



# **Allelopathic interactions in river benthic photrophic biofilms : a multi-approach study**

Joey Allen

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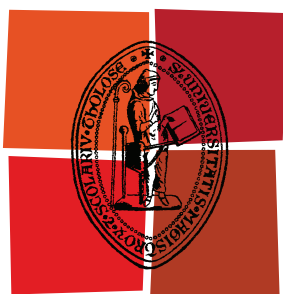
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# THÈSE

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Joey ALLEN

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Interactions allélopathiques au sein de biofilms phototrophes de rivière:  
Étude multi-approche d'une interaction ciblée.

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**Unité de recherche :**

EcoLab (UMR5245)

**Directeur(s) de Thèse :**

Joséphine LEFLAIVE, Maître de conférences, Université Toulouse III Paul Sabatier

Loïc TEN-HAGE, Maître de conférences, Université Toulouse III Paul Sabatier

**Rapporteurs :**

Élisabeth GROSS, Professeur, Université de Lorraine

Jean-Luc MOUGET, Professeur, Université du Maine

**Membre(s) du jury :**

Chris BOWLER, Directeur de recherche, École Normale Supérieure, Paris (président)

Christophe ROUX, Professeur, Université Toulouse III Paul Sabatier (examineur)

Fayza DABOUSSI, Directeur de recherche, INSA Toulouse (membre invité)



## Résumé

Les interactions allélopathiques sont définies comme la libération par un organisme de composés chimiques inhibant les compétiteurs. Dans les milieux aquatiques, elles sont reconnues comme un facteur de la dynamique des communautés de phytoplancton. Malgré les conditions favorables créées par l'organisation des microorganismes en biofilms, l'allélopathie a été beaucoup moins étudiée dans les communautés de microorganismes phototrophes benthiques.

L'objectif de cette thèse était d'étudier des interactions allélopathiques, au travers d'un cas particulier, entre les microorganismes dans les biofilms phototrophes d'eau douce. Ainsi le travail s'est focalisé sur les effets allélopathiques d'une algue verte filamenteuse, *Uronema confervicolum* Lagerheim, sur des diatomées benthiques. L'hypothèse principale est que, dans certaines conditions, *U. confervicolum* libère suffisamment de composés allélopathiques pour inhiber significativement le développement des diatomées épiphytes. Cette hypothèse soulève trois questions (i) quels sont les composés allélopathiques, (ii) leur production dépend-elle des conditions environnementales et (iii) quel est l'effet des composés allélopathiques produits sur les diatomées ? Cette interaction a été étudiée au travers de multiples approches. L'activité d'extraits de biomasse algale et de filtrat de culture a été testée sur la croissance, la photosynthèse, l'adhésion et la motilité des diatomées. Les techniques de fractionnement guidé par bioessais et de profilage métabolomique ont été utilisées pour identifier les composés allélopathiques produits. Les effets des facteurs environnementaux (limitations en nutriment, apports de lumière et de gaz) et de la phase de croissance sur la production et la libération de différents composés allélopathiques ont également été étudiés. Une étude approfondie de l'effet sur les diatomées des composés allélopathiques anti-adhésion a été réalisée en combinant le profilage métabolomique, la transcriptomique et la microscopie électronique à balayage.

Les extraits d'*U. confervicolum* ont montré un effet négatif sur la croissance, la photosynthèse, l'adhésion et la motilité des diatomées. Deux acides gras polyinsaturés (acides linoléique et  $\alpha$ -linolénique) ont été identifiés comme des composés allélopathiques inhibant la croissance et la photosynthèse des diatomées. L'inhibition de l'adhésion des diatomées est, par contre, causée par d'autres composés allélopathiques non identifiés. L'augmentation de l'intensité lumineuse a accru la production de composés allélopathiques, à l'instar des composés défensifs des plantes. Cependant, aucune des théories classiques sur les défenses chimiques des plantes n'a permis d'expliquer l'ensemble des résultats obtenus sur les questions de régulation. En plus de l'inhibition de l'adhésion, le filtrat d'*U. confervicolum* inhibe la formation de la matrice constituée de substances polymériques extracellulaires, impacte le métabolisme énergétique et induit une modification globale du transcriptome et du métabolome des diatomées. Une partie des gènes et métabolites les plus fortement impliqués dans la réponse des diatomées à l'inhibition de l'adhésion ont été identifiés.

Ce travail a permis de mieux évaluer l'activité allélopathique de l'algue verte filamenteuse *U. confervicolum* et son impact potentiel sur la structure et le fonctionnement des biofilms phototrophes. D'autres approches seront nécessaires, à l'avenir, pour mieux comprendre le rôle global de l'allélopathie dans des biofilms phototrophes complexes.

## Abstract

Allelopathic interactions are defined as the release, by an organism, of chemicals that inhibit competitors. In aquatic environments, they are recognised as a driver of phytoplankton community dynamics. Despite the favourable conditions created by the organisation of microorganisms in biofilms, allelopathy has been much less studied in benthic phototrophic microorganism communities.

The objective of this thesis was to increase our comprehension of allelopathic interactions amongst phototrophic microorganisms in biofilms by analysing several aspects of a specific allelopathic interaction. The model chosen was the allelopathic effect of *Uronema confervicolum* Lagerheim, a filamentous green alga isolated from river biofilms, on diatoms from the same environment. The main hypothesis was that, at least in specific environmental conditions, *U. confervicolum* produce and release sufficiently large amounts of allelopathic compounds to inhibit significantly the development of epiphytic diatoms. Three questions arised from this hypothesis: (i) what are the allelopathic compounds implied, (ii) do their production and release depend on environmental factors and (iii) what is the effect of these compounds on epiphytic diatoms? This interaction has been studied using multiple approaches. Activity of algal biomass extracts and culture filtrates have been tested on diatom growth, photosynthesis, adhesion and motility. Bioassay-guided fractionation and metabolomic profiling have been used to identify the allelopathic compounds produced. The effect of environmental factors (nutrient limitation, light and gas supply) and growth phase on the production and release of the different allelopathic compounds have also been studied. In-depth study of the effect of anti-adhesion allelochemicals on diatom by combining metabolomic profiling, transcriptomics and scanning electron microscopy have been carried out.

The extracts of *U. confervicolum* exhibited negative effects on diatoms growth, photosynthesis, adhesion and motility in bioassays. Two polyunsaturated fatty acids (linoleic and  $\alpha$ -linolenic acids) have been identified as allelopathic compounds affecting growth and photosynthesis but not adhesion ability of diatoms. The inhibition of diatom adhesion was caused by other allelopathic compounds, unidentified so far. Increasing light intensity enhanced the production of *U. confervicolum* allelopathic compounds as predicted by plant defence theories, but the regulation of allelopathic activity was not completely explainable by any classic plant defence theory. Finally, beside an inhibition of diatom adhesion *U. confervicolum* filtrate was also found to inhibit the formation of diatom extracellular polymeric substances matrix, affect energy metabolism and induce global modification of diatom transcriptome and metabolome. Some of the genes and metabolites implied in the response of diatoms to adhesion inhibition have been identified.

These results indicate the strong potential of the filamentous green alga *U. confervicolum* to impact biofilm community by its allelopathic activity. Results on the allelopathic activity of this alga and the effect on diatoms in laboratory experiments provide the basis for further experiments to access the role of allelopathy in complex biofilms. These findings will encourage researchers working on biofilms to consider allelopathy when phototrophic biofilm ecology is studied.

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## Avant-propos

Dans les milieux aquatiques, les interactions chimiques sont impliquées dans de nombreux processus, tels que les interactions proie-prédateurs et la défense contre les herbivores (Brönmark and Hansson, 2012). L'évidence de l'importance de ces interactions dans la structuration des communautés se fait de plus en plus forte (Ianora *et al.*, 2006, 2011; Pohnert, 2008; Pohnert *et al.*, 2007). En particulier, les interactions allélopathiques, c'est à dire l'inhibition des compétiteurs par la production et la libération dans l'environnement de composés chimiques (Rice, 1984), ont été fortement étudiées dans les milieux aquatiques (Gross, 2003; Leflaive and Ten-Hage, 2007; Legrand *et al.*, 2003). Dans ces milieux, l'allélopathie peut influencer significativement le fonctionnement de l'écosystème (*e.g.* Czárán *et al.*, 2002; Keating, 1977).

Lorsque l'allélopathie est étudiée dans les milieux aquatiques, la distinction est rarement faite entre les organismes planctoniques et les organismes benthiques constitutifs des biofilms. Pourtant l'organisation en biofilm est de nature à influencer fortement la nature, l'intensité et l'impact des interactions allélopathiques (cf. chapitres 1-3). Ainsi, afin de déterminer si les interactions allélopathiques jouent un rôle important dans l'écologie des biofilms phototrophes, cette thèse se focalise sur les interactions allélopathiques entre micro-algues formant des biofilms. Le terme de micro-algue est utilisé ici d'un point de vue fonctionnel et écologique pour désigner tous les microorganismes phototrophes procaryotes (cyanobactéries) et eucaryotes (*e.g.* algues vertes unicellulaires et filamenteuses, diatomées), sans considération taxonomique.

L'allélopathie est un processus délicat à étudier du fait de la nécessité de séparer les effets de composés allélopathiques des effets liés à l'exploitation des ressources. Le biofilm est également un système extrêmement complexe, avec de forts gradients de nutriments et de lumière. Il abrite généralement une grande diversité d'espèces ce qui multiplie les possibilités d'interactions allélopathiques (figure 1 a). Il a donc été nécessaire d'utiliser un système simplifié pour étudier cette interaction (figure 1 b).

Le système simplifié utilisé est basé sur l'interaction entre une algue verte filamenteuse, *Uronema confervicolum* Lagerheim, produisant des composés allélopathiques (Leflaive *et al.*, 2008b, 2008a) et des diatomées benthiques choisies comme cibles des composés allélopathiques. *U. confervicolum* est connue pour ses propriétés allélopathiques (Leflaive *et al.*, 2008b, 2008a). Les algues vertes filamenteuses sont fréquemment colonisées par des diatomées épiphytes (Zulkifly *et al.*, 2013). Toutefois, il semblerait que ce ne soit pas le cas pour *U. confervicolum* (R. Le Cohu, communication

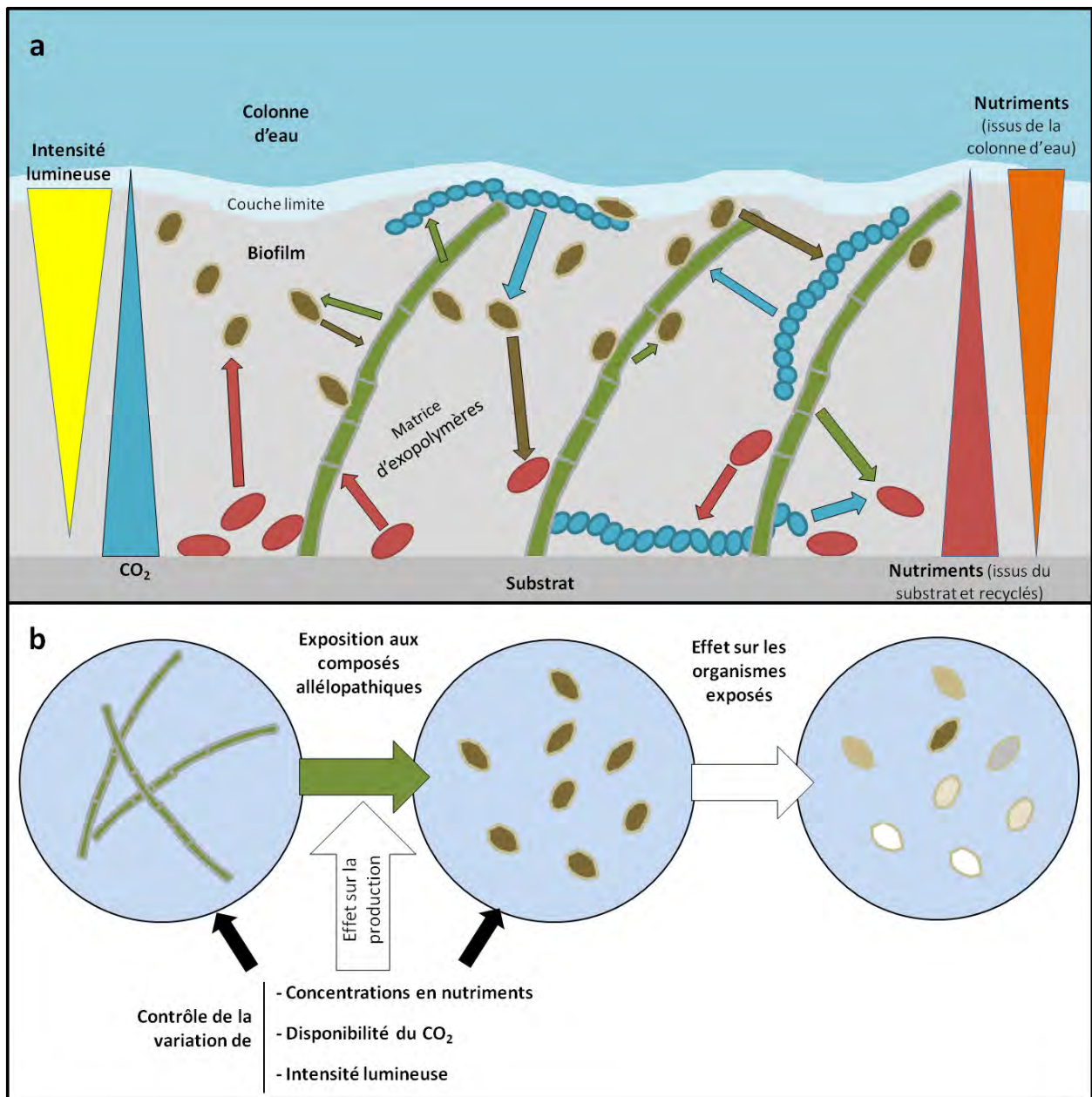


**Figure 1 : Représentation schématique d'un biofilm phototrophe (a) et du système simplifié utilisé pour l'étude des interactions allélopathiques (b). Les flèches de couleur représentent les transferts éventuels de composés allélopathiques entre les organismes. Les organismes hétérotrophes n'ont pas été représentés.**

personnelle). L'inhibition des diatomées par des composés allélopathiques pourrait être à l'origine de cette absence.

Des diatomées de trois espèces différentes, *Fistulifera saprophila* (Lange-Bertalot & Bonik) Lange-Bertalot, *Gomphonema parvulum* (Kützing) Kützing et *Nitzschia palea* (Kützing) W. Smith ont été choisies comme cibles de l'allélopathie d'*U. confervicolum*.

Ce système simplifié permet de mettre en évidence l'existence d'interactions allélopathiques et d'étudier l'effet des conditions environnementales sur la production de composés allélopathiques et les conséquences de ces interactions sur la cible des composés allélopathiques. Les résultats issus de

l'étude de ce système doivent permettre une meilleure compréhension des interactions ayant lieu au sein du biofilm.

Dans le **premier chapitre** de ce manuscrit, après une présentation du type d'interaction (l'allélopathie) et du modèle biologique (le biofilm phototrophe), une synthèse bibliographique détaille les **interactions allélopathiques impliquant le microphytobenthos**. Cette synthèse présentera notamment les raisons pour lesquelles les interactions allélopathiques jouent un rôle potentiellement plus important dans les milieux microbiens benthiques que dans les milieux planctoniques. Le **deuxième chapitre** sera consacré à l'étude de la **production des composés allélopathiques** par *Uronema confervicolum*. Dans cette partie je décrirai tout d'abord les effets d'extraits d'*U. confervicolum* sur des diatomées benthiques et l'identification d'acides gras polyinsaturés comme composés allélopathiques produits par *U. confervicolum*. Le **troisième chapitre** abordera la question de la **régulation de la production de composés allélopathiques** en utilisant tout d'abord l'exemple des acides gras polyinsaturés, puis celui d'autres composés allélopathiques, également produits par *U. confervicolum* et inhibant l'adhésion des diatomées. Cette inhibition de l'adhésion par les composés allélopathiques produits par *U. confervicolum* fera l'objet du **quatrième chapitre** de ce manuscrit. Cette partie sera consacrée à l'effet et à la **réponse des diatomées à l'exposition aux composés allélopathiques** anti-adhésion produits par *U. confervicolum*. Pour finir, l'ensemble des résultats fera l'objet d'une discussion générale dans le cinquième chapitre. Des perspectives pour de futures recherches sur l'allélopathie au sein de biofilms phototrophes seront présentées.



# Chapitre I

## Introduction générale



## I. Introduction générale

### I.1 L'allélopathie

L'allélopathie est un processus connu de longue date, en effet, d'après Willis (2007) ce type de processus était déjà observé dans l'antiquité. La définition de l'allélopathie - et le terme en lui-même - remonte aux travaux de Molisch (1937), consacrés aux interactions chimiques entre plantes (*Der Einfluss einer Pflanze auf die andere: Allelopathie*). Cependant, les limites du concept d'allélopathie restent floues. La définition de l'allélopathie la plus reconnue est celle de Rice « *any direct or indirect harmful or beneficial effect by one plant (including microorganisms) on another through the release of chemicals that escape into the environment* » [tout effet direct ou indirect, positif ou négatif, d'une plante (microorganismes inclus) sur une autre par la production de composés chimiques libérés dans l'environnement] (Rice, 1984). Il est généralement accepté que l'allélopathie désigne aussi des interactions chimiques entre invertébrés sessiles et entre bactéries. Certains auteurs étendent ce concept d'allélopathie : par exemple Fujii et Hiradate (2007) définissent l'allélopathie comme « *an action of natural bioactive chemicals produced by plant to other life* », d'autres incluent les interactions de type proie-prédateur (Rizvi and Rizvi, 1992). A l'opposé, d'autres auteurs conservent la limite de l'allélopathie comme interaction entre compétiteurs (Gross, 2003). La quasi-totalité des interactions allélopathiques décrites dans la littérature concernent des cas d'inhibition, bien que la différence entre un effet positif et négatif soit souvent une question de concentration (Belz *et al.*, 2007).

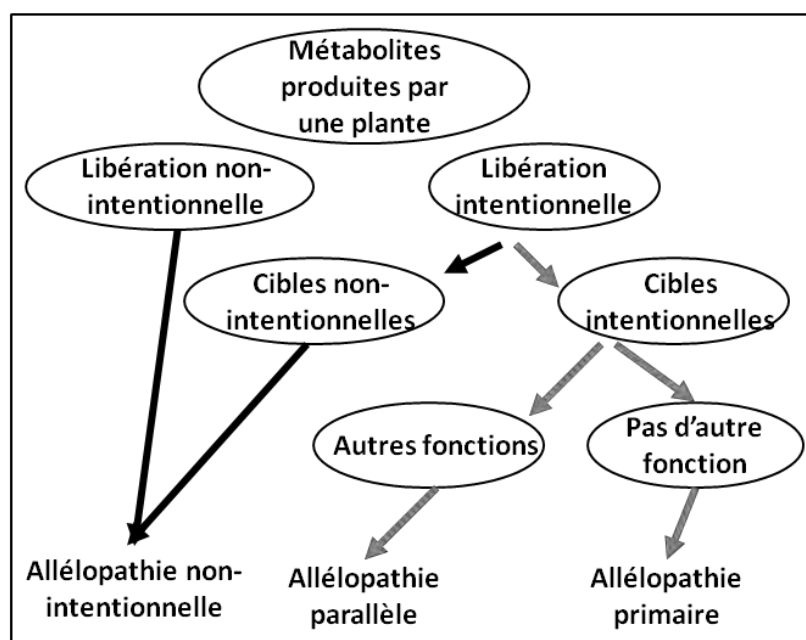


Figure 2 : Les trois différentes catégories écologiques de l'allélopathie. D'après Sinkkonen (2006).

D'un point de vue écologique, l'allélopathie est définie comme une forme de compétition par interférence, c'est-à-dire une limitation de la capacité des compétiteurs à accéder aux ressources, par opposition à la compétition par exploitation, c'est-à-dire la déplétion des ressources par leur exploitation directe (Vance, 1984).

Plusieurs types d'allélopathie peuvent être distingués (Sinkkonen, 2006 ; figure 1). En effet, des composés produits par un organisme peuvent inhiber d'autres organismes sans que cela soit la fonction de la production de ces composés (allélopathie 'non-intentionnelle'). Par exemple les composés phénoliques de feuilles de divers arbres peuvent inhiber des micro-algues aquatiques (Bährs *et al.*, 2012; Tsuda *et al.*, 2005). Cette inhibition n'apporte aucun bénéfice à l'organisme émetteur. La production de ces composés inhibiteurs n'a pas été sélectionnée durant l'évolution de l'espèce émettrice pour sa fonction allélopathique contre les microalgues. La fonction la plus probable de ces composés est certainement la défense contre l'herbivorie ou une activité allélopathique contre d'autres plantes terrestres. D'autre part, un composé allélopathique peut avoir d'autres fonctions en plus de sa fonction d'allélopathie (allélopathie parallèle), *e.g.* la défense contre l'herbivorie et allélopathie des aldéhydes polyinsaturés produits par les diatomées (Jüttner, 2001; Leflaive and Ten-Hage, 2009). Dans ce cas, on peut supposer que les deux fonctions apportent un bénéfice à l'organisme émetteur et donc participent au maintien de la fonction allélopathique. Des composés impliqués dans le métabolisme primaire peuvent avoir aussi une fonction allélopathique (Pohnert, 2012 et **chapitres 2 et 3**). Finalement, lorsque le seul rôle connu des composés est une fonction allélopathique, on parle d'allélopathie 'primaire' (Sinkkonen, 2006). Il est souvent difficile de déterminer la fonction principale de composés allélopathiques, par exemple de nombreux composés cyanobactériens ont été identifiés, dans un premier temps, pour leur toxicité mais exhibent également une activité allélopathique. Déterminer si la fonction de ces composés est l'allélopathie ou la défense reste complexe (Bajpai *et al.*, 2013; Brutemark and Engström-Öst, 2013; Holland and Kinnear, 2013; Pinheiro *et al.*, 2013; Valdor and Aboal, 2007).

Tous les composés produits par un organisme et ayant une activité inhibitrice ne sont pas des composés allélopathiques. Plusieurs critères ont été établis pour démontrer qu'un composé chimique est un composé allélopathique (Sinkkonen, 2006; Willis, 1985) :

- l'organisme supposé émetteur affecte l'organisme cible (l'inhibition observée ne doit pas pouvoir être causée par d'autres facteurs).
- le composé allélopathique (ou un précurseur) doit être, activement ou non, libéré dans l'environnement.

- la quantité de composé qui atteint la cible doit être suffisamment importante pour causer l'effet observé.

En règle générale, les tests visant à mettre en évidence une interaction allélopathique correspondent à un compromis entre la réalité biologique et l'exclusion de la compétition par exploitation (figure 3 ; Gross *et al.*, 2007). Dans certains cas, un effet particulièrement fort ou ne pouvant pas être confondu avec un effet lié à la déplétion des nutriments permet d'établir le caractère allélopathique d'un organisme (*e.g.* Vanellander *et al.*, 2012). Cependant, il est rare de pouvoir déterminer avec certitude la réalité de l'interaction allélopathique, mais l'utilisation de multiples expérimentations différentes peut permettre de réduire très fortement le doute.

Pour une étude plus approfondie de l'allélopathie, il est souvent nécessaire d'identifier les composés impliqués. L'identification des composés allélopathiques repose sur deux méthodes principales: Le fractionnement guidé par bioessais (bioassay guided fractionation ; **chapitre II**) et l'utilisation du profilage métabolomique (Prince and Pohnert, 2010 ; **chapitre III.2**). Ces méthodes permettent

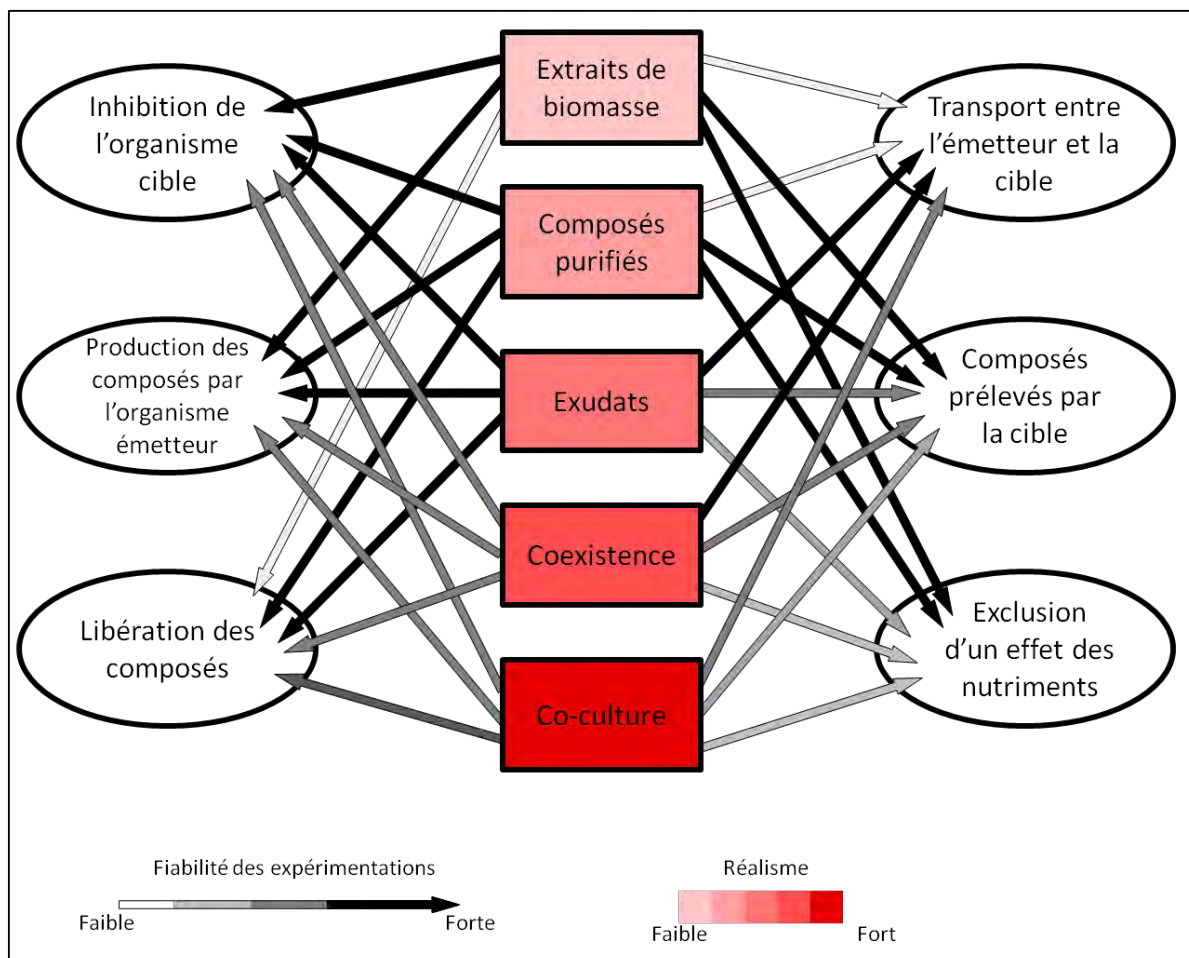


Figure 3 : Représentation schématique du niveau de preuve extractible des tests classiquement réalisés pour étudier l'allélopathie d'organismes aquatiques, d'après Gross *et al.* (2007).

l'identification de composés bioactifs. Pour établir la nature allélopathique des composés identifiés, il faut respecter les critères cités précédemment. Il est possible de déterminer l'existence d'interactions allélopathiques sans passer par l'identification des composés impliqués. La méthode qui pourrait être la plus fiable pour refléter les conditions normales de l'interaction entre les organismes tout en excluant un effet lié aux nutriments serait d'étudier l'effet de la densité de l'organisme cible sur sa sensibilité à l'organisme émetteur (Weidenhamer, 2006). Cette méthode a fait ces preuves avec des plantes terrestres (Weidenhamer, 2006), mais n'a jamais été testée avec des microorganismes aquatiques.

#### *Effet de l'allélopathie sur le fonctionnement des écosystèmes : Pourquoi étudier l'allélopathie ?*

Une grande partie des études consacrées à l'allélopathie s'attache plus à la recherche de nouveaux herbicides bio-sourcés et à la sélection de plantes d'intérêt agronomique (par leur activité allélopathique) qu'à l'étude de l'allélopathie dans un contexte naturel et à la compréhension de son rôle dans le fonctionnement des écosystèmes. Pourtant de nombreux résultats tendent à montrer une grande importance de l'allélopathie dans la structuration des communautés et le fonctionnement des écosystèmes.

La pression de sélection exercée par l'exposition aux composés allélopathiques mène à l'émergence de souches (ou variétés) résistantes à ces composés allélopathiques. Le potentiel allélopathique peut donc ne pas être visible à un moment donné, mais peut être révélé lorsque l'organisme est déplacé vers une communauté composée d'organismes « naïfs » vis-à-vis des composés allélopathiques produits. Ainsi, l'allélopathie est impliquée dans un grand nombre de cas d'invasion biologique par des espèces exotiques (« novel weapon hypothesis » ; Callaway and Ridenour, 2004).

En plus de son implication dans les invasions biologiques, l'allélopathie aurait d'autres impacts importants sur les écosystèmes. En effet, dans des lacs peu profonds, la production de composés allélopathiques par les macrophytes et le biofilm phototrophe favoriserait le maintien d'une eau peu turbide, due à une faible quantité de phytoplancton (Hilt and Gross, 2008; Wu *et al.*, 2014). Dans les milieux aquatiques en général, l'allélopathie participerait au maintien des efflorescences algales (blooms). Bien que la production de composés allélopathiques ne permette pas d'expliquer la formation de ces blooms algaux (Jonsson *et al.*, 2009), elle serait en mesure de les prolonger en empêchant d'autres espèces algales de se développer. Cependant, l'allélopathie est très probablement impliquée dans les successions écologiques. En effet, Keating (1977) a montré que les cyanobactéries formant des efflorescences algales successives dans des lacs eutrophisés étaient

systématiquement inhibées par les composés allélopathiques produits par leurs 'successeurs'. De même, les cyanobactéries succédant à des diatomées produisaient des composés allélopathiques inhibant ces diatomées (Keating, 1978).

A l'inverse de ces cas où l'allélopathie a tendance à renforcer la dominance d'une espèce, l'allélopathie, dans certaines conditions, peut promouvoir la coexistence de différentes espèces par la création de réseaux de compétition circulaire (« circular competitive networks », on parle aussi de relation non-hiérarchique ou intransitive). Le principe de ces réseaux est qu'au lieu d'une relation hiérarchique classique (espèce A > espèce B > espèce C et A > C dans un environnement donné) la relation est d'ordre non-hiérarchique (A > B, B > C, C > A ; à la manière du jeu « pierre-feuille-ciseaux ») (Czárán *et al.*, 2002).

Ce type de relation peut apparaître du fait de la production de composés allélopathiques. L'organisme produisant des composés allélopathiques (A) sera dominant face à un organisme sensible (S) inhibé par les composés allélopathiques (donc A>S). A sera dominé par un organisme résistant (R), ce dernier n'ayant pas à assumer le coût de production des composés allélopathiques (donc R>A). Cependant, si l'on conçoit que la protection contre les composés allélopathiques a

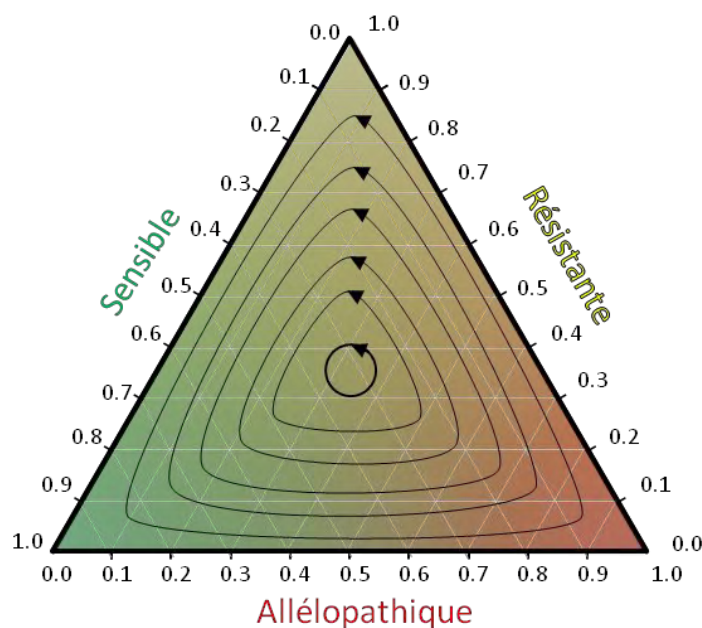


**Figure 4 : Représentation schématisée de la dynamique d'une communauté composée d'une espèce produisant des composés allélopathiques, d'une espèce résistante et d'une espèce sensible. Les valeurs sur les axes représentent l'abondance relative de chaque espèce. Les flèches représentent les trajectoires des communautés. D'après Czárán *et al.*, (2002).**

également un coût en terme de limitation de croissance, alors S, qui n'a pas à assumer ce coût, aura une croissance plus forte que R (en l'absence de A) donc S > R (Czárán *et al.*, 2002 figure 4).

De tels systèmes ont été mis en évidence chez des animaux marins sessiles (bryozoaires, spongiaires et ascidies) (Jackson and Buss, 1975) et ont pu être créés artificiellement avec un modèle bactérien (Kerr *et al.*, 2002). Par contre, aucun cas de compétition non-hiérarchique n'a jamais été décrit chez les plantes ou les micro-algues (Wilson, 2011).

## I. 2 Les biofilms phototrophes

Dans les milieux aquatiques, les biofilms phototrophes sont des assemblages de microorganismes phototrophes et hétérotrophes inclus dans une matrice extracellulaire et se développant à l'interface entre la colonne d'eau et un support. Les biofilms phototrophes se développent généralement sur toutes les surfaces immergées exposées à la lumière comme, entre autres, les rochers, sédiments et plantes aquatiques (Pouličková *et al.*, 2008; Stevenson, 1996 ; figure 5)

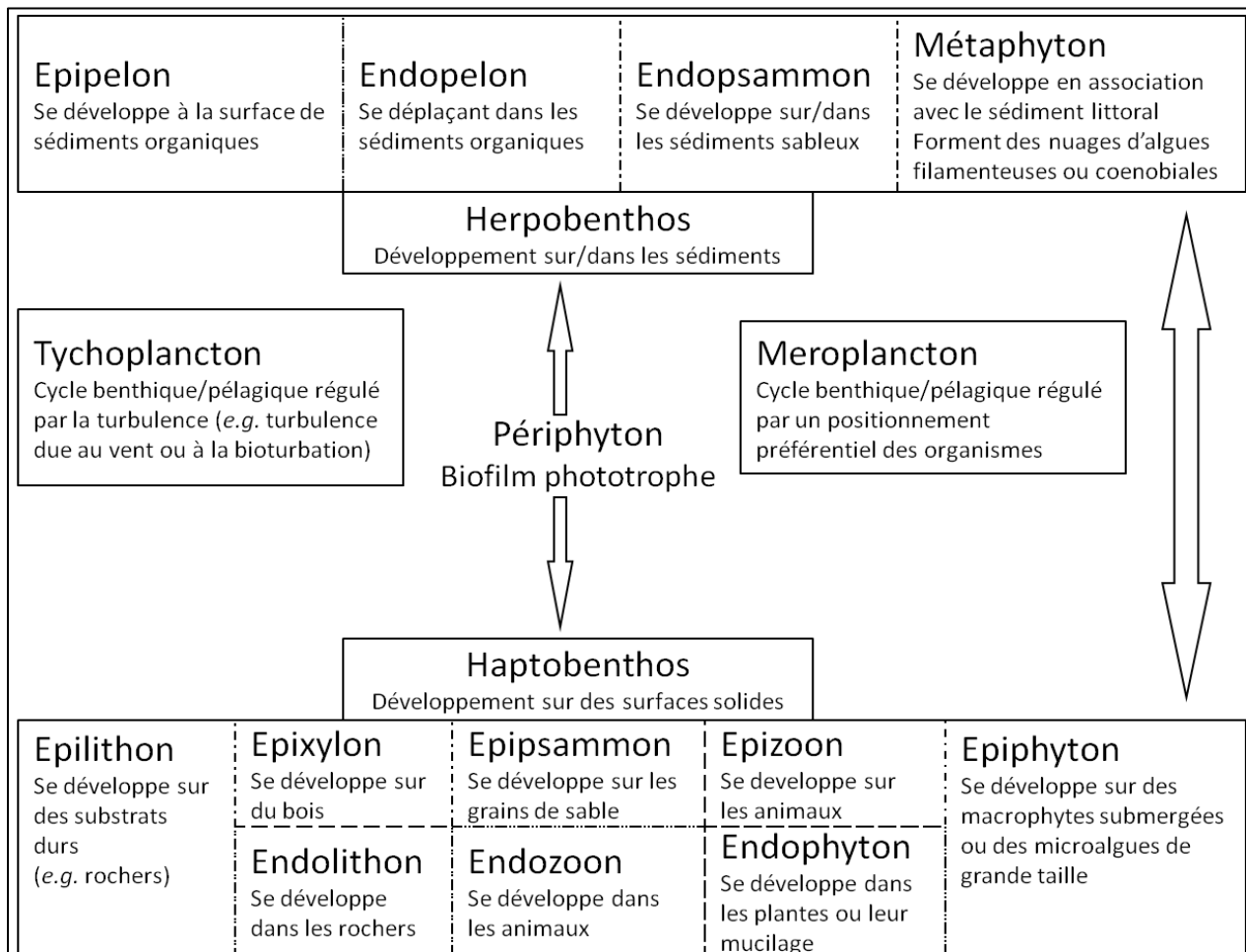


**Figure 5 : Terminologie associée aux différents types de biofilms phototrophes selon la nature du substrat. D'après Pouličková *et al.* (2008).**

Le rôle fondamental du biofilm phototrophe dans de nombreux écosystèmes aquatiques rend la compréhension de son fonctionnement particulièrement importante. Le biofilm phototrophe peut représenter la majeure partie de la production primaire de l'écosystème dans la zone littorale des lacs (Liboriussen and Jeppesen, 2003; Vadeboncoeur *et al.*, 2002) et dans les rivières dans lesquelles le courant est trop important pour permettre le développement du phytoplancton (Vannote *et al.*, 1980). En dépit de son importance pour le fonctionnement et le suivi de milieux aquatiques (Lowe

and Pan 1996), de sa diversité et sa différence marquée avec le système planctonique, le biofilm phototrophe a reçu relativement peu d'attention (Vadeboncoeur *et al.*, 2002).

Même dans les milieux aquatiques dépendant principalement des apports de carbone allochtones, tels que les ruisseaux forestiers, le biofilm phototrophe joue un rôle important. En effet, même si la production primaire des biofilms phototrophes ne contribue que faiblement à l'apport de carbone organique dans ces milieux, ils sont une source importante d'acides gras essentiels pour les organismes détritivores (Danger *et al.*, 2013). Le biofilm contribue de manière importante aux processus hydrologiques et biogéochimiques en augmentant le stockage transitoire de l'eau, en captant les particules organiques en suspension et les polluants (par exemple les éléments traces métalliques) et en limitant la libération de nutriments à partir des sédiments (Battin *et al.*, 2003; Coutaud *et al.*, 2014; Poulíčková *et al.*, 2008). De plus, les biofilms phototrophes sont le siège d'une importante biodiversité. Cette biodiversité est particulièrement remarquable chez les diatomées. McGregor *et al.* (2006) a identifié jusqu'à 73 espèces de diatomées sur un site, la richesse spécifique des diatomées d'un biofilm dépasse fréquemment les 20 espèces (Biggs and Smith, 2002; Jyrkänkallio-Mikkola *et al.*, 2016). La diversité est aussi d'ordre génétique, par exemple, différents génotypes de *Phormidium* sp. ont pu être isolés sur 1cm<sup>2</sup> de biofilm (Wood *et al.*, 2012). Leur forte diversité (et le fait qu'ils soient fixés) fait des biofilms des indicateurs très utilisés pour l'évaluation de la qualité écologique des cours d'eau (Lowe and Pan 1996). Cela renforce l'intérêt de comprendre les mécanismes à l'origine de cette diversité. Comprendre les mécanismes permettant la coexistence de nombreuses espèces est une question centrale en écologie. Dans le cas des organismes phototrophes benthiques, la compétition est très forte pour une poignée de ressources différentes (lumière, azote, phosphore, CO<sub>2</sub> dissous, silice pour les diatomées, vitamines). Les modèles classiques, ne peuvent expliquer cette forte diversité (McCormick, 1996) (*cf* « paradox of the plankton », Hutchinson, 1961). Les fluctuations des conditions environnementales contribuent à expliquer cette diversité (McCormick, 1996), mais d'autres facteurs pourraient jouer un rôle important.

Il est primordial de prendre en compte le fait que le biofilm ne correspond pas seulement à un ensemble d'organismes posés les uns à côté des autres sur un support, mais que le développement en biofilm a des conséquences importantes. La simple diminution de la distance entre les organismes renforce les interactions et notamment la compétition pour l'espace. Cette organisation en biofilm induit également des gradients de ressources : la lumière diminue très rapidement entre la surface du biofilm et le support, le gradient de nutriments dépend de la nature du support et de l'épaisseur du biofilm (McCormick, 1996). Ces différents gradients physicochimiques transforment le biofilm en une communauté hautement structurée (Stoodley *et al.*, 2002) que l'on peut considérer comme des « paysages microbiens » caractérisés par une forte hétérogénéité spatiale (Battin *et al.*, 2007). Il a été

proposé un modèle d'organisation du biofilm (basé sur l'étude de biofilms bactériens) « en champignons » (Costerton *et al.*, 1995), alimenté par des « canaux » permettant le déplacement de l'eau et de substances dissoutes au sein du biofilm (De Beer *et al.*, 1994, 1996). Dans les biofilms matures, la reminéralisation par les hétérotrophes de la matière organique produite par les phototrophes permet un cycle des nutriments très rapide et donc une forte production primaire.

Le biofilm n'est pas seulement composé des microorganismes, bien au contraire, plus de 90% de la masse sèche du biofilm peut être constituée des **substances polymériques extracellulaires (EPS)**. Les EPS forment la matrice du biofilm, ils sont constitués principalement de polysaccharides, de protéines et d'acides nucléiques produits par les organismes du biofilm (Flemming and Wingender, 2010). Cette matrice a de nombreuses fonctions (adhésion du biofilm au substrat, protection contre la dessiccation) mais surtout elle apporte de la cohésion. En effet, c'est la matrice d'EPS qui permet la structuration tridimensionnelle du biofilm et donc la création de micro-habitats très différents. La présence de cette multitude d'habitats pourrait expliquer en partie la forte biodiversité des biofilms. La cohésion du biofilm a aussi comme conséquence de stabiliser les interactions, créant des consortiums stables d'organismes pouvant ainsi coopérer (Flemming, 2009).

Malgré cette stabilisation des interactions par les EPS, les biofilms sont des systèmes dynamiques caractérisés par des cycles de phase d'accrétion (accumulation de biomasse) suivis par des phases d'érosion (détachement du biofilm) (Biggs, 1996). Le détachement du biofilm donne lieu de nouveau à un processus de colonisation, maintenant la communauté en perpétuelle évolution. Un exemple de conséquences concrètes de l'organisation en biofilm est illustré par le fait qu'une même souche de diatomée est plus résistante aux herbicides lorsqu'elle se développe en biofilm que lorsqu'elle est cultivée sous forme planctonique (Larras *et al.*, 2013). Ces avantages de l'organisation en biofilm, en font, d'après Flemming et Wingender (2010), "the most successful forms of life on earth".

Ainsi il est convenu que les organismes organisés en biofilm interagissent beaucoup plus fortement entre eux qu'avec ceux de la colonne d'eau. Le biofilm est donc à considérer comme un microenvironnement séparé de la colonne d'eau bien qu'interagissant fortement avec elle.

### I.3 L'allélopathie dans les biofilms

Il existe deux principaux obstacles limitant l'efficacité de la libération de composés allélopathiques et donc l'existence d'organismes aux propriétés allélopathiques dans les milieux aquatiques planctoniques (Jonsson *et al.*, 2009; Lewis, 1986). Tout d'abord, les distances entre cellules restent toujours relativement importantes (plusieurs fois le diamètre d'une cellule). De cette distance importante résulte une forte dilution des composés lorsqu'ils atteignent une cellule cible. Le coût métabolique de l'allélopathie est donc très fort, à l'exception des blooms où la densité algale devient très importante (Jonsson *et al.*, 2009). De plus, pour les organismes planctoniques, les bénéfices de la production par une cellule de composés allélopathiques seront partagés par tous les organismes non affectés par ces composés et situé à proximité (de la même espèce ou non). Le processus de sélection naturelle favorisera le développement d'individus produisant peu ou pas de composés allélopathiques au sein de la population de micro-algues planctoniques allélopathiques (Lewis, 1986). Le développement sous forme de biofilm réduit l'effet de ces deux obstacles. En effet, les particularités du mode de vie benthique par rapport au planctonique sont en mesure d'impacter fortement les interactions chimiques, et, étant donné l'intense compétition dans les biofilms, les interactions allélopathiques en particulier. La simple réduction de la distance physique entre les cellules facilite les interactions chimiques car les organismes se retrouvent exposés à de plus fortes concentrations de composés allélopathiques. Du fait de cette plus forte exposition, les micro-algues benthiques sont en général plus résistantes aux composés allélopathiques. D'autre part, la plus grande structuration des communautés benthiques modifie l'influence des interactions allélopathiques. En effet, Kerr *et al.* (2002) ont mis en évidence que l'allélopathie permettait une coexistence de différentes espèces uniquement lorsque les organismes (en l'occurrence des bactéries) étaient organisés en biofilms. D'après Durrett et Levin (1997) les espèces produisant des composés allélopathiques ne peuvent avoir évolué que dans des milieux spatialement structurés, tels que les biofilms. L'article de synthèse qui suit est consacré aux interactions allélopathiques impliquant des microorganismes phototrophes benthiques. Il s'agit de la première synthèse consacrée aux interactions allélopathiques dans les biofilms phototrophes depuis plus de 15 ans (Juttner, 1999). Cette synthèse rassemble les informations et réflexions supportant l'hypothèse d'une grande importance de l'allélopathie dans les biofilms. Dans cet article, les organismes marins et dulçaquicoles sont tous deux considérés car les processus impliqués sont globalement similaires. La grande diversité des espèces parmi les trois groupes majeurs de microorganismes phototrophes benthiques (*i.e.* cyanobactéries, diatomées, algues vertes) et la diversité de nature chimique des composés allélopathiques impliqués confirment que l'allélopathie est commune dans les biofilms. La diversité des composés allélopathiques impliqués pourrait témoigner de la coévolution des espèces

allélopathiques et de leurs cibles. Malgré un faible nombre d'études, plusieurs résultats montrent un impact sur les communautés de composés allélopathiques de micro-algues benthiques (Leão *et al.*, 2012; Scholz and Liebezeit, 2012). La dernière partie de cet article explique comment l'importance de l'allélopathie dans les biofilms peut avoir été sous-estimée du fait des méthodes utilisées non-adaptées à l'étude des interactions allélopathiques dans ces systèmes. En effet, la diffusion des composés allélopathiques ayant principalement lieu par diffusion au sein de la matrice de substances extracellulaires et par les canaux du biofilm, l'étude de ces interactions dans des conditions réalistes est très complexe.

Allelopathic interactions involving benthic phototrophic  
microorganisms

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# Allelopathic interactions involving benthic phototrophic microorganisms

Joey L. Allen, Loïc Ten-Hage and  
Joséphine Leflaive\*

ECOLAB, Université de Toulouse, CNRS, INPT, UPS,  
Toulouse, France.

## Summary

**As a way to prevent resource depletion by other species, many phototrophic aquatic microorganisms produce inhibitory compounds. This process, known as allelopathy, has been widely studied in planktonic environments, where it is recognized as being a driving force of planktonic communities. However, in benthic environments, biofilms provide very particular micro-environments. The present review focuses on allelopathic interactions involving benthic phototrophic prokaryotes and micro-eukaryotes ('microalgae'), which generally form biofilms, and includes any interaction involving benthic microalgae either as the emitter or as the target in both marine and freshwater habitats.**

**To support our hypothesis on the importance of allelopathy in biofilms due to the particularities of biofilms, we show that (i) reported allelopathic species and compounds are diverse and numerous in the three major groups of benthic phototrophic microorganisms, (ii) allelopathic benthic species could affect community composition, (iii) allelopathy in biofilms is currently underestimated because of the lack of suitable methods.**

**As benthic primary producers represent an important source of organic carbon in some streams and littoral areas, these interactions could impact the whole ecosystem in these areas, probably more than in areas dominated by planktonic communities.**

## Introduction

Plant allelopathy has been known since ancient Greece and Rome (Willis, 2007), even though the term is much more recent (Molisch, 1937). It may be defined as '*any direct or indirect harmful or beneficial effect by one plant*

*(including microorganisms) on another through production of chemical compounds that escape into the environment'* (Rice, 1984). In this review, 'allelopathy' includes any chemically mediated inhibition of competitors. Toxins have been excluded when their effect is limited to predatory organisms. Allelopathy is a case of interferential competition (one species alters the ability of another to exploit the resources) distinct from exploitative competition where one species affects another only through the resource depletion resulting from resource utilisation (Vance, 1984). Though initially studied in plants, allelopathy appears to play an important role in phototrophic aquatic microorganism communities (Gross, 2003). Aquatic microorganisms can be either free in the water column or fixed on a substrate. The term 'benthic' refers here to organisms that are fixed onto a substrate. In the aquatic environment, every surface exposed to light will become rapidly covered by a biofilm, therefore benthic microalgae are generally included in biofilms. The term 'microalgae' in this review includes both prokaryotic and eukaryotic photosynthetic microorganisms.

Biofilms (phototrophic and heterotrophic) present certain specificities which make them propitious for the development of chemical interactions (Wahl *et al.*, 2012; Fig. 1). According to Durrett and Levin (1997), such spatially-structured environments are the only ones where allelopathy could evolve. Biofilms are characterized by a high density of organisms embedded in a matrix of extracellular polymeric substances. In phototrophic biofilms primary production and remineralisation occur alongside each other. Channels within the biofilm promote mass transfer of nutrient, gases and chemicals (De Beer *et al.*, 1996). Therefore, biofilm organisms interact more with other biofilm organisms than with the water column at least in the case of mature biofilms. This might not be true in the early stages of colonisation.

Considering that the effect of allelopathy depends on the density of target cells (Weidenhamer, 2006), the high cell density in biofilms protects organisms against dilute allelopathic compounds (Fig. 1), and this could partially explain the protective effect of biofilms (Larras *et al.*, 2013). Hilt (2006) has found that epiphytic biofilm species are often more resistant in co-culturing experiments than planktonic species. Benthic microalgae are likely to be exposed to more concentrated chemicals

Received 14 March, 2016; accepted 15 June, 2016. \*For Correspondence. E-mail: josephine.leflaive@univ-tlse3.fr; Tel. +33 5 61 55 85 49; Fax +33 5 61 89 01.

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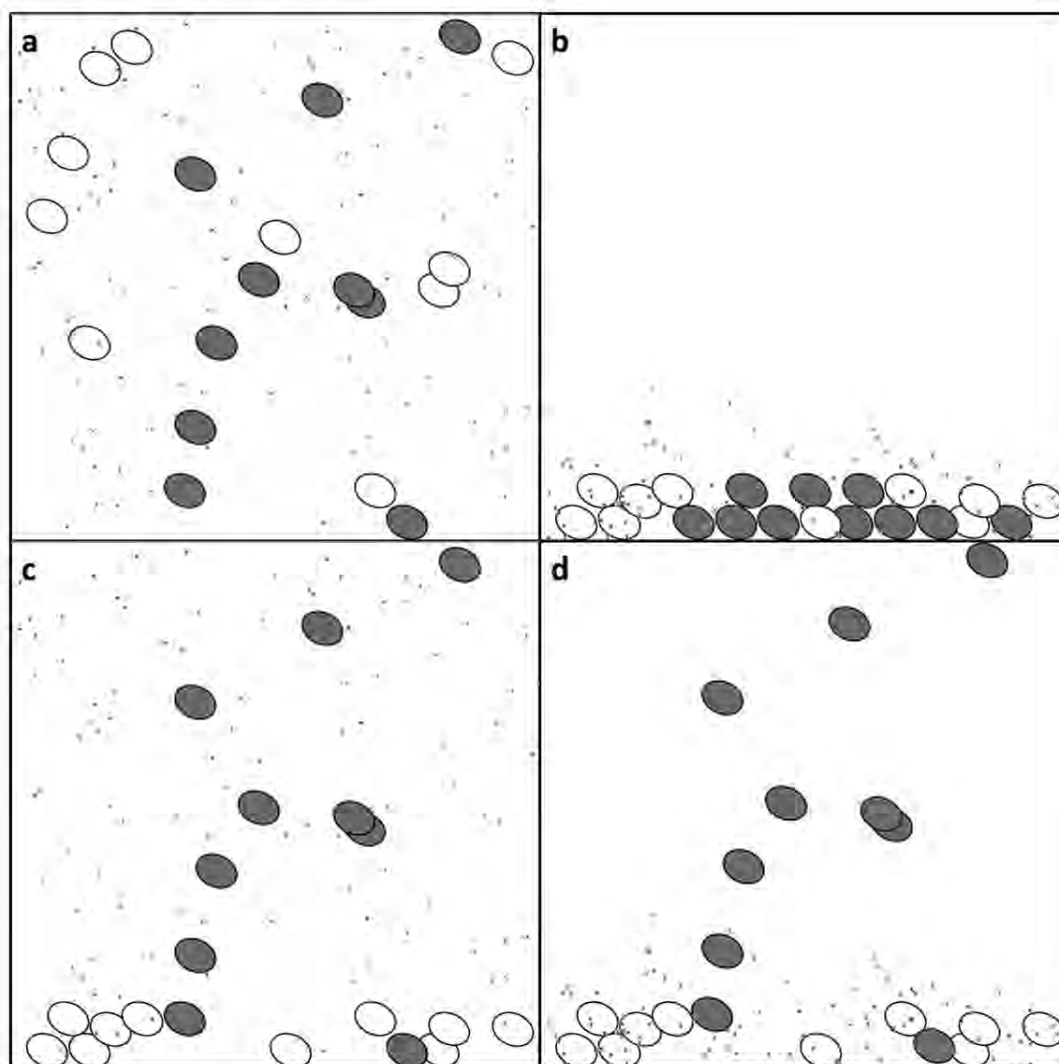


**Fig. 1.** Importance of benthic or planktonic organisation for allelopathic interactions (grey circles: emitter species cells, open circles: target species cells, crosses: allelopathic compounds). In all situations the number of cells and the number of molecules are the same but the target cells are exposed to a variable concentration of allelopathic compound.

- a. Two planktonic species and a water soluble allelochemical.
- b. Two benthic species and a lipophilic allelochemical.
- c. A benthic species and a planktonic species producing a water soluble allelochemical.
- d. A benthic species and a planktonic species with adsorption of the allelochemicals on the biofilm.

exuded by neighbouring cells. Possible frequent exposure to allelochemicals would cause a high selective pressure leading to the appearance of resistant strains. Furthermore, allelochemicals from the water column may become concentrated in the biofilms. For example, biofilms strongly adsorb microcystins produced by the cyanobacterium *Microcystis aeruginosa* (Wu *et al.*, 2010). This accumulation process may favour the allelopathic inhibition of biofilm (Fig. 1).

The benthic or planktonic way of life may also have consequences on the chemical nature of allelopathic compounds. For planktonic species (Fig. 1), allelochemicals

have to be somewhat water soluble to facilitate their transfer to the target cells. Because of dilution, concentrations of excreted molecules decrease very rapidly as the distance from the cell increases, so allelopathy mostly occurs only during blooms when cell density is sufficient for the allelochemicals to reach high concentrations (Jonsson *et al.*, 2009). In the enclosed environment of the biofilm (Fig. 1), lipophilic allelochemicals could become highly concentrated, while remaining practically absent from the surrounding water (Gross *et al.*, 1994). It has been stated that allelochemicals might have been selected for their lipophilicity which is a way to limit dilution (Jüttner, 1999).

Moreover, biofilms often grow in flowing water, therefore soluble compounds would be quickly advected away. Lipophilicity of allelochemicals would therefore be a great advantage for benthic organisms as it limits the loss of chemicals by dilution. This statement is also supported by studies on the effect of herbicides on biofilms. Indeed, although benthic growth provides a certain protection for the cell against herbicides, the protective effect is lowered when the herbicides are lipophilic (Larras *et al.*, 2013) suggesting that lipophilic allelochemicals would be more effective against organisms in biofilms. In fact most of the allelochemicals identified from benthic organisms are lipophilic (Fischerellins or fatty acids for instance) (Table 1).

Despite the particularities and the important contribution of phototrophic biofilms to ecosystem processes, notably to primary production (Liboriussen and Jeppesen, 2003), most studies about chemical interactions between photosynthetic microorganisms have focused on planktonic species. During the last two decades, the importance of such interactions in shaping communities has become more obvious (Ilanora *et al.*, 2006; Pohnert *et al.*, 2007). Though several reviews have considered allelopathy in phototrophic microorganisms (e.g. Gross, 2003; Legrand *et al.*, 2003), only one, published more than 15 years ago, has focused on biofilm phototrophic organisms (Jüttner, 1999).

The present review aims to gather information to access the importance of allelopathy as a factor controlling biofilm structure and function. Biofilm microalgae are in competition among themselves for light, nutrients and space but also with the heterotrophic component of biofilms (bacteria and fungi), planktonic microalgae, macroalgae and macrophytes (aquatic plants) (Fig. 2). Indeed, epiphytic biofilms strongly reduce macroorganism access to light, gases and nutrients (Dodds, 1991; Wahl *et al.*, 2012). All these interactions are considered in this review (Fig. 2, Table 2) and examples provided. The focus has been made on primary producers as they represent the source of organic carbon for most aquatic ecosystems, therefore interactions among microheterotrophs are not included. Furthermore, interactions among biofilm heterotrophs have been largely reviewed elsewhere (Bowman, 2007; Egan *et al.*, 2008; Dobretsov *et al.*, 2013). However, it must be noted that the particular features of biofilms and the methodological issues involved (see below) also apply to heterotrophic microorganisms in biofilms.

### High diversity of reported allelopathic interactions

#### *Interactions between biofilm phototrophs*

Allelopathic species are found among the three main algal groups with biofilm-forming species: cyanobacteria, diatoms and green algae. The interactions are

summarized in Table 2. Dinoflagellates are not included because only one study has considered benthic dinoflagellates and no allelopathic effects were observed (Sugg and VanDolah, 1999). Cyanobacterial toxins have been extensively studied because of the health hazards they represent. Other metabolites produced by cyanobacteria have raised considerable interest because of their industrial or pharmaceutical applications (for review see Berry *et al.*, 2008). Research into the roles of these compounds (e.g. allelopathy, defence) is late compared to their screening, but the allelopathic properties of some species (e.g. *Fischerella* sp.) have been intensively studied for several decades (Flores and Wolk, 1986). Smith and Doan (1999) stated that six genera, all containing benthic species, (*Fischerella*, *Nostoc*, *Calothrix*, *Anabaena*, *Scytonema* and *Oscillatoria*), have been commonly found to produce allelopathic compounds. Since publication of their review (Smith and Doan, 1999), several other genera have been added to the list (Table 1), with some new cases being recently reported (Leao *et al.*, 2010).

Interspecific chemical interactions in diatoms have been poorly studied despite their diversity and abundance in all aquatic habitats. However, recent studies have revealed that allelochemicals from benthic diatoms are diverse: eicosapentaenoic acid, a polyunsaturated fatty acid and 2E,4E-decadialenal, a polyunsaturated aldehyde (Leflaive *et al.* 2008a; Leflaive and Ten-Hage, 2009), polyphenolic compounds (Pouvreau *et al.*, 2007), cyanogen bromide (BrCN) a halogenated compound (Vanellander *et al.*, 2012). All these compounds are produced by benthic diatoms and target co-occurring benthic diatoms.

Fewer screenings to discover new chemicals have been carried out with biofilm-forming green algae compared to diatoms and cyanobacteria. However, several studies have demonstrated that filamentous green algae (FGA) use chemicals to ensure their dominance (Leflaive *et al.*, 2008b; Trochine *et al.*, 2011). Biofilm forming green algal allelochemicals have rarely been identified, but it has been shown that one species, *Uronema confervicolum*, probably produces an array of different allelopathic compounds (Allen *et al.*, 2015).

#### *Benthic microalga–planktonic microalga interactions*

Allelopathic inhibition of planktonic organisms by benthic algae has been described in several studies (Table 2), despite the fact that allelochemicals produced by benthic microalgae would be highly diluted in the overlaying water, making the cost to the benthic microalgae of inhibiting planktonic algae very high. Considering the lack of apparent relevancy of such phenomena, these interactions could be explained as cases of unintentional allelopathy (Sinkkonen, 2006). The primary target could possibly be another benthic organism, but, in favourable

**Table 1.** Examples of allelopathic benthic microalgae, their target, the allelopathic compounds when identified and the methods employed.

| Green Algae                         |                                                                  |                                               |                                     |                                                 |
|-------------------------------------|------------------------------------------------------------------|-----------------------------------------------|-------------------------------------|-------------------------------------------------|
| Emitter                             | Target                                                           | Compound                                      | Effect                              | Methods                                         |
| <i>Spirogyra</i> sp.                | <i>Elodea nuttallii</i>                                          | –                                             | None                                | Co-culturing                                    |
| <i>Spirogyra</i> sp.                | Phytoplankton                                                    | –                                             | None                                | Co-culturing, filtrates                         |
| <i>Spirogyra</i> sp.                | Phytoplankton                                                    | –                                             | Inhibition                          | Co-culturing                                    |
| <i>Spirogyra</i> sp.                | <i>Oscillatoria</i>                                              | –                                             | Stimulation                         | Biomass extracts                                |
| <i>Cladophora glomerata</i>         | <i>Nitzschia fonticola</i>                                       | –                                             | Lower photosynthetic rate           | Extracts                                        |
| <i>Cladophora</i> sp.               | Phytoplankton                                                    | –                                             | Inhibition                          | Co-culturing, filtrates*                        |
| <i>Cladophora</i> sp.               | <i>Desmodesmus</i> spp.                                          | –                                             | Inhibition                          | Co-culturing                                    |
| <i>Uronema confervicolum</i>        | <i>N. palea</i> , <i>F. saprophila</i>                           | $\alpha$ -linolenic [5.9]linoleic acids [6.8] |                                     | Biomass extracts                                |
| <i>Uronema confervicolum</i>        | <i>Desmodesmus quadricolor</i>                                   |                                               | Colony induction, growth inhibition | Biomass extracts, filtrates*                    |
| Diatoms                             |                                                                  |                                               |                                     |                                                 |
| Emitter                             | Target                                                           | Compound                                      | Effect                              | Methods                                         |
| <i>Nitzschia pellucida</i>          | Benthic diatoms                                                  | Cyanogen bromide (BrCN) [1.1]                 | Chloroplast bleaching, cell lysis   | Co-culture, filtrates, pure compounds*          |
| <i>Nitzschia pellucida</i>          | Marine bacteria                                                  | Halogenated compounds                         | Quorum sensing inhibition           | Co-culture, pure compounds*                     |
| <i>Haslea ostrearia</i>             | Benthic diatoms                                                  | Marrerin (polyphenolic pigment) <sup>a</sup>  | Growth inhibition                   | Purified compounds*                             |
| <i>Phaeodactylum tricornutum</i>    | Marine bacteria                                                  | Eicosapentaenoic acid [5.6]                   |                                     | Purified compounds*                             |
| Periphyton biofilm (mainly diatoms) | <i>Microcystis aeruginosa</i>                                    | Indole [2.1], 3-oxo-a-ionone [1.6]            |                                     | Biomass extracts                                |
|                                     |                                                                  |                                               |                                     | Co-culture, extracts, pure compounds*           |
| Cyanobacteria                       |                                                                  |                                               |                                     |                                                 |
| Emitter                             | Target                                                           | Compound                                      | Effect                              | Methods                                         |
| <i>Nodularia harveyana</i>          | <i>Arthrospira (Spirulina) laxissima</i> , Benthic cyanobacteria | Norharmane [3.2]                              |                                     | Medium extracts                                 |
| <i>Nostoc spongiaeforme</i>         | Planktonic green algae                                           | ND                                            |                                     | Purified compounds                              |
| <i>Lyngbya aestuarii</i>            | <i>Lemna minor</i>                                               | ND, nostocine A [–2.69]                       | Induction of ROS                    | Purified compounds                              |
| <i>Phormidium</i>                   | Phytoplankton                                                    | Fatty acid (2.5 dimethyldecanoic) [5.6]       |                                     | Biomass extracts                                |
| <i>Fischerella muscicola</i>        | Phytoplankton                                                    | –                                             |                                     | Biomass extracts                                |
| <i>Lyngbya bouillonii</i>           | Coral                                                            | Fischerellin A & B [5.7]                      |                                     | Biomass extracts*                               |
| Cyanobacterial mats                 | Diatoms, bacteria                                                | –                                             |                                     | Co-culture                                      |
| <i>Oscillatoria</i> sp.             | <i>Chlorella vulgaris</i>                                        | Portoamide A & B (cyclic peptide)             |                                     | Biomass extracts                                |
| <i>Oscillatoria</i> sp.             | <i>Ankistrodesmus falcatus</i>                                   | –                                             |                                     | Filtrate, biomass extracts, purified compounds* |
| <i>Scytonema holmannii</i>          | Eukaryotic microorganisms                                        | Cyanobacterin [4.5]                           |                                     | Filtrates*                                      |
|                                     |                                                                  |                                               |                                     | Purified compound                               |

Referenced articles are those which report and allelopathic effects of the emitter, against a competitor, and not those that identified the compound. ND, not determined. Asterisk indicates when the methods used were sufficient to clearly demonstrate an allelopathic effect. Species in bold: marine or brackish water organisms. Numbers in brackets indicate the log  $K_{ow}$  (Octanol/Water partition coefficient) of the compounds (based on Pubchem computed coefficient: [www.ncbi.nlm.nih.gov/pccompound](http://www.ncbi.nlm.nih.gov/pccompound)).

<sup>a</sup>No log  $K_{ow}$  but high water solubility and very low solubility in organic solvent (Pouvreau *et al.*, 2006).

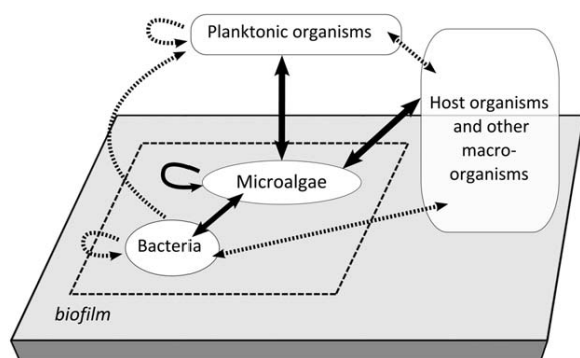


**Fig. 2.** Examples of allelopathic interactions in aquatic environments involving benthic microorganisms. Solid arrows indicate the interactions considered in the review, dashed arrows indicate allelopathic interactions where benthic phototrophic microorganisms are not involved.

conditions for benthic microalgae to form a thick biofilm, the amount of compound that escapes from the biofilm could be sufficient to inhibit planktonic algae, particularly in shallow water with low flow, where biofilms can reach a high level of biomass and productivity. Some studies show that biofilms can limit the density of planktonic phototrophic microorganisms (Dondajewska and Budzyska, 2009), but other studies suggest that the effects of FGA-dominated biofilms are not chemically mediated but are rather linked to resource depletion (Irfanullah and Moss, 2005). Nevertheless, Wu *et al.* (2011; 2014) have reported that the cyanobacterium *Microcystis aeruginosa* is inhibited by diatom-dominated biofilm allelochemicals. Trochine *et al.* (2011) have observed a decrease in phytoplankton density when exposed to FGA. Cell-free filtrate of benthic FGA cultures induces the formation of colonies by the planktonic green alga *Desmodesmus quadricapsa* (Leflaive *et al.*, 2008b). Colony formation reduces the buoyancy of this alga and increases its sedimentation rate (Lüring, 2003). This mechanism could be a way for *U. confervicolum* to reduce competition for light by planktonic algae. The last three examples exclude involvement of nutrient depletion, supporting the hypothesis of possible unintentional allelopathic inhibition of planktonic algae by benthic microalgae.

#### *Benthic microalga–microheterotroph interactions*

Even though phototrophic microorganisms generally represent the major part of phototrophic biofilm biomass, heterotrophic microorganisms are an important component of phototrophic biofilms and play an important role in chemical interactions. Heterotrophs allow nutrient cycling, therefore they are necessary for the high productivity of biofilms. Furthermore bacteria are often involved in mutualistic relationships with phototrophs. Competition between bacteria and microalgae is high in

#### *Allelopathy in phototrophic biofilms* 5

phosphorous-limited conditions due to greater P immobilisation by bacteria than by algae (Danger *et al.*, 2007). Yet, antibacterial, quorum-sensing inhibitors and antifungal compounds have often been isolated from biofilm phototrophs (Volk and Furkert, 2006; Syrpas *et al.*, 2014) some of them being naturally released into the medium (Gastineau *et al.* 2012). Such compounds might be specifically targeted against 'useless' or pathogenic bacteria or fungi, and allow the emitter to select the bacterial community by inhibiting some strains and stimulating others (Gantar *et al.*, 2011; Morrow *et al.*, 2011). Another possibility is that such compounds are produced by organisms with relatively slow growth rate that will not take advantage of higher nutrient cycling as can be found in terrestrial allelopathy (Vokou *et al.*, 2006).

Benthic microalgae can also be the target of allelochemicals produced by benthic bacteria. They have notably been shown to produce compounds preventing the adhesion of microalgae and the formation of biofilms. For example, marine bacteria of diverse genera, isolated from the surface of macroalgae exhibit activity against diatoms (Kumar *et al.*, 2011; Silva-Aciades and Riquelme, 2008). Rao *et al.* (2007) have determined that a low density of *Pseudoalteromonas tunicata* ( $10^2$  to  $10^3$  cells  $\text{cm}^{-2}$ ), similar to that occurring on macroalgal thalli, could be sufficient to inhibit the settlement of target organisms. This suggests that the chemical interaction between *P. tunicata* and benthic diatoms, described in laboratory experiments, may actually occur in natural environments. The ecological role of these compounds could be part of a mutualistic relationship between macroalgae and bacteria (Egan *et al.*, 2013).

#### *Benthic microalga–macroorganism (macrophytes, macroalgae, sessile invertebrates) interactions*

Fouling by microalgae can also be directly inhibited by macroalgal metabolites. Extracts of numerous marine macroalgae inhibit diatom-dominated biofilm formation, though the targeted species are different for each extract (Lam *et al.*, 2008). Many studies have revealed that allelochemicals are surface-associated in macroalgae (Lane *et al.*, 2009; Andras *et al.*, 2012). Freshwater macrophytes have been found to allelopathically inhibit epiphytic FGA (Hilt *et al.*, 2012). In a mesocosm study, macrophytes inhibited biofilm growth, and, although the respective roles of exploitative competition and allelopathy were not determined, allelopathy was recognized as an important factor (Gette-Bouvarot *et al.*, 2015). Corals have also shown their ability to inhibit fouling by microalgal competitors (Bak and Borsboom, 1984; Maida *et al.*, 2006).

In benthic environments, the large number of allelopathic interactions and diversity of compounds described in the three major groups of benthic algae is consistent

Table 2. Examples of studies reporting or searching for allelopathic interactions involving benthic microalgae.

| Emitter                   | Benthic microalgae                                           |                                                                                  |                                                             |                          |                                                                                                 | Target                                                                                                                      |                                                                |                |
|---------------------------|--------------------------------------------------------------|----------------------------------------------------------------------------------|-------------------------------------------------------------|--------------------------|-------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------|----------------|
|                           | Green algae                                                  | Diatoms                                                                          | Cyanobacteria                                               | Dinoflagellates          | Bacteria                                                                                        | Planktonic microalgae                                                                                                       | Others                                                         | Macroorganisms |
| <i>Benthic microalgae</i> |                                                              |                                                                                  |                                                             |                          |                                                                                                 |                                                                                                                             |                                                                |                |
| Green algae               | -                                                            | Leflaive <i>et al.</i> (2008a);<br>Dodds (1991);<br>Allen <i>et al.</i> (2015)   | Mohamed (2002)                                              | -                        | -                                                                                               | Leflaive <i>et al.</i> (2008b); Trochine <i>et al.</i> (2011); Dondajewska and Budzynska (2009); Irfanullah and Moss (2005) | Irfanullah and Moss (2005)                                     |                |
| Diatoms                   | -                                                            | Vanelslander <i>et al.</i> (2012);<br>Pouvreau <i>et al.</i> (2007)              | Scholz and Liebezeit (2012)                                 | -                        | Desbois <i>et al.</i> (2010);<br>Syrpas <i>et al.</i> (2014);<br>Gaslineau <i>et al.</i> (2012) | Wu <i>et al.</i> (2011)                                                                                                     | -                                                              |                |
| Cyanobacteria             | -                                                            | Scholz and Liebezeit (2012);<br>Dobretsov <i>et al.</i> (2011)                   | Hirata <i>et al.</i> (2003);<br>Gross <i>et al.</i> (1994)  | -                        | Dobretsov <i>et al.</i> (2011);<br>Gantar <i>et al.</i> (2011);<br>Morrow <i>et al.</i> (2011)  | Hirata <i>et al.</i> (2003); Lopes and Vasconcelos (2011);<br>Barreiro and Vasconcelos (2014); Leao <i>et al.</i> (2010)    | Entzeroth <i>et al.</i> (1985); Tiliyanov <i>et al.</i> (2007) |                |
| Dinoflagellates           | -                                                            | -                                                                                | -                                                           | Sugg and VanDolah (1999) | -                                                                                               | -                                                                                                                           | -                                                              |                |
| Others                    |                                                              |                                                                                  |                                                             |                          |                                                                                                 |                                                                                                                             |                                                                |                |
| Bacteria                  | -                                                            | Wigglesworth-Cooksey and Cooksey (2005); Silva-Aciaras and Riquelme (2008)       | -                                                           | -                        | -                                                                                               | -                                                                                                                           | -                                                              |                |
| Planktonic microalgae     | Leflaive <i>et al.</i> (2008b);<br>Song <i>et al.</i> (2013) | Lam <i>et al.</i> (2008); Bak and Borsboom (1984);<br>Maida <i>et al.</i> (2006) | Barreiro and Vasconcelos (2014);<br>Erhard and Gross (2006) | -                        | -                                                                                               | -                                                                                                                           | -                                                              |                |
| Macroorganisms            | Hilt <i>et al.</i> (2012);<br>Erhard and Gross (2006)        | -                                                                                | -                                                           | -                        | -                                                                                               | -                                                                                                                           | -                                                              |                |

References in bold: marine or brackish water organisms.

with the hypothesis of an important role of allelopathy in biofilms. The high diversity of compounds indicates that allelopathic species have been selected during evolution, therefore allelopathic compounds produced are important for the producer. However, other functions cannot be excluded.

### Role of allelopathy in structuring communities

The importance of allelopathy in population dynamics and species coexistence has been demonstrated by mathematical modelling (Czárán *et al.*, 2002; Roy and Chattopadhyay, 2007; Mougi, 2013). The models are based on the hypothesis that the production of allelochemicals allows allelopathic species to outcompete sensitive species, but reduces their growth rate due to the cost of producing allelochemicals and defending themselves against the autotoxicity of allelochemicals. When allelopathic species have outcompeted sensitive ones, the benefit of allelochemical production is low and the cost is still high. Therefore species (or strains) resistant to allelochemicals would outcompete allelopathic species because they do not have to support the cost of producing the allelochemicals. Since allelopathic species density is reduced, sensitive species are no longer controlled by allelochemicals and would outcompete resistant species because they do not support the cost of protection against allelochemicals. These relationships lead to a cyclic or stable co-existence. The existence of such circular competitive networks has also been proposed to explain species coexistence (Laird and Schamp, 2006). Such networks lack experimental evidence (Wilson, 2011) except in sessile invertebrate communities (Jackson and Buss, 1975) and in bacterial communities (Kerr *et al.*, 2002). For microbial communities, a spatially-structured environment (e.g. a biofilm) is necessary for the promotion of species co-existence by allelopathy (Durrett and Levin, 1997; Kerr *et al.*, 2002). Further research in this field is needed since experimental evidence with phototrophic organisms is still lacking.

Allelopathy has been shown to increase biodiversity in benthic microalgae (Leao *et al.*, 2012). Leao *et al.* (2012) have tested the effect of the benthic *Oscillatoria* sp. allelochemicals on the natural phytoplankton community. In their study growth rates of several taxa decreased, including *Microcystis* except a particular genotype of *Microcystis*. This reveals that intra-specific variability is promoted by allelopathy and could be a population defence mechanism to allelochemicals.

As suggested by modelling, the production of allelochemicals can counterbalance slow growth and low nutrient uptake rates and can allow the emitter to outcompete other species in certain nutritive conditions or to co-exist in others. This has been demonstrated for

the benthic cyanobacterium *Oscillatoria* sp. that had lower growth and nutrient uptake rates than the green alga *Ankistrodesmus falcatus*, but outcompeted this species in low and high nitrogen conditions due to a strong allelopathic effect observed with filtrate (Barreiro and Vasconcelos, 2014). A second example is provided by a study by Scholz and Liebezeit (2012) who have carried out co-culture experiments. In two- and three-species experiments, the N<sub>2</sub>-fixing cyanobacteria outcompeted diatoms in all but one case. The result of a six-species culture (three diatoms and three cyanobacteria) was more surprising: one diatom species dominated after 30 days and diatom density was two to tenfold higher than cyanobacteria density after 120 days. Supernatant extract exhibited anticyanobacterial activity, therefore the unexpected diatom persistence and dominance could be explained by the production of allelochemicals.

Biofilm allelopathy could also play a role in stabilisation of clear-water states. Indeed, phototrophic biofilms have been experimentally used to control the planktonic cyanobacterium *Microcystis aeruginosa* (Wu *et al.*, 2011; 2014).

### Do we have suitable methods to study allelopathic interactions involving benthic microorganisms?

Choosing the design of an experiment when studying allelopathy is a trade-off between ecological relevance and exclusion of exploitative competition (Gross, 2003). To demonstrate allelopathic interactions it should be shown that one or several chemicals could induce deleterious effects on a target species and that, in their natural environment, target cells are exposed to sufficiently large quantities of this compound (Sinkkonen, 2006). The last point is, here, the most important because for benthic algae, the natural environment is the biofilm rather than the surrounding water. Most of the methods used to study allelopathy in plankton have also been applied to benthic microalgae while many are not suited to the context of the benthic environment. For example the use of cell-free culture filtrate is one of the most frequently used methods for the study of planktonic and benthic organisms (e.g. Barreiro and Vasconcelos, 2014). Considering the interactions between two benthic microorganisms, the concentration of allelochemicals in the biofilm will be much higher in the vicinity of the target organisms where the emitter is also present, than in cell-free culture filtrate even if the allelochemical is water soluble. Hence, allelopathic interactions will be underestimated when model organisms are benthic algae.

The same problem occurs with co-culturing experiments. The technique consists of separating the competing species with a membrane permeable to the allelochemicals. This method is very useful as it can be

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used *in situ* (Hilt *et al.*, 2012). Its drawbacks are that benthic organisms, which are naturally in close contact with each other, are separated, and this may lower their exposure to the allelochemicals. As for the previous method, the membrane prevents direct cell-to-cell transfer of allelochemicals and targets are exposed to lower concentrations than if they are inside the biofilm. Moreover, it does not eliminate exploitative competition.

Considering these two points, for more relevancy, benthic organisms (emitters and targets) should not be separated in experiments. This kind of experiment is generally referred to as 'co-existence' experiments. For instance, the agar diffusion assay (ADA) is a classic method (Flores and Wolk, 1986). The ADA consists in growing the emitter on solid medium. Once colonies of the emitter have grown, they are overlaid by agar containing the indicator strain. The diffusion of allelochemicals through the medium leads to the formation of an inhibition area. The conditions used to culture algae on a solid medium are far from natural but are more ecologically relevant for biofilm organisms than for planktonic ones. The diffusion process within mucilaginous matrices will not be very different from that occurring in the agar medium (Jüttner, 1999).

Co-existence experiment does not allow separating clearly interferential chemical interactions (i.e. allelopathy) and exploitative competition. To be achieved, this requires considering the dynamics and the aspect of the cultures in detail. Indeed, in some cases, the effects of allelopathy can be very different from those induced by nutrient limitation. Rapid cell lysis, chloroplast bleaching within a few hours or formation of colonies, aggregates or abnormal cells are the likely result of an allelopathic effect. For instance, Vanelislander *et al.* (2012) have observed massive cell death within 24 h with a small quantity of emitter cells, which was certainly linked to allelopathic activity. Not every allelochemical provides such 'spectacular' effects easily recognizable as allelopathic in origin. In such cases, a good solution to separate the effects of nutrient competition from allelopathy is to study the density-dependency of allelopathy. This method have been developed for plants in a terrestrial environments (Weidenhamer, 2006). It is based on the observation that '*in a given soil volume containing a finite amount of phytotoxin, plants growing at low density have a larger amount of the toxin available per plant*' (Weidenhamer, 2006). This statement has been made for plants, but similar patterns can be observed in algae. Indeed it has been observed that extracts of the allelochemical producing FGA *Uronema confervicolum* had less effect on the diatoms *Nitzschia palea* and *Fistulifera saprophila* at  $1.10^6$  cell mL<sup>-1</sup> or a higher cell density than when diatom cell density was  $5.10^5$  cell mL<sup>-1</sup> (personal observations by JL). An increasing number of cells

leads to a decreasing amount of allelopathic compounds per target cell and, therefore, to a reduced effect of the compounds. The use of increasing density of the target alga and a constant density of the emitter leads to a decreasing effect of allelopathy and an increasing growth rate (compared to less dense cultures). If there is no allelopathic effect, the growth rate will be constant or will decrease with an increase in the density of the organisms, as competition will be enhanced.

All the above methods are used to determine whether allelopathic interactions occur between benthic microorganisms. Many studies use biomass extracts which could be tested in different bioassays. This method has been used by Scholz and Liebezeit (2012) for the screening of allelopathic activities of benthic diatoms and cyanobacteria. Biomass extracts showed antialgal activity, phytochemical screening indicated the presence of typical families of allelochemicals. However, the authors have not been able to link these results to possible allelopathic activity because of the lack of evidence that the compounds could actually be transmitted to a target cell. Indeed biomass extract would also contain compounds that are never released from the cell. Providing evidence that allelochemicals are excreted when they do not diffuse into the surrounding medium is very difficult. Lipophilic adsorbents have been used by Gross *et al.* (1994) to accumulate the chemical compounds that are transferred only within the biofilm. Allelochemicals produced by a benthic cyanobacterium were found bound to C-18 adsorbent beads and in the biomass, but not in the medium, indicating direct contact transmission or low diffusion of the allelochemicals.

## Conclusions

In this review, we have gathered arguments in support of the statement that allelopathy is an important process in biofilms, indeed, it may play a more important role in benthic than in planktonic organisms. It may thus seem paradoxical that allelopathic interactions have been less described in benthic than in phytoplanktonic microalgae. Besides the technical difficulties when working with fixed organisms, because of the co-evolution between emitters and targets, allelopathy may not be visible at a given moment even if it is responsible for the community structure observed. Co-evolution of allelopathic species and targets would also promote the production of novel weapons against resistant microalgae. This could explain the very large array of allelochemicals produced by benthic organisms (from the low molecular weight cyanogen bromide to much more complex compounds like the portoamides). Therefore, we probably have access to only a very small proportion of the great diversity of compounds produced by benthic microalgae.

However, priority should not be given to screenings and allelochemical identification but to the development of experiments with complex communities, as is done in ecotoxicology (e.g. McClellan *et al.*, 2008). In this way biofilms could be used as model communities for general questions on allelopathic interactions and particularly to study the influence of allelopathy on community structure (difficult to test on terrestrial plants).

The development of 'omics' should allow rapid progress in allelopathy research. Particularly, metabolomics could be very useful for allelochemical identification (Prince and Pohnert, 2010). These methods have shown their efficiency in terrestrial environments (Jandová *et al.*, 2015). Moreover, metabolomics allows the study of allelochemicals even at ultralow concentrations (Weston *et al.*, 2015). Metabolomics and proteomics can also enable a better understanding of target responses to allelochemical exposure (Poulson-Ellestad *et al.*, 2014). Transcriptomics could be used for the same purpose as in ecotoxicology (Guo *et al.*, 2016). Progress should be made to find new methodologies suited to the particularities of biofilms such as cell-to-cell transfer and low diffusion.

Allelopathy should be recognized as one of the driving forces underlying biofilm composition and researchers should not only focus on abiotic factors and trophic interactions but also consider interferential competition. Simple assays for allelopathy can easily be included when biofilm communities are studied.

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## Chapitre II

*Uronema confervicolum* (Chlorophyta), une algue verte  
benthique potentiellement allélopathique



## II. *Uronema confervicolum* (Chlorophyta), une algue verte benthique potentiellement allélopathique

### II.1 Présentation du modèle – les algues vertes filamenteuses.

Les algues vertes filamenteuses ne correspondent pas à un groupe phylogénétique précis. Parmi les algues vertes (Chlorophyta) on trouve des formes filamenteuses dans divers classes : Chlorophyceae (e.g. *Oedogonium grande*), Ulvophyceae (e.g. *Uronema confervicolum*), Cladophorophyceae (e.g. *Cladophora glomerata*) et Zygnematophyceae (e.g. *Spirogyra porticalis*). Ces formes partagent un même mode de vie (filaments fixés) et des caractéristiques écologiques proches. On les retrouve dans les milieux marins et dulçaquicoles.

Les algues vertes filamenteuses se développent principalement dans la zone eulittorale des lacs (Lowe, 1996) et en rivière lorsque l'intensité lumineuse est forte (Biggs, 1996). En règle générale, les algues vertes filamenteuses sont associées aux surfaces fortement éclairées (Hill, 1996) et de très forts développements (figure 6a) peuvent en être observés dans des milieux fortement enrichis en phosphore (Stevenson *et al.*, 2012). Certaines algues vertes filamenteuses sont généralement fortement colonisées par des bactéries et des microalgues épiphytes, particulièrement des diatomées (figure 6b), formant un biofilm autour des filaments (Lowe, 1996; Zulkifly *et al.*, 2013; figure 6b). La présence d'algues vertes filamenteuses accroît fortement la surface disponible pour les organismes épiphytes (Zulkifly *et al.*, 2013). La surface fonctionnelle de la zone littorale des lacs peut ainsi être augmentée d'un facteur 2000 par la présence d'algues vertes filamenteuses, en particulier de l'espèce *Cladophora glomerata*. Les algues vertes filamenteuses créeraient une multitude de niches écologiques et seraient de ce fait des espèces clefs dans les écosystèmes aquatiques (Lowe, 1996; Zulkifly *et al.*, 2013). Le développement d'épiphytes sur les filaments a cependant de nombreuses conséquences négatives pour les algues vertes filamenteuses, notamment la réduction de l'accès à la lumière, aux nutriments et aux gaz dissous (Dodds, 1991; Dodds and Gudder, 1992).

Les algues vertes filamenteuses sont généralement associées à une faible abondance de phytoplancton (Irfanullah and Moss, 2005a) dont elles pourraient être responsables (Peretyatko *et al.*, 2007). Plusieurs hypothèses ont été proposées pour expliquer cet effet : la consommation des nutriments par les algues vertes filamenteuses, la création de refuges pour la méiofaune qui augmenterait la pression d'herbivorie sur le phytoplancton ou encore un effet allélopathique des algues vertes filamenteuses contre le phytoplancton (Dondajewska and Budzynska, 2009; Irfanullah and Moss, 2005a, 2005b; Trochine *et al.*, 2011). En effet, certaines algues vertes filamenteuses

produiraient des composés inhibant le développement des diatomées épiphytes (Dodds, 1991). Mais l'hypothèse d'un effet allélopathique est controversée du fait notamment de résultats indiquant une absence d'effet (Irfanullah and Moss, 2005b) ou même une stimulation de cyanobactéries par les exsudats de l'algue verte filamenteuse *Spirogyra* sp. (Mohamed, 2002).

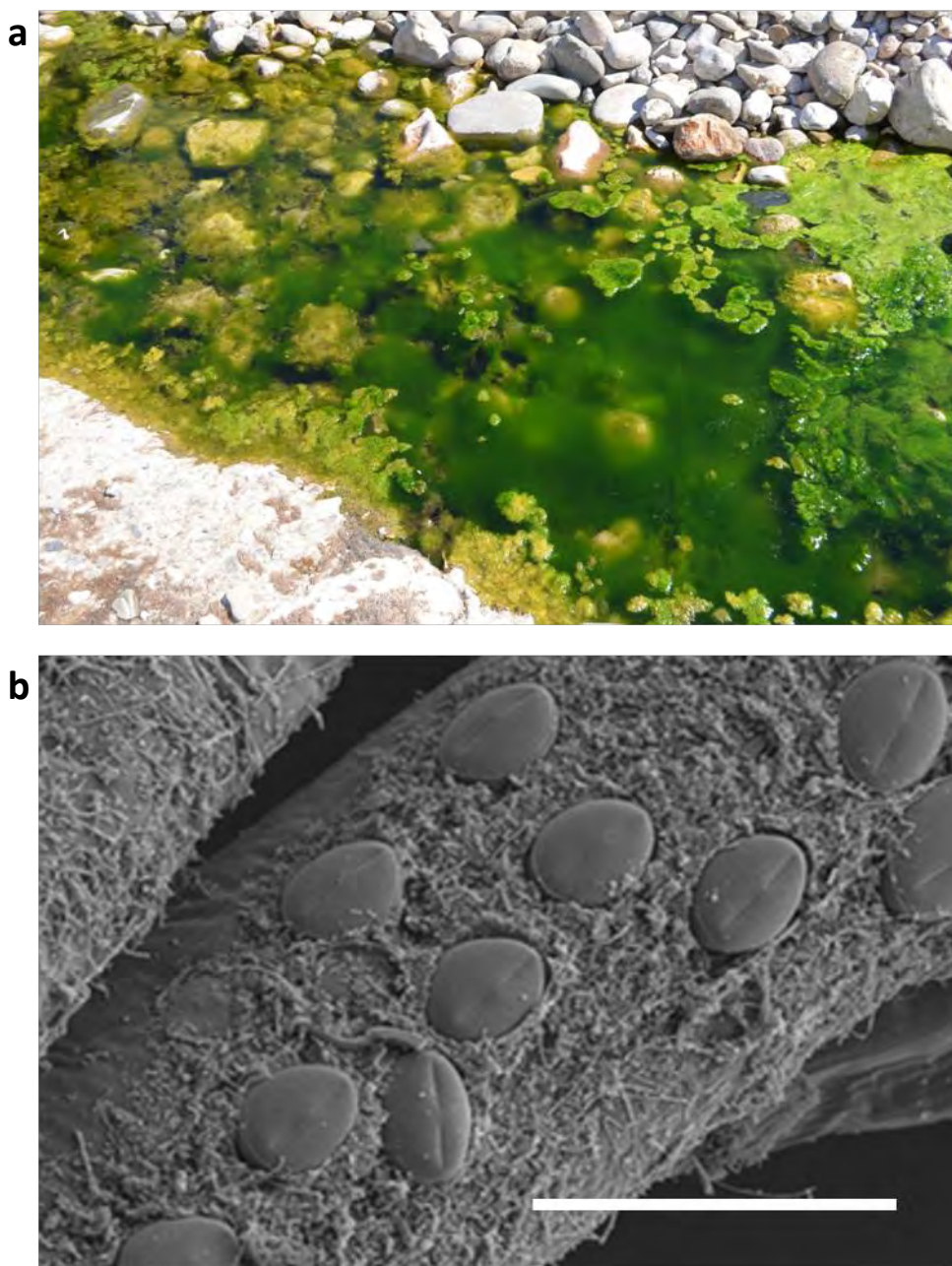


Figure 6 : (a) Ecosystème aquatique dominé par des algues vertes filamenteuses (photographie par J. Leflaive, Rivière Tarn) et (b) Image en microscopie électronique à balayage de filaments de *Cladophora glomerata* colonisé par des diatomées. Barre d'échelle : 50  $\mu$ m. Extrait de Zulkifly *et al.* (2013).

## II.2 Activité allélopathique d'*Uronema confervicolum* : implication d'acides gras polyinsaturés.

Contrairement à d'autres espèces d'algues vertes filamenteuses, très peu de diatomées épiphytes sont observées sur les filaments d'*U. confervicolum* *in situ* ou en co-culture (observations personnelles). Des études précédentes menées au sein du même groupe de recherche ont mis en évidence la présence de composés bioactifs dans des extraits d'*U. confervicolum* (Leflaive *et al.*, 2008). Un screening réalisé utilisant des tests de diffusion sur agar avait également indiqué le fort potentiel allélopathique de cet organisme. Ces observations ont permis de suspecter un effet allélopathique de cette algue verte dirigé contre les diatomées. Le but de l'étude présentée dans ce chapitre était de vérifier l'hypothèse de l'existence d'une telle interaction, de caractériser ces effets et d'identifier le ou les composés impliqués.

Pour évaluer dans quelle mesure les composés allélopathiques produits par *U. confervicolum* affectent les diatomées benthiques, différents tests ont été réalisés. Les paramètres mesurés reposent d'une part sur des fonctions communes à tout organisme photosynthétique (croissance et photosynthèse) et d'autre part sur des fonctions particulièrement liées au mode de vie benthique (adhésion et motilité sur un substrat). Ces paramètres ont été mesurés sur la diatomée *Fistulifera saprophila*. Cette diatomée, une espèce assez ubiquiste, a été isolée dans le même type de milieu qu'*U. confervicolum* et dans le même bassin versant.

Les tests ont été réalisés à partir d'extraits de la biomasse d'*U. confervicolum*. Ces extraits ont été obtenus par une extraction chimique utilisant successivement du cyclohexane et de l'acétone afin d'extraire un large spectre de molécules apolaires et polaires. Des tests de croissance et d'adhésion ont aussi été réalisés à partir du milieu de culture d'*U. confervicolum* contenant les substances exsudées.

L'**inhibition de la croissance** a été estimée par la mesure de l'absorbance à 680 nm de cultures de *F. saprophila* après 3 jours d'exposition à différentes concentrations d'extraits. La croissance est un paramètre assez intégrateur car toute inhibition d'une fonction importante conduit généralement à une réduction de la croissance. L'effet sur l'**activité photosynthétique** a été évalué par la mesure fluorimétrique de l'efficacité de séparation des charges du photosystème II (Genty *et al.*, 1989), à l'aide d'un PhytoPAM (Walz) équipé d'une fibre optique. La **capacité des diatomées à adhérer** à un substrat a été mesurée par le comptage des cellules adhérant au fond des puits d'une plaque 24-puits après 4 h d'incubation. Des **dosages de l'oxyde nitrique** (NO), un composé gazeux produit par les diatomées et lié à l'inhibition de leur adhésion (Thompson *et al.*, 2008), ont été réalisés. Un test d'adhésion a également été réalisé avec une pré-exposition à un inhibiteur de la synthèse de NO.

Enfin, une mesure de l'**inhibition de la motilité** des diatomées a été basée sur le phototactisme de *F. saprophila*.

Pour identifier les composés impliqués, une procédure de **fractionnement guidé par bio-essais** de l'extrait contenant les composés bioactifs a été utilisée. Le fractionnement a été réalisé par une chromatographie liquide haute performance (HPLC) semi-préparative. Les différentes fractions obtenues ont été testées pour identifier celles qui conservent ou non l'activité biologique recherchée. Le bioessai utilisé pour vérifier l'activité des fractions était un test d'inhibition de croissance. Les fractions ont ensuite été analysées par UHPLC couplé à des détecteurs UV et DEDL (détecteur évaporatif à diffusion de lumière). La démarche suivie pour l'identification des composés allélopathiques est résumée dans la figure 7.

De cette démarche, il est ressorti que des composés isolés spécifiquement dans les fractions actives étaient deux acides gras polyinsaturés : l'acide linoléique et l'acide  $\alpha$ -linoléique, identifiés en comparant leurs temps de rétention en UHPLC avec des standards. Afin de confirmer que l'effet inhibiteur provenait bien de ces composés, les effets de standards de ces deux acides gras sur la croissance, l'efficacité photosynthétique et l'adhésion ont été testés. De plus, ces deux acides gras ont été dosés dans le milieu de culture.

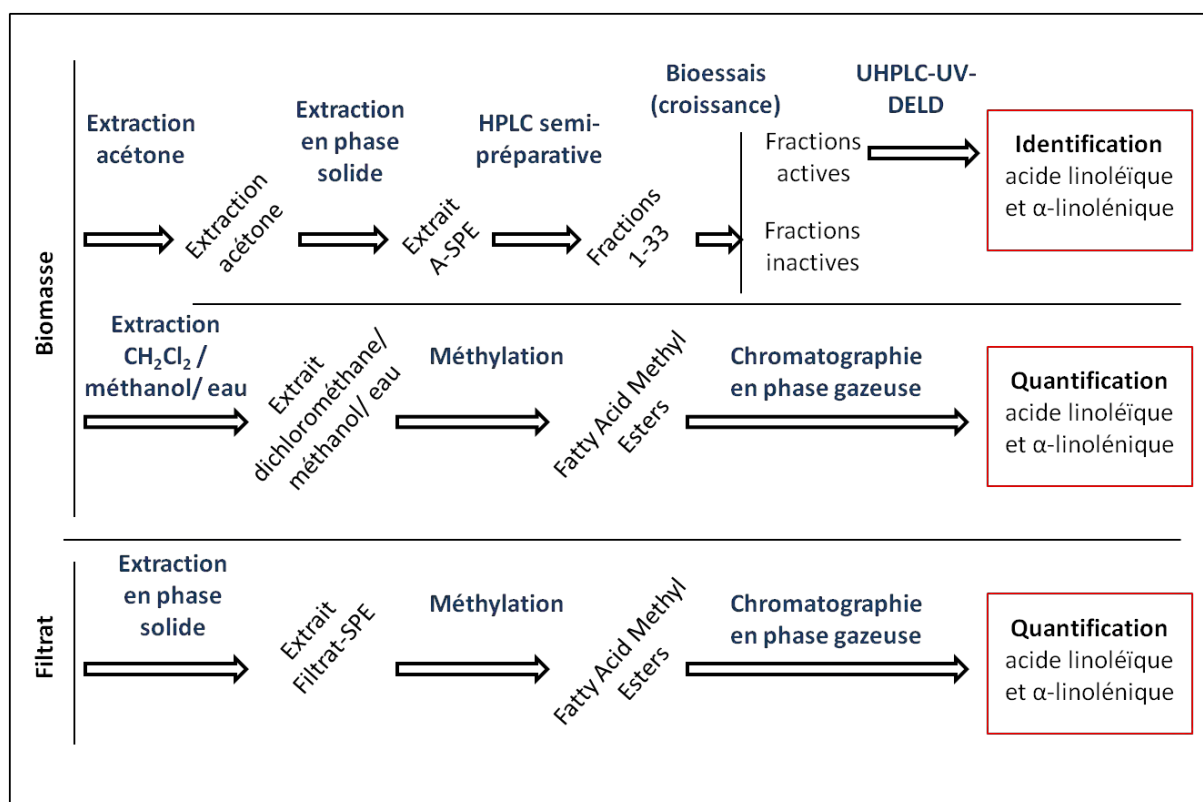


Figure 7 : Procédure expérimentale pour l'identification des composés allélopathiques.

Les résultats ont révélé une activité forte des extraits totaux pour tous les paramètres mesurés, avec des effets significatifs observés à partir de 5 à 20  $\mu\text{g ml}^{-1}$  d'extrait brut. La croissance des diatomées a été inhibée par les standards des deux acides gras identifiés dans les fractions actives à des concentrations à partir de 0.25  $\mu\text{g ml}^{-1}$ , tandis qu'aucun effet sur l'adhésion des diatomées n'a été constaté. Les deux acides gras ont été détectés dans le milieu de culture, mais à de très faibles concentrations (0,76 et 0,66  $\mu\text{g l}^{-1}$ ) et ils étaient présents dans la biomasse à de fortes concentrations (0,60 et 2,54  $\mu\text{g g}^{-1}$ ). Le filtrat n'a pas eu d'effet sur la croissance mais a inhibé fortement l'adhésion des diatomées (80% d'inhibition).

Ces résultats indiquent que l'acide linoléique et l'acide  $\alpha$ -linoléique participent très probablement à l'inhibition de la croissance observée avec les extraits d'*U. confervicolum*. Les faibles concentrations mesurées dans le milieu de culture peuvent s'expliquer par le caractère hydrophobe de ces composés. Les composés hydrophobes diffusent peu dans le milieu mais peuvent cependant être fortement concentrés à proximité immédiate du biofilm (Gross *et al.*, 1994). Cet aspect est également discuté dans le chapitre suivant (III.1). Par contre, les effets observés sur l'adhésion sont plus probablement causés par d'autres composés étant donné que les acides gras seuls n'inhibent pas l'adhésion des diatomées. De plus, le test d'adhésion réalisé avec le filtrat d'*U. confervicolum* a montré également un fort effet inhibiteur du filtrat. L'inhibition de l'adhésion est liée à une activation de la production d'oxyde nitrique (NO).

Cette étude illustre la complexité des interactions allélopathiques, avec au minimum trois composés allélopathiques différents produits par *U. confervicolum* et inhibant diverses fonctions des diatomées. Pour compléter ces données et pouvoir utiliser *U. confervicolum* comme espèce allélopathique modèle, il est à ce stade primordial de comprendre l'importance réelle des acides gras polyinsaturés dans l'allélopathie d'*U. confervicolum* et d'identifier les composés inhibant l'adhésion des diatomées (Chapitre III).



Impairment of benthic diatom adhesion and  
photosynthetic activity by allelopathic compounds from  
a green alga: involvement of free fatty acids?

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# Impairment of benthic diatom adhesion and photosynthetic activity by allelopathic compounds from a green alga: involvement of free fatty acids?

Joey L. Allen · Loïc Ten-Hage · Joséphine Leflaive

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**Abstract** The role of chemical interactions in shaping microbial communities has raised increasing interest over the last decade. Many benthic microorganisms are known to develop chemical strategies to overcome competitors, but the real importance of chemical interactions within freshwater biofilm remains unknown. This study focused on the biological and chemical mechanisms of an interaction involving two benthic microorganisms, an allelopathic filamentous green alga, *Uronema confervicolum*, and a common diatom, *Fistulifera saprophila*. Our results showed that functions critical for benthic phototrophic microorganisms were inhibited by *U. confervicolum* extracts. Growth, cell motility, adhesion, and photosynthetic activity were impaired at extract concentrations ranging between 5 and 20  $\mu\text{g ml}^{-1}$ . The adhesion inhibition was mediated by intracellular nitric oxide (NO) induction. A bioassay-guided fractionation of the extract with HPLC helped to identify two C18 fatty acids present in the growth-inhibiting fractions: linoleic (LA) and  $\alpha$ -linolenic (LNA) acids. These compounds represented 77 % of the total free fatty acids of *U. confervicolum* and were present in the culture medium (1.45  $\mu\text{g l}^{-1}$  in total). Both could inhibit the diatom growth at concentrations higher than 0.25  $\mu\text{g ml}^{-1}$ , but had no effect on cell adhesion. The discrepancy between the effective concentrations of fatty acids and the concentration

found in culture medium may be explained by the presence of high-concentration microenvironments. The compounds involved in adhesion inhibition remain to be identified. Though further experiments with complex biofilms are needed, our results suggest that *U. confervicolum* may participate to the control of biofilm composition by inhibiting diatom adhesion.

**Keywords** Allelopathy · Adhesion · Benthic diatom · Biofilm · Filamentous green algae · Free fatty acids · Nitric oxide

## Introduction

At the base of aquatic food webs, phototrophic microorganisms face intense competition for the exploitation of common limiting resources while they are under high grazing pressure (Sterner 1989). They develop various strategies to overcome these issues, including morphological adaptations or high growth rates. The production and release of bioactive metabolites seems also to be a widespread strategy which received much attention during the last decade (Ianora et al. 2006; Pohnert et al. 2007). The inhibition of competing organisms by the release of chemicals is a process known as allelopathy (Rice 1984). Allelopathic interactions have been reported in marine and aquatic ecosystems, for both benthic and planktonic microorganisms (Leflaive and Ten-Hage 2007). It has been shown to play a role in species succession (Keating 1977) and in the control of specific composition in planktonic communities (Takamo et al. 2003; Vardi et al. 2002). Biofilms, i.e., complex communities of micro- and meso-organisms embedded in an extracellular polysaccharide matrix (Lock 1993), are favorable environments for the development of allelopathic interactions (Jüttner 1999). Indeed, close distances between cells and the presence of a matrix facilitate

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J. L. Allen · L. Ten-Hage · J. Leflaive  
Université de Toulouse, INP, UPS, EcoLab (Laboratoire d'Ecologie Fonctionnelle et Environnement), 118 Route de Narbonne,  
31062 Toulouse, France

J. L. Allen · L. Ten-Hage · J. Leflaive (✉)  
CNRS, EcoLab, 31062 Toulouse, France  
e-mail: josephine.leflaive@univ-tlse3.fr

the transport of allelochemicals from emitter to receptor. Though less studied in biofilms than in planktonic communities, allelopathy seems to be widespread among benthic microorganisms. For instance, a temporal survey of freshwater epilithic biofilms suggested the frequent presence of inhibiting compounds within biofilms (Leflaive et al. 2008a). Twenty percent of the cyanobacterial mats sampled in Taiwan exhibited inhibiting activities against phototrophic microorganisms (Jüttner and Wu 2000). Moreover, among the allelopathic compounds described in literature, many are produced by benthic microorganisms, like nostocine A from the cyanobacteria *Nostoc spongiaeforme* (Hirata et al. 2003) or fischerellin A from the cyanobacteria *Fischerella muscicola* (Hagmann and Jüttner 1996). The chemical nature of allelopathic compounds is very diverse; they can be phenolic compounds (Pouvreau et al. 2007), fatty acids (DellaGreca et al. 2010), or halocarbons (Vanellander et al. 2012) for instance.

Among photosynthetic microorganisms, filamentous green algae (FGA) can grow either fixed to a substrate, as a part of biofilms, either in floating mats. In both cases, FGA are involved in competitive interactions with phytoplanktonic species and benthic/epiphytic species for nutrients, light, or space on substratum. Even when nutrients are not limiting, competition still occurs for light because of shading linked to the presence of phytoplankton or epiphytes (Dodds 1991). From this viewpoint, a parallel may be established with aquatic plants which have similar interactions with photosynthetic microorganisms (Gross 2003). In such a context, several FGA, as some aquatic plants, have been shown to produce allelopathic compounds targeting various organisms. However, their chemical nature remains unknown. For instance, volatiles from *Spirogyra longata* exhibited antibacterial activity against model strains (*Escherichia coli* and *Staphylococcus aureus*) (Kamenarska et al. 2000). Extracts from *Cladophora glomerata* impacted negatively the photosynthesis rate in the epiphyte diatom *Nitzschia fonticola* (Dodds 1991). Phytoplanktonic species were also shown to be the target of the allelopathic compounds released by FGA. For instance, a strain belonging to the genus *Cladophora* inhibited the growth of several *Desmodesmus* species (Dondajewska and Budzyska 2009). Similarly, extracts from *Uronema confervicolum* induced negative effects in *Desmodesmus quadricornis* (Leflaive et al. 2008c). These effects, involving the production of reactive oxygen species and the induction of mitogen-activated protein (MAP) kinase, varied from morphological changes (colony formation) to cell lysis for the higher concentrations of extracts. Colony formation was expected to provoke higher sedimentation rates (Lürding and Van Donk 2000) and thus lower shading effects. These extracts could also inhibit or decrease the growth and the photosynthetic activity of benthic/epiphytic algae, such as

the diatom *Nitzschia palea* (Leflaive et al. 2008b; Leflaive and Ten-Hage 2009). The production of allelopathic compounds by *U. confervicolum* was controlled by intrinsic (growth phase) and extrinsic (light, nutrients) factors.

The present study fits into a broader project, the aim of which is to specify the role of chemical—or more specifically allelopathic—interactions in shaping benthic communities in freshwater biofilms. Since FGA can reach high densities in mature biofilms in summer or late summer (Biggs 1996) and may be involved in allelopathic interactions, as seen above, we have chosen to focus on an interaction between an FGA strain and a benthic strain.

*U. confervicolum*, a common FGA previously shown to produce inhibiting compounds (Leflaive et al. 2008c), was taken as a model of allelopathic algae. Though the interaction between *U. confervicolum* and a phytoplanktonic species was well described, very little is known when the target is a benthic or epiphytic species. Yet, the latter presents some specificity linked to their way of life. Indeed, benthic/epiphytic diatoms like the ubiquitous *Fistulifera saprophila* are able to adhere to a substrate and to move on it (Leflaive and Ten-Hage 2011). Such functions are essential for the diatom ecology, in the same way as morphology is critical for planktonic cells. The first aim of the study was thus to determine if such functions were affected by *U. confervicolum*. The second aim was to attempt to identify the chemical nature of the compounds from *U. confervicolum* involved in the allelopathic effect. This is essential when studying chemical interactions because it may help to predict the mode of action and the concentration pattern in the environment and it allows in some cases working with pure compounds.

## Material and methods

### Algal cultures

The two algal strains used in the study, the green algae *U. confervicolum* (uro co 01 t) and the diatom *F. saprophila* (fis sa 07g) were isolated from diatom-dominated biofilms from the rivers Tarn and Garonne, respectively (southwestern France). Even if they were not isolated in the same location, the two algae share the same habitat and can be found at both locations. In culture, *F. saprophila* forms biofilms and floating aggregates. Stock cultures of these strains were maintained in 100-ml Erlenmeyer flasks on COMBO medium (Kilham et al. 1998) in a temperature-controlled chamber at 18 °C (light/dark 16:8, 30  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ). All the experiments were performed in those conditions. Mass

cultures of *U. confervicolum* were run in 500-ml Erlenmeyer flasks bubbled with air and filled with 400 ml of COMBO medium.

#### Chemicals

4-Amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM diacetate, 5 mM stock in DMSO) was purchased from Molecular Probes-Invitrogen, sodium nitroprusside (SNP, 100 mM fresh stock in water) was purchased from Merck, and the arginine analog NGmonomethyl-L-arginine (NMMA, 100 mM stock in water) and the fatty acids  $\alpha$ -linolenic (LNA) and linoleic (LA) were purchased from Sigma-Aldrich.

#### Chemical extractions

*U. confervicolum* cells were harvested by filtration (GF/C, pore size 1.2  $\mu\text{m}$ ), weighted, and extracted three times for a few hours with cyclohexane then acetone (around 6 ml per gram of fresh mater). The extracts were evaporated to dryness, weighed, resuspended in cyclohexane or methanol to a final concentration of 10  $\text{mg ml}^{-1}$ , and then they were stored at  $-20^\circ\text{C}$  until use. The extract with cyclohexane, containing more hydrophobic compounds than the acetone extract, was not used in the present study. The acetone extract was pre-purified with solid-phase extraction (SPE) in a protocol established after the results of preliminary experiment in order to preserve the biological activity. LiChrolut EN (200 mg of sorbent, 3 ml, Merck) syringe barrels were conditioned with two volumes of methanol, washed with two volumes of water, and loaded with 5 mg of acetone extract diluted in 6 ml of water. The columns were then rinsed with two volumes of 75 % aqueous methanol and the inhibiting compounds were eluted with two volumes of 100 % methanol. The eluates were pooled, evaporated to dryness, and resuspended in methanol to a final concentration of 10  $\text{mg ml}^{-1}$ . These extracts will be referred as A-SPE extracts (A for acetone).

#### Growth inhibition assay

The test was run in triplicate in 96-well microplates, each well containing 200  $\mu\text{l}$  of COMBO medium inoculated with exponentially growing cultures of *F. saprophila* at  $2 \times 10^5$  cells  $\text{ml}^{-1}$ . The plates were incubated under the same conditions as stock cultures. The cells were exposed to a range of final concentrations of A-SPE extracts (0, 10, 20, 50, 100, and 250  $\mu\text{g ml}^{-1}$ ) after evaporation of the solvent. Algal growth was determined by measurements of optical density (OD, 680 nm, multi-well plate reader Asys

UVM 340, Salzburg, Austria) after 3 days. In parallel, algal cells were observed with an inverted microscope equipped with a  $\times 40$  objective (Motic). The percent of growth inhibition was calculated as follows for each replicate:

$$\% \text{ of growth inhibition} = 100 \times (\text{OD}_C - \text{OD}) / \text{OD}_C$$

Where  $\text{OD}_C$  is the mean OD for controls.

#### Motility assay

The effects of A-SPE extracts on *F. saprophila* motility were measured as described in Leflaive and Ten-Hage (2011). Briefly, the principle was to determine the number of cells moving to a small lit area (0.014  $\text{mm}^2$ ) of a Sedgewick Rafter counting cell placed under a microscope (Eclipse 80i Nikon) equipped with a camera (Nikon, software NIS). A photograph was taken after 15, 30, 45, and 60 min. This was repeated three times for controls and for cells exposed to 5, 10, 20, and 50  $\mu\text{g ml}^{-1}$  of A-SPE extract. The number of cells visible on photographs was counted and the results expressed as cell number increase ( $P$  in percent):

$$P \text{ in } \% = 100 \times (C_i - C_0) / C_0$$

Where  $C_i$  is the number of visible cells at  $t_i$  ( $i=15, 30, 45$ , or 60) and  $C_0$  the number of cells visible at  $t_0$ .

#### Adhesion assay

Two experiments were performed to study the effects of A-SPE extract on *F. saprophila* capability to adhere to surfaces. The first one aimed to test the hypothesis that the extracts could inhibit cell adhesion, the second one aimed to determine if adhesion inhibition could involve the induction of nitric oxide (NO) by using a NO-synthase inhibitor (NMMA). These experiments were run in triplicate in 24-well plates, each well containing 1 ml of culture medium ( $2 \times 10^5$  cells  $\text{ml}^{-1}$ ). Plates were incubated under the same conditions as stock cultures. In the first experiment, *F. saprophila* was incubated in a plate in the presence of a range of A-SPE extracts concentrations (5, 10, 20, 50  $\mu\text{g ml}^{-1}$ ). After inoculation of the plate, the cells were allowed to settle and fix for 4 h. Then, well contents were gently shaken by pipetting and non-adherent cells were removed. The remaining cells, still fixed to the bottom of the wells, were counted in at least 20 fields of 0.11  $\text{mm}^2$  using an inverted microscope (Axiovert 10, Zeiss). In a second experiment, *F. saprophila* cells ( $2 \times$

$10^5$  cells  $\text{ml}^{-1}$ ) were pre-incubated during 30 min in the dark with 0.5 mM NMMA before the addition of A-SPE extracts at a final concentration of  $20 \mu\text{g ml}^{-1}$ . The adhesion assay was then performed as described above, with a control, cells exposed to extracts at  $20 \mu\text{g ml}^{-1}$ , and cells exposed to extracts at  $20 \mu\text{g ml}^{-1}$  and pre-exposed to NMMA. The percent of adhesion inhibition was calculated as follows for each replicate:

$$\% \text{ of adhesion inhibition} = 100 \times (D_C - D) / D_C$$

Where  $D_C$  is the mean cellular density in controls and  $D$  is the mean cellular density in the treatments.

The effect of spend medium on adhesion was also tested. *F. saprophila* cells were incubated 4 h in 100 % spend medium. Nitrogen ( $\text{NaNO}_3$ ) and phosphorous ( $\text{KH}_2\text{PO}_4$ ) were added to spend medium to get the same concentrations as COMBO medium. COMBO medium was used as control.

#### Oxide nitric assay

Since NO production is known to be involved in adhesion impairment in diatoms (Thompson et al. 2008), we determined if NO was produced when *F. saprophila* was exposed to A-SPE extract. Algal cells were incubated in the dark with  $10 \mu\text{M}$  DAF-FM for 60 min before a washing step (centrifugation 5 min at  $5000\times g$ ). The cells were then incubated in the dark for 30 min to allow de-esterification before another washing step. The old medium was then replaced by fresh COMBO medium containing either A-SPE extracts (2, 5, and  $10 \mu\text{g ml}^{-1}$ ) or SNP (NO donor, positive control, 0.5 mM). To quantify NO accumulation, DAF-FM fluorescence was measured after 5 h with a fluorescence microplate reader (Synergy MX, BioTek, Winooski, USA) using a 485-nm excitation filter and a 538-nm emission filter.

#### Photosynthetic activity assay

Photosynthetic efficiency of *F. saprophila* cells exposed to A-SPE extract was derived from fluorescence-based quantum yield measurements. Diatom cells were incubated during 1 week in 24-wells plates (1 ml of COMBO medium per well, inoculation with  $8 \times 10^6$  cells  $\text{ml}^{-1}$ ) in order to form dense biofilms. *F. saprophila* biofilms were then exposed to A-SPE extract (10, 20, and  $50 \mu\text{g ml}^{-1}$ ) as follows: at  $t=0$ , 0.5 ml of culture medium was removed and replaced with 0.5 ml of medium containing the extract. The plates were kept in the same conditions than stock cultures. The effective quantum efficiency of charge separation of photosystem II (PSII) (yield) was measured at  $t=0$ , 1, 3, 5, and 24 h with a PhytoPAM fluorimeter (Walz, Effeltrich, Germany). After

3 min of dark adaptation, the biofilms were illuminated with a measuring light at  $32 \mu\text{mol m}^{-2} \text{s}^{-1}$  (MF64). This light intensity was chosen because it was very close to incubation conditions (Schmitt-Jansen and Altenburger 2008). The yield was calculated according to (Genty et al. 1989)

$$\text{Yield} = (F'_m - F) / F'_m$$

where  $F'_m$  is the maximum fluorescence after saturation pulse and  $F$  was the steady-state fluorescence.

Four measures were made per well, each treatment was run in triplicate.

#### Analytic and semi-preparative HPLC

In an attempt to identify the compounds involved in the growth inhibition of *F. saprophila*, A-SPE extract was fractionated with reverse-phase semi-preparative HPLC. Biomass extracts were used instead of medium extracts to have sufficient amount of bioactive compound to carry out bioassay-guided fractionation. Indeed, the concentration of inhibiting compounds in the spend medium was low since it could not inhibit the diatom growth (data not shown). Fractionation was performed with the AutoPurification System from Waters which includes 2767 Sample Manager, 2545 Binary Gradient Module, System Fluidics Organizer, and 2489 UV/Vis Detector. The collection of detected fractions was automatic in tubes and driven by MassLynx Software with the FractionLynx Application Manager. The detection wavelengths used were 228 and 415 nm with 33 fractions collected. The wavelengths of detection had been chosen after preliminary assays with A-SPE extract to allow the observation of all the detected molecules. The analytical and preparative columns were XBridge C18  $5 \mu\text{m}$  (in  $4.6 \text{ i.d.} \times 150$  and  $19 \text{ i.d.} \times 150 \text{ mm}$ ) columns. The separation was achieved at room temperature. The mobile phase was a gradient of water (0.1 % trifluoroacetic acid (TFA)) and acetonitrile (0.1 % TFA) (Table 1) with a flow rate of  $20 \text{ ml min}^{-1}$ . The preparative column was loaded with  $900 \mu\text{l}$  of A-SPE extract.

The collected fractions were checked on Acquity UPLC-photo diode array detector (PDA)-evaporative light scattering detector (ELSD) from Waters driven by the Empower version 2 software. The column was an Acquity CSH C18  $1.7 \mu\text{m}$  ( $2.1 \times 50 \text{ mm}$ ). The wavelengths of detection were 228 and 415 nm. ELSD detection was added to allow the detection of lipids (Bravi et al. 2006).

The flow rate was  $0.4 \text{ ml min}^{-1}$ . The chromatographic separation was achieved with a gradient of mobile phase as described in Table 1 and the temperature reached  $45^\circ\text{C}$ . The injection volume was  $2 \mu\text{l}$ . The ELSD conditions were a 40-

**Table 1** Gradients of mobile phases used in preparative and analytic HPLC

|                  | Time (min) | % water (0.1 % TFA) | % acetonitrile (0.1 % TFA) |
|------------------|------------|---------------------|----------------------------|
| Preparative HPLC | 0          | 60                  | 40                         |
|                  | 1.5        | 60                  | 40                         |
|                  | 13         | 10                  | 90                         |
|                  | 18         | 0                   | 100                        |
|                  | 18.5       | 60                  | 40                         |
| Analytic HPLC    | 0          | 60                  | 40                         |
|                  | 0.5        | 60                  | 40                         |
|                  | 8          | 0                   | 100                        |
|                  | 8.5        | 60                  | 40                         |

psi gas pressure, a gain of 500, and a drift tube set point temperature of 50 °C.

The collected fractions were next evaporated to dryness and resuspended in 90 µl of methanol. Their biological activity was controlled with a growth inhibition assay, as described above, with 1.25 µl of each fraction per well. The plates were observed daily with an inverted microscope equipped with a ×40 objective (Motic) and the presence of dead cells was noted. The inhibitory effect of the fractions was noted as null (–), weak (+, lower density than controls), and strong (++ , lower density plus cell lyses).

#### Free fatty acid quantification in algal biomass and culture medium

Triplicates of 800 ml of *U. confervicolum* culture (two 400-ml culture flasks) were used for the quantification of fatty acids in biomass and culture medium. Algal biomass was separated from the medium by filtration (GF/C, pore size 1.2 µm) before lyophilization. Fatty acids were extracted from the medium using SPE cartridge conditioned as described above (LiChrolut EN 200 mg of sorbent, 3 ml, Merck) and eluted with 6 ml of 100 % methanol. The internal standard heptadecanoic acid was added to the extract (2 µg) or to 10 mg of algal freeze-dried biomass (10 µg). Algal biomass was then extracted with methanol–water–dichloromethane (2.5–2–2.5 ml).

The medium and biomass extracts were directly methylated in boron trifluoride methanol solution 14 % (SIGMA, 1 ml) and heptane (1 ml) at RT for 5 min. After addition of water (1 ml), fatty acid methyl esters (FAMES) were extracted with heptane (3 ml), evaporated to dryness, and dissolved in ethyl acetate (20 µl). FAME (1 µl) were analyzed by gas–liquid chromatography (Lillington et al. 1981) on a Clarus 600 PerkinElmer system using a FAMEWAX RESTEK fused silica capillary columns (30 m×0.32 mm i.d, 0.25 µm film thickness) and a flame ionization detector (FID). Oven temperature was programmed from 110 to 220 °C at a rate of 2 °C

per min and the carrier gas was hydrogen (0.5 bar). The injector and the detector were at 225 and 245 °C, respectively.

#### Biological assays with free fatty acids

The growth and adhesion inhibition activities of linoleic (LA) and α-linolenic acids (LNA) on *F. saprophila* were tested as described for A-SPE extracts. LA and LNA were diluted in ethanol at 2500, 500, 125, and 25 µg ml<sup>–1</sup>. For growth inhibition assay, 2 µg of these solutions was evaporated in a microtube under sterile conditions at room temperature. One milliliter of COMBO medium inoculated with 2×10<sup>5</sup> cells ml<sup>–1</sup> was then added. For adhesion inhibition assay, 4 µg ml<sup>–1</sup> of the fatty acid solutions was diluted in 2 ml of COMBO medium without evaporation phase. Controls received the same volume of ethanol. In both assay, final LNA and LA concentrations were 5, 1, 0.25, and 0.05 µg ml<sup>–1</sup>. Algal growth and adhesion were measured as described for A-SPE extracts.

#### Statistical analyses

The data were compared statistically using one-way ANOVA followed by the Fischer post hoc comparison test (Statistica program for Windows, version 8.0). Repeated-measures ANOVA were also used for experiments with several measures over time. For all statistical analyses, significance was inferred at  $p \leq 0.05$ .

## Results

#### Biological effects of *Uronema* extracts

Analysis of variance revealed a significant effect of A-SPE extract on the growth of *F. saprophila* (Fig. 1a). A slight negative effect was observed at 5 µg ml<sup>–1</sup>, and growth was totally inhibited after 3 days of exposure at 35 µg ml<sup>–1</sup>

( $p < 0.001$ ). At high concentrations, cell lyses were observable from 24 h of exposure. The potential effects of extracts on cell motility were next determined in a test based on *F. saprophila* phototaxis. In control conditions, cells were indeed attracted to the light as revealed by the increase in cell number (Fig. 1b). When cells were exposed to A-SPE extract, the increase of cell number was much lower. Results of repeated-measures ANOVA indicated that this effect was strongly significant ( $p = 0.0039$ ) (supplementary material 1). After 60 min, the lowest value was obtained for cells exposed to the concentration  $50 \mu\text{g ml}^{-1}$ . There was no significant difference between the other concentrations of extract. The adhesion capabilities of *F. saprophila* were strongly impaired within a few hours by A-SPE extract, as shown in Fig. 2a ( $p < 0.001$ ). This inhibition of adhesion was observable from  $10 \mu\text{g ml}^{-1}$  and was dose-dependent, with a maximal value of 73 % for  $50 \mu\text{g ml}^{-1}$  of A-SPE extract. It appeared that this effect was strongly reduced when the cells were incubated in the presence of both extract ( $20 \mu\text{g ml}^{-1}$ ) and NMMA (0.5 mM), an inhibitor of NO synthase (Fig. 2b). In parallel, the production of NO in cells exposed to A-SPE extract was determined with the fluorescent probe DAF-FM, with the NO donor SNP as a positive control. Analysis of variance indicated a significant increase in DAF-FM fluorescence ( $p < 0.01$ ) independently of the concentration applied (Fig. 2c). Spend medium induced an 80 % decrease in the adherent cell number.

The potential effects of A-SPE on the photosynthetic activity of *F. saprophila* were tested in an experiment performed on *F. saprophila* biofilms. Before the application of the extract, the yield value of biofilms was in mean  $0.55 \pm 0.01$  ( $n = 12$ ). Because of slight differences between the yield values before the beginning of the experiment, the corresponding yield values at  $t_0$  have been subtracted from the data. This helped to better observe the dynamic of the yield after the addition of the extract (Fig. 3). Repeated-measures ANOVA performed on these data indicated a significant effect of treatment ( $p = 0.004$ ) (supplementary material 2). The yield of biofilms exposed to 20 and  $50 \mu\text{g ml}^{-1}$  of A-SPE extract significantly decreased after 3 and 5 h of exposure. No difference was observed between treatments after 24 h, the mean yield value was then  $0.56 \pm 0.01$ .

#### Identification of putative inhibiting compounds

In order to try to identify the compounds involved in the observed biological activities, the A-SPE extract was fractionated using semi-preparative HPLC. The purity of all the fractions was checked with analytical ultra high performance liquid chromatography (UHPLC). None of the isolated fraction was pure. However the number of

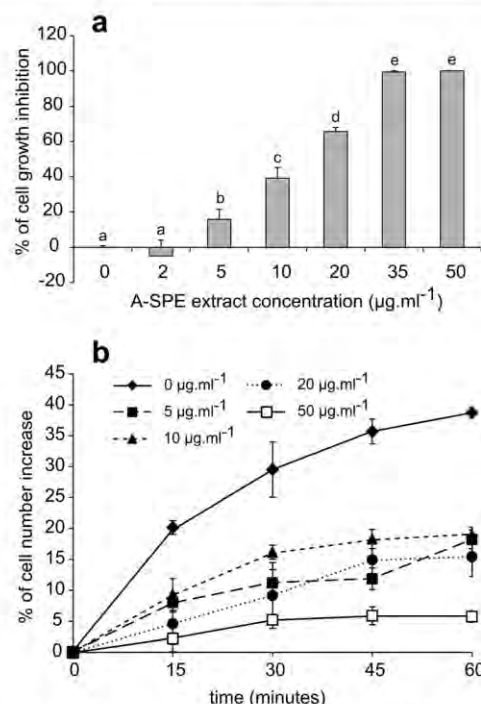


**Fig. 1** Effect of A-SPE extracts on *F. saprophila* growth and motility. **a** Percent of growth inhibition compared to controls, after a 3-day incubation. **b** Percent of *F. saprophila* cell number increases in a lit area of a slide after exposure to various concentrations of A-SPE extracts. Vertical bars represent standard error ( $n = 3$ ). Letters indicate statistically homogeneous groups

detected compounds was strongly lowered compared to the initial extract. For instance, the chromatograms of A-SPE extract can be compared to those of fraction 18 (Supplementary material 3a-f). The biological activity of the 33 fractions obtained was tested with a growth inhibition assay with *F. saprophila*. The results of microscopic observations during 1 week of exposure are summarized in Table 2. Absence of growth and cell deaths were observed in the fractions 8, 18, and 21. A unique peak was visible with ELSD in the fractions 18 and 21, at 4.61 and 5.23 min, respectively (see Supplementary material 3f for instance). The same peaks were visible in A-SPE extract. Those peaks were not detectable in UV-Visible. This was in accordance with previous results of thin-layer chromatography suggesting the involvement of fatty acids in the biological activity of *U. confervicolum* (data not shown). We therefore decided to use several standards of fatty acids in order to identify these two peaks. The standards were chosen after results from literature. Pure dilutions of LA and LNA were injected in UHPLC, in the same conditions as the A-SPE extract and fractions. The two fatty acids were clearly detectable with ELSD (example in Supplementary material 3g). The retention times were 4.62 and 5.23 min for LNA and LA, respectively, which

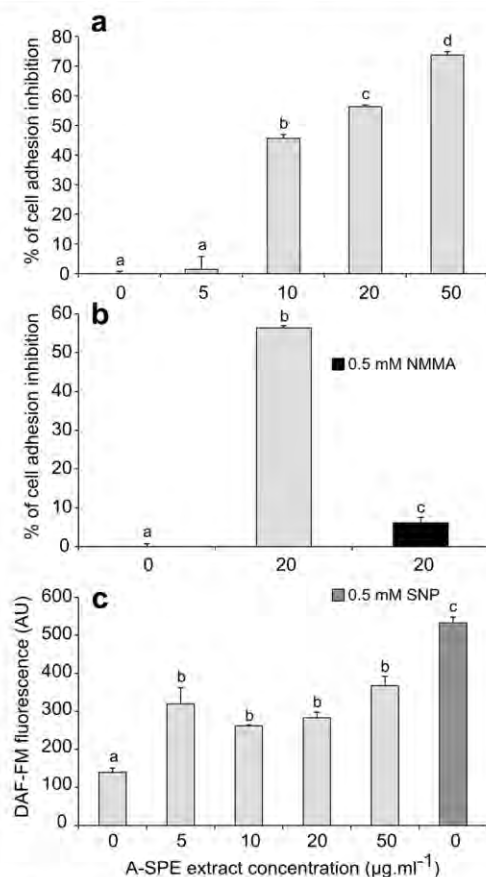


**Fig. 2** Effect of A-SPE extracts on *F. saprophila* adhesion: percent of cell adhesion inhibition of *F. saprophila* cells exposed to A-SPE extracts alone (a) or with the NO-synthase inhibitor NMMA (b). Fluorescence of the nitric oxide indicator DAF-FM in *F. saprophila* cells exposed to A-SPE extract or to the NO-donor SNP (c). Vertical bars represent standard error ( $n=3$ ). Letters indicate statistically homogeneous groups

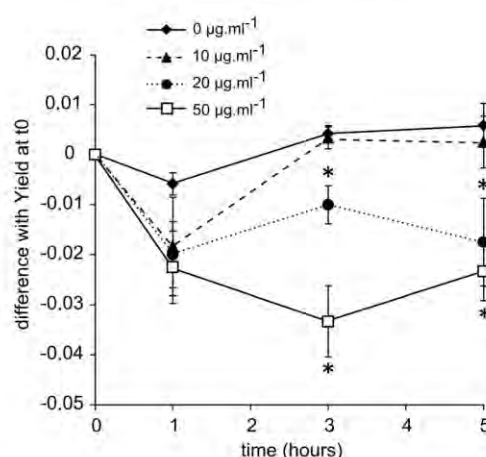


**Fig. 3** Temporal variation of yield values of *F. saprophila* biofilms exposed to A-SPE extracts. To facilitate comparisons, the initial yield values were subtracted so that all  $t_0$  values are set to 0. Vertical bars represent standard error ( $n=3$ ); asterisks indicate values significantly different from the control value (0  $\mu\text{g}\cdot\text{ml}^{-1}$ )

indicates that LNA was present in fraction 18 and LA in fraction 21.

#### Free fatty acid concentrations

LA and LNA were found in *U. confervicolum* biomass at high concentrations, respectively,  $0.60 (\pm 0.24)$  and  $2.54 (\pm 1.07) \mu\text{g}\cdot\text{mg}^{-1}$ . The concentration of the other free fatty acids detected was  $0.85 (\pm 0.29) \mu\text{g}\cdot\text{mg}^{-1}$ . LA and LNA corresponded to  $15 (\pm 1)$  and  $62 (\pm 5)\%$  of the total free fatty acids. LA and LNA were also found in the culture medium, at lower concentrations,  $0.38 (\pm 0.15)$  and  $0.33 (\pm 0.12) \mu\text{g}\cdot\text{l}^{-1}$ , respectively. Previous experiments with fatty acid standards dissolved in COMBO medium at known concentrations had shown that the extraction method provides a yield of about 50 % (data not shown). The estimated concentrations of LA and LNA should thus be around  $0.76$  and  $0.66 \mu\text{g}\cdot\text{l}^{-1}$ , respectively.

#### Biological effects of free fatty acids

Analysis of variance revealed a significant effect of both fatty acids on the growth of *F. saprophila* at concentrations higher than  $0.05 \mu\text{g}\cdot\text{ml}^{-1}$  (Fig. 4a). There was almost no dose effect between  $0.25$  and  $5 \mu\text{g}\cdot\text{ml}^{-1}$ , for both fatty acids. At the tested concentration, no effect of LA and LNA on *F. saprophila* adhesion could be observed (Fig. 4b).

#### Discussion

The aim of this study was to investigate the specific response of a benthic/epiphytic diatom to the allelopathic compounds produced by a filamentous green alga, *U. confervicolum*. The use of the term “allelopathic compounds” rather than “allelochemicals” (Leflaive and Ten-Hage 2007) is justified by previous results of agar diffusion assays showing that inhibiting compounds are actually released by *U. confervicolum* cells (Leflaive et al. 2008c and unpublished results). These exuded bioactive compounds were active against the green alga *D. quadrispina* and the diatom *F. saprophila*. In our experiments, we used extracts of biomass rather than culture filtrate because the concentration of bioactive compounds was much higher in there. Differences in nutrient concentration when using filtrate could also induce changes in growth in an experiment lasting 3 days even if macronutrients are added at the beginning. In previous experiments, filtrates did not have any effect on growth (data not shown). The strain we used was not fully axenic, so the effect could be attributed to the alga or to associated bacteria. Microscope

**Table 2** Results of the biological assay performed on the fractions from A-SPE extract after 1 week of incubation

| Fraction | Growth inhibition | Fraction | Growth inhibition | Fraction | Growth inhibition |
|----------|-------------------|----------|-------------------|----------|-------------------|
| 1        | –                 | 12       | –                 | 23       | –                 |
| 2        | –                 | 13       | –                 | 24       | –                 |
| 3        | –                 | 14       | +                 | 25       | +                 |
| 4        | –                 | 15       | –                 | 26       | –                 |
| 5        | –                 | 16       | –                 | 27       | –                 |
| 6        | –                 | 17       | –                 | 28       | –                 |
| 7        | –                 | 18       | ++                | 29       | –                 |
| 8        | ++                | 19       | +                 | 30       | –                 |
| 9        | +                 | 20       | –                 | 31       | –                 |
| 10       | +                 | 21       | ++                | 32       | –                 |
| 11       | +                 | 22       | –                 | 33       | –                 |

The fractions exhibiting the highest activity are in italics

– no or very weak effect, + effect on cell density, ++ strong effect on cell density and cell lyses

observations indicated that bacterial biomass remained extremely low compared to algal biomass, especially during the exponential growth phase when the algae were sampled. Moreover, the identified allelochemicals,  $\alpha$ -linolenic and linoleic acids, have been found to be produced by *U. confervicolum* in a previous study (Lang et al. 2011). This is less clear for the compounds involved in adhesion inhibition; they may have a

bacterial origin. Bacteria may favor the release of LA, LNA, and the unidentified compounds via the lysis of algal cells. A similar process may occur in the natural environment.

#### Interaction *U. confervicolum*/*F. saprophila*

Our results demonstrated that growth and several functions (adhesion, motility, and photosynthesis) of the diatom were severely impacted by the exposure to *U. confervicolum* extracts. The PhytoPAM used here to assess photosynthetic activity of *F. saprophila* biofilms brought information about the effective quantum efficiency of separation charge of photosystem II (yield), which can be used for monitoring the efficiency of photochemical processes in photosystem II (PSII) (Roháček and Barták 1999). A decrease in this parameter may be linked to a direct impairment of the PSII (Juneau et al. 2002). However, a decreasing yield could also be a response to a general toxicity, such as the one induced by DNA-damaging agents (Tetu et al. 2013). Photosynthesis is commonly affected by allelopathic compounds, especially those produced by plants (see review by Reigosa et al. 1999). In some cases the PSII is the specific target of the compound, such for the fischerellin A (Hagmann and Jüttner 1996). In other cases (e.g., phenolic compounds), reduced rates of photosynthesis are the results of physiological cascades initiated by a non-specific permeability of cell membranes (Einhellig 2004). In our case, we could not determine if the PSII is the specific target of allelopathic compounds. It is also possible that the observed effects were linked to a poor physiological state of

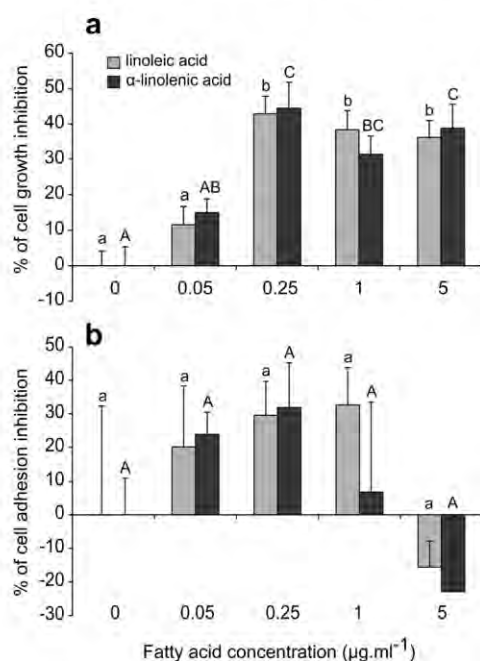


**Fig. 4** The effect of free fatty acids on *F. saprophila* growth after a 3-day incubation revealed by the percent of growth inhibition compared to controls (a) and on *F. saprophila* cell adhesion after a 4-h incubation (b). Vertical bars represent standard error ( $n=3$ ). Letters indicate statistically homogeneous groups

cells, due to impairment of other function by allelopathic compounds.

The other parameters we focused on are essential for the benthic way of life of the studied diatom. Raphid diatoms like *F. saprophila* live fixed, within biofilms. They adhere to substrates through the secretion of extracellular polymeric substances (EPS) from a slit in the cell wall called the raphe (see review by Wetherbee et al. 1998). EPS also allow gliding motility (Underwood and Paterson 2003), cells can migrate to the places that provide the best conditions. Adhesion and motility were both inhibited when cells were exposed to 10 and 5  $\mu\text{g ml}^{-1}$  of A-SPE extract, respectively. We detected the presence of NO in diatom cells shortly after exposure to the extracts, with no obvious dose effect. Otherwise, the adhesion inhibition at 20  $\mu\text{g ml}^{-1}$  was decreased by almost 90 % when the cells were incubated with a NO-synthase inhibitor, the NMMA. In a previous study, we had shown that the adhesion and motility of *F. saprophila* cells was impaired in the presence of a NO donor (Leflaive and Ten-Hage 2011). Put together, these results indicate that induction of NO by a NO-synthase-like activity is a part of the mechanism of adhesion and motility inhibition by *U. confervicolum* allelopathic compounds. NO is a multifunctional messenger involved in plants in gene regulation in photosynthesis, cell death, and basic cellular metabolism (Wendehenne et al. 2004). It is also known to be involved in diatom adhesion. Indeed, it plays a role in diatom selectivity in adhering to surfaces: intracellular NO concentrations are higher when the surface is not suitable for adhesion (Thompson et al. 2008). Moreover, cells with artificially high concentrations of NO were shown to have reduced adhesion strength to several surfaces (Thompson et al. 2008). Vardi et al. (2008) also demonstrated that overexpression of a protein involved in NO generation could reduce the adhesion ability of marine diatom. Here, the effects of A-SPE extracts were very quick. The adhesion assay was performed within 4 h, without pre-incubation of cells with the extracts. A very similar response was observed when *F. saprophila* cells were exposed to a polyunsaturated aldehyde produced by some diatom species, the 2E-4E-decadialenal (Leflaive and Ten-Hage 2011). This compound could induce rapid NO production in some diatoms (*F. saprophila* (Leflaive and Ten-Hage 2011), *Phaeodactylum tricornutum* (Vardi et al. 2006)). Though NO is directly involved in adhesion, it is also commonly produced in stress responses (del Rio et al. 2004). In the context of *U. confervicolum*–*F. saprophila* interaction, it is thus not possible to determine if the observed effects on adhesion and motility are the specific effects of the allelopathic compounds or if they are only the consequence of a global response to stress and decrease in physiological state. Growth of

*U. confervicolum* can be the result of either vegetative division or asexual reproduction by sporulation (Ramanathan 1964). The spores settle down and they become attached to the substrate. During this period, they are in competition with the other benthic algae for space. The release of allelopathic compounds from *U. confervicolum* may prevent the attachment of epiphytic cells to its filaments or to the substratum in their vicinity in order to avoid shading and competition for the spores. This would explain why *U. confervicolum* filaments collected in the field or co-cultured with diatoms are most often lacking epiphytic cells (personal observations). In this study, we tested only the primary adhesion of diatoms. After this first step, adhesion may become stronger via processes such as mechanisms that cross-link EPS components (Vreeland et al. 1998). It would be interesting to determine if the allelopathic compounds from *U. confervicolum* could also affect secondary adhesion and provoke the release of diatom cells. With longer exposure, it may also be relevant to determine if the EPS production would be modified by the extract, since they participate to the processes of adhesion and motility. The adhesion of diatom was also inhibited by spend medium. Since the adhesion assay lasts only 4 h, this excludes an effect of nutrient limitation.

#### Allelopathic compounds identification

Algal extracts contain a very high number of molecules, including some intracellular compounds which are not involved in allelopathic interactions and may induce non-relevant effects in the target species. It is thus important to purify complex extracts (at least partly) and to determine the chemical nature of the involved compounds before addressing their mode of action and their effects in complex biofilms. We followed a classical bioassay-guided fractionation method. Growth inhibition tests were chosen for bioassays because they represent an integrative measurement (impairment of principal function of the organisms would lead to an impairment of growth) and they were easier and less time-consuming than the other tests we performed with A-SPE extract. After the first fractionation, the complexity of the samples was significantly reduced. Though several fractions showed slight biological activity (see Table 2), we focused on the most active fractions. Two of them (fractions 18 and 21) seemed to contain very few compounds. Some of the peaks were shared with other fractions showing no growth inhibition, which suggested that they were not involved in the biological effect. Interestingly, each of these fractions contained a unique compound detectable only with ELSD detector. This detection method has proven to be very efficient in detecting fatty acids (Bravi et al. 2006). Previous experiments with

*U. confervicolum* had pointed to the possibility that growth inhibition could involve fatty acids (data not shown). Since the C18  $\alpha$ -linolenic and linoleic acids are among the main fatty acids of *U. confervicolum* (Lang et al. 2011) and have both shown inhibiting effects on the growth of microalgae (e.g., Chiang et al. 2004; Yamada et al. 1993), we used LA and LNA standards in UHPLC and demonstrated that these fatty acids were present in the bioactive fractions (see Supplementary material 3). LA and LNA were also detected in culture medium which confirmed that they were excreted by *U. confervicolum* cells. The biological activity of these two fatty acids was very similar, they both could inhibit *F. saprophila* growth at concentrations above  $0.05 \mu\text{g ml}^{-1}$ , but had no effect on cell adhesion. Though the two fractions were not pure, LA and LNA are likely involved in some biological activity of *U. confervicolum* extracts. Their concentrations in culture medium ( $<1 \mu\text{g l}^{-1}$ ) were much lower than those which were effective in the biological assays ( $>50 \mu\text{g l}^{-1}$ ). This discrepancy may be explained by several points. The ratio *U. confervicolum* biomass/medium volume in cultures may have been too low to allow significant accumulation of fatty acids. However, in the vicinity of *U. confervicolum* filaments, the concentrations of fatty acids may have been much more elevated than the mean concentration value in the whole culture volume. This is very important since in the considered interaction, the distances between the two partners are very low especially when considering the inhibition of adhesion of epiphytic diatoms on *U. confervicolum* filaments. Another point is linked to the low solubility of fatty acids in water. Wu et al. (2006) proposed that these compounds may form micelles which may stick to cell surface and form a high-concentration microenvironment. Another possibility is the presence of synergistic effects. LA and LNA may be components of a cocktail of compounds involved in the allelopathic interaction. This has been shown in the interaction between the cyanobacteria *Microcystis* and the cladoceran *Daphnia magna*: the toxin microcystin had no effect alone but it significantly enhanced the toxic effects of  $\gamma$ -linolenic acid (Reinikainen et al. 2001). The difficulty of dealing with synergistic compounds is a well-known disadvantage of the bioassay-guided fractionation method (Byers 1992).

The inhibitory effects of polyunsaturated fatty acids (PUFA) on algae have been known for decades. One of the first algal allelopathic compounds described in literature, “chlorellin,” from *Chlorella vulgaris* (Pratt et al. 1944), is a mixture of C18 fatty acids (DellaGreca et al. 2010; Scutt 1964). PUFAs, and especially C18 like LA, LNA, and oleic acid, exhibited toxicity against various green algae and cyanobacteria (see reviews by Ikawa 2004; Watson 2003). *Botryococcus braunii* (Chlorophyceae) was thus shown to favor its dominance in phytoplanktonic community through the production of a mixture of free fatty acids (Chiang et al.

2004). The effective concentrations of LA and LNA have been shown to range between  $0.5 \mu\text{g ml}^{-1}$  (cyanobacteria *Phormidium tenue* (Yamada et al. 1993)) and  $8\text{--}14.5 \mu\text{g ml}^{-1}$  (24-h  $\text{EC}_{50}$  for the green alga *Monoraphidium contortum* (Wu et al. 2006)). The diatom strain used in the present study (growth inhibition at  $0.25 \mu\text{g ml}^{-1}$ ) appeared to be much more sensitive than green algae of previous studies. Though additional studies with more diatom species are needed to confirm this, this may be explained by the higher content of green algae in polyunsaturated C18, as suggested by Lang et al.'s (2011) results. Green algae may have developed mechanisms of resistance, which is suggested by the fact that producers of fatty acids are less sensitive than non-producers, as shown by DellaGreca et al. (2010). The mode of action of fatty acids has been described by Wu et al. (2006). They demonstrated that the toxicity of fatty acids is linked to damages of plasma membranes which lead to changes in membrane permeability, dissociation of phycobilins from thylakoids, and cell lysis. Increase in membrane permeability may explain the observed effect of A-SPE extracts in photosynthesis activity (Einhellig 2004). To confirm this hypothesis, we used sodium dodecyl sulfate (SDS), which is a surfactant, as a way to increase membrane permeability. SDS and LNA tested in the same conditions than the extracts both reduced the photosynthetic yield, but contrary to the extracts, the effect was stronger 24 h after exposure (supplementary material 4). This is consistent with the hypothesis that the photosynthesis inhibition was probably due to an impairment of membrane integrity by PUFA contained in the extracts.

## Conclusion

Our results indicate that *U. confervicolum* produces and releases free fatty acids (mainly LA and LNA) that are able to reduce the growth and photosynthetic activity of the benthic/epiphytic diatom *F. saprophila*. The effective concentrations of these fatty acids were close to environmental concentrations of free fatty acids ( $1\text{--}2 \mu\text{g ml}^{-1}$  (Ikawa 2004)), which indicates that they may play a significant role in controlling the composition of benthic phototrophic communities in natural environments. LA and LNA are unlikely to be involved in the effects of *U. confervicolum* extracts on the adhesion and motility of the diatom. This suggests that the green alga releases a mixture of several metabolites having different biological activities. The chemical nature of the anti-adhesion compounds remains to be determined. Specific bioassays will be applied to the fractions obtained with semi-preparative HPLC in this aim.

On the whole, our results indicate that chemical interactions may favor the high densities of FGA observed in biofilms previously dominated by diatoms. To confirm this,

more studies with complex biofilms exposed to *U. confervicolum* exudates will be needed.

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# **Impairment of benthic diatom adhesion and photosynthetic activity by allelopathic compounds from a green alga: involvement of free fatty acids?**

Joey L. Allen, Loïc Ten-Hage and Joséphine Leflaive\*

\*Corresponding author: [josephine.leflaive@univ-tlse3.fr](mailto:josephine.leflaive@univ-tlse3.fr)

Université de Toulouse; INP; UPS; EcoLab (Laboratoire d'Ecologie Fonctionnelle et Environnement);  
118 Route de Narbonne, F-31062 Toulouse, France.

CNRS; EcoLab; F-31062 Toulouse, France.

## **Supplementary Material**

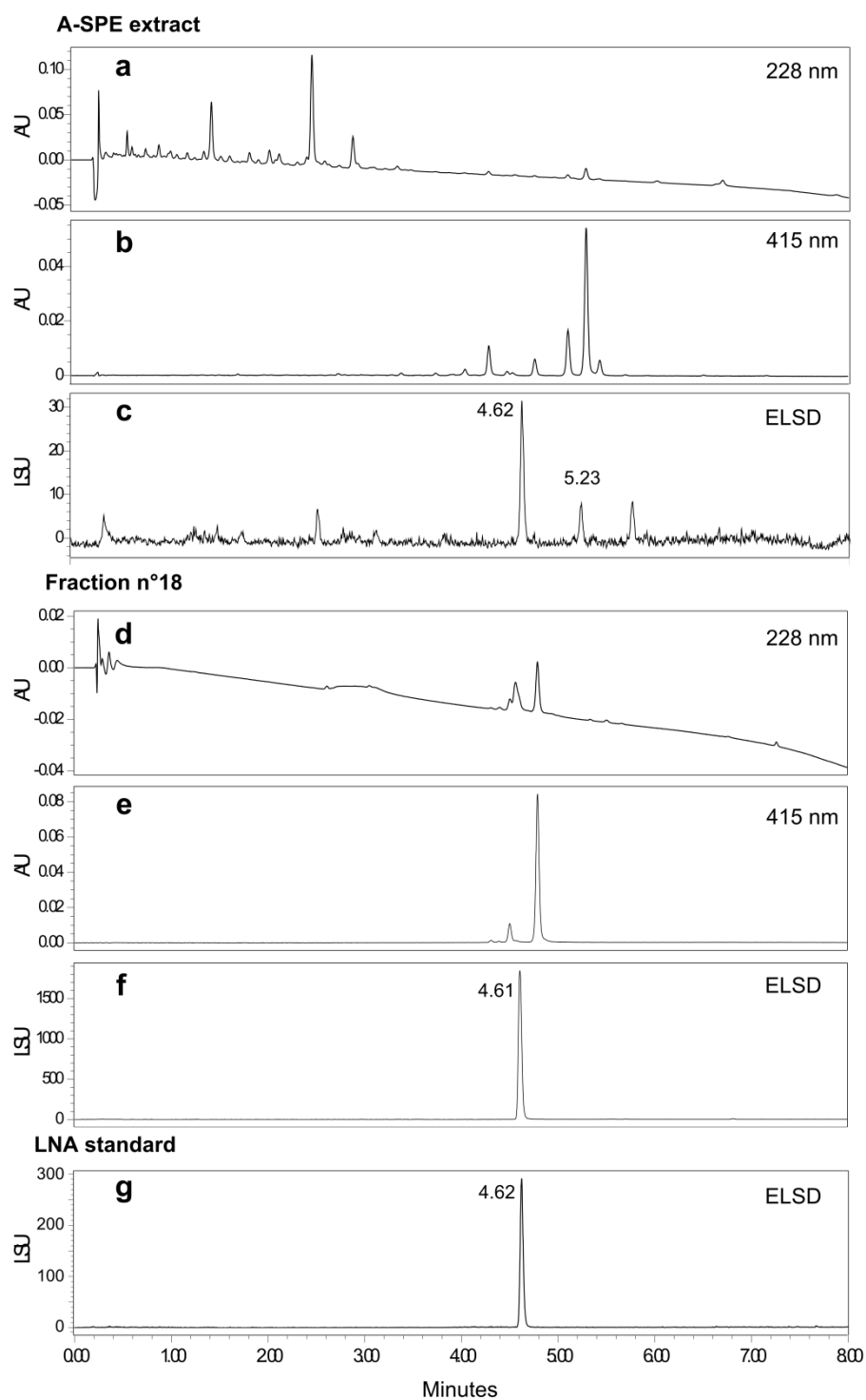
**Supplementary Material 1:** Results of repeated-measures ANOVA for the motility assay. Significant p-values are in bold

| Effect           | <i>p</i> values | Degrees of freedom | <i>F</i> |
|------------------|-----------------|--------------------|----------|
| Treatment        | <b>0.0039</b>   | 2                  | 16.03    |
| Time             | <b>0.00001</b>  | 3                  | 18.43    |
| Time x Treatment | <b>0.0318</b>   | 6                  | 3.02     |

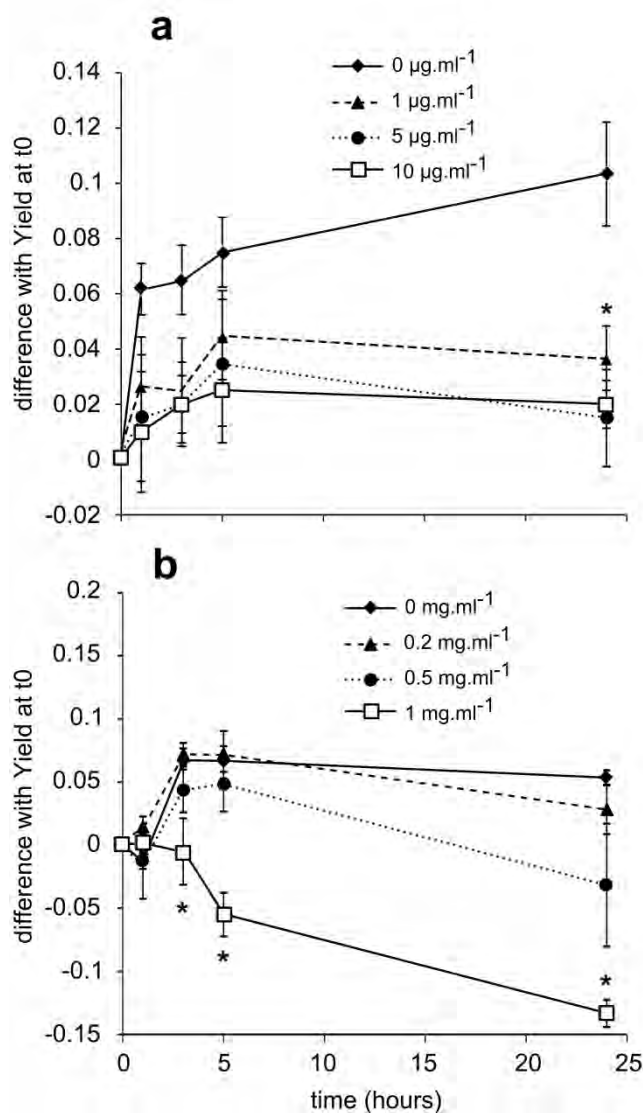
**Supplementary Material 2:** Results of repeated-measures ANOVA for the photosynthetic activity assay. Significant p-values are in bold

| Data             | Effect           | <i>p</i> values | Degrees of freedom | <i>F</i> |
|------------------|------------------|-----------------|--------------------|----------|
| Yield – yield t0 | Treatment        | <b>0.004</b>    | 3                  | 10.08    |
|                  | Time             | 0.084           | 2                  | 2.89     |
|                  | Time x Treatment | 0.163           | 6                  | 1.79     |

**Supplementary Material 3:** Chromatograms obtained with analytical UHPLC with a UV-visible detector set on 228 (**a, d**) and 415 (**b, e**) nm and an ELSD detector (**c, f, g**). The analyzed samples were A-SPE extract (**a-c**), the 18<sup>th</sup> fraction (**d-f**) obtained after semi-preparative HPLC and a standard of  $\alpha$ -linolenic acid (**g**)



**Supplementary material 4:** Temporal variation of yield values of *F. saprophila* biofilms exposed to  $\alpha$ -linolenic acid **(a)** and to sodium dodecyl sulfate (SDS) **(b)** which is a surfactant targeting the cellular membranes in order to compare their effects on the diatom photosynthetic activity. To facilitate comparisons the initial yield values were subtracted so that all t0 values are set to 0. Vertical bars represent standard error (n=3), asterisks indicate values significantly different from the control value ( $0 \mu\text{g}.\text{ml}^{-1}$ ).





## Chapitre III

### *Uronema confervicolum* (Chlorophyta) : Composés allélopathiques et régulation de leur production



### III. *Uronema confervicolum* (Chlorophyta) : Composés allélopathiques et régulation de leur production

#### III.1 Régulation de la production d'acides gras polyinsaturés allélopathiques.

La production de composés allélopathiques représente un coût métabolique non négligeable pour l'organisme émetteur. Ce coût et les conséquences, en termes de réduction de croissance, dépendent fortement des conditions environnementales. Le bénéfice associé à la production de ces composés est également influencé par ces mêmes conditions. Notre hypothèse est que la production des composés allélopathiques est régulée par les conditions environnementales.

Deux principales théories ont été établies pour prédire l'effet de **variables environnementales** sur la production de **métabolites secondaires**. D'une part, la **Growth-Differentiation Balance Hypothesis** (GDBH) prédit une optimisation de leur production basée sur les coûts, avec la priorité toujours donnée à la croissance (Herms and Mattson, 1992; Loomis, 1932; Stamp, 2004). La GDBH affirme que tout facteur qui limite la croissance plus qu'il ne limite la photosynthèse favorise les processus de différenciation. Les processus de différenciation incluent la production de métabolites secondaires riches en carbone. Cette théorie est critiquée car seuls les coûts de productions des composés sont pris en compte, alors que les avantages que l'organisme peut tirer de la production des métabolites secondaires (réduction de la compétition, diminution de la prédation), sont eux aussi affectés par les conditions environnementales (Hamilton *et al.*, 2001). D'autre part, l'**Optimal Defence Theory** (ODT) suppose que la production de métabolites secondaires ne dépend pas seulement du coût de la production, mais surtout du bénéfice que l'organisme retirera de la production des métabolites secondaires (Hamilton *et al.*, 2001). Il faut noter que l'ODT inclut aussi des processus de concentration des défenses chimiques dans les parties les plus sensibles d'une plante (Stamp, 2003). L'ODT est souvent réduite à cette sous hypothèse. Ici elle est utilisée dans son sens le plus général, *i.e.* la prise en compte des bénéfices de la libération de métabolites secondaires (Hamilton *et al.*, 2001).

La régulation de la production de composés allélopathiques dépend fortement de la composition chimique de ces composés. Par exemple, la Carbon-Nutrient Balance Hypothesis (CNBH, Bryant *et al.*, 1983) prédit qu'une plante produira des métabolites secondaires riches en azote (N ; *e.g.* alcaloïdes) lorsque celui-ci est présent en excès et des métabolites secondaires pauvres en azote (*e.g.* polyphénols) lorsqu'il est limitant. Dans le cas de l'interaction entre *U. confervicolum* et les diatomées benthiques, les composés allélopathiques inhibant la croissance sont des acides gras (*cf.*

Chapitre II), soit des molécules constituées uniquement de C, H et O et n'intégrant ni N, ni P dans leur composition. Ce modèle biologique ne nous permet donc pas de tester la CNBH (il faudrait pour cela avoir identifié au moins un composé allélopathique riche en N). Par contre, il est possible de tester l'hypothèse d'une régulation uniquement par les ressources (GDBH) ou par les besoins de l'organisme en défenses chimiques contre les compétiteurs (ODT). Le cas de l'inhibition de l'adhésion des diatomées par *U. confervicolum*, impliquant probablement des composés allélopathiques non-identifiés, sera traité dans la partie III.2.

D'après la GDBH, une limitation de la croissance par les nutriments aura donc tendance à entraîner une augmentation de la production de ces acides gras aux fonctions allélopathiques. De même, une forte intensité lumineuse et une forte disponibilité du CO<sub>2</sub> dissous, en permettant une augmentation de l'assimilation de carbone par la photosynthèse, favoriseront aussi la production de composés allélopathiques. Toutefois, ces composés ont vraisemblablement d'autres fonctions en plus de l'allélopathie (allélopathie parallèle).

Les expérimentations réalisées et présentées ici avaient donc pour but de comprendre les mécanismes de régulation de production des composés allélopathiques. Notre objectif était de déterminer si l'une de ces deux théories, initialement formulées dans le contexte des métabolites secondaires chez les plantes, s'appliquerait au contexte des interactions allélopathiques entre microalgues benthiques.

Quatre expérimentations ont été mises en œuvre :

1. La première a été réalisée afin de mettre en évidence l'adsorption des acides gras au niveau des biofilms.
2. La seconde a permis d'évaluer la production des composés allélopathiques (acides gras) au long du développement de l'algue verte en milieu fermé. D'après la GDBH, la diminution rapide des nutriments disponibles devrait conduire à l'augmentation de la production de ces composés avec le temps, alors que d'après l'ODT la production resterait constante car le bénéfice issu de la production de composés allélopathiques ne devrait pas changer avec le temps.
3. La troisième expérimentation a été conçue pour déterminer si un processus de rétrocontrôle négatif pourrait réguler la concentration du milieu en composés allélopathiques.
4. Enfin, la quatrième visait à étudier l'effet de la limitation en nutriments (N et P) et de la vitesse de croissance sur la production de composés allélopathiques. D'après la GDBH, la production de composés allélopathiques devrait être plus forte dans des conditions de lumière élevée et de forte limitation par les nutriments. D'après l'ODT, elle serait plus forte

lorsque l'intensité lumineuse est faible, car le besoin - élimination des épiphytes - est plus fort.

Pour toutes ces expérimentations, les acides gras ont été dosés dans le milieu de culture filtré et la biomasse. Les concentrations de cinq acides gras ont été mesurées : les deux acides gras précédemment identifiés comme allélopathiques: LA et LNA, deux acides gras saturés : les acides palmitique (hexadécanoïque, C16:0) et stéarique (octadécanoïque, C18 :0) et un autre acide gras polyinsaturé pour lequel on ne connaît pas d'activité allélopathique : l'acide hexadécatriénoïque (C16 :3).

Les résultats du test d'adsorption des acides gras ont montré que 99% des acides gras allélopathiques (LA et LNA) récupérés lors des extractions l'ont été dans la biomasse et seulement 1% dans le milieu de culture. Il n'est cependant pas possible de déduire directement de ces résultats un taux d'adsorption des acides gras à la surface des filaments d'*U. confervicolum*, car le test d'adsorption a été réalisé avec un biofilm de *Gomphonema parvulum*. Cette diatomée a été utilisée car elle ne produit pas elle même les acides gras utilisés. Ces résultats suggèrent toutefois que l'adsorption des acides gras sur la biomasse algale est loin d'être négligeable.

Les résultats obtenus dans les expériences 2 et 4 montrent un découplage entre la production d'acides gras (AG présents dans la biomasse) et la libération (basée sur la quantification des AG dans le milieu).

Concernant la régulation, la concentration d'AG dans le milieu de culture n'augmente pas avec l'âge de la culture et donc avec la biomasse. On observe un fort pic de libération de LNA après 14 jours puis la concentration revient à son niveau de base. Il semble y avoir un mécanisme (rétrocontrôle négatif) régulant la concentration des acides gras dans le milieu ce qui pourrait expliquer l'absence d'augmentation de la concentration d'AG avec l'augmentation de la biomasse algale. La lumière favorise fortement la production et la libération des acides gras. Globalement, les effets des conditions environnementales observés ne sont totalement conformes à aucune des théories proposées (cf. figure 7 de l'article).



Regulation of fatty acid production and release in  
benthic algae: could parallel allelopathy be explained  
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**Article soumis à *Oikos*.**



# **Regulation of fatty acid production and release in benthic algae: could parallel allelopathy be explained with plant defence theories?**

Joey L. Allen, Loïc Ten-Hage and Joséphine Leflaive

EcoLab, Université de Toulouse, CNRS, INPT, UPS, Toulouse, France

## **Abstract**

Free fatty acids (FFAs) produced by microalgae have multiple functions. Among these, there are inhibitory compounds against competitors (allelochemicals). The competitive advantage of the release of allelochemicals depends on environmental factors, but the effect of the environmental factors on allelopathic compound production is still poorly known.

Our study was designed to investigate how growth phase, algal population density, nutrient limitation and carbon assimilation affect production and release of allelopathic FFAs among other FFAs. The model species used was *Uronema confervicolum*, a benthic filamentous green alga that produces two allelopathic FFAs (linoleic and  $\alpha$ -linolenic acids). FFAs have been quantified in algal biomass and in culture medium.

Our results reveal that the release of the different FFAs is independent of FFA cellular content and that light is the major factor affecting positively the production of allelopathic FFA.

The results have been interpreted using two theories on plant defence: the growth-differentiation balance hypothesis and the optimal defence theory. Both were insufficient to explain the observed effect of environmental factors on allelopathic FFAs.

## **Introduction**

Allelopathy, *i.e.* the inhibition of competing organisms through the release of specific chemicals (Rice, 1984) is known to be very important in aquatic phototrophic microorganisms (Gross, 2003a). It strongly influences species successions in lakes (Keating, 1977). Allelopathic interactions in aquatic microorganisms are under the control of biotic and abiotic environmental factors impacting the production and release of chemicals. For example, allelopathy of the haptophyte *Prymnesium parvum* is enhanced by unbalanced N:P ratio and by high aeration but decreased by high light intensity (Graneli and Salomon, 2010). Allelochemical production by planktonic and benthic green algae is also affected by nutrients and light (Barreiro and Hairston, 2013; Leflaive and Ten-Hage, 2009). Higher temperature, higher light, phosphorous or magnesium deprivation increase the

allelopathic activity of the cyanobacterium *Cylindrospermopsis raciborskii* (Antunes *et al.*, 2012). Similar responses have been observed in other cyanobacteria (Ray and Bagchi, 2001).

The production of other bioactive compounds by aquatic phototrophic microorganisms, such as toxins, is also affected by environmental factors (*e.g.* Pinna *et al.*, 2015). The effect of environmental factors is generally interpreted through plant defence related theories (Pavia and Toth, 2008). Ecological theories focusing on the interactions between available resources and chemical defences have been widely applied to toxin production by dinoflagellates (Hardison *et al.*, 2012, 2013), planktonic cyanobacteria (Van de Waal *et al.*, 2009) and benthic cyanobacteria (Heath *et al.*, 2016). They have also been used to predict how allelopathy among aquatic macrophytes is regulated by environmental factors (Bauer *et al.*, 2009; Gross, 2003b). The Growth-Differentiation Balance Hypothesis (GDBH) states that the production of secondary metabolites (*i.e.* compounds that are not involved in the primary metabolism including energy metabolism and growth) would be enhanced by any environmental factor that reduces growth more than it reduces photosynthesis (Herms and Mattson, 1992; Loomis, 1932; Stamp, 2004). When growth is limited by any stress, photosynthetates that accumulate can be used to produce allelochemicals. Allelopathic potential would therefore be high in optimal conditions for photosynthesis (high irradiance and CO<sub>2</sub> availability) in nutrient poor, or unbalanced nutrient conditions. The results of a study from Gross (2003b) on allelochemical production by freshwater macrophytes were partially consistent with this hypothesis. In the framework of the GDBH, the Carbon-Nutrient Balance Hypothesis (CNBH) (Bryant *et al.*, 1983) states that secondary metabolites would be built up from the most abundant element. According to the CNBH, in nutrient-limited conditions allelochemicals or defence compounds are made only of carbon, oxygen and hydrogen (carbon-based metabolites) like fatty acids or polyunsaturated aldehydes. On the contrary, in a nitrogen-rich environment, allelochemicals or defence compounds would include N in their composition (peptidic compounds, cyanogenic compounds) (Herms and Mattson, 1992). This model has been applied to cyanobacterial toxins with different N-contents: a toxin with higher N-content was produced in higher quantity in N-enriched conditions (Van de Waal *et al.*, 2009).

The main issue with GDBH is that it only considers the cost of defence, but not the benefits. Another of the main plant defence theories, the optimal defence theory (ODT) gives more importance to the needs of a plant for defences (Pavia and Toth, 2008; Wäckers and Bonifay, 2004). Most studies have considered only one of the consequences of ODT: the different allocation of defence compounds between different parts of a plant (Pavia and Toth, 2008) therefore, ODT is not often considered when microorganisms are studied (Ianora *et al.*, 2011). However, the differential allocation among the plant organs is just one of the 'subhypothesis' derived from ODT (Stamp, 2003). Indeed, ODT states that the level of secondary metabolite production is not only controlled by production costs

but also by the costs / benefits balance of the production of secondary metabolites (Hamilton *et al.*, 2001). This theory could be extended to allelopathy assuming that the benefits of allelopathy correspond to the decrease in competition and to a better access to resources (light, nutrients, and space).

In freshwater, allelopathic compounds have very diverse chemical identities, for example fatty acids, peptides, pyrrolidine and alkaloids (Leao *et al.*, 2009; Leflaive and Ten-Hage, 2007). Among these, fatty acids or their derivatives are frequent (Ikawa, 2004). They are produced for example by the planktonic green alga *Chlorella vulgaris* (DellaGreca *et al.*, 2010), by diatom-dominated biofilms (Jüttner, 2001) and by the filamentous green alga *Uronema confervicolum* (Allen *et al.*, 2015). Besides their role in allelopathic interactions, some of these compounds are part of the primary metabolism of their producers. It is an example of parallel allelopathy where one compound has several identified functions including an allelopathic one (Sinkkonen, 2006). In this prospect, their production is regulated by several factors. For example, changes in light intensity, temperature, salinity, and nutrient concentrations as well as a source of organic carbon influence cellular accumulation of fatty acids in cultured microalgae (Kim and Hur, 2013; Śliwińska-Wilczewska *et al.*, 2016; Solovchenko, 2012; Van Wagenen *et al.*, 2012). Stream periphyton fatty acid profiles are strongly influenced by light and nutrients (Hill *et al.*, 2011). The existence of different regulation systems for the production of multifunction compounds may in some cases lead to non-adaptive concentrations regarding the allelopathic function (Sinkkonen, 2006).

The aim of this study was to determine to what extent the regulation of allelopathic compound production in aquatic microorganisms could be related to the need for their allelopathic function, as hypothesized in the ODT, or could be linked to the available resources, as stated in the GDBH. To test these hypotheses, we have used the benthic green alga *Uronema confervicolum* as a model organism. This species is known to produce linoleic (LA) and  $\alpha$ -linolenic acids (LNA), two polyunsaturated fatty acids (C18:2 $\omega$ 6 and C18:3 $\omega$ 3 respectively) with allelopathic properties against benthic epiphytic diatoms (Allen *et al.*, 2015). Since LA and LNA are multifunctional compounds, it is necessary to distinguish allelopathy (extracellular activity) from the other functions of fatty acids (intracellular activity).

For this purpose, the two free fatty acids (FFA) with allelopathic properties (LA and LNA) and three additional FFA, without known allelopathic properties (C16:0 hexadecanoic acid, C18:0 stearic acid, and C16:3 hexadecatrienoic acid) have been quantified in the culture medium and in the biomass of *U. confervicolum*. The alga has been sampled at different culture phases and in various conditions in terms of growth phase, growth rate, nutrient limitation and cell density. ODT predicts that

allelopathy will be higher when light or nutrient are low because such situation stimulates the competition for resources. GDBH predicts that the production of allelopathic FFA will be higher in case of nutrient limitation but will also increase with light (because there is more photosynthetates available).

## Material and methods

### *Algal cultures*

The algal strains used in the study were the green alga *Uronema confervicolum* (uro co 01 t strain), isolated from biofilms sampled in the river Tarn (Southwestern France), and the diatom *Gomphonema parvulum* strain SAG1032-1, supplied by the SAG (Sammlung von Algenkulturen Gottingen, Germany). Stock cultures of these strains were maintained in 100-mL Erlenmeyer flasks on COMBO medium (Kilham *et al.* 1998) in a temperature-controlled chamber at 18 °C (light:dark 16:8, 30  $\mu\text{mol.m}^{-2}.\text{s}^{-1}$ ). For the experiments 500-mL Erlenmeyer flasks bubbled with air and filled with 400 mL of COMBO medium were placed in the same conditions after inoculation with  $2.5 \cdot 10^6$  cells of *U. confervicolum*. These culture conditions are thereafter referred as standard conditions.

### *Fatty acid adsorption on biomass*

This experiment was designed to evaluate the potential adsorption of FFA on algal biomass. The adsorption of LA and LNA on a biofilm of the diatom *Gomphonema parvulum* was determined. This diatom was chosen because it contains neither PUFA used (Lang *et al.*, 2011). *G. parvulum* was cultured axenically in 500-mL flasks filled with 400 mL of COMBO medium renewed regularly. After 6 weeks, 350 mL of medium was discarded before the addition of 0.4 mg of LA and 1.3 mg of LNA (purchased from Sigma-Aldrich) in 10  $\mu\text{L}$  of ethanol (corresponding to approximately half of the mean total amount of LA and LNA in a culture flask of *U. confervicolum*). After 4h, biomass and medium were separated by filtration (GF/F, 0.7 $\mu\text{m}$  pore size). Biomass was freeze-dried and weighed ( $\pm 0.1$  mg). FFA concentrations were determined in both filtrate and biomass.

### *Growth phase experiment*

This experiment was designed to study the production and release of FFA during the successive growth phases corresponding to algal growth in batch cultures. *U. confervicolum* was cultured in standard conditions. After 4, 7, 10, 12, 14, 17, 20, 24, 31, 38 and 47 days of culture, two randomly chosen flasks were pooled for each of three replicates. Whole algal biomass and medium were harvested and separated by filtration (GF/F, pore size 0.7  $\mu\text{m}$ ). Algal biomass was weighed after freeze-drying. FFA concentrations were determined in both algal biomass and culture medium, using 800 mL per replicate. For the first date, 3 flasks were used per replicate in order to have enough

biomass for subsequent analysis. Freeze-dried powdered biomasses from the 12, 14, 17, 24 and 47 sampling days were analysed to determine carbon, nitrogen and phosphorous concentrations.

#### *Density effect experiment*

This experiment aimed to determine if, in a given culture condition, the release of allelochemicals was proportional to the algal biomass (*i.e.* constant production per cell when available resources remain constant) or if it was regulated so that their concentration remained constant. In this prospect, the same initial biomass of *U. confervicolum* was cultured in different volumes of culture medium. If the same amount of allelochemicals was released by the same biomass, the concentration of allelochemicals should vary. In the experiment  $2.5 \times 10^6$  cells of *U. confervicolum* were added in either one 1000-mL flask filled with 800 mL, two 500-mL flasks filled with 400 mL or four 250-mL flasks filled with 200 mL (in triplicates), placed in standard conditions. At the beginning of exponential growth, algal growth rate was not affected by the volume of culture media. The number of flasks in each condition was adjusted to perform FFA analyses on equivalent culture volumes. After 12 days, biomass and medium were separated as in the growth phase experiment and FFA concentrations were determined in the filtrate.

#### *Abiotic factor experiment*

This experiment was designed to study the effect of resource availability and limitations on FFA production. In this experiment, we manipulated *U. confervicolum* growth rate (3 levels) and nutrient concentration and limitation (4 levels). The different growth rates were obtained by modifying light and aeration conditions. The 3 levels, thereafter referred as 'growth' conditions, were high light level ( $60 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ ) and air bubbling (high growth, HG,  $0.0308 \text{ d}^{-1}$ ), low light ( $30 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ ) and air bubbling (medium growth, MG,  $0.0130 \text{ d}^{-1}$ ) and low light ( $30 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ ) and no air bubbling (low growth, LG,  $0.0071 \text{ d}^{-1}$ ). For each growth condition, the growth rate and sampling date were determined by a preliminary experiment. Algae were cultured in 250-mL flasks filled with 200 mL in high growth and in low growth conditions in COMBO medium. A triplicate was regularly sampled, all the biomass was freeze-dried and weighted. The growth curves obtained (fig. S1) were used to determine the sampling dates in HG (11 days and 23 days) and LG conditions (39 days and 78 days), data from the growth phase experiment were used for MG condition (28 days and 49 days). Cultures were stopped at mid-exponential growth phase (when half on the maximum biomass is reached) and at the beginning of the stationary phase. To obtain the 4 nutrient conditions, the culture media used were COMBO medium, nitrogen-depleted COMBO medium (nP,  $\text{NO}_3$  was reduced by 20%), phosphorous-depleted COMBO medium (Np, phosphorous was reduced by 40%), and nitrogen- and phosphorous-depleted COMBO medium (np, where N was reduced by 20% and P by 40%). For each

nutrient condition, nutrient limitation was determined as described in Danger *et al.* (2007). The algae, inoculated at  $1 \times 10^7$  cells.mL<sup>-1</sup>, were cultured during 34 days in standard condition in each of the four media described above before addition of N, P or both to the media. Absorbance (680 nm, multi-well plate reader Asys UVM 340, Salzburg, Austria) was measured after 8 days. An increase of absorbance, while no increase in the control after the addition of a nutrient, indicates that this nutrient is the limiting factor. These nutrient limitation assays performed for each nutrient condition indicated that *U. confervicolum* was N-limited in NP, nP and np treatments and P-limited in Np treatment (fig. S2). At each sampling date, FFAs were quantified in culture medium and biomass. C, N and P content of algal biomass were determined on all samples from the mid-exponential phase.

#### *Sample processing*

For all experiments whole algal biomass and medium were harvested and separated by filtration (GF/F, pore size 0.7 µm). Fatty acids were immediately extracted from medium (800 mL) using Solid-Phase Extraction (SPE) cartridges (LiChrolut EN 200 mg of sorbent, 3 mL, Merck) conditioned with 2 volumes of methanol, washed with 2 volumes of water, and eluted with 6 mL of 100% methanol. Algal biomass was weighed after freeze-drying. C and N were measured in freeze-dried powdered biomasses with a ThermoFisher Flash 2000 elemental analyzer (ThermoFisher). Total phosphorous was measured after persulfate oxidation in acid condition using ammonium molybdate spectrophotometric method.

#### *Free fatty acid quantification in algal biomass and culture medium*

FFA concentrations were determined in culture medium, using 800 mL per replicate, and in algal biomass. 2 µg and 10 µg of the internal standard heptadecanoic acid were added to the SPE extract and to algal freeze-dried biomass, respectively. Algal biomass was then extracted with methanol-water-dichloromethane (2.5-2-2.5 mL).

The medium SPE and biomass extracts were directly methylated in boron trifluoride methanol solution 14 % (SIGMA, 1 mL) and heptane (1 mL) at room temperature for 5 min. After addition of water (1 mL), fatty acid methyl esters (FAMES) were extracted with heptane (3 mL), evaporated to dryness and dissolved in ethyl acetate (20 µL). FAME (1 µL) were analyzed by gas-liquid chromatography on a Clarus 600 Perkin Elmer system using a Famewax RESTEK fused silica capillary column (30 m x 0.32 mm i.d, 0.25 µm film thickness) and a flame ionization detector (FID). Oven temperature was programmed from 110°C to 220°C at a rate of 2°C per min and the carrier gas was hydrogen (0.5 bar). The injector and the detector were at 225°C and 245°C respectively.

#### *Statistics*

The FFA concentrations at each date (growth phase experiment) and for each treatment (density effect experiment) were compared statistically using one-way ANOVA and a Tukey's HSD post-hoc test for multiple comparisons. Comparisons of the FFA composition in culture medium and biomass were achieved with PERMANOVA. Three-way ANOVA (with growth phase, growth conditions and nutrient levels as factors) were used for total FFA content and each FFA proportion comparison. Tukey's HSD post-hoc test was used for multiple comparisons. Data were transformed in order to fit to normality and homoscedasticity. When these conditions could not be achieved, the Kruskal-Wallis test was used instead of ANOVA. Correlation coefficients were calculated and tested between elemental composition and FFA concentrations. For all tests significance threshold was set at  $\alpha = 0.05$ .

## Results

The most abundant fatty acids produced and released by *U. confervicolum* were LNA, LA, C16:0, C18:0 and C16:3 (Tables S1, S2) representing respectively (in standard conditions after 24 days) 21.7%, 60.5%, 10.8%, 2.5% and 4.5% of FFA in biomass (total concentration  $42.01 \mu\text{g}.\text{mg}^{-1}$ ) and 3.2%, 6.1%, 50.9%, 35.9% and 3.9% of FFA in culture medium (total concentration  $10.51 \mu\text{g}.\text{L}^{-1}$ ).

### *Fatty acid adsorption on biomass*

231  $\mu\text{g}$  of LNA and 60  $\mu\text{g}$  of LA were recovered after SPE from the medium of biofilm-free flasks and 220  $\mu\text{g}$  and 123  $\mu\text{g}$  of LNA and LA were recovered from flasks with *G. parvulum* biofilm. For the latter, only 1.04 % ( $\pm 0.21$  %) of these fatty acids were extracted from the medium, the other 98.96% were extracted from the diatom biomass.

### *Growth phase and density effect on allelochemical production and release*

During the growth of *U. confervicolum*, LA concentration in the culture medium remained nearly constant from the first sampling date (4 days) to 38 days (fig. 1a). Between 38 and 47 days a significant three-fold increase (from  $1.36 \mu\text{g}.\text{L}^{-1}$  to  $4.45 \mu\text{g}.\text{L}^{-1}$ ) was observed ( $p < 0.001$ ). LA content in algal biomass increased with culture age ( $p = 0.0014$ ): it was low before 12 days and higher after this date. The highest concentration was obtained at 47d with  $5.14 \mu\text{g}.\text{mg}^{-1}$  of dry algal biomass. A high level of LA content was also reached at 14 days ( $3.71 \mu\text{g}.\text{mg}^{-1}$ ). Considering the 4d-38d period, LA contents in the medium and the biomass were not significantly correlated ( $p = 0.19$ ). The highest medium concentration of LNA ( $2.70 \mu\text{g}.\text{L}^{-1}$ ) was reached after 14 days of cultivation and the lowest after 31 days (fig. 1b). Medium LNA concentration was not significantly correlated with culture age ( $p = 0.23$ ) or algal biomass ( $p = 0.21$ ). LNA content in algal biomass peaked after 14 days as observed in the medium. LNA contents in the medium and the biomass were not significantly correlated

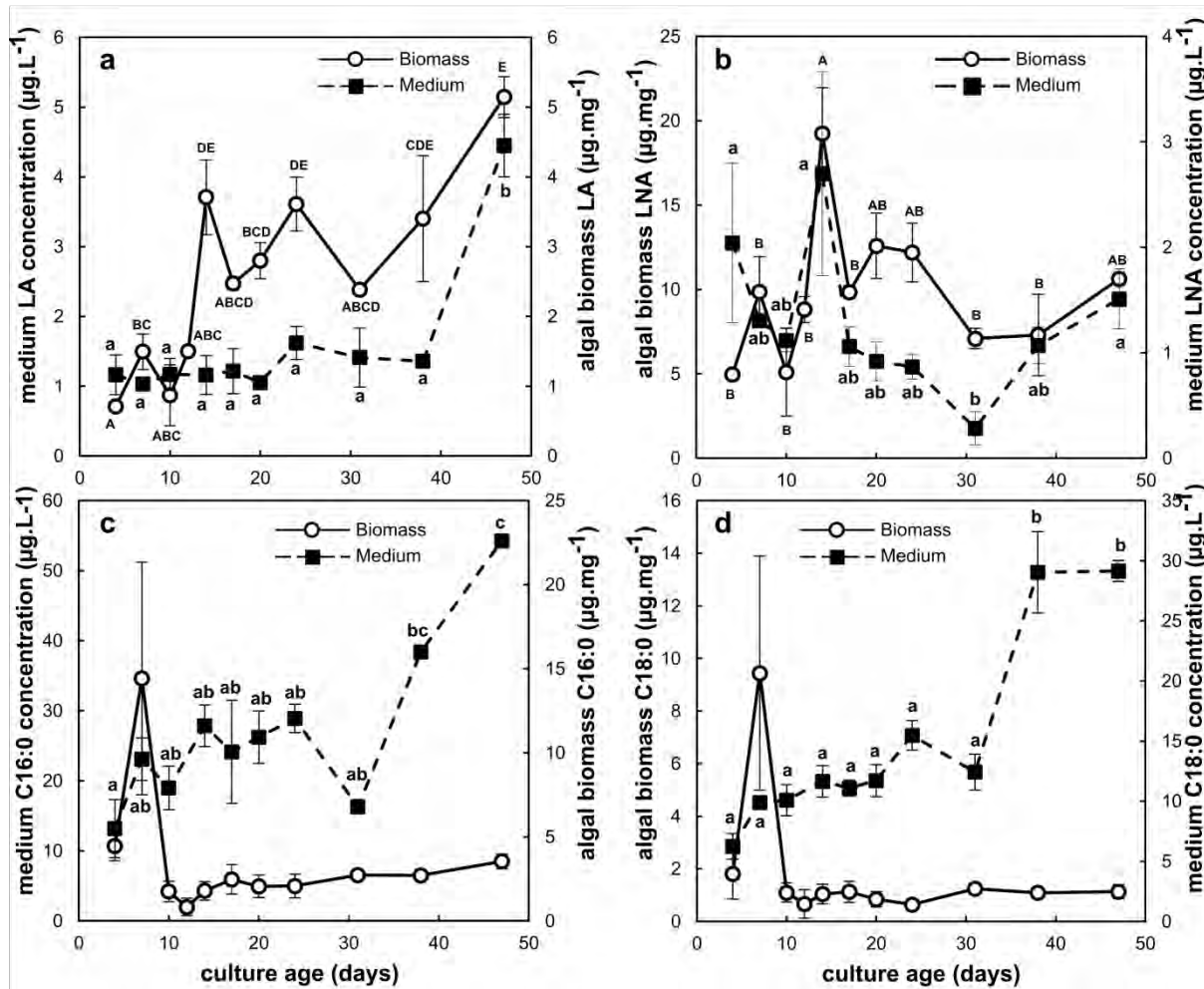


Fig. 1. Concentration of linoleic (a) and,  $\alpha$ -linolenic acid (b) Hexadecanoic acid (c) and Stearic acid (d) in *U. confervicolum* biomass (open circles) and medium (closed squares). Vertical bars represent standard error (n=3). Letters indicate statistically homogeneous groups ( $p < 0.05$ ).

( $p = 0.18$ ). LA and LNA contents in the biomass were highly correlated ( $p < 0.001$ ), whereas for medium concentrations no correlation was found ( $p = 0.51$ ).

The medium concentration of both saturated fatty acids studied (C16:0, C18:0) increased with culture age ( $p < 0.001$  for both), with a decrease between 24 and 31 days, followed by a rapid increase (fig. 1c-d). Their biomass content peaked after 7 days, rapidly decreased between 7 and 10 days and then stayed at a relatively low level.

The quantities of dissolved allelopathic FFA per biomass unit were very high at the first sampling time (4 days), and strongly decreased until 31 days of cultivation (fig. 2). By day 31, this ratio increased again as algal growth slowed down and the concentration of both allelopathic FFA in the medium increased. In the 'density effect' experiment, flasks of different volume (200, 400 and 800 mL) were inoculated with the same quantity of *U. confervicolum* cells. The concentrations of both allelopathic FFA in medium from flasks of different volume were not significantly different ( $p = 0.21$  and  $0.23$ ) (data not shown). The amounts of LA and LNA per unit of biomass were significantly affected by the

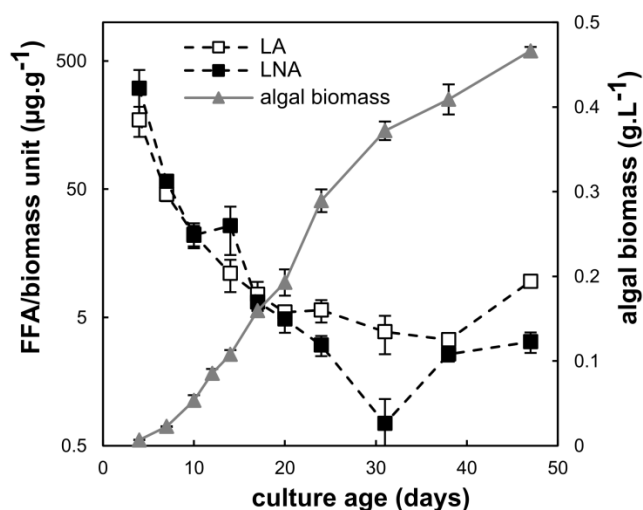


**Fig. 2.** Mean amount of linoleic acid (LA, open squares) and  $\alpha$ -linolenic acid (LNA, closed squares) in *U. confervicolum* medium per unit of algal biomass and in *U. confervicolum* biomass (grey triangles). Vertical bars represent standard error (n=3).

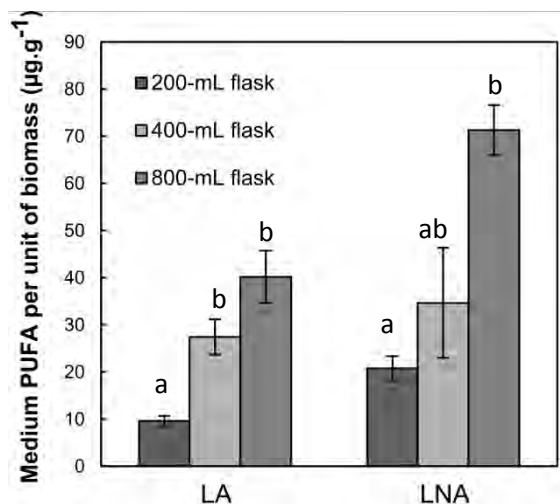


**Fig. 3.** Mean medium amount of linoleic acid (LA) and  $\alpha$ -linolenic acid (LNA) per unit of *U. confervicolum* biomass after 11 days of growth in 200, 400 or 800 ml of COMBO medium. Vertical bars represent standard error (n=3). Letters indicate statistically homogeneous groups.

flask volume ( $p = 0.042$  and  $0.0093$  for LNA and LA per biomass unit) (fig. 3). The quantities of LA and LNA in each flask were strongly related to the culture volume ( $p=0.00094$  and  $0.0010$ ).

#### Effect of abiotic factors

FFA concentrations in medium and biomass were determined at mid-exponential phase and at the beginning of stationary phase in three different 'growth conditions' and four different culture medium. Both were less affected by abiotic factors in the medium than in the biomass (table 1). Growth phase affected total FFA concentration in the culture medium, as it decreased from  $45.0 \pm 0.2 \mu\text{g.L}^{-1}$  to  $32.0 \pm 0.1 \mu\text{g.L}^{-1}$  between the mid-exponential phase sampling and beginning stationary phase sampling ( $p < 0.001$ , table S1). The same pattern was observed with FFA in biomass. The FFA compositions were affected neither in medium nor in biomass. However the part of C18:0 in the excreted FFA and C16:3 in the biomass increased between the two phases.

Nutrient condition affected only C18:0 among excreted FFA ( $p = 0.03$ ). C18:0 was

higher in np, and lower in NP treatment. In contrast, FFA composition, total FFA and C16:0 concentrations in the biomass were affected by nutrient condition (table 1).

Growth conditions did not affect total FFA concentration in the medium. The fatty acid composition of algal exudates did not change significantly due to the three factors studied, but the interaction between growth and nutrient conditions was very significant ( $p = 0.001$ ). In culture medium, growth conditions affected only LA and LNA concentrations, which were significantly higher in high growth

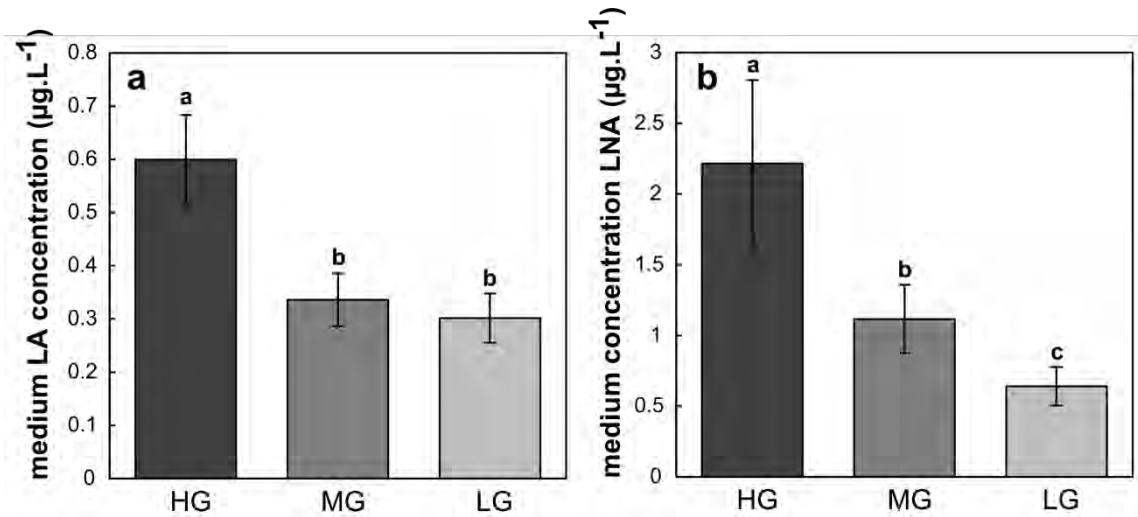


Fig. 4. Concentration of linoleic acid (a) and  $\alpha$ -linolenic acid (b) in *U. confervicolum* culture medium with different growth conditions. HG: high growth (high light and aeration). MG: medium growth (low light, high aeration). LG: low growth (low light and low aeration). Vertical bars represent standard error (n=3). Letters indicate statistically homogeneous groups.

|         |             | Factors                      |                              |                              | Interactions between factors |                              |                              |                |
|---------|-------------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|----------------|
|         |             | phase                        | nutrients                    | growth                       | Phase<br>×                   | Phase<br>×                   | Nutrient<br>×                | Nutrients<br>× |
|         |             |                              |                              |                              | nutrient                     | growth                       | growth                       | phase × growth |
| medium  | composition | 0.07                         | 0.09                         | 0.07                         | 0.67                         | 0.15                         | <b>1.00 10<sup>-03</sup></b> | 0.75           |
|         | total FA    | <b>1.60 10<sup>-04</sup></b> | 0.27                         | 0.21                         | 0.53                         | 0.82                         | 0.37                         | 0.62           |
|         | LA          | 0.14                         | 0.71                         | <b>0.02</b>                  | 0.39                         | 0.24                         | <b>0.02</b>                  | 0.70           |
|         | LNA         | 0.40                         | 0.96                         | <b>1.64 10<sup>-03</sup></b> | 0.92                         | 0.36                         | <b>2.93 10<sup>-05</sup></b> | 0.39           |
|         | C16:0       | 0.18                         | 0.08                         | 0.27                         | 0.68                         | 0.54                         | 0.22                         | 0.70           |
|         | C18:0       | <b>0.03</b>                  | <b>0.03</b>                  | 0.57                         | 0.47                         | <b>0.05</b>                  | <b>3.14 10<sup>-05</sup></b> | 0.75           |
|         | C16:3       | 0.21                         | 0.83                         | 0.23                         | -                            | -                            | -                            | -              |
| biomass | composition | 0.06                         | <b>0.05</b>                  | <b>1.00 10<sup>-03</sup></b> | <b>0.03</b>                  | <b>9.99 10<sup>-04</sup></b> | <b>1.00 10<sup>-03</sup></b> | 0.13           |
|         | total FA    | <b>9.20 10<sup>-16</sup></b> | <b>2.13 10<sup>-05</sup></b> | <b>6.23 10<sup>-10</sup></b> | 0.1                          | <b>0.01</b>                  | <b>2.60 10<sup>-05</sup></b> | 0.93           |
|         | LA          | 0.24                         | 0.6                          | <b>7.4 10<sup>-09</sup></b>  | -                            | -                            | -                            | -              |
|         | LNA         | 0.13                         | 0.60                         | <b>2.43 10<sup>-03</sup></b> | -                            | -                            | -                            | -              |
|         | C16:0       | 0.06                         | <b>0.05</b>                  | <b>5.67 10<sup>-05</sup></b> | 0.27                         | <b>2.87 10<sup>-03</sup></b> | <b>5.83 10<sup>-05</sup></b> | 0.24           |
|         | C18:0       | 0.13                         | 0.12                         | <b>3.63 10<sup>-08</sup></b> | 0.73                         | 0.20                         | <b>0.01</b>                  | 0.16           |
|         | c16:3       | <b>6.88 10<sup>-03</sup></b> | 0.75                         | <b>6.91 10<sup>-05</sup></b> | -                            | -                            | -                            | -              |

Table 1. Probability values from PERMANOVA (for compositions) with the proportion of each FFA and 3-way ANOVA (for the others) on total amount of FFA, and the proportion of each FFA in *U. confervicolum* culture medium and biomass. The three factors were the phase (two levels: mid-exponential growth end of exponential growth), the initial concentrations in N and P (nutrients, four levels), growth (the different growth rates obtained by the HG, MG and LG treatments, see fig. 4 caption, 3 levels). For ANOVA Data were transformed in order respect normality and homoscedasticity. When these conditions could not be achieved, Kruskal-Wallis test were used instead of ANOVA (no interaction tested in that case). Value in bold indicates significant effect ( $p < 0.05$ ).

conditions (fig. 4). In biomass, growth conditions strongly impacted FFA composition and total amounts of each FFA (table 1). LA was higher in MG, LNA and C16:0 were higher in LG and lower in MG, C18:0 and C16:3 were higher in HG (table S2).

| Mid exponential growth |                         |                         |                         |                     |             |
|------------------------|-------------------------|-------------------------|-------------------------|---------------------|-------------|
|                        | LA                      | LNA                     | C16                     | C18                 | C16:3       |
| LA                     | <u>0,02 (-0,27)</u>     | <b>&lt;0,001 (0,1)</b>  | <b>0,51</b>             | <b>0,89</b>         | <b>0,49</b> |
| LNA                    | <i>&lt;0,001 (2,09)</i> | <u>0,15</u>             | <b>0,91</b>             | <b>0,91</b>         | <b>0,13</b> |
| C16                    | <i>&lt;0,001 (0,46)</i> | <i>&lt;0,001 (0,14)</i> | <u>0,02 (0,45)</u>      | <b>0,66</b>         | <b>0,52</b> |
| C18                    | <i>&lt;0,001 (0,06)</i> | <i>&lt;0,001 (0,02)</i> | <i>0,05</i>             | <u>0,02 (4,57)</u>  | <b>0,36</b> |
| C16:3                  | <i>&lt;0,001 (0,17)</i> | <i>&lt;0,001 (0,07)</i> | <i>&lt;0,001 (0,25)</i> | <i>0,003 (1,27)</i> | <u>0,37</u> |

| Late/end of exponential growth |                         |                         |                         |             |                         |
|--------------------------------|-------------------------|-------------------------|-------------------------|-------------|-------------------------|
|                                | LA                      | LNA                     | C16                     | C18         | C16:3                   |
| LA                             | <u>0,81</u>             | <b>&lt;0,001 (0,17)</b> | <b>0,39</b>             | <b>0,42</b> | <b>&lt;0,001 (0,57)</b> |
| LNA                            | <i>&lt;0,001 (1,98)</i> | <u>0,94</u>             | <b>0,83</b>             | <b>0,42</b> | <b>&lt;0,001 (3,19)</b> |
| C16                            | <i>&lt;0,001 (0,28)</i> | <i>&lt;0,001 (0,14)</i> | <u>0,78</u>             | <b>0,50</b> | <b>0,01 (0,68)</b>      |
| C18                            | <i>&lt;0,001 (0,07)</i> | <i>&lt;0,001 (0,03)</i> | <i>&lt;0,001 (0,12)</i> | <u>0,81</u> | <b>0,90</b>             |
| C16:3                          | <i>0,86</i>             | <i>0,89</i>             | <i>0,11</i>             | <i>0,58</i> | <u>0,85</u>             |

**Table 2. Probability value of correlations (coefficient indicated in bracket for significant ( $p<0.05$ ) correlations) between the different FFA in the medium (in bold, upper right part of each table), in *U. confervicolum* biomass (in italic, lower left part of each table) and between biomass and medium for each FFA (underlined values).**

|         |          | C              | N              | P     | C:N            | C:P   | N:P           |
|---------|----------|----------------|----------------|-------|----------------|-------|---------------|
| medium  | total FA | -3.09          | -2.51          | -3.28 | 1.59           | 0.05  | 1.23          |
|         | LA       | -0.23          | -0.02          | 1.00  | 0.04           | -0.01 | -0.04         |
|         | LNA      | 0.48           | 1.72           | 6.44  | -0.81          | -0.05 | -0.15         |
|         | C16      | -0.15          | 0.46           | 2.23  | -0.04          | -0.02 | 0.32          |
|         | C18      | -3.41          | <b>-5.57**</b> | -8.85 | <b>2.84**</b>  | 0.08  | 0.43          |
|         | C16:3    | 0.22           | 0.9            | -4.1  | -0.42          | 0.04  | <b>0.66*</b>  |
| biomass | total FA | <b>23.87**</b> | <b>16.97*</b>  | 10.89 | -7.25          | 0.01  | 4.03          |
|         | LA       | <b>4.97**</b>  | 2.87           | 3.15  | -1.12          | -0.01 | 0.71          |
|         | LNA      | <b>14.55**</b> | <b>10.60*</b>  | 9.61  | <b>-4.73*</b>  | -0.02 | 2.13          |
|         | C16      | <b>2.86**</b>  | <b>2.98**</b>  | -4.84 | <b>-1.24**</b> | 0.05  | <b>1.13**</b> |
|         | C18      | <b>0.39*</b>   | 0.11           | 1.48  | -0.02          | -0.01 | -0.04         |
|         | C16:3    | <b>1.34**</b>  | 0.73           | 1.5   | -0.31          | -0.01 | 0.1           |

**Table 3. Coefficients (slope) of linear regression of total fatty acid and the five individual FFA on *U. confervicolum* biomass C, N and P content and C:N, C:P and N:P ratio in algal biomass. \*\* highly significant correlation ( $p<0.01$ ), \* significant correlation ( $p<0.05$ ).**

For the two allelopathic FFA studied and C16:3, the concentrations in the medium were not correlated (or negatively correlated for LA) with the concentrations in the biomass, while, for C16:0 and C18:0 the medium and the biomass concentrations were positively correlated in the mid-exponential phase (table 2). A PCA (fig. 5) highlights that samples from different growth phase and growth conditions are quite well separated on the basis of their FFA concentrations in the medium and in the biomass. LA, LNA and C16:3 concentrations in the medium were co-linear, and they were independent of concentration of all FFAs in the biomass. Medium concentrations of LA and LNA were

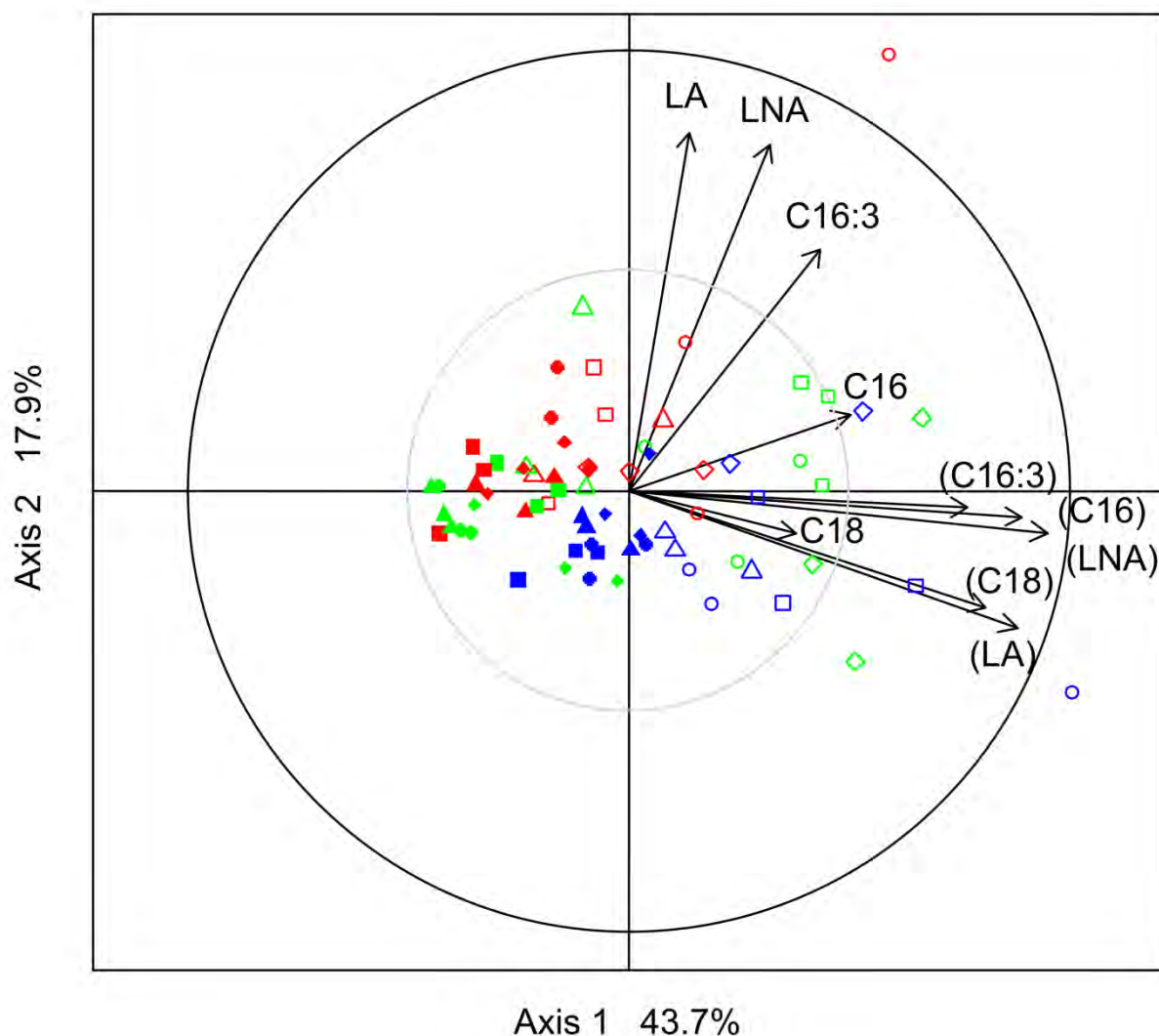


**Fig. 5. Variable and sample ordination after principal component analysis. Arrows represent variables. Variable in brackets are those measured in *U. confervicolum* biomass, the others were measured in the medium. Variable measured are LA: linoleic acid, LNA:  $\alpha$ -linolenic acid, C16: hexadecanoic acid, C18: stearic acid, C16:3: and hexadecatrienoic acid. Symbols represent each sample. Circles: NP treatment, squares: nP treatment, diamonds: Np treatment, triangles: np treatment, open symbols: mid-exponential growth sampling, closed symbols: Late/end of exponential growth sampling. Red symbols: 'high growth' treatment, blue symbols: 'medium growth' treatment, green symbols: 'low growth' treatment.**

correlated with C16:3 medium concentration in late exponential phase, but not related with the other variables (table 2, fig. 5).

C, N and P composition of algal biomass were determined for all treatments at the mid-exponential phase sampling (fig. S3). The total concentration of fatty acids in the medium was not correlated with algal elemental composition (C, N, P, table 3). N-content influenced the composition of excreted FFA ( $p = 0.031$ ) by lowering the concentration of C18 ( $p = 0.005$ ). The concentration of fatty acids in algal biomass was correlated with C and N contents ( $p=0.001$  and  $0.026$ ) and negatively correlated with C/N ratio (but  $p=0.051$ ).

## Discussion

### *Distinguishing allelopathy and other functions of allelochemicals*

Allelopathy mediated by FFA is a case of parallel allelopathy i.e., allelopathy is not the unique function of the allelochemical (Sinkkonen, 2006). FFAs are involved in both primary metabolisms and allelopathy. It is common that metabolites have multiple functions (reviewed by Pohnert, 2012). For instance brevetoxins produced by the dinoflagellate *Karenia brevis* are involved in grazer deterrence and are also a stock of carbon and nitrogen (Pinna *et al.*, 2015). Haemolytic compounds from the Raphidophyceae *Fibrocapsa japonica* have been found to be simple FFAs (Fu *et al.*, 2004). In the case of parallel allelopathy, it could be expected that the regulation of allelochemicals will not always be adaptive considering the allelopathic function. Measuring excreted FFA is the only way to evaluate the regulation of the allelopathic function mediated by these compounds. Indeed, by definition, allelochemicals have to be externalized to be effective. Excreted compounds could be adsorbed on surfaces, including algal biomass (Wu *et al.*, 2010). Our 'adsorption experiment' showed that a high quantity of FFA could be adsorbed on a diatom biofilm. A diatom was chosen here because this species does not produce LA and LNA (Lang *et al.*, 2011), but the different cell surface properties, and the surface/volume ratio of the diatom cells could have increased adsorption compared with *U. confervicolum* filaments. It is likely that medium FFA accounts for only a minor part of the extracellular FFA of *U. confervicolum* culture. Most of it is certainly adsorbed on cell surface. This indicates that we should consider the extracellular pool of FFA as divided into medium FFA and adsorbed FFA. Adsorbed FFA and intracellular FFA were not distinguishable. In our experiments, medium FFA concentration could be linked to the extracellular FFA: biomass ratio more than to the extracellular FFA amount. In any case, an increase of medium concentration of a FFA would be due to an increase of FFA liberation per algal cell. The importance of allelochemical adsorption has been shown with Fischerellins produced by the cyanobacterium *Fischerella muscicola* (Gross *et al.*, 1994). It is also quite similar to what is found with macroalgae expressing surface associated allelochemicals (Andras *et al.*, 2012; Lane *et al.*, 2009). Adsorption of allelochemicals is rarely taken into account. The production of allelochemicals is often presented as a unit of compounds quantified in the medium per unit of algal biomass (e.g. DellaGreca *et al.*, 2010). However, when the compound is strongly adsorbed by algal biomass, the measure already depends on algal biomass.

The externalisation of allelopathic compounds could be the result of passive mechanisms: passive diffusion or damaged cells (Jüttner, 2001), or the result of active exudation controlled by the cell. In the case of passive mechanisms, compound concentration in the medium would be highly correlated with algal density. Moreover, in the case of passive diffusion the concentrations of FFA in both biomass and culture medium would have been positively correlated. The results of the 'abiotic factor

experiment' revealed that for these two allelochemicals, their concentration in the biomass was negatively correlated or not correlated with their concentration in the medium. Moreover, the amount of LA and LNA stayed relatively constant while biomass increased in the 'growth phase experiment'. These results indicate that there are two pools of these FFA, the extracellular 'allelopathic' pool and the intracellular pool, with specific regulation mechanisms.

To our knowledge, C16:3 has never been reported as an allelopathic compound, but, as for the two allelopathic FFA, the medium concentration of C16:3 was not correlated with biomass C16:3 concentration. Nevertheless, an allelopathic function could be expected as the cytotoxic effect of FFA on algae is generally high with highly unsaturated FFA (Wu *et al.*, 2006). C16:0 and C18:0 have not been identified as *U. confervicolum* allelochemicals.

#### *Regulation of the release of allelopathic compounds*

Growth differentiation balance hypothesis (GDBH) states that the production of carbon-based secondary metabolites increases when growth is limited and excess of photosynthetates are available for the synthesis of secondary metabolites (Stamp, 2004). Optimal defence theory (ODT) does not take into account only the cost of defence (or allelopathy), but also the benefits of the investment in such compounds (Hamilton *et al.*, 2001; Stamp, 2003). GDBH assumes that priority is always given to growth, while ODT postulates that resources could be allocated to secondary functions if the benefits of these secondary functions are higher than those of an allocation to growth. FFA could be easily induced because they could be stored in triglyceride and rapidly released (Pohnert, 2012). In our study, we observed a strong increase in medium LA concentration after 38 days of growth and a less strong increase for LNA between 31 and 47 days, corresponding to high C:N and C:P ratios in algal biomass (fig. 6) as it is predicted by the GDBH. However, a peak in LNA concentration (and for LA, in a lesser extent) was observed around the 14<sup>th</sup> day of the experiment

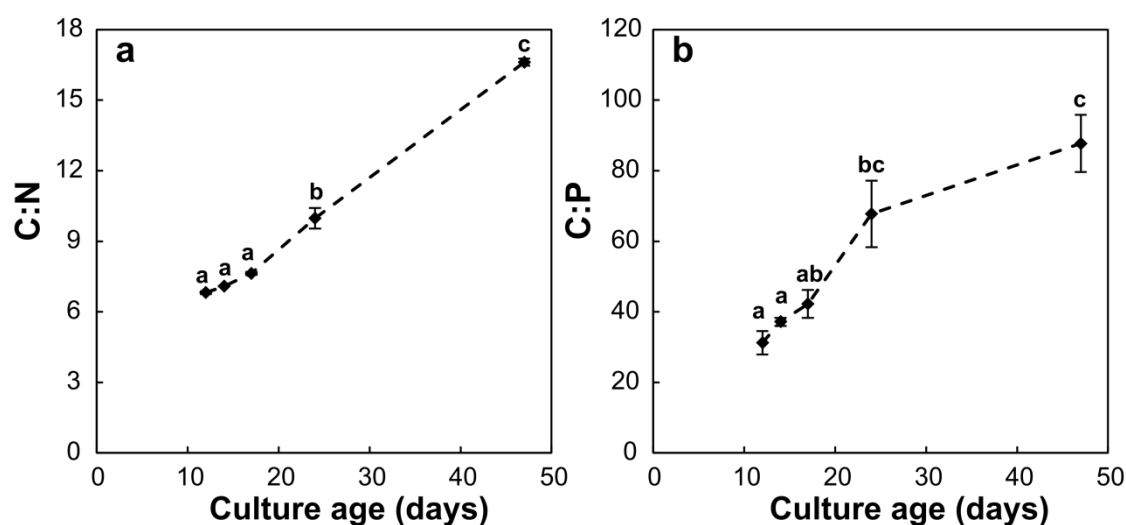


**Fig. 6.** C:N (a) and C:P (b) ratio in *U. confervicolum* biomass. Algae were cultured in standard conditions. Vertical bars represent standard error (n=3). Letters indicate statistically homogeneous groups.

We suppose that this peak began at the time when nutrients were no longer available in the medium, according to previous results with growth and nutrient concentration measurement in the same culture condition (Leflaive and Ten-Hage, 2009). So, stored nutrients began to be used by the alga (increase of C:N and C:P ratio after this date, fig. 6). The alga could have released this allelopathic compound to inhibit competitors at this 'critical' point. At a certain nutrient level, carbon could be allocated to allelopathic compounds in order to limit the depletion of nutrients by other species. This hypothesis is consistent with the ODT, since allelopathic FFA may have increased with the need of allelopathic compounds to exclude competitors.

The stability of the allelopathic FFA concentration in the culture medium, while biomass increased, besides the phenomenon of absorption, could imply a regulation process. The 'density effect' experiment was designed to test the hypothesis of a regulation of FFA exudation through a negative feedback process. The cultivation time was sufficiently short to keep *U. confervicolum* in exponential growth with a similar growth rate in each treatment. In this experiment, a lack of regulation of the exudation would have resulted in a constant FFA quantity (and different concentrations) as there were no differences in algal biomass. According to GDBH, the amount of FFA excreted in the medium should have been the same whatever the volume of the flask containing the algae because the culture conditions were identical (at least at the beginning of exponential phase). For the same reason, according to the benefits based approach (ODT), the concentration of FFA should remain the same because the need for effective allelochemicals was constant. This implies the existence of a negative feedback control of FFA exudation. Our results are consistent with this later hypothesis.

GDBH predicts that the production of carbon-based allelochemicals will be highest in optimal conditions for photosynthesis (our HG condition). When applying ODT to allelopathy, an increase of allelochemicals in low light conditions (mimicking shading by planktonic or epiphytic competitors) could be expected. In our 'abiotic factor experiment', the highest concentrations in allelopathic FFA were reached in HG conditions, which is consistent with the GDBH and some previous results. Indeed, increase of allelopathy with higher light intensity has been observed with the cyanobacterium *Cylindrospermopsis raciborskii* (Antunes *et al.*, 2012). On the contrary, allelopathy of the haptophyte *Prymnesium parvum* decreases with high light (Graneli and Salomon, 2010) and allelopathy of the green alga *Chlamydomonas reinhardtii* has been found to be higher with low light (Barreiro and Hairston, 2013). Results obtained by Gross (2003b) on allelopathic phenolic compounds exuded by *Myriophyllum spicatum* were more contrasted. Indeed, increasing light levels increased the amount of exuded phenolic compounds (supporting GDBH), but the proportion of phenolic compounds in the total organic carbon exuded was higher in low light. Therefore, the allocation of

carbon to allelopathy decreased with decreasing light, but less than the allocation to other exuded compounds. This was consistent with the ODT.

Light limitation and CO<sub>2</sub> limitation both reduce growth rate. However, according to Van de Waal *et al.* (2009), growth limitation by light or by CO<sub>2</sub> availability has different effects on secondary metabolites production. In our study, we did not directly assess whether light or CO<sub>2</sub> availability limited growth in each treatment. However, since additional aeration without additional light allowed a higher growth in MG treatments than in LG, we could assume that growth rate was limited by CO<sub>2</sub> in LG treatments. Similarly since additional light without additional aeration allows a higher growth in HG condition than in MG, we could assume that growth rate was limited by light in MG condition. The highest quantities of LA, LNA, C16:0 and C18:0 in algal biomass at the beginning of the stationary phase were obtained in the intermediate condition where the ratio CO<sub>2</sub> availability/light was the highest. This highlights the fact that CO<sub>2</sub>/light ratio is a factor to consider when studying regulation of allelopathy. In our case, for both allelopathic FFA, this amount of excreted FFA was higher in HG than in MG and higher in MG than in LG. Therefore allelochemical release depended more on the growth rate (resulting from light and CO<sub>2</sub> levels) than on the CO<sub>2</sub>/light ratio.

Nutrient limitation reduces growth more than photosynthesis. According to GDBH, an increased production of allelochemicals is therefore expected when cells are nutrient-limited. The effect of nutrient limitation on the benefits of the release of allelochemicals is difficult to predict. Actually, nutrient limitation could increase the benefits of inhibiting competitors because it would limit depletion of nutrients by other species. But target organisms are generally more sensitive to allelochemicals under nutrient sufficient conditions than under nutrient stress (Leflaive and Ten-Hage, 2009), so the benefits of allelochemicals would be higher in nutrient rich conditions. Our results on the effect of nutrients are not consistent with any of these theories. Actually, LA and LNA concentrations in HG condition were the highest in the treatment with the highest nutrient level (NP) and were much lower in the unbalanced Np and nP treatments. These results confirm the results obtained on the same species and another green alga (Leflaive and Ten-Hage, 2009), but are contradictory to numerous results. Indeed, higher allelopathic activity under P- or N-depleted conditions was observed with different microalgae (Antunes *et al.*, 2012; Graneli and Salomon, 2010; Ray and Bagchi, 2001). Particularly, limiting P concentrations has often been pointed out to increase toxicity (Hardison *et al.*, 2013) and allelopathy (Von Elert and Jüttner, 1996). DellaGreca *et al.* (2010) found an increased per cell production of chlorellin (an allelopathic mixture of FFA) in P-limited growth. Here, the different nutrient levels have not affected the release of allelopathic FFA. It could be due to low differences between nutrient levels (20% and 40% lower N or P, respectively). Many studies used higher nutrient-level differences (DellaGreca *et al.*, 2010; Leflaive and Ten-Hage, 2009).

However, lower nutrient-levels would have led to very a different final amount of biomass, and therefore to incomparable conditions.

We measured C, N and P cellular concentrations in order to determine whether allelopathic FFA were affected by biomass elemental composition. Indeed, Pinna *et al.* (2015) have showed that C-rich toxin production by the marine dinoflagellate *Ostreopsis cf ovata* was positively correlated with high cellular C:N and C:P ratio. In culture medium, only C18:0 was affected by N content and C: N ratio and C16:3 was positively affected by N: P ratio. N and C contents had strong effects on biomass FFA concentrations for all FFA. However, P or N deprivation not only limits growth but could also slow it down. Algal growth would depend on protein synthesis, therefore it depends on N availability and on the rate of protein synthesis that depends directly on P availability (as the majority of P is used to make ribosome). The assimilation of carbon would depend on proteins involved in photosynthesis. Therefore N or P depletion limits the assimilation of carbon and its allocation to secondary metabolisms. (Flynn *et al.*, 2010). Moreover, the measurements of biomass C, N and P content do not take into account the loss of these elements through excretion of compounds.

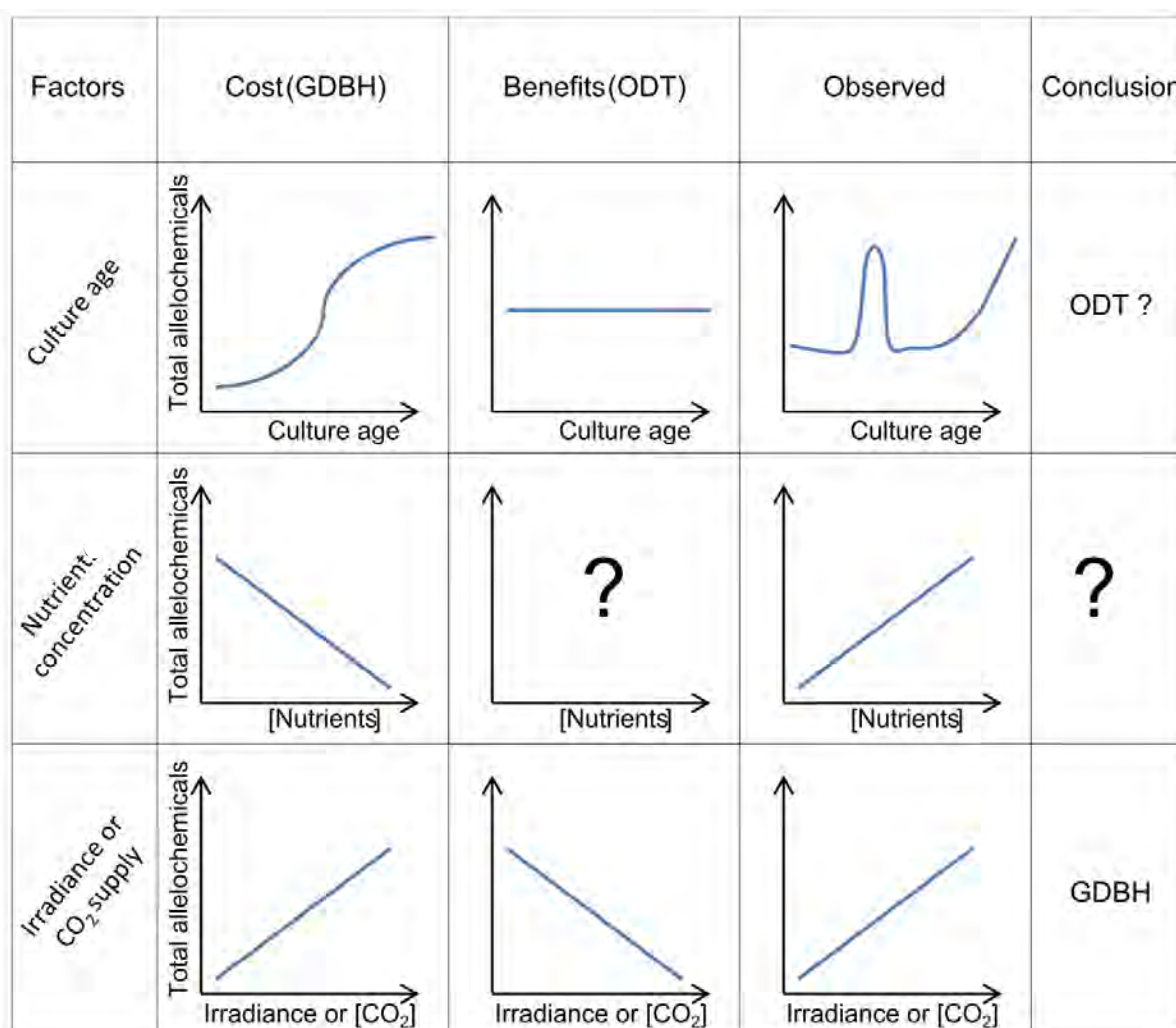


Fig. 7. Schematic representation of the effect of different factors on allelochemicals according to growth-differentiation balance hypothesis (GDBH), optimal defence theory (ODT) and the results of the present study. 81

## Conclusion

This study is the first to chemically quantify the effects of abiotic factors on the production of allelopathic compounds by benthic filamentous green algae. Growth was the major factor, affecting positively the release of allelopathic FFA. Both GDBH and ODT were insufficient to explain the observed patterns of regulation (fig. 7). The results of some of our experiments could be explained by either theory, but neither can explain all the results. However, benefits of allelopathy are rather hard to estimate, the evaluation of benefits still needs to be refined to completely test the hypothesis. We focussed on two allelopathic FFA, but *U. confervicolum* also produces other unidentified compounds with allelopathic activity (Allen *et al.*, 2015). It is possible that resources are differentially allocated to different kind of metabolites with a secondary function, depending on environmental factors. Such trade-offs have been recently highlighted in marine seaweeds exposed to competitors (Rasher and Hay, 2014). Moreover, in a complex environment the diversity of interactions (competition with other species and other trophic interactions) could have led to non-adaptive processes regarding interactions between *U. confervicolum* and diatoms. Our results highlight the limits of some ecological theories that are well suited for simple systems but not to complex interactions with many feed-back mechanisms and allocation trade-offs. Further research is needed as elucidating the regulation of allelopathic compound production could be a way to access how these interactions are important and how much organisms could invest in these interactions.

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### Supplementary material for

Allen, J.L., Ten-Hage, L., and Leflaive, J. Regulation of fatty acids production and release in benthic algae: could parallel allelopathy be explained with plant defence theories? – *Oikos* 000: 000-000.

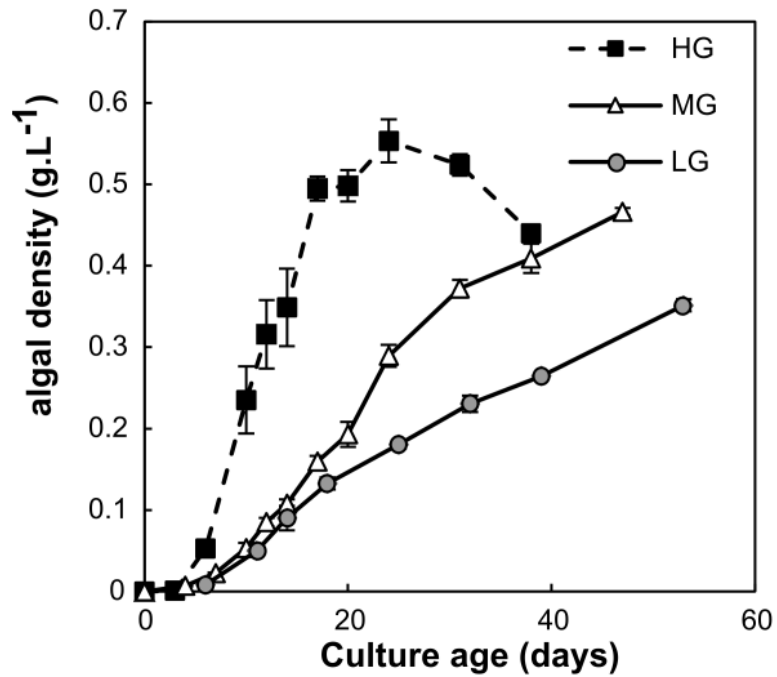


**Figure S1:** Growth curves of the algae cultured either in 'high growth' condition (HG, high light and air bubbling), 'medium growth' condition (MG, low light and air bubbling) or in 'low growth' (LG, low light and no air bubbling). Vertical bars represent standard error (n=3).

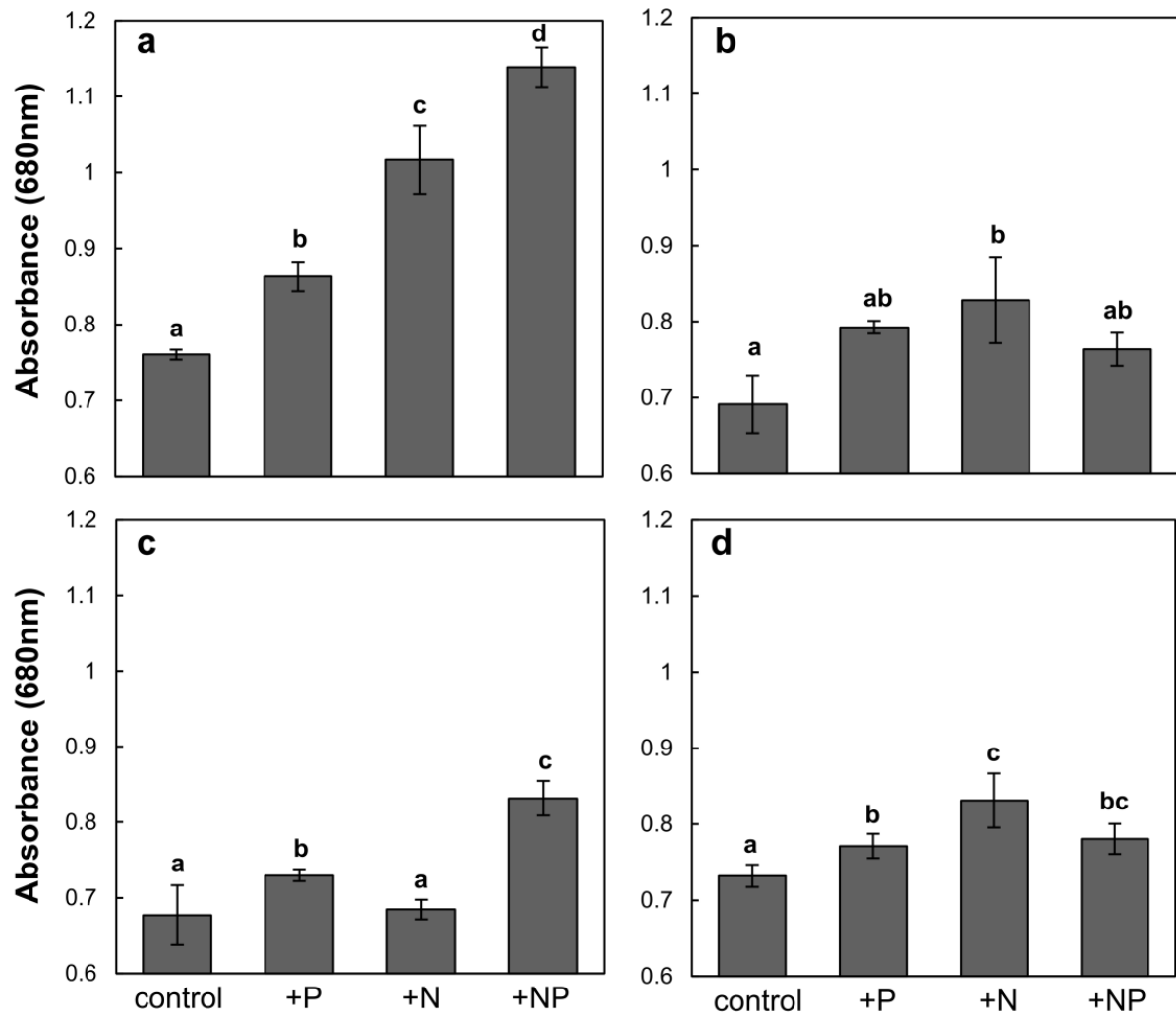


**Figure S2:** Absorbance measured after 42 days of cultivation in NP (a), nP (b), Np (c) and np (d) conditions. 8 days before measurement, demineralised water (control), phosphorous (+P), nitrogen (+N) or both (+NP) were added to the cultures. Vertical bars represent standard error (n=3). Letters indicate statistically homogeneous groups.

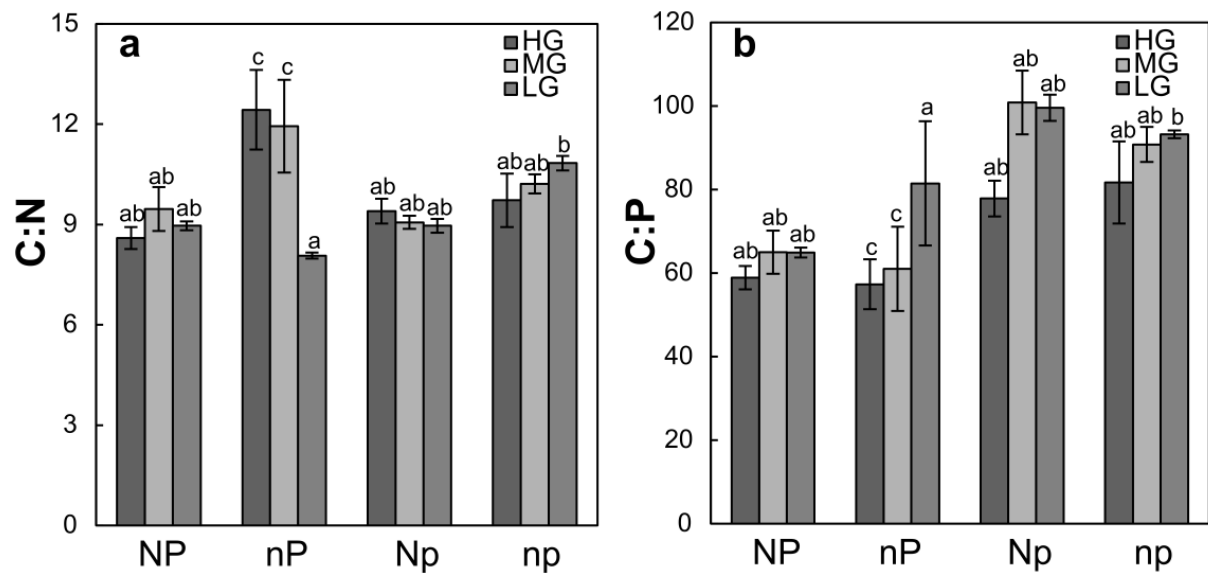


**Figure S3.** C, N and P content in algal biomass. (a, c, e) algae were harvested in mid-exponential growth from cultures with different 'growth' conditions (HG, MG and LG) and in four different medium (NP, nP, Np and np). (b, d, f) algae were cultured in standard conditions. Vertical bars represent standard error (n=3).

Supplementary material

|       |    | Mid exponential growth |                       |                        |                       | Late/end of exponential growth |                        |                        |                        |                       |    |
|-------|----|------------------------|-----------------------|------------------------|-----------------------|--------------------------------|------------------------|------------------------|------------------------|-----------------------|----|
|       |    | NP                     | nP                    | np                     | Np                    |                                | NP                     | nP                     | np                     | Np                    |    |
| LA    | HG | 4.49 (± 0.4)           | 2.43 (± 0.104)        | 3.53 (± 0.926)         | 3.33 (± 0.609)        | b                              | 3.53 (± 0.179)         | 1.52 (± 0.171)         | 2.18 (± 0.717)         | 2.72 (± 0.346)        | b  |
|       | MG | 9.11 (± 1.92)          | 9.47 (± 0.668)        | 6.88 (± 0.936)         | 7.33 (± 0.536)        | a                              | 6.37 (± 0.456)         | 5.59 (± 0.262)         | 5.92 (± 0.549)         | 5.85 (± 0.134)        | a  |
|       | LG | 6.19 (± 0.769)<br>a    | 8.93 (± 0.901)<br>a   | 3.48 (± 0.418)<br>b    | 8.46 (± 0.343)<br>ab  | a                              | 1.18 (± 0.568)<br>ns   | 2.68 (± 0.300)<br>ns   | 1.75 (± 0.184)<br>ns   | 3.56 (± 0.691)<br>ns  | b  |
| LNA   | HG | 24.3 (± 3.13)          | 12.7 (± 0.375)        | 19.6 (± 5.7)           | 16.2 (± 2.27)         | ns                             | 11.7 (± 1.100)         | 5.5 (± 0.780)          | 6.38 (± 2.14)          | 8.95 (± 1.96)         | b  |
|       | MG | 25.4 (± 7.18)          | 24.2 (± 1.19)         | 19.7 (± 2.39)          | 25.1 (± 3.17)         | ns                             | 13.9 (± 0.507)         | 14.3 (± 0.767)         | 15.8 (± 1.51)          | 15.5 (± 0.425)        | a  |
|       | LG | 21 (± 2.79)<br>ab      | 22.1 (± 2.4)<br>ab    | 12.6 (± 1.53)<br>b     | 33.2 (± 1.89)<br>a    | ns                             | 7.52 (± 0.690)<br>ab   | 11 (± 1.05)<br>ab      | 6.11 (± 0.995)<br>b    | 14.7 (± 3.01)<br>a    | b  |
| C16   | HG | 3.17 (± 0.229)         | 1.93 (± 0.0926)       | 2.83 (± 0.51)          | 2.96 (± 0.246)        | b                              | 1.83 (± 0.0557)        | 1.28 (± 0.11)          | 1.91 (± 0.304)         | 2.22 (± 0.258)        | b  |
|       | MG | 4.54 (± 1.04)          | 4.05 (± 0.152)        | 3.98 (± 0.266)         | 5.3 (± 0.132)         | a                              | 2.5 (± 0.0492)         | 2.19 (± 0.0834)        | 2.83 (± 0.160)         | 3.36 (± 0.0982)       | a  |
|       | LG | 5.35 (± 0.647)<br>a    | 6.17 (± 0.192)<br>ab  | 2.42 (± 0.238)<br>b    | 5.51 (± 0.368)<br>a   | a                              | 1.55 (± 0.166)<br>b    | 2.2 (± 0.125)<br>b     | 1.54 (± 0.109)<br>b    | 2.86 (± 0.644)<br>a   | b  |
| C18   | HG | 0.67 (± 0.0576)        | 0.42 (± 0.0229)       | 0.453 (± 0.0158)       | 0.603 (± 0.0952)      | b                              | 0.344 (± 0.0349)       | 0.287 (± 0.0416)       | 0.208 (± 0.0281)       | 0.303 (± 0.0067)      | b  |
|       | MG | 1.05 (± 0.155)         | 0.838 (± 0.168)       | 0.617 (± 0.113)        | 0.626 (± 0.0925)      | a                              | 0.596 (± 0.0255)       | 0.474 (± 0.0198)       | 0.458 (± 0.0653)       | 0.497 (± 0.0341)      | a  |
|       | LG | 0.471 (± 0.0276)<br>a  | 0.462 (± 0.0425)<br>a | 0.201 (± 0.0224)<br>b  | 0.726 (± 0.0527)<br>a | c                              | 0.178 (± 0.018)<br>a   | 0.228 (± 0.0131)<br>ab | 0.171 (± 0.0215)<br>b  | 0.343 (± 0.0382)<br>a | c  |
| C16:3 | HG | 1.68 (± 0.19)          | 0.948 (± 0.129)       | 1.19 (± 0.172)         | 1.15 (± 0.0618)       | ns                             | 0.762 (± 0.105)        | 0.452 (± 0.0241)       | 1.49 (± 0.882)         | 0.658 (± 0.116)       | ns |
|       | MG | 1.91 (± 1.03)          | 1.69 (± 0.123)        | 1.16 (± 0.11)          | 2.1 (± 0.363)         | ns                             | 0.571 (± 0.0482)       | 0.561 (± 0.0395)       | 0.553 (± 0.0596)       | 0.768 (± 0.0853)      | ns |
|       | LG | 1.21 (± 0.361)<br>ns   | 1.65 (± 0.312)<br>ns  | 0.961 (± 0.0259)<br>ns | 1.88 (± 0.193)<br>ns  | ns                             | 0.448 (± 0.0967)<br>ns | 0.549 (± 0.141)<br>ns  | 0.413 (± 0.0471)<br>ns | 0.745 (± 0.209)<br>ns | ns |

Table S1. Mean (±SE) FFA concentration in *U. confervicolum* biomass. HG: high growth (high light and aeration); MG: medium growth (low light, high aeration); LG: low growth (low light and low aeration); NP: COMBO medium (control); nP: N-depleted COMBO medium; np N and P depleted COMBO medium; Np: P depleted COMBO medium. Letters indicate statistically homogeneous groups (p<0.05). ns: not significant.

### III. *Uronema confervicolum* : Composés allélopathiques et régulation de leur production

|       |    | Mid exponential growth |                  |                   |                   |           | Late/end of exponential growth |                   |                   |                  |           |
|-------|----|------------------------|------------------|-------------------|-------------------|-----------|--------------------------------|-------------------|-------------------|------------------|-----------|
|       |    | NP                     | nP               | np                | Np                |           | NP                             | nP                | np                | Np               |           |
| LA    | HG | 0.721 (± 0.216)        | 0.631 (± 0.332)  | 0.431 (± 0.226)   | 0.234 (± 0.0117)  | <b>a</b>  | 0.822 (± 0.0859)               | 0.358 (± 0.179)   | 0.256 (± 0.0195)  | 0.381 (± 0.023)  | <b>a</b>  |
|       | MG | 0.337 (± 0.129)        | 0.51 (± 0.0927)  | 0.282 (± 0.0502)  | 0.0748 (± 0.0551) | <b>ab</b> | 0.0596 (± 0.0344)              | 0.309 (± 0.0724)  | 0.217 (± 0.0476)  | 0.143 (± 0.0924) | <b>b</b>  |
|       | LG | 0.137 (± 0.0761)       | 0.209 (± 0.105)  | 0.185 (± 0.0269)  | 0.375 (± 0.0315)  | <b>b</b>  | 0.347 (± 0.103)                | 0.14 (± 0.0756)   | 0.299 (± 0.0276)  | 0.492 (± 0.206)  | <b>ab</b> |
|       |    | ns                     | ns               | ns                | ns                |           | ns                             | ns                | ns                | ns               |           |
| LNA   | HG | 4.32 (± 2.87)          | 0.793 (± 0.793)  | 2.64 (± 1.51)     | 0.890 (± 0.295)   | ns        | 2.84 (± 1.6)                   | 0.878 (± 0.6)     | 0.726 (± 0.143)   | 1.1 (± 0.274)    | <b>a</b>  |
|       | MG | 0.645 (± 0.0859)       | 1.44 (± 0.383)   | 0.319 (± 0.0505)  | 0.303 (± 0.206)   | ns        | 0.35 (± 0.31)                  | 0.935 (± 0.0926)  | 0.111 (± 0.111)   | -                | <b>b</b>  |
|       | LG | 0.0708 (± 0.0708)      | 1.28 (± 0.617)   | 0.651 (± 0.164)   | 2.60 (± 0.909)    | ns        | 0.179 (± 0.179)                | 0.459 (± 0.236)   | 1.05 (± 0.195)    | 1.41 (± 0.564)   | <b>ab</b> |
|       |    | ns                     | ns               | ns                | ns                |           | ns                             | ns                | ns                | ns               |           |
| C16   | HG | 4.07 (± 0.325)         | 2.65 (± 0.509)   | 3.01 (± 0.868)    | 3.41 (± 0.103)    | ns        | 3.89 (± 0.541)                 | 1.99 (± 0.395)    | 2.25 (± 0.698)    | 1.95 (± 0.281)   | ns        |
|       | MG | 5.35 (± 1.62)          | 5.4 (± 1.26)     | 3.03 (± 0.71)     | 2.38 (± 0.57)     | ns        | 2.37 (± 0.653)                 | 4.12 (± 1.43)     | 1.34 (± 0.366)    | 1.42 (± 0.204)   | ns        |
|       | LG | 3.13 (± 0.862)         | 5.27 (± 1.58)    | 2.95 (± 0.88)     | 3.65 (± 0.401)    | ns        | 3.17 (± 1.02)                  | 1.64 (± 0.86)     | 1.76 (± 0.451)    | 2.92 (± 0.715)   | ns        |
|       |    | ns                     | ns               | ns                | ns                |           | ns                             | ns                | ns                | ns               |           |
| C18   | HG | 4.93 (± 1.30)          | 8.72 (± 2.14)    | 4.86 (± 0.848)    | 5.30 (± 0.940)    | ns        | 3.89 (± 0.46)                  | 5.06 (± 0.201)    | 5.26 (± 0.372)    | 5.44 (± 0.106)   | ns        |
|       | MG | 3.77 (± 0.483)         | 3.96 (± 0.186)   | 3.91 (± 0.594)    | 8.01 (± 3.11)     | ns        | 3.88 (± 1.04)                  | 4.31 (± 1.04)     | 3.77 (± 0.186)    | 4.74 (± 0.739)   | ns        |
|       | LG | 7.00 (± 1.15)          | 7.99 (± 3.13)    | 6.07 (± 1.40)     | 4.75 (± 0.520)    | ns        | 4.26 (± 0.595)                 | 3.58 (± 1.82)     | 5.3 (± 0.711)     | 4.6 (± 0.851)    | ns        |
|       |    | ns                     | ns               | ns                | ns                |           | ns                             | ns                | ns                | ns               |           |
| C16:3 | HG | 0.85 (± 0.617)         | 0.273 (± 0.0427) | 0.257 (± 0.0911)  | 0.273 (± 0.120)   | <b>ab</b> | 0.807 (± 0.245)                | 0.0999 (± 0.05)   | 0.147 (± 0.0165)  | 0.21 (± 0.0683)  | <b>a</b>  |
|       | MG | 0.41 (± 0.18)          | 1.27 (± 0.294)   | 0.549 (± 0.249)   | 1.67 (± 1.52)     | <b>a</b>  | 0.202 (± 0.115)                | 0.452 (± 0.103)   | 0.0315 (± 0.0315) | -                | <b>b</b>  |
|       | LG | -                      | 0.215 (± 0.164)  | 0.0759 (± 0.0431) | 0.792 (± 0.207)   | <b>b</b>  | 0.113 (± 0.0629)               | 0.0584 (± 0.0299) | 0.206 (± 0.0269)  | 0.341 (± 0.0718) | <b>ab</b> |
|       |    | ns                     | ns               | ns                | ns                |           | ns                             | ns                | ns                | ns               |           |

Table S2. Mean (±SE) FFA concentration in *U. confervicolum* medium (μg.L<sup>-1</sup>). MG: medium growth (low light, high aeration); LG: low growth (low light and low aeration); NP: COMBO medium (control); nP: N-depleted COMBO medium; np N and P depleted COMBO medium; Np: P depleted COMBO medium. Letters indicate statistically homogeneous groups (p<0.05). ns: not significant.

### III.2 Production de composés allélopathiques anti-adhésion.

D'après les résultats présentés dans le chapitre II, *U. confervicolum* produirait, en plus des acides gras cités précédemment, d'autres composés allélopathiques inhibant spécifiquement la capacité d'adhésion des diatomées. Cette constatation amène une nouvelle question : la régulation de la production et de la libération des composés inhibant l'adhésion est-elle la même que pour les acides gras allélopathiques, ou bien est-ce que la production de composés allélopathiques permet de maintenir un même niveau global d'activité allélopathique lorsque la libération d'acides gras est faible ?

De plus, pour une meilleure compréhension du rôle de ces composés, il est nécessaire de pouvoir les identifier. L'étude présentée dans cette partie est consacrée au travail réalisé sur les facteurs régulant l'activité allélopathique anti-adhésion de l'algue verte filamenteuse *U. confervicolum* et à une tentative d'identification des composés impliqués dans cette inhibition.

Pour cette étude, nous avons utilisé du filtrat d'*U. confervicolum* en culture dans différentes conditions de lumière et d'aération et les échantillons de biomasse des expérimentations 2 (prélèvements au cours d'une culture) et 4 (cultures dans des conditions environnementales différentes) de la partie III.1. Une extraction à l'acétone a été réalisée pour récupérer les composés allélopathiques. Les variations de la production de composés anti-adhésion ont été évaluées grâce à

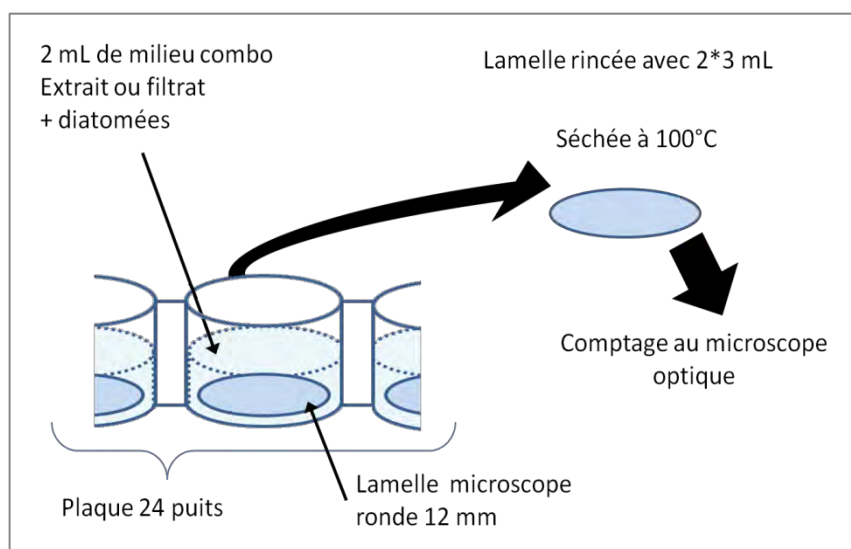


Figure 8 : Schéma de la procédure expérimentale du test d'adhésion réalisé.

un test d'adhésion des diatomées sur une lamelle de verre (figure 8).

La stratégie utilisée pour l'identification des composés allélopathiques impliqués est l'utilisation du profilage métabolomique (Prince and Pohnert, 2010; Scognamiglio *et al.*, 2015; Weston *et al.*, 2015).

Cette méthode consiste à établir un « profil métabolique » en utilisant une méthode séparative (dans notre cas une chromatographie liquide haute performance, HPLC). Cela permet de séparer les différents métabolites (repérés par les différents pics sur le chromatogramme), mais n'autorise pas une identification directe des composés. La comparaison des profils d'échantillons ayant différents niveaux d'activité permet de relier les différences d'intensités des pics (donc les différences de concentrations d'une molécule) au niveau d'activité des échantillons. Cette démarche aboutit à la sélection d'un petit nombre de métabolites à identifier, *a priori* impliqués dans l'activité allélopathique observée. La figure 9 illustre ce propos avec deux chromatogrammes issus d'extraits de biomasse d'*U. confervicolum* présentant des activités d'inhibition d'adhésion contrastées.

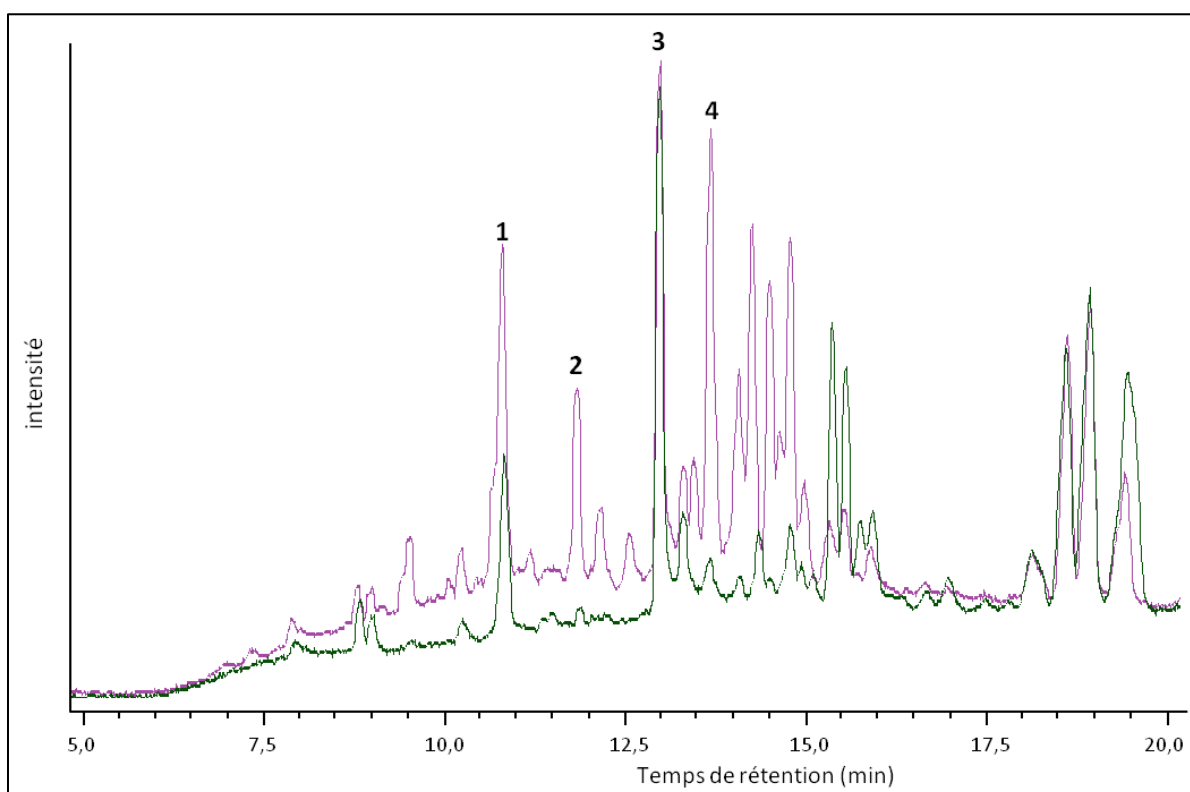


**Figure 9 : Profils métabolomiques obtenus par HPLC couplée à un détecteur à barrette de diodes de deux extraits d'*U. confervicolum* avec une forte (ligne violette) et une faible (ligne verte) activité allélopathique anti-adhésion. Les pics 1 et 3 sont communs aux deux échantillons : les molécules correspondant à ces pics ne sont certainement pas impliquées dans l'effet observé. Les pics 2 et 4 sont beaucoup plus forts dans l'échantillon ayant une forte activité : ils peuvent correspondre à un composé allélopathique impliqué dans l'effet observé.**

**NB :** Cette figure est présentée pour illustrer le principe du profilage métabolomique pour l'identification de composés allélopathiques. Les données de cette figure sont celles d'une expérience préliminaire et non les données utilisées pour déterminer la nature des composés allélopathiques inhibant l'adhésion.

Dans le cas de l'activité allélopathique d'*U. confervicolum*, afin d'obtenir des profils métabolomiques contrastés, nous avons utilisé la variabilité de l'activité allélopathique (effet anti-adhésion) induite par les différentes conditions de culture. Les différences dans les profils métabolomiques ont été reliées à l'activité d'inhibition de l'adhésion en utilisant une régression sPLS (sparse Partial Least

Square ; Lê Cao *et al.*, 2008, 2011). Cette méthode statistique permet la sélection d'un petit nombre de métabolites potentiellement liés à l'effet d'inhibition de l'adhésion observé.

Les résultats issus des expérimentations montrent un effet négatif de l'intensité lumineuse sur l'activité anti-adhésion du filtrat ( $p = 0.016$ ), une augmentation de la concentration de composés allélopathiques inhibant l'adhésion dans la biomasse avec l'âge de la culture, un effet positif de l'intensité lumineuse sur l'activité anti-adhésion des extraits de biomasse ( $p = 0.003$ ) et une absence d'effet significatif de la concentration initiale en nutriments. Quatre métabolites potentiellement impliqués dans l'inhibition de l'adhésion des diatomées ont été repérés. Ils n'ont pas pu pour le moment être identifiés. Cependant, le travail d'identification est toujours en cours. Il est possible que les composés inhibant l'adhésion ne fassent pas partie des composés détectés lors du profilage métabolomique, car ce dernier est toujours partiel, les composés détectés dépendant des méthodes utilisées pour la séparation et la détection des métabolites (Prince and Pohnert, 2010).

Dans l'expérience de culture en batch (milieu fermé), l'accumulation de biomasse a entraîné une diminution de l'intensité lumineuse et des nutriments. Ainsi, l'augmentation de l'activité anti-adhésion des extraits pourrait être due à l'un ou l'autre de ces facteurs. Etant donné que, nos résultats ont mis en évidence par ailleurs que la forte intensité lumineuse augmente la production des composés ayant une activité allélopathique anti-adhésion, l'augmentation de l'activité anti-adhésion avec le temps est plus probablement due à la déplétion des nutriments. L'absence d'effet « nutriments » avec les échantillons issus de l'expérimentation 4 (voir III.1) peut s'expliquer par des différences trop faibles dans les concentrations en nutriments dans les milieux de départ. Ainsi la **production** de composés allélopathiques anti-adhésion augmente avec la lumière et diminuerait, probablement, avec la concentration en nutriments. Cela correspond à la théorie GDBH pour un composé fortement carboné. Par contre, les résultats obtenus avec le filtrat montrent que ces composés sont plutôt **libérés** en condition de lumière faible. Ainsi, ces composés s'accumuleraient dans des conditions favorables à leur production, mais seraient libérés dans les conditions où leur effet serait plus bénéfique pour l'organisme émetteur, ce qui correspondrait plus à l'ODT. Ici encore il apparaît que ces théories développées dans le cadre des défenses chez les plantes ne s'appliquent que partiellement à la problématique des interactions allélopathiques chez les microorganismes. Cela peut s'expliquer par une régulation séparée de la production et de la libération.



Production of anti-adhesion allelopathic compounds by  
a filamentous green alga.

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## Production of anti-adhesion allelopathic compounds by a filamentous green alga.

Joey L. Allen<sup>1</sup>, Gérald Culioli<sup>2</sup>, Loïc Ten-Hage<sup>1</sup> and Joséphine Leflaive<sup>1\*</sup>

<sup>1</sup>ECOLAB, Université de Toulouse, CNRS, INPT, UPS, Toulouse, France

<sup>2</sup>Laboratoire MAPIEM, Université de Toulon, La Garde, France

### Abstract

For biofilm forming benthic diatoms, the ability to adhere to a surface is of vital importance. We already described that the filamentous green alga *Uronema confervicolum* produces allelopathic compounds (*i.e.* chemicals released to inhibit competitors) that inhibit specifically diatom adhesion. The present study focuses on the regulation by abiotic environmental factors of the production of anti-adhesion allelopathic compounds. Three experiments have been carried out to study the effect of environmental factors on the inhibition of the adhesion of the diatom *Fistulifera saprophila* using (i) filtrate of *U. confervicolum* cultured in three different light and aeration conditions, (ii) algal biomass extracts of *U. confervicolum* cultured in batch cultures during 12 to 47 days and (iii) algal biomass extracts of *U. confervicolum* cultured in different light, aeration and nutrient conditions. A metabolomic profiling-based approach was used for the identification of allelopathic compounds. High light decreased the allelopathic anti-adhesion activity of the filtrate while it increased the anti-adhesion activity of the extracts of algae cultured in nutrient rich conditions. Culture age increased the allelopathic anti-adhesion effect. Our results suggest an accumulation of the compounds when conditions are favourable for their production and higher release in less favourable conditions. Four metabolites were determined as putative allelopathic compounds but not yet identified.

### Introduction

Adhesion is a particularly important function for benthic diatoms as they generally colonize every light-exposed surface, even exposed to high flow. This ability to adhere allows diatoms to develop as epiphytes on other phototrophic organisms (Wetherbee *et al.*, 1998). The development of epiphytes can be particularly harmful for colonized organisms which therefore have developed various strategies to avoid surface colonisation (Harder, 2009). Among these strategies the production of allelopathic compounds (chemical compounds produced by an organism to inhibit potential competitors) is common in benthic environment (Allen *et al.*, 2016). The modes of action of allelopathic compounds are diverse: enzyme inhibition, membrane disruption, photosystem inhibition (Gross, 2003). However, allelopathic compounds could also inhibit the adhesion ability of

the target. Indeed, in a previous study, we have identified that the filamentous green alga (FGA) *Uronema confervicolum* inhibited diatom growth, photosynthesis and adhesion. While the growth and photosynthesis inhibition have been found to be notably due to two polyunsaturated fatty acids, these compounds were not responsible of the adhesion inhibition (Allen *et al.*, 2015). We hypothesised that the inhibition of diatoms was due to other allelopathic compounds still unidentified so far.

Abiotic environmental factors are known to influence the production of secondary metabolites. Particularly, light intensity or P-deprivation have been often pointed out to affect the production of allelopathic compounds (*e.g.* Granéli and Hansen, 2006; Leflaive and Ten-Hage, 2009).

The present study focuses on the allelopathic inhibition of diatom adhesion by *U. confervicolum* and the abiotic factors that could influence the production of anti-adhesion allelopathic compounds. The variability of the allelopathic activity induced by the environmental conditions allows the use of metabolomic profiling to approach the identification of the involved allelopathic compounds (Prince and Pohnert, 2010; Weston *et al.*, 2015).

## Material and methods

The filamentous green alga *Uronema confervicolum* (uro co 01t strain) and the diatom *Fistulifera saprophila* (fis sa 07g strain), isolated from biofilms in south-western France rivers, were cultured in 500-ml flasks filled with 400 ml of medium in a temperature-controlled chamber at 18°C (light:dark 16:8). In standard conditions, algae were cultured in COMBO medium (Kilham *et al.*, 1998), the light intensity was set at 30  $\mu\text{mol.m}^{-2}.\text{s}^{-1}$  and cultures were aerated through air bubbling. In the first experiment, 3 ml of filtrate were sampled in three culture flasks of *U. confervicolum* after 3, 20 and 31 days of cultivation in three different 'growth rate conditions': a 'high growth' condition (HG) obtained by increasing light intensity to 60  $\mu\text{mol.m}^{-2}.\text{s}^{-1}$ , a medium growth condition (MG) corresponding to the light and aeration of standard conditions and a 'low growth' condition (LG) obtained by limiting the aeration to only the diffusion of gases through the cellulose caps of the culture flasks. In the second experiment, *U. confervicolum* was cultured during 12, 14, 17, 20, 24, 31, 38 and 47 days in standard conditions before biomass harvesting. In the third experiment, *U. confervicolum* was cultured in four different culture media: COMBO medium (NP), nitrogen-depleted (-20%) COMBO medium (nP), phosphorous-depleted (-40%) COMBO medium (Np) and N- and P-depleted COMBO medium (np), and, with a factorial design, the FGA was also cultivated in the three different 'growth rate conditions' (HG, MG and LG, each 'growth rate condition' was associated

with the four culture media) as described above. Algal biomass was harvested when cultures reached mid-exponential growth phase and at the end of exponential growth phase (as described in Allen *et al.* submitted). For each sample, freeze-dried powdered algal biomass samples were extracted three times by sonication in 25 ml acetone and 3 h extraction at room temperature. Extracts were filtrated (GF/F filters) before evaporation to dryness and re-suspended in methanol to a final dry extract concentration of 10 mg.ml<sup>-1</sup>.

For adhesion assays, 10 µl of extract was evaporated to dryness and dissolved with 4 µl of ethanol followed by 2 ml of COMBO medium in a 24-well plate. A glass microscope cover slip (ø 12mm) was placed at the bottom of each well. *F. saprophila* (4.10<sup>5</sup> cells) was then inoculated. After 4 h in the dark at 18°C, glass cover slips were rinsed two times with 3 ml of distilled water to remove non adhered cells. Diatoms were then fixed by heating at 100°C. Adhered cells were counted under light microscope (Eclipse 80i Nikon, 600 × magnification). Adhesion inhibition was calculated as follows:

Adhesion inhibition (%) = 100 x (D<sub>c</sub> - D)/D<sub>c</sub>, where D<sub>c</sub> is the mean cellular density in controls and D is the mean cellular density in the treatments.

Result of the adhesion assays with filtrate were analysed using ANCOVA. Results of adhesion assays with filtrate were analysed using either two-way (time x growth conditions) or three-way ANOVA (time x nutrient conditions x growth conditions). Data were transformed when necessary to fit normal distribution.

#### *Metabolomic profiling*

The metabolomic profiling was carried out on an Elite LaChrom (VWR-Hitachi, Fontenay-sous-Bois, France) chromatographic system coupled with an ion trap mass spectrometer (Esquire 6000, Bruker Daltonics, Wissembourg, France). Chromatographic separation was achieved on a reversed-phase column (150 × 3 mm, 2.6 µm, Kinetex Phenyl-Hexyl, Phenomenex, Le Pecq, France) maintained at 30°C and the injected sample volume was 10 µl. The mobile phase consisted of acidified water (A) and acetonitrile (B) (containing each 0.1% of formic acid) and the flow rate was set at 0.5 ml.min<sup>-1</sup>. The elution gradient started at 10% B and kept for 2 min, then to 100% B at 12 min and kept for 13 min; then back to 10% B over 0.1 min and maintained 9.9 min. The electrospray interface (ESI) with nebulizing gas (N<sub>2</sub>) pressure at 40 psi, drying gas (N<sub>2</sub>) flow at 8 l.min<sup>-1</sup> and drying temperature at 350°C was operated in positive mode (capillary voltage at 4000 V). Mass spectra were acquired in the full scan range *m/z* 50 to 1200. The mass spectrometer was controlled using Esquire Control version 6.1 and acquired data were handled with Data Analysis version 4.3 (Bruker Daltonics). Several pooled

samples were prepared by sampling and mixing a small amount of each sample. These pooled samples were used as quality controls (QCs) and, in order to ensure analytical repeatability, these QCs were injected at the beginning, at the end and every four samples within the sequence run. Control samples (media blanks) were also prepared and were randomly injected within the sequence. These blanks allowed the further subtraction of contaminants or components coming from the growth media and the analytical procedure. At the end of the sequence, raw LC/MS data were converted into netCDF files and processed for peak finding, integration and alignment using the open source XCMS package (version 1.38.0) in the R 3.1.0 environment (*matchedFilter* method, signal-to-noise ratio: 5, group band-width: 3; the other parameters were default settings). The resulting variables list was further processed by the CAMERA package (version 1.18.0) and three successive filtering steps (signal/noise ratio, coefficient of variation and coefficient of the autocorrelation between variables in a same given pcgroup) using an in-house script were run on R.

#### *Metabolomic data analysis*

For variable (metabolite) selection, partial least square (PLS) and multilevel sparse partial least square regression (sPLS; Lê Cao *et al.*, 2008) were performed using adhesion inhibition values as the response variable. Only variables with a positive correlation with the inhibitory activity of the extract were conserved after the analysis. Multivariate analyses were performed with the statistical software 'R' using the package 'mixOmics'.

### **Results and discussion**

The filtrate of the FGA *U. confervicolum* inhibited diatom adhesion (Fig 1a). This confirms that the inhibitory compound(s) is(are) released in the environment, and therefore can be considered as allelopathic compounds (Sinkkonen, 2006). In our experiment with filtrates, an effect of nutrient depletion by the FGA could not be excluded. However, the fact that similar results were obtained with filtrate and extracts (Fig. 1b-d), and the short exposure time in the adhesion assay (4 h) suggest that the observed effect was more likely due to chemical inhibition than to resource depletion.

The filtrate expressed higher activity after 20 days than after 31 and 3 days (Fig 1a), while the anti-adhesion activity of biomass extracts increased significantly with the cultivation time (Fig 1b). In batch culture, the amount of available nutrients and light decreases when cultivation time and algal biomass increase. The filtrate activity was the highest in MG conditions and lowest in HG conditions ( $p=0.016$ ).

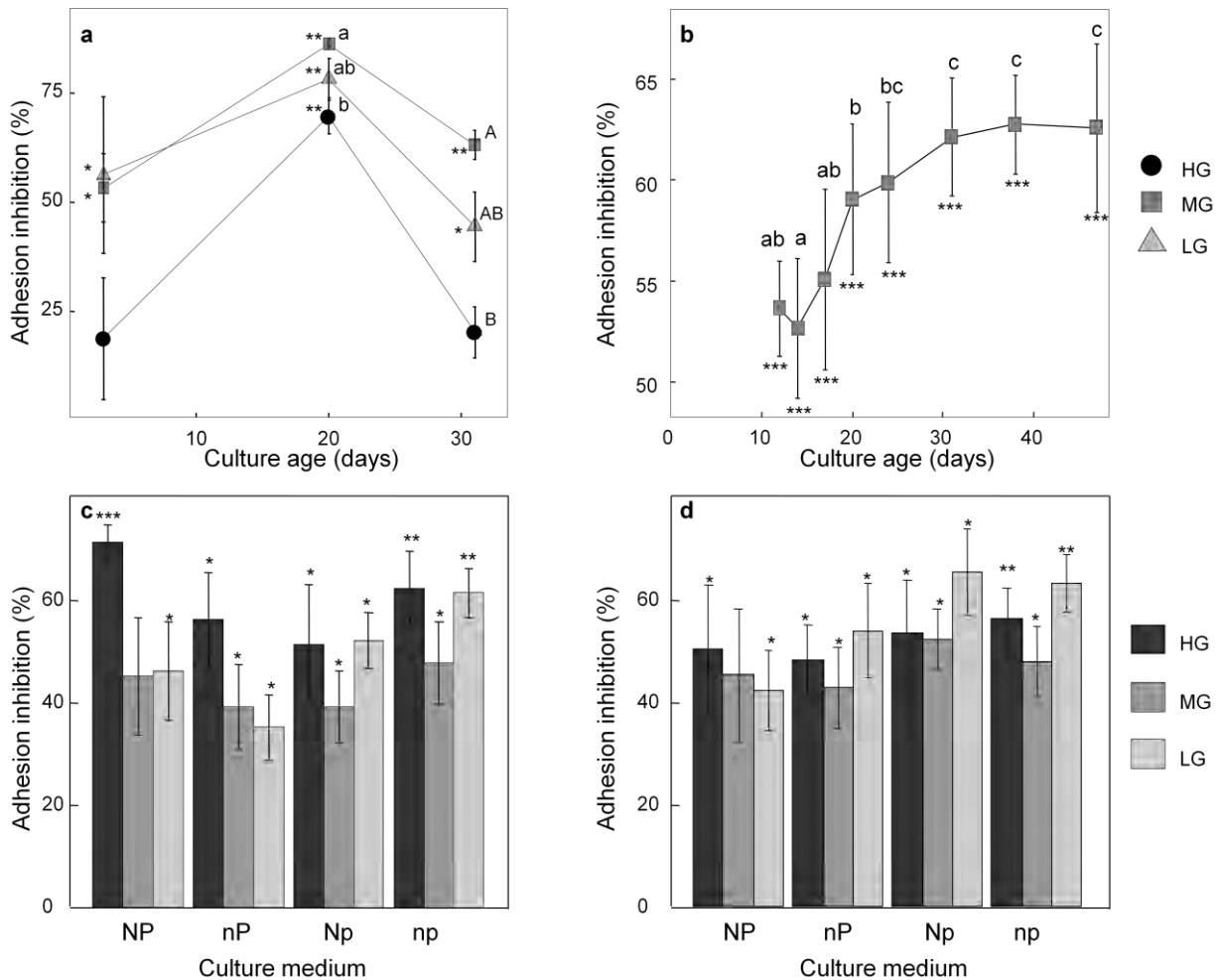


**Figure 1.** Inhibition of the adhesion of the diatom *G. parvulum* exposed to (a) filtrate or (b-d) biomass extracts of *U. confervicolum* after different cultivation time (a, b) and in different 'growth rate conditions' (a, c and d) and cultured in different media (c, d). c: mid-exponential phase culture; d: end of exponential phase culture. HG: high growth (high light and aeration). MG: medium growth (low light, high aeration). LG: low growth (low light and low aeration). NP: COMBO medium (control); nP: N-depleted COMBO medium; np N and P depleted COMBO medium; Np: P depleted COMBO medium. Vertical bars represent standard error (n=3). Letters indicate statistically homogeneous groups. Asterisk indicates significant difference with the control (\*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$ ).

The third experiment (Fig 1c-d) was specifically designed to test the effect of light, CO<sub>2</sub> availability and nutrients on the production of allelopathic compound. A significant effect of growth conditions ( $p = 0.003$ ) indicates a lower activity of MG extracts and higher in HG, at the opposite of the results obtained with filtrates where MG culture filtrate exhibited the higher activity.

These results reveal the importance of the light and dissolved gas availability. Anti-adhesion compounds were mostly produced in high light condition or in a lesser extent with lower light and with low CO<sub>2</sub> availability. In contrast the compounds were mostly released in MG where light was likely the factor that limited growth. This suggests an accumulation of the compounds when conditions are favourable for their production and higher release in less favourable conditions. Nutrient concentration had no clear effect on the production of allelochemicals while nutrient

limitation and unbalanced nutrient ratio have often been pointed out as factors that enhance allelopathy (e.g. Granéli and Hansen, 2006).

In the second experiment we have observed an increase of extract anti-adhesion activity with increasing culture age in batch culture. In batch culture, light intensity and nutrient availability decrease with increasing culture age. Since light intensity has been found to increase the production of anti-adhesion allelopathic compound, the increase of anti-adhesion activity in batch culture was probably due to nutrient depletion. The absence of effect of nutrients in the third experiment was likely caused by insufficient differences between nutrient conditions.

The results on the production of allelopathic compounds fit well with Growth-Differentiation Balance Hypothesis (GDBH, Herms and Mattson, 1992). GDBH states that high light and nutrient depletion increase the production of carbon-based secondary metabolites. However, the release of anti-adhesion compounds occurred mostly in unfavourable conditions for their production. This is in accordance with an alternative plant defence theory, the Optimal Defence Theory (Hamilton *et al.*, 2001). The identification of anti-adhesion allelopathic compound would allow us to test these hypotheses.

The variability of the activity of the extracts allowed the use the metabolomic profile to select putative allelochemicals (Prince and Pohnert, 2010; Weston *et al.*, 2015). Metabolomic profiling was realised separately on the extracts of the second and third experiment. The most likely allelopathic compounds were selected on the basis of the multilevel sPLS regression. The selected metabolites were the M326T97 and M796T1023 with the extracts of the second experiment, and M227T272 and M289T373 with the extracts of the third experiment (table 1). These metabolites expressed the highest variable importance in the projection (VIP) for metabolites according to the PLS performed. Metabolites with negative correlation with the adhesion inhibition were not considered in the

| Metabolite id | <i>m/z</i> | Retention time (s) | VIP  |
|---------------|------------|--------------------|------|
| M326T97       | 326        | 97                 | 2.37 |
| M796T1023     | 796        | 1023               | 2.06 |
| M227T272      | 227        | 272                | 2.25 |
| M289T373      | 289        | 373                | 2.33 |

**Table 1. Metabolites selected as the potential allelopathic compounds. VIP: Variable Importance in the projection obtained with the PLS model.**

potential allelochemical list (table 1). Although metabolomic profiling is a global approach, all the chemicals could not be detected using a single approach (Prince and Pohnert, 2010). Therefore other compounds could have been involved but not detected.

To conclude, environmental factors affected differentially production and release of anti-adhesion allelopathic compounds. Compound

identification has not been achieved but a shortlist of four metabolites potentially responsive of the anti-adhesion allelopathic activity of *U. confervicolum* against diatoms has been selected.

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## Chapitre IV

Effets de composés allélopathiques produits par  
*Uronema confervicolum* sur les micro-algues benthiques



#### **IV. Effets de composés allélopathiques produits par *Uronema confervicolum* sur les micro-algues benthiques**

Après avoir étudié les aspects liés à la production de composés allélopathiques, en utilisant les diatomées uniquement comme indicateur de l'effet allélopathique dans le cadre de bioessais, il est nécessaire pour appréhender l'interaction allélopathique dans son ensemble d'étudier comment les composés allélopathiques produits par *U. confervicolum* affectent les diatomées benthiques. Pour cela, le choix a été fait de se concentrer sur l'effet allélopathique anti-adhésion, particulièrement intéressant du fait de sa relation avec le mode de vie benthique. De plus, l'étude de la réponse aux composés allélopathiques anti-adhésion présente l'avantage de permettre éventuellement de mieux comprendre les mécanismes de l'adhésion des diatomées, un processus encore trop partiellement élucidé.

Les diatomées benthiques ont une importance fondamentale dans la structure et le fonctionnement de la plupart des biofilms phototrophes dont elles sont généralement le groupe algal dominant que ce soit en lac ou en rivière (Stevenson *et al.*, 1996). Les diatomées benthiques représentent également un excellent outil de bioindication, notamment pour l'évaluation de la qualité écologique des milieux aquatiques (Lowe and Pan 1996).

Comme pour tous les microorganismes formant des biofilms, l'adhésion est un processus primordial pour les diatomées. Grâce à leur capacité d'adhérer rapidement, les diatomées sont, avec les bactéries, les premiers colonisateurs des substrats nouvellement immergés. Une grande partie de l'intérêt portée à l'adhésion des diatomées vient du fait que cette capacité à adhérer est responsable de pertes économiques importantes par le développement de biofilms sur des surfaces artificielles telles que les coques des bateaux (Schultz *et al.*, 2011). De plus, l'adhésion des diatomées a également un impact dans les milieux naturels, notamment, de par la stabilisation des sédiments (Pouličková *et al.*, 2008; Wigglesworth-Cooksey *et al.*, 2001) et l'effet sur les organismes utilisés comme supports par les diatomées (Harder, 2009). La compréhension des mécanismes d'adhésion chez les diatomées et la recherche de solutions permettant de limiter ce phénomène sur les surfaces artificielles est donc l'objet de nombreuses études (*e.g.* Cooksey *et al.*, 2009; Hunsucker and Swain, 2016; Molino and Wetherbee, 2008; Willis *et al.*, 2014). Pourtant, les mécanismes de l'adhésion des diatomées restent peu connus : d'après Wetherbee *et al.* (1998) l'adhésion des diatomées repose sur le « complexe d'adhésion » (adhesion complex ; figure 10) constitué des filaments d'actine à l'intérieur de la cellule, de substances adhésives à l'extérieur (faisant partie des EPS) et de protéines

transmembranaires faisant le lien entre les deux. La nature exacte de ces deux dernières composantes est toujours inconnue (Dugdale *et al.*, 2005, 2006, Willis *et al.*, 2013, 2014).

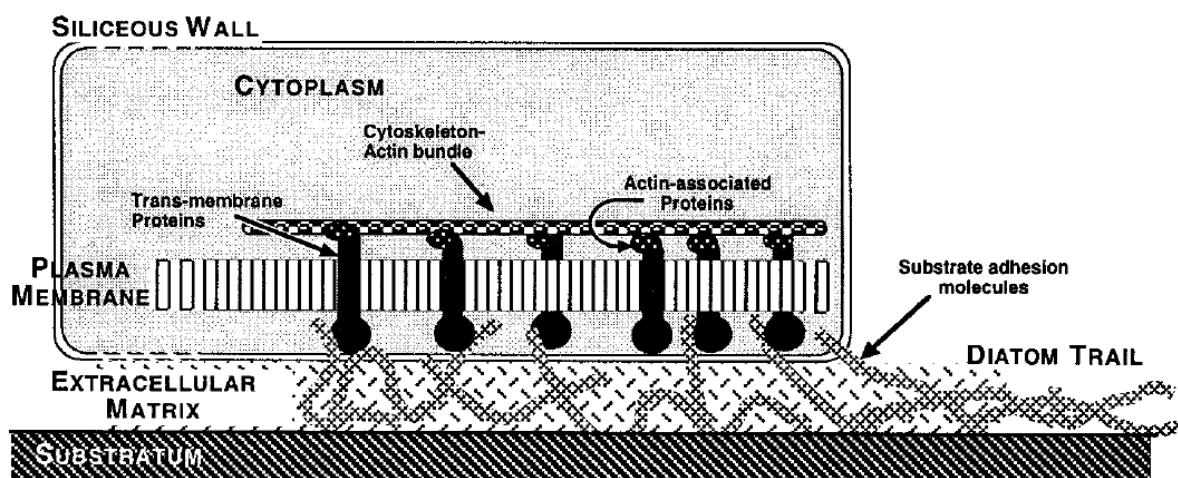


Figure 10 : Représentation du complexe d'adhésion des diatomées. Extrait de Wetherbee *et al.* (1998).

*Effet de composés allélopathiques anti-adhésion sur une diatomée benthique : étude par profilage métabolomique et transcriptomique.*

Le but de cette étude est de déterminer **spécifiquement** la réponse d'une diatomée aux composés allélopathiques inhibant l'**adhésion**. Pour cela, le choix a été fait d'utiliser le filtrat d'*U. confervicolum* plutôt que des extraits de biomasse. En effet, le filtrat n'a d'effet observable que sur l'adhésion des diatomées (chapitre II), alors que les extraits ont des effets sur de nombreuses fonctions du fait de la présence d'autres composés allélopathiques (acides linoléique et  $\alpha$ -linoléique), ainsi que des molécules n'étant pas excrétées naturellement par l'algue mais pouvant interférer avec le métabolisme de la diatomée. Dans l'étude présentée au chapitre III, le choix de l'utilisation des extraits a été motivé par le besoin d'une grande quantité de composés pour pouvoir réaliser les analyses. Dans le cas actuel, la priorité était d'éviter les interférences avec d'autres composés non impliqués dans l'inhibition de l'adhésion.

Afin de limiter la dégradation des composés allélopathiques par des microorganismes hétérotrophes durant les incubations, il a été choisi d'utiliser **une souche axénique de diatomées**. Il faut néanmoins considérer que la réponse de la diatomée, en présence du cortège bactérien associé aurait pu être sensiblement différente. Plusieurs espèces de diatomées en culture axénique ont été exposées au filtrat d'*U. confervicolum* dans des expériences préliminaires : *Gomphonema parvulum* (souche SAG1032-1), *Fistulifera saprophila* (souche fis.sa 02g) et *Nitzschia palea* (souche CPCC-160). Les

capacités d'adhésion de la souche de *G. parvulum* étant beaucoup plus affectées par le filtrat que celles des deux autres souches, celle-ci a été choisie pour l'étude présentée ici.

Cette étude combine deux approches :

- La première est une **approche ciblée**, visant à vérifier l'hypothèse que l'inhibition de l'adhésion est liée à un effet au niveau de la matrice d'EPS.
- Une deuxième **approche, globale**, non ciblée et donc plus exploratoire, a été utilisée. Elle est basée sur deux méthodes: le profilage métabolomique (voir chapitre III) et la transcriptomique. Cette dernière méthode consiste en une étude globale de l'expression des gènes grâce à un séquençage des ARNm.

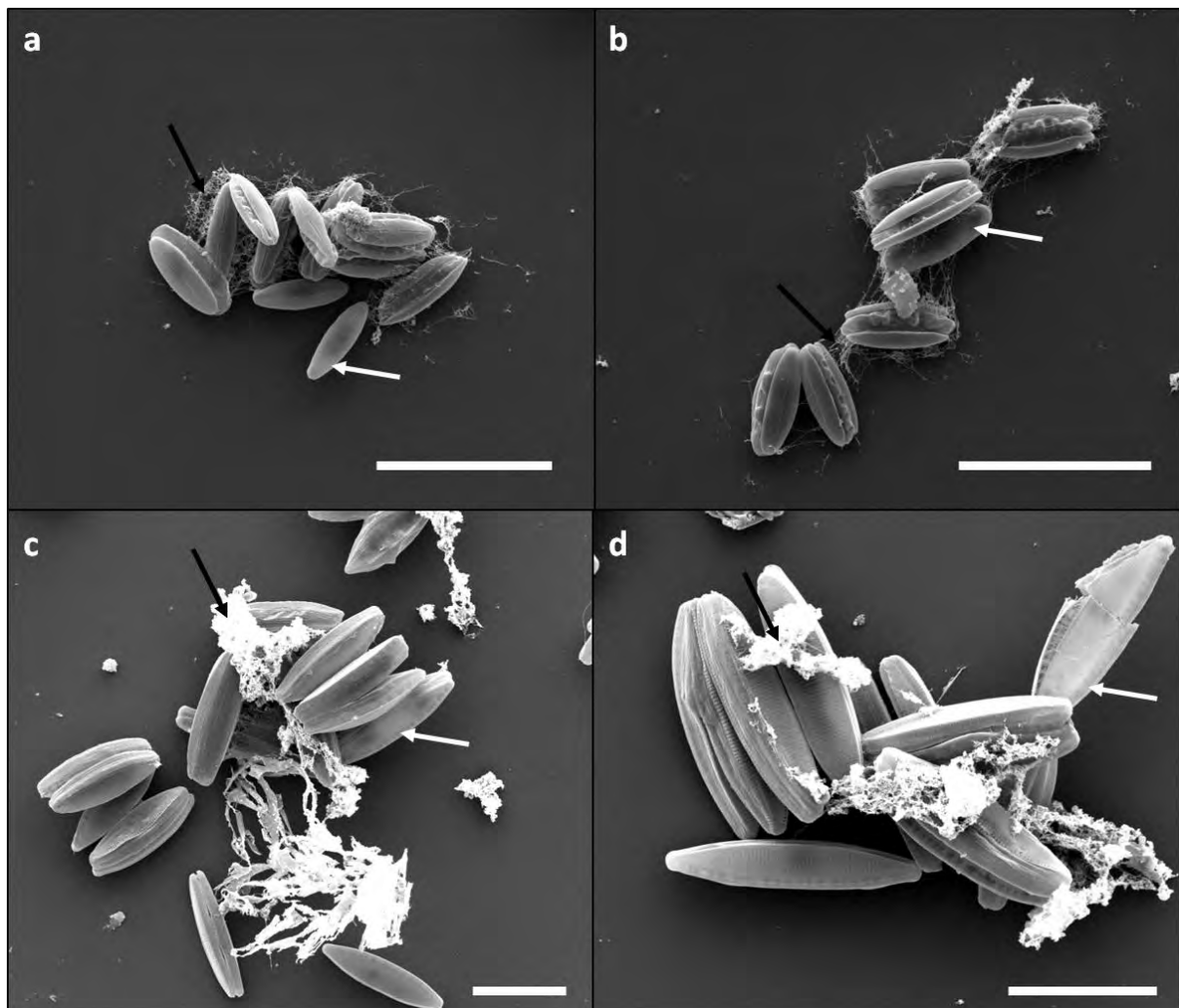


Figure 11 : Observation au microscope électronique à balayage de cellules de *F. saprophila* (a - b) et *N. palea* (c - d) exposées pendant 26 h au filtrat de *U. confervicolum* (a, c) et leurs contrôles (b, d). Barre d'échelle : 10 μm. Les flèches noires indiquent les diatomées, les flèches blanches indiquent les EPS.

Les résultats obtenus dans la première approche confirment le lien entre l'effet observé sur l'adhésion des cellules et la formation de la matrice d'EPS. En effet, l'observation en Microscopie Electronique à Balayage (MEB) des cellules de *G. parvulum* adhérentes et de leurs EPS montre une **forte diminution de la présence des exopolymères** au voisinage des cellules en réponse au filtrat d'*U. confervicolum* (voir figure 2 de l'article, p. 119). En plus de ces résultats, une expérience similaire a été réalisée sur les deux souches de diatomées peu (*F. saprophila*) ou pas (*N. palea*) impactées par le filtrat d'*U. confervicolum*. Dans ces deux cas, aucune modification visible des EPS n'a été observée (figure 11).

Ces premiers résultats suggèrent que les composés allélopathiques excrétés par *U. confervicolum* pourraient soit **réduire la production d'EPS** par *G. parvulum*, soit **affecter la liaison EPS-cellules ou EPS-substrat**. Dans ce dernier cas, les EPS seraient éliminées au cours de la préparation des lamelles et de fait non observables.

L'analyse non ciblée a révélé qu'en plus d'affecter l'adhésion et les EPS, le filtrat d'*U. confervicolum* a un fort impact au niveau du métabolome et du transcriptome de la diatomée. Dans l'approche par **profilage métabolomique**, une démarche d'identification des composés a été réalisée uniquement pour les métabolites présentant un intérêt du fait de leur différence d'expression entre les conditions. Ainsi, le composé sur lequel l'effet est le plus marqué a pu être identifié comme étant la **carnitine (figure 12)**. Ce composé serait principalement impliqué dans le métabolisme énergétique.

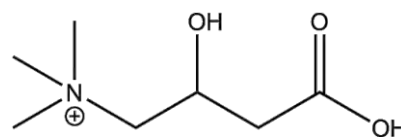


Figure 12 : Formule développée de la carnitine.

L'identification des autres métabolites est toujours en cours.

L'**analyse transcriptomique** a mis en évidence que, de manière étonnante, l'expression de gènes liés à la photosynthèse et au métabolisme énergétique est stimulée tandis que les gènes liés à l'adhésion (facteurs de coagulation) et au cycle cellulaire sont inhibés, cette dernière observation était plus attendue.

Cette étude a permis de caractériser des gènes directement et indirectement reliés au processus d'adhésion. Les composés allélopathiques inhibant l'adhésion impactent également la production ou le maintien des EPS ainsi qu'une grande partie du métabolome et du transcriptome de la diatomée. En plus de l'effet négatif sur l'adhésion et les EPS, ces composés ont un impact important sur le métabolisme énergétique. Cet impact correspondrait plus, d'après l'analyse du transcriptome, à une stimulation. La photosynthèse et des fonctions liées à l'adhésion semblent aussi stimulées. Nous avons proposé l'hypothèse que cette stimulation est liée à la réponse des diatomées à une plus faible adhésion (figure 13). La stimulation du métabolisme observée serait due à une plus forte production

de substances adhésives pour compenser la plus faible adhésion de ces substances. La plus forte allocation d'énergie au processus d'adhésion peut, au final, limiter la croissance, conformément à l'inhibition observée de gènes liés au cycle cellulaire.

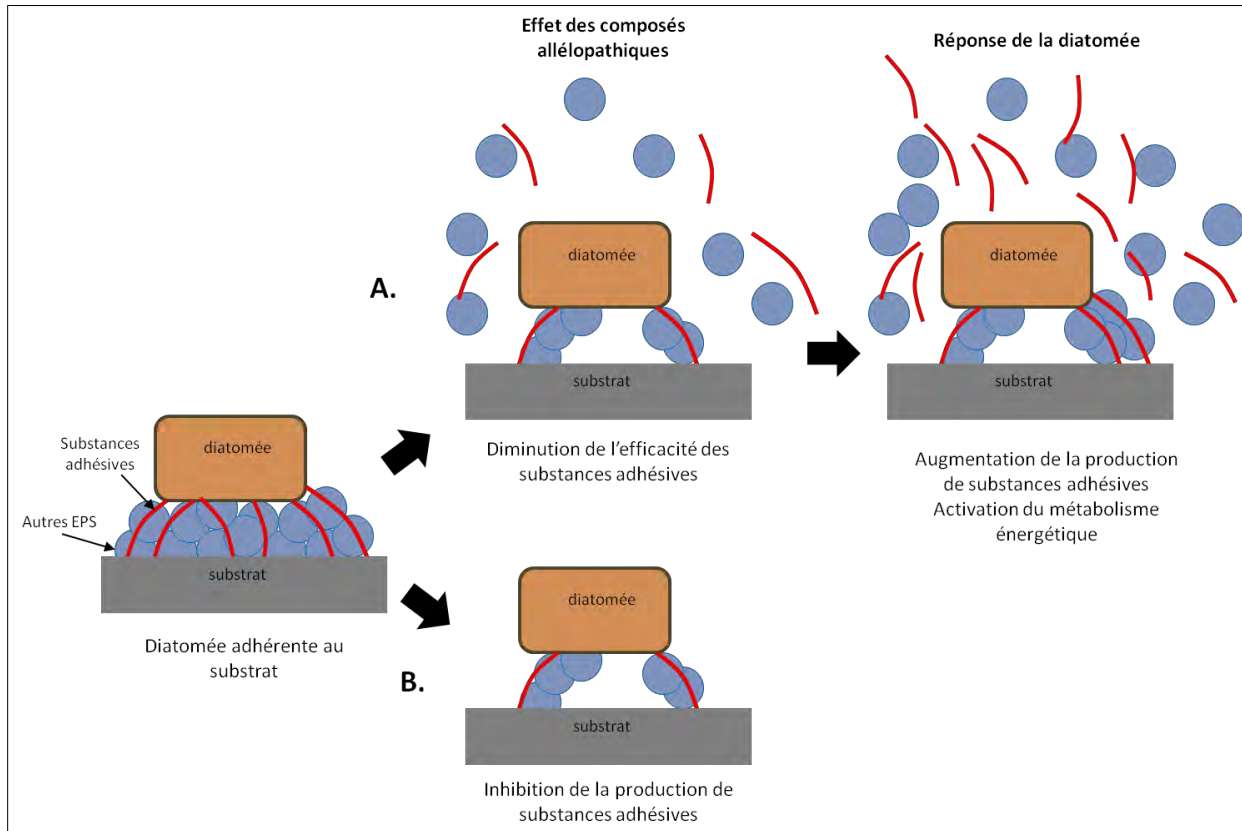


**Figure 13 : Représentation schématique de l'effet des composés allélopathiques anti-adhésion sur des diatomées et réponse des diatomées selon les deux hypothèses proposées : A. effet sur la liaison EPS-cellules et EPS-substrat, B. inhibition de la production d'EPS.**



Diatom metabolomic and transcriptomic responses to  
adhesion inhibition by allelopathic compounds.

**Article en préparation.**



### **Diatom metabolomic and transcriptomic responses to adhesion inhibition by allelopathic compounds.**

Joey L. Allen<sup>1</sup>, Christophe Roux<sup>2</sup>, Gérald Culioli<sup>3</sup>, Loïc Ten-Hage<sup>1</sup> and Joséphine Leflaive<sup>1</sup>

<sup>1</sup>ECOLAB, Université de Toulouse, CNRS, INPT, UPS, Toulouse, France

<sup>2</sup>Laboratoire de Recherche en Sciences Végétales, CNRS-Université Toulouse III, Castanet-Tolosan, France

<sup>3</sup>Laboratoire MAPIEM, Université de Toulon, La Garde, France

#### **Abstract**

Benthic diatoms generally form biofilms, *i.e.* an assemblage of surface-associated microorganisms embedded in an extracellular polymeric substances (EPS) matrix. They often develop as epiphyte on other algae. While for benthic diatoms the ability to adhere to a surface is of vital importance, for organisms that are colonised, epiphytic diatoms represent a harmful competitor. Despite the ecological importance and economic impact of diatom fouling, the mechanisms of diatom adhesion are still poorly known. We have already described that the filamentous green alga *Uronema confervicolum* produces allelopathic compounds (*i.e.* chemicals released to inhibit competing organisms) that inhibit specifically diatom adhesion. In this study we have investigated the inhibition of adhesion of the benthic diatom *Gomphonema parvulum* in response to the exposure to *U. confervicolum* allelochemicals. We also use this system to decipher the mechanisms involved in diatom adhesion by studying the effect of the exposure to *U. confervicolum* allelochemicals on genes expression and metabolites production of the diatom *Gomphonema parvulum*.

An axenic strain of this benthic diatom was exposed to cell-free culture filtrate of *U. confervicolum* during periods of 2, 26 and 50 hours. Adhesion assays were carried out and EPS were also quantified. In order to identify mechanisms involved in response to allelochemicals, transcriptomic and metabolomic profiling were performed on treated and control diatom cultures.

The results revealed that allelopathic compounds reduce EPS matrix formation as well as adhesion. Important modifications of metabolome and transcriptome were observed. Notably, one of the most affected metabolites was identified as carnitine. Interestingly, while cell-adhesion and cell cycle related genes were inhibited in response to allelochemicals, photosynthesis and energy metabolism related genes were stimulated. This study highlights genes directly and indirectly related to diatom adhesion. The inhibition of adhesion may probably be due to the effect on EPS, and the effect on general metabolism a response of diatoms to low adhesion.

## Introduction

In aquatic ecosystems, phototrophic benthic organisms colonise quickly every light-exposed surface. They form biofilms on the colonised surfaces through the production of an extracellular polymeric substance matrix. This phenomenon, named 'biofouling', occurs even on other phototrophic organisms (epibiosis) which are colonised for instance by benthic diatoms (Lam *et al.*, 2008; Wahl *et al.*, 2012). The development of such epiphytic biofilm is harmful to the colonised organisms as it reduces the access to light, gases and nutrients (Dodds, 1991; Harder, 2009). Biofouling also causes severe issues due to the development of biofilms on artificial substrates such as ship hulls (Schultz *et al.*, 2011). Diatoms (with bacteria) are the first colonisers of newly immersed surfaces, therefore they play a key role in biofilm formation. They also represent an important part of primary production in low to mid-order rivers (Stevenson *et al.*, 1996) and strongly contribute to sediment stabilisation (Pouličková *et al.*, 2008; Wigglesworth-Cooksey *et al.*, 2001).

Epiphytic development requires the benthic diatoms to adhere strongly to their substrate, particularly because they often develop in habitats with high flow (low order river, intertidal zone). Diatom adhesion depends on the "adhesion complex" that is composed of the actin cytoskeleton inside the diatom cell, connector proteins through cell membrane and extracellular adhesive strands comprised in the extracellular polymeric substance (EPS) matrix (Wetherbee *et al.*, 1998). The exact nature of the two last components is still unknown and is an active research field. The amino-acid composition of adhesive material of two marine diatoms has been recently determined (Poulsen *et al.*, 2014). Dugdale *et al.* (2005, 2006b, 2006a) used atomic force microscopy to characterize adhesive nanofibers from diatoms. Different adhesive nanofibers were found to be made of the same single protein with a length of 97 amino-acid in the diatom *Toxarium undulatum* (Dugdale *et al.*, 2006a). A similar organization of modular proteins was found in the pennate diatom *Phaeodactylum tricornutum* with a longer repeated domain of approximately 336 amino-acids (Dugdale *et al.*, 2006b). However the protein was not identified. Willis *et al.* (2014) and Buhmann *et al.* (2014) both identified putative adhesion proteins of the marine diatom *Phaeodactylum tricornutum* using genome data. These results were obtained by comparison of *Phaeodactylum tricornutum* sequences with known adhesion proteins from other organisms (plants, animals, and bacteria).

Due to the harmful consequences of surface colonisation by diatoms, many macroorganisms have developed strategies to limit diatom biofouling on their surfaces (Harder, 2009). A frequently observed strategy to limit biofouling is the production of allelopathic compounds inhibiting potential epiphytes. This process has been observed with marine macroalgae, corals, macrophytes (Bak and Borsboom, 1984; Erhard and Gross, 2006; Lam *et al.*, 2008). While allelochemicals affect their targets

in very different ways, some organisms produce allelopathic compounds that specifically inhibit the ability of diatoms to adhere to a substrate (Wigglesworth-Cooksey and Cooksey, 2005). Indeed, the filamentous green alga *Uronema confervicolum* has been found to produce allelopathic compounds with anti-adhesion properties (Allen *et al.*, 2015).

The present study aims to understand the mechanisms by which the allelopathic compounds produced by *U. confervicolum* affect the ability of diatoms to adhere to a substrate. The recent development of ‘-omics’ methods provides an excellent tool for the comprehension of the mode of action of allelochemicals (Duke *et al.*, 2013; Poulson-Ellestad *et al.*, 2014; Zheng *et al.*, 2016). Furthermore, while the mechanisms of diatom adhesion are poorly known to date, the study of the effect of allelopathic compounds on adhesion at the transcriptome and at the metabolome level would highlight the genes and metabolites that are involved in the adhesion of diatoms.

## Material and methods

### *Production of allelochemicals*

The filamentous green alga *U. confervicolum* was inoculated in forty-two 500-ml Erlenmeyer flasks filled with 400 ml of COMBO medium (Kilham *et al.*, 1998) with  $2.5 \times 10^6$  cells. Algal cultures were placed at 18°C in a growth chamber with a 16:8 light:dark cycle (light intensity:  $30 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ ) with constant aeration by air bubbling. After 21 days, corresponding to the maximum filtrate anti-adhesion activity determined previously (Allen *et al.* in prep.), algal biomass and culture medium were separated by filtration through GF/F filters (0.7  $\mu\text{m}$  pore size). Cell-free culture filtrate was then placed at -20°C until use. Just before use, the culture filtrate was sterilised by filtration through 0.22  $\mu\text{m}$ -pore size filters (GPWP type, Millipore). Total N and P concentrations were quantified in the filtrate (data not shown) and  $\text{NaNO}_3$  and  $\text{KH}_2\text{PO}_4$  were added to reach N and P concentrations similar to those of COMBO medium.

### *Exposure of G. parvulum to allelochemicals*

The experiment was run in 500-ml flasks filled with 400 ml of nutrient enriched *U. confervicolum* filtrate or with COMBO medium (control). Flasks were inoculated with  $3 \times 10^5 \text{ cell.ml}^{-1}$  of *G. parvulum* and placed in a growth chamber in the same conditions as above but aeration was limited to gas diffusion through cellulose caps. After 2 h, 26 h and 50 h, about 350 ml of culture medium were pumped out without re-suspending diatoms that had settled at the bottom of the flask. Diatoms were then scrapped using a sterile cell scraper. The remaining 50 ml of medium containing the diatoms were transferred in 50-ml tubes, placed on ice while a subsample was counted under light microscope (Eclipse 80i Nikon) using a Malassez counting chamber. For EPS quantification six samples (three treated, three controls) were used at each time point. For transcriptomics and metabolomics

each sample was divided in three subsamples. A subsample of  $4 \times 10^5$  cells was used immediately to carry out adhesion assay. For transcriptomics, a subsample corresponding to  $3 \times 10^7$  cells was centrifuged (10 min, 4°C, 1 400 g). Cell pellets were transferred to 2-ml tubes and immediately frozen in liquid nitrogen. For metabolomic profiling, a subsample corresponding to at least  $6 \times 10^7$  cells was filtered through GF/C filters. Filters and cells were enveloped in aluminium foil and immediately frozen in liquid nitrogen. Three replicates were harvested after 2 h for transcriptomic analyses, fifteen replicates were harvested after 26 h and fifteen others after 50 h for transcriptomic and metabolomic profiling. All frozen samples were conserved at -80°C until further processing. Sterile COMBO medium (50 ml) was filtrated through GF/C filters. These filters were used as blanks for the metabolomic profiling. All the filters and the aluminium foil had been placed in a muffle furnace (6 h at 500°C) before the experiment to remove any organic compounds that could false the results.

#### *Adhesion assay*

A glass microscope cover slip ( $\varnothing$  12 mm) was placed at the bottom of the wells of a 24-well plate. Each well was filled with 2 ml of COMBO medium and  $4 \times 10^5$  cells of *G. parvulum*. After 4 h in the dark at 18°C, glass cover slips were rinsed two times with 3 ml of deionised water to remove non adhered cells. The glass cover slips were dried and diatoms fixed by heating at 100°C. Adhered cells were counted under light microscope (Eclipse 80i Nikon, 600 × magnification). Adhesion inhibition (%) was calculated as  $100 \times (D_c - D)/D_c$ , where  $D_c$  is the mean cellular density in controls and D is the mean cellular density in the treatments.

#### *Extracellular polymeric substances analysis*

For extracellular polymeric substances (EPS) analysis, immediately after cell harvesting, diatoms were centrifuged (10 min, 4°C, 1 400 g). Cell pellets were placed in 2 ml-tubes and rinsed with 1 ml of Phosphate Buffered Saline (PBS) solution. EPS were extracted with the extraction buffer (1% EDTA solution in Tris-buffer (0.1 M) adjusted to pH 8.5 with HCl) during 1.5 h at 4°C under constant agitation. Samples were centrifuged (4 000 g, 15 min) the supernatant (S) was used for extractable bound EPS quantification. A sample of cells was observed under light microscope to ensure the integrity of cells after EPS extraction. Carbohydrates and proteins were measured in the EPS extracts as described by Verneuil *et al.* (2015). Briefly, carbohydrates were quantified in S using the anthrone method. 200  $\mu$ l of a 2 g.l<sup>-1</sup> anthrone solution in sulfuric acid were added to 100  $\mu$ l of S. A glucose solution in extraction buffer (from 0.01 g.l<sup>-1</sup> to 1.0 g.l<sup>-1</sup>) was used for calibration. After 30 min at 60°C samples were cooled for 10 min at room temperature before OD measurement at 620 nm (Asys UVM 340, Salzburg, Austria). Protein quantification was performed using the bicinchonique acid (BCA) method with the Pierce BCA Protein Assay kit (Thermo scientific, Rockford, USA) following

manufacturer guidelines. BSA solution ( $0.1 \text{ g.l}^{-1}$  to  $1.0 \text{ g.l}^{-1}$ ) diluted with the extraction buffer was used as standard for calibration.

#### *Preparation of scanning electron microscopy samples*

A glass microscope cover slip was placed at the bottom of each well of a 24-well plate. Each well was then filled with 2 ml of sterile *U. confervicolum* filtrate or COMBO medium and inoculated with  $6 \times 10^5$  cells of *G. parvulum*. The plates were placed in the cultivation chamber for 2 h, 26 h and 50 h. At the end of this incubation time, medium was carefully removed and replaced by sodium cacodylate buffer containing 2% formaldehyde for 2 h. Samples were then dehydrated with an ascending gradient of ethanol (50%, 70%, 80%, 95% and 100%, 15 min each) before critical point drying. Samples were platinum-coated before observation with an ESEM Quanta 250 FEG Scanning Electron Microscope (SEM).

#### *Chemical extraction*

Frozen filters containing the diatom cells were ground with liquid nitrogen-cooled mortar and pestle and extracted with 30 ml of cooled acetone:methanol (1:1). Extracts were filtered through muffled GF/F filters, evaporated to dryness and re-suspended in methanol to a final concentration of 10 mg of dry extract per ml.

#### *Metabolomic profiling*

The metabolomic profiling was carried out on an Elite LaChrom (VWR-Hitachi, Fontenay-sous-Bois, France) chromatographic system coupled with an ion trap mass spectrometer (Esquire 6000, Bruker Daltonics, Wissembourg, France). Chromatographic separation was achieved on a reversed-phase column ( $150 \times 3 \text{ mm}$ ,  $2.6 \mu\text{m}$ , Kinetex Phenyl-Hexyl, Phenomenex, Le Pecq, France) maintained at  $30^\circ\text{C}$  and the injected sample volume was  $10 \mu\text{l}$ . The mobile phase consisted of acidified water (A) and acetonitrile (B) (containing each 0.1% of formic acid) and the flow rate was set at  $0.5 \text{ ml.min}^{-1}$ . The elution gradient started at 10% B and kept for 2 min, then to 100% B at 12 min and kept for 13 min; then back to 10% B over 0.1 min and maintained 9.9 min. The electrospray interface (ESI) with nebulizing gas ( $\text{N}_2$ ) pressure at 40 psi, drying gas ( $\text{N}_2$ ) flow at  $8 \text{ l.min}^{-1}$  and drying temperature at  $350^\circ\text{C}$  was operated in positive mode (capillary voltage at 4000 V). Mass spectra were acquired in the full scan range  $m/z$  50 to 1200. The mass spectrometer was controlled using Esquire Control version 6.1 and acquired data were handled with Data Analysis version 4.3 (Bruker Daltonics). Specific identification of molecular formula of putative biomarkers was done on a Q-ToF Impact II mass spectrometer (Bruker Daltonics). Several pooled samples were prepared by sampling and mixing a small amount of each sample. These pooled samples were used as quality controls (QCs) and, in

order to ensure analytical repeatability, these QCs were injected at the beginning, at the end and every four samples within the sequence run. Control samples (media blanks) were also prepared and were randomly injected within the sequence. These blanks allowed the further subtraction of contaminants or components coming from the growth media and the analytical procedure. At the end of the sequence, raw LC/MS data were converted into netCDF files and processed for peak finding, integration and alignment using the open source XCMS package (version 1.38.0) in the R 3.1.0 environment (*matchedFilter* method, signal-to- noise ratio: 5, group band-width: 3; the other parameters were default settings). The resulting variables list was further processed by the CAMERA package (version 1.18.0) and three successive filtering steps (signal/noise ratio, coefficient of variation and coefficient of the autocorrelation between variables in a same given pcgroup) using an in-house script were run on R. Commercial standard of carnitine (Sigma-Aldrich, Saint Louis, USA) was analysed in the same conditions as experimental samples.

#### *RNA extraction*

The RNeasy plant mini kit (QIAGEN, Hilden, Germany) was used for RNA extraction. RTL extraction buffer (450 µl) and muffled glass beads (500 µl; 0.50 - 0.75 mm diameter) were added to the frozen samples. Cells were disrupted using a TissueLyser bead mill (QIAGEN) according to manufacturer instructions for yeast samples. RNA was then extracted according to manufacturer instructions.

#### *RNA-seq analysis*

The libraries were prepared according to Illumina's protocols using the Illumina TruSeq RNA Sample Prep Kits v2 (Illumina Inc., San Diego, USA) using the protocol recommended by the manufacturer. Libraries were quantified by qPCR using the KAPA Library Quantification Kit (PN11 KK4824- KAPA Biosciences, Nanterre, France) to obtain an accurate quantification. RNA-seq experiments were performed on the Illumina HiSeq2500 using a 2 x 200 bp pair-end strategy with the Illumina TruSeq SBS sequencing kits v4 (PN FC-401-3001, HiSeq2500). Sequencing was performed at the GeT (Genome & Transcriptome Core facilities, Toulouse, France, <http://get.genotoul.fr/>). Raw reads were trimmed using CLC software (v9.1, CLC Bio Workbench, Qiagen, Denmark) before assembly process, using same CLC software with default settings. The final assembly has a total length of 58.5 Mb with 44580 non redundant virtual transcript (NRVT, thereafter 'sequences' – mean 1.318 kb, N50= 2006 kb). Annotations were derived by comparing the NRVTs to the public Refseq protein database with the blastx algorithm. Based on the blast results, all protein ORFs (complete or incomplete) encoded by *G. parvulum* were predicted by the OrfPredictor tool using default settings (Min *et al.*, 2005). The Gene Ontology (GO) terms were assigned to each NRVT using InterProScan program (Quevillon *et al.*, 2005). The annotation of Eukaryotic Orthologous Groups (KOG) and function classes was derived

using the webMGA tool (Wu *et al.*, 2011). Potential InterPro domains harbored in NRVTs were predicted based on InterProScan hits. Raw sequencing reads, assembly and annotations have been released in NCBI-Gene Expression Omnibus (in progress).

Trimmed reads were mapped to the Gompho\_CDS1 assembly to define transcript accumulation patterns by RNA-seq functionality of the CLC Genomic Workbench suite. To find the genes significantly differentially expressed during symbiosis, RPKM (Mortazavi *et al.*, 2008 ; Reads Per Kilobase of exon per Million fragments mapped) and proportion-based test statistics (Baggerly *et al.*, 2003) with a False Discovery Rate (FDR, Benjamini and Hochberg, 1995) correction for multiple testing were calculated (settings: minimum mapped read length fraction = 0.95; minimum similarity = 0.98). According to CLC recommendation, genes were considered as significantly differentially expressed when meeting the requirements of "total difference reads mapped" >10 (parameter proposed by CLC WorkBench to select only significantly expressed genes) and FDR corrected  $p < 0.05$ . Gene set enrichment was performed by using the unconditional GStats test of Falcon and Gentleman (2007) based on Gompho\_CDS1 annotations. Briefly, the test measures the extent to which the annotation categories of features (GO terms and InterPro domains) in up ( $\geq 2$ ) or down ( $\leq 2$ ) differentially expressed gene lists are over or under-represented relative to those of the features in the total Gompho\_CDS1 gene repertoire (44580 NRVTs).

#### *Data analyses*

Student t-tests were used to determine statistically significant differences in adhesion assays and in EPS analyses. Significance threshold was set at  $\alpha = 0.05$ . Global changes in metabolome and transcriptome were evaluated using both unsupervised (Principal Component Analysis, PCA) and supervised (partial least square – discriminant analysis, PLS-DA) multivariate analyses. For the metabolomic data, Variable Importance in the Projection scores (VIP) associated with the PLS-DA and sparse PLS-DA (sPLS-DA ; Lê Cao *et al.*, 2011). Optimal number of selected variable to lower error rate was performed as described in Lê Cao *et al.*, (2011). A PLS-DA was performed on transcriptomic RPKM data. Sparse partial least square (sPLS ; Lê Cao *et al.*, 2008) was used to integrate metabolites and sequences (NRVTs) expression for variable selection as described in Gonzalez *et al.* (2012). All multivariate analyses were performed using mixOmics package in R software

Statistically significant enrichment of annotation terms was determined using hypergeometric test: the "p-value" corresponds to the tail probability of the hyper geometric distribution. Significance threshold was set at  $\alpha = 0.01$ .

Clusterisation on gene expression profiles was realised using k-means clustering to form two separate groups at each time point of the experiment. Different profiles were generated by the different associations between one group for each time point. Only significantly differentially

expressed genes were used for cluster formation. Log-transformed RPKM difference data were used for clustering.

## Results

### *Diatom adhesion*

An inhibition of the adhesion of *G. parvulum* cells exposed to *U. confervicolum* filtrate was observed at the three time points of the experiment (2 h, 26 h and 50 h). It was significant after 26 h and 50 h ( $p = 0.0142$  and  $5.34 \cdot 10^{-5}$  respectively) and a strong tendency at 2 h ( $p = 0.075$ ) (figure 1a).

### *Extracellular polymeric substances*

No significant differences in EDTA-extractable EPS protein content were observed between diatom cultures exposed to *U. confervicolum* filtrate and controls, but a not significant decreasing trend was observed at 26 h (figure 1b). A significant decrease in EDTA-extractable EPS polysaccharides was observed for the 26 h sample ( $p = 0.023$ ) but not for 2 h and 50 h samples (figure 1c).

SEM images (figure 2) revealed a strong decrease of cell- or surface-bounded EPS at the three time points of the experiment. In the SEM images of control samples, EPS were visible next to most of the diatom cells, with larger amounts of EPS surrounding diatom aggregates. In the samples exposed to *U. confervicolum* filtrate, only very few amounts of EPS are visible. The glass cover slip looked “clean” around diatom cells (figure 2).

### *Metabolomics and transcriptomics*

PCA and PLS-DA reveal that *U. confervicolum* filtrate has induced a strong global modification of *G. parvulum* metabolome and transcriptome (figure 3). At the metabolome level, the PLS-DA revealed that 95 variables (metabolites) have a Variable Importance in the Projection score (VIP) over 0.7 indicating important variable (Tenenhaus, 1998). Seven metabolites with the high importance to

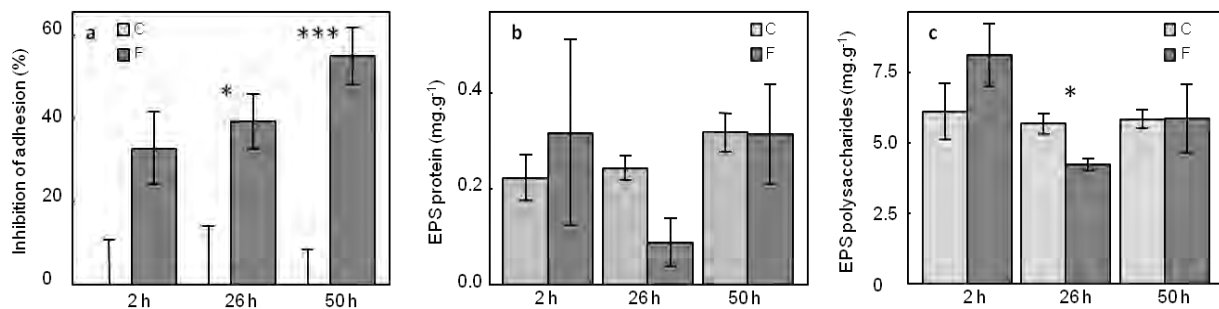


**Figure 1.** Effect of allelopathic compounds on *G. parvulum* adhesion (a), EPS protein content (b) and EPS sugar content (c) after 2, 26 and 50 h of exposure. Mean  $\pm$  SE ( $n = 3$ ). Asterisk indicates significant difference with the control.

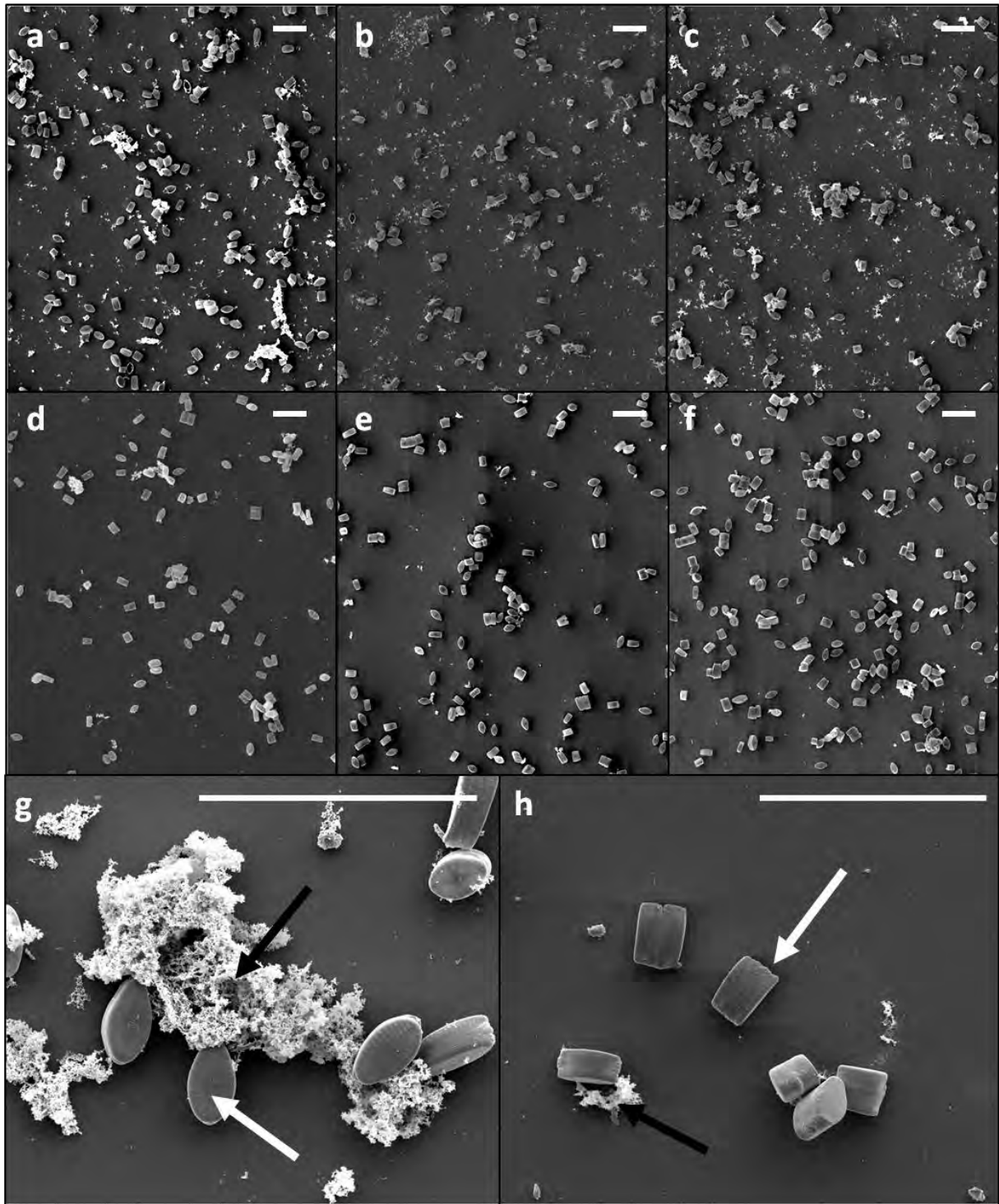


Figure 2. Scanning electron microscopy images of *G. parvulum* cells in control conditions (a-c and g) and exposed to *U. confervicolum* filtrate (d-f and h) after 2h (a, d), 26h (b, e, g and h) and 50h (c, f) of exposure. White arrows indicate diatom cells. Black arrows indicate extracellular polymeric substances. Scale bars represent 25  $\mu\text{m}$ .

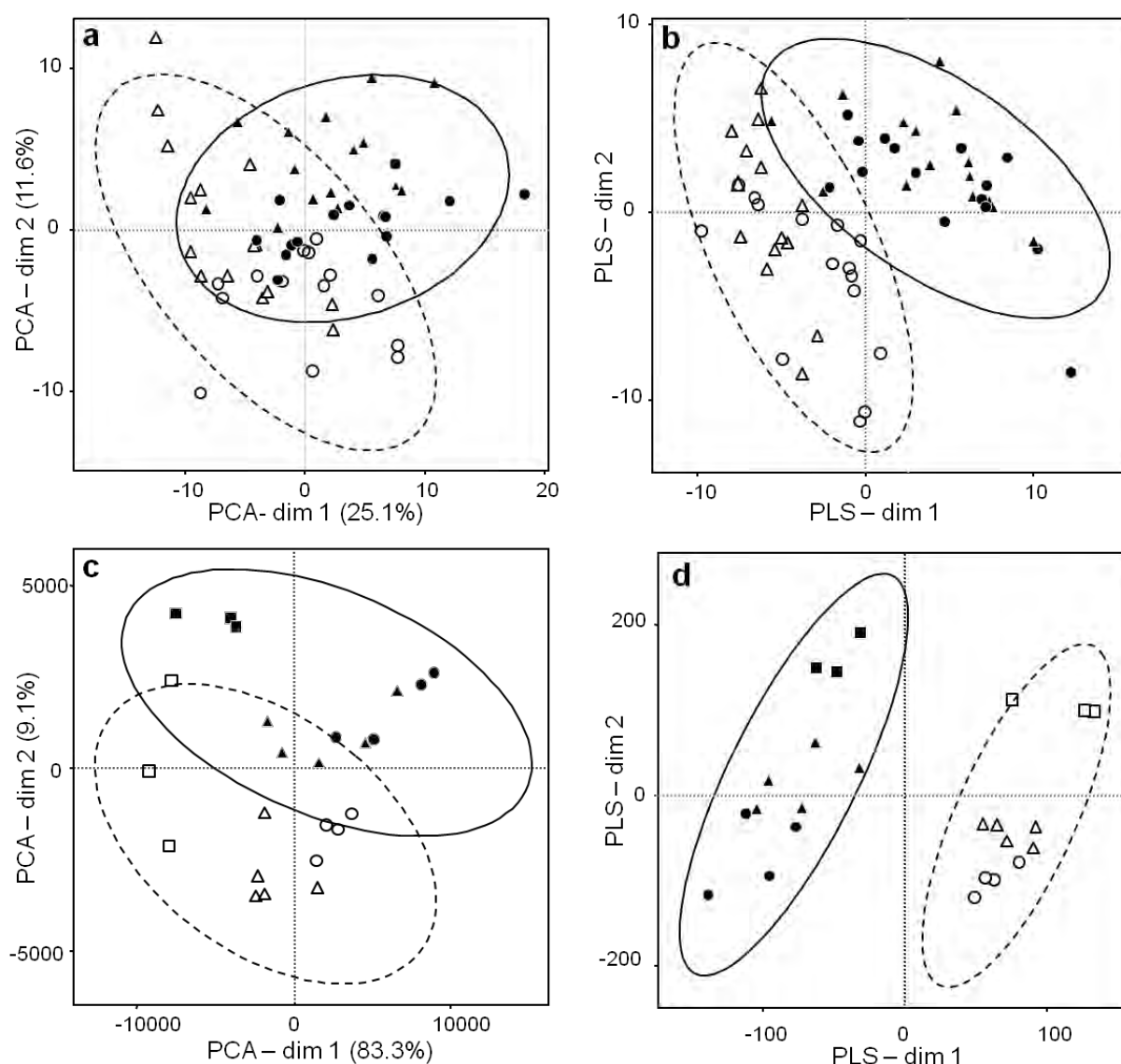


**Figure 3.** Global effect of allelochemicals on *G. parvulum* metabolome and transcriptome revealed by PCA and PLS-DA. (a) PCA and (b) PLS-DA individuals plot with the metabolomic profiles; (c) PCA and (d) PLS-DA individuals plot with transcriptomic RPKM data. Square: 2 h sample; circle: 26 h sample; triangle: 50 h sample; open symbol: control sample; closed symbol: exposed sample.

separate treated and control sample (determined as VIP > 2 and selection in the sPLS-DA optimised for variable selection) are presented in table 1. These seven selected metabolites were induced by the exposure to the filtrate. The formula of the metabolite M162T65 has been identified by HRMS (High-Resolution Mass Spectrometry) as  $C_7H_{15}NO_3$ . Its fragmentation pattern by HRMS/MS indicated that this compound was probably carnitine. This hypothesis was fully supported by the injection of a commercial standard which showed the same retention time and MS data. With this knowledge, carnitines derivatives were specifically researched. In particular, fragmentation patterns of acylcarnitines were reported to show a characteristic fragment ion at  $m/z$  85 in common. Thus, a second metabolite (M204T68) was putatively identified as acetylcarnitine on the basis of its

molecular formula and HRMS/MS fragmentation. This compound is repressed ( $p = 0.02$ ) and have a relatively low VIP (VIP = 1.07).

At the transcriptome level, 18 208 sequences (over 44 580; 41%) were significantly differentially expressed in at least one time point (FDR  $P$  value  $\leq 0.05$  and  $|\text{difference}| \geq 10$ ) (see supporting information table S1.A). Enrichment analyses were performed for each time point of the experiment on Gene Ontology Biological process and InterPro Domains annotations. Considering the sequences with a fold change higher than 2 or lower than -2, 32 'biological processes' were over-expressed and 15 repressed in at least one time point, 114 InterPro Domains were over-expressed and 52 repressed in at least one time point (SI tables S1.C1, C2, C3). One biological process was over-expressed and one was repressed at the three time points (table 2). Five domains were over-expressed and one repressed at the three time points (table 2). The 50 most positively and negatively differentially expressed genes for each time point are displayed in supporting information. PLS-DA were used to select the sequences that best distinguished control and exposed samples at each time point and globally, these sequences are displayed in SI table S2 B1-4.

**Table 1. Selected metabolites induced or repressed by the exposure to allelochemicals. Asterisks indicate metabolites selected using sPLS-DA analysis.**

| Metabolite ID | $m/z$ | Retention time (s) | Induced/Repressed (I/R) | VIP – 26 h | VIP – 50 h | VIP – global | Putative compound             |
|---------------|-------|--------------------|-------------------------|------------|------------|--------------|-------------------------------|
| M 162 T 65    | 162   | 65                 | I                       | 2.47*      | 2.16*      | 2.47*        | Carnitine ( $C_7H_{15}NO_3$ ) |
| M 384 T 77    | 384   | 77                 | I                       | 2.59*      | 0.92       | 1.80         |                               |
| M 348 T 78    | 348   | 78                 | I                       | 2.37*      | 1.13       | 1.77         |                               |
| M 406 T 78    | 406   | 78                 | I                       | 2.31*      | 1.23       | 1.91         |                               |
| M 521 T 980   | 521   | 980                | I                       | 2.12       | 2.04       | 2.23*        |                               |
| M 802 T 951   | 802   | 951                | I                       | 2.03       | 1.59       | 1.92*        |                               |
| M 313 T 997   | 313   | 997                | I                       | 1.85       | 1.91       | 2.03*        |                               |

**Table 2. Gene lists for enriched GO terms and InterPro domains at 2h, 26h and 50h in response to fractions from Uronema vs. control condition.**

| Induced/Repressed (I/R) | Function                                       | P.value 2 h | P.value 26 h | P.value 50 h |
|-------------------------|------------------------------------------------|-------------|--------------|--------------|
| R                       | GO:0006144 purine nucleobase metabolic process | 6.15E-04    | 2.51E-12     | 1.53E-04     |
| I                       | GO:0000050 urea cycle                          | 0.01        | 2.50E-07     | 1.65E-08     |
| R                       | IPR001054 Adenylyl cyclase class-3             | 1.87E-13    | 0            | 4.52E-07     |
| I                       | IPR022644 Orn                                  | 1.62E-03    | 7.07E-04     | 0.01         |
| I                       | IPR011249 Metalloenzyme, LuxS                  | 3.37E-03    | 6.52E-03     | 6.52E-05     |
| I                       | IPR001518 Argininosuccinate synthase           | 4.04E-03    | 0.01         | 4.67E-04     |
| I                       | IPR023329 Chlorophyll a                        | 4.24E-03    | 6.66E-16     | 5.55E-16     |

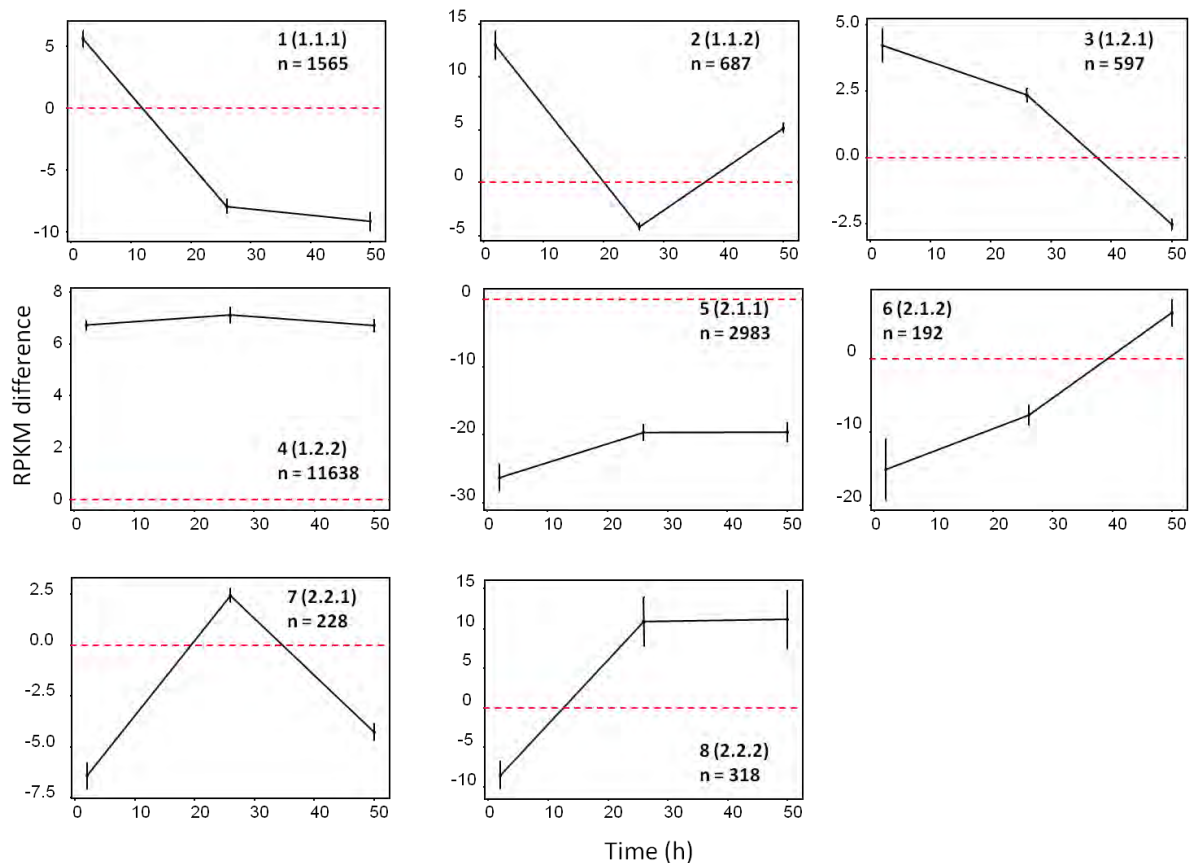


**Figure 4.** Expression profiles of the eight groups of significantly differentially expressed sequences. Clusters are produced according to the expression profiles. n: number of sequences in each cluster. Error bars represent standard error.

The sequences that were differentially expressed in at least one time point were clustered according to their expression profiles (figure 4). Eight clusters were formed. The clusters containing the higher number of sequences represented consistently induced sequences (cluster 4; n = 11638) and consistently repressed sequences (cluster 5; n = 2983). Numerous sequences have a 'decreasing profile' with over-expression at the first time point of the experiment and repression at the last one (clusters 1 and 3; n = 1565 and 597 respectively). Less sequences have the inversed profile (clusters 6 and 8; n = 192 and 318 respectively).

Metabolomic profiling and transcriptomics data were integrated using multilevel sPLS in order to investigate how *G. parvulum* metabolome expression can be linked to gene expression profiles. Highly correlated sequences and metabolites were determined. Sequences and metabolites from the first component of the sPLS model were highly affected by the exposure to *U. confervicolum* filtrate (figure 5) The 20 genes most highly correlated with gene expression are listed in SI table S2.C.

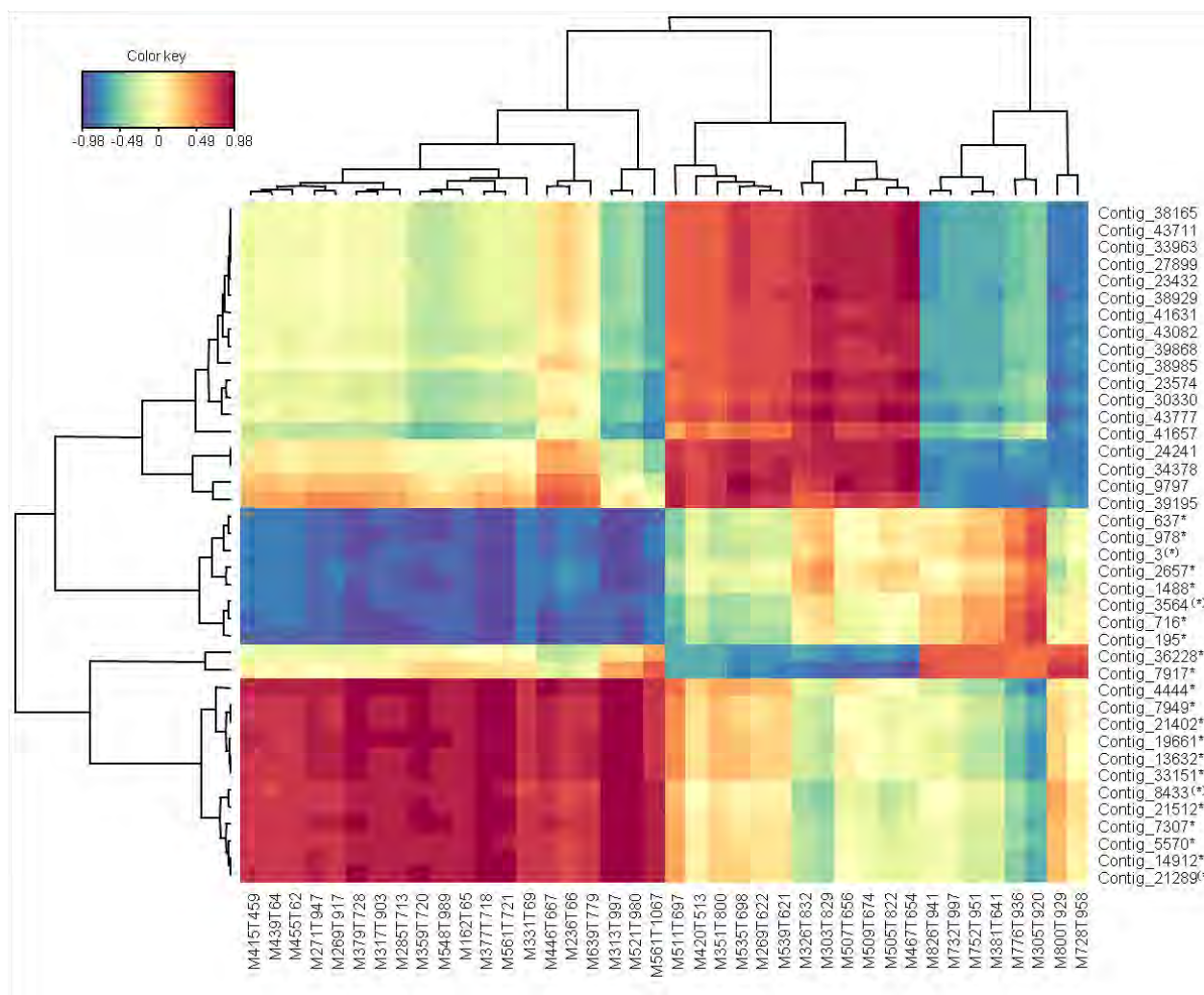


**Figure 5. Clustered image map representing correlations between metabolites and gene expression (heat map). Red colour indicates positive correlation, blue indicates negative correlation. Dendrographs represent relations among genes (left) and among metabolites (top). \*: significantly differentially expressed gene at 26 h and 50 h. (\*): significantly differentially expressed gene at 26 h but not at 50 h. Represented values are the first two components of multilevel sPLS regression model.**

## Discussion

### *General effect of anti-adhesion allelopathic compounds*

Our study reveals the strong effect of allelochemicals produced by the filamentous green alga *U. confervicolum* on the diatom *G. parvulum*, at the intracellular level. Indeed, besides the known effect on diatom adhesion (Allen *et al.*, 2015) it also strongly affects diatoms EPS matrix and induced strong modifications of diatom metabolome and transcriptome in a very short time (2 h of exposure for the first experimental time point). Diatom cell adhesion strongly depends on calcium ion availability (Geesey *et al.*, 2000). However, the fact that adhesion assays were carried out in fresh, nutrient rich medium during 4 h after the exposure to the filtrate and the fact that similar results were obtained in a previous study using algal biomass extracts (Allen *et al.*, 2015) exclude the role of

nutrient limitation in the observed effect. Metabolomic profiling and transcriptomics give different information, at the early and final steps of diatom response (Weston *et al.*, 2015). Untargeted metabolomic profiling provides only partial information depending on the method used. Here the extraction method (methanol-acetone) allows the recovery of a large panel of compounds. The separation technique used separates more efficiently lipophilic compounds, but the identification of carnitine, a polar compound, confirms the large panel of the investigated metabolome. Other studies have shown that exposure to allelopathic species affects diatom metabolome, proteome and transcriptome. Allelopathic compounds produced by dinoflagellate *Karenia brevis* affected metabolome and proteome of the marine diatom *Thalassiosira pseudonana* and to a lesser extent of another diatom, *Asterionellopsis glacialis* (Poulson-Ellestad *et al.*, 2014). Another study investigated the effect of a dinoflagellate, *Alexandrium tamarense*, on the marine diatom *Phaeodactylum tricornutum* (Zheng *et al.*, 2016). The global response of *G. parvulum* to the exposure to allelochemicals is a stimulation of genes related to photosynthesis and metabolism of amino-acids, sugars and lipids and an inhibition of cell cycle (based on GO biological process enrichment analyses, SI table S1.C1-3). While cell cycle inhibition is a common feature, our results are globally contrasting with other studies on the effect of allelopathic compounds on diatoms. Indeed, the latter have shown inhibition of photosynthesis and energy metabolism related genes (Poulson-Ellestad *et al.*, 2014; Zheng *et al.*, 2016). Moreover, the metabolomic profiling has shown a higher concentration of carnitine, a betaine, in allelopathic compounds exposed diatoms, while Poulson-Ellestad *et al.* (2014) observed an inhibition of betaine. All these contrasting results reveal the originality of the mode of action of the allelopathic compounds in *U. confervicolum* filtrate that does not inhibit diatom growth in a measurable magnitude but inhibit specifically diatom adhesion (Allen *et al.*, 2015).

#### *Effect on the 'adhesion complex'*

The adhesion of diatoms is supposed to involve an 'adhesion complex' constituted of extracellular adhesive material (included in the EPS) and transmembrane proteins linking extracellular adhesive to the cytoskeleton, which is the last part of the complex (Wetherbee *et al.*, 1998). Therefore the observed inhibition of diatom adhesion by the anti-adhesion allelopathic compounds of *U. confervicolum* could involve the inhibition of the three parts of the complex. As SEM images have revealed a strong effect on diatom EPS, an effect on the extracellular part of the complex seems very likely. Transcriptomic analyses highlighted that genes identified as coagulation factors (von Willebrand factor type D, coagulation factor 5) were globally repressed after 26 h and 50 h. In contrast, several coagulation factors (high molecular weight kininogen, von Willebrand factor type A) were significantly over-expressed. The enhancement of adhesion-related genes expression could be

due to the response of the diatom to a lower adhesion or to a direct inhibition by the allelopathic compounds.

The second part of the adhesion complex proposed by Wetherbee *et al.* (1998) is a connector protein similar to animal integrins. In our data set 19 sequences were annotated as “integrin”, four of them were significantly differentially expressed with fold-change above 2 / below -2. One gene (contig\_17123) annotated as integrin or cadherin was comprised in the 50 genes with the highest VIP at 50 h. This sequence is a good candidate for gene coding diatom connector protein between extracellular adhesive and cytoskeleton.

Cytoskeleton is the only well described component of the adhesion complex. However diatom cytoskeleton is quite different to other organisms. Many actin-related proteins are not common with other organisms, therefore diatom cytoskeleton is relatively poorly known (Aumeier *et al.*, 2015). Anti-adhesion allelopathic compounds appear to affect diatom cytoskeleton in the present experiment. Particularly, a gene coding for a dynein (contig\_21541) involved in microtubule motor activity was strongly over-expressed (change-fold > 4.6) at 2 h and 26 h and expressed a strong VIP at 26 h.

#### *Other effects: a response of diatoms to weaker adhesion to surfaces?*

The exposure to the anti-adhesion allelochemicals has also effects that could not be directly related to the adhesion process. Particularly, the most affected metabolite was identified as carnitine (Bremer, 1983; van der Hooft *et al.*, 2015). Carnitine is involved in the energy metabolism by transferring acyl and acetyl group in mitochondria. The finding of acetyl-carnitine, repressed by the exposure to anti-adhesion allelochemicals suggests a conversion of acetyl-carnitine to carnitine linked to energy metabolism.

Another interesting result is that the sequence (contig\_3376) with the highest importance in projection (SI table S2.B1) when all time points were considered together was identified as a fructose-1,6-bisphosphate aldolase. A fructose bisphosphate aldolase has been found associated with the cytoskeleton in the marine diatom *P. tricornutum* (Allen *et al.*, 2012). The authors suggested that this protein was involved in *P. tricornutum* adhesion and motility (Allen *et al.*, 2012). Our results are consistent with this hypothesis.

However, this effect could also be linked to the global effect on diatom energy metabolism. Indeed, energy metabolism was affected by the exposure to anti-adhesion allelopathic compounds. This was revealed by the stimulation of carnitine (table 1), a compound that is linked to the regulation of energy metabolisms (Van der Hooft *et al.*, 2015). The enrichment analysis indicates mostly a stimulation of energy metabolism. Our hypothesis is that allelopathic compounds do not inhibit the production of diatom adhesive material but inhibit their fixation to the substrate and the diatom. In

this hypothesis the allelopathic compound would act on EPS polymerisation or on the production of substances that cross-link and stabilise adhesive material. The low amounts of EPS on the glass support (figure 2) can be due to a detachment of excreted EPS and not to a lower production. The stimulation of energy metabolism can be, according to this hypothesis, the expression of the response of the diatom: a higher production of adhesive material. The activation of excretion related genes (*e.g.* contig\_4444 with an organic acid transmembrane transporter activity, linked to the effects on metabolome, figure 5 and SI table S2.C) also support this hypothesis. This hypothesis explains the low differences between treated and control samples when EPS were quantified, compared to the strong difference observed in SEM. SEM sample preparation could have removed weakly bounded EPS, while in the samples used for EPS quantification the over-production and the lost of EPS could have led to lower differences in the measured amount of EPS.

## Conclusion

Anti-adhesion allelopathic compounds produced by *U. confervicolum* affect diatom adhesion, EPS production or stability and a large part of diatom metabolome and transcriptome. Surprisingly, beside a negative effect on adhesion and EPS related functions, anti-adhesion allelopathic compounds stimulate energy metabolism, photosynthesis and several functions that are related to the adhesion process. We have hypothesised that this stimulation was a part of the response of the diatom to cope with the weaker adhesion when exposed to allelopathic compounds. The higher allocation of energy to the adhesion process would limit growth, accordingly an inhibition of cell cycle related genes was observed.

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Supporting information S1:

**Table S1A:** transcriptomic analysis of Gomphonema genes in response to *U. confervicolum* filtrate (U) compared to Control condition (C).

**Table S1B:** Differentially expressed genes present at the different time points.

**Table S1C.1:** Gene lists for enriched GO terms and InterPro domains at 2h in response to fractions from *U. confervicolum* (F) vs control condition (C).

**Table S1C.2:** Gene lists for enriched GO terms and InterPro domains at 26h in response to fractions from *U. confervicolum* (F) vs control condition (C).

**Table S1C.3:** Gene lists for enriched GO terms and InterPro domains at 50h in response to fractions from *U. confervicolum* (F) vs control condition (C).

Supporting information S2:

**Table S2 A1:** List of the 50 most up- and down-regulated genes at 2 h.

**Table S2 A2:** List of the 50 most up- and down-regulated genes at 26 h.

**Table S2 A3:** List of the 50 most up- and down-regulated genes at 50 h.

**Table S2 B1:** List of the 50 genes with the highest importance in the multilevel PLS-DA model projection. All the times are taken into account.

**Table S2 B2:** List of the 50 genes with the highest importance in the multilevel PLS-DA model projection at 2h.

**Table S2 B3:** List of the 50 genes with the highest importance in the multilevel PLS-DA model projection at 26h.

**Table S2 B4:** List of the 50 genes with the highest importance in the multilevel PLS-DA model projection at 50h.

**Table S2 C:** List of the 20 differentially expressed genes highly related with effect on the metabolome.

## Supporting information S1C

**Table supplement 1C.1: Gene lists for enriched GO terms and InterPro domains at 2 h in response to fractions from *U. confervicolum* vs control condition.** The "Hypergeometric tests on annotations" function of CLC Genomics Workbench was applied. The "Full set" refers to the total number of genes with the corresponding annotation in Gomphonema gene repertoire 44580 model genes). The "in Subset" indicates the number of fungal genes with the corresponding annotation observed in subsets of induced ( $\geq 2$ ) or repressed ( $\leq -2$ ) genes after the different treatments. The "Expected in subset" corresponds to values that would have been expected if the subset was a random draw from the full set. The "p-value" corresponds to the tail probability of the hyper geometric distribution. Only data with p-value  $\leq 0.01$  are listed. The table below list the feature ID corresponding to the enriched categories extracted from table S1A.

| InterPro domains                                                      |          |           |                    |          |
|-----------------------------------------------------------------------|----------|-----------|--------------------|----------|
| Genes overexpressed ( $\geq 2$ )                                      |          |           |                    |          |
| Category                                                              | Full set | In subset | Expected in subset | p-value  |
| IPR016040 NAD(P)-binding domain                                       | 349      | 88        | 56                 | 4.65E-06 |
| IPR027413 GroEL-like equatorial domain                                | 29       | 15        | 5                  | 8.66E-06 |
| IPR024370 PBP domain                                                  | 10       | 8         | 2                  | 1.37E-05 |
| IPR001846 von Willebrand factor, type D domain                        | 79       | 27        | 13                 | 5.21E-05 |
| IPR000717 Proteasome component (PCI) domain                           | 20       | 11        | 3                  | 6.80E-05 |
| IPR002076 GNS1                                                        | 22       | 11        | 4                  | 2.09E-04 |
| IPR010989 t-SNARE                                                     | 4        | 4         | 1                  | 6.44E-04 |
| IPR001563 Peptidase S10, serine carboxypeptidase                      | 21       | 10        | 3                  | 6.70E-04 |
| IPR022644 Orn                                                         | 7        | 5         | 1                  | 1.62E-03 |
| IPR011643 High-CO <sub>2</sub> inducible periplasmic                  | 10       | 6         | 2                  | 1.91E-03 |
| IPR002557 Chitin binding domain                                       | 70       | 21        | 11                 | 2.29E-03 |
| IPR005804 Fatty acid desaturase, type 1                               | 17       | 8         | 3                  | 2.58E-03 |
| IPR026444 Secretion system C-terminal sorting domain                  | 5        | 4         | 1                  | 2.81E-03 |
| IPR006634 TRAM                                                        | 21       | 9         | 3                  | 3.08E-03 |
| IPR011249 Metalloenzyme, LuxS                                         | 25       | 10        | 4                  | 3.37E-03 |
| IPR002301 Isoleucine-tRNA ligase                                      | 11       | 6         | 2                  | 3.64E-03 |
| IPR024969 Rpn11                                                       | 8        | 5         | 1                  | 3.75E-03 |
| IPR012394 Aldehyde dehydrogenase NAD(P)-dependent                     | 3        | 3         | 0                  | 4.04E-03 |
| IPR022127 Protein of unknown function DUF3661, vacuolar transmembrane | 3        | 3         | 0                  | 4.04E-03 |
| IPR000926 GTP cyclohydrolase II, RibA                                 | 3        | 3         | 0                  | 4.04E-03 |
| IPR001721 Threonine dehydratase, C-terminal regulatory domain         | 3        | 3         | 0                  | 4.04E-03 |
| IPR005723 ATPase, V1 complex, subunit B                               | 3        | 3         | 0                  | 4.04E-03 |
| IPR001518 Argininosuccinate synthase                                  | 3        | 3         | 0                  | 4.04E-03 |
| IPR023329 Chlorophyll a                                               | 78       | 22        | 12                 | 4.24E-03 |
| IPR001208 Mini-chromosome maintenance, DNA-dependent ATPase           | 15       | 7         | 2                  | 5.09E-03 |
| IPR007318 Phospholipid methyltransferase                              | 6        | 4         | 1                  | 7.36E-03 |
| IPR015931 Aconitase                                                   | 6        | 4         | 1                  | 7.36E-03 |
| IPR018506 Cytochrome b5, heme-binding site                            | 24       | 9         | 4                  | 8.74E-03 |
| IPR013831 SGNH hydrolase-type esterase domain                         | 41       | 13        | 7                  | 9.04E-03 |
| IPR000909 Phospholipase C, phosphatidylinositol-specific , X domain   | 17       | 7         | 3                  | 0.01     |
| IPR006275 Carbamoyl-phosphate synthase, large subunit                 | 10       | 5         | 2                  | 0.01     |
| IPR000092 Polyprenyl synthetase                                       | 10       | 5         | 2                  | 0.01     |
| IPR017946 PLC-like phosphodiesterase, TIM beta                        | 4        | 3         | 1                  | 0.01     |
| IPR025333 Protein of unknown function DUF4239                         | 4        | 3         | 1                  | 0.01     |
| IPR004529 Phenylalanyl-tRNA synthetase, class IIc, alpha subunit      | 4        | 3         | 1                  | 0.01     |

**Repressed genes ( $\leq 2$ )**

| Category                                                           | Full set | In subset | Expected in subset | p-value  |
|--------------------------------------------------------------------|----------|-----------|--------------------|----------|
| IPR001054 Adenylyl cyclase class-3                                 | 437      | 29        | 5                  | 1.87E-13 |
| IPR000232 Heat shock factor (HSF)-type, DNA-binding                | 127      | 9         | 2                  | 2.93E-05 |
| IPR001017 Dehydrogenase, E1 component                              | 6        | 3         | 0                  | 3.64E-05 |
| IPR013632 DNA recombination and repair protein Rad51, C-terminal   | 3        | 2         | 0                  | 4.53E-04 |
| IPR022629 S-adenosylmethionine synthetase, central domain          | 3        | 2         | 0                  | 4.53E-04 |
| IPR025714 Methyltransferase domain                                 | 33       | 4         | 0                  | 7.05E-04 |
| IPR007130 Diacylglycerol acyltransferase                           | 4        | 2         | 0                  | 9.00E-04 |
| IPR009082 Signal transduction histidine kinase, homodimeric domain | 42       | 4         | 1                  | 1.77E-03 |
| IPR016087 Chalcone isomerase                                       | 6        | 2         | 0                  | 2.21E-03 |
| IPR001932 Protein phosphatase 2C (PP2C)-like domain                | 48       | 4         | 1                  | 2.90E-03 |
| IPR017978 GPCR, family 3, C-terminal                               | 26       | 3         | 0                  | 3.94E-03 |
| IPR001544 Aminotransferase, class IV                               | 9        | 2         | 0                  | 5.18E-03 |
| IPR015412 Autophagy-related, C-terminal                            | 10       | 2         | 0                  | 6.42E-03 |
| IPR023394 SEC7-like, alpha orthogonal bundle                       | 31       | 3         | 0                  | 6.51E-03 |
| IPR013194 Histone deacetylase interacting                          | 11       | 2         | 0                  | 7.79E-03 |
| IPR000477 Reverse transcriptase                                    | 12       | 2         | 0                  | 9.27E-03 |
| IPR009075 Acyl-CoA dehydrogenase                                   | 1        | 1         | 0                  | 0.01     |
| IPR002208 SecY                                                     | 1        | 1         | 0                  | 0.01     |
| IPR005822 Ribosomal protein L13                                    | 1        | 1         | 0                  | 0.01     |
| IPR006223 Glycine cleavage system T protein                        | 1        | 1         | 0                  | 0.01     |
| IPR001699 Transcription factor, T-box                              | 1        | 1         | 0                  | 0.01     |
| IPR001096 Peptidase C13, legumain                                  | 1        | 1         | 0                  | 0.01     |
| IPR016009 tRNA (guanine-N1-)-methyltransferase                     | 1        | 1         | 0                  | 0.01     |
| IPR002143 Ribosomal protein L1                                     | 1        | 1         | 0                  | 0.01     |
| IPR000306 FYVE zinc finger                                         | 14       | 2         | 0                  | 0.01     |

**GO Biological Process****Genes overexpressed ( $\geq 2$ )**

| Category                                             | Full set | In subset | Expected in subset | p-value  |
|------------------------------------------------------|----------|-----------|--------------------|----------|
| GO:0006413 translational initiation                  | 20       | 13        | 3                  | 1.76E-06 |
| GO:0006457 protein folding                           | 59       | 20        | 10                 | 9.24E-04 |
| GO:0000162 tryptophan biosynthetic process           | 52       | 18        | 9                  | 1.25E-03 |
| GO:0009097 isoleucine biosynthetic process           | 13       | 7         | 2                  | 2.38E-03 |
| GO:0006428 isoleucyl-tRNA aminoacylation             | 10       | 6         | 2                  | 2.42E-03 |
| GO:0051252 regulation of RNA metabolic process       | 5        | 4         | 1                  | 3.34E-03 |
| GO:0006694 steroid biosynthetic process              | 21       | 9         | 4                  | 4.19E-03 |
| GO:0006020 inositol metabolic process                | 3        | 3         | 1                  | 4.63E-03 |
| GO:0006525 arginine metabolic process                | 26       | 10        | 4                  | 6.48E-03 |
| GO:0000050 urea cycle                                | 20       | 8         | 3                  | 0.01     |
| GO:0007264 small GTPase mediated signal transduction | 13       | 6         | 2                  | 0.01     |
| GO:0006544 glycine metabolic process                 | 33       | 11        | 6                  | 0.01     |

**Repressed genes ( $\leq -2$ )**

| Category                                                                        | Full set | In subset | Expected in subset | p-value  |
|---------------------------------------------------------------------------------|----------|-----------|--------------------|----------|
| GO:0007067 mitosis                                                              | 3        | 3         | 0                  | 3.03E-06 |
| GO:0007165 signal transduction                                                  | 54       | 7         | 1                  | 1.16E-05 |
| GO:0006144 purine nucleobase metabolic process                                  | 131      | 8         | 2                  | 6.15E-04 |
| GO:0019551 glutamate catabolic process to 2-oxoglutarate                        | 3        | 2         | 0                  | 6.29E-04 |
| GO:0006555 methionine metabolic process                                         | 5        | 2         | 0                  | 2.06E-03 |
| GO:0055114 oxidation-reduction process                                          | 167      | 8         | 2                  | 2.93E-03 |
| GO:0006661 phosphatidylinositol biosynthetic process                            | 7        | 2         | 0                  | 4.24E-03 |
| GO:0000122 negative regulation of transcription from RNA polymerase II promoter | 8        | 2         | 0                  | 5.60E-03 |
| GO:0000256 allantoin catabolic process                                          | 1        | 1         | 0                  | 0.01     |
| GO:0006278 RNA-dependent DNA replication                                        | 1        | 1         | 0                  | 0.01     |
| GO:0002939 tRNA N1-guanine methylation                                          | 1        | 1         | 0                  | 0.01     |

**Table supplement 1C.2: Gene lists for enriched GO terms and InterPro domains at 26 h in response to fractions from *U. confervicolum* vs control condition.** The "Hypergeometric tests on annotations" function of CLC Genomics Workbench was applied. The "Full set" refers to the total number of genes with the corresponding annotation in Gomphonema gene repertoire 44580 model genes). The "in Subset" indicates the number of fungal genes with the corresponding annotation observed in subsets of induced ( $\geq 2$ ) or repressed ( $\leq -2$ ) genes after the different treatments. The "Expected in subset" corresponds to values that would have been expected if the subset was a random draw from the full set. The "p-value" corresponds to the tail probability of the hyper geometric distribution. Only data with p-value  $\leq 0.01$  are listed. The table below list the feature ID corresponding to the enriched categories extracted from table S1A.

| InterPro domains                                                            |          |           |                    |          |
|-----------------------------------------------------------------------------|----------|-----------|--------------------|----------|
| Genes overexpressed ( $\geq 2$ )                                            |          |           |                    |          |
| Category                                                                    | Full set | In subset | Expected in subset | p-value  |
| IPR023329 Chlorophyll a                                                     | 78       | 31        | 5                  | 6.66E-16 |
| IPR006275 Carbamoyl-phosphate synthase, large subunit                       | 10       | 8         | 1                  | 2.26E-08 |
| IPR011643 High-CO <sub>2</sub> inducible periplasmic                        | 10       | 6         | 1                  | 1.92E-05 |
| IPR001576 Phosphoglycerate kinase                                           | 5        | 4         | 0                  | 1.13E-04 |
| IPR010788 Violaxanthin de-epoxidase                                         | 9        | 5         | 1                  | 1.66E-04 |
| IPR023005 Nucleoside diphosphate kinase, active site                        | 6        | 4         | 0                  | 3.21E-04 |
| IPR022644 Orn                                                               | 7        | 4         | 0                  | 7.07E-04 |
| IPR009448 UDP-glucose:Glycoprotein Glucosyltransferase                      | 4        | 3         | 0                  | 1.30E-03 |
| IPR021827 Nucleoporin Nup186                                                | 4        | 3         | 0                  | 1.30E-03 |
| IPR001478 PDZ domain                                                        | 122      | 18        | 9                  | 2.09E-03 |
| IPR000131 ATPase, F1 complex, gamma subunit                                 | 9        | 4         | 1                  | 2.27E-03 |
| IPR001849 Pleckstrin homology domain                                        | 35       | 8         | 2                  | 2.43E-03 |
| IPR006424 Glyceraldehyde-3-phosphate dehydrogenase, type I                  | 5        | 3         | 0                  | 3.08E-03 |
| IPR000941 Enolase                                                           | 10       | 4         | 1                  | 3.58E-03 |
| IPR006685 Mechanosensitive ion channel MscS                                 | 16       | 5         | 1                  | 3.81E-03 |
| IPR000782 FAS1 domain                                                       | 30       | 7         | 2                  | 3.98E-03 |
| IPR023394 SEC7-like, alpha orthogonal bundle                                | 31       | 7         | 2                  | 4.83E-03 |
| IPR002395 HMW kininogen                                                     | 2        | 2         | 0                  | 4.91E-03 |
| IPR002327 Cytochrome c, class IA                                            | 2        | 2         | 0                  | 4.91E-03 |
| IPR003805 Cobalamin biosynthesis CobS, cobalamin-5-phosphate synthase       | 2        | 2         | 0                  | 4.91E-03 |
| IPR006058 2Fe-2S ferredoxin, iron-sulphur binding site                      | 2        | 2         | 0                  | 4.91E-03 |
| IPR022485 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate synthase    | 2        | 2         | 0                  | 4.91E-03 |
| IPR011266 Bacterial fibrinogen-binding adhesin, fibrinogen-binding domain 2 | 2        | 2         | 0                  | 4.91E-03 |
| IPR028110 Protein of unknown function DUF4499                               | 2        | 2         | 0                  | 4.91E-03 |
| IPR002016 Haem peroxidase, plant                                            | 6        | 3         | 0                  | 5.84E-03 |
| IPR000409 BEACH domain                                                      | 6        | 3         | 0                  | 5.84E-03 |
| IPR014758 Methionyl-tRNA synthetase                                         | 6        | 3         | 0                  | 5.84E-03 |
| IPR022155 Protein of unknown function DUF3684                               | 6        | 3         | 0                  | 5.84E-03 |
| IPR011249 Metalloenzyme, LuxS                                               | 25       | 6         | 2                  | 6.52E-03 |
| IPR016024 Armadillo-type fold                                               | 235      | 27        | 16                 | 7.76E-03 |
| IPR022805 Phosphoenolpyruvate carboxylase, bacterial                        | 7        | 3         | 0                  | 9.70E-03 |
| IPR017926 Glutamine amidotransferase                                        | 7        | 3         | 0                  | 9.70E-03 |
| IPR001494 Importin-beta, N-terminal domain                                  | 7        | 3         | 0                  | 9.70E-03 |
| IPR009053 Prefoldin                                                         | 7        | 3         | 0                  | 9.70E-03 |
| IPR005112 dDENN domain                                                      | 13       | 4         | 1                  | 0.01     |
| IPR000048 IQ motif, EF-hand binding site                                    | 44       | 8         | 3                  | 0.01     |
| IPR004240 Nonaspanin (TM9SF)                                                | 36       | 7         | 3                  | 0.01     |
| IPR002919 Trypsin Inhibitor-like, cysteine rich domain                      | 21       | 5         | 1                  | 0.01     |
| IPR001650 Helicase, C-terminal                                              | 55       | 9         | 4                  | 0.01     |
| IPR012313 Zinc finger, FCS-type                                             | 3        | 2         | 0                  | 0.01     |
| IPR001518 Argininosuccinate synthase                                        | 3        | 2         | 0                  | 0.01     |
| IPR011333 BTB                                                               | 8        | 3         | 1                  | 0.01     |

| IPR004179 Sec63 domain                                  | 8        | 3         | 1                  | 0.01     |
|---------------------------------------------------------|----------|-----------|--------------------|----------|
| <b>Repressed genes (<math>\leq 2</math>)</b>            |          |           |                    |          |
| Category                                                | Full set | In subset | Expected in subset | p-value  |
| IPR001054 Adenylyl cyclase class-3                      | 437      | 40        | 6                  | 0        |
| IPR001846 von Willebrand factor, type D domain          | 79       | 11        | 1                  | 9.93E-09 |
| IPR021720 Malectin                                      | 89       | 10        | 1                  | 3.74E-07 |
| IPR005046 Protein of unknown function DUF285            | 148      | 11        | 2                  | 6.12E-06 |
| IPR009269 Protein of unknown function DUF926            | 2        | 2         | 0                  | 1.85E-04 |
| IPR001763 Rhodanese-like domain                         | 32       | 4         | 0                  | 9.02E-04 |
| IPR011706 Multicopper oxidase, type 2                   | 15       | 3         | 0                  | 1.01E-03 |
| IPR007272 Sulphur transport domain                      | 5        | 2         | 0                  | 1.80E-03 |
| IPR000421 Coagulation factor 5                          | 6        | 2         | 0                  | 2.68E-03 |
| IPR014830 Glycolipid transfer protein domain            | 6        | 2         | 0                  | 2.68E-03 |
| IPR009050 Globin-like                                   | 7        | 2         | 0                  | 3.72E-03 |
| IPR000727 Target SNARE coiled-coil domain               | 28       | 3         | 0                  | 6.39E-03 |
| IPR013519 Integrin alpha beta-propellor                 | 10       | 2         | 0                  | 7.76E-03 |
| IPR013216 Methyltransferase type 11                     | 30       | 3         | 0                  | 7.76E-03 |
| IPR017938 Riboflavin synthase-like beta-barrel          | 31       | 3         | 0                  | 8.51E-03 |
| IPR012336 Thioredoxin-like fold                         | 139      | 6         | 2                  | 0.01     |
| IPR013255 Chromosome segregation protein Spc25          | 1        | 1         | 0                  | 0.01     |
| IPR027120 Structural maintenance of chromosomes Smc2    | 1        | 1         | 0                  | 0.01     |
| IPR016137 Regulator of G protein signalling superfamily | 1        | 1         | 0                  | 0.01     |

**GO Biological Process**

| <b>Genes overexpressed (<math>\geq 2</math>)</b> |          |           |                    |          |
|--------------------------------------------------|----------|-----------|--------------------|----------|
| Category                                         | Full set | In subset | Expected in subset | p-value  |
| GO:0009765 photosynthesis, light harvesting      | 58       | 24        | 4                  | 1.45E-13 |
| GO:0000050 urea cycle                            | 20       | 10        | 1                  | 2.50E-07 |
| GO:0006886 intracellular protein transport       | 70       | 13        | 5                  | 9.70E-04 |
| GO:1901605 alpha-amino acid metabolic process    | 2        | 2         | 0                  | 4.89E-03 |
| GO:0006094 gluconeogenesis                       | 69       | 11        | 5                  | 7.87E-03 |
| GO:0015979 photosynthesis                        | 28       | 6         | 2                  | 0.01     |
| GO:0005198 structural molecule activity          | 3        | 2         | 0                  | 0.01     |

| <b>Repressed genes (<math>\leq 2</math>)</b>         |          |           |                    |          |
|------------------------------------------------------|----------|-----------|--------------------|----------|
| Category                                             | Full set | In subset | Expected in subset | p-value  |
| GO:0006144 purine nucleobase metabolic process       | 131      | 15        | 1                  | 2.51E-12 |
| GO:0044763 single-organism cellular process          | 112      | 12        | 1                  | 1.09E-09 |
| GO:0007067 mitosis                                   | 3        | 3         | 0                  | 1.07E-06 |
| GO:0006661 phosphatidylinositol biosynthetic process | 7        | 2         | 0                  | 2.16E-03 |
| GO:0007165 signal transduction                       | 54       | 4         | 1                  | 2.27E-03 |
| GO:0006809 nitric oxide biosynthetic process         | 1        | 1         | 0                  | 0.01     |

**Table supplement 1C.3: Gene lists for enriched GO terms and InterPro domains at 50 h in response to fractions from *U. confervicolum* vs control condition.** The "Hypergeometric tests on annotations" function of CLC Genomics Workbench was applied. The "Full set" refers to the total number of genes with the corresponding annotation in *Gomphonema* gene repertoire 44580 model genes). The "in Subset" indicates the number of fungal genes with the corresponding annotation observed in subsets of induced ( $\geq 2$ ) or repressed ( $\leq -2$ ) genes after the different treatments. The "Expected in subset" corresponds to values that would have been expected if the subset was a random draw from the full set. The "p-value" corresponds to the tail probability of the hyper geometric distribution. Only data with p-value  $\leq 0.01$  are listed. The table below list the feature ID corresponding to the enriched categories extracted from table S1A.

| InterPro domains                                           |          |           |                    |          |
|------------------------------------------------------------|----------|-----------|--------------------|----------|
| Genes overexpressed ( $\geq 2$ )                           |          |           |                    |          |
| Category                                                   | Full set | In subset | Expected in subset | p-value  |
| IPR023329 Chlorophyll a                                    | 78       | 35        | 6                  | 5.55E-16 |
| IPR016040 NAD(P)-binding domain                            | 349      | 63        | 27                 | 2.27E-10 |
| IPR006275 Carbamoyl-phosphate synthase, large subunit      | 10       | 9         | 1                  | 9.34E-10 |
| IPR010788 Violaxanthin de-epoxidase                        | 9        | 7         | 1                  | 5.26E-07 |
| IPR000782 FAS1 domain                                      | 30       | 12        | 2                  | 1.06E-06 |
| IPR001576 Phosphoglycerate kinase                          | 5        | 5         | 0                  | 2.80E-06 |
| IPR006361 Uroporphyrinogen decarboxylase HemE              | 5        | 5         | 0                  | 2.80E-06 |
| IPR011249 Metalloenzyme, LuxS                              | 25       | 9         | 2                  | 6.52E-05 |
| IPR011771 Magnesium-chelatase, subunit H                   | 8        | 5         | 1                  | 1.29E-04 |
| IPR006424 Glyceraldehyde-3-phosphate dehydrogenase, type I | 5        | 4         | 0                  | 1.70E-04 |
| IPR005147 tRNA synthetase, B5-domain                       | 5        | 4         | 0                  | 1.70E-04 |
| IPR003819 Taurine catabolism dioxygenase TauD              | 13       | 6         | 1                  | 2.31E-04 |
| IPR009014 Transketolase, C-terminal                        | 24       | 8         | 2                  | 3.08E-04 |
| IPR010255 Haem peroxidase                                  | 14       | 6         | 1                  | 3.78E-04 |
| IPR012394 Aldehyde dehydrogenase NAD(P)-dependent          | 3        | 3         | 0                  | 4.67E-04 |
| IPR001518 Argininosuccinate synthase                       | 3        | 3         | 0                  | 4.67E-04 |
| IPR007318 Phospholipid methyltransferase                   | 6        | 4         | 0                  | 4.78E-04 |
| IPR000941 Enolase                                          | 10       | 5         | 1                  | 5.07E-04 |
| IPR002123 Phospholipid                                     | 22       | 7         | 2                  | 1.01E-03 |
| IPR007378 Tic22-like                                       | 7        | 4         | 1                  | 1.05E-03 |
| IPR025333 Protein of unknown function DUF4239              | 4        | 3         | 0                  | 1.76E-03 |
| IPR001841 Zinc finger, RING-type                           | 173      | 25        | 13                 | 1.89E-03 |
| IPR003333 Mycolic acid cyclopropane synthase               | 8        | 4         | 1                  | 1.96E-03 |
| IPR004328 BRO1 domain                                      | 8        | 4         | 1                  | 1.96E-03 |
| IPR028055 Membrane insertase YidC                          | 8        | 4         | 1                  | 1.96E-03 |
| IPR000771 Ketose-bisphosphate aldolase, class-II           | 9        | 4         | 1                  | 3.32E-03 |
| IPR023210 NADP-dependent oxidoreductase domain             | 34       | 8         | 3                  | 3.79E-03 |
| IPR022418 Porphobilinogen deaminase, C-terminal            | 5        | 3         | 0                  | 4.15E-03 |
| IPR004480 Monothiol glutaredoxin-related                   | 5        | 3         | 0                  | 4.15E-03 |
| IPR001342 Homoserine dehydrogenase, catalytic              | 5        | 3         | 0                  | 4.15E-03 |
| IPR014748 Crononase, C-terminal                            | 5        | 3         | 0                  | 4.15E-03 |
| IPR001563 Peptidase S10, serine carboxypeptidase           | 21       | 6         | 2                  | 4.26E-03 |
| IPR002919 Trypsin Inhibitor-like, cysteine rich domain     | 21       | 6         | 2                  | 4.26E-03 |
| IPR000092 Polyprenyl synthetase                            | 10       | 4         | 1                  | 5.19E-03 |
| IPR002076 GNS1                                             | 22       | 6         | 2                  | 5.47E-03 |
| IPR006685 Mechanosensitive ion channel MscS                | 16       | 5         | 1                  | 5.93E-03 |
| IPR001806 Small GTPase superfamily                         | 2        | 2         | 0                  | 6.02E-03 |
| IPR002496 Phosphoribosyl-AMP cyclohydrolase domain         | 2        | 2         | 0                  | 6.02E-03 |
| IPR004398 RNA methyltransferase, RsmD                      | 2        | 2         | 0                  | 6.02E-03 |
| IPR013784 Carbohydrate-binding-like fold                   | 2        | 2         | 0                  | 6.02E-03 |
| IPR003428 Mitochondrial glycoprotein                       | 2        | 2         | 0                  | 6.02E-03 |

|                                                                          |     |    |    |          |
|--------------------------------------------------------------------------|-----|----|----|----------|
| IPR002395 HMW kininogen                                                  | 2   | 2  | 0  | 6.02E-03 |
| IPR014984 HopJ type III effector protein                                 | 2   | 2  | 0  | 6.02E-03 |
| IPR003754 Tetrapyrrole biosynthesis, uroporphyrinogen III synthase       | 2   | 2  | 0  | 6.02E-03 |
| IPR004788 Ribose 5-phosphate isomerase, type A                           | 2   | 2  | 0  | 6.02E-03 |
| IPR002042 Uricase                                                        | 2   | 2  | 0  | 6.02E-03 |
| IPR002327 Cytochrome c, class IA                                         | 2   | 2  | 0  | 6.02E-03 |
| IPR003805 Cobalamin biosynthesis CobS, cobalamin-5-phosphate synthase    | 2   | 2  | 0  | 6.02E-03 |
| IPR016867 Glycine cleavage repressor GcvR                                | 2   | 2  | 0  | 6.02E-03 |
| IPR006372 Bacteriochlorophyll                                            | 2   | 2  | 0  | 6.02E-03 |
| IPR004188 Phenylalanine-tRNA ligase, class II, N-terminal                | 2   | 2  | 0  | 6.02E-03 |
| IPR022485 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate synthase | 2   | 2  | 0  | 6.02E-03 |
| IPR007590 CWC16 protein                                                  | 2   | 2  | 0  | 6.02E-03 |
| IPR005322 Peptidase C69, dipeptidase A                                   | 2   | 2  | 0  | 6.02E-03 |
| IPR002130 Cyclophilin-like peptidyl-prolyl cis-trans isomerase domain    | 62  | 11 | 5  | 7.65E-03 |
| IPR008972 Cupredoxin                                                     | 6   | 3  | 0  | 7.81E-03 |
| IPR014102 Phytoene desaturase                                            | 6   | 3  | 0  | 7.81E-03 |
| IPR002016 Haem peroxidase, plant                                         | 6   | 3  | 0  | 7.81E-03 |
| IPR002650 Sulphate adenylyltransferase                                   | 6   | 3  | 0  | 7.81E-03 |
| IPR023005 Nucleoside diphosphate kinase, active site                     | 6   | 3  | 0  | 7.81E-03 |
| IPR013657 UAA transporter                                                | 24  | 6  | 2  | 8.64E-03 |
| IPR002035 von Willebrand factor, type A                                  | 56  | 10 | 4  | 0.01     |
| IPR012336 Thioredoxin-like fold                                          | 139 | 19 | 11 | 0.01     |
| IPR022644 Orn                                                            | 7   | 3  | 1  | 0.01     |
| IPR018215 ClpP, active site                                              | 7   | 3  | 1  | 0.01     |
| IPR001216 Cysteine synthase                                              | 7   | 3  | 1  | 0.01     |
| IPR000889 Glutathione peroxidase                                         | 13  | 4  | 1  | 0.01     |

**Repressed genes ( $\leq 2$ )**

| Category                                                  | Full set | In subset | Expected in subset | p-value  |
|-----------------------------------------------------------|----------|-----------|--------------------|----------|
| IPR001846 von Willebrand factor, type D domain            | 79       | 10        | 1                  | 4.23E-07 |
| IPR001054 Adenylyl cyclase class-3                        | 437      | 23        | 7                  | 4.52E-07 |
| IPR005046 Protein of unknown function DUF285              | 148      | 12        | 2                  | 3.82E-06 |
| IPR000421 Coagulation factor 5                            | 6        | 3         | 0                  | 7.33E-05 |
| IPR021720 Malectin                                        | 89       | 8         | 1                  | 7.74E-05 |
| IPR009269 Protein of unknown function DUF926              | 2        | 2         | 0                  | 2.44E-04 |
| IPR007065 HPP                                             | 2        | 2         | 0                  | 2.44E-04 |
| IPR007956 Malonyl-CoA decarboxylase                       | 2        | 2         | 0                  | 2.44E-04 |
| IPR009292 Protein of unknown function DUF947              | 2        | 2         | 0                  | 2.44E-04 |
| IPR015916 Galactose oxidase, beta-propeller               | 23       | 4         | 0                  | 4.12E-04 |
| IPR022629 S-adenosylmethionine synthetase, central domain | 3        | 2         | 0                  | 7.24E-04 |
| IPR013216 Methyltransferase type 11                       | 30       | 4         | 0                  | 1.17E-03 |
| IPR007130 Diacylglycerol acyltransferase                  | 4        | 2         | 0                  | 1.43E-03 |
| IPR011706 Multicopper oxidase, type 2                     | 15       | 3         | 0                  | 1.50E-03 |
| IPR017940 ABC transporter, integral membrane type 1       | 83       | 6         | 1                  | 1.92E-03 |
| IPR006652 Kelch repeat type 1                             | 36       | 4         | 1                  | 2.33E-03 |
| IPR025705 Beta-hexosaminidase subunit alpha               | 7        | 2         | 0                  | 4.86E-03 |
| IPR024079 Metalloproteinase, catalytic domain             | 29       | 3         | 0                  | 0.01     |
| IPR009071 High mobility group box domain                  | 85       | 5         | 1                  | 0.01     |
| IPR013194 Histone deacetylase interacting                 | 11       | 2         | 0                  | 0.01     |
| IPR017938 Riboflavin synthase-like beta-barrel            | 31       | 3         | 0                  | 0.01     |
| IPR000232 Heat shock factor (HSF)-type, DNA-binding       | 127      | 6         | 2                  | 0.01     |

## GO Biological Process

Genes overexpressed ( $\geq 2$ )

| Category                                                      | Full set | In subset | Expected in subset | p-value  |
|---------------------------------------------------------------|----------|-----------|--------------------|----------|
| GO:0009765 photosynthesis, light harvesting                   | 58       | 24        | 5                  | 3.55E-11 |
| GO:0000050 urea cycle                                         | 20       | 12        | 2                  | 1.65E-08 |
| GO:0006094 gluconeogenesis                                    | 69       | 17        | 6                  | 9.36E-05 |
| GO:0006782 protoporphyrinogen IX biosynthetic process         | 7        | 5         | 1                  | 1.05E-04 |
| GO:0055114 oxidation-reduction process                        | 167      | 30        | 15                 | 1.69E-04 |
| GO:0006979 response to oxidative stress                       | 25       | 9         | 2                  | 2.01E-04 |
| GO:0000162 tryptophan biosynthetic process                    | 52       | 13        | 5                  | 5.31E-04 |
| GO:0015995 chlorophyll biosynthetic process                   | 14       | 6         | 1                  | 8.35E-04 |
| GO:0006525 arginine metabolic process                         | 26       | 8         | 2                  | 1.50E-03 |
| GO:0006694 steroid biosynthetic process                       | 21       | 7         | 2                  | 1.76E-03 |
| GO:0006508 proteolysis                                        | 140      | 23        | 13                 | 3.21E-03 |
| GO:0006000 fructose metabolic process                         | 52       | 11        | 5                  | 5.72E-03 |
| GO:0006779 porphyrin-containing compound biosynthetic process | 5        | 3         | 0                  | 6.34E-03 |
| GO:0006544 glycine metabolic process                          | 33       | 8         | 3                  | 7.58E-03 |
| GO:0006644 phospholipid metabolic process                     | 2        | 2         | 0                  | 8.11E-03 |
| GO:0045454 cell redox homeostasis                             | 15       | 5         | 1                  | 8.17E-03 |
| GO:0006633 fatty acid biosynthetic process                    | 28       | 7         | 3                  | 0.01     |
| GO:0051205 protein insertion into membrane                    | 6        | 3         | 1                  | 0.01     |
| GO:0016117 carotenoid biosynthetic process                    | 6        | 3         | 1                  | 0.01     |
| GO:0006631 fatty acid metabolic process                       | 11       | 4         | 1                  | 0.01     |
| GO:0006098 pentose-phosphate shunt                            | 36       | 8         | 3                  | 0.01     |

Repressed genes ( $\leq -2$ )

| Category                                                                        | Full set | In subset | Expected in subset | p-value  |
|---------------------------------------------------------------------------------|----------|-----------|--------------------|----------|
| GO:0006144 purine nucleobase metabolic process                                  | 131      | 8         | 2                  | 1.53E-04 |
| GO:0044763 single-organism cellular process                                     | 112      | 7         | 1                  | 3.51E-04 |
| GO:0006555 methionine metabolic process                                         | 5        | 2         | 0                  | 1.38E-03 |
| GO:0030001 metal ion transport                                                  | 21       | 3         | 0                  | 1.87E-03 |
| GO:0000122 negative regulation of transcription from RNA polymerase II promoter | 8        | 2         | 0                  | 3.77E-03 |
| GO:0000478 endonucleolytic cleavage involved in rRNA processing                 | 1        | 1         | 0                  | 0.01     |



## Chapitre V

### Discussion générale et perspectives



## V. Discussion générale et perspectives

### V.1. Synthèse des résultats obtenus.

Le travail réalisé au cours de cette thèse a permis de mettre en évidence la production de plusieurs types de composés allélopathiques par l'algue verte filamenteuse *Uronema confervicolum* :

- deux acides gras polyinsaturés, l'acide linoléique (LA) et l'acide  $\alpha$ -linolénique (LNA), ayant une activité contre la photosynthèse et la croissance des diatomées ont été identifiés grâce à une procédure de fractionnement guidé par bioessais, la vérification de l'activité de standards de ces deux acides gras et l'extraction et le dosage ciblé de ces deux molécules dans le milieu de culture.
- des composés non-identifiés inhibant l'adhésion des diatomées et ayant un impact sur la matrice de substances polymériques extracellulaires (EPS). L'exposition à ces composés impacte également le métabolisme énergétique notamment par une augmentation de la concentration cellulaire en carnitine et une stimulation des gènes impliqués dans la photosynthèse et le métabolisme énergétique. L'implication potentielle de plusieurs métabolites dans l'activité d'inhibition de l'adhésion a été déterminée par une méthode basée sur l'utilisation des profils métabolomiques. L'identification de ces métabolites est en cours.

Deux hypothèses, la Growth-Differentiation Balance Hypothesis (GDBH) et l'Optimal Defence Theory (ODT) ont été testées pour comprendre les mécanismes de régulation de la production de composés allélopathiques. Alors que sur certains aspects les résultats peuvent être expliqués par l'une ou l'autre des hypothèses, aucune ne permet de les expliquer dans leur ensemble.

Pour cette étude, nous avons utilisé plusieurs approches complémentaires. Tout d'abord, pour l'étude de l'activité des extraits, plusieurs paramètres ont été mesurés : la croissance, la photosynthèse, l'adhésion et la motilité. Seule la croissance, considérée comme le paramètre le plus intégrateur, a été utilisée lors de la démarche de fractionnement guidé par bioessais. L'utilisation de différents bioessais a permis de réaliser que les composés identifiés étaient probablement responsables de l'effet observé sur la croissance et la photosynthèse, mais pas de l'inhibition de l'adhésion observée. Ainsi la mesure de paramètres multiples a permis la mise en évidence de la production d'autres composés.

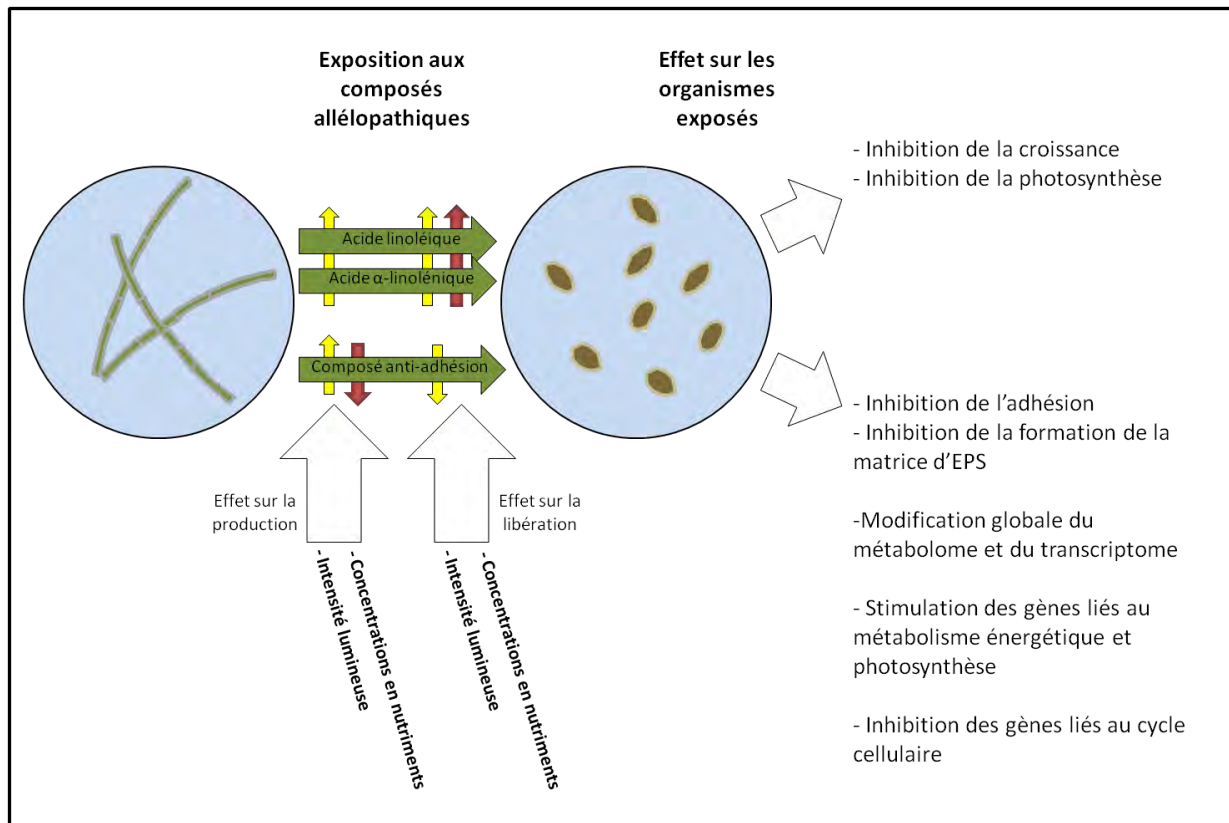


**Figure 14 : Synthèse des principaux résultats obtenus.**

L'étude réalisée sur l'activité allélopathique d'hyphomycètes aquatiques (annexe) illustre aussi l'utilité de l'emploi de différents bioessais. En effet, dans cette étude certaines souches n'ont d'effets que dans les tests sur milieu solide, d'autres qu'en milieu liquide. Cela indique l'implication de plusieurs composés aux propriétés chimiques distinctes (annexe).

Une autre méthode, basée sur le profilage métabolomique, a été choisie pour essayer d'identifier les composés impliqués dans l'inhibition de l'adhésion. L'utilisation du profilage métabolomique aurait pour avantage de limiter la dégradation des composés et de nécessiter une masse d'extrait nettement inférieure (Prince and Pohnert, 2010; Weston *et al.*, 2015). Cette méthode avait surtout l'avantage de pouvoir être combinée à l'étude de l'effet des facteurs environnementaux sur l'activité allélopathique.

Pour l'étude de la réponse de la diatomée aux composés allélopathiques inhibant l'adhésion, le choix a été fait d'utiliser une approche non ciblée (mis à part les dosages et observations des EPS, justifiés par la nature de l'effet observé). Cette approche globale a consisté en une analyse de la réponse de la diatomée au niveau du transcriptome, correspondant à la première étape de la réponse de la diatomée, et au niveau du métabolome, correspondant au stade final de la réponse. D'autres données 'omiques' peuvent être utilisées pour ce type de questions, telles que la protéomique. Cependant, les données protéomiques auraient été plus redondantes avec les données de

transcriptomique que ne le sont celles de métabolomique. Il faut noter que les données de métabolomiques sont très partielles : seulement certaines catégories de molécules sont observées du fait d'une limitation au niveau de l'extraction (extraction principalement des composés apolaires), de la séparation (les composés les plus polaires éluent très rapidement mais sont plus difficile à séparer) et de la détection ( $m/z$  entre 50 et 1200 élimine les composés les plus lourds et les composés difficilement ionisables) alors que la transcriptomique est beaucoup plus globale.

Dans cette étude, il n'a pas été possible de déterminer avec certitude si l'inhibition de l'adhésion observée était due à une inhibition de la production d'EPS ou à la diminution de la liaison entre les EPS et le substrat (lamelle de verre) et entre les EPS et les cellules. Les autres résultats supportent plutôt la seconde hypothèse. En effet, le fait que peu de différences aient été observées dans les dosages d'EPS pourrait indiquer que c'est la préparation des échantillons pour le MEB qui a éliminé les EPS, dans les échantillons exposés aux composés allélopathiques inhibant l'adhésion. La stimulation de gènes liés au métabolisme énergétique et à l'excrétion indiquerait une plus forte production de substances extracellulaires pour compenser la faible adhésion. Les EPS seraient donc dispersées dans le milieu environnant au lieu de rester liées au biofilm. Pour pouvoir valider ou invalider cette dernière hypothèse il a été envisagé de réaliser un dosage du carbone organique total dans le milieu de culture des diatomées exposées au filtrat et leur témoin. Cependant, l'ajout de filtrat apporte aussi du carbone organique. Il serait donc nécessaire de déterminer précisément la nature de ce carbone organique ou de trouver des composés produits uniquement par les diatomées, une analyse des sucres (méthodologie en développement au plateau d'analyse physico-chimique d'EcoLab) pourrait permettre de vérifier cette hypothèse.

L'implication dans la réponse de la diatomée des gènes et des métabolites repérés par cette approche exploratoire pourra ensuite être vérifiée par des méthodes plus ciblées : dosage spécifique des métabolites sélectionnés, RT-qPCR pour les gènes identifiés et production de mutants n'exprimant pas les gènes identifiés comme liés à l'adhésion (Willis *et al.*, 2014).

## **V.2. Diversité des interactions allélopathiques dans un biofilm phototrophe.**

L'un des objectifs primordiaux en écologie chimique est la compréhension du rôle des interactions chimiques dans les écosystèmes. Il est d'autant plus important de comprendre le rôle des interactions allélopathiques que ce processus est très commun et diversifié, du moins dans les biofilms phototrophes (partie I.3). En effet, de nombreux composés allélopathiques, de nature chimique très variée, ont été identifiés dans les groupes les plus communs de micro-algues

benthiques. Cependant, cela pouvait paraître moins évident pour les algues vertes filamenteuses que pour les diatomées ou les cyanobactéries (chapitre I). Les nouveaux résultats apportés pendant cette thèse confirment non seulement que les algues vertes filamenteuses produisent elles aussi des composés allélopathiques, mais aussi qu'une seule souche est capable de produire plusieurs composés allélopathiques différents. En effet, l'allélopathie de l'algue verte filamenteuse *Uronema confervicolum* est basée sur deux acides gras polyinsaturés (dont les effets sont proches mais avec des différences dans leur régulation) et sur d'autres composés (non identifiés à l'heure actuelle) aux effets, aux propriétés chimiques et aux modes de régulation différents de ceux des acides gras polyinsaturés allélopathiques. Peu de cas similaires de plusieurs activités allélopathiques pour une même micro-algue ont été reportés jusqu'à présent dans la littérature. Il faut toutefois noter que séparer l'effet de plusieurs composés allélopathiques peut s'avérer délicat. Dans notre étude, sans de telles différences sur le mode d'action et la diffusion dans le milieu, l'existence d'autres composés n'aurait pas été suspectée. Cela semble plus commun chez les cyanobactéries, connues pour produire une grande diversité de composés bioactifs (e.g. Berry et al., 2008), tels que le genre *Fischerella* (Leão et al., 2012).

A côté de ces travaux centrés sur l'interaction *U. confervicolum*-diatomées, nous avons mis en évidence l'existence d'interactions allélopathiques entre des hyphomycètes aquatiques provenant de ruisseaux forestiers et des diatomées (annexe). C'est la première fois que ce type d'interaction entre hyphomycètes aquatiques et microorganisme photosynthétique était rapporté. Plusieurs espèces d'hyphomycètes, vraisemblablement associées à des composés allélopathiques différents, étaient impliquées. Notre hypothèse est que ces interactions chimiques inhibitrices participeraient, entre autres mécanismes, à la dominance des décomposeurs sur les producteurs primaires dans ces ruisseaux forestiers. Cela souligne encore une fois l'importance de la compréhension de l'allélopathie pour l'étude du fonctionnement de tous les types d'écosystèmes aquatiques.

### V.3. Transposition au milieu naturel

Les effets allélopathiques observés l'ont été en laboratoire en milieux contrôlés. C'est d'ailleurs le cas pour la plupart des cas d'interactions allélopathiques entre organismes aquatiques rapportés dans la littérature. En effet, étant donné que la démonstration de l'activité allélopathique d'un organisme nécessite d'exclure tout autre facteur confondant, il est nécessaire de travailler en conditions contrôlées. La question qui se pose alors est de déterminer dans quelle mesure l'activité allélopathique observée est transposable au milieu naturel. Les éléments de discussion présentés dans la suite de cette partie visent à évaluer dans quelle mesure les informations apportées par l'étude de notre système simplifié issus de biofilms phototrophes (figure 1 p. 2) pourraient nous

permettre en retour de mieux comprendre les interactions au sein du système complexe qu'est le biofilm phototrophe.

#### *Cas des acides gras polyinsaturés*

Bien que les acides gras polyinsaturés LA et LNA aient été initialement identifiés dans des extraits de biomasse algale, des dosages dans le milieu de culture ont mis en évidence que ces composés étaient également excrétés par les cellules, ce qui a permis de conclure quant à leur rôle allélopathique possible (chapitre II). Cependant, les concentrations de LA et LNA mesurées dans le milieu se sont révélées très faibles, bien inférieures aux concentrations nécessaires pour inhiber la croissance des diatomées utilisées dans le bioessai. Notre hypothèse est que l'activité allélopathique d'*U. confervicolum* via les acides gras polyinsaturés repose sur l'accumulation de ces composés au niveau du biofilm. Cette accumulation a été mise en évidence dans le chapitre III, en dosant les acides gras retenus sur un biofilm de diatomées. Ces composés sont donc très probablement en mesure d'inhiber les diatomées se développant en épiphytes sur les filaments d'*U. confervicolum*. L'effet allélopathique causé par les acides gras polyinsaturés serait dans ce cas dépendant du contact entre l'algue verte filamenteuse et les diatomées. Cette interaction est proche de ce qui est observé entre des macroalgues et des diatomées marines. En effet, il a été démontré que des composés allélopathiques produits par des macroalgues étaient généralement associés à la surface de l'algue (Andras *et al.*, 2012; Lane *et al.*, 2009). Par contre, il est très improbable que la libération d'acides gras permette à *U. confervicolum* d'inhiber des algues plus distantes, telles que des algues planctoniques, du fait de la faible diffusion des composés.

#### *Cas des composés inhibant l'adhésion des diatomées*

Concernant l'effet anti-adhésion, son induction par le filtrat prouve que les composés bioactifs impliqués sont bien libérés. Il est tout de même nécessaire d'exclure la possibilité que l'effet observé soit dû à une déplétion des nutriments, particulièrement les macronutriments (N et P) et le calcium, important pour le processus d'adhésion (Cooksey, 1981; Geesey *et al.*, 2000). Le fait que les mêmes effets anti-adhésion aient été observés avec le filtrat enrichi en N et P, les extraits (donc sans impact sur les nutriments) et que cet effet perdure après que les diatomées exposées au filtrat aient été placées dans du milieu COMBO frais, montre clairement l'absence de lien avec une déplétion de nutriments. Dans ce cas, les composés diffusent plus dans le milieu, mais les diatomées en contact avec les filaments d'*U. confervicolum* ou à proximité directe seront plus fortement impactés car les composés seront fortement dilués après leur libération dans le milieu.

### *Possibilité d'une synergie ?*

Les acides gras polyinsaturés, inhibant la photosynthèse et la croissance, et les composés inhibant l'adhésion impactent donc les mêmes organismes (les diatomées épiphytes) et pourraient agir de façon synergique. En effet, de fortes synergies peuvent être observées entre différents composés allélopathiques produits par une même espèce, comme c'est le cas pour les portoamides A et B produits par la cyanobactérie *Oscillatoria* sp. qui individuellement ont peu d'activité mais une activité beaucoup plus forte lorsqu'ils sont en mélange (Leão *et al.*, 2010). La synergie entre plusieurs facteurs de stress serait, d'après Liess *et al.* (2016), un phénomène général. La mise en évidence d'activités allélopathiques basées sur la synergie de plusieurs composés se heurte à des problèmes méthodologiques, notamment lors de l'utilisation de méthodes de fractionnement guidé par bioessais. Dans notre cas, les composés allélopathiques produits par *U. confervicolum* affectent des fonctions différentes, mais l'étude approfondie de l'activité anti-adhésion a révélé également une stimulation du métabolisme énergétique. Cette stimulation pourrait être due à une compensation des effets des composés allélopathiques anti-adhésion. La présence des acides gras allélopathiques limiterait donc la capacité de la diatomée à compenser l'effet simultané des composés anti-adhésion. Une hypothèse expliquant l'action des acides gras polyinsaturés sur les diatomées est qu'ils endommageraient les membranes (Ikawa, 2004). Ceci pourrait faciliter la pénétration d'autres composés chimiques et de ce fait, augmenter leur effet. D'autre part, l'exposition des diatomées au filtrat d'*U. confervicolum* stimule le métabolisme photosynthétique, au contraire, l'exposition aux acides gras diminue l'efficacité photosynthétique. Il serait intéressant de comprendre l'interaction des deux effets. L'hypothèse d'une synergie entre les deux activités allélopathiques d'*U. confervicolum* pourrait être facilement testée en comparant l'effet du filtrat, l'effet des acides gras et l'effet du mélange des deux sur l'adhésion, la croissance et la photosynthèse.

### *Coût et bénéfices de la production des composés allélopathiques*

L'un des principaux obstacles au maintien de la production de composés allélopathiques dans une population est que les bénéfices de l'allélopathie sont partagés par tous les organismes autour de l'émetteur (« distributed benefits » ; Lewis, 1986). Ce partage des avantages de la production de composés allélopathiques favorise les organismes dans la population qui n'en produisent pas, car n'ont pas à en assumer le coût mais en ont tout de même les bénéfices. Ce processus aurait tendance à faire diminuer la production de composés allélopathiques par la population (Lewis, 1986). Toutefois, dans le cas d'*U. confervicolum*, plusieurs facteurs permettent de supposer que le partage des bénéfices est réduit. D'une part, l'organisation structurée du biofilm limite l'effet de distribution des bénéfices de l'allélopathie (Durrett and Levin, 1997). En effet, les souches produisant moins de composés allélopathiques forment rapidement des zones dans lesquelles l'allélopathie est réduite,

les cellules ne profitent donc pas des composés allélopathiques émis par d'autres organismes. D'autre part, les acides gras allélopathiques sont composés essentiellement de carbone, et en condition de lumière forte - condition dans laquelle se développent les algues vertes filamenteuses - le carbone peut être fixé en excès. En effet, jusqu'à 90% du carbone fixé peut être excrété par les algues vertes filamenteuses (Cheney and Hough, 1983). Les résultats présentés dans le chapitre III montrent une augmentation de la production lorsque les conditions permettent une plus forte assimilation de CO<sub>2</sub>. Cette production supplémentaire ne représente que peu ou pas de coût pour l'algue.

#### *Résistance des cibles ?*

Pour réaliser les expérimentations, je me suis focalisé sur l'utilisation de souches sensibles aux activités allélopathiques d'*U. confervicolum*, mais de nombreuses souches se sont révélées résistantes aux acides gras allélopathiques, à l'activité anti-adhésion (voir figure 11 chapitre IV) ou aux deux à la fois. Pour les chapitres II et III, les diatomées étaient utilisées en tant qu'indicateurs de la présence de composés allélopathiques. Il convenait donc de choisir les souches les plus sensibles. Pour le chapitre IV, le choix s'est porté vers la souche la plus sensible afin de maximiser l'observation de la réponse aux composés allélopathiques conduisant à une perte d'adhésion. Le design idéal pour cette expérimentation aurait été d'utiliser deux souches de diatomées, l'une résistante et l'autre sensible, afin de déterminer quels changements dans le métabolome et le transcriptome sont réellement liés à l'inhibition de l'adhésion. Cela n'a pas pu être réalisé pour des raisons techniques, la quantité d'ARN extrait à partir des échantillons de *N. palea* (résistantes aux composés allélopathiques inhibant l'adhésion) étant trop faible pour le séquençage des ARN.

Cette existence de souches résistantes peut remettre en question l'utilité de la production de composés allélopathiques pour *U. confervicolum*. Cependant, il faut remarquer que l'inhibition de l'adhésion est particulière du point de vue des conséquences sur les diatomées. En effet, les diatomées sensibles sont rapidement emportées par le courant et ne subissent donc plus les effets des composés allélopathiques. La pression de sélection qui en résulte serait donc moins forte que dans le cas de composés provoquant des dommages plus conséquents pour les diatomées.

#### **V.4. Impact fonctionnel et structurel sur les communautés ?**

L'effet sur les communautés de la présence d'espèces émettant des composés allélopathiques n'a pas pu être directement étudié durant cette thèse. Cependant, certains résultats obtenus permettent de soulever de nouvelles perspectives.

*Allélopathie, exclusion compétitive et coexistence*

L'activité allélopathique peut rendre *U. confervicolum* plus compétitive vis-à-vis d'autres espèces que ce que l'on pourrait estimer en se focalisant sur la capacité de cette algue à exploiter les ressources. Il a été montré, dans une étude de Barreiro et Vasconcelos (2014), que l'allélopathie pouvait changer l'issue de la compétition entre deux espèces en entraînant la dominance d'une espèce ayant une croissance plus faible mais inhibant celle à croissance rapide. L'allélopathie, en réduisant la capacité de l'espèce impactée par les molécules libérées à exploiter les ressources, peut permettre la dominance d'une espèce à croissance plus lente. Mais, en créant des systèmes dans lesquels aucune espèce n'est dominante face à toutes les autres (réseaux de compétition non-hiérarchique). L'allélopathie peut avoir aussi des conséquences bien plus complexes au niveau des écosystèmes.

En effet, de nombreux travaux de modélisation indiquent que l'allélopathie pourrait promouvoir la biodiversité par la création de ces réseaux de compétition non-hiérarchique. Etant donné que dans un réseau de compétition non-hiérarchique aucune espèce n'est plus compétitive que toutes les autres à la fois, le phénomène d'exclusion compétitive est évité lorsque plusieurs espèces sont en compétition pour une même ressource limitante (Czárán *et al.*, 2002; Roy and Chattopadhyay, 2007; Mougi, 2013). Ce mécanisme a été confirmé expérimentalement par Kerr *et al.* (2002) en utilisant une communauté artificielle constituée d'une souche de bactéries produisant de la colicine, d'une souche de bactéries sensibles et d'une souche résistante à la colicine. Cependant, cela ne prouve pas l'occurrence de ce mécanisme dans des communautés naturelles. L'étude des interactions entre invertébrés marins sessiles par Jackson et Buss (1975) suggère l'existence de réseaux de compétition non-hiérarchique dans ces communautés, mais l'implication de ce caractère non-hiérarchique pour le maintien de la coexistence des espèces présentes n'y a pas été vérifiée. Il apparaît que le système *U. confervicolum* – diatomées pourrait être utilisé pour tester cette hypothèse. La croissance d'*U. confervicolum* et de diatomées sensibles et résistantes aux composés allélopathiques (et isolées à partir d'un même biofilm) pourrait être suivie en co-culture deux à deux afin de déterminer si leur relation correspond à une situation non-hiérarchique. Si tel est le cas, on pourra suivre la dynamique des trois espèces en co-cultures pour vérifier le maintien de la coexistence des trois espèces.

*Importance relative de la compétition par interférence et par exploitation*

Si les résultats obtenus nous permettent d'affirmer qu'*U. confervicolum* peut très probablement avoir une activité allélopathique contre les diatomées même en dehors des conditions contrôlées du laboratoire, il est par contre difficile d'estimer l'importance relative de l'allélopathie par rapport à la compétition par exploitation directe des ressources. Cette question a de l'importance, car si l'effet de l'allélopathie est négligeable vis-à-vis de l'effet de l'exploitation, alors les relations entre les espèces et la dynamique des communautés dépendront uniquement de leur capacité à exploiter les

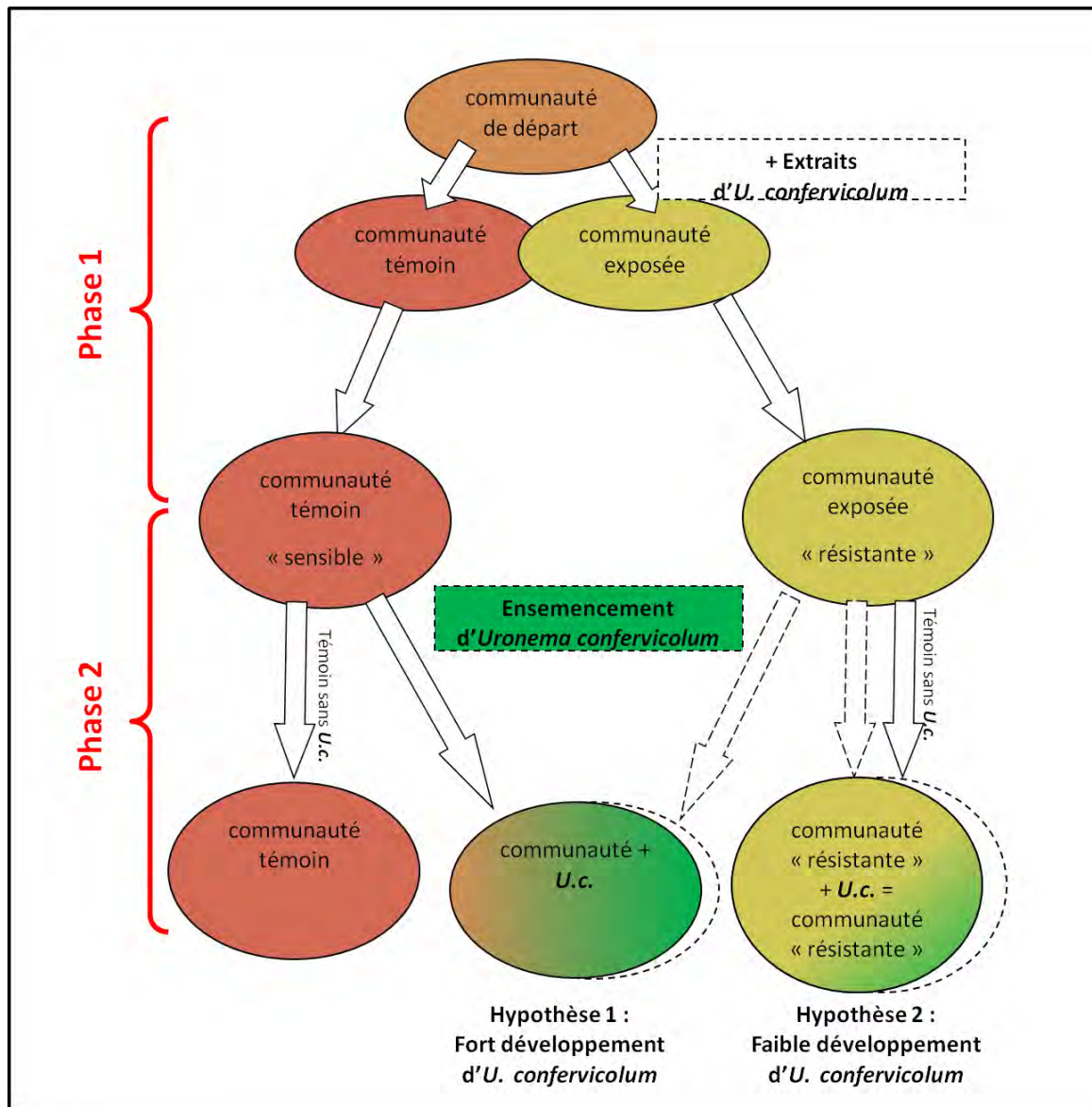


**Figure 15 : Représentation de la démarche proposée pour la détermination de l'importance de l'allélopathie dans un biofilm complexe.**

ressources. Par contre, si l'effet de l'allélopathie n'est pas négligeable, alors les processus décrits ci-dessus seraient une explication envisageable de la forte diversité spécifique que l'on peut rencontrer au sein de biofilms.

Il serait possible de tester l'importance de la production de composés allélopathiques pour le développement d'*U. confervicolum* au sein de biofilms complexes artificiels ou naturels. Le principe serait de comparer le développement d'*U. confervicolum* avec et sans activité allélopathique.

Pour cela, l'idée est de s'inspirer de ce qui est fait en écotoxicologie avec l'étude des « pollution induced community tolerance » (PICT ; Blanck *et al.*, 1988). La démarche proposée est basée sur l'adaptation de la communauté aux composés allélopathiques. Elle est constituée de deux phases : une première phase durant laquelle la communauté est exposée artificiellement aux composés

allélopathiques, et une deuxième phase dans laquelle la communauté est exposée directement à l'organisme produisant des composés allélopathiques. La première phase a pour but de créer une communauté résistante aux composés allélopathiques par l'inhibition des souches sensibles (figure 15). Pour une résistance à tous les composés allélopathiques des extraits de biomasse pourraient être utilisés. Il serait aussi possible de tester uniquement l'effet du filtrat, des acides gras ou de la combinaison filtrat-acides gras pour tester l'effet de composés allélopathiques spécifiques. L'hypothèse est que l'exposition aux composés allélopathiques entrainera une modification des communautés en favorisant fortement les souches peu sensibles aux composés allélopathiques produits par l'algue (Leão *et al.*, 2012). Ainsi, à la fin de cette phase deux communautés différentes seront issues de la première utilisée : une communauté exposée « résistante » aux composés allélopathiques et une communauté témoin, théoriquement plus sensible aux composés allélopathiques.

La seconde phase est celle qui permettrait réellement de déterminer l'importance de l'allélopathie pour l'organisme émetteur. Elle consiste en une comparaison du développement de l'organisme émetteur en culture avec la communauté « sensible » et avec la communauté « résistante ». Un développement plus rapide en culture avec la communauté sensible qu'avec la communauté « résistante » indiquerait que l'allélopathie est un facteur important du développement de l'organisme émetteur (hypothèse 1, figure 15). Un développement sensiblement égal signifierait que l'allélopathie n'a pas d'impact important rendant *U. confervicolum* meilleur compétiteur (hypothèse 2 figure 15). Un autre paramètre à mesurer serait la composition des différentes communautés afin de vérifier si la composition de la communauté « sensible » se rapproche de la composition de la communauté « résistante » après l'ajout de l'émetteur dans la communauté « sensible ». Si c'est le cas, cela montrerait que l'allélopathie peut être un facteur important dans la dynamique des communautés. L'une des difficultés sera de prendre en compte l'évolution des communautés liées seulement à la maturation du biofilm et non pas à un stress. Il faudrait également réussir à obtenir une communauté suffisamment contrastée pour avoir un effet assez marqué des composés allélopathiques et des communautés « sensibles » et « résistantes » réellement différentes.

#### **V.5. Autres questions liées à l'allélopathie dans les biofilms.**

La meilleure compréhension de l'effet de l'allélopathie sur les biofilms phototrophes pourrait permettre d'étudier les interactions entre l'allélopathie et d'autres facteurs, tels que la présence de polluants (herbicides, métaux). En effet, le stress induit par la présence de composés allélopathiques pourrait renforcer l'impact des polluants (Liess *et al.*, 2016; Zuo *et al.*, 2015). D'autre part, certaines caractéristiques permettant une résistance aux composés allélopathiques (par exemple un ratio

surface/volume réduit, d'après Lyczkowski and Karp-Boss, 2014) pourraient également conférer une plus grande résistance à certains polluants. Ainsi, les communautés les plus exposées à des composés allélopathiques pourraient être plus polluto-résistantes et l'effet de l'allélopathie sur la structure et le fonctionnement des biofilms réduit dans les zones les plus polluées. La démarche décrite précédemment pourrait être complétée avec des expositions à d'autres facteurs de stress afin de tester ces hypothèses.

Nous avons testé l'impact de facteurs environnementaux abiotiques sur la production et la libération de composés allélopathiques par d'*U. confervicolum*. Les facteurs biotiques pourraient aussi avoir un effet sur la production et la libération des composés allélopathiques, plusieurs hypothèses pourraient être testées :

- La présence de compétiteurs (*e.g.* des diatomées) pourrait, par l'intermédiaire de signaux chimiques, stimuler la production de composés allélopathiques.
- La présence de prédateurs pourrait entraîner une plus forte allocation des ressources vers des composés de défense au détriment de l'activité allélopathique
- La présence de macrofaune (consommant ou non *U. confervicolum*) pourrait entraîner par bioturbation une plus forte libération des composés allélopathiques dans le milieu.

L'interaction de l'allélopathie avec d'autres facteurs environnementaux biotiques serait à prendre en compte dans la transposition des résultats obtenus en conditions contrôlées vers des systèmes plus complexes.

## Conclusion

L'ensemble des travaux réalisés au cours de cette thèse représente une étude poussée d'une interaction très spécifique. Bien que les interactions dans un biofilm phototrophe complexe ne sauraient se résumer à l'interaction allélopathique entre deux espèces, cette approche est nécessaire dans un premier temps avant de pouvoir étudier l'ensemble du système. La mise en évidence de plusieurs composés allélopathiques différents impactant différentes fonctions chez les diatomées justifie cette approche. Cette étude a notamment permis de caractériser plusieurs composés allélopathiques, d'autres étant toujours en cours d'identification. Ces résultats rendent possible l'évaluation de l'importance de l'allélopathie par rapport à la compétition par interférence. Des marqueurs chimiques et moléculaires de la réponse des diatomées à l'exposition aux composés allélopathiques ont été identifiés. Tous ces marqueurs de « production » (acides linoléique et  $\alpha$ -linoléique, composés inhibant l'adhésion) et de « réponse » (carnitine, gènes spécifiques) pourront être utilisés pour étudier l'allélopathie dans un système plus complexe. L'efficacité des méthodes dites 'omiques' (métabolomique et transcriptomique) pour l'étude des interactions chimiques est mise en évidence dans cette thèse, notamment, ils permettent de mettre en évidence des effets des composés allélopathiques qui seraient passés inaperçus avec des méthodes classiques, mais qui peuvent prendre plus d'importance si les interactions et les facteurs de stress sont multiples.

Au delà des questions liées spécifiquement aux biofilms phototrophes, il s'avère que ces derniers pourraient être utilisés comme modèles pour l'étude générale du rôle de l'allélopathie et plus largement de la compétition par interférence. En effet, ils sont de relativement bons modèles expérimentaux et sont propices aux interactions chimiques.

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## **Annexe**



Allelopathic inhibition of primary producer growth and  
photosynthesis by aquatic fungi.

**Article soumis à *Fungal Ecology* en tant que 'short communication'**



**Allelopathic inhibition of primary producer growth and photosynthesis by aquatic fungi.**Joey L. Allen<sup>1</sup>, Joséphine Leflaive<sup>1</sup>, Charlotte Bringuier<sup>1</sup>, Loïc Ten-Hage<sup>1</sup>, Eric Chauvet<sup>1</sup>,Julien Cornut<sup>2</sup> and Michael Danger<sup>2</sup><sup>1</sup>ECOLAB, Université de Toulouse, CNRS, INPT, UPS, Toulouse, France<sup>2</sup>Laboratoire Interdisciplinaire des Environments Continentaux (LIEC), Université de Lorraine, CNRS, Metz, FranceAbstract

Autochthonous primary production is generally much reduced in forested headwater streams. Several hypotheses have been proposed for explaining this observation, among them, the low light intensity, or the strong constraints exerted by stream current. Allelopathic inhibition of competitors is a common ecological process in aquatic environments. Aquatic hyphomycetes are known to chemically inhibit bacteria and fungi (including other aquatic hyphomycetes) but a possible allelopathic effect of aquatic hyphomycetes on primary producers has never been tested. The inhibitory effect of twelve aquatic hyphomycete species was tested on three diatom species. Nine aquatic hyphomycete species exhibited anti-diatom activity. Up to 100% diatom growth inhibition was observed. Our study reveals that such allelopathic interactions might be common in streams and probably involve an array of fungal compounds. We propose that the generally reduced primary production observed in forested headwater streams is, among other factors, due to the inhibition of primary producers by allelopathic compounds released by aquatic hyphomycetes.

Introduction

Forested headwater streams are generally characterized by low primary production (Fisher and Likens, 1973; Vannote *et al.*, 1980). The low abundance of primary producers (PP) has most often been explained by shading from riparian trees (Hill *et al.*, 1995; Vannote *et al.*, 1980). However, in deciduous forest the incoming solar radiation intensity may be sufficient to sustain PP growth at least from autumn to spring, *i.e.* when leaves are shed. Alternative hypotheses for slow algal development, at least in some contexts, rely on stream water current (e.g. Peterson and Stevenson, 1989). Finally, PP may also be strongly nutrient-limited in these generally oligotrophic streams (Naiman *et al.*, 2000). More recently, competitive exclusion between heterotrophic microbial

decomposers and algae has also been proposed to explain the low abundance of PP in forested streams (Danger *et al.*, 2013). Yet, if bacterial decomposers exhibit higher nutrient uptake rates than most PP due to their smaller size (Currie and Kalff, 1984; Danger *et al.*, 2007), this discrepancy is less likely for fungal decomposers because their cells are larger. Indeed, aquatic hyphomycetes, which are among the most important microbial decomposers in headwater streams (Gessner *et al.*, 2007), develop hyphae that are generally larger than most diatom cells occurring in such aquatic ecosystems.

In this study, we propose a supplementary hypothesis that has, to our knowledge, never been tested, for explaining the low amount of primary producers in headwater streams: we hypothesized that, in certain circumstances, primary production could be altered by chemical compounds released from aquatic hyphomycetes. This process, known as allelopathy, is well documented among aquatic microorganisms (Allen *et al.*, 2016; Gross, 2003), but such interactions have never been investigated between aquatic hyphomycetes and PP.

### Material & methods

The aquatic hyphomycete species used in this study were *Alatospora acuminata* Ingold (ALAC), *Anguillospora crassa* Ingold (ANCR), *Anguillospora filiformis* Greathead (ANFI), *Arbusculina moniliformis* (Descals) Descals & Marvanová (ARMO), *Articulospora tetracladia* Ingold (ARTE), *Clavariopsis aquatica* de Wildeman (CLAQ), *Flagellospora curvula* Ingold (FLCU), *Heliscus lugdunensis* Saccardo & Thérry (HULU), *Tetrachaetum elegans* Ingold (THEL), *Tetracladium marchalianum* de Wildeman (TEMA), *Tricladium chaetocladium* Ingold (TRCH) and *Tricladium splendens* Ingold (TRSP) (See Supplementary Information). All strains were isolated from forested headwater streams in south-western France. For Test 1, aquatic hyphomycetes were grown on 2% malt extract agar in Petri dishes incubated in the dark at 15°C. For Tests 2 and 3, seven aquatic hyphomycete strains (ALAC, ANCR, ARMO, CLAQ, HULU, THEL and TRCH) were cultivated in 50 mL of the mineral medium described in Arce Funck *et al.* (2015) with five alternative sources of organic carbon: glucose (5 g.L<sup>-1</sup>), cotton-strips, or twelve leaf litter discs (ø 12 mm) of alder (*Alnus glutinosa* (L.) Gaertn.), maple (*Acer pseudoplatanus* L.) or oak (*Quercus robur* L.). To reduce the release of leachates which could also interact with diatom growth, leaf discs and cotton-strips were autoclaved in 40 mL of deionized water prior to being placed in sterilised culture medium.

The diatom strains used were *Fistulifera saprophila* (Lange-Bertalot & Bonik) Lange-Bertalot (isolated from biofilms in the river Garonne, south-western France), *Nitzschia palea* (Kützing) W. Smith

(purchased from the Canadian Phycological Culture Centre, Waterloo, Canada), and *Gomphonema parvulum* (Kützinger) Kützinger (purchased from the Sammlung von Algenkulturen, Göttingen, Germany). All diatoms were cultured in axenic conditions with COMBO medium (Kilham *et al.*, 1998) in a temperature-controlled chamber at 18°C with a 16:8 light:dark cycle and 30  $\mu\text{mol.m}^{-2}.\text{s}^{-1}$  light intensity. All experiments were carried out in these conditions.

Test 1: To determine whether aquatic hyphomycetes produce allelopathic compounds, 6 mm-diameter cores were cut in the active margin of each aquatic hyphomycete solid culture. Cores were placed in 90 mm-diameter Petri dishes on solidified COMBO medium (1% agar) on which diatoms ( $4 \times 10^6$  cells for *F. saprophila* and *G. parvulum* or  $2 \times 10^6$  cells for *N. palea*) were previously homogeneously spread. A sterile malt extract agar core (control) was placed on each Petri dish. The diatom photosynthetic efficiency of photosystem II (PSII yield), which is strongly linked to their physiological state (Baker, 2008), was measured with a Phyto-PAM fluorimeter (Walz, Effeltrich, Germany) next to each core after three days for *F. saprophila* and *G. parvulum* or five days for *N. palea*.

Test 2: To determine whether inhibitory compounds from leaf litter could be released due to its decomposition by aquatic hyphomycetes, liquid cultures of aquatic hyphomycetes were filtrated through sterile PolyEtherSulfone filters (0.22  $\mu\text{m}$  pore size, Nalgene, Rochester, USA). Diatoms were exposed to aquatic hyphomycete filtrates in 96-well plates. Each well was filled with 120  $\mu\text{L}$  of filtrate and 60  $\mu\text{L}$  of twice concentrated COMBO medium. Diatoms were inoculated at densities of either  $2 \times 10^5 \text{ cell.mL}^{-1}$  for *F. saprophila* and *G. parvulum* or  $1 \times 10^5 \text{ cell.mL}^{-1}$  for *N. palea*. After five days, optical density (OD, 680 nm, Asys UVM 340, Salzburg, Austria), which is related to diatom biomass, of diatom cultures exposed to the filtrates of the seven aquatic hyphomycetes and photosynthetic yield of diatoms exposed to four of them (i.e., ARMO, CLAQ, THEL and TRCH) were measured.

Test 3: To understand the differences between the results of Test 1 and 2, glucose-, cotton fabric- and alder-grown ARMO and TRCH filtrates were solidified by mixing rapidly 1 mL of filtrate with 0.5 mL of 2X COMBO medium containing 2% low melting point SeaPlaque agarose (Cambrex, Rockland, USA) at 35°C. Cores of solidified filtrates were used as cores of fungal colonies in Test 1. Only *F. saprophila* was used as target species.

All experiments were run in triplicate. The effect of each aquatic hyphomycete species was tested against control using Student *t*-test with log transformed data to fit normal distribution when necessary. A 2-way ANOVA (using carbon source and diatom species as factors) was used to determine the effect of leaf litter alone.

## Results

Test 1: Of the twelve aquatic hyphomycete species tested, eight had a significant effect, always negative, on PSII yield for at least one diatom species (fig. 1). One aquatic hyphomycete (ARMO) inhibited the three diatoms. ARMO had a strong effect on all diatoms (more than 65% of inhibition,  $p < 0.001$ ). Most of the aquatic hyphomycetes specifically inhibited one or two diatoms but not the others (fig. 1).

Test 2: The carbon source used in liquid culture alone (without aquatic hyphomycetes) had a significant effect ( $p < 0.001$ ) on the measured OD by diatoms. Indeed, it was higher with oak, maple and alder than with glucose (fig. 2, controls). The most active filtrates OD were those from TRCH and ANCR, while the filtrates with the lower inhibitory activity were produced by ALAC and ARMO. OD of the three diatoms tested was inhibited by more than 70% by all filtrate types from TRCH except that produced from cotton fabric. ANCR filtrates also had strong effects, principally on *G. parvulum* and *N. palea*. Some rare stimulatory effects were observed (fig. 2). The photosynthetic yield of all diatoms tested was significantly reduced by glucose-, alder-, maple- and oak-grown TRCH filtrates but not of cotton fabric-grown TRCH filtrate. Maple- and oak-grown CLAQ filtrates significantly inhibited *G. parvulum* photosynthesis but had no effect on other diatoms. ARMO and TEEL filtrates had no effect on diatom photosynthesis (fig. 3).

Test 3: ARMO and TRCH were used as they have the highest effect in Test 1 and 2 respectively. PSII yield of *F. saprophila* cultivated on solid medium and exposed to solidified aquatic hyphomycete filtrates was significantly inhibited by glucose-grown ARMO filtrate (fig. 4). ARMO filtrate cores had higher inhibitory activity than TRCH filtrate cores ( $p = 0.036$ ).

## Discussion

While several studies have reported inhibitory effects of aquatic hyphomycetes on bacteria and fungi (e.g. Gulis and Stephanovich, 1999; Sati and Arya, 2010) and chemical inhibition among aquatic hyphomycetes (Ferreira *et al.*, 2010; Shearer and Zare-Maivan, 1988; Treton *et al.*, 2004) or between aquatic hyphomycetes and co-occurring bacterial strains (Gulis and Suberkropp, 2003), the allelopathic effect of aquatic hyphomycetes on stream PP had never been studied to our knowledge. Our results show that most of the aquatic hyphomycete species tested (8 on 12 tested in solid cultures) expressed inhibitory activity against at least one diatom species. Moreover some aquatic hyphomycete species, ARMO and TRCH particularly, had a strong activity against all the diatoms tested in test 1 and 2, respectively. Considering these results, our hypothesis that allelopathic

interactions may be an important factor that limits PP in headwater streams seems to be supported. The inhibition of PP could help fungi in reducing the competition for limiting nutrients with PP in the generally oligotrophic forested headwater streams. However, we cannot exclude that the observed inhibition could also be due to parallel allelopathy (Sinkkonen, 2006), *i.e.* a side effect of allelochemicals produced to inhibit other organisms, *e.g.* other aquatic microbial decomposers.

The activity of the aquatic hyphomycetes tested varied among target diatom species. While ALAC and FLCU only inhibited *F. saprophila*, ANCR only inhibited the two other diatoms, suggesting the involvement of different compounds. Similarly, the inhibitory activity depended on whether diatoms were in liquid or solid cultures, but this varied among species. For instance, ARMO allelopathic compounds inhibited diatoms in solid cultures, when diatoms were exposed to solid culture cores or to solidified filtrate, while TRCH inhibited diatoms in liquid cultures, but solidified filtrate had no effect. This might be due to different chemical properties of compounds affecting their diffusion within the agar, suggesting that various compounds with distinct chemical properties are involved.

Leaf litter leachates, especially labile phenolic compounds, are considered as potential inhibitors of aquatic PP (Bährs *et al.*, 2012; Tsuda *et al.*, 2005). We hypothesized that additional inhibitory compounds originating from the leaf litter could also be released during the decomposition process ensured by aquatic hyphomycetes. To test this hypothesis, the leaf discs were initially autoclaved in deionized water to remove labile compounds. As a result, no inhibitory effect of leaf litter alone was observed, suggesting that most inhibitory compounds were lost with the leachates (Danger *et al.* 2013). The slight stimulation effect of leaf litter on algal growth may be due to a supply of growth factors by the leaf litter. No leaf litter effects were observed: glucose-grown aquatic hyphomycetes inhibited diatoms as much as leaf litter-grown ones. While fungal biomass was not estimated, the lower effect of cotton fabric-grown aquatic hyphomycetes is likely due to their lower and delayed growth on this substrate (Cornut *et al.*, submitted).

Our study highlights that aquatic hyphomycetes commonly produce diverse allelochemicals that inhibit PP. Aquatic hyphomycete and diatom species used are common in streams and tests performed were not likely to overestimate the effect of aquatic hyphomycetes therefore this inhibitory effect could occur in streams. Whether and in which proportion these compounds participate to reduce PP in forested headwater streams remains to be determined, but such a process certainly adds to the hypotheses currently proposed to explain the reduced development of algal biofilms in forested headwater streams.

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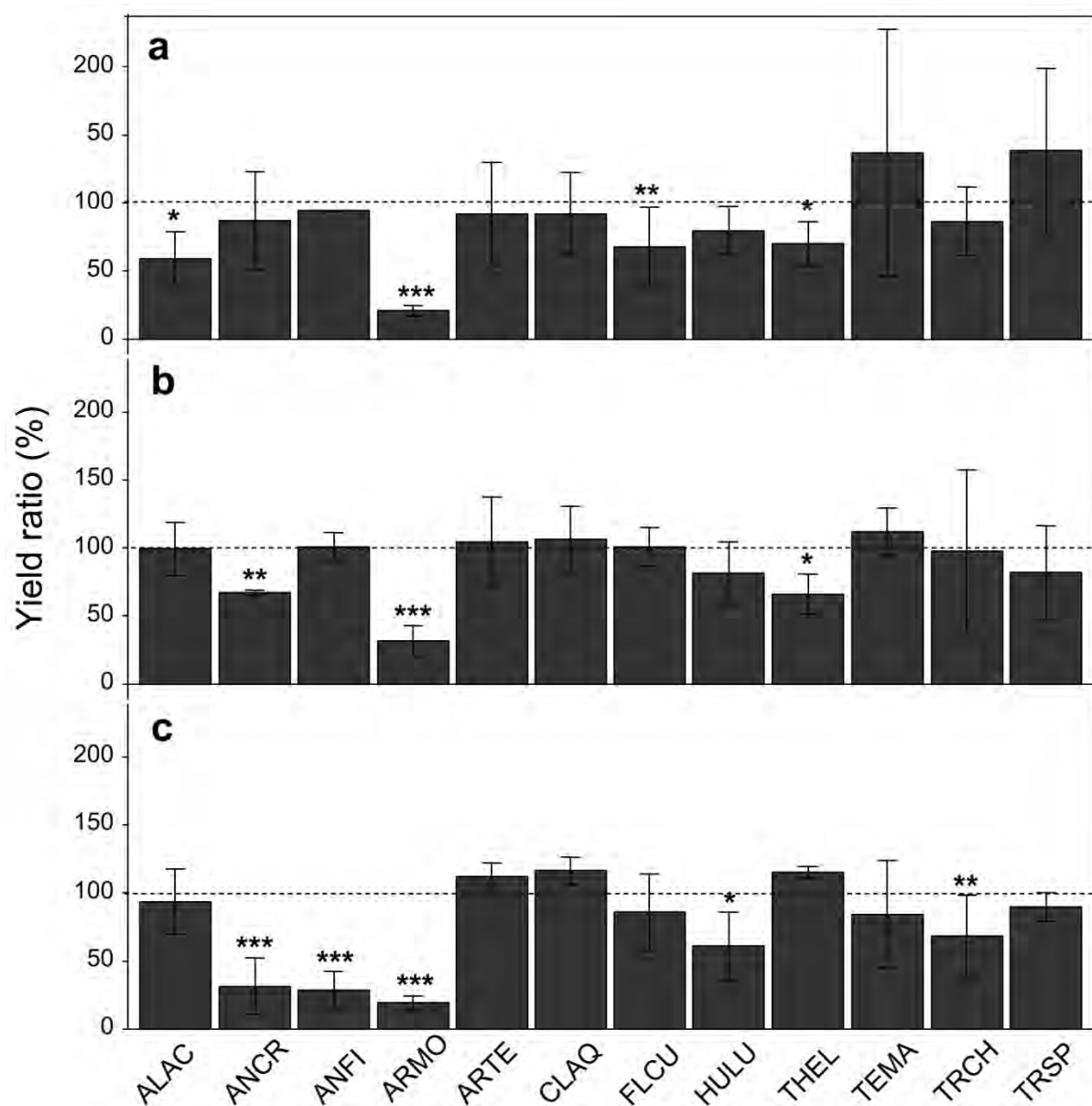


Fig 1. PS II yield of *F. saprophila* (a), *G. parvulum* (b) and *N. palea* (c) after exposure to twelve aquatic hyphomycete culture core, measured by PAM fluorimetry and expressed as % of PS II yield in control (Test 1). Mean  $\pm$  SE ( $n = 3$ ). Dashed line represents control (100%). Asterisk indicates significant difference with the control (\*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$ ).

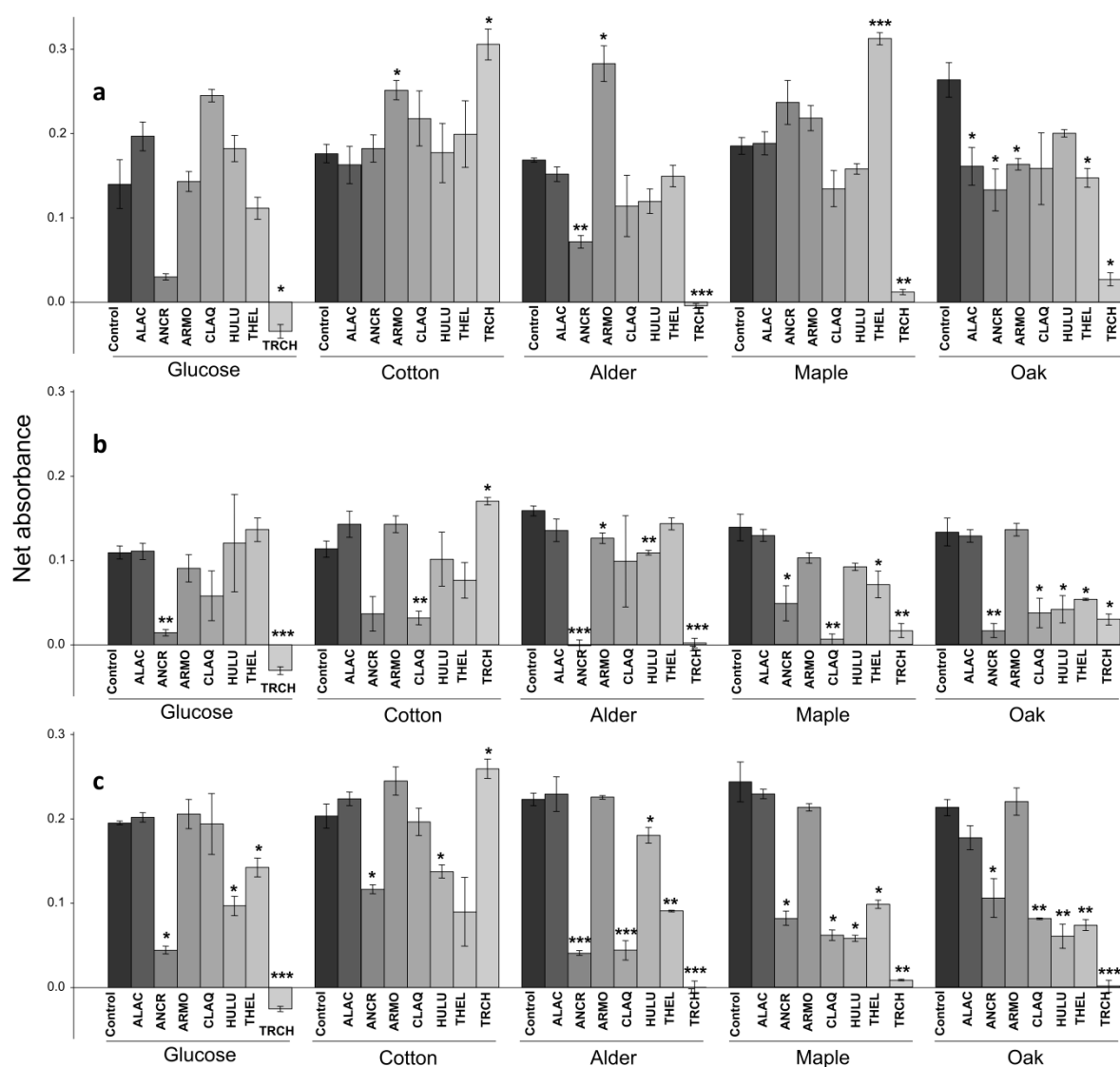


Fig. 2. Net absorbance value (680 nm) of *F. saprophila* (a), *G. parvulum* (b) and *N. palea* (c) after exposure to filtrates of seven aquatic hyphomycete cultures produced from five carbon sources (Test 2). Mean  $\pm$  SE ( $n = 3$ ). Asterisk indicates significant difference with the control (\*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$ ).

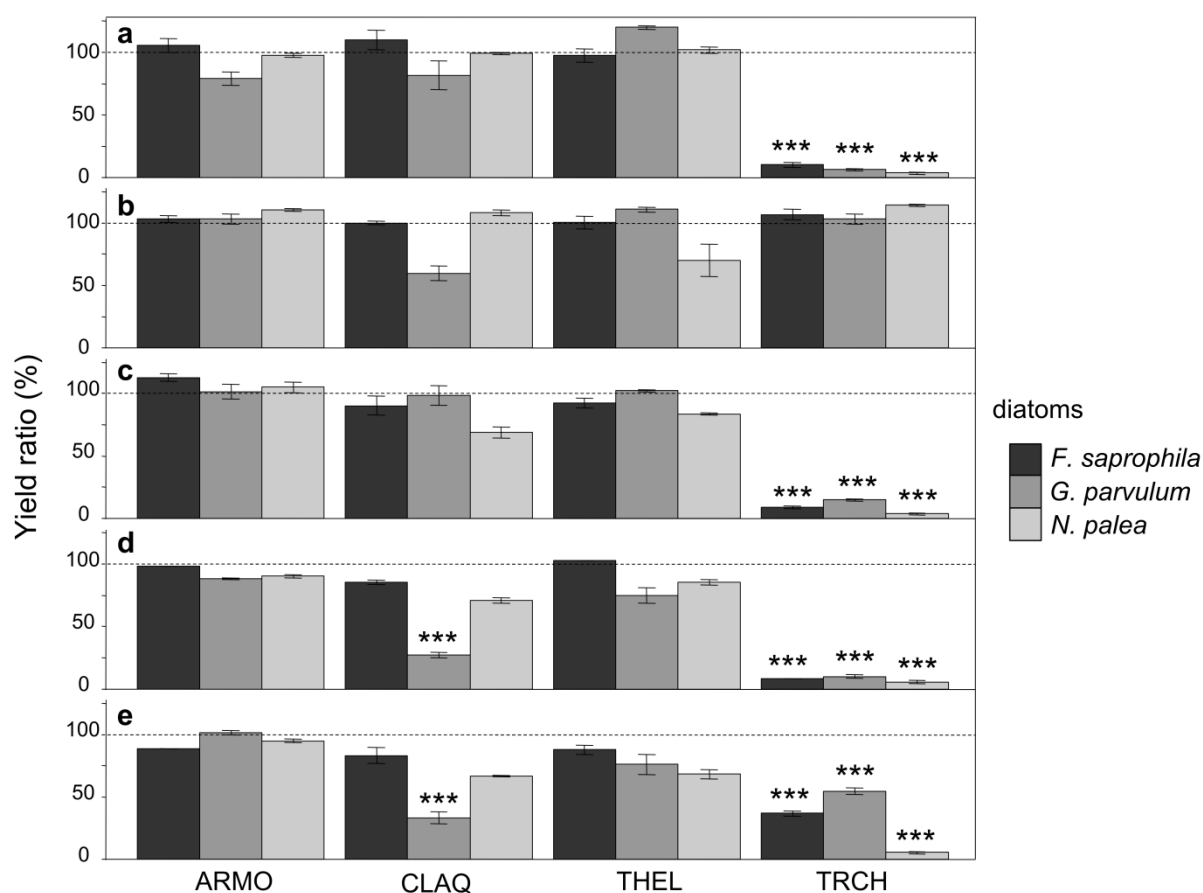


Fig 3. PS II yield of *F. saprophila* (black), *G. parvulum* (grey) and *N. palea* (light grey) measured by PAM fluorimetry and expressed as % of PS II yield in control, after exposure to filtrates of four aquatic hyphomycete cultures produced from five carbon sources (Test 2): Glucose (a), cotton fabric (b), and leaf litter of alder (c), maple (d) and oak (e). Dashed line represents control (100%). Mean  $\pm$  SE ( $n = 3$ ). Asterisk indicates significant difference with the control (\*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$ ).



Fig. 4. PS II yield of *F. saprophila* after exposure to solidified aquatic hyphomycete culture filtrate, measured by PAM fluorimetry and expressed as % of PS II yield in control (Test 3). Dashed line represents control (100%). Mean  $\pm$  SE ( $n = 3$ ). Asterisk indicates significant difference with the control (\*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$ ).

Table S1. List of the strains used.

| Species                                                               | Strain number                                       |
|-----------------------------------------------------------------------|-----------------------------------------------------|
| Aquatic hyphomycetes                                                  |                                                     |
| <i>Alatospora acuminata</i> Ingold (ALAC)                             | 180-1658                                            |
| <i>Anguillospora crassa</i> Ingold (ANCR)                             | 130-1659                                            |
| <i>Anguillospora filiformis</i> Greathead (ANFI)                      | 130-1650                                            |
| <i>Arbusculina moniliformis</i> (Descals) Descals & Marvanová (ARMO)  | 182-1692                                            |
| <i>Articulospora tetracladia</i> Ingold (ARTE)                        | 180-1647                                            |
| <i>Clavariopsis aquatica</i> de Wildeman (CLAQ),                      | 180-1651                                            |
| <i>Flagellospora curvula</i> Ingold (FLCU)                            | 180-1662                                            |
| <i>Heliscus lugdunensis</i> Saccardo & Thérny (HULU)                  | 180-1645                                            |
| <i>Tetrachaetum elegans</i> Ingold (THEL)                             | 180-1653                                            |
| <i>Tetracladium marchalianum</i> de Wildeman (TEMA)                   | 183-1670                                            |
| <i>Tricladium chaetocladium</i> Ingold (TRCH)                         | 180-1646                                            |
| <i>Tricladium splendens</i> Ingold (TRSP)                             | 180-1649                                            |
| Diatoms                                                               |                                                     |
| <i>Fistulifera saprophila</i> (Lange-Bertalot & Bonik) Lange-Bertalot | fis.sa 07g                                          |
| <i>Nitzschia palea</i> (Kützinger) W. Smith                           | CPCC-160 (Canadian<br>Phycological Culture Centre)  |
| <i>Gomphonema parvulum</i> (Kützinger) Kützinger                      | SAG1032-1 (Sammlung von<br>Algenkulturen Gottingen) |

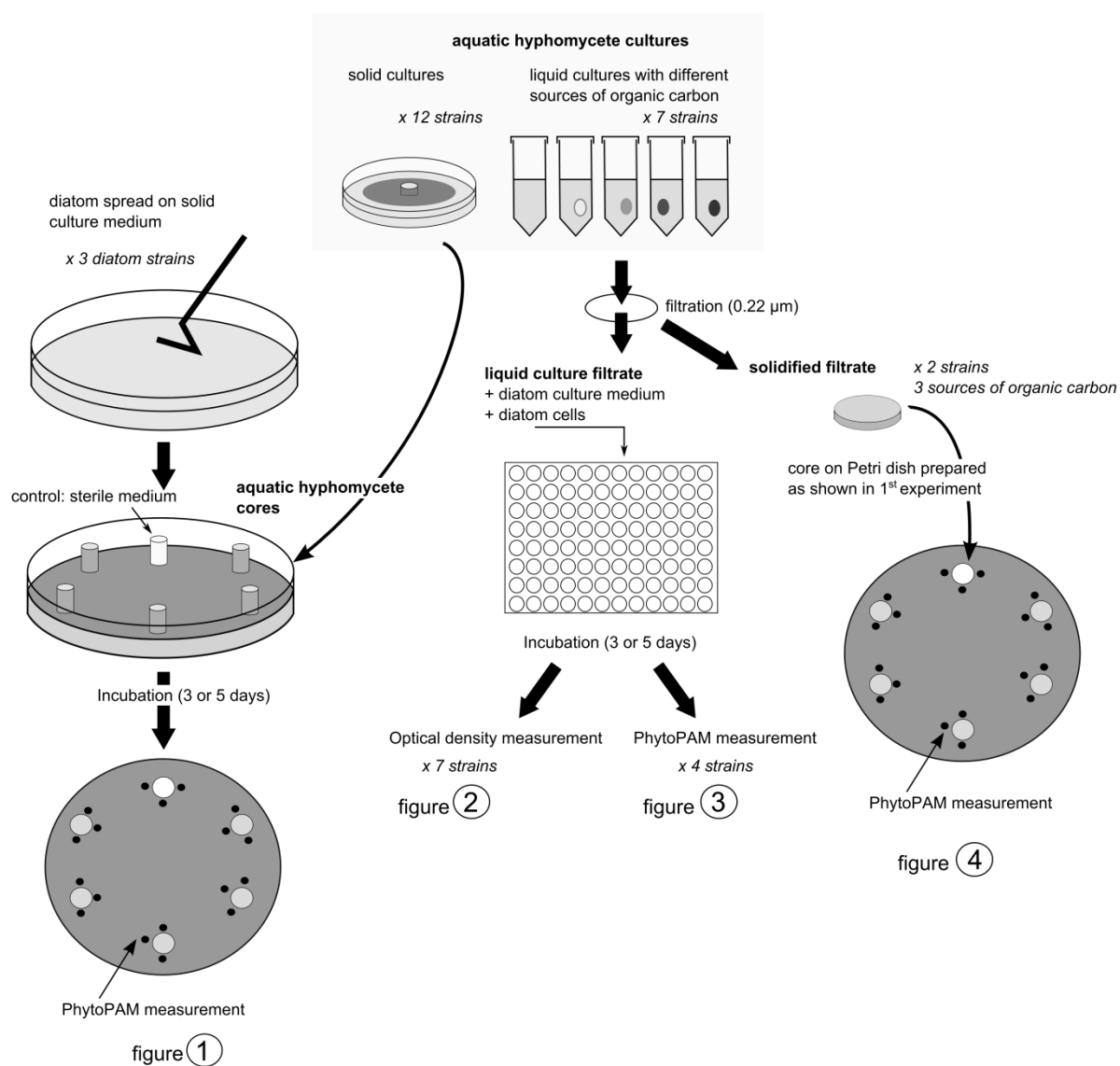


Figure S1. Schematic representation of the experimental procedures in Test 1 (left panel), test 2 (medium panel) and Test 3 (right panel)

Les interactions allélopathiques sont définies comme la libération par un organisme de composés chimiques inhibant les compétiteurs. Dans les milieux aquatiques, elles sont reconnues comme un facteur de la dynamique des communautés de phytoplancton. Malgré les conditions favorables créées par l'organisation des microorganismes en biofilms, l'allélopathie a été beaucoup moins étudiée dans les communautés de microorganismes phototrophes benthiques.

L'objectif de cette thèse était d'étudier des interactions allélopathiques, au travers d'un cas particulier, entre les microorganismes dans les biofilms phototrophes d'eau douce. Ainsi le travail s'est focalisé sur les effets allélopathiques d'une algue verte filamenteuse, *Uronema confervicolum* Lagerheim, sur des diatomées benthiques. L'hypothèse principale est que, dans certaines conditions, *U. confervicolum* libère suffisamment de composés allélopathiques pour inhiber significativement le développement des diatomées épiphytes. Cette hypothèse soulève trois questions (i) quels sont les composés allélopathiques, (ii) leur production dépend-elle des conditions environnementales et (iii) quel est l'effet des composés allélopathiques produits sur les diatomées ? Cette interaction a été étudiée au travers de multiples approches. L'activité d'extraits de biomasse algale et de filtrat de culture a été testée sur la croissance, la photosynthèse, l'adhésion et la motilité des diatomées. Les techniques de fractionnement guidé par bioessais et de profilage métabolomique ont été utilisées pour identifier les composés allélopathiques produits. Les effets des facteurs environnementaux (limitations en nutriment, apports de lumière et de gaz) et de la phase de croissance sur la production et la libération de différents composés allélopathiques ont également été étudiés. Une étude approfondie de l'effet sur les diatomées des composés allélopathiques anti-adhésion a été réalisée en combinant le profilage métabolomique, la transcriptomique et la microscopie électronique à balayage.

Les extraits d'*U. confervicolum* ont montré un effet négatif sur la croissance, la photosynthèse, l'adhésion et la motilité des diatomées. Deux acides gras polyinsaturés (acides linoléique et  $\alpha$ -linolénique) ont été identifiés comme des composés allélopathiques inhibant la croissance et la photosynthèse des diatomées. L'inhibition de l'adhésion des diatomées est, par contre, causée par d'autres composés allélopathiques non identifiés. L'augmentation de l'intensité lumineuse a accru la production de composés allélopathiques, à l'instar des composés défensifs des plantes. Cependant, aucune des théories classiques sur les défenses chimiques des plantes n'a permis d'expliquer l'ensemble des résultats obtenus sur les questions de régulation. En plus de l'inhibition de l'adhésion, le filtrat d'*U. confervicolum* inhibe la formation de la matrice constituée de substances polymériques extracellulaires, impacte le métabolisme énergétique et induit une modification globale du transcriptome et du métabolome des diatomées. Une partie des gènes et métabolites les plus fortement impliqués dans la réponse des diatomées à l'inhibition de l'adhésion ont été identifiés.

Ce travail a permis de mieux évaluer l'activité allélopathique de l'algue verte filamenteuse *U. confervicolum* et son impact potentiel sur la structure et le fonctionnement des biofilms phototrophes. D'autres approches seront nécessaires, à l'avenir, pour mieux comprendre le rôle global de l'allélopathie dans des biofilms phototrophes complexes.