CONTRIBUTION OF ANTIOXIDANT ENZYMES TO TOXICITY ASSESSMENT IN FLUVIAL BIOFILMS

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PhD thesis

Contribution of antioxidant enzymes to toxicity assessment in fluvial biofilms

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Dirigida per:

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

CERTIFIQUE:

Que aquest treball titulat "**Contribution of antioxidant enzymes to toxicity assessment in fluvial biofilms**", que presenta Chloé Bonnineau, per a l'obtenció del títol de Doctora, ha estat realitzat sota la seva direcció i que compleix els requeriments per poder optar a Menció Europea.

Dra. Helena Guasch
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A Mathieu,

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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 16 de desembre de 2009. En base a les condicions establertes en l'esmentada sessió, tot seguit es mostra que es compleixen els mínims de qualitat i quantitat d'articles sol·licitats per presentar la tesi com a compendi d'articles. En el meu projecte de tesi presento cinc articles dels quals en sóc la primera autora: dos estan publicats, un es troba en revisió i dos estan en preparació. Les referències dels articles, i en cas que ja hagin estat publicats, les àrees temàtiques, factors d'impacte (FI) i el rang de les revistes segons l'ISI web de l'any 2009 són els següents:

1. Bonnineau, C., Bonet, B., Corcoll, N., Guasch, H., 2011. Catalase in fluvial biofilms: a comparison between different extraction methods and example of application in a metal-polluted river. Ecotoxicology 20, 293-303.

- Àrea temàtica: Environmental Sciences
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2. Referència: Bonnineau, C., Tlili, A., Faggiano, L., Montuelle, B., Guasch, H. The use of antioxidant enzymes in freshwater biofilms: temporal variability vs. ecotoxicological responses. In preparation.

3. Referència : Bonnineau, C., Gallardo Sague, I., Urrea, G., Guasch, H. Light history modulates antioxidant and photosynthetic responses of biofilms to natural stressors (light) and chemical stressors (herbicides). Submitted to Ecotoxicology.

4. Referència: Bonnineau, C., Guasch, H., Proia, L., Ricart, M., Geiszinger, A., Romaní, A.M., Sabater, S. 2010. Fluvial biofilms: a pertinent tool to assess β-blockers toxicity. Aquatic Toxicology 96, 225-233.

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5. Referència: Bonnineau, C., Scholz, S., Guasch, H., Schmitt-Jansen, M. Exploring the potential of a Functional Gene Array (FGA) for fluvial biofilms. In preparation.

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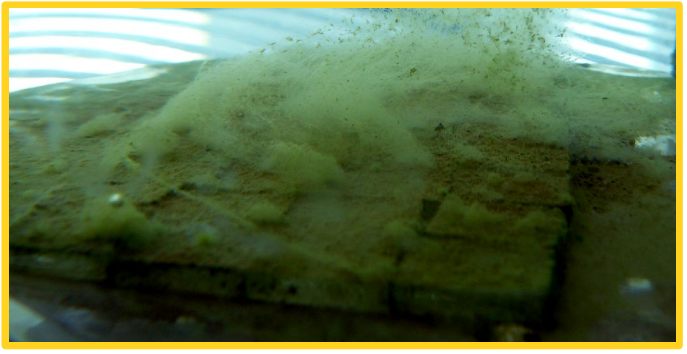


Summary

Nowadays, European water bodies are undergoing increasing pressure and demand due to human use. Consequently, preservation and/or restoration of freshwater ecosystems have become an essential concern for human societies. To evaluate the impact of contamination on aquatic ecosystems, different tools have been developed dedicated, for instance, to toxicants quantification in water and sediment or to the evaluation of ecosystem status in response to pollutants. In this context, molecular biomarkers were especially developed to detect sub-lethal effects of contaminants, provoked, for instance, by low concentrations of chemicals. Ecotoxicological tests performed at community level also highlighted the importance of interactions within species in response to contamination and the higher ecological relevance of such tests compared to single-species toxicity tests. Therefore this thesis focused on the use of molecular biomarkers at community-level to assess chemicals toxicity. Biofilms were chosen as an important community of freshwater ecosystem since they play an important role as primary producers and are recognized to be pertinent biological indicators. Different biomarkers targeting various levels of biological organization are already used to account for biofilm status (e.g. photosynthetic parameters, pigment composition or bacterial mortality). To our knowledge, there is no specific parameter of stress response among these classical indicators. Thus, the use of antioxidant enzymes as molecular biomarkers of oxidative stress for the whole biofilm has been proposed in the present study.

Chapter I: Catalase in fluvial biofilms: a comparison between different extraction methods and example of application in a metal-polluted river.

This study focused on the development of an extraction method for the antioxidant enzyme catalase that provided the basis for the study of other antioxidant enzymes. Biofilms grown in the laboratory were used to select the best extraction method among trituration, ultrasonication, homogenization and homogenization followed by glass bead disruption. The latter method appeared to be the best compromise for catalase

extraction within biofilms. In addition, this method was applied to field biofilms from a metal-polluted river. A negative correlation was found between catalase activity and metal gradient while none was found for photosynthetic efficiency, a classical biomarker. This result highlighted the relationship between catalase activity and contamination. The use of microcosms was then proposed to further investigate cause-effect relations between antioxidant enzymes activities and water contamination.

Chapter II: The use of antioxidant enzymes in freshwater biofilms: temporal variability vs. ecotoxicological responses.

The temporal variation of a biomarker is an estimate of its "natural" range of variation which may be a pertinent baseline to estimate the importance of changes occurring during toxicant exposure. In the present study, the temporal variations of three antioxidant enzymes: catalase, ascorbate peroxidase and glutathione reductase were studied in control biofilms and those exposed to increasing concentrations of the herbicide oxyfluorfen in microcosms. In control communities, biofilm ageing was related to an increase in biomass (chlorophyll-*a*, weight) and a shift in pigment composition indicative of a decrease in diatoms and an increase in green algae and cyanobacteria relative abundances. In addition, a peak in ascorbate peroxidase was observed at the end of the exponential growth phase while catalase and glutathione reductase seemed to play a more important role in slow- rather than in exponentially--growing biofilms. Effects of oxyfluorfen after chronic exposure were characterized mainly by a higher ascorbate peroxidase and catalase activities in biofilms exposed to low (3 to 15 µg L^{-1}) and high (30 to 150 µg L^{-1}) concentrations, respectively. Moreover, after five weeks of exposure eukaryal communities were structured by oxyfluorfen and both bacterial and eukaryal richnesses decreased in samples exposed to 150 µg L^{-1}. These structural changes were supported by a higher catalase activity in response to a sudden increase in oxyfluorfen concentrations in biofilms chronically exposed to 75 and 150 µg L^{-1} than in controls (biofilms not chronically exposed to contaminant). These results suggest that species with a higher antioxidant capacity were likely to be selected during chronic exposure to oxyfluorfen. Therefore, the

chronic effect of this toxicant on community structure may be enhanced in multiple stress situations, as often observed in the field, due to an expected loss of species.

Chapter III: Light history modulates antioxidant and photosynthetic responses of biofilms to natural stressors (light) and chemical stressors (herbicides).

In this chapter the influence of a confounding factor, that is light intensity, on biofilms response to oxidative stress induced by herbicides was investigated. Biofilms were grown in microcosms under sub-optimal, saturating, and high light intensities and showed characteristics common to shade/light adaptation. Nevertheless, this adaptation was not supported by differences in activities of the antioxidant enzymes: catalase, ascorbate peroxidase, glutathione reductase or superoxide dismutase. Then, the exposure of mature communities to different light intensities or different herbicides (glyphosate, AMPA, copper and oxyfluorfen) showed that exposure to previous stress, such as the limitation or the excess of light, influenced the capacity of biofilms to cope with further natural or chemical stressors. Indeed, short-term exposure to a sub-optimal light intensity provoked a decrease in catalase activity of biofilms adapted to high light intensity but not in those adapted to saturating light intensity. Short-term exposure to a light intensity higher than the one during colonization provoked photoinhibition in biofilms adapted to sub-optimal and saturating conditions and an activation of ascorbate peroxidase activity only in those adapted to sub-optimal light intensity. Concerning herbicides exposure, high-light adapted biofilms were more tolerant to glyphosate than shade-adapted biofilms in terms of photosynthetic efficiency, while the degradation product of glyphosate AMPA was not found to be toxic to biofilms at concentrations of up to 500 mg L^{-1}. Copper exposure provoked a decrease in protein content in all biofilm communities and an increase in ascorbate peroxidase of shade-light adapted biofilms. Finally, oxyfluorfen provoked an increase in ascorbate peroxidase activities of biofilm communities regardless of their light history. These results highlighted both positive and negative interactions between natural and chemical stressors and indicated that physical habitat characteristics would strongly influence community resistance to disturbances.

Chapter IV: Fluvial biofilms: a pertinent tool to assess β-blockers toxicity.

Catalase activity was included in a set of biomarkers to assess the toxicity of the emerging contaminants: propranolol, metoprolol and atenolol. Indeed, these three β-blockers, regularly detected at the ng L^{-1} range in rivers, are molecules designed to be biologically active and little is known about their toxicity towards aquatic ecosystems. Although these molecules belong to the same class of pharmaceuticals, they had different effects on biofilms. Propranolol was the most toxic of the three β-blockers tested, affecting principally the algal component, while metoprolol was mainly toxic for bacteria. Atenolol toxicity was lower but affected all the biofilm compartments. The higher toxicity of propranolol and metoprolol may be due to a better absorption within biofilms. These differential effects on non-target species may question the classification by mode of action in target species of such contaminants. In addition, since β-blockers are mainly found in mixtures in rivers, their differential toxicity could have potential relevant effects on the interactions between algae and bacteria within river biofilms.

Chapter V: Exploring the potential of a Functional Gene Array (FGA) for fluvial biofilms.

Microarrays allow the activity of thousands of genes to be detected simultaneously and could therefore contribute to improve a multi-biomarker approach. Nevertheless, this technology requires *a priori* knowledge of genetic sequences. Consequently, its application to communities composed of many non-sequenced species, such as biofilms, involves numerous challenges. In the present study, a specific approach based on consensus sequences was used to design a functional gene array (FGA) dedicated to the study of gene expression in autotrophic river biofilm communities. To do so, consensus sequences from up to 6 microalgal species (diatoms and chlorophytes) were used to design 1554 probes for 83 functional genes corresponding to several essential biochemical and stress response pathways (including the antioxidant enzymes) of microalgae. Results of the hybridization at different temperatures (55, 60 and 65ºC) of

RNA extracted from *Chlamydomonas reinhardtii*, *Scenedesmus vacuolatus* and multi-species biofilms to the FGA showed that sequence divergence affected signal intensity. Nevertheless, a hybridisation temperature of 55ºC was found to allow a good compromise between cross-hybridisation and specificity. This experiment was a proof-of-concept to illustrate the potential of an FGA for biofilms. Though this FGA may already be useful for ecotoxicological laboratory experiments performed with similar biofilm communities, more genetic information is needed to extend the number of genes on the FGA and thus explore the functional diversity of biofilms.

In conclusion, catalase, ascorbate peroxidase and glutathione reductase were found to be biomarkers of oxidative stress in biofilms while no evidence of activation or inhibition of superoxide dismutase were observed. Catalase and ascorbate peroxidase activities followed a unimodal pattern while no specific pattern of variations could be described for glutathione reductase activity. These antioxidant enzymes activities were also influenced by confounding factors such as biofilm age, colonization light or exposure light. For instance, oxyfluorfen exposure provoked an increase in catalase in old biofilms (73 days) while in younger biofilms (28 days) this toxicant induced ascorbate peroxidase. Though antioxidant enzymes activities can be influenced in the same extent by natural factors and toxicants, they provided valuable information to understand the effects of chemicals on biofilms. In particular, this thesis pointed out the interest of determining antioxidant enzymes activities patterns throughout a gradient of oxidative stress in short-term toxicity tests to compare antioxidant capacity of different biofilms communities. This thesis also provided additional evidence of the interest of a multi-biomarker approach at community-level to assess toxicity of emerging pollutants on non-target communities. To provide a prognostic tool helping in the selection of pertinent biomarkers, the feasibility of measuring gene expression in biofilm communities using a functional gene array was shown. Further investigations in biofilm transcriptomics are then suggested to apply this technique to toxicity assessment.

Resum

Avui en dia, l'elevada demanda i la pressió exercida sobre els cursos d'aigua europeus segueixen incrementant. En conseqüència, la conservació i/o la restauració dels ecosistemes d'aigua dolça segueixen preocupant a la societat. Per tal d'avaluar l'impacte de la contaminació en els ecosistemes aquàtics, la comunitat científica ha desenvolupat diferents eines per mesurar la concentració de tòxics a l'aigua i als sediments i l'estat de l'ecosistema que rep aquests contaminants. Dins d'aquest marc conceptual, s'han utilitzat biomarcadors moleculars per detectar els efectes sub-letals que la contaminació provoca. Estudis ecotoxicològics a nivell de comunitat van demostrar la importància de les interaccions entre especies en la resposta a la contaminació, posant en evidència l'elevada rellevància ecològica d'aquests estudis si els comparem amb assajos estàndards realitzats amb mono-cultius. En conseqüència, aquesta tesi es centra en el us de biomarcadors moleculars a nivell de comunitat per avaluar els efectes dels contaminants. Es van utilitzar comunitats del perífiton, o biofilm perquè juguen un paper important en els ecosistemes fluvials com a productors primaris, i han estat reconeguts com bons indicadors biològics dels rius. Per determinar l'estat del biofilm, s'han utilitzat tradicionalment diversos biomarcadors tals com l'eficiència fotosintètica, la composició de pigments o la mortalitat dels bacteris, entre d'altres. Aquests biomarcadors clàssics no inclouen paràmetres específics de la resposta a l'estrès. Així doncs, en aquesta tesi es proposa l'us dels enzims antioxidants com a biomarcadors de l'estrès oxidatiu en el biofilm.

Capítol I: L'activitat catalasa dels biofilms de rius: comparació entre mètodes d'extracció i un exemple d'aplicació en un riu contaminat per metalls.

En aquest estudi, es desenvolupa un mètode d'extracció de l'enzim antioxidant catalasa que servirà de base per a futurs estudis d'altres enzims antioxidants. Biofilms colonitzats al laboratori van ser utilitzats per seleccionar el millor mètode d'extracció entre trituració, ultra-sonicació, homogeneïtzació i homogeneïtzació seguida de disrupció cel·lular amb boles de vidre. El millor mètode d'extracció va ser la

homogeneïtzació seguida de disrupció cel·lular amb boles de vidre. L'aplicació d'aquest mètode en biofilms de rius va revelar una correlació negativa entre la activitat catalasa i el gradient de metalls a l'aigua del riu. Per altra banda, no es va trobar cap relació amb la contaminació metàl·lica a l'utilitzar biomarcadors més clàssics tals com l'eficiència fotosintètica. En conclusió, aquest resultat evidencia la relació existent entre l'activitat catalasa i la contaminació metàl·lica. Es proposa l'us de microcosmos per futures investigacions de les relacions causa-efecte entre la contaminació de l'aigua i els enzims antioxidants.

Capítol II: L'us dels enzims antioxidants en biofilms de riu: variabilitat temporal vs. resposta ecotoxicològica.

La variabilitat temporal d'un biomarcador permet avaluar el seu rang "natural" de variació, el qual podria esdevenir la línia de base pertinent per entendre la importància dels canvis provocats per l'exposició a un tòxic. En aquest capítol es van estudiar, en microcosmos, les variacions temporals de tres enzims antioxidants: la catalasa, l'ascorbat peroxidasa i la glutatió reductasa, en biofilms controls i d'altres exposats a concentracions creixents de l'herbicida oxifluorfè. En les comunitats control es va poder relacionar l'envelliment del biofilm amb l'increment de biomassa (clorofil·la-*a*, pes) i amb el canvi en la composició de pigments associats amb canvis en la composició de la comunitat; disminució en la proporció de diatomees i increment d'algues verdes i cianobacteris. L'activitat ascorbat peroxidasa va incrementar al final de la fase de creixement exponencial, mentre que el paper de la catalasa i la glutatió reductasa esdevingueren més importants durant la fase posterior de creixement més lent. L' exposició crònica a oxifluorfè va causar un increment de l'activitat ascorbat peroxidasa en biofilms exposats a baixes concentracions del tòxic (de 3 a 15 $\mu g\ L^{-1}$), mentre que l'activitat catalasa augmentà en biofilms exposats a concentracions més elevades (de 30 a 150 $\mu g\ L^{-1}$). Després de cinc setmanes d'exposició es va poder constatar que l'oxyfluorfè estructurava la comunitat eucariota del biofilm i que la concentració més elevada (150 $\mu g\ L^{-1}$) provocava una reducció en la riquesa bacteriana i eucariota dels biofilms. Per altre banda, aquests canvis estructurals estaven

relacionats amb una major activitat catalasa en resposta a un increment de oxyfluorfè en els biofilms exposat de manera crònica a 75 i 150 µg L^{-1} que en els controls (aquells no exposats amb anterioritat). Aquests resultes suggereixen una selecció d'espècies més tolerants a l' estrès oxidatiu. La pèrdua de biodiversitat deguda a la exposició crònica a l'herbicida oxifluorfè podria esdevenir especialment problemàtica en situacions de estrès múltiple.

Capítol III: Les condicions de llum durant la colonització modulen les respostes antioxidants i fotosintètiques en biofilms exposats a estrès natural (llum) i químic (herbicides).

En aquest capítol es va investigar la influencia de la llum en la resposta de biofilms a l'estrès oxidatiu degut a herbicides. El creixement de biofilms en microcosmos en condicions de llum d'intensitat sub-òptima, saturant o alta provocà una adaptació de les comunitats mostrant característiques típiques de comunitats de sol o d'ombra. Si més no, aquesta adaptació no comportà cap diferència remarcable en les activitats dels enzims antioxidants estudiats: la catalasa, l'ascorbat peroxidasa, la glutatió reductasa i la superòxid dismutasa. Més endavant, l'exposició d'aquestes comunitats a diferents intensitats de llum o diferents herbicides (glifosat, AMPA, coure i oxifluorfè) va permetre comprovar que l'adaptació a un estrès previ, com podien ser l'excés o limitació de llum, influïa sobre la capacitat del biofilm a respondre a un segon estrès natural o químic. De fet, l'exposició a una llum inferior a la de la colonització va provocar un lleuger increment d'eficiència fotosintètica i una reducció de la catalasa en biofilms de sol (aquells adaptats a una intensitat de llum alta) però no en els adaptats a una intensitat de llum saturant. L'exposició a una llum més elevada que la de la colonització va provocar una inhibició de la fotosíntesis en biofilms d'ombra o aquells adaptats a una intensitat de llum saturant. Per altra banda, es va observar una activació de l' activitat ascorbat peroxidasa en les comunitats d'ombra. En relació amb l'exposició als herbicides, biofilms d'ombra eren més sensibles al glifosat que els de sol, mentre que el producte de degradació del glifosat, l'AMPA, no va generar toxicitat per sota dels 500 mg L^{-1}. L'exposició al coure va provocar un increment de la activitat

de l'ascorbat peroxidasa en biofilms d'ombra i un descens en la concentració de proteïnes tant en biofilms d'ombra com en els de sol. Finalment l'efecte de l'oxifluorfè no diferí tampoc entre biofilms de sol o d'ombra, provocant, en tots els casos, un increment de l'ascorbat peroxidasa. En conclusió, l'estudi posa en evidència que poden donar-se interaccions tant positives com negatives entre l'estrès ambiental i l'estrès químic. Els resultats obtinguts posen també en evidència que les característiques físiques de l'hàbitat (que modulen l'ambient lumínic) poden influir sobre la resistència de la comunitat a les pertorbacions.

Capítol IV: Els biofilms de rius: un eina pertinent per l'estimació de la toxicitat dels β-bloquejants.

La mesura de l'activitat catalasa va ser inclosa en una sèrie de biomarcadors utilitzats per avaluar la toxicitat de diversos contaminants emergents: el propranolol, el metoprolol i l'atenolol. Aquestes tres β-bloquejants han estat detectats regularment en rius a nivell de ng L^{-1}. Són molècules dissenyades per ser biològicament actives i la seva toxicitat sobre els ecosistemes aquàtics és poc coneguda. Encara que aquestes molècules pertanyeran al mateix grup de compostos farmacèutics, l'efecte causat sobre el biofilm va ser diferent. El propranolol va ser el més tòxic i va causar més toxicitat sobre el compartiment algal del biofilm mentre que el metoprolol va ser més tòxic pels bacteris. L'atenolol va ser poc tòxic però va afectar tots els compartiments del biofilm. La millor absorció del propranolol i del metoprolol al biofilm podria explicar la seva major toxicitat. Finalment, els efectes diferencials observats sobre espècies no-diana, plantegen la idoneïtat de la classificació actual d'aquests contaminants, basada en el seu mode de acció en especies diana. A més, donat que els diferents β-bloquejants es troben normalment en barreja, aquesta toxicitat diferencial podria tenir efectes importants en les interaccions entre algues i bacteris en els biofilms fluvials.

Capítol V: Exploració del potencial d'una Xip de Gens Funcionals (Functional Gene Array: FGA) per biofilms de rius.

Els micro-xips poden permetre la detecció simultània de l'activitat de milers de gens i així contribuir a millorar una aproximació amb múltiples biomarcadors. Si més no aquesta tecnologia requereix el coneixement *a priori* de les seqüències gèniques, fet que en dificulta l'aplicació en comunitats, la majoria de les espècies de les quals no han estat seqüenciades. L' aproximació basada en seqüències de consens, presentada en aquest capítol, es va utilitzar per dissenyar un xip de gens funcionals (FGA) per a l'estudi de l'expressió gènica en biofilms fluvials. Seqüències de consens de fins a 6 espècies micro-algals (diatomees i clorofícies) van ser utilitzats per dissenyar 1554 sondes per 83 gens funcionals. Aquests gens corresponen a processes bioquímics i de resposta a l'estrès (incloent els enzims antioxidants). La hibridació de RNA extret de *Chlamydomonas reinhardtii*, *Scenedesmus vacuolatus* i de biofilm a diferents temperatures (55, 60 i 65 ºC), va permetre mostrar la influencia de les divergències entre seqüències en la intensitat del senyal. L'experiment mostrà que el millor compromís entre hibridació creuada i especificitat es donava a la temperatura d'hibridació de 55ºC. L'estudi permet validar la pertinència d'utilitzar una FGA per estudiar les variacions en l'expressió gènica dels biofilms provocats per la seva exposició als tòxics. En estudis posteriors, l'ampliació del nombre de gens en la FGA permetrà explorar de manera més completa la diversitat funcional del biofilm i els efectes que la contaminació hi provoca.

En conclusió, es va trobar que la catalasa, l'ascorbat peroxidasa i la glutatió reductasa eren biomarcardors de estres oxidatiu en biofilms però no es va poder demostrar per la superòxid dismutasa. Es va determinar un patrò unimodal de resposta de la catalasa i de l'ascorbat peroxidasa al llarg de un gradient de estres oxidatiu. Els experiments realitzats no van permetre elucidar aquest patró de variació per la glutatió reductasa. Diferents factors com l'edat del biofilm, la llum de colonització o

d'exposició poden afectar l'activitat d'aquests enzims antioxidants. Per exemple, biofims envellits (73 dies) van respondre a una exposició a l'oxifluorfè incrementant la catalasa però en biofilms més joves (28 dies) va ser l'ascorbat peroxidasa la que va incrementar. Tot i que les activitats antioxidants poden veure's influenciades amb la mateixa mesura per factors naturals i contaminants, aquestes activitats ens permeten entendre millor l'efecte dels contaminants en el biofilm. Cal remarcar que aquesta tesi il·lustra l'interès de determinar els patrons de variació de les activitats antioxidants al llarg de un gradient de estrès oxidatiu en assajos de toxicitat aguda. Aquests assajos es poden utilitzar per comparar la capacitat antioxidant entre diferents comunitats. Aquesta tesi també proporciona evidències que mostren l'interès de l'aproximació multi-biomarcador a nivell de comunitat per avaluar la toxicitat dels contaminants emergents sobre espècies no-diana. Al verificar la possibilitat de mesurar l'expressió gènica un biofilms utilitzant un xip de gens funcionals, la recerca duta a terme també proporciona una eina pronòstica que pot ajudar a la selecció dels millors biomarcadors. Per tant, es recomana la realització, en un futur, d'investigacions de transcriptòmica en biofilms per tal de validar la seva aplicació en estudis de toxicitat.

Résumé

De nos jours, l'utilisation anthropique grandissante des cours d'eau européens entraîne une augmentation de la pression sur les écosystèmes aquatiques. La préservation et/ou la restauration de ces écosystèmes sont ainsi devenues des préoccupations essentielles pour les sociétés humaines. Pour évaluer l'effet de la pollution sur l'écosystème aquatique, différents outils ont été développés dans le but, par exemple, de quantifier les polluants présents dans l'eau et les sédiments ou de définir le statut écologique d'un écosystème en réponse à l'introduction d'un composé toxique. Parmi ces outils, les biomarqueurs moléculaires ont été utilisés, entre autres, pour détecter les effets sublétaux de polluants présents à faibles concentrations. En parallèle, des tests écotoxicologiques réalisés avec des communautés ont aussi mis en évidence l'importance des interactions entre espèces lors de l'exposition à un polluant. Ces tests ont donc une pertinence écologique plus importante que les tests standards réalisés avec une seule espèce. En conséquence, cette thèse s'intéresse à l'utilisation de biomarqueurs moléculaires dans les communautés aquatiques pour évaluer la toxicité de différents polluants. Le biofilm (aussi appelé périphyton) a été choisi comme communauté d'étude du fait de son rôle important dans les rivières notamment comme producteur primaire mais aussi parce qu'il est reconnu comme un indicateur biologique pertinent de l'état des écosystèmes aquatiques. Différents biomarqueurs sont traditionnellement utilisés pour estimer l'état du biofilm (e.g. l'efficacité photosynthétique, la composition pigmentaire, la mortalité bactérienne). Cependant, à notre connaissance, il n'existe pas parmi ces indicateurs classiques de marqueurs spécifiques de la réponse au stress. C'est pourquoi cette thèse propose l'utilisation des enzymes antioxydantes comme biomarqueurs du stress oxydatif chez le biofilm.

Chapitre I : La catalase des biofilms de rivière : une comparaison entre différentes méthodes d'extraction et un exemple d'application dans une rivière polluée par des métaux.

Ce chapitre est centré sur le développement d'une méthode d'extraction de la catalase du biofilm qui servira ensuite de bases pour les études d'autres activités antioxydantes. Des biofilms cultivés en laboratoire ont donc été utilisés pour déterminer la méthode d'extraction la plus appropriée entre la trituration, la sonication, l'homogénéisation et l'homogénéisation suivie d'un cassage de cellules additionnel à l'aide de billes de verre. Cette dernière méthode s'est révélée la meilleure pour l'extraction de la catalase du biofilm et a été appliquée à des biofilms échantillonnés dans une rivière polluée par des métaux. Une corrélation négative a été mise en évidence entre l'activité catalase et le gradient de concentration des métaux tandis qu'aucune n'a été trouvée pour le biomarqueur plus classique qu'est l'efficacité photosynthétique. Ce résultat montre l'existence d'une relation entre l'activité de la catalase et la pollution. L'utilisation de microcosmes est alors proposée pour les études suivantes sur les relations de cause à effet entre les activités antioxydantes et la pollution aquatique.

Chapitre II : L'utilisation d'enzymes antioxydantes des biofilms de rivière : variation temporelle vs. réponses écotoxicologiques.

La gamme de fluctuations temporelles d'un biomarqueur peut-être une bonne estimation de sa gamme de variations « naturelles » et peut donc servir de ligne de base pour évaluer l'importance des changements dus à l'exposition à un polluant. Dans ce chapitre, les variations temporelles des enzymes antioxydantes catalase, ascorbate peroxydase et glutathion réductase de biofilms contrôles et de biofilms exposés à différentes concentrations de l'herbicide oxyfluorfène ont été étudiées en microcosmes pendant 5 semaines. Dans les communautés contrôles, le vieillissement du biofilm a été relié à une augmentation de la biomasse (chlorophylle-*a*, masse) et à un changement dans la composition pigmentaire indiquant une diminution de l'abondance relative en diatomées et une augmentation de celle en algues vertes et en

cyanobactéries. De plus, la fin de la phase de croissance exponentielle a été caractérisée par une augmentation de l'activité de l'ascorbate peroxydase tandis que la catalase et la glutathion réductase semblaient jouer un rôle plus important dans la phase de croissance ralentie. L'exposition chronique à l'oxyfluorfène a entraîné une augmentation de l'activité de l'ascorbate peroxydase dans les biofilms exposés à de faibles concentrations (de 3 à 15 µg L^{-1}) et de la catalase dans ceux exposés à de fortes concentrations (de 30 à 150 µg L^{-1}). De plus, après cinq semaines d'exposition, les communautés eucaryotes du biofilm ont été structurées par l'oxyfluorfène et les richesses bactérienne et eucaryote ont été réduites dans les biofilms exposés à 150 µg L^{-1}. En association avec ces changements structurels, l'activité catalase des biofilms chroniquement exposés à 75 et 150 µg L^{-1} étaient plus hautes que celle des contrôles (biofilms non-exposés de manière chronique au polluant) après une augmentation brutale de la concentration en oxyfluorfène. Ces résultats suggèrent une sélection d'espèces plus résistantes au stress oxydatif lors de l'exposition chronique à l'oxyfluorfène. Malgré cet avantage, la perte de biodiversité risque d'être problématique notamment dans des situations de stress multiples.

Chapitre III : L'intensité lumineuse de croissance des biofilms module leur réponse aux stress naturel (lumière) et chimiques (herbicides).

Dans ce chapitre, l'influence d'un facteur environnemental : l'intensité lumineuse, sur la réponse des biofilms au stress oxydatif induit par des herbicides a été étudiée. La culture de biofilms en microcosmes sous une intensité lumineuse sous-optimale, saturante ou élevée a révélé des caractéristiques typiques de photo-adaptation (à l'ombre ou à la lumière) pour chaque type de communauté. Néanmoins, ces adaptations n'étaient pas associées à des différences dans les activités antioxydantes de la catalase, de l'ascorbate peroxydase, de la glutathion réductase ou de la superoxide dismutase. Ensuite, l'exposition de ces communautés matures à des intensités lumineuses différentes ou à des herbicides (glyphosate, AMPA, cuivre, oxyfluorfène) a montré l'influence que peut avoir un stress préliminaire, tel que l'excès ou la limitation de lumière, sur la capacité des biofilms à répondre au stress suivant qu'il soit naturel ou

chimique. De fait, la courte exposition à une intensité lumineuse sous-optimale a provoqué une diminution de l'activité de la catalase chez les biofilms adaptés à une intensité lumineuse élevée mais pas chez ceux adaptés à une intensité saturante. De plus la courte exposition des biofilms à une intensité lumineuse plus élevée que pendant la culture a provoqué une inhibition de la photosynthèse chez les biofilms adaptés à une intensité sous-optimale ou saturante mais, dans ces conditions, une augmentation de l'activité de l'ascorbate peroxydase n'a été observée que chez ceux adaptés à une intensité sous-optimale. Concernant l'exposition aux herbicides, les biofilms adaptés à une intensité lumineuse sous-optimale présentèrent une sensibilité plus élevée au glyphosate que ceux adaptés à une intensité lumineuse élevée. Aucun effet toxique n'a été observé chez les biofilms après une exposition au produit de dégradation du glyphosate, l'AMPA, à des concentrations allant jusqu'à 500 mg L^{-1}. L'exposition au cuivre a provoqué une diminution de la concentration en protéines chez tous les biofilms et une augmentation de l' activité de l' ascorbate peroxydase chez les biofilms adaptés à une intensité lumineuse sous-optimale. Finalement, une augmentation de l'activité de l'ascorbate peroxydase a été observée le long du gradient de concentrations de l'oxyfluorfène chez tous les biofilms, indépendamment de leur intensité lumineuse de culture. Ces résultats montrent qu'ils existent des interactions à la fois négatives et positives entre les facteurs de stress naturels et chimiques et indiquent que les caractéristiques physiques de l'habitat peuvent influencer fortement la réponse des communautés aux perturbations.

Chapitre IV : Les biofilms de rivière : un outil pertinent pour évaluer la toxicité des β-bloquants.

Plusieurs biomarqueurs du biofilm, incluant l'activité de la catalase, ont été utilisés pour évaluer la toxicité pour les biofilms de rivière des polluants émergents suivants : propranolol, métoprolol et atenolol. De fait, ces trois β-bloquants, régulièrement détectés dans les rivières à des concentrations de l'ordre du ng L^{-1}, sont des molécules biologiquement actives et leur toxicité envers les écosystèmes aquatiques est peu connue. Bien que ces molécules appartiennent à la même classe de produits

pharmaceutiques, elles ont eu des effets différents sur les biofilms. Le propranolol s'est révélé le plus toxique des β-bloquants, affectant principalement le compartiment algal du biofilm, tandis que le métoprolol a eu un impact plus important sur les bactéries. La toxicité de l'atenolol était plus faible mais a affecté tous les compartiments du biofilm. La plus forte toxicité du propranolol et du métoprolol est probablement liée à leur meilleure absorption par le biofilm. Ces effets différentiels sur des espèces non-cibles peuvent remettre en question la classification de ce type de polluants suivant leur mode d'action sur les espèces cibles. De plus, ces β-bloquants se trouvent souvent en mélange dans les rivières et cette toxicité différentielle pourrait alors avoir d'importants effets sur les interactions entre algues et bactéries des biofilms.

Chapitre V : L'exploration du potentiel d'une puce à ADN de gènes fonctionnels pour le biofilm.

L'utilisation de puces à ADN permet de détecter simultanément l'activité de milliers de gènes et peut donc contribuer à la sélection de biomarqueurs de toxicité pertinents. Cependant cette technologie requiert la connaissance *a priori* des séquences génétiques et son application à une communauté composée d'espèces dont le génome n'a pas été séquencé, comme le biofilm, implique donc la résolution de nombreux challenges. Dans ce chapitre, une approche spécifique, basée sur les séquences consensus entre différents génomes, a été utilisée pour construire une puce à ADN de gènes fonctionnels (Functional Gene Array : FGA) pour l'étude de l'expression des gènes des communautés autotrophes des biofilms de rivière. Pour cela, des séquences consensus obtenues après la comparaison des séquences génétiques appartenant à 6 espèces de micro-algues (diatomées et chlorophycées) ont été utilisées pour élaborer 1554 sondes nucléiques correspondant à 83 gènes impliqués dans des processus biochimiques ou de réponse au stress (incluant les enzymes antioxydantes). L'hybridation à différentes températures (55, 60 et 65 ºC) d'ARN extrait de *Chlamydomonas reinhardtii*, de S*cenedesmus vacuolatus* et de biofilm a montré que la divergence de séquences entre l'ARN extrait et les sondes affectait l'intensité du signal. Cependant une température d'hybridation de 55ºC a permis d'obtenir un bon compromis entre l'hybridation croisée

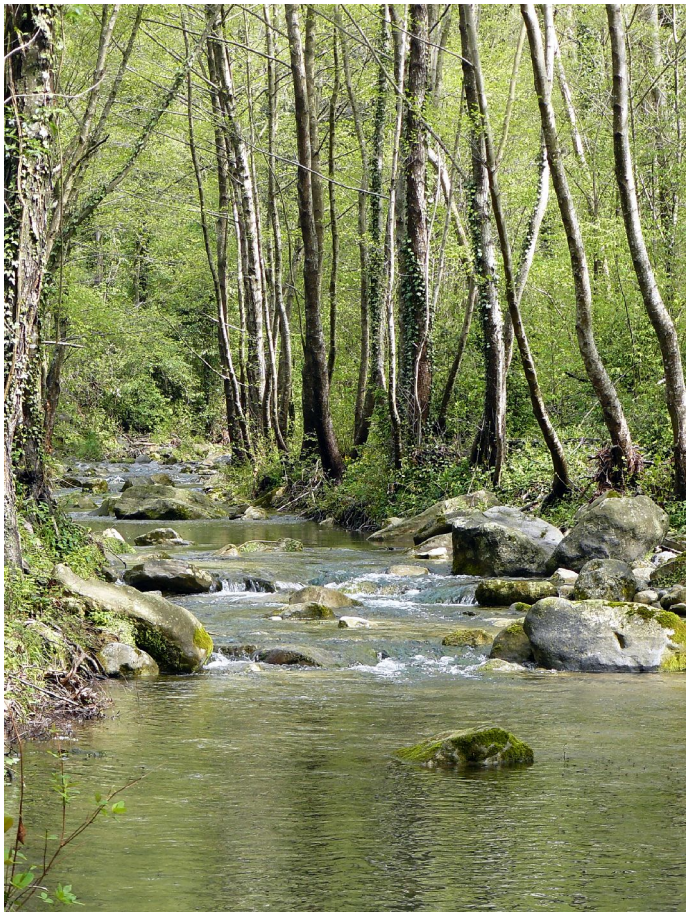
et la spécificité. Cette expérience a permis de valider la pertinence de cette approche pour étudier l'expression des gènes chez le biofilm. Si cette FGA peut déjà être utilisée, en particulier pour des tests écotoxicologiques réalisés avec des communautés similaires cultivées en laboratoire, plus d'information génétique est nécessaire pour étendre le nombre de gènes sur la FGA et ainsi explorer de manière plus complète la diversité fonctionnelle du biofilm.

En conclusion, les résultats de cette thèse montrent que la catalase, l'ascorbate peroxydase et la glutathion réductase sont des biomarqueurs du stress oxydatif dans le biofilm mais il n'a pas été possible de le montrer pour la superoxide dismutase. Alors que les variations de la catalase et l'ascorbate peroxydase le long d'un gradient de stress oxydatif s'ajustent à un modèle unimodal, le modèle de variation de la glutathion reductase n'a pas pu être défini. Différents facteurs tels que l'âge du biofilm, l'intensité lumineuse durant la colonisation ou l'exposition peuvent affecter ces activités antioxydantes. Par exemple, une activation de la catalase a été observée chez des biofilms âgés (73 jours) en réponse à une exposition à l'oxyfluorfen tandis que chez des biofilms plus jeunes (28 jours) cette exposition a induit l'activation de l'ascorbate peroxydase. Bien que les activités antioxydantes puissent être affectées, dans la même mesure, par des facteurs naturels et par des polluants, leur étude permet de mieux comprendre l'effet de la pollution sur les biofilms. En particulier, cette thèse illustre l'intérêt de déterminer les patrons de variation des activités antioxydantes le long d'un gradient de stress oxydatif lors de tests de toxicité aiguë. Ces test peuvent être utilisés pour comparer la capacité antioxydante de différentes communautés. Cette thèse montre également l'intérêt de combiner de multiples biomarqueurs avec des tests écotoxicologiques sur des communautés pour évaluer la toxicité de polluants émergents envers des espèces non-cibles. La possibilité de mesurer l'expression génétique dans les biofilms grâce à une puce à ADN de gènes fonctionnels est un premier pas vers la réalisation d'un outil pronostique, pouvant aider à la sélection des biomarqueurs les plus pertinents. Néanmoins, l'application de cette technique à

l'évaluation de la toxicité dans les biofilms nécessite un approfondissement des recherches sur la transcriptomique des biofilms.

General

Introduction



Blue gold is the name given to water by some science-fiction authors in a future where its scarcity and its contamination make it an inestimable jewel (Martinigol and Hyman 2002). In the last few years, it has seemed that these scenarios were not so unrealistic. For instance, in 2010, the waste reservoir of the alumina plant of Ajka (Hungaria) broke and thousands tons of contaminated red muds were released in the environment. This strong and sudden alkaline metallic contamination extinguished all aquatic life in the Marcal river turning the "blue living gold" in "red dead mud". Though accidental spills are spectacular, most common contamination in Europe is due to chronic inputs of various pollutants from urban waste, industry and agricultural areas. In addition to this chemical contamination, the aquatic ecosystem often undergoes other anthropogenic pressures such as habitat modification (Matthaei et al. 2010). Decrease in fish abundance or algal bloom are some of the visible effects of these multiple-stress situations that affect both structure and function of aquatic ecosystems. Nevertheless, the impact of chronic contamination is rather gradual and subtle and it may be difficult to discriminate the changes due to contamination from those due to natural environmental variations (Moore 2002).

Advances in chemistry, ecology and ecotoxicology allowed the development of efficient methods and tools to detect contaminants, even at low concentrations, and to determine their effects and consequences on the aquatic ecosystem. These advances also allowed establishing causal relationships between pollutants and biological impairments. In ecology, effects of disturbances at ecosytem-level were generally assessed by investigating changes in community structure (biodiversity, community composition) while single-species tests used in ecotoxicology allowed to better understand how chemicals affect the functioning of biological elements.

In this context, this thesis explores the interest of molecular biomarkers at community-level to assess chemicals toxicity. In particular, the present thesis aims to develop and validate the antioxidant enzymes activities (AEA) as biomarkers of oxidative stress in freshwater biofilms and to show their interest in ecotoxicological tests to assess the impact of both priority and emerging pollutants, with a focus on

emerging organic compounds. To our knowledge, only one study reported the use of these enzymes in biofilms (Guasch et al. 2010). A complementary and innovative approach is also introduced to explore the use of transcriptomic tools in biofilms.

Scientific development in toxicity assessment in aquatic ecosystems

Scientific research underpins water management and provided the tools implemented in the Water Framework Directive (WFD, 2000/60/EC) adopted in 2000 by the European Union (EU). The goal of the WFD is to achieve a "good status" for all of Europe's surface waters and groundwater by 2015. To this end, first, the chemical and the ecological status of water bodies in Europe has to be determined by combining the detection of priority pollutants recognized as harmful for the aquatic environment (chemical status) and the estimation of the structure and functioning of biological indicators (ecological status). Based on these data, "slight" deviations from reference conditions would characterize a "good" ecological status and "moderate" deviations a "moderate" ecological status. Scientists are then in charge of determining the reference conditions of water bodies as well as the meaning of "slight" and "moderate". Thanks to the extensive monitoring recommended by the WFD, knowledge on structure, function and ecological status of European rivers has increased in the last 10 years (Hering et al. 2010).

In particular, molecular biomarkers have been developed to assess sub-lethal effects of chemicals and understand their impact on organism functioning. Moreover community ecotoxicology has been developed to assess toxicity at higher degree of ecological relevance than single-species tests.

1. Molecular biomarkers

In the WFD, most of the evaluation of biological elements has been focused on structural indicators (e.g. abundance, taxonomic indices) that have been recognized as pertinent indicators of common types of impact (e.g. eutrophication, organic pollution, acidification) (Hering et al. 2010). Nevertheless, chronic exposure to low concentrations of chemicals has also been shown to have subtle and gradual impacts on communities by provoking sub-lethal effects and thus altering community functioning or resilience (Moore 2002, Moss 2008). Investigating effects of chemicals exposure at sub-cellular levels has been indeed very useful to determine chemicals mode of action but also the consequences of exposure on organisms. This approach resulted on the description of various biomarkers of exposure and effects of contaminants (see for example: Bayley et al. 1999; Chan 1995; Forget et al. 2003; Matozzo et al. 2008). A common definition of biomarkers refers to biochemical, physiological or histological indicators of exposure or toxicant effects (Huggett et al. 1992; Forbes et al. 2006). Nevertheless, most of the studies focused on molecular biomarkers. Indeed, since 1980, 4,697 research articles or reviews published in "environmental sciences" contained "biomarker" in the title, abstract or keywords; among them 2,934 also contained "molecular biomarker", 1,346 "physiological biomarker" and 401 "histological biomarker" (SCOPUS).

The development of molecular biomarkers is historically linked with medicine and vertebrate biology. They were first developed in fish but are now also used in macroinvertebrates, aquatic plants and algae. The added value of molecular biomarkers in ecological risk assessment and ecotoxicology has been discussed many times (e.g. Brain and Cedergreen 2009; Forbes et al. 2006; Lam and Gray 2003; Montserrat et al. 2003; Sarkar et al. 2006). Most of the issues debated concerned the specificity and the interpretation of molecular biomarker variations.

Changes in molecular biomarkers indicate an interaction between an environmental perturbation and the organism. In the field, this perturbation may result

from the combination of various stressors, including known and unknown chemicals. Some biomarkers are specific of certain chemical compounds and can then be used to detect their presence in complex mixtures. For instance, the glycol-lipid protein vitellogenin has been shown to be a specific biomarker for estrogens and antiestrogens in juvenile and adult fish and can then specifically indicate fish exposure to endocrine disrupting chemicals (Hutchinson et al. 2006). One of the most investigated biomarkers is the cytochrome P4501A (CYP1A) in fish. It is specifically induced by certain classes of pollutants (polycyclic aromatic hydrocarbons: PAHs, coplanar polychlorobiphenyls: PCBs, polychlorinated dibenzofurans and dibenzodioxins) even under variable natural conditions since its induction has been related to contaminants level in the environment in the majority of the field studies reviewed by Bucheli and Fent (1995). However, few biomarkers are specific of exposure and even those may be influenced by inter-species variations or environmental factors such as variations in light intensity, flow velocity or temperature among others. Peakall (1994) considered acetylcholinesterase (AChE) as a gold standard biomarker due to its specific inhibition in response to organophosphates and carbamates but Pfeifer et al. (2005) demonstrated that AchE activity is influenced positively by temperature and negatively by salinity in blue mussels (*Mytilus* sp.). This natural variability has to be taken into account when interpreting biomarkers variations in the field. In the laboratory, the use of microcosms experiments may reduce the influence of confounding factors and thus ease the interpretation of biomarkers changes.

Variations at molecular level in response to toxicant exposure are expected to occur before changes at individual, population or community level are visible. Therefore molecular biomarkers may provide early warning of perturbations and thus allow preventive measures to be taken to avoid ecologically relevant impairments. Indeed changes in molecular biomarkers have been observed at lowest concentrations and after shorter exposure than modifications occurring at higher level of biological organization. Xenobiotics can be excreted from cells by conjugation with glutathione, this detoxification is catalysed by the glutathione-S-transferase (GST). Various studies

showed that GST in aquatic plants is a rapid biomarker which induction can occur after less than 24h of exposure to levels expected to impair growth (Brain and Cedergreen 2009). However, various authors highlighted the difficulties to link biomarker response with impairments at higher levels of biological organization. Though very high levels of vitellogenin were related with kidney failure (Herman and Kincaid 1988) and disruption in blood dynamics and function (Scholz and Gutzeit 2000), the impact of lower levels are not well defined and a modest increase in vitellogenin may not predict quantifiable impairment (Hutchinson et al. 2006). Indeed, it is difficult to determine whether biomarker variations result from cell damage or from homeostasis maintenance. In particular, metabolic enzymes, stress proteins or antioxidant enzymes are expected to respond to chemical stress as long as cell damage is repairable. Then, their activity is expected to stabilize/decrease when toxicity provokes irreversible damages in the cell machinery. Consequently, the response of molecular biomarkers to chemical exposure has often been described as unimodal throughout exposure time or pollutant gradient (Brain and Cedergreen 2009). For example, Ortiz-Delgado et al. (2008) reported, in fish *Sparus aurata* exposed to TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) during 20 days, a unimodal pattern throughout gradient of exposure for the activity of the enzyme EROD (ethoxyresorufin O-deethylase) which reflects cytochrome P450 induction. While intermediate levels of pollutant may be characterised by an increase in molecular biomarker response, the same level may be found in both reference conditions and extremely high levels of pollutant (Amiard-Tricquet 2011). The extrapolation of molecular biomarker changes to higher levels of biological organization is limited and biomarkers may be seen rather as prognostic tools (“signposts”) than as deterministic predictors (“traffic lights”; Hutchinson et al. 2006).

Previous investigations clearly questioned the interest of molecular biomarkers in routine monitoring due to their limited interpretation in complex situations. Nevertheless, molecular biomarkers may provide valuable information to understand how toxicity affects an organism (mode of action) but also in which extent this

organism is able to cope with toxicity. Investigating the time- and dose-range of biomarkers response may be especially useful to discriminate homeostasis from cell damages. In addition, no biomarker can by itself be fully informative, a multi-biomarker approach including various levels of biological organization seems essential to link molecular toxic effects with ecologically relevant ones (Ernst and Peterson 1994).

2. From single-species to community ecotoxicology

Ecotoxicological studies compare exposed and non-exposed biota under controlled conditions to determine the toxicity of chemicals. These studies are regularly used to update the list of priority pollutants defined by the WFD and thus to take into account the increasing number of chemicals released into the aquatic environment (Schwarzenbach et al. 2006). Most of these ecotoxicological studies are performed with single species and, therefore, have a limited ecological relevance as they do not account for interactions within the ecosystem or complex communities. Indeed, chemicals are likely to provoke direct effects on the different individuals of a community but also indirect effects due to these interactions (Clements and Newman 2002).

Community ecotoxicology, as defined by Clements and Newman (2002), aims to separate anthropogenic disturbance from natural variability by integrating the response of various species. Experiments can be conducted at different scales depending on the specific objectives and the logistical considerations. At an ecosystem scale, sites can be selected according to the presence/absence of a disturbance ("natural" experiments) or the disturbance can be introduced by the researcher in the system (ecosystem manipulation; Diamond 1986). Both types of experiment allow a realistic comparison of community responses between a reference and an impacted site, for instance. In addition, they allow experiments to be conducted at large spatio--temporal scale, including large communities. However, they may not allow cause-

-effect relationships to be demonstrated or underlying mechanisms to be understood due to their low replication and experimental control and the presence of confounding variables (e.g. pre-treatment differences between experimental units). In addition, the modification of an ecosystem by releasing toxicants into the natural environment poses obvious ethical problems (Clements and Newman 2002).

On a smaller scale, as in microcosm and mesocosm experiments, natural or laboratory communities are exposed to a disturbance under controlled conditions. While microcosms are generally located indoors, mesocosms are bigger systems, placed outdoors and partially bounded to natural ecosystems (Clements and Newman 2002). Previous studies introduced the use of indoor laboratory channels as a suitable microcosm for growth and toxicant exposure of different communities such as biofilms (Serra et al. 2009a; Ricart et al. 2009; Sabater et al. 2007). These systems are a compromise between single-species tests and field experiments in terms of both realism and experimental control (Schmitt-Jansen et al. 2008b; Relyea and Hoverman 2006). Since communities are composed of a high diversity of individuals and species, their use in ecotoxicological tests results in a higher variability than in single-species tests. However, due to this same diversity, the use of communities allows a wider range of direct and indirect effects to be detected than single-species tests. In addition, the experimental control and replication is higher in microcosm or mesocosm experiments than in experiments at an ecosystem scale. This feature limits the effect of confounding variables and allows the effect of one particular variable (e.g. toxicant concentration) to be isolated and thus specific cause-effect relationships to be demonstrated. In addition, a higher number of replicate treatment units can be included in microcosm experiments, making the realisation of relatively complex experimental designs easier, such as the factorial ones (Clements and Newman 2002).

Molecular biomarkers in community ecotoxicology

By its complexity a community offers numerous points of view on the effects of a toxicant. Structural measures such as abundance or species distribution may inform particularly on how chemicals affect biodiversity and may particularly reflect chronic impact of contaminants. To link structural changes with ecologically relevant effects, measures of ecosystem processes such as primary production or nutrient cycling can be especially useful. Nevertheless, toxicants are expected to affect various processes at different levels of biological organization before affecting community structure and ecosystem functioning. In addition some pollutants and particularly emerging compounds may not directly affect biomass or composition, but rather metabolism and, therefore, community functioning (Geiszinger et al. 2009). In this context, molecular biomarkers may be especially useful to understand how chemicals affect community metabolism.

Since complexity increases with the number of species, molecular biomarkers within communities are expected to be more variable than within single-species. Then, their application in community requires to carefully investigate their sensitivity, specificity and natural variation.

As demonstrated for single-species, a battery of biomarkers is essential to evaluate chemical hazards (Ernst and Peterson 1994). Molecular biomarkers in communities should be then associated with other community metrics. Indeed, using biomarkers at different levels of organization may allow both the acute and chronic effects of a toxicant to be captured. For instance, in the conceptual framework established for biofilm communities by Guasch et al. (2010), after the study of different scenarios of copper exposure, physiological responses are expected after acute exposure while changes in community composition may result from chronic exposure.

1. Freshwater biofilms

Rivers are complex ecosystems, composed of different interacting compartments (e.g. hyporheic, benthic, pelagic). Among them, freshwater biofilms (also known as periphyton) play an important role as primary producers and have been recognized as pertinent biological indicators. In addition, these communities are cultivated in laboratory relatively easily and in a short-time allowing experiments to be performed with a high degree of ecological relevance and under controlled conditions.

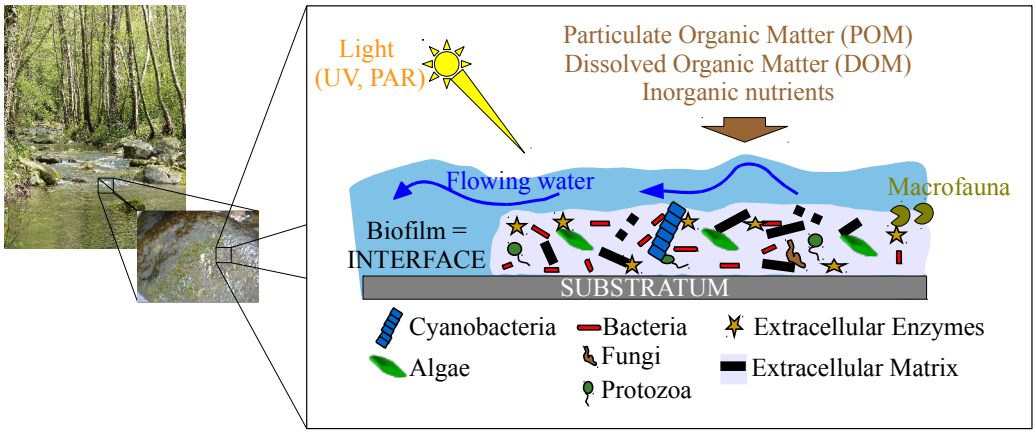


Figure 1. Schematic view of a freshwater biofilm (modified after Romani et al. 2010).

Structure, composition and formation

Freshwater biofilms are complex and structured benthic communities. The numerous species of these communities form a 3D structure as they live closely together in a matrix composed of extracellular polymeric substances (EPS matrix; Fig. 1). Biofilms can be found in various micro-habitats as they are able to attach to different solid substrates (cobbles, wood, sand, etc.). In this micro-ecosystem, two functional components can be distinguished. Green algae, diatoms and cyanobacteria form the autotrophic component of biofilms, while bacteria, fungi and protozoa compose the heterotrophic one (Romaní 2010). Bacteria are the first to colonize the substrata by developing an organic matrix. Next, small adnate diatoms are recruited

from the water column and settle in the prepared surface. Then, apically attached colonial diatoms are recruited, followed by filamentous green algae and cyanobacteria (Biggs 1996). All the micro-organisms within the biofilm interact strongly together. For instance, exudates from algae are used as organic substrates by bacteria (Romaní and Sabater 1999a). As the biofilm grows, it becomes thicker and diffusion is reduced. This structure creates marked gradients of light, dissolved oxygen and nutrients among other factors that structure and partition the community (Dodds et al. 1999; Wetzel 1993).

A tight link to fluvial ecosystems

Freshwater biofilms play an important role in aquatic ecosystems in primary production as well as in carbon and nutrient cycling. In mid-size order streams, biofilms are considered as the main primary producers (Vannote et al. 1980). They are also the primary harvesters of phosphorus and nitrogen. The heterotrophic compartment is responsible for the degradation of macro-molecules by releasing extracellular enzymes in the EPS matrix. The degraded macro-molecules can then be uptaken and used by all the micro-organisms within biofilms. Finally, biofilms are at the basis of the trophic chain in rivers, being important sources of energy for invertebrates and herbivorous fish (Stevenson et al. 1996). Freshwater biofilms are thus tightly linked to the aquatic ecosystem. On the one hand, environmental characteristics (e.g. light availability, temperature, flow velocity, etc.) shape biofilm structure and function. For instance, light limitation, as in shaded streams, will favour communities dominated by diatoms while under light excess, i.e. in clear shallow water, communities dominated by green algae are more likely to be present (Hill 1996). On the other hand, biofilm functioning influences the main processes in streams (Romaní et al. 2004).

Biofilms as biological indicators

Structure and function of periphyton may then reflect physical, chemical and biological disturbances within aquatic ecosystems. Indeed, biofilms are recognized as pertinent indicators of integrated ecosystem health (Sabater and Admiraal 2005). Diatom indices are routinely used to assess status of rivers and monitoring of different biofilm activities (photosynthesis, heterotrophic activities) has shown to provide an insight into the "health" status of aquatic ecosystems. Furthermore, biofilms are characterized by high species richness with a reliable amount of sensitive and discriminating species. These features, combined with a short generation time, make them pertinent early warning systems of disturbances within the ecosystem (Sabater et al. 2007).

Freshwater biofilms are likely to be affected by river contaminants as shown by various studies (Guasch et al. 2003; Lawrence et al. 2005). Moreover, previous ecotoxicological tests on biofilms showed that effects of chemicals at community level may differ from the ones observed in standard mono-species tests. For instance, Ricart et al. (2009) found biofilm communities to be more sensitive to the herbicide diuron than single-species tests. Indeed, after 8 days of exposure, they observed an EC_{50} for diatom biovolume (i.e. the concentration for which diatom biovolume is reduced by 50%) of 0.09 µg L^{-1}, while in previous single-species tests on different algal species, EC_{50} for growth ranged between 4 and 30 µg L^{-1} (Gatidou and Thomaidis 2007; Ma 2002; Podola and Melkonian 2005). However, the biofilm structure may also provide protection and thus better resistance of biofilm communities to chemicals. For example, Franz et al. (2008) found multi-species biofilms more resistant to the biocide triclosan than the two suspended micro-algae: *Scenedesmus vacuolatus* and *Nitzchia palea*. They found EC_{50}s for photosynthesis (i.e. the concentration for which photosynthetic efficiency is reduced by 50%) of 900 µg L^{-1} of triclosan for communities and of 3.7 and 390 µg L^{-1} of triclosan for *S. vacuolatus* and *N. palea* in suspension, respectively. As illustrated by these examples, biofilm communities are more than the random association of non-interacting species. Therefore, applying

community ecotoxicology to biofilms may provide a more realistic insight into the potential effects of a chemical in ecosystems than single-species tests.

2. Potential molecular biomarkers in biofilms: antioxidant enzymes activities (AEA) and gene expression

Antioxidant enzymes activities (AEA)

Oxidative stress is a common form of stress due to the accumulation of reduced forms of atmospheric oxygen called Reactive Oxygen Species (ROS) in cells. Among them, singlet oxygen ($^1O_2$) is formed by the direct excitation of oxygen while superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (HO^-) result from the transfer of one, two or three electrons, respectively, to oxygen (Edreva 2005). ROS are by-products of normal metabolism, such as photosynthesis or respiration. In plants and algae, chloroplasts are the main source of ROS (Edreva 2005); for instance, excited chlorophyll-*a* in chloroplast can react with ground-state oxygen and provoke O_2^- formation (Ledford and Niyogi 2005). ROS are also likely to be produced in pathways involving enzymes such as glycolate oxidase, xanthine oxidase or amine oxidase among others (Mittler 2002). ROS production can also be enhanced by environmental stresses (drought, extreme temperatures, etc.), metal or organic pollutants (Pinto et al. 2003; Geoffroy et al. 2003).

The accumulation of ROS is dangerous for the cell as they are highly reactive molecules that may cause unrestricted oxidation of cellular components (Mittler 2002). Indeed, ROS may provoke protein denaturation, lipid peroxidation, pigment breakdown or DNA damages (Valavanidis et al. 2006). They can also interfere in metabolism by inhibiting enzymes or unbalancing the $NADP^+$/NADPH ratio, for instance. Finally, oxidative burst can provoke the leakage of cellular contents and the oxidative destruction of the cell (Edreva 2005; Asada 2006). Nevertheless, recent studies showed that ROS also play a positive role in the cells, especially in signal

transduction and pathogen defence. In particular H_2O_2, the most stable ROS, is considered to play an important role in acclimation to high-light stress or regulation of antioxidant enzymes (Ślesak et al. 2007).

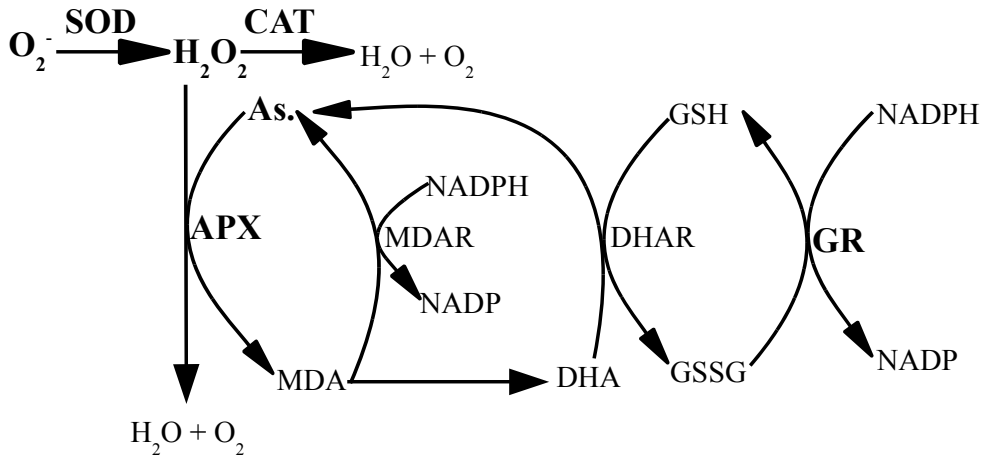


Figure 2. Pathway for scavenging of superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2) by the antioxidant enzymes: superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR). Other abbreviations: As., ascorbate; MDA, monodehydroascorbate; MDAR, MDA reductase; DHA, dehydroascorbate; DHAR, DHA reductase; GSH, glutathione; GSSG, oxidized glutathione (Modified from Mittler 2002; Noctor and Foyer 1998).

Consequently, cells have evolved different mechanisms to tightly regulate ROS levels. Non-enzymatic mechanisms involve quenching of ROS by molecules, such as glutathione, carotenoids or ascorbate. Nevertheless, major ROS scavenging mechanisms are the antioxidant enzymes. The superoxide dismutase (SOD, EC 1.15.1.1) catalyses the dismutation of the most reactive ROS: O_2^- in O_2 and H_2O_2 (Scandalios 1993); this former is then scavenged by catalase (CAT, EC 1.11.1.6, Chelikani et al. 2004) and ascorbate peroxidase (APX, EC 1.11.1.11, Lesser 2006) (Fig. 2). The glutathione reductase (GR, 1.8.1.7) participates in this reaction by regenerating the co-factor needed by APX (ascorbate-glutathione cycle, Fig. 2, Mittler 2002). These enzymes have different characteristics both in location and response. SOD and APX are present in the chloroplast, cytosol, mitochondria, peroxisomes and apoplast while CAT is principally found in peroxisomes (Mittler 2002). Enzyme

activities are sensitive to different factors, such as pH, temperature, substrate concentration. A unimodal response of enzyme activities is usually observed throughout a gradient of these factors; the peak of activity defines thus the optimal conditions specific to each enzyme (Copeland, 2000). For instance, APX has a higher affinity for H_2O_2 than CAT. Therefore, fine modulation of ROS might be done by APX, whereas CAT might be activated to remove ROS excess during stress (Mittler 2002).

By their important and various roles in redox homeostasis, antioxidant enzyme activities (AEA) may indicate both the occurrence of stress and the capacity of the cells to cope with it. Different organic pollutants are likely to provoke oxidative stress either directly or indirectly by disturbing cell metabolism (Kappus and Sies 1981; Rutherford and Krieger-Liszkay 2001). In ecotoxicology, AEA have been successfully used as biomarkers of oxidative stress in different aquatic organisms, as reviewed by Valavanidis et al. (2006). In algae, previous studies have shown that AEA are good indicators of cell response to oxidative stress (Geoffroy et al. 2002; Dewez et al. 2005). Geoffroy et al. (2004) showed that CAT was a more sensitive biomarker of toxicity than photosynthetic efficiency in *Scenedesmus obliquus* exposed to flumioxazin. To our knowledge, the only study reporting the measurement of AEA in freshwater biofilms pointed out that AEA were more sensitive biomarkers of copper toxicity than photosynthetic parameters (Guasch et al. 2010). Indeed, antioxidant enzymes are expected to react quickly to avoid the oxidative burst in cells and so be early warning systems.

Gene expression

While DNA contains the information coded by the genes for performing all processes a cell is able to do, RNA is the transcripted part of the DNA; that is, the genes effectively used by the cell. RNA transcription is regulated by a number of transcription factors (generally DNA interacting proteins) that are activated or

inactivated by the state of the cell and the presence (or absence) of signalling molecules, for example, substrate or toxic compounds. Though different mechanisms of regulation control the translation of RNA in active proteins, variations in the amount of a particular RNA transcript generally correspond to similar variations in the concentration of the protein encoded by this transcript. Therefore, variations in RNA transcript levels can be directly related to changes in processes encoded by the corresponding genes (Brock and Madigan 1991). A transcriptomic analysis allows RNA levels to be measured by extracting the RNA from a target biological system and hybridising it to probes of known genes. Then, the quantity of transcripts hybridised to one probe can be quantitatively measured (e.g. by Real Time Polymerase Chain Reaction: RT-PCR; Dorak 2006) or qualitatively compared to a reference (e.g. microarray; Snape et al. 2004). Microarrays contain thousands of probes spotted and can thus be used to compare simultaneously the variation levels of thousands of genes between a control and an exposed sample, for instance (Neumann and Galvez 2002). Thus, a transcriptomic analysis can give a snapshot of the state of hundreds of processes in a cell.

Since *a priori* knowledge of genetic information is needed, the application of transcriptomic analysis in biofilm communities is difficult. Indeed, most of the organisms in the community are not genetically characterised, nor is their exact distribution known (Zhou and Thompson 2002). However, Functional Gene Arrays (FGAs) based on consensus sequences from genes of key processes may partially overcome these limitations and allow gene expression of communities to be monitored (Wu et al. 2001). Consensus sequences used to design FGAs probes result from the alignment of various genes from known species and are also expected to be found in non-sequenced organisms (He et al. 2008). Such an array already exists for microbial communities (He et al. 2007). In the present study, the possibility of transcriptomic analysis for freshwater biofilms was explored.

3. The multi-biomarker approach

As biofilm communities are assemblages of different components with distinct functions in the ecosystem, the use of component-specific or species-specific biomarkers may point out some direct and indirect underlying mechanisms of toxicity, but also specific impairment in biofilm functioning. For example, Ricart et al. (2009) highlighted the indirect effect of the herbicide diuron, inhibitor of photosynthesis, on bacteria within biofilm communities.

Throughout the present study, different biomarkers were used with the aim of selecting a set of biomarkers of biofilm pertinent for toxicity assessment. Some were related to physiological functions of the autotrophic (e.g. photosynthetic parameters, pigment concentration) or the heterotrophic (peptidase activity) component of biofilm, whereas others concerned the structure of the entire biofilm (biomass) or of specific components (e.g. diatom composition, bacterial mortality, eukaryotic and prokaryotic diversity) (Fig. 3). The contribution of AEA as specific biomarkers of oxidative stress to the information obtained from these other biomarkers was evaluated in the present study.

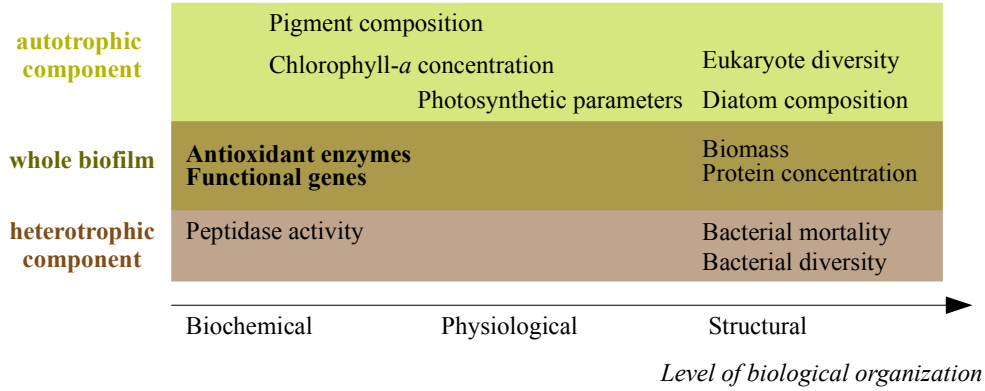


Figure 3. The different biomarkers used throughout this study, listed according to their level of biological organization and their location in biofilm community.

Biomarkers of the autotrophic component of biofilms focused on both structure and function. The abundance of photosynthetic organisms within a biofilm can be estimated through chlorophyll-*a* concentration (Sabater et al. 2007) and the relative

abundance of the different algal groups can be evaluated by other group specific pigments, such as for instance, fucoxanthin for diatoms, chlorophyll b for green algae or zeaxanthin for cyanobacteria (Jeffrey et al. 1997). Different photosynthetic parameters are often measured to assess the functioning of the autotrophic component. The photosynthetic efficiency and capacity correspond to the effective and maximal photosynthesis, respectively (Schmitt-Jansen and Altenburger 2008a). The photochemical and non-photochemical quenchings indicate the part of the light energy allocated to photochemical processes (e.g. calvin cycle) and non-photochemical ones (e.g. heat dissipation) respectively (Muller et al. 2001). The taxonomic composition of the diatom community has been widely used as a water quality indicator for different reasons. Diatoms are ubiquitous and abundant in various types of water bodies, and their specific siliceous structure allows a reliable taxonomical identification. In addition, diatom community assemblages correspond to specific water characteristics, due to their marked autoecological preferences for pH, nutrient content, habitat and light (Sabater and Admiraal 2005). Nevertheless, few studies related chemical pollution to diatom indices and caution is recommended when using diatom indices for toxicity detection (Sabater et al. 2007). Finally, the analysis of eukaryotic diversity provides structural information on the whole autotrophic component (Tlili et al. 2008).

Both structural and functional parameters are also commonly employed to characterize the heterotrophic component. The ratio between live and dead bacteria is a common measure of bacterial mortality. The analysis of the bacterial diversity provides structural information on the heterotrophic component. One of the heterotrophic component's functions is the degradation of macro-molecules in the EPS matrix via extracellular enzymes. Thus, the activity of these extracellular enzymes can indicate both heterotrophic activity and substrata availability (Romaní and Sabater 1999b). The enzyme leucine-aminopeptidase catalyzes protein degradation providing smaller peptides that micro-organisms within biofilms can assimilate more easily. This enzyme activity has also been shown to reflect algae-bacteria interactions (Francoeur and Wetzel 2003; Romaní and Sabater 1999a).

4. Organic contaminants used in the present study

Organic pollutants refer to compounds containing carbons. Herbicides and insecticides are usually found in agricultural areas while surfactants, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and others chemicals from industry (detergents, dyes, oils, solvents, resins, etc.) are more common in urban and industrial areas (Hoagland et al. 1996). In addition to these toxicants, the occurrence of pharmaceuticals in rivers has increased in the last decade (Heberer 2002). Concentrations of organic pollutants in rivers are usually relatively low, even below detection limits. However, short peaks of concentration can be observed usually related to specific events, such as accidental spillage or runoff events (Hoagland et al. 1996).

The chronic exposure of aquatic organisms to low concentrations of mixtures of organic pollutants is of major concern. First, some emerging pollutants have been shown to have significant toxicological effects on aquatic organisms at a nanogram per litre range (Sanderson et al. 2004). In addition, the toxicity of a mixture of pollutants with similar modes of action is likely to be higher than the toxicity of each compound alone, even though their concentrations are below detection limits (Arrhenius et al. 2004). Organic pollutants, such as herbicides or pharmaceuticals, are designed to be biologically active and may have expected and unexpected effects on biota. Moreover, these molecules are likely to be uptaken and/or degraded by organisms with the risk of bioaccumulation and/or toxic metabolite formation (Cáceres et al. 2008; Geyer et al. 1984; Singer et al. 2004).

Different toxicity tests were conducted in this thesis, focusing on the impact of organic contaminants on freshwater biofilms. Since previous studies pointed out the peroxidizing potential of oxyfluorfen towards non-target species, it was principally used as a “model” toxicant in this study to evaluate the potential of antioxidant enzyme activities as molecular biomarkers of oxidative stress. Glyphosate is a common herbicide found in rivers, therefore, it is likely to affect biofilms and its toxicity was

assessed. Finally the multi-biomarker approach at community-level was used to assess toxicity of four emerging contaminants: the degradation product of glyphosate AMPA and three pharmaceuticals: the β-blockers propranolol, metoprolol and atenolol.

Oxyfluorfen

Oxyfluorfen is a neutral diphenyl-ether herbicide (CAS: 42874-03-3, 2-chloro--1-(3-ethoxy-4-nitrophenoxy)-4-(trifluoromethyl) benzene) used to control pre- and post-emergent annual broadleaf and grassy weeds in a wide variety of crops (Scrano et al. 1999). Though oxyfluorfen authorization by EU expired at the end of December 2010, application for re-inclusion in the authorized list of substances has been submitted and it may be used until December 2011 (EU Pesticides database). Due to its stability and persistence in soil (Das et al. 2003), dissolved or soil-adsorbed oxyfluorfen may reach the aquatic ecosystem during runoff events. For instance, Hladik et al. (2009) reported concentrations of up to 1 μg g^{-1} of oxyfluorfen in suspended sediment samples of a Californian river collected during a storm event. Non-target species may then be exposed to this toxicant.

Due to its specific mode of action, oxyfluorfen is particularly toxic for photosynthetic organisms. It inhibits chlorophyll-*a* biosynthesis and provokes the accumulation of protoporphyrin IX (an intermediate of chlorophyll-*a* biosynthesis pathway) in the cytoplasm; as a potent photosensitizer this molecule generates high levels of oxidative stress (Aizawa and Brown 1999; Duke et al. 1991).

Previous studies highlighted toxic effects of oxyfluorfen on freshwater fish and micro-algae. It was found to be toxic for the fish *Oreochromis niloticus* with a LC_{50} (i.e. the concentration for which mortality increased by 50%) of 3 mg L^{-1} after 96h of exposure (Hassanein et al. 1999). Sub-lethal effects observed on this fish species concerned the inhibition of acetylcholinesterase activity (Hassanein 2002), the induction of stress proteins (hsp70; Hassanein et al. 1999) and the activation of some antioxidant enzyme activities in liver (Peixoto et al. 2006). Growth and photosynthetic

efficiency of the micro-algae *Scenedesmus obliquus* were also inhibited by oxyfluorfen, with an EC_{50} for growth (i.e. the concentration for which growth is reduced by 50%) of 15 µg L^{-1} (Geoffroy et al. 2003). In addition, oxyfluorfen exposure led to a significant activation of antioxidant enzyme activities in this algal species at concentration down to 7.5 µg L^{-1} (Geoffroy et al. 2002).

Glyphosate and AMPA

Glyphosate is a herbicide used worldwide. Due to its broad spectrum it is used in both crop and non-crop areas to control vegetation and kill unwanted weeds. Its concentration in rivers is in the microgram per litre range (Battaglin et al. 2005). In the natural environment glyphosate is quickly degraded to aminomethylphosphonic acid (AMPA) by bacteria (Mallat and Barceló 1998). This degradation product is more persistent and often found in rivers in areas where glyphosate is used (Pesce et al. 2008).

While the mode of action and toxicity of glyphosate has been widely studied, less information is available on AMPA. Glyphosate reduces the biosynthesis of aromatic amino acids (phenylalanine, tyrosine and tryptophane) by inhibiting the enzyme 5-enolpyruvyl-shikimate-3-phosphate synthase. Aromatic aminoacids are essential for secondary metabolism, such as, for instance, ubiquinones biosynthesis. Therefore, the inhibition of this pathway affects the whole cell metabolism (Herrmann 1995).

Glyphosate toxicity towards fish, invertebrates or micro-algae was found to depend on the physico-chemical characteristics of water (hardness, alkalinity, pH, temperature) (Henry et al. 1994; Neskovic et al. 1996). The active compound glyphosate was found to be less toxic to fish than the formulation of the herbicide, probably due to the higher toxicity of the surfactant. Indeed, a LC_{50} of 620 mg L^{-1} of glyphosate was reported for *Cyprinus carpio* L. after 96 h of exposure (Neskovic et al. 1996), whereas LC_{50} ranged between 4 and 30 mg L^{-1} for *Salmo gairdneri*,

Oncorhynchus nerka, *Ictalurus punctatus* or *Lepomis microchirus* exposed to the formulation Roundup® (Abdelghani et al. 1997; Servizi et al. 1987; Williams et al. 2000). Survival of invertebrates and particularly of zooplankton was affected by glyphosate exposure in the same concentration range as fish. For instance, after 48h of exposure to different glyphosate formulations LC_{50} for *Daphnia magna* ranged between 3 to 218 mg L^{-1} depending on the water characteristics (Folmar et al. 1979; Henry et al. 1994; Servizi et al. 1987). Sub-lethal effects of glyphosate on growth, reproduction of fish or invertebrates were rarely observed (Folmar et al, 1979; Henry et al. 1994; Morgan and Kiceniuk 1992; Tate et al. 1997). Due to the potential use of glyphosate as a carbon or nitrogen source by algae, low concentrations of glyphosate were found to have positive effects on planktonic algal growth while higher concentrations had deleterious effects. For instance, the growth and photosynthetic rate of the planktonic microalgae *Scenedesmus quadricauda* were stimulated after exposure to 0.02 mg L^{-1} of glyphosate and inhibited after exposure from 2 to 20 mg L^{-1} (Wong 2000). Similar results were found for *Selenastrum capricornutum,* exposed to the Roundup formulation of glyphosate (Abdel-Hamid et al. 1996). The EC_{50} for growth ranged from 7 to 410 mg L^{-1} depending on the algal species and the glyphosate formulation tested (Anton et al. 1993; Bozeman et al. 1989; Sáenz et al. 1997). Freshwater biofilms were also found to be affected by glyphosate (Vera et al. 2009) with EC_{50} for carbon assimilation (i.e. the concentration for which carbon assimilation is reduced by 50%) ranging between 8.9 and 89 mg L^{-1} depending on the origin of the community (Goldsborough et Brown 1988). Pesce et al. (2009) showed that environmentally relevant concentrations of glyphosate (10 µg L^{-1}) have no effects on the algal biomass of biofilm communities. However, in samples collected in summer, changes in algal community composition and eukaryotic community diversity were observed (Pesce et al. 2009).

To our knowledge, no studies have been conducted to assess AMPA toxicity towards aquatic ecosystems besides its frequent occurrence in rivers. Nevertheless, Bonnet et al. (2007) performed a series of bioassays to assess toxicity of AMPA. The

growth of the eukaryote *Tetrahymena pyriformis* (ciliate) was not affected after 9h of exposure to concentrations up to 5 g L^{-1} while the bioluminescence of the prokaryote *Vibrio fisheri* was inhibited by 50% after 15 min. of exposure to 53.4 mg L^{-1} of AMPA.

These previous studies indicate that community history may condition biofilm sensitivity to glyphosate. Therefore, in the present study toxicity of glyphosate (*N*-(Phosphonomethyl)glycine, monoisopropylamine salt solution, CAS: 38641-94-0) and AMPA ((Aminomethyl)phosphonic acid, CAS: 1066-51-9) were assessed on different biofilm communities using a multi-biomarker approach.

β-blockers

β-blockers are human pharmaceuticals widely used in therapy against hypertension or heart failure. Due to their incomplete degradation in sewage treatment plants, concentrations at the nanogram per litre range can be found in rivers and littoral waters of Europe and North America. Huggett et al. (2002) reported a concentration of propranolol of up to 1.9 µg L^{-1} in North America. In the River Llobregat (Spain), some β-blockers (propranolol, metoprolol, atenolol and sotalol) have been detected, with maximum values of 60, 180, 670 and 1820 ng L^{-1} respectively (Muñoz et al. 2009). The arrival of these products may lead to chronic contamination, with unknown impacts on aquatic ecosystems.

Previous studies revealed the toxicity of some β-blockers, particularly for fish and algae. However, the mode of action of these pharmaceuticals in non-target species has not been elucidated. An exposure of 14 days to 0.5 mg L^{-1} of the β-blocker propranolol provoked a significant reduction in the growth of the fish *Oryias latipes* (medaka) as well as changes in some hormone levels (testosterone, plasma estradiol). Longer exposure (4 weeks) to environmentally relevant concentrations of propranolol (0.5 and 1 µg L^{-1}) also affected the reproduction success of this fish (Huggett et al. 2002). Cleuvers (2005) pointed out the particular sensitivity of micro-algae to β-blockers by comparing results of standard toxicity tests on *Daphnia magna, Lemna*

minor and *Desmodesmus subspicatus.* This last species was the most sensitive, with an EC_{50} for growth of 0.7 mg L^{-1} of propranolol. Escher et al. (2006) also illustrated the phytotoxicity of 4 β-blockers in a non-target effect study based on the inhibition of the photosynthesis of green algae.

These previous studies indicate that β-blockers may be emerging contaminants. In the present thesis, the toxicity assessment of the three β-blockers: propranolol hydrochloride (CAS: 3506-09-0), atenolol (CAS: 29122-68-7) and metoprolol tartrate (CAS: 56392-17-7) was conducted using biofilm communities. This experiment was performed to illustrate the potential of a multi-species system combined with a multi--biomarker approach to assess the toxicity of emerging contaminants.

Objectives

The main objective of this study is to develop and evaluate the use of antioxidant enzymes as biomarkers of oxidative stress in biofilm communities.

The specific objectives of this study are:

1. To set up an appropriate method for extraction and activity measurements of antioxidant enzymes from biofilms.
2. To characterize the pattern of temporal variability of antioxidant enzyme activities (AEA) in biofilms commonly used as controls in long-term exposure experiments.
3. To test the potential of AEA as biomarkers of oxidative stress in biofilms by assessing the short- and long-term effects of a toxicant inducing oxidative stress (the herbicide oxyfluorfen) on biofilms.
4. To determine how environmental factors can influence AEA response. Specifically, to study the influence of light history on AEA response of biofilms exposed to herbicides.
5. To estimate the potential of a community level multi-biomarker approach, including AEA, to determine the effects of emerging contaminants (the pharmaceuticals β-blockers).
6. To explore the possibilities of other molecular biomarkers at community level. Specifically, to evaluate the possibility of measuring gene transcription in biofilm communities.

Hypotheses

Based on current knowledge and methodologies on antioxidant enzyme metabolism in algae and on biofilm ecotoxicology the following hypotheses have been formulated:

1. AEA of biofilms are biomarkers of oxidative stress induced by contaminants.
2. AEA of biofilms follow a unimodal pattern throughout exposure gradient.
3. AEA of biofilms are influenced by environmental factors such as biofilm age or light.
4. Biofilms grown under high oxidative stress (high light intensity, presence of a peroxidising chemical) are more tolerant to oxidative stress induced by chemicals.
5. A multi-biomarker approach at community-level reveals unexpected effects of emerging chemicals on non-target organisms.
6. Gene expression can be measured in biofilm communities using custom DNA array.

Project development

The development of this project involved mainly experimental research on biofilms grown in indoor microcosms.

The first step was methodological, dedicated to the selection of the most appropriate extraction method for antioxidant enzymes from freshwater biofilms. Four commonly used methods were compared focusing on catalase (CAT) activity and the most appropriate one was then applied to the extraction of other antioxidant enzymes: ascorbate peroxidase (APX), superoxide dismutase (SOD) and glutathione reductase (GR).

Then the potential of AEA as biomarkers of oxidative stress induced by toxicants was evaluated by measuring CAT and APX activities in biofilms throughout a five-week exposure to a toxicant expected to provoke oxidative stress: the herbicide oxyfluorfen. The experiment also allowed the natural variability of CAT and APX throughout time to be explored and compared to other biomarkers of biofilm ageing.

In a following experiment, the role of light intensity as a confounding factor was explored. Biofilms grown under different light intensities were used to assess CAT, APX, GR and SOD activities changes in response to 6h of exposure to other light intensities or to the herbicides: oxyfluorfen, copper or AMPA. These toxicity tests allowed investigating the role of pre-exposure conditions (different light intensities) on biofilm capacity to answer to further stress.

To illustrate the potential of a multi-biomarker approach at community level in toxicity assessment, a set of biofilm biomarkers, including CAT activity, was used to assess the toxicity of three emerging contaminants: the β-blockers: propranolol, metoprolol and atenolol.

The last part of the project explored the possibility of using transcriptomic analyses within freshwater biofilms as a perspective for the development of other molecular biomarkers within biofilms. A functional gene array for biofilms was developed and its specificity was tested using biofilms and two micro-algae

(*Chlamydomonas reinhardtii* and *Scenedesmus vacuolatus*). This last experiment was done at the Department of Bioanalytical Ecotoxicology of the Helmholtz Centre for Environmental Research – UFZ (Germany) under the supervision of Dr. Schmitt-Jansen and Dr. Scholz.

Methodology used

The different methodologies used throughout the project are outlined below and described in detail in the specific chapters:

Antioxidant enzymes:

- Protein extraction (comparison of different methods)
- Protein quantification (Bradford 1976)
- CAT, APX, SOD and GR activity measurements by spectrophotometry (Aebi 1984; McCord and Fridovich 1969; Nakano and Asada 1981; Schaedle and Bassham 1977)

Biomarkers of the autotrophic component of biofilm:

- Measurement of photosynthetic parameters (efficiency, capacity, photochemical quenching, non-photochemical quenching) using PhytoPAM (Pulse Amplitude Modulated) fluorometer (Bilger and Björkman 1990; Genty et al. 1989; Schreiber et al. 1986)
- Pigment extraction and analyses by HPLC (Jeffrey et al. 1997)
- Chlorophyll-*a* extraction and analyses by spectrophotometry (Jeffrey and Humphrey 1975)

Microarray:

- RNA extraction, integrity and quality check on agarose gel
- RNA labelling and hybridisation on microarrays
- Microarray scanning

Physical and chemical water analyses:

- Dissolved oxygen concentration, pH, conductivity and temperature of water in channel, measured using probes
- Soluble reactive phosphorus (SRP) analysis following Murphy and Riley (1962)
- Analysis of β-blockers concentration in water by HPLC (Delamoye et al. 2004)

Statistical analyses:

The majority of the statistical analyses were performed with the software R 2.6.2 (R Development Core Team, 2008). Methodologies used involved:

- Tests of significance (ANOVA, Mann-Whitney)
- Linear and non-linear regression (Scholze et al. 2001)
- NEC and EC_{50} calculations (values and confidence intervals) (Liber et al. 1992)
- Multivariate analyses (simple, between and within Principal Component Analyses; Redundancy Direct Analyses)
- Clustering

In addition to these methods, other techniques performed by collaborators were used in this project in order to obtain complementary data. These include metal analyses in water, determination of eukaryotic and bacterial diversity by DGGE analysis, measurement of peptidase activity and bacterial live/dead ratio, determination of taxonomic diatom composition, diatom biovolume measurement and calculation of diversity index.

Chapter I

Catalase in fluvial biofilms:

a comparison between different extraction methods and example of application in a metal-polluted river

Bonnineau, C., Bonet, B., Corcoll, N., Guasch, H. 2010

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Abstract

Antioxidant enzymes are involved in important processes of cell detoxification during oxidative stress and have, therefore, been used as biomarkers in algae. Nevertheless, their limited use in fluvial biofilms may be due to the complexity of such communities. Here, a comparison between different extraction methods was performed to obtain a reliable method for catalase extraction from fluvial biofilms. Homogenization followed by glass bead disruption appeared to be the best compromise for catalase extraction. This method was then applied to a field study in a metal-polluted stream (Riou Mort, France). The most polluted sites were characterized by a catalase activity 4 to 6 times lower than in the low-polluted site. Results of the comparison process and its application are promising for the use of catalase activity as an early warning biomarker of toxicity using biofilms in the laboratory and in the field.

Keywords: biofilm, catalase, extraction, biomarker, metal.

1. Introduction

Oxidative stress is a common form of stress due to the accumulation of reactive oxygen species (ROS) in cells. H_2O_2 is a ROS produced by organisms in normal metabolism such as photosynthesis or respiration but also in stress metabolism induced by natural or chemical disturbances (Mittler 2002). In cells, levels of H_2O_2 have to be tightly regulated. On the one hand, H_2O_2 accumulation may cause great damage to the cell due to its high capacity of unrestricted oxidation (Edreva 2005). On the other hand, H_2O_2 is an essential signalling molecule in different pathways as, for instance, in defence reactions against pathogens or regulation of cell expansion in higher plants (Laloi et al. 2004). Among the antioxidant mechanisms developed by cells to maintain redox homeostasis, the antioxidant enzyme catalase (CAT, EC 1.11.1.6) is one of the most efficient enzymes in degrading hydrogen peroxide (H_2O_2) in water and oxygen (Lesser 2006) and has been extensively studied (Chelikani et al. 2004). CAT enzymes are predominantly located in peroxisomes of the cells (De Duve and Baudhuin 1966; Stabenau 1984). Due to its relatively low affinity for H_2O_2, CAT is mainly involved in the removal of excess H_2O_2 during important oxidative stress (Mittler 2002). As a change in redox balance can be dramatic for the cell, changes in CAT level may reflect environmental disturbances. CAT inhibition may suggest the presence of disturbances that reduce the cell's ability to cope with oxidative stress, while an increase in CAT activity may suggest an active response of cells to oxidative stress. Both CAT inhibition and activation provide useful information to detect environmental disturbances and estimate cell resistance to oxidative stress. CAT activity may be inhibited by different factors, such as a very high concentration of H_2O_2 (Lardinois et al. 1996) or an excess of light. Moreover, environmental perturbations (osmotic stress, changes in temperature) can reduce the usually high turnover of CAT and, therefore, decrease its activity (Lesser 2006). CAT activity, like other antioxidant enzyme activities, may complement the information obtained by classical endpoints (growth, mortality) and be successfully used in toxicity assessment

in algae (Contreras et al. 2009; Geoffroy et al. 2004; Liu and Xiong 2009; Nie et al. 2009; Qian et al. 2008, 2009). For example, Dewez et al. (2005) pointed out that CAT was a more sensitive biomarker of fludioxionil toxicity than photosynthetic parameters in *Scenedesmus obliquus* (Dewez et al. 2005). In addition, Geoffroy et al. (2004) highlighted the higher sensitivity of CAT than other antioxidant enzymes (ascorbate peroxidase, glutathione reductase) in *Scenedesmus obliquus* exposed to the herbicide flumioxazin.

Although these studies emphasized the interest of CAT as a biomarker of oxidative stress in toxicity assessment, they were performed on mono-specific cultures of algae and, therefore, do not allow the effects of these toxicants to be understood at community level. Freshwater ecology studies performed at community level (e.g. using freshwater biofilms) provide a more realistic approach to assess the effects of toxicants (Sabater et al. 2007). Biofilms are complex communities composed of green algae, diatoms (brown algae), cyanobacteria, bacteria, protozoa and fungi; these micro--organisms live closely together embedded in an extracellular matrix (Romaní 2010). The extraction of CAT in such a community is challenging due to the diversity in cells walls and micro-organism size. For instance, silicate skeleton and cellulosic cell walls of diatoms and green algae, respectively (Mackie and Preston 1974; Soininen 2007), may be more difficult to break than the cell walls of bacteria composed of peptidoglycans and/or phospholipids (Schleifer and Kandler 1972). An efficient extraction method should maximize the quantitative extraction of CAT from all the different micro-organisms of biofilm communities. To our knowledge, only two previous studies related the use of antioxidant enzymes in biofilms (Bonnineau et al. 2010; Guasch et al. 2010). Although both studies highlighted the potential of antioxidant enzymes as biomarkers of toxicity for copper (Guasch et al. 2010) or for the β-blocker propranolol (Bonnineau et al. 2010), the method used for extraction in these studies (homogenization with a glass tissue grinder) presented limitations. In Guasch et al. (2010) a high amount of biofilm surface (100 cm^2) was required for extraction. The reduction of the amount of biofilm needed for CAT extraction would

improve the feasibility of the method both in the laboratory, where space is limited, and in the field, where biofilm biomass is subject to strong variations (Romaní and Sabater 2001). In the second study, the use of the same extraction method but with less biomass led to highly variable CAT measurements, probably due to a low concentration of CAT in the final extract (Bonnineau et al. 2010). Both examples also illustrate the need to improve the method of CAT extraction in biofilm communities by minimizing the amount of biomass needed and maximizing CAT concentration in enzymatic extracts.

The main objective of the present study was to find an appropriate method for the extraction of CAT from fluvial biofilms. More specifically, the study aimed to:

1. Improve the extraction procedure in order

    - to maximize the quantity of protein extracted and the specific CAT activity in the enzymatic extract

    - to minimize the amount of biomass required.

2. Test the applicability of the selected procedure to assess exposure effects in naturally occurring biofilms.

The present study focused on CAT activity as it is the main antioxidant enzyme in plant (Geoffroy et al. 2003) and has been shown to be especially sensitive to toxicant exposure in algae (Geoffroy et al. 2004). However previous studies had shown the importance of other antioxidant enzymes as biomarkers of oxidative stress (Contreras et al. 2009; Geoffroy et al. 2004; Liu and Xiong 2009; Nie et al. 2009; Qian et al. 2008, 2009). The extraction method developed for CAT is expected to provide basis for the study of other antioxidant enzymes within biofilms.

Different methods (trituration, ultrasonication, homogenization and homogenization followed by glass bead disruption) were selected based on current methods used for antioxidant enzyme extraction in microalgae or bacteria (Choo et al. 2004; Janknegt et al. 2007; Tang et al. 1998; Wang et al. 2006) or used for DNA extraction from soil bacterial communities (Bäckman et al. 2003). These extraction

techniques require a priori a lower amount of biomass and a smaller volume of extraction buffer than the homogenization with a glass tissue grinder used in previous studies on biofilms (Bonnineau et al. 2010; Guasch et al. 2010). Extraction by ultrasonication and by trituration were compared and the best extraction methods between these two was compared to extraction by homogenization and homogenization followed by glass beads disruption. Efficiencies of methods in terms of protein extraction and CAT extraction were compared as well as their reproducibility. The comparison was done using biofilms grown in the laboratory under controlled conditions. CAT activities were then measured in natural biofilms growing in a metal--polluted river (Riou Mort, France) and compared to a more classical endpoint: photosynthetic efficiency.

2. Material and methods

2.1 Method comparison in laboratory biofilms

The comparison between the different methods of extraction was performed with laboratory biofilms under realistic experimental conditions. After colonization, biofilms were briefly exposed either to their colonization light intensity or to strong light intensity and after exposure they were sampled for CAT extraction. As CAT is subject to photoinhibition, strong light intensity exposure is expected to reduce its activity (Feierabend and Engel 1986). The sensitivity of the extraction methods: trituration, homogenization and homogenization followed by glass bead disruption, for detecting CAT activity under such an extreme situation was also tested.

2.1.1 Biofilm colonization

Colonization was performed in crystallizing dishes as described previously (Bonnineau et al. 2010). Briefly, biofilm communities colonized 1 cm^2 of sandblasted glass substrata installed in crystallizing dishes of 2 L. During the entire colonization process, an aquarium pump enabled circulation of water to simulate flowing water at constant velocity. Biofilms were incubated at 19ºC and under a 12/12 h day–night

cycle (120 µmol photons m^{-2} s^{-1}). An inoculum of biofilm, obtained by scraping cobbles from the Llémena River (NE Spain, Serra et al. 2009a), was added weekly to each dish. Dechlorinated tap water was used as culture media and changed twice a week. Nitrogen content of water was at 25 µmol of N L^{-1} (Serra et al. 2009b). Therefore, at each water renewal phosphate was added to a final nominal concentration of 158 µg L^{-1} (1.64 µmol of P L^{-1}) to avoid nutrient depletion and P or N limitation (Hillebrand and Sommer 1999).

2.1.2 Experimental set up

After 3 weeks of colonization, each glass substrata was transferred into a vial containing 10 mL of media. Samples were then incubated for 8h under colonization light intensity or strong light intensity (900 µmol photons $m^{-2}s^{-1}$). The other parameters were similar to colonization. A single-speed orbital mixer (KS260 Basic, IKA®) was used to maintain constant agitation. Control biofilms (referred to as controls) and biofilms exposed to strong light intensity (referred to as exposed biofilms) were sampled after 2 and 8 hours of exposure, one sample consisted of 2 glass substrata of 1 cm^2. Samples exposed to the same treatment and extracted by the same method were considered as experimental replicates. Control biofilms were used to compare extraction methods by trituration (4 replicates) and ultrasonication (3 replicates) while exposed biofilms were used to compare extraction methods by trituration (3 replicates), homogenization (3 replicates) and homogenization and glass beads (2 replicates).

2.1.3 Biofilm sampling

For each sample, the biofilm was removed from the glass substrata with a cell scraper (Nunc, Wiesbaden, Germany) and put into an eppendorf tube. After centrifugation at 2300 g and at 10ºC for 5 min, the excess water was removed, and samples were weighted (wet weight) and frozen immediately in liquid nitrogen. Finally, samples were stored at -80ºC until protein extraction and enzymatic assays had been carried out.

2.1.4 Protein extraction

The present study focused on comparing mechanical extraction techniques and not chemical ones. The same extraction buffer (containing 100 mM Na_2HPO_4/KH_2PO_4, pH 7.4, 100 mM KCl, 1 mM EDTA) was thus used in the different protocols adapted from existing methods. Samples were kept on ice during extraction by the different methods described as follows.

Trituration under liquid nitrogen (adapted from Choo et al. 2004 and Wang et al. 2006): the frozen sample was placed in a mortar and ground to powder with a pestle adding liquid N_2 when needed. Then, the powder was transferred to an eppendorf tube where 200 µL of extraction buffer were added for 100 mg of wet weight of sample.

Ultrasonication (adapted from Janknegt et al. 2007): the frozen sample was resuspended in 400 µL of extraction buffer for 100 mg of wet weight of samples and sonicated applying 2 pulses of 30 s and 25 µm of amplitude (Labsonic 2000, B.BRAUN).

Homogenization (adapted from Bäckman et al. 2003): 200 µL of extraction buffer were added for each 100 mg of wet weight of sample, and then samples were homogenized by applying 2 pulses of 30 s of homogenizer (DIAX900, Heidolph) with 1 min interval on ice.

Homogenization and disruption with glassbeads (adapted from Bäckman et al. 2003 and Tang et al. 1998): after homogenization, as described above, 100 mg of glass beads (≈500µm of diameter) were added for each 100 mg of wet weight of samples and further cell disruption was performed through 3 pulses of 30 s of beadbeater (MP FastPrep-24, $v = 4\ m\ s^{-1}$) with 5 min intervals on ice.

After cell disruption, homogenates were centrifuged at 10.000 g and 4ºC for 30 min. Supernatants were used as the enzyme source. For each sample, the protein content of supernatant was measured spectrophotometrically (Elx800, BioTek Instruments) in triplicates (referred to as analytical replicates) by the method of

Bradford (Bradford 1976) using dye reagent concentrate from Bio-Rad (Laboratories GmbH, Munich, Germany) and bovine serum albumin as a standard.

2.1.5 Catalase activity measurement

CAT activity was measured spectrophotometrically (UV/Vis Lambda Bio 20, Perkin Elmer) according to Aebi (Aebi 1984) by following the linear decrease in absorbance at 240 nm corresponding to the decomposition of H_2O_2 by CAT. In direct enzymatic assay, substrate concentration has to be in excess throughout the entire assay to avoid limiting the reaction rate. The compromise between substrate concentration and protein quantity defines a linear range of protein quantities for which specific activity is maintained (Palmer 1991). As CAT is inhibited by a high amount of H_2O_2 (Chelikani et al. 2004), the optimal substrate concentration would be the one leading to the highest specific activity and not necessarily the highest one. In this context, a preliminary test with three samples of control biofilm extracted by trituration allowed the optimal substrate concentration and the optimal protein content to be determined. Among the different final concentrations of H_2O_2 tested (2, 10, 15, 20, 25 and 30 mM), 20 mM of H_2O_2 was found to be the optimal one. The three quantities of protein tested (5, 10 and 20 µg) led to similar specific CAT activity and were all in the linear range of protein quantity. Therefore, for each sample, CAT activity was measured in triplicates (referred to as analytical replicates), as follows: the 800 µL reaction mixture contained in a final concentration: 80 mM potassium phosphate buffer (pH 7.0) and the enzyme extract (between 5 and 10 µg of proteins). The reaction was started by adding H_2O_2 at a final concentration of 20 mM. Enzymatic activity was measured after monitoring the decrease in absorbance at 25ºC for 2 min, at the end of which linearity was shown. The specific CAT activity is the amount of H_2O_2 converted per unit of time and per unit of protein in one sample and is expressed in µmol H_2O_2 mg protein^{-1} min $^{-1}$. The total CAT activity is the total amount of H_2O_2 converted per unit of time by one sample and is expressed as µmol H_2O_2 min^{-1} (extinction coefficient: 0.039 cm² µmol^{-1}). Both were calculated in the present study.

2.2 Field study

2.2.1 Study site

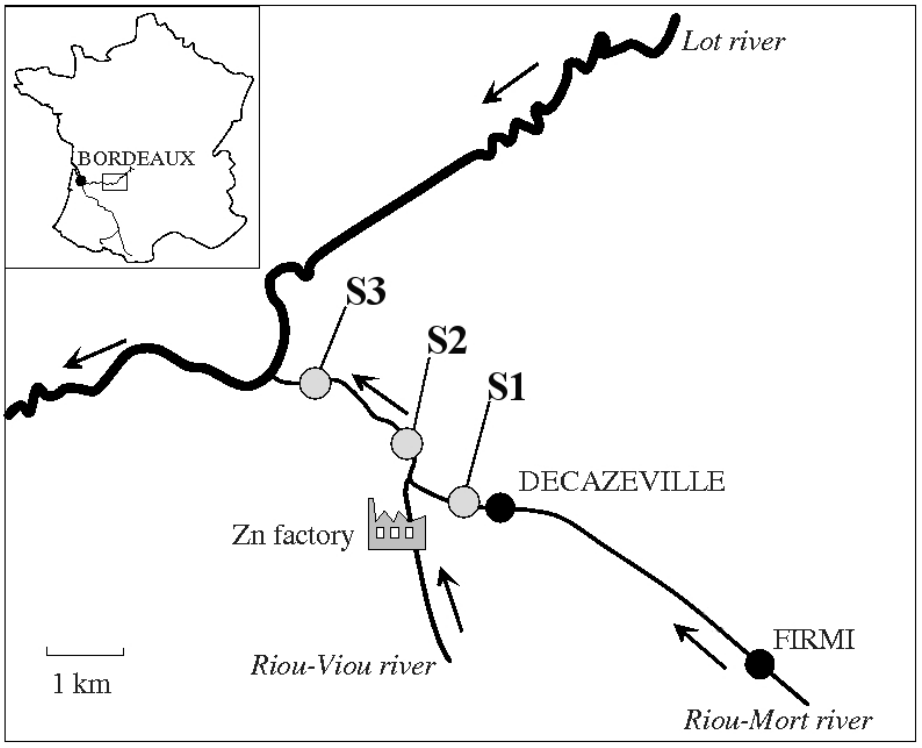

Figure 1. Localisation of sampling sites (S1, S2, S3) on the Riou Mort (adapted from Morin et al. 2008)

This field study was carried out in the Riou Mort (SW Aveyron department, France), a small tributary of the Lot River located in an industrial basin. This stream is highly contaminated by different metals from its confluence with the Riou-Viou, a stream carrying seepage from a former active zinc factory (Morin et al. 2008). Sampling was performed at three sites, one before the confluence with Riou-Viou (S1, Fig. 1) and two after it (S2, S3, Fig. 1).

2.2.2 Sampling

At each sampling site, biofilm and water samples for metal analysis were collected in triplicate. Water samples (5 mL) were immediately filtered (Whatman nylon filters 0.2 µm) and acidified with 1% of HNO_3 (65 % suprapure, Merck) before

measurements of total dissolved metal concentration. For CAT activity, three samples of biofilm were collected by scraping 4 cm^2 from different cobbles with a cell-scraper (Nunc, Wiesbaden, Germany). Since centrifugation in the field was not possible, the excess of water was eliminated using absorbent paper. Dried samples were frozen immediately in liquid nitrogen, and samples were stored at -80ºC until extraction.

2.2.3 Protein extraction and CAT activity measurement

Protein extraction and quantification were performed as indicated above (2.1.4), the extraction method used was homogenization followed by glass bead disruption. CAT activity measurements were performed in microtiter plates (UV-Star 96 well plate, Greiner®). The use of microtiter plates in enzymatic assay reduces the assay time and allows a high number of samples to be measured in parallel. Moreover, CAT assay in microtiter plates based on the measure of the decrease in absorbance of H_2O_2 has been previously validated with *E. coli* by Li and Schellhorn (2007). Preliminary tests with field biofilms from S1 allowed the optimal substrate concentration and protein quantity to be determined. Among the different final concentrations of H_2O_2 tested (10, 15, 20, 30 and 40 mM), 30 mM of H_2O_2 was found to be the optimal one (leading to the highest specific CAT activity). Among the different quantities of protein tested (0.5, 1, 2, 4, 8 and 10 µg), 2 µg was found to be in the linear range of response of CAT activity. After adaptation to the microtiter plate volume, the 250 µL reaction mixture contained 80 mM of potassium phosphate buffer (pH 7.0) and 2 µg of proteins in a final concentration. Reaction was started by adding H_2O_2 in a final concentration of 30 mM. The decomposition of H_2O_2 was determined by measuring the linear decrease in absorbance at 25ºC for 2 min using a microtiter plate reader Synergy4 (BioTek®). Total (µmol H_2O_2 min^{-1}) and specific (µmol H_2O_2 mg $protein^{-1}$ min^{-1}) CAT activity were calculated.

2.2.4 Measure of photosynthetic efficiency

Estimation of photosynthetic efficiency was done by measuring the chlorophyll–*a* fluorescence of biofilm with a PhytoPAM (Pulse Amplitud Modulated) fluorometer (version EDF, Heinz Walz GmbH). In order to obtain replicate measurements, chlorophyll-*a* fluorescence was measured from three cobbles per site. Measurements were done in vivo in dark conditions; the distance between the optical fiberoptics and the sample surface being set at 4 mm. The fluorescence signal recorded at 665 nm was used to calculate the effective PSII quantum yield parameter according to Genty et al. (1989). The effective PSII quantum yield is an indicator of the efficiency of PSII and is expressed in relative units of fluorescence (r.u.).

2.2.3. Metal analysis

The concentration of dissolved metals in water was determined by inductively coupled plasma mass spectroscopy ICP-MS (7500c Agilent Technologies, Inc. Wilmington, DE). The detection limits were 148.91 µg Al L^{-1}, 70.75 µg Fe L^{-1}, 86.46 µg Zn L^{-1}, 0.00 µg Cd L^{-1}, 0.00 µg Ni L^{-1}, 0.00 µg Cu L^{-1} and 0.00 µg Pb L^{-1}. The accuracy of the analytical method was checked periodically using certified references for water (SPS-SW2 Batch 113, Oslo, Norway), the uncertainties (half width of the 95% confidence intervals) were of 1, 1, 1, 0.02, 0.3, 1 and 0.1 µg L^{-1} for Al, Fe, Zn, Cd, Ni, Cu and Pb, respectively.

2.3 Data analyses

All statistical analyses were done using R 2.6.2 (R development Core Team, 2008). Mean values are always presented with the corresponding standard errors.

2.3.1 Methods comparison in laboratory biofilms

To estimate the influence of sampling time, specific CAT activity of samples collected after 2h and 8h of exposure and extracted by trituration were compared. Statistical differences were tested by a Mann-Whitney test (Bauer 1972; Hollander and Wolfe 1973). To determine the significance of the results, the U value obtained from the test was compared to the critical value of the U-distribution table for $\alpha = 0.1$.

The relationship between the total quantity of protein extracted and the initial wet weight of samples was estimated by calculating the Spearman coefficient and corresponding p-value (Hmisc package, Harrell, 2007).

To compare method efficiencies, differences in total quantity of protein extracted, quantity of protein extracted per wet weight, specific and total CAT activities were tested using the Mann-Whitney test as described earlier. Control samples extracted by ultrasonication and by trituration were first compared, and then exposed samples extracted by trituration, homogenization and homogenization with glass beads, were compared two at a time.

In the present study, the intra-sample variability of the quantity of protein extracted per wet weight and of the specific CAT activity was defined as the closeness of agreement between analytical replicates performed under the same conditions and was measured as the standard deviation divided by the mean of the analytical replicates of one sample and expressed in percentage. The inter-samples variability of each method in terms of quantity of protein extracted per wet weight and specific CAT activity was defined as the closeness of agreement on these variables between samples from a same treatment. Inter-samples variability was measured as the standard deviation divided by the mean of the samples of one method and expressed in percentage.

To estimate light effect on CAT activity, specific CAT activity of control and exposed samples extracted by trituration were also compared by a Mann-Whitney test as explained previously.

2.3.2 Application to field study

To integrate mixture effects of metals present in the field, the cumulative criterion unit (CCU) was calculated for each sampling point as described by Guasch et al. (2009). The following equation was used:

$$CCU = \sum \frac{m_i}{c_i}$$

where m is the dissolved metal concentration and c the criterion value for each metal *i*. According to water hardness in the Riou Mort (around 100 mg $CaCO_3$ L^{-1}), criterion values for each metal were: 1000 µg L^{-1} for Fe, 96 µg L^{-1} for Ni, 106 µg L^{-1} for Zn and 1.1 µg L^{-1} for Cd. Concentrations below detection limits (Al, Cu and Pb) were not included in the calculation. Differences between sampling sites in terms of photosynthetic efficiency, specific CAT activity and CCU were tested by a Mann--Whitney test as explained previously. Spearman coefficient and its associated p-value were calculated to estimate linear correlation between specific CAT activity or photosynthetic efficiency and CCU or metal concentration in water.

3. Results

3.1 Methods comparison in laboratory biofilms

3.1.1 Biofilm colonization

Physical and chemical parameters were stable during colonization. Temperature was 19.7 ± 0.2 ºC, dissolved oxygen concentration: 9.2 ± 0.2 mg L^{-1}, water conductivity: 494 ± 8 µS and pH: 8.4 ± 0.1 (n=36 for all parameters). Water used during this experiment had been previously characterized (Serra et al. 2009b); therefore, only phosphate concentration was measured. Total phosphate depletion was never observed during colonization although phosphate concentrations after water changes (87 ± 12 µg L^{-1}, n=9) declined to low levels before water changes (7 ± 1 µg L^{-1}, n=9).

3.1.2 Sensitivity and repeatability of measurements of protein content and specific CAT activity

The quantity of biofilm obtained after scraping 2 cm^2 of substrata was on average 234 ± 24 mg (n=15) of wet weight. This quantity was sufficient for protein extraction and CAT measurement for all extraction methods tested. A positive correlation was found between the quantity of protein extracted and the wet weight of the samples ($\rho = 0.79$, $p < 0.05$), including results from all methods tested, except those from extraction by ultrasonication (Fig. 2).

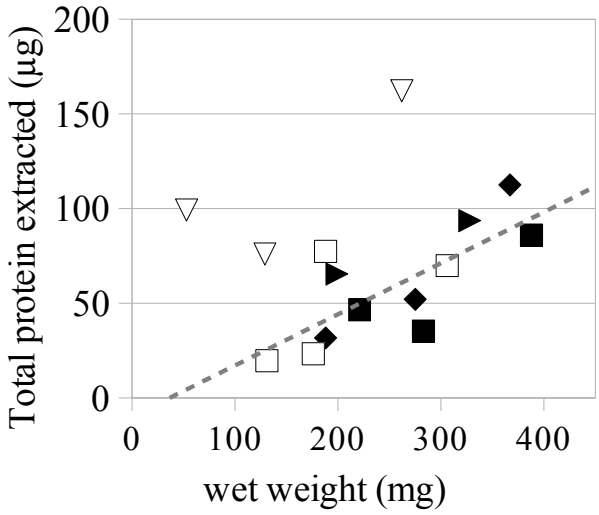

Figure 2. Total protein extracted in function of wet weight of control samples (in white) extracted by trituration (□) or by ultrasonication (∇) and of exposed samples (in black) extracted by trituration (■), homogenization (♦) or homogenization and glass beads (►). The positive correlation between total protein extracted and wet weight of samples extracted by trituration, homogenization and homogenization and glass beads is shown by a grey dotted line.

No significant differences between methods were found for intra-sample variabilities of quantity of protein extracted per wet weight and specific CAT activity ($U > 0$ in all comparisons, $\alpha = 0.1$). For the measure of protein content, intra-sample variability was below 12 % for all methods. For the measure of specific CAT activity, intra-sample variability was below or around 10 % for all methods except for samples extracted by ultrasonication (intra-sample variability = 20.2 ± 11.9 %). This relatively high percentage is mainly due to one sample reaching 44.0 % for a specific CAT activity = 15 µmol H_2O_2 mg protein^{-1} min^{-1} (Table 1).

Table 1. Intra- and inter-samples variabilities (in percentage of the mean) of the measurements of the quantity of protein extracted per wet weight and of the specific CAT activity of control biofilms extracted by trituration and ultrasonication and of exposed biofilms extracted by trituration, homogenization and homogenization followed by glass bead disruption.

Treatment	Extraction method	Protein extracted / wet weight (µg g^{-1})		Specific CAT activity (µmol H_2O_2 mg prot.$^{-1}$ min^{-1})	
		Intra-sample variability	Inter-samples variability	Intra-sample variability	Inter-samples variability
Control	Trituration	9.3 ± 4.1	55.5	9.2 ± 1.9	18.8
	Ultrasonication	2.6 ± 1.9	71.3	20.2 ± 11.9	2.7
Exposed to strong light intensity	Trituration	11.7 ± 7.1	28.7	4.2 ± 1.5	18.9
	Homogenization	3.6 ± 1.2	33.6	8.4 ± 3.4	65.8
	Homogenizaton + glass beads	3.4 ± 1.7	10.0	10.1 ± 4.4	26.8

Intra- and inter-samples variabilities refer to variabilities within analytical and experimental replicates, respectively. For intra-sample variability mean and standard error of experimental replicates are indicated.

3.1.3 Comparison of different extraction procedures

Analysis of results obtained by extraction by trituration showed no differences ($U = 3$, $\alpha = 0.1$) in CAT response between the two different sampling times (data not shown). Consequently, samples collected after 2h and after 8h were not differentiated when comparing the different extraction methods.

Extraction by ultrasonication was compared to extraction by trituration in control samples. Neither the total quantity of protein nor the protein concentration in samples extracted by ultrasonication were significantly different from the values obtained for samples extracted by trituration (Table 3). However, the protein quantity extracted per biomass was significantly higher in samples extracted by ultrasonication than in those extracted by trituration (Table 2 and 3). Though total CAT activity was not significantly different between samples extracted by one or the other method (Table 3), specific CAT activity was 2.8 times lower in samples extracted by ultrasonication than in those extracted by trituration (Table 2 and 3).

Extractions by homogenization and by homogenization followed by glass bead disruption were compared to extraction by trituration in samples exposed to strong light intensity. No significant differences were found between extraction by trituration and extraction by homogenization in terms of total quantity of protein extracted, quantity of protein extracted per wet weight, protein concentration and specific and total CAT activities (Table 3). The additional step of disruption with glass beads led to a significant increase in quantity of protein extracted per wet weight, in protein concentration and in specific and total CAT activities compared to extraction by trituration (Table 2 and 3).

Table 2. Quantity of protein extracted per wet weight, total protein extracted, protein concentration (conc.), total and specific CAT activity of control biofilms extracted by trituration and ultrasonication and of exposed biofilms extracted by trituration, homogenization and homogenization followed by glass bead disruption.

Treatment	Extraction method	Protein extracted / wet weight (µg g^{-1})	Total protein extracted (µg)	Protein conc. (µg mL^{-1})	Total CAT activity (µmol H_2O_2 min^{-1})	Specific CAT activity (µmol H_2O_2 mg $prot.^{-1}$ min^{-1})
Control	Trituration	230 ± 64	47 ± 15	115.2 ± 32.0	2.1 ± 0.7	46.1 ± 4.3
	Ultrasonication	1027 ± 423	112 ± 26	256.7 ± 105.7	1.7 ± 0.4	15.6 ± 0.2
Exposed to strong light intensity	Trituration	185 ± 31	56 ± 15	92.8 ± 15.4	1.4 ± 0.4	25.9 ± 2.8
	Homogenization	221 ± 43	65 ± 24	110.7 ± 21.5	3.8 ± 2.6	46.1 ± 17.5
	Homogenization + glass beads	307 ± 22	79 ± 14	153.5 ± 10.9	4.1 ± 0.1	53.8 ± 10.0

For all variables mean and standard error (SE) of experimental replicates are indicated.

Table 3. U values from Mann-Whitney test resulting from the comparison of the two-at-a-time extraction methods in terms of quantity of protein extracted per wet weight, total protein extracted, protein concentration (conc.), total and specific CAT activity of experimental replicates.

Methods compared	Protein extracted / wet weight	Total protein extracted	Protein conc.	Total CAT activity	Specific CAT activity
Trituration - Ultrasonication	**0**	1	2	6	**0**
Homogenization - Trituration	4	4	4	4	2
Homogenization + glass beads - Trituration	**0**	1	**0**	**0**	**0**
Homogenization + glass beads - Homogenization	1	2	1	2	2

Significant values are indicated in bold ($\alpha = 0.1$).

Regarding the quantity of protein extracted per wet weight, homogenization followed by glass bead disruption led to the lowest inter-samples variability followed by homogenization, trituration (mean of inter-samples variability for control and exposed samples: 42.1 ± 13.1 %) and ultrasonication. Regarding specific CAT activity measurements, the lowest inter-samples variability was observed in samples extracted by ultrasonication followed by those extracted by trituration (mean of inter-samples variability for control and exposed samples: 18.8 ± 0.1 %), homogenization followed by glass bead disruption and simple homogenization.

3.2 Field study

Inter-samples variabilities of specific CAT activity measurements from field samples were 26.4, 121.4 and 33.7 % for samples from S1, S2 and S3, respectively. The highest inter-samples variability (121.4 %) was observed for the lowest specific CAT activity measured (26.2 ± 22.5 μmol H_2O_2 min^{-1} mg $proteins^{-1}$ observed at S2). Inter-samples variabilities of quantity of protein extracted per wet weight were 33.0, 33.4 and 5.8 % for samples from S1, S2 and S3, respectively.

The three study sites presented high differences in both metal concentrations and specific CAT activity and smaller differences in photosynthetic efficiency (Table 4). Concentrations of Al, Cu and Pb were below detection limits at all sites. Pollution at S1 was mainly due to iron, and high concentrations of Zn, Ni and of the heavy metal cadmium were found at S2 and S3 (Table 4). Consequently CCU values were significantly different in all sites ($U = 0$ when comparing S1-S2, S1-S3 and S2-S3, $\alpha = 0.1$), S1 was characterized by an intermediate CCU value and S2 and S3 by high CCU values (Table 4). The total quantity of protein extracted, the quantity of protein extracted per wet weight as well as the specific and total CAT activities followed the inverse pattern, values of those variables in biofilm from S1 were significantly higher than those observed in S2 ($U = 0$, $\alpha = 0.1$) and S3 ($U = 0$, $\alpha = 0.1$; Table 4). Biofilms from S2 and S3 had similar total quantity of protein extracted and similar total and specific CAT activities but the quantity of protein extracted per wet weight was significantly higher in S3 than in S2 ($U = 0$, $\alpha = 0.1$). A significant negative correlation was found between CAT activity and CCU in water ($\rho = -0.86$, $p < 0.05$). Similar correlation was also found between specific CAT activity and Ni, Zn and Cd concentrations in water (for all: $\rho = -0.82$, $p < 0.05$), whereas the correlation between CAT and Fe concentration in water was not significant. Small, but significant differences ($U = 0$ when comparing S1-S2, S1-S3 and S2-S3, $\alpha = 0.1$) were found in photosynthetic efficiencies of biofilms from each site (Table 4). However, no significant correlations were found between photosynthetic efficiency and CCU or Ni, Zn or Cd concentrations in water ($p > 0.1$).

Table 4. Concentration of metals dissolved in water and their corresponding CCU as well as the quantity of protein extracted per wet weight, the total quantity of protein extracted and the total and specific CAT activities for each site.

		S1	S2	S3
Metals dissolved in water	Fe (µg L^{-1})	4532 ± 46.1	3303.7 ± 10.0	3018 ± 57.3
	Ni (µg L^{-1})	5 ± 0.3	29 ± 0.1	14.2 ± 0.8
	Zn (µg L^{-1})	b.d.l.	943.6 ± 17.6	555.5 ± 12.9
	Cd (µg L^{-1})	b.d.l.	13.9 ± 0.5	12.2 ± 0.4
	CCU	4.6 ± 0.0	25.1 ± 0.6	19.5 ± 0.5
Biofilm variables	Photosynthetic efficiency	0.329 ± 0.002	0.286 ± 0.003	0.359 ± 0.004
	Total protein extracted (µg)	635.2 ± 231.6	32.1 ± 5.6	40.7 ± 20.8
	Protein extracted / wet weight (µg g^{-1})	521.3 ± 99.2	149.3 ± 35.3	218.1 ± 8.9
	Total CAT activity (µmol H_2O_2 min^{-1})	119.4 ± 50.7	0.7 ± 0.6	1.6 ± 1.1
	Specific CAT activity (µmol H_2O_2 mg $prot.^{-1}$ min^{-1})	169.3 ± 25.8	26.2 ± 22.5	35.8 ± 8.5

b.d.l: below detection limit.

4. Discussion

4.1 Methods comparison in laboratory biofilms

The measurement of antioxidant enzyme activities in biofilms is an estimation of the capacity of the whole community to respond to oxidative stress. Previous studies highlighted the difficulties to extract antioxidant enzymes efficiently using glass tissue grinder since this instrument requires a high volume of sample (Bonnineau et al. 2010, Guasch et al. 2010). The methods described in the present study allowed the extraction and activity measurement of the antioxidant enzyme CAT from a smaller amount of

starting material corresponding to 2 cm^2 of laboratory biofilm and 4 cm^2 of field biofilms.

For all extraction methods, measurements of the quantity of protein extracted per wet weight and the CAT activity led to a low intra-sample variability indicating a good agreement between analytical replicates and so a good repeatability of these measurements. None of the extraction methods selected had a significant influence on intra-sample variability, showing a good repeatability of this measure in biofilms from laboratory. Nevertheless, a lower repeatability was observed for samples with low specific CAT activity. Therefore, it may be difficult to observe significant changes in samples for which specific CAT activity is lower than 15 µmol of H_2O_2 mg $protein^{-1}$ min^{-1}. Combining good extraction efficiency with a low detection limit is essential to detect a drastic decrease in CAT activity. For example, in this study, effects of strong light intensity on CAT activity were estimated with samples extracted by trituration. A strong photoinhibition led to significantly lower levels of CAT activity in exposed samples, close to detection limit, indicating that the use of a more efficient extraction method would allow more precision in this type of measurement. Moreover, this result confirms previous findings on CAT photoinhibition in algae (Lesser et al. 2006), showing the sensitivity of this enzyme to environmental factors.

First ultrasonication and trituration were compared. Extraction by ultrasonication led to a more efficient extraction of protein than extraction by trituration. Indeed, ultrasonication is a very vigorous process that is expected to completely disrupt cells and allow the release of cell walls and membrane-bound proteins (Cumming and Iceton 2001). Though ultrasonication may cause an increase in temperature and denature proteins (Janknegt et al. 2007), in the present study both methods preserve CAT integrity equally, as shown by the similar total CAT activity obtained in samples extracted by both methods. This result also showed that ultrasonication increased protein extraction but not CAT extraction, the proportion of CAT per protein was then smaller in samples extracted by ultrasonication than in

samples extracted by trituration. In addition, extraction by utrasonication required more extraction buffer than trituration, as the ultrasonic probe had to be covered by buffer during cell disruption, hence a similar protein concentration in samples extracted by ultrasonication and by trituration. Therefore, CAT concentration was lower in enzymatic extracts obtained by ultrasonication than in those obtained by trituration, as also shown by the lowest specific CAT activity. Without an additional concentration step, extraction by ultrasonication may not allow the detection of a CAT concentration as low as that detected in samples extracted by trituration. Moreover, ultrasonication was found to be a less reproducible method in terms of protein extracted per wet weight than trituration. Therefore, ultrasonication is not recommended for protein extraction of freshwater biofilms to perform CAT activity measurement.

Secondly, extraction by trituration, homogenization and homogenization followed by glass beads disruption were compared. The first two methods gave similar results in terms of protein extraction and CAT activities but trituration was a more reproducible extraction method than simple homogenization. The additional step of disruption by glass beads increased CAT extraction significantly, as both quantity of protein extracted per wet weight and total CAT activity were higher in samples extracted by homogenization and glass beads than in samples extracted by trituration. In addition, strength of trituration depends on the operator, while homogenization by a machine and especially cell disruption by glass beads is not operator-dependent; this difference may explain the higher reproducibility of extraction of protein by homogenization and glass beads than extraction by trituration. Moreover, this two-step extraction may be more appropriate for complex communities, such as biofilms. Homogenization may break preferentially the assemblage of biofilm and the biggest cells, while the extraction with glass beads may facilitate the disruption of cell membranes of smaller cells, such as bacteria or diatoms (Cumming and Iceton 2001).

Trituration was then found to be a more appropriate method than ultrasonication for CAT extraction in control biofilms whereas in exposed

communities extraction by homogenization followed by glass beads disruption was found to be better than extraction by trituration or simple homogenization. Though further experiments in control biofilms may be needed to confirm this last result. Homogenization followed by disruption with glass beads appears as the most complete method to extract CAT from freshwater biofilms. Further steps of optimization focusing on this method may improve even more CAT extraction by determining the optimal frequency and duration of time of beadbeater pulses, for instance. Extraction of other antioxidant enzymes (such as ascorbate peroxidase, glutathione reductase, glutathione-S-transferase, superoxide dismutase) by this method is likely to be successful as the same protocol is often used for extraction of different antioxidant enzymes.

4.2 Field study

The field study allowed the three sites to be characterized. Site S1 had moderate metal pollution while sites S2 and S3 were highly polluted according to CCU cut-off values defined by Guasch et al. (2009). Specific CAT activity was 4 to 6 times lower in the highly polluted sites than in the moderately polluted site while variations in photosynthetic efficiency were small between sites (-13 % at S2 and +10 % at S3 compared to S1). A strong correlation was found between CAT and CCU but photosynthetic efficiency was not correlated to CCU or to the concentration of any of the metals. In sites affected by chronic contamination tolerant communities are likely to be found (Soldo and Behra 2000). As photosynthesis is an essential process for community survival, photosynthetic efficiency is likely to be similar between different communities adapted to their environment, as shown in this field study.

Metals are expected to provoke oxidative stress in algae and periphyton (Pinto et al. 2003), so CAT was expected to participate in the response to chronic exposure to metals. High antioxidant enzyme activity was expected at S1 due to iron toxicity (Cassin et al. 2009). The decrease in CAT along the metal toxicity gradient found in this study was in accordance with Tripathi et al. (2006), who reported an induction of

antioxidant enzyme activities under mild oxidative stress and an inhibition under intense oxidative stress. The presence in S2 and S3 of Zn, Cd and of higher concentrations of Ni may be the cause of stronger oxidative stress leading to a decrease in CAT activity. Communities adapted to high metal concentrations presented a low CAT activity but might have developed other mechanisms to cope with oxidative stress induced by metals (pigments, thiols, etc.) or to limit metal toxicity (extracellular detoxification, reduced uptake, sequestration by phytochelatins, etc; Gaur and Rai 2001). The application to a broader range of concentrations in future studies will contribute to corroborate our observations. Nevertheless to confirm a cause-effect relationship the use of microcosms may be a pertinent alternative to a field study as this system allows a gradient of contamination to be simulated under controlled conditions (Clements and Newman 2002).

4.3 First steps towards the use of catalase as a biomarker of stress in biofilm communities

The use of complex communities in ecotoxicological studies allows a realistic approach, although biomarkers of such communities might lack precision (Clements and Newman 2002). The aim of this study was mainly to select an appropriate method for extraction of CAT from biofilm and to evaluate its feasibility and applicability in laboratory and field experiments. In the present study, in both laboratory and field, reproducibility of CAT activity measurements was good (26.8 % to 33.7 %) for almost all samples (except those from S1) and in the usual range found for other biomarkers in microcosms (Giddings and Eddlemon 1979). The measurement of CAT activity is, therefore, reproducible enough to allow CAT activity to be used as a biomarker in biofilms. Nevertheless, three to five replicates per group or sampling site are needed to be able to detect changes lower than 50% by ANOVA analysis.

The response of CAT from freshwater biofilms to usual factors inducing oxidative stress was also validated in the present study. In the field experiment, CAT activity was found to be a better biomarker of metal exposure than photosynthetic

efficiency. Though these examples illustrate the potential of CAT as an indicator of oxidative stress, they also showed that CAT is sensitive to both environmental (light) and chemical (metals) stressors. This limitation might be problematic in field studies where environmental parameters such as light or temperature are subject to variations. Therefore, further research may focus on natural variations of CAT in biofilms from different sites as well as laboratory experiments to determine the impact of specific factors on biofilm CAT activity. From this perspective, CAT in biofilm may not only be considered as an indicator of oxidative stress (as in the laboratory experiment), but also reflect biofilm communities strategies to respond to oxidative stress (as in the field study).

As a complement to this enzymatic approach, the study of variations in gene expression of CAT and other antioxidant enzymes may bring insight into the effects of toxicants on antioxidant enzymes regulation. Indeed it may allow to distinguish between changes at enzyme level (e.g. increase in activity but not in gene expression) and those at cell level (e.g. increase in activity and in gene expression). However the study of gene expression requires a priori knowledge of genetic sequences (Neumann and Galvez 2002). Hence, its application to a community containing mainly non-sequenced organisms, such as biofilms, would involve the resolution of numerous challenges.

5. Conclusion

After comparison of different extraction methods, the present study showed that extraction by homogenization followed by glass bead disruption was the most appropriate for CAT extraction from low amounts of biofilm samples. This method was successfully applied for the extraction of CAT from field biofilms where CAT was found to be strongly correlated with metal pollution. These preliminary results are encouraging and further applications in ecotoxicology will contribute to support the use of antioxidant activities as biomarkers of toxicity within fluvial biofilms.

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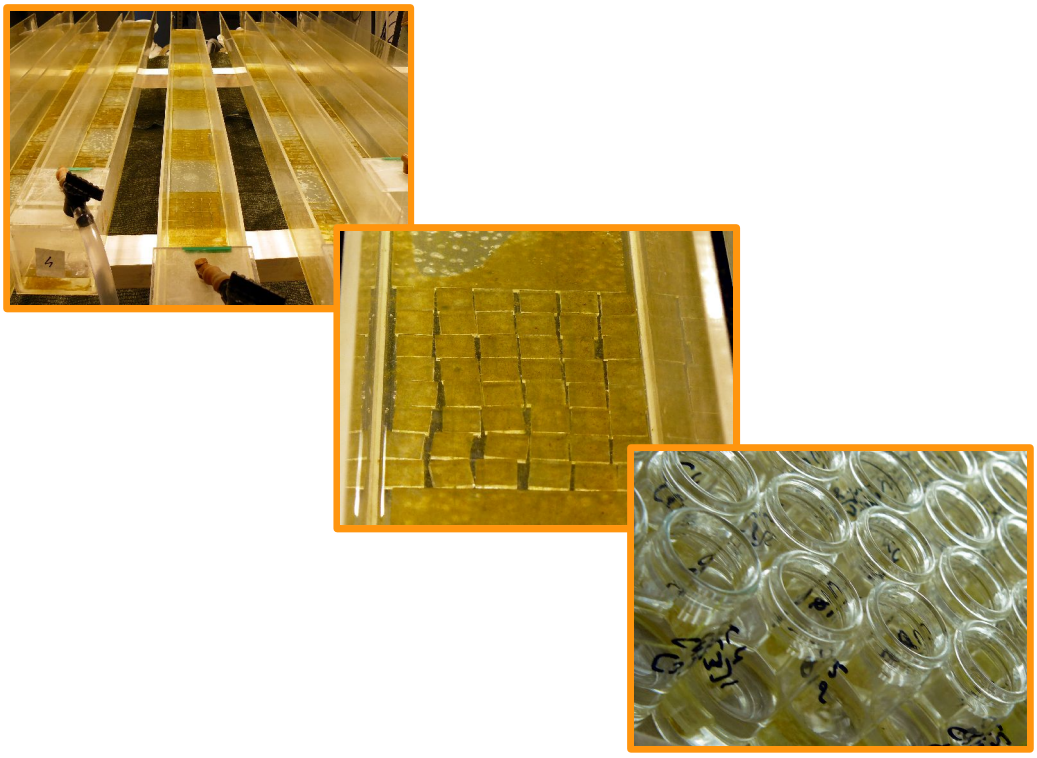
Chapter II

The use of antioxidant enzymes in freshwater biofilms: temporal variability vs. toxicological responses



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Abstract

In freshwater ecosystems, biofilm communities are used as pertinent indicators of ecological status. Most of the parameters used to assess this status focuses on primary production, algal growth or heterotrophic activity, among others. In the present study, the use of antioxidant enzyme activities (AEA) as biomarkers of oxidative stress in freshwater biofilms was presented. Oxidative stress can be provoked by both natural factors (e.g. light or temperature excess) and anthropogenic pollution, the activation or inhibition of AEA in biofilms may indicate their ability to cope with this type of stress. In the present study, AEA were found to change along the dynamic process of biofilm formation. A significant increase in ascorbate peroxidase (APX) activity was observed during the exponential growth. During the slow growth phase, catalase (CAT) and glutathione reductase (GR) seemed to have a more important role. AEA were also affected by the exposure to increasing concentrations of oxyfluorfen during five weeks. APX activity was mainly stimulated in biofilms exposed to low concentrations of oxyfluorfen (3 to 15 µg L^{-1}) while CAT activity was mainly stimulated in those exposed to higher concentrations (30 to 150 µg L^{-1}). However, the ranges of variability of AEA in controls and exposed communities were the same, highlighting the difficulty of a direct interpretation of AEA values. Chronic exposure to oxyfluorfen did not provoke clear effects on pigment composition suggesting that algal group composition was not affected. Nevertheless, after five weeks of exposure, eukaryal communities were structured clearly by toxicant concentration and both eukaryal and bacterial richness were reduced in communities exposed to the highest concentration. The study of AEA responses in short-term toxicity tests was more useful than direct AEA values to discriminate between control and chronically exposed communities. In addition to the structural changes observed, pre-exposure to high oxyfluorfen concentrations led to higher CAT capacity to respond to a sudden increase in concentration (short-term toxicity tests) than controls, suggesting a selection of species with higher antioxidant capacity. Based on our results, continuous inputs of low oxyfluorfen concentrations are expected to affect natural communities. Indeed, the

chronic effect of this toxicant on the community structure may be enhanced in multiple stress situations, as often observed in the field, due to an expected loss of species.

Keywords: catalase, periphyton, maturity, oxyfluorfen, tolerance acquisition.

1. Introduction

In freshwater ecosystems, biofilm communities are now recognized as pertinent indicators of perturbations (Sabater and Admiraal 2005). These complex communities, composed of algae, bacteria, fungi, and protozoa, are embedded in a matrix constituted by extra-polymeric substances (EPS). They live attached to different types of substrates (cobbles, wood, sand, etc.) and are the main primary producers in open streams (Romaní 2010; Stevenson et al. 1996). To assess biofilm status, different structural and functional variables are usually determined. They include community composition (mostly of diatoms), photosynthesis, biomass and heterotrophic activity (Sabater et al. 2007; Weitzel 1979). To complete the information given by these indicators, we propose the use of antioxidant enzyme activities (AEA) in biofilms as indicators of stress and particularly of oxidative stress induced by toxicants. In fact antioxidant enzymes participate in the regulation of reactive oxygen species (ROS) levels to avoid their accumulation and the resulting oxidative stress (Mittler 2002). Previous studies highlighted the interest of AEA as sensitive markers of stress induced by organic and inorganic toxicants. Dewez et al. (2005) showed that the catalase (CAT) activity was a more sensitive biomarker of fludioxionil toxicity than photosynthetic parameters in *Scenedesmus obliquus*. In freshwater biofilms, AEA were found, by Guasch et al. (2010), to be more sensitive to copper toxicity than photosynthetic parameters. The present study focused on three important antioxidant enzymes: CAT, ascorbate peroxidase (APX) and glutathione reductase (GR). CAT (EC 1.11.1.6) and APX (EC 1.11.1.11) catalyse the transformation of hydrogen peroxide in water and oxygen mainly in peroxisomes and chloroplast, respectively (Chelikani et al. 2004; Lesser 2006). GR (1.8.1.7) participates in this reaction by regenerating the co-factor needed by APX (ascorbate-glutathione cycle, Mittler 2002).

Determining the impact of environmental factors at community level is challenging, since temporal variations affect function and structure of communities strongly. Biofilm communities are indeed dynamic; a general pattern of biomass accrual followed by a loss phase is observed in streams due to processes of attachment,

colonization, exponential growth, senescence and sloughing (Biggs 1996). Biofilm formation in rivers starts by the development of an organic matrix by bacterial flora, followed by the recruitment of small adnate diatoms, then apically attached diatoms and, finally, filamentous green algae immigrate and colonize the biofilm (Hudon and Bourget 1981; Peterson and Stevenson 1990). In all communities, succession is a common process susceptible to affect community functioning. For instance, Sabater and Romaní (1996) found a higher respiratory activity in younger rather than in mature biofilms from an undisturbed Mediterranean stream. Romaní et al. (2008) also observed that the release of extracellular bacterial enzymes allowing organic matter compound degradation in the EPS matrix was higher at the beginning of the biofilm formation than at the end of colonization. These examples illustrate how functional biomarkers chosen to reflect perturbations may change due to temporal variability.

Though the effects of temporal variations in ecotoxicology is reduced by using same age communities in toxicity assessment (Clements and Newman 2002), the temporal variation of a biomarker is an estimate of its "natural" range of variation which may be a pertinent scale to interpret the importance of further variations related to disturbances. To our knowledge, patterns of AEA in freshwater biofilms throughout time are unknown. Enzyme activities are sensitive to different factors, such as pH, temperature or substrate concentration. A unimodal response of enzyme activities is usually observed throughout the gradient of these factors; the peak of activity defines thus the optimal conditions specific to each enzyme (Copeland 2000). Consequently, changes in biofilm environmental conditions due to growth or chemical exposure are expected to provoke variations in AEA.

Entrance of chemicals in rivers can affect biofilm development but also the functioning of complex and already well-established biofilm communities (Guasch et al. 2003). Temporal variability is expected to be lower in well-established communities; thus, to test the impact of a toxicant on a fully active community, ecotoxicological tests are better performed on mature communities (Clements and Newman 2002). The present study focused, therefore, on mature biofilm communities

and their transition from an exponential growth phase to a slow growth phase. Temporal variability of AEA throughout this transition was compared to more traditional biofilm metrics, such as pigment composition, photosynthetic efficiency and biomass variables (Sabater et al. 2007). The pigment composition is both a structural and a functional indicator. Marker pigments of some species allow the relative abundance of the different algal groups to be estimated (Jeffrey et al. 1997). In addition, some pigments, such as carotenoids, are specific to oxidative stress response and may support and complete the information obtained by AEA measurements (Pinto et al. 2003).

To study the response of AEA to chemical disturbance, biofilm communities were exposed to increasing concentrations of oxyfluorfen throughout the transition from exponential to slow growth. This diphenyl-ether herbicide was chosen as a "model" toxicant known to provoke oxidative stress in algae. It actually inhibits chlorophyll-*a* biosynthesis and provokes the accumulation in the cytoplasm of protoporphyrin IX, a potent photosensitizer that generates high levels of singlet oxygen and so oxidative stress (Aizawa and Brown 1999; Duke et al. 1991). In *Scenedesmus obliquus* an increase in CAT, APX and GR activities was observed after 24h of exposure to oxyfluorfen (Geoffroy et al. 2003). Changes in AEA after acute and chronic exposure to oxyfluorfen were compared to changes in more traditional metrics as described earlier.

The chronic exposure of a community to a critical level of a chemical is expected to exert a selective pressure on the community by selecting more resistant individuals to the chemical To validate this hypothesis, the bacterial and eukaryal diversity of biofilms exposed during five weeks to increasing concentrations of oxyfluorfen were determined and the AEA response of biofilms chronically exposed to oxyfluorfen was measured in short-term toxicity tests.

The objectives of the present study were then:

1. to characterize the pattern of temporal variation of AEA in mature biofilms
2. to estimate the effects of a toxicant inducing oxidative stress towards biofilm communities using a multi-biomarker approach including AEA
3. to describe structural changes in the community composition occurring after chronic exposure
4. to determine the influence of chronic exposure on biofilms antioxidant capacity to answer to a sudden increase in oxidative stress (induced by oxyfluorfen).

2. Material and methods

2.1 Microcosm setup

Colonization and exposure were performed in an indoor microcosm system consisting of nine recirculating channels previously described by Serra et al. (2009a). Briefly, biofilms were allowed to colonize sandblasted glass substrata of 1.4 and 17 cm^2 installed in the bottom of each channel. In each channel, 10 L of dechlorinated tap water was used as a culture media and changed three times a week; aquarium pumps allowed water recirculation. At each water renewal, phosphate was added to a final nominal concentration of 92 μg L^{-1} to avoid nutrient depletion and P or N limitation. A cooling bath maintained the water temperature at 20ºC. Once a week during the first five weeks of colonization, an original inoculum of biofilm, obtained from the river Llémena (NE Spain, Serra et al. 2009a) was added to each channel. Light was provided by halogen lamps (80-120 μmol photons m^{-2} s^{-1}) with a light regime of 12h:12h light:dark.

After five weeks of colonization, biofilms were exposed to increasing concentrations of oxyfluorfen (CAS: 42874-03-3) following an exponential design (Ricart et al. 2009). Three channels were used as controls and the remaining 6 channels were exposed to 3, 7.5, 15, 30, 75 or 150 μg L^{-1} of oxyfluorfen. Oxyfluorfen was added

in each channel from a stock solution at 15 mg L^{-1} in 2.5 % acetone in order to obtain 0.025 % acetone in each channel, acetone was also added in a similar amount in control channels. At each water renewal, toxicant and/or acetone (when appropriate) were added to compensate for potential degradation of the toxicant and ensure a maximal exposure. Samplings were performed during two periods, biofilms sampled on days 33, 36, 38, 39 and 41 after the start of the colonization were expected to be in exponential growth phase while those sampled on days 59, 66 and 73 were expected to be in slow-growth phase. Exposure to oxyfluorfen started on day 38, two samplings were performed on that day: one just before the exposure and one six hours after the exposure. Consequently, the exposure durations for biofilms sampled on days 39 and 41 were 24 and 72h, respectively. For biofilms sampled on days 59, 66 and 73, exposure durations were three, four and five weeks, respectively. During this last time period (day 59 to 73), no biofilms were exposed to 30 µg L^{-1} of oxyfluorfen due to technical limitations. All samples (except the one after 6h of exposure) were collected at the same moment of the day to limit the impact of potential daily variations. At each sampling and from each channel, three samples (each consisting of three 1.4 cm^2 glass substrata) were collected randomly for AEA measurements and five (1.4 cm^2 glass substrata each) for the measurement of photosynthetic efficiency. For pigment analyses, three samples (1.4 cm^2 glass substrata each) were collected at all sampling times except after six hours of exposure. Just before the start of the exposure (day 38) and five weeks after the exposure (day 73), one sample (17 cm^2 glass substrata) was also collected from each channel for DGGE analysis.

2.2 Short-term toxicity tests

To test the capacity of antioxidant response of biofilms after five weeks of exposure to oxyfluorfen, short-term toxicity tests, based on AEA, were performed. Biofilms from the different channels were exposed to increasing concentrations of oxyfluorfen (0, 1.5, 15, 75, 150 and 1000 µg L^{-1}) in a microcosm set-up previously described (Bonnineau et al. 2010). Briefly, nine glass substrata of 1.4 cm^2 were used for each concentration. Each glass substrata was incubated in a vial containing 10 mL

of colonization medium and the corresponding toxicant concentration. Samples were incubated under the same conditions as the colonization, using a single-speed orbital mixer (KS260 Basic, IKA®) to maintain constant agitation. After six hours of exposure, for each concentration and each channel, three samples, each consisting of three glass substrata, were collected for AEA measurements.

2.3 Biofilm parameters

2.3.1 AEA

Sampling, protein extraction and AEA measurements were performed as described previously (Bonnineau et al. 2011). For each sample, biofilm was removed from glass substrata with a cell scraper (Nunc, Wiesbaden, Germany), put into an eppendorf tube and centrifuged (2300 g, 10ºC, 5 min) to remove the excess of 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 sample. Samples were first homogenized (2 pulses of 30 s 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 30 s of beadbeater (MP FastPrep-24, v=4 m s^{-1}) with 2 min intervals on ice. After cell disruption, homogenates were centrifuged at 10.000g and 4ºC for 30 min. Supernatant was used as the enzyme source. The protein concentration of supernatant was measured in triplicates for each sample by the method of Bradford (1976) using Coomassie Brilliant Blue G-250 dye reagent concentrate (Bio-Rad, Laboratories GmbH, Germany) and bovine serum albumin as a standard. The final concentration of protein was then expressed in µg mg^{-1} of biofilm wet weight.

AEA measurements were performed in microtiter plates (UV-Star 96 well plate, Greiner®), changes in absorbance were followed using a microtiter plate reader

Synergy4 (BioTek®). For all assays the optimal protein concentration was determined by using between 0.5 and 6.5 µg of proteins.

CAT activity was measured spectrophotometrically by following the decomposition of H_2O_2 at 240 nm and 25ºC during 2 min (Aebi 1984). The optimal substrate concentration was determined by using 5, 10, 15, 20, 25 and 35 mM H_2O_2. The 250 µL reaction mixture contained in final concentration 80 mM of potassium phosphate buffer (pH 7.0) and 2 µg of proteins. The reaction was started by adding 35 mM of H_2O_2. CAT activity was calculated as µmol H_2O_2 mg prot.$^{-1}$min^{-1} (extinction coefficient, ε: 0.039 cm² µmol^{-1}).

Oxidation of sodium ascorbate by APX was measured at 290 nm and 25ºC for 2 min according to Nakano and Asada (1981). The optimal substrate concentration was determined by using 0.5, 1, 2, 3, 4 and 5 mM H_2O_2. The 250 µL reaction mixture contained in final concentration: 80 mM of potassium phosphate buffer (pH 7.0), 150 µM of sodium ascorbate and 2 µg of proteins. The reaction was started by adding 4 mM of H_2O_2. APX activity was calculated as µmol ascorbate mg prot^{-1} min^{-1} (ε: 2.8 cm² µmol^{-1}).

The oxidation of NADPH by GR was determined by measuring the decrease in absorbance at 340 nm and 25ºC for 2 min (Schaedle and Bassham 1977). The optimal cofactor concentration was determined by using 0.15, 0.20, 0.25 and 0.30 mM NADPH. The 200 µL reaction mixture contained in final concentration: 100 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM oxidized glutathione and 4 µg of proteins. The reaction was started by adding 0.25 mM NADPH. GR activity was calculated as nmol NADPH mg prot^{-1} min^{-1} (ε: 6.22 cm² µmol^{-1}).

2.3.2 Photosynthetic parameters

For each sample, in vivo photosynthetic efficiency determination was performed using a PAM (Pulse Amplitude Modulated) fluorometer. For technical reasons, measurements from day 33 to 41 were performed using a PhytoPAM (HEINZ WALZ, Effeltrich, Germany) while a MiniPAM (HEINZ WALZ, Effeltrich, Germany)

was used for measurements from day 59 to day 73. The distance between the optical fiberoptics and the sample surface was set at 2 mm. The fluorescence signal was determined by the emitter-detector unit (PHYTO-EDF). After light adaptation, five saturating pulses of actinic light (PAR=120) were applied to the samples to obtain the fluorescence signal at the steady-state (F), the maximal fluorescence yield (Fm') of an actinic-adapted sample and the minimal fluorescence yield (Fo'). These parameters were used to calculate the photosynthetic efficiency (Ph. eff.) following Genty et al. (1989). All calculations were done using the fluorescence signal recorded at 665 nm and are given as relative units of fluorescence.

2.3.3 Pigment analysis by high pressure liquid chromatography (HPLC)

Samples were stored in 15 mL tubes at -80ºC until further analyses. Pigment extraction was performed by ultrasonication as described by Dorigo et al. (2007). Determination of lipophilic pigment composition of biofilm was performed by HPLC as described by Tlili et al. (2008). The injection volume was 100 µL of purified biofilm extract and pigments were separated on a 4.6 x 250 mm column (Waters Spherisorb ODS5 25 µm). Pigment identification was done based on their retention time and absorption spectrum according to the scientific committee for oceanic research (SCOR, Jeffrey et al. 1997). For each sample, the relative abundance (expressed as the percentage of the sum of the areas for all the pigments in the sample) of each pigment was calculated. In addition, standard of chlorophyll-*a* was used to quantify its concentration in each sample, final concentrations are given in µg cm^{-2} of biofilm.

2.3.4 DNA extraction—amplification and denaturing gradient gel electrophoresis (DGGE) analysis

For each sample, biofilm was removed from glass substrata with a cell scraper (Nunc, Wiesbaden, Germany) and put into a 15 mL tube. Samples were then centrifuged for 30 min at 10.000 g and 4°C to remove the excess of water and stored at -80ºC. Nucleic acids extraction, PCR amplification of eukaryotic 18S rRNA gene fragments and bacterial 16S rRNA gene fragments and their DGGE analysis were

performed as described by Tlili et al. (2008). Samples collected at 0h and after 5 weeks of exposure were loaded on the same gel to allow comparison between samples.

2.4 Statistical analyses

The R software (R development Core Team 2008; Ihaka et Gentleman, 1996), the 'ade4' package (Dray and Dufour 2007), the 'proxy' package (Meyer and Butcha 2010) and the 'vegan' package (Oksanen et al. 2010) were used to perform statistical analysis. The statistical significance for all the analyses was set at $p < 0.05$.

For each sampling time and each biological variable, a mean was calculated from the different samples collected from one channel. These mean values (one per channel and per time) were then used in further analyses as independent replicates.

2.4.1 Temporal variation

The temporal variation of biofilms was studied using biofilms from the three unexposed channels sampled at day 33, 36, 38 (t = 0h only), 39, 41, 59, 66 and 73.

To determine the different growth phases of biofilms, chlorophyll-*a* concentration was adjusted to the following sigmoidal model:

$$\text{chlorophyll-}a = K / (1 + \exp(- r * (\text{day} - d_0)))$$

where K is the carrying capacity (maximal chlorophyll-*a* concentration reached), r the growth rate and d_0 the time when maximal growth rate is achieved. The transition from the exponential growth phase to the slow growth phase is observed at the inflexion point i.e. at day d_0 (Romaní 2010).

Differences between colonization time in terms of AEA were estimated by analysis of variance (ANOVA) and post hoc analysed by a Tukey test.

Then the temporal variation of all the biological variables was studied using a multivariate approach. Two tables were constructed (with samples as rows and biological variables as columns). The pigment matrix contained the relative abundance of each pigment and the function-biomass matrix contained the AEA, the photosynthetic efficiency and the biomass variables (concentration of chlorophyll-*a* in

µg cm^{-2} of biofilm, concentration of protein in µg mg^{-1} of biofilm wet weight and biofilm wet weight in mg). Relative abundance of pigments was normalized using an arcsine square root transformation while other biological variables were log-transformed to verify the normality hypothesis.

To understand the temporal variation of each matrix and to determine the importance of differences between the replicated channels, four between-PCAs (Principal Component Analysis) were carried out. In this particular PCA, the factor information is added to each sample, i.e. the sampling day for the factor time or the channel number for the factor channel. Then to reveal the differences between groups, one can look for the dispersion between the gravity centres of the groups formed by these factors. The percentage of variance explained by one factor is the ratio between the sum of the eigenvalues of the between-PCA and the sum of the eigen-values of the PCA (Dolédec and Chessel 1987; Dray and Dufour 2007).

To measure the concordance between the two matrices (pigment and function-biomass), a co-inertia analysis was performed. This multivariate technique, which belongs to the «data coupling» approach, analyses co-structure by maximizing covariance between two matrices (Dray et al. 2003a). This method is especially of interest when different types of variables are compared (here percentages and absolute values) or when different ordination methods are used for the two sets of variables (Dolédec and Chessel 1994). To estimate the degree of concordance between matrices, the RV-coefficient was calculated as the total co-inertia (sum of eigenvalues of a co--inertia analysis) divided by the square root of the product of the squared total inertias (sum of the eigenvalues) from the individual PCAs (Robert and Escoufier, 1976). The RV-coefficient was expressed in percentage. A high coefficient indicates simultaneous variations (either positive or negative) of the two sets of variables while a low coefficient indicates independent variations (Dray et al. 2003b). A Monte-Carlo permutation test on the sum of the eigenvalues of the co-inertia analysis was also performed to assess the significance of the RV-coefficient (Heo et Gabriel, 1998).

2.4.2 Oxyfluorfen exposure

2.4.2.1 Long-term exposure

To study the effects of oxyfluorfen on biofilms, biological variables measured in all channels (three controls and six exposed to increasing concentrations of toxicant) at days 38 (t = 6h), 39, 41, 59, 66 and 73 were used to construct two tables as previously. The pigment matrix contained the relative abundance in pigments normalized using an arcsine square root transformation, the function-biomass matrix contained the AEA, photosynthetic efficiency and biomass variables (concentration of protein in µg mg^{-1} of biofilm wet weight and biofilm wet weight in mg), log-transformed. To better reveal the pattern of variations due to the toxicant exposure, the effect of time was removed from the two sets of variables by carrying out a within-PCA on each matrix. In this particular case of PCA, the mean of the samples in a same group, i.e. collected at the same time, is subtracted to each sample of this group for each variable. All the group centers are, therefore, at the origin of the factorial map and samples are represented with the maximal variance around this origin. Therefore, the patterns of variation obtained at each sampling time can be compared among them. The percentage of variance explained by the within-PCA (the intra-group variance) corresponds to the variance due to other factors than time; it is calculated as the ratio between the sum of the eigen-values of the within-PCA and the sum of the eigen--values of the simple PCA (Dray and Dufour 2007).

To compare the variability of AEA from control and from exposed biofilms during all the exposure period, variances of AEA of biofilms exposed to different oxyfluorfen concentration were compared between them. To do so, a distance matrix was built based on Euclidean distance. Then a permutation-based test of multivariate homogeneity of group dispersions (variance) was performed, each group corresponding to a concentration of oxyfluorfen (Anderson et al. 2006).

Oxyfluorfen was expected to provoke structural changes in biofilm communities only after chronic exposure. Hence, comparison of bacterial and

eurkaryal diversities of biofilms from the different channels were performed for samples collected on day 38, just before exposure, and on day 73, five weeks after exposure. To do so, DGGE profiles were compared for presence or absence of bands by calculating the dissimilarity index of Jaccard; matrices were then used to perform the average method of hierarchical cluster analysis (HCA). In addition, richness was also calculated for each sample (as the number of bands present).

2.4.2.2 Short-term toxicity tests

To test the influence of chronic exposure to higher oxyfluorfen concentrations on the AEA response of biofilms to acute exposure to oxyfluorfen, a two-way ANOVA was performed. The AEA for which the interaction term of the two-way ANOVA was significant were selected for further analysis. For each channel, a one-way ANOVA followed by a Tukey test, as a *post hoc* analysis, were performed on these AEA to reveal the differences between samples after acute exposure to different concentrations of oxyfluorfen

3. Results

3.1. Biofilm colonization

Physical and chemical conditions were stable during the five weeks of colonization (day 0 to 38) and the five weeks of exposure (day 39 to 73), although small differences were observed between the two periods. Mean values (and standard errors) of all channels for colonization and exposure periods, respectively, were the following: temperature was: 20.5 ± 0.1 ºC and 20.2 ± 0.1, dissolved oxygen concentration: 8.88 ± 0.03 and 9.26 ± 0.02 mg L^{-1}, pH: 8.58 ± 0.03 and 8.64 ± 0.04, water conductivity: 418 ± 4 and 404 ± 5 $\mu S \cdot cm^{-1}$ (n = 93 and n = 102, respectively) and flow: 1.45 ± 0.01 and 1.42 ± 0.02 L min^{-1} (n = 117 and n = 84, respectively). Water used during this experiment has already been characterized by Serra et al. (2009b) for NO_3 (1.68 ± 0.14 mg L^{-1}), NO_2 (0.07 ± 0.01 mg L^{-1}) and NH_4 (<0.1 mg L^{-1}) among others (n = 20 for all). In the present experiment, only phosphate concentration was

measured. During the colonization period, phosphate concentration was of 68.8 ± 6.4 µg L^{-1} (n = 42) just after water changes and phosphate addition and declined to low levels (17.0 ± 2.7 µg L^{-1}, n = 42) before water changes. During the exposure period phosphate concentrations were similar after and before water changes (22.9 ± 1.8 and 27.4 ± 3.9 µg L^{-1}, respectively, n = 54). Total phosphate depletion was not observed neither during colonization nor during exposure periods.

3.2 Temporal variations of biological variables in unexposed biofilms

3.2.1 Chlorophyll-a

Chlorophyll-*a* concentration in unexposed biofilms increased during the 10 weeks of the experiment and could be successfully adjusted to a sigmoidal growth curve with K = 17.2 µg chlorophyll-*a* cm^{-2}, d_0 = 43.6 days and r = 0.10 day^{-1} (Fig. 1). Biofilms collected between day 33 and 41 of colonization (< d_0) were in exponential growth phase while samples collected between 59 and 73 days were in slow growth phase. The loss phase was not reached during this experiment as indicated by the d_0 values and the continuous increase of biomass along the experiment (Fig. 1).

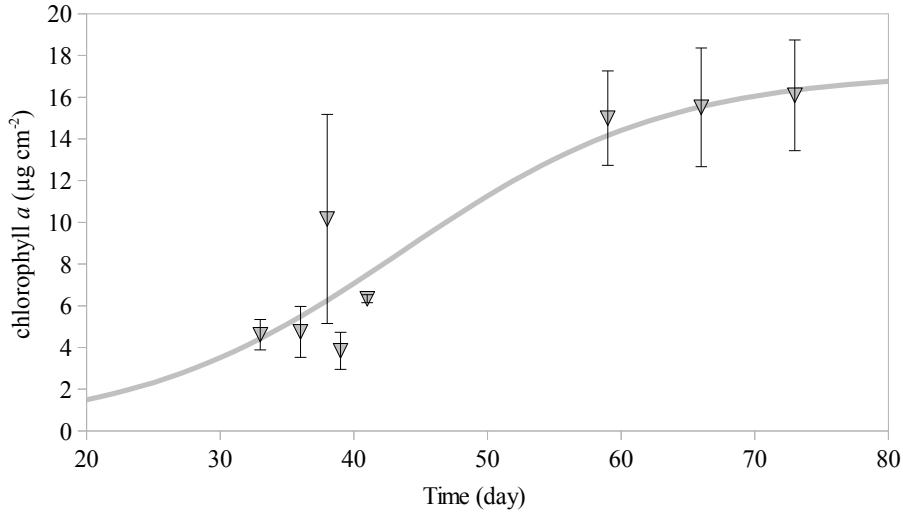


Figure 1. Chlorophyll-a concentration (µg cm^{-2}) in channels throughout time. Triangles correspond to the means (and standard errors) of the different measurements. The plain line shows the growth model fitted to the data.

3.2. Temporal variations of biological variables in unexposed biofilms

3.2.2 AEA

Temporal variation of AEA was observed in unexposed biofilms (Fig. 2). Although CAT and GR activities seemed to increase throughout time, these differences were not significant ($p > 0.05$). The maximum CAT and GR activities were observed at day 59 and correspond to an increase by 142 ± 54 and 163 ± 16% of activities at day 33, respectively. APX activities from biofilms collected at days 39 and 41 were significantly higher (by 142 ± 20 and 138 ± 47 %, respectively) than APX activity of biofilms from day 33 ($F = 4.7$, $p < 0.05$).

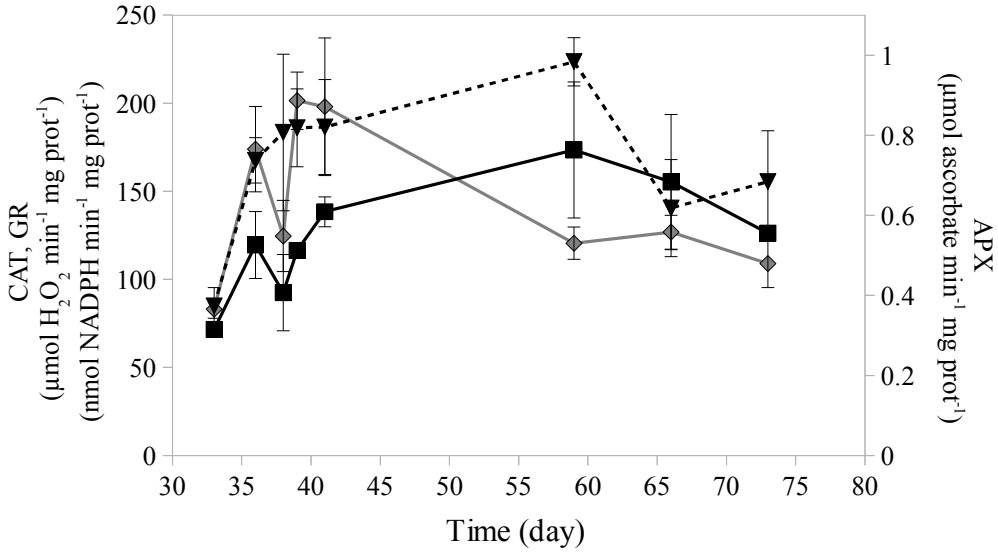


Figure 2. Mean of CAT (■, black plain line), GR (▼, black dashed line) and APX (♦, grey line) activities in biofilms from the three control channels throughout time. Bars indicate standard errors.

3.2.3 Multivariate approach

Multivariate analyses highlighted a high temporal variability for all biological variables. Results of the two between-PCAs showed that the factor time explained 52.2 % of the total variance in the pigment matrix (relative abundance of pigments) and 56.7 % of the total variance in the function-biomass matrix (AEA, photosynthetic and biomass variables). The inter-channel variability was also estimated and results of the between-PCAs showed that the factor channel explained 9.9 % and 11.9 % of the total variance in the pigment and function-biomass matrices, respectively.

Concerning the co-inertia analyses of the pigment and biomass-function matrices, a significant RV coefficient of 44.2 % was obtained (permutation test, $p < 0.05$). This result indicated a good degree of concordance between the two matrices, meaning high similarity of variation between the two sets of variables. The separation of samples was strongly driven by the axis 1, which explained 64.5 % of the variance while the axis 2 explained 24.5 %. Samples from day 33 to 41 were separated from those from day 59 to 73 along the first axis, whereas the second axis accounted for variability within each group of samples (Fig. 3).

Table 1. Code and corresponding pigment name.

Code	Pigment name	Code	Pigment name
ANT	antheraxanthin	NEO	Neoxanthin
bbCAR	β,β-carotene	PHEPa	pheophytin a
CAR	Carotenoid P468	PHERa	pheophorbide a
CHLa	chlorophyll-*a*	tNEO	trans-neoxanthin
CHLb	chlorophyll b	VIO	Violaxanthin
CHLc	chlorophyll c	ZEA	Zeaxanthin
DIAD	diadinoxanthin	U1	unknown 1
DIADcI	diadinochrome I	U2	unknown 2
DIADcII	diadinochrome II	U3	unknown 3
DIAT	diatoxanthin	U4	unknown 4
FUC	fucoxanthin	U5	unknown 5
LUT	lutein		

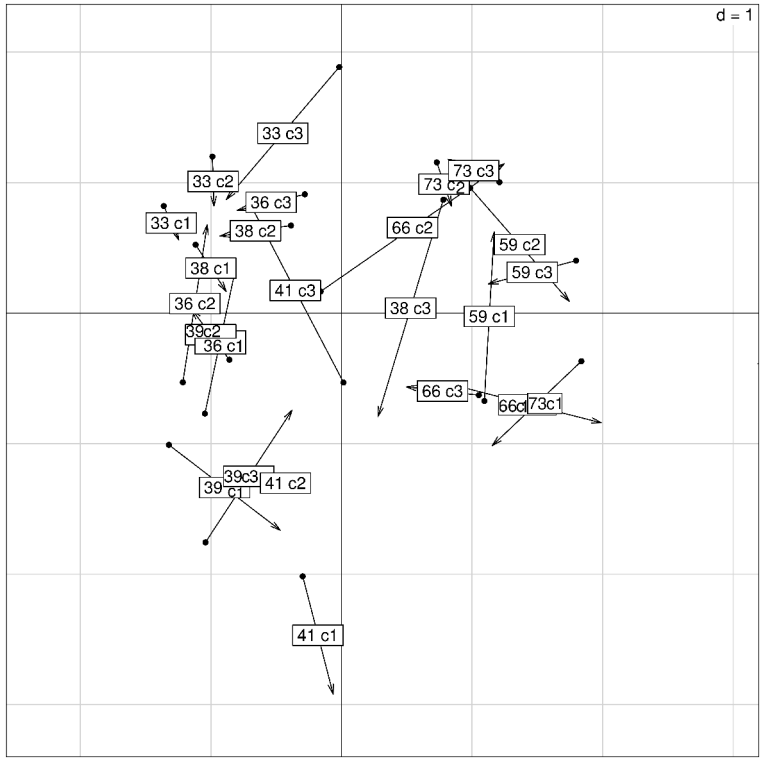


Figure 3. Ordination of the samples by the co-inertia analysis. For each sample, ordination by PCA of the pigment matrix corresponded to the beginning of the arrow and by the PCA of function-biomass matrix to the end of the arrow. For each sample, the sampling day is indicated followed by the channel (c1, c2 or c3).

Biofilms from day 33 to 41 were characterized by a higher protein concentration and a higher APX activity (Fig. 4); these biological variables were associated with a higher relative abundance of two unknown pigments (U1, U2), diadinochrome II, chlorophyll c and violaxanthin (Fig. 4). Various pigments (lutein, β,β-carotene, chlorophyll b, diatoxanthin, fucoxanthin and U3, U4) were characterized by low scores (< 0.05) on the axis 1 of the co-inertia indicating a low variability of these pigments in biofilms along this axis (Fig. 4). Biofilms from day 59 to 73 were then characterized by a higher biomass (chlorophyll-*a* and wet weight) and higher CAT and GR activities (Fig. 4); these biological variables were also associated with a higher relative abundance of chlorophyll-*a,* carotenoid P468, zeaxanthin, and anteraxanthin (Fig. 4).

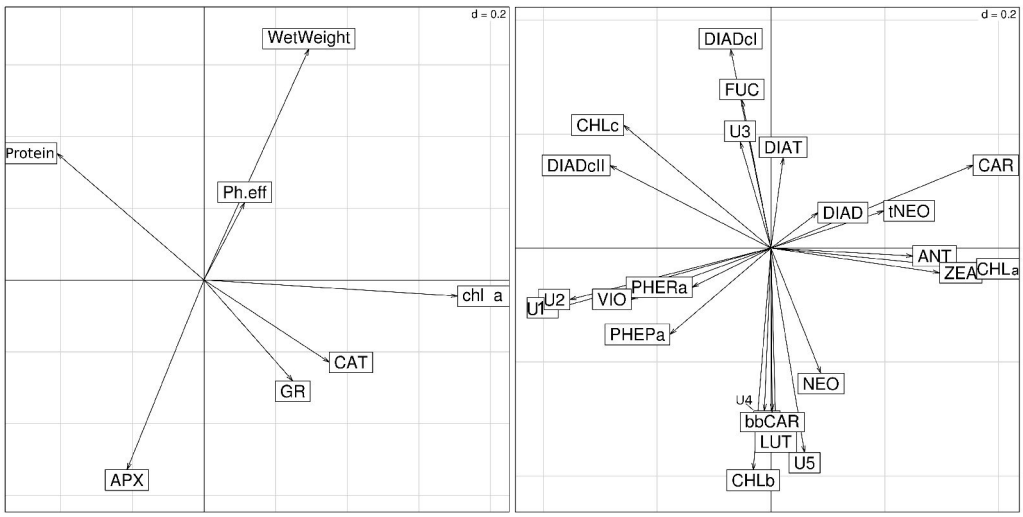


Figure 4. Normed coefficients of the different variables on the axes of the co-inertia analyses. The correspondence between pigment codes and complete names is given on Table 1. On the left graph Ph. eff. stands for photosynthetic efficiency.

Variability in biofilms from day 33 to 41 was higher along the second axis than in older biofilms. In addition, samples from day 33 to 41 were distributed chronologically along the second axis, the time being negatively correlated with this axis (Fig. 3). Inside this group of samples, the oldest ones (day 39-41) were characterized by higher AEA (Fig. 4), which were associated with higher relative abundance of chlorophyll b, lutein, β,β-carotene, neoxanthin and two unknown pigments (U4, U5; Fig. 4). The youngest biofilm samples (day 33) were characterized by higher wet weight and protein concentration (Fig. 4) and also by a higher relative abundance of diadinochrome I, fucoxanthin and chlorophyll c (Fig. 4). The scores of each variable on the co-inertia axes are available in the supporting information (Table A1; Table A2).

3.3. Exposure to oxyfluorfen

3.3.1. Relative abundance of pigments

During the five weeks of exposure to different concentrations of oxyfluorfen, the relative abundance of pigments in biofilms was slightly affected. The first two-axes of the within-PCA explained 30.5 % of the variance (wPCA1: 19.4 %, wPCA2: 11.1 %). The intra-group variance of the pigment matrix was 58.3 %, meaning that more than half of the variance was due to other factors than time. Nevertheless, relationships between oxyfluorfen exposure and patterns of changes in the pigments matrix were unclear. The patterns of biological response observed after different times of exposure were different, and never concentration-dependent. Moreover, samples exposed to oxyfluorfen were, in most of the cases, within control variability (Supporting information, Fig. A1).

3.3.2 AEA, photosynthetic efficiency and biomass variables

Results of the within-PCA show that during the five weeks of exposure to different concentrations of oxyfluorfen, biomass variables (protein concentration and wet weight) were almost unaffected by the toxicant while AEA and photosynthetic efficiency were slightly more affected (Fig. 5B).

The enzymatic activities of control biofilms during the exposure period (day 38 to 73) were between 102.2 and 197.1 µmol H_2O_2 mg prot.$^{-1}$ min^{-1}, 0.369 and 1.120 µmol ascorbate mg prot^{-1} min^{-1}, 124.4 and 265.9 nmol NADPH mg prot^{-1} min^{-1} for CAT, APX and GR activities, respectively. Lower and higher activities were observed in exposed biofilms as for this period their CAT, APX and GR activities ranged between 87.5 and 274.3 µmol H_2O_2 mg prot.$^{-1}$ min^{-1}, 0.272 and 1.136 µmol ascorbate mg prot^{-1} min^{-1}, 95.7 and 330.2 nmol NADPH mg prot^{-1} min^{-1}, respectively. Nevertheless, the variances of AEAs of biofilms exposed to different oxyfluorfen concentration were not significantly different ($F = 1.68$, $p > 0.05$, $n = 999$ permutations), indicating that AEAs had a similar range of variation overtime in both control and exposed communities.

Based on the within-PCA results, other factors than time explained about half of the variance (intra-group variance: 51.3 %). The first two-axes of the within-PCA explained 28.3 % of the variance (wPCA1: 14.1 %, wPCA2: 11.5 %). The patterns of biological response changed throughout exposure and were never concentration-dependent, but some general trends could be observed (Fig. 5A). Samples from biofilms exposed from 30 to 150 µg L^{-1} were mainly distributed along axis 1 (Fig. 5A) which was negatively correlated with APX activity and positively correlated with photosynthetic efficiency, CAT and GR activities (Fig. 5B). Samples from biofilms exposed from 3 to 15 µg L^{-1} were mainly distributed along the axis 2 (Fig. 5A), which was negatively correlated with GR and APX activities and positively correlated with CAT activity and photosynthetic efficiency (Fig. 5B).

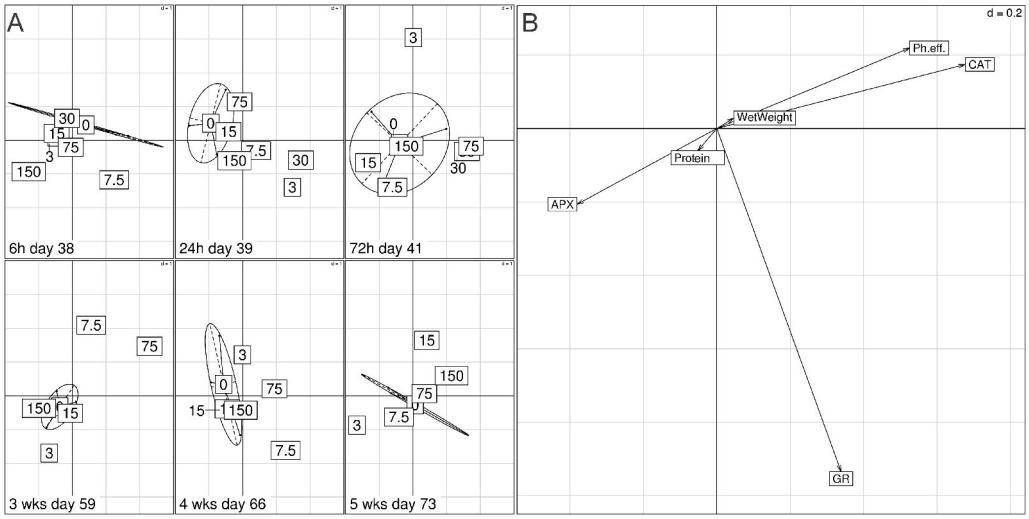


Figure 5. Results of the within-PCA of the function-biomass matrix of samples exposed to oxyfluorfen. (A) Factorial maps: samples collected the same day are represented in different factorial maps. The times of exposure (6h, 24h, 48h, 3, 4 or 5 weeks) are indicated followed by the sampling day (from 39 to 73); wks stands for weeks. The sample labels correspond to the oxyfluorfen concentration. (B) Normed coefficients of the variables on the first two axes of the within-PCA of the function--biomass matrix of exposed biofilms. Ph. eff. stands for photosynthetic efficiency.

3.3.3 Eukaryotic community structure

Concerning the eukaryal diversity, 36 bands were detected in the biofilm sample as a whole. After five weeks of colonization (t = 0h) the number of bands detected was 16 or 17, whereas after ten weeks, the number of bands detected ranged from 23 to 27 (average: 25). Biofilms exposed to 150 µg L^{-1} of oxyfluorfen during the last five weeks of growth had the lowest number of bands detected (23) within ten-week old samples. Cluster analysis was performed using the presence/absence of bands within each sample (Fig. 6A). The eukaryotic community was very similar between the different channels at the beginning of the exposure (t = 0h). Based on the bar plot of node heights (Fig. 6A), six groups were separated. First, samples collected at t = 0h were clearly different from those collected after five weeks of exposure. Within the chronically-exposed samples, five groups with similar eukaryotic community were observed. The first one contained samples exposed to 150 µg L^{-1} of oxyfluorfen, the second one the samples exposed to 75 µg L^{-1}, a third group was formed by the controls, and the two others by samples exposed to 3 µg L^{-1} and by samples exposed to 7.5 and 15 µg L^{-1}, respectively (Fig. 6A).

3.3.4 Bacterial community structure

Concerning the bacterial diversity, 54 bands were detected in the biofilm sample as a whole. After five weeks of colonization (t = 0h) the number of bands detected ranged from 25 to 27 (average: 26), whereas after ten weeks the number of bands detected ranged from 30 to 35 (average: 32). Biofilms exposed to 150 µg L^{-1} of oxyfluorfen during the last five weeks of growth had the lowest number of bands detected (30) within ten-week old samples. Cluster analysis was performed using the presence/absence of bands within each sample (Fig. 6B). Based on the bar plot of node heights (Fig.6B), three groups can be separated after cluster analysis. First, samples from t = 0h were clearly separated from chronically-exposed samples; then, in this former group, biofilms exposed to 15 and 75 µg L^{-1} of oxyfluorfen were separated from the other ones (Fig. 6B).

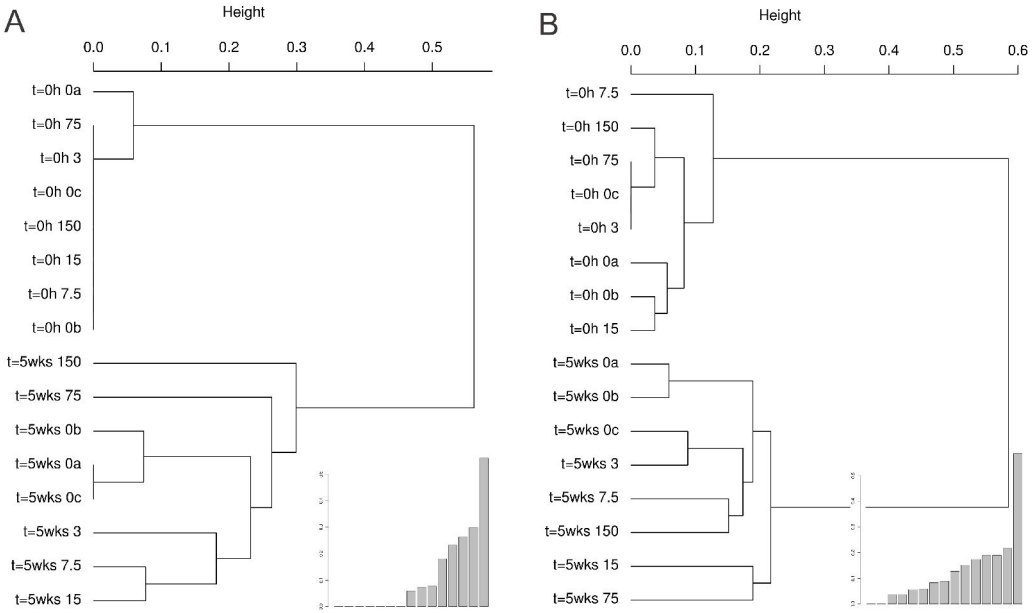


Figure 6. Cluster analysis of the biofilms eukaryal (A) and bacterial (B) community structure. For each clustering, the bar plots of the node heights are shown. t=0h indicates biofilms sampled on day 38 just before exposure and t=5wks indicates biofilms sampled on day 73 after five weeks of exposure. For each sample oxyfluorfen concentration is indicated; 0a, 0b, 0c correspond to control.

3.3.5 Short-term toxicity tests

After five weeks of exposure, a main effect of the chronic exposure to oxyfluorfen was observed on CAT, APX and GR activities. A main effect of the acute exposure was only observed for CAT activity (Table 2). In addition, the interaction term between acute and chronic exposure was significant for CAT and APX activities (Table 2). This last result suggests an influence of chronic exposure on the CAT and APX response of biofilms to acute exposure to oxyfluorfen.

Table 2. F and p-value from two-way ANOVA analysis of biofilms as influenced by oxyfluorfen concentration during chronic (0, 3, 7.5, 15, 75, 150 µg L^{-1}) and acute (0, 1.5, 15, 75, 150, 1000 µg L^{-1}) exposure.

Source	CAT		APX		GR	
	F	p-value	F	p-value	F	p-value
Chronic exposure to oxyfluorfen	**7.37**	**< 0.05**	**5.44**	**< 0.05**	**7.58**	**< 0.05**
Acute exposure to oxyfluorfen	**3.05**	**< 0.05**	1.32	> 0.05	0.67	> 0.05
Chronic x Acute	**4.39**	**< 0.05**	**2.04**	**< 0.05**	0.86	> 0.05

Values in bold indicate significant result ($p < 0.05$).

CAT activities of biofilms from the different channels (chronically exposed and controls) not exposed to oxyfluorfen during the short-term ecotoxicological tests were not significantly different ($F = 1.33$, $p > 0.05$) whereas the response patterns throughout the acute oxyfluorfen gradient differed between biofilms chronically exposed to different concentrations of oxyfluorfen. After acute exposure, CAT activity of control biofilms (not exposed to chronic contamination by oxyfluorfen) showed a unimodal response throughout oxyfluorfen gradient with a maximum of activity reached after exposure to 15 µg L^{-1}. Indeed, CAT activity increased by 61.0 ± 5.8, 33.2 ± 4.5 and 21.5 ± 4.7 % in control biofilms exposed at 15 µg L^{-1}, compared to non-exposed communities (Fig. 7). In communities chronically exposed to 3, 7.5 and 15 µg L^{-1}, no differences were found between communities exposed 6h to oxyfluorfen and non-exposed ones (Fig. 7). In biofilms chronically exposed to 75 µg L^{-1}, CAT

activity increased significantly by 27.1 ± 6.2 % after acute exposure to 1000 µg L^{-1}. In communities chronically exposed to 150 µg L^{-1}, CAT activity increased significantly by 75.6 ± 4.2, 91.0 ± 17.2, 88.3 ± 8.5 and 129.6 ± 10.5 % after acute exposure to 15, 75, 150 and 1000 µg L^{-1} of oxyfluorfen, respectively (Fig. 7). Consequently, after acute exposure to 1000 µg L^{-1} of oxyfluorfen, biofilms chronically exposed to 150 µg L^{-1} presented a CAT activity 2.5 times significantly higher than biofilms not chronically exposed ($F = 55.47$, $p < 0.05$).

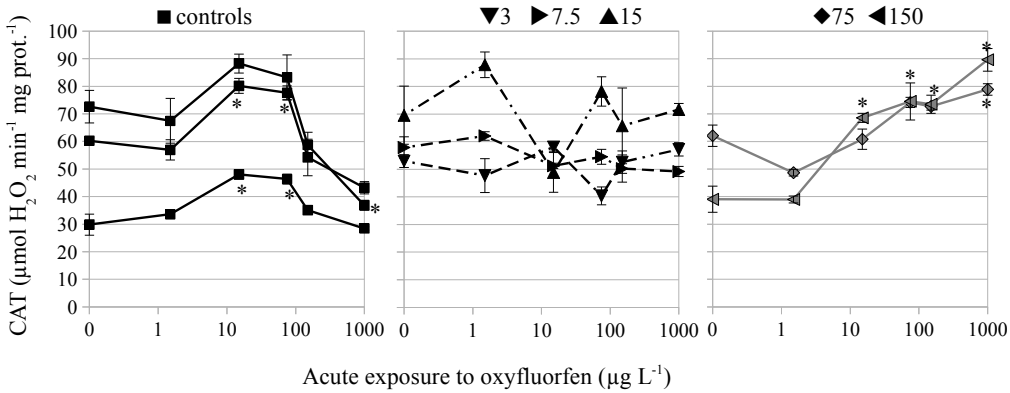


Figure 7. CAT activity after 6h of exposure to oxyfluorfen in biofilm control (■) and in biofilms exposed during 5 weeks to 3 (▼), 7.5 (▶), 15 (▲), 75 (◆) or 150 (◀) µg L^{-1} of oxyfluorfen.

In control biofilms and in communities chronically exposed to 3, 7.5, 75 and 150 µg L^{-1} of oxyfluorfen, no significant differences were observed in APX activity between communities exposed 6h to oxyfluorfen and non-exposed ones (Fig. 8). In biofilms chronically exposed to 15 µg L^{-1} of oxyfluorfen, a significant decrease (by 43.6 ± 7.0 %) in APX activity was observed after acute exposure to 1.5 µg L^{-1} of oxyfluorfen (Fig. 8).

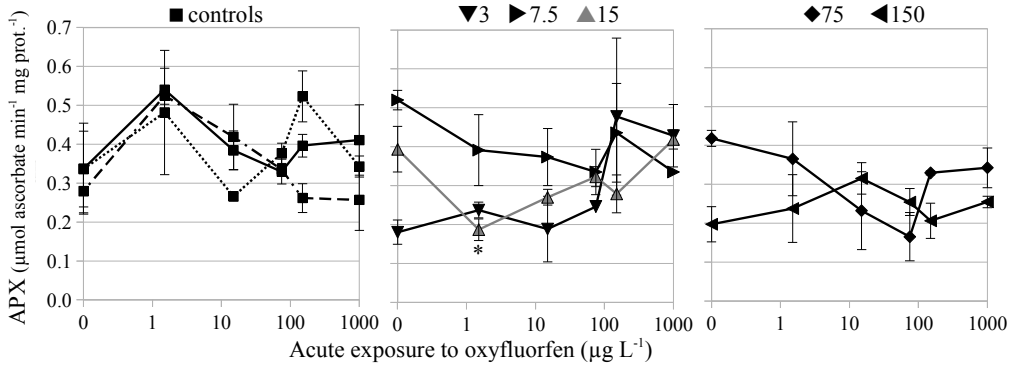


Figure 8. APX activity after 6h of exposure to oxyfluorfen in biofilm control (■) and in biofilms exposed during 5 weeks to 3 (▼), 7.5 (▶), 15 (▲), 75 (◆) or 150 (◀) µg L^{-1} of oxyfluorfen.

4. Discussion

4.1 Temporal variations of biological variables in unexposed biofilms

The use of microcosms to study ecosystems under controlled conditions is common in aquatic ecology (Taub 1997). The system presented here (recirculating channels) allowed active and complex biofilm communities to be maintained during 10 weeks with a good reproducibility. In fact, the factor channel accounted for less than 12 % of the variance in unexposed biofilms (Giddings and Eddlemon 1979).

Temporal variations of biological variables of unexposed biofilms revealed both structural and functional differences between the exponential (day 33 to 41) and slow-growth phases (day 59 to 73). Moreover, a higher variability was found within

exponentially growing biofilms than within slow-growing biofilms. Various authors (Besemer et al. 2007; Sabater and Romaní 1996) reported similar results. For instance, Besemer et al. (2007) observed a decrease in Operational Taxonomic Units turnover (i.e the appearance of new bacterial species) throughout time during biofilm colonization in a mesocosm experiment. Sabater and Romaní (1996) reported a sharp increase in bacterial densities and ectoenzymes activities at the beginning of biofilm colonization in a shaded stream. Therefore, our results and those reported in the literature suggest that the changes occurring during biofilm development may be faster in exponentially growing biofilms due to the higher growth rate while communities in the slow-growth phase may have reached a steady-state.

As expected, older biofilms were characterized by a higher biomass (chlorophyll-*a* and wet weight) than exponentially growing biofilms (Romaní and Sabater 1999a). However, the proportion of proteins in total biofilm biomass decreased throughout time, indicating temporal changes in biofilm composition. As biofilm ages, it becomes thicker and the proportion of molecules different from proteins (e.g. polysaccharides from EPS matrix) may increase (Fernandes da Silva et al. 2008).

The shift from the exponential to the slow growth phase was also characterized by changes in biofilm composition. The complexity of the communities increased with time as shown by the higher bacterial and eukaryal richness at the end of the experiment, in accordance with previous studies (Lear et al. 2008; Sabater and Romaní 1996). Temporal variation in pigment composition during exponential growth suggested a decrease in the proportion of diatoms (fucoxanthin, diadinochrome I, chlorophyll c) and an increase in the proportion of green algae (chlorophyll b, lutein, β,β-carotene, neoxanthin) throughout time. Moreover, slow-growing biofilms were characterized by a higher proportion of cyanobacteria (zeaxanthin). These results indicate a classic succession in biofilms in agreement with previous observations (Biggs 1996).

A temporal shift in the pool of antioxidant enzymes used for oxidative stress management was also observed in biofilms. On the one hand, APX seemed to play a more important role in exponentially than in slow growing biofilms with a significant peak of activity observed at the end of the exponential growth phase. On the other hand, though CAT and GR activities seemed to be independent from time, these enzymes may have a more important role during the slow-growth phase due to the reduction in APX activity. Temporal variations of AEA may be explained by both inter-specific variations and changes in the biofilm micro-environment. In fact, these two aspects are likely to be linked as changes in micro-environment may favour the species with the most appropriate AEA pattern. For instance, communities dominated by diatoms are likely to have a low CAT activity as the occurrence of CAT in diatoms has rarely been observed (Branco et al. 2010; Wilhelm et al. 2006; Winkler and Stabenau, 1995). Besides, as biofilm ages it becomes thicker and marked gradients of light and oxygen can be observed. Therefore, the upper layers of biofilm are characterized by oxygen supersaturation resulting from a high photosynthetic activity while in the bottom layers, where light can be strongly attenuated, photosynthesis and oxygen concentrations are reduced (Carlton et Wetzel; 1987; Dodds, 1989). These changes may create micro-zones with different levels of oxidative stress. The balance between these zones may then determine the oxidative stress level of the whole community. Since on the first sampling of this study, colonization was already on the late exponential phase, a high oxygen concentration and pH may be expected within biofilms. Therefore the balance between the different zones may point towards a high level of oxidative stress. In this case, the stability of AEA (especially of GR and CAT) may reflect a saturation of the antioxidant defence system.

4.2 Effects of oxyfluorfen

Oxyfluorfen was chosen as a “model” toxicant known to inhibit chlorophyll-*a* biosynthesis and to induce oxidative stress in algae (Geoffroy et al. 2003). In the present study, no decrease in relative abundance of chlorophyll-*a* was observed in exposed biofilms but variations in AEA could be related to toxicant exposure. The

activation of one or another enzyme to cope with oxidative stress induced by oxyfluorfen seemed concentration dependent. Indeed, exposure to low oxyfluorfen concentration (3 to 15 µg L^{-1}) led to an increase in APX, whereas exposure to higher concentration (30 to 150 µg L^{-1}) stimulated CAT activity. This result may be related to the higher affinity of APX for hydrogen peroxide. Hence, APX is expected to be activated at lower levels of ROS than CAT (Mittler 2002). This result may be specific to biofilm communities as Geoffroy et al. (2002, 2003) observed an activation of CAT at lower oxyfluoren concentrations (7.5 to 22.5 µg L^{-1}) than APX (15-22.5 µg L^{-1}) in *Scenedesmus obliquus* exposed to this toxicant.

Although the set of functional and structural biofilm biomarkers measured throughout exposure was slightly affected by oxyfluorfen, the communities were strongly structured after five weeks of exposure, as shown by the clustering of the DGGE profiles. Oxyfluorfen was expected to affect mainly the eukaryotic community due to its direct effect on chlorophyll-*a* biosynthesis. Indeed, the eukaryotic community was structured in a concentration-dependent manner by oxyfluorfen. These changes could not be attributed to changes in the relative proportion of algal groups, as indicated by the lack of effects on pigments. It suggests that species resistant to oxyfluorfen were likely to be found in all algal groups. In addition, the highest concentration of oxyfluorfen had a negative impact on algae and bacteria, as indicated by the lowest bacterial and eukaryal richness observed. This result also pointed out the potential indirect effect of oxyfluorfen on the bacterial community in biofilms. In agreement with this idea, Ricart et al. (2009) described how toxic exposure of biofilms to a photosynthesis inhibitor (the herbicide diuron) indirectly affected the bacterial community. In this study, the interaction of bacteria with the potential primary target organisms was the basis of the chain of effects that diuron caused on biofilms. Another study highlighted the importance of the bacterial-algal link in fluvial biofilms by assessing the ecotoxicological effects of the bactericide triclosan on these communities. Though triclosan directly damaged the heterotrophic compartment by reducing bacterial viability, the algal component was also affected through the

inhibition of photosynthetic efficiency and the reduction of diatom viability. This result was attributed to the common use of space and resources within the biofilm (Ricart et al. 2010). These examples and our results emphasize the importance of indirect effects of pollutants on the aquatic system. Indeed, the toxicity of chemicals is not simply a consequence of their direct toxic effect, but it might extend to other trophic levels.

The structural changes observed in biofilm communities after chronic exposure to oxyfluorfen were linked to an enhancement of CAT capacity to answer to higher levels of oxidative stress caused by this same toxicant. Therefore chronically-exposed biofilms may better tolerate oxidative stress at intermediate oxyfluorfen concentrations and may be able to cope with higher levels of oxidative stress than control biofilms. Differences in the ability to reduce oxidative damage may, for instance, be due to various mechanisms, such as an increase in ROS excretion (Choo et al. 2004). These patterns of response of CAT activity showed that oxyfluorfen chronic exposure induced the selection of species with capacity to cope with higher oxidative stress levels, being probably one of the mechanisms of adaptation to oxyfluorfen exposure. This result highlights then the importance of toxicant chronic exposure on the development of biofilm structure and function and thus on the adaptation of the community as demonstrated on several occasions (e.g. Berard et al. 2002; Dorigo et al. 2007; Schmitt-Jansen and Altenburger 2005a; Tlili et al. 2010). Nevertheless further studies are needed, in particular, to demonstrate the link between an increase in CAT capacity to answer to oxidative stress and a better tolerance to peroxidizing compounds.

Previous studies reported concentrations of oxyfluorfen between 0.1 and 1 $\mu g\ L^{-1}$ (estimated concentrations in San Joaquin River based on sediment data, US EPA/OPP 2001) with a maximum of 541 $\mu g\ L^{-1}$ being observed after an accidental spillage (US EPA/OPP 2001). Then, based on our result, oxyfluorfen may represent a risk in the environment. In particular chronic exposure to environmental concentrations may provoke changes in community structure as in the present study eukaryotic diversity was affected after chronic exposure to 3 $\mu g\ L^{-1}$ of oxyfluorfen. Chronic

exposure may thus affect the community by selecting resistant species and provoking a decrease in biofilm biodiversity. Scenarios of chronic contamination by oxyfluorfen may be especially problematic in multiple stress situations. Indeed, species tolerant to other types of stresses (other herbicides, temperature increase, etc.) might disappear due to oxyfluorfen exposure and, therefore, the resistance of the whole community might be lowered (Vinebrooke et al. 2004).

4.3 AEA: temporal variation vs. ecotoxicological effects

Knowing the magnitude and typology of changes occurring in non-exposed biofilms throughout the duration of the experiment facilitates the evaluation of the effects caused by toxicant exposure. In the present study, while some extreme values of AEA were detected in exposed biofilms, it has not been possible to determine activity thresholds indicative of adverse effects. This limitation was due to the high variability observed in both non-exposed and exposed communities but also to the unimodal pattern of variation of AEAs. Indeed, differences between exposed and non-exposed communities were better highlighted by comparing the pattern of AEA response throughout oxyfluorfen gradient after acute exposure. Therefore, the estimation of the concentration range leading to the maximal short-term response may be especially useful to discriminate between controls and exposed communities.

5. Conclusion

Since the pool of AEA dedicated to oxidative stress management changed as biofilm aged, AEA of biofilms may depend strongly on biofilm age. This natural variability may prevent the direct interpretation of biofilms'AEA, especially in the field where biofilm age is rarely known. Nevertheless, AEA brought valuable information on the oxidative effects of oxyfluorfen when the classical biomarker: chlorophyll-*a* was not found sensitive to this toxicant. In particular, the use of AEA in short-term ecotoxicological tests revealed the functional changes provoked by chronic exposure to oxyfluorfen, that is a higher CAT capacity to respond to higher levels of oxidative stress (induced by oxyfluorfen) in biofilms chronically exposed to the same compound (oxyfluorfen). Then, the estimation of the range of concentration leading to the maximal AEA may be an interesting indicator of previous exposure to peroxidizing compounds and/or capacity to cope with oxidative stress. Chronic contamination by oxyfluorfen at environmentally relevant concentration is of concern as it may reduce algal and bacterial diversity in particular by selecting species more resistant to oxidative stress.

Acknowledgements

We thank Berta Bonet for technical help during sampling. Financial support was provided by the Spanish Ministry of Science and Education (FLUVIALMULTISTRESS-CTM2009-1411-CO2-01) and the EC Sixth Framework Program (KEYBIOEFFECTS MRTN-CT-2006-035695).

Supporting information

Table A1. Normed coefficients of the following variables: AEA, photosynthetic and biomass variables on the axes of the co-inertia analysis (CO1, CO2).

Variables	CO1	CO2
CAT	0.35	-0.23
APX	-0.21	-0.53
GR	0.25	-0.29
Ph. eff.	0.11	0.22
Wet weight biofilm	0.29	0.64
Protein conc. (µg mg^{-1})	-0.41	0.35
chlorophyll-a conc. (µg cm^{-2})	0.71	-0.05

Table A2. Normed coefficients of the relative abundance of pigments on the axes of the co-inertia analysis (CO1, CO2).

Pigments		CO1	CO2
code	complete name		
ANT	antheraxanthin	0.25	-0.01
bbCAR	β,β-carotene	0.00	-0.29
CAR	carotenoid P468	0.36	0.15
CHLa	chlorophyll-*a*	0.36	-0.04
CHLb	chlorophyll b	-0.03	-0.39
CHLc	chlorophyll c	-0.26	0.22
DIAD	diadinoxanthin	0.08	0.06
DIADcI	diadinochrome I	-0.07	0.35
DIADcII	diadinochrome II	-0.28	0.14
DIAT	diatoxanthin	0.02	0.16
FUC	fucoxanthin	-0.05	0.26
LUT	lutein	0.01	-0.32
NEO	neoxanthin	0.09	-0.22
PHEPa	pheophytin a	-0.18	-0.15
PHERa	pheophorbide a	-0.14	-0.07
tNEO	trans-neoxanthin	0.20	-0.06
VIO	violaxanthin	-0.25	-0.09
ZEA	zeaxanthin	0.30	-0.04
U1	unknown 1	-0.37	-0.10
U2	unknown 2	-0.35	-0.09
U3	unknown 3	-0.05	0.19
U4	unknown 4	-0.01	-0.28
U5	unknown 5	0.06	-0.36

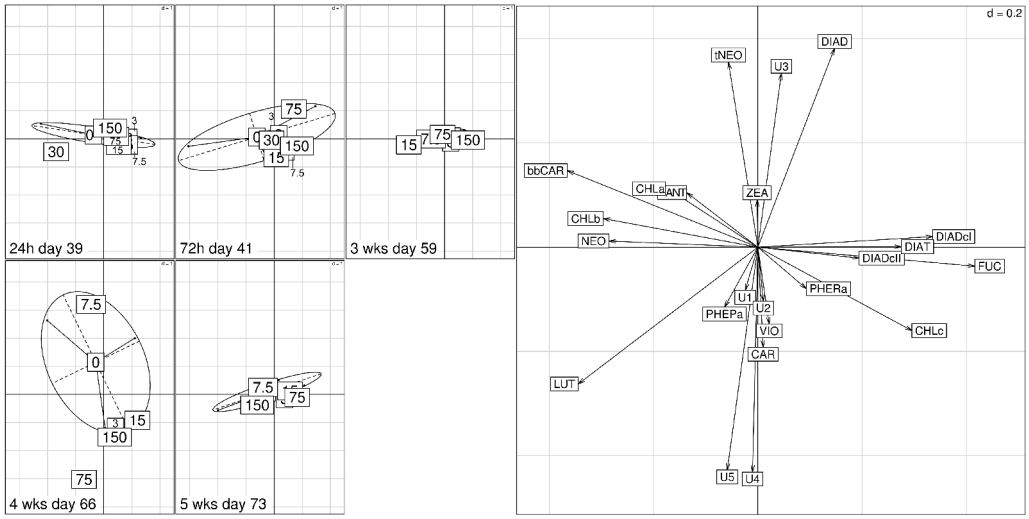
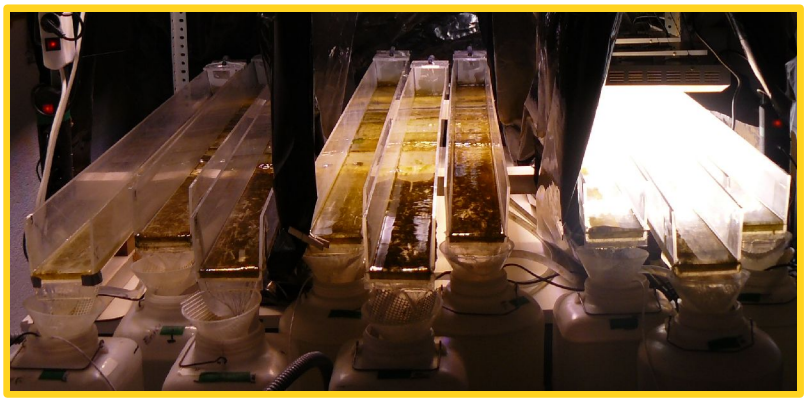


Figure A1. Results of the within-PCA of the pigment matrix of biofilms exposed to oxyfluorfen. (A) Factorial maps: samples collected the same day are represented in different factorial maps. The times of exposure (24h, 48h, 3, 4 or 5 weeks) are indicated followed by the sampling day (from 39 to 73); wks stands for weeks. The sample labels correspond to the exposure concentration of oxyfluorfen. (B) Normed coefficients of the variables on the first two axes of the within-PCA. The correspondence between pigment codes and complete names is given on Table A2.

Chapter III

Light history modulates antioxidant and photosynthetic responses of biofilms to natural stressors (light) and chemical stressors (herbicides)



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Abstract

The influence of light adaptation on the response of freshwater biofilms to natural and chemical stressors was investigated. Biofilms grown in microcosms under sub-optimal, saturating, and high light intensities showed characteristics common to shade/light adaptation. This light history modulated antioxidant and photosynthetic responses of biofilms to the stress caused by short-term exposure to sudden light changes or to herbicides. The response to high light stress was characterized by an increase in ascorbate peroxidase (APX) activity and photochemical quenching combined with a decrease in photosynthetic efficiency in shade-adapted biofilms. Exposure to sub--optimal light intensity led to a decrease in catalase (CAT) activity and non--photochemical quenching in biofilms adapted to high light intensity. Glyphosate exposure led to a stronger decrease in photosynthetic efficiency of shade-adapted biofilms (EC_{50} = 11.7 mg L^{-1}) than of high-light adapted communities (EC_{50} = 35.6 mg L^{-1}). Copper exposure led to an activation of APX only in biofilms adapted to the lowest light intensity (EC_{50}=1949 µg L^{-1}) while a decrease in protein content was observed in all biofilms. Oxyfluorfen toxicity was independent of light history provoking an increase in APX activity (EC_{50} = 10369 µg L^{-1} in high-light adapted biofilms). While light history influenced biofilms'response to glyphosate (co-tolerance), it had little or no influence on response to copper or oxyfluorfen. Moreover shade-adapted biofilms were more sensitive to an increase in light intensity than high-light adapted ones to a reduction in light intensity. Therefore, not only previous exposure to contaminants, but also physical habitat characteristics would influence community resistance to disturbances strongly.

Keywords: shade/light adaptation, periphyton, glyphosate, copper, oxyfluorfen, co-tolerance.

Abbreviations: ROS, reactive oxygen species; AEA, antioxidant enzyme activity; SOD, superoxide dismutase; CAT, catalase; APX, ascorbate peroxidase; GR, glutathione reductase; AMPA, aminomethylphosphonic acid; SUB, sub-optimal; SAT, saturating; HL, high light; Ph. eff., photosynthetic efficiency; Ph. cap., photosynthetic capacity; qP, photochemical quenching; NPQ, non photochemical quenching following Bilger and Bjorkman (1990); qN, non photochemical quenching following Schreiber et al. (1986).

1. Introduction

River biofilms (also known as periphyton) are important primary producers in aquatic ecosystems (Stevenson et al. 1996); in mid-sized streams these benthic communities are regarded as the principal source of energy (Vannote et al. 1980). These complex communities are made up of two components: an autotrophic component constituted of algae and cyanobacteria and an heterotrophic component composed of bacteria, fungi and protozoa. All these micro-organisms live and interact closely together in an extracellular matrix (Romaní 2010). Though other factors play an important role in the ecosystem (grazing pressure, flow velocity, temperature, etc.), light is the first energy source for the autotrophic community (Hill 1996) and its quantity and quality are closely related with temporal variability of primary production in rivers (Hill and Knight 1988). Different factors can influence light quality and quantity reaching biofilms (riparian vegetation, water turbidity, biofilm thickness, etc., Hill 1996) and biofilms can colonize a wide range of benthic light environments from turbid rivers under dense canopy cover to open shallow and clear streams (Wetzel 2005). In addition, in all these environments, light quantity and quality fluctuate throughout the day due to cloudy spells or movements in the riparian vegetation among others (Hill 1996). Biofilm communities, such as phytoplankton, respond to light fluctuations by photoacclimation, that is light or shade acclimation (Boston and Hill 1991; Falkowski 1981). Acclimation and adaptation to light conditions are both characterized by physiological changes in order to maximize light harvested and minimize photo damage (Falkowski and LaRoche 2004). Among the changes observed, variations in pigment composition either at short or long term have been reported; the de-epoxydation of diadinoxanthin in diatoxanthin in the xantophyll cycle allows diatoms to dissipate excess of energy within minutes (Laviale et al. 2009) while the concentration in antenna pigments is usually found to decrease with increasing light irradiance (Hill and Boston 1991). Biofilm thickness and downward micro migration of some motile diatoms have also been reported in biofilms as protective mechanisms against light excess (Hill and Boston 1991; Kromkamp et al. 1998). In

addition, light intensity plays an important role in the structure of the biofilm community; diatoms are expected to dominate under shaded conditions, the community is predicted to be more heterogeneous including diatoms, cyanobacteria and chlorophytes under intermediate intensities while under high light intensities chlorophytes are expected to dominate (Steinman et al. 1989).

The diversity in benthic light environments is a natural feature but it can also result from anthropogenic disturbances and be associated with chemical contamination leading to multiple stress situations (Matthaei et al. 2010). In these situations the physiological processes allowing an organism to attenuate the detrimental effects of one stressor (the physiological tolerance) may be constrained by the environmental conditions (the ecological tolerance; Niinemets and Valladares 2008). Consequently in such situations the ecological tolerance may be lower or higher than the physiological one (Niinemets and Valladares 2006). Agricultural areas are an example of such situations in which the entrance of herbicides can co-occur with the modifications of river banks (Allan 2004). Indeed, these modifications can greatly change light intensity in the aquatic environment. The resulting excess or limitation of light during growth can be regarded as previous stress that may constrain the response to further forms of stress, such as the entrance of herbicides in the river. This problem is of special concern as these chemicals are designed to be effective in autotrophic organisms. Previous studies showed that light history modulates the toxicological response of biofilms exposed to herbicides (Guasch et al. 2003; Laviale et al. 2010); for instance, biofilms from open sites were found to be more sensitive to atrazine than biofilms from shaded rivers in a study by Guasch and Sabater (1998). These studies mainly focused on impacts of herbicides on photosynthesis or pigment composition while other parameters, such as the antioxidant enzymes, also likely to be affected by both herbicides and light, were not addressed. Antioxidant enzymes are the first barrier against oxidative stress that can be both induced by excess of light and some herbicides (Geoffroy et al. 2003; Guasch et al. 2010; Ledford and Niyogi 2005). Indeed, antioxidant enzymes participate in redox homeostasis by scavenging Reactive Oxygen

Species (ROS, O_2^1: singlet oxygen, O_2^- : superoxide anion, H_2O_2: hydrogen peroxide, HO^-: hydroxyl radical) and avoiding their accumulation and the resulting oxidative stress (Mittler 2002). The present study focused on four important antioxidant enzymes: the superoxide dismutase (SOD), the catalase (CAT), the ascorbate peroxidase (APX) and the glutathione reductase (GR). SOD (EC 1.15.1.1) catalyses the dismutation of the most reactive ROS: the superoxide anion in oxygen and hydrogen peroxide (Scandalios 1993); the latter is then scavenged by CAT (EC 1.11.1.6, Chelikani et al. 2004) and APX (EC 1.11.1.11, Lesser 2006). GR (1.8.1.7) participates in this reaction by regenerating the co-factor needed by APX (ascorbate-glutathione cycle, Mittler 2002).

In this context, biofilms adapted to different light conditions may have different strategies to cope with oxidative stress and may present different sensitivity towards herbicides that induce it. Actually two hypotheses are possible:

1. The ecological tolerance acquired under stress conditions is higher than the physiological one. In this case, biofilms grown under higher light intensity are expected to develop more effective physiological processes to cope with oxidative stress than biofilms grown under lower light intensity. The presence of improved physiological processes may then enhance the tolerance to oxidative stress due to herbicides exposure in biofilms grown under higher light intensity (co-tolerance phenomenon; Vinebrooke et al. 2004).

2. The ecological tolerance acquired under stress conditions is lower than the physiological one. In this case, the cost (in terms of energy or species richness) of the physiological processes developed by biofilms to cope with oxidative stress induced by growth light conditions will increase with the light intensity. At one point (the highest light intensity), the cost may become too important for the community to be able to cope with the co-occurrence of stressors: oversaturating light and herbicides exposure causing oxidative stress. In such a case a decrease in tolerance is expected.

To explore these hypotheses, different points were experimentally addressed in the present study. First biofilms were grown in microcosms at different light intensities simulating shaded, saturating and oversaturating light conditions. To reveal patterns of adaptation, these communities were characterized in terms of diatom community composition, photosynthetic parameters, chlorophyll a and antioxidant enzymes activities (AEA). Secondly, to reveal differences in sensitivity to chemical and natural stressors, the responses of these communities to different light intensities and to herbicides were measured. The different light intensities were chosen to reflect sudden changes in river banks leading to an increase in light intensity (e.g. removal of riparian vegetation) or a decrease in light intensity (e.g. entrance of organic matter or sediments provoking water turbidity). These sudden changes are expected to cause stress in non-adapted communities. To estimate the impact of herbicides on the different communities, classical dose-response tests were performed in which exposed samples from each community were compared to their corresponding controls. This common procedure in ecotoxicology allows the toxicological responses between different test organisms and endpoints to be compared; in our case, the communities adapted to different light conditions and the biomarker responses (Clements and Newman 2002). The four herbicides selected (copper, oxyfluorfen, glyphosate and aminomethylphosphonic acid: AMPA) were expected to provoke oxidative stress or to affect photosynthesis. Copper is known to inhibit photosynthesis and to increase ROS (Serra et al. 2010; Pinto et al. 2003). Oxyfluorfen, through the inhibition of chlorophyll a biosynthesis, provokes oxidative stress and photosynthesis inhibition in microalgae (Geoffroy et al. 2003). Glyphosate is known to reduce photosynthesis in biofilms (Goldsborough and Brown 1988) probably by inhibiting aromatic aminoacids biosynthesis and the dependent secondary metabolism (Herrmann 1995), while the effect on algae of its main degradation product, AMPA, remains unknown.

2. Material and methods

2.1 Biofilm colonization

Colonization was performed in an indoor microcosm consisting of nine recirculating channels previously described by Serra et al. (2009a), 10L of dechlorinated tap water were recirculated in each channel. A cooling bath maintained the water temperature at 19ºC. Water was completely replaced three times a week, and to avoid nutrient depletion, phosphate was added to a final concentration of 92 µg L^{-1}. During the last three weeks of colonization, silicate was also added daily in each channel to a final concentration of 15 µg L^{-1} to compensate for a potential limitation. Biofilms colonized sandblasted glass substrata of 1cm^2 installed on the bottom of each channel. Once a week, an original inoculum of biofilm, obtained from the river Llémena (NE Spain, Serra et al. 2009a) was added to each channel. In order to determine the initial diatom community composition, 10 mL of inoculum added at week 1, 3 and 4 of colonization were stored at room temperature after adding 1mL of alcohol. LEDs were used to provide light between 450-660 nm with a regime of 12h:12h light:dark. During the whole colonization period, three channels were incubated under a suboptimal light intensity of 25 µmol photons m^{-2} s^{-1}, three others under a saturating light intensity of 100 µmol photons m^{-2} s^{-1} and the three last ones under a high light intensity of 500 µmol photons m^{-2} s^{-1}. Biofilms grown in these different channels will be hereafter referred to as sub-optimal biofilms (SUB), saturating biofilms (SAT) and high light biofilms (HL) (Fig. 1).

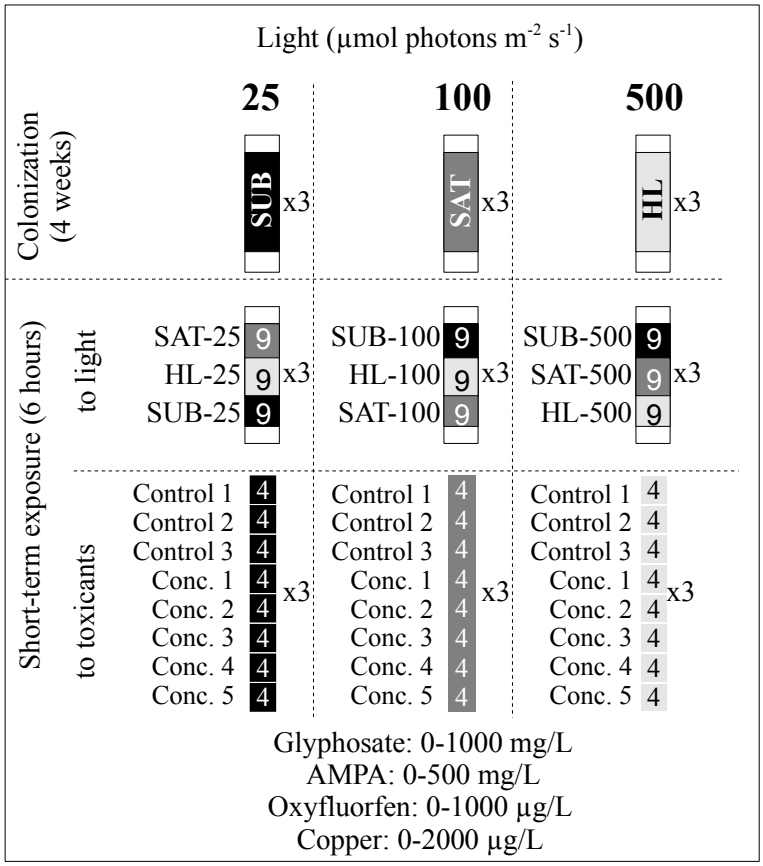

Figure 1. Design of colonization and short-term experiments. Biofilms were grown under different light intensities (SUB biofilms in black, SAT in dark grey and and HL in light grey), for each light condition three channels were colonized for each biofilm community. Thereafter biofilms from the different channels were exposed for 6h to three different light intensities and also to five increasing concentrations of four toxic substances. The numbers 4 and 9 correspond to the number of glass substrata incubated under each condition while x3 indicates that experiments were conducted in triplicates.

2.2 Diatom community composition

After four weeks of colonization, one colonized substrata was collected from each channel and stored at room temperature in 10 mL channel water and 1 mL of alcohol. After scraping biofilm from glass substrata (Nunc, Wiesbaden, Germany) an

aliquot of algal suspension from each sample was prepared using hot hydrogen peroxide. Samples were then mounted on slides using Naphrax (r.i.: 1.74) and up to 400 valves from each slide were counted on random transects under a light microscope (Nikon Eclipse 600 W) using phase-contrast and Nomarski differential interference contrast optics at a magnification of 1000x. Diatoms were mainly identified following Krammer and Lange–Bertalot (Krammer and Lange-Bertalot 1985-1991). In case of doubtful taxonomy, the monographs of Lange-Bertalot (1993, 2001) and Krammer (2000) were also used. Finally biovolume, taxa richness and the Shannon-Wiener index were calculated for each sample. The biovolume of the diatom community was estimated after measuring length, width, and height of 10–30 individuals per species and applying a geometric formula to obtain their volume, using BIOVOL software (Hillebrand et al. 1999; Kirschtel 1996). The total biovolume of the sample was then calculated from the respective composition and densities per surface area of the species in the samples.

2.3 Experimental set-up for short-term exposure to different light intensities

After four weeks of colonization, 18 colonized substrata were taken from each channel and distributed between two other channels incubated under the two other light intensities (Fig. 1). Consequently, biofilms adapted to the three different light conditions were exposed to their colonization light intensity and to two other light intensities (100 and 500 µmol photons m^{-2} s^{-1} for SUB biofilms, 25 and 500 for SAT, 25 and 100 for HL; Fig. 1). After 6h of exposure to the different light intensities, 9 glass substrata colonized by SUB, SAT and HL biofilms were collected from each channel.

2.4 Experimental set-up for short-term exposure to different herbicides

Four acute toxicity tests were performed with the following herbicides: glyphosate (*N*-(Phosphonomethyl)glycine, monoisopropylamine salt solution, CAS: 38641-94-0), AMPA ((Aminomethyl)phosphonic acid, CAS: 1066-51-9), oxyfluorfen (CAS : 42874-03-3) and copper ($CuCl_2$, copper titrisol, CAS : 7447-39-4).

Glyphosate concentrations (0.01, 1, 10, 100 and 1000 mg L^{-1}) were chosen according to previous studies on glyphosate effect on biofilm and algae (Goldsborough and Brown 1988, Wong 2000). AMPA is the product of microbial degradation of glyphosate (Rueppel et al. 1977) and few studies have assessed its impact on algae. AMPA concentrations (0.01, 0.5, 5, 50 and 500 mg L^{-1}) were then chosen to be similar to glyphosate molar concentrations. Oxyfluorfen (1.5, 15, 75, 150 and 1000 µg L^{-1}) and copper (20, 65, 200, 650 and 2000 µg L^{-1}) concentrations were determined according to previous studies (Serra et al. 2010).

Acute toxicity tests were performed on 4 week-old biofilm in a microcosm set-up previously described (Bonnineau et al. 2010). Briefly, each glass substrata was incubated in a vial containing 10 mL of colonization medium and the corresponding toxicant concentration. Samples were incubated during 6 h under the same conditions as the colonization, using a single-speed orbital mixer (KS260 Basic, IKA®) to maintain constant agitation.

For each channel and each toxicant a dose-response was performed including 3 controls and 5 concentrations; for each control or concentration, 4 glass substrata were incubated (Fig. 1), 3 were used for AEA determination and 1 for photosynthetic parameters measurements and chlorophyll-*a* quantification.

2.5 Biofilm biomarkers

2.5.1 AEA

Sampling, protein extraction and AEA measurements were performed as described previously (Bonnineau et al. 2011). Biofilm was removed from glass substrata with a cell scraper (Nunc, Wiesbaden, Germany), put into an eppendorf tube and centrifuged (2300 g, 10ºC, 5 min) to remove the excess of 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 30 s 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 30 s of beadbeater (MP FastPrep-24, v = 4 m s^{-1}) with 2 min intervals on ice. After cell disruption, homogenates were centrifuged at 10000 g and 4ºC for 30 min. Supernatant was used as the enzyme source (Bonnineau et al. 2011). The protein concentration of supernatant was measured in triplicates for each sample by the method of Bradford (1976) using Coomassie Brilliant Blue G-250 dye reagent concentrate (Bio-Rad, Laboratories GmbH, Germany) and bovine serum albumin as a standard. Protein content was then standardized by biofilm surface and expressed in µg cm^{-2}.

AEA measurements were performed in microtiter plates (UV-Star 96 well plate, Greiner®), changes in absorbance were followed using a microtiter plate reader Synergy4 (BioTek®). For all assays the optimal protein concentration was determined by using 1, 1.5, 2, 4, 6, 8 and 10 µg of proteins. All AEA were calculated as specific activities (i.e. per µg or mg of proteins).

CAT activity was measured spectrophotometrically by following the decomposition of H_2O_2 at 240 nm and 25ºC during 2 min (Aebi 1984). The 250 µL reaction mixture contained in final concentration: 80 mM of potassium phosphate buffer (pH 7.00), 30mM of H_2O_2 and 2µg of proteins. The optimal substrate concentration was determined by using 5, 10, 15, 20, 25, 35 and 40 mM H_2O_2. CAT activity was calculated as µmol H_2O_2 mg $protein^{-1}$ min^{-1} (extinction coefficient, ε: 0.039 cm^2 μmol^{-1}).

Oxidation of sodium ascorbate by APX was measured at 290 nm and 25ºC for 2 min according to Nakano and Asada (1981). The 250 µL reaction mixture contained in final concentration: 80 mM of potassium phosphate buffer (pH 7.00), 4 mM of H_2O_2, 150 µM of sodium ascorbate and 2 µg of proteins. The optimal substrate

concentration was determined by using 0.5, 1, 1.5, 2, 2.5, 3 and 4 mM H_2O_2. APX activity was calculated as μmol ascorbate mg protein^{-1} min^{-1} (ε: 2.8 cm² μmol^{-1}).

The oxidation of NADPH by GR was determined by measuring the decrease in absorbance at 340 nm and 25ºC for 2 min (Schaedle and Bassham 1977). The 200 μL reaction mixture contained in final concentration: 100 mM Tris-HCl (pH 7.50), 1 mM EDTA, 1 mM oxidized glutathione, 0.25 mM NADPH and 4 μg of proteins. The optimal cofactor concentration was determined by using 0.05, 0.1, 0.15, 0.2, 0.25 and 0.3 mM NADPH. GR activity was calculated as μmol NADPH mg protein^{-1} min^{-1} (ε: 6.22 cm² μmol^{-1}).

SOD activity was measured by the xanthine/xanthine oxidase assay (McCord and Fridovich 1969, Peskin and Winterbourn 2000). The 200 μL reaction mixture contained in final concentration: 50 mM of sodium-phosphate buffer (pH 8.00), 0.1 mM diethylene triamine, 0.1 mM hypoxanthine , 0.05 mM WST-1 (sodium salt of 4-[3-(4iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate), 6 mU mL^{-1} of xanthine oxidase and 1 μg of proteins. SOD competes with WST-1 for superoxide anions (O_2^{-}) generated by the xanthine/xanthine oxidase system. WST-1 reduction was measured at 450 nm and 25ºC during 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}.

2.5.2 Photosynthetic parameters

Colonized glass substrata were collected for in vivo photosynthetic parameters determination using a PhytoPAM (Pulse Amplitude Modulated) fluorometer (HEINZ WALZ, Effeltrich, Germany). The distance between the optical fiberoptics and the sample surface was set at 2mm. The fluorescence signal was determined by the emitter-detector unit (PHYTO-EDF). After 20 minutes of dark-adaptation, a weak measuring light was applied in order to obtain the minimal (Fo) and maximal (Fm) fluorescence yield of dark-adapted sample. After light adaptation, 5 saturating pulses of actinic light (PAR=120) were applied to the samples to obtain the fluorescence

signal at the steady-state (F), the maximal fluorescence yield (Fm') of an actinic--adapted sample and the minimal fluorescence yield (Fo'). These parameters were used to calculate the photosynthetic efficiency (Ph. eff.), the photosynthetic capacity (Ph. cap.), the photochemical quenching (qP) and the non-photochemical quenchings following Bilger and Bjorkman (1990; NPQ) and following Schreiber et al. (1986; qN) as described in Table 1. All calculations were done using the fluorescence signal recorded at 665 nm and are given as relative units of fluorescence. The relative contribution of the different algal group (green algae, cyanobacteria, diatoms) to the overall fluorescence was also estimated, based on the internal reference excitation spectra of a pure culture, which had been previously validated for periphyton communities by Schmitt-Jansen and Altenburger (2008a). Photosynthesis irradiance (P-I) curves were obtained by measuring the relative electron transport rate (rETR) along light irradiance gradient (Laviale et al. 2009). P-I data from each channel were then fitted to the following inhibition model: $rETR = rETR_m (1-e^{(-\alpha I/rETRm)}) e^{(-\beta I/rETRm)}$ (Platt et al. 1980). The fitted curves were used to estimate the initial slope of the P-I curve (α) and the inhibition parameter (β). Light saturation (I_{sat}) and light inhibition parameter (I_b) were calculated as $rETR_m / \alpha$ and $rETR_m / \beta$, respectively, where $rETR_m$ is the maximum photosynthetic rate (Henley 1993).

Table 1. Formulas used to calculate the different photosynthetic parameters.

Parameters	Equations	References
Ph. eff.	(Fm'-F)/Fm'	Genty et al. (1989)
Ph. cap	(Fm-Fo)/Fm	
qP	(Fm'-F)/(Fm'-Fo')	Schreiber et al. (1986)
qN	1 - [(Fm' - Fo') / (Fm – Fo)	
NPQ	(Fm - Fm')/ Fm'	Bilger and Bjorkman (1990)

2.5.3 Chlorophyll-a concentration

After exposure glass substrata were collected into falcon tubes, immediately frozen in liquid nitrogen and stored at -80ºC until chlorophyll-*a* extraction. The chlorophyll-*a* content was extracted with 90% acetone for 12 h. Sonication (Ultrasonic bath, J.P Selecta) during 2 min improved the pigment extraction and the chlorophyll-*a* concentration was subsequently estimated from spectrophotometric measurements (spectrophotometer UV-1800, Shimadzu) following the method described in Jeffrey and Humphrey (1975).

2.6. Calculation

Mean values are always presented with their corresponding standard errors. The statistical significance for all the analyses was set at $p < 0.05$.

2.6.1 Characterization of biofilm communities

Analyses of diatom community composition data were performed using PRIMER–6 (Clarke and Gorley 2005). From the total number of taxa identified, only those taxa with relative abundances greater than 4% were included in the statistical analysis in order to minimize the influence of rare taxa. The similarity between diatom community assemblages from SUB, SAT and HL biofilms was tested using the ANOSIM routine (Clarke 1993), which tests a priori grouping designations against 1,000 random group designations in ordination space. Finally, indicator taxa for each type of biofilm were determined by the IndVal analysis (Dufrène and Legendre 1997) by using Pc–Ord v.5 statistical package (McCune and Mefford 1999).

Differences between SAT, SUB and HL biofilms after colonization in terms of photosynthetic parameters, parameters from P-I curves, chlorophyll-*a*, protein content and AEA were estimated by analysis of variance (ANOVA) and *post hoc* analysed by a Tukey test using R 2.6.2 (R Development Core Team, 2008).

2.6.2 Short-term exposure to different stressors

Following data analyses were performed using R 2.6.2 (R Development Core Team 2008). To analyse the results from the short-term exposure to light or herbicides, a two-step procedure was used to focus on those biomarkers affected by the stressor and to estimate the influence of colonization light intensity on their response. For each stressor (light, glyphosate, AMPA, copper or oxyfluorfen) and each biofilm community (SUB, SAT or HL biofilm), a one-way ANOVA was performed to determine the biomarkers affected by each stressor. Once the biomarkers affected by the stressor were detected, a two-way ANOVA was performed to determine the influence of colonization light on these biomarkers' response to the stressor, mainly through the calculation of the interaction term.

Concerning the short-term exposure to different light intensities, the results of the one-way ANOVA were also *post hoc* analysed by a Tukey test to reveal differences between controls (biofilms exposed to their own colonization light) and exposed samples. In addition, the biomarker responses in SUB, SAT and HL biofilms exposed to the same light intensity were compared between them using a one-way ANOVA and *post hoc* analysed by a Tukey test.

Concerning the short-term exposure to the different herbicides, a concentration-effect curve was fitted, following a best-fit method, to the response of those biofilm biomarkers, significantly affected by herbicide exposure. Five different types of models (Weibull, log-logistic, Brain-Cousens, Michaelis Menten, asymptotic regression) suitable to describe concentration-response data were independently fitted to each set of data (package drc, Ritz and Streibig 2005). Models having the lowest Akaike Information Criterion (AIC, Akaike 1974) were first selected as AIC is considered to be a measure of the goodness of fit of an estimated statistical model. Then analysis of residuals was performed by plotting the residuals against the mean effects estimated from the fitted regression model. This final selection step allows departures from the fitted model to be detected and provides insight into the validity of

a regression model and error assumption (Scholze et al. 2001). Fitting parameters for each concentration-response curve are available in the supporting information (Table A1). Finally, concentrations leading to an increase or a decrease by 50 % of control value (EC_{50}) and corresponding 95 % confidence interval were calculated by the Effective Dose (ED) command using either asymptotic-based or logarithm scale-based confidence interval (package drc, Ritz and Streibig 2005). In this study, EC_{50} are always presented with their corresponding confidence interval in brackets: []. When none of the model tested could be fitted to the data, results were *post hoc* analysed by a Tukey test to reveal differences between controls and exposed samples.

3. Results

3.1 Characterization of biofilm communities

Physical and chemical conditions were stable during the colonization for all biofilm communities. Mean values (and standard errors), taking into account all channels, were the following: temperature was 19.3 ± 0.1 ºC (n = 116), dissolved oxygen concentration: 9.57 ± 0.03 mg L^{-1} (n = 120), pH: 8.75 ± 0.02 (n = 120), water conductivity: 455.3 ± 3.5 µS cm^{-1}(n = 119) and flow at a rate of 1.13 ± 0.03 L min^{-1} (n=36). Water used during this experiment has already been characterized by Serra et al. (2009b) for NO_3 (1.68 ± 0.14 mg L^{-1}), NO_2 (0.07 ± 0.01 mg L^{-1}) and NH_4 (<0.1 mg L^{-1}) among others (n = 20 for all). In the present experiment, only phosphate concentration was measured. Phosphate concentration was of 35.6 ± 5.2 µg L^{-1} (n = 15) just after water changes and phosphate addition and declined to low levels (8.4 ± 5.4 µg L^{-1}, n=15) before water changes, however total phosphate depletion was not observed during colonization.

The fluorescence signals of periphyton communities showed differences between colonization light intensity. The percentages of fluorescence linked to the different algal groups were similar in SUB and SAT biofilms, whereas HL biofilms showed a higher percentage of fluorescence linked to cyanobacteria and a lower

percentage of fluorescence linked to diatoms. In addition, fluorescence linked to green algae was only detected in HL biofilms from 2 channels out of 3 (Table 3).

Table 2. Indicator value (IndVal) of diatom taxa in each group.

Inoculum (n=3)	
Planothidium frequentissimum (L-B) Round & Bukhtiyarova	90
Amphora inariensis Krammer	100
Amphora libyca Ehrenberg	86
Amphora pediculus (Kut) Grunow	93
Cocconeis placentula Ehrenberg	93
Navicula cryptotenella Lange-Bertalot	90
Navicula subhamulata Grunow	100
Navicula tripunctata (O.F.Mull.) Bory	100
Nitzschia dissipata (Kut.) Grunow	83
SUB biofilms (n=3)	
Achnanthidium biasolettianum (Grunow in Cl. & Grun.) Round & Bukhtiyarova	88
Caloneis bacillum (Grunow) Cleve	77
Nitzschia fonticola Grun. in Cleve et Moller	74
SAT and HL biofilms (n=6)	
Achnanthidium minutissimum (Kütz.) Czarnecki	55
Fragilaria capucina var.mesolepta (Rab.) Rabenhorst	83
Nitzschia palea (Kut.) W. Smith	75

Analyses of diatom communities allowed a total of 68 taxa to be identified; 24 had relative abundances greater than 4% and were included in further statistical analyses. The analysis of similarities (ANOSIM) between diatom communities of the SUB, SAT, HL biofilms and the inoculum revealed a global R=0.66, $p<0.001$, indicating significant differences between the different types of biofilm. Analyses of pairwise comparisons between the groups showed no significant differences between SAT and HL biofilms (R=0.037, $p=0.01$). Consequently, a new ANOSIM analysis was carried out with the 3 following groups: inoculum (group 1), SUB biofilms (group 2)

and SAT and HL biofilms (group 3), this analysis revealed a global R=0.932 (p=0.01), and pairwise R > 0.80 which indicated the consistency of the new 3 groups. The most representative taxa for each group were found by Indicator Species Analysis and are presented in Table 2. Taxa richness was significantly higher in the inoculum (35.5 ± 3.3) than in the biofilms grown in microcosms (Table 3). A similar Shannon-Wiener diversity index was found for the inoculum (2.9 ± 0.3) and SUB biofilms whereas H' was significantly lower for SAT and HL biofilms (Table 3).

Biofilms showed adaptation to the different colonization light intensities. The P-I curves for SUB and SAT biofilms had higher initial slopes than for HL biofilms (Table 3). Though no significant differences were found, I_b for HL biofilms was almost two times higher than I_b for SUB and SAT biofilms (Table 3). Chlorophyll-*a* content and photosynthetic capacity were found to decrease significantly with increasing colonization light intensities (Table 3). Photochemical quenching was significantly 1.4 times lower in SUB biofilms than in SAT biofilms. Unfortunately it was not possible to calculate it for HL biofilms (Table 3). The other photosynthetic parameters (photosynthetic efficiency, qN and NPQ), the AEA and the two indicators of biomass: biovolumes and protein content were similar in SUB, SAT and HL biofilms (Table 3).

Table 3. Characteristics of SUB, SAT and HL biofilms after 5 weeks of colonization under 25, 100 and 500 µmol photons m^{-2} s^{-1}, respectively.

	Biofilm biomarkers	SUB	SAT	HL
Diatom community	**Taxa richness**	**24.7$^{(a)}$ ± 0.9**	**17.3$^{(ab)}$ ± 0.3**	**14.7$^{(b)}$ ± 1.2**
	H'	**2.6$^{(a)}$ ± 0.3**	**1.4$^{(b)}$ ± 0.1**	**1.3$^{(b)}$ ± 0.1**
	Biovolume (mm^3 cm^{-2})	4.65 ± 2.55	6.25 ± 1.96	3.85 ± 1.67
	Density (no diatoms x 10^6 cm^{-2})	25.54 ± 13.03	53.4 ± 17.72	53.37 ± 17.82
Parameters from P-I curves	**α**	**0.49$^{(a)}$ ± 0.02**	**0.37$^{(a)}$ ± 0.04**	**0.22$^{(b)}$ ± 0.03**
	β	0.06 ± 0.02	0.23 ± 0.2	0.06 ± 0.04
	I_{sat} (µmol photons m^{-2} s^{-1})	53 ± 15	234 ± 175	159 ± 62
	I_b (µmol photons m^{-2} s^{-1})	457 ± 68	641 ± 209	742 ± 193
	rETRm	26 ± 6	76 ± 55	33 ± 10
Photosynthetic parameters	Relative fluorescence of green algae (%)	0 ± 0	0 ± 0	13 ± 7
	Relative fluorescence of diatoms (%)	**69$^{(a)}$ ± 1**	**56$^{(a)}$ ± 4**	**41$^{(b)}$ ± 4**
	Relative fluorescence of cyanobacteria (%)	**31$^{(a)}$ ± 1**	**44$^{(ab)}$ ± 4**	**46$^{(b)}$ ± 4**
	Ph. eff.	0.31 ± 0.01	0.31 ± 0.02	0.27 ± 0.02
	Ph. cap.	**0.57$^{(a)}$ ± 0.01**	**0.40$^{(b)}$ ± 0.02**	**0.34$^{(b)}$ ± 0.02**
	qP	**0.57$^{(a)}$ ± 0.03**	**0.77$^{(b)}$ ± 0.04**	**n.d.**
	qN	0.61 ± 0.02	0.39 ± 0.14	0.45 ± 0.13
	NPQ	1.49 ± 0.10	0.89 ± 0.58	1.14[1]
AEA	CAT (µmol H_2O_2 mg $protein^{-1}min^{-1}$)	36.44 ± 2.67	46.93 ± 1.14	41.88 ± 7.15
	APX (µmol ascorbate mg $protein^{-1}min^{-1}$)	0.19 ± 0.02	0.23 ± 0.01	0.25 ± 0.02
	GR (µmol NADPH mg $protein^{-1}min^{-1}$)	0.19 ± 0.01	0.23 ± 0.02	0.34 ± 0.15
	SOD (U µg $protein^{-1}$)	1.67 ± 0.03	1.64 ± 0.04	1.63 ± 0.06
	Chlorophyll a (µg cm^{-2})	**61$^{(a)}$ ± 6**	**42$^{(a)}$ ± 6**	**15$^{(b)}$ ± 5**
	Protein content (µg cm^{-2})	140.2 ± 12.0	177.5 ± 8.1	173.9 ± 15.2

Mean and standard error are indicated for each biomarker. Biomarkers showing significant differences between the different types of biofilm are indicated in bold, different letters indicate significant differences (result of the Tukey test). [1] : only one replicate could be measured

3.2 Short-term exposure to different light intensities

After the exposure of SUB, SAT and HL biofilms to various light intensities (different from the colonization light intensity), chlorophyll-*a* concentration, non--photochemical quenchings (qN, NPQ), GR and SOD activities were not found significantly different from controls i.e. biofilms exposed at their colonization light (results of one-way ANOVAs, data not shown). Consequently, only results concerning photosynthetic efficiency, photosynthetic capacity, photochemical quenching, CAT and APX activities and protein content are presented here (Fig. 2 and 3).

The patterns of variation of the photosynthetic efficiency, the photosynthetic capacity and the photochemical quenching along light gradient were different for each biofilm community. In SUB and SAT biofilms exposed to 500 µmol photons m^{-2} s^{-1}, photosynthetic efficiency decreased significantly by 50 % ($F = 26.6$, $p < 0.05$) and 45 % ($F = 30.8$, $p < 0.05$), respectively, and photosynthetic capacity by 50 % ($F = 19.7$, $p < 0.05$) and 36 % ($F = 22.8$, $p < 0.05$), respectively, while these parameters remained stable under 25 and 100 µmol photons m^{-2} s^{-1} (Fig. 2). In HL biofilms, photosynthetic efficiency increased significantly (+ 31 %) only in samples exposed at 100 µmol photons m^{-2} s^{-1} ($F = 9.0$, $p < 0.05$) while photosynthetic capacity remained stable under exposure to lower light intensities (Fig. 2). In SUB biofilms exposed to 500 µmol photons m^{-2} s^{-1} qP increased significantly by 38 % ($F = 10.7$, $p < 0.05$) while in SAT and HL biofilms this parameter remained stable along light gradient (Fig. 2). Though qP values of HL were stable in samples exposed to 25 and 100 µmol photons m^{-2} s^{-1}, they could not be compared to controls (HL biofilms exposed to 500 µmol photons m^{-2} s^{-1}) due to missing values. It is also worth to note that a decrease in NPQ was observed from 1.14 in HL-500 ($n = 1$) to 0.35 ± 0.2 in HL-100 ($n = 2$) and 0.18 in HL-25 ($n = 1$). However, this result could not be analysed statistically due to many missing values in NPQ calculations.

After exposure to 500 µmol photons m^{-2} s^{-1} photosynthetic efficiencies of SUB and SAT biofilms were significantly lower than the one of HL biofilms ($F = 13.2$,

p< 0.05; Fig. 2). After exposure to 25 µmol photons m^{-2} s^{-1}, the photosynthetic capacity of SUB biofilms was significantly higher than the ones of SAT and HL communities (F = 34.8, p < 0.05; Fig. 2). Finally qP was significantly higher in HL biofilms than in SUB biofilms after exposure to 25 and 100 µmol photons m^{-2} s^{-1} (F = 11.3 and 11. 7, respectively, p < 0.05; Fig. 2). In addition, the interaction term of the two-way ANOVA between colonization light (Lcol) and light of exposure (Lexp): Lcol x Lexp, was significant for both photosynthetic efficiency (F = 4.4, p < 0.05) and capacity (F = 5.5, p < 0.05) but not for qP (F = 0.9, p > 0.05).

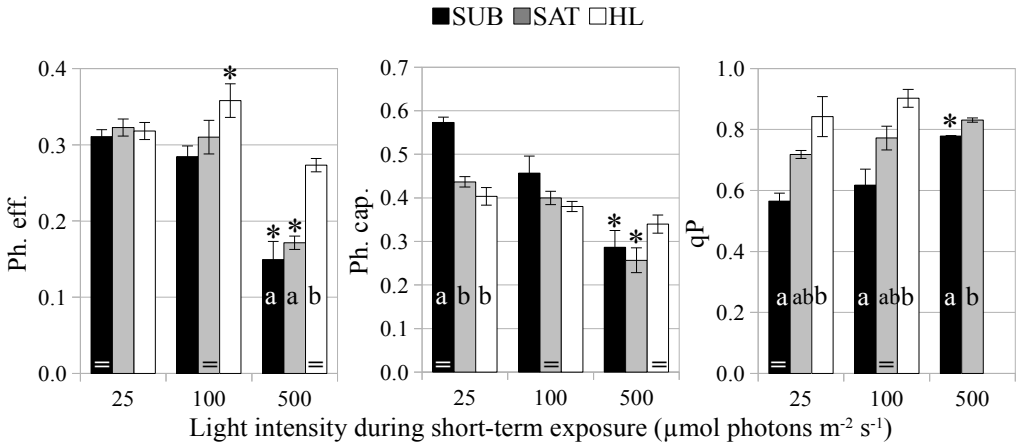

*Figure 2. Photosynthetic efficiency, capacity and qP of SUB (black), SAT (grey) and HL (white) biofilms after 6h of exposure under different light conditions (25, 100, 500 µmol photons m^{-2} s^{-1}). For each community (SUB, SAT or HL): = indicates the control, that is the community exposed to its colonization light, and * indicates, for this community, results significantly different from this control. For each exposure light, the letters on the bars indicate significant differences between SUB, SAT and HL community.*

The patterns of variations of APX, GR activities and of the protein content in biofilm exposed to different light intensities were different for each community. CAT activity remained stable in SUB and SAT biofilms exposed to various light intensities whereas it decreased by 62 % in HL biofilms exposed to 25 µmol photons m^{-2} s^{-1} ($F = 11.9$, $p < 0.05$; Fig. 3). Both APX activity ($F = 11.3$, $p < 0.05$) and protein content ($F = 9.7$, $p < 0.05$) increased significantly by 95 % and 40 %, respectively, in SUB biofilms exposed to 100 µmol photons m^{-2} s^{-1} but remained stable in SAT and HL biofilms exposed to various light intensities.

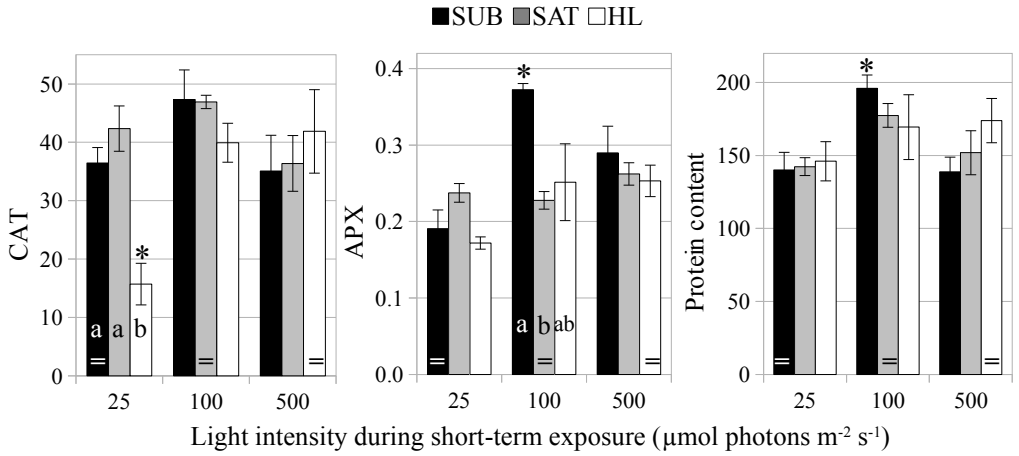

*Figure 3. CAT (µmol H_2O_2 mg protein^{-1} min^{-1}), APX(µmol ascorbate mg protein^{-1} min^{-1}) activities and protein content (µg cm^{-2}) of SUB (black), SAT (grey) and HL (white) biofilms after 6h of exposure under different light conditions (25, 100, 500 µmol photons m^{-2} s^{-1}). For each community (SUB, SAT or HL): = indicates the control, that is the community exposed to its colonization light, and * indicates, for this community, results significantly different from this control. For each exposure light, the letters on the bars indicate significant differences between SUB, SAT and HL community.*

After exposure to 25 µmol photons m^{-2} s^{-1} CAT activity of HL biofilms was significantly lower than the ones of SUB and SAT biofilms ($F = 15.7$, $p < 0.05$; Fig. 3). After exposure to 100 µmol photons m^{-2} s^{-1}, APX activity of SUB biofilms was significantly higher than APX activity of SAT biofilms ($F = 6.9$, $p < 0.05$). Finally, no difference between protein content of SUB, SAT and HL biofilms was found after exposure to any of the light intensity tested. The Lcol x Lexp interaction was significant for CAT ($F = 3.4$, $p < 0.05$) and APX ($F = 3.8$, $p < 0.05$) activities but not for the protein content ($F = 1.4$, $p > 0.05$).

3.3 Short-term exposure to herbicides

Non photochemical quenching: qN, and antioxidant enzymes: CAT and SOD of any of the biofilm community (SUB, SAT or HL) were not significantly affected by any of the herbicides (results of one-way ANOVAs, data not shown). Thereafter, only biomarkers significantly affected by toxicant exposure are presented for each herbicide test.

3.3.1 Glyphosate

Glyphosate provoked a clear decrease in photosynthetic efficiency and capacity in SUB, SAT and HL biofilms (Fig. 4). For photosynthetic efficiency, lower EC_{50}s were obtained for SUB and SAT biofilms (11.7 [-2.5 ; 25.9] and 6.1 [1.1 ; 11.2] mg L^{-1} respectively) than for HL biofilms (35.6 [-167.5; 238.7] mg L^{-1}). Higher EC_{50}s were observed for photosynthetic capacity but following a similar pattern with EC_{50}s of 67.4 [0.5 ; 135.3], 9.8 [1.3 ; 18.3] and 1066.9 [-211.3 ; 4846.9] mg L^{-1} for SUB, SAT and HL biofilms, respectively. The interaction term of the two-way ANOVA between colonization light (Lcol) and herbicide concentration (Tox): Lcol x Tox, was significant for photosynthetic efficiency ($F = 3.3$, $p < 0.05$) and capacity ($F = 2.6$, $p < 0.05$).

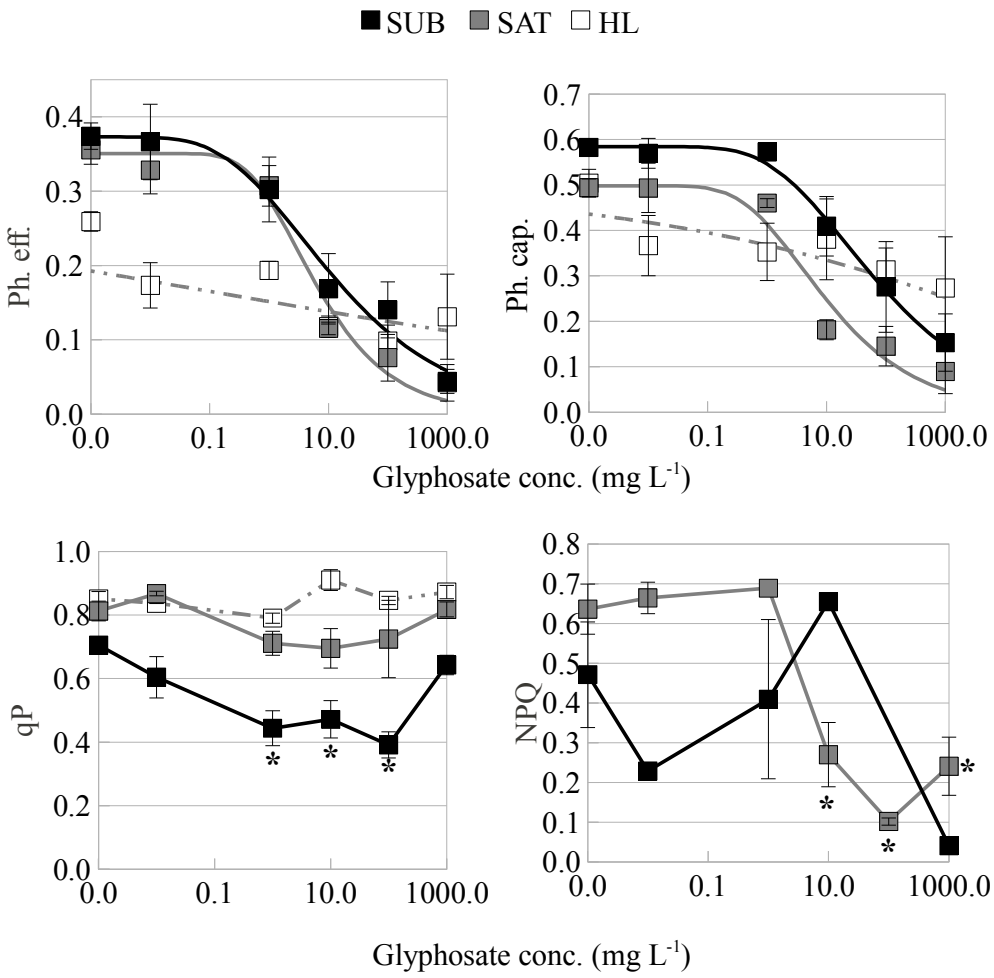

*Figure 4. Photosynthetic efficiency, capacity, qP and NPQ of SUB (black squares), SAT (grey squares) and HL (white squares) biofilms along glyphosate gradient. Only for ph. eff. and cap., lines correspond to model fittings for SUB (black line), SAT (grey plain line) and HL (grey dotted line) biofilms. * : results significantly different from control.*

Quenching mechanisms were also affected by glyphosate exposure. No significant variations of qP in SAT and HL biofilms were detected, whereas in SUB biofilms qP significantly decreased ($F = 10.7$, $p < 0.01$) at intermediate concentrations (from 1 to 100 mg L^{-1}) reaching 44% of inhibition in biofilms exposed to 100 mg L^{-1} (Fig. 4). The Lcol x Tox interaction for qP was also significant ($F = 2.9$, $p < 0.05$).

NPQ could not be calculated for all samples of SUB and SAT biofilms and for none of HL biofilms. A high significant inhibition of NPQ was observed in SAT biofilms exposed to the three highest glyphosate concentrations ($F = 12.9$, $p < 0.01$), whereas in SUB biofilms NPQ was only inhibited at the highest glyphosate concentration (Fig. 4); this difference could not be analysed statistically due to many missing values. Though the NPQ response of SUB and SAT biofilms seems different, the Lcol x Tox term was not significant for NPQ ($F = 1.3$, $p > 0.05$). None of the tested models could be significantly adjusted to qP or NPQ variations along the glyphosate gradient.

Glyphosate was found to interact with Bradford reagent in a concentration-dependent manner, provoking an increase in absorbance at 595 nm that prevented a correct estimation of protein concentration. Therefore, AEA could not be accurately determined for biofilm samples exposed to glyphosate.

3.3.2 AMPA

None of the biomarkers were significantly affected by AMPA exposure in any of the biofilm communities (results of one-way ANOVA, data not shown).

3.3.3 Copper

Copper exposure did not provoke any significant changes either in photosynthetic parameters or in CAT or SOD activities of any of the biofilms communities (results of one-way ANOVA, data not shown), however, GR, APX activities and the protein content were affected by copper.

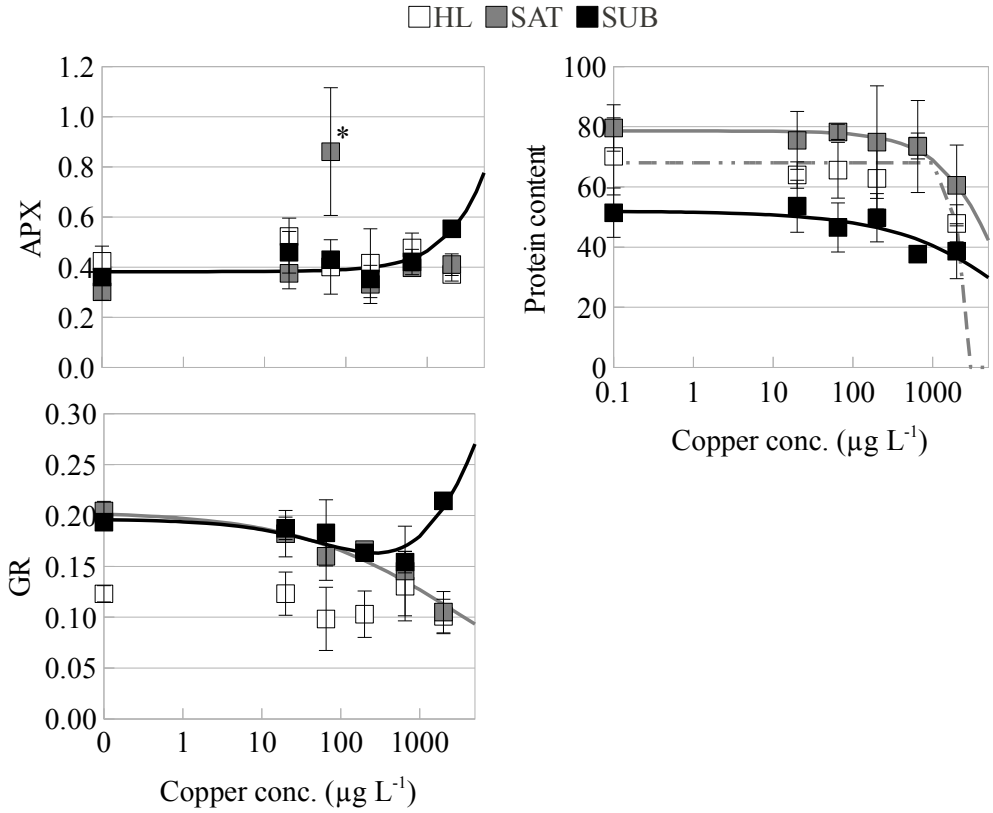

*Figure 5. Antioxidant activities of APX (µmol ascorbate mg protein^{-1} min^{-1}) and GR (µmol NADPH mg protein^{-1} min^{-1}) and protein content (µg cm^{-2}) in SUB (black squares), SAT (grey squares) and HL (white squares) biofilms along copper gradient. Lines correspond to models. * : results significantly different from control.*

APX was activated by copper exposure in both SUB and SAT biofilms while no changes were observed in HL biofilm (Fig. 5). Though a Michaelis-Menten model could be successfully fitted to the increase of APX activity along copper gradient in SUB biofilms (EC_{50} = 1949 [-28116 ; 32014] µg L^{-1}), none of the tested models could be fitted to the trend of the response of SAT biofilms. Indeed the APX response of SAT biofilms was characterized by a significant activation of APX in biofilms exposed to 65 µg L^{-1} ($F = 6.2$, $p < 0.01$; Fig. 5). The Lcol x Tox interaction for APX activity was significant ($F = 3.1$, $p < 0.05$).

Different patterns of GR activity along copper gradient were also observed for each biofilm community. In SUB biofilms, GR was slightly inhibited at intermediate concentrations and activated at the highest copper concentration (Fig. 5); an EC_{50} of 1568 [465 ; 2672] µg L^{-1} was calculated for GR activation. In SAT biofilms, GR was inhibited along the gradient of copper (Fig. 5), reaching 50 % of inhibition at a concentration of 3301 [366 ; 29751] µg L^{-1}. In HL biofilms GR activity remained stable at all concentrations (Fig. 5). However the Lcol x Tox interaction was not significant ($F = 1.8$, $p > 0.05$).

For all the biofilm communities, protein content decreased along the copper gradient (Fig. 5), allowing the calculation of the EC_{50}s: 8054 [-10214 ; 26322], 5507 [-8602 ; 19616] and 2156 [995 ; 3317] µg L^{-1} for SUB, SAT and HL biofilms, respectively. Though the EC_{50}s are different, the Lcol x Tox interaction was not significant ($F = 0.8$, $p > 0.05$).

4.3.4 Oxyfluorfen

Oxyfluorfen provoked only changes in APX activity (Fig. 6) while photosynthetic parameters and the rest of AEA were not significantly affected in any of the biofilm communities (results of one-way ANOVA, data not shown). In all the biofilm communities, APX was activated along the oxyfluorfen gradient. However, none of the models tested could be significantly fitted to the response of SAT biofilms (Fig. 6). The EC_{50} could not be calculated for APX activity of SUB biofilms because the model fitted did not reach 50 % of activation. Indeed the upper limit of the model corresponded to an activity of 0.554 µmol ascorbate mg protein^{-1} min^{-1} (Table A.1) while an activation by 50 % of APX activity would lead to an activity of 0.626 µmol ascorbate mg protein^{-1} min^{-1}. In HL biofilms, the dose leading to an increase by 50 % of APX activity was 10369 [-44415 ; 65153] µg L^{-1}. Moreover, the Lcol x Tox interaction for APX activity was not significant ($F = 1.5$, $p > 0.05$).

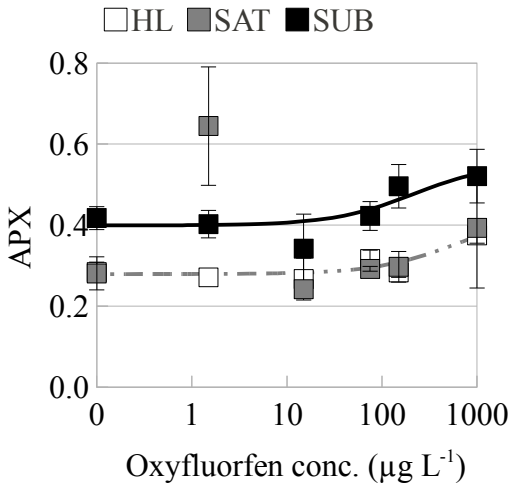

Figure 6. APX activity (µmol ascorbate mg protein^{-1} min^{-1}) along oxyfluorfen gradient in SUB (black squares), SAT (grey squares) and HL (white squares) biofilms. Lines correspond to models.

4. Discussion

4.1 Characterization of biofilm communities

The use of microcosms combined with LED technologies allowed a good control of biofilm colonization conditions and ensured that only the selective pressure (here the light intensity) played a role in the community development.

This set-up allowed obtaining three types of biofilm communities that could be discriminated by their structure or physiology. It is particularly interesting to note that while diatom community composition and photosynthetic capacity separated SUB biofilms from SAT and HL biofilms, parameters indicating shade/light adaptation (α, chlorophyll-*a*) separated the SUB and SAT biofilms from HL biofilms (Table 3). Therefore, it seems that adaptation to sub-optimal light intensity is supported by changes in community structure while adaptation to high light intensity is supported by physiological changes of the community.

Indeed, based on the relative contribution of each algal group to the overall fluorescence, communities grown under light intensity ≤ 100 µmol photons m^{-2} s^{-1} were similar and dominated by cyanobacteria and diatoms while HL communities were characterized by a lower proportion of diatoms and a higher proportion of cyanobacteria and green algae (Fig. 7) in accordance with the prediction of Steinman et al. (1989). The increase in light irradiance decreased the taxa richness and diversity of diatoms strongly (Fig. 7), in agreement with the autoecology of this algal group with preference to low light conditions (Steinman et al. 1989). *Achnanthidium biasolettianum* and *Nitzschia fonticola,* indicators of SUB biofilms, have been described in shaded streams (Roberts et al. 2004), while *Fragilaria* species and *Achnanthidium minutissimum* of the SAT and HL biofilms are typical of open sites (Guasch and Sabater 1998; Roberts et al. 2004; Tornes and Sabater 2010).

Though algal composition was different, all biofilm communities presented similar biovolumes and protein contents indicating that the colonization conditions were not limiting the growth of the different communities. In fact, nutrients were not

limiting, allowing the communities to adapt to their colonization conditions, successfully maintaining equivalent photosynthetic efficiencies. To do so, SUB biofilms presented a higher chlorophyll-*a* concentration in order to harvest a maximum of light energy while HL biofilms had less harvesting pigments to avoid light stress. This difference in chlorophyll-*a* content was directly linked to the differences observed in photosynthetic capacity. These results are in accordance with previous findings on light/shade adaptation of biofilm communities in the field (Stevenson et al. 1996; Laviale et al. 2009).

However, none of these structural or physiological modifications were supported by any changes in AEA as all biofilm communities presented similar CAT, APX, GR and SOD activities. Consequently, these enzymes are unlikely to play a differential role in long-term adaptation to light conditions. The accumulation of other antioxidant molecules as photoprotective pigments (β-carotene, diadinoxanthin and diatoxanthin) may have contributed to light adaptation in our study as described in periphyton adapted to high light conditions by Laviale et al. (2009).

4.2 Short-term exposure to different light intensities

Under field conditions, photoacclimation of biofilm communities to changing light intensity is a common phenomenon allowing organisms to maximize photosynthesis and avoid photodamage (Boston and Hill 1991). In the present study, shade and sun acclimation revealed a limited phenotypic plasticity of biofilms grown under a unique colonization light intensity. Changing light conditions during colonization in the field may allow natural biofilm communities to develop phenotypic plasticity while, in the present study, the strong selection pressure applied by a unique colonization light may limit the photoacclimation capacity of the community.

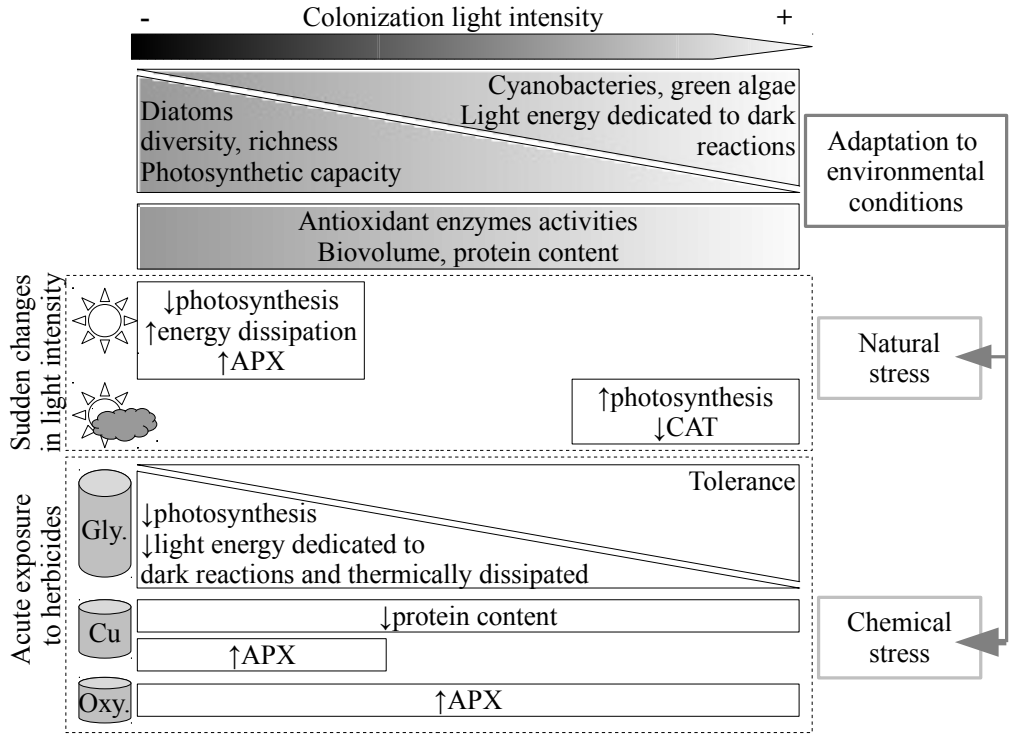

Figure 7. Summary of the main differences between biofilm communities grown under different light intensities (from 25, in black, to 500 µmol photons m^{-2} s^{-1}, in white) after 4 weeks of colonization, after 6h of exposure to an increase (symbolized by a sun) or a reduction in light intensity (symbolized by a sun and a cloud) or after 6h of exposure to increasing concentrations of glyphosate (Gly.), copper (Cu) or oxyfluorfen (Oxy.).

Sun acclimation after short exposure to high light intensity was characterized by photoinhibition in both SUB and SAT biofilms. High light stress provoked stronger effects in SUB than in SAT biofilms (Fig. 2). Though NPQ is one of the main processes in plants and algae to limit photodamage during high light stress (Müller et al. 2001), it was not found to be activated in SUB biofilm communities. Nevertheless, APX was found to be activated under 100 µmol photons m^{-2} s^{-1} (Fig. 3) suggesting an activation of Melher peroxidase reaction. This process allows plants to dissipate excess

of electrons through a "pseudocyclic" electron flow (Neubauer and Yamamoto 1992) and may allow SUB biofilms to maintain a constant photosynthetic efficiency under a slightly higher light intensity (100 µmol photons m^{-2} s^{-1}). This increase in APX activity might be related to an increase in enzyme synthesis as suggested by the increase in protein content observed (Fig. 3). Photochemical quenching was also activated under 500 µmol photons m^{-2} s^{-1}, indicating an increase in light energy dedicated to dark reactions, likely to compensate the effects of the strong photoinhibition (Fig. 2). Shade acclimation was observed in HL biofilms exposed to lower light intensities. It was characterized by a slight increase in photosynthetic efficiency (Fig. 2) and a decreasing tendency of NPQ, indicating that, to maintain the same photosynthetic efficiency, less light energy was heat dissipated (Müller et al. 2001). This change in light utilization may have reduced the oxidative stress in the cells as shown by the decrease in CAT activity observed in HL biofilms exposed to 25 µmol photons m^{-2} s^{-1} (Fig. 3)

The intensity of the colonization light influenced the photosynthetic and the antioxidant (CAT and APX) response of biofilms exposed to different light intensities. AEA variations supported the results obtained by more classical biomarkers as photosynthetic parameters. High-light adapted biofilms were more tolerant to a reduction in light intensity than shade-adapted biofilms to high light exposure (Fig. 7). Indeed, exposure to sub-optimal light intensity of high light-adapted biofilms may be favourable for the community, for example, by reducing the energy cost of oxidative stress response (Fig. 7). Further investigations are required to confirm this hypothesis. On the contrary, high light exposure induced a strong stress for shade-adapted biofilms and thus lowered their photosynthetic activity under this light condition (Fig. 7).

4.3 Short-term exposure to herbicides

Glyphosate affected photosynthesis in biofilms strongly causing functional inhibition but also structural damage at higher concentrations, as shown by the reduction in photosynthetic capacity (Fig. 4). For both parameters and for all types of biofilm, the large confidence interval found illustrates the variability that is usually

found in community data. The lowest EC_{50} found in this study (6.1 mg L^{-1}) was in the same range as the toxicity thresholds reported by Goldsborough and Brown (8.9 to 89 mg L^{-1}; 1988) and indicated a low environmental risk as concentrations of glyphosate found in rivers are usually in the microgram per liter range (Battaglin et al. 2005). The colonization light influenced photosynthetic response of biofilms exposed to glyphosate as HL biofilms were more resistant to glyphosate exposure than SUB and SAT biofilms. Part of this result may be explained by the presence of more tolerant species, such as *Nitzschia palea* in HL biofilms. Pesce et al. (2009) reported in a microcosm experiment that a biofilm community dominated by *Navicula*, *Gomphonema* and *Nitzschia* species was less sensitive to low glyphosate concentration. Nevertheless, physiological adaptation might also play a role in glyphosate response, as SAT and HL biofilms shared similar diatom community but had different tolerance levels to glyphosate. Glyphosate inhibits the biosynthesis of ubiquinones among other processes (Herrmann, 1995); this reduction in ubiquinone production may have a greater impact on a community adapted to low light intensities when more photosynthetic units are required to achieve efficient photosynthesis.

Though both glyphosate and AMPA were tested in similar molar concentrations, the degradation product of glyphosate, AMPA was not found to be toxic to any type of biofilm in a relatively wide range of concentrations (0.01 to 500 mg L^{-1}). Since concentrations of AMPA in rivers are usually in the microgram per liter range (Botta et al. 2009; Popp et al. 2008; Struger et al. 2008), this absence of acute toxicity of AMPA to benthic communities indicates a low environmental risk and confirmed the low toxicity of AMPA to aquatic organisms which is usually accepted (Evrard et al. 2010).

In the present study, antioxidant enzymes were found to be a more sensitive biomarker of copper toxicity than photosynthetic parameters, as observed previously (Guasch et al. 2010). The short incubation time might prevent complete diffusion of copper inside the biofilm 3-D structure and delay the toxic effect expected on photosynthesis. The light intensity during colonization influenced the antioxidant

strategy developed by communities to cope with oxidative stress provoked by copper. As observed in other studies on green algae (Nikookar et al. 2005; Sauser et al. 1997), APX seemed to play an important role in response to copper exposure. Indeed the increase of APX activity in SUB and SAT biofilms at high and intermediate concentrations, respectively (Fig. 5), may be explained by the activation of the ascorbate-glutathione cycle which allows ROS (Mittler, 2002) produced by copper exposure to be scavenged and, therefore, limits protein degradation. Though the influence of colonization light on GR response was not significant, the inhibition of this enzyme in SUB and SAT biofilms (Fig. 5) may reflect an increase in the direct use of glutathione as an antioxidant against copper effect. Therefore, the activation of antioxidant defence in SUB and SAT biofilms may reduce protein degradation provoked by copper while in HL biofilms the absence of antioxidant response may explain the higher protein degradation. However the influence of colonization light on the decrease of protein content in biofilms exposed to copper was not confirmed statistically and further experiments are needed to confirm the potentially higher sensitivity of high-light adapted biofilms to copper. Based on the toxicity thresholds observed in the present study, the acute effects of copper on protein content reduction or on antioxidant response may occur in very polluted sites, for instance close to copper mines, where copper concentrations can reach 1 mg L^{-1} (ATSDR, toxicological profile for copper 2004). Nevertheless, APX may also be activated in moderately polluted sites in which copper concentrations ranged between 30 and 60 µg L^{-1} while no effects were observed at concentrations usually found in unpolluted European rivers (0.6-10.9 µg L^{-1}; Bossuyt and Janssen 2004). Chronic exposure to much lower concentration may also have negative effects on biofilms (Serra and Guasch 2009), however this aspect was not directly addressed in our investigation.

Exposure to oxyfluorfen provoked an activation of antioxidant defences in SUB, SAT and HL biofilms (Fig. 6); similar results had been observed in *Scenedesmus obliquus* exposed 24h to concentrations of oxyfluorfen down to 15 µg L^{-1} (Geoffroy et al. 2003). Based on the EC_{50} for APX activation (≈ 10 mg L^{-1}) calculated in the present

study, the environmental risk of oxyfluorfen is relatively low for fluvial biofilms. Indeed previous studies reported concentrations of oxyfluorfen between 0.1 and 1 µg L^{-1} (estimated concentrations in San Joaquin River based on sediment data, US EPA/OPP 2001) with a maximum of 541 µg L^{-1} being observed after an accidental spillage (US EPA/OPP 2001). In addition, statistical analysis confirmed the absence of interaction between colonization light and APX activity in biofilms exposed to oxyfluorfen, suggesting thus that all communities had the same strategy to cope with oxidative stress induced by oxyfluorfen.

The toxicity thresholds reported in this study are a lot higher than the environmental concentrations of the four herbicides tested, indicating a low environmental risk. Nevertheless, continuous exposure to low concentrations may still present a risk for the environment and further investigation is needed to determine the chronic toxicity of these herbicides on biofilms. Moreover, the interpretation of biomarkers response should be done with caution for those biomarkers not directly related to essential processes for biofilms as AEA. Indeed, while the inhibition of photosynthetic efficiency can be directly related to the energy collection capacity of biofilms, the interpretation of antioxidant enzymes activation or inhibition is more complex. In particular, the link between the amplitude of antioxidant enzymes variations and the health status of biofilms is unknown. For instance, while a 24h exposure to 15 µg L^{-1} of oxyfluorfen reduced by 50% the growth of *Scenedesmus obliquus*, it provoked an increase by only 19 % in APX activity of this green algae (Geoffroy et al. 2002). Thus the health status of biofilms might be impaired more strongly than expected even though AEA variations are inferior to 50%. Consequently, it may be more pertinent to focus on the variations in AEA to understand the antioxidant strategy of biofilms rather than on the EC_{50} of such biomarkers.

Finally, the influence of colonization light on biomarker response to herbicide exposure depended on both the nature of the biomarker and the type of herbicide. Indeed, colonization light influenced photosynthetic response of biofilms exposed to

glyphosate while in biofilms exposed to copper or oxyfluorfen the colonization light had little or no influence on antioxidant response of biofilms (Fig. 7).

4.4 Ecological tolerance to co-occurring stressors

In the present study, the exposure to previous stress, such as the deficiency or the excess of light, constrained the response of biofilms to further natural or chemical stressors indicating that their ecological tolerance differed from their physiological one (Fig. 7). Our results support then the ecological theories concerning the response of plants to multiple co-occurring stressors (i.e. light and water availability) developed from terrestrial ecological studies (Valladeres 2005). Valladares (2005) observed that in some phenotypes of plants high-light stress exacerbated drought–stress effects while, in the present study, it was shade adaptation that influenced the capacity of biofilms to respond to a sudden increase in light intensity or to glyphosate exposure negatively. Effects of high-light adaptation on biofilm response to a sudden reduction in light availability or to copper exposure were less clear.

Vinebrooke et al. (2004) pointed out that the sign and strength of correlations between stressors can strongly influence biodiversity and ecosystem functioning. Actually, the higher resistance to glyphosate observed in biofilms adapted to high light provides an example of co-tolerance. An increase of tolerance to Cd was also described by Navarro et al. (2008) in biofilms adapted to high UV radiation, suggesting that both stress factors had a common tolerance mechanism. Therefore, not only can previous exposure to contaminants lead to co-tolerance phenomena (Blanck et al. 1988), but also physical habitat characteristics would strongly influence community resistance to disturbances. Our results and those reported in the literature (Niinemets and Valladares 2006) highlight the importance of investigating the interaction between co-occurring stressors in understanding community ecology, i.e. the link between biological communities and environmental changes including both natural and anthropogenic stress factors.

4.5 Environmental implications

Based on the results of the present study, in a highly disturbed environment subject to both natural and chemical stressors, such as for instance, the destruction of riparian vegetation combined with the entrance of land washed herbicides, biofilms might be more resistant to herbicides such as glyphosate. These results should be taken into account to understand the lack of algal growth inhibition observed in eutrophic sites receiving high loads of land washed herbicides. Nevertheless, previous studies showed the higher sensitivity of biofilms adapted to high light intensity to other herbicides such as atrazine (Guasch and Sabater 1998). Therefore, as mixtures of herbicides are usually found in rivers, it is not possible to derive general conclusions about the influence of light regime during growth on biofilm toxicity. Moreover, the sudden reduction of light may not cause great negative impact on biofilms from open sites and clear rivers, while the sudden increase in light reaching shade-adapted biofilms may strongly decrease their productivity. Therefore, a combination of both physical and chemical disturbances may have a more dramatic impact on biofilm communities in shaded rivers.

5. Conclusion

The successful colonization of biofilm communities in microcosms under different light intensities revealed a shade/light adaptation of the different communities similar to field observations. Though different communities were obtained, AEA were stable along the range of colonization conditions. Exposure to natural and chemical stressors showed that light and shade adaptation constrained their capacity to cope with further stress factors. Adaptation to high light led to co-tolerance to glyphosate, whereas copper and oxyfluorfen toxicities were independent of light growing conditions. These results illustrate the strong importance of environmental factors modulating community responses to disturbances.

Acknowledgements

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Supporting information

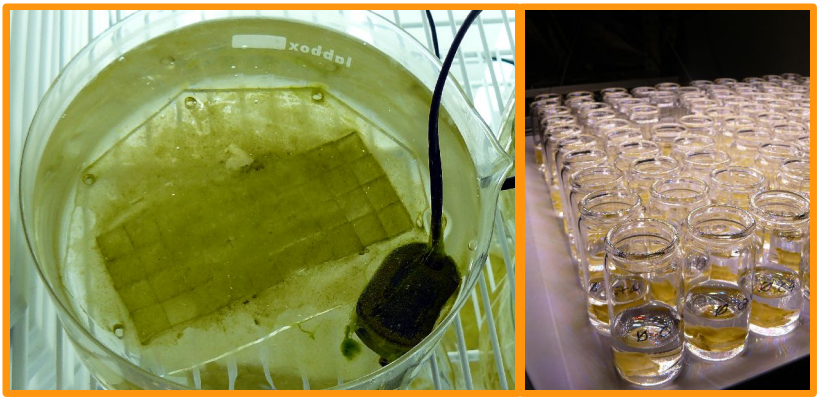
Table A1. Estimated model parameters of the concentration-response curves obtained after exposure to glyphosate (Gly.), copper (Cu) or oxyfluorfen (Oxy.) of SUB, SAT and HL biofilms colonized under 25, 100 and 500 µmol photons m^{-2} s^{-1}, respectively. The same letters in different model equations do not necessarily refer to the same parameters.

Toxic	Biofilm	Parameter	Model	Lower limit	Upper limit	Size of the hormesis effect	Other parameters
Gly.	SUB	Ph. eff.	Weibull 2	c = 0.000	d = 0.373		b = -0.317; e = 3.669
	SAT		Weibull 2	c = 0.000	d = 0.350		b = -0.508; e = 2.983
	HL		Weibull 2	c = 0.000	d = 0.261		b = -0.063; e = 0.105
	SUB	Ph. cap.	Weibull 2	c = 0.000	d = 0.584		b = -0.326; e = 21.886
	SAT		Weibull 2	c = 0.000	d = 0.498		b = -0.412; e = 4.016
	HL		Weibull 1	c = 0.000	d = 0.503		b = 0.114; e = 26884
Cu	SUB	APX	MM	c = 0.382	d = 7.805		e = 89135
	SUB	GR	Brain Cousens	c = 0.000	d = 0.196	f = 0.00025	b = 0.668; e = 555.6
	SAT		Log-logistic	c = 0.000	d = 0.204		b = 0.419; e = 8.106
	SUB	Protein content	Weibull 1	c = 0.000	d = 51.972		b = 0.498; e = 16307
	SAT		Weibull 1	c = 0.000	d = 78.590		b = 0.963; e = 8205
	HL		Weibull 1	c = 0.000	d = 68.048		b = 8.435; e = 2263
Oxy.	SUB	APX	MM	c = 0.399	d = 0.554		e = 216.1
	HL		MM	c = 0.279	d = 0.432		e = 638.2

Model	Functions
Weibull 1	$f(x) = c + (d - c) * (\exp(-\exp(b * (\ln(x) - \ln(e)))))$
Weibull 2	$f(x) = c + (d - c) * (1 - \exp(-\exp(b * (\ln(x) - \ln(e)))))$
MM: Michaelis Menten	$f(x) = c + (d - c) / (1 + (e / x))$
Brain Cousens	$f(x) = c + (d - c + f * x) / (1 + \exp(b * (\ln(x) - \ln(e))))$
Log logistic	$f(x) = c + (d - c) / (1 + \exp(b * (\ln(x) - \ln(e))))$

Chapter IV

Fluvial biofilms: a pertinent tool to assess β-blockers toxicity



Bonnineau, C., Guasch, H., Proia, L., Ricart, M., Geiszinger, A., Romaní, A.M., Sabater, S. 2010. Aquatic Toxicology 96, 225-233

Abstract

Among increasingly used pharmaceutical products, β-blockers have been commonly reported at low concentrations in rivers and littoral waters of Europe and North America. Little is known about the toxicity of these chemicals in freshwater ecosystems while their presence may lead to chronic pollution. Hence, in this study the acute toxicity of 3 β-blockers: metoprolol, propranolol and atenolol on fluvial biofilms was assessed by using several biomarkers. Some were indicative of potential alterations in biofilm algae (photosynthetic efficiency), and others in biofilm bacteria (peptidase activity, bacterial mortality). Propranolol was the most toxic β-blocker, mostly affecting the algal photosynthetic process. The exposure to 531 µg L^{-1} of propranolol caused 85% of inhibition of photosynthesis after 24 h. Metoprolol was particularly toxic for bacteria. Though estimated No-Effect Concentrations (NEC) were similar to environmental concentrations, higher concentrations of the toxic (503 µg L^{-1} metoprolol) caused an increase of 50% in bacterial mortality. Atenolol was the least toxic of the three tested β-blockers. Effects superior to 50% were only observed at very high concentration (707 mg L^{-1}). Higher toxicity of metoprolol and propranolol might be due to better absorption within biofilms of these two chemicals. Since β-blockers are mainly found in mixtures in rivers, their differential toxicity could have potential relevant consequences on the interactions between algae and bacteria within river biofilms.

Keywords: biofilm, β-blocker, biomarkers, multivariate approach.

1. Introduction

Drugs consumption has steeply increased during the last decade and as a consequence the amount of pharmaceutical products that reach freshwater ecosystems has increased. Among these emerging toxicants, β-blockers are widely used in therapy against hypertension or heart failure. As a documented example, from 100 to 250 tonnes of β-blockers are consumed each year in Germany (Cleuvers 2005). The arrival of these products may lead to chronic contamination, with unknown impacts on aquatic ecosystems. Moreover, the degradation of these pharmaceutical products is highly variable depending on the molecule, but also on the type of sewage treatment. Ternes (1998) found that propranolol was removed up to 96% and metoprolol up to 83% through Sewage Treatment Plants (STP), while Vieno et al. (2006) found an 11% removal of metoprolol and a 76% removal of atenolol through a STP in Finland. In any case, β-blockers are not completely removed from sewage effluents and consequently concentrations at the ng L^{-1} range can be found in rivers and littoral waters of Europe and North America. Huggett et al. (2003) reported a concentration of propranolol of up to 1.9 μg L^{-1} in North America. In the River Llobregat (Spain), some β-blockers (propranolol, metoprolol, atenolol and sotalol) have been detected, with maximum values of 60, 180, 670 and 1820 ng L^{-1}, respectively (Muñoz et al. 2009).

In spite of the potential toxicity of some β-blockers, particularly for fish and algae, few studies have been performed on the impact of β-blockers on the aquatic ecosystem. The growth of the fish *Oryias latipes* (medaka) was reduced and its levels of testosterone and plasma estradiol significantly changed after 14 days of exposure to 0.5 mg L^{-1} of propranolol. A reduction of the number of laid eggs was also observed after an exposure of 4 weeks to 0.5 and 1 μg L^{-1} of propranolol (Huggett et al. 2002). Using the *Daphnia magna* immobilization test, the *Lemna minor* and *Desmodesmus subspicatus* growth inhibition tests to assess the toxicity of propranolol, metoprolol and atenolol, Cleuvers (2005) found that *Desmodesmus* was the most sensitive, with an EC_{50} of 0.7 mg L^{-1} for propranolol. Escher et al. (2006) illustrated the phytotoxicity of

4 β-blockers in a non-target effect study based on the inhibition of the photosynthesis of green algae. These single-species tests highlighted the specific acute toxicity of β-blockers towards green algae. However, algae are part of multi-species communities in the environment and a multi-species test involving algae should be the next step (OECD guidelines) in assessing the toxicity of β-blockers. In this paper, therefore, the toxicity of β-blockers has been assessed on fluvial biofilms.

Fluvial biofilms are communities mainly composed of diatoms and green algae as well as of cyanobacteria, bacteria, protozoa and fungi, all embedded in an extracellular matrix (Sabater and Admiraal 2005). Fluvial biofilms are present in different river microhabitats (rocks, sediments, organic debris), and represent an interface between the flowing water and the stream bed (Romaní et al. 2004; Sabater et al. 2002). These characteristics make biofilms pertinent bioindicators of environmental perturbations within the aquatic ecosystem. Several studies have highlighted the biofilm sensitivity to a large panel of toxicants such as heavy metals (Guasch et al. 2002; Ivorra et al. 2002; Pinto et al. 2003), herbicides (Guasch et al. 2003; Gustavson et al. 2003; Leboulanger et al. 2001; Pesce et al. 2008; Schmitt-Jansen and Altenburger 2005b), and pharmaceuticals (Lawrence et al. 2005). Due to this sensitivity, fluvial biofilms can be used as early warning systems for the detection of the effects of toxicants on aquatic systems (Sabater et al. 2007).

Disturbances occurring in the ecosystem would first lead to biochemical and physiological changes within the biofilm that could evolve to community changes if perturbations persist. While changes in community composition have been linked to persistent pollution of river sites (Tornés et al. 2007), the biochemical and physiological changes indicate an initial stress response, and can be used as early indicators of ecosystem damage. Biofilm complexity provides a large panel of functional and structural endpoints to assess toxicity of emerging pollutants. In this study, a biomarker approach has been used to investigate the effect on the whole biofilm community of a 24 h exposure to the three β-blockers metoprolol, atenolol and propranolol.

Because the toxicity mode of action of β-blockers on algal communities is unknown, the set of biomarkers should account for a global status of biofilm, and needs to include their two most important compartments (algae and bacteria). Photosynthetic efficiency was selected as a classical biomarker of autotrophic biofilms (Brack and Frank 1998; Fai et al. 2007; Schmitt-Jansen and Altenburger 2008a). The sensitivity of the different groups of primary producers (cyanobacteria, green algae and diatoms) was estimated through their specific photosynthetic efficiencies (Zhang et al. 2008). To assess toxicity on bacterial compartment, bacterial mortality was determined and the effects on the heterotrophic activity of biofilms (and therefore on the bacteria–-algal relationships; Francoeur and Wetzel 2003) were estimated by means of the extracellular enzyme activities (e.g. peptidase). Furthermore the oxidative stress response was measured as a reflection of the general stress level. Oxidative stress is due to the accumulation of reactive oxygen species (ROS) during metabolic processes, which is enhanced by the presence of heavy metals, herbicides among others (Pinto et al. 2003; Zbigniew and Wojciech 2006). To prevent injuries from ROS accumulation, cells develop antioxidative systems composed of different enzymes and pigments, which can be used as biomarkers of stress from toxicant exposure (Tripathi and Gaur 2004; Tripathi et al. 2006). Catalase is an antioxidant enzyme which scavenges hydrogen peroxide. Geoffroy et al. (2004) observed that catalase activity was a more sensitive biomarker than photosynthetic activity after exposition of *Scenedesmus obliquus* to the herbicide flumioxazin. Most of these biomarkers are functional since the short-time (24 h) exposure is mainly expected to affect the function of biofilms rather than their structure. Altogether, these endpoints compile a set of biomarkers encompassing general metabolic pathways (photosynthetic efficiency, peptidase, bacterial mortality) and stress response mechanism (oxidative stress), allowing responses occurring at molecular level (oxidative stress, peptidase) and at community level (bacterial mortality, photosynthetic efficiency of each algal group) to be detected.

This study aims to illustrate the potential of a multi-species system combined with a biomarker approach to assess toxicity of β-blockers. To do so, the following points are questioned:

1. Does the set of biomarkers allow the toxicity impact on fluvial biofilms of the three analysed β-blockers (same type of chemicals) to be differentiated ?
2. Which β-blocker is the most toxic ?
3. Which biomarker is the most sensitive ?

2. Material and methods

2.1. Chemicals

Pure propranolol hydrochloride (CAS: 3506-09-0), atenolol (CAS: 29122-68-7) and metoprolol tartrate (CAS: 56392-17-7) were purchased from Dr. Ehrenstorfer GmbH® (Germany).

2.2. Biofilm colonization

Biofilm communities were grown on glass substrata installed in crystallizing dishes containing 1.5 L of tap water which had been previously passed through a carbon filter to eliminate chlorine. The original biofilm inoculum was obtained from the river Llémena (NE Spain; Serra et al. 2009a). An aquarium pump allowed constant circulation of water in order to simulate flowing water. The dishes were incubated at 19 ºC and under a 12/12 h day–night cycle, with a photon flux density of about 120 µmol photons m^{-2} s^{-1}. The water was changed twice a week and phosphate was added to a final nominal concentration of 158 µg L^{-1}.

2.3. Diatom community composition

Biofilm samples for diatom examination were collected randomly from 3 crystallizing dishes. Samples were digested with hydrogen peroxide and mounted in permanent slides. Identification and counting was performed as described by Ricart et

al. (2009). Shannon–Wiener diversity index (Shannon and Weaver 1963) was calculated by means of the OMNIDIA package (version 2).

2.4. Acute exposure experiments

Acute toxicity tests were performed on 3 week-old biofilms. Each glass substrata was incubated in a vial containing 10 mL of colonization medium and the corresponding toxicant concentration. Samples were incubated during 24 h under the same conditions as the colonization, using a single-speed orbital mixer (KS260 Basic, IKA®) to maintain constant agitation. Two samplings were performed after 6 h and after 24 h of exposure. Four replicates were used for each endpoint and each concentration (and controls). Four concentrations were tested for each toxicant. Concentrations of propranolol and metoprolol were: 0.9, 90, 900 and 9000 µg L^{-1}. Atenolol concentrations were 0.9, 900, 9000 and 900 000 µg L^{-1}. Concentrations were chosen based on the literature (Munoz et al. 2009; Huggett et al. 2003), atenolol being described as less toxic than propranolol and metoprolol (Cleuvers 2005). Abiotic controls consisted of water samples without incubated biofilms. Real concentrations of the 3 β-blockers were analysed in water of both biotic and abiotic samples, to distinguish between toxicants degraded by photolysis and those being adsorbed and/or absorbed by the biota (Liu and Williams 2007; Piram et al. 2008).

2.5. Photosynthetic efficiency

The chlorophyll fluorescence measurements were carried out by means of a PhytoPAM (Pulse Amplitud Modulated) fluorometer (Heinz Walz GmbH). Four colonised glass substrata for each treatment were collected at random after 6 and 24 h for in vivo chlorophyll fluorescence measurements. These were performed at room temperature and in a dark chamber. The distance between the optical fiberoptics and the sample surface was set at 2 mm. The fluorescence signal was determined by the emitter-detector unit (PHYTO-EDF). Maximal photosynthetic efficiency of PSII (maximal PSII Quantum Yield) was obtained after a 20 min dark-adaption of samples and effective PSII quantum yield (efficiency of PSII) after light-adaptation.

Calculations were done following Genty et al. (1989). The deconvolution of the fluorescence signal into the contributions of the three algal groups and cyanobacteria is based on the internal reference excitation spectra of a pure culture, which has been previously validated for periphyton communities by Schmitt-Jansen and Altenburger (2008a). The maximal and the effective photosynthetic efficiencies were estimated based on the fluorescence signal recorded at 665 nm and given as relative units of fluorescence. The relative contribution of the different algal groups was also used to obtain the effective photosynthetic efficiency for each of them, using the fluorescence signal linked to green algae, cyanobacteria and diatoms.

2.6. Bacterial mortality

Live and dead bacteria were counted with epifluorescence microscopy after double staining with the LIVE/DEAD® Bacteria Viability Kit L7012 (BacLightTM). Two nucleic acid stains composed this kit: the SYTO 9 which stains all cells (excitation/emission 480/500 nm) and the propidium iodide (excitation/emission 490/635 nm) which penetrates cells that have damaged membranes. Sample preparation, staining and counting were done as described by Ylla et al. (2009) using pre-sterilized medium for dilution.

2.7. Peptidase activity

Potential extracellular activity of leucine-aminopeptidase enzyme (EC3.4.11.1) was measured spectrofluorometrically using fluorescent-linked substrate L-leucine-4--methyl-7-coumarinylamide (Leu-AMC) as described by Ricart et al. (2009) and Romaní et al. (2004).

2.8. Protein extraction

Biofilms were removed from the glass substrata with a sterile silicone cell scraper (Nunc, Wiesbaden, Germany) and centrifuged at 2300 × g and 10ºC for 5 min to remove the excess of water. The pellets were frozen immediately in liquid nitrogen and stored at -80 ºC until the enzyme assays were started. Samples were homogenized for 3 min on ice by adding 0.9 mL of homogenization buffer containing

100 mM potassium phosphate buffer (pH 7.4), 100 mM KCl, 1 mM EDTA and 10% (w/v) PVPP (Polyvinylpolypyrrolidone) for 100 mg of wet weight of biofilms. Homogenates were then centrifuged at 10 000 × g and 4ºC for 30 min. Supernatant was used as the enzyme source, the protein content of supernatant was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

2.9. Catalase activity

Catalase activity was measured spectrophotometrically at 240 nm according to Aebi (1984). 800 µL 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 of protein). The H_2O_2 consumption was determined by measuring the decrease in absorbance at 25 ºC for 4 min. Catalase activity was calculated as µmol H_2O_2 mg protein^{-1} min^{-1} using the extinction coefficient of H_2O_2: 0.039 cm^2 µmol^{-1}.

2.10. Analysis of β-blockers in water

After 6 and 24 h of exposure, water from 4 biotic and 4 abiotic samples was filtered through a 0.45 µm nylon filter (Whatman, England) and kept at −20 ºC until HPLC analyses. Control and 0.9 µg L^{-1} samples (nominal concentration) were concentrated prior analyses through tC18 cartridges (Sep-Pak® Vac 3 cc tC18, Waters, Ireland). Cartridges were conditioned with 5 mL of methanol and 5 mL of water before sample enrichment. Then, cartridges were washed with 5 mL of water and dried during 20 min. Elution was done with 8 mL of methanol, eluted samples were then dried under nitrogen stream and reconstituted with 1 mL methanol/water (1:3, v/v).

Samples were analysed by liquid chromatography. The HPLC system consisted of a binary HPLC Pump (1525 Waters), an autosampler (717 Plus Waters) and an UV-detector (Dual λ Abs. Detector 2487 Waters). The methods used were adapted from Delamoye et al. (2004). Flow rate was set at 1 mL min^{-1} and wavelength detection at 227 nm. For controls and samples of a nominal concentration of 0.9 µg L^{-1}, separation was carried out on 3.5 µm C18 column (Symmetry 4.6 mm × 75 mm)

maintained at 30 ºC. Injection volume was 20 µL. Elution was performed with a gradient of acetonitrile and phosphate buffer (10 mM, pH = 3.8) as described in Table 1.

Table 1. Gradient of HPLC elution (in percentage) for the different samples.

Samples concentration	Time (min)	% Acetonitrile	% Phosphate buffer
Control 0.9 µg L^{-1}	0	10	90
	3	10	90
	3.5	20	80
	8	40	60
	12	40	60
	12.5	10	90
	15	10	90
From 90 µg L^{-1} to 900 000 µg L^{-1}	0	15	85
	3	15	85
	3.6	40	60
	10	40	60
	11	15	85
	15	15	85

For samples of nominal concentration between 90 and 900 000 µg L^{-1}, separation was carried out on a 5 µm C18 column (Sunfire 4.6 mm × 150 mm) maintained at 30 ºC. Injection volume was 100 µL. Elution was performed with a gradient of acetonitrile and phosphate buffer (10 mM, pH = 5.00) as described in Table 1. For each β-blocker, the detection limits were 71 µg L^{-1} for propranolol, 42 µg L^{-1} for atenolol, and 20 µg L^{-1} for metoprolol. A value of half the detection limit was attributed to samples whose concentration was below detection limit.

2.11. Data analyses

All statistics analyses were done using R 2.6.2 (R Development Core Team 2008).

The use of multi-species communities implies higher variability than those accounted for by the single-species tests. Thus, two different approaches were used to explore the data. First, No-Effect Concentration was determined independently for each β-blocker and each biomarker to quantify the impact of each toxicant. Measured β-blockers concentrations and all biomarkers responses were used. Biomarker responses corresponding to concentrations below detection limit were used as controls, and then a linear regression was done in the linear range of the remaining points. The linear range was the result of a compromise to maximize the number of points and r^2 (regression coefficient) and to obtain a significant regression ($p < 0.05$). NEC was defined as the intercept between the mean of controls and the linear regression, range for each NEC was determined through inverse regression as described by Liber et al. (1992) and Draper and Smith (1981). When no significant linear regression could be obtained, potential differences between controls and treatments were analysed using analysis of variance (ANOVA), effects were *post hoc* analysed with a Tukey test. For all the analyses, statistical significance was set at $p < 0.05$.

Next, a partial Redundancy Direct Analysis (Vegan package, Oksanen et al. 2008) was performed to integrate the variability due to growth conditions and biofilm formation. Among the ordination method, RDA allows constraining the arrangement of the response variables along the axes. This analysis included the exposure results to the three β-blockers. The biomarkers with too many missing values were excluded from the analysis. The following biomarkers were then used: catalase activity, peptidase activity, life-dead bacterial ratio, photosynthetic efficiency of cyanobacteria and photosynthetic efficiency of green algae. samples containing missing values were also discarded from the analysis (28 samples on a total of 120). Biomarkers responses were scaled. As the gradient of nominal concentration used was very large, concentrations were expressed as ranks (from 0 for control to 5 for the highest concentration). The time of exposure was set as a covariable to integrate results of 6 and 24 h of exposure.

3. Results

3.1. Biofilm colonization

After 3 weeks of colonization, the biofilm had a diatom community composed of 36 species (mean value of three replicates). The most dominant taxa were *Achnanthes minutissima*, *Cymbella microcephala*, *Navicula atomus var. permitis*, *Nitzschia dissipata*, *Nitzschia frustulum* and *Navicula seminulum*. Shannon–Wiener diversity index was 3.7 ± 0.6.

Physical and chemical conditions were stable during the colonization. Water conductivity was at 477 ± 9 µS, pH: 8.46 ± 0.09, dissolved oxygen concentration: 9.11 ± 0.13 mg L^{-1}, and water temperature: 20.6 ± 0.2 ºC (n = 28 for all parameters). Water used during this experiment has been previously characterized by Serra et al. (2009b) for NO_3 (1.68 ± 0.14 mg L^{-1}), NO_2 (0.07 ± 0.01 mg L^{-1}) and NH_4 (<0.1 mg L^{-1}) among others. Then, in this experiment, only phosphorus concentration was measured, the concentration had a mean value of 46.8 ± 2.1 µg L^{-1} (n = 24) just after water changes and phosphorus addition, but declined to low levels (1.5 ± 0.2 µg L^{-1}; n = 20) just before water changes. However complete depletion in phosphorus was never observed during colonization.

Moreover, the biofilm used in these experiments had a normal bacterial mortality with a live/dead bacteria ratio of 48 ± 6%. Mean peptidase activity was 332.8 ± 73.8 nmol AMC cm^{-2} h^{-1} and mean catalase activity was 28.5 ± 10.9 µmol H_2O_2 mg $protein^{-1}$ min^{-1}. The photosynthetic efficiency and capacity of these biofilms were of 0.30 ± 0.05 and 0.47 ± 0.05. The respective photosynthetic efficiencies of cyanobacteria, diatoms and green algae were 0.25 ± 0.05, 0.32 ± 0.11 and 0.33 ± 0.06.

3.2. Measured β-blockers concentrations in water

Propranolol and metoprolol concentrations were below detecttion limits in controls and samples of nominal concentration inferior to 900 and 90 µg L^{-1}, respectively. No atenolol could be detected neither in controls nor in samples of the

lowest nominal concentration (0.9 µg L^{-1}). The concentration of propranolol and metoprolol in water was at least two times lower in the biotic samples than in the abiotic samples, but the concentration of atenolol was similar in the two types of samples (Fig. 1). Moreover, propranolol and metoprolol concentrations were also lower (up to 17 times) than the nominal concentrations in the abiotic samples for all concentrations tested, with an even larger difference at the highest concentration (Fig. 1A and B). The concentration of atenolol in the abiotic samples was close to the nominal one, except for the intermediate concentration (Fig. 1C).

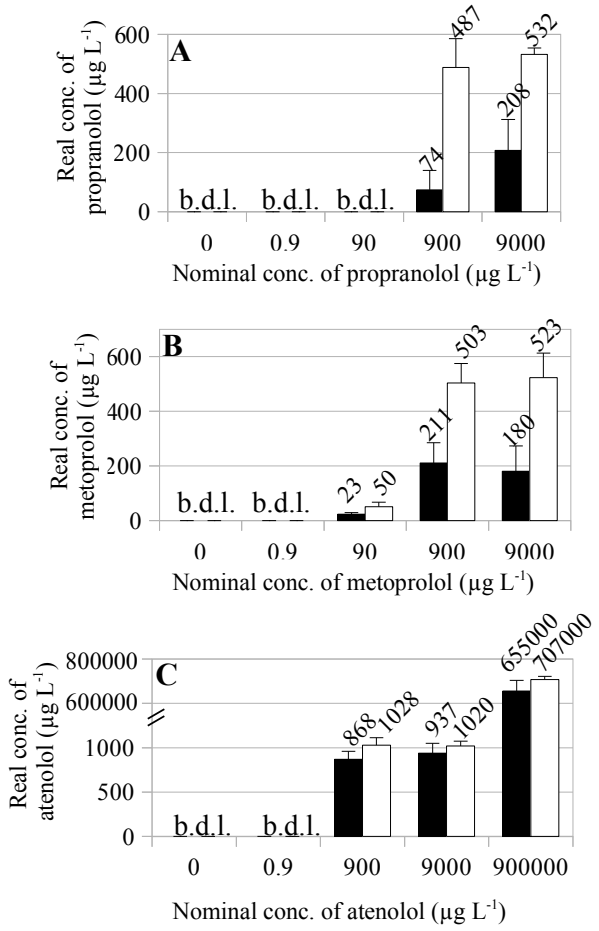


Figure 1. Concentrations of propranolol (A), metoprolol (B) and atenolol (C) of biotic (black bars) and abiotic (white bars) samples after 24h of exposure. On the horizontal axis, nominal concentrations in µg L^{-1} of each β-blocker are shown.

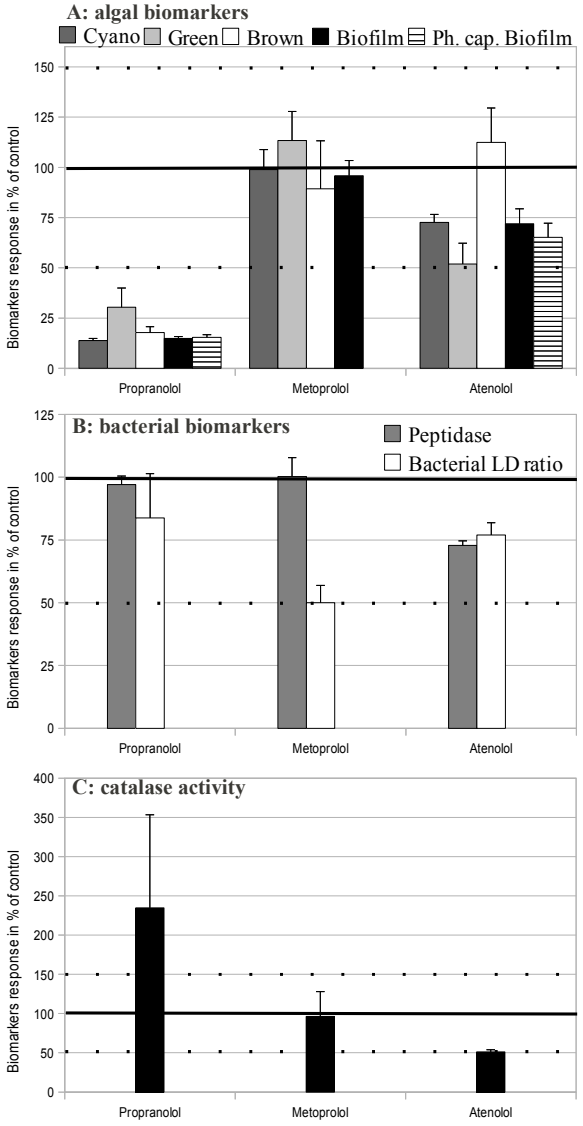


Figure 2: Response of the different biomarkers, expressed in percentage of control, after 24h of exposure to 531 µg L^{-1} of propranolol, 522 µg L^{-1} of metoprolol and 707 000 µg L^{-1} of atenolol. Graph A represents the response of algal biomarkers: photosynthetic efficiency of cyanobacteria (dark grey), green algae (grey), brown algae (white) and all biofilm (black) and photosynthetic capacity of all biofilm (horizontal hatches). Graph B represents bacterial biomarker: peptidase activity (grey) and live/dead ratio of bacteria (white). Graph C represents the catalase activity (black). In all graphs error bars depict the standard error; a plain black line indicates the control (100%) and dotted lines indicated +/- 50% of activation/ inhibition compared to control.

3.3. Effect of the highest β-blockers concentration

After 24 h of exposure, the two highest concentrations tested caused clear effects. However, the three β-blockers tested affected the various biomarkers differently; in most of the cases, the effects observed increased with concentration, here mean data expressed in percentage of control obtained after 24 h of exposure to the highest concentration are presented (Fig. 2).

The algal component was mainly affected by propranolol and atenolol while metoprolol had only a transitory effect on cyanobacteria. Indeed, after 6 h of exposure, the cyanobacteria photosynthetic efficiency was inhibited at 20 ± 4% in samples exposed at the two highest concentrations of metoprolol (503 and 522 µg L^{-1}; data not shown). However, these observations were not confirmed after 24 h of exposure (Fig. 2A). The highest concentration of propranolol (531 µg L^{-1}) caused 86 ± 1% of inhibition of photosynthetic efficiency for cyanobacteria, 70 ± 10% for green algae and 82 ± 3% for diatoms. The photosynthetic capacity of the whole biofilm was inhibited at 85 ± 1% (Fig. 2A). The highest concentration of atenolol (707 000 µg L^{-1}) significantly inhibited the photosynthetic efficiency of cyanobacteria by 27 ± 4% and of green algae by 48 ± 10% (Table 2, Fig. 2A). The photosynthetic efficiency of diatoms was not significantly affected by atenolol exposure (Table 2). Photosynthetic efficiency and capacity of the biofilm was also significantly inhibited by the highest concentration of atenolol (28 ± 7% and 35 ± 7% of inhibition; Fig. 2A).

The bacterial component of biofilm was mainly affected by metoprolol and atenolol rather than by propranolol. After 6 h the peptidase activity was affected by both propranolol and atenolol (data not shown) while after 24 h exposure, only atenolol had a significant impact on peptidase activity (Table 2). The highest concentration of this β-blocker inhibited 27 ± 2% of the peptidase activity (Fig. 2B). Bacterial mortality was significantly enhanced after a 24 h exposure to metoprolol or to atenolol (Table 2). At the highest concentration, metoprolol caused an inhibition of 50 ± 7% of the Live-Dead bacterial ratio. Exposure to the highest atenolol concentration increased

bacterial mortality by 23 ± 5%. Propranolol caused a moderate increase in bacterial mortality (16 ± 18%; Fig. 2B, Table 2). Despite a great increase observed in the catalase activity of samples exposed to propranolol, this antioxidant response was not significant (Fig. 2C; Table 2). Atenolol and metoprolol antioxidant responses were similar. The highest atenolol concentration significantly inhibited 49 ± 3% of the catalase activity after 24 h of exposure (Fig. 2B, Table 2). Six hours of exposure to 503 µg L^{-1} of metoprolol caused a significant inhibition of catalase activity by 37 ± 6% (data not shown); however, after 24 h of exposure no significant changes in catalase activity were observed in any of the concentrations tested (Table 2).

3.4. Determination of the No-Effect Concentrations (NEC)

The impact of the pollutants tested was quantified by means of No-Effect Concentrations as well as by their upper and lower limits (Table 2). Metoprolol affected mainly bacterial mortality while no significant effects on photosynthetic efficiency were found (Table 2). Biomarker responses of biofilms exposed to metoprolol were highly variable; the range obtained for NECs values are the widest observed in this experiment. NECs of propranolol for peptidase activity and photosynthesis-related endpoints ranged between 293 and 300 µg L^{-1} after 6 h, and between 479 and 489 µg L^{-1} after 24 h of exposure (Table 2).

NECs of atenolol for peptidase and catalase activity and photosynthetic biomarkers had wider ranges and higher variability between endpoints than for propranolol, differing also between algal groups and times of exposure (Table 2). Responses of biofilms exposed to propranolol were the least variable. After 6 h of exposure, effects on peptidase activity and photosynthetic efficiency of cyanobacteria showed similar NEC values (Table 2). After 24 h of exposure, no impact was detected on peptidase activity, and NECs values remained similar for the photosynthetic efficiencies of the different algal groups and for photosynthetic capacity.

Table 2. NEC values in µg L^{-1} and corresponding range for each biomarker after 6h and 24h of exposure to metoprolol (M), propranolol (P) and atenolol (A).

		Peptidase act.		Catalase act.		Life-Dead bacterial ratio		Photosynthetic efficiency								Photo-synthetic capacity	
								Cyanobacteria		Green algae		Brown algae		Biofilm			
	Exp. time	p	NEC	p	NEC	p	NEC	p	NEC	p	NEC	p	NEC	p	NEC	p	NEC
M	6h	n.s.	n.s.	***	45 [$7.6x10^{-140}$; $1.8x10^{46}$]	n.s.	0.64 [$2.2x10^{-7}$; 53.5]	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.d.	n.d.
	24h	n.s.	n.s.	n.s.	n.s.	***	0.04 [$4x10^{-126}$; $4.7x10^{14}$]	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
P	6h	***	341 [108;665]	n.s.	n.s.	n.s.	n.s.	***	300 [266;327]	***	n.s.	n.s.	n.s.	***	293 [248;326]	n.d.	n.d.
	24h	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	***	484 [475;491]	***	479 [383;515]	**	489 [451;512]	***	484 [478;489]	***	485 [476; 493]
A	6h	***	1473 [281; $5.81x10^{3}$]	n.s.	n.s.	n.s.	n.s.	**	1495 [0.9; $44x10^{5}$]	***	69 [0.04; $8.52x10^{3}$]	n.s.	n.s.	**	742 [0.06; $1.3x10^{5}$]	n.d.	n.d.
	24h	***	827 [0.8; $1.04x10^{5}$]	***	908 [39; $1.18x10^{4}$]	**	n.s.	**	590 [0.03; $1.8x10^{5}$]	**	2960 [2;$4.3x10^{5}$]	n.s.	n.s.	**	652 [0.3; $4.5x10^{4}$]	***	707 [2; $2x10^{4}$]

The p column indicates the p-value obtained from an ANOVA analysis of the biomarkers response (n=4 for each concentration)
n.s. : non-significant result with a p-value > 0.05 for ANOVA or for linear regression of NEC calculation
*n.d. : non-determined responses; *: p-value < 0.05; **: p-value < 0.01; ***: p-value < 0.001*

3.5. Multivariate analysis of biofilm response to β-blockers exposure

The ordination of the response of the biomarkers along the gradient of β-blockers is described by the RDA (Fig. 3). The first 2 axes explained 27% of the variance observed among all the samples (from the three experiments). The first axis was mainly driven by the gradient of propranolol (score of 0.85), while the second axis was related to metoprolol (score of -0.89). The photosynthetic efficiency of cyanobacteria and green algae had high negative scores on the first axis, indicating that the photosynthetic efficiency decreased with increasing concentrations of propranolol. Catalase activity also increased along the gradient of propranolol (Table 3). The live--dead bacterial ratio (LD) got the highest positive score in the second axis. Hence the diminution of the live-dead bacterial ratio (higher bacterial mortality) increased with metoprolol concentrations. The peptidase (Pep) and catalase (Cat) activities also decreased with increasing concentrations of metoprolol (Table 3). The third axis (not represented) was mainly driven by the gradient of atenolol (score of -0.96). This axis accounted for only 1.5 % of the variance; no clear response of the biomarkers could be highlighted (Table 3). The percentage of variance observed due to the time of exposure was relatively low (4.3%), indicating that few changes occurred between 6h and 24h of exposure.

Table 3. Scores of the different biomarkers on the RDA and PCA axes.

	RDA1	RDA2	RDA3	PC1	PC2	PC3
Pep	-0.175	0.641	-0.462	1.148	-0.738	0.673
Cat	**0.937**	0.501	0.299	-0.108	-1.318	-1.082
LD	0.154	**1.150**	0.088	0.456	0.945	-0.869
Y Bl	**-1.383**	0.169	0.272	0.500	-0.034	0.509
Y Gr	**-0.962**	0.313	-0.002	1.551	0.188	-0.482

Cat corresponds to catalase activity, Pep to peptidase activity, Y to the photosynthetic efficiency of cyanobacteria (Bl) and of green algae (Gr). Highest scores on the two first RDA axes are indicated in bold.

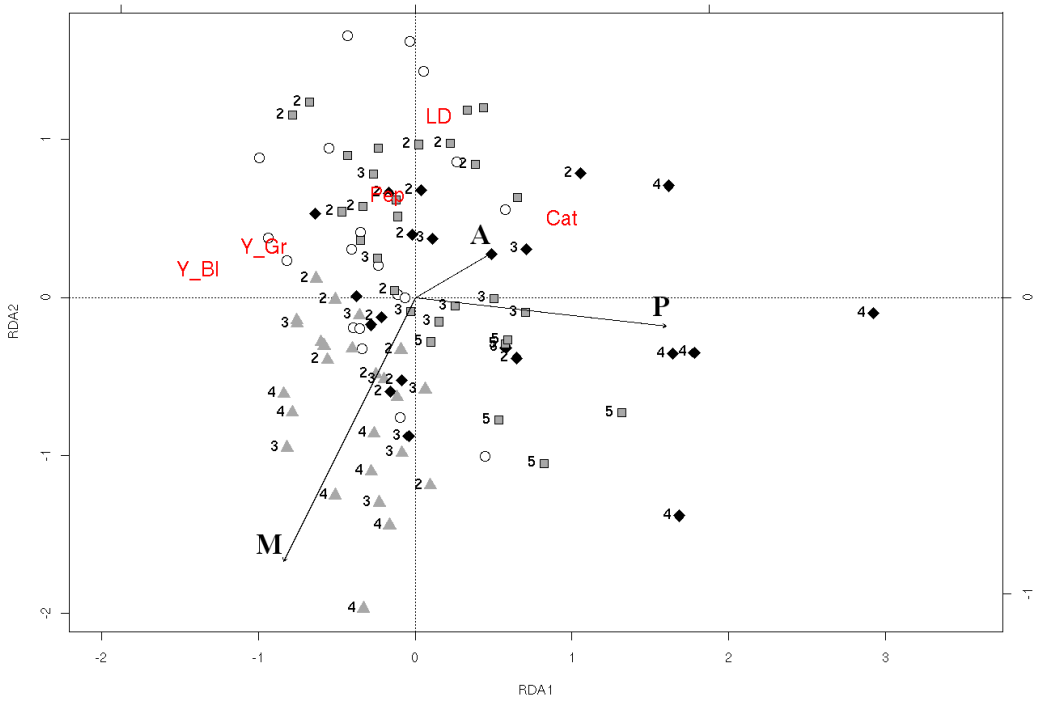


Figure 3. Results of RDA. The gradient of the different toxicants are shown by the arrows: P for propranolol, M for metoprolol and A for atenolol. Symbols corresponds to the different samples: o: control, ♦: samples exposed to propranolol, ▲: to metoprolol, ■: to atenolol. The different biomarkers are indicated in red: Cat for catalase activity, Pep for peptidase activity, Y corresponds to the photosynthetic efficiency of the cyanobacteria (Bl) and the green (Gr) algae respectively. The concentrations ranks 2,3,4,5 are indicated for each sample and correspond to the nominal concentrations of 90, 900, 9 000 and 900 000 µg L^{-1} of β-blocker.

4. Discussion

The whole set of functional and structural biomarkers of fluvial biofilms showed that impacts of the three β-blockers tested were specific. The multivariate analysis separated those samples exposed to propranolol from others exposed to metoprolol or atenolol (Fig. 3). Furthermore, at the highest concentration each β-blocker affected different endpoints (Fig. 2). This result is relevant considering that these compounds are part of the same class of pharmaceuticals, and these molecules are expected to act in a similar way as in humans in all organisms containing β-adrenergic receptors. The mode of action in humans can, in fact, give clues for assessing toxicity on these organisms (Owen et al. 2007). However, the mode of action of these pharmaceuticals in the environment is probably different from that described on human beings. The differences observed in algae and bacteria illustrate the need to produce a different classification that could be used when assessing toxicity of pharmaceuticals on the environment.

HPLC measurements highlighted that differences occurred between nominal and real concentrations for all β-blockers. Concerning atenolol, real concentrations are 9 times lower than the nominal concentration at 9000 µg L^{-1} but are similar to nominal ones at 900 and 900 000 µg L^{-1}. These observations are in contradiction with previous studies in which this compound has been described as generally stable at concentrations up to 10 000 µg L^{-1} (Liu and Williams 2007) and so suggest an operating error. For propranolol and metoprolol, an important difference between nominal and real concentrations is observed at all concentrations, indicating pollutant degradation. The degradation may be due to the light, that causes half-lives (in STP water, under UV radiation) to be around 3–4 h for propranolol and 20–48 h for metoprolol (Piram et al. 2008). Liu and Williams (2007) also observed that the kinetics of propranolol and metoprolol differ with the concentrations, which could explain the similar concentrations obtained at 900 and 9 000 µg L^{-1}. Moreover, biotic pathways of degradation may also occur as biotic and abiotic concentrations differ; the interaction

etween biotic and abiotic pathways of degradation could then lead to similar final concentration of pollutant in the water.

These results illustrate the importance to measure real concentrations in the water and the difficulties to know the real exposure. Indeed, the measure of a toxicant concentration during an assay is a snapshot of the concentration and do not integrate the total concentration during the exposure. In fact, NEC values were derived in our study from measured concentrations of β-blockers in water minimizing therefore an overestimation of toxicity. Degradation and absorption are dynamic processes that occur along all the assay, a kinetic study would have reflected better the exposure of the biofilm. Another option to determine the level of exposure is to determine the internal concentration of pollutant in the biofilm, this method is especially relevant in case of non-metabolised pollutant as metals (Guasch et al. 2009).

Within the biofilm, propranolol affected mainly the algal compartment through inhibition of the photosynthetic process. After 6 h of exposure, cyanobacteria were more sensitive to propranolol than diatoms or green algae, but after 24 h exposure all groups were affected to the same extent, and the highest concentration (531 µg L^{-1}) caused the inhibition of both photosynthetic efficiency and capacity, indicating irreversible damages on the photosynthetic apparatus. Multivariate analysis indicated that high propranolol concentration was associated to high catalase activity. However, significant effects were not detected by the ANOVA and no NEC values could be calculated for this biomarker. This apparent contradiction can be related to the high variability of catalase activity in samples exposed to propranolol. Liu and Williams (2007) have already suggested that the formation of intermediate radicals, which are highly reactive and cause oxidative stress, occurs during the degradation of propranolol. An analysis of the impacts of propranolol on the different antioxidant responses of biofilm could further support our results. Propranolol had little effect on the bacterial compartment of the biofilm, only a transitory effect on peptidase activity could be detected after 6 h of exposure. In conclusion, propranolol affects first the photosynthetic efficiency of cyanobacteria, and later causes irreversible damages to all

photosynthetic groups, which may cause oxidative stress and transitory bacterial response. As algae are essential for primary production in river ecosystems, toxicity of propranolol is of special concern and should be studied more extensively to derive ecotoxicological parameters (NOECs or EC_{50}).

Metoprolol was mainly toxic for bacteria. As shown by the RDA, high concentrations of metoprolol were related with bacterial mortality. The estimated NEC values for bacterial mortality were in the ng L^{-1} range, well within the realistic environmental concentrations. Though caution is required concerning these results (r^2 of regression analysis was below 0.5, and the range for calculated NECs was very large), they indicate the potential chronic effect of metoprolol on biofilm communities.

Atenolol toxicity was very low indeed, even at the highest concentration tested (707 000 µg L^{-1}). In addition, atenolol toxicity was not specific for any of the biomarkers measured, but affected both algae and bacteria. Within the algal compartment, green algae and cyanobacteria were affected by atenolol exposure, while diatoms appeared to be resistant to this toxic. The atenolol effect on the bacterial compartment was expressed in the increase in bacterial mortality and the decrease in peptidase activity at the two highest concentrations. A decrease in peptidase activity implies a reduction in the bacterial ability of hydrolysing peptides of high molecular weight and can be both due to a direct impact of atenolol on the enzymatic activity, but also to the decrease in photosynthetic efficiency that could indirectly affect the bacterial activity (Francoeur and Wetzel 2003). That atenolol at these high concentrations causes global stress both in algae as well as in bacteria was also expressed by the inhibition of catalase activity after 24 h of exposure. Catalase inhibition may be caused by a high level of H_2O_2 due to oxidative stress.

Atenolol was the less toxic of the three β-blockers tested. slight atenolol effects were only observed at the mg L^{-1} range while 531 µg L^{-1} of propranolol caused 85% of inhibition of photosynthesis efficiency of biofilm. These results were consistent with the findings of Cleuvers (2005) who classified atenolol as non-toxic for aquatic

organisms. According to NEC values, metoprolol was the most toxic of the 3 β-blockers tested. However, at the highest concentrations tested (503 µg L^{-1} for metoprolol and 522 µg L^{-1} for propranolol), propranolol determined a greater effect than metoprolol. Therefore propranolol would be the most toxic of the three β-blockers tested, consistently with the conclusions of the risk assessment performed by Cleuvers (2005) on *Daphnia magna* and *Desmodesmus subspicatus*.

The higher toxicity of propranolol and metoprolol could be related to a better absorption by biofilms of these β-blockers. Indeed, higher concentrations of propranolol and metoprolol were found in abiotic samples than in biotic samples while concentrations were similar in these two types of samples for atenolol. Moreover the higher log Kow of propranolol and metoprolol (3.37–3.48 and 1.88–2.28, respectively) than of atenolol (0.16–1.95) support this hypothesis. Therefore, propranolol and metoprolol toxicity might be caused by direct effects on internal metabolism whereas atenolol toxicity might be caused by interactions at the cell periphery.

Propranolol and atenolol are in the lower range of toxicity in comparison to others pharmaceuticals. Their respective NOEC for photosynthetic efficiency of all biofilm were of 484 and 652 µg L^{-1}. Fent et al. (2006) reviewed the NOEC values of 9 different pharmaceuticals (acetylsalicylic acid, salicylic acid, diclofenac, ibuprofen, naproxen, propranolol, clofibric acid, carbamazepine and fluoxetine) for different aquatic organisms to range between 0.001 and 1000 µg L^{-1}. The most toxic of these pharmaceuticals for phytoplankton was the neuroactive compound fluoxetine (NOEC = 0.001 µg L^{-1}) while the less toxic was the anti-inflammatory aproxen (NOEC ≈ 1000 µg L^{-1}). Metoprolol toxicity towards bacteria (≈50% of mortality after 24 h exposure to 523 µg L^{-1}) was also lower than those of some antibiotics (phenazone, amoxicilin and erythromycin). Indeed these antibiotics caused more than the 50% decrease of bacterial adhesion of a complex microbial community after 2 days incubation at 5 µg L^{-1} (Schreiber and Szewzyk 2008). However the toxicity of most of these pharmaceutical compounds has been tested on algae or bacteria and not on the whole biofilm communities, making comparison difficult. Moreover tests at

community level often indicate subtle effects that may become relevant during chronic exposure. For example, Lawrence et al. (2008) showed that a chronic exposure of river biofilm to 10 μg L^{-1} of the antimicrobial agent chlorhexidine leaded to significant changes in periphyton community composition while conventional toxicological tests with cyanobacteria, algae and protozoa did not reveal any significant effect at concentrations up to 100 μg L^{-1}.

In this study, the use of biofilms as multi-species systems, and a wide array of biomarkers have proved useful to distinguish qualitatively the effects of the three β-blockers. Since β-blockers are mainly found as mixtures in rivers, this species--specific toxicity could have potential consequences on interactions between algae and bacteria and furthermore on the whole aquatic ecosystem. Mixtures of β-blockers in the environment are usually found at low concentrations and may especially affect the bacterial compartment of biofilms. Nevertheless, high concentration pulses can affect dramatically the algal compartment. This experiment investigated No-Effect--Concentration after short-term exposure. However, it is unknown whether or not long--term exposure to concentrations lower than NEC would affect fluvial biofilms and so analyses of chronic effects of β-blockers (at low concentrations) on biofilms would be of great interest.

Acknowledgements

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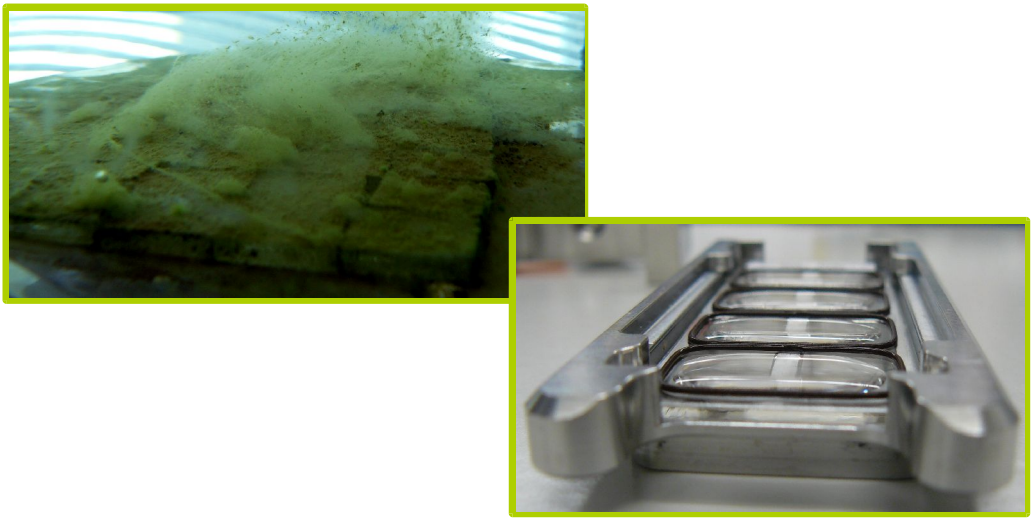
Chapter V

Exploring the potential of a Functional Gene Array (FGA) for fluvial biofilms



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In preparation

Abstract

In the present study, the possibility of studying gene expression in autotrophic river biofilm communities was explored. Microarrays may overcome past limitations on biomarkers research that covered a small number of processes, by providing a system-wide analysis of cell functioning based on the simultaneous detection of the activity of thousands of genes. To this aim, a Functional Gene Array (FGA), based on consensus sequences from genes of key physiological processes, was designed including 83 functional genes chosen to reflect several essential biochemical pathways and specific stress response pathways of microalgae such as the calvin cycle or the antioxidant enzymes. Probes of these genes were designed from consensus sequences from up to 6 microalgal species (diatoms and chlorophytes). Furthermore, species--specific probes were included resulting in 1554 unique oligonucleotide probes for 83 different genes. RNA extracted from *Chlamydomonas reinhardtii*, *Scenedesmus vacuolatus* and multi-species biofilms was then hybridised to oligonucleotide arrays at different hybridisation temperatures (55, 60 and 65ºC). Signal intensity was affected by sequence divergence but results showed that a hybridisation temperature of 55ºC allowed a good compromise between cross-hybridisation and specificity. The methodological developments presented in this study are the first basis towards the application of transcriptomics in toxicity assessment in biofilms. This approach could be especially useful to screen a high number of genes and detect which are affected by a chemical. In addition, it may allow linking oxidative stress response to other metabolic processes in biofims. Nevertheless, this FGA is mainly a prognostic tool indicating which processes susceptible to be affected by a chemical should be investigated at higher level of biological organisation. Due to the limited number of probes and available sequence information the designed FGA is at present recommended for controlled ecotoxicological microcosm or mesocosm experiments, field applications may require further method developments and validations.

Keywords: periphyton, microalgae, cross-hybridisation.

1. Introduction

In river ecosystems, biofilm communities play a major role in carbon and nutrient recycling, primary production or detoxification (Romaní et al. 2004; Romaní 2010). Hence, they are recognized as pertinent indicators of aquatic ecosystem quality (Sabater and Admiraal 2005). Fluvial biofilms are complex communities composed of algae, cyanobacteria, bacteria, fungi and protozoa closely interacting in an extracellular matrix (Romaní 2010). Therefore, various methods are used to determine structural and functional parameters specific to the different biofilm components, and to quantify these processes (Weitzel 1979; Denkhaus et al. 2007) and evaluate the response of the community to different perturbations (Sabater and Admiraal 2005). Among these methods, various molecular biomarkers of exposure to and/or effect of chemicals have been used such as the extracellular enzyme peptidase (e.g. Ricart et al. 2009), the 18S rRNA and 16S rRNA (as indicator of community structure, e.g. Tlili et al. 2008) or some pigments (e.g. Laviale et al. 2010). These molecular biomarkers are expected to be particularly useful to assess sub-lethal effects under realistic contamination scenarios (i.e. chronic contamination at low concentrations) (Moore 2002). Based on previous studies (Bonnineau et al. 2010, 2011; Guasch et al. 2010), the antioxidant enzyme activities (AEA) of biofilm communities are sensitive biomarkers which may provide valuable information on the antioxidant capacity of these communities. Though different regulation mechanisms can exist, enzymatic activity within cells is linked with the number of enzymes that depends on the transcription of the corresponding genes (Brock and Madigan 1991). Therefore the activation of gene transcription is the first step in cell response to a perturbation and may be detected earlier and at lower level of stress than the activity itself. Indeed, Wu and Lee (2008) showed that the activation of the transcription of the genes encoding for the antioxidant enzymes catalase (CAT) and ascorbate peroxidase (APX) occurred after exposure to lower concentrations of copper than the activation of these enzymes in the algae *Ulva fasciata* Delile. This example points out the potential of antioxidant enzymes gene expression as sensitive biomarker of sub-lethal oxidant effect.

Besides, previous studies also showed the importance to use various biomarkers to detect both direct and indirect effects of chemicals on biofilm communities and to assess toxicity of chemicals with unknown modes of action (Franz et al. 2008; Ricart et al. 2009, 2010). Even compounds from a same chemical class may have different impact on non-target species. For instance the β-blocker propranolol was found toxic for the autotrophic component of the biofilms while the β-blocker metoprolol was toxic for the bacterial component (Bonnineau et al. 2010). Therefore it seems more interesting to assess the expression of various genes corresponding to essential processes in biofilms rather to focus on only one.

In this context, microarrays offer a comprehensive tool for system-wide analysis based on the simultaneous detection of the activity of thousands of genes. This technology is based on the hybridisation of RNA or DNA extracted from a target biological system to probes corresponding to known genes (Neumann and Galvez 2002). Since a priori knowledge of genetic information is needed, most common applications are related with the study of gene expression and regulation within single species in laboratory experiments or environmental samples (Jamers et al. 2009). In communities, such as biofilms, the application is, however, difficult, since most of the organisms in the community are not genetically characterised nor is their exact distribution known (Zhou et al. 2002). Functional Gene Arrays (FGAs) based on consensus sequences from genes of key processes may partially overcome these limitations and allow gene expression of communities to be monitored (Wu et al. 2001). Consensus sequences used to design FGAs probes result from the alignment of various genes from known species and are also expected to be found in non-sequenced organisms (He et al. 2008). Therefore, FGAs can be used to characterize communities in terms of their functional attributes, to determine the dominant biological/geochemical processes and their activities in such a community, to study the link between structure and function at community level and at ecosystem level (Andersen et al. 2010). One of the most complete FGAs developed is the GeoChip 2.0 that covers more than 10000 microbial genes in more than 150 functional groups

involved mainly in biogeochemical cycling processes (He et al. 2007). To our knowledge, such an array has never been developed for autotrophic biofilm communities, despite the high ecological relevance of these communities. Therefore, the main objective of the present study was to explore the feasibility of designing a FGA for biofilms based on the existing genome information on microalgae and to characterize some key features (such as specificity, cross-hybridization, hybridization temperature) of this FGA. Due to the scarce genetic information available, the design of such an array for the autotrophic component of biofilms is a considerable challenge, then the application of this FGA in toxicity assessment in biofilms is beyond the scope of this study.

This study would rather focus on the methodological developments essential for further applications of the FGA. First, oligonucleotide probes were designed by selecting pertinent genes chosen to reflect several essential biochemical pathways as well as specific stress response pathways (Box 1). Gene sequences were derived from genome information of species potentially present in biofilms or closely related to biofilm species. Then, a method for extraction of high quality RNA from biofilm samples was reported. Finally, the use of different hybridisation temperatures (i.e. stringency levels) and of different biological targets allowed results of the FGA with respect to cross hybridisation and signal specificity to be evaluated.

2. Material and methods

2.1. Culture of Chlamydomonas reinhardtii, Scenedesmus vacuolatus and biofilm communities

Populations of *Chlamydomonas reinhardtii* Dangeard were cultured in sterile minimum medium in Erlenmeyer flasks at a light intensity of ~ 120 µmol photons s^{-1} m^{-2} in a 14 h light/ 10 h dark cycle at 20 ± 0.5 °C on a rotary shaker by permanent agitation at 200 rpm. Subcultures were inoculated every two weeks.

Populations of the unicellular chlorophyte *Scenedesmus vacuolatus* Shihira & Krauss (strain 211-15) were grown in sterile minimum medium and under controlled conditions as described by Altenburger et al. (2004). To obtain synchronous growth of algal populations, algae were cultivated under synchronizing conditions (illumination of white light of ~ 400 µmol photons s^{-1} m^{-2} in a 14 h light/ 10 h dark cycle at 28 ± 0.5 °C) by daily dilution.

To assign cultural cell densities and cell volume, an electronic cell analyzer (CASY, Schärfe System GmbH, Reutlingen, Germany) was used.

Biofilms were grown on artificial substrates (glass discs of 1.5 cm diameter) in 20 L microcosms under controlled conditions, as described by Schmitt-Jansen and Altenburger (2005b). Microcosms were incubated at 20°C under a 14:10 light:dark cycle and water was mixed by paddles. Water, taken from the stream Parthe (Leipzig, Germany) was refreshed once a week. Mature biofilms (around 5 week-old) were used for RNA extraction.

2.2. RNA Extraction

C. reinhardtii cells and *S. vacuolatus* cells were harvested by centrifuging 50 mL of culture (6 x 10^5 cells mL^{-1} and 2.2 x 10^7 cells mL^{-1}, respectively) at 3300 g for 5 min. Then, 500 µL of Trizol (Invitrogen, Karlsruhe, Germany) and 150 mg of glass beads (0.5 mm diameter, Carl Roth, Karlsruhe, Germany) were added to the algal pellet and cell disruption was performed through 3 pulses (45 s, 30 s, 30 s) of a bead beating system (FastPrep®-24, MP Biomedicals, Illkirch, France; v=6.5 m s^{-1}). Samples were stored for 2 min on ice between each pulse. After 5 min of incubation at room temperature RNA was extracted by adding 100 µl of chloroform to the sample. After centrifugation for 5 min at 12 000 x g and 4°C, the supernatant was transferred to a new tube and chloroform extraction was repeated. The remaining steps were performed according to the manufacturer's instructions for RNA isolation with Trizol reagent. A modified protocol was used for RNA extraction from biofilm communities. For each sample, biofilms from 20 discs was scraped into a 1.5 ml reaction tube and samples

were centrifuged for 5 min at 10000 x g and 16ºC. After removing excess water, samples were weighted (wet weight). Afterwards, cell disruption and chloroform RNA extraction were performed as described above but using 1 mL of Trizol for cell disruption and a similar amount of supernatant and chloroform. Since biofilm samples contain humic matter and polysaccharides from extracellular polymeric substances (EPS), RNA precipitation was done by adding 2 volumes of polyethylene glycol (PEG) solution (30% PEG, 1.6 M NaCl) to 1 volume of supernatant (adapted from Griffiths et al. 2000). After 2 hours of incubation at room temperature and 10 min centrifugation at maximal speed and 4ºC, the RNA pellet was washed twice with 200 µL of cold ethanol, air dried at room temperature and reconstituted in 50 µL of DEPC water (5 min incubation at 55ºC).

In all samples, potential DNA contamination was eliminated by treatment with DNAse I (U μg^{-1} RNA, RNAse-free, Roche) at 25ºC during 15 min. Subsequently, RNA was further purified using the RNeasy Mini Plant Kit (Qiagen®, Hilden, Germany). The manufacturer's protocol was modified for clean-up of total RNA from *S. vacuolatus* and biofilms as follows: in order to improve RNA cleanup, RLC buffer with 10 µL mL^{-1} of β-mercaptoethanol was used instead of RLT buffer. All RNA extracts were stored at -80ºC until labelling. RNA quantity was assessed using Nanodrop® ND-100 UV-VIS spectrophotometer, RNA integrity and quality were checked on 1.5 % agarose gel.

2.3. RNA labelling

300 ng of RNA extracted from *C. reinhardtii* and 900 ng from *S. vacuolatus* and biofilms were used for labelling with Cyanine 3 using the Quick Amp Labelling kit (Agilent Technologies®, Böblingen, Germany). For *S. vacuolatus* and biofilm samples, transcription time was extended by 30 min to 2 h 30 min. Purification of labelled RNA was performed using RNeasy Mini Plant Kit (Qiagen®) as described above.

2.4. Probe design and microarray production

For the functional gene array (FGA) 58 different types of enzymes were selected (Box 1). Gene sequence information available from four chlorophytes (*C. reinhardtii*, *Scenedesmus obliquus* (Turpin) Kützing, *Stigeoclonium helveticum* Vischer, *Oedogonium cardiacum* (Hassall) Wittrock) and two diatoms (*Phaeodactylum tricornutum* Bohlin and *Thalassiosira pseudonana* Hasle and Heimdal) was used to design probes for the FGA. For each gene, available sequences were retrieved from the NCBI database and compared by alignment using Kalign software (EMBL-EBI, Lassmann and Sonnhammer 2005) with default settings (80, 3, 3 for gap open penalty, gap extension penalty and terminal gap penalties, respectively). For each species, sequences of more than 60 bp and with homology to at least one other species (from now on these sequences will be referred to as homologous sequences) were used to design 60mer oligonucleotides using eArray software (Agilent Technologies®). Two different probes were designed for each selected homologous sequence. Depending on the number of homologous regions, up to 148 probes were designed for each gene and printed in duplicate on microarray slides (4 x 44 K format) using the Agilent SurePrint platform (Agilent Technologies®). A list of the probes annotated for gene and species is available in the supporting information (Table A1).

2.5. Hybridisation

For each biological target (*C. reinhardtii*, *S. vacuolatus* and biofilms), three hybridisation temperatures were tested (55ºC, 60ºC and 65ºC). 1.65 µg of labelled RNA was used for hybridisation according to manufacturer indications. After 17h of hybridisation, arrays were washed with stabilization and drying solution (Quick Amp Labelling kit, Agilent Technologies®).

2.6. Microarray analyses and data extraction

Microarrays were scanned at 5 µm resolution using GenePix ® 4400A Scanner (MDS Analytical Technologies GmbH, Ismaning, Germany) at 532 nm. PMTs (Photo Multiplier Value) were auto-adjusted for each slide to a saturation tolerance of 0.01 %

(PMT = 427, 438 and 457 for slides hybridized at 65ºC, 60º C and 55ºC, respectively). Spot identification was performed utilizing the circular feature alignment of GenePix Pro 7 software (Axon) and Agilent GAL files.

2.7. Data analyses

The data discussed in the present study have been deposited in NCBI's Gene Expression Omnibus (Edgar et al. 2002) and are accessible through GEO Series accession number GSE22496. Data analyses were performed using Knime 2.1.2 with R extension for data organization and normalization (Berthold et al. 2008) and R 2.6.2 (R Development Core Team 2008) for other statistical analyses.

F532 (fluorescence intensity at 532 excitation) medians were first log2 transformed and then normalized by quantile normalization (affy package, Gautier et al. 2004). To explore the differences between the samples, a Principal Component Analysis (PCA) on signal intensities was performed (ade4 package, Dray and Dufour 2007). To explore the specificity of FG probes, the percentages of identity between probes of three genes: *atpA*, *atpB* and *rbcL* (coding for ATP synthase CF1 alpha chain, ATP synthase CF1 beta chain and Rubisco large subunit, respectively) obtained from different organisms and *C. reinhardtii* genome were calculated by the BLAST software (Altschul et al. 1997). Since these genes correspond to key processes in autotrophic organisms, various sequences were available from the different organisms chosen for the present study. Probes coding for *atpA* were obtained from *P. tricornutum, S. obliquus* and *S. helveticum*, for *atpB* from *S. obliquus* and *S. helveticum*, for *rbcL* from *S. obliquus*, *S. helveticum* and *O. cardiacum*. Then, Signal to Noise Ratios (SNR) of *C. reinhardtii* targets hybridized to these probes were compared to the corresponding percentages of identity. A SNR of 3 was considered as the minimum value for accurate quantification, as it is commonly accepted (He et al. 2005). The relation between the percentage of probes detected (i.e. not flagged by GenePix Pro 7 software) per gene and per species and the hybridisation temperature was also analysed for each biological target using Spearman correlation (Hmisc package, Harrell 2007).

3. Results

3.1. FG probes design

To design the FGA, 83 genes were selected coding for 57 different enzymes or functions (including their isoforms) involved in processes of energy production, C and N utilization or stress responses (Box 1).

On the array, each gene was represented by different probes from different homologous regions. For example, when aligning sequences coding for the *atpB* gene, one sequence was found to be similar between genes from *C. reinhardtii*, *T. pseudonana*, *O. cardiacum*, *S. helveticum*, *S. obliquus* and *P. Tricornutum,* and an additional sequence was found to be similar between genes from *O. cardiacum*, *S. helveticum*, *S. obliquus* and *P. tricornutum*. Consequently, 10 unique homologous regions were used to design probes for the *atpB* genes: 1 was derived from *C. reinhardtii* genome, 1 from *T. pseudonana*, 2 from *O. cardiacum*, 2 from *S. helveticum*, 2 from *S. obliquus* and 2 from *P. tricornutum*; then 2 different probes were designed from each homologous sequence resulting in 20 unique probes for *atpB* genes. By this process, a total of 1554 unique probes were designed and printed in duplicate on the array.

3.2. RNA extraction from biofilm samples

No RNA could be extracted from biofilms using the protocol described for *C. reinhardtii*. Therefore, a modified protocol, including a precipitation step with a saline solution of PEG instead of ethanol precipitation, was used in order to improve separation between cell debris, humic acids and RNA. Finally 3.8 µg and 13.7 µg of clean RNA ($A_{260/280}$ and $A_{260/230} > 2$) was obtained from 870 mg and 1079 mg of biofilms, respectively. Between 10 and 40 µg of clean RNA were extracted from 50 mL of a culture of *C. reinhardtii* or *S. vacuolatus*.

Box 1. Main pathways targeted by the genes in the FGA for autotrophic biofilm.

Energy	Photosynthesis/respiration/energy		
	chlorophyll synthetase	cytochrome c oxidase	
	phosphoenolpyruvate carboxylase	ATP synthase	
C utilization	Calvin cycle	TCA cycle	Glycolysis
	rubisco large/small subunit	pyruvate dehydrogenase	hexokinase
	phosphoglycerate kinase	citrate synthase	phosphoglucose isomerase
	glyceraldehyde 3 P dehydrogenase	aconitase hydratase	phosphofructokinase
	fructose 1,6 bisphosphatase	isocitrate dehydrogenase	enolase
	transketolase	succinate dehydrogenase	pyruvate kinase
	sedoheptulose 1,7 biphosphatase	malate dehydrogenase	fructose bisphosphate aldolase
	phosphoribulokinase	fumarate hydratase	glyceraldehyde 3P dehydrogenase
			phosphoglycerate kinase/mutase
	Pentosephosphate pathway		Glyoxylate cycle
	glucose 6 phosphate dehydrogenase	ribose 5 phosphate isomerase	isocitrate lyase
	fructose 6 phosphate aldolase	ribulose phosphate 3 primerose	malate synthase
	D-glucono-15-lactonelactonohydrolase	transketolase	
		transaldolase	
N utilization	Transamination/glutamine biosynthesis		
	glutamate-1-semi aldehyde aminotransferase	glutamine synthetase	
	Glutathione biosynthesis		
	glutathione synthetase		
Stress Response	Carotenoids biosynthesis	Antioxidant system	Protection/protein folding
	phytoene desaturase/synthase	catalase	chaperonin
	precursor of lycopene beta cyclase	ascorbate peroxidase	heat shock protein
	beta-carotene ketolase	glutathione-s-transferase	
	carotenoid hydroxylase	glutathione reductase	
	isopentenyl-diphosphate delta isomerase	superoxide dismutase	
Others	cytochrome p450	decarboxylase	
	translation elongation factor	actin	
	uroporphyrinogen	alpha/beta tubulin	
		enoyl-[acyl-carrier-protein] reductase (fabI)	

3.3. Results of FGA hybridisation

3.3.1. Overview of hybridisation results

For all species and all probes tested, hybridisation temperature had slight effects only on intensity distribution (Supporting information, Fig. A1). Normalised microarray data (log2 transformed fluorescence intensities) were analysed by a PCA (Fig. 1).The first PCA axis explained 43.9 % of the total variance and the second one 37.8 %. Samples from *S. vacuolatus* and *C. reinhardtii* had similar scores on the first axis (between 12 and 26) and different ones on the second axis (between -28 and -39 and between 26 and 35, respectively) while scores of samples from biofilms were different from those of monocultures on both axes (between -38 and -42 on axis 1 and between 1 and 5 on axis 2). The separation of the samples hybridised at different temperatures along the PCA axes also indicates that hybridisation temperature did not severely interfere with the relative distribution of signal intensities.

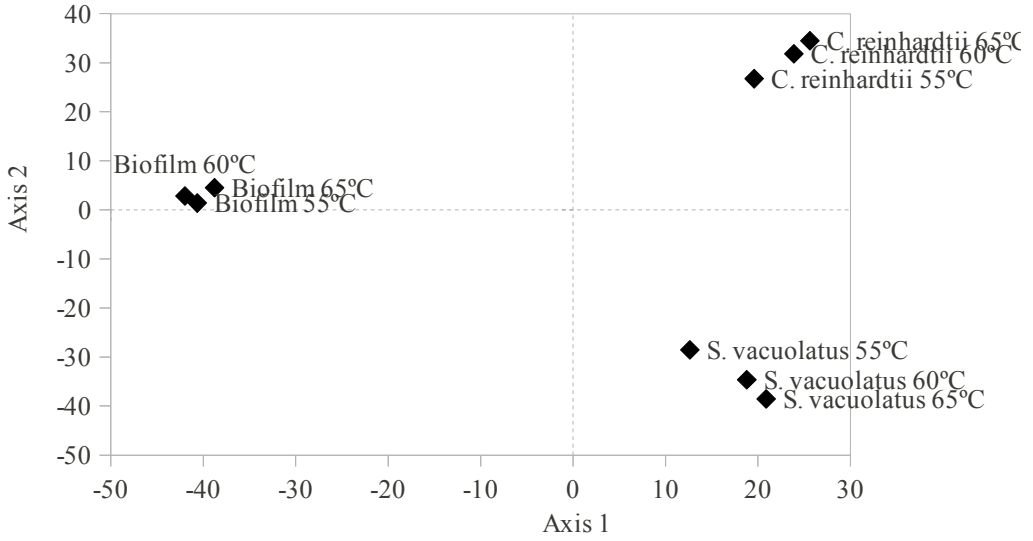

Figure 1. Results of PCA. Diamonds indicate the different target samples: C. reinhardtii, S. vacuolatus and Biofilm. The numbers correspond to the hybridisation temperatures.

3.2 Effects of probe-target identity on signal intensity

SNR of *C. reinhardtii* transcripts against probes of three genes with different percentages of identity are presented in Figure 2. For all genes and all hybridisation temperatures, SNR increased with percentage of identity. SNR for maximal identity was different for each gene: between 45 and 64 for *atpA,* between 28 and 45 for *atpB*, between 33 and 84 for *rbcL*. At the highest hybridisation temperature, only targets presenting 100% of identity with *atpA* probes had a SNR > 3. However, at lower temperature, targets presenting 85 % of identity also had a SNR > 3. At all hybridisation temperatures, targets presenting less than 85 % of identity with *atpB* probes had SNR < 3. At the highest hybridisation temperature, targets presenting a percentage of identity ≥ 90% with *rcbL* probes had a SNR > 3. However, at lower temperature, targets presenting 78 % of identity also had a SNR > 3.

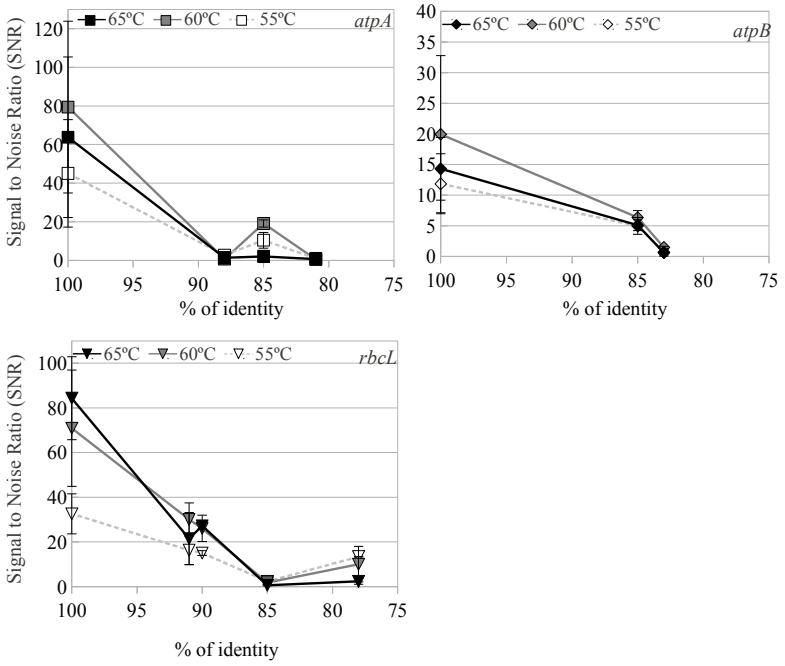

Figure 2. Signal to Noise Ratio of C. reinhardtii mRNA target hybridized to probes corresponding to atpA (square), to atpB (diamond) and to rbcL (triangle). On the x-axis the percentage of identity of these probes with C. reinhardtii genome is indicated. Results of hybridisation performed at 65ºC (black line), 60ºC (plain grey line) and 55ºC (dashed grey line) are shown.

3.3 Hybridisation of the different biological targets to the different types of probes

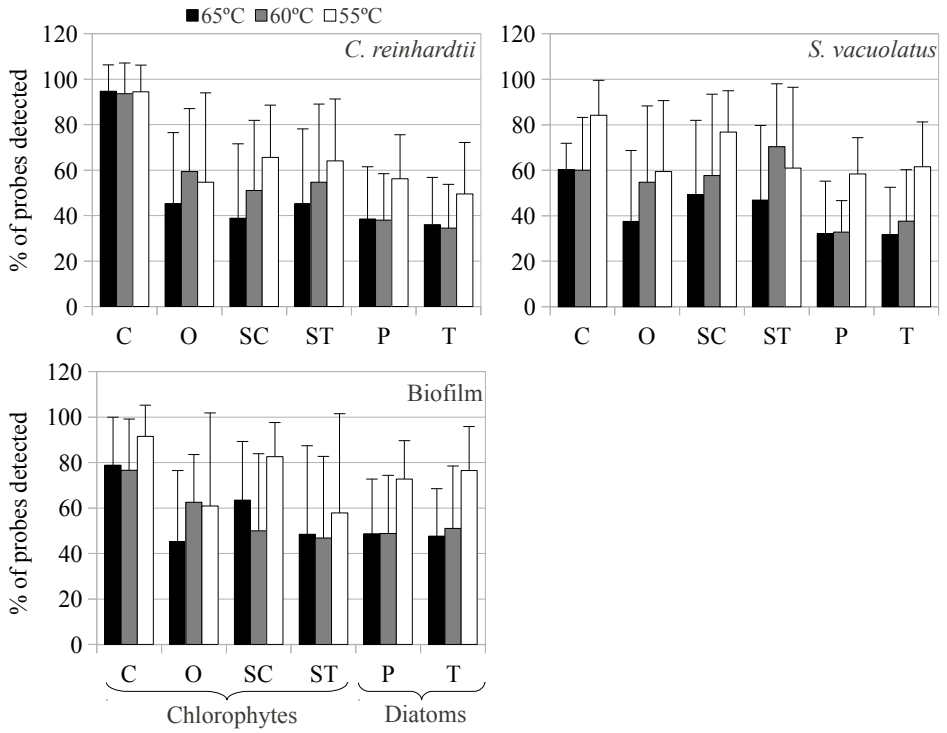

Figure 3. Percentage of probes detected per gene and per species by each target at different hybridisation temperatures: 65ºC (black), 60ºC (grey), 55ºC (white). The x-axis showed the species of origin of the probes:C: C. reinhardtii,O: O. cardiacum, SC: S. obliquus, ST: S. helveticum, T: T. pseudonana, P: P. tricornutum.

For all targets, the percentage of probes detected per gene and per organism was highly variable from gene to gene as shown by the high standard deviations in figure 3.

Nevertheless, some differences could be highlighted between biological targets. First, *C. reinhardtii* targets hybridized on average to 90% of the probes obtained from the same organism and only to 40 - 60 % of probes obtained from other organisms (Fig. 3). Temperature was significantly correlated with the percentage of probes from *P. tricornutum* and *T. Pseudonana,* detected by *C. reinhardtii* target (ρ = -0.35 and -0.25, respectively). *S. vacuolatus* hybridized mostly to targets from

C. reinhardtii, *S. obliquus* and *S. helveticum* (between 50% and 80% of probes from each species detected at all hybridisation temperatures, Fig. 3). A significant correlation was observed between the hybridisation temperature and the percentage of probes from *C. reinhardtii*, *P. tricornutum* and *T. pseudonana* detected by the *S. vacuolatus* target (ρ = -0.46, -0.56 and -0.51, respectively). For all species of origin of the probes, at least 50 % of the probes were detected by the biofilm target, the highest percentage of probes detected was observed for probes from *C. reinhardtii* and *S. obliquus* (Fig. 3). Moreover, a significant correlation was found between hybridisation temperature and percentage of probes from *C. reinhardtii, P. tricornutum* and *T. pseudonana* detected by biofilm targets (ρ = -0.27, -0.41 and -0.47, respectively).

3.4 Genes detected

For all biological targets, all genes were detected at hybridisation temperature of 55ºC, meaning that hybridisation to at least one probe per gene was detected. At higher hybridisation temperatures, fewer genes were detected, for *C. reinhardtii* the probes detected covered 95 % and 96 % of genes at hybridisation temperatures of 60 ºC and 65 ºC, respectively; for *S. vacuolatus* 94 % and 92 % and for biofilm 95% in both cases.

Most of the genes were detected by biofilm target indicating that consensus sequences used for probes design are likely to match the genome of non-sequenced species within these communities. Even under high stringency conditions (hybridisation temperature: 65 ºC) all the probes were detected for some genes related with C and N utilization (phosphoribulokinase, glutamate-1-semialdehyde aminotransferase), with the photosynthetic system (chlorophyll synthetase, ATP synthase), the protein protection (chaperonin) or the cells structure (alpha/beta tubulin). On the contrary, some genes were not detected by biofilm target at hybridisation temperature of 60 and 65ºC, they were those related with C utilization (phosphoglycerate kinase, hexokinase) or carotenoids biosynthesis (precursor of

cyclase lycopene beta cyclase). Moreover between 50 and 85 % of the probes of the genes related with the antioxidant system (catalase, ascorbate peroxidase, Mn and Fe superoxide dismutase, glutathione-S-transferase, glutathione reductase) were detected under high stringency conditions, this percentage was between 80 and 95 % at hybridisation temperature of 55ºC.

4. Discussion

Microarray analysis of biofilms is a challenging task for several reasons.

(1) Extraction of good quality RNA free of substances that may inhibit further labelling or hybridisation reactions is difficult in environmental samples. Micro--organisms in biofilms are embedded in an extracellular matrix composed of extracellular polymeric substances (EPS), so humic acids and polysaccharides can be found in biofilm samples (Neu and Lawrence 1997; Christensen 1989) and are known to interfere with RNA isolation. In addition, biofilm communities are composed of different microorganisms with different cell wall resistance and protocols for RNA extraction should allow extraction of RNA from all of these diverse organisms in a quantitative way. Mechanical disruption of biofilm cells with glass beads has previously been found to be an appropriate method for protein extraction from biofilms (Bonnineau et al. 2011). Combined with Trizol extraction, this method allowed a successful cell disruption and RNA isolation. Since interfering substances in biofilm extracts were found to prevent isolation of RNA from the extract, RNA precipitation with a saline solution of PEG was needed to ensure recuperation of clean RNA. This method has previously been validated for DNA/RNA extraction in other types of environmental samples (Griffiths et al. 2000) and is, therefore, recommended for extraction of high quality RNA from biofilm communities.

(2) Microarray hybridisation is dependent on stringency conditions and probes specificity (Bar-Or et al. 2007). In the present study, different stringency levels (hybridisation temperatures) were tested, but the effect on hybridisation was low as

shown by the distribution of intensities after hybridisation at different temperatures. Thus, the potential cross-hybridisation occurring at low stringency is relatively weak and would not artificially increase the intensity of the most expressed transcripts. Moreover, the percentage of genes detected by each biological target decreased with decreasing hybridisation temperature: only a hybridisation temperature of 55ºC allowed the detection of 100% of the functional genes. Therefore, a temperature of 55ºC seems the best compromise for the biofilm FGA. The impact of specificity on hybridisation was studied using transcripts from *C. reinhardtii,* since for this species enough genetic information was available to estimate percentage of identity with the different types of probes. SNR decreased dramatically with specificity; for the three genes a reduction by 2 to 4 times of SNR was observed for probes sharing less than 90 % of identity with the gene from *C. reinhardtii*. At all stringency conditions tested, transcripts with low specificity (< 90 % of identity) may not influence the level of expression of this particular gene and consequently the rate of false positives may be relatively low.

(3) To ensure a sensitive representation of genes transcription in biofilms, a high diversity of probes has to be maintained for each gene. In the present study, FG probes were not derived from one consensus sequence corresponding to the alignment of various homologous sequences but from all these different homologous sequences. Therefore, each gene on the array was represented by various probes from different species. During gene selection, it was not always possible to obtain full coding cDNA or mRNA sequences for all the species. Thus, for some genes, the number of homologous sequences differed from one species to another. *C. reinhardtii*, *S. vacuolatus* and biofilms presented different patterns of hybridisation on the FGA as indicated by PCA results. This result can either be explained by a differential expression and/or distribution of the different genes within the biological targets and/or by a selective hybridisation to different probes corresponding to the same gene but derived from different species. The second hypothesis is supported by the selective hybridization of each biological target to the different probes as shown by the

percentage of probes detected per gene and per organism. *C. reinhardtii* and *S. vacuolatus* hybridized preferentially to probes from closer organisms (chlorophyte species), while biofilms presented less specific hybridisation. The use of different probes from different species but corresponding to the same gene increased the chance to obtain probes highly similar to RNA extracted from biofilm communities and, therefore, to equally detect all genes of the array. In the present study, this assumption was confirmed, the redundancy in gene function allowed the detection of 100% of the functional genes at the hybridisation temperature of 55ºC. Nevertheless, at higher temperature some of these genes were not detected in the different biological targets. It is likely that these genes might only partially match the probes. These results indicate that to cover a maximum of the genetic diversity of a biofilm community it is essential to obtain probes from various organisms.

The methodological developments presented in this study represent the first basis towards the application of transcriptomics in biofilms. They include an efficient method for RNA extraction from biofilms, a list of oligonucleotides probes corresponding to a part of the biofilm metagenome and a validation of the principle of FGA for the autotrophic biofilm. This proof-of-concept illustrates then the potential of an FGA for biofilm communities to approach diversity and complexity of such samples.

This FGA could be then used in ecotoxicological tests to determine the effects of a chemical on different important processes related to primary production, nutrient utilization or antioxidant protection in biofilms. Such an insight in various molecular processes may especially help in interpreting antioxidant enzymes variations as it would link them with changes of other important processes. This FGA offers a unique opportunity to screen 83 genes and detect which are affected by chemical exposure. Quantification of the changes occurring in the genes detected can further be done by RT-qPCR (Dorak 2006). However, since the extrapolation of genes variations to changes at higher levels of organization is likely to be influenced by many regulation mechanisms, this screening is a prognostic tool to indicate which processes are at risk

and should be investigated at higher levels of biological organization (e.g. protein concentration, enzymatic activity, photosynthesis/respiration). This would also help in better understanding toxicity mechanisms. For instance, an increase of antioxidant enzymes transcripts would indicate an activation of antioxidant defence system, nevertheless if antioxidant activities decrease concomitantly this could indicate an oxidative degradation of these enzymes just after their production. Therefore this FGA could be especially useful to assess toxicity of emerging compounds but also to link changes in different biological processes.

Variations in biofilm community structure may probably be reflected by the hybridisation and would make difficult the comparison of expression gene level between different communities. Therefore this FGA is recommended to be used with similar biofilm communities during short-term experiments to obtain information on processes directly affected by a chemical.

5. Comments and recommendations

The present study highlights that the Functional Gene Array approach can principally be used for gene transcription analysis in fluvial biofilms. A relatively quick and simple method was used to design a FGA for biofilm communities. This FGA may already be used to measure changes in transcription level of 83 genes in biofilm communities. To explore functional diversity of biofilm communities and extend the number of genes on the FGA, more genetic information is needed from species commonly found in biofilms, in particular, on freshwater diatoms. Though metagenome construction is a long and expensive approach, selection of information from a metatranscriptome library from both prokaryotic and eukaryotic organisms composing biofilm communities would probably extend the diversity of biofilm FGAs with respect to genes and species. However, obtaining such a library would involve numerous challenges especially concerning eukaryotes as illustrated by the recent

description of the first metatranscriptome library for eukaryotic marine plankton by John et al. (2009)

Since the genetic information used for probes design was limited, first applications should focus on the difference in level of transcription of genes between similar communities exposed to stressors in acute ecotoxicological tests (e.g. short--term effects due to chemical exposure or changes in environmental parameters). The number (83) and the variety of genes that can be screened with this FGA may be particularly useful to indicate which genes are affected by a toxicant and therefore which processes are potentially at risk. The processes selected by this first screening may be further investigated to scale-up toxicity effects to higher levels of biological organization.

Acknowledgements

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Supporting information

Table A1 is attached to the Thesis as a CD.

Table A1. Probes and their corresponding annotation (gene and species of origin)

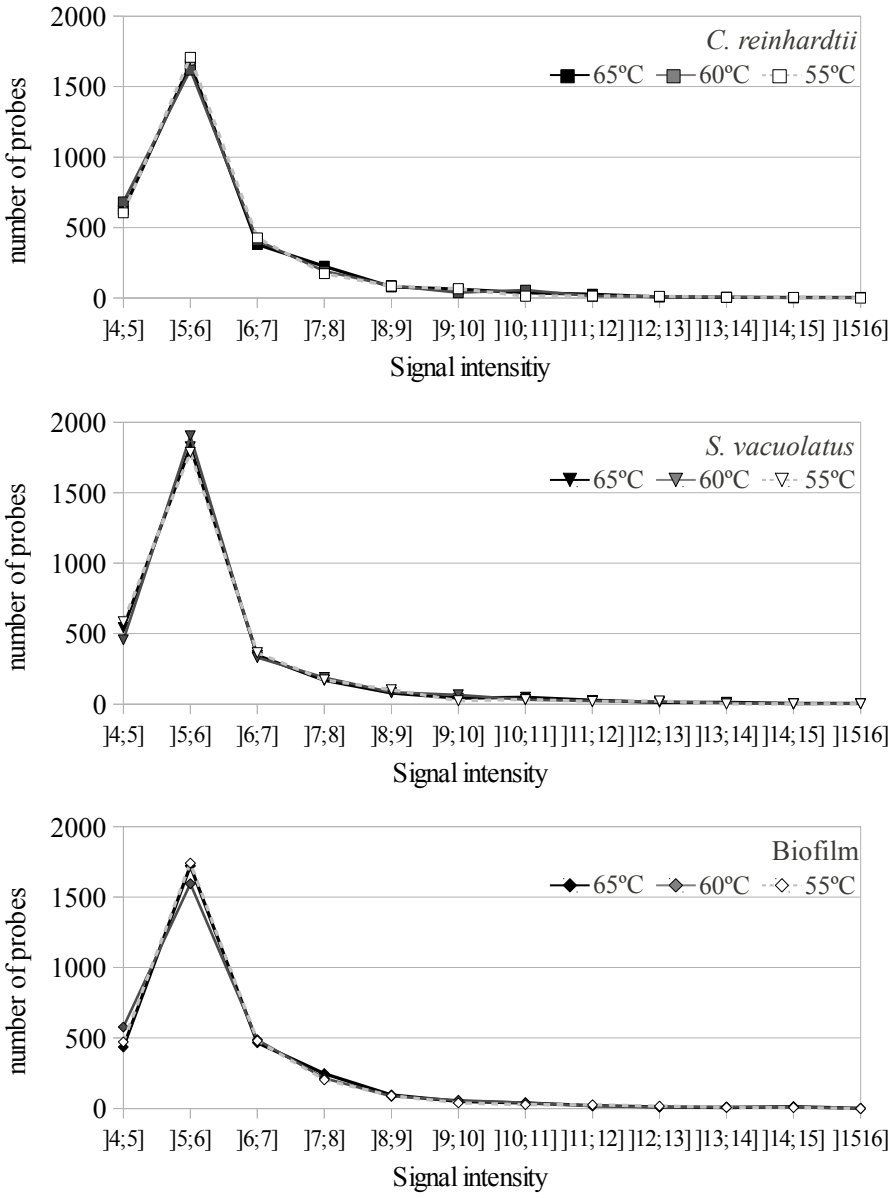
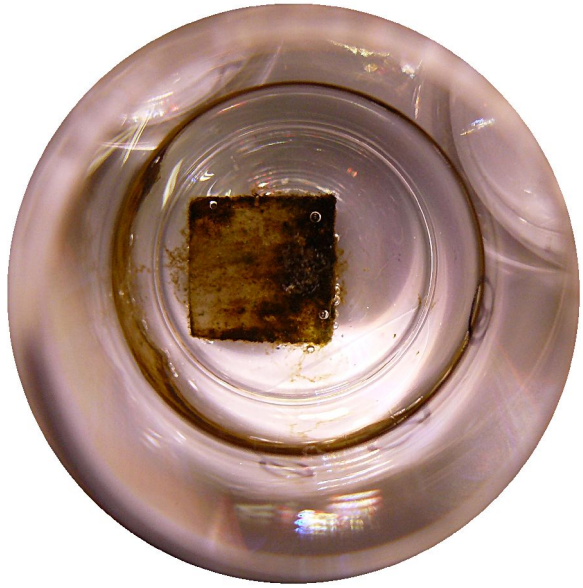


Figure A1. Distribution of intensities of the different genes for C. reinhardtii, S. vacuolatus and biofilm after hybridization at 65ºC (black line), 60ºC (grey plain line) and 55ºC (dashed grey line).

General

Discussion



The different studies presented in this thesis have illustrated the potential of antioxidant enzymes activities (AEA) as biomarkers of oxidative stress in biofilms. In the following section, first, the main findings (AEA characteristics and possible application) of this thesis are discussed. Then the contribution of AEA to a multi--biomarker approach for toxicity assessment at community level is critically evaluated. Finally, the perspectives opened by this thesis, on the use of molecular biomarkers (AEA but also gene expression) in biofilm toxicity assessment are presented.

Antioxidant enzymes in fluvial biofilms

1. AEA variations in response to exposure to oxidative stress

The exposure of biofilms to stressors known to produce oxidative stress (oxyfluorfen, copper and light intensity increase) led to variations in catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) while no variations in superoxide dismutase (SOD) were observed. The activation or inhibition of antioxidant enzymes are the indirect measure of oxidative stress as they indicate a response to a change in reactive oxygen species (ROS) levels (Valavanidis et al. 2006; Kim et al. 2010; Klaper et al. 2009; Machala et al. 2001). Then, the absence of changes in SOD activity questions its potential as biomarker of oxidative stress in biofilms. Since SOD was not activated while variations in other AEA indicated oxidative stress induced by high light intensity, oxyfluorfen or copper exposure, SOD regulation might not be a key mechanism to adjust redox levels within biofilms in these situations. Individual characteristics of the main species forming the biofilm exposed could explained this result as SODs are very diverse within micro-algae (Wolfe-Simon et al. 2008) and previous studies reported that the activation or inhibition of micro-algal SOD in response to oxidative stress was dependent on the stressor (metals, organic pollutants or environmental factors) but also on the species (e.g. Li et al. 2006; Liu and Pang 2010; Pinto et al. 2003; Reis et al. 2011; Tripathi et al. 2004, 2006; Zbgniew et al.

2006). Besides, previous observations have emphasized the importance of SOD in response to metal contamination in both micro-algae (Pinto et al. 2003) and biofilms (Bonet al. personal communication; Guasch et al. 2010). SOD may be especially affected by metal contamination. Indeed Guasch et al. (2010) demonstrated that SOD was affected by acute exposure to copper only in biofilms pre-exposed to copper pulses during colonization while in control ones (not exposed to copper pulses during colonization), SOD was stable after acute exposure to copper as also observed in our study (Chapter III). Though the potential of SOD as biomarkers of oxidative stress was not demonstrated in this thesis, based on previous studies, it may be especially interesting to investigate the specificity of SOD to metal toxicity.

On contrary to SOD, CAT, APX and GR activities were found to be biomarkers of oxidative stress in fluvial biofilms in the present thesis. Both inhibition and activation of these AEA were observed in response to oxidative stress and some specific patterns of variations could be elucidated for CAT, APX and GR responses to acute exposure to changing levels of oxidative stress.

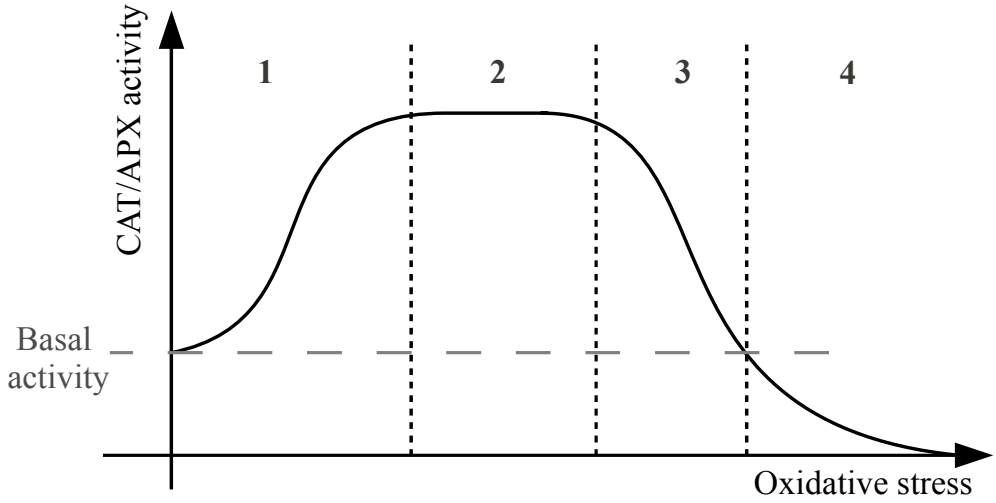


Figure 1. Unimodal pattern of variation of CAT and APX activity in biofilm: 1: increase; 2: saturation; 3: decrease; 4: inhibition

CAT and APX are enzymes directly involved in oxidative stress management by scavenging hydrogen peroxide. The different experiments performed suggested that these enzymes follow a unimodal pattern throughout oxidative stress gradient. This pattern was clearly observed for CAT in mature biofilms exposed to a gradient of oxyfluorfen or propranolol (Chapter II and IV) and suggested for APX by the significant activation of this enzyme at intermediate levels of oxidative stress in shade-adapted biofilms exposed to high light intensities and in biofilms adapted to saturating light intensity exposed to copper (Chapter III). This pattern can be explained by four mechanisms (Fig. 1):

1. An increase in both enzymatic velocity and production leading to an increase of biofilm AEA in a concentration-dependant manner to respond to low and/or moderate levels of oxidative stress
2. Enzymatic production and velocity reach their maximum leading to a saturation in biofilm AEA (plateau)
3. Enzymatic velocity is inhibited by too high concentrations of ROS and/or enzymes are degraded by ROS. Biofilm AEA decreased
4. The antioxidant defence system can not cope with all the ROS which provoke irreversible oxidative damage in cells machinery. Biofilm AEA are inhibited and decrease to 0 as the cells die.

Nevertheless, this unimodal pattern was not observed in all the experiments of this thesis as APX and CAT activities were also found to increase throughout oxidative stress gradient. In particular, CAT activity increased throughout oxyfluorfen gradient during acute exposure of mature biofilms pre-exposed to this chemical (Chapter II). APX activity also increased with increasing concentrations of oxyfluorfen or copper in shade-adapted biofilms and with increasing concentrations of oxyfluorfen only in biofilms adapted to high light intensities (Chapter III). This concentration-dependent increase of activity may correspond to the first part of the unimodal response pattern presented earlier and suggests that levels of oxidative stress in these situations were not

sufficient to inhibit CAT and APX activities. Though APX activity was not inhibited even at high concentrations of copper, a decrease in protein content was observed, indicating potential oxidative damage on proteins but not specifically on APX. Therefore AEA inhibition may be regulated by other factors than oxidative degradation. Further investigations involving direct measurements of ROS, of protein degradation rate and of APX co-factor concentration are needed to understand the regulation of inhibition mechanisms in biofilm AEA and to link the last step of the CAT/APX unimodal model with irreversible oxidative damages. In addition, the activation of CAT and APX, which both scavenge hydrogen peroxide, was found to be complementary. Indeed, an increase in APX activity was observed in biofilms exposed to low concentrations of oxyfluorfen while CAT was stimulated in biofilms exposed to higher concentrations (Chapter II). This result is coherent with the higher affinity of APX for hydrogen peroxide (Mittler 2002).

GR activity was generally found to increase throughout the pollutant gradient (Chapters II and III). However, both inhibition and activation were observed in biofilms exposed to copper, depending on biofilm adaptation to light colonization (adapted to saturating or shaded light conditions, respectively; Chapter III). These different patterns throughout a same gradient of oxidative stress may be explained by the indirect role of GR in oxidative stress management. GR catalyses the transformation of oxidized glutathione (GSSG) in reduced glutathione (GSH) (Noctor et Foyer 1998). GSH can therefore be used to recycle the ascorbate co-factor needed by APX in the water-water cycle (Asada 1999), to inactivate xenobiotics or oxidative compounds by the glutathione-S-transferase or as precursor of phytochelatins biosynthesis among other processes (Noctor et Foyer 1998). Consequently, changes in GR activity may not be directly linked to the ROS levels but to the consumption of GSH by these different processes. Though other enzymes (e.g. monodehydroascorbate reductase, dehydroascorbate reductase) are involved in the maintenance of the GSSG:GSH ratio (Mittler 2002), GR may be an indicator of the evolution of the GSSG:GSH ratio. In this context, an increase in GR activity may reflect an increase in

GSSG consumption while an inhibition may either correspond to a decrease in GSSG available or a decrease in GSH consumption.

Response of all antioxidant enzymes occurred in a similar time scale. Variations in CAT, APX and GR were mainly observed after acute exposure to different toxicants while chronic exposure led to slight differences between AEA of control and exposed biofilms (Chapters II to IV). Therefore, in the present study, differences in response time scales were not observed between antioxidant enzymes but response of all antioxidant enzymes occurred in a relatively short time scale (6h to 24h).

2. Natural variability and modulating factors

To estimate the quality of a biomarker and determine its possible application, it is essential to determine its specificity which, for a biomarker of perturbation, is directly linked with its natural variability in undisturbed situations (Lam 2009). The present thesis highlighted the influence of biofilm age, of light intensity and of community adaptation (to light intensity or to chronic contamination) on AEA responses.

Temporal variability of AEA in biofilms

Biofilms are dynamic communities in which biomolecular composition as well as species distribution change throughout time during community establishment (Chapter II). Variations in AEA were also observed during biofilm growth. In particular, a significant increase in APX activity throughout time characterized exponentially growing biofilms while CAT and GR activity were relatively stable over time. These results suggest that the pool of AEA dedicated to oxidative stress management may change as biofilm ages. Therefore, the activation of one or another enzyme to answer to oxidative stress may also be linked to biofilm age. For instance, exposure to a gradient of oxyfluorfen concentration led to changes in CAT activity in

73 day-old biofilms (Chapter II) and to changes in APX activity in 28 day-old biofilms (Chapter III). In the same way, Guasch et al. (2010) observed that acute copper exposure led to an increase in CAT activity in 35 day-old biofilms while in this thesis acute copper exposure led to an increase in APX activity in 28 day-old biofilms. Thus, as biofilm ages, the main enzyme responsible for hydrogen peroxide scavenging in biofilms is likely to shift from APX to CAT. Since few CAT have been described in diatoms (Branco et al. 2010; Wilhelm et al. 2006; Winkler and Stabenau, 1995), this shift could be explained by temporal changes in biofilm community composition. Moreover, as biofilm ages and gets thicker, oxygen, pH and so oxidative stress increase within biofilms, CAT may be preferentially respond to additional oxidative stress in such cases as its affinity for hydrogen peroxide is lower than APX (Mittler 2002).

Influence of light intensity

Short exposure to a light intensity different from the one throughout colonization provoked variations of CAT and APX activities (Chapters I and III). For instance, a strong increase in light intensity provoked an inhibition of CAT in biofilms (Chapter I), while an activation of APX was observed in shade-adapted biofilms exposed to a slight increase in light intensity (Chapter III). In addition, a strong decrease in light intensity also provoked a decrease in CAT activity in biofilms adapted to high light intensity (Chapter III). These results suggest that CAT and APX are involved in the photo-acclimation process in biofilms as observed for micro-algae (Janknegt et al. 2009). Therefore, AEA in the field may be subjected to daily variations following light fluctuation throughout the day. Further experiments are needed to support this hypothesis. They may be conducted in laboratory under a dynamic light regime or directly in the field.

AEA variability induced by natural or chemical factors

Interestingly, variations in AEA provoked by short-term changes in natural factors (e.g. light or growth) were of the same magnitude as AEA variations caused by toxic exposure. For instance, during exponential growth, APX activity increased by 2.4 times between day 33 and day 39 of colonization (Chapter II) while in biofilms exposed to copper or oxyfluorfen the maximum increments detected in APX activity were between 2 and 3 times control values (Chapter III). Similarly, a strong reduction in light intensity provoked a decrease by 2.5 times in CAT activity of high light-adapted biofilms (Chapter III), whereas in biofilms exposed to oxyfluorfen or propranolol the maximum increments observed in CAT activity were between 2 and 3 times control values (Chapters III and IV). In addition, in biofilms exposed to propranolol, CAT variations were within this range even when biofilms were seriously affected, as indicated by the strong photosynthetic efficiency inhibition. These observations tally with the unimodal pattern of these enzymes suggesting a limited range of variation of AEA from biofilms. In addition, based on these examples it is not possible to define a threshold value corresponding to a critical level of antioxidant enzyme activation.

Long-term adaptation influenced antioxidant enzymes plasticity rather than basal AEA

In the field study reported in this thesis, CAT activity was lower in natural biofilms from sites highly polluted by metals than in those from moderately polluted sites suggesting that AEA activities in the field could indicate disturbed situations. However long-term laboratory experiments realized in this thesis did not confirm this hypothesis for other stressors. Indeed, chronic exposure to different light intensities (Chapter III) or to oxyfluorfen (Chapter II) provoked structural changes in the community, but only slight changes in AEA between the different biofilms (Chapter II and III). Then, adaptation to these situations may involve changes in other protective mechanisms (e.g. antioxidant molecules, ROS excretion, pigments, EPS thickness;

Noctor and Foyer 1998; Choo et al. 2004) which may represent a lower cost of adaptation than changes in AEA (Mouneyrac et al. 2011). Therefore, since biofilm adaptation may result in the maintenance of a basal level of AEA, AEA response to perturbations is likely to be transitory.

Nevertheless, exposure to further stresses of adapted biofilms revealed differences in the short-term response of AEA from the different adapted communities (Chapters II and III). Biofilm adaptation did not concern basal AEA levels but rather antioxidant enzymes plasticity. Consequently, the pattern of AEA response to acute exposure to oxidative stress gradient may be a global indicator of tolerance acquisition and may allow the oxidative stress level undergone by two different communities to be compared.

3. AEA as biomarkers of oxidative stress induced by toxicants in biofilms: interest and limitations.

The present study revealed different characteristics of AEA from biofilms that may influence their use as biomarkers of oxidative stress. While CAT and APX activities followed a unimodal pattern, it has not been possible to determine a "typical" pattern of response for GR activity, probably because this enzyme is indirectly linked to ROS levels and its regulation is very complex. In addition, APX is likely to be activated in exponentially growing biofilms in response to low levels of oxidative stress, whereas CAT is likely to be activated in slow-growing biofilms in response to high levels of oxidative stress.

Time response

Variations in AEA may be observed in a short time (6h to 24h) after stress induction and may then be considered as **early warning systems**. Though variations in AEA are **transitory** and differences in AEA are unlikely to be detected after long-term exposure, comparison of fast AEA responses of different communities throughout a

gradient of oxidative stress may allow the oxidative stress levels undergone by these communities to be compared. Indeed, AEA are **integrative biomarkers** reflecting biofilm history in terms of oxidative stress.

Specificity

AEA are **not specific of a toxicant but are indicators of biofilm status**. However, **no critical threshold values** indicating irreversible oxidative damages could be defined and further studies are needed to link AEA response patterns to the survival capacity of biofilm (e.g. growth, photosynthetic efficiency, mortality).

AEA are ubiquitous in the different species of the biofilms and are thus a **global indicator of biofilm** and are **not species-specific**.

Sensitivity

The present thesis showed that **for some toxicants** (e.g. copper, oxyfluorfen), AEA are **more sensitive biomarkers** than traditional biomarkers (e.g. photosynthetic parameters, pigments), in agreement with previous findings (Dewez et al. 2005; Guasch et al. 2010).

Applications

Finally, **AEA** were found to be **valuable biomarkers of oxidative stress** in **short-term toxicity tests** to:

- **determine the peroxidizing potential of a compound**
- **compare oxidative stress levels undergone by different communities and thus estimate their oxidative stress tolerance.**

In the present study, the measure of CAT and APX activity, in particular, have provided interesting and pertinent information on chemical toxicity towards biofilm communities. Nevertheless, the method developed in this study may be used to extract and measure the activity of other antioxidant enzymes; for instance, measuring the glutathione-S-transferase (GST) activity that participates in xenobiotic detoxification (Torres et al. 2008) may be of great interest.

Contribution of AEA to a community-level multi-biomarker approach to assess chemicals toxicity

1. The meaning of AEA at community level

In a homogeneous population composed of one species, the response of each individual is expected to be similar and the measure of AEA of the population reflects what is happening for each individual. Since antioxidant enzymes are sensitive to various factors (pH, temperature, substrate concentration among others) that may affect their velocity, each enzyme has a velocity maximum for specific optimal conditions (Copeland 2000). The AEA of a cell is then a combination between the concentration of antioxidant enzyme and its velocity; both factors are influenced by environmental conditions including changes in oxidative stress levels. As oxidative stress increases, AEA are expected to increase to scavenge the reactive oxygen species (ROS) and maintain redox homoeostasis. At high level of oxidative stress, ROS are expected to denature proteins, including antioxidant enzymes. Therefore, in these conditions, a decrease in AEA is expected. Consequently, though antioxidant enzyme biosynthesis is regulated by different mechanisms, a unimodal pattern is expected for each enzyme throughout a gradient of oxidative stress.

In a 3D complex community, such as biofilms, individuals may have different micro-environments, and species may have different resistance to oxidative stress and different defence mechanisms (Dodds 1989; Choo et al. 2004), so the measure of AEA at community-level does not reflect the variation of AEA in each individual. Nevertheless, the biofilm as a whole is expected to be adapted to the prevailing level of oxidative stress so the AEA of biofilms are also expected to follow a unimodal pattern throughout an oxidative stress gradient. This assumption follows observations on photosynthesis-irradiance (P-I) curves of biofilms. Though species forming biofilms have different photosynthetic characteristics (Richardson et al. 1983), P-I curves of communities can be calculated based on the assumptions and models developed for

single species (Boston et Hill 1991). This assumption was confirmed in this thesis for CAT and APX activities. The variations of these AEA from biofilms may then result from an average between activation and inhibition of AEA in each biofilms' micro--organism. Thus, the measure of AEA at community level reflects the tendency (activation or inhibition) observed in the majority of individuals and species within the community.

To compare activity of antioxidant enzymes extracted from different samples, specific enzymatic activity is usually normalized by the amount of biomass, like protein concentration or cell density (Janknegt et al. 2007). For biofilm communities the determination of cell density would be technically difficult. In addition, it is unlikely that each cell had the same antioxidant activity, due to the high species diversity within, and to the complexity of biofilms. Therefore, normalizing the antioxidant activity by the total number of cells would be meaningless. Normalization could also be done by surface or weight instead. AEA by surface or by biomass correspond to the antioxidant activity of the whole biofilm including extracellular matrix, dead cells and possible sediments stuck in the biofilm. Although this measure allowed the total antioxidant activity of one sample to be measured, some results may be misleading due to the difficult distinction between active and inactive biomass fractions. For instance, a toxicant may provoke cell death within biofilms without provoking oxidative stress. However, dead cells do not immediately disappear from the biofilm, so a decrease in AEA by biomass or surface would be observed as a result of the decrease in the proportion of active biomass (ratio of live/dead cells).

The normalization by protein concentration may partially overcome this limitation. Enzymatic activity per protein can be understood as the part of resources the biofilm community dedicates to such antioxidant defence. Assuming that an increase in AEA is mostly due to an increase in enzyme concentration, AEA normalized by protein may reflect the proportion of active antioxidant enzymes within the total number of proteins synthesized by the biofilm. Although normalization by protein allows the ratio of active antioxidant enzymes within all proteins to be estimated, it

does not allow variations in antioxidant enzyme concentrations to be distinguished from those in other protein concentrations. Indeed, toxicant exposure may affect the biosynthesis of other proteins. Therefore, variations in AEA normalized by protein may be due to an increase or decrease in other protein concentrations and not to a direct increase or decrease in antioxidant enzymes. For instance, exposure to metals have been found to enhance metallothionein synthesis (Robinson 1989), a decrease in AEA by protein observed in such a case might be an artefact due to the increase in metallothionein concentrations and not a real decrease in the antioxidant capacity of biofilms. Moreover the normalization by protein is dependant on the extraction technique. For instance, ultrasonication is expected to completely disrupt cells and allow the release of cell walls and membrane-bound proteins in addition to other proteins that can be released by other extraction techniques, such as trituration (Cumming and Iceton 2001). As catalase is not a membrane-bound enzyme, the use of ultrasonication may lead to a lower ratio catalase/total of proteins and so to a lower catalase activity normalized per protein than the use of trituration (Chapter I).

In conclusion, the normalization of AEA by surface, biomass or by protein had different limitations that should be taken into account when interpreting the results. The normalization of AEA by surface or biomass may estimate the total antioxidant capacity of biofilms and may be related to the concentration of active antioxidant enzymes per cm^2 or g of biofilm. The normalization of AEA by protein may reflect the proportion of active antioxidant enzymes within the proteins synthesized by biofilms. Furthermore, the protein concentration is a valuable additional information to understand variations in AEA normalized by proteins (Chapter III).

2. Contribution of AEA to a set of biomarkers to assess toxic effects of chemicals within biofilms

The classical biomarkers already used in biofilms are related with their structure and main functions (e.g. diatom composition, photosynthesis) however the good functioning of different molecular processes is essential to guarantee the integrity of these functions. Among these processes, equilibrating redox levels is essential for all aerobic organisms to use oxygen while limiting the hazards of oxygen reduction (Edreva et al. 2004). Since photosynthesis generates inevitably ROS, oxidative stress management is even more important for autotrophic organisms. The few biomarkers related with this process in biofilms are antioxidant pigments such as the diadinoxanthin and diatoxanthin of the xantophyll cycle (Laviale et al. 2010). The activation of the xantophyll cycle in biofilms is tightly linked to diatoms photosynthesis as it allows them dissipating the excess of energy due to light increase. Indeed, most of these pigments are related with the oxidative stress generated by the photosynthesis and thus may only account for oxidative damage affecting the photosynthetic apparatus in autotrophic organisms. Antioxidant enzymes can scavenge ROS, independently of their origin, in all the cells of both the autotrophic and heterotrophic component of the biofilm. AEA changes indicate a perturbation of the redox balance and are biomarkers of oxidative stress in biofilms. AEA are not then specific of a type of oxidative stress but rather indicate the exposure to a compound perturbating the redox balance. In the present study, CAT, APX and GR variations could indicate oxidative stress generated by various toxicants such as oxyfluorfen, copper or propranolol. Since in some cases, AEA were found to be more sensitive biomarkers than photosynthetic parameters, pigments or chlorophyll-*a,* they may allow exposure to toxicant to be detected before the toxic effects reached essential biofilm processes such as photosynthesis. Nevertheless, changes in AEA can not be used to predict damages at higher levels of organization. While an increase in AEA reflects that biofilm is coping with ROS excess, it is not possible to know the outcome of this process on biofilm fitness. Indeed, lots of factors can influence the success of ROS

elimination and this limited extrapolation capacity is a common limitation of molecular biomarkers (Forbes et al. 2006).

Consequently, AEA can mainly be used to elucidate the peroxidizing potential of a chemical and indicate whether biofilm response is active (AEA activation) or passive (AEA inhibition). The complementary use of other biomarkers is essential to link this sub-lethal molecular effects with effects at higher levels of biological organization.

In this context, AEA may be especially useful to complete a biomarkers tool--box for assessing toxicity of emerging compounds with unknown modes-of-action on biofilms. Moreover, measuring patterns of AEA response to an acute gradient of oxidative stress is an interesting tool to compare the capacity to respond to oxidative stress of different biofilm communities. These tests could be used to assess community adaptation in microcosms but also in the field. On Chapter I, a decrease in CAT activity of field biofilms was linked with metal pollution whereas microcosm experiments showed that communities adapted to organic pollution or different light intensity had similar basal AEA. Further investigation is then required to demonstrate the interest of monitoring AEA of natural biofilms to indicate hot-spots of contamination.

The advantages of a multi-biomarker approach at community-level in toxicity assessment

The functioning of aquatic ecosystems is based on tight interactions within and between the different compartments of a river and previous studies showed the high interest of using aquatic communities to assess chemicals toxicity, highlighting in particular the higher ecological relevance of community tests than of single-species tests (Cairns 1983; Clements and Newman 2002). The present study brings additional evidence on the interest of community level investigations to assess chemicals toxicity. In particular, the different experiments showed the importance of the selection of pertinent biomarkers to detect both direct and indirect effects of toxicants and to better assess toxicity of emerging compounds. Moreover, the true replication and high experimental control obtained in community microcosm experiments allowed isolating the effects of one or two factors while preserving a pertinent degree of complexity. With this approach, the impact of multiple stress situations can be understood with a better ecological relevance.

The herbicide oxyfluorfen was expected to be mainly toxic to algae by its mode of action (chlorophyll-*a* biosynthesis inhibitor). However, long-term exposure to oxyfluorfen provoked a decrease in bacterial richness (Chapter II). As previous studies on oxyfluorfen toxicity towards soil bacteria had suggested a positive effect of this herbicide on microbial activities (Das et al. 2003), the negative effect observed in our study is likely to be linked to a strong algal-bacterial relationship in biofilms. In agreement with this idea, other studies highlighted the importance of indirect effects of toxicants in biofilm communities. For instance, the photosynthesis inhibitor, diuron (herbicide), was found to indirectly affect the bacterial component of biofilms (Ricart et al. 2009), while the bactericide triclosan had a negative indirect effect (photosynthesis inhibition) on the algal component of the community (Ricart et al. 2010). Based on these examples and our results, assessing toxicity at community level

seems essential to gain a better overview of the risks of chemicals towards the aquatic ecosystem. Therefore, the use of higher organization systems to assess both direct and indirect effects of pollutants is strongly recommended in agreement with other authors (Fleeger et al. 2003; Clements and Newman 2002; Rohr et al. 2006).

To reduce the complexity of ecotoxicology, the contaminants are usually grouped in "guilds" with similar modes of action. This grouping is based on the assumption, often validated, that contaminants with similar modes of action are expected to have similar effects of the same magnitude on one species (Rohr et al. 2006). However, the result of the present study suggests that grouping chemicals by their mode of action in target species may be inappropriate to assess their effects on non-target species. In the present study, the use of a community-level, multi-biomarker approach to assess the effects of three β-blockers on biofilms revealed differences in the toxic effects of these emerging contaminants. Propranolol was found to be toxic for the algal compartment while metoprolol was found to be more toxic for the bacterial compartment of biofilms (Chapter IV). Indeed, none of the micro-organisms within biofilms had β-adrenergic receptors and, therefore, other chemical characteristics may be taken into account (e.g. the octanol-water partition coefficient: log K_{ow}) to explain the differences in effects observed. Hence, other classifications are needed to assess effects of chemicals and in particular of emerging contaminants towards non-target species. The classification by quantitative structure-activity relationships (QSAR) focusing on the physico-chemical properties of a chemical and the toxicity data available for it, may be of special interest in this context (Bradbury 1995; Colombo et al. 2008).

Aquatic environments undergo physical and chemical stresses due to physical modifications of the environment (dams, channels, etc.) and the entrance of pollutant mixtures, therefore each field situation is virtually a multiple stress scenario in which the different environmental factors exert a selective pressure on the community. Anthropogenic disturbances may be found among these environmental factors and thus influence the community development and function. Since single-species have a

limited plasticity and so a narrower range of adaptation than communities, single--species tests may then overestimate the effects of multiple stress situations on aquatic ecosystems. Then, the pertinence of using single-species tests to account for multiple stresses is questioned. To overcome these limitations, the use of communities may allow a wider range of environmental conditions to be tested. In the present study, the influence of light history on herbicide toxicity was demonstrated for biofilms (Chapter III). Other studies have also pointed out that environmental conditions are susceptible to affect community sensitivity to various toxicants (Guasch and Sabater 1998; Laviale et al. 2010). Moreover efficient microcosm systems allow maintaining aquatic communities under controlled conditions in order to investigate the mechanisms and the consequences of adaptation. In the present thesis, long-term exposure to different levels of light intensities or to different oxyfluorfen concentrations revealed a successful adaptation of biofilms to their environmental conditions (Chapters II and III). Though the functional redundancy characteristic of community allowed an adaptation of the community to these situations, changes in biofilm structure (Chapter II) and AEA response to a gradient of oxidative stress (Chapters II and III) were observed. These changes may influence positively or negatively the response of communities to further stresses and thus may be of importance in the most common scenario of co-occurring, multiple types of stress. These results illustrate the potential of a community approach to assess the effects of multiple stress factors.

To understand the direct and indirect effects of multiple stressors on biofilm communities, it is essential to be able to measure a high number of biomarkers. The methodological development presented in this thesis showed the feasibility of a biofilm functional gene array (FGA) dedicated to the measure of expression of 83 genes from the autotrophic component of biofilms. Though further investigation is needed to improve this FGA, it is proposed as a powerful prognostic tool to assess sub-lethal effects of toxicants on biofilms and to indicate which biofilm functions may be at risk.

Future needs and perspectives

AEA

Though a unimodal pattern could be identified for CAT and APX, no model could be fitted to the data. The realisation of dose-response curves including more concentrations would allow fitting bell-shaped dose-response curve to the data, for instance by combining two sigmoid equations (Motulsky and Christopoulos 2004). This modelling can then help in determining toxicity thresholds (concentrations leading to 50% of activation/inhibition, range of concentration for which the activity is stimulated). In parallel, further investigation is required to better understand the consequences of AEA variations on biofilms main processes.

To complete this approach, the influence of environmental factors likely to modulate antioxidant capacity could be investigated. Among them, drought and salinity are likely to be involved in multiple stress scenarios in the context of climate change and the evaluation of the influence of these parameters on the antioxidant capacity of biofilms may be of great interest.

In addition, further investigations should focus on other antioxidant enzymes. Since glutathione-S-transferase is expected to play an important role in xenobiotic detoxification, the measurement of its activity may then contribute to the understanding of community response to toxicants (Torres et al. 2008).

Most of the experiments of the present thesis were conducted in microcosms, highlighting the interest of such systems to assess chemical toxicity. Nevertheless, the measure of antioxidant activities in field biofilms may provide an insight into their variations *in situ*. While the AEA of biofilms adapted to different situations were similar in microcosms, measuring AEA from different sites may allow this hypothesis to be tested under natural conditions. In addition, bio-assays performed in the laboratory with different natural communities may allow comparing their antioxidant potential by comparing their AEA pattern throughout a gradient of oxidative stress.

Molecular biomarkers

Molecular biomarkers reflect sub-lethal effects of toxicants on biofilms and can then give clue on the potential effects at higher levels of organization. However they have been poorly used in routine monitoring or ecological risk assessment because of the difficulty in providing unambiguous and ecologically relevant indicators of toxicity (Forbes et al. 2006). Some studies highlighted the possibility to link molecular biomarkers variation with population or community fitness, in particular in macroinvertebrates. In river biofilms further investigation is still needed to link variations in molecular biomarkers such as AEA with biofilm main function (photosynthetic capacity, growth, etc.). In this context, transcriptomics may be especially useful as it provides a snapshot of biological complexity (expression of numerous genes) that can be compared with effects at higher level of biological organization. This approach may help in understanding how toxic effects scale-up from molecular reactions to community function.

Finally, this thesis illustrated how molecular biomarkers can be developed and used at community level providing pertinent information on biofilm status. Future developments of molecular biomarkers at community level may also help to understand underlying toxic mechanisms; and these developments might focus on proteomics or metabolomics (Sans-Piché et al. 2010), for example.

General

Conclusions

Methodological developments

1. Homogenization followed by glass bead disruption was the most appropriate method to extract catalase from biofilms after comparison with three commonly used methods (trituration, ultrasonication and homogenization). In addition, this method allowed catalase extraction from a small amount of biofilms (obtained from 2 to 4 cm^2 of colonized substrata) and was successfully applied for the extraction of ascorbate peroxidase, superoxide dismutase and glutathione reductase from fluvial biofilms.

2. The reproducibility of the measurement of catalase activity ranged between 20 and 30 %, indicating a good precision for this biomarker in biofilms.

3. The use of recirculating channels as biofilm microcosms ensured a good replicability within communities.

4. The use of consensus sequences from up to 6 microalgal species (diatoms and chlorophytes) to build a functional gene array (FGA) for biofilms was validated and a hybridisation temperature of 55ºC was found to be the best compromise between cross-hybridisation and specificity for the biofilm FGA.

5. To extract high quality RNA from biofilms, RNA precipitation with a saline solution of PEG is recommended.

Characteristics of biofilm antioxidant enzymes

Patterns of variation

6. The patterns of variation of CAT and APX activities throughout oxidative stress induced by chemicals described a partial or a complete unimodal pattern.

Response to oxidative stress induced by toxicants

7. In three polluted sites of the Riou Mort, catalase activity in river biofilms was found to be negatively correlated with the cumulative criterion units (CCU) for metals and with Ni, Zn and Cd concentrations in water.

8. Acute and chronic exposure to oxyfluorfen provoked an activation of ascorbate peroxidase and/or catalase activities. During acute and chronic exposure to oxyfluorfen, biofilms exposed to low concentrations (3 to 15 µg L^{-1}) were mainly characterized by a higher ascorbate peroxidase activity and those exposed to higher concentrations (30 to 150 µg L^{-1}), by a higher catalase activity.

9. Copper exposure provoked a decrease in protein concentration of biofilm communities regardless of their light history and an increase in ascorbate peroxidase activity of shade-adapted communities.

10. Exposure to β-blockers provoked only slight effects on catalase activity. Exposure to propranolol was principally linked with an increase in catalase activity, while a decrease was observed in biofilms exposed to high concentrations of atenolol.

Influence of confounding factors

11. Both biofilm age and light intensity provoked changes in antioxidant enzyme activities.

12. Catalase, ascorbate peroxidase and glutathione reductase activities were not stable throughout biofilm colonization. Ascorbate peroxidase played an important role at the end of the exponential growth phase while catalase and glutathione reductase were more important during the slow-growth phase. These temporal variations may be linked with biofilm ageing.

13. In response to oxidative stress induced by oxyfluorfen, catalase activity increased in old biofilms (73 days) while ascorbate peroxidase was activated in younger biofilms (28 days)

14. Biofilm acclimation to short changes in light intensity was supported by variations in catalase and ascorbate peroxidase activities. Short-term exposure to high light intensity provoked photoinhibition and an activation of ascorbate peroxidase activity in biofilm, while exposure to low light intensity was characterized by a slight increase in photosynthetic efficiency and a decrease in catalase activity.

15. The adaptation of biofilms to colonization conditions (different light intensities, chronic contamination by oxyfluorfen) was characterized by changes in biofilm structure and function. These changes modulated biofilm capacity to answer to further stress in some cases.

16. The antioxidant enzyme activities of catalase, ascorbate peroxidase, glutathione reductase, or superoxide dismutase were not found to play a role in the adaptation to different colonization light intensities.

17. Copper exposure provoked an increase in ascorbate peroxidase activity of shade-adapted communities.

18 Chronic exposure to oxyfluorfen resulted in an enhanced resistance to oxidative stress in biofilms exposed to 75 and 150 µg L^{-1}. These changes were linked with structural changes in eukaryal communities chronically exposed to different concentrations of oxyfluorfen and with a reduction of both bacterial and eukaryal richness in communities exposed to 150 µg L^{-1}.

The multi-biomarker approach

19. A multi-biomarker approach at community level showed that the effects on biofilms of three pollutants from the same class of compounds (β-blockers) were different.

20. Propranolol was the most toxic of the three β-blockers tested, affecting mainly the algal compartment of biofilm. A 24h-exposure to 531 µg L^{-1} caused 85% of inhibition of photosynthetic efficiency.

21. Metoprolol was mainly toxic for bacteria with NEC values in the ng L^{-1} range for bacterial mortality. A 24h-exposure to 503 µg L^{-1} increased bacterial mortality by 50%.

22. Atenolol toxicity was low and effects superior to 50% were only observed after 24h-exposure to 707 mg L^{-1}.

23. The higher toxicity of propranolol and metoprolol may be due to a better absorption within biofilms.

24. The feasibility of a FGA for biofilms was demonstrated and would improve a multi-biomarker approach by enhancing the number of biomarkers measurable in ecotoxicological tests. Though more genetic information is needed to improve the biofilm FGA, first applications should focus on the difference in gene expression between similar communities exposed to stressors.

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