0.2 µm nylon membrane filters, Whatman) and acidified immediately with 200 µL of HCl (2 M) and 100 µL of NaN3 (2.7 mM). These were analyzed using size-exclusion chromatography in connection to online, high sensitivity organic carbon detection (LC–OCD) used also to analyze humic substances (HS) (Huber and Frimmel, 1992; Huber and Gluschke, 1998).

Suspended solids (SS) were analyzed monthly according to Elosegui and Butturini (2009). Monthly rainfall data of the area was obtained from the website of the Osor Council (http://www.osor.cat/meteorologia.php), which has meteorological monitoring from 2008. Above-canopy global radiation data was obtained from the meteorological station of the Catalan Meteorological Service located 1 km east of the L site (Anglès town). Radiation reaching the streambed was estimated by filtering the series of data of global radiation by light interception coefficients calculated by the Hemiview canopy analysis software (version 2.1, Delta-T Devices Ltd). Hemiview was used to perform image analysis of hemispherical photography determining the gap fraction, contributions of direct and diffuse solar radiation from each sky direction, site factors

and leaf area index. Hemispherical photographs of the canopy were taken twice during the study period (July 2009 and January 2010), and every 50 m in the 3 reaches, with a high resolution digital camera (Nikon D-70s, NIKON Corporation, Tokyo, Japan) fitted to a 180° fisheye (Fisheye-NIKKOR 8 mm, NIKON Corporation, Tokyo, Japan). Global radiation was converted to photosynthetic active radiation (PAR) according to McCree (1972) and expressed as µmol photons m^2 s^{-1}. Water flow (Q) was measured monthly at each site, according to the methods of Gore and Hamilton (1996) using an acoustic Doppler velocity meter (FlowTracker Handheld-ADV®, SonTek, San Diego, CA, U.S.A.).

Analyses of total Fe, Mn and Zn dissolved in water were performed monthly in triplicate using 5 mL of filtered water (0.2 µm nylon membrane filters, Whatman) and acidified immediately with 1% of HNO_3 (65% suprapure, Merck). Analyses were done by inductively coupled plasma mass spectroscopy (ICP-MS 7500c Agilent Technologies, Inc., Wilmington, DE). Detection limits were 0.74 µmol Fe L^{-1}, 0.18 µmol Mn L^{-1} and 0.22 µmol Zn L^{-1}. When the value was below the detection limit, half of the detection limit was used for data treatment (Helsel, 1990). The accuracy of the analytical methods was checked periodically using a certified water reference (reference material for measurement of elements in surface waters: SPS-SW2 Batch 113, Oslo, Norway). Water samples were stored at 4 °C until their analysis in the laboratory. vMINTEQ program (Gustafsson, 2006) was used with the Stockholm humic acid model (Gustafsson, 2001) to model free and labile species. To do this, concentrations of HS were used together with total dissolved concentrations of metals (Zn, Mn, Fe), alkalinity, cations and anions, as well as pH and temperature. Labile metal concentrations were calculated according to Bradac et al. (2010). Calculations were performed with the assumption that Fe was present as precipitated Fe(III) oxides (i.e. ferrihydrite). Speciation analyses were done every two months.

2.2.2. Biofilm parameters

Depending on the time of year, there was more or less biofilm biomass in the stream. Hence, different biofilm surfaces were scraped directly from different pebbles (from 5 to 10) in order to obtain enough biofilm biomass to perform the required analyses. Toothbrushes were used to scrape biofilm samples and Pasteur pipettes to put the scraped biofilm in their respective vials. Biofilms were scraped directly from the surface of pebbles after defining the surface with plastic molds: one of 2 cm^2 and another of 25 cm^2. For each biofilm parameter different pebbles were scraped to obtain replicates. 25 cm^2 was scraped for each replicate of AFDW, 2 cm^2 for each replicate of Chl-a and photosynthetic parameters and between 4 and 24 cm^2 for each replicate of AEA. AFDW and Chl-a were standardized by surface.

Total Fe, Mn and Zn contents in biofilms were analyzed monthly in triplicate using 25 or 50 cm^2 of biofilm surface per replicate (to obtain enough biofilm for the analysis) after scraping three different pebbles (three different natural replicates). Biofilm samples were stored at −20 °C before analysis. Samples were lyophilized, weighed and digested with 4 mL of HNO_3 (65% suprapure, Merck) and 1 mL of H_2O_2 (30% suprapure, Merck) in a high performance microwave digestion unit (Milestone, Ethos Sel) and were thereafter diluted to 15 mL with milli-Q water. After digestion, liquid samples were also measured with ICP-MS. The detection limits were 2.2 µmol Fe L^{-1}, .21 µmol Mn L^{-1} and 0.15 µmol Zn L^{-1}. When the value was below the detection limit, half of the detection limit was also used for data treatment (Helsel, 1990). The accuracy of the analytical methods was checked periodically using certified reference materials trace elements in plankton (CRM 414), Community Bureau of Reference (BCR), Brussels, Belgium).

Total biofilm biomass was measured as ash free dry weight (AFDW). 25 cm^2 was scraped monthly in triplicate for each replicate. Samples were dried for 24 h at 105 °C

in order to calculate dry matter. Afterwards, samples were combusted in an oven at 450 °C (Obersal MOD MF12-124, Spain) for 4 h, and then weighed again to calculate the mineral content. The AFDW was calculated by subtracting the mineral matter from the total dry matter and was expressed in mg cm^{-2}.

Algal biomass was analyzed monthly in triplicate using a biofilm surface of 1 cm2 per replicate following the Jeffrey and Humphrey (1975) method and was expressed as µg Chl-a cm−2. The quotient between optical densities at 430 and 665 nm (referred to in the text as OD 430/665 ratio) (Margalef, 1983) was used as a broad indicator of the proportion of protection pigments (e.g. carotenoids) or degradation products per unit of active Chl-a. Samples were taken monthly (n=13) at each site in triplicate. The ratio between Chl-a and AFDW was also calculated, and referred to as the autotrophic index (AI).

Chlorophyll-a fluorescence parameters. The effective (Φ'_M) and optimal (Φ_M) quantum yield parameters, and the fluorescence signal linked to cyanobacteria (F(Bl)), green algae (F(Gr)) and diatoms (F(Br)), were determined by measuring the biofilm Chl-a fluorescence emissions with a PhytoPAM (pulse amplitude modulated) fluorometer (Heinz Walz GmbH). PhytoPAM uses a set of light-emitting diodes (LED) that excite chlorophyll fluorescence using four different wavelengths (470, 520, 645, and 665 nm), following the procedures described in Corcoll et al. (2011). Due to logistic problems, these parameters were sampled every two months from November to July '10 using 1 cm^2 per replicate.

2.3. Protein extraction and antioxidant enzyme activities (AEA)

Sampling, protein extraction and AEA measurements were performed as described in Bonnineau et al. (2011). Biofilm surface of 4 to 24 cm^2 was scraped in triplicate every two months. The protein concentration of the supernatant was measured and used in

triplicate for each sample following the Bradford (1986) method using Coomassie Brilliant Blue G-250 dye reagent concentrate (Bio-Rad, Laboratories GmbH, Munich, Germany) and bovine serum albumin as a standard. AEA measurements were performed in microtiter plates (UV-Star 96 well plate, Greiner®), and changes in absorbance were followed using a microtiter plate reader Synergy4 (BioTek®). Results of protein concentration are expressed as µg of protein mL^{-1}. For all assays the optimal protein concentration was 2 µg of protein. All AEA were calculated as specific activities (i.e. per µg of protein). For each assay the optimal concentration of substrate or cofactor was determined by testing the concentration in mM of H_2O_2 for the CAT and APX assays, NADPH for the GR assay, GSH for the GST assay and WST-1 for the SOD assay.

SOD activity was measured spectrophotometrically at 450 nm according to McCord and Fridovich (1969) and Peskin and Winterbourn (2000). The 200 µL reaction mixture contained the following in the final concentration: potassium phosphate buffer (50 mM, pH 8.0), diethylene triamine (0.1 mM), hypoxanthine (0.1 mM), water-soluble tetrazolium salt (WST-1 at 0.050 mM final concentration), and the enzyme extract (2 µg protein). 6 mU mL^{-1} of xanthine oxidase was added to start the assay. WST-1 produces a water-soluble formazan dye upon reduction with a superoxide anion. The rate of the reduction with $O_2^{-\bullet}$ is linearly related to the xanthine oxidase activity, and is inhibited by SOD. Therefore, the IC 50 (50% inhibition activity of SOD or SOD-like materials) can be determined by this colorimetric method at 25 °C for 10 min. SOD activity was calculated as U µg $protein^{-1}$ after calculating the specific SOD activity (U) using this formula: (%inhibition*100/50)/(protein concentration*enzymatic extract volume).

SOD competes with WST-1 for superoxide anions ($O_2^{\bullet-}$) generated by the xanthine/xanthine oxidase system. WST-1 reduction was measured at 450 nm and 25

°C for 10 min. One unit of SOD was defined as the amount of sample required for 50% inhibition of WST-1 reduction and SOD activity was expressed as U µg protein^{-1}.

CAT activity was measured spectrophotometrically at 240 nm according to Aebi (1984). 250 µL of reaction mixture was obtained by adding potassium phosphate buffer (pH 7.0) (80 mM final concentration); H_2O_2 (30 mM final concentration) and the enzyme extract (2 µg protein). The H_2O_2 consumption was determined by measuring the decrease in absorbance at 25 °C for 3 min. CAT activity was calculated as µmol H_2O_2 µg protein^{-1} min^{-1} (extinction coefficient, ε: 0.039 M cm^{-1}).

APX activitywas assessed by monitoring the decrease in absorbance at 290 nm, at 25°C and for 2 min, due to ascorbate oxidation, according to Nakano and Asada (1981). 250 µL of reaction mixture was obtained by adding potassium phosphate buffer (pH 7.0) (80 mM final concentration); H_2O_2 (4 mM final concentration); Na-ascorbate (1.5 mM final concentration) and the enzyme extract (2 µg protein). APX activity was calculated as µmol ascorbate µg protein^{-1} min^{-1} (extinction coefficient, ε: 2.8 M cm^{-1}).

GR activity was assessed by monitoring the decrease in absorbance at 340 nm, at 25 °C and for 2 min (Schaedle and Bassham, 1977). 200 µL of reaction mixture was obtained by adding Tris hydrochloride buffer (pH 7.5) (100 mM final concentration) and EDTA (1 mM); oxidized glutathione: GSSG (1 mM final concentration); NADPH (0.25 mM final concentration) and enzyme extract (2 µg protein). GR activity was calculated as µmol NADPH min^{-1} µg^{-1} of protein.

GST activity was assessed by monitoring the increase in absorbance at 340 nm, at 25ºC and for 4 min (Grant et al., 1989). 200 µL of reaction mixture was obtained by adding potassium phosphate buffer (pH 7.4) (100 mM final concentration); CDNB (1-chloro-2,4-dinitrobenzene) (50 mM final concentration); reduced glutathione: GSH (4

mM final concentration) and the enzyme extract (2 µg protein). GST activity was calculated as µmol CDNB conjugate min^{-1} $µg^{-1}$ of protein (extinction coefficient, ε: 9.6 M cm^{-1} and path length was 0.524 cm) after subtracting the Δ340 min^{-1} for the blank reaction from the Δ340 min^{-1} for each sample reaction.

2.4. Statistical analysis

All variables, except pH, were previously log-transformed to reduce skewed distributions. Two way ANOVA (site and time) was used to determine physicochemical and biological differences: i) among sites, ii) between months (time) and iii) different sites having different temporal variations (site*time). To perform this analysis, parameters with three replicates per sampling time (every month or every twomonths) were used (biological parameters, dissolved and accumulated metals and nutrients). For the rest of the physicochemical parameters with only one replicate per sampling time (every month or two months) one way ANOVA was used to explore differences between sites and over the year. Homogeneity of variances and normality of data were checked prior to data analysis. If significant differences were found ($p<0.05$), the ANOVA was followed by a Tukey-b test. Pearson correlations were performed in order to explore the relation between AEA and between metals. All these analyses were done with SPSS v15.0 software.

Finally multivariate analyses were performed in order to obtain an ordination of cases based on their environmental parameters, metals and the corresponding biological responses measured. This was done using the CANOCO soft-ware version 4.5 (Ter Braak and Smilauer, 2002). Detrended correspondence analysis (DCA) was used to determine the maximum gradient length of biofilm metrics, which was 0.86, indicating that linear methods would be appropriate (Ter Braak and Smilauer, 2002). To avoid colinearity, variables were selected based on inspection of variance inflation factors (VIF<20) (Ter Braak and Smilauer, 2002). Moreover, relationships between

environmental parameters, metals and biological variables over the year were assessed with redundancy detrended analysis (RDA). To select only the explanatory variables that significantly explained the distribution pattern of our samples, a forward selection was done at a cut-off point of p=0.1 and the significance of each variable was tested using the Monte Carlo permutation test (999 unrestricted permutations). Probabilities for multiple comparisons were adjusted using the Bonferroni correction. The variance partitioning technique was applied to separate the effects of metal concentrations and physicochemical effects on biological responses (Borcard et al., 1992). We performed a series of RDAs: (1) RDA of response variables constrained by physicochemical variables, (2) RDA of the response variables constrained by metal pollution, (3) partial RDA of the response variables constrained by physicochemical variables with metal pollution as co-variable, and (4) partial RDA of the response variables constrained by metal pollution with physicochemical variables as co-variables.

3. RESULTS

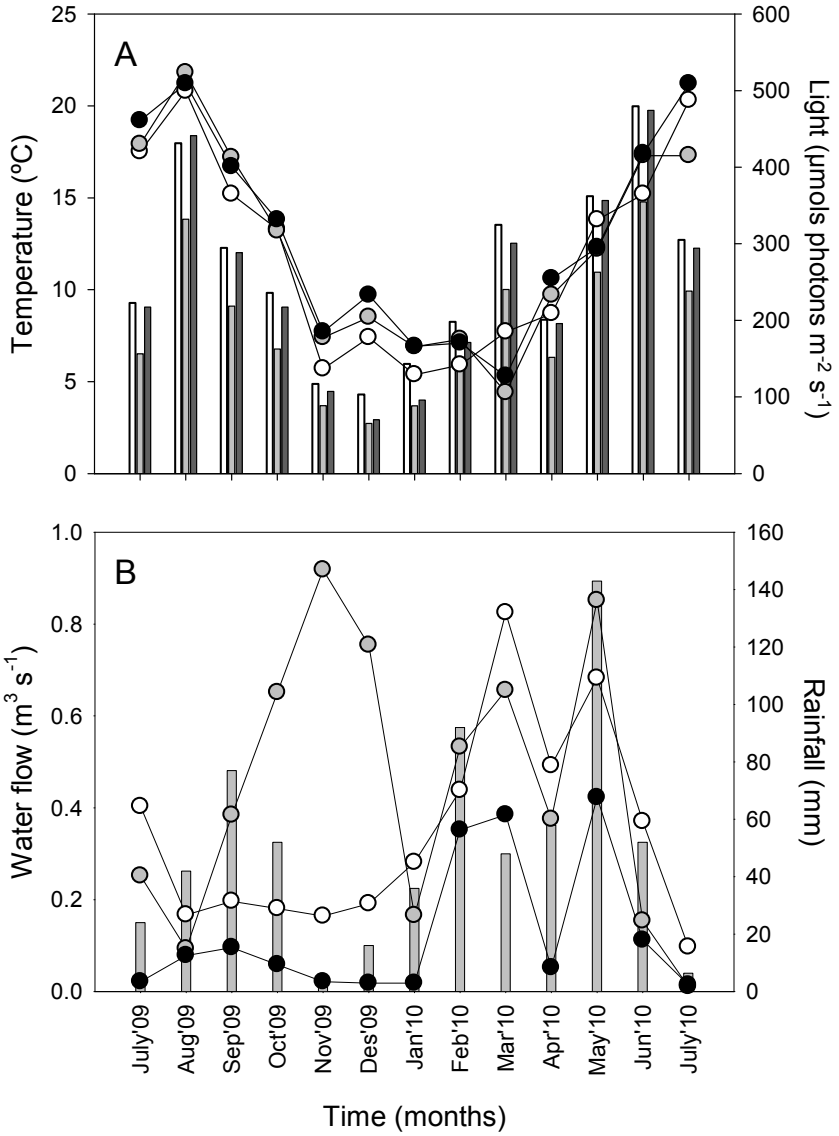
3.1. Physical and chemical parameters

Two-way ANOVA results highlight temporal variability (signifi cant differences between months) and main site-specific characteristics (significant differences between sites). Moreover, the interaction between factors indicated sites that follow different patterns of temporal variation. Sites B, L and M had similar water physicochemical parameters and only differed in pH, SS and nutrient content (higher at the upstream site, B) (Table 1). Water flow was lower at M (downstream of the mine effluent) whereas conductivity and ionic strength were higher in agreement with dissolved Zn, Mn and Fe concentrations (Table 2).

Focusing on temporal variability (factor time of the two-way ANOVA) was significant for most environmental variables following, in some cases, a clear seasonal pattern (e.g.

light, water temperature, dissolved oxygen, conductivity and HS) while nutrients (PO_4^{3-}, NO_2^{-},NO_3^{2-} and NH_4^{+}) had a more scattered temporal pattern among sites (as indicated by the significant interaction between time and site (ANOVA, F=6.07, F = 13.74, F = 5.51, F = 19.86, p < 0.0001, respectively) (Fig. 2A and Table 1)). Zn, Mn and Fe (total dissolved concentration) also had temporal variability, with higher values during summer and lower in spring, the opposite ofwater discharge. Their temporal pattern differed between sites (Figs. 2B, 3 and Table 2). Biofilm Zn content (Zn acc., Table 2 and Fig. 3) followed a similar pattern to water content, either total concentrations or free and labile species (Zn, Zn^{2+} and Zn L.).

Figure. 2. A) Mean temperatures (in lines) and light intensity (in bars); B)mean water flow (in lines) and rainfall (in bars) over the year at each site: non-metal polluted site (B) in white, low polluted site (L) in gray and moderate polluted site (M) in dark gray.



However, biofilm content presented less temporal variation. Mn and Fe biofilm content also showed fewer variations over time and in contrast to Zn these were not related with dissolved concentrations (Fig. 3 and Table 2).Whereas total dissolved Fe was higher at the M site, Fe biofilm content was similar between sites (Table 2). Significant interactions between site and time of dissolved and accumulated metals showed that sites affected seasonal responses but did not affect labile and free metals (ANOVA, $F = 0$, $p = 1$, in all dissolved metals (Zn, Mn and Fe) and $F = 11.90$, $F = 7.73$, $F = 3.86$, $p < 0.0001$ for Zn acc., Mn acc., and Fe acc., respectively).

3.2. Biological parameters

Most biological parameters differed both between sites and sampling times (Table 3 and Figs. 4 and 5), with the exception of algal biomass (Chl-a) and photosynthetic efficiency (Φ'_M), which only differed temporally, and OD, which was higher at the M site but did not change over the year. However the significant interaction between site and time showed that sites affected the seasonality of Chl-a, AFDW and AI (ANOVA; $F = 3.13$, $p < 0.0001$, $F = 5.89$, $p < 0.0001$, $F = 2.64$, $p < 0.001$, respectively). Cyanobacteria and diatom fluorescence (Fo(Bl) and Fo(Br), respectively) differed between sites, being higher at L and B respectively, while green algae fluorescence (Fo(Gr)) did not (Table 3). Moreover, over the year a clear algal succession was observed from diatoms (Fo(Br)) in cold conditions (January) to green algae (Fo(Gr)) in warm conditions (July '10). This seasonal pattern of biofilm communities was more evident in B than in polluted sites (Fig. 4). Sites affected the seasonality of Fo(Gr) and Fo(Br) according to the significant interaction between sites and time (ANOVA, $F = 3.02$, $F = 2.81$, $p < 0.05$, respectively).

3.3. Multivariate analysis

RDA analysis (Fig. 6) showed that among all the measured explanatory variables, the significant ones which made it possible to explain the distribution of sites according to

their biological responses were: temperature, light intensity, PO_4^{3-}, total dissolved Zn concentration and Zn accumulated in the biofilm (Zn and Zn acc. respectively) accounting for a significant part of the variability (62.4%). Samples from the B site were placed in RDA following the seasonality of the system. Summer samplings in July '09, September '09, May '10 and July '10 had the highest values of temperature and light intensity. Autumn sampling (November '09) was located according to the decrease in both temperature and light intensity, and winter samples, on the left side, (opposite water temperature) (Fig. 6). Following the seasonal pattern of B, there was Chl-a (increasing in winter samples) opposite most AEA (SOD, CAT, APX and GR) which increased under warmer conditions (high values of temperature and light intensity). In particular, CAT, GR and APX seemed to follow the light pattern (Fig. 6). However, sites influenced AEA responses as well as protein concentration as the significant interaction between sites and time indicate (ANOVA, F_{GST} = 103,51; F_{CAT} = 15.05, F_{APX} = 36.96, F_{GR} = 9.36, F_{SOD} = 59.75, $F_{proteins}$ = 35.21, $p < 0.0001$).

Samples from L and M were also arranged temporally but they were highly influenced by Zn and Zn acc. (Fig. 6). This group included sampling times characterized by having high Zn and Zn acc. in the biofilm within the lowest water flow period. Under these conditions GST was the only antioxidant system enhanced (Fig. 6). Similarly, protein concentration was also higher in samples with high Zn content. However, this parameter was also related with environmental parameters like light intensity in the RDA.

The variance partitioning technique made it possible to distinguish between the percentage of variance explained by environmental parameters and that explained by metal pollution. Environmental data (temperature, light intensity and PO_4^{3-}) alone accounted for 31.7% of the total explained variation, while metal pollution data (Zn and Zn acc.) alone explained 20.5%. The shared fraction of total variation between

environmental and metal pollution data was 10.2%, so the unexplained variation was 37.6%. Changes in the pigment ratio (OD 430/665 ratio), CAT, APX and GR and proteins, were explained mainly by environmental parameters. GST was explained by metals while total biomass (AFDW), Chl-a, the autotrophic index (AI) variability and SOD, were explained by both environmental parameters and metals (the shared fraction of total variation).

Figure 3. Average and standard deviation of total dissolved metals (µmol L^{-1}) and accumulated in biofilms (mmol g $AFDW^{-1}$) over the year at each site: non-metal polluted site (B) in white, low metal polluted sites (L) in gray and moderate metal polluted site (M) in black.

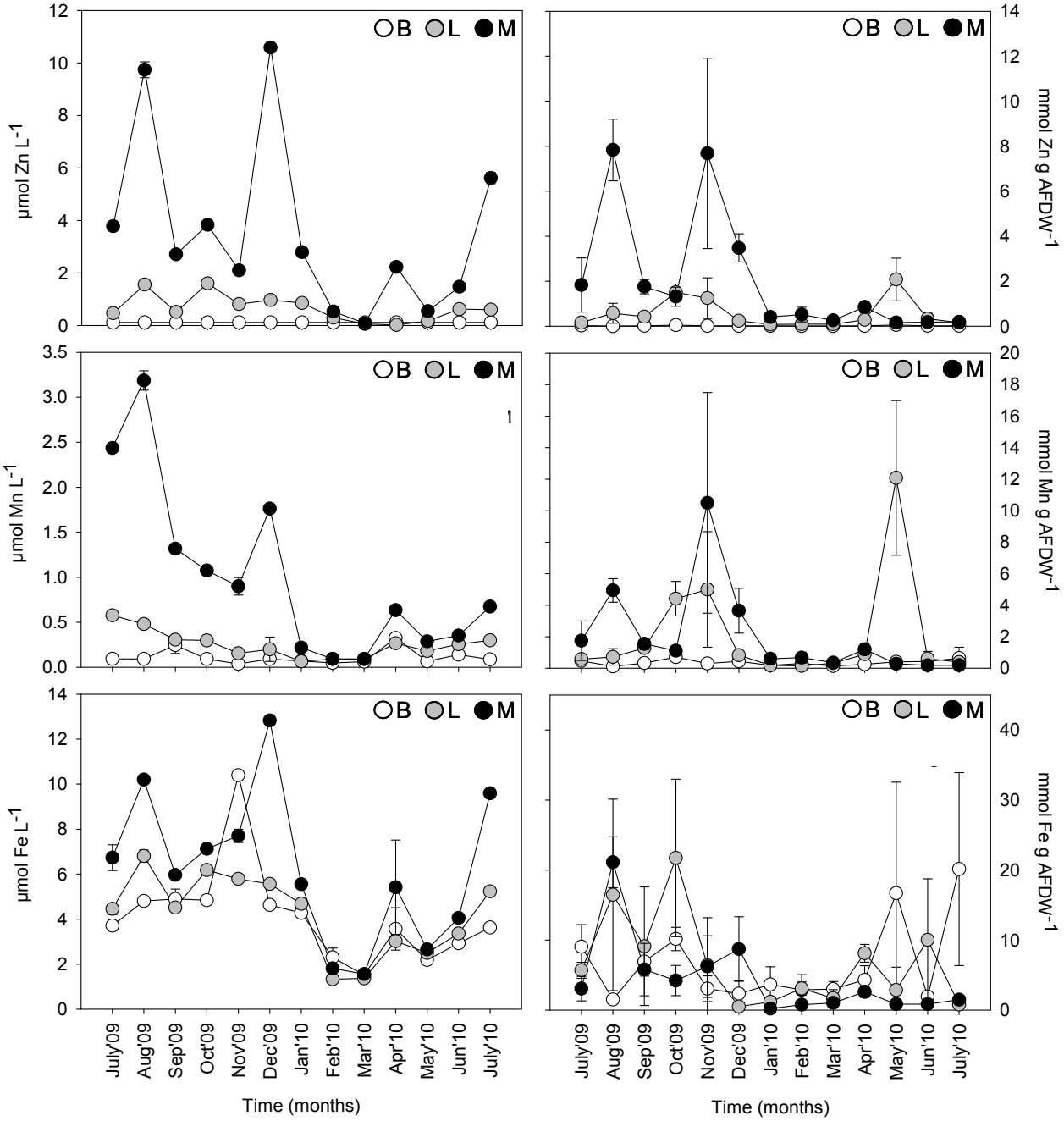


Table 1. Average of physicochemical parameters at the non-metal polluted site (B), low (L) and moderate (M) metal polluted sites and at each sampling time (from July 2009 to July 2010). Parameters are: water pH (pH), oxygen (Oxy), conductivity (*K*), temperature (T), solids in suspension (SS), dissolved organic carbon (DOC), humic acids (HA), phosphate (PO_4^{3-}), nitrite (NO_2^-), nitrate (NO_3^{2-}), ammonium (NH_4^+), ion strength (IS), water flow (Q) and light intensity (light). Different letters indicate significant differences (tukey-b test) between sites and sampling times.

.		pH*	Oxy*	*K**	T*	SS*	DOC*	HA*	PO_4^{3-}**	NO_2^-**	NO_3^{2-}**	NH_4^+**	IS **	Q*	Light *
		u.pH	mg L^{-1}	µS cm^{-1}	ºC	mg L^{-1}	mg L^{-1}	mg L^{-1}	µg L^{-1}	µg L^{-1}	mg L^{-1}	µg L^{-1}	mol L^{-1}	m^3 s^{-1}	µmol photons m^2 s^{-1}
Site	B	8.34^{a}	10.7	273^{b}	12.07	4.82^{a}	2.99	0.73	441^{a}	78^{c}	4.92^{a}	170^{a}	0.003^{c}	0.35^{a}	263
	L	7.81^{c}	10.5	286^{ab}	12.68	1.54^{b}	3.69	0.83	376^{b}	142^{b}	5.08^{a}	92^{b}	0.004^{b}	0.45^{a}	192
	M	8.08^{b}	10.6	347^{a}	13.01	2.06^{b}	3.26	0.86	290^{c}	385^{a}	4.33^{b}	109^{b}	0.005^{a}	0.13^{b}	249
	N / site	*13*	*13*	*13*	*13*	*13*	*7*	*7*	*39*	*39*	*39*	*39*	*39*	*13*	*13*
	F	*15.05*	*Ns*	*3.43*	*ns*	*7.52*	*ns*	*ns*	*83.6*	*275.1*	*8.27*	*26.41*	*326.10*	*6.36*	*ns*
	p-value	*p < 0.0001*	*p > 0.05*	*p < 0.05*	*p > 0.05*	*p < 0.005*	*p > 0.05*	*p > 0.05*	*p < 0.0001*	*p < 0.0001*	*p < 0.001*	*p < 0.0001*	*p < 0.0001*	*p < 0.005*	*p > 0.05*
Time	Jul'09	7.85	9.3^{f}	277^{ab}	18.2^{bc}	2.62	2.61	0.79^{bc}	341^{de}	81^{d}	3.66^{de}	23^{e}	0.004^{c}	0.23	199^{def}
	Aug	7.95	8.7^{f}	407^{a}	21.3^{a}	0.25	*nd*	*nd*	403^{cd}	102^{d}	2.97^{de}	71^{de}	0.005^{a}	0.11	402^{ab}
	Sep	8.16	9.4^{f}	313^{ab}	16.4^{c}	2.93	3.06	0.76^{bc}	329^{ef}	23^{d}	3.67^{de}	0^{e}	0.004^{cd}	0.23	267^{cde}
	Oct	8.17	9.8^{ef}	395^{a}	13.4^{d}	1.56	*nd*	*nd*	279^{efg}	197^{c}	2.82^{e}	51^{de}	0.005^{a}	0.30	205^{def}
	Nov	7.92	11.9^{bcd}	360^{ab}	6.9^{ef}	2.17	2.43	0.58^{bc}	288^{efg}	241^{bc}	3.27^{de}		0.005^{b}	0.37	104^{fg}
	Dec	8.27	9.7^{ef}	387^{ab}	8.5^{ef}	4.37	*nd*	*nd*	262^{fg}	367^{a}	3.28^{de}	50^{de}	0.005^{b}	0.32	80^{g}
	Jan	8.10	12.6^{ab}	256^{ab}	6.4^{f}	5.78	2.88	0.61^{c}	484^{ab}	242^{bc}	7.78^{ab}	4^{e}	0.003^{de}	0.16	109^{fg}
	Feb	8.00	11.3^{cd}	246^{ab}	6.8^{ef}	5.76	*nd*	*nd*	420^{bc}	202^{c}	8.33^{a}	451^{a}	0.003^{ef}	0.44	169^{efg}
	Mar	8.22	13.4^{a}	224^{ab}	5.8^{f}	2.67	5.03	1.22^{a}	290^{efg}	183^{c}	5.90^{c}	289^{b}	0.003^{ef}	0.62	288^{cd}
	Apr	8.12	12.4^{abc}	254^{ab}	9.7^{e}	2.26	*nd*	*nd*	461^{abc}	261^{bc}	6.79^{bc}	208^{c}	0.004^{cd}	0.31	183^{defg}
	May	8.06	10.8^{de}	194^{b}	12.8^{d}	1.86	4.82	0.89^{b}	225^{g}	213^{c}	3.81^{de}	306^{b}	0.002^{f}	0.65	327^{bc}
	Jun	8.14	8.9^{f}	270^{ab}	16.6^{c}	2.91	*nd*	*nd*	512^{a}	209^{c}	5.69^{c}	18^{e}	0.003^{ef}	0.21	436^{a}
	Jul'10	8.03	9.0^{f}	320^{ab}	19.6^{ab}	1.32	2.38	0.72^{bc}	501^{a}	305^{ab}	4.16^{d}	118^{d}	0.005^{ab}	0.04	279^{cde}
	N / time	*3*	*3*	*3*	*3*	*3*	*3*	*3*	*9*	*9*	*9*	*9*	*9*	*3*	*3*
	F	*ns*	*30.65*	*3.18*	*71.78*	*ns*	*ns*	*12.36*	*33.27*	*20.53*	*44.24*	*74.03*	*65.84*	*ns*	*21.36*
	p-value	*p > 0.05*	*p < 0.0001*	*p < 0.01*	*p < 0.0001*	*p > 0.05*	*p > 0.05*	*p < 0.0001*	*p < 0.0001*	*p < 0.0001*	*p < 0.0001*	*p < 0.0001*	*p < 0.0001*	*p > 0.05*	*p < 0.0001*

ns means non-significant.

** Two way ANOVA results;

* One way ANOVA results per site and time;

Table 2. Average of metal concentrations in water (dissolved (M), free (M^{2+}) and labile (M L.)) and accumulated in biofilm (M acc.) at the non-metal polluted site (B), low (L) and moderate (M) metal polluted sites and at each sampling time (from July 2009 to July 2010). Different letters indicate significant differences (tukey-b test) between sites and sampling times. Free and labile Fe species were below the detection limit.

		Zn** µmol L^{-1}	Mn** µmol L^{-1}	Fe** µmol L^{-1}	Zn acc.** mmol g $AFDW^{-1}$	Mn acc.** mmol g $AFDW^{-1}$	Fe acc.** mmol g $AFDW^{-1}$	Zn^{2+}* µmol L^{-1}	Zn.L* µmol L^{-1}	Mn^{2+}* µmol L^{-1}	Mn.L* µmol L^{-1}
Site	B	bdl^{c}	0.11^{c}	4.13^{b}	0.02^{c}	0.36^{b}	6.59	bdl^{b}	bdl^{b}	0.20^{b}	0.29^{b}
	L	0.66^{b}	0.25^{b}	4.21^{b}	0.56^{b}	2.11^{a}	6.74	0.53^{b}	0.65^{b}	0.31^{b}	0.36^{b}
	M	3.54^{a}	1.00^{a}	6.20^{a}	2.04^{a}	2.07^{a}	4.37	1.74^{a}	2.64^{a}	0.58^{a}	0.84^{a}
	n/site	*39*	*39*	*39*	*39*	*39*	*39*	*7*	*7*	*7*	*7*
	F	*22584.46*	*0*	*0*	*68.00*	*16.20*	*ns*	*9.43*	*10.89*	*6.10*	*5.60*
	p-value	*p < 0.0001*	*p = 1*	*p = 1*	*p < 0.0001*	*p < 0.0001*	*p > 0.05*	*p < 0.001*	*p < 0.0001*	*p < 0.01*	*p < 0.01*
Time	Jul'09	1.45^{d}	1.04^{b}	4.97^{d}	0.67^{b}	0.92^{b}	5.93^{abc}	1.30	1.53	0.53	0.80
	Aug	3.80^{a}	1.25^{a}	7.27^{b}	2.80^{a}	1.92^{b}	13.0^{a}	*nd*	*nd*	*nd*	*nd*
	Sep	1.11^{f}	0.62^{d}	5.13^{d}	0.73^{b}	1.05^{b}	7.27^{abc}	0.61	1.17	0.53	0.80
	Oct	1.85^{c}	0.49^{e}	6.05^{c}	0.96^{b}	2.08^{b}	12.0^{ab}	*nd*	*nd*	*nd*	*nd*
	Nov	1.01^{g}	0.36^{f}	7.96^{a}	2.98^{a}	5.26^{a}	5.22^{abc}	0.84	1.09	0.41	0.55
	Dec	3.89^{a}	0.68^{c}	7.67^{ab}	1.24^{b}	1.64^{b}	3.85^{bc}	*nd*	*nd*	*nd*	*nd*
	Jan	1.25^{e}	0.12^{i}	4.84^{d}	0.17^{b}	0.32^{b}	1.68^{c}	1.03	1.31	0.28	0.30
	Feb	0.31^{i}	0.08^{i}	1.81^{h}	0.21^{b}	0.37^{b}	2.24^{c}	*nd*	*nd*	*nd*	*nd*
	Mar	0.08^{j}	0.08^{i}	1.48^{h}	0.12^{b}	0.27^{b}	1.90^{c}	0.17	0.23	0.09	0.13
	Apr	0.79^{h}	0.41^{f}	4.00^{e}	0.38^{b}	0.78^{b}	5.01^{abc}	*nd*	*nd*	*nd*	*nd*
	May	0.28^{i}	0.18^{h}	2.45^{g}	0.76^{b}	4.25^{a}	6.80^{abc}	0.26	0.35	0.29	0.35
	Jun	0.73^{h}	0.25^{g}	3.45^{f}	0.18^{b}	0.40^{b}	4.27^{bc}	*nd*	*nd*	*nd*	*nd*
	Jul'10	2.11^{b}	0.35^{f}	6.15^{c}	0.12^{b}	0.40^{b}	7.47^{abc}	1.35	2.19	0.40	0.54
	n/time	*9*	*9*	*9*	*9*	*9*	*9*	*3*	*3*	*3*	*3*
	F	*0*	*0*	*0*	*13.44*	*9.31*	*4.02*	*ns*	*ns*	*ns*	*ns*
	p-value	*p = 1*	*p = 1*	*p = 1*	*p < 0.0001*	*p < 0.0001*	*p < 0.0001*	*p > 0.05*	*p > 0.05*	*p > 0.05*	*p > 0.05*

ns means non-significant; *nd* means no data; bdl means below detection limit.

** Two way ANOVA results;

* One way ANOVA results per site and time;

Table 3. Average of biological parameters at the non-metal polluted site (B), low (L) and moderate (M) metal polluted sites and at each sampling time (from July 2009 to July 2010). Biological parameters are: algal biomass (Chl-a), pigment ratio (OD), biomass (AFDW), autotrophic index (AI), fluorescence of cyanobacteria (Fo(Bl)), green algae (Fo(Gr)) and diatoms (Fo(Br)), effective and optimal photosynthetic activity (Φ'_M and Φ_M respectively), protein concentration of biofilm (proteins), glutathione-S-transferase (GST), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR) and superoxide dismutase (SOD). Different letters indicate significant differences (tukey-b test from two way ANOVA measurements) between sites and sampling times.

		Chl-a µg cm^{-2}	OD ratio	AFDW mg cm^{-2}	AI	Fo(Bl) %	Fo(Gr) %	Fo(Br) %	Φ'_M	Φ_M	Proteins mg g $AFDW^{-1}$	GST µmol CDNB conjugate min^{-1} ng^{-1} of protein	CAT µmol H_2O_2 µg $protein^{-1}$ min^{-1}	APX µmol Ascorbate µg $protein^{-1}$ min^{-1}	GR µmol NADPH min^{-1} μg^{-1} of protein	SOD U µg $protein^{-1}$
Site	B	58.56	2.22^{a}	1.27^{a}	0.10^{b}	56.55^{b}	14.83	28.24^{a}	0.27	0.80^{a}	11.80^{b}	0.051^{b}	0.25^{a}	$1.87{\cdot}10^{-3a}$	$4.19{\cdot}10^{-4a}$	98.33^{a}
	L	47.81	2.31^{a}	0.90^{b}	0.09^{b}	72.58^{a}	12.25	15.16^{b}	0.24	0.79^{a}	18.07^{b}	0.008^{c}	0.18^{c}	$1.61{\cdot}10^{-3b}$	$2.80{\cdot}10^{-4b}$	125.95^{a}
	M	50.62	1.98^{b}	0.75^{b}	0.23^{a}	55.28^{b}	21.38	22.13^{ab}	0.27	0.68^{b}	42.54^{a}	0.071^{a}	0.22^{b}	$0.53{\cdot}10^{-3c}$	$3.98{\cdot}10^{-4a}$	96.13^{b}
	N /site	*39*	*39*	*39*	*39*	*5*	*5*	*5*	*5*	*5*	*7*	*7*	*7*	*7*	*7*	*7*
	F	*ns* *p > 0.05*	*5.791* *p < 0.005*	*10.79* *p < 0.0001*	*8.49* *p < 0.0001*	*8.85* *p < 0.001*	*ns* *p > 0.05*	*3.57* *p < 0.05*	*ns* *p > 0.05*	*4.75* *p < 0.05*	*36.74* *p < 0.0001*	*209.63* *p < 0.0001*	*17.00* *p < 0.0001*	*191.76* *p < 0.0001*	*12.32* *p < 0.0001*	*130.52* *p < 0.0001*
Time	Jul'09	27.73^{de}	2.19	0.37^{d}	0.25^{abc}	*nd*	*nd*	*nd*	*nd*	*nd*	65.49^{a}	0.088^{a}	0.36^{a}	$1.50{\cdot}10^{-3b}$	$3.13{\cdot}10^{-4b}$	100.70^{c}
	Aug	33.96^{de}	2.26	1.58^{bc}	0.20^{abc}	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
	Sep	38.38^{de}	1.85	0.88^{cd}	0.09^{bc}	*nd*	*nd*	*nd*	*nd*	*nd*	11.42^{c}	0.052^{b}	0.14^{d}	$1.28{\cdot}10^{-3b}$	$4.06{\cdot}10^{-4ab}$	98.25^{cd}
	Oct	27.41^{de}	2.17	0.32^{d}	0.12^{abc}	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
	Nov	50.92^{cd}	2.23	0.65^{d}	0.32^{ab}	53.41	19.05^{ab}	27.54^{b}	0.14^{c}	0.42^{c}	17.24^{c}	0.000^{d}	0.19^{cd}	$1.44{\cdot}10^{-3b}$	$3.28{\cdot}10^{-4b}$	116.53^{b}
	Dec	98.55^{b}	2.11	1.00^{cd}	$0.15a^{bc}$	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
	Jan	70.89^{c}	2.70	1.72^{b}	0.05^{c}	54.86	0.00^{c}	45.14^{a}	0.32^{a}	0.67^{b}	49.14^{b}	0.091^{a}	0.21^{c}	$0.65{\cdot}10^{-3c}$	$2.90{\cdot}10^{-4b}$	84.78^{e}
	Feb	48.51^{cd}	2.29	1.54^{bc}	0.04^{c}	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
	Mar	127.94^{a}	2.12	2.49^{a}	0.05^{c}	68.04	7.60^{bc}	21.70^{b}	0.34^{a}	0.83^{a}	5.57^{c}	0.032^{c}	0.14^{cd}	$0.64{\cdot}10^{-3c}$	$4.56{\cdot}10^{-4a}$	89.97^{de}
	Apr	$35.49d^{e}$	2.08	0.92^{cd}	0.04^{c}	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
	May	16.17^{e}	2.10	0.31^{d}	0.08^{bc}	66.62	22.68^{ab}	10.70^{bc}	0.24^{b}	0.94^{a}	14.17^{c}	0.042^{bc}	0.28^{b}	$0.92{\cdot}10^{-3c}$	$4.85{\cdot}10^{-4a}$	95.64^{cd}
	Jun	27.11^{de}	2.03	0.52^{d}	0.07^{bc}	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
	Jul'10	77.22^{bc}	2.10	0.29^{d}	0.35^{a}	64.42	31.44^{a}	4.14^{c}	0.25^{b}	0.91^{a}	5.91^{c}	0.000^{d}	0.19^{cd}	$2.90{\cdot}10^{-3a}$	$2.83{\cdot}10^{-4b}$	161.08^{a}
	N /time	*3*	*3*	*3*	*3*	*3*	*3*	*3*	*3*	*3*	*3*	*3*	*3*	*3*	*3*	*3*
	F	*23.59* *p < 0.0001*	*ns* *p > 0.05*	*16.12* *p < 0.0001*	*3.81* *p < 0.0001*	*ns* *p > 0.05*	*8.05* *p < 0.0001*	*12.68* *p < 0.0001*	*17.16* *p < 0.0001*	*28.86* *p < 0.0001*	*34.21* *p < 0.0001*	*118.59* *p < 0.0001*	*31.60* *p < 0.0001*	*99.76* *p < 0.0001*	*6.18* *p < 0.0001*	*135.08* *p < 0.0001*

ns means non-significant; *nd* means no data.

Figure. 4. Graphs on the left side show the mean fluorescence of the main algal group at each site (cyanobacteria in gray (Fo(Bl)), green algae in stripes (Fo(Gr)) and diatoms in white (Fo(Br))). Graphs on the right side show the average and standard deviation of effective (Φ'_M) and optimal (Φ_M) photosynthetic efficiencies over the year at each site: non-metal polluted site (B) in white, low metal polluted sites (L) in gray and moderate metal polluted site (M) in black.

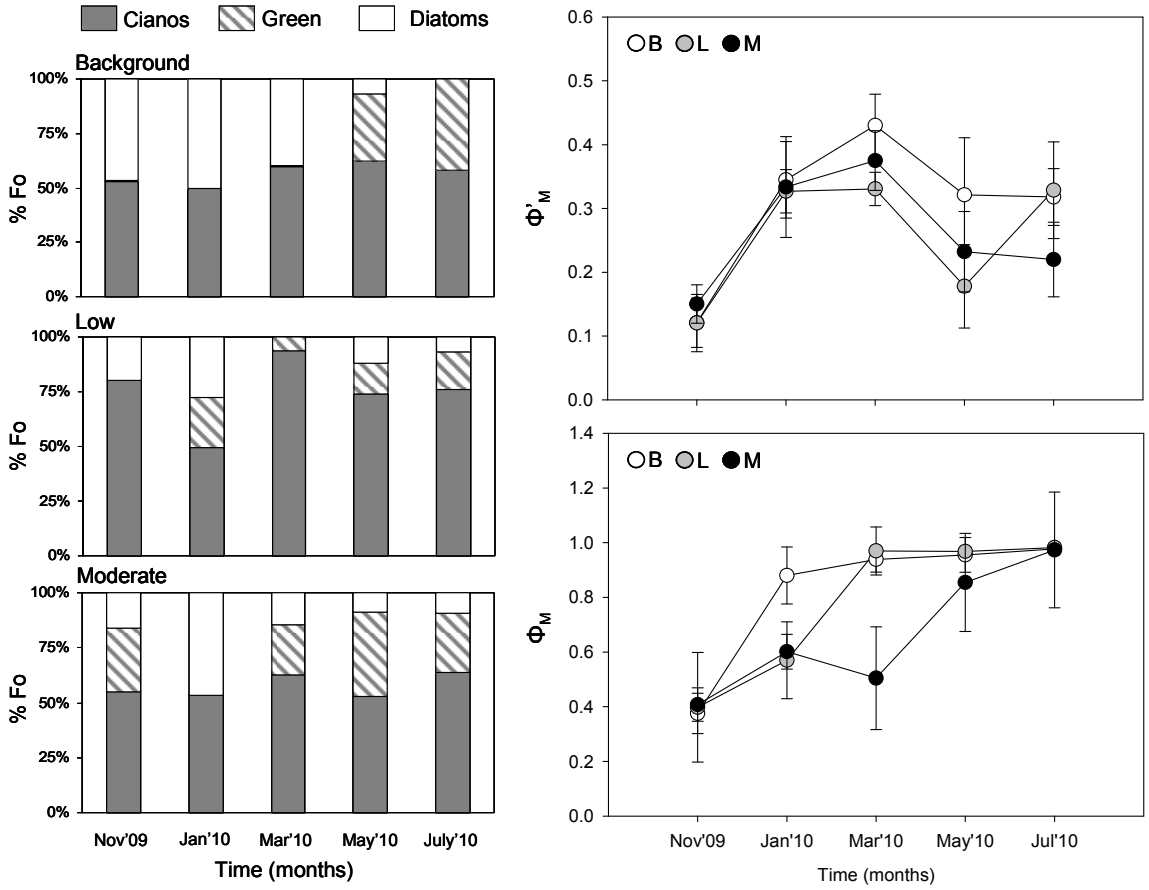


Figure 5. Average and standard deviation of antioxidant enzyme activities and biofilm protein concentration over the year at each site: non-metal polluted site (B) in white, low metal polluted sites (L) in gray and moderate metal polluted site (M) in black. CAT is expressed as µmol H_2O_2 µg protein^{-1} min^{-1}, GR as µmol NADPH min^{-1} µg^{-1} of protein, SOD as U µg protein^{-1}, APX as µmol Ascorbate µg protein^{-1} min^{-1}, GST as µmol CDNB conjugate min^{-1} µg^{-1} of protein and proteins as mg protein g AFDW^{-1}.

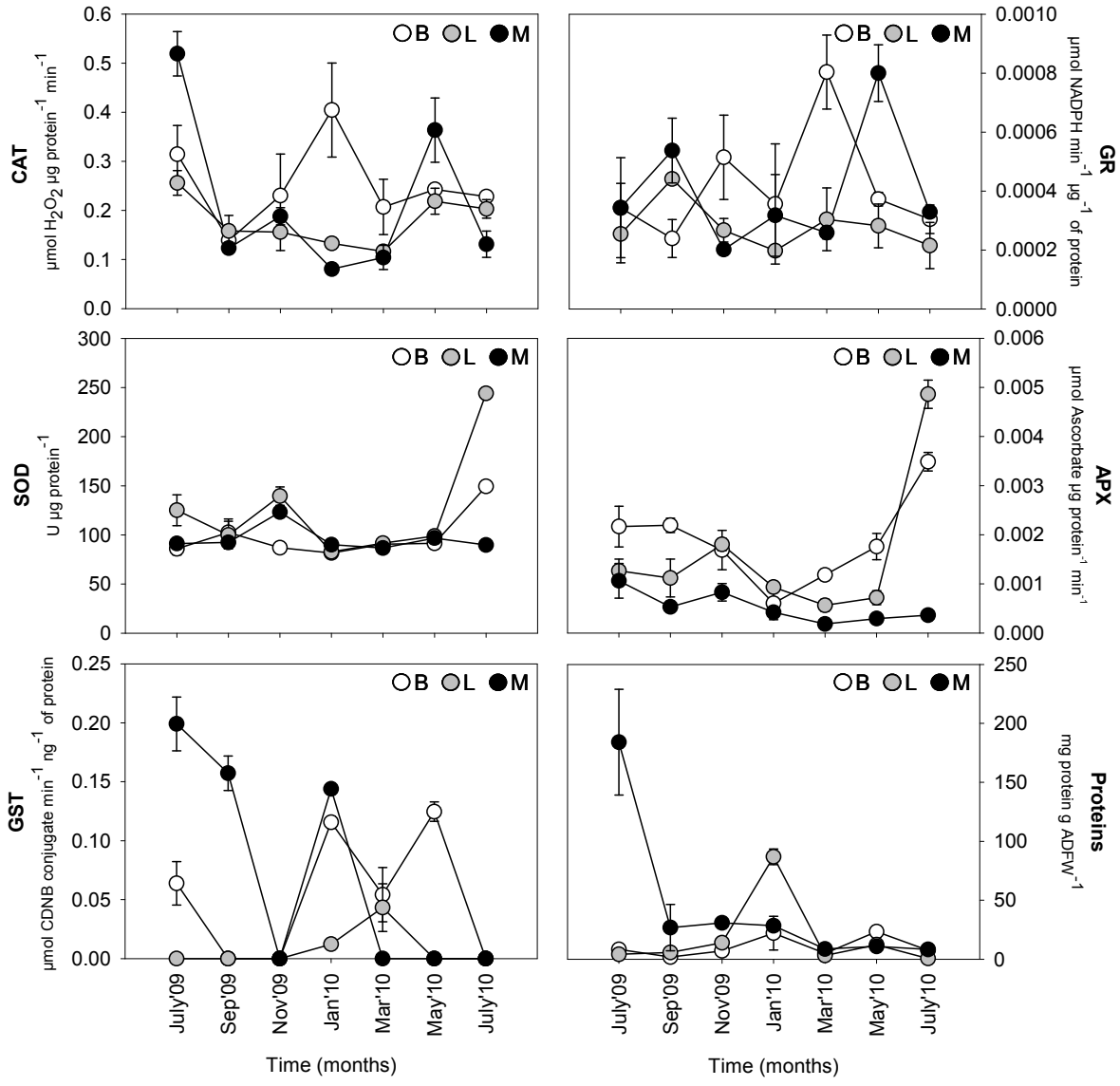
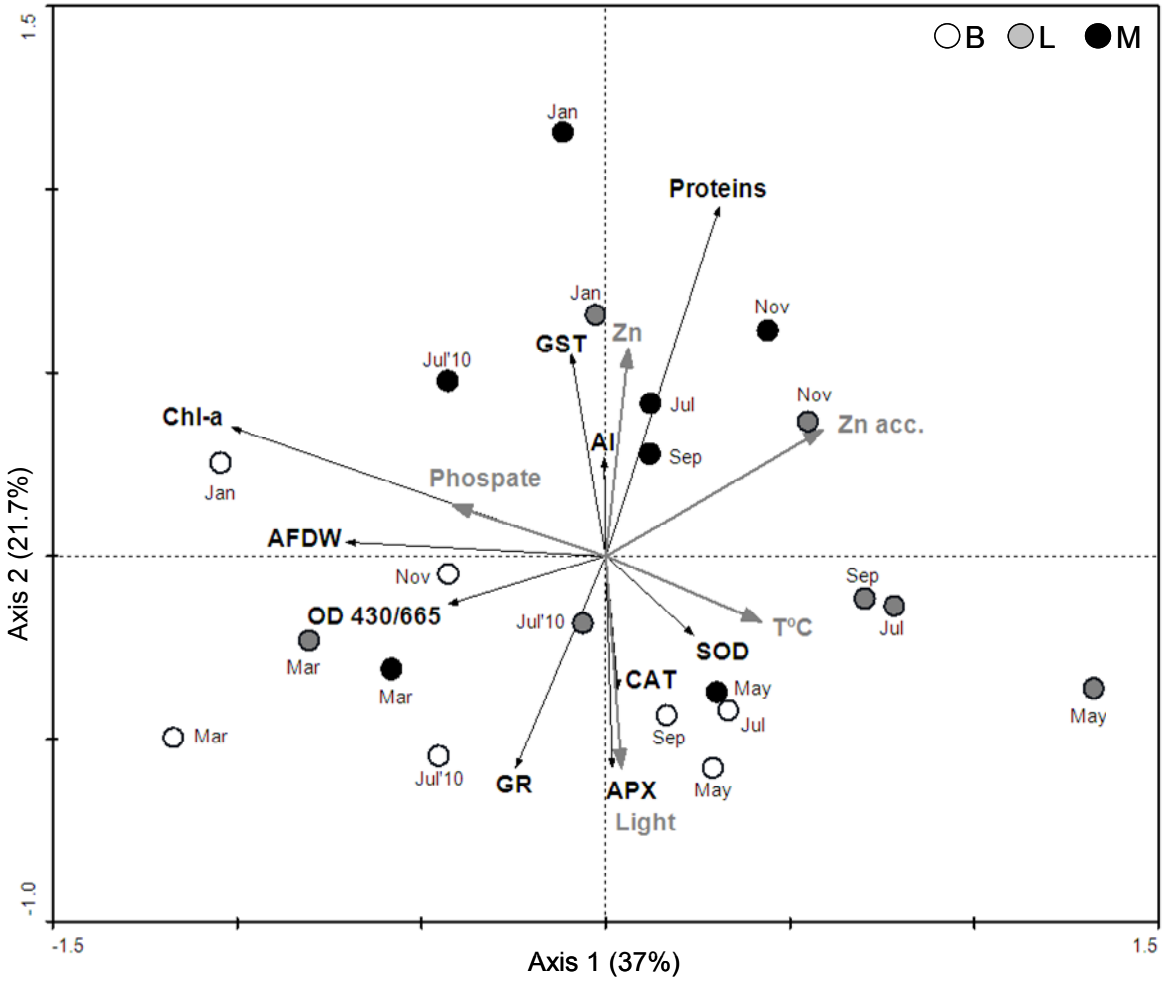


Figure 6. Triplot based on RDA of environmental parameters, metal concentration in biofilm and biological parameters of the annual monitoring in Osor stream which explain 62.4% of total variance. Three sampling sites are represented: non-metal polluted site (B) in white, low metal polluted site (L) in gray and moderate metal polluted site (M) in black at each sampling time from July'09 (referred to as Jul) to July'10. Gray arrows representing the constraint variables give dissolved Zn in water and Zn accumulation in biofilm (Zn and Zn acc., respectively), temperature (TºC), light intensity (Light) and PO_4^{3-} (Phosphate). Black arrows represent biological variables: algal biomass (Chl-a), pigment ratio (OD 430/665), biomass (AFDW), autotrophic index (AI), protein concentration (Proteins), superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione reductase (GR) and glutathione-S-transferase (GST).



4. DISCUSSION

Seasonal pattern in the Osor stream followed the pattern described in other forested Mediterranean streams (Guasch and Sabater, 1995; Acuña et al., 2007). Water temperature followed ambient temperature changes, whereas light intensity was influenced by riparian shade, reducing the amount of light reaching the river bed in

summer and autumn leading to a maximal standing crop of algae (in terms of chlorophyll-a) in winter and early spring. The expected biofilm seasonality succession (Margalef, 1963) was also observed, showing a shift from diatoms (in winter and early spring) to green algae (from late spring to late summer).

AEA responses to environmental changes have been reported in several investigations performed in the laboratory (Butow et al., 1997; Aguilera et al., 2002; Li et al., 2010). In this study we provide, for the first time, community level (fluvial biofilms) data of AEA responses to environmental changes and also to a multiple-stress situation (environmental stress plus metal pollution). Temporal patterns in AEA were linked to seasonality at the non-polluted site (B). APX, CAT, GR and SOD were enhanced under situations of high light intensity and water temperature (Fig. 6) indicating that AEA may contribute to mitigate environmental stress in natural biofilm communities. In agreement with our results, several investigations have reported AEA responses to environmental variability, mainly attributed to the increase of ROS linked to photosynthesis enhancement. AEA seasonality was reported in freshwater dinoflagellates, increasing overall at the end of spring in conjunction with elevated ambient stress conditions (i.e. light intensity) (Butow et al., 1997). A clear seasonal variation was also reported in green and red arctic macroalgae. Under UV-B radiations, GR decreased significantly in early summer, maybe due to direct UV-B radiation damage or predominant use of CAT. Similarly, Aguilera et al. (2002) reported the increase in SOD and CAT at the end of the summer. In contrast, Li et al. (2010) observed an increase of APX in macroalgae under high UV-B conditions as well as SOD maintenance but a decrease in CAT activity. In fluvial biofilms, an increase of APX under intense light was also reported in microcosm experiments (Bonnineau, 2011). In conclusion, these studies support the results observed in our field investigation, which showed a clear seasonality either in biofilm algal groups or AEA

responses. However, since our study was conducted at a community level, and communities change over time, it was difficult to ascertain whether AEA variability was due to photo and/or thermoacclimation occurring at the species level and/or could be attributed to community composition changes in the biofilm and their specific antioxidant enzymes.

In addition to environmental factors which drive functional and structural changes in biofilm communities (Romaní and Sabater, 2001), it is also largely reported that metals cause toxicity in biofilms. In this study, water flow was driving metal toxicity, diluting Zn concentration under high flow and vice versa. This is a common relation between water flow and metals in mining areas (Guasch et al., 2009). Although bioavailability and toxicity of trace metals to aquatic microorganisms are determined by free metal species (Guasch et al., 2009), in this study dissolved (which largely exceed 2.15 μmol L^{-1} legislation limits according to EPA, 2006) and labile Zn could also be considered as good predictors of toxicity because they followed the same pattern as Zn^{2+}. Furthermore Zn accumulation in biofilms made it possible to differentiate the most polluted site (M) from the rest. In contrast, although Mn and Fe were always higher in M, their accumulation did not differ between sites. In addition, their dissolved concentration in water was not expected to cause toxicity based on toxicity thresholds reported by EPA (2006). Thus, the difference observed between sites could be attributed to Zn, either in water or accumulated in biofilms as RDA analysis highlights.

The most polluted site in this study was defined as moderately polluted (M), with maximum Zn values around 11 μmol L^{-1}. Despite exceeding the EPA toxicity threshold, it was lower than in other streams, like in Riou Mort (SW France), where Zn values were 2.5 times higher (Arini et al., 2011) or in River Dommel (Admiraal et al., 1999), around 9 times higher. Thus, this study provides evidence of how Zn pollution defined

as moderate (if compared with other metal polluted rivers) may affect biofilms at a functional and structural level.

The considerable variability of environmental factors combined with multiple interactions of stressors (i.e. metals) in aquatic ecosystems complicates the establishment of definitive causal links between stressors and effects (e.g. reduced biological diversity) (Ricciardi et al., 2009). Bonnineau (2011) talked about the difficulty in differentiating between metal effects and confounding factors, the most challenging aspect of our study. We foresaw and observed clear differences in the structure of biofilm communities between sites (algal community composition, organic matter and algal biomass). Zn toxicity influenced biological responses at polluted sites (L and M), masking the seasonality observed at B. Diatoms were less abundant at polluted sites in favor of cyanobacteria, as other studies have reported (Tlili et al., 2011; Corcoll et al., 2012), losing the expected seasonal succession observed at B (at the non metal-polluted site). A reduction in diatom diversity has been described (Morin et al., 2011; Corcoll et al., 2012) but also an increase in community tolerance (Tlili et al., 2011). Furthermore, high values of the autotrophic index were reported at M, which is in agreement with the observations of Tlili et al. (2011), who attributed this change to a protection mechanism (greening effect) caused by metals.

No differences between sites were observed on photosynthetic efficiencies. This was attributed to an adaptation process, in which species sensitive to metals were replaced by tolerant ones, leading to a new community with similar functionality (in terms of photosynthesis) to the original one (Garcia-Meza et al., 2005; Serra et al., 2009; Corcoll et al., 2012). These results contrast with the AEA responses that were affected by chronic Zn exposure. In fact, elevated ROS levels, controlled by AEA, occur at metal

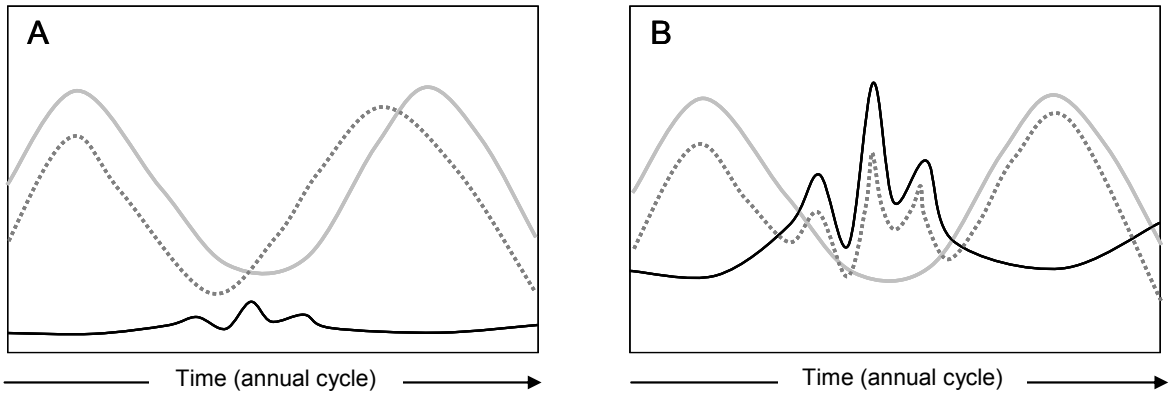
concentrations that may not be inhibitory to the photosynthetic activity of algae (Szivak et al., 2009), highlighting their higher sensitivity.

It is also important to note that, in periods of the year with low metal concentration due to the water flow dilution effect, metal polluted sites (L and M) had similar biological responses to B (Figs. 6 and 7A). In contrast, when water flow was low, the highest values of metals were observed (from July '09 to January at the M site and November to January at the L site) and GST showed an enhancement attributed to metal toxicity (Figs. 6 and 7B). Maharana et al. (2010) reported an increase of GST in the marine brown algae (Padina tetrastromatica) under metal polluted conditions while Pereira et al. (2009) suggested that GST was not the main antioxidant defense in algae (Ulva sp.). Protein concentration also moved in the same direction as accumulation of Zn (Fig. 6). However, we could not affirm that protein concentration will be a metal indicator as in other studies (Le Faucheur et al., 2005; Bonnineau et al., 2011). In spite of being correlated with GST, based on the RDA, this parameter was also statistically related with environmental parameters (e.g. light intensity). In contrast with our expectations, we did not find any clear CAT response. CAT increased in marine brown algae (P. tetrastromatica) as well as in biofilms in a field experiment with similar conditions to our background metal polluted site, and also higher values of CAT were found in Ulva sp. during spring and autumn under high concentrations of Ni and Cu (Pereira et al., 2009; Maharana et al., 2010; Bonnineau et al., 2011). AEA responses are very complex, thus a set of enzymes rather than just one or two are required to assess biological responses under field conditions.

Loss of heterotrophic and autotrophic richness of biofilm communities has been described in Osor stream due to Zn (Tlili et al., 2011; Corcoll et al., 2012). This loss in diversity of species could tentatively be linked with the observed loss of AEA ability to

respond to environmental stress in metal-polluted sites. In the framework of the biodiversity–ecosystem functioning (B–EF) theory (Kominoski et al., 2010), Zn pollution effects on biofilms could be comparable to resource diversity effects (bottom up) restricting their capacity to cope with further stress.

Figure 7. Hypothetical response of antioxidant enzyme activities (AEA) over a year in field conditions. The gray line represents environmental conditions, the black line represents metal concentration and the gray dotted line represents antioxidant enzyme activity response. The only difference between A andB is the increase in metal concentration in the stream from non-metal polluted or low metal concentrations to moderate concentrations (shown in this study). Panel A shows a pattern of AEA affected by environmental conditions under non-metal or low metal polluted conditions. Panel B shows the effect of moderate metal polluted conditions over the AEA pattern.



5. CONCLUSION

This study presents, for the first time, the AEA responses of fluvial biofilm communities in the field over an annual cycle. Following our objectives, AEA variability could be partially attributed to metal pollution (e.g. light) but also to differences in metal concentrations between sites. Moreover it has been observed that AEA follow a seasonal pattern, like the algal group succession at the non metal-polluted site. Water flow, which in this study determines metal concentration, dilutes metal inputs differentially therefore provoking different biological responses over the year at sites

with low or moderate metal pollution, masking the seasonality observed at the non metal-polluted site.

AEA have been successfully tested as biomarkers of organic and inorganic pollutants under laboratory conditions. However, field investigations have only addressed metal-pollution effects on AEA seasonality. A similar approach might be performed in further studies to test AEA as biomarkers of organic pollution.

We recommend a combination of physicochemical measurements (i.e. metal content in water and in biofilm) and the use of AEA as an effect based approach to investigate single and combined effects of metals and environmental stress under field conditions.

Acknowledgments

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CHAPTER 2

Antioxidant enzyme activities responses as biomarker of Zn pollution in a natural system: an active bio-monitoring study

ABSTRACT

This study aimed to explore the use of antioxidant enzyme activities (AEA) and biofilm metal accumulation capacity in natural communities as effect-based indicator of metal exposure in fluvial systems. To reach this, an active biomonitoring using biofilm communities was performed during 5 weeks. After colonization over artificial substrata in a non-polluted site, biofilms were translocated to 4 different sites with different metal pollution in the same stream. The evolution of environmental parameters as well as biofilm responses (AEA, algal biomass, optical densities 430:665 ratio and metal accumulation in biofilm) were analysed over time. Physicochemical parameters were different between sampling times as well as between the most polluted site and the less polluted ones, mainly due to Zn pollution. In contrast, AEA and metal accumulation in biofilms allowed us to discriminate the high and moderate metal pollution sites from the rest (low and non-metal polluted sites). Zn, the metal with the highest contribution to potential toxicity, presented a fast and high accumulation capacity in biofilms.

According to the multivariate analysis, AEA showed different responses. While catalase (CAT) and ascorbate peroxidase (APX) variability was mainly attributed to environmental stress (pH, temperature and phosphate concentration), glutathione-S-transferase (GST) changes were related to metal pollution and reductase (GR) and superoxide dismutase (SOD) to both stress factors. AEA and metal accumulation are proposed as sensitive effect-based field methods, to evaluate biofilm responses after acute metal exposure (e.g. an accidental spill) due to their capacity to respond after few hours, but also in routinely monitoring due to their persistent changes after some weeks of exposure. These tools could improve the Common Implementation Strategy (CIS) of the Water Framework Directive (WFD) as expert group request.

1. INTRODUCTION

In 2009, the Common Implementation Strategy (CIS) of the Water Framework Directive (WFD) established the expert group Chemical Monitoring and Emerging Pollutants (CMEP). This expert group was established to take up the challenges arising from the revision of the list of priority substances as well as from the requirements stemming from Directive 2009/90/EC (also called QA/QC Directive). In particular, CMEP has to deal with aspects related to standardization and quality assurance issues stemming from the implementation of Commission Directive 2009/90/EC, such as methods to assess bioavailability for metals. A specific task of the CMEP activity is to elaborate specific guidance on the use of alternative effect-based monitoring methods, such as the use of biomarkers. These methods are required for investigative monitoring and to better evaluate the link between the chemical and ecological status and the effects of the mixture of pollutants and emerging pollutants. Moreover, they are needed to better understand the real environmental quality of the aquatic ecosystems through the evaluation and detection of the effects of pollutants on the aquatic organism, which can detect pollutants that cannot be detected with routine chemical analysis (EC. Guidance document n.25).

Zn, one of the most wide spread metals due to both natural and anthropogenic causes, is present in a wide range of concentrations in aquatic systems. Zn concentrations which exceed levels marked by the European and American legislation are often found. In Spain, Zn occurrence led to the establishment of Zn Environmental Quality Standards (EQS) (annual average values (AA)) of 30 μg L^{-1} for soft water and 500 μg L^{-1} for hard water (from RD 60/2011 by means of the Water Framework Directive (WFD) (Directive 2000/60/EC)). The maximum concentration for water quality criteria in the USA is within this range; 120 μg L^{-1} according to the Environmental Protection Agency (EPA, 2006).

Zinc (Zn), apart from being a micronutrient, can also be toxic at higher concentrations when accumulated in the biota. Its toxic effects have been largely reported in cultures, microcosms and natural systems (Shehata et al. 1999; Behra et al. 2002; Morin et al. 2007; Guasch et al. 2010; Corcoll et al. 2012). Oxidative stress of Zn has also been reported due to its capacity to induce the production of reactive oxygen species (ROS) (Sauser et al. 1997; Nagalaskshmi and Prasad 1998; Collén et al. 2003; Valavanidis et al. 2006; Tripathi et al 2006; Qian et al. 2011; Guasch et al. 2010; Bonnineau et al. 2011).

Antioxidant enzyme activities (AEA) are common mechanisms in the organism to regulate the reactive oxygen species (ROS) produced by organisms as a result of metabolic processes, such as respiration or photosynthesis (Asada, 2006). Furthermore, AEA play an important role in cellular defence strategy against oxidative stress caused by toxicants like metals (Collén et al., 2003; Tripathi et al., 2006; Valavanidis et al., 2006). Since AEA are able to respond to acute and chronic metal exposure, some authors have proposed their use at both temporal scales (Valavanidis et al. 2006; Guasch et al. 2010; Maharana et al. 2010; Bonnineau et al. 2010). In addition, AEA require less time to be analysed than taxonomy (Bonet et al. 2012).

AEA response to stress is usually not linear and may also change after chronic exposure due to adaptation. Bonnineau (2011) suggested that AEA follow a unimodal pattern with different patterns of activity: increase, saturation, decrease and inhibition. Most AEA studies have been performed in cultures and with single species in the field (Sauser et al. 1997; Geofroy et al. 2004, Li et al. 2006; Tripathi et al. 2006; Pereira et al. 2009), and with less frequency in microcosm systems with complex communities, like biofilms (Bonnineau et al. 2010; Guasch et al. 2010; Bonnineau et al. 2011; Bonet et al. 2012). However, they offer a poor and lower level of ecological realism (Clements

et al. 2002).The use of biofilms as bioindicators is due to their capacity to respond to organic and inorganic pollution after both acute and chronic exposure (Soldo and Behra 2000; Morin et al. 2007; Sabater 2007; Dorigo et al. 2010; Romaní 2010). Few studies, to our knowledge, have been carried out in natural freshwater systems using biofilms to evaluate AEA responses to metal pollution (Bonnineau et al. 2010; Bonet et al. 2013).

AEA responses are dependent on time, dose, toxicant, as well as on organisms (from single species to complex communities). Hence, their interpretation can be complex. In Chapter 3, which was partly conducted in the same stream as this study, we showed an induction of CAT and GST after a short active bio-monitoring experiment (6 and 24h). In contrast, higher metal contamination in Riou Mort (SW France) reduced all the AEA analysed (CAT, APX, GR and GST). Under chronic conditions, during an annual monitoring in this stream (the Riera d'Osor), AEA, like the algal biofilm community, showed an interesting seasonal variation in sites with no or low levels of metal pollution, whereas this seasonality was reduced in the most polluted site. Furthermore, it showed that glutathione-S-transferase (GST) was the most sensitive AEA to Zn pollution changes over the year (Bonet et al. 2013). Changes in autotrophic and heterotrophic biofilm communities, attributed to Zn pollution, were also reported in Tlili et al. (2011) and Corcoll et al. (2012).

In contrast to AEA patterns over time, biofilm metal accumulation in microcosm experiments was linear (Bonet et al. 2012) over a 5-week period. However, do biofilms have an unlimited metal accumulation capacity? Can biofilms accumulate all metals present in water and do accumulation kinetics depend on metal form (free, labile or dissolved)?

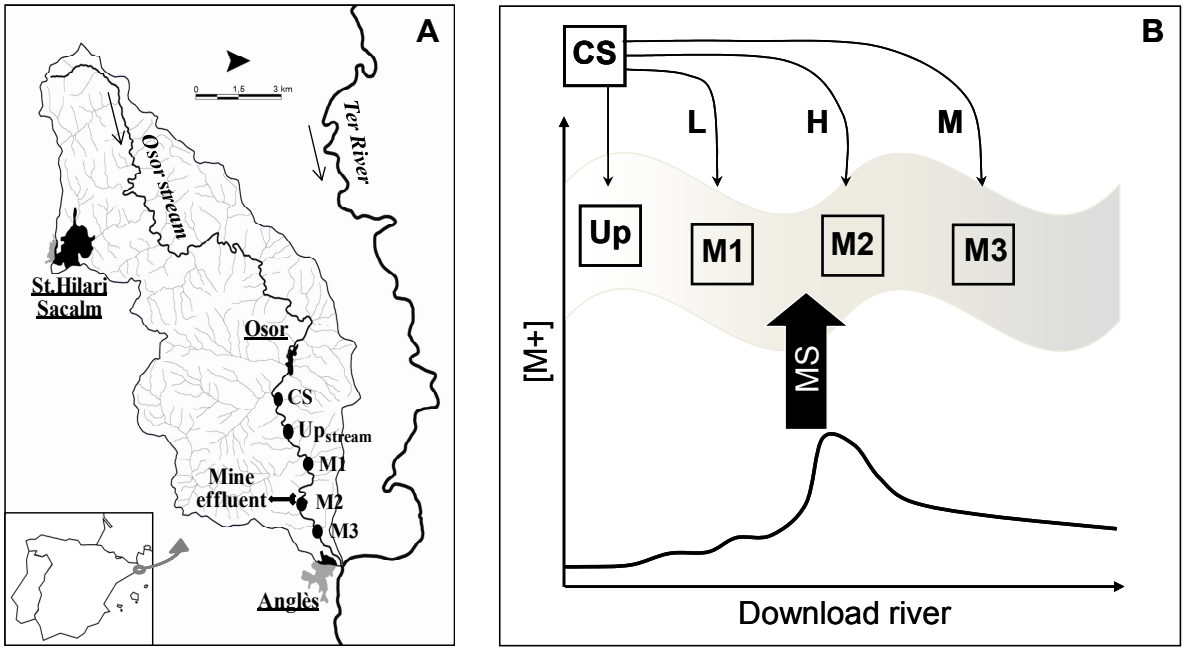
Hence, in this study we explore, on the one hand, AEA responses to metal pollution and, on the other, biofilm metal accumulation capacity to validate their applicability as tools in an effect-based monitoring method as demanded by CMEP. To achieve both objectives a translocation experiment was performed in a metal-polluted fluvial stream, the Riera d'Osor. Natural communities (fluvial biofilms) were translocated from one site to another, allowing their biological responses to be quantified (De Kock and Kramer, 1994). This approximation provides a high degree of realism in ecotoxicology (Ivorra et al. 1999) and allows stress responses under complex aquatic field situations to be evaluated (Rotter et al. 2011). Biofilms were translocated from a colonization site to different sites with different metal pollutions. Physicochemical and biological parameters of each site were analysed to know the main differences between and within the reference site and polluted sites after translocation. Specifically, the set of AEA analysed was: catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), glutathione-S-transferase (GST) and superoxid dismutase (SOD). Due to differences between sites, mainly attributed to metal concentration, we expected to observe different magnitudes and types of biological responses and metal accumulation capacities. We also expected that functional responses would occur in a short period of time after translocation. After some days, when biofilm adapted to metal pollution, stable AEA were expected in polluted sites, but in different ones from the reference site due to differences in metal exposure history. Biofilm metal accumulation capacity would tend to increase over time, and differences between metals were also expected.

2. MATERIAL AND METHODS

2.1. Study site

Translocations were conducted in the Riera d'Osor. A 5Km stretch was selected starting after the village of Osor and ending before the town of Anglès, including the

Figure 1. The Riera d'Osor (NE Iberian Peninsula, Ter river basin). A) Location of the sampling sites form upstream to downstream: colonization site (CS), non-metal polluted site (Up), mining 1 (M1), mining 2 (M2) after the entrance of mine effluent and mining 3 (M3). Figure B) shows translocation experimental design. Letters L, H and M mean the increase of Zn pollution in water, low, high and moderate, respectively.



mining area (Fig.1A). The Riera d'Osor is a small tributary of the river Ter located in the north east of Catalonia (NE Spain) (Fig. 1A). This stream is affected by effluents and runoffs from a former mine that extracted sphalerite ((Zn, Fe)S) and galena (PbS). In spite of the fact that mining activities finished in 1980, no environmental rehabilitation was carried out and the stream is still receiving the input of a continuous mine effluent (referred to in the text and figure 1A as mine source (MS)) and also diffuse metal inputs from mine run-over of metal-polluted landfills. Despite metal pollution, this stream is relatively well preserved, with well-developed riparian vegetation and low urban pressures. Its hydrology has been altered due to the diversion of part of the stream water flow for electric power production.

Four sites with different metal concentrations were selected: the non-metal polluted site called *upstream*, before the mine source, as the reference site (referred to as Up), *mining 1* (referred to as M1) placed after Up site and upstream of the main source, *mining 2* (referred to as M2), located just after the mine source with continuous inputs of metals, and *mining 3* (referred to as M3) further downstream with less metal concentrations due to metal precipitation and storage in the sediment (Tlili et al. 2011; Corcoll et al. 2012).

2.2. Experimental set-up and sample collection

The experiment started on 12 April and lasted until 21 June, 2009. Biofilm was grown on artificial substrates to reduce the heterogeneity that occurs on natural substrates (Cattaneo et al. 1997). Small (1.2 x 1.2 cm) and large (8.5 x 2 cm) sand-blasted glass substrata were fixed to cement cobbles (40 x 40 cm) with silicon sealant. These cement cobbles were placed horizontally on the streambed in the colonization site (CS), in the middle of the stream (Fig. 1A). After 5 weeks of biofilm colonization, artificial substrata were moved from CS to the four selected sites (Up, M1, M2 and M3) in similar light and water current conditions. After translocations, water and biofilm samples were collected after 6h, 1, 3, 7, 21 and 35 days. To avoid translocation effects being confused, Up site, with similar physicochemical conditions and with a good ecological status like CS, was used as the reference site to follow the biological evolution of translocated biofilms (Fig. 1B). With this translocation design, different changes in Zn exposure were achieved: i) low Zn pollution in M1, ii) moderate Zn pollution in M3 and iii) high Zn pollution in M2. No rain was expected during the translocation experiment. However, a heavy rain event of 46 mm occurred (according to Met Office, 2007) just three days before.

2.3. Physicochemical and biological parameters

Temperature, dissolved oxygen, pH and electrical conductivity (WTW METERS, Weilheim, Germany) were measured *in situ* at each sampling period. To analyse chemical water parameters (per triplicate) including nutrients, anions and cations, dissolved organic carbon (DOC) and total dissolved metals, water samples were filtered (0.2 µm nylon membrane filters, Whatman). Immediately after being filtered, DOC samples were acidified with 200 µL of HCl (2M) and 100 µL of NaN_3 (2.7mM) and total metal dissolved samples were acidified with 1% of HNO_3 (65% suprapure, Merck). All water samples were kept in the fridge (at 4ºC) or frozen (at -20ºC) until analysis. PO_4^{3-} was analysed following the Murphy and Riley method (1992). NO^{2-}, NO_3^{2-}, and NH_4^+ were analysed using ion-chromatography (761 Compact IC, Metrohm, Herisau, Switzerland) (Hach, 1992). DOC was measured on a Shimadzu TOC 5000. Suspended solids (SS) were analysed one time per site and sampling time according to Elosegui and Butturini (2009). Light was also measured in situ at each sampling site over substrates (LI-COR Inc., Lincoln, NE, USA).

Total dissolved metal concentration was analysed by inductively coupled plasma mass spectroscopy (ICP-MS 7500c Agilent Technologies, Inc. Wilmington, DE). Detection limits were 5.92 µg Zn L^{-1}, 1.01 µg Cd L^{-1}, 35 µg Fe L^{-1}, 0.19 µg Al L^{-1}, 5.95 µg Ni L^{-1}, 0.82 µg Cu L^{-1} and 1.20 µg Pb L^{-1}. When the value was below the detection limit, half of the detection limit was used for data treatment (Helsel, 1990). The accuracy of the analytical methods was checked periodically using a certified water reference (Reference material for measurement of elements in surface waters: SPS-SW2 Batch 113), Oslo, Norway).

In order to integrate the mixture effects of metals present in the Riera d'Osor, the metal concentrations were used to calculate Cumulative Criterion Units (CCU) 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.org.gov/waterscience/criteria/wqctable) used to investigate the responses of aquatic organisms to metals (Clements et al., 2000; Guasch et al. 2010; Morin et al. 2012). There are three categories of CCU: *background* category (CCU < 1), *low* category (1 < CCU < 2) which indicate low potential toxicity and *moderate* category (2 < CCU < 10) which indicate moderate potential toxicity.

Total metal accumulation in biofilms was analysed at each site in triplicate using 34 cm^2 of scraped biofilm (two big substrata, 8.5 x 2 cm). Biofilm samples were stored at -20ºC before analysis. Then biofilms were lyophilized, weighed and digested using 4mL of HNO_3 (65% suprapure, Merck) and 1mL of H_2O_2 (30% suprapure, Merck) in a high performance microwave digestion unit (Milestone, Ethos Sel). Then, they were diluted to 15mL with milli-Q water, and the subsequent liquid samples were treated as dissolved metal water samples. Detection limits were 0.19 µg Zn L^{-1}, 0.75 µg Cd L^{-1}, 2.65 µg Fe L^{-1}, 0.10 µg Al L^{-1}, 5.40 µg Ni L^{-1}, 1.81 µg Cu L^{-1} and 0.80 µg Pb L^{-1}. Half of the detection limit was used for data treatment when the value was below the detection limit (Helsel, 1990). The accuracy of the analytical methods was checked periodically using certified reference materials (Trace Elements in Plankton (CRM 414), Community Bureau of Reference (BCR), Brussels, Belgium).

Algal biomass was analysed using 1.5 cm^2 of biofilm surface following the Jeffrey and Humphrey (1975) method and was expressed as µg Chl-a cm^{-2}. The ratio between the optical densities of 430 and 665 nm (referred to in the text as OD 430:665 ratio) was used as a broad indicator of the proportion of protection pigments (e.g. carotenoids) or degradation products per unit of active Chl-a (Margalef, 1983).

Protein extraction and antioxidant enzyme activities (AEA)

Three glasses of 8.5 cm^2 were collected at each sampling site and time. Biofilm was scraped directly from a glass substratum, put into an eppendorf tube and stored in liquid nitrogen until arrival at the laboratory. AEA samples were stored at -80ºC until their analysis. Extraction and quantification of protein and AEA measurements were performed as described in Bonnineau et al. (2011) and Bonet et al. 2013. The optimal protein concentration used was 4 µg. All AEA were calculated as specific activities (i.e. per µg of proteins). For each assay the optimal concentration of substrate or cofactor was optimized, thus the concentrations used here were 30 mM of H_2O_2 for CAT assay, 4 mM of H_2O_2 for the APX assay, 0.25 mM of NADPH for the GR assay, 5 mM of GSH for the GST assay and 0.05 mM of WST-1 SOD assay (0.05 mM).

2.4. Statistical analysis

One-way ANOVA was used to evaluate the main physicochemical and biological differences between sites at each sampling time (6h, 1, 3, 7, 21 and 35 days). If significant differences were found, ($p<0.05$), the ANOVA was followed by a Tukey-b test. Homogeneity of variances and normality of data were checked prior to data analysis. All these analyses were done with SPSS v15.0 software.

Sigma Plot v.11.0 was used to perform linear regressions between metal concentrations and their corresponding accumulation in biofilm along the river at each sampling time and the slope defined as metal accumulation capacity of biofilm. Slopes were then plotted versus time to describe their temporal pattern. Sigma Plot v.11.0 was also used to obtain the best fit in each sampling site of AEA changes over time to mathematical models: lineal, sigmoidal or quadratic. These models were selected in order to evaluate all phases of the unimodal response: increase, saturation and decrease and/or inhibition, respectively. While temporal patterns in the reference site

(Up) are expected to respond to successional changes and/or oxidative stress caused by environmental changes, deviations from these patterns in metal-polluted sites (M1, M2 and M3) were attributed to metal pollution.

Multivariate analyses were performed using the CANOCO software version 4.5 (Ter Braak and Smilauer, 2002). All variables (except pH) were previously transformed using $\log_{10}(x + 1)$ to reduce skewed distributions. Principal component analysis (PCA) was used to analyse the variability of the physicochemical and metal concentration water parameters throughout the experiment. Moreover, a redundancy detrended analysis (RDA) was used to evaluate biological responses in relation to environmental variables and metal pollution in water. Prior to performing RDA, a detrended correspondence analysis (DCA) was applied to determine the maximum gradient length of biofilm parameters (AEA, algal biomass and metal accumulation in biofilm), which was 0.36, indicating that the linear method would be appropriate. To avoid co-linearity, variables were selected based on inspection of variance inflation factors (VIF < 20) (Ter Braak and Smilauer, 2002). 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 $p = 0.1$. Cut-off point and probabilities for multiple comparisons were corrected using the Bonferroni correction. The significance of the RDA axes was assessed using the Monte Carlo permutation test (999 unrestricted permutations).

The variance partitioning technique was applied to separate the effects of metal concentration (Zn, Fe and Al) on biological parameters distribution from other physicochemical parameters (pH, temperature and PO_4^{3-}) when pertinent (Borcard et al. 1992). This approach allowed us to determine the fractions of the explained variance that were related to each data subset or shared by both. To perform the

variance partitioning, a series of RDAs was done: (1) RDA of response variables constrained by physicochemical variables, (2) RDA of the response variables constrained by metal pollution in water, (3) partial RDA of the response variables constrained by physicochemical variables with metal pollution as co-variables, and (4) partial RDA of the response variables constrained by metal pollution with physicochemical variables as co-variables. Accordingly, the explanatory variables were grouped into two subsets: (a) physicochemical variables and (b) metals.

3. RESULTS

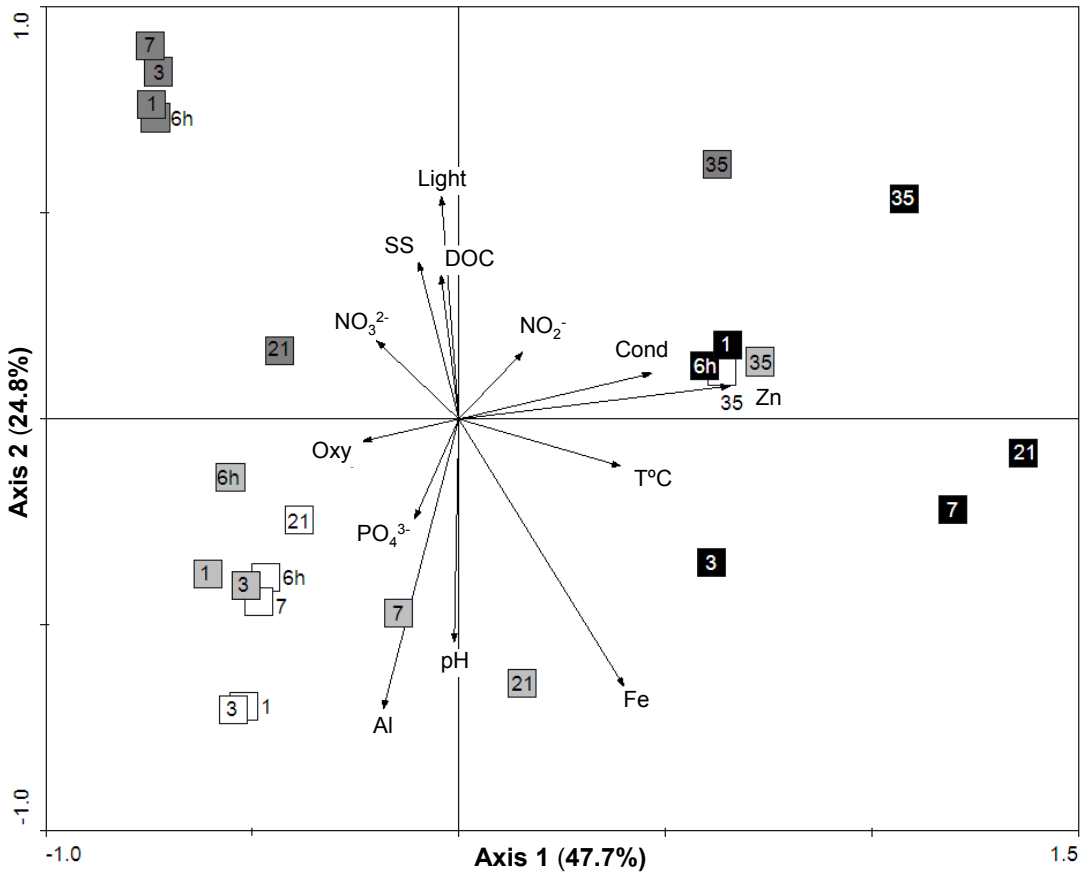
3.1. Physicochemical data

All the physicochemical data including the environmental parameters and metals in water of the experimental sites are summarized in the annex 1. Colonization site (CS) and upstream site (Up), the reference site used in the translocation experiment were similar, differing only in the concentration of total dissolved Fe in water (ANOVA, $F = 8.12$, $p < 0.01$, $n = 18$). The metal concentration of the mine source (MS) (the main metal effluent placed before M2 site) was 1221.96 ± 402.31 µg Fe L^{-1} and 2240.53 ± 1080.35 µg Zn L^{-1}.

In the experimental sites (Up, M1, M2 and M3), total Al, Fe and Zn dissolved in water were measurable. The other metals (Ni, Cu, Cd and Pb) were always below detection limit (annex 1). Thus, CCU was performed with Al, Fe and Zn. Up, M1 and M3 sites had the background category (0.47, 0.57 and 0.24 CCU, respectively) while M2 had moderate potential toxicity (2.41 CCU). Fe contributed most, being 74.1 and 62.7 % in percentage at Up and M1, respectively. In M3, metal contribution was due to both Fe and Zn, which contributed 50 and 48.8 %, respectively. In contrast, in the M2 site the main contribution was due to Zn with 76.8 %. Al contribution was below 15 % at all sites.

Physicochemical PCA shows the ordination of sampling points within the first two axes (Fig. 2). The first axis explains 47.7% of variance and is a Zn axis separating the most polluted site (M2) from the others. Furthermore allow to separate earlier samplings (6 and 24 hours) from the rest. The second axis of the PCA explains 24.8% of variance and separates sites according Al and Fe and environmental parameters like pH and light. The rain event three days before the last sampling caused an increase in metal concentration at Up, M1 and M3 on day 35 while values decreased in M2 (Fig. 2 and annex 1).

Figure 2. Biplot of the principal component analysis (PCA) based on the different physicochemical parameters at each sampling time (6h, 1, 3, 7, 21 and 35 days) and site: non-polluted site in white (Up), mining 1 (M1) in light gray, mining 2 (M2) in black and mining 3 (M3) in dark gray.



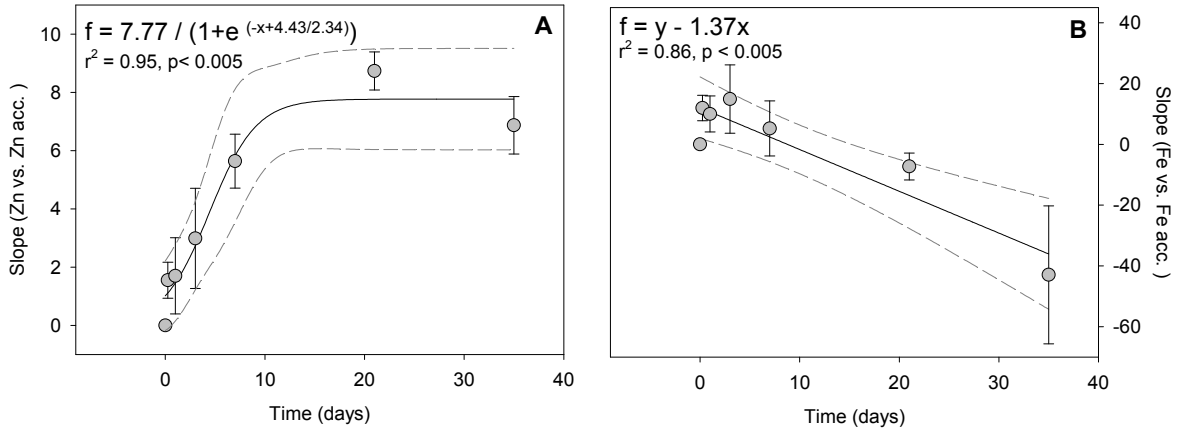
The most polluted site, M2, had lower nutrient (PO_4^{3-}, NO_3^{2-}, NO_2^-) concentrations. Dissolved organic carbon (DOC) was similar between sites, but lower in M2 at the end

of the experiment (annex 1). M3 had the lowest pH (ANOVA, $F = 5.16$, $p < 0.005$, $n = 6$) and M1 showed the highest Al values (annex 1). Finally, the Up site had much lower metal concentrations (corresponding to background metal contents) and higher nutrient concentrations than polluted sites.

3.2. Metal accumulation

Among the metals that were not detectable dissolved in water, Ni, Cu and Pb were found accumulated in biofilms at the end of the experiment (annex 2). Zn, Fe and Al were detectable in all water samples, thus regression with their respective metal accumulation in biofilms, referred to as biofilm metal accumulation capacity, could be explored. Zn accumulation capacity increased quickly from 6h to 7 days reaching a plateau from that day onwards (Fig. 3A). Fe acc. capacity in biofilm presented a negative linear regression over time (Fig. 3B), whereas Al did not present a clear pattern.

Figure 3. Metal accumulation capacity of biofilm over the active bio-monitoring experiment. A) regression of total Zn dissolved in water versus Zn accumulated slopes over time which follow a sigmoidal fit, and B), total Fe dissolved in water versus Fe accumulation slopes over time which follow a lineal fit. Dash gray lines indicate a 95% of confidence band.

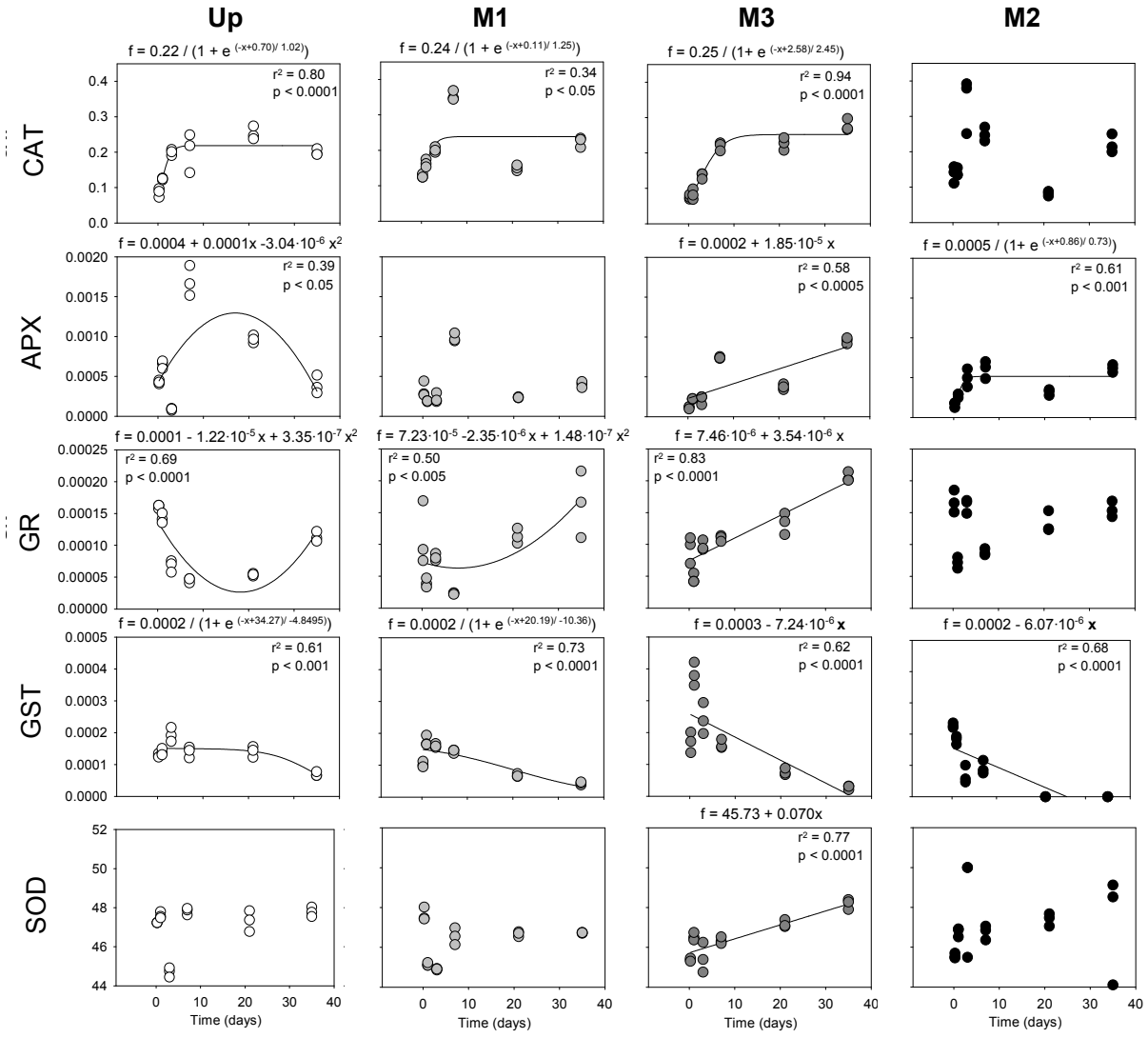


3.3. Temporal variation of AEA

Based on the fitting of AEA over time, a predictable pattern of temporal variation (significant fitting) was observed in most sites but M2 showed more scattered temporal changes (Fig. 4). CAT activity followed a sigmoidal curve which reached a plateau from 7 days on at all sites, except in M2. APX showed different fits at each site. In the Up site, APX followed a quadratic curve, decreasing its activity from 21 days on. In contrast in M3, it followed a linear increase over time while in M2, it followed a sigmoidal curve, reaching a plateau after 7 days. At the M1 site no fitting was possible. GR activity in Up and M1 followed an increasing quadratic curve after 21 days while M3 followed a linear increase. In M2, as in the case of CAT activity, it was not possible to establish any adjustment. In the case of GST, the Up and M1 sites were fitted with a sigmoidal curve, decreasing from 7 days on, whereas the most polluted sites, M2 and M3, followed a linear decrease reaching a completed depletion of its activity in the most polluted site (M2). Concerning the SOD, it was only possible to establish a fitting in M3 following a linear increase curve. Similarities and/or differences of curves among sites are corroborated by the ANOVA test performed at each sampling time at each site (Annex 3).

Temporal changes were not fitted to any of the tested mathematical models, either for chl-a or for pigment degradation (OD ratio). Chl-a, only differed at 3 and 7 days, being higher in polluted sites than Up site while pigment degradation (OD ratio) did not differ throughout the experiment (Annex 3).

Figure 4. Antioxidant enzyme activities (AEA) evolution over time at each site following the increase of metal pollution: non-metal polluted site (Up) in white, mining 1 (M1), before mine source in light grey, mining 3 (M3) downstream of mine source in dark grey and mining 2 (M2) after mine source in black. Parameters are: catalase (CAT) express in µmol H_2O_2 µg protein^{-1} min^{-1}, ascorbate peroxidase (APX) express in µmol Ascorbate µg protein^{-1} min^{-1}, glutathione reductase (GR) express in µmol NADPH µg^{-1} protein min^{-1}, glutathione-S-transferase (GST) express in µmol CDNB conjugate µg^{-1} protein min^{-1}, superoxide dismutase (SOD) express in U µg protein^{-1}. Sigmoidal, quadratic or lineal adjust between AEA over time are indicated in each graph.

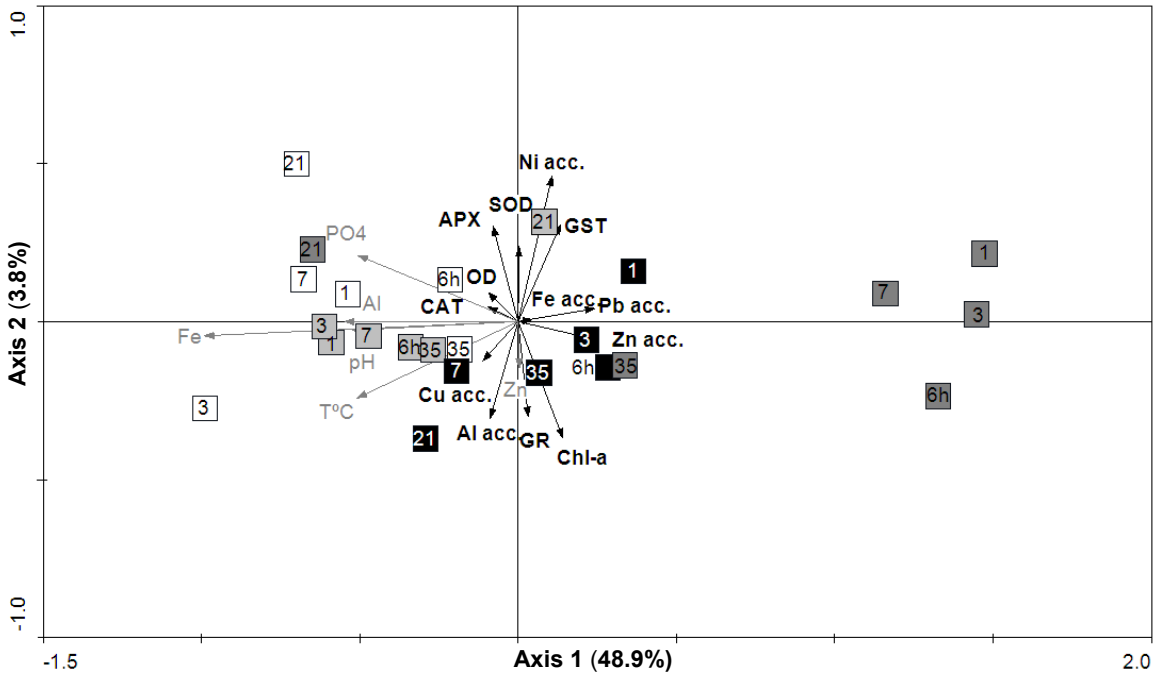


3.4. RDA of physicochemical conditions and biofilm responses

Based on the RDA, pH, temperature, PO_4^{3-}, dissolved Zn, Fe and Al explain 55.3 % of the total variance attributed to physicochemical parameters (Fig. 5). The first axis, which explains 48.9 % of variance, arranges biofilms samplings according to Al, Fe,

temperature, pH and PO_4^{3-} concentration. The second axis explains only 3.8 % of variance and was attributed to Zn pollution. The first axis of RDA shows that biofilms from M2, the most polluted site, were overall Zn influenced according to the increase in Zn acc. in biofilm as well as the enhancement of GR and chl-a. Although dissolved Pb in water was below the dissolved detection limit, M2 had an important Pb acc.. Biological responses of biofilms in M3 were also influenced by metals, but at 21 days where strongly influenced by PO_4^{3-}. Up and M1 overlapped and were strongly influenced by environmental parameters, Al and Fe. Under these conditions CAT, APX, OD ratio were stimulated.

Figure 5. Triplot based on the redundancy analysis (RDA) of environmental parameters, metal concentration in biofilm and biological parameters of the translocation experiment in la Riera d'Osor which explain 55.36 % of total variance. Sampling sites are: non-metal polluted site (Up) in white, mining 1 (M1) in light gray, mining 2 (M2) in black and mining 3 (M3) in dark gray at their respective time (6h, 1, 3, 7, 21 and 35 days). Gray arrows represent the constraint variables give total dissolved Zn, Fe and Al in water, pH, TºC (temperature) and PO_4^{3-} (phosphate). Black arrows represent biological parameters: algal biomass (Chl-a), optical density 430:665 ratio (OD), superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione reductase (GR), glutathione-S-transferase (GST) and metal accumulation in biofilm (Al acc., Fe acc., Zn acc., Ni acc., Cu acc. and Pb acc.).



Variance partitioning shows that environmental data (pH, temperature and PO_4^{3-}) alone account for 7.2 % of the total explained variation, while dissolved Zn, Fe and Al in water explain 15.5 %. The shared fraction of total variation between environmental data and metals was 32.6 %, so the unexplained variation was 44.7 %. With respect to unconstrained variance, the percentage of variance explained by environmental parameters and metals in water was different from the response variables (Table 1). Most of the biological responses CAT, APX Ni acc. were explained by environmental parameters while GST, Zn acc. and Pb acc. were explained by metals. The rest of the biological parameters were explained by both, environmental parameters and metals (Table 1).

Table 1. Results of the partial Redundancy Analysis (RDA) using the whole set of biofilm parameters (CAT, APX, GR, GST, SOD and Chl-a), environmental parameters (pH, temperature and phosphate concentration) and total Zn, Fe and Al dissolved in water. In the left-hand column, bold numbers highlight values with fraction of total variance > 10%. On the right-hand column (percentage of fractions of explained variance) bold numbers highlight values that were biased towards certain groups of biofilm variables (> 70 %).

	Fraction of total variance		Fraction of total variance (%)	
Biological parameters	Physical and Chemical	Metals	Physical and Chemical	Metals
Chl- a	6.21	**10.5**	37.16	62.84
OD	**16.33**	**11.09**	59.56	40.44
CAT	**34.56**	3.3	**91.28**	8.72
APX	**28.01**	6.12	**82.07**	17.93
GR	**11.02**	**15.17**	42.08	57.92
GST	**11.32**	**26.63**	29.83	**70.17**
SOD	**17.74**	**19.18**	48.05	51.95
Al a cc.	**27.58**	**30.31**	47.64	52.36
Fe a cc.	**10.71**	**23.57**	31.24	68.76
Ni a cc.	**14.77**	3.35	**81.51**	18.49
Cu a cc.	**17.85**	**36.08**	33.10	66.90
Zn a cc.	4.61	**15.68**	22.72	**77.28**
Pb a cc.	4.74	**16.5**	22.32	**77.68**

4. DISCUSSION

The aims of this study were to explore the spatial and temporal AEA responses of biofilm communities in a metal-polluted stream and the biofilm metal accumulation capacity to validate them as tools of the effect-based monitoring method. Active biomonitoring allowed us to assess biological responses to temporal variation of environmental parameters, including different metal pollution scenarios. The three selected metal-polluted sites showed different physicochemical conditions. Therefore, it was possible to attribute specific biofilm responses to different metal-pollution conditions and to discriminate between different biofilm capacities to accumulate metals according to the measured metals. In addition, the rain event that occurred at the end of the translocation experiment (three days before the last sampling) offered interesting observations because it influenced stream discharge as well as nutrient and metal concentrations.

4.1. Metal pollution

The maximum value of Zn concentration in this study was found in M2 and was 609.42 μg Zn L^{-1}, which exceeds both European and US legislation limits, according to the Water Framework Directive (WFD) (Directive 2000/60/EC) and US Environmental Protection Agency (EPA, 2006). The maximum value of Al was 23.25 μg L^{-1} found at M1 and the maximum value of Fe was 755.17 μg L^{-1} found at the M2 site. These concentrations are not worrying since they are low and biological effects are not expected. Moreover, Al and Fe are not included as priority pollutants or as “specific pollutants” in European and American legislation. In fact, due to their high affinity to form hydroxide compounds, Al and Fe precipitate easily and are not assimilable by biota. At the same time, these hydroxide compounds can act as a trap due to their ability to adsorb metals, like Zn.

The most metal-polluted site (M2), presented the highest Zn, Al and Fe concentrations. However, CCU in the M2 site indicated a moderate potential metal toxicity, mainly due to Zn. In the M1 site, despite presenting the highest values of dissolved Al, potential metal toxicity was mainly due to Fe (according to CCU) like in the Up site. In M3, potential metal toxicity was attributed to both Zn and Fe (~ 50 % each one), even though Fe was only detectable from 21 days on. At the end of the experiment (35 days), total Zn concentration decreased in the most polluted site (M2) maybe due to dilution caused by the rain event (Guasch et al. 2010; Bonet et al. 2013), but increased in the other sites probably due to runoff contributions (Meylan et al. 2003) (Fig. 2).

4.2. Biofilm responses

Biofilms showed a fast Zn accumulation capacity, detectable after a few hours of translocation. Throughout the duration of the experiment, the fast accumulation period (from 6 hours to 10 days, approximately) was followed by a slower metal accumulation phase leading to a saturation of Zn accumulation after 10 days. It is interesting to highlight that after 21 days, translocated biofilms reached maximum Zn accumulation (5753 μg g DW^{-1}), in the range of values reported in natural Zn polluted biofilms around 11768 μg g DW^{-1} in July 2009 and 1961 μg g DW^{-1} in July of 2010, (Bonet et al. 2013).

The decrease in Zn acc. between 21 and 35 days at all sites could be attributed to the rain event which can cause a “washing” effect on biofilms. This highlights the dynamics of fluvial systems where alternations in the periods of accumulation and release of metals between the water phase and biofilms are to be expected. In contrast to Zn, a negative relationship between dissolved and accumulated Fe was observed throughout translocation. This negative slope could be related with the higher tendency of Fe to precipitate, as commented above.

The autotrophic compartment of biofilm is mainly composed by green algae, diatoms and cyanobacteria. In the same active bio-monitoring experiment, Tlili et al. (2011) and Corcoll et al. (2012) reported a clear decrease in diatom abundance in the most Zn-polluted site (M2) in favour of cyanobacteria and green algae. Furthermore, not only was a reduction in diatom diversity and biovolume, and an increase in malformed diatoms described under these Zn conditions (Morin et al. 2012; Corcoll et al. 2012), but also an increase in biofilm community tolerance (Tlili et al. 2011). Thus, the observed variation of algal biomass at all sites after 3 and 7 days could be due to this shift in biofilm composition.

AEA variations at the Up site might be attributed to environmental changes rather than metal pollution. Indeed, AEA are known to respond in the face of environmental stress (e.g. light, pH, temperature) to reduce the increase in ROS in organisms. In a former study performed in the same stream over a year, APX, CAT, GR and SOD were enhanced under spring and summer conditions with high light intensity and water temperature (Bonet et al. 2013). AEA variations due to environmental parameters have also been reported with single species in natural systems (Butow et al. 1997; Aguilera et al. 2002; Li et al. 2010) and biofilms in a microcosm study (Bonnineau 2011). Environmental parameters are not the only factors that may modulate AEA responses. Community changes can also contribute since each species may have its own defence mechanisms. However, in this study we did not expect huge community changes in the Up site because of similar environmental conditions to the colonization site (CS).

In spite of the fact that physicochemical variables separate M2 from the rest of the sites (PCA results), the relationship between biological responses and physicochemical parameters (RDA) allowed to separate the most polluted sites M2 and M3, from both Up and M1. According to our results, Up and M1, showed similar environmental and

metallic conditions explaining why AEA evolution over time followed similar patterns. Differences in AEA patterns between M2 and M3 could be explained by the higher pH and NO_3^{2-} in M3. Furthermore, at the end of the experiment, M3 presented higher Fe, Al and Cu accumulation values in biofilm than M2. Although Al and Cu in water were below detection limits and Fe in water was only detectable at the end of the experiment, all of these metals were accumulated in biofilms from M3. The same trend was also observed for Pb in M2. This underlines the great sensitivity of biofilm to metal pollution, even when metals are not detectable with chemical water analysis (Bradac et al. 2010; Corcoll et al. 2012). Biofilm acts as a "biological passive sampler" and has the advantages that are described for bioindicators, such as integrating over time a potentially fluctuating level of contaminants. Whereas a specific analysis of water may miss the actual level of metals, biofilms that live in the environment do not.

CAT, APX and Ni acc. variations were attributed to other processes (e.g. environmental and nutrient concentration) rather than to the metal gradient (according to the variance partitioning technique of RDA). GST was related with metal pollution whereas SOD and GR activities were correlated to both environmental parameters and metal pollution. Similarly, CAT, APX, GR and SOD values were also attributed to environmental changes rather than to metal pollution in a previous annual monitoring performed in the Riera d'Osor (Bonet et al. 2013). In contrast to our observations, other authors showed that these AEA were sensitive to metal exposure either by using algal cultures or biofilm communities in microcosms studies (Sauser et a. 1997; Tripathi et al. 2006; Bonet et al. 2012). Indeed GST, was correlated to both to environmental factors and metal toxicity (according to RDA), the influence of metals on the GST activity was twice as high as the influence of environmental factors one, indicating that GST measurements should be considered for the evaluation of Zn exposure. In fact, in the most Zn-polluted site, M2, GST was completely inhibited from 21 days on. Moreover,

GST followed the opposite pattern to the Zn accumulation capacity of biofilms. In contrast to our observations, Pereira et al. (2009) showed that GST activity under chronic metal pollution was not the main antioxidant defence against metals in some algae (*Ulva sp.*), while Maharana et al. (2010) reported an increase in this activity in others (*Padina tetrastromatica*) suggesting that GST may differ depending on the studied specie or species. In a previous study, both GST responses (increase and decrease) due to metal pollution, mainly Zn pollution, were observed in the same period of different years (July 2009 and 2010) (Bonet et al. 2013). Thus, the use of different single species or communities has to be considered because each species have their own metabolic pathways and defence.

AEA, like functional parameters, have shown different responses to metal exposure. In contrast, common functional parameters analysed, such as photosynthetic activities, did not show differences at the chronic level maybe due to their adaptation to metal pollution (Garcia-Meza et al. 2005; Serra et al. 2009).

The observed differences in AEA responses over time between early and later samplings (acute vs. chronic effects) at each site could be explained in two ways. Firstly, temporal variations of AEA may naturally occur. In addition, enzymes do not vary in a synchronous way, and it is quite common that while some may be induced, others do not vary or may even be depleted (Regoli et al. 2011). In fact, biochemical relationships between AEA are very complex and they can interact between one another. SOD transforms superoxide (O_2^-) to hydrogen peroxidase (H_2O_2). CAT and APX catalyze H_2O_2 into non-damaging substances (i.e. water). The sigmoidal curve of CAT activity versus the decrease in APX might indicate that CAT was able to reduce environmental stress rather than APX during persistent stress (Mittler 2002). At the same time, APX are involved in the glutathione-ascorbate cycle (Mittler 2002), jointly

with GST and GR. The increase in APX and GR at M3 could indicate a good antioxidant defence under these conditions while the decrease in GST might indicate the inhibition of this activity. These biochemical relationships should be taken into consideration when analysing how AEA respond in the face of environmental parameters as well as multiple-stress factors (environmental and metal pollution). Secondly, AEA variability of early samplings observed in this study reflected an acute response due to the new metal polluted conditions at the translocation sites (antioxidant defence). Whereas the variability observed after some days of exposure might point out an adaptation process to metal pollution. In fact, some AEA values found at the end of this study were in agreement with annual monitoring performed in this stream using biofilm communities directly from the stream bed and thus adapted to each site's conditions (Bonet et al. 2013).

5. CONCLUSIONS

This paper has examined the application of effect-based active biomonitoring using AEA and metal accumulation in freshwater biofilm. We consider that this approach is useful to evaluate and discriminate biological differences between sites with background potential metal toxicity and sites with moderate potential metal toxicity from hours to weeks and/or months and give a realistic vision of the ecological status. This study highlights the great sensitivity of biofilm as a "biological passive sampler" of metal pollution rather than simple chemical water analysis due to its important capacity to accumulate metals even when metal concentrations in water are below detection limits. In addition, this study has shown that some of the measured AEA (i.e. GST) are sensitive to metal pollution. We would recommend that, instead of using one or two antioxidant enzymes as biological indicators of metal pollution, it would be better to use a set of AEA to assess biological responses under field conditions due to their complementary activities and relationship. Moreover, AEA metal responses could be

better explained by analysing ROS concentrations in biofilm or non-enzymatic compounds which cope also with ROS (e.g. glutathione (GSH) and carotenoids).

In the light of our findings, effect-based evaluation using AEA and metal accumulation of biofilms in combination with physicochemical analysis permit early and sensitive assessment of metal pollution, and would be great tools to improve investigative monitoring and enhance the Common Implementation Strategy (CIS) of the WFD.

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Annex 1. Average and standard deviations (avg ± SD) of physicochemical parameters at each sampling time in each site: non-metal polluted site (Up), mining 1 (M1), mining 2 (M2) and mining 3 (M3). ANOVA and tukey-b test indicate significant differences between site at each sampling time (n = 3). *bdl* means below detection limit. *ns* means no significance. NH_4^+, Pb, Cu, Cd and Ni dissolved in water were below detection limit.

Parameters	Site	6h	1 day	3 days	7 days	21 days	35 days
pH	Up[a]	8.15	8.42	8.18	7.88	8.05	8.15
	M1[a]	8.30	8.53	8.39	7.87	7.96	7.75
	M2[a]	8.30	8.28	8.19	8.06	8.20	7.86
	M3[b]	8.02	7.95	7.27	7.78	7.79	7.28
	ANOVA	*F = 4.75, p > 0.05, (n = 6)*					
Cond	Up	200	198	230	246	255	231
	M1	199	199	228	252	1086	257
	M2	245	242	275	383	592	404
	M3	207	206	244	244	209	228
	ANOVA	*ns*					
Oxy	Up	8.70	9.73	8.96	9.25	9.42	8.67
	M1	8.54	9.46	9.51	8.88	9.29	7.37
	M2	8.63	9.47	9.31	9.15	9.30	8.81
	M3	8.74	9.67	9.39	9.48	9.36	8.74
	ANOVA	*ns*					
TºC	Up	16.60	17.60	19.70	17.90	17.00	19.30
	M1	17.70	17.90	18.20	18.60	16.90	20.00
	M2	18.00	17.30	18.20	19.30	19.70	19.10
	M3	17.70	16.20	17.30	17.80	17.90	18.90
	ANOVA	*ns*					
SS mg L^{-1}	Up	3.17	2.91	4.97	4.03	3.35	22.83
	M1	4.14	2.98	3.66	3.64	1.94	4.94
	M2	2.89	2.55	2.98	1.30	0.71	5.03
	M3	5.83	6.20	3.94	5.84	3.31	14.33
	ANOVA	*ns*					
DOC mg L^{-1}	Up	2.83 ± 0.21	2.83 ± 0.00	4.20 ± 0.14	4.28 ± 1.95	2.76[a] ± 0.04	3.82[a] ± 0.30
	M1	3.07 ± 0.11	3.07 ± 0.00	3.81 ± 0.75	4.24 ± 2.17	2.78[a] ± 0.06	4.60[ab] ± 0.39
	M2	2.85 ± 0.21	2.85 ± 0.00	3.86 ± 0.96	4.42 ± 2.37	2.14[c] ± 0.04	3.64[a] ± 0.20
	M3	4.02 ± 2.08	4.02 ± 0.00	4.24 ± 1.09	4.87 ± 2.99	2.63[b] ± 0.05	5.50[b] ± 1.12
	ANOVA	*ns*	*ns*	*ns*	*ns*	*F = 118.36, p > 0.0001*	*F = 5.56, p > 0.05*
PO_4^{3-} mg L^{-1}	Up	0.22 ± 0.01	0.29b ± 0.00	0.34[b] ± 0.03	0.46[c] ± 0.02	0.65[a] ± 0.03	0.32[b] ± 0.01
	M1	0.20 ± 0.01	0.25a ± 0.02	0.27[a] ± 0.02	0.35[b] ± 0.01	0.41[c] ± 0.02	0.43[c] ± 0.01
	M2	0.16 ± 0.00	0.24a ± 0.01	0.25[a] ± 0.01	0.26[a] ± 0.01	0.17[d] ± 0.01	0.23[a] ± 0.01
	M3	0.13 ± 0.09	0.24a ± 0.02	0.27[a] ± 0.01	0.33[b] ± 0.04	0.52[b] ± 0.02	0.30[b] ± 0.02
	ANOVA	*ns*	*F = 8.63, p > 0.01*	*F = 14.01, p > 0.005*	*F = 38.42, p > 0.0001*	*F = 288.10, p > 0.0001*	*F = 119.33, p > 0.0001*

Parameters	Site	6h	1 day	3 days	7 days	21 days	35 days
NO_2^- mg L^{-1}	Up	0.02 ± 0.00	0.03 ± 0.03	0.05b ± 0.00	0.01 ± 0.01	0.04 ± 0.01	0.21[b] ± 0.04
	M1	0.03 ± 0.02	0.02 ± 0.00	0.03[a] ± 0.01	0.02 ± 0.00	0.02 ± 0.00	0.15[b] ± 0.02
	M2	0.01 ± 0.00	0.01 ± 0.00	0.03[a] ± 0.01	0.01 ± 0.01	0.02 ± 0.01	0.03[a] ± 0.00
	M3	0.02 ± 0.00	0.03 ± 0.01	0.03[a] ± 0.00	0.02 ± 0.00	0.05 ± 0.04	0.18[b] ± 0.02
	ANOVA	*ns*	*ns*	*F = 9.86, p > 0.005*	*ns*	*ns*	*F = 31.46, p > 0.0001*
NO_3^{2-} mg L^{-1}	Up	2.99a ± 0.03	2.73c ± 0.10	2.44c ± 0.02	2.06 ± 0.33	4.56a ± 0.20	3.51 ± 0.80
	M1	2.29b ± 0.22	2.26a ± 0.11	2.17b ± 0.01	1.82 ± 0.08	1.53c ± 0.05	3.37 ± 0.08
	M2	1.94c ± 0.19	2.36ab ± 0.03	2.05a ± 0.10	1.73 ± 0.04	0.99d ± 0.11	2.17 ± 1.74
	M3	2.55b ± 0.05	2.63bc ± 0.23	2.40c ± 0.03	2.25 ± 0.27	3.97b ± 0.24	3.57 ± 0.11
	ANOVA	*F = 26.58, p > 0.0001*	*F = 7.66, p > 0.01*	*F = 30.91, p > 0.0001*	*ns*	*F = 335.24, p > 0.0001*	*ns*
Al µg L^{-1}	Up	2.46[a] ± 1.01	8.82[a] ± 4.87	15.62 ± 4.90	5.48[b] ± 0.62	bdl	bdl
	M1	6.88[b] ± 0.45	23.25[b] ± 11.01	6.35 ± 0.38	8.13[c] ± 0.66	13.49 ± 1.54	bdl
	M2	1.43[a] ± 2.31	1.02[a] ± 1.36	19.21 ± 29.92	0.18[a] ± 0.15	bdl	bdl
	M3	bdl[a]	bdl[a]	bdl	bdl[a]	bdl	bdl
	ANOVA	*F = 15.77, p > 0.001*	*F = 9.36, p > 0.01*	*ns*	*F = 226.37, p > 0.0001*	*F = 227.56, p > 0.0001*	*ns*
Fe µg L^{-1}	Up	267.17[bc] ± 6.69	277.97[a] ± 7.37	295.57[b] ± 6.94	316.20[b] ± 2.11	321.90[b] ± 3.61	296.23[b] ± 2.42
	M1	251.40[b] ± 1.10	277.83[a] ± 21.83	283.93[b] ± 2.05	334.40[b] ± 10.17	348.93[c] ± 7.94	327.27[c] ± 5.06
	M2	278.07[c] ± 19.50	302.47[a] ± 10.38	355.37[c] ± 28.11	572.07[c] ± 19.23	755.17[d] ± 10.89	472.70[d] ± 3.36
	M3	bdl	bdl[b]	bdl[a]	bdl[a]	265.47[a] ± 7.67	229.17[a] ± 4.16
	ANOVA	*F = 435.12, p > 0.0001*	*F = 342. 48, p > 0.0001*	*F = 327.31, p > 0.0001*	*F = 1369.77, p > 0.0001*	*F = 2600.79, p > 0.0001*	*F = 2115.02, p > 0.0001*
Zn µg L^{-1}	Up	bdl	bdl	bdl	bdl[a]	bdl[a]	108.10[a] ± 4.16
	M1	bdl	bdl	bdl	10.81[a] ± 0.29	31.28[b] ± 3.84	132.07[a] ± 10.21
	M2	106.59 ± 8.42	122.73 ± 4.75	148.03 ± 11.20	402.30[b] ± 30.68	605.63[c] ± 15.40	354.67[b] ± 7.91
	M3	bdl	bdl	bdl	bdl[a]	bdl[a]	119.17[a] ± 14.91
	ANOVA	*F = 445.79, p > 0.0001*	*F = 1786.10, p > 0.0001*	*F = 499.55, p > 0.0001*	*F = 500.79, p > 0.0001*	*F = 4222.56, p > 0.0001*	*F = 414.00, p > 0.0001*

Annex 2. Average and standard deviation (avg ± SD) of metal accumulation in biofilms expressed in µg gDW^{-1} at each sampling sites: non-metal polluted site (Up), mining 1 (M1), mining 2 (M2) and mining 3 (M3). ANOVA followed by tukey-b test indicate significant differences between site at each sampling time (n = 3). *bdl* means below detection limit. *ns* means no significance. Cd acc. was below detection limit in all cases.

	Site	6h	1 day	3 days	7 days	21 days	35 days
Al acc.	Up	27073.70 ± 2109.71	27704.06 ± 540.43	29370.78^{b} ± 1877.35	22784.10 ± 4063.36	16754.23 ± 1822.49	16575.47^{a} ± 1260.07
	M1	30971.97 ± 2433.01	25257.61 ± 2595.11	30025.52^{b} ± 4295.42	15954.73 ± 5822.82	16465.18 ± 953.98	27446.24^{b} ± 4939.35
	M2	30094.31 ± 3460.51	27137.77 ± 2467.59	24534.37^{ab} ± 4490.90	22038.69 ± 1279.91	15599.00 ± 3390.73	13374.26^{a} ± 3331.04
	M3	27320.24 ± 4533.84	24260.74 ± 573.37	19803.33^{a} ± 3574.52	19812.20 ± 3076.28	20455.83 ± 2548.95	29206.02^{b} ± 2356.58
	ANOVA	*ns*	*ns*	*F = 4.96, P > 0.05*	*ns*	*ns*	*F = 17.32, p > 0.001*
Fe acc.	Up	25758.22 ± 1892.22	24947.83^{c} ± 334.72	24499.75^{a} ± 327.97	23478.28 ± 5507.73	15539.62 ± 1236.26	13322.69^{a} ± 1476.05
	M1	26761.83 ± 1250.72	22175.84^{ab} ± 1571.00	25076.70^{b} ± 2550.93	24837.66 ± 10526.55	13940.59 ± 1740.92	19448.88^{b} ± 4011.30
	M2	25319.84 ± 1956.63	23836.12^{bc} ± 693.54	20845.99^{ab} ± 2603.36	20220.05 ± 1128.30	13012.93 ± 2386.47	10602.28^{a} ± 2673.91
	M3	22845.36 ± 2940.52	20967.96^{a} ± 756.17	18095.11^{a} ± 1641.87	17902.74 ± 2339.07	17943.54 ± 2187.33	22621.44^{b} ± 1565.91
	ANOVA	*ns*	*F = 10.13, p > 0.005*	*F = 8.09, p > 0.01*	*ns*	*ns*	*F = 13.03, p > 0.005*
Ni acc.	Up	7.37 ± 0.76	9.03a^{b} ± 0.91	6.72 ± 4.61	97.74 ± 140.19	18.41^{a} ± 6.79	6.56^{a} ± 2.72
	M1	9.50 ± 0.65	7.12^{a} ± 1.17	7.62 ± 0.77	101.90 ± 77.98	26.13^{ab} ± 12.86	1.34^{b} ± 0.97
	M2	9.45 ± 0.65	9.24^{ab} ± 1.22	10.21 ± 0.96	42.29 ± 9.51	38.43^{b} ± 5.66	9.10^{a} ± 2.42
	M3	10.12 ± 1.84	10.29^{b} ± 0.94	10.41 ± 1.03	18.33 ± 4.02	10.38^{a} ± 1.50	10.98^{a} ± 0.71
	ANOVA	*ns*	*F = 4.61, p > 0.05*	*ns*	*ns*	*F = 6.98, p > 0.05*	*F = 14.16, p > 0.001*
Cu acc.	Up	17.77 ± 1.08	21.92 ± 1.80	16.56 ± 3.30	34.16 ± 45.58	4.93^{a} ± 2.29	2.94^{a} ± 1.02
	M1	23.89 ± 1.21	19.82 ± 5.20	15.88 ± 0.59	34.13 ± 26.12	2.98^{a} ± 0.39	13.26^{bc} ± 2.74
	M2	19.26 ± 1.70	17.08 ± 2.80	17.10 ± 1.58	5.30 ± 1.77	5.48^{a} ± 3.82	9.47^{ab} ± 5.35
	M3	18.59 ± 4.37	22.25 ± 5.53	13.66 ± 1.16	11.70 ± 1.95	11.37^{b} ± 1.61	17.63^{c} ± 0.84
	ANOVA	*ns*	*ns*	*ns*	*ns*	*F = 6.91, p > 0.05*	*F = 12.23, p > 0.005*

	Site	6h	1 day	3 days	7 days	21 days	35 days
Zn acc.	Up	144.84^{a} ± 5.69	157.64^{a} ± 1.16	157.32^{a} ± 21.33	81.07^{a} ± 74.15	97.08^{a} ± 5.11	83.92^{a} ± 8.04
	M1	196.00^{ab} ± 8.26	227.89^{a} ± 38.57	485.36^{b} ± 153.90	132.83^{a} ± 13.80	965.88^{b} ± 99.40	639.88^{b} ± 237.06
	M2	359.77^{c} ± 52.73	472.22^{b} ± 81.18	836.23^{c} ± 185.07	2517.09^{b} ± 533.47	5733.95^{c} ± 571.46	1974.59^{c} ± 325.37
	M3	255.68^{b} ± 42.11	419.51^{b} ± 73.96	565.35^{bc} ± 97.48	612.49^{a} ± 141.01	633.79^{ab} ± 60.75	436.99^{ab} ± 9.26
	ANOVA	*F = 21.98, p > 0.0001*	*F = 20.05, p > 0.0001*	*F = 13.81, p > 0.005*	*F = 50.82, p > 0.0001*	*F = 240.10, p > 0.0001*	*F = 50.52, p > 0.0001*
Pb acc.	Up	49.52 ± 21.27	47.71 ± 1.64	43.05^{a} ± 8.67	23.34^{a} ± 13.40	19.98^{a} ± 2.57	22.44^{a} ± 0.43
	M1	49.17 ± 1.38	55.79 ± 1.92	116.25^{ab} ± 32.27	26.31^{a} ± 11.85	151.98^{bc} ± 8.17	127.41^{b} ± 6.33
	M2	60.22 ± 22.52	74.12 ± 17.18	75.20^{ab} ± 17.83	138.25^{b} ± 38.68	228.09^{c} ± 61.11	160.88^{b} ± 75.98
	M3	52.22 ± 5.10	72.10 ± 22.45	134.06^{b} ± 50.76	125.00^{b} ± 37.26	121.49^{b} ± 29.36	140.42^{b} ± 51.12
	ANOVA	*ns*	*ns*	*F = 5.01, p > 0.05*	*F = 14.36, p > 0.001*	*F = 19.10, p > 0.001*	*F = 5.44, p > 0.05*

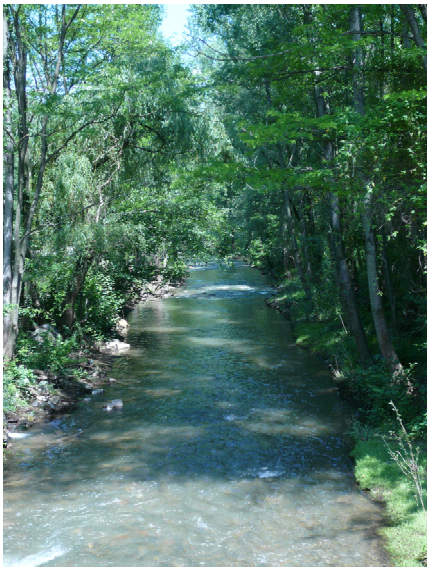
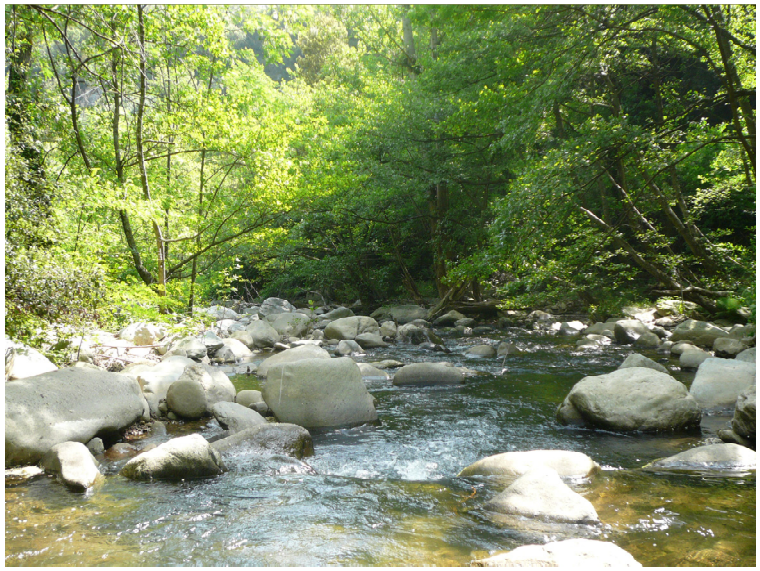
Annex 3. Average and standard deviations (avg ± SD) of biological parameters at each sampling time in each site: non-metal polluted site (Up), mining 1 (M1), mining 2 (M2) and mining 3 (M3). ANOVA and tukey-b test indicate significant differences between site at each sampling time (n = 3). *bdl* means below detection limit and *ns* means no significant.

Parameter	Site	6h	1 day	3 days	7 days	21 days	35 days
Chl-a µg Chl-a cm^{-2}	Up	41.70 ± 9.47	52.87 ± 36.70	72.48^{b} ± 25.82	12.78^{b} ± 1.22	43.39 ± 4.87	37.28 ± 4.38
	M1	50.19 ± 1.73	32.84 ± 25.33	117.35^{ab} ± 55.90	19.04^{b} ± 2.94	32.71 ± 0.50	76.45 ± 37.46
	M2	83.48 ± 39.09	46.82 ± 8.34	113.67^{ab} ± 5.97	70.63^{a} ± 15.08	42.10 ± 12.02	78.54 ± 20.88
	M3	84.24 ± 29.25	38.67 ± 25.91	186.46^{a} ± 15.67	95.23^{a} ± 21.73	23.84 ± 12.91	54.62 ± 13.05
	ANOVA	*ns* *p < 0.05*	*ns* *p < 0.05*	*F = 6.58, p > 0.05*	*F = 27.14, p > 0.0001*	*ns* *p < 0.05*	*ns* *p < 0.05*
OD ratio 430/665	Up	0.04 ± 0.02	0.00 ± 0.00	0.06 ± 0.05	0.15 ± 0.04	0.03 ± 0.01	0.02 ± 0.01
	M1	0.04 ± 0.01	0.00 ± 0.00	0.06 ± 0.08	0.05 ± 0.02	0.04 ± 0.01	0.03 ± 0.01
	M2	0.02 ± 0.00	0.01 ± 0.01	0.03 ± 0.02	0.04 ± 0.03	0.03 ± 0.01	0.02 ± 0.01
	M3	0.02 ± 0.01	0.01 ± 0.01	0.02 ± 0.01	0.07 ± 0.09	0.06 ± 0.05	0.03 ± 0.02
	ANOVA	*ns* *p < 0.05*	*ns* *p < 0.05*	*ns* *p < 0.05*	*ns* *p < 0.05*	*ns* *p < 0.05*	*ns* *p < 0.05*
CAT µmol H_2O_2 µg protein^{-1} min^{-1}	Up	0.09^{b} ± 0.01	0.12^{b} ± 0.00	0.20^{b} ± 0.01	0.20^{b} ± 0.05	0.25^{a} ± 0.02	0.20^{b} ± 0.01
	M1	0.13^{a} ± 0.01	0.16^{a} ± 0.01	0.20^{b} ± 0.01	0.35^{a} ± 0.01	0.15^{b} ± 0.01	0.22^{b} ± 0.01
	M2	0.14^{a} ± 0.02	0.14^{ab} ± 0.01	0.34^{a} ± 0.08	0.25^{b} ± 0.02	0.08^{c} ± 0.01	0.22^{b} ± 0.03
	M3	0.08^{b} ± 0.01	0.08^{c} ± 0.01	0.14^{b} ± 0.01	0.22^{b} ± 0.01	0.23^{a} ± 0.02	0.28^{a} ± 0.02
	ANOVA	*F = 13.82, p > 0.005*	*F = 29.52, p > 0.0001*	*F = 14.30, p > 0.001*	*F = 14.78, p > 0.001*	*F = 92.59, p > 0.0001*	*F = 10.60, p > 0.005*
APX µmol Ascorbate µg protein^{-1} min^{-1}	Up	$4.28\cdot10^{-4a}$ ± $2.26\cdot10^{-5}$	$6.51\cdot10^{-4a}$ ± $4.72\cdot10^{-5}$	$0.86\cdot10^{-4b}$ ± $1.21\cdot10^{-5}$	$16.9\cdot10^{-4a}$ ± $18.9\cdot10^{-5}$	$9.67\cdot10^{-4a}$ ± $4.89\cdot10^{-5}$	$3.90\cdot10^{-4c}$ ± $11.5\cdot10^{-5}$
	M1	$3.29\cdot10^{-4a}$ ± $9.65\cdot10^{-5}$	$1.88\cdot10^{-4c}$ ± $0.60\cdot10^{-5}$	$2.26\cdot10^{-4b}$ ± $5.96\cdot10^{-5}$	$9.83\cdot10^{-4ab}$ ± $5.30\cdot10^{-5}$	$2.36\cdot10^{-4b}$ ± $0.59\cdot10^{-5}$	$3.99\cdot10^{-4c}$ ± $3.79\cdot10^{-5}$
	M2	$1.62\cdot10^{-4b}$ ± $3.24\cdot10^{-5}$	$2.77\cdot10^{-4b}$ ± $2.55\cdot10^{-5}$	$4.97\cdot10^{-4a}$ ± $11.2\cdot10^{-5}$	$6.07\cdot10^{-4c}$ ± $10.8\cdot10^{-5}$	$3.19\cdot10^{-4c}$ ± $3.68\cdot10^{-5}$	$6.16\cdot10^{-4b}$ ± $4.79\cdot10^{-5}$
	M3	$1.12\cdot10^{-4b}$ ± $1.54\cdot10^{-5}$	$2.22\cdot10^{-4bc}$ ± $0.52\cdot10^{-5}$	$2.14\cdot10^{-4b}$ ± $5.57\cdot10^{-5}$	$7.41\cdot10^{-4bc}$ ± $1.11\cdot10^{-5}$	$3.73\cdot10^{-4b}$ ± $3.48\cdot10^{-5}$	$9.53\cdot10^{-4a}$ ± $3.81\cdot10^{-5}$
	ANOVA	*F = 23.05, p > 0.0001*	*F = 187.65, p > 0.0001*	*F = 18.62, p > 0.001*	*F = 55.42, p > 0.0001*	*F = 268.03, p > 0.0001*	*F = 45.88, p > 0.0001*
GR µmol NADPH µg^{-1} protein min^{-1}	Up	$16.1\cdot10^{-5}$ ± $0.32\cdot10^{-5}$	$14.2\cdot10^{-5a}$ ± $0.76\cdot10^{-5}$	$6.81\cdot10^{-5c}$ ± $0.92\cdot10^{-5}$	$4.52\cdot10^{-5c}$ ± $0.37\cdot10^{-5}$	$5.39\cdot10^{-5b}$ ± $0.21\cdot10^{-5}$	$11.3\cdot10^{-5b}$ ± $0.78\cdot10^{-5}$
	M1	$11.2\cdot10^{-5}$ ± $4.99\cdot10^{-5}$	$3.97\cdot10^{-5c}$ ± $0.71\cdot10^{-5}$	$7.98\cdot10^{-5bc}$ ± $0.56\cdot10^{-5}$	$2.30\cdot10^{-5d}$ ± $0.12\cdot10^{-5}$	$11.3\cdot10^{-5a}$ ± $1.17\cdot10^{-5}$	$16.4\cdot10^{-5ab}$ ± $5.22\cdot10^{-5}$
	M2	$16.7\cdot10^{-5}$ ± $1.74\cdot10^{-5}$	$7.16\cdot10^{-5b}$ ± $0.86\cdot10^{-5}$	$16.1\cdot10^{-5a}$ ± $1.10\cdot10^{-5}$	$8.78\cdot10^{-5b}$ ± $0.51\cdot10^{-5}$	$13.4\cdot10^{-5a}$ ± $1.68\cdot10^{-5}$	$15.5\cdot10^{-5ab}$ ± $1.23\cdot10^{-5}$
	M3	$9.34\cdot10^{-5}$ ± $2.08\cdot10^{-5}$	$4.62\cdot10^{-5c}$ ± $0.75\cdot10^{-5}$	$9.77\cdot10^{-5b}$ ± $0.80\cdot10^{-5}$	$11.0\cdot10^{-5a}$ ± $0.47\cdot10^{-5}$	$13.3\cdot10^{-5a}$ ± $1.68\cdot10^{-5}$	$20.5\cdot10^{-5a}$ ± $7.44\cdot10^{-6}$
	ANOVA	*ns* *p < 0.05*	*F = 111.84, p > 0.0001*	*F = 68.15, p > 0.0001*	*F = 303.81, p > 0.0001*	*F = 23.80, p > 0.0001*	*F = 5.78, p > 0.05*

Parameter	Site	6h	1 day	3 days	7 days	21 days	35 days
GST µmol CDNB conjugate μg^{-1} protein min^{-1}	Up	$1.29 \cdot 10^{-4c}$ ± $0.57 \cdot 10^{-5}$	$1.39 \cdot 10^{-4b}$ ± $0.97 \cdot 10^{-5}$	$1.93 \cdot 10^{-4ab}$ ± $2.18 \cdot 10^{-5}$	$1.40 \cdot 10^{-4a}$ ± $1.72 \cdot 10^{-5}$	$1.41 \cdot 10^{-4a}$ ± $1.70 \cdot 10^{-5}$	$0.70 \cdot 10^{-4a}$ ± $0.67 \cdot 10^{-5}$
	M1	$1.02 \cdot 10^{-4c}$ ± $0.88 \cdot 10^{-5}$	$1.75 \cdot 10^{-4b}$ ± $1.62 \cdot 10^{-5}$	$1.61 \cdot 10^{-4b}$ ± $0.62 \cdot 10^{-5}$	$1.44 \cdot 10^{-4a}$ ± $0.56 \cdot 10^{-5}$	$0.68 \cdot 10^{-4b}$ ± $0.49 \cdot 10^{-5}$	$0.43 \cdot 10^{-4b}$ ± $0.47 \cdot 10^{-5}$
	M2	$2.28 \cdot 10^{-4a}$ ± $0.75 \cdot 10^{-5}$	$1.82 \cdot 10^{-4b}$ ± $1.31 \cdot 10^{-5}$	$0.68 \cdot 10^{-4c}$ ± $2.86 \cdot 10^{-5}$	$0.92 \cdot 10^{-4b}$ ± $2.16 \cdot 10^{-5}$	0.00^{c} ± $0.24 \cdot 10^{-5}$	0.00^{c} ± $0.96 \cdot 10^{-5}$
	M3	$1.70 \cdot 10^{-4b}$ ± $3.18 \cdot 10^{-5}$	$3.83 \cdot 10^{-4a}$ ± $3.63 \cdot 10^{-5}$	$2.42 \cdot 10^{-4a}$ ± $4.88 \cdot 10^{-5}$	$1.62 \cdot 10^{-4a}$ ± $1.40 \cdot 10^{-5}$	$0.76 \cdot 10^{-4b}$ ± $1.05 \cdot 10^{-5}$	$0.28 \cdot 10^{-4b}$ ± $0.64 \cdot 10^{-5}$
	ANOVA	*F = 29.97, p > 0.0001*	*F = 77.67, p > 0.0001*	*F = 17.42, p > 0.001*	*F = 11.18, p > 0.005*	*F = 126.60, p > 0.0001*	*F = 77.29, p > 0.0001*
SOD U µg $protein^{-1}$	Up	47.23^{a} ± 0.00	47.61^{a} ± 0.17	44.70^{b} ± 0.24	47.82^{a} ± 0.17	47.33 ± 0.53	47.78 ± 0.24
	M1	47.64^{a} ± 0.33	45.14^{b} ± 0.07	44.90^{b} ± 0.03	46.55^{b} ± 0.42	46.67 ± 0.12	46.73 ± 0.02
	M2	45.56^{b} ± 0.12	46.77^{b} ± 0.22	48.50^{a} ± 2.60	46.75^{b} ± 0.36	47.40 ± 0.32	47.25 ± 2.74
	M3	45.37^{b} ± 0.07	46.54^{b} ± 0.19	45.46^{ab} ±0.75	46.34^{b} ± 0.17	47.17 ± 0.19	48.19 ± 0.25
	ANOVA	*F = 121.59, p > 0.0001*	*F = 107.11, p > 0.0001*	*F = 5.06, p > 0.05*	*F = 14.09, p > 0.001*	*ns p < 0.05*	*ns p < 0.05*

CHAPTER 3

Antioxidant enzyme activities responses in two Zn polluted streams: an active bio-monitoring

ABSTRACT

Effects of metal exposure (mainly zinc (Zn)) in biofilm communities were investigated in two mine basins, Riera d'Osor (NE Catalonia, Spain) and Riou Mort (SW Bordeaux, France), both of which offer the opportunity to study the effects of metal pollution on natural communities by means of active monitoring (i.e. translocation experiments). Riou Mort was more mineralized and metal polluted than Riera d'Osor, and thus presented higher conductivity. In both cases, biofilms were transferred from sites having either background or moderate levels of metal pollution to sites with higher pollution levels. This generated three classes of metal concentration: low, moderate and high. Physical, chemistry and biological parameters, including antioxidant enzyme activities (AEA) (i.e. catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), glutathione-S-transferase (GST) and superoxide dismutase (SOD)) and other classical endpoints like effective photosynthetic efficiency (Φ'_M) and algal biomass (chl-a), were analysed 6h and 24h after translocation.

In the Riou Mort, with higher Zn pollution (from 10 to 776 µg Zn L^{-1} dissolved in water and from 757 to 5954 µg Zn g DW^{-1} accumulated in biofilm), a general reduction of AEA (i.e. CAT, GR and GST) was observed. Only APX was enhanced under the lowest metal concentration change investigated. At this study-site, the highest Zn increase caused the enhancement of APX activity and chl-a, whereas photosynthetic efficiency decreased under the lowest Zn increase conditions. In Riera d'Osor with lower Zn pollution (115 µg Zn L^{-1} dissolved in water and 151 to 416 µg Zn g DW^{-1} accumulated in biofilm) a decrease in APX, GR, SOD and Φ'_M was observed whereas CAT and GST were enhanced.

We concluded that AEA are able to reflect fast (from 6 to 24h) responses to differences in Zn pollution depending on the magnitude of Zn pollution. Thus, the use of AEA in

biofilms is proposed as a sensitive effect-based biomarker of metal toxicity in fluvial systems.

1. INTRODUCTION

Metals are the most common non-biodegradable pollutants reported at elevated concentrations around the world (Mallick and Mohn, 2003). Zinc (Zn) is one of the most widespread metals due to both natural and anthropogenic origins. Furthermore Zn is present in a wide range of concentrations throughout aquatic ecosystems. In addition, Zn can be a micronutrient at low concentrations while toxic at higher concentrations. Its toxic effects are largely reported using different experimental approaches (Morin et al. 2007; Guasch et al. 2010; Bonnineau et al. 2011; Corcoll et al. 2011; Bonet et al. 2012).

It is well known that trace metals, such as Zn, induce oxidative stress due to the generation of reactive oxygen species (ROS) (Collén et al. 2003; Tripathi et al 2006; Quian et al. 2011; Bonnineau et al. 2011; Bonet et al. 2012). However, organisms have physiological mechanisms to cope with the toxic effects of these reactive species. Antioxidant enzyme activities (AEA), apart from being involved in metabolic processes such as photosynthesis and respiration, prevent an increase in ROS and maintain them in equilibrium after toxic exposure to organic and inorganic pollutants. Thus, some authors have suggested the use of AEA as biomarkers of acute and chronic toxic exposure due to their capacity to respond to both temporal scales (Valavanidis et al. 2006; Guasch et al. 2010; Bonnineau et al. 2012; Bonet et al. 2012). Until now, some studies have tested AEA in cultures and also in the field, but mainly at the species level (Sauser et al. 1997; Geoffroy et al. 2004, Li et al. 2006; Tripathi et al. 2006; Pereira et al. 2009) and with less frequency in microcosms at the community level by using biofilms (Bonnineau et al. 2010; Guasch et al. 2010; Bonnineau et al. 2011; Bonet et al. 2012). Fewer studies have been done in natural ecosystems (Bonnineau et al. 2011a; Bonet et al. 2013). All these studies, although different in terms of ecological relevance (Clements et al. 2002), provide a significant insight into the relationship between metal exposure and AEA. Overall, all these studies conclude that AEA responses are time,

dose, toxicant (organic or inorganic) as well as organism (from single species to complex communities) dependent (Bonet et al. 2012).

Biofilms have characteristics that make them good bioindicators of organic and inorganic pollution after both acute and chronic exposure (Sabater 2007; Romaní 2010; Corcoll et al. 2011; Tlili et al. 2011; Bonet et al. 2012). In contrast to taxonomical studies commonly addressed in periphyton studies (Morin et al. 2011) very few studies have been focused on biofilm AEA (Guasch et al. 2010; Bonnineau et al. 2011a; Bonet et al. 2012). Evaluation of AEA changes may provide earlier information about short-term (within hours) effects of chemical exposure while requiring less analysis time compared to taxonomical approaches (Bonet et al. 2012).

Mine basins have been largely investigated as ecotoxicogical case-studies (Duong et al. 2008). In this paper, two metal polluted systems located in former mining basins mainly affected by Zn, Riera d'Osor (NE Catalonia, Spain) and Riou Mort (SW Bordeaux, France), were investigated. In Riera d'Osor, since the end of mine activities in 1980 no environmental management has been carried out and the stream is still receiving the input of continuous mine effluents, as well as diffuse metal inputs from mine run-off from metal-polluted landfills. Recent studies have reported the effects of Zn pollution in biofilm communities focusing either on their autotrophic or heterotrophic components (Morin et al 2011; Tlili et al. 2011; Corcoll et al. 2012). Ecological effects of metal pollution in the Riou Mort have been studied since 1994 and this experimental watershed was labelled CNRS/PEVS in 2002. Although a significant decrease in waste emissions from the Zn industry has been reported since the end of Zn ore treatment in 1987, it still exhibits high metal concentrations (Audry et al. 2004; Coynel et al. 2007), several times higher than in Riera d'Osor at the most polluted site (2.5 times higher in July 2009, Arini et al. 2011; Corcoll et al. 2012). To date, the majority of studies performed in Riou Mort have evaluated structural changes in biofilm communities,

mainly of diatom assemblages (Gold et al. 2002; Morin et al. 2007; Duong et al. 2008; Morin et al. 2008; Arini et al. 2011; Arini et al. 2012).

Thus, the aim of this study was to explore the AEA responses of biofilms to Zn pollution after acute exposure in the field. To reach this goal, active bio-monitoring was performed in both streams (Rotter et al. 2011). It was based on the translocation of aquatic communities (fluvial biofilms) from one site to another, allowing the quantification of their responses in the field (De Kock and Kramer, 1994; Ivorra et al. 1999). Physicochemical and biological characteristics of both streams, Riou Mort and Riera d'Osor, were analysed to assess the main differences between and within streams. Biological parameters such as a set of AEA (catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), glutathione-S-transferase (GST) and superoxide dismutase (SOD)) and photosynthetic efficiency were analyzed as well as algal biomass. In order to find out the effect of metal pollution changes, all parameters were analyzed 6 and 24 hours after being translocated from their original sites to metal polluted sites within each stream.

The main objective of this study was to assess the magnitude and different types of biological responses while taking into account the different physicochemical conditions at both streams, mainly attributed to metal concentration, which is higher in Riou Mort than in Riera d'Osor. Functional responses were anticipated, rather than variations in algal biomass, between origin and translocation sites in each stream after a short period of time due to the increased metal exposure conditions.

2. MATERIAL AND METHODS

2.1. Study sites

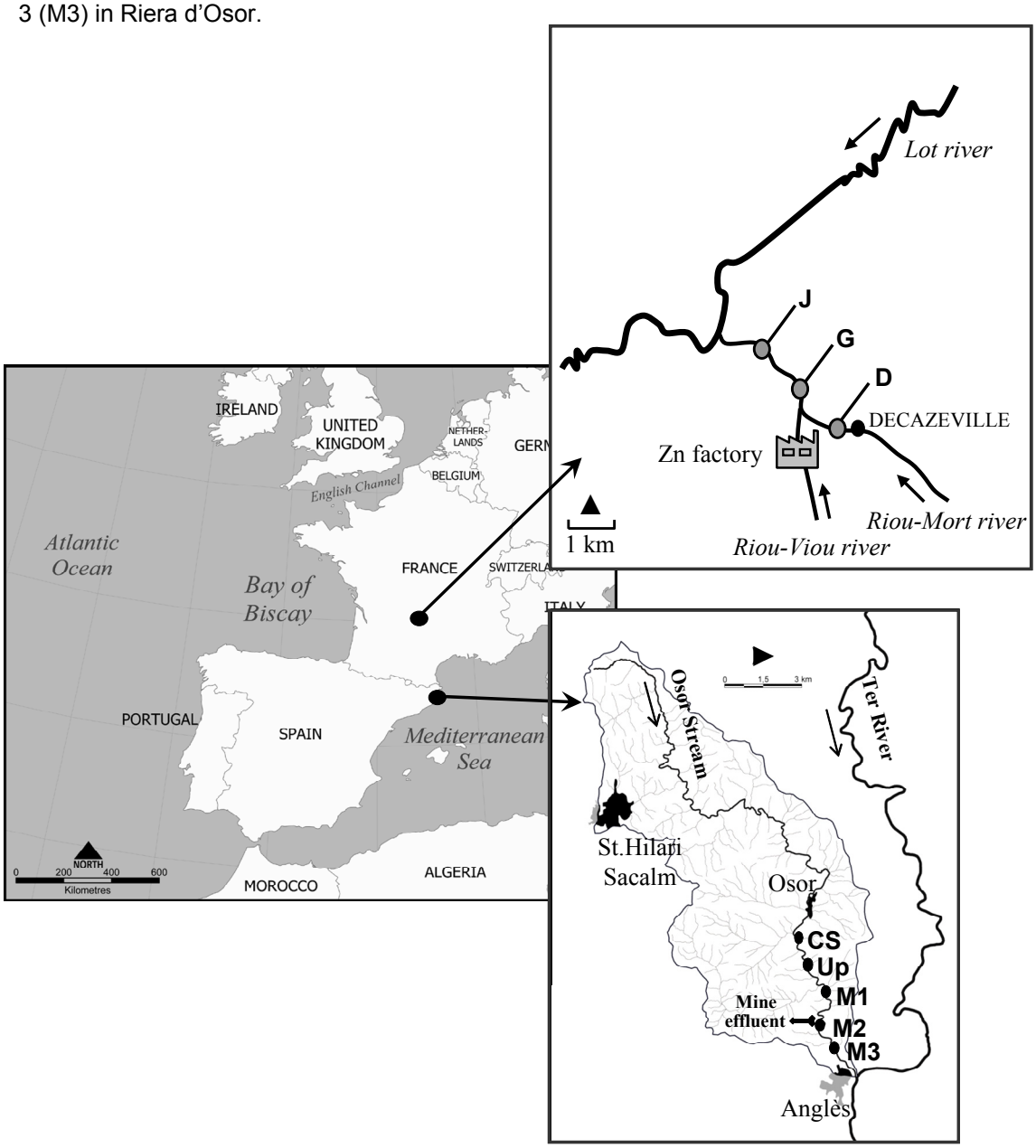
Translocation experiments were conducted in the Riou Mort and Riera d'Osor (Fig. 1). Riou Mort is a small tributary of the River Lot, located in the metal industry basin of

Decazeville (SW, Bordeaux, France). Three sites with different types and levels of metal pollution were selected: Decazeville (D) upstream of a Zn factory, located in the urban zone and with a background level of Zn pollution, Le Grange (G) with the highest Zn values because of its location downstream of the Zn factory and the confluence of a non-Zn polluted tributary (Riou Viou) and, further downstream, Joanis (J), with moderate levels of Zn pollution (Coynel et al. 2009; Bonnineau et al. 2011a). Riera d'Osor is a small tributary of the River Ter located in the north-east of Catalonia (NE Spain) (Fig. 1). This stream, in comparison with Riou Mort, is relatively well preserved. It has well developed riparian vegetation and low urban pressures although hydrology has been altered due to the diversion of part of the stream discharge for electric power production. Four sites with different Zn concentrations were selected: a reference site situated upstream, before the Zn mine source (Up); further downstream but still situated upstream of the main source and with low Zn pollution, a site designated as mining 1 (M1); the third site was located just after the mine source (referred to as MS) with continuous inputs of Zn provoking high Zn pollution (mining 2 (M2)); and, finally, mining 3 (M3) with moderate Zn pollution due to metal precipitation and storage in the sediment (Tlili et al. 2011; Corcoll et al. 2012; Bonet et al. 2013).

2.2. Experimental set-up and sample collection

While similar in most aspects of the experimental design, translocation experiments were slightly different between the two streams due to experimental problems. For biofilm sampling, in Riera d'Osor artificial substrata, heavy stoneware (40x40cm) with attached sand-blasted glass substrata (70 units of 8.5 x 2 cm and 80 units of 1.2 x 1.2 cm), was used whereas the translocation in Riou Mort was performed with natural substrata (pebbles). 5 days after putting artificial substrata in the Riou Mort, strong rain caused a flow peak, reaching 30.5 m^3 s^{-1} (http://www.hydro.eaufrance.fr/).

Figure 1. Localization of Riou Mort (SW, Bordeaux, France) and Riera d'Osor (NE Catalonia, Spain) and sampling sites of each stream, Decazeville (D), La Grange (G) and Joanis (J) in Riou Mort and colonization site (CS), Upstream (Up), mining 1 (M1), mining 2 (M2) and mining 3 (M3) in Riera d'Osor.



This event caused the loss of the artificial substrata and a reset in the natural biofilm colonization of the stream. Although artificial substrata could not be used in Riou Mort, differences between colonized substrata used in Riou Mort (pebbles) and artificial substrata used in Riera d'Osor (sand-blasted glass substrata) were not expected to influence our results since biofilm communities were similar in terms of maturity, sampled after 5 weeks of colonization in both cases (see below). Both experiments

were performed in June (June 2008 at Riou Mort and June 2009 at Riera d'Osor) and a similar set of physicochemical and biological parameters were measured after 6 and 24 hours of biofilm translocations.

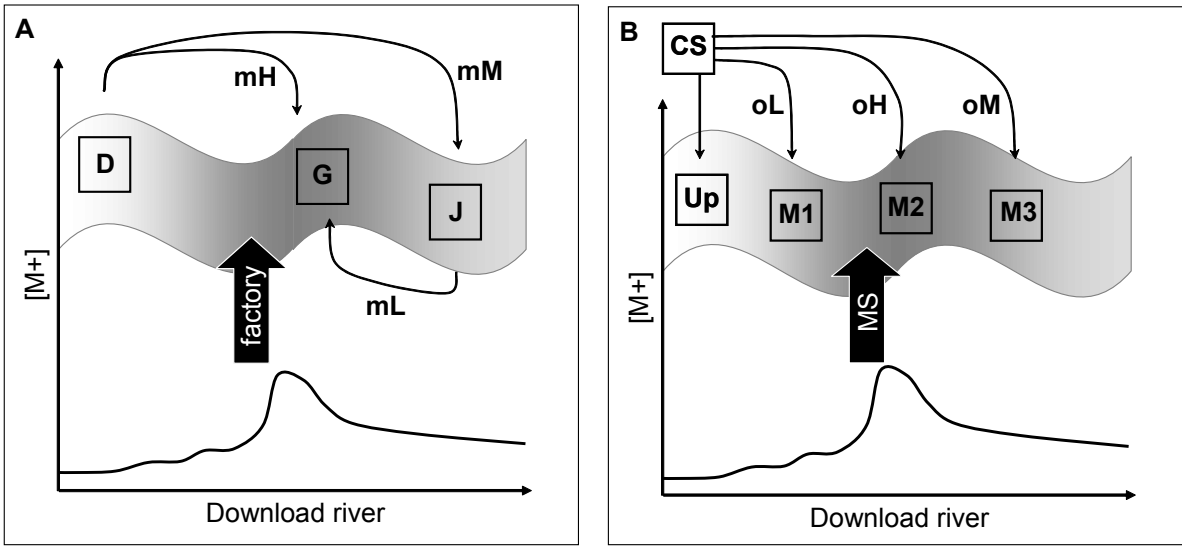
In both cases, the place where biofilms were colonized will be referred to as origin site in the text and the destination as translocation site, while the abbreviations L, M and H, low, moderate and high respectively, are used to indicate the expected relative change in metal concentrations between origin and translocation sites. In Riou Mort, origin sites were Decazeville (D) (non-Zn polluted) and Joanis (J) (a moderately polluted site), and translocation sites were Joanis (J) and Le Grange (G) (high Zn polluted sites). In total, three translocations were performed. Three pebbles translocated from (i) Decazeville to Le Grange caused a high increase in metal exposure conditions referred to as mH, (ii) from Decazeville to Joanis, caused a moderate increase in metal conditions referred to as mM and (iii) from Joanis to Le Grange created the lowest increase in Zn pollution conditions referred to as mL (Fig. 2A). In Riera d'Osor, artificial substrata were previously colonized upstream in the same stream for 5 weeks, at the colonization site (referred to as CS). Subsequently, artificial substrata were moved from CS to the four selected sites (Up, M1, M2 and M3). To account for possible translocation effects Up was considered as a non-Zn polluted site. Thus, different changes in Zn exposure were achieved (i) from Up to M1 (low Zn pollution) referred to as oL, (ii) from Up to M2 (high Zn pollution) referred to as oH, and (iii) from Up to M3 (moderate Zn pollution) referred to as oM (Fig. 2B).

2.2.1. Physicochemical parameters

Temperature, dissolved oxygen, pH and electrical conductivity were measured *in situ* (WTW METERS, Weilheim, Germany) at all locations. Water samples (10 mL) were filtered (0.2 µm nylon membrane filters, Whatman) to analyse phosphates (PO_4^{3-}) (Murphy and Riley, 1962), anions (Cl^-, NO^{2-}, NO_3^{2-}, CO_3^{2-} and SO_4^{2-}) and cations (K^+,

Na^+, NH_4^+, Ca^{2+} and Mg^{2+}) using ion-chromatography (761 Compact IC, Metrohm, Herisau, Switzerland) (Hach, 1992). Samples for dissolved organic carbon (DOC) were acidified immediately with 200 µL of HCl (2 M) and 100 µL of NaN_3 (2.7 mM) and were analysed following APHA, (1989). Suspended solids (SS) were analysed according to Elosegui and Butturini (2009). Light intensity was also measured *in situ* at each sampling site over biofilm substrates (LI-COR Inc., Lincoln, Nebraska, USA) and expressed as µmols photons m^{-2} s^{-1}.

Figure 2. Experimental design of translocation conducted in A) Riou Mort and B) Riera d'Osor. Arrows indicate translocations and expected metal concentration changes in each stream. In Riou Mort translocations were performed from: Joanis (J) to Le Grange (G) giving low metal concentration changes (mL), Decazeville (D) to Joanis (J) giving a higher change in metal exposure (mM) and Decazeville (D) to Le Grange (G), giving the highest change (mH). In Riera d'Osor translocations were performed from: upstream (Up) to mining 1 (M1), mining 3 (M3) and mining 2 (M2) giving low (oL), moderate (oM) and higher (oH) relative metal concentration changes respectively. Grey intensity is used to highlight metal pollution gradients.



Total dissolved metal concentrations were analysed at each site in triplicate using 5 mL of filtered water (0.2 µm nylon membrane filters, Whatman) and acidified immediately with 1% of HNO_3 (65% suprapure, Merck). Analyses were done by inductively coupled plasma mass spectroscopy (ICP-MS 7500c Agilent Technologies, Inc. Wilmington, DE). Detection limits for Riou Mort were 3.41 µmol Zn L^{-1}, 3.02 µmol Cd L^{-1}, 1.54 µmol

Fe L^{-1}, 4.20 µmol Al L^{-1}, 3.95 µmol Ni L^{-1}, 4.38 µmol Cu L^{-1} and 3.78 µmol Pb L^{-1}. In Riera d'Osor they were 5.92 µmol Zn L^{-1}, 1.01 µmol Cd L^{-1}, 35 µmol Fe L^{-1}, 0.19 µmol Al L^{-1}, 5.95 µmol Ni L^{-1}, 0.82 µmol Cu L^{-1} and 1.20 µmol Pb L^{-1}. When the value was below the detection limit, half of the detection limit was used for data treatment (Helsel, 1990). The accuracy of the analytical methods was checked periodically using a certified water reference (Reference material for measurement of elements in surface waters: SPS-SW2 Batch 113, Oslo, Norway). Water samples were stored at 4ºC until analysis in the laboratory.

Total metal accumulation in biofilms was analyzed at each site in triplicate. 20 cm^2 and 34 cm^2 of biofilm surface were scraped in Riou Mort and in Riera d'Osor respectively (two big substrata, 8.5 x 2 cm). Biofilm samples were stored at -20ºC prior to analysis. Samples were lyophilized, weighed and digested with 4mL of HNO_3 (65% suprapure, Merck) and 1mL of H_2O_2 (30% suprapure, Merck) in a high performance microwave digestion unit (Milestone, Ethos Sel) and were thereafter diluted to 15mL with milli-Q water. After digestion, liquid samples were treated as water samples following the procedure described above. Detection limits for Riou Mort were 0.29 µmol Zn L^{-1}, 0.35 µmol Cd L^{-1}, 10.43 µmol Fe L^{-1}, 4.34 µmol Al L^{-1}, 0.30 µmol Ni L^{-1}, 0.62 µmol Cu L^{-1} and 4.67 µmol Pb L^{-1}. In Riera d'Osor they were 0.19 µmol Zn L^{-1}, 0.75 µmol Cd L^{-1}, 2.65 µmol Fe L^{-1}, 0.10 µmol Al L^{-1}, 5.40 µmol Ni L^{-1}, 1.81 µmol Cu L^{-1} and 0.80 µmol Pb L^{-1}. Half of the detection limit was also used for data treatment when the value was below the detection limit (Helsel, 1990). The accuracy of the analytical methods was checked periodically using certified reference materials (Trace Elements in Plankton (CRM 414), Community Bureau of Reference (BCR), Brussels, Belgium).

2.2.2. Biological parameters

For all biological parameters analysis, three replicates were collected at each site and sampling time (6 and 24h). In Riou Mort the biofilm was collected from three arbitrarily

selected pebbles at each sampling site. Using a toothbrush, the biofilm was scraped off a 4 cm^2 area of the upper part of each pebble's substratum, defined by a plastic mould. In Riera d'Osor the biofilm was collected using individual glass.

Algal biomass (chl-a) was analysed using a biofilm surface of 4 cm^2 and 1.5 cm^2 in Riou Mort and Riera d'Osor respectively (Jeffrey and Humphrey 1975) and was expressed as µg Chl-a cm^{-2}.

The effective quantum yield (Φ'_M) was determined by measuring the biofilm chl-a fluorescence emission with the PhytoPAM (Pulse Amplitude Modulated) fluorometer (Heinz Walz GmbH) at excite chlorophyll fluorescence of 665nm, following the procedures described in Corcoll et al. (2011). In Riou Mort 4 cm^2 were scraped while in Riera d'Osor individual small substrata (1.2 x 1.2 cm) were taken and placed in transparent glass vials filled with 10 mL of water from the corresponding sampling site.

Protein extraction and antioxidant enzyme activities (AEA)

At both streams catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR) and glutathione-S-transferase (GST) activities were measured while SOD was also analysed at Riera d'Osor. A minimum of biomass is required to perform the AEA analysis. Therefore, biofilm samples were scraped from a surface area of 17 to 34 cm^2 (depending if there was more or less biomass in the system) and were punt into an eppendorf tube and sotred in liquid nitrogen until arrival at the laboratory. AEA samples were stored at -80ºC until their analysis. Extraction and quantification of protein and AEA measurements were performed as described in Bonnineau et al. (2011) and Bonet et al. 2013.

For assays, the optimal protein concentration was 2 µg and 4 µg of proteins in samples from Riou Mort and Riera d'Osor, respectively. All AEA were calculated as specific

activities (i.e. per µg of proteins). For each assay the optimal concentration of substrate or cofactor was optimized. Thus the concentrations used here were 30 mM of H_2O_2 for CAT assay in both streams, 3 mM of H_2O_2 in Riou Mort and 4 mM of H_2O_2 in Riera d'Osor for the APX assay, 0.25 mM of NADPH for the GR assay in both streams, 2 mM of GSH in Riou Mort and 5 mM of GSH in Riera d'Osor for the GST assay and 0.05 mM of WST-1 for SOD assay in both streams. CAT activity was calculated as µmol H_2O_2 µg protein^{-1} min^{-1}, APX activity as µmol Ascorbate µg protein^{-1} min^{-1}, GR activity as µmol NADPH µg protein^{-1} min^{-1} µg^{-1}, GST as as µmol CDNB conjugate µg protein^{-1} min^{-1} and SOD activity was expressed as U µg protein^{-1}.

2.3. Statistical analysis

One-way ANOVA was used to determine the main physicochemical and biological differences between sites at Riou Mort and Riera d'Osor. Two-way ANOVA (site and time) was used at each study site to determine significant biological differences between origin site and translocation sites at different times (6 and 24 hours) in both streams. A posteriori comparison was carried out with the Tukey-b test using 95% confidence limits. Homogeneity of variances and normality of data were checked prior to the ANOVA. In Riou Mort biofilms from D site (origin site) were compared with biofilms translocated to DG (mH) and DJ (mM) and from J (origin site) to G (mL). In Riera d'Osor biofilms from Up site (origin site) were compared with biofilms translocated to M1 (oL), M2 (oH) and M3 (oM). Pearson correlations were also analysed between all measured variables. All these analyses were done with SPSS v15.0 software.

3. RESULTS

3.1. Physicochemical conditions

All physicochemical parameters are summarized in Tables 1 and 2. The main physicochemical differences between both streams were conductivity, water

temperature, dissolved oxygen (in % of saturation), solids in suspension and DOC, which were higher in Riou Mort (Table 1). Riou Mort also had a higher nutrient load, overall nitrogen forms (Table 1) and more mineralized water than Riera d'Osor, with a higher concentration of majority ions (Table 1).

Metal water contents (total dissolved concentrations) at the background origin sites (Up from Riera d'Osor and D from Riou Mort) did not have detectable levels of Zn, Cd, Ni, Cu or Pb while the moderate polluted origin site (J in Riou Mort) had Zn, Cd, Fe, Al and Ni (Table 2). In Riera d'Osor, Zn was only detectable at M2 site but significantly below the concentration found at the polluted sites of Riou Mort G and J (Table 2). Cd was present at the polluted sites of Riou Mort (G and J). Fe and Al were higher in Riou Mort than Riera d'Osor, particularly at the origin site (D), probably due to geological differences (Table 2).

Focusing on biofilm metal content (total accumulated concentration), Zn accumulation (Zn acc.) did not differ between Riera d'Osor sites and was in the same range as Riou Mort's origin site (D) while it was 6 and 8 times higher at J and G respectively than at the other sites. Pb acc. in Riera d'Osor was also in the same range as the background origin site of Riou Mort (D) but was 4 times higher at G and J (Riou Mort). Fe acc. was also similar between sites but higher at D and G (Riou Mort). Cd acc., Ni acc. and Cu acc. were measurable only in Riou Mort being higher in G than J. Al acc. was in the same range in both streams (Table 2).

Table 1. Average and standard deviation of physicochemical parameters at each sampling site and stream. Riera d'Osor sites are: upstream (Up), mining 1 (M1), mining 2 (M2) and mining 3 (M3). Riou Mort sites are: Decazeville (D), Le Grange (G) and Joanis (J). Conductivity is expressed in µS cm^{-1}, temperature in ºC, light in µmols photons m^2 s^{-1}, dissolved oxygen (in mg/L and % saturation). Solids in suspension (SS), Dissolved Organic Carbon (DOC), nutrients (PO_4^{3-}, NO_2^-, NO_3^{2-} and NH_4^+), anions (Cl^- and SO_4^-) and cations (Na^+, K^+, Ca^{2+}, Mg^+) are expressed in mg L^{-1}. ANOVA has been performed to detect significant differences between sites from both streams and when this was significant a tukey-b test was performed using $p < 0.05$. *ns* means no significant.

Parameters	Riera d'Osor				Riou Mort			ANOVA	
	Up	M1	M2	M3	D	G	J	F	p-value
Conductivity	199.15^{a} ± 1.20	198.95^{a} ± 0.07	243.50^{a} ± 2.12	206.35^{a} ± 0.92	1544.50^{c} ± 44.55	1023.00^{b} ± 0.00	1043.00^{b} ± 50.91	947.57	0.001
TºC	17.10^{a} ± 0.71	17.80^{a} ± 0.14	17.65^{a} ± 0.49	16.95^{a} ± 1.06	22.15^{ab} ± 3.04	24.90^{b} ± 0.00	22.55^{ab} ± 2.19	9.24	0.005
pH	8.29 ± 0.19	8.42 ± 0.16	8.29 ± 0.01	7.99 ± 0.05	8.10 ± 0.23	8.36 ± 0.08	8.34 ± 0.16	ns	
Oxy	9.22 ± 0.73	9.00 ± 0.65	9.05 ± 0.59	9.21 ± 0.66	8.50 ± 1.71	9.85 ± 0.00	10.49 ± 0.08	ns	
% Oxy	98.65^{a} ± 9.40	97.20^{a} ± 7.21	97.25^{a} ± 5.02	97.35^{a} ± 4.60	116.90^{ab} ± 2.69	120.90^{b} ± 0.00	124.15^{b} ± 4.31	10.29	0.005
SS	3.04^{ab} ± 0.19	3.56^{ab} ± 0.82	2.72^{a} ± 0.24	6.01^{ab} ± 0.27	7.45^{b} ± 2.09	6.18^{ab} ± 0.87	4.04^{ab} ± 1.92	4.82	0.05
Light	27.33^{a} ± 1.76	546.17^{ab} ± 210.72	287.21^{ab} ± 32.70	211.27^{a} ± 24.09	812.00^{ab} ± 837.21	385.20^{ab} ± 399.09	1634.50^{b} ± 34.65	4.45	0.05
DOC	2.83^{a} ± 0.13	3.07^{a} ± 0.07	2.85^{a} ± 0.13	4.02^{b} ± 1.32	7.45^{d} ± 0.34	5.53^{c} ± 0.06	5.87^{c} ± 0.29	68.13	0.001
PO_4^{3-}	0.026^{a} ± 0.039	0.022^{a} ± 0.03	0.020^{a} ± 0.04	0.019^{a} ± 0.09	0.029^{a} ± 0.02	0.047^{a} ± 0.03	0.91^{b} ± 0.48	23.90	0.001
NO_2^-	0.02^{a} ± 0.02	0.02^{a} ± 0.02	0.01^{a} ± 0.00	0.03^{a} ± 0.01	1.05^{b} ± 0.15	1.79^{c} ± 0.61	1.99^{c} ± 0.65	39.55	0.001
NO_3^{2-}	2.86^{a} ± 0.16	2.28^{a} ± 0.15	2.15^{a} ± 0.26	2.59^{a} ± 0.16	0.41^{a} ± 0.10	28.03^{b} ± 7.84	27.38^{b} ± 8.08	69.27	0.001
NH_4^+	0.00^{a} ± 0.00	0.06^{a} ± 0.07	0.00^{a} ± 0.00	0.05^{a} ± 0.12	0.47^{b} ± 0.37	0.49^{ab} ± 0.21	0.94^{b} ± 0.63	5.64	0.001
Cl^-	10.88^{a} ± 0.38	10.23^{a} ± 0.60	11.20^{a} ± 0.76	10.75^{a} ± 0.64	17.85^{c} ± 0.06	14.47^{b} ± 0.89	17.54^{c} ± 0.22	96.28	0.00
SO_4^-	44.45^{ab} ± 44.20	69.46^{ab} ± 89.45	49.75^{ab} ± 33.24	25.49^{a} ± 15.50	112.64^{b} ± 0.81	71.85^{ab} ± 3.95	65.04^{ab} ± 2.51	2.76	0.05
Na^+	14.41^{a} ± 1.43	14.25^{a} ± 2.27	15.47^{a} ± 1.45	14.29^{a} ± 0.20	37.69^{c} ± 7.82	29.86^{b} ± 1.92	33.92^{bc} ± 1.83	40.15	0.00
K^+	0.99 ± 0.50	0.81 ± 0.68	0.81 ± 0.65	0.63 ± 0.58	17.59 ± 2.61	13.38 ± 0.83	13.61 ± 0.30	ns	
Ca^{2+}	31.53^{a} ± 6.28	28.59^{a} ± 3.58	39.19^{a} ± 4.20	31.84^{a} ± 5.55	161.30^{c} ± 15.21	136.50^{b} ± 23.24	140.85^{b} ± 1.94	101.34	0.00
Mg^+	5.79^{a} ± 3.02	4.27^{a} ± 0.59	6.02^{a} ± 1.88	4.41^{a} ± 0.36	163.28^{c} ± 40.71	119.72^{b} ± 12.95	114.58^{b} ± 0.83	76.02	0.00

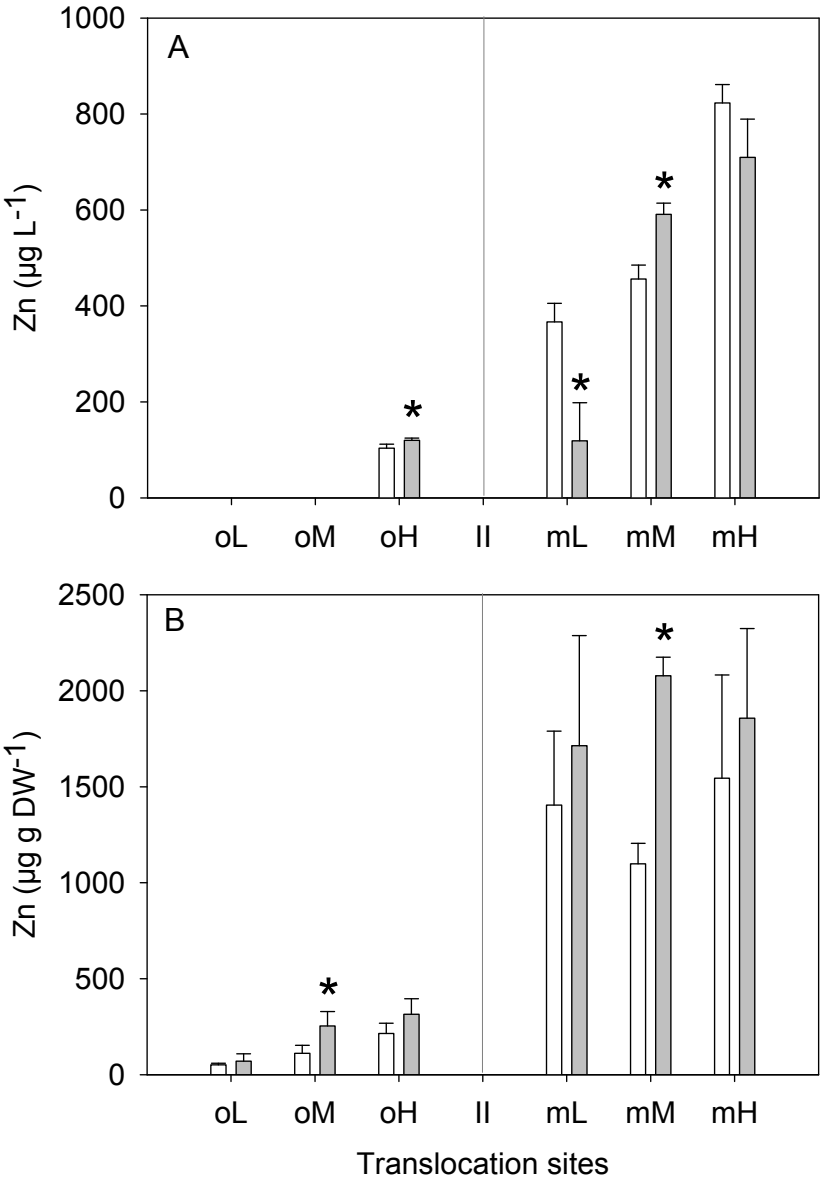
Table 2. Average and standard deviation of total dissolved metals in water and accumulated in biofilms (acc.) expressed in µg L^{-1} and µg gDW^{-1}, respectively, at each sampling site and stream. Riera d'Osor sites are: upstream (Up), mining 1 (M1), mining 2 (M2) and mining 3 (M3). Riou Mort sites are: Decazeville (D), Le Grange (G) and Joanis (J). ANOVA has been performed to detect significant differences between sampling sites within streams and when this was significant a tukey-b test was performed using $p < 0.05$ (N = 6). *bdl* means below detection limit and *ns* means no significant.

	Riera d'Osor				Riou Mort			ANOVA	
	Up	M1	M2	M3	D	G	J	F	p-value
Zn	*bdl*^{a}	*bdl*^{a}	114.66^{b} ± 10.75	*bdl*^{a}	10.07^{a} ± 3.04	776.45^{d} ± 82.99	533.55^{c} ± 92.28	322.87	0.001
Cd	*bdl*^{a}	*bdl*^{a}	*bdl*^{a}	*bdl*^{a}	bdl^{a}	14.18^{b} ± 1.28	13.15^{b} ± 0.76	429.20	0.001
Fe	272.57^{ab} ± 8.64	264.62^{ab} ± 20.02	290.27^{ab} ± 19.33	*bdl*^{a}	4280.08^{d} ± 332.46	3181.25^{c} ± 131.88	2881.83^{b} ± 71.18	715.42	0.001
Al	5.64^{a} ± 4.69	15.06^{b} ± 11.36	1.23^{a} ± 1.71	*bdl*^{a}	113.05^{c} ± 34.45	66.03^{bc} ± 34.83	46.49^{bc} ± 15.98	18.52	0.001
Ni	*bdl*^{a}	*bdl*^{a}	*bdl*^{a}	*bdl*^{a}	*bdl*^{a}	115.25^{ab} ± 139.93	82.94^{b} ± 102.71	5.31	0.001
Cu	*bdl*	*bdl*	*bdl*	*bdl*	*bdl*	*bdl*	*bdl*	ns	
Pb	*bdl*	*bdl*	*bdl*	*bdl*	*bdl*	*bdl*	*bdl*	ns	
Zn acc.	151.24^{a} ± 7.92	211.94^{a} ± 30.46	415.99^{a} ± 86.84	333.51^{a} ± 101.22	757.71^{a} ± 231.22	5954.09^{c} ± 637.04	4864.44^{b} ± 95.77	102.51	0.001
Cd acc.	*bdl*^{a}	*bdl*^{a}	*bdl*^{a}	*bdl*^{a}	0.97^{a} ± 0.20	88.09^{c} ± 15.57	68.37^{b} ± 3.11	130.13	0.001
Fe acc.	25353^{ab} ± 1294	24469^{ab} ± 2815	24578^{ab} ± 1544	21907^{a} ± 2178	40934^{c} ± 1769	41147^{c} ± 13822	37306^{bc} ± 3548	6.36	0.001
Al acc.	27389 ± 1420	28115 ± 3855	28616 ± 3138	25791 ± 3341	28579 ± 8976	29468 ± 7327	26368 ± 1767	ns	
Ni acc.	*bdl*^{a}	*bdl*^{a}	*bdl*^{a}	*bdl*^{a}	33.47^{b} ± 7.34	95.56^{c} ± 9.78	85.55^{c} ± 26.36	72.27	0.001
Cu acc.	*bdl*^{a}	*bdl*^{a}	*bdl*^{a}	*bdl*^{a}	40.21^{a} ± 2.38	139.79^{c} ± 13.34	84.53^{b} ± 0.10	24.30	0.001
Pb acc.	48.62^{a} ± 13.53	52.48^{a} ± 3.92	67.17^{a} ± 19.46	62.16^{a} ± 18.18	69.12^{a} ± 7.21	276.49^{b} ± 50.54	224.54^{b} ± 39.11	16.35	0.001

As a result of the performed translocations at Riera d'Osor, Zn increase in oH translocation was around 110 µg L^{-1} though significantly different between sampling times (ANOVA, $F = 8.36$, $p < 0.05$, $n = 3$). The increase in Zn acc. was 60, 182 and 264 µg Zn gDW^{-1} at oL, oM and oH respectively, and also significantly different between 6 and 24h at oM (ANOVA, $F = 8.23$, $p < 0.05$, $n = 3$) (Fig. 3). In Riou Mort, the increase of Zn in water caused by translocations was on average 250, 500 and 800 µg L^{-1} and 1500, 1600 and 1700 µg Zn gDW^{-1} in the biofilm in mL, mM and mM translocations,

respectively. An increase of both Zn and Zn acc. was also observed in mM after 24h (ANOVA, F = 39.33 and F = 137.16, $p < 0.005$, n = 3, respectively). In contrast, in mL Zn conditions, Zn changes in water were lower after 24 h (ANOVA, F = 23.65, $p < 0.005$, n = 3) (Fig.3).

Figure 3. Increase of Zn in water (A) and accumulated in biofilms (B) from their respective origin site at 6h (in white) and 24h (in grey) in both streams: Riera d'Osor (o) and Riou Mort (m). Translocations were performed from upstream to mining 1, mining 3 and mining 2 in Riera d'Osor giving low, moderate and high metal conditions (oL, oM and oH, respectively). In Riou Mort translocations were performed form Joanis to Le Grange, Decazeville to Joanis and Decazeville to Le Grange giving low, moderate and high metal conditions (mL, mM and mH, respectively). Asterisks indicate differences at 6 and 24h (ANOVA, $p < 0.05$, n = 3).



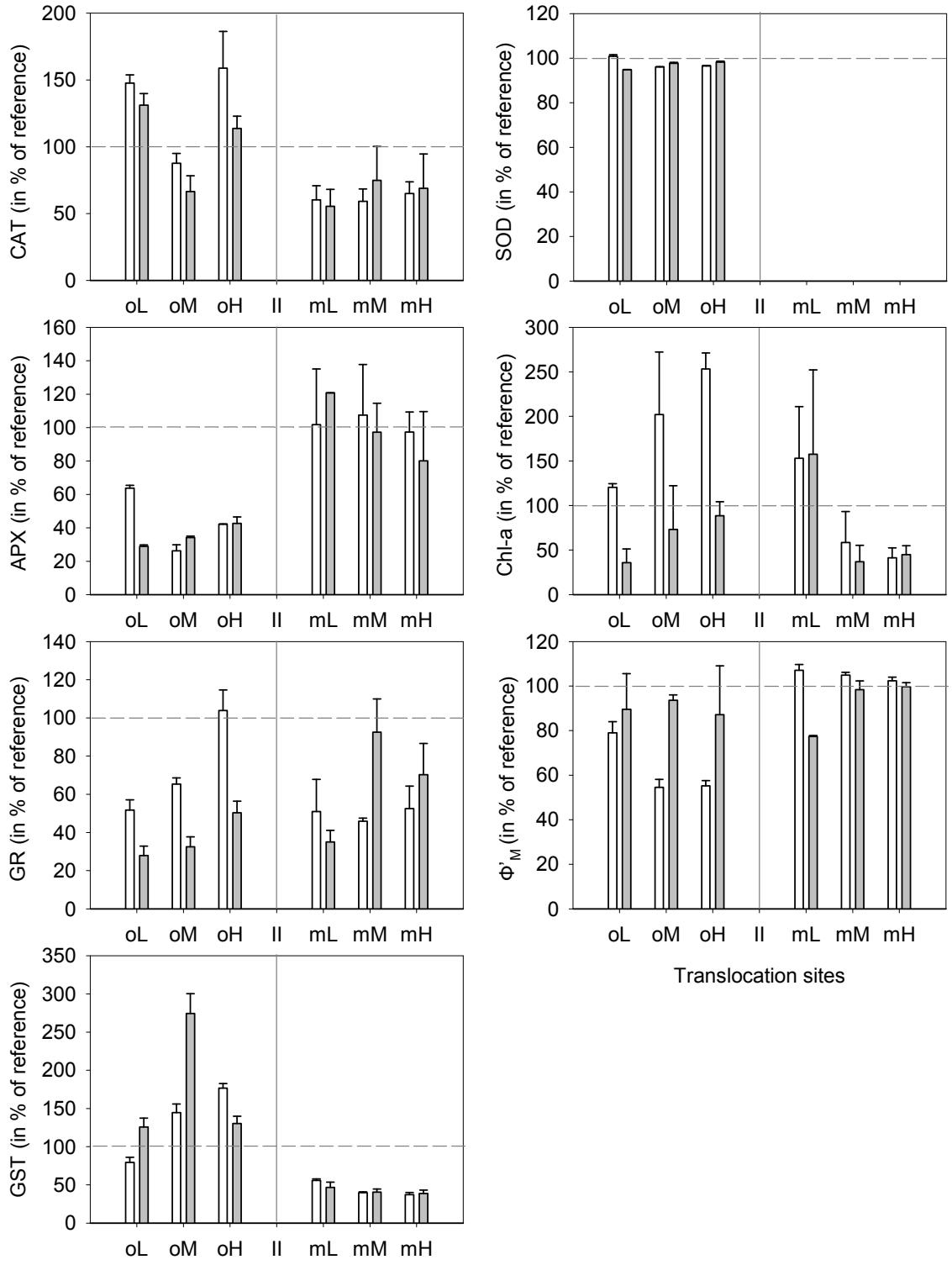
3.2. Biological responses

As expected, origin sites (Up in Riera d'Osor and D in Riou Mort) had different biological attributes. GR and GST were lower in Riera d'Osor (ANOVA, F = 13.28, F = 51.05, $p < 0.001$, n = 3, respectively). Riou Mort had higher Chl-a (ANOVA, F = 22.88, $p < 0.001$, n = 3). APX and Φ'_M were similar between streams (annex 1).

After translocation, in Riera d'Osor, transferred biofilms from Up to polluted sites showed either increases or decreases in the measured AEA activities (Fig. 4 and annex 1). APX, GR and SOD decreased compared to the origin site, while GST increased. CAT increased except under oM metal conditions. Φ'_M was reduced under all polluted conditions, oL, oM and oH, and Chl-a was not affected. In contrast to Riera d'Osor, Riou Mort AEA decreased in most cases (Fig. 4 and annex 1). Biofilms exposed to mL, mM and mH metal conditions (from J to G, D to J and D to G, respectively) underwent a significant decrease in CAT, GR and GST enzyme activities. An exception was APX, which was enhanced under mL conditions. In contrast Φ'_M was reduced under mL conditions but it was higher at all Riou Mort translocation sites after 6h than after 24h. Finally, chl-a was reduced under mH and mM conditions (Fig. 4 and annex 1).

Correlations were calculated between all measured variables. In Riou Mort, total dissolved Zn was positively correlated with APX (r^2= 0.66, $p<0.05$), and negatively correlated with Chl-a (r^2 = 0.74, $p < 0.05$). None of the other variables showed any significant correlations.

Figure 4. Biological parameters expressed as a percentage of their respective reference sites at 6h (in white) and 24h (in grey) in both streams. Translocations were performed from upstream to mining 1, mining 3 and mining 2 in Riera d'Osor giving low, moderate and high metal conditions (oL, oM and oH, respectively). In Riou Mort translocations were performed form Joanis to Le Grange, Decazeville to Joanis and Decazeville to Le Grange giving low, moderate and high metal conditions (mL, mM and mH, respectively). *nd* means no data.



4. DISCUSSION

4.1. Zn pollution

The aim of this paper was to evaluate biofilm responses to Zn exposure. Nevertheless, possible effects of the co-occurring metals in river water cannot be completely discarded. In Riou Mort, Cd, Ni, Al and Fe were also present. Al and Ni were not different between sites, thus their toxicity, if any, did not change due to translocation. Cd and Fe differed between sites. Fe, although coming from natural sources, was above toxicity thresholds, and higher at the origin site, thus we cannot discard effects on biofilms (Bonnineau et al. 2011). However, translocations did not cause an increase in biofilm Fe content, thus translocation effects were not expected. Regarding Cd, effects on biofilms were not expected based on former studies that demonstrated that the effect of a similar Zn+Cd mixture could be mainly attributed to Zn (Wang et al. 1995; Ivorra et al. 2002; Corcoll et al. 2012). Focusing on biofilm metal content, changes in biofilm Cu and Pb content were observed. While Pb effects on algae are not expected at low concentration, Cu is described as a phytotoxic metal because it directly affects the PSII (Tripathi et al. 2006; Serra et al. 2009; Guasch et al. 2010; Bonnineau 2011). Therefore, Cu could have contributed, at least partially, to the observed toxicity in our study. However, regarding our results, this hypothesis could not be confirmed due to the co-occurrence of Cu with Zn at the studied sites. In Riera d'Osor, apart from dissolved Zn in water, Al and Fe were also present. Al in water was higher at M1, more probably because of urban runoff rather than mining sources. Fe (dissolved and accumulated) was present at most sites and higher at Up, M1 and M2. However concentrations were too low to cause toxicity. For all these reasons, metal toxicity in this study was mainly attributed to Zn (dissolved and accumulated in biofilms).

A short period of metal exposure (6 and 24 h) to different levels of metal pollution was enough to increase biofilm metal contents. In Riera d'Osor, in spite of the fact that Zn in

water was only detectable at M2 (the most polluted site), Zn acc. in biofilm was measurable at all translocation sites. These results reflect the fact that measurements of metal concentrations in biofilm matrices are more sensitive than measurements in grab water samples, highlighting the interest of this parameter in monitoring of rivers with low levels of metal pollution (Guasch et al. 2010). In Riou Mort the increase in total dissolved Zn (1.5, 53 and 77 times higher than their respective origin sites) caused an increase of three orders of magnitude in biofilm Zn content. Overall, we can conclude that the main metal that changed after the translocations was Zn, thus it is expected to be the main driver of biological responses as discussed above.

4.2. Biological responses after biofilm translocations

The range of Zn found in both streams, dissolved in water (from 10 to 776 μg L^{-1}) and the amount accumulated in the biofilm (from 150 nearly 6000 μg Zn g DW^{-1}) after a short period of exposure (6 and 24 hours), was enough to produce clear physiological responses. Most biological biofilm parameters (i.e. AEA) were affected after translocation to metal-polluted sites. Moreover, different biological responses could be distinguished between streams according to their Zn pollution, lower in Riera d'Osor than in Riou Mort.

Under low Zn pollution (Riera d'Osor), the effects were mainly at physiological level, not affecting algal biomass. CAT and GST of biofilm communities were able to cope with the oxidative stress by increasing their activities while APX, GR, SOD and Φ'_M decreased (with respect to the origin site, Up). The variability of CAT activity under low Zn conditions (oL, oM and oH in Riera d'Osor and mL in Riou Mort) could be due to other factors such as environmental stress. In fact, it has been reported that environmental parameters can affect the AEA (Butow et al. 1997; Aguilera et al. 2002; Li et al. 2010; Bonnineau 2011). On the other hand, after this short period of time, a decrease in algal biomass was only observed under the highest Zn pollution scenarios

(the mM and mH translocations in Riou Mort) indicating that high metal toxicity might cause algal mortality. As expected, algal biomass did not change in the mL translocation because the origin site (J) already had high values of Zn (in water and accumulated in biofilm) and biofilm communities were pre-adapted (Tlili et al. 2011).

In agreement with this point, APX was able to respond to moderate Zn stress in a pre-adapted community (in mL conditions) and Φ'_M was slightly inhibited, indicating that metal increase caused transient physiological damage (Corcoll et al. 2012). The inhibition of CAT, GST and GR caused by Zn to non-adapted biofilms could be due to the direct effects of Zn on biofilm metabolism, but also due to cell mortality. GST showed the expected uni-modal enzyme response to increasing toxicity: increasing with low Zn concentration (Riera d'Osor) thus indicating a fast antioxidant response able to cope with the stress caused by low metal exposure, and decreasing under high Zn concentrations (Riou Mort) once the antioxidant capacity had been overwhelmed. A decrease in GST activity has been attributed to high ROS in biofilm (i.e. H_2O_2, De Franco et al. 2009). In agreement with these results, a positive relationship between Zn and GST was also observed at an annual monitoring in Riera d'Osor (Bonet et al. 2013).

In contrast to the CAT and GST inductions observed in Riera d'Osor, APX was clearly inhibited. This APX inhibition could be explained by direct Zn toxicity but also by the metabolic relationship with other enzymes such as GR (ascorbate-glutathione cycle, Mittler 2002). A decrease in GR activity (observed in both systems) may be due to the reaction of –SH groups with free ion metals, which could lead to a depletion in cellular GSH poll. GSH is a prominent cellular antioxidant (Nagalakshmi and Prasad 2001) used to recycle the ascorbate (APX co-factor) (Asada 1999), to inactivate oxidative compounds by the GST or as a precursor of phytochelatins biosynthesis, among other processes (Noctor et Foyer 1998). Therefore, the reduction in GR induces a lack of co-

factor concentration and APX may be affected due to metal effects as well as due to physiological requirements. Finally, SOD activity, only measured in Riera d'Osor, was reduced under all Zn exposure scenarios. In agreement with our results, Sauser et al. (1997) also reported a decrease in SOD after a few hours (~20h) of metal exposure. Nevertheless, at 80 hours it reached a plateau state. In contrast, an increase in SOD after metal exposure was reported by Mallick (2004).

Overall, the set of AEA analyzed in this study (SOD, CAT, APX, GR and GST) could be used as indicators of oxidative stress caused after some hours of Zn exposure and would allow us to distinguish between different levels of Zn pollution scenarios: low and high.

AEA activation could be considered as a direct enzyme induction to remove the excess of ROS produced by metals, as well as environmental factors. In contrast, the AEA inhibition could be attributed to several causes. First, it could be attributed to the direct effects of Zn (inhibition). Secondly, it could indicate that other protective mechanisms of cells like amino acids which can chelate metals in the cytoplasm, scavenging hydroxyl radicals, maintaining water balance and reducing metal uptake (Mallick 2004) could cope with oxidative stress rather than AEA. Also it could be attributed to lipid membrane peroxidation and their consequent cell death as we have observed in Riou Mort.

5. CONCLUSION

From the evaluation of physicochemical and biological parameters of different metal pollution scenarios we can conclude that:

- Translocation experiments, as an active bio-monitoring approach, allow us to assess the short-term effects of metal pollution on biofilms.

- Biofilm metal contents are more sensitive to metal pollution increasing after a few hours and thus are more indicative of metal pollution than water metal analysis. Biofilms are able to accumulate metals even when water metal concentrations are below detection limits. Thus a combination of water and biofilm analysis better reflects the real metal pollution of streams.
- AEA show different responses according to different metal concentrations, decreasing under higher metal conditions in Riou Mort while a more diverse response was observed under low metal pollution.

Hence from this study we propose active bio-monitoring including metal concentration analyses in water and biota (biofilms) and biological parameters (AEA) as effect based analysis useful to improve the environmental risk assessment at chemical and biological level after an accidental spill, as well as to evaluate the effect of point-sources of pollution such as the entrance of a polluted tributary or outflow water from waste water treatment plants.

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Annex 1. Average and standard deviation of biological parameters at origin and translocation sites. In Riera d'Osor: low (oL), moderate (oM) and higher (oH) metal change translocations from upstream (Up) to mining 1 (M1), mining 3 (M3) and minig 2 (M2). In Riou Mort: from Joanis (J) to Le Grange (G) (low metal increase (mL)), from Decazeville (D) to Joanis (J) (moderate metal increase (mM)) and from Decazeville (D) to Le Grange (G) (high metal increase (mH)). For each stream, two-way ANOVA results (F and p value) were used to detect significant differences ($p < 0.05$, $n = 6$) between sites (origin and translocation sites) and samplings times (6h and 24h). *nd* means no data and *ns* means no significant.

		CAT	APX	GR	GST	SOD	Chl-a	Φ'_M
Riera d'Osor	**Up**	0.11^{a} ± 0.01	$5.40\cdot10^{-4a}$ ± $1.74\cdot10^{-5}$	$1.52\cdot10^{-4a}$ ± $5.30\cdot10^{-1}$	$1.34\cdot10^{-4a}$ ± $0.28\cdot10^{-5}$	47.42^{a} ± 0.12	47.29 ± 19.25	0.32^{a} ± 0.00
	oL (M1)	0.15^{c} ± 0.00	$2.59\cdot10^{-4b}$ ± $6.40\cdot10^{-5}$	$076\cdot10^{-4b}$ ± $3.03\cdot10^{-5}$	$1.39\cdot10^{-4a}$ ± $0.53\cdot10^{-5}$	46.39^{b} ± 0.18	41.52 ± 16.69	0.27^{b} ± 0.01
	oM (M3)	0.08^{b} ± 0.01	$1.67\cdot10^{-4c}$ ± $35.8\cdot10^{-5}$	$0.70\cdot10^{-4b}$ ± $0.94\cdot10^{-5}$	$2.77\cdot10^{-4c}$ ± $0.32\cdot10^{-5}$	45.96^{c} ± 0.08	61.46 ± 2.36	0.23^{b} ± 0.00
	oH (M2)	0.14^{c} ± 0.01	$2.20\cdot10^{-4bc}$ ± $0.49\cdot10^{-5}$	$1.20\cdot10^{-4a}$ ± $0.62\cdot10^{-5}$	$2.05\cdot10^{-4b}$ ± $0.40\cdot10^{-5}$	46.17^{bc} ± 0.07	65.15 ± 21.74	0.22^{b} ± 0.04
	ANOVA	*36.23 < 0.001*	*94.36 < 0.001*	*20.84 < 0.001*	*69.83 < 0.001*	*81.74 < 0.001*	*ns*	*12.76 < 0.001*
	6h	0.11 ± 0.01	$2.58\cdot10^{-4}$ ± $3.72\cdot10^{-5}$	$1.33\cdot10^{-4}$ ± $1.96\cdot10^{-5}$	$1.57\cdot10^{-4}$ ± $1.23\cdot10^{-5}$	46.45 ± 0.14	64.90 ± 17.27	0.27 ± 0.01
	24h	0.13 ± 0.01	$3.35\cdot10^{-4}$ ± $24.8\cdot10^{-5}$	$0.75\cdot10^{-4}$ ± $3.75\cdot10^{-1}$	$2.20\cdot10^{-4}$ ± $1.19\cdot10^{-5}$	46.52 ± 0.07	42.80 ± 11.72	0.25 ± 0.02
	ANOVA	*17.56 < 0.001*	*20.16 < 0.001*	*47.69 < 0.001*	*61.09 < 0.001*	*ns*	*ns*	*ns*
Riou Mort	**J**	0.12 ± 0.01	$3.82\cdot10^{-4}$ ± $29.8\cdot10^{-5}$	$5.04\cdot10^{-4}$ ± $21.4\cdot10^{-5}$	$3.11\cdot10^{-4}$ ± $1.06\cdot10^{-5}$	*nd*	46.07 ± 0.37	0.29 ± 0.01
	mL (G)	0.10 ± 0.00	$5.49\cdot10^{-4}$ ± $13.1\cdot10^{-5}$	$2.54\cdot10^{-4}$ ± $2.62\cdot10^{-5}$	$1.97\cdot10^{-4}$ ± $1.54\cdot10^{-5}$	*nd*	126.74 ± 24.35	0.27 ± 0.01
	ANOVA	*6.89 < 0.05*	*7.61 < 0.05*	*14.13 < 0.01*	*31.49 < 0.001*	*ns*	*ns*	*19.7 < 0.005*
	6h	0.12 ± 0.02	$4.97\cdot10^{-4}$ ± $27.6\cdot10^{-5}$	$4.21\cdot10^{-4}$ ± $20.5\cdot10^{-5}$	$2.41\cdot10^{-4}$ ± $1.14\cdot10^{-5}$	*nd*	83.31 ± 18.86	0.28 ± 0.01
	24h	0.10 ± 0.02	$4.34\cdot10^{-4}$ ± $10.8\cdot10^{-5}$	$3.37\cdot10^{-4}$ ± $1.77\cdot10^{-5}$	$2.67\cdot10^{-4}$ ± $0.66\cdot10^{-5}$	*nd*	89.50 ± 42.84	0.28 ± 0.00
	ANOVA	*ns*	*6.53 < 0.05*	*ns*	*ns*	*ns*	*ns*	*19.7 < 0.005*
	D	0.13^{a} ± 0.00	$4.97\cdot10^{-4}$ ± $1.97\cdot10^{-5}$	$4.18\cdot10^{-4a}$ ± $7.31\cdot10^{-5}$	$5.56\cdot10^{-4a}$ ± $3.68\cdot10^{-5}$	*nd*	195.37^{a} ± 38.73	0.28 ± 0.01
	mM (J)	0.08^{b} ± 0.01	$4.42\cdot10^{-4}$ ± $5.60\cdot10^{-5}$	$2.54\cdot10^{-4b}$ ± $0.74\cdot10^{-5}$	$2.20\cdot10^{-4b}$ ± $0.66\cdot10^{-5}$	*nd*	84.40^{b} ± 0.12	0.28 ± 0.01
	mH (G)	0.08^{b} ± 0.01	$5.09\cdot10^{-4}$ ± $5.08\cdot10^{-5}$	$2.82\cdot10^{-4b}$ ± $4.25\cdot10^{-5}$	$2.23\cdot10^{-4b}$ ± $1.14\cdot10^{-5}$	*nd*	91.95^{b} ± 18.31	0.29 ± 0.01
	ANOVA	*5.63 < 0.05*	*ns*	*6.83 < 0.01*	*56.98 < 0.001*	*ns*	*8.73 < 0.005*	*ns*
	6h	0.10 ± 0.01	$5.21\cdot10^{-4}$ ± $5.48\cdot10^{-5}$	$2.96\cdot10^{-4}$ ± $7.90\cdot10^{-5}$	$3.32\cdot10^{-4}$ ± $6.78\cdot10^{-5}$	*nd*	122.57 ± 21.89	0.26 ± 0.01
	24h	0.10 ± 0.00	$4.43\cdot10^{-4}$ ± $5.52\cdot10^{-5}$	$3.39\cdot10^{-4}$ ± $0.49\cdot10^{-5}$	$3.34\cdot10^{-4}$ ± $3.03\cdot10^{-5}$	*nd*	125.24 ± 36.55	0.30 ± 0.01
	ANOVA	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*102.4 < 0.001*

Chapter 4

Antioxidant enzyme activities as biomarkers of Zn pollution in fluvial biofilms

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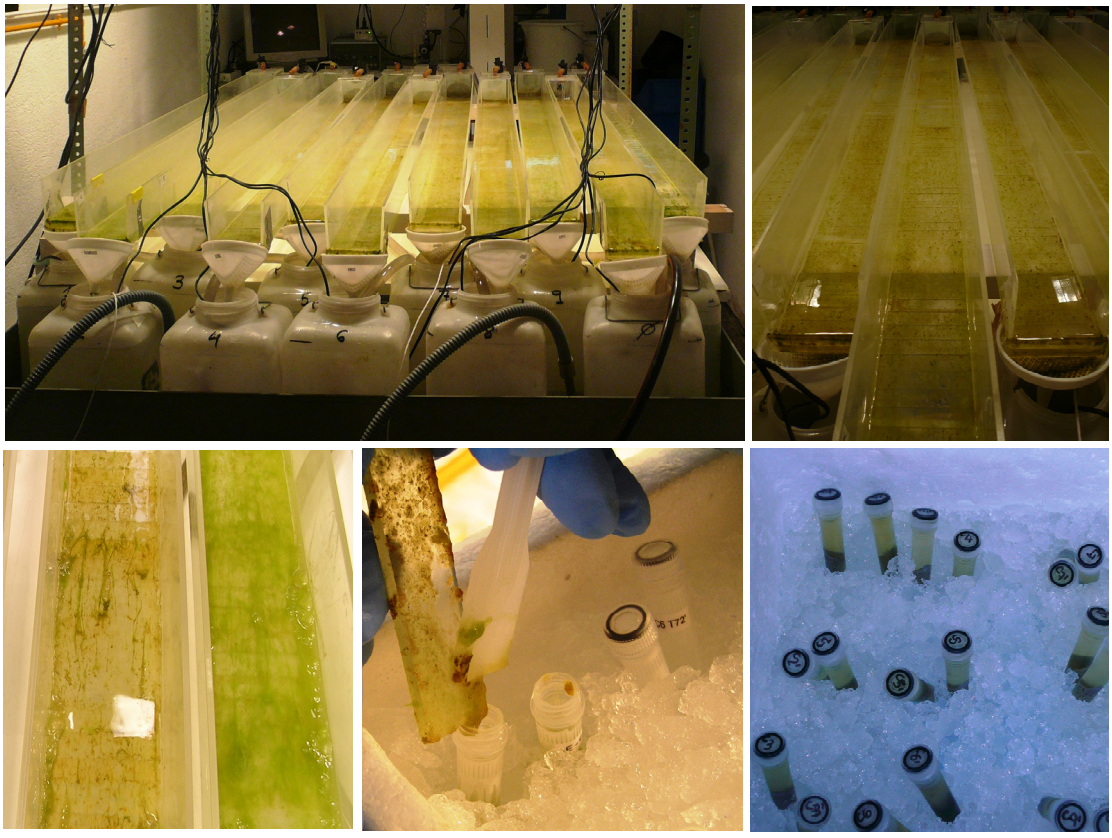


ABSTRACT

The potential of the antioxidant enzyme catalase (CAT) and ascorbate peroxidase (APX) as molecular biomarkers of Zn toxicity in freshwater biofilms has been explored in this study jointly with other classical functional and structural endpoints (photosynthetic parameters, algal group composition and bioaccumulation). Biofilms were colonized in an indoor microcosm system for 5 weeks and then exposed to Zn for 5 weeks. To evaluate Zn effects, biofilms were sampled 5 and 3 days before exposure, just before exposure (time 0), and after 6 hours, 1, 3, 7, 21 and 35 days of metal exposure. Most endpoints measured were affected by Zn exposure (320 μgZn L^{-1}) during both periods of exposure. APX was the only functional parameter responding after a few hours of Zn exposure, highlighting its use as an early toxicity biomarker. Structural changes began after 3 days of exposure, starting with a decrease in algal biomass and an increase in the OD 430:665 ratio. Structural changes in biofilm communities were observed after 1 week, leading to a shift from diatoms to cyanobacteria and green algae-dominated communities. CAT activity was thereafter enhanced (after three weeks of exposure) and attributed not only to a direct effect of Zn bioaccumulation but also to an indirect effect of the community composition changes driven by chronic metal exposure. It can be concluded that biofilm antioxidant enzyme activities may provide evidence of early stress caused by metal exposure and also provide information about the mechanism of community adaptation. This information can be of great interest to improve current tools used for risk assessment.

1. INTRODUCTION

Biofilms, also known as phytobenthos or periphyton, are consortia of microorganisms (algae, bacteria, cyanobacteria, fungi and protozoa) attached to a surface and embedded in an extracellular matrix of polymeric substances (Romaní 2010). It is the first compartment of the trophic chain in most fluvial ecosystems (Sabater et al. 2007). The capacity to accumulate metals present in the water column is one of their singular properties (Ivorra et al. 2000). Depending on their oxidation states, metals can be highly reactive, and therefore toxic to biofilm organisms (Pinto et al., 2003; Töpperwien et al., 2007; Guasch et al. 2010a). Time exposure is also important when wishing to explore metal effects, since metal bioaccumulation in biofilms increases over time and therefore increases metal toxicity. After a short period of time (from hours to days), metals can produce functional effects such as a decrease in photosynthetic activity and after a long period of time (from weeks to months), structural effects (e.g. a shift in algal groups) are expected (Corcoll et al. 2011; Tlili et al. 2011).

Nowadays, there are metrics such as biotic indices, which have been shown to be of great value for assessing pollution effects in freshwater systems (Platts et al. 1983; Tison et al. 2008). On the other hand, there are few indicators that may be considered specific to metal pollution. Several studies have indicated a change in the structure of biofilms when exposed to metal pollution (Peres et al. 1997; Dickman 1998; Ivorra 2000; Torres et al. 2000; Gold 2002; Gomez and Licursi 2003; Gold et al. 2003). For instance, diatom deformities (Dickman 1998, Falasco et al. 2009), size reduction of diatoms as well as the global community appeared to be excellent specific indicators of metal contamination (Morin et al. 2011). However, this approach involves an additional counting effort which is hardly compatible with routine biomonitoring (Morin el al. 2007). Moreover, biotic indices are mostly based on structural changes occurring at community level which are expected to integrate biotic responses over relatively long periods of time (from weeks to months depending on the life time of the organisms

investigated). Furthermore, some organisms have the ability to repair damage induced by initial toxic insults and make adjustments to their biological responses, thus increasing the chance of false negatives (Lam 2009). Hence, it is of great interest to develop functional metal toxicity bioindicators focusing on both, early responses and chronic effects, in order to complement the information provided by classical biotic indices such as biomass or photosynthetic parameters and try to understand the relation between them.

Metals are present in many aquatic systems of the world due to both natural and anthropogenic sources. Zn, one of the most toxic metals, presents a wide range of concentration. This metal can be found at high concentrations dissolved in water, accumulated in the biota and also in sediments (Behra et al. 2002; Blanck et al 2003; Holding et al. 2003; Besser et al. 2007; Farag et al. 2007; Morin et al. 2007, Guasch et al. 2010b; Tlili et al 2011). It is a known fact that Zn is both a micronutrient for life and a toxicant at higher concentrations. Acute effects of Zn on biofilms have been reported at concentrations ranging from 7 mg Zn L^{-1} to 195 mg Zn L^{-1} (Admiraal et al. 1999; Blanck et al 2003). Chronic effects on biofilms have been reported at lower concentrations ranging from 50 µg Zn L^{-1} to 2.5 mg Zn L^{-1} (Genter et al. 1987). However, indirect effects, such as the reduction of nutrient uptake in biofilms, have also been found at much lower concentrations (6-25 µg Zn L^{-1}) under oligotrophic conditions (Paulsson et al. 2002), similar to toxicity values (15 µg L^{-1}) reported in free-living algae (*Selenastrum capricornutum*), (Posthuma et al. 2001). A higher uptake of Zn was measured in algal biomass compared to other tested metals (Shehata et al. 1999).

Metals and other pollutants (e.g. organic ones) can produce several toxic effects on algae and bacteria, the two main compartments of biofilm. One of them is the enhancement of reactive oxygen species (ROS) (Weckx and Clijsters, 1997; Collén et al. 2003; Pinto et al., 2003; Zbigniew and Wojciech, 2006; Bonnineau et al. 2010),

which are commonly produced during metabolic processes, such as photosynthesis and respiration (Asada 2006). It has been reported that Zn may cause an increase in ROS in autotrophic organisms (Cuypers et al. 2001; Tripathi and Gaur, 2004), mainly superoxide anion radical ($O_2^{\cdot -}$), hydroxyl radical (·OH) and hydrogen peroxidase (H_2O_2). To prevent ROS effects, cells have antioxidative systems composed of different enzymatic and non-enzymatic mechanisms. The induction of these systems is considered to play an important role in the cellular defence strategy against oxidative stress caused by toxic metal concentrations (Collén et al. 2003; Geoffroy et al. 2003; Tripathi et al 2006; Valavanidis et al. 2006). Among these enzymatic systems, antioxidant enzyme activities (AEA), such as catalase (CAT) and ascorbate peroxidase (APX), can transform peroxides into non-reactive species. In the case of Zn, the inhibition of some AEA (Van Assche and Clijster, 1990), the induction of other AEA (Vangronsveld and Clijsters, 1994) and also other responses such as the production of phytochelatins (Nagalakshmi and Prasad, 1998; Le Faucheur et al. 2005) have been reported.

Until now many studies related to AEA have been performed in mono-specific cultures (Nagalakshmi and Prasad, 1998; Collén et al. 2003; Geoffroy et al. 2004; Tripathi et al. 2006). It is well known that culture studies have relatively low ecological relevance in contrast to studies performed with communities (Depledge et al. 1995; Lam 2009). Hence, a more realistic approximation is required. Biofilm ecotoxicological studies, in spite of their complexity, provide a community ecotoxicology approach and allow acute effects on entire communities, and also response after chronic exposure including the evaluation of direct and indirect toxic effects. To our knowledge, few studies have been focused on AEA in biofilms (Bonnineau et al. 2010; Guasch et al. 2010a; Bonnineau et al. 2011) and these studies highlighted the potential of AEA as biomarkers of toxicity.

Within this scenario, this study sets out to explore the use of antioxidant enzyme activities as “early warning biomarkers” as well as chronic functional biomarkers of Zn exposure on biofilm communities. To reach this goal, a microcosm study was carried out under controlled experimental conditions. Different time periods were investigated: 5 and 3 days before exposure, just on exposure (0 hours) and after 6 hours, 1, 3, 7, 21 and 35 days of exposure. Catalase (CAT) and ascorbate peroxidase (APX) were analysed. Other classical endpoints such as biofilm biomass, photosynthetic parameters, main algal groups of biofilm and the bioaccumulation of Zn were also analysed for comparison.

Functional changes were expected after acute exposure (e.g. inhibition of photosynthetic parameters) while structural ones (e.g. shift in algal composition) and higher Zn bioaccumulation were expected after chronic exposure. Moreover, AEA were expected to respond in both exposure periods, revealing whether the response was transitory or persistent.

2. MATERIAL AND METHODS

2.1. The microcosm setup and experimental design

The experiments were carried out in the indoor microcosm river system described in Serra et al. (2009). In this experiment, six Perspex channels were used and the bottom of each channel was covered with sandblasted glass substrata. The light intensity was between 100 and 120 µmol photons m^{-2} s^{-1} and the flow was around 1 L min^{-1}. The microcosm system was used for biofilm colonization during 5 weeks and afterwards biofilms were exposed to Zn for 5 weeks more. Sandblasted glass substrata of two different sizes (8.5 cm x 2 cm and 8.5 cm x12 cm) were used to facilitate biofilm colonization and obtain sampling units with different amounts of biomass depending on the requirements of the different measurements performed. Sampling was established 5 and 3 days before exposure, just before metal addition, and after 6 hours, 1, 3, 7, 21

and 35 days of exposure. Each channel represented an experimental unit so all samples were taken per triplicate.

2.2. Colonization and Zn exposure of biofilm communities

Biofilm colonization was achieved by introducing aliquots of fluvial biofilms obtained from the Llémena River, a small calcareous tributary of the Ter River (NE Spain; Serra et al. 2009). This river is relatively unpolluted by metals and nutrients (Catalan Water Agency, ACA) making it possible to avoid communities already pre-selected for Zn tolerance. A new aliquot was provided weekly to each channel during the first four weeks of colonization. Water from the channels was completely replaced three times a week, and 30 μg L^{-1} of phosphate (nominal concentration) was added as KH_2PO_4 (Merck, Darmstadt, Germany) at each water renewal to avoid nutrient depletion.

After five weeks of colonization, Zn addition was started. Three channels were used as controls (referred to as controls in the text), and the other three used for Zn additions (400 μg Zn L^{-1} nominal concentration (referred to as Zn treatment in the text). This concentration was selected because it is environmentally realistic and we have found it in two field studies carried out in a mining area: Riou Mort (SW France) (Bonnineau et al. 2011) and the other in the Osor stream (NE Catalonia, Spain) (Tlili et al. 2011; Morin et al. 2011) with the aim of comparing and contrasting biological results (AEA) later on. Zn solution was prepared from the metal stock standard from 1000 mg Zn titrisol ($ZnCl_2$ in HCl 0.06%) of Merck (Darmstadt, Germany). This solution was prepared with deionised water (18MΩ Q-H_2O grade Barnstead Nanopure). In order to obtain metal equilibrium, Zn was added to the carboys and kept at room temperature for 24 hours before water renewal.

2.3. Physical and chemical analyses

Temperature, pH, dissolved oxygen and conductivity were measured at all sampling times in each channel before and after each water renewal using a multi-parametric probe (WTW METERS, Weilheim, Germany). Water samples were taken to measure phosphate concentration throughout the experiment and were analyzed by the Murphy and Riley method (1962). DOC and alkalinity were analyzed following APHA (1989). Anions (Cl-, NO^{2-}, NO_3^- and SO_4^{2-}) and cations (K^+, Na^+, NH_4^+, Ca^{2+} and Mg^{2+}) were analyzed using ion-chromatography (761 Compact IC, Metrohm, Herisau, Switzerland).

Total dissolved Zn concentrations were measured throughout the experiment at all sampling times defined above (see section 2.1). Measurements were carried out in triplicate. Samples were immediately filtered (Whatman nylon filters 0.2 µm) and acidified with 1% of HNO_3 (65% suprapure, Merck). Analyses were done by inductively coupled plasma mass spectroscopy ICP-MS (7500c Agilent Technologies, Inc. Wilmington, DE). The detection limit was 2.61 µg Zn L^{-1}. When the value was below the detection limit, half of the detection limit was used for data treatment (Helsel, 1990). The accuracy of the analytical methods was checked periodically using a certified water reference (Reference material for measurement of elements in surface waters: SPS-SW2 Batch 113), Oslo, Norway).

2.4. Biofilm biomarkers

Algal biomass and pigment degradation. Chlorophyll-a (Chl-a) concentration, used as a measure of algal biomass, was analysed using one glass substrata (17cm^2) following the Jeffrey and Humphrey (1975) method. Chl-a concentration was expressed as µg cm^{-2}. Pigment degradation was calculated as the quotient between optical densities at 430 and 665 nm (referred in the text as OD 430/665 ratio) (Margalef, 1983). This ratio is a broad indicator of the proportion of protection pigments (e.g. carotenoids) or degradation products per unit of active Chl-a.

Chlorophyll-a fluorescence parameters. The chlorophyll-a in vivo fluorescence parameters were measured with the PhytoPAM (Pulse Amplitud Modulated) fluorometer (Heinz Walz GmbH), which uses a set of light-emitting diodes (LED) that excite chlorophyll fluorescence using four different wavelengths (470, 520, 645, and 665 nm). Three glass substrata (17cm^2) from each channel were analyzed using this technique and all the measurements were based on the procedure described by Corcoll et al. (2011). The effective quantum yield of photosystem II (PSII) (referred to as Φ'_M) and the maximal quantum yield of PSII (referred to as Φ_M) were calculated from the fluorescence signal recorded at 665 nm and given as relative units of fluorescence (Genty et al. 1989). The deconvolution of the overall fluorescence signal (Fo) into the contributions of three main algal groups composing the biofilm, was based on the internal reference excitation spectra of a pure culture which had previously been validated for biofilm communities (Schmitt-Jansen and Altenburger, 2008). The fluorescence attributed to cyanobacteria, referred 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), were used for evaluating the relative contribution of each group to the whole community and for following their temporal changes .

Zn bioaccumulation. Zn content in biofilms was analyzed using a glass substrata surface of 51cm^2. Biofilm samples were stored at -20ºC before analysis. Samples were lyophilized, weighed and digested with 4mL of HNO_3 (65% suprapure, Merck) and 1mL of H_2O_2 (30% suprapure. Merck) in a high performance microwave digestion unit (Milestone, Ethos Sel) and were thereafter diluted to 15 mL with milli-Q water. After digestion, liquid samples were treated as water samples following the procedure described before (section 2.3). The detection limit was 2.55 µg Zn g DW^{-1}. When the value was below the detection limit, half of the detection limit was also used for data treatment (Helsel, 1990). The accuracy of the analytical methods was checked

periodically using certified reference materials (Trace Elements in Plankton (CRM 414), Community Bureau of Reference (BCR), Brussels, Belgium).

Antioxidant enzyme activities. Sampling, protein extraction and AEA measurements were performed as described in Bonnineau et al. (2011).

Two glass substrata (17cm^2 each) of biofilm were sampled from each channel (considered as pseudo-replicates). Biofilm was removed from glass substrata with a cell scraper (Nunc, Wiesbaden, Germany), put into an eppendorf tube and centrifugated (2300 g, 10ºC, 5 min.) to remove the excess water. The samples were weighted (wet weight) and frozen immediately in liquid nitrogen. Samples were stored at -80ºC until protein extraction and enzymatic assays had been carried out.

For protein extraction, 200 µL of extraction buffer (100 mM Na_2HPO_4/KH_2PO_4, pH 7.4, 100 mM KCl, 1 mM EDTA) were added for each 100 mg of wet weight of sample. Samples were first homogenized (2 pulses of 30s of the homogenizer DIAX900, Heidolph) and then disrupted by adding 100 mg of glass beads (≈ 500 µm of diameter) for each 100 mg of wet weight of sample and performing 3 pulses of 30s of beadbeater (MP FastPrep-24, v = 4 m s^{-1}) with 2 min intervals on ice. After cell disruption, homogenates were centrifuged at 10.000 g and 4ºC for 30 min. Supernatant was used as the enzyme source (Bonnineau et al. 2011).

The protein concentration of supernatant was measured and used in triplicates for each sample following the Bradford method (1976) using dye reagent concentrate from Bio-Rad (Laboratories GmbH, Munich, Germany) and bovine serum albumin as a standard. For each essay, the volume of supernatant used was based on the previously estimated total protein content in order to obtain 10 µg of protein.

CAT activity ($2H_2O_2 \rightarrow 2H_2O + O_2$) was measured spectrophotometrically at 240 nm according to Aebi (1984). 800 µL of reaction mixture was obtained adding potassium phosphate buffer (pH 7.0) (80 mM final concentration); H_2O_2 (20 mM final concentration) and the enzyme extract (10 µg protein). The H_2O_2 consumption was determined by measuring the decrease in absorbance at 25°C for 4 min. CAT activity was calculated as µmol H_2O_2 µg protein^{-1} min^{-1} (extinction coefficient, ε: 0.039 cm² µmol^{-1}).

APX activity ($C_6H_8O_6 + H_2O_2 \rightarrow C_6H_6O_6 + 2\ H_2O$) was assessed by monitoring the decrease in absorbance at 290 nm, at 25°C and for 2.5 min, due to ascorbate oxidation, according to Nakano and Asada (1981). 1000 µL reaction mixture were obtained adding potassium phosphate buffer (pH 7.0) (80 mM final concentration); H_2O_2 (5 mM final concentration); Na-Ascorbate (30 mM final concentration) and the enzyme extract (10µg protein). APX activity was calculated as µmol Ascorbate µg protein^{-1} min^{-1} (ε: 2.8 cm² µmol^{-1}).

2.5. Statistical analysis

At each sampling time, the one way ANOVA test was used to detect significant differences ($p<0.05$) between control and Zn treatment. The set of biofilm endpoints included were: Chl-a, pigment degradation (OD 340/665 ratio), maximal quantum yield (Φ_M) and effective quantum yield (Φ'_M), fluorescence of cyanobacteria (Fo(Bl), green algae (Fo(Gr)) and diatoms (Fo(Br)), Zn bioaccumulated, catalase (CAT) and ascorbate peroxidase (APX) activities. Pearson correlations between biological endpoints were used to highlight their similarities and differences and also to explore Zn exposure effects between all sets of biological. These analyses were performed using the SPSS software. Regression analysis ($y = a + bx$) was used to study the relationship between Zn bioaccumulation on biofilm and time exposure. This analysis was performed using Sigma Plot 11.0 software.

3. RESULTS

Physical and chemical water parameters

Physical and chemical conditions remained stable during the whole experiment. During colonization, dissolved oxygen was 8.89 ± 0.03 mg L^{-1}, water temperature 19.44 ± 0.43ºC, pH 8.35 ± 0.03 and conductivity 327.24 ± 19.60 µS cm^{-1} (Avg ± SD, n=78). Nutrient concentrations were 30.26 ± 1.99 µg L^{-1} phosphate; 4.35 ± 0.61 mg L^{-1} nitrate; 0.057 ± 0.094 mg L^{-1} ammonia (Avg ± SD, n = 15) and DOC was 4.92 ± 1.65 mg L^{-1} (Avg ± SD, n = 3). Alkalinity 1.47 ± 0.12 meq L^{-1} (Avg ± SD, n = 14). The corresponding major cations and anions were 3.35 ± 0.24 mg K^+ L^{-1}, 25.02 ± 3.18 mg Na^+ L^{-1}, 41.06 ± 7.67 mg Ca^{2+} L^{-1}, 8.11 ± 1,04 mg Mg^{2+} L^{-1} (n = 38), 89.24 ± 6.45 mg Cl^- L^{-1} and 117.6 ± 7.1 mg SO_4^{2-} L^{-1} (Avg ± SD, n = 26).

During Zn exposure, physical and chemical conditions were similar to those during colonization and did not differ between treatments (Table 1). Zn concentrations after water renewal were 9-15% above expected values (400 µg Zn L^{-1}, nominal concentration). Metal concentration decreased to average values of 133.5 µg Zn L^{-1} after three days (before water renewal) giving an average exposure value of 320 µg Zn L^{-1} (Table 1).

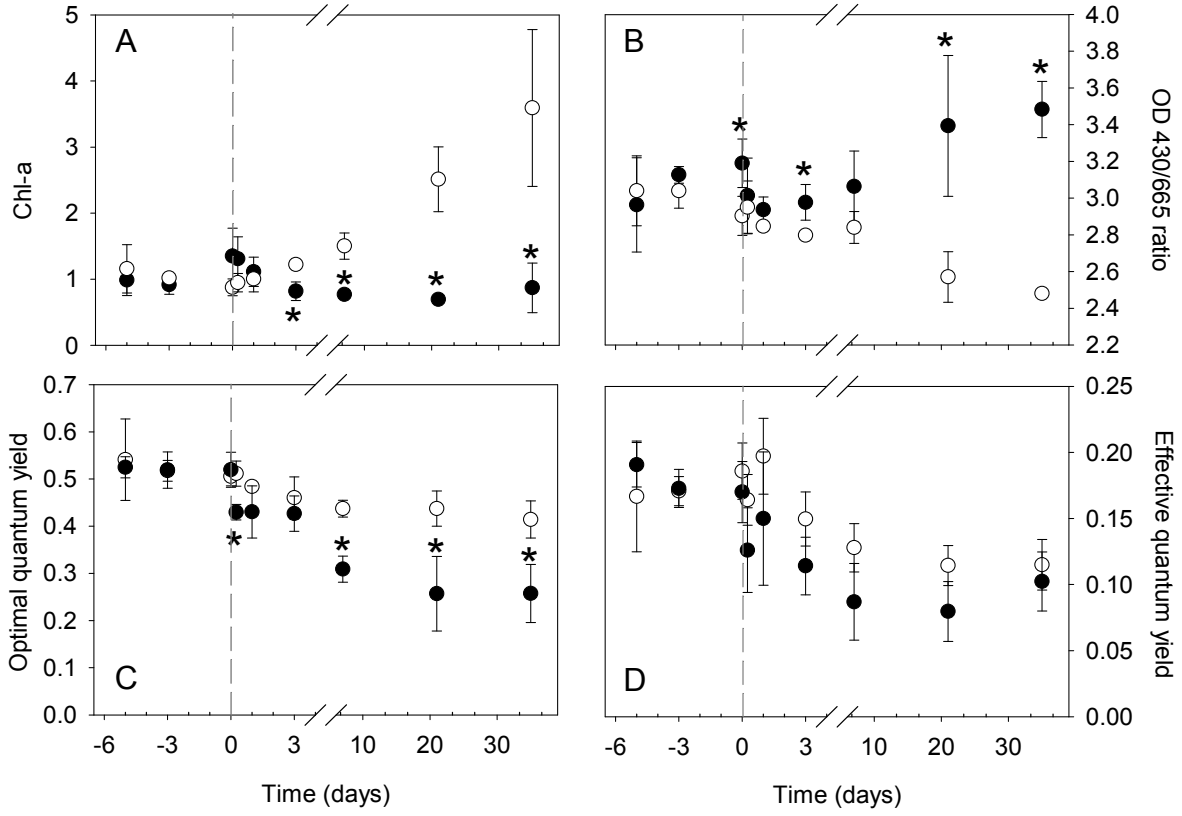
Table 1. Average (Avg) and standard deviation (±SD) of physical and chemical conditions during Zn exposure (from time 0 until week 5). Dissolved oxygen, temperature (TºC), pH, conductivity (n=60), phosphate (n=15) and dissolved metal concentration (n=27).

Parameter	Control	Zn
Oxygen (mg L^{-1})	9.46 ± 0.29	9.07 ± 0.17
T (ºC)	18.50 ± 0.99	18.33 ± 1.22
pH (u.pH)	8.64 ± 0.19	8.54 ± 0.13
Conductivity (µS cm^{-1})	307.00 ± 22.40	342.50 ± 19.60
PO_4^{3-} (µg L^{-1})	23.94 ± 3.79	23.32 ± 4.04
Zn in water (µg L^{-1})	4.65 ± 4.17	317.50 ± 129.60

Non-exposed biofilms

Algal biomass (chl-a) was around 1 µg cm^{-2} before exposure and the first days of the experiment and increased from week 1 onwards reaching 3.6 µg cm^{-2} at the end of the experiment (Fig. 1). Pigment degradation as well as $\Phi_{M,}$ and Φ'_M followed the opposite pattern to Chl-a ($r = -0.84$, $p < 0.001$, $r = -0.63$, $p < 0.01$, $r = -0.52$, $p < 0.05$, $n = 18$ respectively) (Fig. 2B, 2C and 2D), while Fo(Bl), Fo(Gr) and Fo(Br) were well correlated with Chl-a ($r = 0.90$, $r = 0.84$, and $r = 0.811$, $p < 0.01$, $n = 18$ respectively), (Fig. 2). CAT ranged from 0.049 to 0.057 µmol H_2O_2/min/µg prot and APX from 0.31 to 0.62 µmol Na-Asc/min/µg prot during the whole experiment (Fig. 3). APX was negatively correlated with Φ'_M ($r = -0.501$, $p < 0.01$, $n = 27$).

Figure 1. Changes in No Zn (white dots) and Zn (black dots) treatment over time in the following endpoints: A) Chl-a, B) OD 430/665 ratio, C) optimal (Φ_M) and D) effective quantum yield (Φ'_M). Values are average and standard deviation of 5 and 3 days before exposure (-5 and -3d respectively), just before exposure (0d), and after 6 hours, 1, 3, 7, 21 and 35 days of exposure. The scratch line indicates the beginning of Zn exposure. For each endpoint and sampling time, an ANOVA has been performed to detect significant differences between treatments indicated



Accumulation of Zn

Zn bioaccumulation (n = 24) followed a linear regression model, (r^2 = 0.94, $p < 0.0005$) being statistically different from controls from 6h to the end of exposure (ANOVA, F = 10.04, $p < 0.05$) and reaching 1571.24 ± 323.07 µg Zn g^{-1} DW at the end of the experiment (Fig. 4). $\Phi_{M,}$ and Φ'_M followed the same pattern of temporal variation as controls but with lower values, being $\Phi_{M,}$ statistically different from controls and reaching 38% below them (Fig. 1). In contrast, pigment degradation ratio (OD 430/665) showed an opposite pattern to controls, increasing over time and reaching values 40% above controls. Although algal biomass was stable during the whole exposure period, Fo(Bl), Fo(Gr) and Fo(Br) showed different patterns of variation, revealing the effects of Zn exposure on the community composition.

Effects of Zn

The magnitude of Zn effects increased over time, increasing progressively the response of most of the measured endpoints. Zn bioaccumulation reached values 180 times higher than controls (Fig. 4). This increase in bioaccumulation corresponded with an important reduction in algal biomass (33% after 3 days, 49% after 7 days; 72 after 21 days and 76% below controls after 35 days; ANOVA, F = 19.35, $p < 0.05$, F = 36.17, $p < 0.01$, F = 40.92, $p < 0.01$ and F = 14.34, $p < 0.05$ respectively) (Fig. 1A). Similarly, Φ_M was 16, 29, 41 and 38% below controls throughout the experiment (ANOVA, F = 20.34, $p < 0.05$, F = 45.99, $p < 0.005$, F = 12.87, $p < 0.05$ and F = 13.93, $p < 0.05$ respectively over time) (Fig. 1C). On the other hand, OD 430/665 ratio increased over time reaching values 6, 32 and 40% above controls after 6 hours, 21 and 35 days respectively (ANOVA, F = 9.91, $p < 0.05$, F = 12.00, $p < 0.05$ and F = 125.17, $p < 0.05$, respectively) (Fig. 1B).

Treatment effects on the community composition (Fo corresponding to different groups of algae and cyanobacteria) were not observed until day 7. Fo(Br) was 51%, 89% and

88% below controls (ANOVA, F = 11.29, p < 0.05, F = 7.67, p < 0.05 and F = 15.85, p < 0.05 respectively) (Fig. 2C) and Fo(Gr) was 47 and 42% below controls at the end of exposure (ANOVA, F = 8.50, p < 0.05 and F = 14.00, p < 0.05 for days 21 and 35, respectively). APX activity had a bi-phasic response being higher than controls just at day 1 (ANOVA, F = 18.47, p < 0.05), similar to them after 3 and 7 days and lower at longer exposures reaching values of 32 and 39% below controls after 21 and 35 days (ANOVA, F = 13.14, p < 0.05, F = 7.79, p < 0.05 respectively) (Fig. 3B). CAT was unaffected until day 21 and increased afterwards (ANOVA, F = 16.20, p < 0.05) (Fig.3A). Furthermore CAT was correlated with Zn bioaccumulation over the experiment (r = 0.60, p < 0.01, n = 24), whereas APX was not.

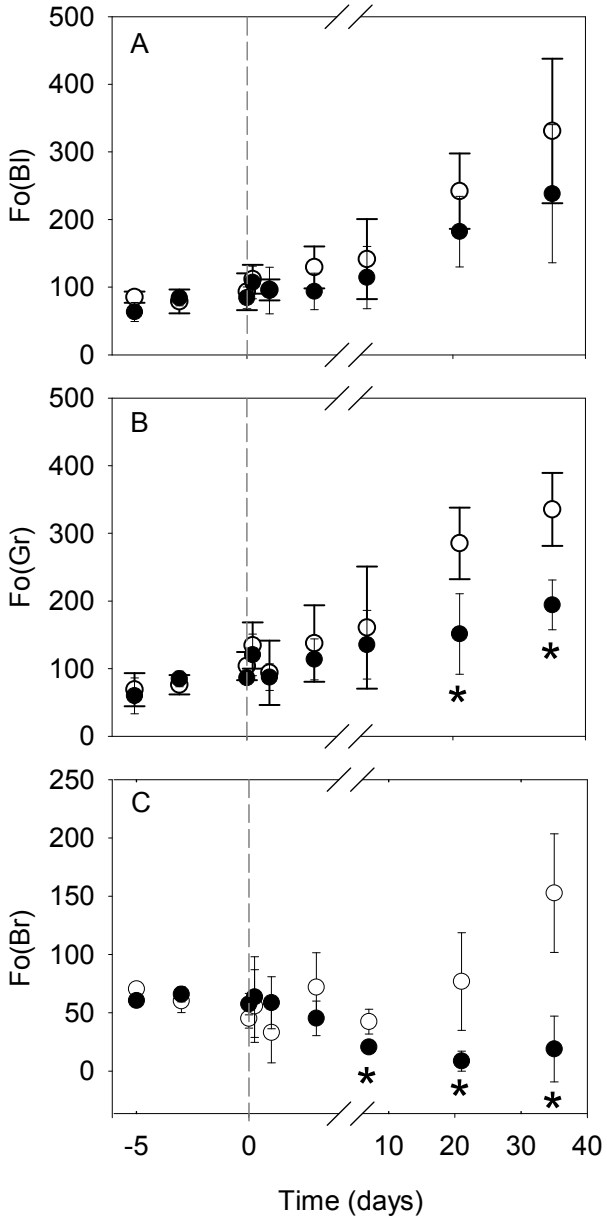


Figure 2. Changes in No Zn (white dots) and Zn (black dots) treatment over time in relative abundance of biofilm algal community: A) cyanobacteria (Fo(Bl)), B) green algae (Fo(Gr)) and C) diatoms (Fo(Br)). Values are average and standard deviation of 5 and 3 days before exposure (-5 and -3d respectively), just before exposure (0d), and after 6 hours, 1, 3, 7, 21 and 35 days of exposure. The scratch line indicates the beginning of Zn exposure. For each endpoint and sampling time, an ANOVA has been performed to detect significant differences between treatments indicated by * (p<0.05; n=3).

Figure 3. Changes in No Zn (white dots) and Zn (black dots) treatment over time in antioxidant enzyme activities: A) catalase (CAT) and B) ascorbate peroxidase (APX). Values are average and standard deviation of 5 and 3 days before exposure (-5 and -3d respectively), just before exposure (0d), and after 6 hours, 1, 3, 7, 21 and 35 days of exposure. The scratch line indicates the beginning of Zn exposure. For each endpoint and sampling time, an ANOVA has been performed to detect significant differences between treatments indicated by * ($p < 0.05$; $n = 3$).

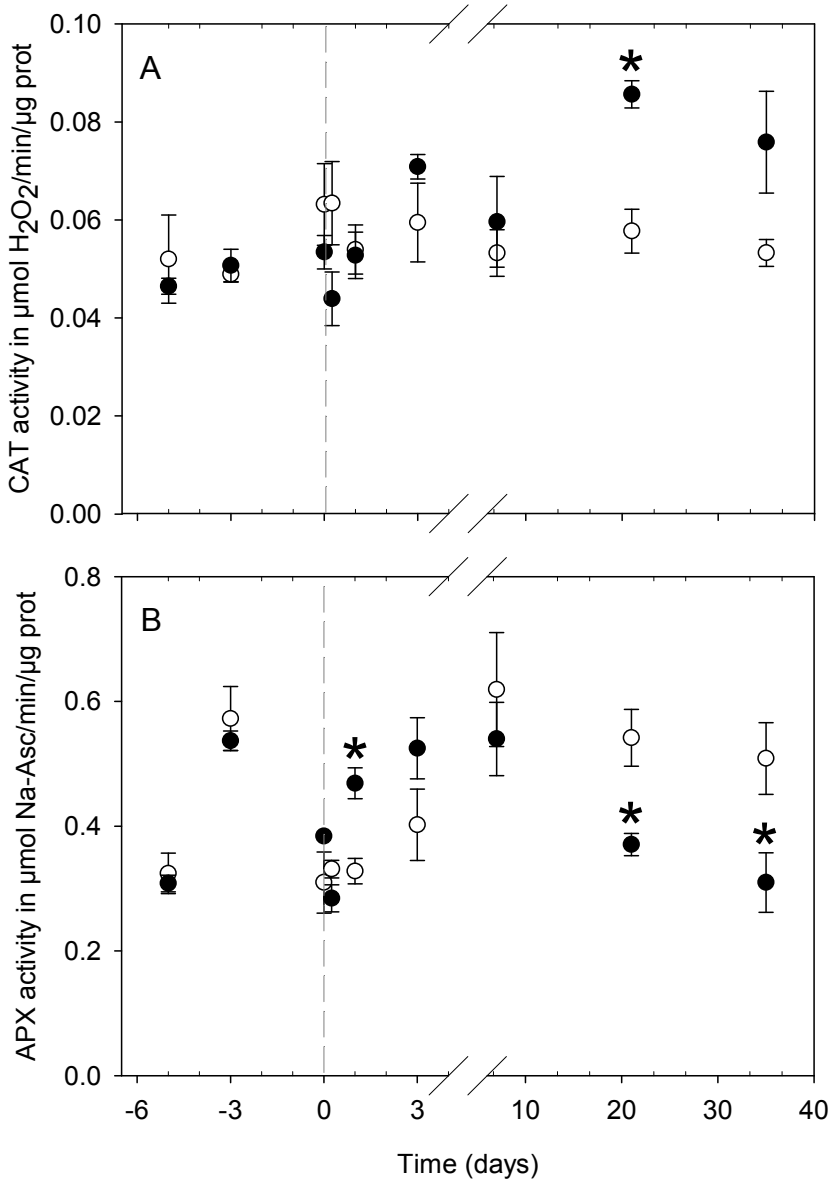
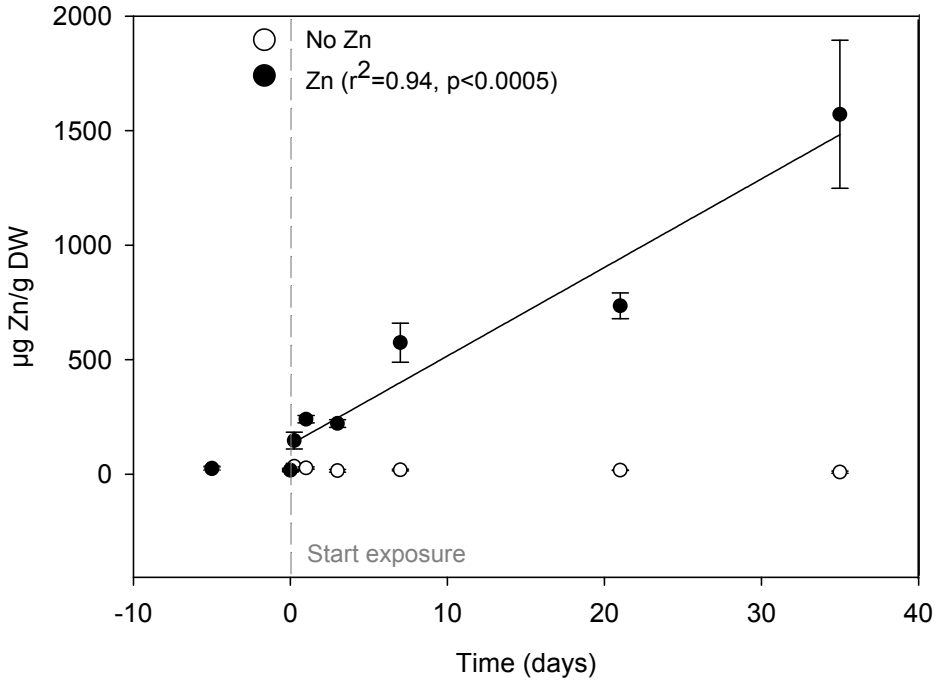


Figure 4. Accumulation of Zn per dry weight over time in No Zn (white dots) and Zn (black dots) treatment values are in average and standard deviation (n = 3) of 5 and 3 days before exposure (-5 and -3d respectively), just before exposure (0d), and after 6 hours, 1, 3, 7, 21 and 35 days of exposure. The scratch line indicates the beginning of Zn exposure. For each treatment a regression analysis has been performed but was only significant in Zn treatment.



4. DISCUSSION

Zn exposure (320 µg Zn L^{-1}) caused many alterations to the biofilm community, observed in most of the measured endpoints. However, the type and magnitude of response depended on the time of exposure.

In contrast with our results, short-term effects of Zn on biofilms have been observed at higher concentrations, ranging between 450 µg Zn L^{-1} and 40 mg Zn $^{-1}$ (Admiraal et al. 1999; Blank et al. 2003; Guasch et al. 2003). These values, much higher than those described in free-living organisms, are explained by the structure of biofilms reducing Zn penetration into the cells by the presence of polysaccharide matrix (EPS) (Sabater et al. 2007; Romaní 2010).

As we expected, functional responses were first observed and followed thereafter by structural changes. It is important to point out that the APX activity was the first functional endpoint showing a clear response, reaching values 43% above controls after 24h of exposure. It was more sensitive than Φ'_M, a functional endpoint commonly used to assess effects of chemicals on the photosynthetic activity of biofilms (Corcoll et al. 2011). In agreement with our results, Geoffroy et al. (2004) found AEA to be more sensitive than photosynthesis in algal cultures (*Scenedesmus obliquus)* exposed to the herbicide flumioxazin, and Guasch et al. (2010a) with biofilms exposed to Cu. The fact that no photosynthetic parameters were affected during the first hours of Zn exposure period could be related to the capacity of biofilm to enhance metal detoxifying mechanisms such as AEA to deal with Zn toxicity. According to our results, it has been suggested that APX is the main enzyme for H_2O_2 removal and its increase indicates increased availability of ROS (Nagalaskmi et al. 1998). In fact, APX is present in almost all cellular compartments (chloroplast, cytosol, mitochondria, perosixome and apoplast) (Mittler 2002; Barros et al. 2003).

By increasing the time of exposure, and therefore Zn bioaccumulation, and following physiological alteration, structural damage was detected (after three days) as a reduction in algal biomass and an increase in accessory pigments (e.g. carotenoids). These changes could explain the decrease in Φ_M observed slightly later (on day 7). It has been extensively reported that oxidative stress due to chemical stress can induce an increase in carotenoids in a large number of plants and algae (Ledford and Niyogi, 2005). In this study, an increase in OD 430/665 ratio, indicative of pigment changes, could be related to a failure of the photosynthetic apparatus of algae and at the same time could explain a decrease in the maximal quantum yield (Φ_M) of the communities growing under Zn exposure (Corcoll et al. 2011; Tlili et al. 2011). However, a detailed pigment analysis should be performed to support this hypothesis.

Community composition changes were observed after one week of exposure leading to a dominance of cyanobacteria and green algae over diatoms. This secondary succession with these marked community composition changes is an indicator of persistent and damaging effects of the toxicant on the community structure with important implications for many ecosystem functions (Romaní 2010). Among them, this community had a higher CAT activity. Li et al. (2006) reported that a progressive Zn bioaccumulation may explain the increase of CAT in cultures of *Pavlova viridis*. Also in natural ecosystems, seasons with high metal concentration in water and bioaccumulation in algae (*Ulva* sp.) had a positive correlation between metals and CAT activity (Pereira et al. 2009) as found in this study.

Despite that CAT and APX have a high affinity to remove H_2O_2, they are localized in different cell compartments and their activities are also regulated differently. On one hand, CAT is mainly localized in peroxisomes which can proliferate due to oxidative stress and its activity is not dependant of any co-factor. On the other hand, APX has a broad distribution within cells and its activity depends on the availability of ascorbic acid (Mittler 2002). Moreover, what may also occur is that other mechanisms, such as the production of phytochelatins (Gaur and Rai 2001; Pawlik-Skowronska 2003; Le Faucheur et al. 2005; Perales-Vela et al. 2006) or carotenoids (Ledford and Niyogi, 2005), might help to cope with metal toxicity. These other mechanisms might aid antioxidant enzymes (CAT in this study) to reduce the ROS produced by Zn.

5. CONCLUSIONS

The AEA of biofilms showed a fast response (after 24h of exposure) previous to any community composition change, supporting its use as an early warner of toxic exposure. However, this response was within the range of variability of controls. Recent studies (Bonnineau PhD. Thesis) revealed that biofilm AEA are not only affected by toxic exposure, presenting temporal changes associated with community

succession processes (Bonnineau PhD. Thesis). This aspect highlights the necessity for analysing AEA together with other indicators of exposure, such as metal bioaccumulation.

In contrast with early AEA responses driven by the APX activity, CAT showed a clear increase, above the range of variability of controls, after several weeks of exposure. This increase was associated with a secondary succession characterized by a strong inhibition of growth and the replacement of diatoms by greens and cyanobacteria. Whether CAT increase was a direct effect of exposure or was driven by the secondary succession process caused by Zn and the different algal capacities to activate one or other AEA to reduce ROS is difficult to untangle and requires further investigation. On the other hand, CAT increase was linked with many deleterious effects of toxic exposure, also including the progressive accumulation of Zn, indicating that CAT activity at community level was contributing to cope with the excess of ROS generated by toxic exposure. In this case, AEA provide information about the mechanisms of community adaptation rather than providing direct evidence of chemical exposure. This information can be of great interest for improving current tools used for risk assessment, providing the basis to assess functional effects of metal toxicity in real exposure scenarios.

ACKNOWLEDGEMENT

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GENERAL DISCUSSION

In this Thesis a set of AEA was investigated. While some AEA showed a clear relationship to metal exposure, this relationship was less clear with others. On the other hand, some AEA were mainly related to environmental and biological seasonality.

Based on the annual cycle (Chapter 1), and focusing on the temporal patterns observed in the non-metal polluted site, CAT, APX and GR were enhanced under warm and high-light conditions when green algae were abundant in the biofilm community (Table 1). Similarly, results obtained from the field experiment (Chapter 2), despite being of a shorter duration, related the CAT and the APX variability to environmental changes (Table 1). It is interesting to point out that CAT and APX remained almost constant in the experiment performed on microcosms, supporting our field observation, since light and temperature conditions were constant and not expected to cause stress under the experimental conditions established.

It is well known that natural communities respond to environmental conditions leading to seasonal succession (Margalef 1963). However, there has been no information until now on the link between seasonal succession and community responses to oxidative stress.

Table 1. Summary of environmental and biological parameters that influenced the natural variability of AEA in non-metal polluted sites. The results were obtained from an annual cycle and a translocation experiment performed in the Riera d'Osor. Fo(Gr) refers to the fluorescence linked to green algae. An ✓ indicates a relationship (either positive or negative) and the intensity (in bold) the magnitude. An Ø indicates lack of relationship.

	Light	Temperature	pH	Fo(Gr)
CAT	**✓**	**✓**	**✓**	✓
APX	**✓**	**✓**	**✓**	✓
GR	**✓**	✓	✓	✓
GST	Ø	Ø	Ø	Ø
SOD	Ø	✓	✓	Ø

The results obtained in this Thesis show that the replacement of species in the community is also linked to changes in AEA responses to environmental stress, indicating that differences in AEA between species may also contribute to their competitiveness within the community.

As expected, AEA responded to metal exposure but this response was not simple, involving different enzymes depending on the magnitude and duration of exposure and, therefore, the amount of stress caused. The experimental studies were performed at different temporal scales, from short- (up to 24h) to long-term exposure (up to 35 days). While physiological responses were expected in the first case, changes in community composition due to adaptation were expected after several weeks, and these changes might complicate the interpretation of the physiological parameters investigated.

Following the expected results, AEA responses were clear after short-term metal exposure. Focusing on this temporal scale, it is important to highlight that the transfer of metals from the media to biofilms was very fast. Figure 1 summarizes values of metal contents dissolved and accumulated in biofilms from the different experimental studies performed. Within this short period of time, accumulation of Zn reached 150-225 μg g DW^{-1} in the microcosms, up to 200-500 μg g DW^{-1} in the Riera d'Osor and one order of magnitude higher, around 5000-6000 μg g DW^{-1}, in the Riou Mort. Comparing laboratory and field results, metal accumulation was much higher in the field. This difference indicated that metal accumulation was not only caused by dissolved metal contents in water. Among other possible factors, particle-bound metals may play an important role in the transfer of metals from bulk water to biofilms in natural systems (Arini et al. 2011). This aspect may explain the observed differences in metal accumulation between laboratory investigations and field studies. Whereas metals were artificially added to the solution in the first case, metals could come from the water as wlla s from suspended solids or colloids in the second case. This second

point could also explain why metal accumulation in the Riera d'Osor occurred in sites where metals in the water were below detection limit (Fig. 1).

Given that biofilm metal contents are good indicators of exposure after a short period of time, these values have been used in this section to explore the link between metal exposure and AEA responses (Chapters 2 and 3). This exploration has also been based on the assumption that a general pattern could be established. Thus, metal accumulation data of different biofilm communities from different river sites were plotted against each AEA investigated. Following this procedure, most activities with the exception of CAT followed a predictable pattern of variation (Fig. 2 and 3). It is important to highlight that CAT responded after translocation to metal-polluted sites in the two study cases (the Riera d'Osor and the Riou Mort). However, responses were very different between sites, thus a general trend could not be established (Fig. 2). On the other hand, APX increased linearly reaching the highest values at the maximum Zn accumulation values measured, SOD decreased linearly within the range investigated, GR increased following a sigmoidal model, whereas GST reached the maximum values at intermediate Zn accumulation values (Fig. 2). Overall, this set of AEA, despite having differences in sensitivity depending on the magnitude of exposure, is proposed as an effect-based monitoring tool for the detection of the impairment caused by metal contamination episodes of short duration, such as an accidental metal spill. A linear positive response is expected for APX, GR and GST activities and negative for SOD up to 600 μg Zn g DW^{-1}. On the other hand a GST reduction is expected under high metal conditions. GR will also respond linearly up to 6000 μg Zn g DW^{-1} whereas APX will still increase at a higher concentration (Fig. 3). Caution should be taken, however, with other confounding factors. As mentioned above, environmental factors may cause similar effects on CAT, APX and GR. Thus, environmental parameters, like light intensity and water temperature or community composition, should also be measured

and taken into account for the comparison between reference and impacted biofilm communities.

Figure 1. Summary of average and standard deviations of total metal concentration dissolved in the water (µg L^{-1}) on the left side, and accumulated in biofilm (µg gDW^{-1}) on the right side, after 6 and 24 hours of exposure in the Riera d'Osor, the Riou Mort and the microcosm experiment. *bdl* stands for "below detection limit". The intensity of greyness indicates the increase of Zn pollution at each site: upstream (Up), mining 1 (M1), mining 3 (M3), mining 2 (M2), Decazeville (D), Joanis (J), Le Grange (G), control channels (Crt.) and Zn channels (Zn).

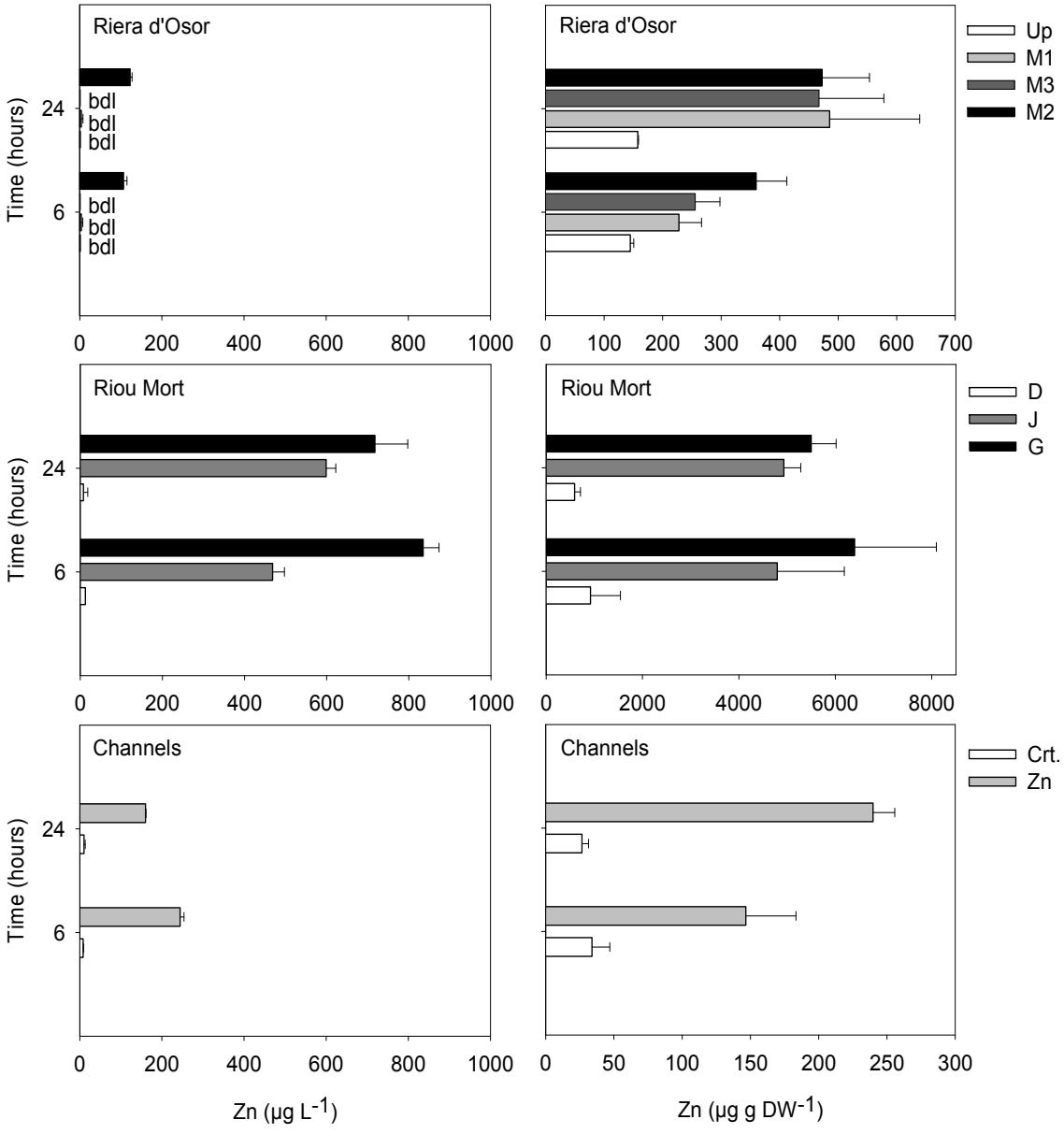


Figure 2. Acute AEA responses in function of Zn accumulation (µg Zn g DW^{-1}) in the Riera d'Osor (in white) and the Riou Mort (in grey). Catalase (CAT) did not fit any model. Ascorbate peroxidase (APX) and superoxide dismutase (SOD) followed a linear model, GR a sigmoidal and GST a quadratic one. CAT is expressed in µmol H_2O_2 µg $protein^{-1}$ min^{-1}, APX in µmol Ascorbate µg $protein^{-1}$ min^{-1}, GR in µmol NADPH μg^{-1} protein min^{-1}, GST µmol CDNB conjugate μg^{-1} protein min^{-1} and SOD in U µg $protein^{-1}$. Medium-dash dark grey lines indicate 95% confidence while short-dash light grey lines indicate the 95% prediction.

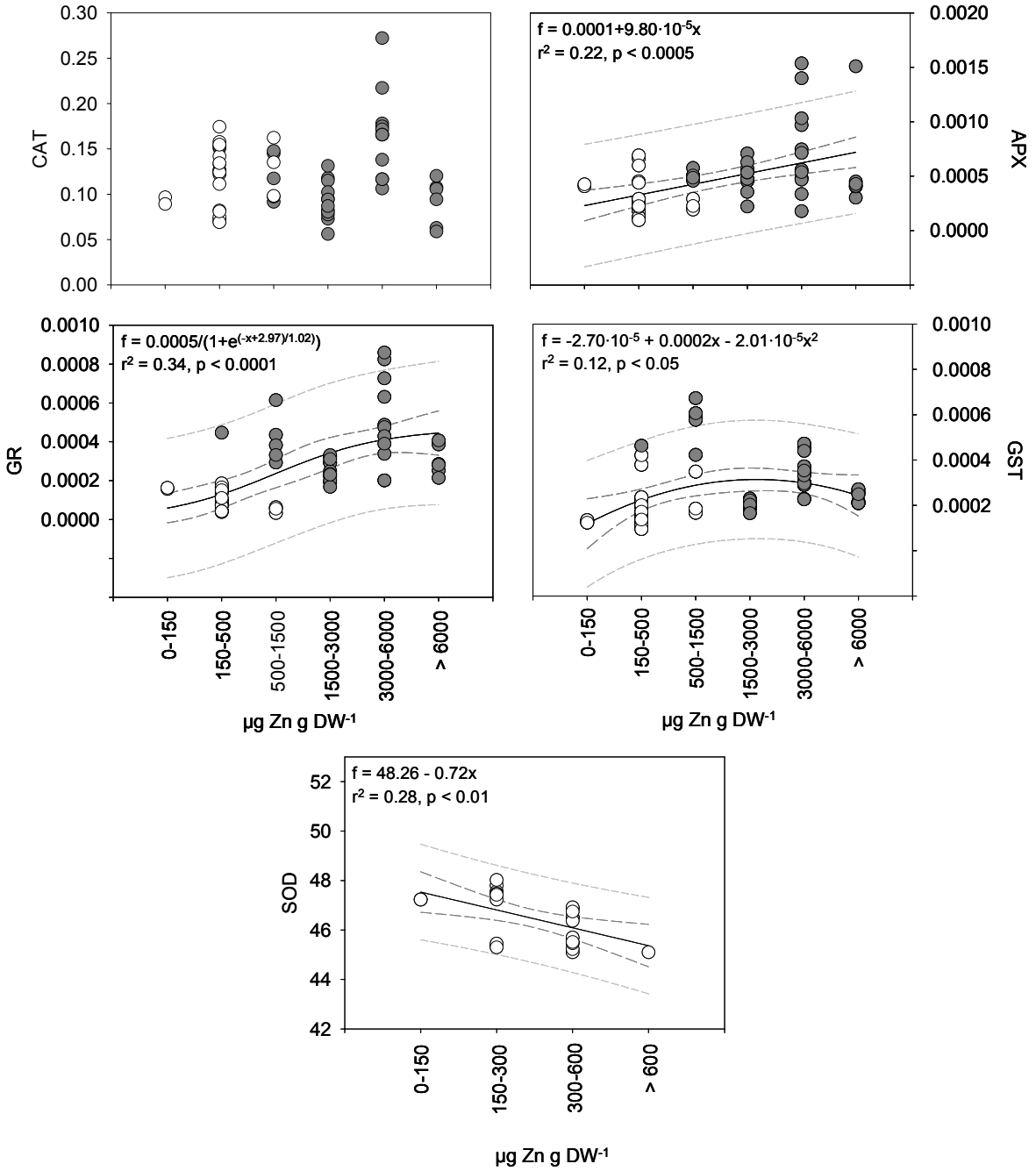
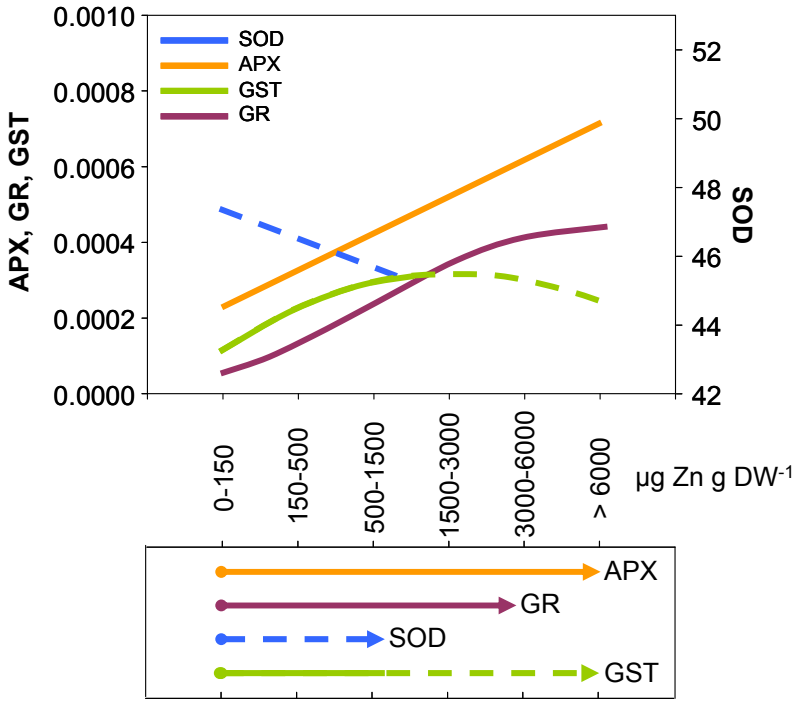


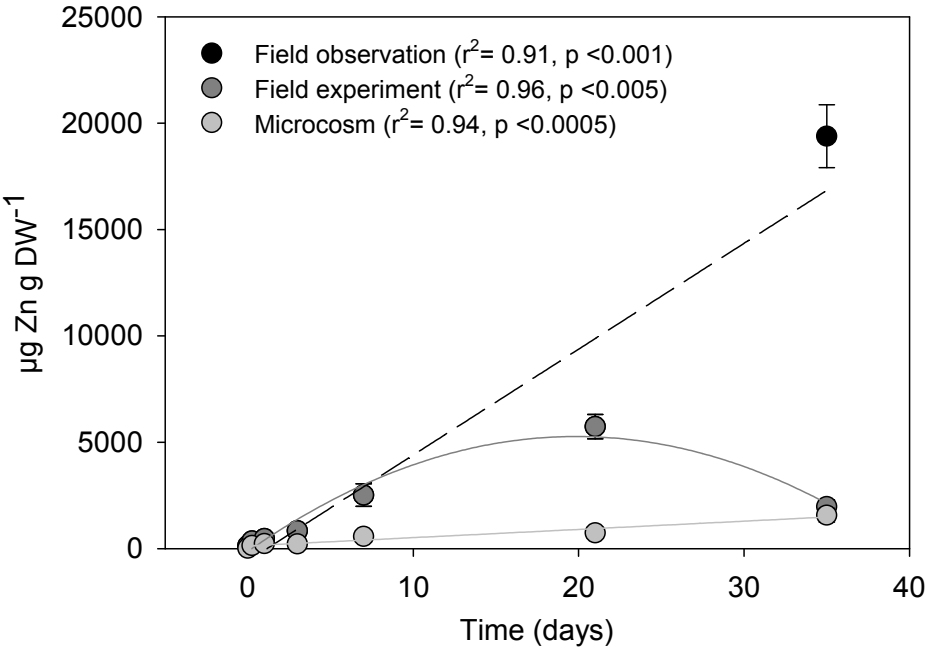
Figure 3. Acute AEA responses in function of Zn accumulation in field biofilm communities (µg Zn g DW^{-1}). A) Ascorbate peroxidase (APX) and superoxide dismutase (SOD) follow a linear model, glutathione reductase (GR) follows a sigmoidal model and glutathione-S-transferase (GST) a quadratic model. B) Approximate range of linear response for each enzyme. APX in µmol Ascorbate µg $protein^{-1}$ min^{-1}, GR in µmol NADPH $µg^{-1}$ protein min^{-1}, GST µmol CDNB conjugate $µg^{-1}$ protein min^{-1} and SOD in U µg $protein^{-1}$. Solid lines indicate positive responses while dash lines indicate a negative one.



It is well known that periphyton communities are able to adapt to environmental changes, including exposure to metals for a long period of time (Guasch et al. 2003; Sabater et al. 2007). Following the responses discussed above, biofilms are expected to accumulate higher amounts of metals if exposure continues. Figure 4 summarizes the patterns of accumulation after several weeks observed in different studies (Chapter 1, 2 and 4). In agreement with the pattern observed within the first hours of exposure, metal accumulation was lower in the microcosms than in the field. On the other hand, metal contents decreased during the last weeks of exposure in the field, and this reduction was attributed to the dilution and cleaning caused by a natural perturbation: a rain event. As discussed in Chapter 1, natural systems are dynamic, thus the pattern

observed is not surprising. However, metal accumulation may reach much higher values. Samples taken during the same period of the year from natural substrata accumulated up to 20000 µg Zn g DW^{-1}, indicating that accumulation may increase linearly under stable exposure conditions.

Figure 4. Zn accumulation patterns in biofilms over time during later spring-early summer period. Microcosm (light grey) and field experiment (dark grey) data are based on real Zn accumulation values while the field observation (black) is used to draw the hypothetical evolution of Zn accumulation under stable conditions.



Focusing on AEA after chronic exposure, the patterns observed were very complex and the values found in the metal-polluted sites were within the range measured in reference sites. Nonetheless, the analysis of data allowed for the discrimination between environmental variability and metal exposure effects, the latter inhibiting GST. However, caution should be taken before using AEA as biomarkers of chronic metal pollution since metal effects can be confused with biofilm responses to environmental stress. On the other hand, the comparison between reference and metal-impacted sites provided interesting and unexpected results. The most consistent result was the lack of

seasonality in metal-polluted sites compared to reference sites observed in the annual monitoring (Chapter 1). AEA contributed to cope with oxidative stress caused by the environment and this important functionality was lost, or at least masked by the effects caused by metal exposure.

The set of studies presented highlights the different responses that AEA may have to metal pollution depending on the magnitude and duration of exposure (Table 2). While most AEA responded to low but acute metal exposure, these responses were less consistent when either the concentration or time increased, highlighting the need to investigate a larger set of parameters.

Table 2. Summary of acute and chronic AEA responses to metal pollution. An ✓ indicates a relationship (either positive or negative) and the magnitude highlighted in bold. An Ø indicates the lack of relationship and a – the lack of data.

	Acute		Chronic
	Low metal pollution (Riera d'Osor and channels)	High metal pollution (Riou Mort)	Low metal pollution (Riera d'Osor)
CAT	**✓**	**✓**	Ø
APX	**✓**	✓	Ø
GR	**✓**	**✓**	✓
GST	**✓**	**✓**	**✓**
SOD	**✓**	-	✓

GENERAL CONCLUSIONS

The usefulness of AEA as biomarkers of metal (Zn) pollution can be discussed due to their variability. AEA showed more consistent results in acute than in chronic studies, responding according to the metal pollution level (low, in the Riera d'Osor and high in the Riou Mort). AEA were more sensitive than other functional parameters (e.g. photosynthetic parameters). Furthermore, metal accumulation was also very sensitive even after a short period of time and revealed that even when metals in water were below detection limits they can quickly be accumulated in biofilms and trigger biological effects at a functional level. AEA and metal accumulation provided information that water chemistry could not. Thus, the "triad approach" adapted to biofilms could be useful for investigative biomonitoring as well as for detecting the biological effects of accidental spills on fluvial biofilms.

In contrast to the AEA responses observed after acute exposure, investigative monitoring performed at long term showed a higher variability of AEA responses, partially attributed to environmental changes (except GST). Due to the high AEA sensitivity to environmental factors, a set of AEA could be also useful to assess other impacts on aquatic systems, such the ones that may be produced by climate change (e.g. biological effects of drought/flood events, increase in UV, and temperature). In fact, annual investigative monitoring allowed it to be shown (i) how AEA followed the seasonality of the system (due to environmental factors as well as biofilm community changes) and (ii) how this functional seasonality was masked or affected by Zn pollution. Under chronic conditions, the best tool used in this Thesis was metal accumulation in biofilm. Metal accumulation in biofilms allowed us to detect pollution effects or situations that happened, which could not be detected only by chemical analyses, but which still caused effects on the biota. Furthermore, physiological and species composition changes confirmed that exposure to low metal concentration has already caused damage on the community, a rather common situation in human-impacted fluvial systems.

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