Effets de mélanges de pesticides sur les biofilms périphytiques d'eau douce

S. Kim Tiam

Thèse

Présentée pour l'obtention du grade de

Docteur de l'Université de Bordeaux

Ecole doctorale : « Sciences et Environnements»

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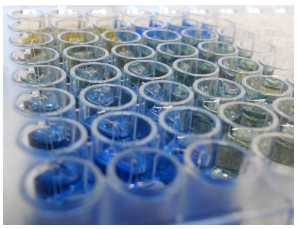
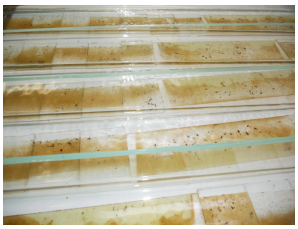


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Résumé

On considère généralement les biofilms comme des indicateurs biologiques d'alerte, les organismes les composant ayant des temps de génération relativement courts et présentant une grande diversité de *preferenda* environnementaux et de sensibilité aux altérations anthropiques. Dans ces travaux, les effets de pesticides sur les biofilms de rivière ont été étudiés à différentes échelles de représentativité, allant de mélanges complexes à faible dose en utilisant des extraits d'échantillonneur passif POCIS (Polar Organic Chemical Integrative Sampler) à des molécules testées seules, en passant par des mélanges simples.

L'exposition chronique et à faible dose aux extraits de POCIS a révélé des impacts au niveau de la croissance, de la structure (assemblages de diatomées) et du fonctionnement du biofilm en lien avec son exposition passée. De plus les expériences utilisant des molécules testées seules (pesticides et métabolites) et les mélanges simples ont permis de caractériser la toxicité relative des composés présents dans les extraits de POCIS en lien avec leur mode d'action et d'explorer la réponse de descripteurs encore peu utilisés en écotoxicologie comme la construction de Rapid Light Curves (RLCs).

Ce travail confirme la pertinence de l'utilisation des extraits d'échantillonneurs passifs comme le POCIS pour mieux appréhender les effets des pesticides en mélanges sur le biofilm de rivière ainsi que l'intérêt des RLCs en tant que descripteur précoce d'exposition aux pesticides.

Abstract

Biofilms can be regarded as biological warning systems, because they are generally composed of short generation time organisms presenting a large range of environmental *preferenda* and various sensibilities to anthropogenic disturbance. In the present study, effects of pesticides on river biofilms have been studied at different levels of representativeness, from complex mixtures at low dose represented by extracts of the passive sampler POCIS (Polar Organic Chemical Integrative Sampler) to molecules tested alone, via simple mixtures.

Chronic exposure to low dose of POCIS extracts revealed impacts on growth related, structural (diatom assemblages) and functional parameters related to biofilm exposure history. Moreover experiments using single molecules and simple mixtures allowed to characterised the relative toxicity of compounds present in the POCIS extracts in link with their specific mode of action and explore the response of descriptors rarely used in ecotoxicology field like the construction of Rapid Light Curves (RLCs).

This work confirms the relevance of the use of passive sampler extracts as POCIS in order to better understand the effects of pesticides in mixture on river biofilms as well as the interest of RLCs as early descriptors of pesticides exposure.

SOMMAIRE

Abréviations

2,4-D	2,4-dichloro phénoxyacétique
ACP	analyses en composantes principales
AEAs	activités enzymatiques antioxydantes
AFDM	ash-free dry weight
AFNOR	association française de normalisation
ANOVA	analysis of variance
APX	ascorbate péroxydase
CAT	catalase
CE_X	concentration inhibant X% du paramètre étudié par rapport aux témoins
Chl *a*	Chlorophylle a
C_{POCIS}	concentration dans le POCIS
C_w	concentration dans l'eau
DCE	Directive Cadre sur l'Eau
DCPMU	dichlorophenyl-methylurea
DEA	atrazine-deséthyl
DET	terbuthylazine-deséthyl
DGT	Diffusive Gradient in Thin-Films
DIA	désisopropyl-atrazine
DIA-d5	désisopropyl-atrazine deutérée
DMN	desmethyl norflurazon
DW	dry weight
EDA	effect directed analyses
EPS	extracellular polymeric substance
ERA	Environmental Risk Assessment
EROs	espèces réactives de l'oxygène
ESA	ethanesulfonic acid
ESI	électrospray ionisation
ETR	electron transport rate
F_0	émission de fluorescence de base
$F_{0'}$	émission de fluorescence juste avant l'application du flash lumineux
F_m	fluorescence maximale
$F_{m'}$	émission de fluorescence pendant l'application du flash lumineux
Fs_{665}	fluorescence de chlorophylle à mesurée à 665nm
Fs_{Bl}	signal de fluorescence lié au groupe des cyanobactéries
Fs_{Br}	signal de fluorescence lié au groupe des diatomées
Fs_{Gr}	signal de fluorescence lié au groupe des algues vertes
F_v/F_m	rendement quantique maximal de la photochimie du Photosystème II
GC	chromatographie en phase gazeuse
GF/C	glass fiber filter
GR	glutathion réductase
GST	glutathion-S-transférase
HAP	hydrocarbures aromatiques polycycliques
HPLC	chromatographie liquide à haute performance
HSD	honestly significant difference
I	valeur de l'irradiance incidente
IBD	indice biologique diatomées
I_k	intensité lumineuse à partir de laquelle la relation n'est plus linéaire dans les courbes RLC
IPS	Indice de polluo-sensibilité

Irstea	Institut national de Recherche en Sciences et Technologies pour l'Agriculture et l'Environnement
K_{ow}	coefficient de partage octanol-eau
K_{sw}	coefficient de partage eau-échantillonneur
k_u	constante de la pente d'accumulation
LA	lumière actinique
LC	chromatographie en phase liquide
LC_{50}	concentration nécessaire pour induire 50% de mortalité
LDPE	Low-Density Polyethylene
LOEC	plus faible concentration provoquant un effet mesurable
LSS	flash de lumière sursaturante
MESCO	Membrane-Enclosed Silicone Collector
ML	lumière modulée
MOD	matière organique dissoute
M_{POCIS}	masse de l'adsorbant
MS/MS	spectrométrie de masse en tandem
MSSC	matière sèche sans cendre
NFZ	norflurazon
NOEC	concentration ne provoquant pas d'effet observé
N_{POCIS}	masse d'analyte dans le POCIS
NQE	normes de qualité environnementale
OA	oxanilic acid
P680	cœur du centre réactionnel constitué par des chlorophylles a spéciales
PAM	pulse amplitude modulation
PAR	photosynthetically active radiation
PAT	Programme d'Action Territoriale
PCB	polychlorobiphényles
PE	POCIS extracts
PEC	Predicted Environmental Concentration
PICT	acquisition de tolérance induite
PNEC	Predicted No Effect Concentration
POCIS	Polar Organic Chemical Integrative Sampler
PoToMAC	Potentiel Toxique dans les Milieux Aquatiques Continentaux
PPCP	produit pharmaceutique et de soin personnel
PRC	composés de référence et de performance
PS	poids sec
PSI	photosystème I
PSII	Photosystème II
Q_A	quinone fortement liée au cœur du Photosystème II
Q_B	plastoquinone
rETR	vitesse relative de transfert des électrons au niveau du Photosystème II
$rETR_{max}$	valeur maximale du rETR
RLC	rapid light curve
R_s	taux d'échantillonnage
SAU	Surface Agricole Utile
SPE	extraction sur phase solide
SPMD	Semi-Permeable Membrane Devices
SR	Silicone Rubber
SSD	modèle de distribution de sensibilité des espèces
t	temps d'exposition

$t_{1/2}$	limite de linéarité
TBZ	tébuconazole
TWA	time-weighted average
UR MALY	unité de recherche Milieu Aquatique écologie et pollution de Lyon
UR REBX	unité de recherche Réseaux Épuration et qualité des eaux de Bordeaux
US EPA	agence américaine de protection de l'environnement
YES	Yeast Estrogen Screen
ZABR	Zone Atelier du Bassin du Rhône
α	pente initiale de la courbe RLC
Φ_{PSII}	rendement quantique efficace de la photochimie du Photosystème II

Chapitre I : Introduction

La première trace de l'utilisation de pesticides date de la Grèce antique, avec notamment l'utilisation du soufre comme agent de fumigation. Les premiers pesticides de synthèse quant à eux ont fait leur apparition dans les années 1930, avec le développement d'une part de la chimie organique et d'autre part de la recherche sur les armes chimiques au cours de la Première Guerre mondiale. En parallèle les pratiques agricoles se transforment à partir du milieu du XIX^{ème} siècle, avec l'émergence de la mécanisation et de la protection chimique pour améliorer la productivité des cultures. Dans un premier temps, on assiste au développement de l'utilisation de composés inorganiques comme le soufre ou le sulfate de cuivre, puis les composés organiques de synthèse émergent avec la commercialisation du DDT (insecticide) en 1943 ou du 2,4-D (herbicide) en 1946. De nos jours l'utilisation de pesticides à grande échelle est devenue la norme dans l'agriculture.

Cependant cette utilisation massive de produits phytosanitaires entraine une contamination de l'environnement et en particulier du compartiment aquatique. Les pesticides et leurs produits de dégradation peuvent alors y atteindre des concentrations susceptibles de provoquer des effets toxiques chez les organismes y étant exposés et une dégradation de la qualité de la ressource en eau.

Cette thèse s'inscrit en grande partie dans le cadre du programme ANR PoToMAC (Potentiel Toxique dans les Milieux Aquatiques Continentaux: échantillonnage passif des pesticides et relations exposition/impacts sur les biofilms, 2012-2015) et plus précisément dans la tâche 4 du projet : Apport des techniques d'échantillonnage passif à l'étude du potentiel toxique des pesticides sur des communautés périphytiques en laboratoire.
L'objectif de cette thèse était donc d'évaluer les effets de pesticides en mélange à faible dose sur les biofilms de rivière, en particulier en utilisant l'échantillonneur passif POCIS (Polar Organic Chemical Integrative Sampler) et ainsi pouvoir proposer des pistes pour le développement d'indicateurs permettant de mettre en évidence les pollutions de type toxique.

L'évaluation du risque environnemental est basée sur la détermination de la toxicité (généralement aiguë) de substances seules sur des organismes modèles. Cette démarche est cependant très éloignée des conditions pouvant être rencontrées par les organismes dans l'environnement puisque les espèces y vivent en interaction les unes avec les autres et que les molécules y sont présents sous forme de cocktail. Les impacts de mélanges complexes de

molécules peuvent être étudiés via l'utilisation d'extraits d'échantillonneurs passifs, cette démarche a l'avantage de prendre en compte les problématiques des effets de mélange et des produits de dégradation. La grande majorité des travaux disponibles dans la littérature utilisant cette approche sont réalisés sous forme de tests de toxicité aiguë. En revanche très peu d'études se sont penchées sur les effets de ces extraits d'échantillonneurs passifs à long terme et à faible dose, qui pourtant permettraient d'appréhender les effets chroniques de mélange de contaminants.

Lors de cette thèse, l'outil POCIS a été utilisé dans un premier temps sur la rivière Morcille dont le profil de contamination est bien connu et ce grâce à plusieurs projets menés sur le bassin versant et impliquant entre autres Irstea (programmes de recherche Cemagref « PestExpo » et Onema-Cemagref action 26 « Remédiation de l'effet de pesticides » ayant précédé l'ANR PoToMAC). Lors de cette étude les méthodes classiquement utilisées dans notre laboratoire ont été appliquées pour l'évaluation des effets des extraits de POCIS sur le biofilm. Après avoir considéré les extraits de POCIS comme une « boîte noire » dans un contexte de contamination bien connu, nous nous sommes intéressés aux composés échantillonnés par l'outil et plus précisément à la toxicité relative des polluants ; en particulier en ce qui concerne les métabolites et composés parents. Pour finir nous nous sommes interrogés sur la représentativité de la fraction échantillonnée par le POCIS, et ce dans le cadre d'un bassin versant encore peu étudié à ce jour (le bassin versant du Trec-Canaule) ce qui en parallèle permettra de tester la validité du POCIS pour différents contextes de contamination.
Tout au long de cette thèse nous avons travaillé avec des paramètres classiquement utilisés pour évaluer les effets de toxiques (pesticides organiques, métaux, produits pharmaceutiques) sur du biofilm (densités de diatomées, analyses taxonomiques, mesures de fluorescence, biomasse, activités enzymatiques antioxydantes), nous avons également évalué l'intérêt de descripteurs peu utilisés à ce jour dans le cadre de l'écotoxicologie (RLCs : Rapid Light Curves) et enfin nous avons cherché à développer des outils adaptés au modèle biologique diatomée (biologie moléculaire).

Cette thèse est donc organisée en 8 chapitres, dont fait partie cette introduction (chapitre I). Le chapitre suivant (chapitre II) présente des éléments de contexte. Les sources, la présence dans l'environnement et les techniques d'échantillonnage des pesticides seront abordées, suivis de la démarche actuelle d'évaluation du risque environnemental, des études

écotoxicologiques et de l'état des connaissances sur les effets de pesticides sur le biofilm de rivière ainsi que sur le couplage des tests biologiques avec les échantillonneurs passifs. Le chapitre III expose les matériels et méthodes utilisés tout au long de la thèse. Les chapitres suivants développent les résultats obtenus au travers des différentes expériences visant à caractériser les effets d'un mélange « réaliste » de pesticides sur des biofilms de rivière aux histoires d'exposition différentes (chapitre IV), à comparer les impacts aigus de pesticides et d'un produit de dégradation utilisés seuls et combinés (chapitre V) et évaluer les effets à long terme de pesticides et d'un produit de dégradation (chapitre VI) et enfin à s'interroger sur la représentativité de la fraction échantillonnée par le POCIS en comparant les effets d'une exposition chronique à un extrait d'échantillonneur passif et d'un mélange reconstitué sur du biofilm de rivière (chapitre VII). Le chapitre VIII a pour but de dresser un bilan général de ces différents résultats et de proposer des pistes de recherche pour le développement ultérieur d'outils dans le cadre de l'évaluation de la qualité de l'eau face à des pollutions toxiques complexes. Les annexes présentent les travaux relatifs au développement des outils de biologie moléculaire adaptés aux diatomées.

Chapitre II : Généralités

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2.1 L'eau : une ressource à protéger

L'eau recouvre 72% de la surface de la Terre, mais seulement 2,5% de l'ensemble de la ressource serait de l'eau douce (Rodda et Shiklomanov 2003). Le développement démographique, économique et social des populations humaines et de leurs activités exerce des pressions sur les ressources en eau. L'agriculture est de loin le plus grand consommateur d'eau douce, environ 70% de l'ensemble des prélèvements d'eau douce sont destinés à l'irrigation pour l'agriculture. Cette demande grandissante pour les ressources en eau représente un challenge, non seulement du point de vue quantitatif mais aussi qualitatif.
Le milieu aquatique est susceptible d'être affecté par un éventail très large de pollutions, qu'elles soient de nature physique, chimique ou biologique (Tableau 1).

Type de pollution	Nature	Origines
Physique	Rejets d'eau chaude	Centrales thermiques, nucléaires
	MES	Rejets urbains, érosion des sols
Chimique	Matière organique	Effluents domestiques, agricoles, agroalimentaires
	Fertilisants (nitrate, phosphate)	Agriculture, lessives
	Métaux	Industries, agriculture, déchets
	Pesticides (herbicides, fongicides, insecticides…)	Industries, agriculture
	Organochlorés (PCB, solvants)	Industries
	Composés organiques de synthèse	Industries
	Détergents	Effluents domestiques
	Hydrocarbures	Industrie pétrolière, transports
Biologique	Bactéries, virus, champignons…	Effluents urbains, agricoles

Tableau 1: Origine et nature des différents types de pollution (d'après Chouteau 2004)

Dans le cadre de la mise en place de la Directive Cadre sur l'Eau (DCE), un premier état des lieux a été réalisé par les agences de l'Eau. L'état d'une masse d'eau de surface s'apprécie sur deux aspects :

- état (ou potentiel) écologique : sur 6 classes, de « très bon » à « mauvais » ou « indéterminé » ;
- état chimique : sur 3 classes, « bon », « mauvais » ou « indéterminé ».

Le bon état écologique prend en compte la qualité de l'ensemble des compartiments écologiques : eau, faune, flore, habitat. Pour les masses d'eau artificielles ou fortement modifiées, on parle de bon potentiel. Les valeurs de référence sont dans ce cas moins exigeantes. L'état chimique est quant à lui évalué en fonction des concentrations mesurées pour 41 substances identifiées qui ne doivent pas dépasser les normes en vigueur dites « normes de qualité environnementales ».

L'état des lieux de 2009 indique que 41,5% des masses d'eau de surface sont en bon ou très bon état (ou potentiel) écologique et que 43,2% des masses d'eau de surface atteignent le bon état chimique.

Sur les 41 substances faisant l'objet d'une norme de qualité environnementale, 18 sont des produits phytosanitaires.

2.2 Les pesticides

2.2.1 Familles et propriétés

Il existe deux grandes classifications des pesticides organiques de synthèse ; ces composés peuvent être regroupés par famille chimique ou par cible. La classification en famille rassemble des produits ayant des groupements fonctionnels identiques. On distingue ainsi parmi les grandes familles chimiques les organophosphorés, les organochlorés, les carbamates et thiocarbamates et les pyréthroïdes de synthèse.

Une classification peut également être établie par cible. Les pesticides se répartissent alors en trois catégories principales : les herbicides, les fongicides et les insecticides qui s'attaquent respectivement aux « mauvaises herbes », aux champignons et aux insectes. Nous pouvons également noter l'existence d'autres catégories comme les rodonticides (rongeurs), les nématicides (nématodes), les molluscicides (limaces, escargots) ou les corvicides (corbeaux).

2.2.2 Statistiques des usages

Malgré la baisse constante du tonnage des substances actives vendues entre 1999 et 2011, la France reste le premier consommateur de pesticides au niveau européen avec plus de 62 000 tonnes de substances actives vendues en 2011 (UIPP, 2012). La grande majorité des substances actives vendues en France sont des produits de synthèse avec 48 800 tonnes en 2011 contre 13 900 pour les produits inorganiques (cuivre et soufre) (Figure 1).

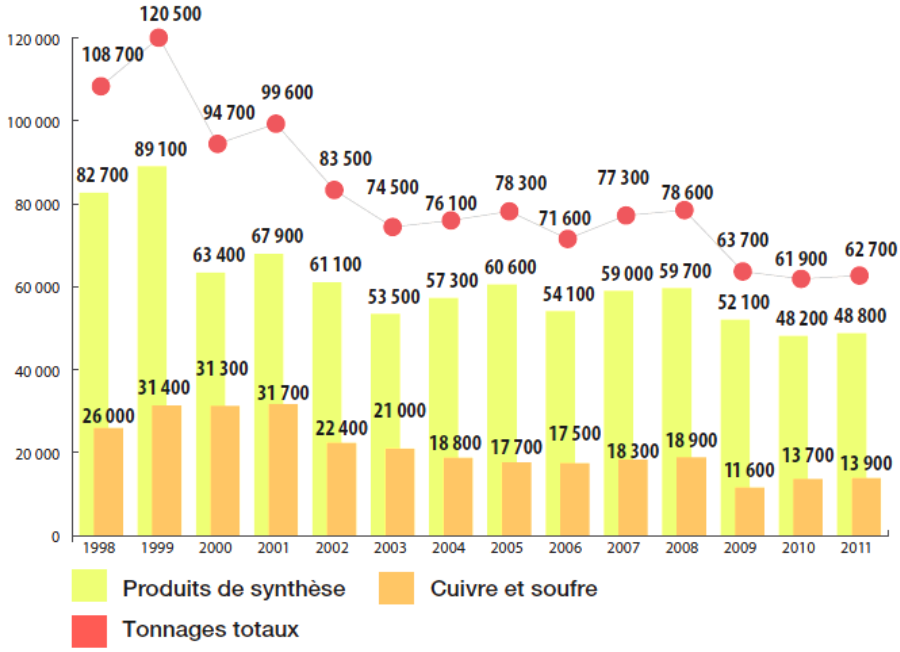

Figure 1: Tonnages des substances actives vendues de 1998 à 2011 (en tonnes) (d'après le rapport d'activité 2011-2012 de l'union des industries de la protection des plantes, UIPP 2012)

Le chiffre d'affaires du secteur des produits phytosanitaires à travers le monde représentait en 2011 un total de plus de 44 milliards de dollars. L'Europe arrive en tête avec 27,7 % de ce chiffre d'affaires suivie par l'Asie (26,4 %), l'Amérique latine (22,9 %), l'Amérique du Nord (19,1 %) et l'Afrique (4,0 %).

2.2.3 Présence dans l'environnement

D'après le rapport 2013 du service de l'observation et des statistiques (SOeS 2013), des pesticides ont été décelés sur plus de 90 % des points de mesure du réseau hydrographique français en 2011. Une majorité de points présente des concentrations moyennes annuelles en pesticides inférieures à 0,5 µg/l (seuil pour la somme des molécules phytosanitaires lors d'un prélèvement à ne pas dépasser pour de l'eau potable, Décret n°2001-1220 2001). Les points au-delà de ce seuil se situent dans les régions céréalières, de maïsiculture ou de viticulture, notamment dans le bassin parisien, en Adour-Garonne et le long du Rhône, ou à tradition maraîchère, comme en Martinique et Guadeloupe. Les fortes valeurs relevées sur ces deux îles sont surtout dues à la présence de chlordécone.

Dix-sept points présentent une moyenne annuelle supérieure à 5 µg/l. La plupart de ces points se situent dans les zones de grande culture du nord de la France, du bassin parisien et du Sud-Ouest (Figure 2).

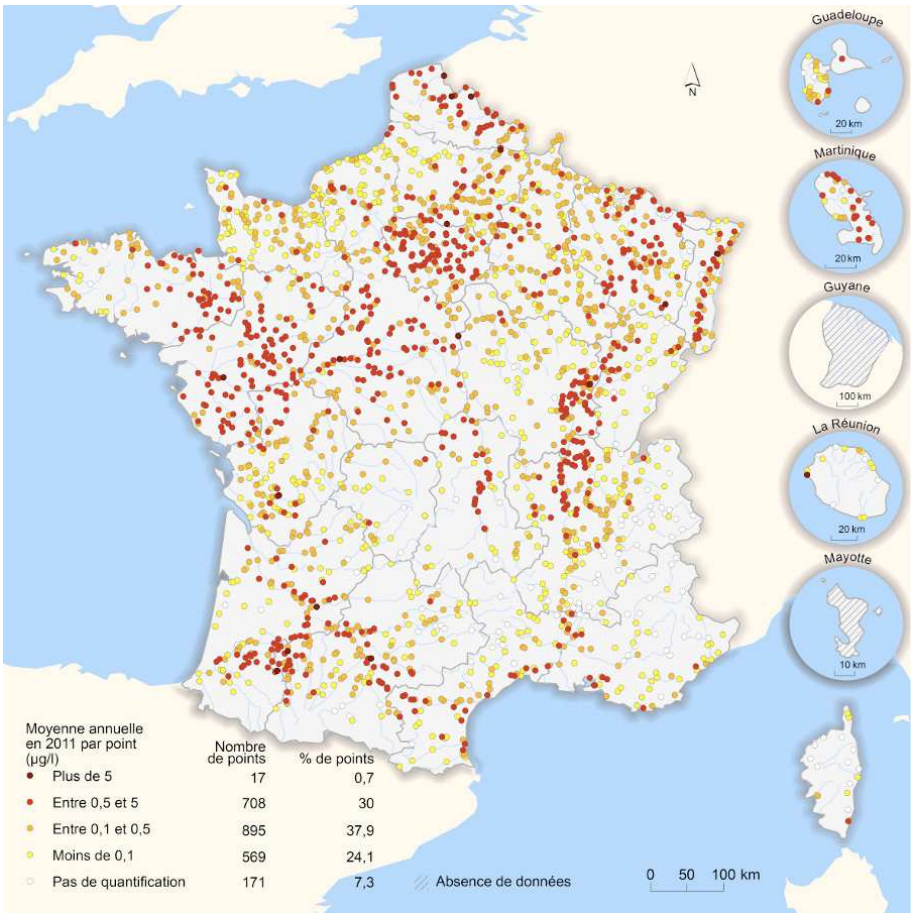

Figure 2: Concentration totale moyenne en pesticides en 2011 (d'après le rapport 2013 du service de l'observation et des statistiques, SOeS 2013)

La Figure 3 présente les 15 pesticides les plus quantifiés dans les cours d'eau de France métropolitaine en 2011. Ces composés sont en majorité des herbicides ou leurs produits de dégradation, deux sont des métabolites et trois sont des substances interdites d'utilisation.

L'AMPA, métabolite de l'herbicide glyphosate, est décelé sur près de la moitié des analyses où il est recherché et occupe la 1ère place de ce classement, juste devant sa molécule mère, présente sur le tiers de ces recherches.

L'atrazine prouve sa forte persistance dans le milieu et sa lente dégradation depuis son interdiction fin 2003. Mais sa présence semble diminuer ces dernières années au profit de son métabolite la déséthyl atrazine. Le diuron, malgré son interdiction d'usage entrée en vigueur fin 2008, est toujours très quantifié dans les cours d'eau.

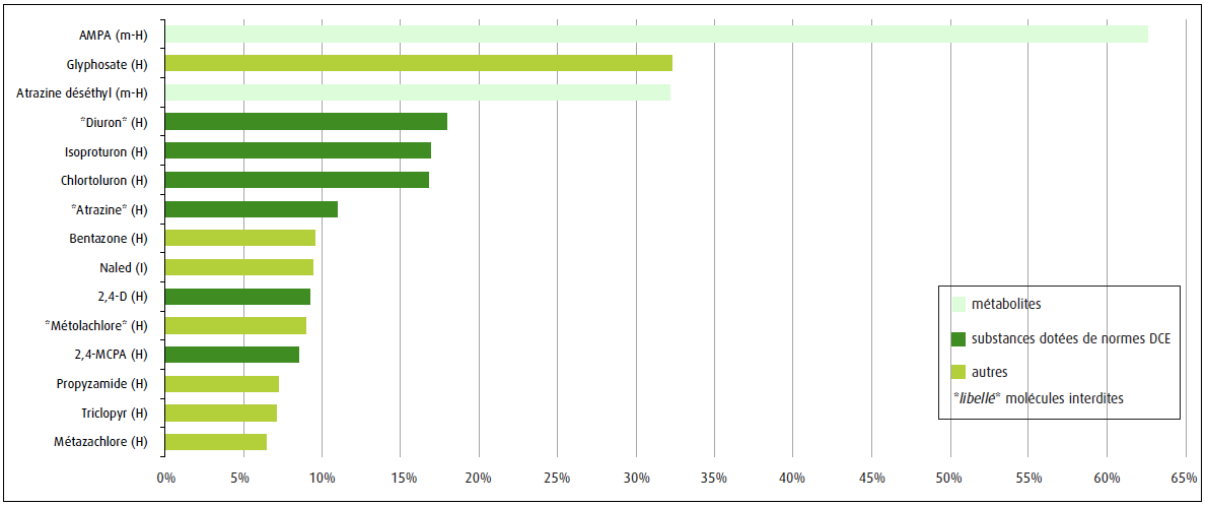

Figure 3 : Les pesticides les plus quantifiés en France métropolitaine en 2011 (d'après le rapport 2013 du service de l'observation et des statistiques, SOeS 2013)

Dix des quinze pesticides les plus quantifiés sont encore autorisés ; parmi eux on retrouve des herbicides à usages proches : le chlortoluron et l'isoproturon sont en effet principalement associés aux grandes cultures (blé tendre, orge d'hiver). D'autres ont des champs d'action plus vastes comme le glyphosate, le 2,4-D (traitements généraux) ou le propyzamide (utilisable en grandes cultures mais aussi en viticulture, en arboriculture et en maraîchage).

Les données présentées ci-dessus ont été acquises grâce à l'échantillonnage ponctuel basse fréquence (généralement trimestrielle), qui est à ce jour la stratégie utilisée en routine dans les réseaux de surveillance nationaux.

2.2.4 L'échantillonnage passif pour caractériser l'exposition aux pesticides dans les milieux aquatiques

Dans le cadre de la détermination des niveaux de contamination des masses d'eau, il existe deux grandes techniques d'échantillonnage : l'échantillonnage actif (ponctuel ou moyenné) et l'échantillonnage passif intégratif. L'échantillonnage actif ponctuel offre une image de la contamination environnementale à un instant donné. Il se révèle donc insuffisant pour assurer un suivi précis de la contamination des masses d'eau, notamment lors de fluctuations rapides des niveaux de contamination (cas des pesticides). L'échantillonnage actif ponctuel haute fréquence ou intégratif est une alternative d'intérêt tout particulier du point de vue de la représentativité de l'information acquise ; cependant cette solution n'est pas généralisable au niveau national de par les coûts engendrés (multiplication des échantillons, des analyses, logistique complexe,…). Une alternative réside dans l'utilisation

de l'échantillonnage passif. L'avantage de telles techniques repose sur leur caractère intégratif. Ici l'outil va échantillonner en continu les contaminants présents dans le milieu, l'information obtenue se traduit alors par des concentrations pondérées dans le temps. La Figure 4 illustre cette représentativité temporelle des échantillonnages ponctuels et passifs. Outre sa meilleure représentativité concernant l'exposition réelle de l'écosystème aquatique, l'échantillonnage passif permet d'abaisser de manière significative les seuils de détection grâce à la préconcentration des analytes *in situ*.

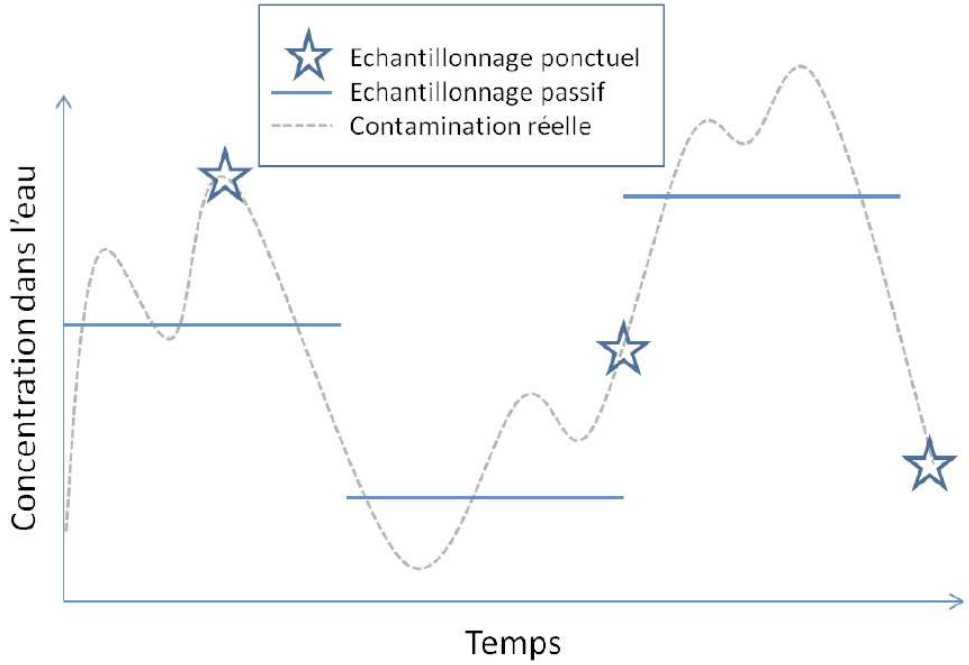

Figure 4: Représentativité temporelle des échantillonnages ponctuels et intégratifs (d'après Fauvelle 2012)

Parmi les produits phytosanitaires susceptibles d'atteindre l'environnement aquatique, ceux appartenant à la catégorie des pesticides polaires sont les plus préoccupants puisqu'ils font partie des pesticides les plus fréquemment retrouvés dans les rivières. Le Polar Organic Chemical Integrative Sampler (POCIS) a été développé il y a une dizaine d'années (Alvarez 1999, Alvarez et al. 2004) pour échantillonner de manière passive les composés organiques polaires. Il est constitué d'un adsorbant microporeux emprisonné entre deux membranes de polyethersulfone (PES, diamètre des pores 0,1µm), le tout étant retenu par deux anneaux en inox (Figure 5).

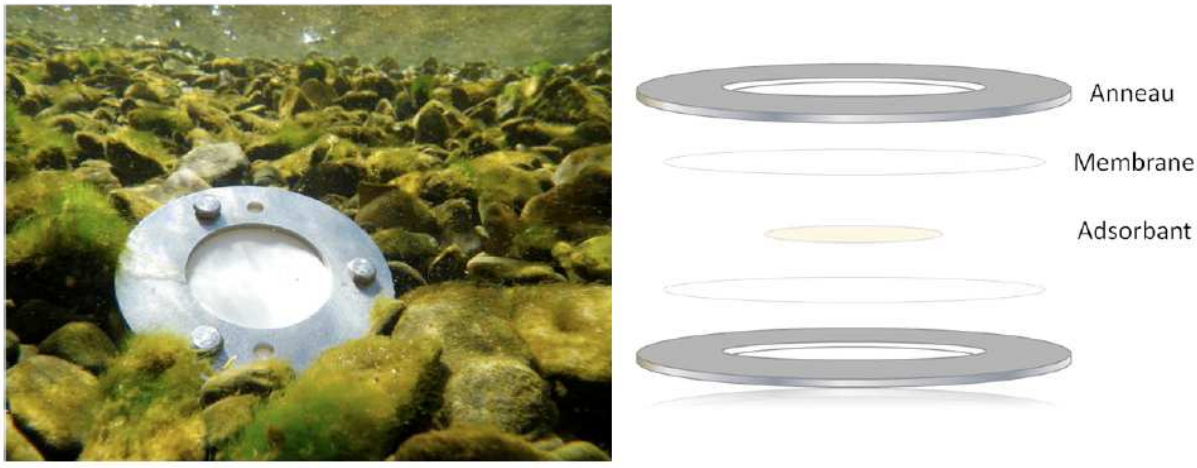

Figure 5: Polar Organic Chemical Integrative Sampler (POCIS) (d'après Fauvelle 2012)

Parmi les configurations commerciales, il a été montré que le POCIS « pharmaceutique » était le plus adapté à l'échantillonnage des pesticides polaires (Mazzella et al. 2007), ce dernier contient de la phase adsorbante Oasis HLB (copolymère hydrophile-hydrophobe). Cet outil peut potentiellement échantillonner les molécules dont le log K_{ow} (coefficient de partage octanol-eau) est compris entre 0 et 4. Le (Tableau 2) présente la capacité du POCIS à échantillonner différentes familles chimiques de pesticides en fonction de leur polarité. Ainsi le POCIS est particulièrement adapté à l'échantillonnage des composés appartenant à la famille des chloroacetanilides comme le métolachlore ou l'acétochlore. Au contraire l'outil ne permet pas d'échantillonner de manière efficace les aryloxyacides (log K_{ow} <1) comme l'acide 2,4-dichloro phénoxyacétique (2,4-D) ou les organophosphorés dont le log K_{ow} est supérieur à 3 comme le chlorpyriphos.

Polarité	herbicides	fongicides	insecticides
Log Kow <1	- - Aryloxyacides sulfonylurées Tricétones (Mazzella et al. 2007, Fauvelle et al. 2012)		
1≤Log Kow ≤3	++ Triazines Phenylurées (Alvarez et al. 2004, Mazzella et al. 2007, Lissalde et al. 2011, Ibrahim et al. 2013 , Martínez Bueno et al. 2009)	++ Morpholines Strobilurines (Lissalde et al. 2011, Assoumani et al. 2013)	+ Carbamates (Lissalde et al. 2011, Martínez Bueno et al. 2009) Organophosphorés (Alvarez et al. 2004, Lissalde et al. 2011)
Log Kow >3	++ Chloroacetanilides (Mazzella et al. 2007, Lissalde et al. 2011, Ibrahim et al. 2013, Vercraene-Eairmal et al. 2010, Fox et al. 2010) - Dinitroanilines Diphenylethers (Mazzella et al. 2007)	+ Triazoles (Charlestra et al. 2012 , Ibrahim et al. 2013)	- Organophosphorés (Lissalde et al. 2011)

Tableau 2: Capacité du POCIS à échantillonner différentes familles chimiques de pesticides en fonction de leur polarité (++ : composé très bien échantillonné par le POCIS, + : composé convenablement échantillonné par le POCIS, - : composé très mal échantillonné par le POCIS).

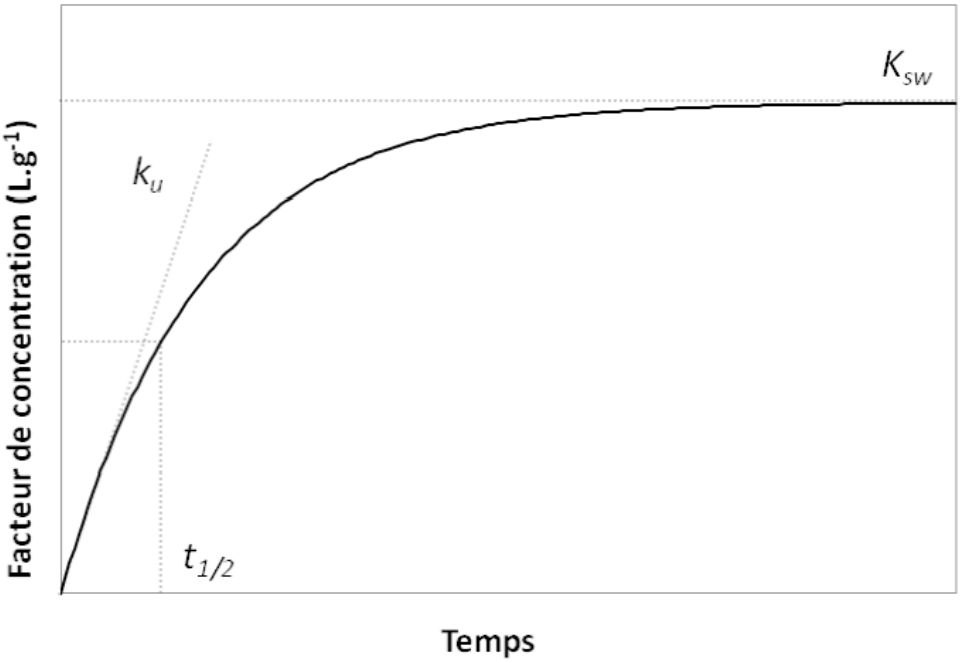

Figure 6: Accumulation des contaminants dans un échantillonneur passif (d'après Vrana et al. 2005)

L'accumulation des composés se fait par diffusion depuis le milieu vers la phase réceptrice selon une cinétique de premier ordre. Cette cinétique comprend deux composantes : une première phase intégrative pendant laquelle le facteur de concentration est proportionnel à la durée d'exposition ($t < t_{1/2}$), puis un régime d'équilibre où le facteur de concentration tend vers un équilibre échantillonneur-eau (K_{sw}) et devient indépendant du temps ($t >> t_{1/2}$) (Figure 6). Les composés d'intérêt sont préalablement étalonnés au laboratoire de manière à déterminer la constante k_u (la constante de la pente d'accumulation). Pour pouvoir retrouver les concentrations du milieu, il est donc nécessaire de se placer dans la partie intégratrice de la cinétique. Dans ce cas, la capacité de la phase réceptrice (adsorbant dans le cas du POCIS) est loin de la saturation et la relation suivante -dérivée des lois de diffusion de Fick- s'applique alors :

$$C_w = C_{POCIS} / (k_u \times t)$$

Où C_w et C_{POCIS} représentent la concentration d'analyte dans l'eau et le POCIS respectivement, k_u la constante de la pente d'accumulation à l'origine ($t \leq t_{1/2}$) et t le temps d'exposition. Si on introduit la masse de l'adsorbant M_{POCIS}, l'équation peut être réarrangée de la manière suivante afin d'obtenir une relation simple dans laquelle intervient le taux d'échantillonnage R_s:

$$C_w = (C_{POCIS} \text{ x } M_{POCIS}) / (R_s \text{ x } t)$$

La relation peut alors être simplifiée comme suit :

$$C_w = N_{POCIS} / (R_s \text{ x } t)$$

Où N_{POCIS} représente la masse d'analyte dans le POCIS.

Cependant l'accumulation des composés dépend des paramètres physico chimiques du milieu (vitesse de courant, température,…), les constantes R_s déterminées au laboratoire ne sont alors plus applicables directement. L'utilisation de composés de référence et de performance (PRC) a été proposée (Booij et al. 1998, Huckins et al. 2002, Mazzella et al. 2007) pour prendre en compte ce phénomène. Les PRC sont introduits dans l'échantillonneur passif avant son exposition dans le milieu, ce sont des molécules deutérées dont les constantes de désorption (préalablement étudiées au laboratoire) servent à corriger la mesure des concentrations des composés accumulés *in situ*.

2.3 Evaluation du risque environnemental et réglementation

Le document d'assistance technique sur l'évaluation des risques (EC 2003) définit les quatre étapes sur lesquelles se base l'évaluation du risque environnemental :

- l'identification du danger
- l'établissement de la relation effet-concentration
- l'évaluation de l'exposition
- la caractérisation du risque

Ces différentes étapes sont réalisées par substance et pour chacun des trois compartiments continentaux (l'environnement aquatique, l'environnement terrestre et l'air) ainsi que pour l'environnement marin.

Données disponibles	Facteur de sécurité
Au moins une valeur de L(E)C_{50} pour chacun des trois niveaux trophiques (poisson, *Daphnia* et algue)	**1000**
Une NOEC (poisson ou *Daphnia*)	**100**
Deux NOECs pour des espèces représentant deux niveaux trophiques (poisson et/ou *Daphnia* et/ou algue)	**50**
Des NOECs pour au moins trois espèces (normalement poisson, *Daphnia* et algue) représentant trois niveaux trophiques	**10**
Méthode de modèle de distribution de sensibilité des espèces (SSD)	**5-1 (A justifier au cas par cas)**
Données de terrain ou modèles environnementaux	**Révisé cas par cas**

Tableau 3: Facteurs de sécurité pour le calcul des $PNEC_{aquatique}$ pour les substances individuelles, appliqués aux espèces les plus sensibles disponibles (modifié d'après EC 2003)

L'évaluation de l'exposition environnementale peut se faire à partir de données prédites (modélisées) ou directement mesurées dans l'environnement. Ces données permettent alors le calcul d'une concentration prédite dans l'environnement, la PEC (Predicted Environmental Concentration). La partie concernant l'évaluation des effets a quant à elle pour objectif le calcul d'une concentration prédite sans effet, la PNEC (Predicted No Effect Concentration). Cette PNEC est calculée pour chaque substance à partir des données issues de tests de toxicité auxquelles est appliqué un facteur de sécurité. Ce facteur prend en compte les incertitudes liées entre autres à la variabilité intra et interspécifique (variabilité biologique), à l'extrapolation de la toxicité à court terme à la toxicité à long terme et à l'extrapolation des données de laboratoire aux impacts sur le terrain. Comme présenté dans la Tableau 3, le nombre et de la nature des données conditionnent donc la valeur de ce facteur. Par exemple si au moins une valeur de L(E)C_{50} (Concentration nécessaire pour induire 50% de mortalité (L : lethal) ou d'effet (E : efficace) par rapport aux témoins) est disponible pour chacun des trois niveaux trophiques (poisson, *Daphnia* et algue), le facteur de sécurité de 1000 sera alors appliqué à la plus faible des trois valeurs de L(E)C_{50}. A l'opposé ce facteur peut être réduit à une valeur comprise entre 5 et 1 si un modèle de distribution de sensibilité des espèces (SSD) a été réalisé.

Comme présenté sur la Figure 7, une fois les valeurs de PEC et PNEC obtenues pour une substance particulière, le rapport PEC/PNEC est calculé ; deux cas de figure se présentent alors. Si ce rapport est inférieur à 1 des tests de toxicité supplémentaires ainsi que des mesures de réduction du risque pour cette substance ne sont pas nécessaires. En revanche si le rapport PEC/PNEC est supérieur à 1, des démarches sont entreprises pour faire diminuer ce ratio en réalisant de nouvelles études sur la substance ou en adoptant des mesures de réduction du risque.

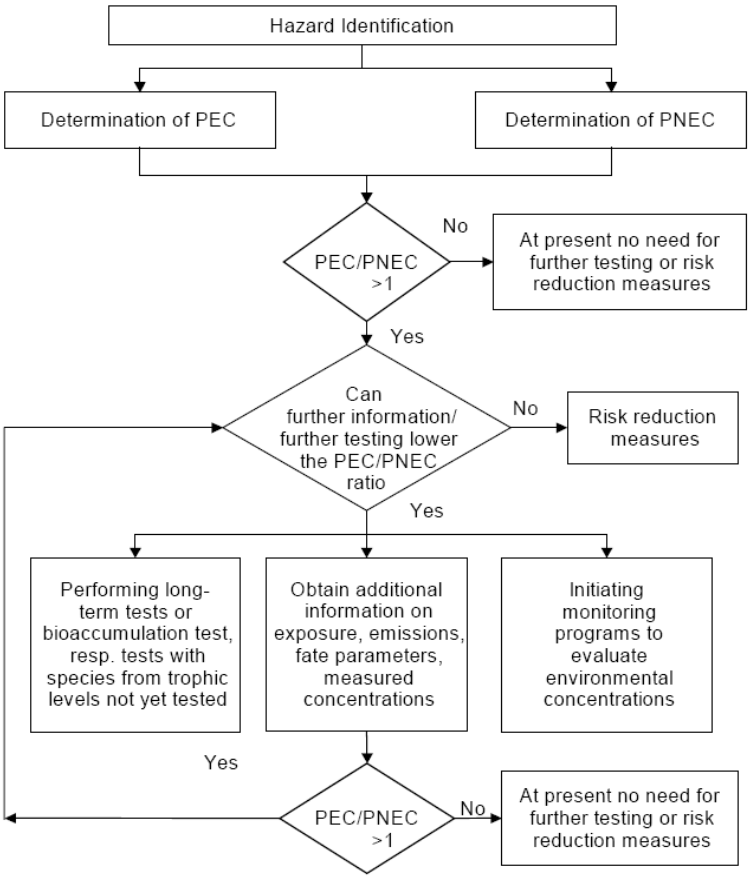

Figure 7: Procédure générale dans l'évaluation du risque environnemental (d'après EC 2003)

D'autre part, les normes de qualité environnementale (NQE) ont été mises en place dans le cadre de la Directive Cadre européenne sur l'Eau (2000/60/EC) pour 33 substances ou groupes de substances prioritaires plus huit substances supplémentaires. Dix-huit de ces 41 substances sont des produits phytosanitaires. Une NQE représente la concentration d'un polluant ou d'un groupe de polluants qui ne doit pas être dépassée afin de protéger la santé humaine et les écosystèmes et est basée sur le calcul des PNEC.

Il est postulé que le non dépassement des PNEC assure la protection du compartiment aquatique dans son ensemble, cependant comme nous l'avons vu ces PNEC sont basées sur des tests de toxicité monospécifiques et sont établies substance par substance : ce sont là les incertitudes et les limites liées à cette démarche. En effet cette dernière est pauvre du point de vue de la représentativité environnementale puisque les espèces vivent en interaction les unes avec les autres et que les composés sont présents sous forme de cocktail dans l'environnement aquatique. Les effets de mélanges ne sont en particulier donc pas pris en compte dans l'évaluation du risque environnemental actuelle.
Les tests de toxicité monospécifiques établis substance par substance sont donc peu réalistes, cependant nous allons voir que les études écotoxicologiques peuvent couvrir un large panel d'échelles de représentativité.

2.4 Les études écotoxicologiques : un équilibre entre réalisme et réductionnisme

Truhaut a été le premier à donner une définition de l'écotoxicologie (1977). L'écotoxicologie est alors définie comme la branche de la toxicologie concernant l'étude des effets toxiques causés par les substances naturelles ou les composés d'origine synthétique sur les constituants des écosystèmes animaux (incluant les humains), végétaux et micro-organismes, dans un contexte intégré. Pour établir le plan d'expérience d'une étude écotoxicologique, il convient de trouver un équilibre entre réalisme et réductionnisme, pour cela l'expérimentateur peut jouer sur différentes échelles de représentativité :

- ✓ <u>Echelle temporelle</u> : dans le domaine de l'écotoxicologie, deux grands modèles d'expérience existent : les tests d'exposition aiguë et chronique. Les premiers visent à déterminer des relations dose-effet de composés modèles, les organismes sont exposés à des doses croissantes de toxiques pendant une période brève (de quelques heures à quelques jours) (Sprague 1969). Ces tests permettent d'obtenir des paramètres comme la CE_X (concentration inhibant X% du paramètre étudié par rapport aux témoins), la NOEC (concentration ne provoquant pas d'effet observé) ou encore la LOEC (plus faible concentration provoquant un effet mesurable) (Newman et Unger 2003). Ces tests sont rapides, peu coûteux et robustes. Cependant ils n'offrent pas une grande représentativité du point de vue écologique puisque les organismes sont rarement exposés à des doses de contaminants telles que celles utilisées pour ces tests, de plus seuls les effets aigus sont visibles dans ce genre de tests. Au contraire les tests de contamination chronique offrent une plus grande représentativité du point de vue

écologique, dans ces tests les organismes peuvent être exposés à de plus faibles concentrations de contaminants pendant de longues périodes de temps (de quelques jours à quelques semaines) (Suter 1993). Ces tests nécessitent cependant des moyens humains, financiers et techniques importants.

- ✓ Echelle spatiale : Il est possible de distinguer les études *in situ* et les études menées dans des conditions contrôlées de laboratoire. Les premières permettent d'appréhender les phénomènes d'une manière extrêmement réaliste ; puisque les réponses des organismes sont directement étudiées dans le milieu naturel. Cependant très peu voire aucun paramètre n'est contrôlé par l'expérimentateur, les paramètres abiotiques comme la température, la vitesse de courant, l'ombrage peuvent grandement influer sur les réponses des organismes, la difficulté étant de pouvoir différencier la variabilité naturelle de celle liée à la contamination. Dans ce cas, il peut donc être difficile de relier contamination et effets observés. Des études en conditions semi contrôlées existent, avec par exemple l'utilisation de rivières pilotes ; il est alors possible de contrôler certains paramètres comme la vitesse du courant ou la concentration en nutriments, il devient alors plus facile d'attribuer les effets observés à un contaminant.

  Les expériences en conditions contrôlées de laboratoire permettent quant à elles de faire plus facilement le lien entre contamination et effets toxiques, puisqu'il est possible dans de telles expositions de contrôler un grand nombre de paramètres (luminosité, vitesse du courant, température, concentration en nutriments, niveaux de contamination,…) (Forbes et al. 1997). Les essais en laboratoire permettent de simplifier au maximum les conditions d'exposition, ils peuvent être réalisés dans un espace réduit avec peu de matériel et sont reproductibles… mais pauvres du point de vue de la représentativité spatiale puisque dans la plupart des cas ils sont réalisés dans des unités expérimentales de type bécher, erlenmeyer, tubes à essai,…

  Un bon compromis entre les études *in situ* et les essais en laboratoire réside dans l'utilisation de canaux artificiels. En effet, ces systèmes expérimentaux modélisant une rivière à dimensions réduites permettent le contrôle d'un grand nombre de paramètres tout en offrant une certaine représentativité spatiale, ils peuvent de plus être implantés soit directement sur le terrain soit utilisés au laboratoire (Gulp et al. 1996).

- ✓ Echelle organisationnelle : les réponses vis-à-vis d'un toxique peuvent être appréhendées à différents niveaux d'organisation biologique, de la cellule à l'écosystème en passant par la population et la communauté. Les tests mono spécifiques sont souvent utilisés dans le cadre de la législation, ils présentent l'avantage d'être rapides, peu coûteux et robustes ; cependant ils n'offrent pas une grande représentativité écologique puisque la sensibilité peut varier très fortement d'un organisme à un autre et ce même au sein d'une même classe algale. A l'opposé, les tests plurispécifiques offrent un meilleur réalisme écologique puisqu'ils intègrent des organismes pouvant avoir des *preferenda* environnementaux et des sensibilités aux toxiques variées. L'utilisation des communautés est d'ailleurs mise en avant pour étudier les effets des contaminants (Clements et Rohr 2009). Dans ce contexte les biofilms de rivière sont particulièrement intéressants puisqu'ils intègrent une grande diversité d'espèces formant une communauté, ils sont d'ailleurs de plus en plus préconisés pour l'évaluation d'effets de polluants variés (Sabater et al. 2007).

- ✓ Echelle d'exposition aux toxiques : l'expérimentateur peut choisir d'étudier les effets d'un seul toxique jusqu'aux effets d'un mélange environnemental complexe et inconnu en passant par tous les mélanges simples possibles. Selon le rapport de l'agence américaine de protection de l'environnement (US EPA 1987), un mélange simple est un mélange contenant deux composés identifiables ou plus, mais assez peu pour que la toxicité du mélange puisse être convenablement caractérisée. Un mélange complexe est quant à lui défini comme un mélange comprenant un tel nombre de composés qu'une estimation de sa toxicité induit trop d'incertitudes et d'erreurs pour être utile. En général, les études d'effets de produits chimiques seuls visent à comprendre les mécanismes de toxicité et/ou à déterminer la toxicité relative d'un produit (notamment dans le cadre de la législation). Ces tests sont peu coûteux et simples à mettre en œuvre ; cependant ils sont pauvres du point de vue de la représentativité de la l'exposition « réelle » des organismes car les contaminants sont rarement présents seuls dans l'environnement mais au contraire se retrouvent sous forme de mélanges complexes.

  Les premières études s'intéressant aux effets de mélanges ont défini quatre types de mécanismes pour l'action conjointe des toxiques (Bliss 1939, Hewlett et Plackett 1964). Deux de ces mécanismes postulent la non interaction des toxiques du mélange et prédisent ainsi des effets additifs (similaire et indépendant). Lorsque les effets

observés sont différents de ceux prédits par ces deux modèles, on parle alors de synergie ou d'antagonisme, qui représentent le cas où les effets observés sont respectivement plus et moins importants que les effets attendus dans le modèle additif. Ces études permettent de comprendre les mécanismes d'action de substances modèles et ainsi de prédire les effets de mélanges associés dans le cas de mélanges simples (généralement au plus une dizaine de molécules).

Un niveau important de représentativité de contamination environnementale est atteint avec l'utilisation d'extraits d'échantillonneurs passifs ou bien encore avec l'exposition à des échantillons environnementaux (par exemple : effluent complexe prélevé sur le terrain).

Le niveau de représentativité vis à vis de l'exposition aux toxiques peut donc être appréhendé par le nombre de composés pris en compte mais aussi par les concentrations considérées. En effet, les impacts d'un seul toxique, d'un mélange simple ou d'un mélange complexe peuvent être étudiés à des concentrations très élevées ou bien à des concentrations réalistes par rapport à la contamination environnementale. Dans le premier cas, c'est le potentiel toxique du composé ou du groupe de composés qui est appréhendé puisque ce sont les effets aigus qui sont observés. Au contraire les expositions à des concentrations proches de celles mesurées dans l'environnement permettent de faire le lien entre le potentiel toxique (déterminé à des concentrations élevées) et les effets observables *in situ*.

Le choix entre ces différentes échelles de représentativité dépend des objectifs de l'expérience (veut-on appréhender la toxicité d'un mélange réaliste de pesticides ou comprendre les mécanismes de toxicité de mélanges existants de contaminant modèles à modes d'action différents ?) et des moyens disponibles (humains, financiers, techniques, organisationnels).

L'approche visant à utiliser des extraits d'échantillonneur passif à faibles concentrations sur du biofilm de rivière atteint un niveau de représentativité élevé puisque l'expérimentateur travaille ici avec des communautés biologiques et un mélange toxique environnemental complexe et réaliste. Cette démarche permet en particulier de travailler sans *a priori* sur la nature des toxiques présents dans l'extrait et d'intégrer les effets de mélange.

Cependant elle a aussi des limites puisque c'est une approche au cas par cas et qui n'identifie pas le/les contaminant(s) responsable(s) de la toxicité observée puisque les extraits de POCIS

sont regardés comme une « boîte noire ». Ainsi seule la toxicité globale de l'extrait est appréhendée.

2.5 Utilisation des biofilms comme communauté modèle

2.5.1 Définitions

Le terme biofilm périphytique ou périphyton désigne à l'origine l'ensemble de la microflore (microalgues, bactéries, champignons) se développant sur tout type de substrat immergé, qu'il soit vivant ou inerte (Wetzel 1983). Les protozoaires, les organismes zooplanctoniques et les macroinvertébrés se développant également à la surface du substrat ne sont donc pas considérés comme faisant partie intégrante du biofilm dans cette définition.
Ces différents organismes sont enrobés dans une matrice composée d'exo polysaccharides sécrétés par les composantes algale et bactérienne (Figure 8). Cette matrice assure la cohésion du biofilm et joue un rôle dans la protection face aux facteurs environnementaux.

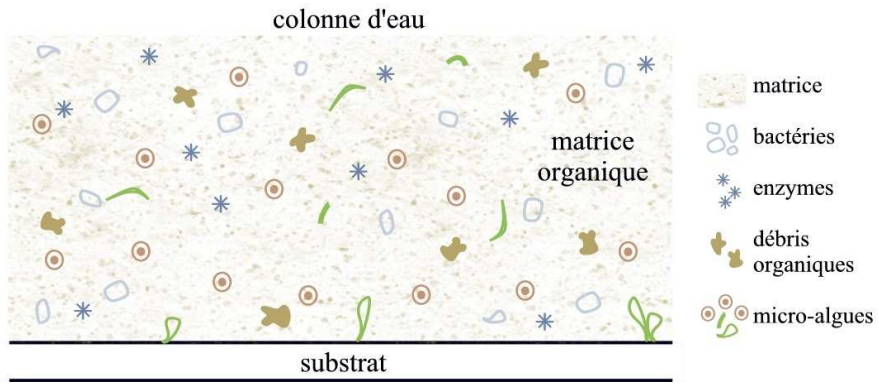

Figure 8: Représentation schématique des différentes composantes d'un biofilm périphytique (d'après Morin 2006)

Le périphyton est désigné sous différentes terminologies selon le substrat sur lequel il se développe :

- ✓ L'épiphyton colonise les végétaux (macrophytes, lentilles d'eau,…).
- ✓ L'épilithon colonise les supports minéraux grossiers (cailloux, rochers, galets,…).
- ✓ L'épipélon colonise la vase.
- ✓ L'épipsammon colonise le sable.

Dans cette thèse, les différentes études ont porté sur le périphyton colonisant les supports minéraux grossiers c'est-à-dire l'épilithon. Les termes périphyton et biofilm seront utilisés indifféremment pour désigner l'épilithon dans ce manuscrit.

2.5.2 Dynamique de colonisation

Dans un premier temps, le dépôt d'une couche de matière organique et de bactéries facilite l'installation de petites espèces de diatomées adhérentes et mobiles, ce sont les espèces pionnières. Les espèces érigées se développent alors avec l'apparition d'espèces à court pédoncule puis moins attachées au substrat et enfin les espèces filamenteuses se développent à leur tour. Au fur et à mesure de la croissance du biofilm, l'architecture tridimensionnelle se complexifie donc pour former à maturité un biofilm dont l'épaisseur est conditionnée par le pool d'espèces présentes dans le milieu, le type de substrat mais aussi les conditions environnementales (comme la lumière, le courant, le broutage, ou les concentrations en nutriments et en toxiques) durant les stades de colonisation (Figure 9).

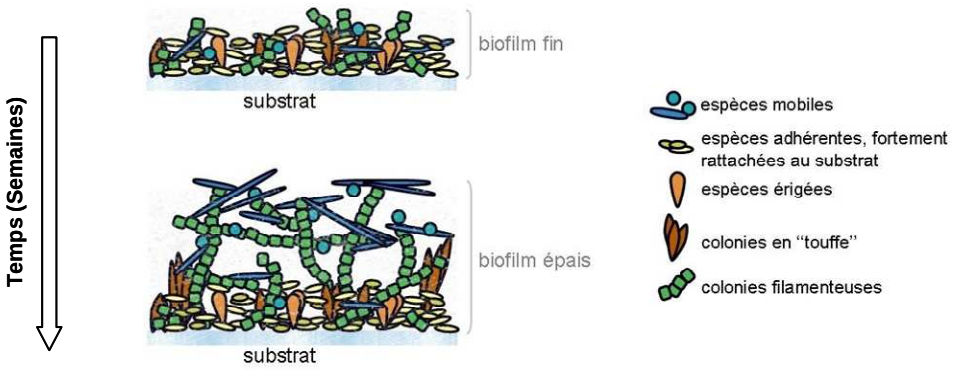

Figure 9: Architecture tridimensionnelle d'un biofilm (d'après Morin 2006)

2.5.3 Rôle écologique dans les cours d'eau

Le biofilm joue un rôle essentiel dans le fonctionnement des écosystèmes aquatiques, et particulièrement dans les systèmes lotiques de petite taille, les organismes qui s'y développent sont responsables de la majorité de la production énergétique via la production primaire et le cycle des nutriments (Battin et al. 2003).

Le biofilm est ainsi le siège de processus auto et hétérotrophes intervenant dans les cycles bio-géochimiques majeurs (Costerton et al. 1994). Au sein du biofilm, les bactéries hétérotrophes sont capables de métaboliser la matière organique dissoute (MOD), rendant ainsi possible son transfert vers les niveaux trophiques supérieurs (Romaní et al. 2004). Les biofilms sont des sites majeurs d'assimilation et de stockage du carbone organique dissous, ils contribuent ainsi au cycle du carbone mais aussi à ceux de l'azote ou du phosphore. Ils sont aussi à la base de la chaîne alimentaire où ils servent de source de nourriture pour de nombreux organismes brouteurs (gastéropodes ou certains poissons).

Parmi les composantes de ce biofilm, les diatomées sont utilisées de manière privilégiée dans le cadre de la bioindication et des études écotoxicologiques. Ces algues brunes unicellulaires dont la taille varie de quelques µm à plus de 500µm possèdent une paroi siliceuse qui se compose de deux unités imbriquées : l'épivalve et l'hypovalve. Les formes et les ornementations caractéristiques des valves sont utilisées pour l'identification jusqu'au niveau de l'espèce lors de l'examen microscopique. Les diatomées sont des organismes ubiquistes, possédant un temps de génération court ; les différentes espèces présentant par ailleurs des *preferenda* environnementaux et des sensibilités aux toxiques variées. Ces caractéristiques ont conduit à leur utilisation actuelle dans plusieurs indices rendant compte de la qualité générale des eaux comme l'Indice Biologique Diatomées (Coste et al. 2009). D'autre part, de récentes études ont pu montrer leur potentiel dans l'évaluation des pollutions toxiques, notamment dans le cas des contaminations métalliques (Morin 2006, Arini 2011).

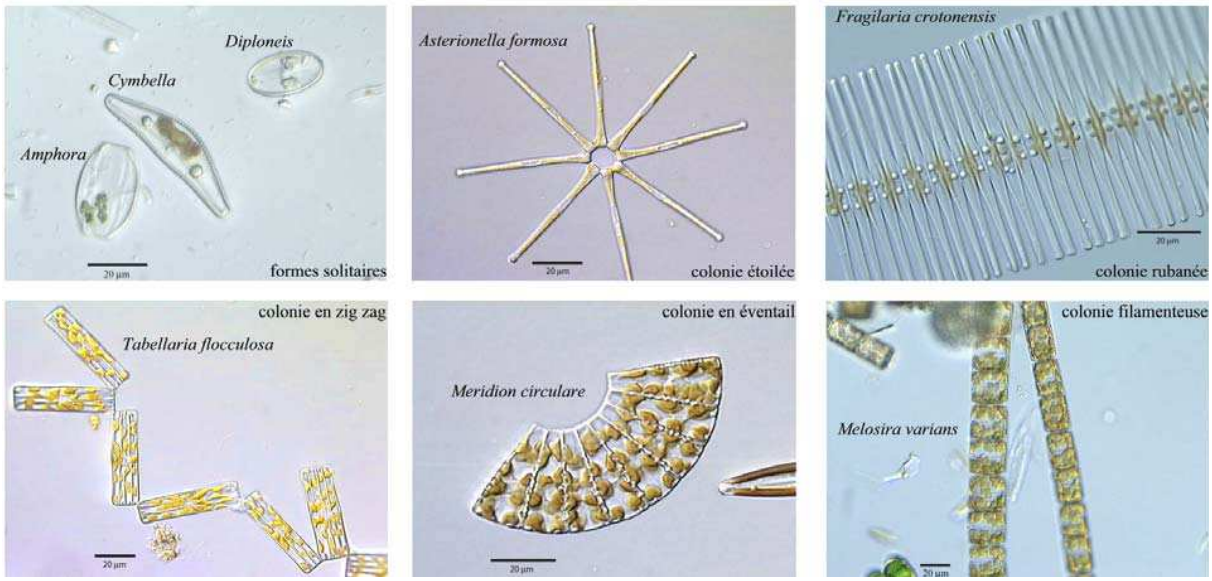

Figure 10: Différentes espèces de diatomées sous formes solitaires et coloniales (photographies de Jason Oyadomari, source : http://www.bio.mtu.edu/, dans Morin, 2006)

2.5.4 Des biofilms sensibles aux pesticides

Les pesticides peuvent avoir de multiples effets sur le biofilm de rivière, que ce soit sur la croissance, au niveau structurel ou fonctionnel.

- ✓ Croissance du biofilm

La grande majorité des études, qu'elles soient réalisées au laboratoire ou *in situ*, ont mis en évidence des effets négatifs de pesticides sur la croissance du biofilm et ce à différentes échelles d'organisation. Des impacts ont pu être observés sur la

composante algale dans son ensemble ou encore sur une classe algale en particulier. Debenest et al. (2009) ont pu observer une diminution de concentration en chlorophylle a après avoir exposé du biofilm pendant 6 jours à 5 et 30µg/l d'isoproturon; reflétant ainsi l'impact de l'herbicide sur la croissance de la composante algale globale. Certaines études se sont attachées à déterminer l'impact des pesticides sur le groupe des diatomées en particulier : Pérès et al. (1996) ont ainsi mis en évidence une réduction marquée de la densité en diatomées après 34 jours d'exposition à 5µg/l d'isoproturon.

- ✓ Structure du biofilm

Les impacts au niveau structurel peuvent être évalués à différentes échelles de complexité et par diverses méthodes, les études disponibles dans la littérature se sont penchées sur la détermination relative des groupes algaux (généralement algues vertes, diatomées et cyanobactéries), la caractérisation de la diversité procaryote et eucaryote ou encore la structure des communautés de diatomées.

Ainsi Schmitt-Jansen et Altenburger (2005) ont pu montrer une évolution de la structure du biofilm avec un changement de dominance du groupe algal des diatomées vers celui des chlorophytes suite à l'exposition à l'isoproturon pendant deux semaines.

Dans une étude *in situ*, Dorigo et al. (2010) ont quant à eux pu mettre en évidence des profils de communautés procaryotes et eucaryotes différents en fonction du niveau de contamination en pesticides des différentes stations. Tlili et al. (2008) ont également observé des changements dans les profils de communautés procaryotes et eucaryotes au terme d'une expérience de contamination chronique et de pics de diuron en microcosmes.

Un effort particulier a été mis sur la caractérisation des effets des toxiques (dont des pesticides) sur le groupe des diatomées de par leur utilisation actuelle et en routine dans le biomonitoring. Morin et al. (2010a) ont étudié le potentiel de récupération de communautés périphytiques sur une rivière impactée par un bassin versant viticole ; les auteurs ont mis en évidence un changement de composition spécifique chez les diatomées pour trois stations situées sur la rivière Morcille étant soumises à des niveaux croissants de pression pesticide avec des abondances relatives plus

importantes de certaines espèces comme *Achnanthidium minutissimum* (Kützing) Czarnecki, *Eolimna minima* (Grunow) Lange-Bertalot ou *Navicula lanceolata* (Agardh) Ehrenberg au niveau de la station de référence par rapport aux stations contaminées. Lors d'une autre étude *in situ*, Ricart et al. (2010a) ont montré une relation entre des pesticides de type triazines et les distributions de communautés de diatomées. Cependant, *in situ* il reste encore difficile de séparer les effets attribuables aux pesticides et ceux dus aux facteurs environnementaux (température, lumière, vitesse de courant, taux d'oxygène dissous, trophie) et aux autres pollutions toxiques telles que les contaminations métalliques par exemple ; ceux-ci peuvent donc représenter des facteurs de confusion importants dans l'interprétation de la réponse biologique attribuable à la pression pesticide. Les études au laboratoire sont donc indispensables pour comprendre les modifications induites par les produits phytosanitaires sur le biofilm, et pour pouvoir ainsi être capable d'appréhender et de reconnaitre les effets attribuables à une contamination en pesticides dans le milieu naturel. Certaines études se sont donc intéressées aux effets de ces composés sur les assemblages de diatomées en conditions contrôlées de laboratoire : Roubeix et al. (2011) ont par exemple observé des différences de composition spécifique de diatomées avec un développement plus important de certaines espèces comme *Planothidium frequentissimum* (Lange-Bertalot) Lange-Bertalot, *Planothidium lanceolatum* (Brébisson ex Kützing) Lange-Bertalot, *Halamphora montana* (Krasske) Levkov, *Surirella brebissonii* Krammer & Lange-Bertalot et *Nitzschia gracilis* Hantzsch suite à une exposition au métolachlore.

- ✓ Impacts physiologiques

Dans la littérature, des impacts à divers niveaux physiologiques ont été étudiés suite à l'exposition de biofilms à des pesticides.

De nombreuses études se sont intéressées aux effets des pesticides sur les capacités photosynthétiques de la composante algale du biofilm, ces dernières peuvent être mesurées via les mesures de fluorescence chlorophyllienne en lumière modulée (PAM : pulse amplitude modulation) qui sont de plus en plus utilisées grâce aux récents développements et démocratisation des appareils de mesure. Une diminution des capacités photosynthétiques du biofilm lors de tests d'exposition aiguë à l'atrazine (Guasch et al. 1997), à l'isoproturon (Schmitt-Jansen et Altenburger 2007, Laviale et al. 2010) ou encore au diuron et à ses métabolites (Pesce et al. 2006) a été décrite.

Les pesticides peuvent induire un stress oxydant via la production d'espèces réactives de l'oxygène toxiques pour la cellule, cette dernière dispose de différents moyens de régulation du stress oxydant dont l'activation d'enzymes antioxydantes comme la catalase, l'ascorbate péroxydase, la glutathion réductase ou la glutathion-S-transférase. Récemment les activités enzymatiques antioxydantes (AEAs) ont été proposées comme marqueurs du stress oxydant induit par les toxiques pour le biofilm de rivière (Bonnineau et al. 2013). Les auteurs ont notamment pu montrer lors d'un test de toxicité aiguë à l'oxyfluorfène une plus importante activité de la catalase pour des biofilms chroniquement exposés à cet herbicide par rapport à des biofilms non préalablement exposés. Dans une autre étude, Bonnineau et al. (2012) ont observé une augmentation de l'activité de l'ascorbate péroxydase suite à l'exposition à ce même herbicide.

- ✓ Tolérance acquise des communautés

D'autres études utilisent le concept PICT ou l'acquisition de tolérance induite proposée par Blanck et al. (1988) pour évaluer l'impact des pesticides sur le biofilm de rivière. Le concept PICT est basé sur la théorie selon laquelle si une substance est toxique, elle va exercer une pression de sélection sur la communauté, entrainant une tolérance plus importante de cette communauté vis-à-vis de cette substance comme illustré dans la Figure 11. Il est donc possible de mettre en évidence une exposition passée à des pesticides en évaluant la sensibilité du biofilm en l'exposant à des concentrations en toxique croissantes. Deux paramètres sont couramment utilisés lors de la construction des ces courbes dose-réponse : les capacités de photosynthèse et de respiration. Plusieurs travaux ont révélé la pertinence de cette méthode face à des contaminations au diuron et à ses métabolites (Tlili et al. 2011, Pesce et al. 2010b, Dorigo et al. 2010) ou à la prométryne (herbicide, Rotter et al. 2011).

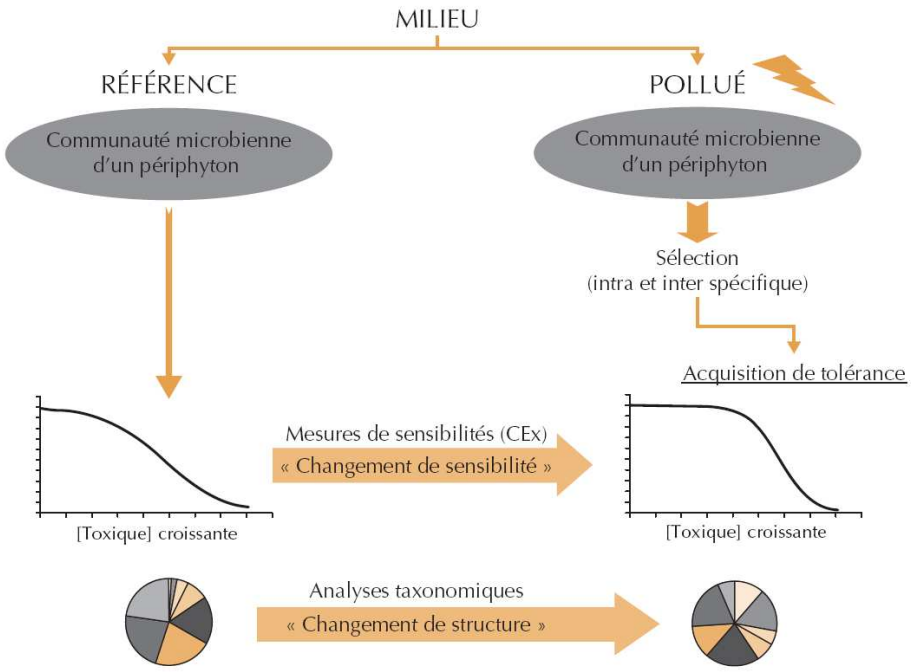

Figure 11 : Le concept PICT (d'après Pesce et al. 2008)

2.6 L'utilisation d'extraits d'échantillonneurs passifs pour les études écotoxicologiques

Comme vu précédemment, les échantillonneurs passifs ont été initialement développés dans le but d'intégrer de manière continue la concentration de molécules dans le milieu grâce à des dispositifs sélectifs d'une catégorie de composés chimiques. Ils ont aussi été utilisés en combinaison avec des tests biologiques dans le but d'appréhender une certaine « toxicité environnementale ». Ce type d'approche permet en particulier d'intégrer les problématiques liées aux effets de mélange et de pouvoir travailler sans connaître au préalable la composition de l'extrait. Concernant le milieu aquatique, il est possible d'utiliser divers échantillonneurs passifs en fonction de la polarité des substances suspectées ; le POCIS est par exemple particulièrement adapté pour échantillonner les pesticides et les PPCP (produit pharmaceutique et de soin personnel), le DGT (Diffusive Gradient in Thin-Films, Davison et Zhang 1994, Zhang et Davison 1995) pour les contaminants métalliques, le MESCO (Membrane-Enclosed Silicone Collector, Paschke et al. 2006) et le SR (Silicone Rubber, Rusina et al. 2010) pour les PCB (polychlorobiphényles) ou encore le SPMD (Semi-Permeable Membrane Devices, Huckins et al. 1990) et les membranes LDPE (Low-Density Polyethylene, Müller et al. 2001) pour les hydrocarbures aromatiques polycycliques (HAP) (Figure 12).

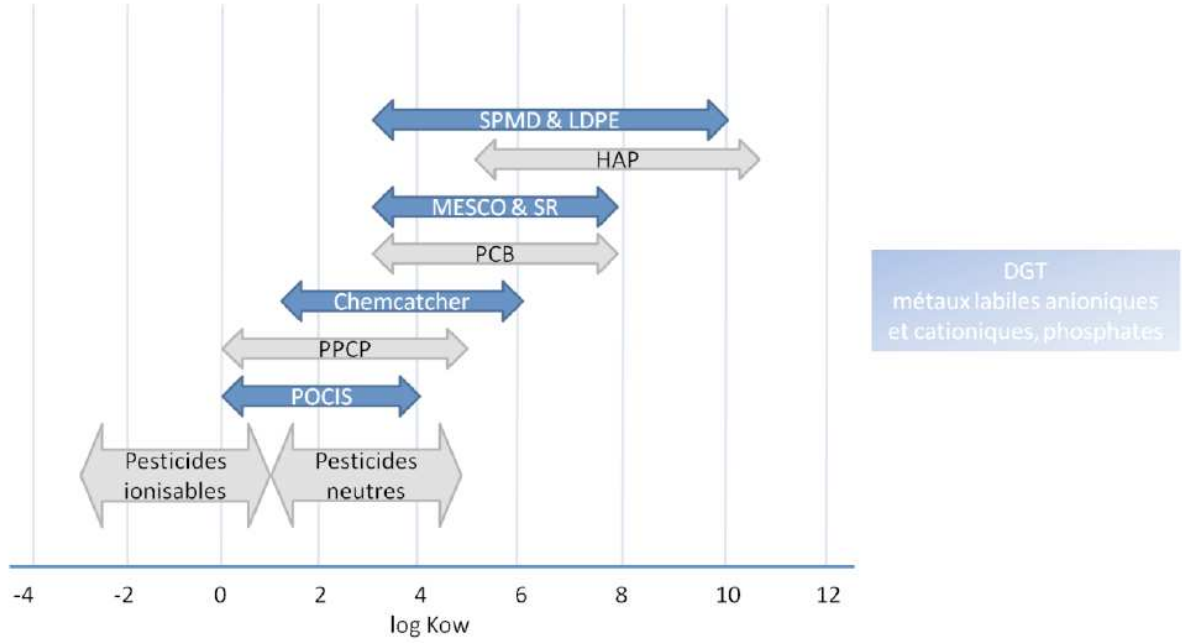

Figure 12: Domaine d'application des principaux échantillonneurs passifs (en bleu) et gamme de polarité de quelques familles de contaminants organiques (en gris). Applicabilité du Diffusive Gradient in Thin-film (DGT) spécifiée pour les composés inorganiques (d'après Fauvelle 2012)

Le couplage des extraits d'échantillonneurs passifs et des études écotoxicologiques peut permettre d'une part d'appréhender le potentiel toxique d'un mélange environnemental complexe lorsque les effets des extraits sont évalués à des concentrations aiguës et d'autre part d'étudier des impacts plus réalistes avec l'exposition à des concentrations de l'ordre de grandeur de celles mesurées dans le milieu.

2.6.1 Toxicité aiguë des extraits d'échantillonneurs passifs

La grande majorité des études appréhendant la toxicité des extraits d'échantillonneurs passifs a été conduite sous la forme de bioessais. Généralement, les extraits peu ou pas dilués (c'est-à-dire contenant des composés dont les concentrations sont très largement supérieures aux concentrations pouvant être rencontrées dans l'environnement) sont utilisés lors de tests *in vitro* ou *in vivo*.

Cette approche a été largement utilisée dans le contexte de la problématique des perturbateurs endocriniens, notamment en utilisant le test Yeast Estrogen Screen (YES). Vermeirssen et al. (2005) ont ainsi pu mettre en évidence l'activité œstrogénique d'extraits de POCIS provenant d'un site en aval d'une station d'épuration. D'autres études ont également utilisé ce test pour mettre en évidence une activité œstrogénique d'extraits de POCIS provenant de sites impactés

par des stations d'épuration (Balaam et al. 2010, Liscio et al. 2009) ou des activités d'élevage (Matthiessen et al. 2006).

L'utilisation d'extraits d'échantillonneurs passifs couplés aux tests biologiques a aussi été utilisée pour d'autres types de contamination comme par exemple les cas de pollutions aux pesticides, aux polychlorobiphényles (PCB) ou encore aux hydrocarbures aromatiques polycycliques (HAP). Allan et al. (2012) ont ainsi observé l'apparition d'anomalies morphologiques sur des embryons de poisson zèbre (*Danio rerio*) exposés à des extraits de LDPE modifié (Lipid-free tubing) immergés dans une zone portuaire où les concentrations en HAPs sont particulièrement importantes. Roig et al. (2007) ont pu mettre en évidence les effets de métaux lourds et de composés organiques en exposant des bactéries (*Vibrio fischeri*) et des algues vertes (*Pseudokirschneriella subcapitata*) à des extraits de DGTs et de SPMDs.

Deux études se sont penchées sur les effets de pesticides en mélange sur les biofilms de rivière en utilisant des extraits de POCIS : Pesce et al. (2011b) et Morin et al. (2012b) ont ainsi observé des impacts des extraits sur les capacités photosynthétiques du biofilm en relation avec l'exposition passée des communautés illustrant ainsi le concept PICT.

La toxicité à laquelle sont exposés les organismes aquatiques dans le milieu naturel a été appréhendée en utilisant un couplage de divers échantillonneurs et tests biologiques en fonction du type de contamination suspectée. Ce type d'approche permet en particulier d'intégrer les problématiques liées aux effets de mélange et de pouvoir travailler sans *a priori* sur la composition de l'extrait. Cependant en plus de ces études sur les effets aigus des extraits, les travaux s'intéressant aux effets à faible dose et à long terme sont complémentaires car plus réalistes d'un point de environnemental. Ce changement d'échelle temporelle (de l'exposition aiguë vers l'exposition chronique) et d'échelle d'exposition aux toxiques (des concentrations aiguës vers des concentrations réalistes du point de vue de la contamination environnementale) pourraient permettre de mieux prendre en compte les effets chroniques des toxiques sur les organismes aquatiques dans l'évaluation du risque environnemental.

2.6.2 Toxicité des extraits à concentrations environnementales

A ce jour très peu de travaux ont été réalisés pour caractériser spécifiquement les effets toxiques à faible dose et à long terme des mélanges complexes de contaminants,

incluant ceux extraits d'échantillonneurs passifs principalement à cause des coûts engendrés par les expériences conduites sur plusieurs semaines et les difficultés techniques associées. A notre connaissance seules deux études ont suivi cette démarche, l'une en utilisant des extraits de SPMDs et l'autre de POCIS.

Petty et al. (2000) ont déployé des SPMDs sur un site localisé à proximité d'une usine de traitement des eaux fortement contaminé aux HAP, PCB et pesticides organochlorés puis ont exposé des truites arc en ciel (*Oncorhynchus mykiss*) aux extraits pendant 11 jours. Les auteurs ont ainsi pu mettre en évidence une augmentation de l'activité enzymatique au niveau du foie, une perturbation des systèmes de neurotransmission et une perturbation potentielle du système endocrinien chez les poissons exposés aux extraits.

Une étude s'est intéressée aux effets de pesticides en mélange à faible dose et à long terme sur le biofilm de rivière : Morin et al. (2012b) ont utilisé des canaux artificiels pour exposer des communautés périphytiques à des extraits de POCIS. Les résultats n'ont pas montré de changement sur la structure des communautés mais cependant une diminution globale de la tolérance avec une diminution de la pression de contamination. Cette étude a permis de souligner l'importance de maintenir les niveaux de contamination dans les systèmes expérimentaux et a mis en évidence l'intérêt que pouvait apporter une telle démarche de manière à mieux prendre en compte les effets chroniques de toxiques dans l'évaluation du risque environnemental.

Chapitre III : Matériels et méthodes

Liste des figures

3.1 Sites d'étude

Deux sites d'étude aux profils de contamination en pesticides différents ont été choisis lors de ce travail de thèse : le bassin versant de la Morcille située dans le Beaujolais et celui du Trec-Canaule situé dans le Lot et Garonne.

3.1.1 La rivière Morcille

Le bassin versant de l'Ardières-Morcille situé dans le Haut-Beaujolais couvre environ 220 km^2, ce site est étudié depuis de nombreuses années dans le cadre de différents programmes de recherche (programme de recherche Cemagref-« PestExpo », Onema-Cemagref action 26 « Remédiation de l'effet de pesticides », Zone Atelier du Bassin du Rhône (ZABR)) qui ont permis, entre autres, de mettre en évidence une forte contamination du milieu aquatique par des produits phytosanitaires. La Morcille (8km) est un petit affluent de l'Ardières caractérisé par son bassin versant (9,5km^2) fortement viticole, essentiellement forestier en amont et planté de vignes en aval (Figure 13).

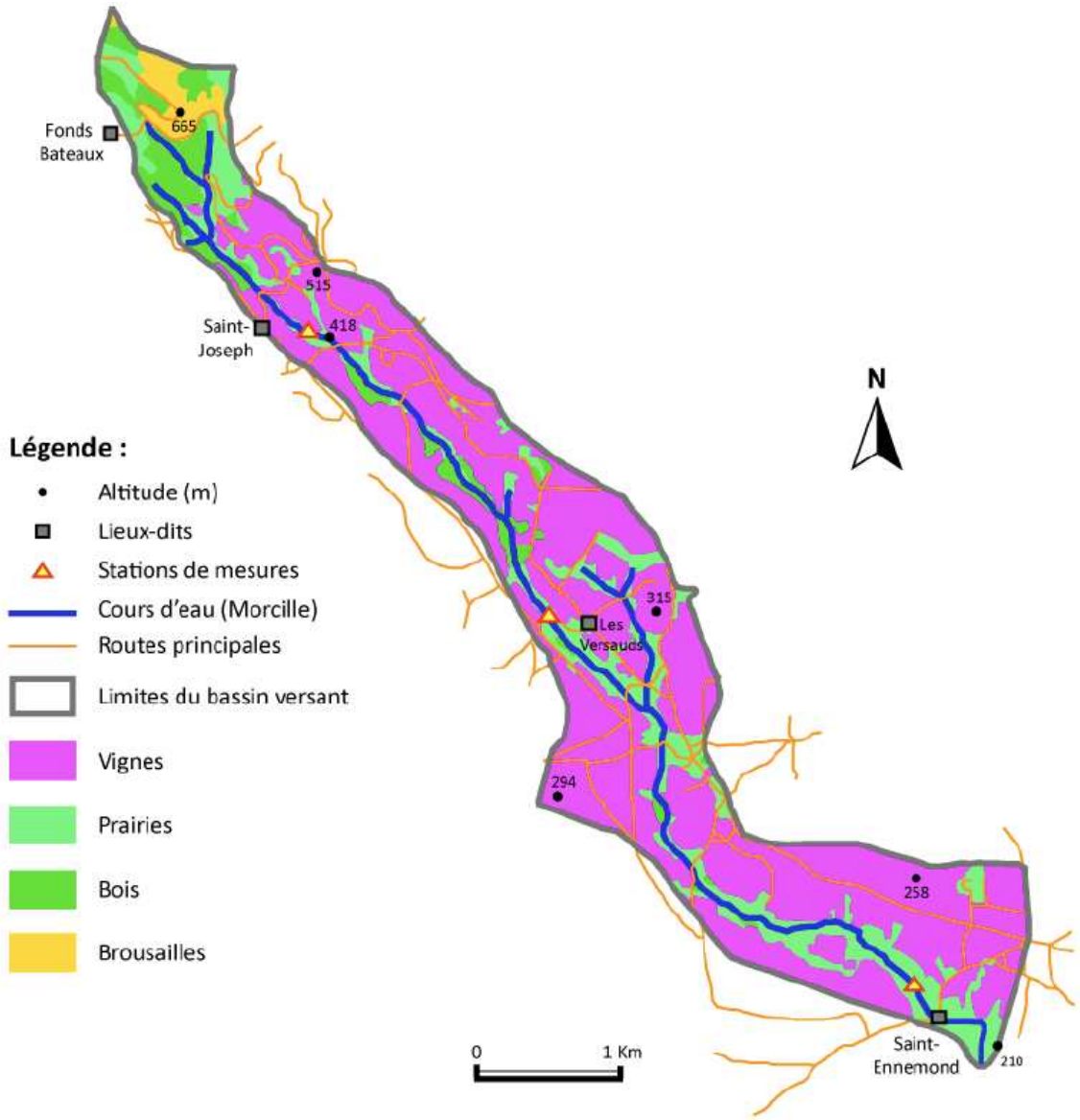

Figure 13: Assolement du bassin versant de la Morcille (Source : Marquier 2007)

Il en résulte un gradient de pression viticole amont-aval le long de la rivière Morcille (6,7% ; 51,6% et 79% de la surface du sous-bassin occupé par des vignes; respectivement pour les stations St Joseph, Les Versauds et St Ennemond) qui s'accompagne d'un gradient croissant des teneurs en pesticides retrouvées dans le cours d'eau (pesticides majoritairement à action herbicide mais aussi quelques fongicides : Montuelle et al. 2010). La contamination persiste toute l'année mais on observe un pic au printemps et pendant une partie de l'été (généralement de fin mars à début août). Les herbicides sont plutôt utilisés au printemps (avant la croissance des plantes) alors que les fongicides le sont plutôt durant l'été.

Les concentrations peuvent atteindre des niveaux importants (de l'ordre de plusieurs µg/l, Montuelle et al. 2010, Morin et al. 2012b, Rabiet et al. 2010) notamment lors des épisodes pluvieux et des crues.

D'autre part, un gradient amont-aval est également observé avec des métaux comme l'arsenic et le cuivre (Rabiet et al. 2008), le carbone organique dissous et les composés inorganiques azotés et phosphorés (Dorigo et al. 2007), comme c'est souvent le cas dans les bassins versants agricoles.

3.1.2 La rivière Trec

Le bassin versant du Trec-Canaule (202 km^2) fait partie de la petite région agricole des « Coteaux Nord du Lot-et-Garonne ». Il est étudié depuis quelques années dans le cadre du Programme d'Action Territoriale (PAT) Trec et Canaule qui a débuté en fin d'année 2008. Ce programme a pour objectif l'amélioration de la qualité de l'eau et du réseau hydrographique, il comporte une trentaine d'actions dont le suivi de la qualité des rivières. Il a permis d'établir une cartographie de l'occupation des sols ainsi que de générer des données sur la contamination en produits phytosanitaires des rivières (Figure 14).

Figure 14: Localisation des rivières le Trec et la Canaule appartenant au bassin versant du Trec-Canaule ainsi que du point de prélèvement « Trec » (carte Géoportail)

L'ensemble du bassin versant subit une pression agricole particulièrement marquée puisque la Surface Agricole Utile (SAU) représente près de 60% de la superficie totale du bassin versant. Les grandes cultures (blé et maïs) y représentent la majorité des activités agricoles (67% de la

SAU), on y trouve aussi des surfaces fourragères (10% de la SAU) ainsi que des cultures spécialisées comme les cultures maraîchères, l'arboriculture ou la viticulture (7, 4 et 1% de la SAU respectivement).

Ce bassin versant englobe deux cours d'eau : le Trec et la Canaule. Le Trec est un affluent en rive droite de la Garonne, qu'il rejoint à Marmande après un parcours de 24,4km.

Des concentrations particulièrement élevées en pesticides ont été mesurées à la station Trec aval Longueville située après la confluence entre le Trec et la Canaule avec une moyenne de 8,59 µg/l en pesticides totaux pour l'année 2009. Au sein des molécules les plus souvent détectées pour cette année, nous retrouvons 15 herbicides, 2 produits de dégradation, 1 fongicide et 1 insecticide. Parmi ces 15 herbicides, la plupart sont essentiellement utilisés pour le désherbage des grandes cultures. Le 2,4-D (herbicide), la bentazone (herbicide), la diméthénamide (herbicide), le métolachlore (herbicide), l'imidaclopride (insecticide) et le déséthyl atrazine (produit de dégradation de l'atrazine) sont les molécules avec la fréquence de détection la plus élevée (Duprat 2010).

3.2 Echantillonnage des communautés naturelles et des pesticides

3.2.1 Echantillonnage des communautés naturelles

Lors de cette thèse, deux techniques ont été utilisées pour échantillonner les communautés naturelles : la pose de substrats artificiels et le grattage de supports naturels.

Dans le premier cas, des lames de verre placées à l'intérieur de cagettes en plastique ont été utilisées comme support de colonisation pour les biofilms (Figure 15).

Figure 15: Supports artificiels pour la colonisation des biofilms.

Les cagettes ont été immergées pendant 28 jours sur les différents sites d'étude, puis transportées au laboratoire dans l'eau du site correspondant. Les lames ont ensuite été conservées dans l'eau du site enrichie avec les nutriments du milieu WC (Guillard et Lorenzen 1972) et soumises à une photopériode 12h/12H à 18°C jusqu'à utilisation lors des expérimentations en conditions contrôlées de laboratoire (sous 7 jours).

L'échantillonnage sur supports naturels a été réalisé par grattage de cailloux issus du site, les biofilms ont alors été remis en suspension dans l'eau du site de prélèvement, soumis à une photopériode 12h/12H à 18°C jusqu'à utilisation du biofilm lors des expériences d'exposition aux pesticides.

3.2.2 Echantillonnage des pesticides

Les pesticides ont été échantillonnés à l'aide du dispositif POCIS présenté précédemment et par des prélèvements d'eau ponctuels.

La préparation des POCIS ainsi que leur analyse a été réalisée par le laboratoire de chimie d'Irstea Bordeaux selon le protocole développé par Lissalde et al. (2011). Deux types de POCIS ont été utilisés lors de cette thèse, les POCIS « quantitatifs » et « accumulatifs ». Les premiers contiennent un composé de référence et de performance (PRC), la DIA-d5 (désisopropyl-atrazine deutérée) (Mazzella et al. 2007), ils sont utilisés pour obtenir une estimation moyenne des concentrations en pesticides dans les eaux. Les POCIS «accumulatifs» sont quant à eux destinés aux tests de toxicité qui seront réalisés au laboratoire et ne renferment pas de PRC. Les échantillonneurs passifs ont été placés à l'intérieur de cagettes en plastique et immergés pour une durée de 2x14 (POCIS « quantitatifs ») ou 28 jours (POCIS « accumulatifs ») sur les sites d'étude puis conservés à 4°C jusqu'à l'étape d'extraction permettant de récupérer les contaminants présents dans la phase pour le dosage ou les tests de toxicité. Pour les POCIS « quantitatifs », la phase (200mg) est alors récupérée dans des tubes d'extraction sur phase solide (SPE) de 3ml, l'élution est réalisée avec 6 ml de méthanol puis 6 ml d'un mélange méthanol/acétate d'éthyle (75/25). L'éluat est alors évaporé à 60°C en chaleur continue puis repris dans 1ml d'acétonitrile pour le dosage.

La phase (600mg) des POCIS « accumulatifs » est récupérée dans des tubes SPE de 6ml, l'élution est alors réalisée avec 10 ml de méthanol puis 10 ml d'un mélange méthanol/acétate d'éthyle (75/25), l'éluat est évaporé à sec et repris dans 10ml d'eau minérale. L'opération est répétée pour tous les POCIS « accumulatifs », les solutions sont alors rassemblées pour constituer la solution de contamination qui servira pour les tests de toxicité. Cette solution sera ensuite diluée selon les concentrations désirées lors des expériences.

Les calculs sont basés sur la détermination du volume total échantillonné (V_{tot}) calculé à partir du volume journalier théorique échantillonné par le POCIS « accumulatif » (e.g. 200mL/jour pour 600mg de phase) ($V_{POCIS,th}$), du nombre (n) et de la durée d'exposition (t) des POCIS sur le terrain par la relation :

$$V_{tot} = V_{POCIS,th} \times n \times t$$

Ainsi pour être concentré d'un facteur *F* par rapport à la concentration environnementale moyenne recherchée, le nombre *n* de POCIS devra être repris dans le volume $(\frac{1}{F})$ x V_{tot}.

Pour obtenir une concentration équivalente à la concentration environnementale moyenne, il faudra donc reprendre les *n* POCIS dans un volume égal au V_{tot} (avec $F = 1$, par exemple pour réaliser une exposition à des concentrations réalistes par rapport à la contamination environnementale). Au contraire pour réaliser une exposition à des concentrations élevées, les *n* POCIS devront être repris dans un volume beaucoup moins important. Pour obtenir par exemple une solution en moyenne 300 fois plus concentrée par rapport à la concentration environnementale, alors $F = 300$ et les *n* POCIS devront être repris dans un volume égal à $(\frac{1}{300})$ x V_{tot}.

Une solution « Témoin solvant » sera également utilisée lors des tests de toxicité. Cette solution est obtenue selon le même protocole que pour les POCIS « accumulatifs » en ajoutant de la phase HLB vierge (en même quantité) dans les cartouches d'élution.

Des analyses sur les échantillons d'eau ont également été réalisées de manière à quantifier les pesticides non échantillonnés par le POCIS ou dont le R_s n'est pas déterminé. La phase d'extraction sur phase solide a été réalisée avec les systèmes Visiprep™ et Visidry™ (Supelco) : 100 ml d'échantillon sont filtrés sur filtres GF/F (0,7 µm, Whatman), le pH est ajusté à 7 puis 10 µl d'une solution d'étalons internes sont ajoutés avant l'étape d'extraction. Les cartouches sont conditionnées avec 5 ml de méthanol et 5 ml d'eau ultra pure. 50 ml d'échantillon d'eau sont alors passés sur cartouche et 5 ml d'eau ultra pure (avec 5% de méthanol) sont utilisés pour rincer la cartouche. Les cartouches sont alors séchées 15 min sous jet d'azote et stockées à 4°C avant l'étape d'élution. L'élution est réalisée avec 3 ml de méthanol puis 3 ml d'un mélange méthanol/acétate d'éthyle (75/25). Finalement, 10 µl d'un mélange contenant les standards analytiques sont ajoutés avant évaporation à sec. L'extrait est alors reconstitué avec 1 ml de l'éluant initial HPLC (acétonitrile/eau ultra pure 90/10).

3.3 Expérimentations en conditions contrôlées de laboratoire

3.3.1 Conditions générales d'exposition

Les conditions d'exposition et les différents systèmes expérimentaux utilisés lors de cette thèse seront détaillés dans la partie « Matériels et Méthodes » spécifique à chacun des

chapitres suivants. Brièvement, les expositions de biofilms aux extraits de POCIS ont été réalisées en canaux artificiels alors que les expositions aux molécules seules ou en mélange simple ont été réalisées soit en microplaques soit dans des unités expérimentales type flacons en verre.

3.3.2 Paramètres physicochimiques

Les paramètres physicochimiques tels que le pH, la conductivité, la concentration en oxygène dissous et la température ont été suivis au cours des différentes expériences à l'aide du pH-mètre pH 3110 (WTW), du conductimètre LF 340 (WTW) et de l'oxymétre Oxi 340i (WTW). La température a été déterminée en moyennant les valeurs données par les trois appareils. Les concentrations en nitrates, orthophosphates et silice ont été déterminées par le laboratoire de chimie des eaux d'Irstea Bordeaux selon les normes NF EN ISO 13395 10/96 à l'aide de l'Auto-analyser Evolution II Alliance pour les nitrates et selon les normes NF T90-023 09/82 et NF T 90-007 02/01 pour les orthophosphates et la silice respectivement et en utilisant le spectromètre Lambda 2 Perkin Elmer.

3.3.3 Dosage des pesticides

Le dosage des pesticides dans les extraits issus de POCIS et SPE (eaux) provenant de la Morcille, du Trec et des expérimentations en canaux a été effectué par les laboratoires de chimie d'Irstea Bordeaux (UR REBX) et Irstea Lyon (UR MALY), d'après la méthode développée par Lissalde et al. (2011). Les échantillons ont été analysés par chromatographie liquide couplée à un spectromètre de masse en tandem (HPLC–ESI–MS/MS) au moyen d'une chaîne HPLC Ultimate 3000 (Dionex) équipée avec une colonne Gemini NX C18, 10 mm x 2 mm, 3 µm (Phenomenex) et un triple quadrupole API 2000 (AB Sciex), ainsi que par chromatographie gazeuse couplée à un spectromètre de masse en tandem (GC–MS/MS) du type Quantum GC (Thermo) équipé avec une colonne Rxi-5MS 30 m x 0.25 mm x 0.25 µm (Restek). Les concentrations en pesticides dans les eaux de la Morcille ont été également déterminées après extractions SPE sur cartouches Oasis HLB à l'Irstea de Lyon, suivies par une analyse HPLC–ESI–MS/MS avec une chaîne HPLC 1100 (Agilent) et un triple quadrupole API 4000 (AB Sciex). La séparation chromatographique a été réalisée avec une colonne analytique Atlantis T3, 3µm, 2.1 mm x 100 mm (Waters, France) (Assoumani et al. 2013).

Les méthodes analytiques ont été validées en termes de linéarité de la fonction d'étalonnage, de spécificité, du taux de récupération de l'extraction et de la limite de quantification selon la norme française NF T90-210.
De plus, des blancs laboratoire (SPE et POCIS) et terrain (POCIS uniquement) ont été réalisés régulièrement, ainsi que des contrôles qualité lors des séquences d'analyse ou des étapes d'extractions (eau minérale dopée).

3.3.4 Evaluation de la croissance des communautés

3.3.4.1 Estimation de la densité de diatomées

Le comptage des diatomées est réalisé à partir des échantillons formolés sous grossissement x400 grâce à un microscope photonique LEITZ à l'aide d'une cellule de comptage de type Nageotte (Marienfeld, Germany). Les échantillons sont passés au vortex puis homogénéisés par pipetages successifs et éventuellement dilués au besoin selon leur concentration. Un aliquote de 200µL est déposé au centre de la cellule de comptage et recouvert par une lamelle planée. Les cellules vivantes et mortes sont dénombrées dans 10 champs de la zone quadrillée (1,25 µL chacune, 0,5 mm de profondeur). La distinction entre les cellules vivantes et mortes a été estimée par observation de la turgescence et de la couleur des chloroplastes.
La densité des diatomées exprimée en individus par cm^2, est alors calculée selon la formule suivante :

$$N = ((A \times B) / (10 \times 1{,}25.10^{-3})) / (C/S)$$

N = densité moyenne en cellules par cm^2
A = nombre de cellules dénombrées dans 10 champs
B = facteur de dilution
S = Surface grattée en cm^2
C = Volume de reprise en ml
10 = nombre de champs comptés
$1{,}25.10^{-3}$ = volume d'un champ en mL

3.3.4.2 Mesure de la concentration en chlorophylle a

La concentration en chlorophylle a est estimée par la technique de fluorescence en lumière modulée à l'aide du fluorimètre Phyto-PAM (Heinz Walz GmbH, Germany). Dans

une large gamme de teneur en chlorophylle, l'intensité de fluorescence est proportionnelle à la concentration en chlorophylle. Les mesures ont été réalisées avec la calibration par défaut de l'appareil.

3.3.4.3 Mesure de la biomasse

Le poids sec (PS) et la matière sèche sans cendre (MSSC) ont été déterminés suivant la norme NF EN 872. Les filtres en fibre de verre (taille des pores : 1,2 µm ; GF/C Whatman), sont passés à l'étuve à une température de 100°C pendant une heure. Ils sont ensuite pesés après équilibration à température ambiante dans un dessiccateur pendant au moins 15 minutes (détermination de m_{filtre}). Les échantillons sont alors filtrés sous vide, passés à l'étuve à 105°C pendant une heure, puis pesés après équilibration à température ambiante dans un dessiccateur pendant au moins 15 minutes (détermination de $m_{étuve}$).Les échantillons sont alors passés une heure au four à 500°C puis pesés après équilibration à température ambiante dans un dessicateur pendant au moins 15 minutes (détermination de m_{four}).
Le PS et la MSSC sont déterminés selon les formules suivantes :

$$PS\ (mg/cm^2) = (m_{étuve} - m_{filtre}) / S$$

et

$$MSSC\ (mg/cm^2) = PS \times (1-((m_{four} - m_{filtre})/(m_{étuve} - m_{filtre}))$$

$m_{étuve}$ = masse du filtre après filtration et passage à l'étuve en mg
m_{filtre} = masse du filtre avant filtration en mg
m_{four} = masse du filtre après passage au four en mg
S = surface grattée en cm^2

3.3.5 Analyse qualitative et quantitative de la structure des communautés

3.3.5.1 Groupes algaux

Les groupes algaux sont déterminés par la technique de fluorescence en lumière modulée à l'aide du fluorimètre Phyto-PAM (Heinz Walz GmbH, Germany). La distinction entre les différents groupes algaux est basée sur la mesure de la fluorescence émise après excitation à différentes longueurs d'ondes. Les quatre longueurs d'ondes utilisées (470, 520, 645 et 665 nm) sont choisies de manière à permettre une différenciation optimale entre cyanobactéries, algues vertes et diatomées. En effet, pour un échantillon ne contenant que des cyanobactéries presque aucun signal ne sera détecté avec une longueur d'excitation de 470nm

(pas de chlorophylle b) alors qu'un large signal sera détecté pour une longueur d'excitation de 645nm (due à l'absorption de l'allophycocyanine). Un échantillon d'algues vertes montrera quant à lui un large signal à une longueur d'onde d'excitation de 470nm (présence de chlorophylle b) et un faible signal avec une longueur d'onde d'excitation de 520nm. Au contraire les diatomées montreront un signal non seulement à 470nm mais aussi à 520nm d'excitation due à l'absorption de la chlorophylle c, de la fucoxanthine et des caroténoïdes.

3.3.5.2 Détermination de la composition spécifique des communautés de diatomées

Ces analyses ont été effectuées par le laboratoire de phyto-écologie d'Irstea Bordeaux (UR REBX).

L'identification des diatomées présentes dans les échantillons de périphyton (préalablement fixés) est réalisée d'après l'examen microscopique de leur squelette siliceux. Le protocole normalisé AFNOR (NF EN 13946) consiste en une première étape de digestion de la matière organique par un traitement des échantillons à l'eau oxygénée (H_2O_2, 30%) bouillante de 10 minutes. Un deuxième traitement, à l'acide chlorhydrique (HCl, 35%) bouillant pendant 5 minutes est parfois nécessaire pour obtenir un nettoyage satisfaisant des frustules. Ensuite, les résidus d'eau oxygénée et d'acide sont éliminés par des cycles successifs de centrifugations (5 minutes à 2500g) et de rinçages à l'eau distillée. Une partie aliquote est déposée sur une lamelle propre et déshydratée par séchage. La lamelle est ensuite retournée sur une goutte de résine réfringente, le Naphrax® (Brunel Microscopes Ltd, UK), déposée au préalable sur une lame, le chauffage permet de chasser les bulles d'air et d'aboutir à la fabrication de lames permanentes.

L'identification des diatomées se fait par l'observation en microscopie photonique (au grossissement x1000) des lames préparées, selon la norme NF EN 14407 (2004). L'effort de détermination porte sur 400 valves au minimum.

3.3.6 Analyse du fonctionnement des communautés

3.3.6.1 Activités enzymatiques antioxydantes

Les enzymatiques antioxydantes sont utilisées comme biomarqueur du stress oxydant du biofilm (Bonnineau 2011). Quand la balance redox est déséquilibrée, différentes enzymes interviennent pour réguler la production des espèces réactives de l'oxygène (EROs) (Figure 16).

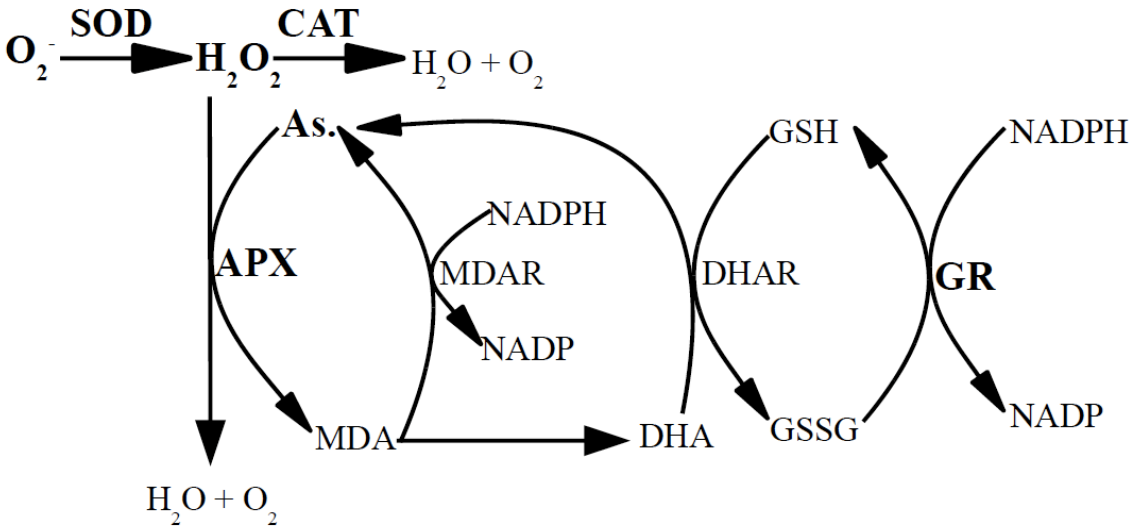

Figure 16: Système de détoxification du radical superoxyde (O_2^-) et du peroxyde d'hydrogène (H_2O_2) par les activités enzymatiques antioxydantes : superoxyde dismutase (SOD), catalase (CAT), ascorbate peroxydase (APX) et glutathion réductase (GR). Autres abréviations: As., ascorbate; MDA, monodéhydroascorbate; MDAR, MDA réductase; DHA, déhydroascorbate; DHAR, DHA réductase; GSH, glutathion; GSSG, glutathion oxydé (dans Bonnineau 2011, modifié d'après Noctor et Foyer 1998)

Le dosage des activités enzymatiques antioxydantes est réalisé pour la catalase (CAT), la glutathion réductase (GR), la glutathion-S-transférase (GST) et l'ascorbate péroxydase (APX) selon le protocole développé par l'équipe de l'Institut d'Ecologie Aquatique de l'Université de Girona en Espagne (Bonnineau et al. 2013, Bonet et al. 2013).

Après échantillonnage, les biofilms sont centrifugés à 2300 g pendant 5 min pour éliminer l'excès d'eau, immédiatement plongés dans l'azote liquide puis conservés dans l'azote liquide jusqu'à l'étape d'extraction.

Les enzymes étant des protéines très sensibles à la chaleur, les échantillons doivent être conservés le plus possible dans la glace pendant toutes les étapes, de l'extraction jusqu'au dosage enzymatique.

- ✓ Extraction des protéines totales

200µL de tampon d'extraction (100 mM Na_2HPO_4/KH_2PO_4, pH 7,4, 100 mM KCl, 1 mM EDTA) sont ajoutés pour chaque 100 mg d'échantillon humide, deux pulses de 30s avec une minute d'intervalle dans la glace sont alors appliqués aux échantillons à l'aide d'un homogéniseur (PT 1600E, Kinematica). Les échantillons sont ensuite vortexés trois fois 30s avec deux min d'intervalle dans la glace après l'ajout de billes de verre dans une proportion de 100mg de billes pour 100mg de biofilm (masse humide). Les échantillons sont alors

centrifugés pendant 30 min à 10 000 g et le surnageant contenant l'extrait de protéines récupéré.

- ✓ Dosage des protéines totales

Le dosage des protéines totales a été réalisé avec le lecteur Synergy4 (BioTek®) selon la méthode de Bradford (1976). Trois réplicats analytiques sont réalisés pour chaque échantillon. 5µl de surnageant et 200µl de réactif de Bradford (Bio-Rad Laboratories GmbH, Germany) sont introduits dans chaque puits de la microplaque. Le sérum d'albumine bovine est utilisé comme standard analytique. L'absorbance du Bleu de Coomassie est modifiée par les liaisons avec l'arginine et les acides aminés hydrophobes présents dans les protéines. La teinte passe du rouge (absorbance à 470nm) au bleu (absorbance à 595nm) en présence de protéines.

- ✓ Détermination des activités enzymatiques antioxydantes (AEAs)

Les mesures d'AEAs ont été réalisées en microplaques (UV-Star 96 well plate, Greiner®), l'évolution de l'absorbance a été suivie à l'aide du spectromètre Synergy4 (BioTek®). Chaque dosage enzymatique a été réalisé avec 2µg de protéines. Les AEAs étant dépendantes de la concentration en substrat, une étape d'optimisation est nécessaire pour chaque biofilm d'origine et/ou d'exposition aux pesticides différents. Plusieurs concentrations en substrat ont donc été testées pour chaque activité enzymatique et pour les différents biofilms.

- <u>L'activité de la catalase (CAT)</u> a été mesurée en suivant la décomposition de H_2O_2 à 240 nm et 25ºC pendant 2 min (Aebi 1984). La concentration optimale en substrat a été déterminée en testant les concentrations suivantes: 10, 15, 20, 30 et 40 mM de H_2O_2. La réaction est réalisée dans un volume final de 250µl, chaque puits contient l'extrait enzymatique (2µg prot), un tampon Na_2HPO_4/KH_2PO_4 (pH 7,0) (80 mM concentration finale) et H_2O_2. La réaction est initiée avec l'ajout de H_2O_2. L'activité de la catalase est calculée en µmol H_2O_2 µg $prot^{-1}min^{-1}$ (coefficient d'extinction ε = 0,039 cm^2 μmol^{-1}).

- <u>L'activité de la glutathion réductase (GR)</u> a été déterminée en suivant l'oxydation de NADPH à 340 nm et 25ºC pendant 2 min (Schaedle et Bassham 1977). La concentration optimale en substrat a été déterminée en testant les concentrations: 0,10 ; 0,15 ; 0,20 ; 0,25 et 0,30 mM de NADPH. La réaction est réalisée dans un volume final de 200µl, chaque puits contient l'extrait enzymatique (2µg prot), un tampon Tris-HCl (pH 7,5) 100 mM, EDTA

1mM, 1 mM de glutathion oxydé (GSSG) et NADPH. La réaction est initiée avec l'ajout de NADPH. L'activité de la GR est calculée en µmol NADPH µg $prot^{-1}min^{-1}$ (coefficient d'extinction ε = 6,22 cm^2 μmol^{-1}).

-L'activité de la glutathion-S-transférase (GST) a été mesurée en suivant la formation du conjugué CDNB-glutathion à 340 nm et 25ºC pendant 4 min (Grant et al. 1989). La glutathion-S-transférase combine le CDNB avec le glutathion oxydé (GSSG) pour former un conjugué CDNB-glutathion qui absorbe à 340 nm. Le conjugué peut aussi se former par voie non-enzymatique il est donc important de mesurer l'absorbance de blanc (sans extrait enzymatique). La concentration optimale en substrat a été déterminée en testant les concentrations: 0,875 ; 3,5 ; 7 ; 8,75 ; 12,25 et 15,75 mM de GSH. La réaction est réalisée dans un tampon KH_2PO_4/K_2HPO_2 (pH 7,4), 100mM, CDNB et dans un volume final de 200 µL. La réaction est initiée avec l'ajout de GSH. L'activité de la GST est calculée en µmol GST µg $prot^{-1}min^{-1}$ (coefficient d'extinction ε = 9.6 cm^2 μmol^{-1}).

-L'activité de l'ascorbate péroxydase (APX) a été mesurée en suivant la décomposition de H_2O_2 à 290 nm et 25ºC pendant 1,5 min (Nakano et Asada 1981). La concentration optimale en substrat à été déterminée en testant les concentrations suivantes: 1, 2, 3, 4 et 5 mM de H_2O_2. La réaction est réalisée dans un volume final de 250µl, chaque puit contient l'extrait enzymatique (2µg prot), du tampon Na_2HPO_4/KH_2PO_4 (pH 7,0) (80 mM concentration finale), Na-Ascorbate (150 µM) et H_2O_2 pour initier la réaction. L'activité de l'ascorbate péroxydase est calculée en µmol H_2O_2 µg $prot^{-1}min^{-1}$ (coefficient d'extinction ε = 2,8 cm^2 μmol^{-1}).

3.3.6.2 Mesure de la fluorescence chlorophyllienne en lumière modulée

Les organismes photosynthétiques sont capables de convertir l'énergie lumineuse en énergie chimique. Les photons sont capturés au niveau des antennes collectrices des photosystèmes, l'énergie d'excitation est alors transmise aux centres réactionnels où s'effectue la conversion de l'énergie lumineuse en énergie chimique. Lorsqu'une molécule de chlorophylle reçoit de l'énergie lumineuse, elle passe alors dans un état excité instable qui correspond à un niveau énergétique supérieur. Cette énergie est alors transmise de proche en proche jusqu'au cœur du centre réactionnel constitué par des chlorophylles a spéciales que l'on appelle P680. Lorsque la P680 est excitée, elle peut transmettre un électron via une phéophytine à une quinone fortement liée au cœur du PSII (Q_A), puis à une plastoquinone qui

s'attache temporairement au PSII (Q_B). P680 oxydée récupère alors son électron d'une tyrosine de la protéine D1.

La lumière absorbée par l'appareil photosynthétique n'est pas totalement transformée en énergie chimique, elle peut être dissipée sous forme de chaleur ou bien réémise sous forme de fluorescence. Le transfert d'énergie d'une molécule de chlorophylle excitée à une autre molécule de chlorophylle se fait en 10^{-11} secondes, les phénomènes qui conduisent à la dissipation de l'énergie sous forme de chaleur ou de fluorescence ont lieu quant à eux en 10^{-9} secondes. La probabilité de transfert de l'excitation à une autre molécule de chlorophylle est donc beaucoup plus grande que celle d'une dissipation de l'énergie sous forme de chaleur ou de fluorescence.

Ces trois voies sont compétitives, ainsi à travers l'étude de l'une d'entre elles (la fluorescence dans notre cas) nous allons pouvoir obtenir des informations sur les deux autres.

Les mesures de fluorescence en lumière modulée ont été réalisées à l'aide d'un fluorimètre Phyto-PAM (Heinz Walz GmbH, Germany). Le flux lumineux émis par fluorescence dépend de la lumière absorbée par le PSII, ce qui rend difficile les mesures dans le cas où la luminosité varie (en plein champ par exemple). L'utilisation d'une lumière modulée permet de pallier ce problème : en effet l'application d'une lumière modulée provoque l'émission d'une florescence modulée. La lumière modulée utilisée pour les mesures est produite par une diode et filtrée pour couper les longueurs d'onde au dessus de 680nm, seule la florescence modulée selon les mêmes caractéristiques est alors amplifiée (Figure 17).

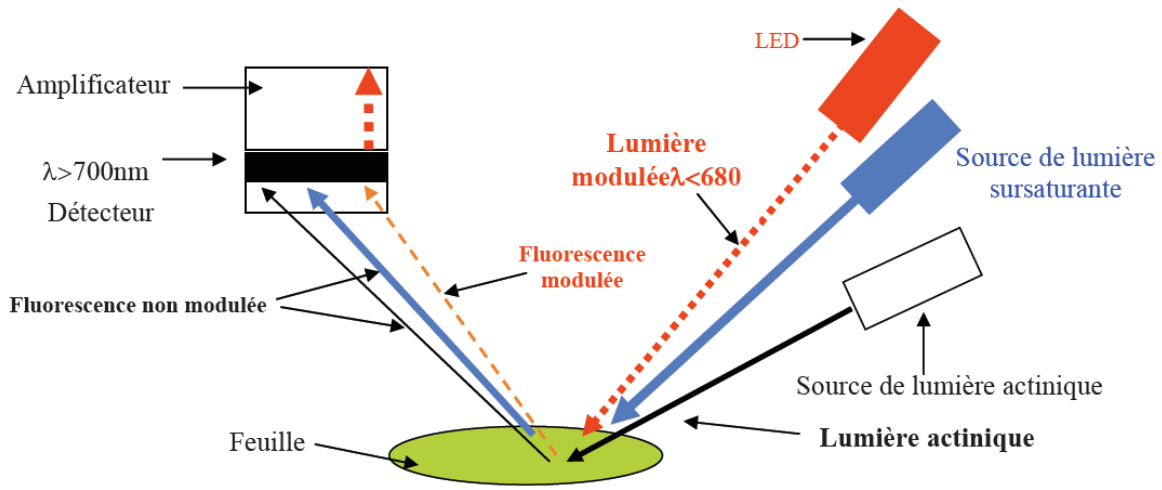

Figure 17: Schéma de la technique de mesure de l'émission de la fluorescence modulée de la chlorophylle d'une feuille. La lumière modulée est émise par une LED à une longueur d'onde inférieure à 680 nm. Il lui correspond une fluorescence modulée qui, seule, est amplifiée par un amplificateur sélectif accordé sur la

fréquence de modulation de la lumière modulée. La lumière actinique produit aussi une émission de fluorescence par la feuille. Cette émission n'est pas amplifiée. Une source de lumière sursaturante non modulée ferme tous les centres et produit aussi de la fluorescence non modulée qui n'est donc pas amplifiée. La lumière sursaturante est aussi une lumière actinique. (d'après Cornic 2007)

Le fluorimètre Phyto-PAM permet de mesurer le rendement quantique de la photochimie du photosystème II qui représente la probabilité pour qu'une excitation induite par un photon se désactive par la voie photochimique. Si l'échantillon est adapté à l'obscurité, on mesure le rendement quantique maximal de la photochimie du PSII (F_v/F_m), chez les plantes supérieures il est compris entre 0,84 et 0,80. Si les mesures sont réalisées sous la lumière on mesure alors le rendement quantique efficace de la photochimie du PSII (Φ_{PSII}) dans la plupart des cas F_v/F_m est supérieur à Φ_{PSII}, cependant il existe des exceptions chez les diatomées. Les mesures de fluorescence permettent aussi de calculer la vitesse relative de transfert des électrons au niveau du PSII (rETR).

La variation de la vitesse relative de transfert des électrons au niveau du PSII (rETR) en fonction de l'irradiance est étudiée pour estimer les capacités photosynthétiques ainsi que les paramètres de saturation lumineuse d'un échantillon.

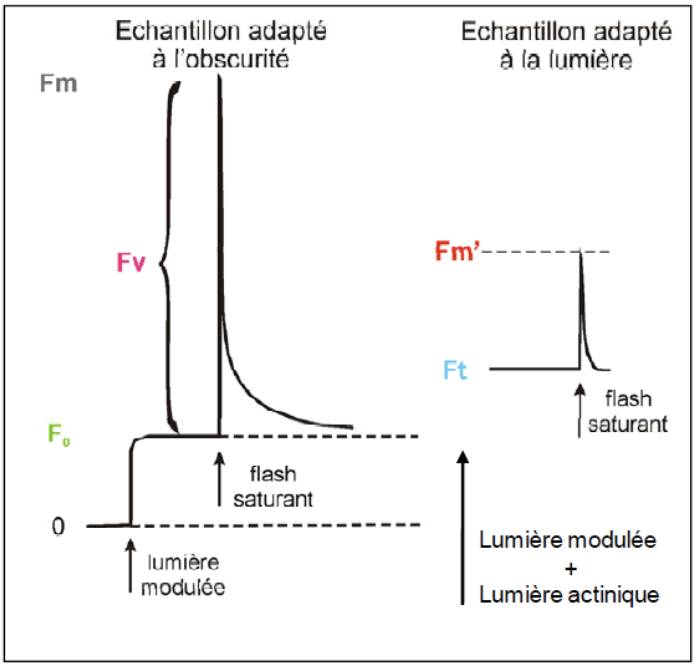

Figure 18: Principe de la technique de mesure de la fluorescence chlorophyllienne en lumière modulée (d'après Gévaert 2001)

En pratique ces paramètres sont mesurés comme suit :

- ✓ Le rendement quantique optimal du PSII (F_v/F_m) :

L'adaptation de l'échantillon à l'obscurité permet le retour à un état ouvert de tous les centres réactionnels (accepteurs primaires Q_A oxydés). Une lumière modulée (ML) d'intensité constante et faible, et qui ne permet pas la mise en route des mécanismes photochimiques, est appliquée sur l'échantillon. Cette lumière produit une émission de fluorescence de base F_0. Si nous appliquons alors un flash de lumière sursaturante (LSS) tous les centres réactionnels se ferment, la voie photochimique est ainsi bloquée. La dissipation de l'énergie en excès sous forme de chaleur quand à elle est négligeable car plus lente à se mettre en place : nous mesurons alors la fluorescence maximale F_m. Le rendement quantique optimal de la photochimie du PSII (F_v/F_m) est alors calculé de la manière suivante :

$$F_v/F_m = (F_m - F_0) / F_m$$

- ✓ Le rendement quantique efficace du PSII (Φ_{PSII}) :

Lorsqu'on échantillon est exposé à une lumière actinique (LA), les processus photochimiques se mettent en route, après plusieurs minutes d'acclimatation à cette lumière un équilibre s'établit entre le nombre de centres réactionnels ouverts et fermés. Si nous appliquons alors un flash de lumière sursaturante, les centres réactionnels ouverts se ferment : nous mesurons alors $F_{0'}$ et $F_{m'}$, correspondant respectivement à l'émission de fluorescence juste avant et pendant l'application du flash lumineux.

Le rendement quantique efficace de la photochimie du PSII (Φ_{PSII}) est alors calculé de la manière suivante :

$$\Phi_{PSII} = (F_{m'} - F_{0'}) / F_{m'}$$

- ✓ La vitesse relative de transfert des électrons au niveau du PSII (rETR) :

Ce paramètre est couramment utilisé pour estimer l'activité photosynthétique, il est définit par l'équation suivante :

$$rETR = \Phi_{PSII} \times I \times 0,5 \times 0,84$$

où I et Φ_{PSII} correspondent respectivement à la valeur de l'irradiance incidente et au rendement photosynthétique efficace comme définit précédemment. Le facteur 0,5 est lié à l'hypothèse selon laquelle l'énergie lumineuse est également répartie entre le photosystème I et le photosystème II, alors que seule la contribution de la fluorescence émise par le PSII est prise en compte lors des mesures (Maxwell et Johnson 2000). En effet, à température ambiante l'émission de fluorescence chlorophyllienne provient essentiellement de l'antenne

collectrice du photosystème II (PSII), l'émission de fluorescence par le photosystème I (PSI) est faible par rapport à celle émise par le PSII et ne représente au plus que de 10 à 20% de l'émission totale. Le facteur 0,84 correspond à la fraction de lumière absorbée (Björkman et Demmig 1987).

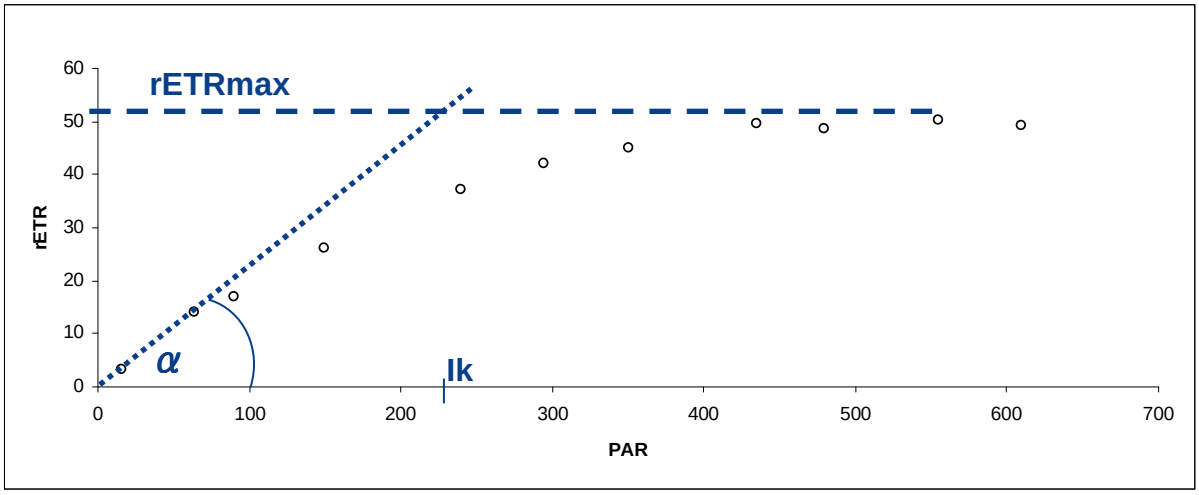

Figure 19: Exemple de courbe étudiant la variation du rETR en fonction de l'irradiance et de ses paramètres photosynthétiques associés

La réalisation de courbes étudiant la variation du rETR en fonction de l'irradiance (Figure 19) permet d'extraire différents paramètres :

- La pente initiale de la courbe (α) représente l'efficacité photosynthétique à faible irradiance.
- L'intensité lumineuse à partir de laquelle la relation n'est plus linéaire (I_k).
- La valeur maximale du rETR représente l'activité photosynthétique maximale ($rETR_{max}$).

3.4 Analyses statistiques

Différentes analyses statistiques ont été réalisées à travers les expériences menées tout au long de cette thèse, elles seront spécifiées dans la partie « Matériels et méthodes » de chacun des chapitres suivants.

Afin de révéler les différences statistiques entre échantillons les analyses ANOVA ont été utilisées suivies d'un test post-hoc HSD de Tuckey (pour les données normalement distribuées) à l'aide du logiciel STATISTICA 6.1 (Statsoft). Dans le cas de la distribution non

normale des données les tests non paramétriques de Kruskall Wallis et de comparaison 2 à 2 de Mann Whitney ont été appliqués.

Les courbes dose-réponse ainsi que les EC_{10}, EC_{25} et EC_{50} ont été calculées en utilisant la macro REGTOX EV 7.0.5. (Vindimian 2003) sur Excel et la fonction *drm* de la librairie *drc* pour R (Ritz et Streibig 2005).

Les différences entre échantillons au regard de l'abondance relative des espèces de diatomées ont été décrites à l'aide d'analyses en composantes principales (ACP) et de dendrogrammes. Les ACP ont également été utilisées pour mettre en évidence les différences entre échantillons à partir de jeux de données contenant des variables biologiques comme le biovolume, la densité cellulaire, le poids sec, la matière sèche sans cendre ou encore le rendement photosynthétique. Les ACP et les dendrogrammes ont été réalisés à l'aide des fonctions *dudi.pca* et *hclust* respectivement de la librairie *Ade4* pour R (Dray et Dufour 2007).

Chapitre IV : Effets d’un mélange réaliste de pesticides sur des biofilms de rivière aux histoires d’exposition différentes

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A ce jour, deux grands types d'approches ont été utilisés pour décrire les impacts de mélanges de contaminants à des concentrations réalistes sur le périphyton. Les approches de translocation offrent une représentativité importante puisque les organismes sont directement soumis à la contamination *in situ*. Cependant il peut parfois être difficile de comprendre les relations de causalité entre la présence de ces contaminants et les réponses des biofilms puisqu'un grand nombre de paramètres n'est pas connu de l'expérimentateur. Au contraire les expositions au laboratoire permettent de contrôler certains de ces paramètres (lumière, température, vitesse du courant, concentrations en toxiques,...). Néanmoins les études menées jusque là ne se sont intéressées, au mieux, qu'à un cocktail restreint de contaminants.

Le premier objectif de ce travail de thèse consiste à évaluer les réponses de biofilms de rivière aux histoires d'exposition différentes face à un mélange complexe de pesticides constitué par des extraits de POCIS.

Pour cela une expérience en conditions contrôlées de laboratoire a été réalisée. Dans un premier temps des supports artificiels ont été posés sur la rivière Morcille (site présenté au chapitre III) au printemps 2011 pour permettre la colonisation par les biofilms naturels. Deux sites aux concentrations en pesticides contrastées ont été choisis : la station St Joseph (située en amont) peut être considérée comme un site de référence, au contraire le site de St Ennemond (située en aval) subit des contaminations chroniques en pesticides. En parallèle les POCIS ont été exposés sur le site contaminé aux mêmes dates.
Les biofilms ont alors été exposés en canaux artificiels aux extraits de POCIS. Plusieurs modalités ont été réalisées, les biofilms originaires de St Joseph ont été exposés aux extraits de POCIS ou maintenus dans de l'eau sans pesticide pour modéliser l'apparition d'une contamination aux pesticides ou non. Les biofilms de St Ennemond ont, quand à eux, été exposés aux extraits de POCIS ou placés dans de l'eau sans pesticides pour modéliser un maintien ou une levée de pression toxique respectivement.

Différents paramètres ont été suivis tout au long des 13 jours d'exposition en canaux. Tout d'abord la tolérance acquise des biofilms *in situ* a été évaluée via des tests d'inhibition à court terme basés sur l'activité photosynthétique et réalisés avec les extraits de POCIS.
Les impacts au cours du temps des extraits de POCIS sur les biofilms soumis aux différents traitements ont été évalués par des mesures de biomasse, de fluorescence en lumière modulée

(détermination des groupes algaux et rendement photosynthétique), de densités de diatomées et par des analyses taxonomiques.

Les résultats obtenus montrent que la sensibilité aux extraits de POCIS des biofilms de l'amont est supérieure à celle des biofilms de l'aval, révélant l'histoire d'exposition *in situ* des biofilms aux pesticides et soulignant ainsi le concept PICT (ou l'acquisition de tolérance induite proposée par Blanck et al. 1988). L'exposition chronique aux extraits de POCIS en canaux artificiels a mis en évidence des effets significatifs sur la croissance au regard de la biomasse (PS et MSSC) et de la densité en diatomées ainsi que sur la structure du biofilm avec des impacts sur les niveaux de fluorescence et la composition des assemblages de diatomées. La réponse de certains de ces paramètres varie en fonction de l'origine des biofilms, mettant ainsi en évidence le rôle crucial de leur exposition passée. De plus, au vu des résultats obtenus lors de cette expérience il est apparu que l'utilisation des niveaux de fluorescence pour mesurer la croissance globale du biofilm et le pourcentage relatif des différents groupes algaux doit être considérée avec précaution dans les études s'intéressant aux inhibiteurs du PSII. Nous avons aussi souligné que les conclusions à propos des assemblages de diatomées doivent considérer la sélectivité des échantillonneurs passifs d'une part, et le rôle de l'immigration d'espèces d'autre part.
Notre étude montre l'importance de la prise en compte des effets chroniques de pesticides en mélange dans les études sur le risque environnemental et la pertinence de l'utilisation des extraits d'échantillonneurs passifs (comme le POCIS) dans cette approche.

Environmental effects of realistic pesticide mixtures on natural biofilm communities with different exposure histories

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Abstract

Impact of the changes in toxicant pressure to attached microbial communities from different origins is generally assessed using translocation experiments. Nevertheless these approaches do not allow to clearly identify to what extent the toxicant of interest is responsible for the observed effects on biofilms, since abiotic (light, temperature, current velocity…) and biotic (species drift) parameters are not controlled and multi contaminations often occur. This study deals with the use of Polar Organic Chemical Integrative Sampler (POCIS) extracts to assess the impact of low-dose pesticide mixtures on natural biofilm communities originating either from a chronically contaminated or a reference field site. To investigate how biofilm communities from the wild, pre-exposed to pesticides *in situ* or not might respond to environmentally realistic changes in pesticide pressure, they were exposed to either clean water or to POCIS extracts in order to represent toxic pressure with a realistic pesticide mixture directly isolated from the field. Chronic effects of low-dose of pesticides were assessed after 0, 3, 7 and 13 days of exposure in artificial channels. Diatom growth kinetics, dry weight, ash-free dry mass, algal fluorescence-related parameters, effective quantum yield of Photosystem II and diatom community structure were determined. The use of POCIS extracts allowed us to highlight chronic impacts of low doses of a mixture of pesticides on natural communities with regard to biofilm exposure history.

Key words

Pesticides, periphyton, passive samplers, diatom assemblages, low-dose exposure, mixture effects, environmental risk assessment.

1. Introduction

The European Union's Water Framework Directive has set the target to achieve good ecological status for all aquatic environments in Europe by 2015. In this context, there is an increasing need to develop appropriate diagnostic tools for water quality assessment. In France, in collaboration with Water Agencies and Environment Ministry services, Irstea (ex Cemagref) has developed several diatom indices to estimate global water quality like the "Indice de Polluosensibilité Spécifique" (IPS, Cemagref 1982) or the Biological Diatom Index (BDI, Coste et al. 2009), both routinely used for monitoring applications in several European countries. Nevertheless, the indices currently used for general water quality assessment - including the IPS and BDI - were not designed to assess specific toxic alterations (metals, synthetic organic pollutants) and are not really suitable for a sensitive diagnosis of this type of pollution. Thus, pesticides are of particular concern because of the established contamination of European water systems (Herrero-Hernández et al. 2013, Dubois et al. 2010) and the potential toxic effects they can produce on aquatic organisms (DeLorenzo et al. 2001). Diatoms and biofilms have shown their potential utility for bioindication with regard to pesticide pollution. For example, Roubeix et al. (2011) observed structural impact at the community level and morphological abnormalities after exposure in microcosms to metolachlor (a herbicide belonging to the chloroacetanilide group). Other studies have assessed the effects of pesticides on functional descriptors such as photosynthetic (Laviale et al. 2011) or antioxidant enzyme activities (Bonnineau et al. 2013).

The traditional approach to environmental risk assessment consists of evaluating the toxicity of a single compound on single species. But this reasoning leads to a lack of ecological relevance because organisms belong to complex biological communities and chemicals are present in mixtures in the environment. The development of new tools is thus needed to reach a more environmentally realistic and integrative approach in risk assessment studies. Attached microbial communities could respond to such needs as they play a fundamental role in the ecological functioning of river systems, owing to their key position in the trophic web and their important contribution to primary production. Moreover, such communities interact strongly with dissolved substances such as pesticides present in water and are likely to respond quickly to contaminant pressures making river biofilms useful early warning systems for the detection of the effects of toxicants with a variety of methods. In general, physiological approaches may be appropriate for the detection of acute effects whereas persistent or chronic effects should affect other biofilm indicators, for example growth or

biomass-related factors, or community composition (in particular the diatom community) (Sabater et al. 2007).

In order to assess impact of changes of toxic pressure on biofilms most of the studies have been using translocation approaches (Morin et al. 2010a, Arini et al. 2012a). In a general way, reference biofilms transferred to contaminated sites presented characteristics of communities originated from polluted sites after some weeks. In the other way, recovery of communities after translocation from a contaminated to a less contaminated site has been observed from weeks to months. Nevertheless in these *in situ* studies, impacts of toxicants are often difficult to separate from abiotic and biotic factors. It is so necessary to complete *in situ* studies with laboratory experiments.

Passive sampling devices like the Polar Organic Chemical Integrative Sampler (POCIS) are useful tools for monitoring trace levels of chemicals in aquatic environments since they concentrate several organic chemicals from large volumes of water (Mazzella et al. 2010). This high concentration of compounds makes the POCIS a powerful instrument for the assessment of the extract toxicity via biological testing. The use of the POCIS in combination with bioassays has the advantage of being more relevant from an ecotoxicological perspective because of the pollutant mixtures it provides. Moreover, this approach gives an estimation of an integrative measure of the toxic potential of a group of compounds including unknown toxicants (a non *a priori* approach). For these reasons, most studies have dealt with biological testing of POCIS extracts, more often establishing dose response curves (evaluation of the acute toxicity). While POCIS extracts in combination with bioassays have been well documented with compounds like endocrine disruptors or photosystem II inhibitors (Vermeirssen et al. 2010, Matthiessen et al. 2006, Balaam et al. 2010), evaluation of potential extract toxicity in long-term studies is still in its infancy. The first and only study dealing with chronic low-dose effects of passive sampler extracts on biofilms was conducted by Morin et al. (2012b). The experiment was a first attempt to evaluate whether chronic effects of pesticides in a mixture could be approached by the use of POCIS extracts. The study highlighted the methodological issues of dealing with low contaminant doses in long-term experiments, particularly the difficulty of controlling the concentration of contaminants in large volume of water; but nevertheless reported the promising perspectives of the approach for further ecotoxicology studies. Taking into account the issues pointed out and the knowledge acquired by Morin et al. (2012b), the present study investigated how changes in pesticide pressure may be approached by the use of POCIS extracts in chronic exposure

conditions close to *in situ* levels and discussed future possible applications of such approaches to better include chronic exposure in Environmental Risk Assessment procedures.

To assess the effects of pesticides in mixture, the use of PE in channels is so representing an intermediate situation between field studies where confusion factors are numerous and laboratory experiments where the realism regarding the toxicant exposure is low.

2. Material and methods

2.1. Study site and sampling procedure

The study was carried out on the Morcille River, located in the Beaujolais vineyard area of eastern France. The Morcille River has been extensively studied over the past decades (Rabiet et al. 2010, Montuelle et al. 2010, Dorigo et al. 2007). The area is subjected to strong agricultural pressure - essentially exerted by vineyards - and is characterized by an increasing multi-contaminant gradient. Pesticides overall and to a lesser extent metal and nutrient concentrations increase from upstream to downstream.

Glass slides fixed in perforated plastic boxes were used as artificial substrates to allow biofilm colonisation for 4 weeks (24th May-21th June 2011) at the 2 stations located upstream (reference site) and downstream (the more contaminated site).

“Quantitative” POCIS were used for pesticide quantification of the majority of the compounds found in the water (Mazzella et al. 2010, Lissalde et al. 2011), while grab sampling was used to determine concentration of compounds that were not calibrated by POCIS in the present study (average of the 5 grab samples taken from 24th May-21th June 2011).

Performance and Reference Compound (PRC) was introduced in “Quantitative” POCIS. The devices were immersed in the current at upstream and downstream stations for two weeks and then replaced by new ones for two extra weeks for pesticide quantification and the characterisation of biofilms *in situ* past exposureAfter collection, all POCIS were kept at -4°C until extraction and chemical analysis.

“Accumulative” POCIS used for the toxicity tests (Morin et al. 2012b) were immersed at the downstream station during the biofilm colonisation period to concentrate pesticides. After 4 weeks in the river, the glass slides and POCIS were brought back into the laboratory. The biofilms were put in aquariums filled with water from their respectively sites supplemented by sufficient nutrients to allow growth for one week before the beginning of the channel experiment.

2.2. Laboratory experimental conditions

2.2.1. Acute toxicity testing

In order to characterize the initial tolerance of upstream and downstream biofilms to pesticides, acute toxicity tests were carried out after one week in the lab. Short-term photosynthetic bioassays were performed in triplicate using measurement of the optimal quantum yield (F_v/F_m) as endpoint (defined in 2.4.1) on upstream and downstream biofilms. Biofilms were exposed to semi-logarithmic series of dilutions of the pre-concentrated extracts. For each replicate, 140 cm^2 of biofilm were scraped from glass sides and resuspended in 28 mL of mineral water; then 1.4 mL of biofilm were exposed to 1.4 mL of toxicant (2.8 mL final volume) to reach the same volume in every assay. Six concentrations of POCIS extracts (from d0 to d5), a control (ultrapure water) and an extraction blank obtained with the same extraction procedure as for POCIS extraction (Blank) were tested. Only pure Blank was tested corresponding to the highest exposure concentration (d0). Biofilms were exposed for 24h at 19-20°C under artificial light (30 $\mu mol.m^{-2}.s^{-1}$). After 30 minutes in the dark, fluorescence F_v/F_m emitted by the plant component of the biofilm suspensions was measured by fluorimetry.

2.2.2. Channel experiments

After one week in the lab in aquariums filled with water from their respectively originated site, upstream and downstream biofilms were exposed to POCIS extracts (PE) for 13 days in artificial glass channels in WC culture medium (Guillard and Lorenzen 1972). The colonised glass slides were laid directly on the bottom of the artificial channels. Upstream (not contaminated) biofilms were exposed to PE at low concentrations in the range of those measured in the contaminated downstream section of the Morcille river (and in the range of that applied for the more dilute solutions d4 and d5 in the acute toxicity testing) in order to model toxic pressure appliance (Upstream with PE) or placed into clean water in order to represent reference conditions (Upstream without PE). Downstream biofilms were exposed to PE in order to model toxic pressure maintenance (Downstream with PE) or replaced into clean water in order to mimic removal of toxic pressure (Downstream without PE). Each condition was performed in triplicate. Experimental channels were maintained at a temperature of 19 to 20°C during the 13 days of exposure with a photon flux density of 30 $\mu mol.m^{-2}.s^{-1}$, a 12:12 h light:dark cycle and under continuous water flow.

In order to fix the contamination level and to avoid decrease drop in pesticide concentrations (Morin et al. 2012b), 4 POCIS-equivalent extracts were used for spiking the channel volume (9L) one day before the beginning of the experiment. Then, the channels were spiked again on

days 3 and 6 with one POCIS-equivalent to ensure contamination levels in channels throughout the experiment (13 days). Nutrients were added on day 6 to ensure sufficient concentrations to allow biofilm growth. Nutrients concentrations and physicochemical conditions were monitored throughout the experiment.

Biofilm samples were collected by scraping glass slides with a razor blade and resuspended in a known volume of mineral water for further analyses. Samples were collected in triplicate on days 0, 3, 7 and 13 for diatom densities, effective quantum yield of PSII (Φps_{II}), fluorescence of Chl *a* measured at 665nm (Fs_{665}), fluorescence signals linked to cyanobacteria (Fs_{Bl}), diatom (Fs_{Br}), and green algae (Fs_{Gr}) group determination. Taxonomic composition was determined on days 0 and 13; dry weight (DW) and ash-free dry mass (AFDM) were determined on day 13. pH, temperature, conductivity, oxygen, nitrates, orthophosphates and silica concentrations were recorded in water channels throughout the 13 days of the experiment.

2.3.Physico-chemical parameters and pesticide analysis

The method developed by Lissalde et al. (2011) was used for the analysis of POCIS extracts. The solid receiving phase contained in the POCIS (Oasis HLB sorbent, Waters) was recovered in ultrapure water. Then all samples were analyzed by HPLC–ESI–MS/MS (HPLC Ultimate 3000, Dionex, equipped with a Gemini NX C18 column, 10 mm x 2 mm, 3 µm, Phenomenex, and API 2000 triple quadrupole, AB SCIEX equipped with an electrospray ionization source) and GC–MS/MS (Quantum GC, Thermo, equipped with a Rxi-5MS column 30 m x 0.25 mm x 0.25 µm, Restek). Pesticide concentrations in the water phase were determined from spot samples by solid-phase extraction on Oasis HLB cartridges followed by liquid chromatography tandem mass spectrometry (LC/MS/MS API 4000, Applied Biosystem, France). Chromatographic separation used an Atlantis T3 analytical column, 3µm particle size, 2.1 mm x 100 mm from Waters (France), at a flow rate of 300 µL min^{-1} with a mobile phase consisting of acidified acetonitrile and water. Injection volume was 20 µL (Assoumani et al. 2013).

Analytical methods were validated in terms of calibration linearity, specificity, extraction recovery, and quantification limit according to the French standard NF T90-210. Additionally, SPE and POCIS blanks were run, and the recoveries of two levels of spiked mineral water (e.g. 0.02 and 0.2 µg/L for LC-MS/MS analysis) were evaluated for each batch. Two calibrating standards (e.g. 5 and 25 µg/L, every 10 samples) and analytical blanks were periodically checked.

pH meter (pH 3110, WTW), conductimeter (LF 340, WTW) and oxymeter (Oxi 340i, WTW) were used to determine pH, conductivity and oxygen concentration respectively. The temperature was obtained by averaging the values recorded by the 3 devices. Nitrate concentration was calculated according to French standard (NF EN ISO 13395 10/96) using Auto-analyser Evolution II Alliance. Orthophosphate and silica were determined following standard procedures (NF T90-023 09/82 and NF T 90-007 02/01 respectively) with a Perkin Elmer Lambda 2 spectrophotometer.

2.4. Biofilm response analysis

2.4.1. In vivo fluorescence measurements

In vivo fluorescence measurements used Pulse Amplified Modulated fluorometry (PhytoPAM, Heinz Walz GmbH, Germany) at four different excitation wavelengths (470, 520, 645 and 665 nm) characterizing and discriminating different groups of algae. For example green algae show a large signal when excited at 470 nm due to Chl *b* and a low signal at 520 nm whereas diatoms display strong signal at 470 and 520 nm when excited in relation to Chl *c*, fucoxanthin and carotenoids. For cyanobacteria low signal is recorded at 470 nm while excitation at 645 nm is particularly strong (Walz 2003). By deconvolution of the signals it is then possible to estimate the fluorescence signals linked to the 3 main algal groups: cyanobacteria (Fs_{Bl}), diatoms (Fs_{Br}), and green algae (Fs_{Gr}). Measurements recorded at an excitation wavelength of 665 nm (Fs_{665}) estimate the fluorescence related to the photosynthetic component of the entire biofilm as 665 nm corresponds to the maximum excitation wavelength of the Chl *a* molecule. Fluorescence was measured over the same surface area for all samples and is expressed in relative units (ru).

Optimal quantum yield (F_v/F_m) and effective quantum yield (ΦpsII) were recorded at an excitation wavelength of 665 nm. F_v/F_m was measured after 30 min of adaptation to dark and was calculated according to (Genty et al. 1989):

$$F_v/F_m = (F_m-F_0) / F_m$$

with F_0 the minimum fluorescence determined after a weak far red modulated light and F_m the maximum level of fluorescence measured during a saturating white light pulse.

Samples were exposed to actinic light to determine their effective quantum yield. ΦpsII was calculated according to (Genty et al. 1989):

$$\Phi ps_{II} = (F_m'-F_t) / F_m'$$

with F_t the minimum fluorescence determined after weak far red modulated light and $F_{m'}$ the maximum level of fluorescence measured during a saturating flash of white light while the sample was under actinic light.

2.4.2. Dry weight and ash-free dry mass

On day 13, the dry matter and the organic matter content were evaluated by calculating the dry weight (DW) and the ash-free dry weight (AFDM) following European standard NF EN 872. For each sample, 50 cm^2 of biofilm were scraped from glass slides and resuspended in 10 mL of mineral water. Biofilm suspensions were filtered through individual, previously dried, 25 mm GF/C Whatman glass fiber filters (1.2 µm pore size). Each filter was weighed, after 1h drying at 105°C to calculate the DW, then at 500°C for 1h, and weighed again to calculate the mineral matter. AFDM was calculated by subtracting the mineral matter weight from the total weight of dry matter.

2.4.3. Diatom analysis

2.4.3.1. Diatom cell density

The cells in each sample were counted using a Nageotte counting chamber (Marienfeld, Germany). 200 µL of sample were placed in the counting chamber and the total number of dead and live cells was recorded in 10 fields of the gridded area (1.25 µL each, 0.5 mm depth) under light microscopy at 400x magnification. Distinction between dead and alive organisms was estimated by the observation of the turgescence and colour of the chloroplasts as described in Morin et al. (2010b).

2.4.3.2. Taxonomic analyses

Diatoms were identified from permanent slides prepared following European standard permanent slides NF EN 13946, and they were identified at 1,000 x magnification to the lowest taxonomic level possible using standard references (Hofmann et al. 2011).

2.5. Data analysis

The effects of pesticide exposure on photosynthetic efficiency (n=3), fluorescence levels (Fs_{665}, Fs_{Bl}, Fs_{Gr} and Fs_{Br}, n=3) and diatom densities (n=3) were tested by one-way ANOVA analysis using STATISTICA 6.1 (StatSoft). The ANOVA was followed by a Tukey–HSD test. Homogeneity of variance was checked prior to data analysis. Due to the non-

homogeneity of variance for DW, AFDM and Φps_{II}, statistical differences on these parameters were analysed by Kruskall Wallis test, followed by 2 by 2 Mann-Whitney comparisons.

For the acute toxicity tests, EC_{25} (concentration needed to decrease F_v/F_m of 25% compared to the control) was calculated using REGTOX EV 7.0.5. (Vindimian 2003).

3. Results

3.1. *In situ* pesticide exposure and initial tolerance of biofilms

3.1.1. Pesticide contamination levels in the river

The main pesticides quantified *in situ* at the two sampling sites on the Morcille river are presented in Figure 20.

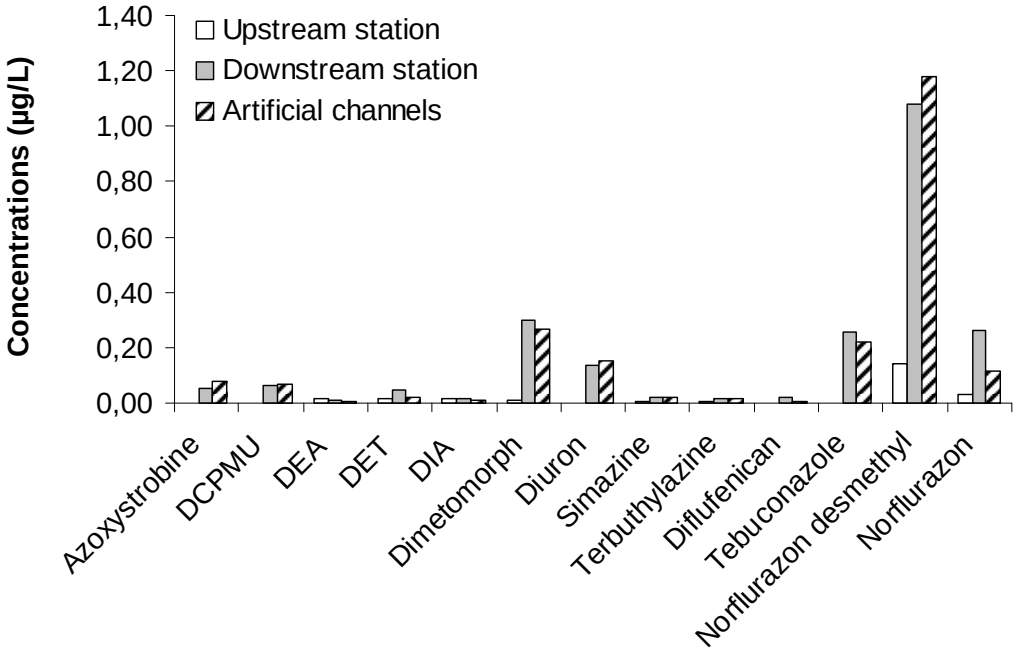


Figure 20 : Concentrations (µg/L) estimated at upstream and downstream stations of the Morcille River in spring 2011 (24th May-21th June) and nominal concentrations applied in contaminated treatment (with PE) in the artificial channel experiment. Azoxystrobine, DCPMU, DET, DIA, dimetomorph, diuron, simazine and terbuthylazine are *In situ* 1 month time -weighted average (TWA) concentrations estimated with POCIS. Desmethyl norflurazon, norflurazon, diflufenican and tebuconazole concentrations were estimated by punctual sampling. White and grey bars represent in situ concentrations at upstream and downstream station respectively; hatched bars represent concentrations in PE contaminated treatment during channel experiment.

The concentrations from 24th May to 21th June 2011, corresponding to the 4 weeks of biofilm colonisation, were very low upstream (reference site, total pesticide concentration = 240 ng/L) in comparison to the downstream site (contaminated site, 2260 ng/L).

POCIS analysis revealed the presence of fungicides (dimetomorph, azoxystrobine, tebuconazole), herbicides (norflurazon, diuron, terbuthylazine, simazine, diflufenican) and herbicide metabolites (desmethyl norflurazon, deisopropylatrazine, DIA; dichlorophenulmethylurea, DCPMU; desethylterbuthylazine, DET; desethylatrazine, DEA) at the downstream site of the Morcille River from 24th May to 21th June 2011. Norflurazon, desmethyl norflurazon, dimetomorph, tebuconazole and diuron were the most concentrated compounds (1080, 265, 298, 259 and 138 ng/L respectively), representing 90% of the total pesticide and metabolite concentration. The contamination profile observed in spring 2011 was characteristic of typical vineyard area contamination and similar to that reported in previous investigations at the site (Rabiet et al. 2010, Montuelle et al. 2010, Dorigo et al. 2007). We can note the persistence of diuron despite its prohibition in France since December 2008 due to its long lifetime in the soil.

3.1.2. Exposure history in field

Pollution-Induced Community Tolerance (PICT) was assessed on upstream and downstream biofilms on day 0 in order to evaluate the relative sensitivity of communities to PE in relation to their *in situ* exposure history during the colonisation period.

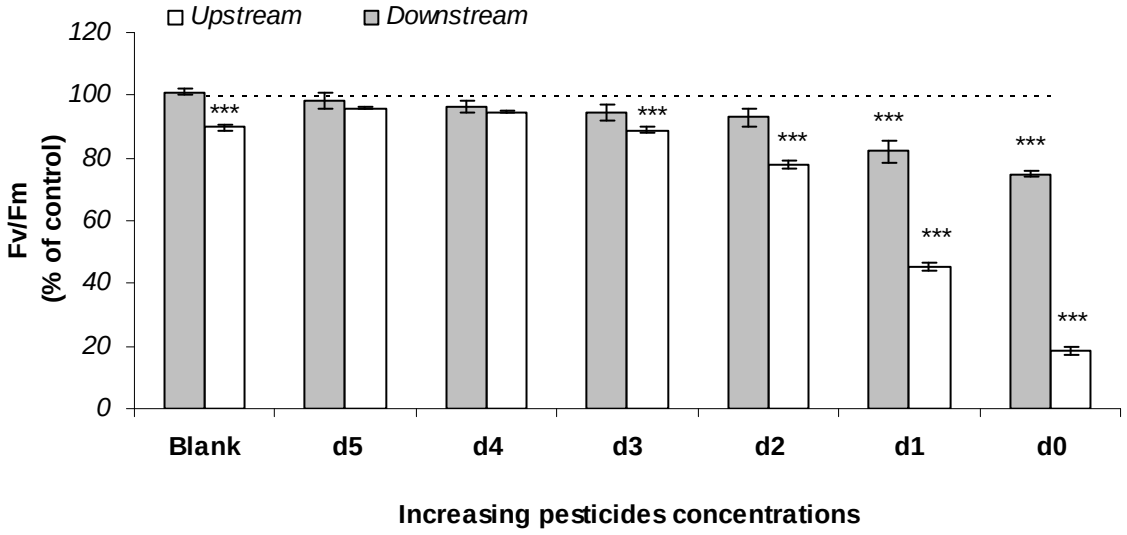
Table 4 : Pesticide concentrations (µg/L) used for the short term toxicity test with successive dilutions (from d0 to d5; PICT tests). Each dilution is expressed in function of environmental concentrations (mean of total pesticides concentrations) at the downstream site of Morcille River estimated with POCIS in spring 2011 (24th May-21th June). 1X corresponds to environmental concentrations.

Substance group	Substance name	d0	d1	d2	d3	d4	d5
Triazine	Simazine	3.65	1.15	0.36	0.11	0.04	0.01
	DIA (Deisopropylatrazine)	1.80	0.57	0.18	0.06	0.02	0.01
	DEA (Desethyl atrazine)	0.55	0.17	0.06	0.02	0.01	0.00
	DET (Terbuthulazine desethyl)	3.90	1.23	0.39	0.12	0.04	0.01
	Terbuthylazine	2.45	0.77	0.24	0.08	0.02	0.01
Phenylurea	DCPMU	11.80	3.73	1.18	0.37	0.12	0.04
	Diuron	25.60	8.09	2.56	0.81	0.26	0.08
Strobilurin	Azoxystrobine	13.10	4.14	1.31	0.41	0.13	0.04
Morpholine	Dimetomorph	45.50	14.39	4.55	1.44	0.45	0.14
Triazole	Tebuconazole	37.75	11.94	3.78	1.19	0.38	0.12
Carboxamide	Diflufenican	1.32	0.42	0.13	0.04	0.01	0.00
Pyridazinone	Norflurazon	20.00	6.32	2;00	0.63	0.20	0.06
	Desmethyl norflurazon	201.00	63.56	20.10	6,36	2.01	0.64
Concentration factor compared to environmental contamination		**156X**	**49X**	**16X**	**5X**	**1.6X**	**0.5X**

The final pesticide concentrations used for the short-term toxicity test with successive dilutions are presented in Table 4, the most dilute solutions (d4 and d5) were close to

environmental concentrations (estimated by quantitative POCIS and spot sampling, reported in Figure 20) and the strongest solution (d0) corresponded to 100- to 300-fold realistic concentrations.

Figure 21 : F_v/F_m (expressed as % of respective controls ± standard deviation) of upstream and downstream biofilm communities exposed to serial dilutions of POCIS extracts (dx) or extraction blank (Blank) at day 0 for 24h. White and grey bars represent biofilms originated from upstream and downstream of the river respectively. Stars indicate statistical difference from controls (n=3, *p<0.05, ** p<0.01, ***p<0.001)



Photosynthetic efficiency (F_v/F_m) expressed as percentage of the control is plotted versus PE dilutions at day 0 for upstream and downstream biofilms (Figure 21). Statistical analysis revealed that F_v/F_m was significantly different from control for d0, d1, d2, d3 and extraction blank (Blank) for upstream communities with a particular very strong inhibition for the highest pesticide concentration (82±1% for d0). On the other hand F_v/F_m was significantly different from the control only for the 2 highest pesticide concentrations (d0 and d1) for downstream biofilms and F_v/F_m was only reduced by 25±1% for the highest concentration (d0). The significant inhibition of F_v/F_m (10±1% for Blank) for upstream biofilms exposed to extraction blank (Blank) highlights the very high sensitivity of upstream biofilms.

From these F_v/F_m values, EC_{25} were calculated and proved to be 8 times higher for downstream communities than for upstream communities with EC_{25} equal to 0.06 ± 0.01 PE *versus* 0.48 ± 0.14 PE for upstream and downstream communities respectively. These values of EC_{25} correspond to 19 ± 3 X and 149 ± 43 X for upstream and downstream communities

respectively with 1X corresponding to *in situ* concentrations at the downstream site of the river (mean of the different compounds sampled in the POCIS ± SD).

3.2. Evaluation of pesticides chronic effects on biofilms contaminated by POCIS extracts in channel experiments.

3.2.1. Experimental conditions

The nominal concentrations of the main pesticides applied in the channels in reference to the *in situ* contamination context are presented in Figure 20. Appropriate dilutions of PE were performed in order to reach pesticide concentrations measured at the downstream station estimated by "quantitative" POCIS and spot sampling in spring 2011 (24th May-21th June) for a total pesticide concentrations of about 2260 ng/L, with the presence of desmethyl norflurazon, norflurazon, dimetomorph, azoxystrobine, tebuconazole, diuron, diflufenican, terbuthylazine, simazine, DIA, DCPMU, DET and DEA.

Physico-chemical parameters and nutrient concentrations along the artificial channel experiment are presented in Table 5 (means for each parameter over the 13 days of experiment). No significant differences were observed between treatments over the 13 days of the experiment; temperature, conductivity and pH presented mean values of 21 ± 0.2 °C, 367.5 ± 35 µS/cm and 7.6 ± 0.2 respectively.

Table 5 : Mean physico-chemical parameters (± standard deviation) in artificial channels over the 13 days for the different treatments (Upstream without PE, upstream with PE, downstream without PE and downstream with PE).

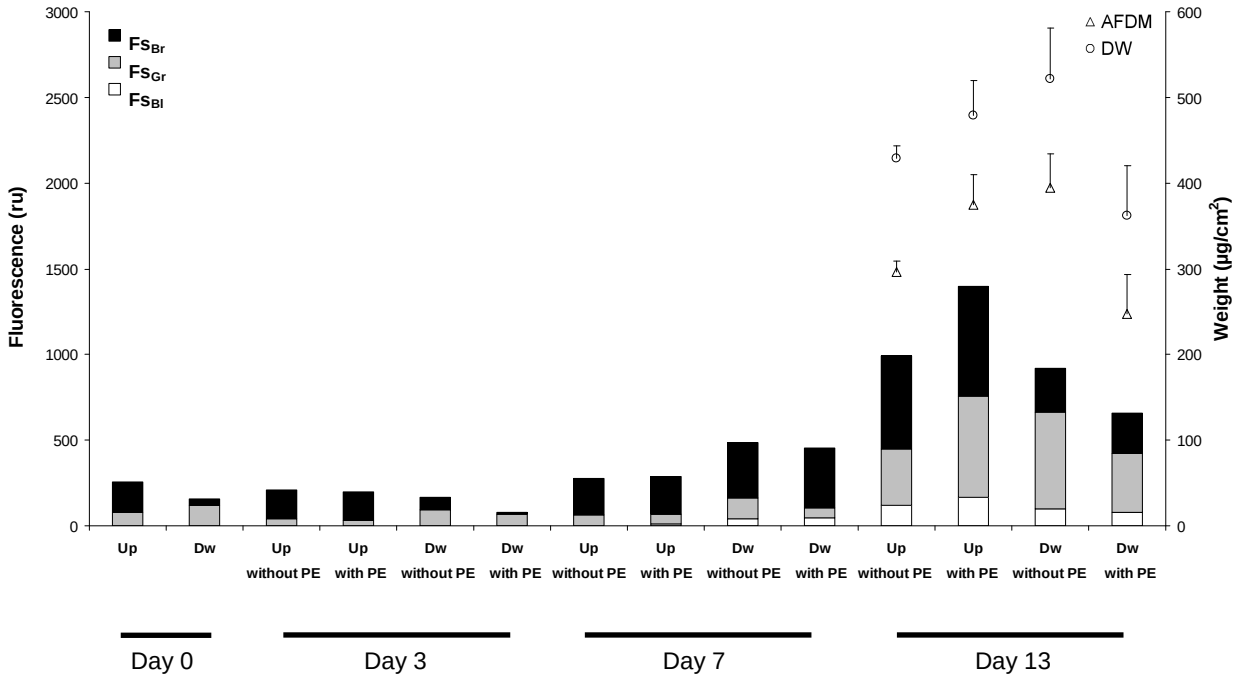
	Temp (°C)	pH	Cond. (µS/cm)	O_2 (mg/l)	N-NO_3 (mg/l)	P-PO_4 (mg/l)	Si (mg/l)
Upstream without PE	21.2 ± 0.3	7.5 ± 0.2	374.0 ± 96.9	7.7 ± 0.5	62.2 ± 11.5	3.0 ± 1.5	5.9 ± 0.3
Upstream with EP	21.4 ± 0.2	7.6 ± 0.2	365.7 ± 92.5	7.1 ± 1.0	66.0 ± 10.0	3.0 ± 1.3	6.4 ± 0.1
Downstream without PE	21.5 ± 0.2	7.7 ± 0.2	377.8 ± 96.3	7.7 ± 0.5	59.2 ± 13.0	2.7 ± 1.4	5.9 ± 0.3
Downstream with PE	21.4 ± 0.2	7.6 ± 0.3	352.5 ± 70.6	7.4 ± 0.2	54.0 ± 12.4	2.3 ± 1.3	6.3 ± 0.1

3.2.2. Photochemical efficiency and fluorescence levels

No significant difference of effective quantum yield (ΦpsII) was observed between upstream biofilms with or without PE and between downstream biofilms with or without PE over the 13 days of exposure in the channels (data not shown). ΦpsII remained stable (mean for all treatments of 0.45 ± 0.01) over the whole experimental duration, nevertheless with a slight decrease on day 7 for upstream with and without PE (ΦpsII = 0.36 ± 0.01; mean for upstream with and without PE).

Regarding fluorescence of Chl *a* measured at 665nm (Fs_{665}), fluorescence signals linked to cyanobacteria (Fs_{Bl}), diatoms (Fs_{Br}), and green algae (Fs_{Gr}), all values increased through the 13 days of the experiment in all treatments (Figure 22). At the end of the experiment, no significant difference was shown for Fs_{Bl} and Fs_{Br} levels with or without exposure to PE for upstream and downstream biofilm. In contrast, significant differences were observed for Fs_{Gr} in regards to PE exposure or not. Thus, on day 13, for upstream biofilm, Fs_{Gr} was higher in the treatment with PE compared to the treatment without PE, while we observed a lower Fs_{Gr} for downstream biofilm exposed to PE compared to non-exposed biofilms.

Figure 22 : Fluorescence levels (Fs) and biofilm weight in function of time exposure to the different treatments. Fluorescence levels related to cyanobacteria Fs_{Bl}, green algae Fs_{Gr} and diatom groups Fs_{Br} are expressed in relative units of fluorescence and are represented by the white, grey and black areas respectively. Dry weight (DW) and ash-free dry mass (AFDM) of total biofilm recorded at day 13 only are expressed in µg/cm^2 and represented by circle and triangle respectively. Up: Upstream biofilm; Dw: Downstream biofilm.

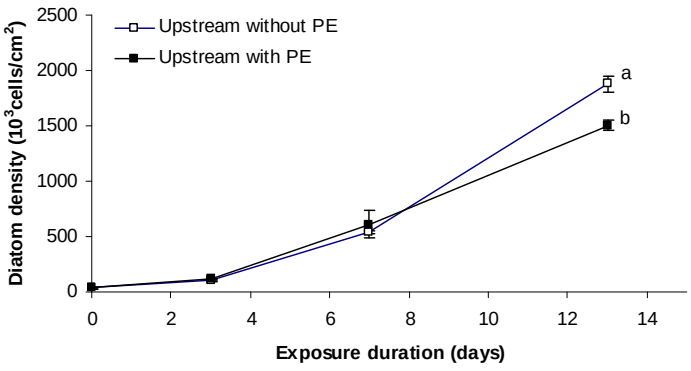


3.2.3. Diatom density and biofilm biomass

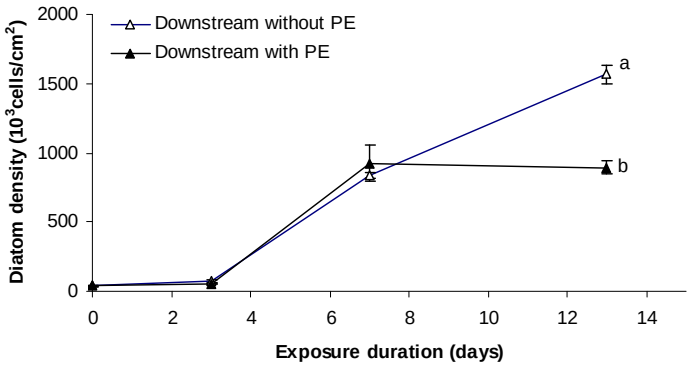
Diatom densities, over the 13 days of direct exposure to PE in artificial channels are plotted in Figure 23. Statistical analysis revealed that diatom density different from day 0 to day 7 was not significantly firstly between the downstream without PE and downstream with PE treatments and secondly between upstream without and upstream with PE. Significant differences in diatom cell densities were observable on day 13 between exposed and non-exposed biofilms independently of biofilm origin.

Figure 23 : Total diatom density ± standard deviation versus exposure time for the biofilms originated from upstream (a) or downstream (b) sites of Morcille River and exposed to the different treatments (Upstream with PE, Upstream without PE, Downstream with PE and Downstream without PE), significant differences between a and b (*p* value < 0.05). Solid squares and solid triangles represent contaminated conditions. Open squares and triangles represent conditions without pesticide. PE = POCIS extracts

(a)



(b)



For downstream biofilms the diatom cells density was 1.8 times higher without PE compared to treatment with PE (1 570 ± 250 .10^3 cells/cm^2 for downstream without PE versus 890 ± 150 .10^3 cells/cm^2 for downstream with PE). Moreover, downstream diatom growth was null between day 7 and 13 for downstream biofilms with PE treatment (920 ± 150 .10^3 cells/cm^2 at day 7 versus 890 ± 150 .10^3 cells/cm^2 at day 13). For upstream biofilms, the diatom cell density was 1.2 times higher without PE compared to treatment with PE (1 876 ± 72 .10^3 cells/cm^2 for upstream control versus 1 504 ± 44 .10^3 cells/cm^2 for upstream with PE). For biofilms of both origins, note that diatom growth initiated at day 3 and densities followed the same trend with a similar order of magnitude. Dry weight and ash-free dry matter were recorded at the end of the experiment for the different exposure scenarios (Figure 22). Statistical analyses revealed differences in DW and AFDM after 13 days of exposure to the different treatments in channels. Moreover, these two parameters followed different trends depending on biofilm origin. Exposure to PE led to a significant increase of AFDM for upstream biofilm compared to non-exposed upstream biofilm (380 ± 30 µg/cm^2 and 300 ± 10 µg/cm^2 respectively for upstream biofilm with and without PE). In contrast, downstream biofilm showed an opposite trend with lower values of DW and AFDM for biofilm exposed to PE compared to non-exposed biofilm. In this case, DW and AFDM were on average 1.4 and 1.6 times higher for non-exposed biofilms compared to exposed biofilms.

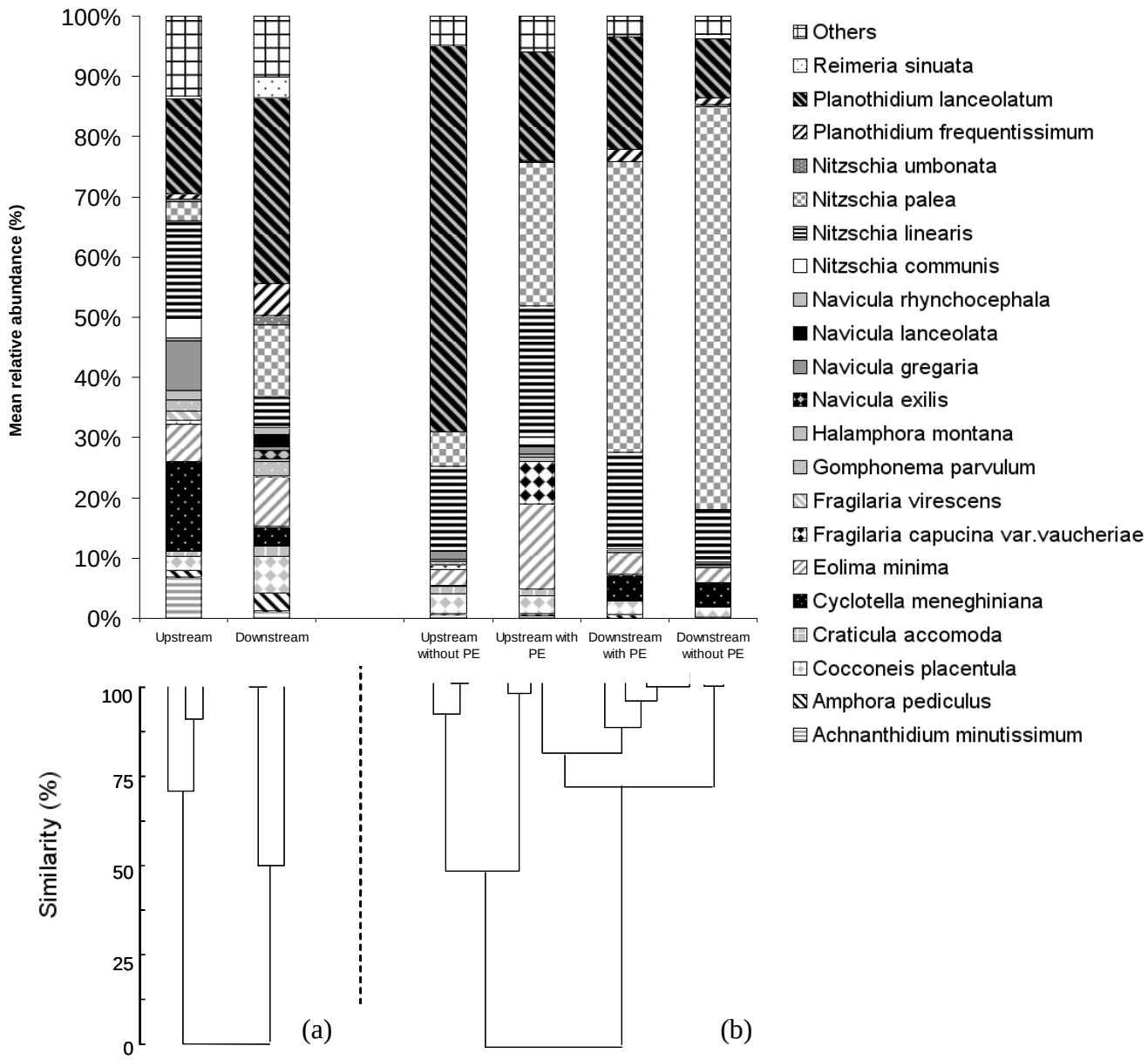
3.2.4. Diatom assemblages

A total of 69 species were identified. The 21 species occurring at more than 2% relative abundance in at least one sample are presented in Figure 24. Sample clustering clearly differentiated diatom communities at day 0 depending on the origin of the biofilms (Figure 24a). Species richness was on average higher for upstream biofilms than downstream biofilms (36 ± 4 and 30 ± 3 respectively). Upstream biofilms were dominated by *Nitzschia linearis* var.

linearis (Agardh) W.M.Smith (16% relative abundance), *Planothidium lanceolatum* (Brebisson ex Kützing) Lange-Bertalot (16% relative abundance) and *Cyclotella meneghiniana* Kützing (15% relative abundance).

Downstream biofilm compositions were characterized by *Planothidium lanceolatum* (31% relative abundance), *Nitzschia palea* (Kützing) W.Smith (12% relative abundance) and *Eolimna minima* (Grunow) Lange-Bertalot (8% relative abundance). Assemblages always presented a large abundance of *P. lanceolatum*, with higher relative abundance at the contaminated site (31% vs 16% relative abundance for downstream and upstream respectively). Moreover *Achnanthidium minutissimum* (Kützing) Czarnecki was more abundant in upstream biofilm than in downstream (7% and 1% relative abundance for upstream and downstream biofilms respectively).

Figure 24 : Mean relative abundances of the 21 dominant species (i.e. representing more than 2% relative abundance in at least one sample) at day 0 (a) and day 13 (b).



After 13 days in the channels, the split already observed at day 0 between upstream and downstream biofilms was still observed (Figure 24b). Species richness decreased with time of exposure in the laboratory for all treatments (36 ± 4 and 30 ± 3 at day 0 for upstream and downstream biofilms) but without significant difference between conditions at the end of the experiment (19 ± 1 and 21 ± 3 for upstream without PE and upstream with PE respectively and 15 ± 1 and 14 ± 3 for downstream without PE and downstream with PE respectively).

Planothidium lanceolatum showed a high relative abundance in all treatments at day 13 (reaching 64% upstream without PE treatment).

The cluster dendogram for day 13 (Figure 24b) clearly grouped biofilms from the upstream site by treatments (exposure or not to PE). Even though present in large amounts for all treatments, *Planothidium lanceolatum* was less abundant in upstream biofilms exposed to pesticides than in upstream biofilms maintained under control conditions (18% vs 64% for upstream with PE and upstream without PE respectively).

This lower relative abundance of *Planothidium lanceolatum* upstream with PE compared to upstream without PE coincides, in upstream biofilm with PE treatment, with an increase in the relative abundance of *Eolimna minima* (3% and 14% for upstream without and upstream with PE respectively) and *Nitzschia palea* (6% and 24% for upstream without and upstream with PE respectively).

Concerning downstream biofilm exposed to POCIS extracts or not, no strong difference was observed in taxonomic composition. For both treatments the assemblages were dominated by *Nitzschia palea*, *Planothidium lanceolatum*, *Nitzschia linearis*, *Cyclotella meneghiniana*, *Eolimna minima* and *Cocconeis placentula* Ehrenberg var. placentula.

4. Discussion

4.1. Passive samplers as a tool to take into account chronic pesticide exposure in environmental risk assessment (ERA)

The use of passive sampler extracts could be a powerful tool in environmental risk assessment, since the devices take into account the issues associated to the presence of a mixture of known compounds, unknown compounds, and metabolites. Passive sampler extracts can thus be used for the evaluation of both acute and chronic effects of environmental pollution. The combination of passive sampler extracts with biological testing at acute exposure levels has already shown its relevance and is well documented in the literature, with the majority of studies focusing on responses of model organisms (Vermeirssen et al. 2010, Matthiessen et al. 2006, Balaam et al. 2010). In our study, PE were used to highlight the exposure history of biofilms in the river according to the Pollution-Induced Community Tolerance (PICT) concept. The PICT approach illustrates the difference of tolerance to a toxicant between pre-exposed and non-exposed communities. This concept, introduced by Blanck et al. (1988), is based on the idea that when a natural community is exposed to a particular toxicant the more sensitive organisms disappear; and then when this community is exposed again to this toxicant its tolerance will be increased because of the disappearance of

the sensitive species. It is therefore of particular interest to reveal the past *in situ* toxicant exposure of communities. At the beginning of our experiment EC_{25} were equal to 19 ± 3 X versus 149 ± 43 X for upstream and downstream communities respectively (with 1X corresponding to *in situ* concentrations at the downstream site of the river). EC_{25} was 8 times higher for downstream communities than for upstream communities reflecting the order of magnitude of the difference of *in situ* exposure levels with pesticide concentrations averaging 10 times higher at the downstream site than at the upstream site. In this study, the photosynthetic efficiency versus exposure to increasing concentrations of PE was chosen to assess the *in situ* pre-exposure history of biofilms. The results showed the difference of tolerance between upstream and downstream biofilms linked to different levels of pesticide contamination *in situ* as revealed by the time weighted average pesticide concentrations measured with "Quantitative" POCIS at the two sites during the biofilm colonisation period. Since diatom assemblages clearly differed between upstream and downstream sites, one may argue that the increasing tolerance observed downstream was mainly due to the development of more tolerant species in this contaminated river section.

PE were then used in the channel experiment for the assessment of chronic and long-term effects of pesticide mixtures on these two communities with different *in situ* exposure histories.

Exposure to PE in channels revealed effects at environmentally realistic concentrations on biofilm structural and growth-related endpoints. Cocktails of contaminants even at low concentrations were thus proven to induce adverse effects on the natural communities of the aquatic ecosystems. These results showed the importance of working on the effects of chronic and low dose mixtures; and in this challenging work, passive samplers like POCIS appear to be of particular interest since they concentrate huge volumes of water, which is an essential condition for running long-term experiments. Currently environmental risk assessment focuses on responses to model organisms to acute exposure; such an approach is needed and has advantages (e.g. fast, low cost, easy to perform) but leads to a lack of ecological relevance. This is why it could be particularly interesting to include chronic testing since both approaches give complementary information. Regarding biofilms, short-term treatment leads to physiological alterations and while the long-term effects are changes in community structure (Sabater et al. 2007).

4.2. Duration required to reveal pesticide effects

The significant decrease of diatom density observed on day 13 on upstream biofilms exposed to PE compared to non-exposed biofilms highlighted the adverse effects of a mixture of pesticides at realistic environmental concentrations as produced using POCIS extracts. As pointed out in the literature, diatoms are an algal group particularly sensitive to pesticide exposure and thus show great potential for the development of new tools in biomonitoring programs focussing on toxic pollution. The responses of diatoms and more generally of aquatic organisms to chronic exposure to environmental concentrations of mixtures of toxicants are poorly documented. To our knowledge only one study has focused on low dose effects of mixtures of pesticides on river biofilms (Morin et al. 2012b). In this study, authors exposed biofilms to PE from Morcille River but did not observe any impact of toxicants on diatom density even after 2 weeks of exposure. They attributed this result to probable difficulties in maintaining toxicants at environmentally realistic levels as very low concentrations were applied averaging 0.5 ± 0.1 µg l^{-1}(total pesticides concentrations).

Previous studies showed the impact of the exposure to single pesticide on diatoms growth but after longer exposure durations than in our study. For example (Pérès et al. 1996) reported the effects of low doses of herbicide with a decrease in densities between 87 and 96% compared to the controls after 34 days of direct exposure to 5µg/L of isoproturon (a phenylurea herbicide). In our study dealing with environmentally relevant concentrations, time to observed effect was situated between 7 and 13 days of exposure. We therefore recommend running this type of experiment for 2 weeks. Future works could focus on effects between day 7 and day 13 with a shorter time-scale in order to better characterize growth kinetics.

The negative correlation observed after 13 days of exposure in channels between AFDM and diatom densities suggests a positive impact of pesticides on the global biofilm growth but that the growth occurs in components of the biofilm other than diatoms. This could be linked to other algal groups being favoured by low pesticide pressure like green algae or cyanobacteria (discussed below) and/or non-algal biofilm components like bacteria, fungi, microfauna or the extracellular polymeric substance (EPS) matrix. The increase of EPS production has already been observed as a response to physical stress, Artigas et al. (2012) observed a higher EPS content for biofilm colonised in an intermittent river compared to a biofilm growing in a permanent river. Changes in EPS production were also studied under exposure to metal and organic compounds. Fang et al. (2002) showed that marine biofilms increased the production of extracellular polymeric substances by up to 100%, when exposed to metals and chemicals, such as Cd(II), Cu(II), Pb(II), Zn(II), Al(III), Cr(III), glutaraldehyde, and phenol. This

increase could be linked to the role of EPS as a protective shield for the cells against the adverse effects of the external environment.

Fluorescence levels are often used in field or laboratory studies to assess global biomass or biofilm growth (Serra et al. 2009, Barranguet et al. 2000). Using fluorescence levels as a proxy of the biofilm growth, we can conclude to a global growth of biofilm in 13 days and of the different types of algae as studied in detail here for diatoms.

The interpretation of fluorescence levels was the focus of a recent study (Corcoll et al. 2012a) reporting that fluorescence increase is not always linked to biofilm growth or biomass increase particularly when dealing with PSII inhibitors. When the photosynthetic apparatus is damaged by exposure to pesticides, the cells were proved able to induce the production of molecules of Chl *a* in order to maintain a suitable photosynthetic activity with the total number of cells remaining unchanged (greening effect) (Ricart et al. 2009, Guasch et al. 1997). Fluorescence can also increase without an increase of Chl *a* concentration when photosynthetic organisms are exposed to pesticides. When algae are exposed to PSII inhibitors, the electron transport chain is blocked, the photochemical pattern is then altered and is no longer available to evacuate the energy captured at the collective antenna, a maximum fluorescence peak is then observed (Ralph 2000). Chl *a* concentrations should not be estimated only by fluorescence measurement when dealing with exposure to pesticides targeting PSII in order to avoid global growth misinterpretation but should be confirmed by traditional Chl *a* measurements (determined spectrophotometrically), biomass determination (DW, AFDM) or algae counting. “Real” growth, greening effect and blockage of photosynthetic apparatus by PSII inhibitors can occur together and sometimes it is not possible to distinguish the different effects if only based on fluorescence measurements.

In our experiment the global increase of fluorescence at 665nm (Fs_{665}) and of fluorescence signals linked to cyanobacteria (Fs_{Bl}), diatoms (Fs_{Br}), and green algae (Fs_{Gr}) between day 0 and day 13 is clearly mainly explained by biofilm growth over the 13 days of exposure in channels since the increase was observed under all treatments. These results were also confirmed with the correlated increase of diatom densities and Fs_{Br} ($r^2 = 0.67$) with time exposure for all treatments. Nevertheless looking at differences between treatments we observed that growth is not the only factor explaining fluorescence variations.

When comparing Fs_{Br} to diatom density at day 13 for upstream biofilms, Fs_{Br} is higher when biofilm is exposed to PE than in the treatment without pesticide, but the number of diatoms follows an opposite variation with a higher diatom density in the treatment without pesticides.

These results are likely explained by both phenomena cited above, i.e. an increase of the number of chlorophyll a molecules per diatom cell (greening effect) and/or increase of fluorescence signal due to blockage of the electron transport chain since PSII inhibitors like diuron and simazine have been identified in PE.
Concerning Fs_{Gr}, its higher value at day 13 in treatment with PE compared to treatment without PE is to be viewed with respect to the variations of biomass (higher biomass and Fs_{Gr} for upstream biofilms exposed to PE compared to non-exposed upstream biofilms). If Fs_{Gr} is considered to be related to the growth of green algae (supported by higher biomass with PE than without), the results could suggest the development of tolerant green algae initially present in the upstream biofilm in the treatment with PE.

DW, AFDM, fluorescence levels and diatom density are often used as classical endpoints to detect long-term effects of chemicals on natural biofilms. We highlight here that biomass parameters (DW and AFDM) and fluorescence levels have to be regarded with precaution. DW and AFDM reflect global biofilm growth and responses to toxicant can greatly vary in regards to biofilm origins. Fluorescence levels cannot be directly linked to Chl *a* concentration and users have to be aware of possible misinterpretation as highlighted by Corcoll et al. (2012a). Nevertheless, more global endpoints like diatom densities showed here their suitability in highlighting the impacts of realistic complex pesticide mixtures for reference (upstream biofilms) communities.

The massive growth of *Planothidium lanceolatum* from day 0 to day 13 on upstream biofilms without pesticides was not observed when biofilms were exposed to PE: the relative abundance of this species remained the same as at day 0 (16% day 0 and 18 % at day 13) suggesting that the inhibition of its proliferation was caused by pesticides exposure.
Likewise, this lower relative abundance of *Planothidium lanceolatum* upstream with PE compared to upstream without PE treatment paralleled an increase in the relative abundance of *Eolimna minima* (3% for upstream without PE and 14% for upstream with PE) and *Nitzschia palea* (6% for upstream without and 24% upstream with PE) suggesting the pesticide tolerant character of both species. Very little is known about the relative sensitivities of diatoms to toxicants. *Eolimna minima* had been described as pesticide sensitive in numerous field studies (Hamilton et al. 1987, Pérès et al. 1996, Morin et al. 2009), but as mentioned above, pesticide gradients often parallel metal and/or nutrient gradients making it

difficult to clearly identify the particular effects of pesticides on diatom assemblages in the presence of multi-contamination.

Some recent laboratory studies have investigated the relative sensitivity of single diatom species exposed to pesticides; and have reported *Nitzschia palea* and *Eolimna minima* as relatively tolerant to diuron (Larras et al. 2012, Moisset, Personal communication) and *Planothidium lanceolatum* as relatively sensitive to diuron (Moisset, Personal communication).

These results suggest a change on diatom assemblages with application of pesticide pressure observable after 13 days, indicating selection by the treatment and thus different sensitivities of diatom species in our reference biofilm.

Divergence for upstream communities (non-exposed *in situ* to pesticides) exposed or non-exposed to pesticides in channels (using diatom density, biomass, and fluorescence levels (only Fs_{Gr}) and taxonomic composition as endpoints) appeared from 13 days of experiment. Running experiments for two weeks is long enough (and seems to be the minimum for diatom density and fluorescence levels) when working with low concentration of toxicant to be able to assess the toxic potential of environmentally realistic mixtures.

4.3. Time to recover from pesticide effects

The positive effects of the removal of pesticide pressure on downstream biofilms in regards to diatom growth are significant only at the last sampling time (day 13). Time to observe the effects brought about by the removal of toxic pressure on diatom growth is therefore most likely situated between 7 and 13 days after the placement of biofilms in clean water. Compared with diatom density results for upstream biofilms, the time required for *in situ* pre-exposed communities (downstream) to recover is of the same magnitude as the time required by pesticides to induce their effects on reference communities (upstream).

DW and AFDM variations followed the same pattern as diatom densities for the biofilm from the downstream site with higher values of DW, AFDM and diatom densities when communities where placed in clean water for 13 days highlighting the positive effect on diatom growth and global biofilm growth of removing pesticide toxic pressure. The results were in accordance with the data published for most field studies: negative effects had been observed with toxicant exposure on DW and AFDM. For example Morin et al. (2010a) recorded lower DW and AFDM in a field study on the Morcille River in spring 2008 for

natural communities sampled at the downstream site (chronically pesticide contaminated) compared to the upstream, reference site. Nevertheless, together with the results on upstream biofilms (higher DW and AFDM for exposed compared to non-exposed upstream biofilms) it can be noted that DW and AFDM followed different patterns with in regards to biofilm origin.

As found for upstream biofilms, fluorescence levels produced by the diatom groups at day 13 did not follow the results found during the counting procedure. No significant difference of Fs_{Br} between exposed or non-exposed biofilm was observed whereas diatom density was 1.8 times higher with the non-contaminated treatment. This result was also observed for upstream biofilms and similar hypotheses can be put forward (greening effect and/or induction of fluorescence due to close of PSII reaction centres in the treatments upstream with PE and downstream with PE).
Concerning the fluorescence signal linked to green algae groups, significantly higher values of Fs_{Gr} were recorded in the treatment without pesticides compared to the treatment with pesticides after 13 days of exposure. Like for upstream biofilms, Fs_{Gr} was correlated with biomass, nevertheless the variations of Fs_{Gr} were opposite for upstream and downstream biofilms (higher biomass and Fs_{Gr} for upstream biofilms exposed to PE compared to non-exposed upstream biofilms and lower biomass and Fs_{Gr} for downstream biofilms exposed to PE compared to non-exposed downstream biofilms). These opposite effects of low concentrations of pesticides on Fs_{Gr} in regards to biofilm origins could suggest a difference of sensitivity at day 0 between the upstream and downstream green algae groups. This difference of sensitivity between green algae species had already been reported in several works dealing with planktonic species (Huertas et al. 2010, Ma et al. 2002). The river biofilm research community, has carried out very few studies on green algae leading to a lack of knowledge about the response of this biofilm component to toxicant exposure.

No strong differences in diatom taxonomic composition were observed between downstream biofilms exposed to POCIS extracts or not; in both treatments the assemblages were dominated by *Nitzschia palea*, *Planothidium lanceolatum*, *Nitzschia linearis*, *Cyclotella meneghiniana*, *Eolimna minima* and *Cocconeis placentula* Ehrenberg var. placentula (Figure 24b). So, the trajectory observed for downstream biofilms does not seem to be affected when pesticide pressure is maintained or not. Several hypotheses could explain this similar evolution in diatom composition with in-channel exposure time.

The first cause for this result could be related to POCIS selectivity. Since POCIS preferentially sample compounds with $4 \geq \log K_{ow} \geq 0$, organic compounds outside this range of polarity and of course inorganic compounds are not taken up during sampling. In the case of the Morcille river multi-contamination occurs and the pesticide gradient parallels a metal gradient from upstream to downstream. The cocktail of compounds to which downstream biofilms are exposed *in situ* includes metals. Actually As and Cu have been recorded at concentrations reaching the Predicted No Effect Concentration (PNEC) at the downstream site of the river (Montuelle et al. 2010). When exposing communities from a multi-contaminated site to PE, the pressure exerted by all the compounds not sampled by POCIS but present in the field is removed. 2-4 D, chlorpyriphos (Assoumani et al. 2013) and metals are examples of such compounds. In our case, most concern lies in metal contamination since organic compounds not sampled by POCIS have been found to remain under the detection limit (data not shown) at the downstream site. Our hypothesis is that metal levels at the downstream site of the river exerted a strong selection pressure, so removal of the metal pressure is an important driver explaining the similarity in the evolution of diatom communities between day 0 and day 13. So, the changes in the patterns observed for downstream biofilms do not seem so to be affected by maintainance or removal of the pesticide pressure alone in the case of communities impacted *in situ* by multi-contamination (metals and pesticides) highlighting the influence of the *in situ* exposure history on the responses to pesticide exposure.

On the other hand, the importance of immigration processes in diatom assemblage recovery has to be taken into account. Abiotic drivers are generally considered as the major structuring components for natural communities (Stevenson 1996, Sabater 2009), here the removal or maintenance of pesticide pressure represented by PE did not influence diatom assemblages. This importance of immigration phenomena in diatom communities has been pointed out in a recent study; Morin et al. (2012a) investigated on controlled laboratory conditions the diatom assemblage recovery after chronic copper exposure and did not observe any recovery in assemblages placed under control conditions without external immigrants. In contrast, with contribution of external immigrants, they observed divergence of assemblages with a return to a “control” assemblage within 2 weeks.

The non-divergence of diatom composition after 13 days of experimental exposure in the laboratory for the *in situ* pre-exposed biofilms could also be linked to the initial very high tolerance of downstream biofilms to PE (EC_{25} was equal to 149 X for downstream biofilm). Some studies have demonstrated that acquired tolerance to contaminants may remain quite a

long time after removing toxic pressure (Dorigo et al. 2010). However, Pesce et al. (2010a) observed that a decrease in pesticide exposure can be followed by a slight decrease in biofilm tolerance (as assessed by EC_{50}). Dorigo et al. (2010) ascribed the incomplete recovery to delayed toxicant release from the biofilm matrix. To date, very little is known about tolerance changes of biofilms in response to the reduction of pollution levels, especially in the context of multi-contamination (Pesce et al. 2011a).

Significant differences for downstream communities (pre-exposed *in situ* to pesticides) exposed or not to pesticides in channels were observed with regards to diatom density, biomass, and fluorescence levels (only Fs_{Gr}) but not on taxonomic composition. As discussed above similar diatom assemblages at day 13 could be explained by the impossibility of immigration in laboratory systems and/or by the selective extraction of the POCIS (presence of metal contamination at the downstream station). Nevertheless, removal of pesticide pressure modelled by the replacement of downstream biofilms on clean water led to higher diatom density, DW, AFDM and Fs_{Gr} compared to biofilm exposed to PE at day 13. Two weeks is thus long enough to observe the effects of removal of pesticide pressure on *in situ* contaminated biofilms; furthermore the same range of time has been evaluated for the highlighted effects of low doses of pesticides on upstream biofilm (contamination modelling). Actually, in laboratory experiments dealing with passive sampler extracts, conclusions about diatom assemblage drivers have to be regarded with precaution due to the selectivity of passive samplers and to the major role of immigration.

5. Conclusions and perspectives

The use of extracts from passive sampling devices in biological tests has emerged this last decade (Harman et al. 2012). It especially allows issues related to mixtures, interactions and unknown compounds to be studied. POCIS extracts have been used in a range of acute toxicity tests for the assessment of particular toxicity (e.g. oestrogens, PSII inhibitors) with very promising results but studies evaluating low-dose and long-term effects of POCIS extracts are still in their infancy (Morin et al. 2012b). This study is one of the first to deal with the use of passive sampler extracts for toxicity assessment using chronic exposure on natural biofilm communities. The sensitivity of biofilm communities to PE increases from downstream to upstream, revealing the past history of communities by the *in situ* tolerance induction in the contaminated site of the river as expected from the PICT concept. Chronic exposure to PE in artificial channels at environmental concentrations showed significant

effects on growth-related (dry weight, ash free dry mass and diatom cell densities) and structural (fluorescence levels and diatom composition) parameters; with different trends for some of them depending on the biofilm origin and thus highlighting the crucial importance of exposure history in biofilm responses. The use of fluorescence levels to assess global biofilm growth and relative percentages of different algal groups have been discussed and have to be considered with caution in studies dealing with PSII inhibitors. It has also been highlighted that making conclusions about diatom assemblage drivers has to be done with precaution due to the selectivity of passive samplers and to the major role of immigration which is absent when working in controlled laboratory conditions. Our study showed the importance of taking into account chronic effects of mixtures in risk assessment studies and the relevance of the use of passives sampler (like the POCIS) extracts in this approach.

Acknowledgments

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Chapitre V : Effets aigus de pesticides et d’un produit de dégradation utilisés seuls et combinés

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Dans le chapitre précédent (chapitre IV), nous avons démontré la pertinence de l'échantillonneur passif POCIS pour la mise en évidence des effets chroniques de pesticides en mélange sur des biofilms de rivière aux origines différentes.
Cette approche a l'avantage d'être sans *a priori* sur la composition du cocktail de contaminants présents dans l'extrait, d'intégrer les effets de mélange et de prendre en compte les métabolites. Cependant cette approche « boîte noire » n'apporte pas d'informations sur la toxicité relative des composés identifiés dans l'extrait.

Nous allons donc, dans ce chapitre, nous interroger sur la toxicité relative des composés majoritaires échantillonnés par l'outil dans le contexte de contamination de la rivière Morcille. Dans ce travail, nous prendrons en particulier en compte la problématique des produits de dégradation dont la toxicité est encore trop peu étudiée.

L'objectif de cette étude est de comparer la toxicité de trois composés retrouvés en concentrations importantes dans les extraits de POCIS au printemps 2010 : le tébuconazole, le norflurazon et le norflurazon desméthyle (produit de dégradation principal du norflurazon). La réponse de biofilms de rivière face à ces composés utilisés seuls et en mélange ternaire aux proportions relatives retrouvées sur le site de St Ennemond sur la Morcille au printemps 2010 (5,5 % de norflurazon, 54 % de norflurazon desméthyle et 40,5 % de tébuconazole) a été évaluée. Pour cela, les biofilms ont été exposés à des concentrations croissantes en toxiques en microplaque. Après 48h d'exposition, les effets des toxiques ont été évalués sur les rendements photosynthétiques efficace (Φ_{PSII}) et optimal (F_v/F_m) du PSII et sur la densité et la composition des communautés de diatomées.

Lors de cette étude l'intérêt des tests en microplaque a été souligné puisqu'ils permettent de diminuer les quantités de contaminants utilisés (souvent onéreux). Néanmoins l'utilisation du système microplaque a montré ses limites pour le calcul des densités cellulaires et les analyses taxonomiques de par la difficulté de récupération de l'échantillon (adhésion des cellules aux parois de la microplaque).

Les résultats obtenus ont pu mettre en évidence une plus grande toxicité (F_v/F_m) du norflurazon desméthyle par rapport à son composé parent (le norflurazon) avec une CE_{10} estimée à 58 µg/L (intervalles de confiance $\alpha_{5\%}$: [9 ;254]). Ce résultat souligne en particulier

l'importance de prendre en compte les métabolites dans l'évaluation du risque environnemental.

D'autre part, l'impact du mélange ternaire sur le rendement photosynthétique optimal a été attribué au métabolite de par l'absence de toxicité observée pour le norflurazon et le tébuconazole testés seuls (concentration maximale testée de 3000µg/L pour chacun des composés).

Cette étude est l'une des premières études s'intéressant à la toxicité relative de pesticides et d'un métabolite utilisé seuls et en mélange sur du biofilm de rivière en microplaque. Les résultats obtenus en combinant l'utilisation de la technique de fluorescence en lumière modulée et du système microplaque laissent présager des perspectives intéressantes pour évaluer la toxicité des pesticides seuls et de mélanges simples.

Single and mixture effects of pesticides and a degradation product on fluvial biofilms

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Soumis à « Environmental Monitoring and Assessment »

Abstract

The Morcille River located in the Beaujolais vineyard area (Eastern France), is subjected to strong wine-growing pressure leading to the contamination by a range of herbicides and fungicides of the surrounding fresh water environment. Particularly high concentrations of norflurazon, desmethyl norflurazon and tebuconazole were recorded in spring 2010 at the downstream site on the river. Despite their occurrence in rivers, scare toxicity data are available for these products, in particular in the case of desmethyl norflurazon (main norflurazon degradation product). Further more often toxicity data available only focus on single compounds and are generated by single species toxicity tests linking to a lack of ecological relevance.

Consequently this study was undertaken to evaluate the toxic effects of norflurazon, desmethyl norflurazon and tebuconazole singly and in a ternary mixture on fluvial biofilm. Toxicity tests were performed in microplates for 48h. Photosynthetic endpoints were measured using Pulse Amplified Modulated fluorometry; diatom densities and taxonomic composition were determined. After 48h of exposure, significant effects on optimal quantum yield (F_v/F_m) for desmethyl norflurazon and mixture were observed.

Key words

Pulse Amplified Modulated fluorometry, pesticides, biofilm, metabolites, microplate, mixture.

1. Introduction

The European Union's Water Framework Directive has set the target to achieve good ecological status for all aquatic environments in Europe by 2015. Among environmental pollutants, pesticides are of particular concern and greatly affect water quality management. Providing knowledge and ecotoxicological data about pesticides is a challenge for prioritization of substances. Nevertheless, the question of breakdown products and mixture toxicities is rarely taken into account in water quality management despite recent studies underlining the toxicity of some metabolites (Amorós et al. 2000, Pesce et al. 2010b) or highlighting possible synergic effects of pesticides acting with similar or dissimilar modes of action (Magnusson et al. 2010).

The Morcille River located in the Beaujolais vineyard area (Eastern France) is subjected to strong agricultural pressure, essentially exerted by vineyard treatments. Herbicides and fungicides were found in particularly high concentrations in the surrounding fresh water environment and particularly high levels of norflurazon (a herbicide belonging to the pyridazinones, NFZ), of its main biodegradation product, desmethyl norflurazon (DMN) and of tebuconazole (an azole fungicide, TBZ) were recorded in spring 2010 at the downstream site on the river.

In this context, the objective of this work was to evaluate the single and joint effects of NFZ, DMN and TBZ on river biofilms. Attached microbial communities play a fundamental role in the ecological functioning of river systems, by their key place in the trophic web and their important contribution to primary production. Moreover, river biofilms interact strongly with dissolved substances present in water such as pesticides and are likely to respond quickly to contaminant pressures and can so be regarded as early warning systems and powerfull indicators for ecosystems health (Sabater et al. 2007). Moreover testing effects of toxic compounds on natural communities instead of using classic single species tests is an approach particularly appropriate by integrating high ecological relevance (Clements and Rohr 2009).

Toxicity tests on natural biofilm using Pulse Amplified Modulated fluorometry were performed directly on microplates in order to minimize the volumes of toxicant needed. Decreasing amount of compound in toxicological studies can be a crucial issue in particular when testing expensive substances like metabolites; moreover the use of micro volumes decrease the quantity of waste to treat and so the environmental and monetary coast of the study.

Biofilm was exposed to a range of pesticide concentrations for 48h. Then, photosynthetic parameters (optimal quantum yield: F_v/F_m and effective quantum yield of photosystem II: Φ_{PSII}), diatom densities, and taxonomic composition of diatom communities were determined. Previous studies already showed toxicity of NFZ and TBZ on aquatic organisms (Guseinova et al. 2005), so we hypothesized that these two pesticides might have toxic effects on natural biofilms; and particularly NFZ with regard to its photosynthetic efficiency due to its mode of action (inhibition of carotene synthesis). Moreover based on the scarce data available in the literature, a lower toxicity of DMN compared to its parent compound was expected, due to an increase of polarity from NFZ to DMN and thus an assumed decrease in its ability to influence carotenoid synthesis in the lipophilic chloroplast thylakoid (Wilkinson 1987).

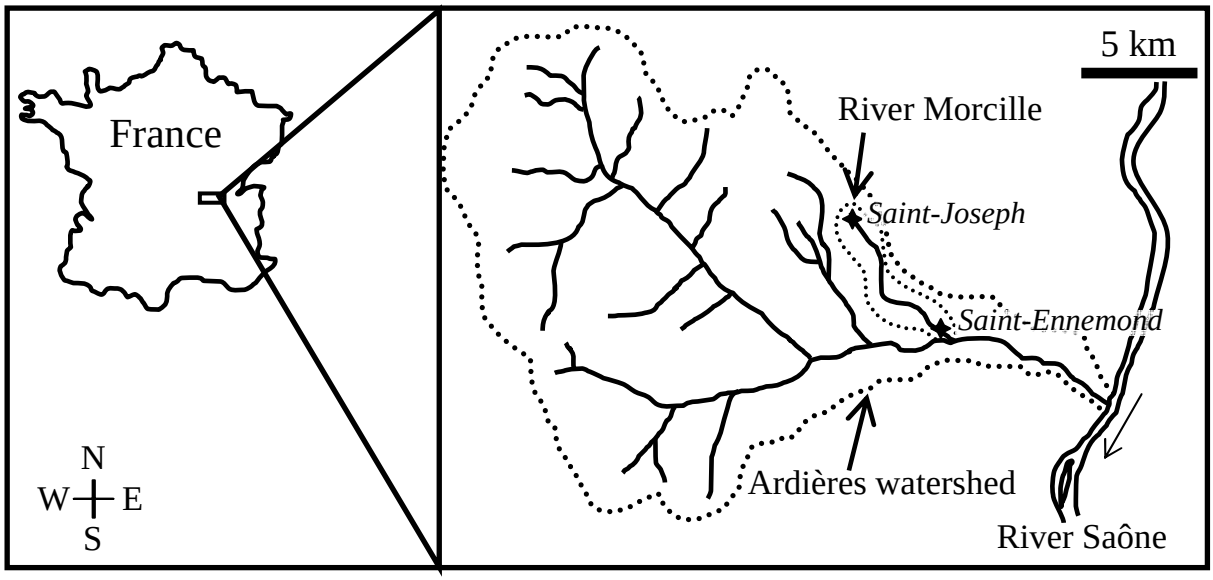
2.Materials and methods

2.1.Experimental design

2.1.1.Biofilm sampling and study site

Biofilms were collected in the Morcille River, located in the Beaujolais vineyard area (eastern France). The Morcille River is subjected to strong agricultural pressure, essentially exerted by vineyards and is characterized by an increasing pesticide gradient from upstream (<0.1 µg/L total pesticides concentrations) to downstream (>1.7 µg/L total pesticides concentrations) (Morin et al. 2012b). Sampling took place in April 2011 at the upstream station (Figure 25) characterized by very low pesticide concentrations (Montuelle et al. 2010).

Figure 25 : Location of the study sites along the Morcille River.



A composite biofilm sample was collected by scraping streambed rocks using a razor blade and was re-suspended in river water from the upstream site. The biofilm was maintained at 19-20°C with a photon flux density of 25 µmol.m^{-2}.s^{-1} and a 12:12 h light:dark cycle before use for the short term bioassay (within one week).

2.1.2.Chemicals

Norflurazon, (CAS reg. 27314-13-2, purity = 94%), desmethyl norflurazon (CAS reg. 23576-24-1, purity > 99%) and tebuconazole (CAS reg. 107534-96-3, purity > 98%) were purchased from Dr. Ehenstarler GmbH, Augsburg. Stock solutions were prepared in ultrapure water at a final concentration of 6000 µg/L.

In order for it to be representative of environmental conditions, mixture composition was chosen according to the concentration ratio of the three products found in the Morcille River at the downstream site in spring 2010: 5.5 % of NFZ, 54% of DMN and 40.5% of TBZ to obtain a stock solution with final total pesticide concentration of 6000 µg/L (327.6 µg NFZ/L, 3246 µg DMN/L and 2426.4 µg TBZ/L).

2.1.3.Short term bioassay

Bioassays were performed in a 96-well microplate with a clear, flat bottom (BD Falcon, Germany). In each well, from 0 to 200 µL of pesticide stock solution was added to 200 µL of biofilm suspended in WC culture medium (10^5 diatom cells/ml) (Guillard and Lorenzen 1972); appropriate volumes of ultrapure water were added to reach a final volume of 400 µL.

Four conditions were run: NFZ tested alone (noted NFZ), DMN tested alone (noted DMN), TBZ tested alone (noted TBZ), and the mixture NFZ, DMN and TBZ as described above (noted Mix). Biofilm was exposed in triplicate to 7 concentrations of total pesticides (C_0=0, C_1=93.75, C_2=187.5, C_3=375, C_4=750, C_5=1500 and C_6=3000 µg/L), and maintained at 19-20°C throughout the 48h of exposure with a photon flux density of 25 µmol.m^{-2}.s^{-1} and a 12:12 h light:dark cycle.

After exposure, optimal quantum yield (F_v/F_m) and effective quantum yield of PSII (Φ_{PSII}) were measured directly on microplates; then biofilms were suspended by successive pipetting, then collected and immediately fixed in lugol for taxonomic determination and counting.

"Control pesticide wells" were used to assess the pesticide stability: 200 µL of the 3 stock solutions (NFZ at 6000 µg/L, TBZ at 6000 µg/L, and DMN at 6000 µg/L) were added at T_0 to empty wells of the microplate to assess in our experimental conditions abiotic degradation of

compounds and well wall adsorption. Pesticide concentrations were determined at T_0 and after 48h.

2.1.4.Pesticide analyses

The 400 µL water samples were dried and then dissolved in 400 µL of ethyl acetate after addition of 4 µL of an internal standard solution (10 ng/µL of atrazine–*d5* and tebuconazole-*d6*), prior to analysis. Determination of pesticides and metabolites was performed with a gas chromatography system TRACE GC ULTRA (THERMO Scientific) coupled with a mass spectrometer Quantum GC (THERMO Scientific) and "triple quadripole" detection. The separation was made with a TR-5MS column (5% phenyl (equiv) polysilphenylenesiloxane, 30m x 0.25mm x 0.25µm, THERMO Scientific). Helium was used as mobile phase at a constant flow rate of 1.2 mL/min. 1 µL of sample was injected on splitless injector at 280°C. The gradient consisted of an isotherm for 0.5 min at 50°C, it then ramped to 190°C at 17°C/min, then to 220°C at 4°C/min, and a last ramp to 300°C at 20°C/min. The temperature was kept constant at 300°C for 8.26 min giving a total running time of 29 min. The source temperature was set at 250°C. The ionization was operated in electron impact mode (70 eV, current of 25 µA), argon was used as collision gas at 1.2 mTorr and acquisition was performed in selected reaction monitoring (SRM) mode with a total cycle time of 200 ms. The SRM transitions were 303/145 and 305/145 for NFZ, 289/145 and 289/173 for DMN, and 250/125 and 252/127 for TBZ. The SRM transitions for the internal standards, atrazine–*d5* and tebuconazole-*d6*, were 205/105 and 256/125, respectively.

2.2.Biofilm response analyses

2.2.1.Photosynthetic efficiency

The optimal quantum yield (F_v/F_m) and the effective quantum yield (Φ_{PSII}) of photosystem II (PSII) were measured using Pulse Amplified Modulated fluorometer (version EDF, Heinz Walz GmbH, Germany). F_v/F_m was measured after 30 min of dark adaptation whereas Φ_{PSII} was measured under light adaptation; F_v/F_m and Φ_{PSII} are described by the following equations (Baker 2008):

$$F_v/F_m = (F_m\text{-}F_0) / F_m \qquad \textbf{(1)}$$

$$\Phi_{PSII} = (F'_m\text{-}F_t) / F'_m \qquad \textbf{(2)}$$

(1) with F_0 the minimum fluorescence determined after a weak far red modulated light and F_m the maximum level of fluorescence measured during a saturating white light pulse.

(2) with F_t the steady-state level of fluorescence under ambient light and F_m the maximum level of fluorescence measured during a saturating white light pulse.

2.2.2.Diatom cell density

Diatom cell densities were determined at T_0 and T_{48h} for the different treatments (NFZ, TBZ, DMN and Mix) for the highest concentration (C_6) and for the Control treatment.

Diatom cells (200 µL of each sample) were enumerated using a Nageotte counting chamber (Marienfeld, Germany). The total number of individuals and the number of dead cells were recorded in 10 fields of the gridded area (1.25 µL each, 0.5 mm depth) under light microscopy at 400x magnification (Leitz photomicroscope). Distinction between dead and live organisms was estimated by observation of the turgescence and colour of the chloroplasts as described by Morin et al. (2010b).

2.2.3.Taxonomic analyses

Taxonomic analyses were performed after 48h of exposure to the different treatments (NFZ, TBZ, DMN and Mix) for the highest concentration (C_6) and for Control treatment.

Diatom identifications were performed after having prepared permanent slides following European standard NF EN 13946. Diatoms were identified at 1,000x magnification to the lowest taxonomic level possible using standard references (Hofmann et al. 2011).

2.2.4.Statistical analysis

The effects of pesticide exposure on photosynthetic efficiency (n=3) and diatom densities (n=3) were tested by one-way ANOVA analyses using Statistica 6.1 (StatSoft, France). The ANOVA was followed by a Tukey–HSD test (*p<0.05, **p<0.01, ***p<0.001). Homogeneity of variance was checked prior to data analysis. EC_{10} and confidence intervals $\alpha_{5\%}$ were calculated using REGTOX 7.0.5 (Vindimian 2003).

3.Results and discussion

3.1.Pesticide stability

Pesticide stability was assessed by comparing concentrations of compounds at T_0 and T_{48h} in the “control pesticide wells” for the highest pesticide exposure concentration for each condition (DMN, NFZ, TBZ and Mix for the C_6 treatments). Due to the physical characteristics of the compounds studied (low K_{ow} values), the latter are not linked to be

adsorbed and so we considered that if no adsorption was noticed in C_6 treatment it was also null in the other treatments.

No decrease was noted for the three compounds between T_0 and T_{48h} in their respective "control pesticide well". The organisms were then considered as having been exposed to the nominal concentration of pesticides and adsorption to the surface wells and photo degradation as was taken to be negligible in our experimental conditions.

Bioaccumulation of compounds by the different biofilm components was not considered in our experiment. Measuring concentrations of pesticides in biofilm is of particular interest to evaluate the exposure history of natural biofilms, nevertheless owing to the complexity of biofilm structure, developing analytical techniques for pesticide concentration determination in this matrix is still at an early stage and requires large amounts of (Byers, personal communication).

3.2.Photosynthetic efficiency

Φ_{PSII} was not significantly different between controls and NFZ, DMN, TBZ or Mix treatments after 48h of exposure, nor was F_v/F_m between controls and NFZ or TBZ treatments (Table 6). In contrast, a marked difference was observed when DMN and Mix treatments were compared to the Control with regard to F_v/F_m (Table 6, Figure 26).

These results show that:

(1) In our experimental conditions, F_v/F_m is a more sensitive parameter to reveal photosynthetic damage after pesticide exposure compared to Φ_{PSII}. This demonstrates the need to study complementary parameters when working with the effects of toxicants on photosynthetic efficiency. This point has already been pointed out by Laviale et al. (2011), who studied the effects of atrazine and isoproturon – two PSII inhibitors – on natural communities; they highlighted that the study of both Φ_{PSII} and F_v/F_m is necessary to avoid a partial view of periphyton photochemical response to these herbicides, and limitations for interpretation of these differential effects.

Table 6 : Mean values and standard errors of the photosynthetic and diatom parameters in function of treatment and time exposure. Φ_{PSII}, F_v/F_m, total diatom densities, mortality, diversity and species richness are given for C_6 for TBZ, NFZ, DMN and Mix treatments. Stars indicate significant difference compared to Control at 48h (n=3, *p<0.05, **p<0.01, ***p<0.001, when p<0.001 p value is given in brackets). n.c. : non calculated and < c.l. : under calculation limit.

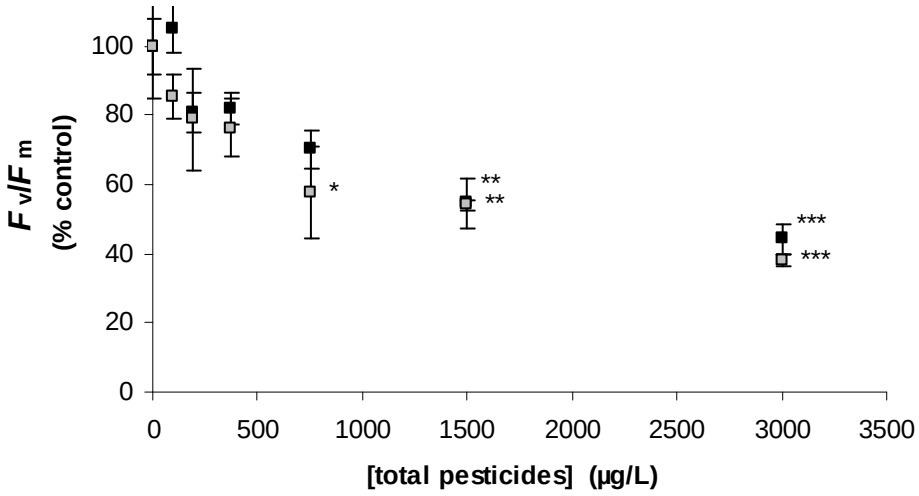
Treatment	Time of exposure (hour)	Φ_{PSII} in C_6=3000 µg/L total pesticides (% control)	F_v/F_m in C_6=3000 µg/L total pesticides (% contrlol)	EC_{10} (µg/L); α 5%	Total diatom densities (10^3 cells/mL)	Mortality (%)	Diversity	Species Richness (total number of species)
Ctr	0	n.c.	n.c.	n.c.	55.6 ± 3.5	23.6 ± 2.9	n.c.	n.c.
Ctr	48	n.c.	n.c.	n.c.	15.3 ± 2.9	21.5 ± 0.1	2.3 ± 0.1	25.7 ± 2.9 (38)
NFZ	48	67.4 ± 5.3	78.2 ± 6.9	< c.l.	33.1 ± 24.6	13.5 ± 10.2	2.1 ± 0.1	22.7 ± 1.6 (32)
TBZ	48	73.5 ± 11.5	72.6 ± 3.9	< c.l.	12.2 ± 1.4	21.6 ± 4.1	2.1 ± 0.1	18.7 ± 1.1* (30)
DMN	48	80.8 ± 3.8	38.2 ± 1.6*** (0.0007)	58; [9;254]	13.8 ± 9.9	19.7 ± 5.0	2.2 ± 0.1	18.7 ± 1.5* (29)
Mix	48	64.1 ± 22	44.2 ± 4.2*** (0.0006)	125; [24;413][a] 68; [16;185][b]	15.8 ± 3.0	19.8 ± 1.9	2.0 ± 0.2	19.7 ± 2.2 (31)

[a] Total pesticide concentration ; [b] DMN concentration.

(2) F_v/F_m and Φ_{PSII} were not sensitive enough to reveal NFZ or TBZ toxicity on the natural community in our experiment.

As a fungicide, TBZ does not act directly on the algal compartment of the biofilm (non-target organisms). Nevertheless, the use of photosynthetic fluorescence parameters can reveal the effects of chemicals affecting metabolic processes not directly linked to photosynthetic electron transport (for example any cellular process downstream of PSII) (Corcoll et al. 2012a). For example Ricart et al. (2010b) showed reduction of effective quantum yield (Φ_{PSII}) by up to 25% with increasing concentrations of the antimicrobial agent triclosan on biofilms. In another study, Bonnineau et al. (2010b) underlined the toxicity of β-blockers (metoprolol, propranolol and atenolol) on fluvial biofilms with regard to photosynthetic efficiency. In our experiment F_v/F_m and Φ_{PSII} were not relevant parameters to reveal the expected indirect effects of TBZ on the photosynthetic apparatus.

Figure 26 : Optimal quantum yield of PSII (F_v/F_m) (expressed as % control ± standard error) of biofilm community exposed to DMN and Mix treatment for 48h. PSII yield is expressed in function of the total pesticides concentrations. Stars indicate statistical difference from controls (n=3, *p<0.05, **p<0.01, ***p<0.001). Grey squares represent DMN treatments and black squares represent Mix treatments.



In contrast, we were expecting to see toxicity effects of NFZ on biofilm photosynthetic efficiency due to its mode of action (inhibitor of the carotenoid biosynthesis) but photosynthetic damage was not revealed after acute NFZ exposure. Some authors have shown the effects of NFZ on photosynthesis efficiency of single species and natural biofilms. For example, Tschiersch et al. (2002) showed a decrease of photosynthetic activity for cultures of *Euglena gracilis*; Frankart et al. (2003) showed a decrease of F_v/F_m after NFZ exposure of

Lemna minor. Pesce showed significant effects of NFZ on natural biofilm on F_v/F_m and determined a CE_{50} close to 100 µg/L (personal communication). This difference of sensitivity could be explained by experimental conditions and variability of natural biofilms (e.g. global community structure, ratio between algal groups, taxonomic composition, life history of the biofilm, etc.).

Optimal quantum yield (F_v/F_m) versus toxicant concentrations for DMN and Mix 48h-treatments is presented in Figure 26 for the whole range of contamination.
Significant differences in F_v/F_m were observed for DMN and Mix treatment between the controls and the highest concentrations after 48h of exposure. F_v/F_m was significantly different in C_4, C_5 and C_6 for DMN treatment (57.9±13.2, 53.9±1.6 and 38.2±1.6 % of control respectively) and in C_5 and C_6 for Mix treatment (54.5±7.3 and 44.2±4.2% of control respectively).
In order to compare the relative toxicity of DMN and Mix treatments, optimal quantum yield (F_v/F_m) was expressed versus total pesticide concentrations and versus DMN concentrations for both treatments and the corresponding EC_{10} (toxicant concentration needed in order to decrease F_v/F_m to 10% compared to the control) were calculated.
EC_{10} were respectively 58 µg DMN/L, $\alpha_{5\%}$ [9;254] for DMN treatment, 125 µg/L total pesticide, $\alpha_{5\%}$ [24;413] and 68 µg DMN/L, $\alpha_{5\%}$ [16;185] for MIX treatment and were not significantly different from each other (Table 6).

To our knowledge this is the first study demonstrating DMN toxicity on photosynthetic efficiency. Very little is known about the chemical properties and mode of action of DMN and toxicological data are scarce. Nevertheless this compound was reported to decrease chlorophyll content in the alga *Scenedesmus acutus* (Sandmann et al. 1981).
Moreover, we observed greater toxicity of DMN than of its parent compound on F_v/F_m (no effect of NFZ was noted at concentrations up to 3000 µg/L). These results underline the necessity to take in account metabolite issues in pesticide toxicity assessment, as highlighted in another study dealing with the ecotoxicity of pesticides and transformation products (Sinclair and Boxall 2003). In this study the authors identified several transformation products that were more toxic than the parent compound, for example 4-chlorophenol was found to be more toxic to algae than its parent compound the 2,4-D (a herbicide belonging to the alkylchlorophenoxy family). They pointed out four main reasons to explain the greater toxicity of the biotransformation product than the parent compound *i.e. "(1) the presence of a*

pesticide toxicophore; (2) the fact that the product is the active part of a propesticide; (3) the product is accumulated to a greater extent than the parent compound; or (4) the product has a more potent mode of action than the parents" (Sinclair and Boxall 2003). Of course explaining differences of toxicity between metabolite and its parent compound and more generally between two toxicants requires having as much toxicological data as possible for each of the compounds involved (e.g. EC_{50}, mechanisms of action of products or mechanisms of tolerance of species exposed). These data are often missing and therefore the scientific community has to continue its efforts to supply such information to the relevant databases.

3.3.Diatom growth

Significant differences in cell densities between Controls at T_0 and the different treatments after 48h of exposure (Ctr, NFZ, DMN, TBZ and Mix) were observed for the C_6 treatments; densities at T_0 were 3.6 times higher than densities at T_{48h} (Table 6). This decrease of cell number could be explained by the fact that biofilm may have become deposited on the bottom of the microplate due to the absence of agitation. When sampling at $T_{48h,}$ part of the cells may have remained adhering to the microplate leading to the decrease in overall cell density observed between T_0 and $T_{48h.}$

Statistical analysis revealed that densities at T_{48h} were not significantly different in control treatment and in NFZ, DMN, TBZ and Mix treatments. Pesticide treatments did not seem to have an effect on diatom density after 48h of exposure. This can be explained by the short time of exposure to toxicants. However, similar tendencies have been reported in the case of algae exposure to other toxicants. For instance Kim Tiam et al. (2012) observed the effect of high concentrations of cadmium (100 µg Cd/L) on the growth of *Eolimna minima* only after 7 days of exposure. In the same way, Morin et al. (2008) reported differences in biofilm diatom densities between control and lower cadmium contaminated treatments (10 µgCd/L) after 6 weeks of exposure.

Percentages of dead cells were not significantly different in controls (T_0 and T_{48h}) compared to contaminated conditions (Table 6). Apoptosis being the ultimate response of cells to toxicant, we can assume that during our experiment the cell damage caused by the pesticides and the metabolite did not exceed critical levels and could still be repaired by other mechanisms involving, for instance, antioxidant enzymes (Bonnineau et al. 2010a).

Although no effect was observed on diatom density or mortality, sub-lethal effects can occur as shown by the decrease of F_v/F_m in DMN and Mix treatments compared to the Control.

3.4.Diatom community structure

Due to the very low diatom concentrations in the samples, counting the 400 frustules as required by NF T90-354 was not feasible; around 200 frustules were counted in each replicate sample. The total number of species recorded per treatment (i.e. composite sample obtained by pooling replicates) was higher in the controls (38 taxa) than in the contaminated treatments (30.5 ± 0.7). Average values of species richness and diversity were equal to 21.1± 3 and 2.1± 0.1 respectively over the treatments.

Statistical analysis revealed that community composition was not significantly different in controls and with NFZ, DMN, TBZ and Mix treatments after 48h of exposure. Communities were dominated by three main species (relative abundance >60% in all samples): *Rhoicosphenia abbreviata, Achnanthidium minutissimum* and *Planothidium lanceolatum*. *Reimeria sinuata, Eolimna minima* and *Nitzschia palea* were also frequently found in samples in lower proportions.

Very little is know about diatom species sensitivity to pesticides, nevertheless some species have already been shown to tolerate triazines like *Achnanthidium minutissimum* or *Eolimna minima* (Pérès et al. 1996, Herman et al. 1986, Kasai et al. 1993, Munoz et al. 2001, Seguin et al. 2001) or a number of pesticides (triazine and urea) like *Nitzschia palea* (Kasai et al. 1993, Dorigo et al. 2004).

All samples were dominated by species belonging to the low-profile guild (79.1±1.6%) as defined by Passy (2007) (*Achnanthidium minutissimum, Planothidium lanceolatum, Reimeria sinuata,* and *Rhoicosphenia abbreviata*). Species from this guild were shown to cope with pesticides (Berthon et al. 2011, Roubeix et al. 2011). The fact that pesticide-tolerant species were already dominant in the inocula, although collected in a very low pesticide concentration site, may explain why no changes in community composition were observed between contaminated and control conditions; and may suggest species selections occurring from very low pesticides concentrations.

4.Conclusions

Studies dealing with pesticides and water quality management cannot be complete and realistic without taking into account metabolites and mixture effects. In this study, the single and joint effects of NFZ, DMN and TBZ were evaluated on natural biofilm; the major implications of this work are:

- Toxicity tests in microplates are of particular interest for decreasing the amounts of chemical (often expensive) needed and is already used in routine studies of single algae species with growth inhibition as endpoint in numerous studies. Nevertheless, physiological information is rarely investigated in this kind of study (Magnusson et al. 2010). To our knowledge this was the first study using Pulse Amplified Modulated fluorometry for toxicity tests in microplates directly on natural biofilms (more realistic than single species tests).
- However, the use of microplates showed some limitations for density calculation and taxonomic analyses (loss of part of sample on the microplate).
- DMN showed higher toxicity on photosynthetic efficiency (F_v/F_m) than its parent compound (NFZ); highlighting the importance of taking metabolites into account in toxicity assessment.
- The Mix treatment significantly affected F_v/F_m. In regards to the absence of toxicity of NFZ and TBZ when tested alone, the toxic effect observed in Mix treatment is probably due to DMN only.

This is one of the first studies dealing with metabolite and mixture toxicity of pesticides on natural biofilms in microplate; the results obtained using Pulse Amplified Modulated fluorometry and microplates together as an experimental unit presage interesting perspectives in the field of toxicity evaluation of pesticides whether as single substances or mixtures.

Acknowledgements

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Chapitre VI : Effets à long terme de pesticides et d’un produit de dégradation

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Lors du chapitre précédent nous nous sommes intéressés à la toxicité aiguë des composés majoritaires retrouvés dans les extraits de POCIS à travers l'étude de paramètres classiquement utilisés sur le biofilm (rendements photosynthétiques, densité et taxonomie des diatomées) après 48H d'exposition. Cependant les techniques de fluorescence en lumière modulée permettent de réaliser de nombreuses analyses susceptibles de mettre en évidence les effets de composés sur le biofilm de rivière. Parmi elles, la construction de courbes lumière photosynthèse (Rapid Light Curves : RLCs) est utilisée pour appréhender les capacités photosynthétiques dans le domaine de l'écophysiologie. Elles ont été largement utilisées pour décrire l'adaptation d'un échantillon à des conditions lumineuses particulières. Néanmoins de telles courbes ne constituent pas un outil exploité en écotoxicologie, malgré leur potentiel pour une évaluation précoce et sensible d'impacts physiologiques sur les organismes.

L'objectif de ce chapitre est d'évaluer la toxicité relative de trois composés retrouvés dans les extraits de POCIS : le diuron, le DCPMU (produit de dégradation majoritaire du diuron) et le norflurazon. Le choix s'est porté sur ces toxiques particuliers car, en plus de leur présence dans les extraits de POCIS, ils possèdent différents modes d'action et vont ainsi avoir un effet plus ou moins direct sur l'appareil photosynthétique. Le diuron et le DCPMU sont des inhibiteurs du photosystème II (PSII), un impact rapide de ces composés est ainsi attendu sur les capacités photosynthétiques du biofilm. Des effets plus indirects sont susceptibles d'être induits par le norflurazon puisque ce composé est un inhibiteur de la synthèse des caroténoïdes, pigments jouant un rôle entre autre dans la collecte de l'énergie lumineuse et la photoprotection.
Les impacts des deux pesticides et du produit de dégradation ont été évalués à travers la mesure de paramètres dit « classiques » comme les rendements photosynthétiques (Φ_{PSII} et F_v/F_m) ou la fluorescence de base (F_0) et la construction des RLCs.

Pour cela, du biofilm a été prélevé d'un site de référence (St Joseph sur la Morcille) puis cultivé au laboratoire sur des lames de verre. Les biofilms ont alors été exposés à une gamme de diuron (de 0,3 à 33,4 µg/L), de DCPMU (de 1 à 1014 µg/L) et de norflurazon (de 0,6 à 585 µg/L). Les réponses des biofilms ont été évaluées après 1, 5, 7 et 14 jours d'exposition directe.

Les résultats ont révélé une plus grande sensibilité des paramètres extraits des RLCs par rapport aux paramètres de fluorescence dits « classiques ». L'impact des toxiques a en effet pu être mis en évidence pour les trois composés dès le premier temps de prélèvement (24h d'exposition) au lieu de cinq jours d'exposition pour les « paramètres de fluorescence classiques » (F_0, F_v/F_m et Φ_{PSII}).

De plus des réponses différentes en fonction du mode d'action des composés ont été notées : les impacts du norflurazon se sont avérés faibles après 5 jours d'exposition mais avec une augmentation considérable de leur intensité avec le temps d'exposition. Au contraire les effets du diuron et du DCPMU ont été du même ordre de grandeur du cinquième ou quatorzième jour d'exposition. Par ailleurs la toxicité relative des composés s'est avérée temps mais aussi paramètre dépendante. En effet le norflurazon peut être considéré comme plus toxique que le diuron et le DCPMU au regard de la fluorescence de base (F_0) alors qu'une tendance opposée a été observée avec les rendements photosynthétiques après 5 et 7 jours d'exposition.

Ces résultats soulignent donc l'importance du temps d'exposition et des paramètres choisis dans l'évaluation du risque environnemental. Nous pouvons aussi noter que des effets ont été observés pour le diuron et le norflurazon à la concentration la plus faible testée (0,3 et 0,6 µg/L pour le diuron et le norflurazon respectivement). Ces impacts sur le biofilm de rivière à des concentrations réalistes d'un point de vue de la contamination environnementale pourraient supposer des effets de tels composés sur les organismes aquatiques et en particulier puisque les toxiques ne sont pas présents seuls mais en cocktail dans l'environnement aquatique.

Notre étude met donc en évidence l'intérêt des RLCs comme descripteurs précoces d'une exposition aux produits phytosanitaires.

Herbicides toxicity in river biofilms assessed by Pulse Amplitude Modulated (PAM) fluorometry

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En préparation

Abstract

The use of Rapid Light Curves (RLCs) as a toxicity end point for river biofilms compared to "classical fluorescence parameters" -minimal fluorescence (F_0), optimal and effective quantum yields of photosystem II (F_v/F_m and Φ_{PSII})- was examined in this study. Measurements were realized after exposure to five concentrations of diuron (from 0.3 to 33.4 µg L^{-1}), DCPMU (diuron main degradation product, from 1.0 to 1014 µg L^{-1}) and norflurazon (from 0.6 to 585 µg L^{-1}) with the lowest exposure concentrations corresponding to levels encountered in chronically contaminated sites. Biofilm responses were evaluated after 1, 5, 7 and 14 days of exposure to the different toxicants.

Parameters calculated from RLCs (α, ETR_{max} and I_k) revealed to be early markers of pesticide exposure since significant effects of contaminated treatments on these parameters were observed from the first day of exposure whereas at day 1 classical fluorescence endpoints (F_0 and F_v/F_m) were only significantly different from Control in DCPMU treatment.

Moreover responses on both "classical fluorescence parameters" and RLC endpoints were highly time dependant and related to the mode of action of the different compounds.

Key words

Pesticides, periphyton, chlorophyll fluorescence, dose-response curves, rapid light curves, environmental risk assessment.

1.Introduction

The European Union's Water Framework Directive has set the target to achieve good ecological status for all aquatic environments in Europe by 2015. Among environmental pollutants, pesticides are of particular concern and greatly affect water quality management. Increasing knowledge and providing ecotoxicological data about pesticides is so a challenge in order to reach this goal.

The Morcille River located in the Beaujolais vineyard area (Eastern France) is subjected to strong agricultural pressure, essentially exerted by vineyard treatments. Herbicides and fungicides were found in particularly high concentrations in the surrounding freshwater environment and particularly high levels of diuron (a phenylurea herbicide), of its main biodegradation product, 1-(3,4-dichlorophenyl)-3-methyl urea (DCPMU) and norflurazon (a herbicide belonging to the pyridazinones, NFZ) were recorded during several years at the downstream site on the river (Montuelle et al. 2010, Rabiet et al. 2010, Morin et al. 2012b).

Diuron is a phenylurea herbicide which blocks electron transfer from Photosystem II (PSII) to Photosystem I (PSI) by binding itself to the D1 protein constitutive of PSII instead of the second electron acceptor (Q_b) (Trebst and Draber 1986, Zer and Ohad 1995). Diuron thus prevents the reoxidation of the primary electron acceptor (Q_a) by blocking the electron transfer from Q_a to Q_b; which leads to an increase of the minimal fluorescence and a decrease in variable fluorescence (Ralph 2000).

DCPMU is the main degradation product of diuron; it is obtained by *N*-demethylation under aerobic conditions. The loss of the methyl group appears to cause a slight but not dramatic decrease in the binding affinity to the Q_b receptor site of D1 (Dewez et al. 2002; Neuwoehner et al. 2010). A slightly lower toxicity of DCPMU compared to the parent compound for photosynthetic organisms is so expected. Actually Pesce et al. (2010b) observed a lower toxicity of DCPMU comparing to its parent compound on river biofilms. Nevertheless, the studies assessing effects of metabolites products are still scarce and in particular the relative toxicity of DCPMU and diuron seems to be highly dependent of selected organisms and endpoints (Gatidou and Thomaidis 2007, Dewez et al. 2002).

Norflurazon (noted NFZ) is a herbicide belonging to the pyridazinones which inhibits carotenoids biosynthesis by exerting its primary inhibitor action on the reaction catalysed by phytoene synthase (Sandmann et al. 1980). Carotenoids are photosynthetic pigments exerting a crucial role in light harvesting and photoprotection (Rowan 1989). Exposure to NFZ is expected to lead to indirect effects on fluorescence levels by inhibiting renewal of carotenoid pool essential to the correct functioning of the photosynthetic apparatus. Its effects have been

well studied in regards to growth related parameters or by pigment measurements for microalgae or plants, but very little is known on its indirect effects on photosynthesis capacities and in particular on river biofilms (Sandmann et al. 1981, Frankart et al. 2003, Wilkinson 1987, Wilson and Koch 2013).

Pulse Amplitude Modulated (PAM) fluorometry is a powerful tool for monitoring the effects of chemicals on photosynthetic organisms based on the measurement of *in vivo* chlorophyll (Chl) fluorescence (Juneau et al. 2007). It allows the estimation of various parameters, among which the minimal fluorescence (F_0), the effective quantum yield of photosystem II photochemistry (Φ_{PSII}) and the optimal quantum yield of PSII (F_v/F_m) have been the most commonly used to study both short and long term effects of herbicides on photosynthetic activity and related physiological activities (Corcoll et al. 2012a). F_0 has been used as a proxy of algal biomass since chlorophyll fluorescence can be proportional to total chlorophyll content (Serôdio et al. 1997). Φ_{PSII} gives a measure of the proportion of the PSII absorbed light that is used in photochemistry and integrates all the processes downstream of PSII which are dependant of the actual test conditions, e.g. light and temperature (Baker 2008). In comparison, F_v/F_m reflects the number of functional PSII, thereby illustrating the sample physiological state (Baker 2008). In particular, F_v/F_m and Φ_{PSII} have been shown to be well appropriate when working with PSII inhibitors (e.g. Laviale et al. 2010, Laviale et al. 2011) but have been strongly recommended to combine with other parameters when working with compound acting on other target than PSII (Bonnineau et al. 2012).

The electron transport rate (ETR) represents a measure of the capacity for photosynthetic activity and can be used to compare plant species. Rapid Light Curves (RLCs) are relating the photosynthetic electron transport rates (ETR) with incident photon irradiance (White and Critchley 1999). This method offers a convenient way for characterizing the photoacclimation status of photosynthetic organisms exposed to different light environments, including diatom dominated biofilms (Serôdio et al. 2005, Roberts et al. 2004). Report of the use of RLCs in order to highlight toxicant impact is scarce (Luís et al. 2013). Nevertheless it offers promising perspectives particularly when working with toxicants with less specific mode of action than PSII inhibitors.

Consequently the first objective of this study was to compare the toxicity of diuron, DCPMU and norflurazon on river biofilms, particularly of DCPMU and norflurazon since few ecotoxicological data are available for these products. The second objective of the study was to evaluate the pertinence of RLCs and classical endpoints used in toxicological studies dealing with fluorescence measurements. For this purpose, field-derived biofilms were

exposed to a range of concentrations, from ecologically relevant to acute ones; the evolution of biofilms responses was assessed in function of time exposure (from 24 h to 14 d) in regards to "classical endpoints" and RLCs parameters.

2.Materials and methods

2.1.Biofilms sampling and culture

Biofilms were collected in the Morcille River, located in the Beaujolais vineyard area (eastern France). Sampling took place in March 2013 at a pristine site located on the upstream of the river characterized by very low pesticide concentrations (Montuelle et al. 2010). Biofilm was collected by scraping streambed rocks using a razor blade and was re-suspended in river water from the upstream site. The biofilm was inoculated into an aquarium containing 8 L of WC culture medium (Guillard and Lorenzen 1972) under continuous water flow insured by a classic aquaria pump (Rena Flow 600 BF, 580L/H) and left for 2 weeks to allow biofilm colonisation on artificial substrates (glass slides 26x26 mm) previously placed on the bottom of the aquaria. The culture aquarium was maintained in a thermoregulated incubator at 20 °C with a photon flux density of 50 µmol m^{-2} s^{-1} and a 12:12 h light:dark cycle during the colonisation period.

2.2.Experimental set-up

Diuron (CAS reg. 330-54-1, purity = 98%), DCPMU (CAS reg. 3567-62-2, purity = 97.7%) and NFZ (CAS reg. 27314-13-2, purity = 94%) were purchased from Dr. Ehenstarler GmbH, Augsburg; stock solutions were prepared in ultrapure water after solvent evaporation. The stock solutions prepared were analyzed by HPLC–ESI–MS/MS (HPLC Ultimate 3000, Dionex, equipped with a Gemini NX C18 column, 10 mm x 2 mm, 3 µm, Phenomenex); the different exposure concentrations were calculated from the analyses of the respective stock solutions.

After 2 weeks of colonisation, glass slides colonized by biofilms were removed from the culture aquaria and introduced in single experimental units in WC media (final volume=50 mL). Biofilms were directly exposed to 5 concentrations of diuron (C_1=0.3, C_2=1.1, C_3=3.3, C_4=10.6 and C_5=33.4 µg L^{-1}), DCPMU (C_1=1.0, C_2=32.1, C_3=101.4, C_4=320.7 and C_5=1014.0 µg L^{-1}) and norflurazon (C_1=0.6, C_2=18.5, C_3=58.5, C_4=185.0 and C_5=585.0 µg L^{-1}). These concentrations were chosen in order to observe effects on photosynthesis up to 50% of inhibition based on EC_{50} calculated from bioassays realized on river biofilms (Pesce et al. 2010b). The lowest exposure conditions were close to concentrations encountered in

chronically polluted rivers (Montuelle et al. 2010). Each condition was realized in triplicate and six control conditions (Ctr) were made (biofilms in the experimental units in WC media without pesticides). Experimental units were maintained in the thermoregulated incubator during the 14 days of experiment with the same conditions than during the colonisation period (20 °C, 50 µmol m^{-2} s^{-1}, and 12:12 h light:dark cycle). Water was changed several times during the experiment (days 2, 6, 9 and 13) in order to avoid nutrient limitation and to maintain pesticides concentrations. Minimal fluorescence (F_0), optimal quantum yield (F_v/F_m) and effective quantum yield of PSII (Φ_{PSII}) were determined for all samples after 1, 5, 7 and 14 d of exposure. RLCs were realized at the same sampling times for Ctr and intermediate (C_2), high (C_4) and very high (C_5) concentrations of the 3 tested chemicals (diuron, NFZ and DCMPU).

2.3.Biofilm responses analyses

All fluorescence measurements were realized using a Pulse Amplified Modulated fluorometer (PhytoPAM version EDF, Heinz Walz GmbH, Germany).

"Classical fluorescence parameters"

F_0 and F_v/F_m were measured after 30 min of dark adaptation whereas Φ_{PSII} was measured under ambient light. F_v/F_m and Φ_{PSII} are described by the following equations (Baker 2008):

$$F_v/F_m = (F_m-F_0) / F_m \quad \textbf{(1)}$$

$$\Phi_{PSII} = (F_m'-F_t) / F_m' \quad \textbf{(2)}$$

(1) with F_0 the minimal fluorescence determined after a weak far red modulated light and F_m the maximum level of fluorescence measured during a saturating white light pulse.

(2) with F_t the steady-state level of fluorescence under ambient light and F_m' the maximum level of fluorescence measured during a saturating white light pulse.

Rapid light curves

Rapid light curves were constructed by exposing the samples to 10 sequential actinic light levels increasing from 64 to 610 µmol.m^{-2}.s^{-1} (i.e the maximum light level range provided by the PhytoPAM) after a 5 min adaptation period at 64 µmol.m^{-2}.s^{-1} corresponding to light intensity of the first step of the curve. Φ_{PSII} was calculated as described above after each illumination step of 10s. RLCs were constructed and fitted using the Phyto-Win Software V 1.45 (Heinz Walz GmbH, Germany). For each level of actinic light, the relative electron transport rate (ETR) was calculated by the software according to the following equation:

$$ETR = \Phi_{PSII} \text{ x PAR x } 0.5 \text{ x } 0.84 \quad \textbf{(3)}$$

(3) with PAR the photosynthetically active radiation applied during each illumination step, 0.84 the assumed absorptivity (Björkman and Demmig 1987) and 0.5 the factor due to the assumption that half of the quanta of the incident PAR are distributed to PSII (Maxwell and Johnson 2000).

The RLCs were then fitted according to a modified version of the photosynthetic model of Eilers and Peeters (1988) in order to calculate the photosynthetic efficiency at low intensity (α), the maximal electron transport rate (ETR_{max}) and I_k the onset of light saturation (Henley 1993).

2.4.Statistical analyses

For each fluorescence parameter, the effects of time of exposure and pesticides concentration were tested by two-way ANOVA analyses. The effects of pesticides exposure were then tested at each exposure time by one-way ANOVA analyses. The ANOVA was followed by a Tukey–HSD test. Normal distribution of data was checked prior to data analysis. Statistical analyses were realized using STATISTICA 6.1 (StatSoft).

Dose response curves were constructed for photosynthetic yields (F_v/F_m and Φ_{PSII}) using a three-parameter log-logistic model of the drc package of R (Ritz and Streibig 2005):

$$f(x, (b, d, e)) = d / (1 + \exp\{b(\log(x) - \log(e))\}$$

With e the EC_{50} the concentration producing a response half-way between the upper limit, d, and lower limit equal to 0. The parameter b denotes the relative slope around e.

Due to non convergence of the model, the highest DCPMU concentration was removed from the data set (slight increase of F_v/F_m and Φ_{PSII} values for the C_5 not accepted by the model).

3.Results

3.1 Classical fluorescence parameters

Two-way ANOVA analyses revealed significant effects of exposure time and chemicals for all parameters (F_0, F_v/F_m and Φ_{PSII}, $p < 0.05$).

3.1.1 Photosynthetic yields

The optimal and effective quantum yields of PSII (F_v/F_m and Φ_{PSII}) expressed as % of Ctr for the different treatments and time exposure are presented in Figure 27(a) and Figure 27(b) but the control values slightly increased during the 14 days of experiment; Φ_{PSII} and F_v/F_m increased from 0.47 ± 0.04 to 0.61 ± 0.02 and from 0.49 to 0.02 to 0.061 ± 0.01, respectively.

This is reflecting that the transfer from the culture aquarium to the experimental units did not affect the physiology of the algal component of the biofilm. Mean values of 0.55 ± 0.05 and 0.56 ± 0.07 for F_v/F_m and Φ_{PSII} respectively highlighted a good physiological state over the 14 d of experiment.

Significant effects of diuron exposure on Φ_{PSII} and F_v/F_m were observed from day 5 to day 14 and were of the same order of magnitude whatever the sampling time considered, with impact from the lowest exposure concentration (C_1=0.3µg L^{-1}). Maximal effect was recorded for the highest concentration (C_5 = 33.4µg L^{-1}) at day 5 (61 ± 3 % Ctrl) and 14 (71 ± 1 % Ctrl) for Φ_{PSII} and F_v/F_m respectively.

Effects of DCPMU exposure were significant from day 1 in regards to F_v/F_m and from day 5 in regards to Φ_{PSII}. From day 5, inhibition of Φ_{PSII} and F_v/F_m was of the same order of magnitude whatever the sampling time considered. Maximal effect was recorded for the concentration C_4 (= 320.7µg L^{-1}) at day 7 (63 ± 3 % and 69 ± 5 % Ctrl).

Impacts of NFZ increased with exposure time ($p<0.001$) and NFZ concentration ($p<0.001$). Inhibition of Φ_{PSII} and F_v/F_m was comparable and significant from day 5 (73 ± 8 and 73 ± 7 % Ctrl, respectively). Maximal effect was recorded for the highest concentration (C_5 = 585µg L^{-1}) at day 14 (34 ± 5 and 32 ± 7 % Ctrl).

Φ_{PSII} was globally more affected by toxicant exposure than F_v/F_m as shown by the calculated effect concentrations (Figure 27). Both yields designed diuron as the more toxic compound with the lower EC_{50}. In regards to Φ_{PSII}, NFZ and DCPMU were in the same range of toxicity, nevertheless F_v/F_m was more impacted by NFZ than DCPMU. The mean EC_{50} ranged the compound toxicity as (from the more to the less toxic): diuron >> NFZ > DCPMU.

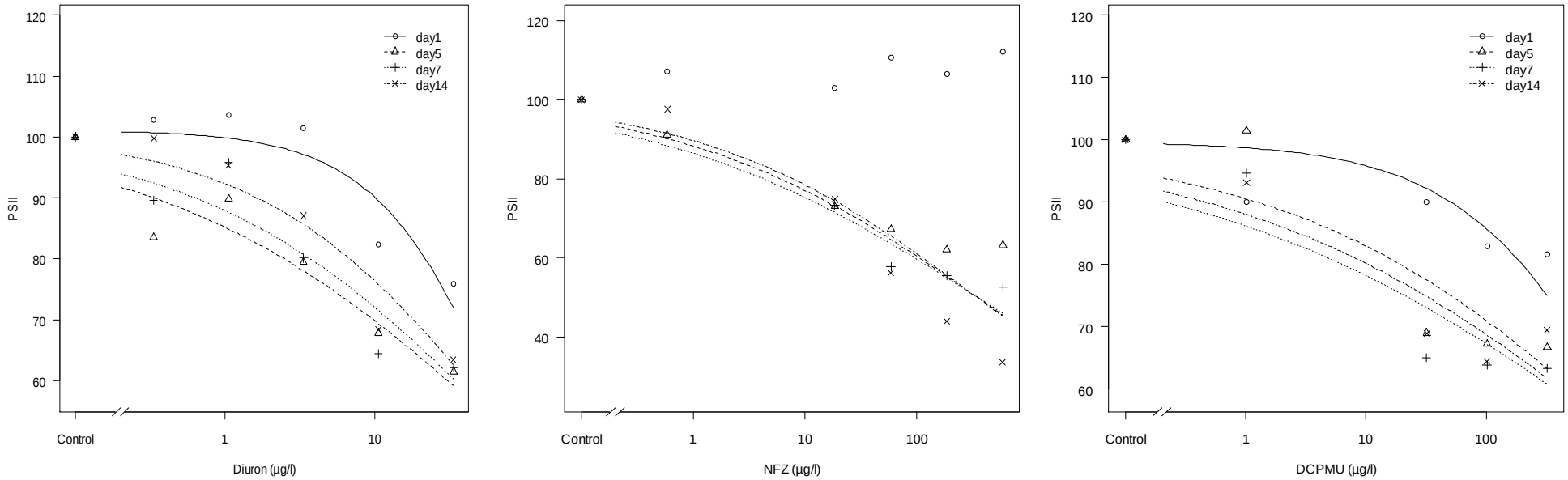
3.1.2 Minimal fluorescence

Values of F_0 for the different chemicals and exposure times are presented in Figure 28. Overall, F_0 significantly increased with time and concentration of chemical ($p<0.001$ for diuron and NFZ and $p<0.01$ for DCPMU).

Maximum values was reached at day 5 for DCPMU and NFZ and at day 7 for diuron and was on the same range of intensity for all compounds (around 2-fold increase in comparison to Ctrl).

Figure 27 : Concentration–response relationships and log-logistic modeling for Φ_{PSII} (a) and F_v/F_m (b) of biofilms exposed to diuron, NFZ and DCPMU for 1, 5, 7 and 14 d. Full and empty squares represent days 1 and 5, full and empty triangles represent days 7 and 14, respectively. Mean values (n=3, SD not represented for clarity) expressed as % Ctrl.

(a)



(b)

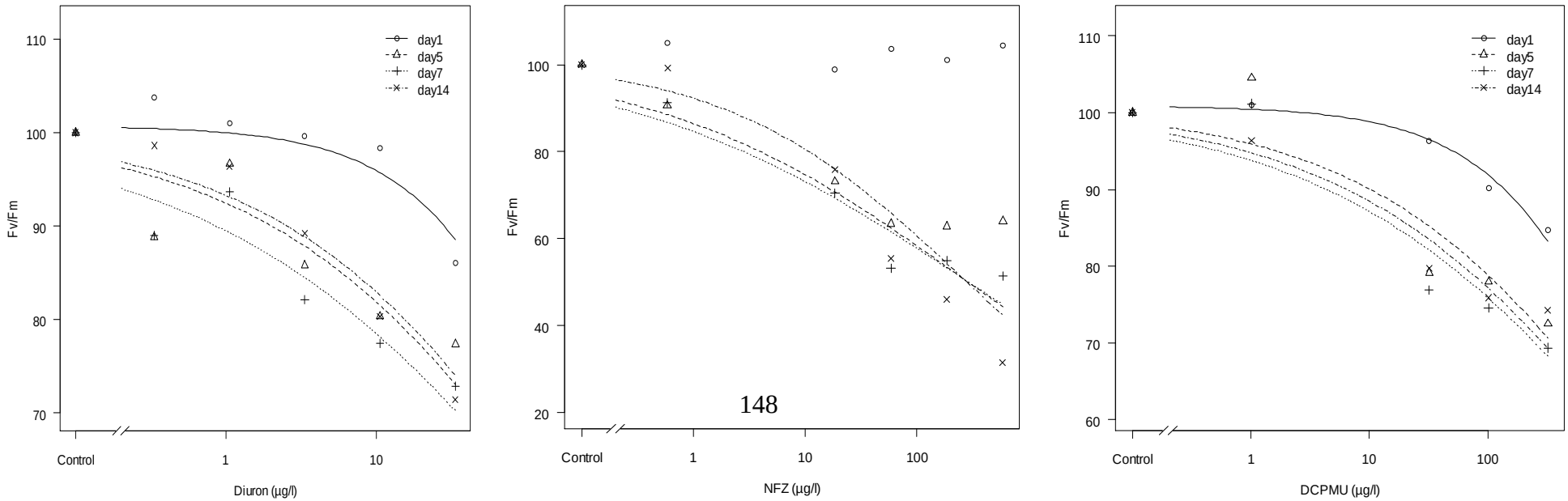
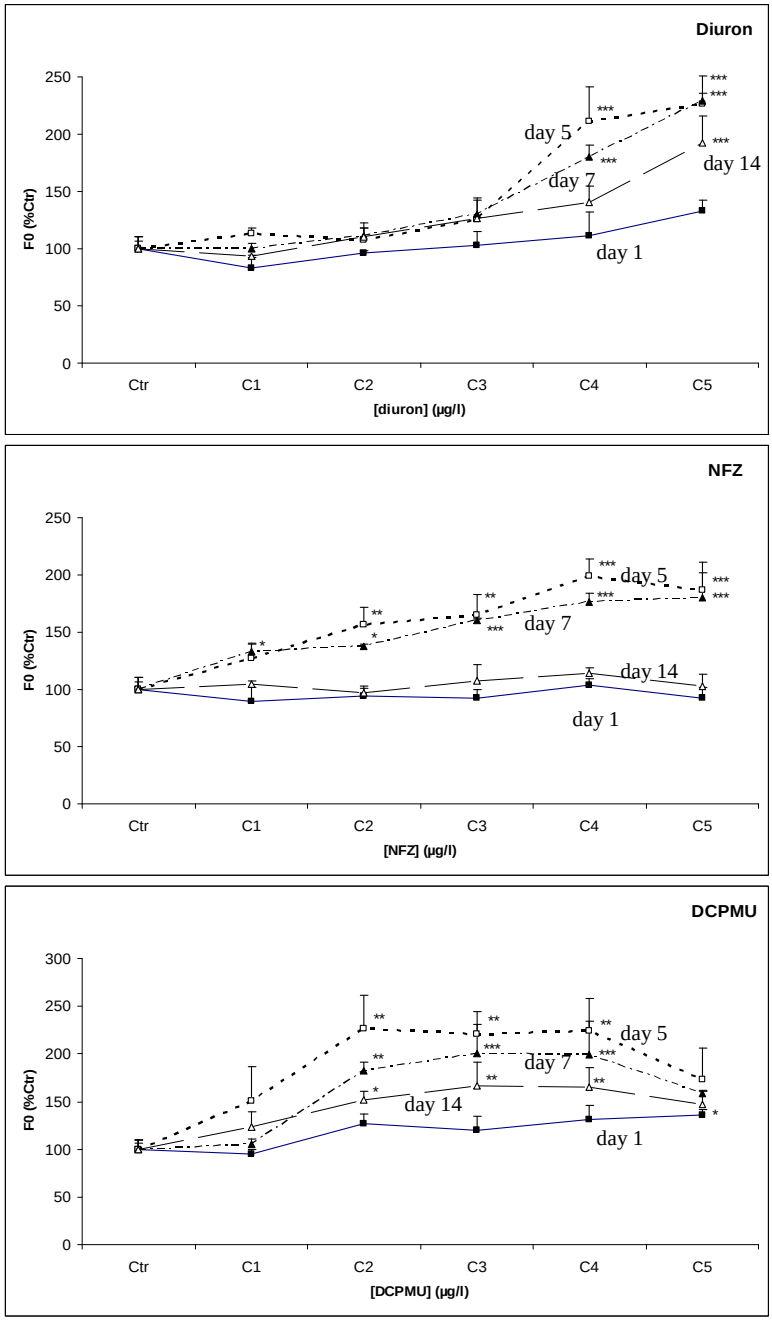


Figure 28 : Minimal fluorescence (F_0) of biofilms exposed to different concentrations of diuron (C_1=0.3, C_2=1.1, C_3=3.3, C_4=10.6 and C_5=33.4 µg L^{-1}), NFZ (C_1=0.6, C_2=18.5, C_3=58.5, C_4=185.0 and C_5=585.0 µg L^{-1}) and DCPMU (C_1=1.0, C_2=32.1, C_3=101.4, C_4=320.7 and C_5=1014.0 µg L^{-1}) for 1, 5, 7 and 14 d. Full and empty squares represent days 1 and 5, full and empty triangles represent days 7 and 14 respectively. Mean values + SD (n=3), expressed as %Ctr. Statistical differences compared to the Ctr for each sampling time indicaded as: *p<0.05; **p<0.01; ***p<0.001.

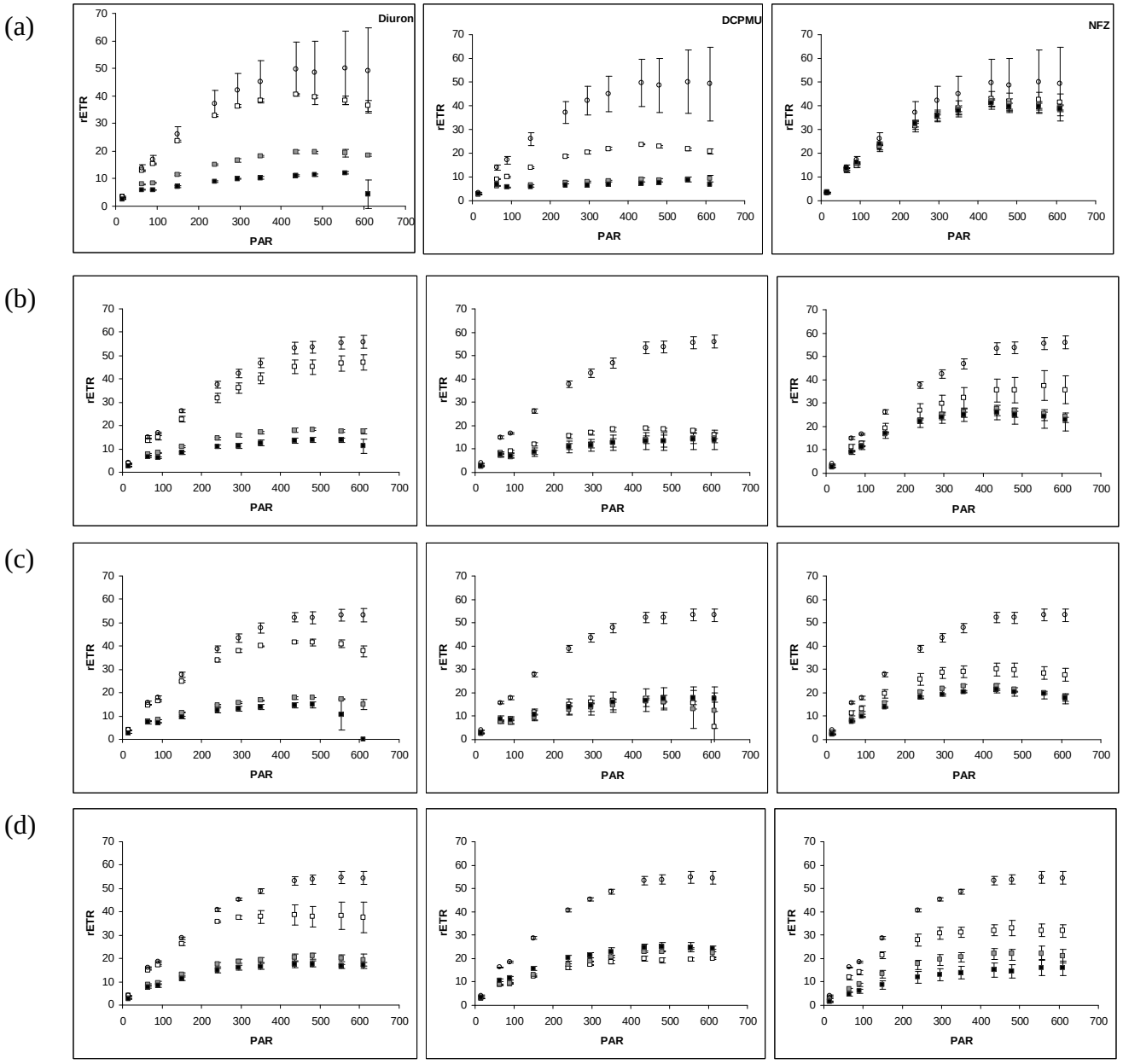


Diuron effect was significant from C_4 (211 ± 30 % of the Ctrl) only after 5 d; the increase of F_0 kept significant until day 14, with a maximum increase of 229 ± 21 % at C_5. In comparison, for DCPMU impact at the C_5 was lower (135 ± 6 %) but effects were maximum and could be detected from C_2 (226 ± 36 %). Effect was maximum after 5 d whatever the tested concentration and remained stable afterwards. NFZ exhibited an intermediate pattern. All concentrations reached their maximum effects at day 7 whatever the tested concentration. Maximum increase was 199 ± 16 % for C_5, but F_0 was significantly affected from C_1 (180 ± 22 %).

3.2 Rapid light curves

Rapid light curves and calculated parameters extracted from RLCs for the 3 treatments at days 1, 5, 7 and 14 are presented in Figure 29 and Table 7. In the Ctrl biofilms, both shape and amplitude of RLCs varied over time as revealed by the statistical analysis of α, ETR_{max} and I_k time courses (ANOVA).

Figure 29 : Rapid Light Curves (RLCs) of biofilms exposed to different concentrations of diuron (C_2=1.1, C_4=10.6 and C_5=33.4µg L^{-1}), NFZ (C_2=18.5, C_4=185.0 and C_5=585.0 µg L^{-1}), DCPMU (C_2=32.1, C_4=320.7 and C_5=1014.0 µg L^{-1}1) during 1 (a), 5 (b), 7 (c) and 14 (d) days. Mean values ± SD. Open circles represent Ctr and white, grey and black squares represent C_2, C_4 and C_5 treatments, respectively.



On average, all parameters increased but α was maximum after 14 d while ETR_{max} and I_k picked at day 3, followed by a decrease. The three parameters were significantly affected by diuron, DCPMU and NFZ exposure (Table 7). At least one parameter decreased significantly after 1 d, whatever the compound.

At day 1 significant differences between diuron contaminated conditions and the Ctr were observed only for I_k and then for all parameters from day 5. Impacts of diuron were particularly marked on ETR_{max} and I_k: from day 5, ETR_{max} was 2 to 6 times lower for the most contaminated treatment (C_5) compared to the Ctr depending of the sampling time considered while this factor was of 2 to 4 times for I_k.

DCPMU showed important impacts on RLCs curves at all sampling times. Impacts were very pronounced since day 1 with significantly lower I_k and ETR_{max} for contaminated conditions compared to the Ctr. Impacts were already very marked for the intermediate exposure treatment (C_2) with an ETR_{max} and Ik 1.4 and 1.3 times lower for C_2 compared to the Ctr respectively. Impacts of DCPMU were persistent with increasing time exposure with significant decrease of ETR_{max}, I_k and α compared to the Ctr.

At day 1, only I_k was significantly impacted by NFZ exposure. Significant differences were observed for all parameters from day 5. α, ETR_{max} and I_k values for the two highest concentrations did not significantly differ between sampling times from day 5 ($p > 0.05$). In contrast significant differences were observed between the intermediate concentration (C_2) and the 2 highest exposure conditions at day 7 (α and ETR_{max}) and at day 14 (ETR_{max} and α).

Table 7 : α, I_k and ETR_{max} calculated from rapid light curves (RLCs) of biofilms exposed to different concentrations of diuron (C_2=1.1, C_4=10.6 and C_5=33.4µg L^{-1}), NFZ (C_2=18.5, C_4=185.0 and C_5=585.0 µg L^{-1}), DCPMU (C_2=32.1, C_4=320.7 and C_5=1014.0 µg L^{-1}) and for control biofilms (Ctr) (without pesticide) at days 1, 5, 7 and 14 of experiment. Mean values ± SD. Statistical differences with Ctr indicated as: *$p < 0.05$.

Diuron

	Alpha				Ik				ETRmax			
Exposure time (d)	Ctr	C_2	C_4	C_5	Ctr	C_2	C_4	C_5	Ctr	C_2	C_4	C_5
1	0.202 ± 0.015	0.203 ± 0.023	0.179 ± 0.002	0.192 ± 0.014	192.7 ± 4.6	193.9 ± 13.8	126.4 ± 6.4*	44.9 ± 21.6*	38.6 ± 2.1	38.9 ± 2.1	22.6 ± 1.1	22.5 ± 13.7
5	0.252 ± 0.007	0.230 ± 0.011	0.184 ± 0.003*	0.179 ± 0.003*	324.9 ± 31.1	297.8 ± 21.8	111.9 ± 6.7*	76.0 ± 6.9*	82.7 ± 9.7	68.6 ± 7.5	20.6 ± 1.2*	13.6 ± 1.5*
7	0.262 ± 0.010	0.252 ± 0.008	0.181 ± 0.001*	0.183 ± 0.003*	220.3 ± 7.2	164.9 ± 6.6*	114.7 ± 2.7*	87.5 ± 7.7*	57.9 ± 3.3	42.3 ± 1.8*	20.8 ± 0.6*	16.1 ± 1.6*
14	0.262 ± 0.004	0.239 ± 0.019	0.193 ± 0.006*	0.182 ± 0.005*	229.5 ± 14.3	171.6 ± 35.5	127.6 ± 13.5*	113.9 ± 7.6*	60.0 ± 3.4	40.1 ± 4.5*	24.7 ± 3.3*	20.8 ± 1.9*

NFZ

	Alpha				Ik				ETRmax			
Exposure time (d)	Ctr	C_2	C_4	C_5	Ctr	C_2	C_4	C_5	Ctr	C_2	C_4	C_5
1	0.202 ± 0.015	0.203 ± 0.028	0.219 ± 0.007	0.230 ± 0.008	192.7 ± 4.6	222.1 ± 13.7*	190.9 ± 0.4	176.2 ± 2.5	38.6 ± 2.1	44.9 ± 5.2	41.8 ± 1.4	40.6 ± 1.4
5	0.252 ± 0.007	0.188 ± 0.020*	0.155 ± 0.001*	0.158 ± 0.003*	324.9 ± 31.1	222.6 ± 37.2	171.9 ± 8.4*	159.2 ± 8.0*	82.7 ± 9.7	42.7 ± 11.7*	26.7 ± 1.4*	25.2 ± 1.3*
7	0.262 ± 0.010	0.192 ± 0.020*	0.142 ± 0.004*	0.138 ± 0.001*	220.3 ± 7.2	154.1 ± 1.9*	155.0 ± 2.9*	147.0 ± 1.7*	57.9 ± 3.3	29.6 ± 2.7*	22.1 ± 0.8*	20.3 ± 0.2*
14	0.262 ± 0.004	0.199 ± 0.011*	0.115 ± 0.009*	0.089 ± 0.010*	229.5 ± 14.3	163.0 ± 2.5*	190.7 ± 2.4	201.7 ± 8.1	60.0 ± 3.4	32.6 ± 0.9*	22.0 ± 0.8*	17.3 ± 2.4*

DCPMU

	Alpha				Ik				ETRmax			
Exposure time (d)	Ctr	C_2	C_4	C_5	Ctr	C_2	C_4	C_5	Ctr	C_2	C_4	C_5
1	0.202 ± 0.015	0.195 ± 0.001	0.227 ± 0.004	0.241 ± 0.021	192.7 ± 4.6	138.7 ± 10.3*	37.5 ± 4.5*	29.5 ± 1.5*	38.6 ± 2.1	26.9 ± 1.9*	8.5 ± 1.0*	7.0 ± 0.3*
5	0.252 ± 0.007	0.187 ± 0.006*	0.196 ± 0.004*	0.212 ± 0.007*	324.9 ± 31.1	111.8 ± 8.3*	73.0 ± 12.5*	65.6 ± 17.7*	82.7 ± 9.7	20.9 ± 1.7*	14.3 ± 2.3*	14.0 ± 4.2*
7	0.262 ± 0.010	0.184 ± 0.007*	0.182 ± 0.004*	0.201 ± 0.019*	220.3 ± 7.2	94.1 ± 5.1*	97.0 ± 17.9*	96.8 ± 22.4*	57.9 ± 3.3	17.3 ± 0.5*	17.7 ± 3.3*	19.8 ± 6.3*
14	0.262 ± 0.004	0.198 ± 0.004*	0.195 ± 0.003*	0.227 ± 0.012*	229.5 ± 14.3	117.1 ± 2.5*	139.6 ± 2.4*	128.1 ± 8.1*	60.0 ± 3.4	23.2 ± 0.9*	27.2 ± 0.8*	28.9 ± 2.4*

4.Discussion

4.1.Diuron, DCPMU and norflurazon relative toxicity assessed by "classical fluorescence endpoints"

In this study we tested the relative toxicity of two herbicides (diuron and norflurazon) and one degradation product (DCPMU) for a range of concentrations (from 0.3 to 33.4µg L^{-1} for diuron, from 1.0 to 1014µg L^{-1} for DCPMU and from 0.6 to 585µg L^{-1} for norflurazon) at different exposure time. Several classical endpoints were assessed in order to test the effects of these 3 compounds on natural biofilms, photosynthetic yields (Φ_{PSII} and F_v/F_m) and minimal fluorescence (F_0).

Both yields designed diuron as the more toxic compound with the lower EC_{50} and F_v/F_m designed DCPMU as the less toxic one, ranging the compounds in the following order of toxicity: diuron >> NFZ > DCPMU.

For diuron, the maximum of inhibition observed in our experiment was about 40% for both endpoints Φ_{PSII} and F_v/F_m. Several studies have been assessing toxicity of diuron by the use of dose response curves and in most of the cases relatively low EC_{50} have been found. For example Magnusson et al. (2010) assessed effect of diuron on the photosynthetic capacities (Φ_{PSII}) of tropical microalgae species and determined EC_{50} of 5.5 ± 0.2 and 5.9 ± 0.2 µg L^{-1} after 3 days of exposure respectively for *Navicula* sp. (Heterokontophyta) and *Nephroselmis pyriformis* (Chlorophyta).

The scientific community agrees on the fact that there is a general lack of knowledge about the toxicity of degradation products and that is of priority for substance regulation (Sinclair and Boxall 2003) In our study, toxic effects of DCPMU -the main diuron degradation product- were observed on both yields. Maximum effects were observed at day 7 with a maximum inhibition of Φ_{PSII} about 32.2% compared to the Ctr. Very few is known about DCPMU toxicity to biofilms, nevertheless Pesce et al. (2010b) determined a EC_{50} around 100µg L^{-1} after 4 hours of exposure, the toxicity observed in our experiment is so relatively low compared to this value.

Higher toxicity of diuron compared to the degradation product was expected, according to the acute toxicity tests performed by Gatidou et al. (2007) on cultures of *Dunaliella tertiolecta* (green algae) and *Navicula forcipata* (diatom), or by Pesce et al. (2010b) on suspensions of natural biofilms. In our study realized on river biofilms in benthic mode we also found a greater toxicity of diuron compared to the DCPMU. According to recent studies (Dewez et al.

2002, Neuwoehner et al. 2010) only slight decrease of toxicity for autotrophic organisms is expected due to the loss of the methyl group between diuron and DCPMU. Nevertheless it has to be noticed that relative toxicity of these two compounds are greatly dependent of the organisms tested since Fernández-Alba et al. (2002) showed that toxicity of diuron and DCPMU were similar when tested with *S. capricornutum* (microalgae) while the degradation product was 125 times more toxic than diuron on *Daphnia magna* (microscopic crustacean).
Effective and optimal quantum yields for biofilms exposed to DCPMU followed a quite different tendency compared to diuron responses. For DCPMU, the high decrease of photosynthetic yields from C_0 to C_2 and then the relative stability of the response with the increase of the concentrations to reach of a plateau between C_2 and C_5 could be associated with a rapid absorption of the DCPMU (WHO 1978) supported by the higher log D_{lipw} (liposome-water partition coefficients) for DCPMU compared to diuron.
Significant impacts of norflurazon exposure on Φ_{PSII} and F_v/F_m were observed from day 5 to day 14. The mean EC_{50} over the experiment were of 323.9 ± 135.5 µg L^{-1} and 573.4 ± 348.7 µg L^{-1} in regards to Φ_{PSII} and F_v/F_m respectively as endpoint. Lower EC_{50} were expected since EC_{50} of NFZ in regards to F_v/F_m was estimated about 100µg L^{-1} by Pesce (personal communication) after 48H of exposure on biofilms in suspension mode. The data found in the literature were highly variable in function of the endpoint considered and organisms tested. For example Wilson and Koch (2013) determined an EC_{50} of 24.9 ± 4.1 µg L^{-1} after 6 days of *Lemna minor* exposure in regards to total frond production. In a different study, Thies et al. (1996) assessed cell volume growth after 14 hours of exposure and determined EC_{50} equal to 12.15 and 182.2 µg L^{-1} for *C. fusca* and *C. sorokiniana* respectively. In the other hand, Nestler et al. (2012) determined that a dose about 1300µg L^{-1} of NFZ was reducing by 50% the photosynthetic capacities of the unicellular freshwater algae *Chlamydomonas reinhardtii* after 24H of exposure.

The much higher EC_{50} values in our experiment for the three compounds than expected from the literature could be explained by the exposure modalities used in this study. Actually we used biofilm previously colonized on artificial substrates to assess diuron, DCPMU and NFZ toxicity in order to be representative of the benthic mode of life of biofilm whereas most of studies constructing dose-response curves -including Magnusson et al. (2010) and Pesce et al. (2010b)- are assessing effects of toxicant on suspended cultures (single species testing) or suspended biofilms (pluri-organisms testing). Biofilms grown on solid substrates are composed of different organisms like bacteria, algae, fungi, and microfauna embedded in an

exopolysaccharide matrix (EPS) building up a tri-dimensional architecture. This matrix having been suggested to protect from toxicants and physical stress (Fang et al. 2002, Artigas et al. 2012). In contrast, suspensions do not benefit from the protective role of this matrix and so the interaction of organisms with toxicant is facilitated, potentially leading to higher toxicity.

In this study we also tested the response of biofilms minimal fluorescence (F_0) to diuron, DCPMU and norflurazon exposure. For all treatments an increase of F_0 with increasing toxicant concentrations was observed.

For diuron, impacts were significant from day 5 and the higher induction of fluorescence was always observed for the higher exposure concentration. Several authors have been studied the evolution of F_0 after exposure to diuron, Ralph (2000) observed similar responses with an increase of F_0 up to 190% of the Ctr after 72H of exposure to 10µg L^{-1} of diuron for the seagrass *Halophila ovalis*.

Exposure to DCPMU lead to a significant increase of F_0 from day 1, the maximum effects were not observed at the maximum concentrations since we noted a rapid increase of F_0 with concentrations followed by a plateau. This particular shape of the curve like for yields could be linked to the rapid absorption of the DCPMU leading to reach of maximal effects at low concentration and then a stability of the response.

For NFZ, the observed increase of F_0 up to 125% of the Ctr for the C_1 at day 5 and 7 revealed the higher toxicity of NFZ compared to diuron and DCPMU at these particular sampling times since EC_{125} were situated between 0.6 and 18.5µg L^{-1} for DCPMU and between 1.1 and 3.3µg L^{-1} for diuron.

In our study impacts have been observed for the lowest exposure concentrations of diuron (0.3 µg L^{-1}) and norflurazon (0.6 µg L^{-1}), with a significant decrease of Φ_{PSII} and F_v/F_m at day 5 and 7 respectively for diuron and a significant increase of F_0 at day 7 for NFZ. These concentrations are in the range of those often encountered in chronically contaminated rivers and so representative of environmentally realistic levels, for example in the Morcille River diuron was recorded at concentrations up to 0.138µg L^{-1} and NFZ to 0.265µg L^{-1} in 2011 (Mazzella, personal communication). Moreover our study was focusing on biofilm communities on benthic mode, since impacts of toxicants are likely to be more important for suspended microalgae than attached ones (Franz et al. 2008), organisms living in the water column are likely to be highly affected by exposure to low concentrations of diuron and

norflurazon highlighting the importance to study impact of pesticides products at environmental concentrations.

4.2.Importance of exposure time in toxicity testing

Environmental risk assessment is based on the analysis of toxicological data extracted from both short and long term experiment. In standardized toxicity tests, exposure duration is defined, allowing comparisons of substances for the same time of exposure. Nevertheless magnitude of effects can be highly variable for compounds with different mode of action.

Significant effects of diuron exposure were observed at day 5, 7 and 14 with effects of the same order of magnitude for these three sampling times (considering Φ_{PSII}, F_v/F_m and F_0), highlighting the rapid inhibition and then a relative stability of impacts that have to be linked with the rapid mode of action of the compound (PSII inhibitor). Inhibition increased with concentrations and maximum inhibition was reached at the highest exposure concentration for both yields (Φ_{PSII} and F_v/F_m); for C_5 Φ_{PSII} was equal to 61 ± 3, 62 ± 2 and 63 ± 2 % of the Ctr at day 5, 7 and 14 respectively, and F_v/F_m to 77 ± 2, 72 ± 2 and 71 ± 1 % of the Ctr at day 5, 7 and 14 respectively. Like for diuron exposure, effects on Φ_{PSII} and F_v/F_m were of the same order of magnitude from day 5 to day 14; nevertheless early effect were detected when biofilms were exposed to DCPMU since significant decrease of F_v/F_m appeared at day 1.

Time considered in the bioassay influenced highly interpretation of the results, for example for DCPMU and diuron considering the three last sampling times we could conclude to higher toxicity of diuron compared to DCPMU in regards to both yields but in the other hand diuron did not impact significantly F_v/F_m at day 1 while DCPMU did. Early effects of DCPMU were also highlighted by minimal fluorescence since significant increase of F_0 compared to the Ctr was observed at day 1 for DCPMU (and not for diuron). The relative toxicity of these two compounds is so highly depending of time considered in the assay.

The importance of time considered in the assay is particularly striking in the case of NFZ. Norflurazon is an inhibitor of carotenoids biosynthesis, this herbicide exerts its primary inhibitor action on the reaction catalysed by phytoene synthase (Sandmann et al. 1980). Carotenoids are photosynthetic pigments exerting a crucial role in light harvesting and photoprotection. When synthesis of carotenoids is inhibited by norflurazon treatment, photoprotection and light harvesting are in a first time assured by the pool of carotenoids present in the cells but rapidly effect of the toxicant will appear and be more and more pronounced with decrease of the pool of carotenoids with time and because of the non replacement of carotenoids. This has been observed when looking at Φ_{PSII} and F_v/F_m; with

more and more pronounced responses with time exposure from day 5 to day 14. Between day 5 and day 14, toxicity of NFZ at the highest exposure concentration was increased by approximately a factor 2. This impressive increase of toxicity with time is likely to be linked to the particular mode of action of NFZ. This result highlights the importance of the time duration taken in account in acute testing and their used in environmental risk assessment especially for compound as norflurazon exhibiting multiplication of toxicity within 2 weeks.

4.3.Complementarity of parameters

Responses of biofilms were dose, time, compound but also parameters dependent. Significant differences were observed between Ctr and contaminated conditions on F_0 and on F_v/F_m at all sampling time; in the other hand impacts for the lower exposure concentration were revealed by both yields for diuron and by F_0 for NFZ. In our study early effects were so better highlighted by F_0 and F_v/F_m and effects at environmental concentration by Φ_{PSII}, F_v/F_m and F_0. Moreover these parameters were more or less sensitive in function of pesticide considered in link with the modes of action of the compounds as diuron and DCPMU act as PSII inhibitors and norflurazon is preventing carotenoids renewal.

4.4.Contribution of rapid light curves approach

RLCs are generally confined to ecological studies; they rely on physiological principles that could, however, greatly contribute to improve our understanding of ecotoxicological impacts on biofilms. Here we demonstrated the potential of approaches based on RLCs to highlight early effects of diuron, DCPMU and NFZ. Indeed impacts of toxicants exposure on biofilms were revealed for the three compounds from day 1 with the impact of at least one parameter extracted from the RLCs (α, ETR_{max} or I_k). These analyses of RLCs lead to the same conclusions than looking at the data from photosynthetic yields (Φ_{PSII} and F_v/F_m) and minimal of fluorescence (F_0). Effects of diuron were of the same order of intensity at the three last sampling times, whereas DCPMU had a rapid and maximum effect of the metabolite at the lower concentrations, with no increase of the response intensity with increasing concentration exposure. For norflurazon, rapid effects of the toxicant appeared and were more and more pronounced with time exposure probably due to the decrease of the pool of carotenoids with time and because of the non replacement of carotenoids (Wilkinson 1987).

Very few studies were assessing the effects of toxicants using RLCs, nevertheless Luís et al. (2013) used this approach in a recent field study in an area strongly affected by acid mine drainage. The authors founded maximum values of α and ETR_{max} for winter at the unimpacted

sites and defined these parameters as early warning signals of biological effects after acute exposure to chemicals, damaging either directly or indirectly PSII.

In our experiment results obtained from RLCs allowed the underlining of pesticide treatments effects from day one and thus for the three compounds diuron, DCPMU and norflurazon. In contrast at day 1, significant differences between Ctr and contaminated conditions were observed only for DCPMU treatment in regards to the "classical fluorescence endpoints" F_0 and F_v/F_m. RLCs construction were shown here to be particularly adapted in order to reveal early effects of herbicides. Moreover only the parameters extracted from the RLCs allowed highlighting the early effects and the maintenance of effects with time exposure and thus for the three compounds tested.

5.Conclusion

We demonstrated here using two pesticides with different modes of action and one degradation product that parameters extracted from RLCs were more sensitive that classical fluorescence parameters since effects of toxicants were observed from the first sampling time (24h of exposure) using RLCs as endpoint instead of 5 days of exposure in regards to "classical fluorescence parameters" (F_0, F_v/F_m and Φ_{PSII}). Moreover we observed different responses in function of the mode of action of compounds not only in function of dose: impacts of NFZ were low at day 5 but intensity of effects increased considerably with time exposure. In contrast the effects observed in diuron and DCPMU treatments were on the same range from day 5 to day 14. Relative toxicity of compound was so time- but also endpoint-dependent. Actually higher toxicity of NFZ compared to diuron and DCPMU was observed in regards to F_0 but the opposite pattern was observed looking at photosynthetic yields as endpoints at the particular sampling time day 5 and 7. These results are so highlighting the importance of the chosen time exposure and endpoint in environmental risk assessment. We can also notice that we observed effects for the diuron and norflurazon applied alone for the lowest exposure; these impacts on river biofilms at environmentally realistic concentrations could suggest effects of such compounds for aquatic organisms and particularly since toxicants are not present alone but found in cocktail in aquatic environments.

Acknowledgments

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Chapitre VII : Effets comparés d’une exposition chronique à un extrait d’échantillonneur passif et d’un mélange reconstitué sur du biofilm de rivière

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Dans le chapitre IV, nous avons évalué le potentiel de l'outil POCIS pour la mise en évidence des effets à faible dose et à long terme de pesticides en mélange. Ce travail nous a permis d'appréhender les réponses de biofilms aux histoires d'exposition différentes face aux extraits de POCIS (EP). L'utilisation des EP s'est avérée particulièrement adaptée pour l'étude d'une exposition réaliste de pesticides en mélange ; nous avons ainsi pu observer l'impact de ces derniers sur la croissance et la structure du biofilm. Dans cette démarche la toxicité globale des extraits est appréhendée, cependant la toxicité relative des composés constituants l'extrait n'est pas étudiée (chapitres V et VI) et les composés responsables de la toxicité observée ne sont pas identifiés.
Concernant ce dernier point, une telle approche constitue le principe des analyses dirigées par les bioessais (EDA ou Effect Directed Analyses). Cette démarche est basée sur la combinaison de procédures de fractionnements, de tests biologiques et d'analyses chimiques. Néanmoins une approche similaire n'est pas envisageable pour des expériences à long terme de par l'ampleur des moyens à apporter (humains, techniques, financiers). Toutefois l'utilisation de mélanges reconstitués d'après la quantification des analytes retrouvés dans les extraits constituerait une première approche dans ce domaine.

Les objectifs de ce chapitre sont (1) d'évaluer les impacts chroniques de pesticides en mélange sur le biofilm de rivière, et ce dans un contexte de contamination différent de celui de la Morcille (notre site d'étude principal dans cette thèse) et (2) de comparer les effets chroniques d'extraits de POCIS et d'un mélange reconstitué basé sur les composés majoritaires identifiés dans les extraits.

Pour cela une expérience en conditions contrôlées de laboratoire a été réalisée. Dans un premier temps des supports artificiels ont été posés sur un site de référence (l'Ourbise) pour permettre la colonisation du biofilm naturel. En parallèle, des POCIS ont été immergés sur le Trec, cette rivière subit une pression agricole particulièrement marquée entrainant des concentrations en pesticides qui peuvent atteindre plusieurs microgrammes par litre (site présenté dans le chapitre III).

Les biofilms ont alors été exposés en canaux artificiels aux extraits de POCIS dilués (EP), à un mélange constitué des pesticides majoritaires identifiés dans les extraits (Mix) ou maintenus dans de l'eau sans pesticide. Une large gamme de paramètres susceptibles de

mettre en évidence les effets de pesticides sur le biofilm a été sélectionnée. Les impacts des extraits de POCIS sur les biofilms soumis aux différents traitements ont été évalués pendant les 14 jours d'exposition par des mesures de biomasse, de fluorescence en lumière modulée (détermination des groupes algaux et rendement photosynthétique), d'activités enzymatiques antioxydantes, de densités de diatomées et par des analyses taxonomiques.

Les résultats obtenus ont permis de mettre en évidence les effets des pesticides à faible dose au niveau de la croissance (PS, MSSC et densités de diatomées), de la structure (composition taxonomique et biovolumes) et du fonctionnement (efficacité photosynthétique et activités enzymatiques antioxydantes) du biofilm. Ces résultats avec ceux du chapitre IV soulignent donc la pertinence du POCIS en tant qu'outil permettant d'étudier les effets de mélanges complexes et réalistes de pesticides sur le biofilm dans des contextes de contamination variés. D'autre part, dans notre expérience les effets des extraits de POCIS ont été similaires à ceux induits par les composés majoritairement identifiés dans les extraits pour l'ensemble des paramètres étudiés. La toxicité chronique observée des extraits provenant du Trec a donc été expliquée par les composés majoritaires. En effet l'analyse en composantes principales (ACP) réalisée au dernier temps d'exposition avec tous les paramètres mesurés (à part la taxonomie des diatomées) a clairement séparé les traitements contaminés aux pesticides (PE et Mix) des témoins. Au contraire l'analyse n'a pas révélé de séparation entre les biofilms exposés aux EP ou aux composés majoritaires identifiés dans l'extrait.

Notre étude montre donc la pertinence de l'utilisation des extraits d'échantillonneurs passifs (comme le POCIS) pour mieux prendre en compte les effets chroniques des mélanges dans l'évaluation du risque environnemental. De plus l'exposition aux EP et aux composés majoritaires ont induit des effets similaires sur le biofilm, ce résultat révèle le potentiel intégratif d'une partie de la toxicité environnementale du POCIS.

Comparative effects of polar passive sampler extracts and reconstituted mixture : Is the toxicity of pesticides mixtures to river biofilm explained by the major identified compounds?

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En préparation

Abstract

Comparative effects of long term exposure to Polar Organic Chemical Integrative Sampler (POCIS) extracts (PE) and to a reconstituted mixture based on the major compounds quantified in the PE were evaluated on river biofilm communities. Biofilms originated from an uncontaminated site were exposed in artificial channels in order to reach environmental realistic concentrations to diluted PE, to the 12 major compounds quantified in the extracts (Mix) or maintained in water without pesticides (Ctr). This study aimed to characterize the effects of chronic and low dose exposure to pesticides on natural biofilm communities and to evaluate if the observed effects due to exposure to PE were explained by the major compounds identified in the extracts. Significant differences between biofilms exposed or not to pesticides were observed in regards to diatom density, biomass (dry weight and ash free dry mass), photosynthetic efficiency (Φps_{II}) and antioxidant enzymatic activities. After 14 days of exposure to the different treatments, the observed trend to the decrease of mean diatom cell biovolumes in samples exposed to pesticides was related to the higher relative abundance of large species like *Cocconeis placentula* or *Amphora copulata* and lower relative abundance of small species like *Eolimna minima* in control biofilms compared to the contaminated ones. Principal component analyses clearly separated contaminated (PE and Mix) from non-contaminated (Ctr) biofilms; on the contrary the analyses did not revealed separation between biofilms exposed to PE or to the 12 major compounds identified in the extract.

Key words

Pesticides, periphyton, passive samplers, effect-directed analyses, low-dose exposure, mixture effects, environmental risk assessment.

1. Introduction

Agricultural activities can lead to a contamination of water bodies by pesticides, and when present in the water such compounds can have important impacts on the biological compartment and more generally on water quality. Pesticides have been shown to have effects on different aquatic organisms like algae, crustacean and on periphytic biofilms (Fernández-Alba et al. 2002 , Debenest et al. 2010). Scientific community has made a particular investment in characterizing responses of biofilms to a large range of pollutants since they are used for biomonitoring for assessing global water quality (i.e. trophic levels) and have shown their potential as early warning systems for detection of the effects of toxicants on aquatic systems (Sabater et al. 2007). Pesticides are present as a cocktail of low concentrated compounds in water bodies. Nevertheless studies assessing toxic effects of pesticide mixtures at environmental realistic concentrations are still scarce, mainly because of cost related and technical difficulties encountered when running such experiment (time consumption, maintain of concentration in experiment system, large amount of water, etc). POCIS allows the concentration of several organic chemicals from large volumes of natural water (Mazzella et al. 2010). This high pre-concentration of compounds makes POCIS a powerful tool to assess the extract toxicity via biological testing and therefore to study mixtures. However, if POCIS extracts in combination with bioassays have been well documented with compounds like endocrine disruptors or photosystem II inhibitors (Vermeirssen et al. 2010, Matthiessen et al. 2006, Balaam et al. 2010), evaluation of potential extracts toxicity by long term studies is still in its infancy. In a recent study, Morin et al. (2012b) have characterized the effects of low dose of pesticides on biofilm communities using POCIS extracts (PE) in long term experiments. In these studies, PE were used as a black box with the advantages to be dealing with mixture issue; this approach gives an estimation of an integrative measure of the toxic potential of a group of compounds including unknown toxicants (without *a priori* approach). In this demarche the global toxicity of the extract is assessed nevertheless compounds responsible for the observed toxicity are not identified. Such approach aimed to identified the toxicity is used in effect-directed analyses (EDA) which is based on a combination of fractionation procedures, bio-testing, and subsequent chemical analyses (Brack 2003, Hecker and Hollert 2009). Consequently, the present study aimed to characterize the effects of chronic and low dose exposure to POCIS extracts in a contamination context by cereal crops on natural biofilm community, and to evaluate whether the observed effects due to exposure to PE were explained by the major compounds identified in the extracts. For that purpose, we

exposed river biofilms originated from an uncontaminated field sampling site to three treatments during 14 days:

1. Biofilms were exposed in artificial channels to POCIS (previously immersed for one month in a pesticide contaminated river) extracts diluted to reach environmentally realistic concentrations.
2. The compounds with nominal concentrations exceeding 20µg/L in the pure PE were chosen to compose the reconstituted mixture (Mix); biofilms were then exposed in the channels to the diluted mixture.
3. The last condition was realized as a control (biofilms without pesticide).

A set of biofilm parameters was selected in order to highlight the variety of perturbations (structural, physiological or growth related) possibly induced by pesticide exposure.

Classical growth related parameters as diatom density, dry weight and ash-free dry mass were recorded. Moreover a focus was made on diatoms species due to their interest in environmental risk assessment.

Therrefore the evolution of the biofilms over time (at day 0, 3, 7 and 14) was followed through diatom densities, effective quantum yield of PSII (Φps_{II}), antioxidant enzymatic activities (catalase: CAT, ascorbate peroxidase: APX, Glutathione reductase: GR and Glutathione-S-transferase: GST), fluorescence signals linked to cyanobacteria (Fs_{Bl}), diatom (Fs_{Br}), and green algae (Fs_{Gr}) groups determination. Taxonomic composition, dry weight (DW) and ash-free dry mass (AFDM) were determined at day 0 and 14.

2. Material and methods

2.1.Study sites and sampling procedure.

Two rivers with different contamination profiles located in the Lot-et-Garonne area, France were chosen for the study. The Trec River, which has been studied since the late 2000s, is subjected to strong agricultural pressure -essentially exerted by cereal crops- and is characterized by resulting important concentrations of pesticides in water (more than 8.5µg/L in 2009 (Duprat 2010). On the contrary the Ourbise River is characterized by low concentrations of pesticides; this river is located in a forested area and is followed by water agencies as a reference station.

Biofilm colonisation

Glass slides fixed in perforated plastic boxes were used as artificial substrates to allow biofilms colonisation for 6 weeks (9^{th} May-18^{th} June 2012) in the Ourbise River (uncontaminated site). Glass slides were brought back into the laboratory and biofilms were

put in aquaria with water from origin site and sufficient nutrients to allow growth for one week before the beginning of the channel experiment.

Pesticides quantification in water

POCIS were used for pesticides quantification in water ("Quantitative" POCIS) (Mazzella et al. 2010, Lissalde et al. 2011). Performance and Reference Compound (PRC) was introduced in "Quantitative" POCIS, the devices were immersed in the Ourbise and Trec stations for two weeks in order to stay in the kinetic part of the accumulation regime and then replaced by new ones for the two weeks left (9th May-5th June 2012) to allow pesticides quantification

Pesticides accumulation for toxicological assessment

"Accumulative" POCIS were immersed in the Trec River (contaminated site) during 4 weeks (9th May-5th June 2012) to allow pesticides sampling in order to lead the following toxicity tests as described in Pesce et al. 2011 and Morin et al. 2012. All POCIS were kept at -4°C before extraction and chemical analyses.

2.2.Channel experiment

Experimental design

Biofilms originated from Ourbise station were exposed to three different treatments: POCIS Extracts (PE), reconstituted Mixture (Mix) or in water without pesticides (Ctr). The reconstituted Mixture was composed by the compounds with nominal concentrations exceeding 20µg/L in the pure PE (see Figure 31). The colonised glass slides were directly placed at the bottom of the artificial channels and experiment was run during 14 days. Biofilms were exposed to PE or Mix treatment at low concentrations in order to evaluate if the complex environmental mixture (PE) and the reconstituted mixture composed by the 12 major compounds found in PE (Mix) had similar effects during chronic exposure on river biofilms. The last condition (biofilms placed into clean water, Ctr) was realized in order to represent reference conditions. Each condition was realized in 5 replicates. Experimental channels were maintained at 19-20°C along the 14 days of exposure with a photon flux density of 30 µmol.m^{-2}.s^{-1}, a 12:12 h light:dark cycle and under continuous flow. Each channel volume (12L) was filled with WC culture medium (Guillard and Lorenzen 1972) in ultrapure water containing either:

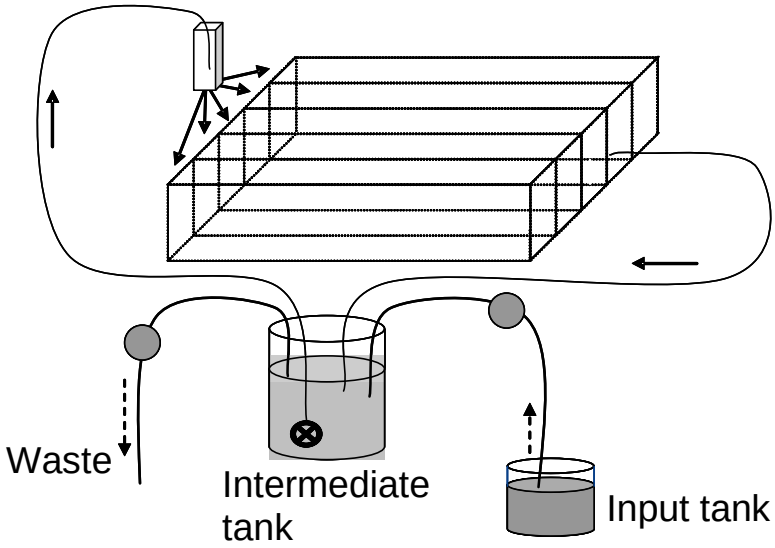
(1) the POCIS extracts diluted to concentrations representative of concentrations encountered in the Trec station (treatment noted: PE).

(2) the reconstituted mixture containing the 12 most concentrated compounds found in PE at concentrations similar to those of the dominant compounds in PE treatment (treatment noted: Mix).

(3) no pesticide (treatment noted: Ctr).

To avoid the rapid decrease of pesticide concentrations due to the adsorption of compounds to the experimental system (Morin et al. 2012b), the channels were put into circulation with water and contaminants (when applicable) one day before the introduction of biofilms (at day-1; one day before the beginning of the experiment). Peristaltic pumps were used for ensuring continuous supply of channels, with a renewal of 3L/day, and stable pesticide concentrations during the 14 days of experiment. The experimental system is described in Figure 30. Supply of the corresponding treatment (PE, Mix or Ctr) diluted in WC medium was distributed in each (replicate) channel and renewed every 2 or 3 days with the appropriate treatment. In total, 20 POCIS-equivalent extracts were used to perform the experiment (from day-1 to day 14) and 57L of water were used for each treatment.

Figure 30 : Experimental set-up of channel systems, with five replicate channels filled by the same tank. The tank was under continuous alimentation delivered by a classic aquaria pump, with a renewal of 3L/day. Peristaltic pumps ensured continuous flow in the channels. Alimentation aquaria were filled with either POCIS extracts (PE), the reconstituted mixture (Mix) or with water without pesticide (Ctr). Channel dimensions are: *l* x *w* x *d* = 74 x 60 x 3 cm. Tanks have 10L capacities. Arrows indicate direction of flow.



Water characterization and biofilm sampling

Physicochemical conditions (pH, temperature, conductivity and oxygen), as well as pesticides (5 sampling) and nutrients (nitrate, orthophosphate and silica) concentrations were monitored throughout the experiment.

Biofilm samples were collected by scraping glass slides with a razor blade and resuspended in mineral water for further analyses. Samples were collected in the five replicates channels at day 0, 3, 7 and 14 for diatom densities, effective quantum yield of PSII (Φps_{II}), antioxidant enzymatic activities, fluorescence signals linked to cyanobacteria (Fs_{Bl}), diatoms (Fs_{Br}), and green algae (Fs_{Gr}) groups determination. Taxonomic composition, dry weight (DW) and ash-free dry mass (AFDM) were determined at day 0 and 14.

2.3.Physico-chemical parameters and pesticides analyses

The method developed by Lissalde et al. (2011) was used for the analysis of both water samples, preconcentrated on solid-phase extraction (SPE) cartridges, and POCIS extracts. The solid receiving phase contained in the POCIS (Oasis HLB sorbent, Waters) was recovered in ultrapure water. Then all samples were analyzed by HPLC–ESI–MS/MS (HPLC Ultimate 3000, Dionex, equipped with a Gemini NX C18 column, 10 mm x 2 mm, 3 µm, Phenomenex, and API 2000 triple quadrupole, AB SCIEX equipped with an electrospray ionization source) and GC–MS/MS (Quantum GC, Thermo, equipped with a Rxi-5MS column 30 m x 0.25 mm x 0.25 µm, Restek). Analytical methods were validated in terms of calibration linearity, specificity, extraction recoveries, and limits of quantifications according to the French standard NF T90-210 (AFNOR 2009). For the quality controls, SPE and POCIS blanks were carried out routinely, and the recoveries of two levels of spiked mineral water (e.g. 0.02 and 0.2 µg/L for LC-MS/MS analysis) were evaluated for each batch. The periodic control of two calibrating standards (e.g. 5 and 25 µg/L, every 10 samples) and analytical blanks were performed as well.

Appropriate probes (pH 3110, WTW, LF 340, WTW, Oxi 340i, WTW) were used in order to determine pH, conductivity and oxygen concentrations respectively as well as temperature. Nitrates concentration was calculated according to French standard (NF EN ISO 13395 10/96, AFNOR 1996) using Auto-analyser Evolution II Alliance. Orthophosphates and silica were determined following standard procedures (NF T90-023, AFNOR 1982) and (NF T 90-007, AFNOR 2001) respectively, with Spectrophotometer Lambda 2 Perkin Elmer.

2.4. Biofilm response analyses

2.4.1. Dry weight and ash-free dry mass

At day 0 and 14, the dry matter and the organic matter content were evaluated by calculating the dry weight (DW) and the ash-free dry weight (AFDM) following European standard NF EN 872 (AFNOR 2005). For each sample biofilm were scraped from glass slides and resuspended in 10 mL of mineral water. Biofilm suspensions were filtered through individual, previously dried, 25 mm GF/C Whatman glass fibre filters (1.2 µm pore size). DW and AFDM were calculated from the differences in filters weight before and after being dried (1h at 105°C, for DW) and then combusted (1h at 500°C, for mineral content, subtracted to DW to obtain AFDM).

2.4.2. Antioxidant enzymatic activities

Protein extraction and antioxidant enzymatic activities (AEAs) measurements were performed using modified protocols developed by Bonnineau et al. (2013) and Bonet et al. (2013).
Biofilms were put into an eppendorf tube and centrifuged (2300 g, 5 min) to remove the excess of water 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 PT 1600E, Kinematica) and then disrupted by adding 100 mg of glass beads (≈500 µm diameter) for each 100 mg of wet weight of sample. Samples were then vortexed 3 times for 30 s with 2 min intervals on ice. After cell disruption, homogenates were centrifuged at 10.000 g for 30 min at room temperature. 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 $\mu g.mg^{-1}$ 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 2 µg of proteins.

Catalase activity (CAT) 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 10, 15, 20, 30 and 40 mM H_2O_2. The 250 µL reaction mixture contained 2 µg of proteins, potassium phosphate buffer (pH 7.0) (80 mM final concentration) and H_2O_2 (40 mM final concentration). CAT activity was calculated as µmol H_2O_2 µg prot.$^{-1}$min^{-1} (extinction coefficient, ε: 0.039 cm² µmol^{-1}).

The oxidation of NADPH by the glutathione reductase (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 0.10, 0.15, 0.20, 0.25 and 0.30 mM NADPH. The 200 µL reaction mixture contained 2 µg of proteins, Tris-HCl buffer (pH 7.5) (100 mM final concentration), EDTA (1 mM final concentration), oxidized glutathione (1 mM final concentration) and NADPH (0.20 mM final concentration). GR activity was calculated as µmol NADPH mg prot^{-1} min^{-1} (ε: 6.22 cm² µmol^{-1}).

Glutathione-S-transferase (GST) was measured by following the increase in absorbance at 340 nm and 25ºC for 4 min (Grant et al. 1989). The optimal cofactor concentration was determined by using 0.875, 3.5, 7, 8.75, 12.25 and 15.75 mM of GSH (reduced glutathione). The reaction is realized in a final volume of 200µL containing 2µg of proteins, potassium phosphate buffer (pH 7.4) (100mM final concentration), GSH (8.75mM final concentration) and CDNB (1-chloro-2,4-dinitrobenzen) (875µM final concentration). GST activity was calculated as µmol CDNB conjugate mg prot^{-1}min^{-1} (ε = 9.6 cm² µmol^{-1}). The conjugate can also be formed by non enzymatic way, it is so important to measure the absorbance of sample without enzymatic extract.

Oxidation of sodium ascorbate by ascorbate peroxidase (APX) was measured at 290 nm and 25ºC for 1.5 min according to (Nakano and Asada 1981). The optimal substrate concentration was determined by using 1, 2, 3, 4 and 5 mM H_2O_2. The 250 µL reaction mixture contained 2 µg of proteins, potassium phosphate buffer (pH 7.0) (80 mM final concentration), sodium ascorbate (150 µM final concentration) and H_2O_2 (5 mM final concentration). APX activity was calculated as µmol ascorbate mg prot^{-1}min^{-1}(ε: 2.8 cm^2 µmol^{-1}).

2.4.3. In vivo fluorescence measurements

In vivo fluorescence measurements were realized by using the Pulse Amplified Modulated fluorometry (PhytoPAM, Heinz Walz GmbH, Germany).

The PhytoPAM allows the measurement at four different excitation wavelengths (470, 520, 645 and 665 nm), this is the basis of distinction and characterization of different groups of algae. By deconvolution of signals it is possible to estimate fluorescence signal linked to the three main algae groups: cyanobacteria (Fs_{Bl}), diatoms (Fs_{Br}), and green algae (Fs_{Gr}).

Effective quantum yield (ΦpsII) was recorded at an excitation wavelength of 665 nm. Samples were exposed to actinic light to determine their effective quantum yield, ΦpsII was calculated according to (Genty et al. 1989):

$$\Phi ps_{II} = (F_m' - F_t) / F_m'$$

with F_t the minimum fluorescence determined after a weak far red modulated light and F_m, the maximum level of fluorescence measured during a saturating white light pulse under actinic light.

2.4.4. Diatoms analyses

2.4.4.1. Diatom cell density

Each sample was counted using a Nageotte counting chamber (Marienfeld, Germany). 200 µL of sample were placed on the counting chamber; the total number of individuals and the number of dead cells were recorded in 10 fields of the gridded area (1.25 µL each, 0.5 mm depth) under light microscopy at 400x magnification. Distinction between dead and live organisms was estimated by the observation of the turgescence and colour of the chloroplast as described in Morin et al. (2010b).

2.4.4.2. Taxonomic analyses

Diatom identifications were performed after having prepared permanent slides following European standard Permanent slides following European standard NF EN 13946 (AFNOR 2003). At least 300 individuals per sample were identified at 1,000x magnification to the lowest taxonomic level possible using standard references (Hofmann et al. 2011).

2.4.4.3. Biovolumes

Theoretical biovolumes of each species were extracted from the database available at https://hydrobio-dce.cemagref.fr/en-cours-deau/cours-deau/Telecharger/indice_biologique_diatomee-ibd/, to calculate mean diatom cell biovolume

per sample. Total biovolume represented by diatoms was calculated from mean diatom cell biovolumes and diatom densities, and expressed as $\mu m^3/cm^2$.

2.5. Data analysis

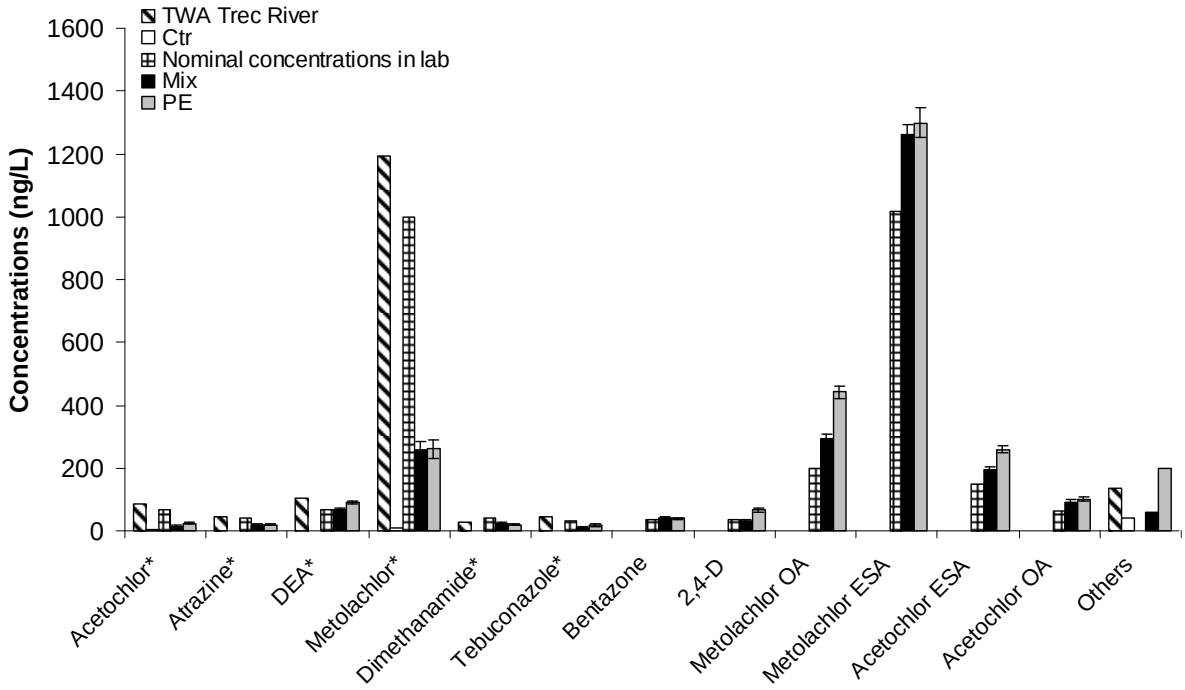
The effects of pesticides exposure on biomass (DW and AFDM, n=5), photosynthetic efficiency (Φps_{II}, n=5), fluorescence levels (Fs_{Bl}, Fs_{Gr} and Fs_{Br}, n=5), antioxidant enzymatic activities (CAT, APX, GR and GST, n=5) and diatoms densities (n=5) were tested by one-way ANOVA analyses using STATISTICA 6.1 (StatSoft) at each sampling time. The ANOVA was followed by a Tukey–HSD test. Homogeneity of variance was checked prior to data analysis. Due to the non homogeneity of variance for DW, AFDM and Φps_{II}, statistical differences on these parameters were analysed by Kruskall Wallis test, followed by 2 by 2 Mann Whitney comparisons.

3. Results and discussion

3.1. Pesticide contamination levels in the rivers estimated by time-weighted average

All pesticides analysed from POCIS in the Ourbise River (reference site) were below the detection limit (data not shown). From all compounds quantified in "Quantitative" POCIS extracts, time-weighted average (TWA) concentrations have been estimated for 18 compounds (no calibration for the other compounds), the major quantified compounds are presented in Figure 31. The Trec River was characterized by very high pesticide concentrations totalizing the sum of the 18 compounds quantified by TWA with 1640 ng/L.

Figure 31 : Concentrations (ng/L) estimated at the Trec station in spring 2012 (9th May-5th June) and mean concentration of the main compounds quantified in the laboratory channel experiment (mean ± SD over the 14 days of experiment, 5 sampling times). Hatched bars represents TWA concentrations and white, gridded, black and grey bars and represent control (Ctr), nominal concentrations, reconstituted mixture (Mix) and POCIS extracts (PE) respectively. Stars indicate compounds estimated by TWA (no calibration for others).



The most concentrated compounds were metolachlor (quantification of total metolachlor), desethyl atrazine (DEA), acetochlor, atrazine, tebuconazole and dimethanamide. Since no calibration is available for metolachlor oxanilic acid (OA), metolachlor ethanesulfonic acid (ESA), acetochlor ethanesulfonic acid (ESA) and acetochlor oxanilic acid (OA), concentration in river of these metabolite products were not estimated nevertheless they were found in important proportion in pure POCIS extract (Figure 31). The encountered pesticides were found to be related to the land uses since the quantified herbicides (metolachlor, acetochlor, atrazine and dimethanamide) are mainly used for the weeding of the important surface of the catchment area used for cereal crops and tebuconazole is a fungicide used as a seed dressing. Moreover the herbicides metabolites (metolachlor ESA, metolachlor OA, acetochlor ESA, acetochlor OA and DEA) were found in PE in higher or similar concentrations than their parent compounds. The important contamination of the aquatic environment by herbicides metabolites has already been described. For example Roubeix et al. (2012a) highlighted that chloroacetanilide herbicides contaminating aquatic systems were often associated with high concentrations of biodegradation products and higher level of DEA compared to atrazine was already recorded in 2009 in the Trec River (Duprat, 2010).

3.2. Laboratory experimental conditions

The nominal and mean concentrations of the main compounds quantified in the channel experiment are presented in Figure 31. The total pesticides concentration (mean over the 14 days of experiment) was of 2830±75 ng/L and 2360±96 ng/L in PE and Mix channels respectively. For the channels spiked with PE, 93% of the total pesticide concentration were represented by the 12 dominant compounds: tebuconazole, acetochlor, atrazine, metolachlor (use of racemic metolachlor), bentazone, 2,4-D, metolachlor OA, metolachlor ESA, acetochlor ESA, acetochlor OA, dimethenamide and desethyl atrazine (DEA). Carbendazine, pyrimicarb, norflurazon and nicosulfuron composed the majority of the remaining 7%. In Mix channel, the 12 compounds selected for the Mix represented 97.5% of the total pesticides concentration.

Concentration of the 12 compounds selected for the Mix was very similar in PE and Mix channels. For example the most concentrated compound metolachlor ESA reached in mean over the all experiment 1300 ± 20ng/L and 1260 ± 15ng/L in the channels contaminated by PE and Mix respectively. Very closed levels between contaminated channels were also recorded for low concentrated compounds also like atrazine (19 ± 2ng/L and 19 ± 2ng/L for PE and Mix treatment respectively) or DEA (90 ± 5ng/L and 69 ± 6ng/L for PE and Mix treatment respectively).

The concentrations recorded along the 14 days of experiment in PE and Mix channels were in the same range as concentrations encountered in the Trec station (TWA Trec) and nominal concentrations. Moreover contamination was very stable in time highlighting the efficiency of the experimental system composed of peristaltic pumps (renewing of 3L/day) in order to ensure stable levels of compounds in the channels. In this experiment, we were so able to reach and maintain pesticides concentrations close to environmentally realistic levels; the decrease often observed when running such laboratory experiments (loss due to adsorption to experimental systems) was prevented by starting up the channels during one day before the beginning of biofilm exposure and by continuous water renewal (3L/day).

Physico-chemical parameters and nutrients concentrations over the experiment are presented in Table 8; no significant difference was observed between treatments over the 14 days of experiment.

Table 8 : Mean physico-chemical parameters (± standard deviation, four sampling times) in artificial channels over the 14 days for the different treatments (PE: POCIS extracts, Mix: Reconstituted mixture and Ctr: condition without pesticide).

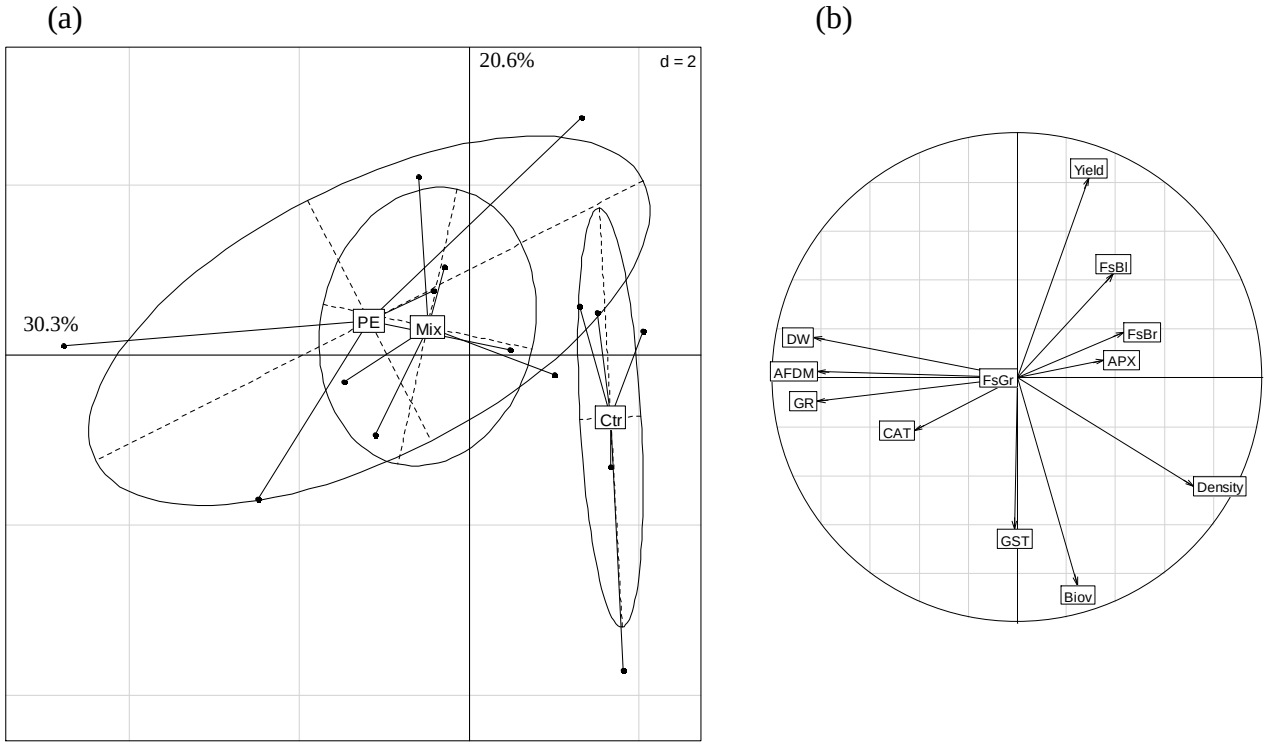
	Temp (°C)	pH	Cond. (µS/cm)	O_2 (mg/L)	N-NO_3 (mg/L)	P-PO_4 (mg/L)	Si (mg/L)
Ctr	22.6 ± 0.6	7.1 ± 0.4	261.8 ± 8.3	7.2 ± 1.5	49.9 ± 6.2	2.7 ± 0.9	1.3 ± 0.4
PE	23.0 ± 0.5	7.1 ± 0.4	254.8 ± 6.0	7.6 ± 0.4	29.9 ± 16.5	2.1 ± 0.8	1.0 ± 0.4
Mix	23.0 ± 0.7	6.6 ± 1.1	259.0 ± 4.5	7.9 ± 0.5	38.3 ± 11.9	2.4 ± 0.9	1.0 ± 0.2

3.3. Biofilm responses to pesticide exposure

3.3.1. Separation of sample after 14 days of experiment

The PCA was realised with all variables apart from diatom taxonomic analyses recorded after the maximal exposure period in order to obtain a global overview of pesticides effects on biofilms (Figure 32).

Figure 32 : (a) Principal component analysis based on biofilm responses to 14 days of exposure to the different treatments (PE, Mix or Ctr) in the channel experiment and (b) related correlation circle. PE = POCIS extracts, Mix= reconstituted Mixture, Ctr= condition without pesticide. Yield= effective quantum yield (ΦpsII), DW=Dry weight, AFDM= Ash-free dry mass, FsBl, FsBr and FsGr: fluorescence signal linked to cyanobacteria, diatoms, and green algae, Biov= mean diatom cell biovolume, density= diatom cell density, CAT=Catalase APX= Ascorbate peroxidase, GR= Glutathione reductase and GST=Glutathione-S-transferase.



Yield: effective quantum yield (ΦpsII), DW: Dry weight, AFDM: Ash free dry mass, Fs_{Bl}, Fs_{Br} and Fs_{Gr}: fluorescence signal linked to cyanobacteria, diatoms, and green algae, Biov: mean diatom cell biovolume, density: diatom cell density, CAT: Catalase APX: Ascorbate peroxidase, GR: Glutathione reductase and GST: Glutathione-S-transferase were the variables selected for the analyses. The first two axes of the PCA explained 50.9% of the variance. The first axis (30.3% of the variance explained by axes 1) clearly separated the samples in function of exposure or not to pesticides. Biofilms exposed to PE and Mix were characterized by high DW and AFDM ; Ctr biofilms were characterized by high diatom density. In regards

to these results from the PCA, exposure to pesticides clearly induced changes on growth related, structural and functional biofilm characteristics. Moreover, to a first sight, PE and Mix treatments seems to have similar effects on biofilms after 14 days of exposure.

3.3.2. Dry weight and ash-free dry mass

Biofilm biomass was measured at the beginning of the experiment (day 0) and after 14 days of exposure to the three different treatments (Table 9). Biomass increased significantly between day 0 and day 14 in all conditions with a mean increase of factor 3.0 and 4.7 for DW and AFDM respectively. At day 14, statistical analyses revealed significant differences between treatments. Both parameters were higher for biofilms exposed to pesticides (PE and Mix treatments) compared to biofilm maintained in water without pesticide (Ctr treatment). DW was significantly higher for PE and Mix treatments and AFDM for Mix treatment only, compared to Ctr biofilms. Moreover, no significant difference was observed between PE and Mix treatment in regards to both DW and AFDM.

In our experiment, the positive impacts of pesticides on the global growth of biofilm (DW and AFDM) is not likely to be explained by better growth of algal component with pesticides exposure but could be due to other components of biofilm. For example an increase in extracellular polymeric substances (EPS) production has already been observed as a response to physical stress (Artigas et al. 2012) or metal and organic compounds (Fang et al. 2002). This increase could be linked to the role of EPS as protective shield for the cells against the adverse influences from the external environment.

3.3.3. Antioxidant enzymatic activities

Catalase (CAT), ascorbate peroxidase (APX), gluthatione reductase (GR) and Glutathione-S-transferase (GST) antioxidant activities over the 14 days of direct exposure to the different treatments in artificial channels are presented in Table 9. Antioxidant enzymes are involved in important processes of cell detoxification during oxidative stress; statistical analyses revealed the impact of pesticides (PE and/or Mix treatments) on APX, GR and GST activities, but no significant differences in CAT activity between treatments.

APX was the first enzymatic activity to be affected, with a significant induction after 3 days of exposure for Mix treatment. In PE treatment, APX was significantly lower than controls from day 7. The impact of pesticides on GR and GST activities were delayed, with the first effects noticeable after 1 week of exposure. GR activity was impacted by PE exposure at day

7 with a significant decrease compared to the Ctr. GST activity of exposed biofilms was statistically higher in both PE and Mix treatments at day 7 than in controls.

Table 9 : Mean values (±SD, n=5) of the different biofilm responses criteria studied: diatom density, dry weight, ash-free dry mass, fluorescence signal linked to cyanobacteria (Fs_{Bl}), green algae (Fs_{Gr}) and diatom (Fs_{Br}) groups, effective quantum yield of PSII (Φps_{II}), mean diatom cell biovolume, total diatom biovolume, catalase (CAT), ascorbate peroxidise (APX), glutathione reductase (GR) and glutathione-S-transferase (GST) activities of biofilms exposed to the different treatments in the channel experiment. Different letters indicate statistically significant differences at each sampling time. ru.: relative unit, nc: non calculated.

	Day 0	Day 3			Day 7			Day 14		
	Ctr	PE	Mix	Ctr	PE	Mix	Ctr	PE	Mix	Ctr
Diatom density										
Diatom density (10^3cells/cm^2)	223 ± 21	82 ± 15 (a)	65 ± 9 (a)	167 ± 19 (b)	336 ± 64	337 ± 31	319 ± 38	423 ± 48 (a)	515 ± 70 (a,b)	628 ± 29 (b)
Biomasses										
Dry weight (mg/cm^2)	0.24 ± 0.01	nc	nc	nc	nc	nc	nc	0.75 ± 0.08 (a)	0.82 ± 0.05 (a)	0.53 ± 0.06 (b)
Ash free dry mass (mg/cm^2)	0.10 ± 0.00	nc	nc	nc	nc	nc	nc	0.49 ± 0.12 (a,b)	0.56 ± 0.05 (a)	0.34 ± 0.04 (b)
Fluorescence levels										
Fs_{Bl} (ru)	202 ± 10	76 ± 13 (a)	48 ± 7 (a)	186 ± 35 (b)	193 ± 23	227 ± 42	215 ± 31	213 ± 10	213 ± 9	214 ± 9
Fs_{Gr} (ru)	0	0	0	0	0	0	0	0	0	0
Fs_{Br} (ru)	520 ± 10	264 ± 29 (a)	174 ± 14 (a)	513 ± 50 (b)	361 ± 36	352 ± 46	439 ± 29	312 ± 33	285 ± 16	292 ± 20
Photosynthetic yield										
ΦpsII	0.33 ± 0.01	0.35 ± 0.02 (a)	0.41 ± 0.02 (a)	0.20 ± 0.02 (b)	0.38 ± 0.03	0.33 ± 0.05	0.35 ± 0.03	0.54 ± 0.03	0.56 ± 0.01	0.56 ± 0.03
Biovolumes										
Mean diatom cell biovolume (µm^3/cell)	1345 ± 110	nc	nc	nc	nc	nc	nc	679 ± 74	614 ± 45	965 ± 169
Total diatom biovolume (10^3 µm^3/cm^2)	296 ± 24	nc	nc	nc	nc	nc	nc	284 ± 39 (a)	323 ± 57 (a)	613 ± 306 (b)
Antioxydant enzymatic activities										
CAT (µmolH_2O_2/µgprot/min)	0.229 ± 0.065	0.162 ± 0.018	0.230 ± 0.066	0.297 ± 0.048	0.170 ± 0.052	0.172 ± 0.028	0.172 ± 0.064	0.126 ± 0.015	0.110 ± 0.016	0.082 ± 0.027
APX (µmolAsc/mgprot/min)	0.344 ± 0.073	0.899 ± 0.283 (a)	2.470 ± 0.417 (b)	1.137 ± 0.250 (a)	0.350 ± 0.067 (a)	0.401 ± 0.058 (a,b)	0.600 ± 0.107 (b)	0.214 ± 0.034 (a)	0.343 ± 0.152 (a,b)	0.326 ± 0.022 (b)
GR (µmolNADPH/mgprot/min)	0.209 ± 0.141	0.205 ± 0.027	0.159 ± 0.023	0.152 ± 0.048	0.119 ± 0.038 (a)	0.415 ± 0.170 (b)	0.305 ± 0.107 (b)	0.464 ± 0.241	0.169 ± 0.059	0.171 ± 0.077
GST (µmolCDNBconjugate/mgprot/min)	0.295 ± 0.028	0.270 ± 0.041	0.283 ± 0.055	0.208 ± 0.011	0.282 ± 0.027 (a)	0.210 ± 0.009 (b)	0.168 ± 0.006 (c)	0.189 ± 0.016	0.210 ± 0.025	0.207 ± 0.031

On the contrary, CAT activity was not statistically different between treatments, however temporal variations were observed. CAT activity of Ctr biofilms increased first from day 0 to day 3 and then decrease from day 3 to day 14, whereas exposure to PE and Mix treatments induced a global decrease of CAT activity. CAT has been shown to be related with metal and herbicide contamination levels (Bonet et al. 2012, Bonnineau et al. 2013); nevertheless from our results we were not able to show the role of CAT in reduction of oxidative stress during exposure to low concentration of pesticides.

AEAs have been used with success in field and laboratory studies to assess diverse types of pollution like metal or pharmaceutical contamination; nevertheless Bonnineau et al. (2013) highlighted the limits of interpretation of AEAs values, due to their temporal variation and the absence of absolute threshold value indicative of oxidative stress induced by the contaminants. In our study in which all biofilms were colonized for 6 weeks in the same station (Ourbise River), pesticide exposure in the channels influenced their growth (DW, AFDM) and biofilm composition, and could explain the confusion of AEAs responses.

3.3.4. In vivo fluorescence measurements

Effective quantum yield (ΦpsII) and fluorescence signals related to the different algal groups in function of exposure duration to the three treatments are presented in Table 9. ΦpsII globally increased for the controls with time exposure (from 0.33±0.01 to 0.56±0.03 between day 0 and day 14) reflecting the adaptation of biofilms to the specific laboratory channels conditions. Significant differences were observed only at day 3 with significant lower values for Ctr compared to the contaminated conditions (0.20±0.02, 0.35±0.02 and 0.41±0.02 for Ctr, PE and Mix respectively). These higher ΦpsII values of contaminated biofilms compared to the controls were unexpected. It was so not possible to highlight pesticide impacts on photosynthetic capacities with ΦpsII as endpoint. This could be linked to the low proportion of PSII inhibitors in the extracts dominated by chloroacetamide and their degradation products.

Based on fluorescence signals, the biofilms were only composed of diatoms (Fs_{Br}) and cyanobacteria (Fs_{Bl}). Originally (day 0), the biofilm from the Ourbise station was characterised by higher fluorescence signals related to diatoms than cyanobacteria. During the 14 days of exposure in the artificial channels, Fs_{Br} and Fs_{Bl} followed different trends. A global decrease of Fs_{Br} was observed for the three treatments, whereas Fs_{Bl} was not statistically different at day 14 comparing to day 0. . At day 3, both Fs_{Br} and Fs_{Bl} decreased dramatically for biofilms exposed to pesticides (PE and Mix) compared to fluorescence levels at day 0, to

the reverse of biofilms remaining stable in water without pesticide. The higher fluorescence signals for Ctr at day 3 coincided with lower values of ΦpsII, supporting the hypothesis of a higher allocation of energy for growth of algal component in Ctr leading to lower values of ΦpsII.

3.3.5. Diatoms analyses

3.3.5.1. Diatom cell density

Diatom densities revealed significant differences at day 3 and day 14 between treatments, as shown in Table 9. In Ctr, a slight decrease in densities was observed between day 0 and day 3 (lag phase due to the change of environmental conditions), followed by classical positive growth. In biofilms exposed to pesticides, the density of diatoms drastically felt down at day 3, with mean values of 82 ± 15 $.10^3$ cells/cm^2 (PE treatment) and 65 ± 9 $.10^3$ cells/cm^2 (Mix treatment), compared to Ctr (167 ± 19 $.10^3$ cells/cm^2). This decrease was concomitant to the lower fluorescence signal of brown algae (Fs_{Br}) observed for contaminated treatments at this sampling time. Then diatom densities increased until the end of the experiment, with lower values for biofilms exposed to pesticides than in Ctr. Although only PE was significantly different from Ctr. PE and Mix treatments were never found statistically different.

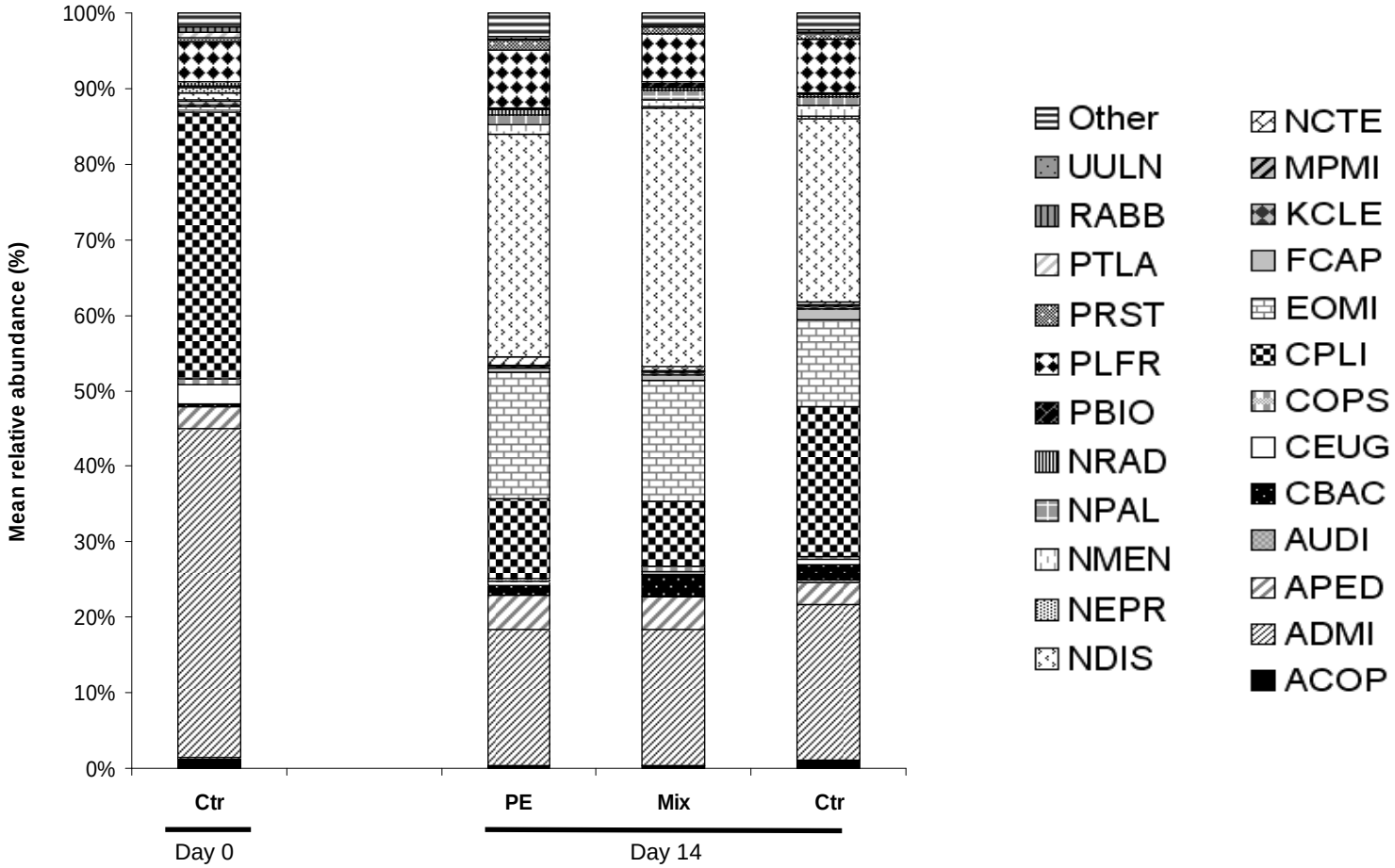
Previous studies showed the impact of the pesticides exposure on diatoms growth, either after longer exposures to a single pesticide (Pérès et al. 1996).

3.3.5.2. Taxonomic analyses

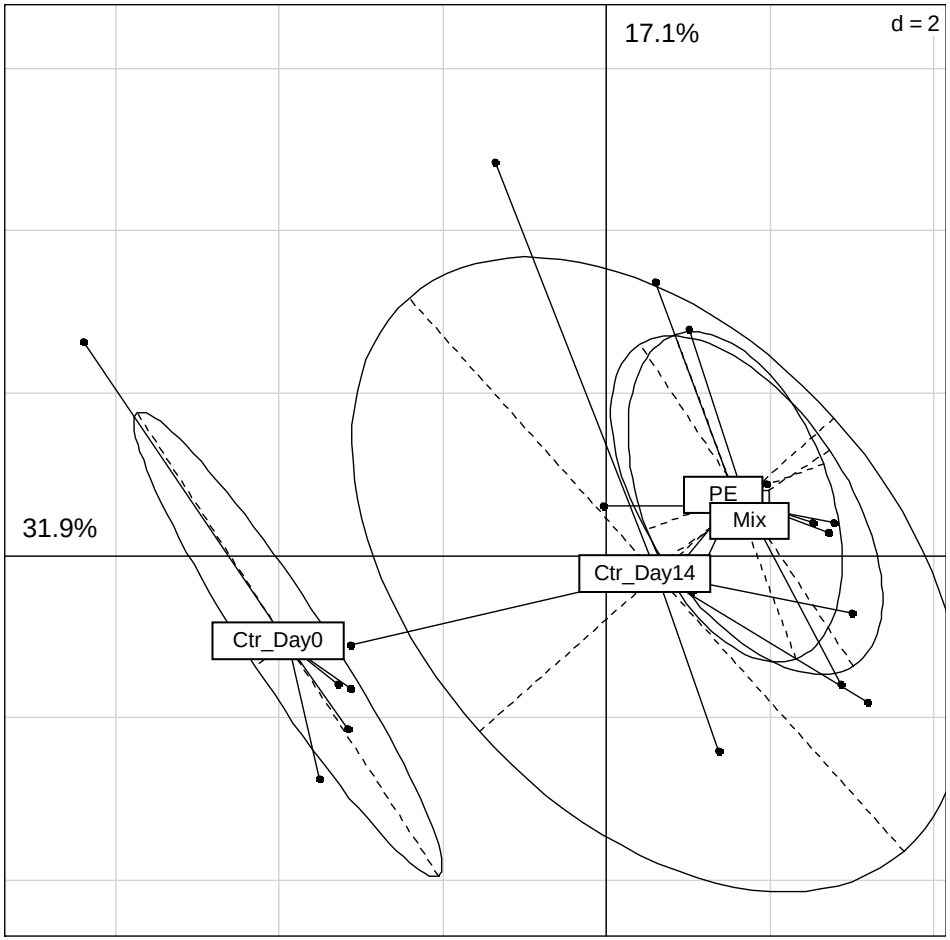
A total of 63 species were identified. The 24 species occurring at more than 1% relative abundance in at least one sample are presented in Figure 33a. At day 0, biofilms were dominated by two species, *Achnanthidium minutissimum* (Kützing) Czarnecki (44% relative abundance) and *Cocconeis placentula* Ehrenberg var. *lineata* (Ehrenberg) Van Heurck (35% relative abundance), followed by *Planothidium frequentissimum* (Lange-Bertalot) (5% relative abundance), *Amphora pediculus* (Kützing) Grunow (3% relative abundance), *Cocconeis euglypta* (Ehrenberg) (2% relative abundance) and *Amphora copulata* (Kützing) Schoeman & Archibald (1% relative abundance).

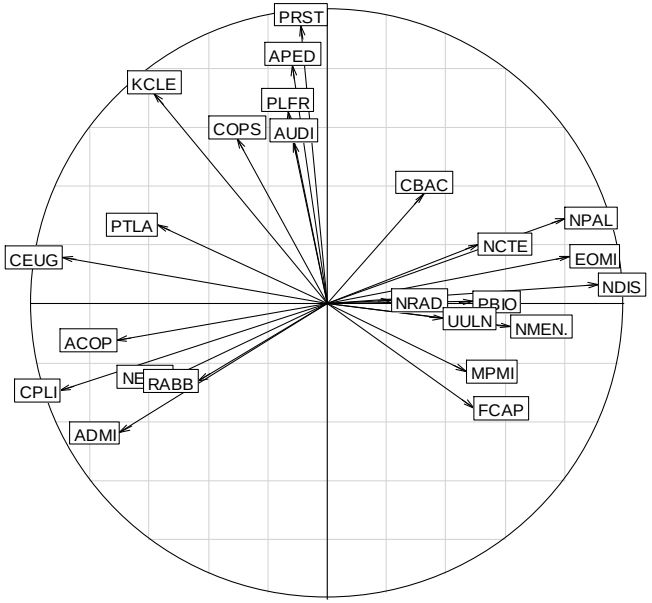
Figure 33 : Mean relative abundances (a) and associated principal component analysis (b) of the 24 dominant species (> 1% relative abundance in at least one sample) at day 0 and after 14 days of exposure to the different treatments. PE: POCIS Extracts; Mix: Reconstituted mixture; Ctr: Control. ACOP: *Amphora copulata*, ADMI: *Achnanthidium minutissimum*, APED: *Amphora pediculus*, AUDI: *Aulacoseira distans*, CBAC: *Caloneis bacillum*, CEUG: *Cocconeis euglypta*, COPS: *Cocconeis pseudothumensis*, CPLI: *Cocconeis placentula var.lineata*, EOMI: *Eolimna minima*, FCAP: *Fragilaria capucina Desmazieres var. capucina*, KCLE: *Karayevia clevei*, MPMI: *Mayamaea permitis*, NCTE: *Navicula cryptotenella*, NDIS: *Nitzschia dissipata*, NEPR: *Neidium productum*, NMEN: *Navicula menisculus var. menisculus*, NPAL: *Nitzschia palea*, NRAD: *Navicula radiosa*, PBIO: *Psammothidium bioretii*, PLFR: *Planothidium frequentissimum*, PRST: *Planothidium rostratum*, PTLA: *Planothidium lanceolatum*, RABB: *Rhoicosphenia abbreviata*, UULN: *Ulnaria ulna*, Other: diatom species representing less than 1% relative abundances in at least one sample.

(a)



(b)


17.1%
d = 2
31.9%
PE
Mix
Ctr_Day14
Ctr_Day0


PRST
APED
KCLE
PLFR
COPS
AUDI
CBAC
PTLA
NPAL
NCTE
CEUG
EOMI
NDIS
NRAD
PRIO
UULN
NMEN.
ACOP
MPMI
NE
RABB
CPLI
FCAP
ADMI

The principal component analyses (PCA) clearly differentiates in Figure 33b diatom communities from day 0 and after 14 days in the channels along Axis 1, highlighting that temporal succession of species prevailed over toxic-induced selection. In particular, relative abundances of the two initially dominant species decreased dramatically at day 14 in all samples; from 44 down to 19% relative abundance for *A. minutissimum* and from 35 down to 13% relative abundance for *C. placentula* (mean of all treatments at day 14).

The PCA did not clearly separate diatom communities at day 14 regarding to the treatment; nevertheless Ctr samples were more dispersed that contaminated ones, confirming that perturbations, here toxic-induced species selection, leads to community homogenisation (e.g. Tison et al. 2005, Passy and Blanchet 2007). At day 14, the assemblages were composed of *Nitzschia dissipata* (Kützing) Grunow var. *dissipata* (29% relative abundance), *A. minutissimum* (19% relative abundance), *Eolimna minima* (Grunow) Lange-Bertalot (15% relative abundance), *C. placentula* (13% relative abundance), *Planothidium frequentissimum* (7% relative abundance) and *Amphora pediculus* (4% relative abundance). In contrast to the decrease of *A. minutissimum* on day 14, of the same order of magnitude in all samples, the relative abundance of *Cocconeis placentula* differed depending on the treatment, with significantly lower relative abundances in biofilms exposed to pesticides (PE and Mix treatments, 11% and 9%) compared to Ctr (20%). Even if species composition was comparable between treatments at day 14 as shown in the PCA, subtle differences in the relative abundances of some species discriminated the 2 treatments with pesticides and the Ctr. As mentioned above for *C. placentula*, species like *Fragilaria capucina* Desmazières var. *capucina* (PE: 5%, Mix: 7% vs. Ctr: 14%) or *Amphora copulata* (4% and 3%, vs. 10%) were more represented in the control channels. In contrast, relative abundances of *Nitzschia dissipata* and *Eolimna minima* were higher in biofilms exposed to pesticides (*N. dissipata*: 29% and 34% vs. 24% in Ctr, *E. minima*: 17% and 16% vs. 11% in Ctr) during 14 days.

Very few is known about relative sensitivity of diatoms to pesticides, for example *Eolimna minima* had been described as pesticide sensitive in several field studies (Hamilton et al. 1987, Pérès et al. 1996, Morin et al. 2009) nevertheless some recent laboratory studies have reported this species as relatively tolerant to elevated concentrations of diuron (Larras et al. 2012, Moisset, personal communication). Moreover it has been shown that the same species could have various tolerances to a particular compound in function of the site it has been isolated. Thus Roubeix et al. (2012b) highlighted different EC_{50} for the diatoms *Encyonema neomesianum* to copper and diuron originated from sites with different levels of pesticides. From the results of our experiment, *C. placentula*, *F. capucina* and *A. copulata* could be

considered as more sensitive to pesticides than *N. dissipata* and *E. minima*. Moreover it has to be noticed that within these species, the larger ones (*C. placentula* and *A. copulata*) were found in higher relative abundance in Ctrl biofilms; on the contrary the small species *E. minima* was more abundant in biofilms exposed to pesticides.

3.3.5.3. Diatom cell biovolume

Mean diatom cell biovolume and total diatom biovolume at day 0 and after 14 days of exposure in channels to the different treatments are presented in Table 9.

A decrease of the mean cell biovolume was observed between day 0 and day 14 for all treatments, from initial values of 1345 ±110 μm^3 to 750 ± 70 μm^3 at day 14 (mean of the 3 treatments). This decrease results from increasing relative abundances of small-sized diatom species like *E. minima*, together with decreasing percentages of large-sized diatom species like *C. placentula* var. *lineata*. Mean cell biovolume significantly decreased with time exposure in PE and Mix treatments (reaching 679 ± 74 and 614 ± 45 μm^3 respectively for PE and Mix at day 14) but not in biofilms maintained in uncontaminated water (965 ± 169 μm^3 at day 14). These differences reflected the slight variations in diatom relative abundances at the end of the experiment, e.g. lower abundances of *E. minima* and *A. pediculus* (two small-sized diatom species, < 100μm^3) and higher abundances of *C. placentula* var. *lineata* (large-sized diatom species, > 2500μm^3) in Ctr biofilms.

Total diatom biovolume of samples -calculated from mean diatom sizes and diatom densities- remained stable from day 0 to day 14 in PE and Mix treatments with an initial biovolume of 296,000 ± 24,000 $\mu m^3/cm^2$ and final values of 284,000 ± 39,000 and 323,000 ± 57,000 $\mu m^3/cm^2$ for PE and Mix treatments respectively. On the contrary, total diatom biovolume in Ctr increased statistically from day 0 to day 14 (613,000 ± 306,000 $\mu m^3/cm^2$ at day 14); moreover at the end of the experiment total diatom biovolume of Ctr was statistically higher than in pesticides contaminated treatments (PE and Mix). These differences can be explained by both the higher mean cell biovolume and the higher diatom density for Ctr biofilms compared to PE and Mix biofilms at day 14.

Reduction of diatom biovolumes with toxicant exposure has already been observed in several studies dealing with metal contamination. For example several authors recorded changing from larger to smaller individuals with increase of metals concentrations in a cadmium/zinc polluted river (Riou-Mort, France) (Morin et al. 2007, Arini et al. 2012a, Arini et al. 2012b). In a translocation study, Corcoll et al. (2012b) also observed the predominance of smaller growth forms in the biofilms in metal-impacted sites. Small-sized individuals are

hypothesized to be less disfavoured than larger ones under toxic exposure, due to reduced cell surface exposed to contaminants (limiting uptake and, thus, toxicity). This descriptor could, therefore, be an indicator potentially applicable to any kind of toxic stress.

3.4. Effects of exposure to low dose of pesticides

The first objective of the study was to evaluate the effects of chronic and low dose exposure of pesticides on river biofilms in a context of pollution by cereal crops and to evaluate if the observed effects of a complex environmental mixture (PE) could be explained by the major compounds quantified in the PE (Mix). Effects of low dose of pesticides on river biofilms using POCIS devices have been previously studied by Morin et al. (2012b). The authors were working on a small river subjected to strong vineyards pressure (Morcille River, eastern France) characterized by high concentration of desmethyl norflurazon (main norflurazon degradation product), norflurazon (carotenoid biosynthesis inhibitor), dimetomorph (lipid synthesis inhibitor), tebuconazole (disruptor of membrane function) and diuron (PSII inhibitor). In this particular context of pesticides contamination due to vineyards activities, they were able to show the interest of such approach and the efficiency of POCIS as a tool in order to highlight effects of low dose of pesticides in river biofilms subjected to a variety of herbicides and fungicides. In our study we showed effects of low dose of pesticides on biofilm with a different contamination profile since the major compounds quantified in POCIS extracts (acetochlor, metolachlor and their metabolites) belonged to the chloroacetamide group. Acetochlor is acting as a lipid synthesis inhibitor and metolachor is a inhibitor of mitosis and cell division, in contrast carotenoid biosynthesis and PSII inhibitors were characterizing the extracts in the study realised by Morin et al. (2012b). The present study is so showing the potential of biofilms as indicators since they respond to a large range pesticide with various modes of action.

Regarding the composition of PE in our study, chloroacetanilide metabolites were found at higher or similar concentrations than parent compounds. Nevertheless they have been shown to have low toxicity compared to their parent compounds even at elevated concentrations. Thus Roubeix et al. (2012a) did not observed inhibition of the growth of the diatom *Nitzschia nana* at concentration up to 100 mg/L of acetochlor OA, acetochlor ESA, metolachlor OA and metolachlor ESA .

Diatom density and biomass could so be considered as global indicators for pesticides exposure, in the other hand structural endpoints seem to be highly dependent of contamination profiles. The POCIS device appears to be a promising tool to better take into account chronic

effects of cocktails in a wide range of contamination contexts, and it proves to be of particular interest for applications in the field of environmental risk assessment.

3.5. Representativeness of POCIS extracts

The second objective of the channel experiment was to evaluate if the major compounds quantified in the PE (Mix) are responsible of the effects of a complex environmental mixture (PE). Extracts of passive sampling devices are increasingly used in order to evaluate toxicity of environmental samples; most of the time acute testing on single species is realized. This approach has the advantage to be integrative for mixture issues, unknown compounds, and metabolite issues; the extracts are so used as a “black box”. Identification of compounds responsible for the observed toxicity can be approached by effect-directed analyses (EDA) methods (Brack 2003, Hecker and Hollert 2009). This approach consists in realizing biological tests with the entire extract and by successive fractioning of the extract until identifying the compound(s) responsible for the observed toxicity. Nevertheless in this approach, biological testing needs to be repeated over successive fractioning steps, which increases the amount of extract needed to run the different testings. In long term experiments, identifying the compounds responsible for the observed toxic effects is a challenge since EDA approach cannot be used because of its extremely time consuming (cannot be easily repeated) process, its need of important volumes of passive sampler extracts, the important number of samples produced and subsequent analyses to run. In this study we did not attempt to identify the specific compound(s) responsible for the toxicity but we designed the experiment in order to investigate if the effects of a complex environmental cocktail could be explained by the mixture of the major compounds quantified the PE. In a previous work we demonstrated the potential of the POCIS as a tool integrative of a part of the environmental toxicity (Morin et al. 2012b). Several scenarios could be expected, first the toxicity of POCIS extracts is higher than toxicity of the major compounds, meaning that other compounds present in PE, but not in the Mix, also contribute to the overall toxicity. These other compound(s) might be identified, or not (unknown compounds because not screened), and could possibly have synergic effects with identified compounds. Secondly lower toxicity of PE compared to the Mix could be observed, and in this situation compounds present in PE but not in the Mix could have antagonistic action, resulting in a decrease of toxicity of PE compared to Mix. Lastly, same toxicity and similar effects observed for PE and Mix: in this scenario, toxicity is explained by the major identified compounds present in the PE and other compounds than the 12 major do not play a key role in the observed effects.

In the present study, PCA (Figure 32) revealed clear separation between control and samples exposed to pesticides but not within contaminated treatments. Similar effects were observed for biofilms exposed to both PE and Mix in regards to photosynthetic efficiency, biomass, diatom density, biovolumes and taxonomic composition. Here the effects of a complex environmental mixture were explained by the major compounds quantified in the PE; POCIS devices could so be considered as a tool integrative of a part of the environmental toxicity.

4. Conclusions and perspectives

After entire POCIS extract chronic effects have been demonstrated, acute testing using effect-directed analyses (EDA) methods can be of interest to identify the compound(s) responsible for the effects observed; nevertheless similar approaches cannot be implemented in long-term experiments because of technical difficulties. This study aimed so to characterize the effects of chronic and low dose exposure to pesticides on natural biofilm community and to evaluate if the observed effects due to exposure to PE were explained by the major compounds identified in the extracts. The design of the experimental system, composed by artificial channels with peristaltic pumps insuring a 3L/day renewal, allowed the accurate control of pesticide concentrations in the channels, essential condition when working with low doses of contaminants. Effects of low dose of pesticides were observed on growth-related (dry weight, ash-free dry mass and diatom cell densities), structural (diatom composition, biovolumes), or physiological endpoints (photosynthetic efficiency and antioxidant enzymatic activities). In our experiment, the effects of PE exposure were similar to the ones induced by the exposure to the major compounds present in PE leading to the conclusion that PE toxicity was mainly due to the major compounds. Principal component analyses clearly separated contaminated (PE and Mix) from non-contaminated (Ctr) biofilms; on the contrary the analyses did not revealed separation between biofilms exposed to PE or to the 12 major compounds identified in the extract. Our study showed the relevance of the use of passive samplers (like the POCIS) extracts in order to better take into account chronic effects of mixtures in evaluation risk assessment studies since effects of POCIS extracts have been highlighted on river biofilms metrics. We also demonstrated that PE effects were similar to those induced by the exposure to the major compounds present, making PE a potential tool integrative of a part of the environmental toxicity.

Acknowledgments

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Chapitre VIII : Conclusions générales et perspectives

L'objectif de cette thèse était d'étudier les effets des pesticides en mélange sur le biofilm de rivière, notamment en évaluant le potentiel de l'outil POCIS pour améliorer la représentativité environnementale de l'exposition lors d'expérimentations en laboratoire, dans le but de proposer des pistes de recherche pour le développement d'outils permettant de diagnostiquer *in situ* les pollutions de type pesticides. Pour ce faire, une large gamme de descripteurs a été testée, allant de paramètres couramment utilisés dans les études pour caractériser le périphyton à des descripteurs spécifiquement développés dans la thèse, en passant par des outils encore peu exploités dans le contexte des pollutions toxiques.

Ce chapitre a pour objectif de faire un bilan des approches utilisées tout au long de cette thèse en présentant les principaux résultats relatifs aux expositions de pesticides sur le biofilm à différents niveaux de représentativité tout en mettant en avant leurs avantages et leurs limites. Dans un second temps les pistes d'investigation et les perspectives proposées par cette thèse pour de futurs travaux de recherche concernant le diagnostic des pollutions toxiques seront présentées.

8.1 Entre réalisme et réductionnisme : des approches complémentaires pour appréhender les effets des pesticides sur le biofilm

En combinant les expérimentations en conditions contrôlées de laboratoire à différentes échelles de temps (à court ou long terme), de concentrations en toxiques (concentrations proches des concentrations environnementales ou bien supérieures) et de complexité (mélange complexe, ternaire, molécules seules), les différents travaux réalisés nous ont permis d'acquérir des informations sur l'évolution de la croissance (densité de diatomées, biomasses), de la structure (composition spécifique des diatomées) et du fonctionnement physiologique (activité photosynthétique, activités enzymatiques) du périphyton face à des expositions aux pesticides. Le biofilm a ainsi été caractérisé à différents niveaux d'organisation, nous nous sommes intéressés aussi bien aux diatomées (densité et structure des communautés) qu'à la composante algale dans son ensemble (groupes algaux et fonctionnement physiologique) et au biofilm dans son entier (biomasse).

8.1.1 Réponses du périphyton en canaux artificiels face à un mélange environnemental de pesticides

A ce jour deux grandes approches ont été utilisées pour caractériser les mélanges de toxiques sur le périphyton. Les études de terrain permettent d'atteindre un niveau important de réalisme puisque les communautés sont directement exposées aux contaminants présents *in*

situ, cependant les facteurs confondants peuvent rendre difficile l'interprétation des résultats. A l'opposé, les expériences au laboratoire permettent de fixer un grand nombre de paramètres (concentration en nutriments, en contaminants, lumière, etc.) ; ce contrôle des conditions expérimentales est néanmoins inévitablement associé à un faible réalisme vis-à-vis de la contamination environnementale puisque les études menées jusque là ne se sont intéressées, au mieux, qu'à un cocktail restreint de contaminants. Les extraits d'échantillonneurs passifs renferment un mélange complexe de composés et sont représentatifs d'une certaine « toxicité environnementale directement extraite du terrain ». En ce sens, le couplage des expériences en canaux artificiels au laboratoire et de l'utilisation des échantillonneurs passifs permettrait de faire le lien entre les conditions contrôlées du laboratoire et la contamination à laquelle sont exposés les organismes dans le milieu aquatique.

Lors de cette thèse les effets de mélanges réalistes (en termes de concentrations imposées et de composition du cocktail) de pesticides sur le biofilm ont donc été étudiés grâce à l'utilisation des extraits d'échantillonneurs passifs en canaux artificiels (Chapitres IV et VII).

Les diatomées : organismes au fort potentiel dans la mise en évidence des contaminations en produits phytosanitaires

Un effort particulier a été mis sur l'étude de la composante diatomique du biofilm de par son utilisation actuelle en routine pour le calcul des indices de qualité générale des eaux (e.g. en France, Cemagref 1982, Coste et al. 2009) et son potentiel avéré dans le cas des pollutions métalliques (Morin et al. 2012c, Gold et al. 2003, Arini et al. 2012a, Duong et al. 2008).

Dans notre étude, un effet négatif a été observé sur les densités de diatomées lors des expositions en canaux artificiels aux extraits de POCIS et ce pour les deux bassins versants à l'étude (la Morcille et le Trec).

L'impact à long terme des pesticides en faible dose sur les diatomées a aussi été observé au regard de la structure des communautés. Ainsi le développement d'espèces comme *Nitzschia palea* (biofilms originaires de St Joseph), *Nitzschia dissipata* (biofilms originaires de l'Ourbise) et *Eolimna minima* (pour les biofilms originaires de St Joseph et de l'Ourbise) semble être favorisé avec l'exposition aux pesticides. Au contraire *Cocconeis placentula*, *Fragilaria capucina* et *Amphora copulata* se développent mieux dans les biofilms originaires de l'Ourbise non exposés aux extraits de POCIS. Ces résultats sont contrastés par rapport à ceux décrits dans la littérature. Par exemple *Eolimna minima* a été décrite comme une espèce sensible aux pesticides dans différentes études *in situ* (Hamilton et al. 1987, Morin et al. 2009, Pérès et al. 1996). Cependant sur le terrain, de nombreux facteurs confondants peuvent rendre

l'identification des relations de causalité difficile (nutriments, métaux, température, pH, lumière...). C'est par exemple le cas sur la Morcille où le gradient en pesticides s'accompagne d'un gradient métallique et d'orthophosphates. Les effets des métaux sont ainsi succeptibles de s'ajouter à ceux induits par les pesticides alors que les nutriments auraient plutôt tendance à moduler les impacts (Barranguet et al. 2002, Guasch et al. 2004, Lozano et Pratt 1994, Morin et al. 2008). C'est pourquoi les études en conditions contrôlées de laboratoires sont nécessaires et complémentaires des études de terrain. Certains travaux se sont ainsi penchés sur la sensibilité relative aux pesticides d'espèces isolées du terrain et ont par exemple décrit *Nitzschia palea* et *Eolimna minima* comme relativement tolérantes au diuron (Larras et al. 2012; Moisset et al., en préparation).

La détermination de la composition spécifique des biofilms a également permis de calculer les biovolumes moyens des diatomées, d'après les dimensions théoriques des espèces inventoriées et leurs abondances relatives dans les échantillons. Ce paramètre s'est avéré affecté par l'exposition aux extraits de POCIS du Trec et une diminution du biovolume moyen a pu être observée suite à l'exposition aux pesticides. Des réductions de biovolumes ont déjà été reportées pour d'autres types de contamination (notamment métalliques) et ce paramètre a été proposé comme indicateur de pollution toxique (Corcoll et al. 2012b, Morin et al. 2007).

Stimulation de la biomasse avec l'exposition aux toxiques à faible dose

Etonnamment des biomasses plus importantes ont été observées quand les biofilms étaient exposés aux pesticides à faible dose. Concernant la contribution de la composante diatomique, nous avons vu que la densité et le biovolume moyen spécifique étaient plus faibles pour les biofilms exposés aux pesticides par rapport aux témoins. Une hypothèse avancée pour expliquer l'augmentation de la biomasse avec l'exposition aux pesticides pourrait être la production d'exo polysaccharides (EPS) par les composantes algale et bactérienne en conditions stressantes. L'induction d'EPS a déjà été observée lors de stress physiques (Artigas et al. 2012) ou chimiques (Fang et al. 2002). Elle jouerait un rôle protecteur vis-à-vis des contaminants en limitant leur diffusion à travers la structure tridimensionnelle du biofilm. L'induction d'EPS en réponse à l'exposition aux extraits de POCIS reste une hypothèse dans notre travail car aucun dosage de ces composés n'a été réalisé ; elle mériterait donc d'être vérifiée avec la détermination des concentrations en EPS dans les biofilms et de leur nature.

Evaluer la croissance algale par la fluorescence : une relation en lien avec le mode d'action des pesticides

Dans notre étude, les mesures de fluorescence en lumière modulée ont été utilisées pour étudier la croissance des différents groupes algaux ; cependant dans le cas d'exposition à des mélanges contenant des inhibiteurs du PSII ces résultats sont à prendre avec précaution puisque ces composés induisent une augmentation de la fluorescence de base, les effets entre la croissance et l'augmentation de la fluorescence de base peuvent donc être difficiles à distinguer (Corcoll et al. 2012a). Ainsi, cette augmentation de la fluorescence de base limiterait l'utilisation de ces descripteurs étendue à cet important groupe de pesticide.

Des réponses contrastées des paramètres physiologiques dans le cas des contaminations chroniques à faible dose

Les réponses des biofilms aux pesticides ont été évaluées sur les capacités photosynthétiques (rendement photosynthétique efficace) et sur les activités enzymatiques antioxydantes (AEAs).

Aucune diminution du rendement photosynthétique n'a été observée suite aux expositions à court terme et à faible dose, la capacité photosynthétique globale du biofilm ne semble donc pas être affectée lors d'expositions à un mélange de pesticides à faible dose aux toxiques. Certaines études ont mis en évidence des impacts sur les capacités photosynthétiques du biofilm face à des contaminations toxiques, cependant dans la plupart des cas les composés étudiés étaient des inhibiteurs de la photosynthèse ayant pour cible le photosystème II comme le diuron (Tlili et al. 2010), l'atrazine (Schmitt-Jansen et Altenburger 2007) ou l'isoproturon (Laviale et al. 2010) et ont été utilisés à des concentrations bien plus importantes (plusieurs dizaines de µg/L) comparées à celles appliquées lors des expériences menées ici. Les inhibiteurs de la synthèse des lipides ont été majoritairement retrouvés dans les extraits provenant du Trec (Chapitre VII); pour les extraits issus de la Morcille le diuron (inhibiteur du PSII) a été retrouvé en proportion importante cependant les concentrations dans les canaux ne dépassaient pas les 0,3µg/L (Chapitre IV). Le mode d'action (non spécifique du photosystème II) et/ou la concentration (faible dose) des composés présents dans les extraits pourraient donc expliquer le fait qu'aucune diminution du rendement photosynthétique n'ait été observée suite aux expositions aux extraits de POCIS. Ce résultat pourrait être expliqué par un processus d'adaptation dans lequel les espèces sensibles sont remplacées par des espèces plus tolérantes conduisant ainsi à une nouvelle communauté dont les fonctions

seraient similaires en termes de photosynthèse à celles de la communauté d'origine (Corcoll et al. 2012b , Serra et al. 2009).

Ces résultats contrastent avec les réponses des activités enzymatiques antioxydantes. En effet des différences entre les biofilms exposés aux pesticides et les biofilms témoins ont été observées sur l'ascorbate peroxydase (APX), la glutathion réductase (GR) et la glutathion-S-transférase (GST). Dans une récente étude, Bonet et al. (2013) ont observé des résultats similaires avec des niveaux d'AEAs affectés par une exposition au zinc alors qu'aucun lien n'avait pu être établi entre les concentrations en métaux et les capacités photosynthétiques du biofilm. Cependant l'interprétation des résultats concernant les AEAs reste complexe lorsque les études sont menées au niveau de la communauté. En effet il est difficile d'attribuer la variabilité de la réponse observée à la mise en place des mécanismes de défense au niveau des espèces ou à la composition de la communauté du biofilm et de leurs activités enzymatiques spécifiques (Bonet et al. 2013).
Certaines études soulignent d'ailleurs la complexité des approches physiologiques (comme l'évaluation des capacités photosynthétiques et des activités enzymatiques antioxydantes) pour faire ressortir les effets d'une contamination chronique sur les biofilms mais ont néanmoins pointé leur efficacité dans la mise en évidence des expositions aiguës (Sabater et al. 2007).

8.1.2 Comprendre la toxicité observée avec l'exposition des biofilms aux extraits de POCIS

Lors de l'utilisation des extraits d'échantillonneur passif dans les tests biologiques, c'est la toxicité globale de l'extrait qui est appréhendée par l'expérimentateur. Les impacts observés sur le biofilm sont ainsi évalués face à un mélange environnemental complexe contenant divers composés présents à des concentrations particulières. Si les effets de mélange sont intégrés en travaillant directement avec des extraits d'échantillonneur passif les questions de la compréhension de ces effets de mélange et de la toxicité relative des composés présents dans l'extrait ne sont en revanche pas abordées. C'est pourquoi il est nécessaire de simplifier les conditions d'exposition du point de vue de la contamination environnementale en travaillant avec des mélanges simples et des molécules seules pour comprendre les mécanismes de toxicité mis en jeu.

Toxicité relative des contaminants échantillonnés par le POCIS

Après avoir utilisé les extraits de POCIS comme vecteur de contamination, nous nous sommes intéressés à quelques contaminants échantillonnés par ce dernier et quantifiés à des concentrations importantes sur notre site d'étude principal (la rivière Morcille). Lors de ce travail l'accent a été mis sur la prise en compte des métabolites, composés qui peuvent être retrouvés et se former à des niveaux élevés dans le compartiment aquatique mais pas encore suffisamment pris en compte dans l'évaluation du risque à ce jour. La toxicité relative du norflurazon, de son métabolite principal (norflurazon desméthyl) et du tébuconazole a été étudiée en microplaques (Chapitre V). Les résultats ont mis en évidence une toxicité plus importante du métabolite par rapport au composé parent, soulignant l'importance des métabolites dans l'évaluation du risque environnemental. Cela a également été montré pour le mélange ternaire dont la toxicité a été attribuée au métabolite norflurazon desméthyl puisque l'exposition au norflurazon et au tébuconazole n'a pas révélé d'impact sur le rendement photosynthétique. L'utilisation du système microplaque a montré ses avantages notamment la diminution des quantités de contaminants qui peut être un élément crucial pour mener des tests de toxicité. Cependant cette approche a aussi révélé ses limites techniques quant à la récupération des échantillons destinés aux comptages et à la taxonomie (adhésion des cellules aux parois des puits).

La dernière étude (Chapitre VI) s'est intéressée à l'évolution temporelle des réponses des communautés périphytiques exposées à des concentrations croissantes en diuron, en DCPMU (produit de dégradation du diuron) et en norflurazon en utilisant les paramètres photosynthétiques classiques et d'autres encore peu étudiés dans le cadre de l'écotoxicologie : les Rapid Light Curves (RLCs). Les résultats ont révélé une toxicité plus importante du diuron par rapport au DCPMU et au norflurazon ainsi qu'une importante variabilité temporelle des réponses en lien avec le mode d'action des composés. Le diuron et le DCPMU ont par exemple montré des impacts rapides et stables dans le temps alors que les effets du norflurazon n'ont cessé d'augmenter au cours des 14 jours d'expérimentation. Ce dernier point a son importance pour l'étude de la toxicité des substances puisque la démarche actuelle de l'évaluation du risque environnemental se base le plus souvent sur des données de toxicité obtenues lors d'expositions aiguës. Il apparaît donc nécessaire de mieux considérer les expositions chroniques dans l'évaluation du risque environnemental pour une meilleure prise en compte des composés ayant un mode d'action indirect par rapport au paramètre de toxicité considéré dans l'étude.

Représentativité des extraits de POCIS

Plusieurs dizaines de molécules ont été identifiées dans les extraits POCIS sur lesquels nous avons travaillé durant cette thèse. Concernant les extraits provenant du Trec (Chapitre VII), les composés de la famille des chloroacétanilides et leurs produits de dégradation se sont avérés être majoritaires. Lors de l'étude d'un mélange environnemental, il est important de savoir si ce sont les composés majoritaires et recherchés *a priori* qui causent la toxicité du cocktail ou si les composés présents en faible proportion jouent aussi un rôle dans la toxicité globale de l'extrait.

Dans notre étude nous avons donc travaillé avec un mélange reconstitué selon les analyses des extraits de POCIS pour tester si la toxicité observée était expliquée par les composés majoritaires recherchés dans nos extraits (Chapitre VII). Les effets à long terme et à faible dose tant au niveau de la croissance, de la structure ou de l'impact fonctionnel se sont révélés similaires entre les EP et le mélange composé des substances majoritaires, ce qui nous a conduit à la conclusion d'une toxicité chronique expliquée principalement par les composés majoritaires recherchés dans les extraits de POCIS. Cette expérience réalisée avec les extraits de POCIS exposés sur le Trec au printemps 2012 est une étude de cas, les résultats obtenus ne peuvent donc pas être généralisés à toutes les contaminations puisqu'ils sont spécifiques du mélange de contaminants échantillonnés par le POCIS sur une période et un site particuliers. Cependant nous avons pu montrer que la fraction échantillonnée par le POCIS, ramenée à des concentrations environnementales réalistes, avait un sens et était pertinente en termes d'effets biologiques.

8.2 Perspectives

L'intérêt de l'utilisation des échantillonneurs passifs pour évaluer les effets de pesticides en mélange complexe sur le biofilm de rivière a été démontré puisque cette approche a permis d'identifier des paramètres rendant compte des effets des pesticides en mélange à faible dose sur les communautés périphytiques. Cette démarche atteint un fort niveau de représentativité écologique et chimique puisque le biofilm est composé d'organismes vivant en communautés et les extraits d'échantillonneurs passifs contiennent de nombreux composés en mélange dont des métabolites. Ce travail ouvre de nombreuses perspectives, les futures études pourront s'orienter selon trois axes qui viseraient à : augmenter la représentativité de l'exposition, mieux identifier et comprendre la toxicité et enfin continuer l'effort de développement de nouveaux descripteurs plus spécifiques des pollutions toxiques appliqués au biofilm.

Augmenter la représentativité

Lors de nos expériences nous avons exposé le périphyton à des concentrations constantes en toxiques. Cependant les concentrations en pesticides dans les rivières peuvent varier fortement en fonction des épisodes de crues ; en particulier sur la Morcille où de brèves mais importantes augmentations des concentrations en contaminants sont observées lors de ces crues (Rabiet et al. 2010). De manière à augmenter la représentativité des conditions d'exposition des communautés dans l'environnement il serait intéressant de simuler des pics de contamination en conditions contrôlées de laboratoire en utilisant par exemple quelques composés retrouvés en concentrations importantes dans le milieu aquatique ; d'autant plus que l'importance du couplage des expositions aiguës et chroniques de polluants sur les biofilms de rivière a été mis en évidence par Tlili et al. (2008) dans le cas de contamination au diuron. Il serait alors intéressant de comparer les réponses des biofilms face à une exposition constante ou à des pics de contamination. Ceci pourrait aussi permettre d'étudier la manière dont le POCIS est capable d'intégrer les pics de contamination.

Les POCIS sont des dispositifs échantillonnant les composés organiques polaires, de par leur nature ils ne sont capables échantillonner qu'une partie du panel de contaminants susceptibles d'atteindre le milieu aquatique. Les futures études devront donc adopter une approche ciblant les différents groupes de substances en utilisant des échantillonneurs passifs complémentaires. Cette démarche serait particulièrement pertinente dans le cas des milieux subissant des contaminations multiples comme la rivière Morcille où le gradient en pesticides est associé à un gradient métallique, de plus comme évoqué précédemment l'utilisation de ces extraits en conditions contrôlées de laboratoire permettrait de minimiser les facteurs confondants comme par exemple les concentrations en nutriments ou l'exposition à la lumière qui peuvent parfois masquer la réponse des biofilms aux toxiques (Guasch et Sabater 1998 , Lozano et Pratt 1994).

En outre, cet outil échantillonne la phase dissoute, or il est communément admis que la toxicité à laquelle sont exposés les organismes aquatiques est représentée par les composés présents dans cette phase dissoute. Néanmoins la question se pose dans le cas des biofilms de rivière puisque les particules ayant des contaminants adsorbés sont susceptibles de se retrouver piégées dans la matrice tridimensionnelle du biofilm et ainsi jouer un rôle significatif dans l'exposition aux toxiques de ce dernier (Morin et al. 2008). Ainsi de manière à caractériser la fraction toxique échantillonnée par le POCIS, différentes modalités

d'exposition du biofilm pourraient être réalisées avec des extraits d'échantillonneurs passifs, de l'eau brute ou de l'eau filtrée.

Identifier et comprendre la toxicité observée

Lorsque les biofilms sont exposés aux extraits de POCIS ces derniers peuvent être assimilés à une « boîte noire », puisqu'ils sont constitués d'un mélange complexe de composés (identifiés ou non) et que c'est la toxicité globale de l'extrait qui est alors évaluée. Pour aller plus loin dans l'étude de la contribution respective des composés majoritaires identifiés dans les extraits de POCIS, il serait envisageable de réaliser des conditions avec des sous mélanges (avec 3 ou 4 molécules) reconstitués d'après la caractérisation des extraits de POCIS, jusqu'aux molécules majoritaires seules de manière à identifier le ou les composés responsables de la toxicité chronique observée. Cette approche permettrait de comprendre plus finement les relations entre exposition et effets mais serait néanmoins extrêmement difficile à mettre en place de par l'importance des moyens expérimentaux nécessaires à sa mise en œuvre (multiplication des échantillons, des analyses biologiques et chimiques, volumes d'eau importants, grandes quantités de biofilm,…).

Une autre alternative résiderait dans la transposition des techniques d'EDA (Brack 2003) sur le biofilm de rivière dans son ensemble ou bien sur une espèce de diatomée particulière (réduction de la variabilité biologique) sous forme de tests de toxicité aiguë. La méthodologie développée dans cette thèse (Chapitre V) pourrait être appliquée ici en utilisant le système microplaque, ce qui permettrait de tester un grand nombre de contaminants et une large gamme de concentrations grâce aux faibles volumes nécessaires pour mettre en œuvre ces essais (réduction des quantités de contaminants et de microalgues utilisées). L'utilisation des microplaques serait d'autant plus pertinente que l'EDA nécessite la multiplication des échantillons, dépendant du nombre de fractions testées, de la gamme de concentrations appliquées et du nombre de réplicats utilisés. Cette approche pourrait permettre d'identifier les composés responsables de la toxicité observée qu'ils aient été identifiés ou non par l'analyse chimique.

Enfin les interactions au sein de l'extrait pourraient être caractérisées sous la forme de courbes dose-réponse réalisées par exemple en microplaque de manière à mettre en évidence les mécanismes d'additivité, de synergie ou d'antagonisme pouvant opérer à l'intérieur de l'extrait.

Développer de nouveaux descripteurs plus spécifiques des pollutions toxiques

Lors de ce travail de thèse, une large gamme de descripteurs susceptibles de répondre à des expositions aux pesticides a été étudiée. L'établissement des courbes évaluant le taux de transport des électrons en fonction d'une irradiance croissante (courbes RLCs) a jusque là très peu été étudié dans le contexte des pollutions toxiques. Cette approche a montré son intérêt puisqu'elle s'est avérée un descripteur précoce d'exposition aux pesticides. En effet des impacts sur la pente initiale de la courbe (α), la valeur d'intensité lumineuse à partir de laquelle la relation n'est plus linéaire (I_k) et le taux de transport relatif des électrons maximal ($rETR_{max}$) ont été observés. Ces paramètres apportent des informations complémentaires sur les capacités photosynthétiques du biofilm. Le α correspond par exemple à l'efficacité photosynthétique à faible irradiance, alors que le $rETR_{max}$ quant à lui renseigne sur les capacités photosynthétiques maximales.

Les futurs travaux devront donc continuer à explorer la réponse des biofilms à une exposition aux pesticides en établissant de telles courbes. En particulier leur sensibilité devra être testée par exemple en exposant les biofilms à des concentrations environnementales en canaux.

Les activités enzymatiques antioxydantes furent proposées comme marqueurs du stress oxydant induit par les toxiques pour le biofilm de rivière (Bonnineau et al. 2013), nous avons donc testé leur application lors d'expositions chroniques aux pesticides. Les résultats se sont avérés compliqués à interpréter de par la forte variabilité naturelle des AEAs et l'absence de valeur-palier rendant compte d'un déséquilibre entre la production et l'élimination des espèces réactives de l'oxygène. De manière à pouvoir plus facilement interpréter les réponses des AEAs à un niveau intégré (e.g. mélange complexe à faible dose) des expériences à une échelle réduite devront être entreprises avec des molécules modèles par exemple sous forme de bioessais.

D'autre part des descripteurs plus spécifiques des diatomées pourront aussi être développés comme par exemple les outils de biologie moléculaire. Cette approche a d'ores et déjà montré son potentiel lors de contamination métallique (Kim Tiam et al. 2012) et a fait l'objet d'un encadrement d'un stage de master 2 dans le cadre du projet ANR PoToMAC (Moisset et al., en préparation). Les résultats obtenus ont permis de mettre en évidence l'impact du diuron sur l'expression de plusieurs gènes impliqués dans la régulation du métabolisme mitochondrial et des photosystèmes et ce pour trois espèces de diatomées d'eau douce (*Eolimna minima*, *Nitzschia palea* et *Planothidium lanceolatum*). Pour de futurs travaux, le séquençage des

gènes codant pour les enzymes antioxydantes comme la catalase, l'ascorbate peroxydase, la glutathion réductase ou encore la glutathion-S-transférase permettrait de mettre en relation les réponses observées au niveau de l'expression génétique et de l'activité enzymatique.

Lors de ce travail de thèse, nous avons donc étudié les effets des pesticides sur le biofilm de rivière, notamment en évaluant le potentiel de l'outil POCIS pour améliorer la représentativité environnementale de l'exposition lors d'expérimentations en laboratoire. L'utilisation des extraits d'échantillonneur passif lors d'expériences en canaux artificiels nous a permis de nous placer dans des conditions d'exposition réalistes par rapport à la contamination environnementale, tant au niveau de la composition du mélange qu'à celui des concentrations auxquelles les organismes sont exposés dans le milieu naturel, tout en nous affranchissant des principaux facteurs confondants qui sont propres aux études *in situ* (variation des concentrations en nutriments, lumière, température…). Ces expériences ont ainsi permis d'identifier des descripteurs de l'exposition chronique à faible dose de pesticides en mélange. Les diatomées se sont avérées particulièrement sensibles à cette exposition puisque des impacts sur la densité, la composition spécifique et les biovolumes ont été observés. D'autre part des impacts sur la biomasse ont été mis en évidence suite aux expositions aux pesticides, l'augmentation des valeurs de PS et MSSC observée dans les conditions contaminées pourrait être liée à une production d'EPS accrue comme mécanisme de défense face aux pollutions toxiques. Lors de ces travaux nous avons eu l'occasion de voir que l'interprétation des mesures de fluorescence de base pouvait être complexe dans le cas où le mélange présente des inhibiteurs du photosystème II.

Quant aux approches physiologiques (rendement photosynthétique et activités enzymatiques antioxydantes), nous avons vu qu'elles pouvaient être mal adaptées et/ou difficiles à interpréter dans le cas des contaminations chroniques (adaptation des communautés pour le rendement photosynthétique et variabilité de la réponse au niveau des communautés pour les AEAs).

Lors de cette thèse, nous avons aussi pu mettre en évidence le potentiel de descripteurs encore peu utilisés dans le domaine de l'écotoxicologie avec l'étude des courbes évaluant le taux de transport des électrons en fonction d'une irradiance croissante (courbes RLCs). Ces descripteurs ont montré leur intérêt puisqu'ils se sont avérés plus précoces que les paramètres classiques de fluorescence (F_0, F_v/F_m et Φ_{PSII}).

En comparant les réponses du biofilm à une exposition aux extraits de POCIS originaires du Trec et à un mélange reconstitué basé sur la composition des produits majoritaires identifiés dans les extraits, nous avons pu montrer que la fraction échantillonnée par le POCIS, ramenée à des concentrations environnementales réalistes, avait un sens et était pertinente en termes d'effets biologiques.

Enfin travailler à une échelle de représentativité moins importante vis à vis l'exposition environnementale et/ou du temps d'exposition nous a permis d'évaluer la toxicité relative des composés majoritairement retrouvés sur le site d'étude principale (la Morcille). Nous avons ainsi pu mettre en évidence l'importance de la durée d'exposition considérée dans l'essai (en lien avec le mode d'action des substances) et de la prise en compte des métabolites dans l'évaluation du risque environnemental.

Le travail réalisé tout au long de cette thèse s'est donc inscrit à différents niveaux de réalisme en termes de contamination environnementale (représentativité du cocktail en termes de composés et de concentration) et de durée d'exposition (expositions aiguës ou chroniques). Ces expériences réalisées à différentes échelles de représentativité sont donc complémentaires pour comprendre les effets des pesticides sur le biofilm de rivière et ainsi pouvoir mieux prendre en compte les pollutions toxiques dans l'évaluation de la qualité des eaux.

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Annexes

Development of q-PCR approaches to assess water quality: Effects of cadmium on gene expression of the diatom *Eolimna minima*.

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Abstract

This study was undertaken to develop molecular tools to assess water quality using diatoms as the biological model. Molecular approaches were designed following the development of a rapid and easy RNA extraction method suited to diatoms and the sequencing of genes involved in mitochondrial and photosystem metabolism. Secondly the impact of cadmium was evaluated at the genetic level by q-PCR on 9 genes of interest after exposure of *Eolimna minima* diatom populations cultured in suspension under controlled laboratory conditions. Their growth kinetics and Cd bioaccumulation were followed.

Population growth rates revealed the high impact of Cd at 100 µg/L with total inhibition of growth. These results are linked to the high bioaccumulation values calculated after 14 days of exposure, 57.0 ± 6.3 µgCd/g dw and 734.1 ± 70 µgCd/g dw for exposures of 10 and 100 µgCd/L respectively.

Genetic responses revealed the impact of Cd on the mitochondrial metabolism and the chloroplast photosystem of *E. minima* exposed to 10 and 100 µgCd/L with induction of *cox1*, 12S, *d1* and *psaA* after 7 days of exposure for the concentration of 100 µgCd/L and of *nad5*, *d1* and *psaA* after 14 days of exposure for both conditions.

This is the first reported use of q-PCR for the assessment of toxic pollution on benthic river diatoms. The results obtained presage interesting perspectives, but the techniques developed need to be optimized before the design of new water quality diagnosis tools for use on natural biofilms.

Key words

Eolimna minima, Diatom, Cadmium, Metal, Quantitative real time PCR.

1.Introduction

Over the past decades, the ever increasing release of agricultural, industrial and domestic waste led to a significant contamination of the environment and particularly of the aquatic compartment.

In regard to the resulting degradation of freshwater quality, various diagnostic tools have been designed. In France, in collaboration with Water Agencies and Environment Ministry services, CEMAGREF has developed several diatom indices to estimate global water quality like the "Indice de Polluosensibilité Spécifique" (IPS, Cemagref 1982) or the Biological Diatom Index (BDI, Coste et al. 2009), both routinely used for monitoring applications in several European countries. Nevertheless, the indices currently used for general water quality assessment - including the IPS and BDI - were not designed to assess specific toxic alterations (metals, synthetic organic pollutants) and are not really suitable for a sensitive diagnosis of this type of pollution. Thus, heavy metals are of particular concern because of the different toxic effects they can produce (Sauvant et al. 1997, Bucio et al. 1995).

Recent works have studied the responses of diatom communities to metal pollution, for example Morin et al. (2008a) observed structural impact at the community level and morphological abnormalities in a metal-polluted stream. Other studies assessed effects of metal-induced oxidative stress on functional descriptors such as photosynthetic (Antal et al. 2004) or antioxidant (Branco et al. 2010) enzyme activities.

However, studies assessing genetic responses of diatoms to contamination exposure are still at the early stage principally because of the lack of available diatom nucleotide sequences in genomic databases and difficulties to access genetic material. Only *Thalassiosira pseudonana* and *Phaeodactylum tricornutum* - two marine diatom species - have as yet been entirely sequenced, and genomic information about freshwater species is still extremely scarce.

Extracting the genetic material of organisms is the first step to obtain sequences of interest. Diatoms require a particular approach for this because of their unique external structure. Diatoms are unicellular algae and their most obvious distinguishing characteristic is their siliceous cell wall called the frustule. Various approaches have been used to break the frustule, but these are often time consuming (Wawrik et al. 2002) and/or need specialized laboratory material such as a French press (Stabile et al. 1990, Davis and Palenik 2008, Hildebrand et al. 1998) or a MiniBeadBeater (Fawley and Fawley 2004). More rapid and straightforward extraction methods are needed, specifically for diatoms. The expression of many genes is involved in the response of organisms to toxicants and can be disturbed by them. Once inside the cell, metals can cause oxidative stress (Wang et al. 2004) affecting the

way in which the mitochondria (Stohs and Bagchi 1995) or photosynthesis function (Knauert and Knauer 2008). The mitochondrial superoxide dismutase (*sodMn*) gene was selected because of its involvement in antioxidant defences. The mitochondrial metabolism was investigated using the cytochrome C oxidase (*cox1*) subunit and the NADH dehydrogenase subunit 5 (*nad5*) genes. In addition, the quantity of mitochondria in the cells was estimated using mitochondrial 12S ribosomal RNA. D1 protein (*d1*) and PsaA protein (*psaA*) are components of PS2 and PS1 respectively and were selected to investigate photosystem metabolism. Cytochrome P450 1A1 (*cyp1A1*) was selected as a biomarker for exposure to polynuclear aromatic hydrocarbons. Two genes commonly used as references in numerous genetic studies were selected as for study in our work: β actin (*act*) and 18S ribosomal RNA. Consequently, the first objective of this work was to develop a diatom-specific extraction method and to shape molecular tools to select and sequence genes of interest to assess metal contamination. We used cultures of *Eolimna minima*, a metal-tolerant freshwater diatom commonly collected within periphytic biofilm samples in running water and especially in metal-contaminated areas (Morin et al, in press). Our attention was particularly focussed on genes involved in mitochondrial and photosystem metabolism and responses of *E. minima* to a gradient of cadmium contamination was then characterised using these selected molecular tools. Cultures of *E. minima* were exposed in the laboratory for 14 days to 10 µgCd/L or 100 µgCd/L. The moderate exposure corresponds to the concentrations reported in cadmium polluted rivers like the river Riou-Mort (South West France) (Feurtet-Mazel et al. 2003) and the highest is comparable to those reported in more highly polluted European rivers (Ivorra et al. 1999). Cell numeration, bioaccumulation and genetic responses were followed 1, 2, 7 and 14 days after contamination.

2.Materials and methods

2.1.Molecular methodological developments

2.1.1.RNA extraction method

In order to access the genetic material of diatoms, a novel RNA extraction method was developed. Designing an efficient method able to break the external silica wall of diatoms without degrading the genetic material was a prerequisite. For this purpose, cultures of the diatom *Eolimna minima* were grown in batches from axenic strains provided by the UTEX algal collection (Texas University, USA). The diatom cells were cultured in sterile Dauta

medium (Dauta 1982) with silica added to a final concentration of 10mg/L in 3L Erlenmeyer flasks. The cultures were maintained at between 17 and 18°C in a thermostatic room with a photon flux density of 160µmol.m^{-2}.s^{-1} and a 12:12 h light:dark cycle. Fresh cultures were inoculated every seven days to promote optimal growth of the diatoms until sufficient cells were obtained.

30 mL (2.10^6 cells/mL) were removed from the *E. minima* cultures and the cells harvested after centrifugation at 3 863g for 5 min at room temperature. The cell pellet was transferred to 2 mL Eppendorf tubes and resuspended in 1 mL of diethyl pyrocarbonate (DEPC) treated water. Then, the diatoms were centrifuged again at 2 419g for 5 min at room temperature and the supernatant was removed. 1 mL of Trizol (invitrogen) and 300 µL of glass beads (0.10 – 0.11mm-diameter, B. Braun Biotech International) were added to the cell pellet. Then the sample was vortexed 3 times for 30 seconds, and the supernatant transferred into a clean Eppendorf tube and placed in a water bath at 30°C for 5 min. After addition of 200 µL of Chloro RECTAPUR (VWR), the sample was vortexed for 10 s and placed in water bath at 30°C for 2 min. The tubes were centrifuged at 11 360g for 5 min at room temperature and the aqueous phase containing the genetic material was transferred into clean tubes. The end of the extraction was realized using Absolultely RNA Miniprep Kit (Stratagène) according to the manufacturer´s instructions:

550 µL of 75% ethanol were added to the aqueous phase, the sample was vortexed, transferred to affinity columns and centrifuged for 1 min at 11 360g at room temperature. Filtrates were removed, 600 µL of low-salt buffer were placed on the column which was centrifuged at 11 360g at room temperature for 2 min. 5 µL of DNAse 1 [1U] and 50 µL of activity buffer were added to the column and placed in a water bath at 37°C for 15 minutes. 600 µL of high-salt buffer were added to the column which was centrifuged for 1 min at 14 500 rpm. 300 µL of low-salt buffer were added and the column was centrifuged for 2 min at 14 500 rpm. The columns were transferred into clean tubes and 30 µL of elution buffer at 60°C were added and centrifuged for 1 min at 12 250g at room temperature. Total RNA was analysed on 1% (w/v) agarose gel with ethidium bromide by UV light on an illuminator.

2.1.2.Reverse transcription of RNA

The first strand of cDNA was synthesised from 14 µL of total RNA (3 to 5 µg) using the Stratascript first strand synthesis system (Agilent). After the addition of 1 µL of oligo(dT) [1µM], 1 µL of random primers [1µM], 0.8 µL of dNTPs [10mM] and 2 µL of 10x first-

strand buffer the reaction was incubated for 5 min at 65°C. Then 1 µL of Stratascript reverse transcriptase [1U/µL] and 0.5 µL of RNase inhibitor [0.5U] were added, the reaction was incubated for 1 h at 42°C in an Eppendorf Mastercycler. The cDNA mixture was conserved at -20°C until it was used in a real-time PCR reaction.

2.1.3.Cloning and molecular characterization of the target genes

Genetic research focussed on seven genes involved in responses to environmental contamination and/or in which expression can possibly be disturbed by environmental factors (*sodMn, nad5, d1, cox 1, psaA, cyp 1A1*, and 12S) and for two reference genes (18S and *act*). Primers were designed by performing ClustalW analysis from protein or nucleic sequences (12S and 18S) from marine diatoms and other phylogenetically related aquatic organisms available in NCBI databases. From these alignments, primer pairs about 20-25 bp long were designed in the most conserved regions for amplification of fragments between 280 and 900 bp.

PCR reactions were performed using these primers following the manufacturer's instructions. After the addition of 1 µL of dNTP [10 mM], 3 µL of $MgCl_2$ [25 mM], 0.2 µL of Taq [5U/µL], 10 µL of activity buffer 5X, 0.5 µL of each primer (upstream and forward primers) [100 µM], 34 µL of DEPC treated water and 1 µL of cDNA, 40 PCR cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min were carried out in an Eppendorf AG thermocycler.

PCR products were analyzed on 1% (w/v) agarose gel with ethidium bromide. The ethidium bromide stained bands were revealed by UV light on an illuminator.

After a purification step using the PCR purification kit (Qiagen) according to the manufacturer's instructions, cDNA was cloned with pGEM®-T, (PROMEGA).

Successful insertion of the fragments was checked by PCR using T7 and SP6 universal primers following the manufacturer's instructions (40 cycles at 94°C for 1 min, 50°C for 1 min and 72°C for 1 min.). All the partial gene sequences obtained were submitted to GenBank under the accession numbers reported in Table1.

2.1.4.q-RT-PCR primer design and real time q-PCR

For each gene, specific q-RT-PCR primer pairs (table 1) were determined using the LightCycler probe design software (version 1.0, Roche).

Real time PCR reactions were performed in a LightCycler (Roche) following the manufacturer's instructions (one cycle at 95°C for 1 min and 50 amplification cycles at 95°C for 5 s, 60°C for 5 s and 72°C for 20 s).

Each 20 µL reaction contained 1 µL of activity buffer (Syber Green I (Roche), *Taq* Polymerase, dNTP), 3.2 µL of $MgCl_2$ [25µM], 2 µL of the gene-specific primer pair at a final concentration of 300 nM for each primer, 12.8 µL of DEPC-treated water and 1 µL of cDNA. Specificity was determined for each reaction from the dissociation curve of the PCR product. This dissociation curve was obtained by following the SYBR Green fluorescence level during gradual heating of the PCR products from 60 to 95°C.

Relative quantification of each gene expression level was normalized according to β-actin gene expression. Relative mRNA expression was generated using the 2−ΔCT method (Livak and Schmittgen 2001).

Table 1. Accession numbers and specific primer pairs for the 9 *E. minima* genes used in our study.

Gene name	Accession number	Primer (5'-3')
sodMn	HM 449706	GGTAGTAGGCGTGCTCCC[a] CCAGGACAACCCGCTC[b]
cox1	HM 449704	CAGTAATTCTCACTGCCCAGC[a] CCGTGTACCCACCGTTG[b]
nad5	HM 449708	TCAACTTGGTTTGCATACATGGC[a] TTGAACTAATCCTGTTGTGGAAGC[b]
d1	HM 449711	ACCACCAAATACACCAGCAAC[a] GCGTCCTTGGATTTTCGTAGC[b]
psaA	HM 449705	CATAAAGCGGCACCCAAAC[a] CTTGGATATAACTGACTCATTAACTCAGG[b]
cyp1A1	HM 449709	ACGAAGGAGTCTCCCATCA[a] CTTGGCCCGAACGATCAG[b]
act	HM 449707	GGCTCCACAAAACCCCAAG[a] GGCGTACCCCTCGTAGAT[b]
12s	HM 449710	CGCGGTAATACGGAGGATGC[a] AGTGCCTTCGCCATCGG[b]
18s	HM 449712	CATTGTCAGAGGTGAAATTCTTGGA[a] CCCCGGAACCCAAAAGT[b]

Abbreviations: *sodMn*-mitochondrial superoxide dismutase; *cox1*-cytochrome C oxidase subunit I; *nad5*-NADH dehydrogenase subunit 5; *d1*-D1 protein; *psaA*-PsaA protein; *cyp1A1*-cytochrome P450 1A1; *act-β*actin, *12s*-mitochondrial ribosomal RNA 12S; *18s*-ribosomal RNA 18S. [a]Upstream primer. [b]Forward primer.

2.1.5.Determination of sensitivity limit

In order to optimize the RNA extraction method, the sensitivity limit was quantified.

Ten dilutions of *E. minima* cultures were prepared to obtain theoretical numbers of cells ranging from 1.8×10^7 cells to 1.8 cells (1.8×10^7; 9×10^6; 1.8×10^6; 9×10^5; 1.8×10^5; 1.8×10^4; 1.8×10^3; 1.8×10^2, 18 and 1.8 cells). Extraction and reverse transcription of RNA were

performed as described previously on these different samples. The resulting cDNA was used for q-PCR with *cox1* and *act* specific primer pairs as described above.

2.2.Experimental protocol to assess cadmium effects on *Eolimna minima*

2.2.1.Exposure conditions

250 mL of *E. minima* culture in suspension in modified Dauta medium were placed in 500-ml Erlenmeyers to reach a final concentration of 10^6 cells/mL. The organisms were directly exposed to three Cd concentrations (C_0=0, C_1=10±3.2 and C_2=96±34.2 µgCd/L, mean values ± standard deviations over 14 days, from $CdCl_2$ stock solution, prepared from a 1000 mg vial, Merck, Germany). The cultures were maintained at between 16 and 18°C along the 14 days of exposure with a photon flux density of 59.19 ± 7.75 $\mu mol.m^{-2}.s^{-1}$ and a 12:12 h light:dark cycle. The Erlenmeyer flasks were closed with sterile cotton wool and kept on an orbital shaker (60 rpm). Triplicate flasks were collected at each of the four different sampling periods (after 1, 2, 7 and 14 days of exposure to Cd) leading to 36 different experimental conditions. Thus 7.5 mL of culture were sampled after the exposure durations assigned for water Cd analyses, intracellular and total Cd concentration analyses and cell counting. Simultaneously, three 100 mL replicates were sampled for genetic analysis and stored at - 80 °C.

2.2.2.Cd analysis

Cadmium concentrations in water and in diatom cells were determined by atomic absorption spectrophotometry (AAS) using a graphite tube atomiser after filtration through a GH polypropylene membrane (0.45 µm pores, 25 mm diameter, PALL) for diatom cell Cd concentration and on a 0.45µm Teflon filter (from CAS) for Cd concentration in water. The analytical method was simultaneously validated for each sample series by analysing standard biological reference materials (TORT 2, Lobster hepatopancreas; DOLT 3 dogfish liver; NCR/CNRC, Ottawa, Canada). 3 mL of nitric acid (65% HNO_3) were added to reference the material before digestion in a pressurized medium (borosilicate glass tube) at 100°C for 3 h. 15 mL of ultra pure water were added and samples were stored at 4°C before analysis.

Samples used for intracellular cadmium determination within diatoms were treated first with EDTA (10 mM), a strong metal complexing ligand, to remove the metal ions adsorbed to the diatom cell walls (Behra et al. 2002). In this case, a drop of EDTA was added to each 2 mL sample and filtered after 10 min. Samples used for total cadmium determination in diatoms skipped this step. After 24h at 44°C, all samples were digested by nitric acid (3 mL of 65%

HNO_3) in a borosilicate glass tube at 100°C for 3 h. The samples were then analysed by atomic absorption spectrophotometry as described previously.

2.2.3.Diatom cell density

1.5 mL aliquots were immediately fixed in formalin (37% formaldehyde, Prolabo, France) for counting. Each sample was counted in triplicate using a Nageotte counting chamber (Marienfeld, Germany). After 10 min ultrasonication, 200 µL of sample were placed on the counting chamber. The total number of individuals and the number of dead cells were recorded in 4 fields of the gridded area (1.25 µL each, 0.5 mm depth) under light microscopy at 400x magnification (Leitz photomicroscope). Distinction between dead and live organisms was estimated by the observation of the turgescence and colour of the chloroplast.

2.2.4.Statistical analysis

Statistical analyses were performed with R 2.12.0 using the Linear Mixed Effect model (library *nlme*). Homogeneity of variances was verified by Leven test and Fisher's LSD was calculated.

To assess the effects cadmium on *E. minima* by the molecular tools described above, expression of the nine target genes sequenced in this study was examined. The expression levels of the nine genes involved in antioxidant defences, or mitochondrial or photosynthetic metabolism were investigated by quantitative real-time PCR as described previously using the q-PCR primers designed in this study.

3.Results and discussion

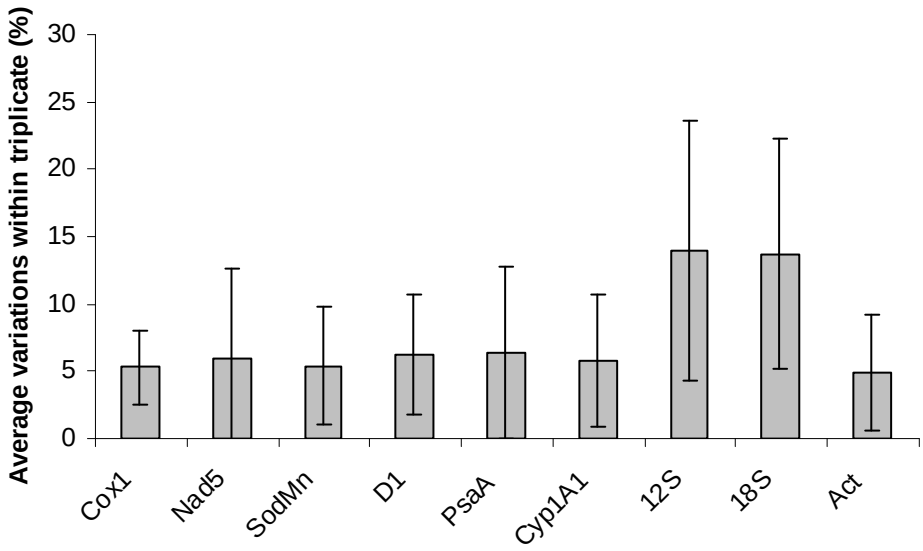
3.1.Methodological developments

3.1.1.Extraction method and optimization

Electrophoresis revealed the presence of large quantities of q-PCR amplification products, underlining the efficiency of this method for breaking open the silicate frustules while preserving the RNA. Moreover, the analysis of triplicates from the cadmium exposure experiment by q-PCR showed the regularity in the threshold cycle of most of the genes (apart from 18S and 12S see below). As shown in figure 1, seven of the nine genes studied showed a percentage of variation within triplicates of under 8%. This homogeneity reveals the high

efficiency of the extraction method and suggests a constant basal level in RNA production from these genes in *E. minima*.

Figure 1. Average variation of threshold cycle within triplicates in percentage over the cadmium exposure experiment for the nine genes studied.



In order to optimize the extraction protocol, the sensitivity limit was determined by performing q-PCR with *act* and *cox1* q-PCR primers. Analysis of dissociation curves of the different amplification products revealed the amplification of cDNA for an initial number of cells in the range 1.8×10^7 to 1.8×10^5. Below this number no amplification was detected through lack of sufficient genetic material. As the minimal number of cells needed to obtain q-PCR amplification was determined at 1.8×10^5 cells for the diatom *E. minima*, the minimum number of cells used in subsequent q-PCR analyses was fixed at 10^7 cells. The RNA extraction method developed in this study showed its high efficiency in the extraction of total RNA for the diatom *E. minima*. Moreover the method is simple and only requires standard laboratory equipment, which is an important advantage compared to existing methods that can require expensive and/or specialised apparatuses to extract RNA like a French pressure cell press (Hildebrand et al. 1998) or MiniBeadBeater (Fawley and Fawley 2004). The RNA extraction method developed is also rapid compared to existing methods using a muffle furnace overnight (Wawrik et al. 2002). Moreover, the method has been successfully tested on some quite different diatom species (*Achnanthidium minutissimum* and *Nitzschia palea*) suggesting it will be applicable to a large range of diatom species.

3.1.2.Nucleotide sequence accession number and q-PCR primers

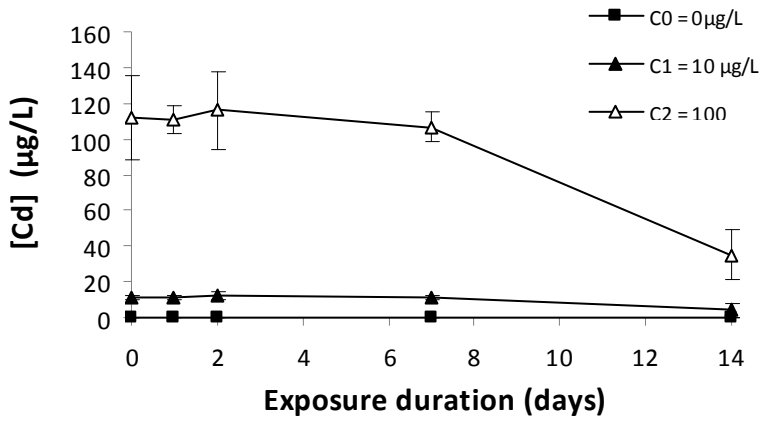
The 9 genes of interest selected in our study were successfully cloned and sequenced, accession numbers of sequenced genes and q-PCR primers are shown in table 1. cDNA sequences of the 9 genes have been deposited in the GenBank database.

3.2.Effects of cadmium on *Eolimna minima*

3.2.1.Cd water concentrations in solution

Cadmium concentration in water for the three different treatments is plotted against time in Figure 2. During the 14 days of exposure, mean Cd concentrations in water were 10±3.2 µg/L and 96±34.2 µg/L for nominal concentrations of 10 and 100 µgCd/L respectively. The cadmium concentrations in water were very stable from days 0 to 7 in both C_1 (11.5±0.6 µgCd/L) and C_2 (111.3±3.8 µgCd/L) treatments. A strong decrease occurred in cadmium concentrations on day 14 with values falling to 4.3±4 µgCd/L and 35.2±14.2 µgCd/L for treatments C_1 and C_2 respectively. This is unlikely to be explained by metal adsorption onto the surface of the experimental units, as no decrease was observed during the first seven days of exposure: bioaccumulation of cadmium by diatoms is more likely responsible for this decrease, as shown by the following results.

Figure 2. Cadmium concentration in water ± standard deviation versus exposure time for the 3 treatments (C_0=0, C_1=10 and C_2=100 µgCd/L). Full and empty triangles represent contaminated conditions. Full squares represent control treatments.



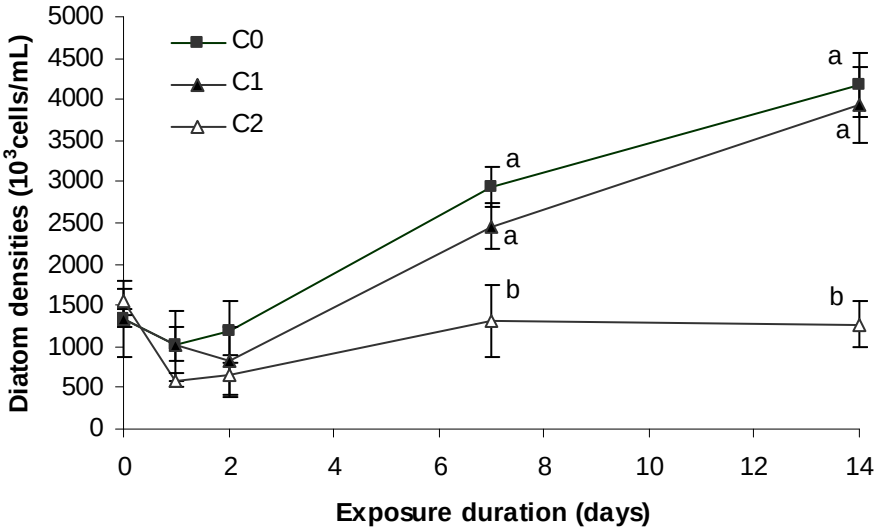
3.2.2.Effect of cadmium on the growth of E. minima

The number density, over the 14 days of direct exposure to the three different concentrations of cadmium, is plotted in Figure 3. Statistical analysis revealed that growth was not significantly different in controls and with a Cd concentration of 10.0±3.2 µg/L over the

whole duration of the experiment. Besides, Gold et al. (2003) and Duong et al. (2008) observed a high proportion of small, adnate species like *Achnanthidium minutissimum*, *Encyonema minutum* and *E. minima* in stations contaminated with around 6 µg/L cadmium. This suggests the tolerance of these species to high levels of cadmium and thus their ability to develop in cadmium-contaminated conditions with around 10 µgCd/L. Nevertheless, densities in contaminated samples were on average lower than in control treatments throughout the 14 days of exposure. It could be interesting to study population kinetics over a longer period to see if, after 14 days of exposure, significant differences are observed between the C_1 treatment and the control as observed by Morin et al. (2008a) who reported differences in biofilm diatom densities between control and 10 µgCd/L-contaminated treatments only after 6 weeks of exposure.

Significant differences in cell densities were observable from day 7 between the controls and the series with the highest contamination of cadmium (2 900 000 ± 250 000 cells/mL for control versus 1 300 000 ± 500 000 cells/mL). After 14 days of exposure, the diatom cell density was 3.2 times higher than with the control treatment (4 200 000 ± 390 000 cells/mL versus 1 300 000 ± 280 000 cells/mL). Moreover, diatom growth was null with treatment C_2. Our results are in accordance with those of Tourtelot (2003) who studied the acute effects of Cd on *Achnanthidium minutissimum.* For this species, referenced as pollution resistant, the rate of growth was null after 14 days of exposure to 100 µgCd/L.

Figure 3. Total density of *Eolimna minima* ± standard deviation versus exposure time for the 3 treatments (C_0=0, C_1=10 et C_2= 100 µgCd/L), significant differences between a and b (p value <0.05). Solid and open triangles represent contaminated conditions. Solid squares represent control treatments.



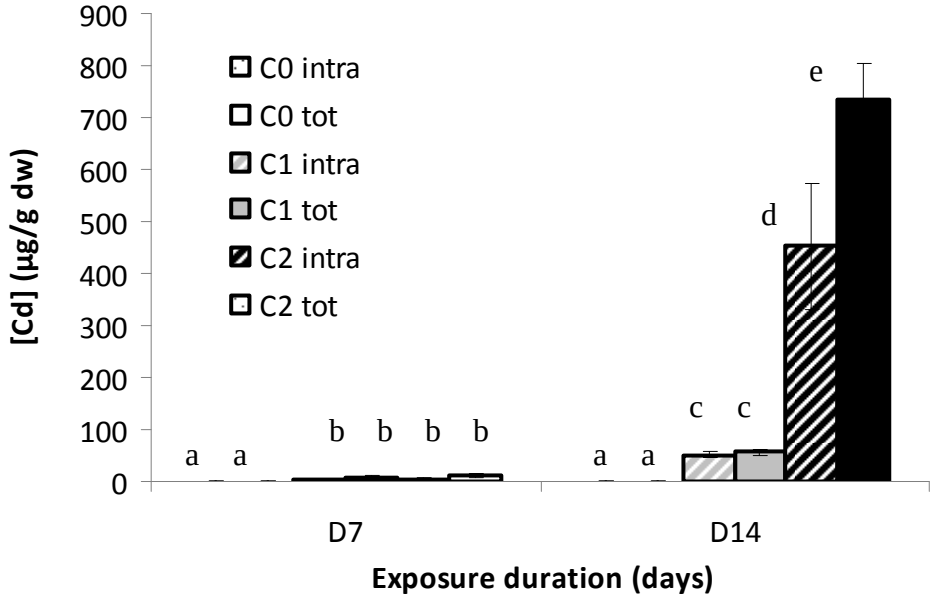
Similar results were observed at the community level, Morin et al. (2008b) exposed bioflms in microcosms to 10 and 100 µgCd/L for 6 weeks and observed a significant reduction in diatom density for biofilms exposed to the higher concentration compared to the control and the low cadmium contamination treatment.

3.2.3.Cd bioaccumulation in diatoms

Intracellular and total bioaccumulation are not given for days 1 and 2 after exposure because of the insufficient quantity of cells in the samples. Intracellular and total Cd concentration in diatoms after 7 and 14 days of exposure to the different treatments (0, 10±3.2 and 96±34.2 µgCd/L) are reported in Figure 4.

Intracellular and total bioaccumulation increased significantly between days 7 and 14 for the 2 contaminated treatments. For the lower contamination concentration (C_1), the intracellular and total concentration increased from 3.6±1.8 to 51.4±5.3 µgCd/g dw and from 6.4±3.4 to 57±6.3 µgCd/g dw respectively. For the higher contamination pressure (C_2), there was an increase from 5.6 ± 2.7 µgCd/g dw to 430.1 ± 86.4 µgCd/g dw and from 11.4 ± 3.0 to 734.1 ± 70 µgCd/g dw respectively, this latter value is in the range of literature values (Morin et al. 2008a, Duong et al. 2008). The increase in cadmium bioaccumulation with exposure time was also shown in environmental samples by Duong et al. (2008). The cadmium content in the biofilm increased gradually for days 14 and then remained constant. This could be due to the saturation of binding sites in the biofilm leading to a limitation of Cd accumulation. In our experiment it is difficult to estimate if the saturation phase is reached after 14 days of exposure. In order to determine the kinetics of saturation and the saturation value for *E. minima*, experiments will have to cover a longer period with a constant contamination pressure.

Figure 4. Intracellular and total Cd bioaccumulation in *E. minima* cultures for the two exposure times and three treatments (C_0=0, C_1=10 and C_2= 100µgCd/L) in µg Cd/g dw. Significant differences between a, b, c, d and e (p value <0.05). White bars represent control treatments, grey bars represent C_1 treatments and black bars represent C_2 treatments. Full and hatched bars represent total and intracellular cadmium bioaccumulation respectively.



After 7 days of exposure a significant difference occurred between the controls and the cadmium-contaminated treatments, but no such difference appeared between moderate and high contamination (C_1 and C_2) or between intracellular and total bioaccumulation.

After 14 days of exposure, total bioaccumulation in the controls was significantly different from that in the cadmium-contaminated treatments (p = 0.008 and p < 0.001 respectively). Total bioaccumulation was significantly higher than intracellular bioaccumulation for contamination C_2 (430.1 ± 86.4 µgCd/g dw versus 734.1 ± 70 µgCd/g dw).

The high values of cadmium bioaccumulated and the large increase between days 7 and 14 for the higher Cd concentration could so explain the total inhibition of growth observed for this treatment.

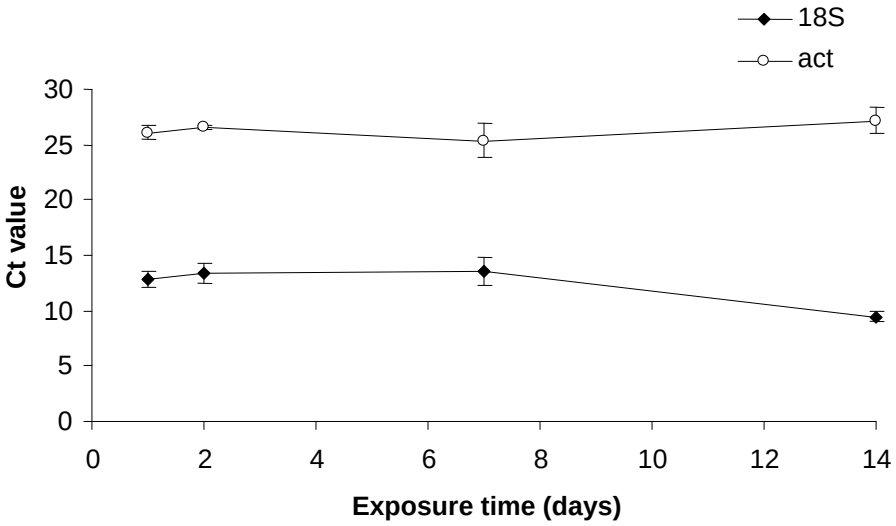
The ratios of adsorbed to absorbed cadmium are difficult to estimate owing to the heterogeneity of the results except for exposure to 100 µgCd/L after 14 days. In order to evaluate this difference better for shorter periods and lower concentrations, sampling volumes would have to be increased. In our study however, increasing sample volumes from 2 to 40 mL still led to very low dry weight measurements (between 2 and 6 mg for day 7 and 14) increasing the incertitude of measurement and errors in bioaccumulation calculations.

Therefore particular attention must be paid to the volumes tested when diatom cultures are considered.

3.2.4.Gene expression levels

In our study, two genes were selected as potential references, β-actin and 18S ribosomal RNA. Both are widely used as references in q-PCR analyses. The average percentage variation in the threshold cycle (Ct) of 18S ribosomal RNA within triplicates was 13.7% against 4.9% for β-actin (figure 1). The Ct value was defined as the number of cycles needed for the amplification signal to reach a specific detection threshold, and so is inversely correlated with the amount of cDNA template present in the PCR amplification reaction. The low stability and the high abundance of 18S rRNA underlined by our results (figure 5) shows the unsuitability of this gene as a reference. Consequently, 18S was finally not kept as a reference gene in our experiments. On the other hand, β-actin showed a high regularity and high threshold cycle values and was thus defined as reference gene in our study. 18S rRNA has also been described as a poor reference gene in other species such as buffalo (*Bubalus bubalis*) (Castigliego et al. 2010) or humans (Radonić et al. 2004). A recent study underlined this point; Siaut et al. (2007) reported that 18S rRNA was a very poor reference gene for *Phaeodactylum tricornutum*, a marine diatom. Here, we chose β-actin as reference gene owing to the stability of its expression, although Siaut et al. (2007) show that the β-actin level appears to be tightly correlated to light with an increase during the light period. In our study, however, the photoperiod was controlled and sampling performed at a fixed time, which avoided this source of error and allowed us to use β-actin as reference gene. Nevertheless it would be interesting to study other reference genes as proposed by Siaut et al. (2007) (*H4*, *RPS*, *Cdka* and *TBP*) for future analyses and particularly in field samples.

Figure 5. Expression of the two potential reference genes 18S and *act* in all treatments. mRNA levels of each reference gene is shown in absolute Ct values during the 14 days of the experiment. Closed and open circle represent 18S and *act* respectively.



Expression of genetic levels during the 14 days of exposure to cadmium at 10.0±3.2 and 96.0±34.2 µg/L are shown in table 2. During the 14 days, an impact resulting in the amplification of 5 of the genes studied was observed (*cox1*, *nad5*, *d1*, *psaA* and 12S). Different genetic responses are expressed as a function of time and concentration of exposure. Differential genetic expression revealed an effect on mitochondrial and photosynthetic metabolism, observed after 7 days of exposure only at the highest contamination pressure, and for both contamination pressures on day 14.

After 7 days of exposure, *cox1*, *d1*, *psaA* and 12S were up-regulated for the exposure concentration of 96.0±34.2 µgCd/L. After 14 days of exposure, there was a strong up-regulation of *nad5*, *d1* and *psaA* for both concentrations. Moreover, a large difference occurred between the 2 treatments: expression of *nad5*, *d1* and *psaA* were 4, 12 and 19 times higher respectively for C_2 than C_1. We can also see that responses of *d1* and *psaA* in the C_2 treatment increased with exposure time by a factor of 4.4 and 6.4 respectively between days 7 and 14.

Table 2. Differential gene expression as compared to actin from *E. minima* after 1, 2, 7 and 14 days of cadmium exposure to 10 and 100 µgCd/L by direct route.

Functions	**Genes**	**Cadmium contaminated experimental units**							
		C1 (10.0 ± 3.2 µg/l)				C2 (96.0 ± 34.2 µg/l)			
		1	2	7	14	1	2	7	14
Mitochondrial metabolism	*cox1*	/	/	/	/	/	/	9.5	/
	nad5	/	/	/	2.5	/	/	/	9.5
	12s	/	/	/	/	/	/	15	/
Oxidative stress	*sodMn*	/	/	/	/	/	/	/	/
Photosynthesis	*d1*	/	/	/	2	/	/	5.5	24
	psaA	/	/	/	2.5	/	/	7.5	48
Xenobiotic metabolisation	*cyp1A1*	/	/	/	/	/	/	/	/

[a]Significant induction and repression factors are indicated by positive and negative values, respectively compared to the control *E.minima*. / : identical to control levels.

The disturbance in the expression of the cytochrome C oxidase (*cox1*) subunit and the NADH dehydrogenase subunit 5 (*nad5*) observed in our study suggests an impact on mitochondrial metabolism. This is in accordance with data in the literature. Indeed, after entering the cell, cadmium can inhibit electron transfer in mitochondrial respiration, and also lead to the formation of reactive oxygen species (ROS) (Wang et al. 2004, Gonzalez et al. 2006). The oxidative stress generated then brings about DNA breaks and induction of apoptotic mechanisms leading to cell death (Bagchi et al. 2000, Chan and Cheng 2003). Nevertheless, the ratio *cox1*/12S was constant throughout the experiment indicating that the number of mitochondria increases to maintain the level of ATP production. This increase of *cox1* expression has already been reported in zebrafish *Danio rerio* (Gonzalez et al. 2006). Electron microscopy studies also in the zebrafish have shown extensive disturbance of the ultrastructure of the mitochondria after metal exposure to methyl mercury (Cambier et al. 2009). In future work it will be of particular interest to use electron microscopy to view *E. minima* after Cd exposure so as to be able to evaluate damage at the mitochondrial level.

Secondly, with regard to the up-regulation of *psaA* and *d1* after 7 days of exposure to 100 µgCd/L and after 14 days for the two contamination levels, it is clearly shown that the photosynthetic system of *E. minima* is strongly affected by exposure to cadmium. The impact of metals on the photosystem is well known and has been studied in numerous works. Photosystem inhibition has been reported by Sudo et al. (2008) at physiological and genetic

levels with, in particular, repression of *d1* and *psaA* in rice leaves after copper stress. Hörcsik et al. (2007) studied the effect of chromium on PS2 activities in *Chlorella pyrenoidosa* cells: they showed the inhibition of PS2 by photodestruction of reaction centres in cells cultivated in the presence of chromium. At the ultrastructural level, Ouzounidou et al. (1997) showed damage to chloroplast structures with changes in the shape and thickness of the thylakoid membranes in wheat (*Triticum aestivum* L.) after exposure to 265 µgCd/L for 7 days.

Moreover, the impact at the genetic level underlined by the up-regulation of *nad5*, *d1* and *psaA* after 14 days of exposure revealed effects at a Cd concentration of 10 µg/L. This result is of particular interest because, at a more integrative level of population kinetics, this effect was not noted. Overall, the above findings stress the importance of considering molecular markers when studying pollution because of the potentially higher sensitivity and the earlier response of genomic markers to toxic pollution compared to more global endpoints like mortality or population kinetics.

No significant difference in genetic expression levels was observed for the genes *cyp1A1* or *sodMn* during the experiment, at either of the concentrations tested. Mitochondrial superoxide dismutase is known to be involved in the oxidative stress response and several studies have shown the up-regulation of this gene after metal contamination. For example Gonzalez et al. (2006, 2005) demonstrated the up-regulation of *sodMn* in the gills of zebrafish after 7 days of direct exposure to 86 ± 0.26 µgCd/L and in another study in skeletal muscle and liver after 21 and 63 days of dietary exposure to methyl mercury for concentrations of 5 to 13.5 µgHg/g dw. In our work, this up-regulation was not observed, several hypotheses can be proposed: the sampling rate may be unsuitable and/or other enzymes of the oxidative stress response may be involved such as catalase, glutathione peroxidise or other types of superoxide dismutases (sodFe, sodCu/Zn or sodNi). Other authors have studied antioxidant enzyme activities, for example Branco et al. (2010) observed an increase in catalase activity compared to controls after 5 days of exposure to Cd from 100 to 300 µgCd/L on the diatom *Nitzschia palea* and in the global superoxide dismutase activity for concentrations from 200 to 300 µgCd/L. In a field study, Bonet et al. (2010) showed a clear inhibition of glutathione-S-transferase at high metal exposure and no difference in superoxide dismutase activity between sites impacted by metals at different levels.

For future analyses it will be interesting to sequence genes coding for catalase, gluthatione peroxidise, glutathione-S-transferase and the other superoxide dismutases (sodFe, sodCu/Zn or sodNi) in order to study their responses to metal exposure together with enzymatic activities. Cytochrome P450 1A1 plays a role in the metabolism of organic compounds such

as PAH or pesticides, which explains the null genetic response observed in our study after cadmium contamination. This gene has been studied and q-PCR primers have been developed for use in future analyses for diatom exposure to compounds like PAH or PCB.

4.Conclusions and perspectives

In this study, a new glass-bead RNA extraction technique for diatoms was successfully developed and optimized. Nine genes of interest were sequenced for *Eolimna minima* allowing the application of q-PCR tools to this species.

Our results underlined the toxicity of Cd towards *E. minima* population kinetics only for the highest concentration, while q-PCR analyses revealed an impact on mitochondrial metabolism and the chloroplast photosystem for both Cd exposure concentrations. Genetic expression of *nad5*, *cox1*, 12S, *d1* and *psaA* by q-PCR could thus constitute an early warning biomarker of metal pollution. Future studies should investigate sequences of genes coding for catalase or glutathion peroxidase in order to study the response to oxidative stress.

The present study is the first reported use of q-PCR on river benthic diatoms and the results obtained are extremely promising. The techniques developed were successfully tested using simplified mixtures of diatom species. Further interesting steps for the early and sensitive assessment of metal pollution would firstly involve validating the results obtained by examining sensitive *vs* tolerant diatom species response levels, then finding or confirming genetic biomarkers for use on natural multispecific biofilms for impact assessment of toxic pollution.

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Contrasted genetic responses of three freshwater diatoms under realistic exposures of diuron

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En préparation

Abstract

This study is based on three major freshwater species of diatoms encountered in the river Morcille, which are exposed to diuron during phytosanitary treatments of vineyard in this region: *Eolimna minima, Nitzschia palea* and *Planothidium lanceolatum.*

In order to assess the impact of pesticides, diatoms were exposed to three concentrations of diuron: 0; 1 and 10µg/L (respectively C_0, C_1 and C_2) during fourteen days in laboratory. Diuron water concentrations in solution, cell numeration, photosynthetic activity and genetic responses were followed 6h, 2, 7 and 14 days after contamination.

Results revealed the toxicity of diuron through the measurements of the optimal quantum yield of the photosystem II (PSII) (decrease of 40 to 50%) and the growth kinetics (complete inhibition of growth for *Planothidium lanceolatum* at 10µg/L).

Genetic responses evidenced the impact of diuron on the mitochondrial metabolism and the chloroplast photosystem II (main target of diuron) on the three species exposed to 1µg/L and 10µg/L of diuron. Thus, the analysis of the gene expression of *psaA, d1, cox1, nad5* and 12s constitute an early biomarker of alert to detect pesticides pollution.

This study revealed different levels of tolerance/sensibility facing diuron contamination for the three species: *Eolimna minima* and *Nitzschia palea* appeared to be more tolerant than *Planothidium lanceolatum*.

The results obtained show that the development of molecular tools and more precisely the biomarkers is an asset to assess organisms' contamination and water quality.

Key words

Diatoms, *Eolimna minima, Nitzschia palea, Planothidium lanceolatum*, Pesticides, Diuron, Pulse-amplitude modulated fluorescence, Quantitative real time PCR.

1.Introduction

The EU Water Framework Directive (2000/60/EC) requires European countries to assess the ecological status of water bodies. This policy of preservation and restoration of aquatic ecosystems aims to achieve good ecological status of water bodies by 2015. Thus, tools and evaluation methods of contamination, also as the characterization of the ecological impacts associated with aquatic system must be improved.

The ever increasing agricultural activities led to the contamination of surface and ground waters, creating strong, long-lasting degradations of aquatic ecosystems. Thus, streams running through these polluted areas are very sensible to contamination by phytosanitary compounds, like diuron, which is frequently found in rivers in France and all through Europe (Loos et al., 2009; SOeS, 2013). Furthermore, it often exceeds the maximum acceptable concentration of 1.8 µg/L fixed by the WFD, even if diuron is prohibited in most European countries, including France, since 2009.

In streams and rivers, the majority of microbial activity is due to periphytic communities which are key players in the general functioning of aquatic ecosystems and more precisely diatoms, which during certain time of the year represent the majority of periphyton (McIntire, 1996). Because of their position at the basis of trophic food webs, they play an important role in major ecological processes such as photosynthesis and nutrient recycling. Furthermore, their capacity of adaptation and tolerance towards environmental factors (light, temperature …) and aquatic pollution, made them relevant bioindicators (Potapova and Charles, 2002). Indeed, facing environmental pressure, communities can adapt irreversibly or temporarily, leading to structure, diversity and function changes.

Over the past decades, the development of the “omics” allowed to highlight, via molecular biology approaches, new genetic descriptors called biomarkers. Facing environmental pollutants they are assets to determine the cellular impacts associated but they can also help to define better the levels of contamination likely to have toxic effects on communities. The use of these new genetic descriptors is currently lacking for the freshwater microalgal compartment. Thus, only few nucleotide sequences of diatoms are available in genomic databases and only two marine diatom species have yet been entirely sequenced (Armbrust et al., 2004; Bowler et al., 2008). Pesticides penetration in cells can induce oxidative stress that might be responsible of DNA breakage or might affect mitochondrial and photosynthetic metabolisms and induce apoptotic mechanisms leading to cell death (Fukuyama et al., 2009; Rutherford and Krieger-Liszkay, 2001; Slaninova et al., 2009). The use of molecular descriptors reflecting these damages could help reveal early and specific cellular effects. Kim

Tiam et al. (2012) successfully used these descriptors in cadmium experimental contamination and highlighted their sensitivity and early response compared to more integrative levels (growth rates). Furthermore, they can complete index methods based on identification of the structure of diatoms communities, still insufficiently sensible to toxics.

This study was undertaken on three freshwater diatoms, commonly encountered on the Morcille River (Beaujolais) which are exposed to pesticides during phytosanitary treatments of vineyards in this region, in particular to diuron (Morin et al., 2010). This herbicide, belonging to the substituted urea family, targets the photosynthesis processes by preventing the formation of oxygen. Diuron binds with high affinity at the Q_B-binding site of the photosystem II, preventing Q_B from binding at this location and blocking the electron transport chain (Zer and Ohad, 1995, Trebst and Draber, 1986). Although its persistence in soil is low (< 1 year), it may last in water more than three years. This substance is mainly detected in the aquatic compartment due to soil leaching and overland flow (Brignon, 2007).

Consequently, an objective of this work was to characterize and study genes involved in mitochondrial and photosynthetic metabolisms. The responses of *Eolimna minima* (Grunow) Lange-Bertalot, *Nitzschia palea* (Kützing) W.Smith, and *Planothidium lanceolatum* (Brébisson ex Kützing) Lange-Bertalot to diuron were assessed in the laboratory during 14 days exposure to 0, 1 or 10 µg/L of diuron. The moderate exposure corresponds to concentrations frequently reported in rivers like the Morcille River and the highest is comparable to those reported during phytosanitary treatment episodes (Rabiet et al., 2010).

Diuron concentrations, pulse-amplitude modulated fluorescence measurements, cell numeration and genetic responses were followed 6 hours, 2, 7 and 14 days after contamination. Specific genetic developments were performed on target genes for the diatom species; these new genetic descriptors were expected to respond earlier, and more efficiently, than current diagnostic tools used for water quality assessment.

2.Materials and methods

2.1.Experimental protocol to assess diuron effects on *Eolimna minima*, *Nitzschia palea* and *Planothidium lanceolatum*

2.1.1. Algal cultures

Three freshwater species were chosen for the study of the impact of diuron: *Eolimna minima, Nitzschia palea* and *Planothidium lanceolatum.* These diatoms are found in abundance at the site of study, the Morcille River (Beaujolais), and are easy to cultivate in laboratory.

Eolimna minima was collected and isolated from periphytic biofilm sampled in the Morcille River. *Nitzschia palea* and *Planothidium lanceolatum*, isolated from freshwater lotic environments, were provided by the Thonon Culture Collection (http://www6.inra.fr/carrtel-collection/, strains reference numbers, respectively TCC583 and TCC615).

2.1.2. Exposure conditions

250 mL of each culture of diatoms, in exponential growth phase, was suspended in 500-mL Erlenmeyer flasks, in modified Dauta medium (supplemented with silica to a final concentration of 10 mg/L) (Dauta, 1982), to reach an initial concentration of 12.10^4 cells/mL. The organisms were directly exposed to three nominal concentrations of diuron (C_0=0, C_1=1 and C_2=10 µg/L) over 14 days, from diuron stock solution (1mg/L, dilution in ultrapure water).

The cultures were maintained between 20 and 22°C along the 14 days of exposure with a photon flux density of 58±8 $\mu mol.m^{-2}.s^{-1}$ and a 12:12 h light:dark cycle. The Erlenmeyer flasks were closed with sterile cotton wool and kept on an orbital shaker (60 rpm). Triplicate flasks were set up leading to a total number of 27 experimental units.

2.1.3. Sampling and kinetic times

Diuron impact on diatoms was assessed after 6 hours, 2, 7 and 14 days of exposure (thereafter called 6h, D2, D7 and D14). At each sampling date, 6 mL of culture suspension were sampled for water diuron analyses, 2 mL for cell counting, 5 mL for pulse-amplitude modulated fluorescence measurements and 35 mL for genetic analysis.

2.2.Laboratory analysis

2.2.1. Diuron analysis

6 mL of culture suspension was filtered with regenerated cellulose membranes (0.45 µm) (Whatman, Versailles, France), and samples were stored at 4°C. 6 mL filtered samples from C_1 were preconcentrated to 1 mL with a SpeedVac SP*D1*21P (Thermo), whereas a dilution of the filtered samples from C_2 was performed. Whatever the sample, an aliquot of 1 mL was spiked with 10 µL of a diuron-d6 (Dr. Ehrenstorfer GmbH, Augsburg, Germany) solution (1 ng/µL) as internal standard, prior to the analysis with a liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS). The chromatographic separation was done on a Gemini-NX C18 3µm, 110A, 100mmx2mm equipped with a SecurityGuard from

Phenomenex (Le Pecq, France) and an HPLC Ultimate 3000 (Dionex, Voisins-le-Bretonneux, France), the quantification was carried out with a triple quadripole mass spectrometer API 2000 (AB Sciex, Les Ulis, France). The two eluents were acetonitrile (A) and ultrapure water with 5 mM ammonium acetate, and a linear gradient was used: 10 % of A for 1 min, then 30 % of A after 4 min, 40 % of A after 8 min, 80 % of A after 9.5 min, followed by 80 % of A until 10.5 min, a decrease of A to 10 % after 11 min. Lastly, the initial composition (10 % of A) was maintained for 4 min. The total running time is 15 min and the flow rate was kept constant at 400 µL/min. Selected reaction monitoring (SRM) mode was used for quantification of both diuron (233>72 and 233>46 SRM transitions) and Diuron-d6 (239>78). Sample injection volume was 50 µL. Further details regarding sample preparation and analysis can be found elsewhere (Lissalde et al., 2011). Instrumental quantification and detection limits were respectively 1 µg/L and 0.3 µg/L.

For the samples with a concentration between the quantification and detection limits, a one-half value of the quantification limit was used in the statistical analyses (Helsel, 1990).

2.2.2. Diatom cell density

The 2 mL aliquots were immediately fixed in formalin (37% formaldehyde, Prolabo, France) for counting. Each sample was counted using a Nageotte counting chamber (Marienfeld, Germany). After homogenisation using a vortex, 200 µL of sample were placed on the counting chamber. The total number of individuals and the number of dead cells were recorded in 10 fields of the gridded area (1.25 µL each, 0.5 mm depth) under light microscopy at 400x magnification (Olympus BX51 photomicroscope). Distinction between dead and live organisms was estimated by the observation of the turgescence and colour of the chloroplast as described in Morin et al. (2010).

2.2.3. Photosynthetic efficiency

The optimal quantum yield of photosystem II (F_v/F_m) was measured using Pulse Amplified Modulated fluorometry (PhytoPAM, Heinz Walz GmbH, Germany). F_v/F_m was measured after 30 min of dark adaptation and is described by the Genty et al. (1989) equation:

$$F_v/F_m = (F_m-F_0) / F_m$$

with F_0 the minimum fluorescence determined after a weak far red modulated light and F_m the maximum level of fluorescence measured during a saturating white light pulse.

2.2.4.Genetic analysis

2.2.4.1. RNA extraction method

In order to access the genetic material of diatoms, RNA extraction was performed. The Falcon tubes containing the samples were put in a water bath at 25°C to defrost then centrifuged at 1850 g for 7 min at room temperature. The supernatant was removed and the cell pellet of diatoms resuspended in 1 mL of Trizol (Invitrogen) and transferred to 1.5 mL microtubes. 300 µL of glass beads (2.5 µm diameter, Sartorius AG) were added to the cell pellet. In order to crush diatoms, the MPFastPrep machine was used (40s at 6m/s). The supernatant was transferred into a clean microtube and placed in a water bath at 37°C for 5 min. After addition of 200 µL of Chloroform RECTAPUR (VWR), the sample was vortexed for 10 s and placed in water bath at 37°C for 2 min. The tubes were centrifuged at 8000 g for 5 min at room temperature and the aqueous phase containing the genetic material was transferred into clean tubes. The end of the extraction was realized using Absolutely RNA Miniprep Kit (Stratagene) according to the manufacturer´s instructions:

550 µL of 75% ethanol were added to the aqueous phase, the sample was mixed, transferred to affinity columns and centrifuged for 1 min at 8000 g at room temperature. Filtrates were removed, 600 µL of low-salt buffer were placed on the column which was centrifuged at 8000 g at room temperature for 2 min. 5 µL of DNAse 1 [1U] and 50 µL of activity buffer were added to the column and placed in a water bath at 37°C for 15 minutes. 600 µL of high-salt buffer were added to the column which was centrifuged for 1 min at 8000 g. 300 µL of low-salt buffer were added and the column was centrifuged for 2 min at 8000g . The columns were transferred into clean tubes and 30 µL of elution buffer at 60°C were added and centrifuged for 1 min at 8000 g at room temperature. Total RNA was analysed on 1% (w/v) agarose gel with ethidium bromide by UV light on an illuminator.

2.2.4.2. Reverse transcription of RNA

The first strand of cDNA was synthesized from 14 µL of total RNA (3 to 5 µg) using the Stratascript first strand synthesis system (Agilent). After the addition of 1 µL of oligo(dT) [1µM], 1 µL of random primers [1µM], 0.8 µL of dNTPs [10mM] and 2 µL of 10x first-strand buffer the reaction was incubated for 5 min at 65°C. Then 1 µL of Stratascript reverse transcriptase [1U/µL] and 0.5 µL of RNase inhibitor [0.5U] were added, the reaction was incubated for 1 h at 42°C in an Eppendorf Mastercycler. The cDNA mixture was conserved at -20°C until it was used in a real-time PCR reaction

2.2.4.3. Cloning and molecular characterization of the target genes

Molecular characterization of the target genes was realized prior to diuron exposure experiment from *Eolimna minima, Nitzschia palea* and *Planothidium lanceolatum* cultures.

Six genes involved in responses to environmental contamination and/or which expression can possibly be disturbed by environmental factors Were characterized. Three genes are involved in the mitochondrial metabolism (*nad5*, 12S, *cox1),* two other genes encoded for major proteins of the photosystems I and II (*psaA* and *d1*, respectively) and one reference gene (β *act*). Primers were designed by performing ClustalW aligments from corresponding sequences belonging to other phylogenetically related aquatic organisms available in databases. From these alignments, primer pairs were designed in the most conserved regions for amplification of fragments between 280 and 900 bp.

PCR reactions were performed, following the manufacturer's instructions. After the addition of 1 µL of dNTP [10 mM], 3 µL of $MgCl_2$ [25 mM], 0.2 µL of Taq [5U/µL], 10 µL of activity buffer 5X, 0.5 µL of each primer (upstream and forward primers) [100 µM], 34 µL of DEPC treated water and 1 µL of cDNA, 40 PCR cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min were carried out in an Eppendorf AG thermocycler.

PCR products were analyzed on 1% (w/v) agarose gel with ethidium bromide.

After a purification step using the PCR purification kit (Qiagen) according to the manufacturer's instructions, cDNA was cloned with pGEM®-T, (PROMEGA).

Successful insertion of the fragments was checked by PCR using T7 and SP6 universal primers following the manufacturer's instructions (40 cycles at 94°C for 1 min, 50°C for 1 min and 72°C for 1 min.

2.2.4.4. q-RT-PCR primer design and real time q-PCR

From the molecular characterization of the target genes, compatible q-RT-PCR primer pairs (Table 1), for the three species, were determined by performing alignments via the ClustalW.

Real time PCR reactions were performed in a LightCycler (Roche) following the manufacturer's instructions (one cycle at 95°C for 1 min and 50 amplification cycles at 95°C for 5 s, 60°C for 5 s and 72°C for 20 s).

Each 20 µL reaction contained 1 µL of activity buffer (Syber Green I (Roche), *Taq* Polymerase, dNTP), 3.2 µL of $MgCl_2$ [25mM], 2 µL of the gene-specific primer pair at a final concentration of 300 nM for each primer, 12.8 µL of DEPC-treated water and 1 µL of cDNA. For negative controls, cDNA was replaced by DEPC-treated water.

Specificity was determined for each reaction from the dissociation curve of the PCR product. This dissociation curve was obtained by following the SYBR Green fluorescence level during a gradual heating of the PCR products from 60 to 95°C.

Relative quantification of each gene expression level was normalized according to β-actin gene expression. Relative mRNA expression was generated using the $2^{-\Delta CT}$ method (Livak and Schmittgen, 2001).

2.3.Statistical analysis

All statistical treatments were performed with the R software (Ihaka and Gentleman, 1996). Prior to analyses, the data were checked for normality and variance equality.

The differences in diuron concentration in suspensions (n=3), as well as the effects of diuron exposure on the optimal quantum yield of photosystem II (n=3), the cell numeration (n=3) and the genetic response (n=3) were tested by the non parametric Kruskall-Wallis test using the (kruskal.test function).

3. Results and discussion

3.1.Effects of diuron on *Eolimna minima*, *Nitzschia palea* and *Planothidium lanceolatum during laboratory contamination*

3.1.1. Diuron water concentrations in solution

Diuron concentration in water was determined for the three species. During the 14 days of exposure, diuron concentration in water was stable during the fourteen days of exposure and close to nominal concentrations. Indeed, mean (± standard error) diuron concentrations over the experiment were 0 µg/L, 1±0.2 µg/L and 10.9±1.3 µg/L for nominal concentrations of 0, 1 and 10 µg/L respectively.

Hartgers et al. (1998) and Jones et al. (2003) argued that minimal concentrations comprised between 1 and 4.5 µg diuron/L are necessary to create impacts. No more than slight effects were thus expected in C_1, at the low limit of this interval. Moreover, in some cases diuron concentration in C_1 was below the nominal concentration (e.g. for *Planothidium lanceolatum* and for *Nitzschia palea* on D7). This phenomenon could be attributed to absorption and/or adsorption by the diatoms, adsorption to the flask walls (even if glass Erlenmeyer were used), (Pesce et al., 2006), or higher bioaccumulation of the pesticide either due to differences in specific uptake capacity (*P. lanceolatum*, compared to the two others diatoms), or linked to the growth kinetics (peak of *N. palea* on D7).

3.1.2. Pulse-amplitude modulated fluorescence measurements

The measurements of the optimal quantum yield of the PSII, over the 14 days of direct exposure to the three different concentrations of diuron, are plotted in Figure 1. The measurements are represented under percentage compared to control conditions, thus, for each time at C_0, the optimal quantum yield measurements equal 100%.

The study of chlorophyll fluorescence through the use of the Phyto-PAM, to evaluate the physiological state of algae, is a method based on the quantitative relationship between chlorophyll fluorescence and the efficiency of photosynthetic energy conversion (H.Walz GMBH, Effeltrich, Allemagne). Under stress conditions, reactive centres are saturated faster and the incoming light is used less efficiently (Laviale, 2008). Thus, variations in the optimal quantum yield reflect the physiological state and potential damages of the organisms. During optimal conditions, the optimal quantum yield of diatoms varies from 0.60 to 0.65 (Laviale, personnal communication).

Mean optimal quantum yield measurements for C_1 for the three species and over all kinetics times were of 99.1±4.6 % compared to the C_0, highlighting that diuron did not have important impact the functioning of the PSII at a concentration of 1±0.2 µg/L of diuron. However, for *Eolimna minima* at C_1, for D2 and D7, the mean optimal quantum yield was significantly lower compared to the C_0 (in mean 92.2±1.1 % of the C_0), this result reflect a possible impact of diuron on photosynthetic capacities at environmentally realistic concentrations .

For the three species at all kinetics times, the optimal quantum yields were significantly lower in C_2 (10.9±1.3 µg/L of diuron) than C_0 and C_1. *Eolimna minima*, *Nitzschia palea* and *Planothidium lanceolatum* showed similar variations of their optimal quantum yield: 6h after contamination the optimal quantum yield decreased of 45.5±6.9 %, highlighting the fast mode of action of diuron (Legrand et al., 2006). Furthermore, from D7, the optimal quantum yield of *Eolimna minima* and *Nitzschia palea* increased to reach 73±8 % at D14, corresponding to an increase of 17.5 %. Thus, adaptive mechanisms seem to be put in place and they reduce diuron impacts significantly. According to Ricart et al.(2009), diuron contamination induces the formation of shade-type chloroplasts which allow compensating for the reduction in photosynthetic efficiency. Conversely, *Planothidium lanceolatum* optimal quantum yield at C_2, for D7 and D14, stayed at a low mean value of 51±7.6 %.

Thus, the diatoms studied can be separated in two groups: *Eolimna minima/Nitzschia palea* which resist better to diuron contamination and *Planothidium lanceolatum* which is more sensible. So far, only few laboratory studies assessed responses of isolated diatoms species to

diuron contamination (Larras et al., 2012). However, diatom responses to other pesticides have been largely assessed in study field and the sensibility/tolerance of diatoms facing these compounds have been described. In a field study conducted by Morin et al. (2009), *Eolimna minima* and *Nitzschia palea* were found in areas with low pesticide inputs whereas *Planothidium lanceolatum* was found in areas with higher pesticide inputs. These results are in opposition with the laboratories results obtained during our study, highlighting the need of combinate laboratory and field studies in order to obtain a better overview of the system by these complementary approaches. Moreover pesticides gradient often go with metallic and/or nutrient gradient (Montuelle et al., 2010) making difficult to clearly identify the particular effects due to pesticides on diatoms assemblages in the case of multi-contamination.

Indeed, the complexity of natural environments can modulate the effects of the pollutant, by creating synergistic effects (e.g. between illumination and pesticides, (Guasch and Sabater, 1998) or lowering the effects of pesticides (for example nutrients stimulating growth Lozano and Pratt, 1994).

3.1.3. Effect of diuron on the growth of Eolimna minima, Nitzschia palea and Planothidium lanceolatum

The density of *Eolimna minima, Nitzschia palea and Planothidium lanceolatum* over the 14 days of direct exposure to the three different concentrations of diuron, are shown in Table 2.

Statistical analysis revealed a clear growth for the three species of diatoms over the 14 days of experimentation at all concentration levels except for *Planothidium lanceolatum* at the C_2.

Globally, *Eolimna minima* and *Nitzschia palea* had the same pattern of growth kinetics. Statistical analysis revealed that growth was not significantly different between controls (C_0) and diuron contaminated conditions of either 1±0.2 µg/L or 10.9±1.3 µg/L over the whole duration of the experiment. Thus, diuron, even at a high concentration, does not impact the growth kinetics of *Eolimna minima* and *Nitzschia palea*.

On the contrary, for *Planothidium lanceolatum*, the control and C_1 conditions showed a positive growth, with classical curves of exponential growth. However, at condition C_2, no growth was observed, the cell number remained low and stable over the 14 days of exposure. Furthermore, the number of dead cells was significantly higher at C_2 (17 ± 2%) compared to conditions C_0 and C_1 (11.4 ± 4%). As observed for photosynthetic measurements, the growth kinetics were impacted negatively by high concentration of diuron. Such results have already

been highlighted in previous studies, as for the diatom *Navicula accomoda* which growth kinetics was impacted by atrazine (Leboulanger et al., 2001).

Numeration of dead cells of *Nitzschia palea* and *Planothidium lanceolatum* increased significantly over the fourteen days of exposure to diuron, highlighting classical mortality of an algal culture. In contrast, the number of dead cells of *Eolimna minima*, at the three concentrations of diuron, was significantly higher compared to the two other species, with an average of 21 ± 3% of dead cells at 6h. This culture was the oldest of the three, but also the most concentrated. It is not surprising then that it contained a highest proportion of dead individuals, highlighting limiting growth conditions. Furthermore, a significant decrease of the number of dead cells was observed after D2. This phenomenon was linked with a revived growth of this species due to the dilution of the strain under experimental conditions, while diuron probably did not inhibit growth kinetics.

To conclude, the growth kinetic of the three diatoms was not impacted either by 1µg/L or by 10 µg/L of diuron, except for *Planothidium lanceolatum* at the highest concentration. This parameter appeared to be less sensible compared to the optimal photosynthetic yield measurements to determine pesticides' impacts.

3.2. Gene expression levels

The five target genes were cloned and sequenced. In order to get Q-PCR primers, compatibles with the three species, alignments were realised with the software ClustalW. For the target genes *psaA*, 12s and *d1* the nucleotide sequences of the three diatoms were used. Nonetheless, for *cox1* and *act* only the nucleotide sequence of *Eolimna minima* was available. Finally, the sequences of *Nitzschia palea* and *Eolimna minima* were used for the gene *nad5*. The q-RT-PCR primers pairs determined (Table 1).

Gene expression levels of *Eolimna minima*, *Nitzschia palea* and *Planothidium lanceolatum*, during the 14 days of exposure to diuron at 1±0.2 and 10.9±1.3 µg/L, are shown in Table 3. Differential gene expression revealed effects of diuron on the photosynthetic and mitochondrial metabolisms after only 6h of exposure, for both concentrations.

3.2.1. Gene expression levels of Eolimna minima

For the exposure concentration of 1±0.2 µg/L of diuron, effects were detected as early as 6h, with a down regulation of the five target genes. From D2 to D7, the gene expression levels of *Eolimna minima* were equal to those of the controls; except for *d1* (PSII) and 12s (down

regulations of 0.5 for both). Finally, on D14, inductions of the genes *psaA, d1, cox1* and 12s were detected (up regulations respectively of 13, 16, 5.5 and 2.5).

Concerning the C_2 treatment (10.9±1.3 µg/L of diuron), an inhibition of the gene expression of *psaA* (PSI), *cox1* and 12s was observed at 6h (same level of down regulation as the C_1 treatment). At D2, all the studied genes are up regulated, on average by 4.2. Conversely, at D7, the gene expression levels were back to normal, to the same levels as the controls. Finally, at D14, up regulations of the genes implicated in the photosystem metabolism (*psaA* and *d1*) and the respiratory chain (*cox1*) appeared.

3.2.2. Gene expression levels of Nitzschia palea

According to the differential gene expression levels, for the condition of contamination C_1, a down regulation of the genes implicated in photosynthesis processes appeared after 6h of exposure to 1 µg/L of diuron. At D2, the genetic expression levels of those genes were back to normal. Conversely, at the same time, a down regulation of the genes implicated in the mitochondrial metabolism was observed (inhibition of *cox1* and *nad5*). At D7, those two genes were then strongly up regulated (inductions respectively of 29 and 9). Finally, at D14, only the gene *psaA* was up regulated.

For the pressure of contamination of 10 µg/L of diuron, and as it is the case with the treatment C_1, an inhibition of the photosynthetic genes was observed at 6h, as well as an induction of *nad5*. This up regulation of the mitochondrial genes was amplified from D2 to D7, to reach levels of 26 and 4.5, respectively for *cox1* and *nad5*. Finally, at D14, the genes *d1*, 12s and *psaA* were up regulated.

3.2.3. Gene expression levels of Planothidium lanceolatum

For the treatment C_1, a down regulation of the gene implicated in the PSI (*psaA)* and the gene 12s was detected after 6h of exposure. At D2, an induction of almost all genes was observed. The gene expression level of *psaA, d1 and 12s* were particularly strongly up regulated (the levels are respectively multiplied by 79, 51.5 and 55, between 6h and D2). From D7 to D14, the gene expression levels were similar to those of the controls.

For the treatment C_2, strong up regulations were measured at 6h (inductions of 109.5 for *cox1*, 22.5 for 12s and 16 for *psaA*) and D2 (inductions of 42 for *d1*, 22.5 for *psaA* and 22 for 12s), for all the studied genes. At D7, the gene expression levels were equal to those of the controls and at D14 a down regulation of *psaA, d1* and 12s was observable.

3.2.4. Gene expression levels: conclusion

Studies about the genetic response of diatoms facing a contaminant are scarce because of the difficulty to access their nucleotide sequences (Kim Tiam et al., 2012). Genetic expression level studies are based on the implication of genes in the organism response facing contamination. Genes from this study were chosen as they are part of the photosynthetic and mitochondrial metabolisms, both potentially impacted by diuron.

The genetic study showed, for the 3 species, a clear impacts on the expression levels of the genes *psaA, d1, cox1, nad5 and* 12s for both pressure of contamination. This highlights the fact that diuron has strong effect on the photosynthetic and mitochondrial metabolisms. Those functions alterations can be linked with the significant decrease of photosynthetic activity for the three diatoms and the growth inhibition of *Planothidium lanceolatum,* at the highest concentration of diuron.

Eolimna minima and *Nitzschia palea* have a common profile of up and down regulations of the five studied genes. A decrease of the general metabolism appears after 6h of exposure to 1 µg/L of diuron. It implies that the respiratory chain and the photosynthesis are rapidly impacted at concentration as low as 1 µg/L of diuron. These results are due to the rapid mode of action of diuron: toxic effects can appear after 15 minutes of exposure (Legrand et al., 2006). At D7, damages are measured; adaptive mechanisms are put in place to counteract diuron's effect. From the previous results in this study, no effect was detected at the exposure condition of 1 µg/L of diuron, neither by photosynthetic parameters nor by the growth kinetic analyses. Thus, genetic responses can reveal the impact of diuron on both mitochondrial and photosynthetic metabolisms at a concentration that did not provoke damages on the classical endpoints used. Concerning the condition of contamination C_2, comparable effects are detected. However, damages appear sooner, at D2 (compared to D7 for the exposure condition C_1), suggesting a dose-dependent effect: for highest concentrations of diuron, adaptive mechanisms are activated earlier. The two diatoms seem to undergo strong impacts at the beginning of the experiment and then, a reaction, through adaptive mechanisms to fight the contaminant, is observed. Those results are in accordance with the recovery observed through photosynthetic measurements: *Eolimna minima* and *Nitzschia palea* undergo a decrease of the optimal quantum yield and then an increase, indicating an improvement of their physiological stage, highlighted here by the genetic expression.

Planothidium lanceolatum has a different functioning pattern: as early as 6h, damages are detected and then a metabolism decrease is observed until the end of the experiment. *Planothidium lanceolatum* seems to behave at the opposite compared to *Eolimna minima* and

Nitzschia palea. Indeed, it reacts from the start of the exposure to contamination but quickly the genetic response is back to the one of the controls. This come down highlights that the disturbance of the two metabolisms studied exceeded critical levels. These results are in accordance with the photosynthetic measurements, the optimal quantum yield measurements for the treatment C_2 stayed low during the 14 days of exposure.

It appears clearly that the photosynthetic metabolism of the three diatoms is strongly impacted by an exposure to diuron through inhibition and then induction of genes *psaA* and *d1* as early as 6h. Diuron impact on the photosynthetic activity at the physiological level is known and is well documented (Debenest et al., 2010; Hartgers et al., 1998; Idedan et al., 2011; Jones et al., 2003). Strong impacts are also detected on the mitochondrial metabolism. There are strong inductions of the genes *cox1* and *nad5*, suggesting an increase of ATP production to counteract the deleterious effects of toxicant (Kim Tiam et al., 2012). Indeed, those two genes are implicated in the functioning of the respiratory chain. Furthermore, the gene 12s is also strongly up-regulated, testifying of an increase of the number of mitochondria (increase of the ratio *cox1*/12s).

Only little literature is available about the genetic impact of diuron on diatoms and more generally about superior plants. Studies based on the exposure of *Thalassiosira pseudonana* by PAHs (organic contamination) Carvalho (2011) or by copper (inorganic contamination) Davis et al. (2006) highlight a common response of the biomarkers: during contamination episode, there is up and/or down regulations of genes implicated in the stress response.

Results from this study can be linked with the one obtain by Kim Tiam et al. (2012) on *Eolimna minima*, showing the impacts of a metallic contamination on the general and photosynthetic metabolisms also as the early up-regulation of the genes for the highest pressure of contamination (100µg Cd/L). The biomarkers used in our study highlight impacts as early as 6h for a diuron pressure of contamination of 1 µg/L. Classical descriptors (photosynthetic activity and cell numeration) didn't highlight these impacts, as also observed by Kim Tiam (2012) for metal contamination. It appears clearly that the improvement of molecular tools and more precisely biomarkers is an asset to follow organism contamination and water quality.

4. Conclusions and perspectives

In the present study molecular tools were developed and successfully used in diuron experimental contamination. The major implications of the work are:

- Five genes of interest were sequenced for *E. minima*, *N. palea* and *P. lanceolatum* allowing the application of q-PCR tools to these species.

- Diuron contamination had impacts on growth rate, optimal quantum yield of the PSII (ΦPSII) and expression of several genes involved in mitochondrial and photosynthetic mechanisms.

- q-PCR tools appeared to be more sensitive than ΦPSII and growth rate analyses to reveal diuron effects

- The study allowed increase knowledge about relative sensitivity of diatom species to diuron in regards to the different endpoints tested. *E. minima* and *N. palea* appeared to be more tolerant than *P. lanceolatum*.

The present study is one of the few study reporting the use of q-PCR on river benthic diatoms and the results obtained are extremely promising for future applications in the field of water quality assessment.

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Figure 1 - Optimal photosynthetic quantum yield of Eolimna minima (a), Nitzschia palea (b) and Planothidium lanceolatum (c) after dark adaptation (30 min), for the 4 sampling times and 3 exposure conditions. C0 = 0 µg/L (black bars), C1 = 1 µg/L (light gray bars) and C2 = 10 µg/L (dark gray bars) of diuron. Differences between times and contamination are marked with different letters at level $p < 0.01$. Mean ± SE are shown.

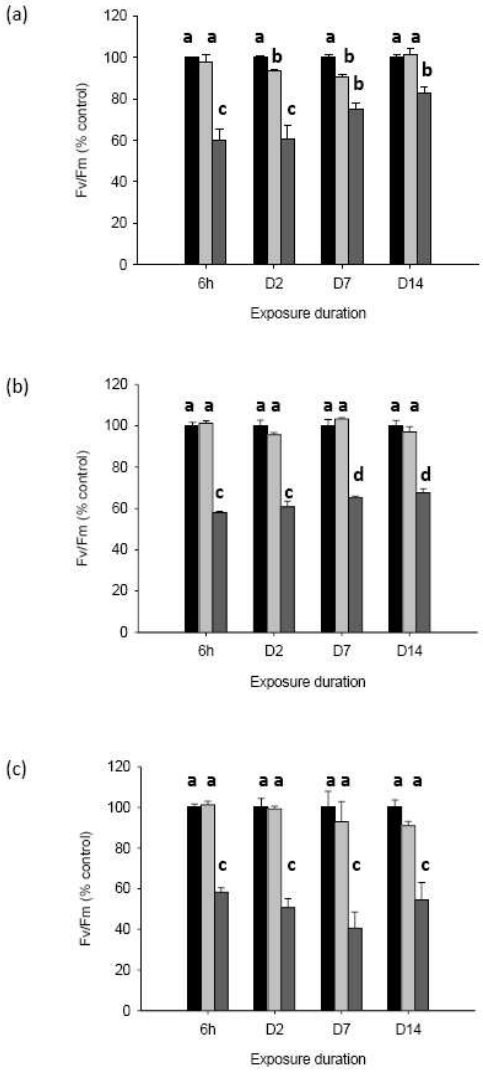

Table 1 - Accession numbers and specific primer pairs for the 6 genes of *Eolimna minima*, *Nitzschia palea* and *Planothidium lanceolatum* used in our study.

Tableau 1 - Accession numbers and specific primer pairs for the 6 genes of *E.minima*, *N.palea* and *P.lanceolatum* used in our study.

Species	Gene name	Primer (5'-3')
All	*psaA*	GGTCAAGAAATTTTAAATGGTGA [a] TAGTGGAACCAACCAGCAAATA [b]
All	*12s*	AGGATGCAAGTGTTATCCGGA [a] CAATATCTACGCATTTCACCGCT [b]
All	*d1*	TCTGCAGTATTCTTAGTATACC [a] CAGCCATGTGGAATGGGTGC [b]
E.minima	*cox1*	ATAGAAGCAGCACCTGAAAGA [a] GTTTTTGGTTATTACCACCTTC [b]
N.palea & P.lanceolatum	*cox1*	CAGTAATTCTCACTGCCCAGC [a] GTTTTTGGTTATTACCACCTTC [b]
E.minima	*nad5*	TGCTATGGAAGGTCCTACTCC [a] CTAATCCTGTTGTGGAAGCAAA [b]
N.palea & P.lanceolatum	*nad5*	TCAACTTGGTTTGCATACATGGC [a] TTGAACTAATCCTGTTGTGGAAGC [b]
E.minima	*act*	CCAACCGCGAGCGCATGACG [a] CGACAGGACGGCCTGGATGTT [b]
N.palea & P.lanceolatum	*act*	GGCTCCACAAAACCCCAAG [a] GGCGTACCCCTCGTAGAT [b]

Abbreviations: *psaA* - PsaA protein ; 12s - ribosomal RNA 12s ; *d1* - D1 protein ; *cox1*- cytochrome C oxidase subunit I ; *nad5* - NADH dehydrogenase subunit 5 ; *act* - β-actin.

a Upstream primer.
b Forward primer.

Table 2 - Total density of *Eolimna minima*, *Nitzschia palea* and *Planothidium lanceolatum* (x 1000cells) ± standard errors and corresponding cell mortality (into parentheses), for the 4 sampling times and 3 exposure conditions. C0 = 0 µg/L, C1 = 1 µg/L and C2 = 10.9 µg/L of diuron. Significant differences between the different times and conditions of contamination are marked with different letters at level $p < 0.05$.

Species	Exposure conditions	Time exposure			
		6 hours	2 days	7 days	14 days
Eolimna minima	C_0	166 ± 16 ^{a} (24±2%)	310 ± 48 ^{b} (26±1%)	1.182 ± 170 ^{c} (14±2%)	1.610 ± 474 ^{c} (13±2%)
	C_1	275 ± 46 ^{b} (21±4%)	521 ± 54 ^{b} (26±2%)	840 ± 150 ^{c} (21±8%)	1.436 ± 613 ^{c} (12±0%)
	C_2	275 ± 33 ^{b} (19±1%)	367 ± 10 ^{b} (21±4%)	1.279 ± 481 ^{c} (15±1%)	1.470 ± 179 ^{c} (13±3%)
Nitzschia palea	C_0	160 ± 17 ^{a} (5±1%)	697 ± 103 ^{b} (9±3%)	1.847 ± 441 ^{c} (13±3%)	1.230 ± 135 ^{c} (12±2%)
	C_1	180 ± 18 ^{b} (3±0%)	636 ± 49 ^{b} (11±6%)	2.172 ± 464 ^{c} (11±1%)	1.496 ± 213 ^{c} (10±2%)
	C_2	198 ± 26 ^{b} (4±2%)	354 ± 42 ^{b} (11±3%)	1.685 ± 181 ^{c} (11±4%)	1.517 ± 144 ^{c} (12±4%)
Planothidium lanceolatum	C_0	98 ± 15 ^{a} (1±0%)	141 ± 37 ^{a} (8±1%)	534 ± 160 ^{b} (9±2%)	515 ± 94 ^{b} (11±5%)
	C_1	86 ± 6 ^{a} (1±0%)	117 ± 19 ^{a} (9±2%)	440 ± 248 ^{b} (6±1%)	660 ± 99 ^{b} (12±2%)
	C_2	158 ± 49 ^{a} (0±0%)	124 ± 9 ^{a} (7±1%)	138 ± 42 ^{a} (14±3%)	148 ± 54 ^{a} (17±2%)

Table 3 - Differential gene expression as compared to actin from *Eolimna minima* (a), *Nitzschia palea* (b) and *Planothidium lanceolatum* (c) after 6h, 2, 7 and 14 days exposure to 1 and 10.9 µg/L of diuron by direct route [a].

(a)

Functions	Genes	Diuron-contaminated experimental units							
		C_1 (1 ± 0.2 µg/L)				C_2 (10.9 ± 1.3 µg/L)			
		6 hours	2 days	7 days	14 days	6 hours	2 days	7 days	14 days
Photosynthetic metabolism	*psaA*	0.5	/	/	13	0.5	4.5	/	4
	d1	0	/	0.5	16	/	4	/	8
Mitochondrial metabolism	*cox1*	0.5	/	/	5.5	0	3.5	/	2.5
	nad5	0	/	/	/	/	4.5	/	/
	12s	0.5	/	0.5	2.5	0.5	4.5	/	/

(b)

Functions	Genes	Diuron-contaminated experimental units							
		C_1 (1 ± 0.2 µg/L)				C_2 (10.9 ± 1.3 µg/L)			
		6 hours	2 days	7 days	14 days	6 hours	2 days	7 days	14 days
Photosynthetic metabolism	*psaA*	0.5	/	/	2	0.5	/	4	3.5
	d1	0.5	/	/	/	0.5	/	/	3
Mitochondrial metabolism	*cox1*	/	0	29	/	/	10.5	26	/
	nad5	/	0	9	/	2.5	6	4.5	/
	12s	/	/	/	/	/	/	2	3

(c)

Functions	Genes	Diuron-contaminated experimental units							
		C_1 (1 ± 0.2 µg/L)				C_2 (10.9 ± 1.3 µg/L)			
		6 hours	2 days	7 days	14 days	6 hours	2 days	7 days	14 days
Photosynthetic metabolism	*psaA*	0.5	39.5	/	/	16	22.5	/	0
	d1	/	51.5	/	/	7	42	/	0
Mitochondrial metabolism	*cox1*	/	8.5	/	/	109.5	5	/	/
	nad5	/	/	/	/	4	/	/	/
	12s	0.5	55	/	/	22.5	22	/	0

a Significant induction (>2) and repression (<0.5) factors are indicated by values, compared to the respective controls *E. minima (a), N. palea (b)* and *P. lanceolatum (c)*. /: identical to control levels.