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Allelochemical interactions between the dinoflagellate *Alexandrium minutum* and the diatom *Chaetoceros muelleri*

Marc Long

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AVEC L'UNIVERSITE DE WOLLONGONG

Par

Marc LONG

Allelochemical interactions between the dinoflagellate *Alexandrium minutum* and the diatom *Chaetoceros muelleri*

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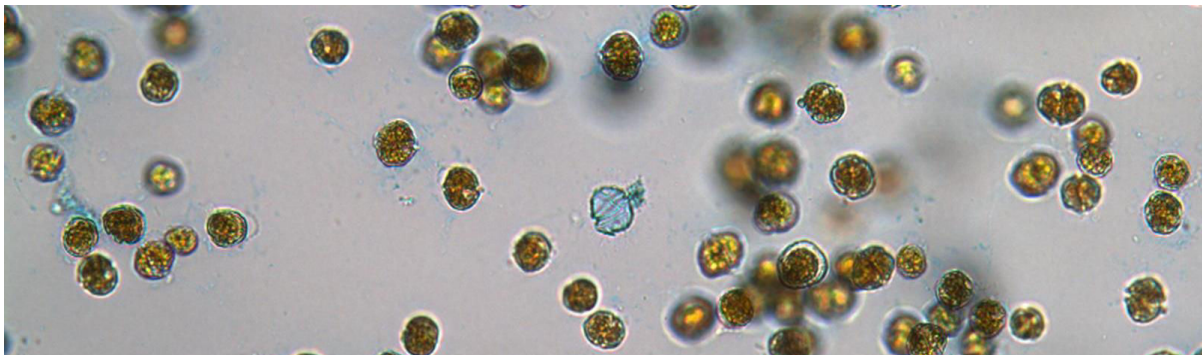
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Allelochemical interactions between the dinoflagellate *Alexandrium minutum* and the diatom *Chaetoceros muelleri*



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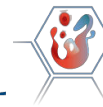
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« Quand on veut être sûr de son coup, Seigneur Dagonet...

On plante des navets.»

Loth (François Rollin) dans Kaamelott, Livre V, Les Repentants

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Table of contents

Abstract	3
Résumé	5
List of abbreviations	7
Chapter 1 - State of the art	9
1 Harmful algal blooms	10
1.1 Phytoplankton.....	10
1.2 Harmful algal blooms	10
1.3 Increase of HABs events	12
1.4 Factors structuring the dynamic of phytoplankton and HABs	14
2 Allelochemical interactions	15
2.1 Chemical ecology	15
2.2 Definition of allelopathy.....	15
2.3 Allelochemical interactions in marine environment.....	16
2.4 Ecological significance.....	17
2.5 Environmental control of allelochemical interactions.....	18
2.6 Microalgae producing allelochemicals	19
3 The genus <i>Alexandrium</i>	20
3.1 Classification	20
3.2 Life cycle	20
3.3 Biology	21
3.4 Toxin production	22
3.5 <i>Alexandrium</i> spp. worldwide geographical distribution.....	22
3.6 <i>Alexandrium</i> spp. outbreaks in Australia.....	23
3.7 <i>Alexandrium</i> spp. outbreaks in France	25
3.8 Environmental control of <i>Alexandrium</i> spp. blooms.....	27
4 <i>Alexandrium</i> spp. allelochemical potency	28
4.1 Allelochemical potency within the genus <i>Alexandrium</i>	28
4.2 Effects of allelochemicals on protists and microbial communities	29
4.3 Allelochemicals mode of action	30
4.4 Effects of allelochemicals on biological membranes	32
4.5 Environmental regulation of <i>Alexandrium</i> spp. allelochemical potency.....	33
4.6 Chemical characterization of <i>Alexandrium</i> spp. allelochemicals	34
4.7 <i>Alexandrium</i> spp. exudates.....	36
5 Biological activities of <i>Alexandrium</i> spp. exudates.....	37
5.1 Hemolytic and cytotoxic activities	37
5.2 Toxicity to bivalves	37
5.3 Toxicity to fish	38
6 Chemical nature of <i>Alexandrium</i> spp. exudates	40
6.1 Characterization of <i>Alexandrium</i> spp. exudates	40

Table of contents

6.2 Characterization of hemolytic and cytotoxic compounds	41
6.3 Characterization of ichthyotoxic compounds	43
Objectives and organization of the thesis	44
Chapter 2 : Unraveling the mechanisms of allelochemical interactions	47
Article 1 – Allelochemicals from <i>Alexandrium minutum</i> induce rapid inhibition of metabolism and modify the membranes from <i>Chaetoceros muelleri</i>	48
Article 2 – Allelochemical interactions between <i>Alexandrium minutum</i> and <i>Chaetoceros muelleri</i> : insights into photosynthesis and membrane integrity	80
Chapter 3 : Development of a bioassay to quantify allelochemical interactions from <i>Alexandrium minutum</i>	103
Article 3 – A rapid quantitative fluorescence-based bioassay to study allelochemical interactions from <i>Alexandrium minutum</i>	104
Chapter 4 : Effects of abiotic factors on allelochemical potency	129
Article 4 – A multi-trait approach reveals the effects of Cu on the physiology of an allelochemical-producing strain of <i>Alexandrium minutum</i>	130
Chapter 5 : What is the chemical nature of <i>Alexandrium minutum</i> allelochemicals ?	163
Chapter 6 – General discussion	191
1 Chemical nature of allelochemicals	192
2 Allelochemicals mode of action	194
3 Interactions between allelochemicals and cytoplasmic membranes	195
4 Interactions between allelochemicals and photosynthetic membranes	197
5 Understanding the mode of action to explain the variability in the sensitivity to allelochemicals	199
6 Variability of allelochemical potency from <i>Alexandrium</i> strains	202
7 The relevance of laboratory studies in an environmental context	203
8 Beyond allelochemical interactions	204
Conclusions and perspectives	207
PhD Outcomes	209
References (Introduction and General discussion)	213
Annexe 1 – Article 1	
Annexe 2 – Article 3	

Abstract

Understanding the factors that favor the establishment and the persistence of harmful algal blooms (HABs) is essential for predicting them, and subsequently mitigating their negative consequences on the marine environment, human health and the economy. Allelochemical potency, i.e. the release of compounds that inhibit competitors, is hypothesized to favor the organisms that produce them and indeed shapes plankton community. The toxic dinoflagellates from the genus *Alexandrium* produce allelochemicals, however, associated interactions are poorly understood. The objective of this thesis is to contribute to our understanding by studying the mechanisms behind the allelochemical interactions between the dinoflagellate *A. minutum* and the diatom *Chaetoceros muelleri*. The results of this work have highlighted that *A. minutum* allelochemicals disrupted membrane functioning of the diatoms within minutes. The cascade of physiological events following the allelochemical interaction that was described in this PhD included permeabilisation of cell membranes, inhibition of photosynthesis, production of reactive oxygen species and modifications of the biochemical composition of membranes. Additionally, this project has highlighted that the allelochemical potency of *A. minutum* can be significantly modulated by environmental parameters, as allelochemical potency of *A. minutum* increased 4-5 times when exposed to toxic concentrations of Cu. Finally, a bioassay developed during this PhD eased the partial isolation of the allelochemicals through guided-fractionation. Although we did not totally purify the allelochemical(s) of *A. minutum*, we isolated a group of hydrophobic candidates whose activity has to be further investigated following their isolation. These results provide a better understanding of the physiological and cellular mechanisms underlying these allelochemical interactions. This PhD clearly highlighted the complexity of allelochemical interactions, but also offers new tools and raises new perspectives to study the ecological consequences of these interactions on marine ecosystems.

Résumé

Comprendre les facteurs favorables à l'établissement et au maintien de proliférations d'algues toxiques et nuisibles est essentiel afin de prévoir et potentiellement atténuer leurs conséquences négatives sur l'environnement marin, la santé humaine ou encore l'économie. L'allélopathie, c'est-à-dire la libération de composés qui inhibent les compétiteurs, favorise l'espèce allélopathique et contribue ainsi à modifier la communauté planctonique. Les dinoflagellés toxiques du genre *Alexandrium* produisent des composés allélopathiques, cependant les interactions allélopathiques sont encore mal comprises. L'objectif de cette thèse était de contribuer à la compréhension des mécanismes d'allélopathie en étudiant l'interaction allélopathique entre le dinoflagellé toxique *Alexandrium minutum* et la diatomée *Chaetoceros muelleri*. Les résultats de ces travaux ont mis en évidence que les composés allélopathiques d'*A. minutum* perturbaient le fonctionnement des membranes des diatomées en quelques minutes. La cascade d'événements physiologiques suivant l'interaction allélopathique décrite dans cette thèse comprend différents effets tels que la perméabilisation des membranes cellulaires, l'inhibition de la photosynthèse, la production d'espèces réactives de l'oxygène et la modification de la composition biochimique des membranes. De plus, ce projet a mis en évidence le fait que l'allélopathie d'*A. minutum* est sous contrôle environnemental, son activité s'est accrue d'un facteur 4 en présence de concentrations toxiques de Cu. Enfin, un bioessai développée au cours de cette thèse a facilité la purification des composés allélopathiques d'*A. minutum* par fractionnement guidé. Bien que nous n'ayons pas totalement purifié le (ou les) composé(s) allélopathique(s), nous avons isolé un groupe de candidats hydrophobes dont l'activité doit être testée après leur isolement. Ces résultats permettent de mieux comprendre les mécanismes physiologiques à la base des interactions allélopathiques, mais révèlent aussi la complexité des interactions allélopathiques. Cette thèse propose également de nouveaux outils et ouvre de nouvelles perspectives pour étudier les conséquences écologiques de ces interactions dans les écosystèmes marins.

List of abbreviations

- AcN** : Acetonitrile
AOM : Algal Organic Matter
- cDOC** : Chromophoric Dissolved Organic Carbon
Cyt b6f : Cytochrome b6f
- DAD** : Diode Array Detector
DCFH-DA : 2',7'-dichlorofluoresceindiacetate (proxy or reactive oxygen species)
DHA : Docosahexaenoic Acid
DiBAC₄(3) : Bis-(1,3-Dibutylbarbituric Acid) Trimethine Oxonol (Proxy of depolarization of cytoplasmic membranes)
DOC : Dissolved Organic Carbon
- EC_{xx}** : Effective Concentration inhibiting xx % of Fv/Fm
ECS : Electro-Chromic Shift
ELSD : Evaporative Light-Scattering Detector
- FA** : Fatty Acids
FDA : Fluorescein Diacetate (Proxy of esterase activity)
FEEM : Fluorescence Excitation-Emission Matrix
FFA : Free Fatty Acids
FSC : Forward Scatter
FTIR : Fourier Transform Infrared Spectroscopy
Fv/Fm : Maximum photosystem II quantum yield
- HAB** : Harmful Algal Bloom
HPLC : High Performance Liquid Chromatography
HPTLC : High-Performance Thin Layer Chromatography
- LC** : Liquid Chromatography
LOEC : lowest-observable-effect concentration
- MeOH** : Methanol
MGDG : Monogalactosyl-diacylglycerol
MS : Mass Spectrometry
- NFA** : Neutral Fatty Acids
NMR : Nuclear Magnetic Resonance
NPQ : Non-Photochemical Quenching
- PAM** : Pulse Amplitude Modulation
PC : Phosphatidylcholine
PFA : Polar Fatty Acids
PK : Polyketide
PQ : Pool of plastoquinones
PSI : Photosystem I
PSII : Photosystem II
PSP : Paralytic Shellfish Poisoning
PST : Paralytic Shellfish Toxin
PUFA : polyunsaturated fatty acids
- Q_B** : Quinone B
- rETR_(PSI or PSII)** : relative electron transport through PSI or PSII
RES : Reactive Electrophile Species
ROS : Reactive Oxygen Species
- SP** : Semi-Preparative
SPE : Solid Phase Extraction
SSC : Side Scatter
STX : Saxitoxins
- TEPs** : Transparent Exopolymer Particles
- Y_{NA}** : Quantum yield of photosystem I on acceptor side
Y_{ND} : Quantum yield of photosystem I on donor side
- Φ_{PSII}** : Quantum yield of photosystem II
Φ_{PSI} : Quantum yield of photosystem I

Chapter 1

State of the art

1 Harmful algal blooms

1.1 Phytoplankton

The word phytoplankton is derived from the greek words “*phuton*” (plant) and “*plangtos*” (drifting) and refers to a heterogeneous group of microscopic photosynthesizing organisms. Phytoplankton (also named as microalgae), like the terrestrial plant, converts sunlight energy into biochemical energy under the process of photosynthesis. Through this process, phytoplankton consumes atmospheric CO₂ and releases O₂. Phytoplankton is referred to as the second lung of the planet, as it produces half of the dioxygen by photosynthesis on earth. Phytoplankton also has an important role in regulating the earth’s climate *via* notably the biological carbon pump (Barton et al., 2013; Carradec et al., 2018). In marine environments, phytoplankton plays a crucial role as it represents the primary producers in the oceans and thus supports the marine food web. However when phytoplankton causes harmful algal blooms (HABs), they can have deleterious effects on the environment.

1.2 Harmful algal blooms

Under certain environmental conditions, phytoplankton can reach high cell densities and sometimes induce discoloration of water bodies. In the case of dinoflagellate blooms, the water becomes brownish/red, thus forming the so called “red tides”. The first red tides events are reported from the bible where the river turned to blood and the fishes that were in the river died; and the river stank, and the Egyptians could not drink of the water of the river (Exodus 7:20-1). The first scientific report of a red tide was done by Darwin in Chile on board of HMS Beagle, the naturalist made some microscopic observations of the discolored waters.

Proliferation of microalgae can severely affect the environment, inducing deleterious effects, they are then called Harmful Algal Blooms (HAB). Harmful effects are broad and include negative aesthetic effects such as discoloration of water (Figure 1) or foam, clogging of desalination systems, oxygen depletion, tissues alterations in aquatic organisms, loss of fisheries stocks or poisoning leading to fisheries closures. HAB can thus have deleterious effects on public health or cause economic losses. Each year, 60 000 humans are intoxicated through ingestion of phycotoxins with an average mortality of 1.5 % (Ajani et al., 2017) and annual economic losses are estimated at \$804 million for Europe and \$79 million for United States (US \$) (Hoagland and Scatasta, 2006).



Figure 1: Water discoloration during 2012 red tide in Wollongong, Australia.
<http://www.illawarramercury.com.au/story/3962154/15-times-armageddon-came-to-wollongong-photos/>

HABs affect the marine environment either by inducing oxygen depletion or *via* the production of diverse phycotoxins. Non-producing toxic microalgae can induce anoxia during senescence of dense algal blooms. Thus algal respiration and bacterial degradation of algal organic matter are responsible for the low oxygen concentrations. Anoxia of waters can finally induce mortality of fish and benthic animals (Granéli et al., 2008). The effect of toxins effects are usually reported for higher trophic level organisms that consume the toxins directly or indirectly. For instance, toxin producing HABs can be toxic through grazing and accumulation by filter feeders (Granéli and Turner, 2007) or through consumption of organisms that have bioaccumulated the toxin (e.g. secondary consumers, predators). Dense blooms are not necessarily needed to induce poisoning (Maso and Garcés, 2006) as they can accumulate through filter feeders and bioaccumulation. However, the most studied toxins are those that can harm humans through the consumption of contaminated organisms, and are classified according to the toxicity induced (e.g. paralytic shellfish toxins (PST), diarrhetic shellfish toxins (DST), amnesic shellfish toxins (DST), neurotoxic

shellfish toxins (NST), ciguatera toxins). In the most severe cases, human intoxication can lead to death.

1.3 Increase of HABs events

The occurrences of HABs are increasing worldwide (Figure 2), and there are many hypotheses to explain this. First of all, the apparent global increase goes on with a public awareness as well as an increase in scientific interest (Hallegraeff, 2004). The increase of human activities in coastal regions requires the monitoring of HABs that increase detection of harmful species (Maso and Garcés, 2006). Moreover the increase of geographical distribution for some species can be explained by introduction from ballast waters or aquaculture practices. This is particularly relevant for the dinoflagellate that can make resistant cysts. Ballast waters are a major vector for global dispersion of phytoplankton (Hallegraeff and Gollasch, 2006), for instance it has been shown with molecular tools that *A. tamarense* has been introduced in Australia by cargo shipping from south-east Asia (Bolch and de Salas, 2007; de Salas et al., 2000). Aquaculture and especially relocation of shellfish stocks may also be a vector. Indeed some toxic algal cells can survive bivalve ingestion (sometimes through encystment) and cells can regrow from feces (Hégaret et al., 2008). Plastic debris can be another vector for phytoplankton and HAB species (Masó et al., 2003; Zettler et al., 2013). A strong correlation has been observed between nitrogen input and HABs in estuarine and marine waters (Anderson et al., 2002), eutrophication is suggested to favor the growth of some HAB species *via* the changes in nutrient ratios (N/P). The role of eutrophication may be even greater for HABs producing allelochemicals, as it can increase their allelochemical production to outcompete other species and form monospecific blooms (Granéli et al., 2008). Thus, the accelerated eutrophication of the coastal environment is a factor that may contribute to the increase in HAB outbreaks. HAB may be stimulated locally under unusual climatic conditions (Clement et al., 2016) linked with global change. Temperature is one of the main parameters that drive phytoplankton growth, as variations of temperature in surface waters influences the structure of phytoplankton communities (Wells et al., 2015). In 2015 and 2016, Chile faced exceptional red tides leading to tonnes of dead shellfish (Figure 3) and salmon (León-muñoz et al., 2018; Montes et al., 2018) along with beaching of whales and sea lions (Häussermann et al., 2017; Pedersen, 2016). Unprecedented climate anomalies (high water surface temperatures) are suspected to be a key factor that enhanced the red tides (Agulhas, 2004; Clement et al., 2016). Other environmental parameters are also likely to influence HAB dynamics; a recent study (Wells et al., 2015) raised

the concern about better investigating the environmental parameters (e.g. metals) that drive HAB blooms and cell toxicity.

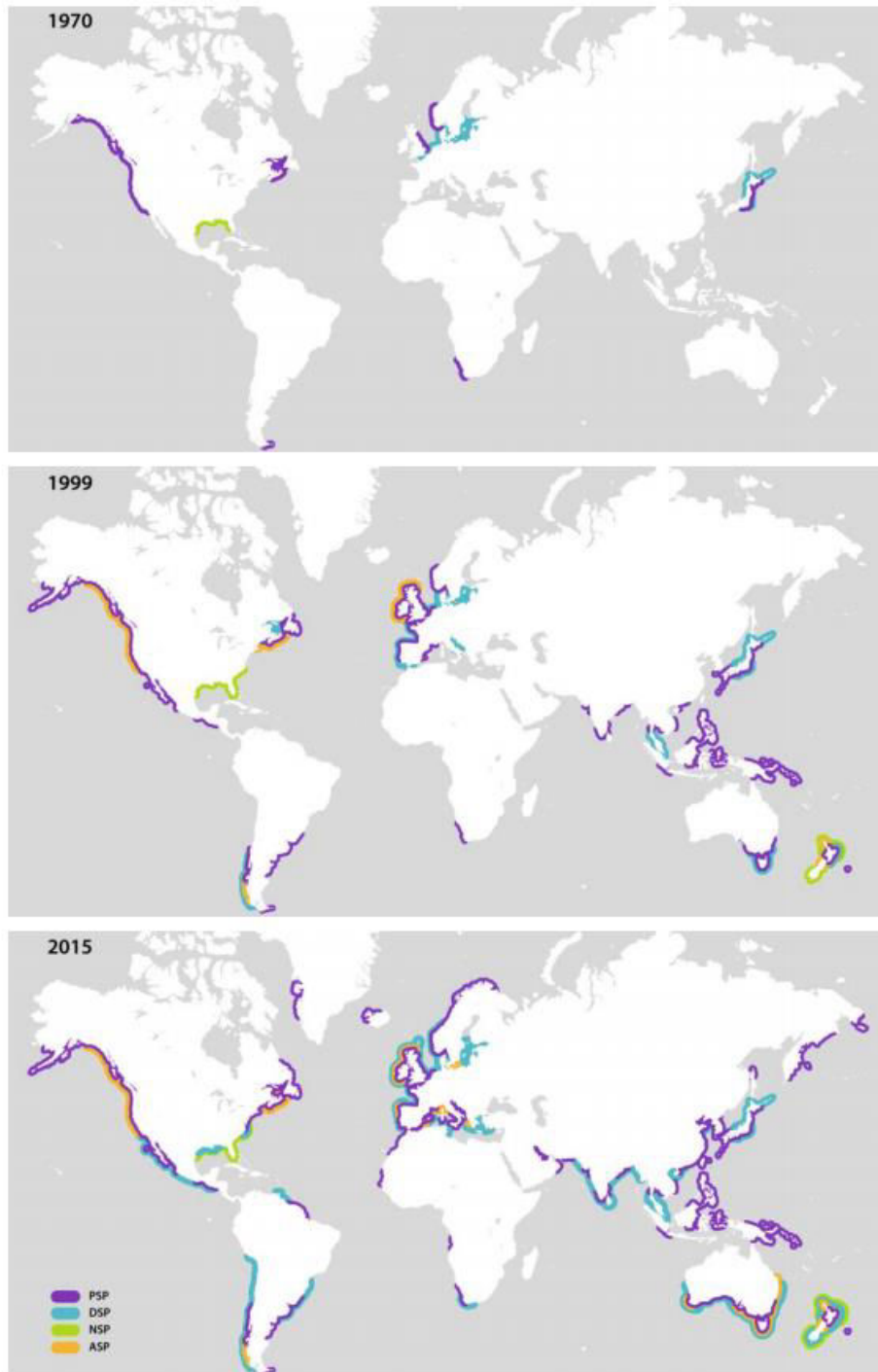


Figure 2: Worldwide increase in number of toxic outbreaks linked with phycotoxins. Lines represent coastal area where toxins were detected at significant levels. PSP: paralytic shellfish poisoning, DSP: diarrhetic shellfish poisoning, NSP: neurotoxic shellfish poisoning, ASP: amnesic shellfish poisoning. From (Bouillot, 2017).



Figure 3: Thousands of clams beached in southern Chile where fish and shellfish have been poisoned by the 2016 red tide. Photograph: Alvaro Vidal/AFP/Getty Images

1.4 Factors structuring the dynamic of phytoplankton and HAB

HAB and more largely plankton communities, are shaped by biotic and abiotic factors. Within the abiotic factors, physical (e.g. temperature, light, stratification, water motion, turbulences, transport) and chemical (e.g. salinity, nutrient, metals) processes appear to be particularly important in the control of algal blooms (Figueiras et al., 2006; Guallar et al., 2017). For instance, retention (i.e. low hydrodynamic) of water is often considered to support blooms of HAB. Another important abiotic factor controlling HAB is the nutrient composition of seawater (Anderson et al., 2002). Nutrient (relative abundances and type of nutrient) will affect the species composition through phytoplankton species preferences. While there seems to be evidence of HAB stimulation by nutrient enrichment, not all organisms respond to this stimuli (Anderson et al., 2002). For instance, some genus like *Alexandrium* seem quite unaffected by nutrient enrichment (Anderson et al., 2012). While the role of abiotic factors in structuring communities has been extensively studied, they only partially explain the protist community composition. In some cases, physico-chemical parameters explain only 30 % of the community variation (Berdjeb et al., 2018) indicating that other factors influence community structure. The authors of this study (Lima-Mendez et al., 2015) have pointed out that the plankton interactome (interactions between organisms) is a thriving force structuring communities. The interactome is shaped with various relationships (e.g. parasitism, symbiosis, competition, predation), and some of interactions are mediated through chemical signaling (i.e.

chemical ecology). Further understanding of the largely unknown chemically mediated interactions between species will help predicting bloom dynamic.

2 Allelochemical interactions

2.1 Chemical ecology

In his article Pohnert (2010) depicts a noisy version of the oceans in contrast to the widespread picture of “silent oceans” often described in the literature. In fact, the author talks about the chemical noise of marine organisms that comes from the release of numerous chemicals into the marine environment. Some of these chemicals are released by organisms as signals/cues to mediate interactions between organisms. The study of chemically mediated interactions forms the field of chemical ecology (Cembella, 2003; Ianora et al., 2006). Marine plankton, including protists, release a wide range of signaling molecules in their surrounding environment (Cembella, 2003; Ianora et al., 2011). These chemical signals control the coexistence and coevolution of species according to their various functions (Cembella, 2003). Chemical signals affect many processes such as sexual responses (i.e. pheromones) (Gillard et al., 2013), resource search (Bondoc et al., 2016; Steinke et al., 2006), cell death (Gallo et al., 2017), grazer-prey interactions (Amato et al., 2018). Some signals can even change the toxic potency of a toxic algae (Selander et al., 2006). The idea that chemical interactions are a driving force shaping plankton communities is emerging (Amato et al., 2018; Amin et al., 2015; Wohlrab et al., 2016). The plankton “chemical language” is, however, largely unspoken as only a few compounds have so far been identified. While chemical ecology is a large discipline, in this project we will focus on allelochemical interactions.

2.2 Definition of allelopathy

Allelopathy was first described in terrestrial environments and these studies mainly referred to negative effects. Three criteria must meet be met to be classified as allelopathy: “(1) the putative aggressor plant or its debris must produce and release chemicals that will be capable of inhibiting growth or any physiological/biological function of other plants when released into the environment, (2) distribution and accumulation of organic complexes [...] must be of sufficient concentration to inhibit nutrient and/or water uptake by roots and /or energy fixation by the sensitive plant, (3) the observed field patterns of plant inhibition cannot be explained solely by physical or other biotic factors” (Blum, 1999; Duke, 2003). Allelopathy as defined by the International Allelopathy Society refers to “the impact of plants upon neighboring plants and/or their associated microflora and/or

macrofauna by the production of allelochemicals; often these allelochemicals typically interfere with plant growth but they may also result in stimulation of growth. The field of allelopathy addresses research on the allelochemicals regulating these interactions, as well as the organisms (including microbes and plants) producing these chemicals, or those directly or indirectly impacted by these associations” (International Allelopathy Society).

While these definitions first applied to terrestrial environments, they are now broadened to the aquatic environments. In contrast to terrestrial environments, differentiation between phototrophic and heterotrophic organisms is not as clear in marine environments (Caron, 2016). Many planktonic organisms can be mixotrophs, meaning that they can be both photosynthetic and heterotroph. Consequently, allelopathy in marine environment cannot simply be defined as interactions between plants. Starting from this definition, some authors only include phytoplankton-phytoplankton interactions (Chan et al., 1980; Gross et al., 2012; Legrand et al., 2003) while other authors broaden the interactions to protists in general and to bacteria, grazers or any organisms competing with protists (Fistarol et al., 2004; Granéli et al., 2006; Poulin et al., 2018; Tillmann et al., 2007). Some authors include positive and negative effects (Cembella, 2003; Eick, 2017; Møgelhøj et al., 2006; Paul et al., 2009), while others only include the negative effects in their definition (Granéli et al., 2006; Legrand et al., 2003; Lundholm et al., 2005; Prince et al., 2008; Ternon et al., 2018). Overall, there is a clear lack of consensus on the strict definition of allelopathy in the marine environment, even the terminology for “allelopathy” in marine environment is varying. Depending on the study, authors prefer to talk about “allelopathy” and “allelopathic interactions” (Kubaneck et al., 2005; Legrand et al., 2003; Poulin et al., 2018; Roy, 2009; Schmidt and Hansen, 2001; Ternon et al., 2018; Vardi et al., 2002), and about “allelochemical interactions” (e.g. Gross et al., 2012; Tillmann et al., 2008), or they use both terminologies. In this project we define allelochemical interactions as the negative effects of a protist on competing protists, bacteria or predators through the release of chemicals, named allelochemicals, in their surrounding environment.

2.3 Allelochemical interactions in marine environment

Allelochemical potency occurs in various microalgal groups including cyanobacteria, dinoflagellates, prymnesiophytes, rapidophytes, or diatoms (Legrand et al., 2003). Concordantly with species diversity, allelochemical diversity is broad (e.g. fatty acids, extracellular peptides, proteinaceous compounds, glycolipids, oxylipins, alkaloids; (Granéli and Turner, 2007; Legrand

et al., 2003)). Most of marine allelochemicals are chemically uncharacterized due to the difficulties of extracting significant amounts of these compounds, and purifying them in complex matrices. Mixture of chemicals released by a mono-culture are usually really complex (Pohnert, 2010) and thus difficult to purify/characterize. One way of characterizing allelochemicals is the description of their basic features: target spectra (organisms affected), activity spectra (physiological functions inhibited), polarity of the compounds, molecular size range, lability, etc.

Allelochemical are usually released in the surrounding environment and become toxic when they come into contact with the target cells. There is evidences of another mode of action, some allelochemical species are toxic through a cell-cell contact (Ternon et al., 2018; Uchida, 2001; Zheng et al., 2016) and may not require the release of allelochemicals. Allelochemicals affect target cells by inducing many different effects such as photosynthesis inhibition (Lelong et al., 2011; Poulson-Ellestad et al., 2014), decrease in swimming velocity (Lim et al., 2014) or immobilization (Tillmann et al., 2007), encystment (Tillmann et al., 2007), grazing inhibition (Kamiyama, 1997), growth inhibition (Arzul et al., 1999; Lim et al., 2014), decreased nutrient uptake (Lyczkowski and Karp-Boss, 2014), compromised membrane integrity (Poulin et al., 2018; Prince et al., 2008), inducing cell lysis (Ma et al., 2011a; Tillmann and John, 2002) and induction of programmed cell death (Vardi et al., 2002). However the precise mode of action is usually unknown. There is a clear lack of knowledge and a need for better characterization of allelochemicals nature and mode of action. Many allelochemicals may have a broader activity spectra and may exhibit other biological activities than strict allelochemical interactions.

2.4 Ecological significance

Allelochemical activity has been defined as an adaptation by which some phytoplankton species could achieve a competitive advantage over other species (Legrand et al., 2003). While bloom succession is modulated by abiotic factors they can also be mediated by biotic factors including allelochemical interactions. Killing other algal cells or inhibiting other algal growth will decrease competition for resources (e.g. nutrients, light, vitamins, trace metals). The bloom dynamic model of species competing for nutrient (Grover and Wang, 2013) suggested that under rich nutrient condition, the species producing allelochemicals will outcompete other species. Under low nutrients conditions, the situation is different and the species with the best nutrient uptakes are more likely to persist. Moreover, allelochemicals with other biological activities (e.g. antibacterial, anti-grazing, antifungal) will also promote the development and persistence of a bloom.

Allelochemical release is also an advantage for mixotrophic species, the immobilization and death of target cells may enhance phagotrophy (Blossom et al., 2012). Phagotrophy may indeed have coevolved with allelochemical activity (Granéli and Turner, 2007). Allelochemical interactions could be one of the key factors favoring the dominance of HAB species over other plankton species during HAB events.

2.5 Environmental control of allelochemical interactions

Some defense and competing mechanisms can be modified by environmental factors to face changing conditions (Lindström et al., 2017; Selander et al., 2006), and are so called induced-responses. These mechanisms can be induced to face an increasing stress but mechanisms can also be inhibited if the resources are limited. Such responses can be modelled (Chakraborty et al., 2018). Modulation of such mechanisms, that can be energetically costly, allow a better allocation of resources by the cells (Selander, 2007). Like the production of other phycotoxins, the production of allelochemicals can be modulated by environmental parameters (Figure 4) (Granéli and Turner, 2007; Legrand et al., 2003). Different abiotic factors like pH, light, nutrients, temperature were shown to modulate allelochemical potency (Granéli and Johansson, 2003; Granéli and Salomon, 2010; Schmidt and Hansen, 2001). Variability of allelochemical potency in response to changing environmental factors show that allelochemicals can be induced-responses, however the knowledge on the environmental control is still scarce. As an example of environmental abiotic control, the prymnesiophyte *Prymnesium parvum* requires light to produce hemolysin (toxin concentration decreases when culture is in the dark), even if the hemolysin is degraded in presence of light (Granéli and Turner, 2007). Production of allelochemicals is, in some situations, indirectly modulated as some allelochemicals may have dual purposes. For instance, the concentration of metals is known to modulate allelochemical activity in terrestrial environments, as some allelochemicals also have chelating properties (Inderjit et al., 2011; Tharayil et al., 2009). To our knowledge, the modulation of allelochemical potency by metals has not been assessed in marine species. Biotic factors such as cell concentration (Granéli and Salomon, 2010; Schmidt and Hansen, 2001; Tillmann et al., 2008), growth phase or physiological state (Arzul et al., 1999; Schmidt and Hansen, 2001), target cells (Kubanek et al., 2005; Tillmann et al., 2008), or allelochemical strain variability (Alpermann et al., 2010; Brandenburg et al., 2018) are also known to influence allelochemical activity. More recently, the production of allelochemicals was shown to be activated by the presence of competitors in co-culture (Ternon et al., 2018).

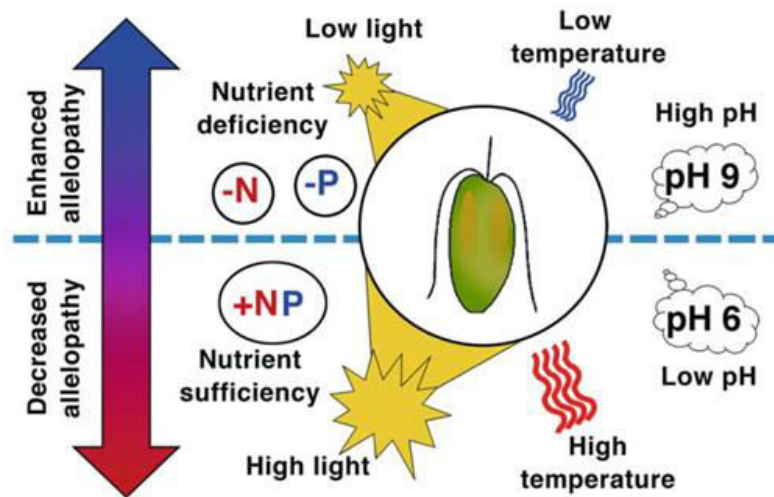


Figure 2: Abiotic factors known to affect allelochemical potency in harmful algae. (Granéli and Turner, 2007)

2.6 Microalgae producing allelochemicals

Allelochemicals are produced by a wide range of organisms (Legrand et al., 2003) including diatoms (Paul et al., 2009), prymnesiophytes (Fistarol et al., 2003; Schmidt and Hansen, 2001), cyanobacterias (Møgelhøj et al., 2006), chlorophytes (Barreiro and Hairston, 2013; Fergola et al., 2007) and dinoflagellates (Poulin et al., 2018; Tillmann et al., 2007). Production of allelochemicals has been widely reported in the toxic dinoflagellate *Alexandrium* spp., with at least 11 species known to produce allelochemicals including *A. minutum*, *A. fundyense*, *A. lusitanicum*, *A. affine*, *A. ostenfeldii*, *A. pseudogonyaulax*, *A. tamarense*, *A. taylori*, *A. tamutum*, *A. tamutum*. *A. pohangense* (Blanco and Campos, 1988; Kim et al., 2016; Lelong et al., 2011; Lyczkowski and Karp-Boss, 2014; Tillmann et al., 2016; Tillmann and John, 2002).

3 The genus *Alexandrium*

3.1 Classification

Within HAB, dinoflagellate (Dinophyceae) are unicellular eukaryotic organisms that account for 75 to 80% of toxic microalgae (Smayda, 1997). Among Dinophyceae the genus *Alexandrium* belongs to the Gonyaulacales order and to the Goniendomataceae family. More than 30 morphologically species belong to this genus (Anderson et al., 2012). Like other dinoflagellates, *Alexandrium* cells have two flagella, one transverse and one longitudinal that allow them to propell and maintain their position in the water (Janson and Hayes, 2007). The genus *Alexandrium* belong to the thecate dinoflagellates, cells are protected by a theca made of cellulosic plates. Morphological descriptions of theca plates usually helps to identify the species.

3.2 Life cycle

Dinoflagellates, including the genus *Alexandrium* have a complex life cycle, which includes two main phases, a haploïdic vegetative phase and a sexual diploïdic phase (Figure 5). Moreover, *Alexandrium* cells have the ability to form cysts when facing adverse environmental conditions. Contrary to vegetative cells, cysts are not motile and tend to sink. Two distinct forms of cysts have been described. The sexual resting cysts come from the fusion of two gametes (one positive and one negative). These resting cysts are thought to remain viable over long periods of time and long exposures to adverse environmental conditions (Doucette et al., 1989), such as winter conditions. When settling to the sediments, they form “banks” of cysts that are durable reserves of cells. Sexual cysts require a dormancy period in order to germinate (Anderson, 1980) contrary to the second form of cysts named temporary cysts (also named pellicle cysts, ecdysal cysts). Temporary cysts usually form when facing a sudden stress (within minutes) like physical stress, thermal choc, nutrient depletion, chemical stress, allelochemical interactions, ingestion by filter feeders etc. (Anderson and Wall, 1978; Doucette et al., 1989; Fistarol et al., 2004; Grzebyk and Berland, 1995; Hégaret et al., 2008; Herzi et al., 2013; Jensen and Moestrup, 1997). Contrary to sexual cysts, no dormancy period is required for temporary cysts to return vegetative cells. Temporary cysts give dinoflagellates an advantage to resist against temporary environmental stress.

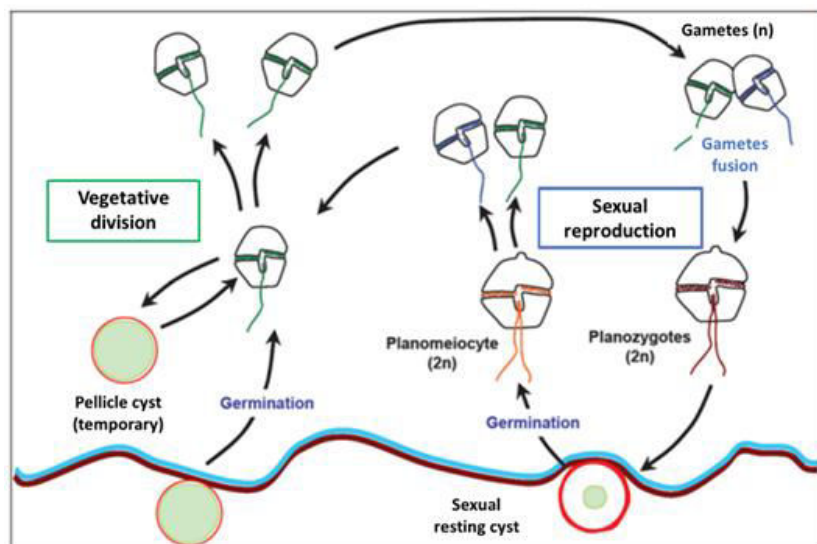


Figure 3: Life cycle of *Alexandrium* spp. from (Haberhorn, 2009).

3.3 Biology

Dinoflagellates of the genus *Alexandrium* have different nutritional strategies, they can either be autotrophic (photosynthetic), heterotrophic or mixotrophic (both auto and heterotrophic) (Blossom et al., 2012; Legrand and Carlsson, 1998). Blooms of *Alexandrium* are usually temporal outbreaks in response to suitable abiotic and abiotic environmental conditions. These outbreaks are enhanced by warm water temperature, sufficient light irradiance, nutrients, shallow waters, water stratification, and confined area (Chapelle et al., 2015). When *Alexandrium* faces the optimal environmental conditions, outbreaks can form high-biomass monospecific blooms (Carreto et al., 1986; Chapelle et al., 2015; Hallegraeff et al., 1988) with cell concentrations up to $10^7 - 10^8$ cells L^{-1} (Chapelle et al., 2015; Pitcher et al., 2007; Rodriguez, 2018; Vila et al., 2001). Most of the *Alexandrium* outbreaks often happen in confined coastal environment that are prone to eutrophication (e.g. harbors, estuaries, lagoons) (Vila et al 2005). These coastal environments are often subjected to anthropisation, and *Alexandrium* blooms can thrive with chemical contaminants. Some studies highlighted the prevalence of dinoflagellates including the genus *Alexandrium* in coastal environments contaminated by metals (Herzi, 2013; Jean et al., 2006, 2005; Tessier et al., 2011). Cells of *Alexandrium* might have developed strategies to face chemical stress such as enhanced exudation or encystment upon metal stress (Herzi et al., 2013).

3.4 Toxin production

The main characteristic of the genus *Alexandrium* is the diversity of toxins produced. This genus can produce saxitoxins, spirolides, goniodomins and other unknown extracellular toxins. Spirolides are lipophilic molecules from cyclic imines groups with neurotoxic properties (Munday et al., 2012). Spirolides, produced by the species *Alexandrium ostenfeldii*, contain many analogs and are fast acting toxins (Munday et al., 2012) that target acetylcholine receptors (Bourne et al., 2010; Hauser et al., 2012). However among the toxins produced by the genus *Alexandrium*, the paralytic shellfish toxins (PST) that are responsible for paralytic shellfish poisoning (PSP) are the most significant and the most extensively described. In the genus *Alexandrium*, PST includes gonyautoxins, saxitoxins and their derivatives. PST are hydrophilic neurotoxins, mainly intracellular, which target the voltage-sensitive sodium channels inducing a blockage of ion transport and action potential of excitable membranes. PST are mainly accumulated by filter feeder bivalves (Bricelj and Shumway, 1998), however other organisms can bioaccumulate PST in their tissues. Human intoxication result from consumption of bivalves that accumulated PST (Bricelj and Shumway, 1998). Indeed PSP outbreaks are responsible for shellfish contamination, economic losses linked with fisheries closure or recreational activities, and human intoxications. Approximately 2000 cases of PSP are reported each year with a mortality rate ranging from 4 to 10% (Sierra-Beltrán et al., 1998). Dinoflagellates of the genus *Alexandrium* can also produce unknown extracellular compounds with different biological activities (as described later). While most of the deleterious effects observed on marine organisms were usually attributed to PST, only a few studies investigated the production of extracellular toxins that can also effect marine organisms. Thus, the deleterious effects observed may not all be attributed to PST.

3.5 *Alexandrium* spp. worldwide geographical distribution

The genus *Alexandrium* is widespread globally in estuarine, coastal, shelf and slope waters from various environments, from temperate (e.g. France and Australia) and tropical regions to southern (Anderson et al., 2012) and northern hemispheres (Tillmann et al., 2016). The genus is present in the Mediterranean sea, where it was first identified in Alexandria in Egypt as *A. minutum* (Halim, 1960). The Mediterranean sea is reported to have the highest diversity of *Alexandrium* species (Anderson et al., 2012). The genus *Alexandrium*, including the species *A. minutum*, have been reported on coasts of every continents. Because of the location of this project, further information

on the distribution of *Alexandrium* in Australia and France are given below, for detailed reports and references on the worldwide distribution, see (Anderson et al., 2012; Lassus et al., 2016).

3.6 *Alexandrium* spp. outbreaks in Australia

Paralytic shellfish poisoning (PSP) events in Australia were first reported in Port Hacking, NSW, in 1945 (Hallegraeff, 1992; Wood, 1953) with a bloom of *A. catenella*. Other *Alexandrium catenella* blooms were reported in Australia in the 1980s, with major blooms in Melbourne, (Ekman et al., 1991), Port Phillip Bay and Sydney (Hallegraeff, 1992). The first occurrence of an *A. minutum* bloom was reported in Port River, Adelaide, SA in 1986-87 (Ekman et al., 1991) (Hallegraeff, 1992). Sequencing of rRNA provided evidences of the non-indigenous origin of *Alexandrium* as “Australian” strains of *A. catenella* are genetically close to Japanese strains and “Australian” strains of *A. minutum* are genetically close to European strains (de Salas et al., 2000; Lilly et al., 2005). Cells of *Alexandrium* spp. and other microalgae may have been introduced *via* ballast waters (Bolch and de Salas, 2007). The genus *Alexandrium* is nowadays widely distributed along Australian coastlines (Ajani et al., 2017; Dias et al., 2015; Farrell et al., 2013; Hallegraeff, 1992) (Figure 6A), from New South Wales (Figure 6B) to Victoria, Western Australia and Tasmania. Tasmania indeed faced an unprecedented bloom of *A. tamarense* in 2012, 200 km of eastern coastline were affected by the HAB. Many fisheries were closed due to shellfish contamination, PST levels in seafood were up to 12.5 times the maximum Australian sanitary standards. Economic losses due to this event were estimated at AUD\$23M (Ajani et al., 2017; Campbell et al., 2013)(Hallegraeff et al., 2017). In the duration of this PhD project (2015 – 2018), several toxic events were reported on the East coast of Tasmania with bivalve contamination (Ajani et al., 2017) and fishery closures (Figure 7). While threshold limit is 0.8 mg eq.STX per kg of bivalve, the maximum concentrations reported reached 24 mg eq.STX per kg of mussel (Ajani et al., 2017). The first cases human illness due to PSP in Australia were reported with this bloom, 3 people were treated and 2 hospitalized (Ajani et al., 2017). More recently, a toxic bloom occurred during the spring 2017 and yielded record levels (180 times the sanitary treshold) of PST contaminations with up to 140 mg eq.STX per kg in mussels from Spring Bay and 150 mg eq.STX per kg in mussels from Bicheno (Tasmanian Biotoxin News; Personal communication).

Chapter 1 – State of the art

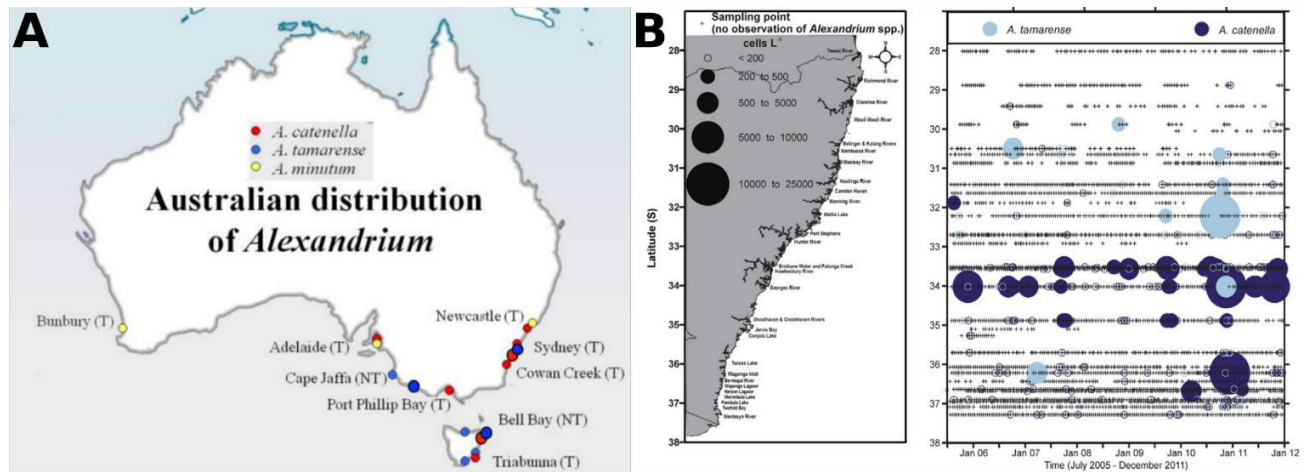


Figure 6: (A) Australian-wide distribution of toxigenic *Alexandrium* species, from "Harmful algal blooms in the Australian region: changes between the 1980s and 2010s". (Hallegraeff, 1992). (B) Distribution and abundance of species of *Alexandrium* spp. (*A. tamarense* and *A. catenella*) along the NSW coastline (July 2005 – December 2011). For the complete version of the figure, please refer to (Farrell et al., 2013)



Figure 7: Permanent warning signs at Freycinet national park, on the east coast of Tasmania during a toxic event in January 2018. Photo : M. Long.

3.7 *Alexandrium* spp. outbreaks in France

In France, the REPHY (Réseau d'Observation et de Surveillance du Phytoplancton et de l'Hydrologie dans les eaux littorales from Ifremer) have monitored HAB since 1992. The main species observed in France are *A. minutum*, *A. catenella* and *A. tamarense*. However other species have been detected: *Alexandrium andersonii*, *A. affine*, *A. insuetum*, *A. leei*, *A. margalefii*, *A. ostensfeldii*, *A. pseudogonyaulax* and *A. tamutum*. First reports of PST contaminated bivalves in France were made in 1988 in the Abers (North-west of Brittany) and in the pond of Thau, Languedoc (Lassus et al., 2016). Microalgae from the genus *Alexandrium* are now present along all French coastlines and outbreaks happen recurrently in the western Mediterranean coastline and in Brittany coastline. A global increase in repartition and outbreak intensity can be observed in some regions, for instance in the west coast of Brittany, *Alexandrium* blooms increased over the last 10 years (Figure 8), with a major event in the bay of Brest in 2012. During summer 2012, *A. minutum* concentrations reached $4 \cdot 10^7$ cells L⁻¹, leading to the first bivalve contamination by PST in the bay of Brest with records in toxin level in shellfish (10 times the international sanitary standards) (Chapelle et al., 2015).

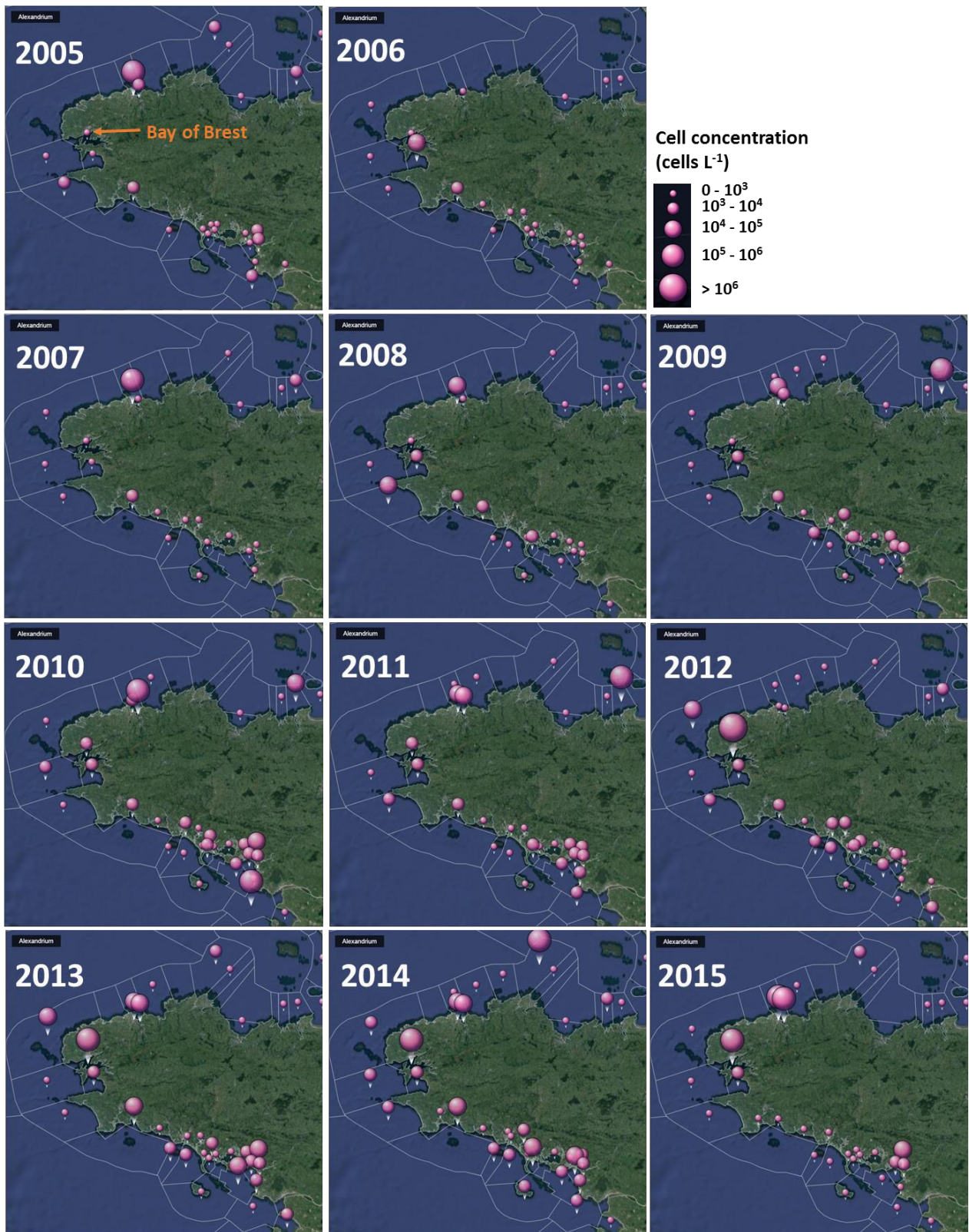


Figure 8: Concentrations of *Alexandrium* sp. in Brittany (France) as reported by the REPHY over the 2005 – 2015 period. <http://envlit.ifremer.fr/var/envlit/storage/documents/parammaps/phytoplankton/index.html#>

3.8 Environmental control of *Alexandrium* spp. blooms

The environmental parameters responsible for the proliferation of *Alexandrium* spp. and causing their toxicity remain very poorly understood. Physicochemical parameters such as temperature, hydrodynamics (Anderson et al., 2012; Guallar et al., 2017), nutrients availability (Hwang and Lu, 2000; Vila et al., 2005), presence of organic compounds (Gagnon et al., 2005) and metals (Herzi et al., 2013; Taroncher-Oldenburg et al., 1999) are known to influence the growth and modulate bloom formation. However biological interactions, such as cell behavior (Anderson et al., 2012), parasitism (Chambouvet et al., 2008; Garcés et al., 2005), predation (Calbet et al., 2003; Collos et al., 2007), allelochemical interactions (Legrand et al., 2003) are also known to influence *Alexandrium* spp. bloom formation. It is difficult to predict HAB at the interface of all the physicochemical and biological processes, especially as these factors are coupled together. The dinoflagellate *Alexandrium* spp. is mainly distributed in coastal and shelf waters or estuarine environments (Anderson et al., 2012; Guallar et al., 2017), which can be associated with human influence and can be subject to contaminations, as for example copper (Cu) contamination (Herzi, 2013; Melwani et al., 2014). For instance, *Alexandrium* spp. bloom in the bay of Brest, France (Chapelle et al., 2015; Guallar et al., 2017) or in the ria de Vigo, Spain (Frangópulos et al., 2004), areas that are subject to Cu contamination (García et al., 2013; Guillou et al., 2000; Lacroix, 2014; Michel and Averty, 1997; Prego and Cobelo-García, 2003; Quiniou et al., 1997). Copper has the ability to affect microalgal physiology, photosynthesis or exudation and overall microalgal growth (Herzi et al., 2013; Juneau et al., 2002; Sabatini et al., 2009). More importantly in the case of HAB, Cu can modulate the toxicity of a toxic diatom *Pseudo-nitzschia* (Maldonado et al., 2002). To our knowledge, only one study investigated the toxic effects of Cu on the genus *Alexandrium* (Herzi et al., 2013), demonstrating growth inhibition, cyst formation and increased exudation of *A. tamrense*. To our knowledge, no study investigated the effect of dissolved Cu on the physiology and allelochemical potency of *A. minutum*.

4 *Alexandrium* spp. allelochemical potency

4.1 Allelochemical potency within the genus *Alexandrium*

Allelochemical activity of the genus *Alexandrium* has also been widely reported. According to the literature, allelochemicals are not related to PST production or even spiroside production but to some unknown extra-cellular compounds (Fistarol et al., 2004; Tillmann et al., 2007; Tillmann and John, 2002). Strains of *Alexandrium* spp. can be either allelochemical producers, PST producers or producers of both toxins. Allelochemicals activity of strains can then be shown through the use of co-cultures, cell-free supernatant or cell-free filtrate (Lelong et al., 2011; Ma et al., 2009; Zheng et al., 2016). Furthermore, activity from whole culture is usually stronger than cell-free extracts (Lelong et al., 2011), likely because of the continuous release of allelochemicals by the culture. Allelochemical activity of *Alexandrium* spp. is classically reported as growth inhibition (Arzul et al., 1999; Hattenrath-Lehmann and Gobler, 2011; Lyczkowski and Karp-Boss, 2014; Zheng et al., 2016) or cell lysis (Tillmann et al., 2008, 2007; Tillmann and John, 2002). However immobilization of motile cells, aberrant behavior, cell deformation (Tillmann et al., 2007), cell bleaching (figure 9) (Lelong et al., 2011; Lyczkowski and Karp-Boss, 2014) or encystment (Fistarol et al., 2004; Tillmann et al., 2007) can also be observed when target cells are in contact with *Alexandrium* spp. allelochemicals. Allelochemical activity in *Alexandrium* spp. cultures is known to be increasing with both algal cell concentration and culture age (Arzul et al., 1999; Lelong et al., 2011; Tillmann et al., 2007; Tillmann and John, 2002). In *A. pseudogonyaulax*, allelochemicals are released to immobilize preys that are trapped in a mucus before feeding on them (Blossom et al., 2012; Blossom et al., 2017). Allelochemical potency is widespread among the genus *Alexandrium*, however, allelochemical variability can be observed at the species level (Tillmann and John, 2002). High variability in the allelochemical potency is also observed among strains from the same species (Arzul et al., 1999; Tillmann and John, 2002), among geographical populations (Brandenburg et al., 2018) or even among clones of the same species (Alpermann et al., 2010). Thus, allelochemical production is likely to be a shared property of the genus *Alexandrium* and the variability observed among species might reflect strain/clone variability.

4.2 Effects of allelochemical interactions on protists and microbial communities

Allelochemicals affect a large range of marine protists including autotrophic and heterotrophic organisms (Fistarol et al., 2004; Tillmann et al., 2008, 2007; Tillmann and Hansen, 2009). Even though allelochemical spectra is large, all species are not affected with the same intensity (Fistarol et al., 2004; Tillmann et al., 2007). Effective filtrate concentrations to induce 50% of target immobilisation/lysis (EC_{50}) were environmentally relevant as they could go down to 20 000 cells L^{-1} (Tillmann et al., 2008). EC_{50} must be lower with culture that continuously release allelochemicals in contrast with filtrates. Moreover, *in-situ*, *Alexandrium* spp. blooms can reach really high cell concentrations in calm waters as they tend to concentrate in horizontal layers or on the water surface (Mouritsen and Richardson, 2003; Ryan et al., 2008). Because the nature and mode of action of allelochemicals are not clear, it is difficult to explain this species-specific action. However it is hypothesized that target cell size influences allelochemical potency, the smaller cells the weaker (Lyczkowski and Karp-Boss, 2014) and that biochemical composition of membranes may also affect cell resistance (Ma et al., 2011a).

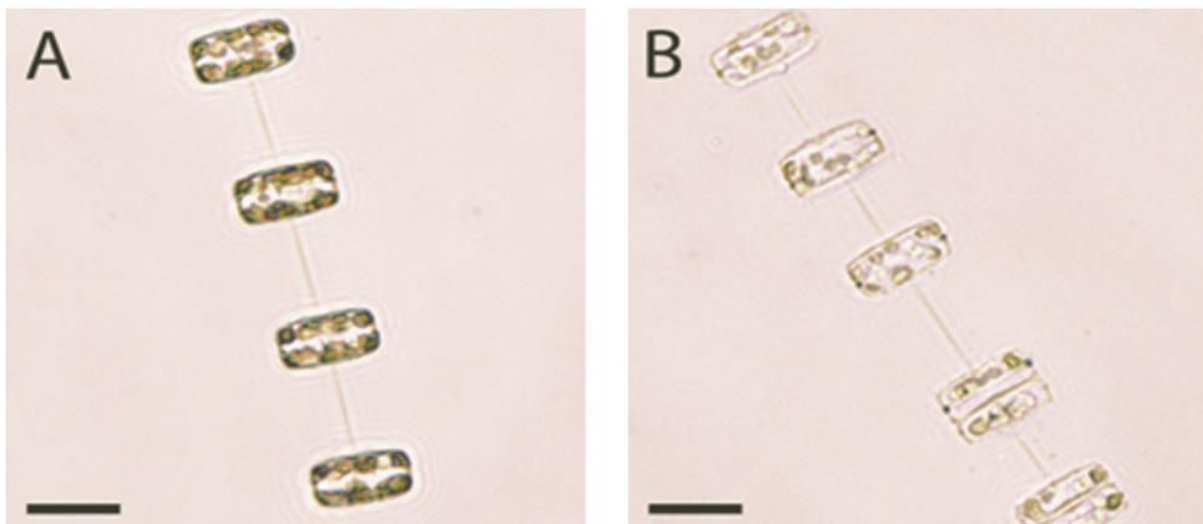


Figure 9: Microphotographs of *Thalassiosira* cf. *gravida* cells from treatments and controls. (A) A healthy chain in a control treatment and (B) a chain exposed to *A. fundyense*. Scale bars 20 μ m. (Lyczkowski and Karp-Boss, 2014).

Allelochemicals do not only affect monocultures, they also affect communities by decreasing growth rates and changing community structure (e.g. relative abundances and dominant species). When natural communities were exposed to a lytic *A. tamarensis* filtrate in controlled conditions, a decrease in populations of ciliates and phytoplankton organisms (e.g. diatoms,

nanoflagellates) was observed except for the small dinoflagellates ($< 30\mu\text{m}$) (Fistarol et al., 2004; Hattenrath-Lehmann and Gobler, 2011; Weissbach et al., 2010). Field observations confirmed the patterns (decrease in nanoflagellates and diatom abundances and increase dinoflagellate densities) observed in laboratory experiments (Hattenrath-Lehmann and Gobler, 2011). Interestingly, filtrate from a non-lytic strain of *A. tamarensis* had positive effects on diatom growth (Weissbach et al., 2010), revealing a non-deleterious allelochemical effect. Toxic effects on predators and competing phytoplankton at high *Alexandrium* concentrations may explain why blooms of *Alexandrium* spp. can reach high densities and persist over time. Moreover it has been demonstrated that allelochemical strains can favor growth over non-allelochemical strains by protecting *A. fundyense* population from grazing (John et al., 2015). Immobilization of prey by allelochemicals may be a hunting strategy enhancing *A. tamarensis* mixotrophy (Weissbach et al., 2010). Altogether, suppression of competitors for nutrients, enhanced mixotrophy and escaping grazer pressure might help *Alexandrium* spp. to outcompete other species and form dense blooms. In the meantime, bacteria associated to microalgal cultures do not seem to be affected (Fistarol et al., 2004; Weissbach et al., 2010), rather their growth is enhanced by the presence of allelochemicals. Lytic activity of cultures persisted when bacterial load is decreased by using antibiotics although full axenia may not have been reached (John et al., 2015; Lelong et al., 2011; Tillmann et al., 2008). The role of bacteria is still to be defined and we cannot exclude that some *Alexandrium* spp. bacteria play a role in allelochemical release.

4.3 Allelochemicals mode of action

Allelochemical activity has been widely reported as inducing cell lysis or encystment of many protists. Aberrant behavior, cell immobilization, cell distortion or bleaching were described as pre-lytic stages (Tillmann et al., 2008, Tillmann et al., 2007, Tillmann and John, 2002) however the precise mode of action of allelochemicals remains unknown.

Mechanisms of allelochemical interactions were studied with molecular tools on the diatom *Phaeodactylum tricorneratum* co-cultured for 24 hours with *A. tamarensis* (Zheng et al., 2016). The growth of *P. tricorneratum* was totally inhibited in co-cultures and cell concentrations dropped by 50 %, revealing cell lysis. Cellular functions of the diatoms were affected by allelochemicals as chloroplast membranes were blurred, thylakoids were disintegrated and mitochondria deformed. Analysis of transcriptional changes revealed that 16 % of expressed genes were upregulated and 12 % of expressed genes downregulated. Transcriptional changes were linked to many processes

of cellular metabolism and highlighted a wide affect on metabolism. Among the many processes involved in this interaction, genes associated to energy process and photosynthesis, especially photosystem II (PSII) and pigment synthesis were down-regulated. Diatom cell cycle was also affected by the interaction as checkpoints of cell division were down regulated, thereby suppressing cell division and explaining absence of culture growth. Similar allelochemical mode of actions were already shown for terrestrial plants (Duke, 2003). Interestingly, genes involved in defense mechanism, exudation of xenobiotics or endocytosis were upregulated. This may highlight defense strategy, for example efflux of allelochemicals, by the diatom. However the study highlighted two possible interactions between *P. tricornutum* and *A. tamarensis* when co-cultured: interaction *via* allelochemicals as well as a cell-cell interaction (through *A. tamarensis* flagella and *P. tricornutum* cell surface receptor). Changes strictly related to allelochemical interaction must be further studied by working with cell free supernatant or filtrates to disentangle the effects of allelochemicals and cell-cell interactions.

The use of transcriptomic is a promising technique, especially as it can be performed in co-cultures. Experimental design is however fundamental to properly unravel the allelochemical mechanisms. In their study, (Zheng et al., 2016) microalgae were sampled after 24 hours when *P. tricornutum* cells were already lysed, it is therefore difficult to distinguish changes related to cells that are dying or cells already dead. Such bias in the experimental design and sampling strategy do not allow any conclusion on the cellular targets and the mode of action of allelochemicals. Future studies should be careful about the kinetic of allelochemical interactions and focus on short-term interactions.

Short-term exposures (minutes to hours) of supernatant on photosystem II were more thoroughly studied on the diatom *C. neogracile* (Lelong et al., 2011). In agreement with previous studies (Tillmann et al., 2007), diatoms morphologic parameters (relative size and relative complexity of the cell, as measured by flow cytometry) were affected. Red auto-fluorescence, which is related to chlorophyll fluorescence, decreased within 30 minutes, highlighting deleterious effects on the photosystem. Effects upon photosynthetic efficiency were visible within minutes, with a decrease in photosynthetic system II efficiency measured by pulse amplitude modulatory fluorometry. Allelochemicals acted on the diatom photosynthetic system especially by decreasing the total number of PSII reaction centers (RCs). With fewer RCs, absorbed photons had less probability of entering the PSII. Therefore, PSII lost efficiency that could be quantified by measuring the maximum Fv/Fm ratio (ratio between the minimum fluorescence state Fo (dark-adapted fluorescent

state) and the maximum fluorescence called Fm). As a consequence of decreased photosynthesis, O₂ production by the diatom was inhibited. Conversely, respiration was increased but to a smaller extent. Furthermore, allelochemicals of *A. catenella* also induced membrane permeability of *Tetraselmis* sp. and *Rhodomonas baltica* within a short time (15 to 30 minutes) as measured by flow cytometry (Granéli and Turner, 2007).

4.4 Effects of allelochemicals on biological membranes

Considering the lytic activity from extracellular compounds released by *Alexandrium* spp., the mode of action of these lytic compounds over biological membranes was further investigated (Ma et al., 2011a). Lytic fractions (isolated by solid phase extraction and liquid chromatography) from *A. tamarense* were tested with different bioassays. When rat neuroendocrine cells PC12 were exposed to lytic fractions, cell membrane became permeable to Ca²⁺ as intracellular Ca²⁺ concentration increased. The use of a Ca²⁺ channel blocker (CdCl₂) demonstrated that Ca²⁺ channels were not involved in the intracellular increase. In another bioassay, lytic activity of different supernatants (*A. tamarense* lytic strain, *A. tamarense* non-lytic strain and *Rhodomonas salina*) were tested over laboratory phosphatidylcholine (PC) and PC/cholesterol liposome. All supernatants induced lysis of liposomes (as measured by the calcein release that was encapsulated in liposomes), however intensity of lytic activity was different between the strains. While the PC liposome lysis was only slightly different between strains (*A. tamarense* strains more lytic than *R. salina*), the activity over PC/cholesterol liposomes was higher and highly strain specific. The lytic strain of *A. tamarense* induced more liposome lysis than the non-lytic strain and *R. salina*. Affinity of lytic compounds for cholesterol was confirmed in a bioassay where *R. salina* was incubated with both lytic compounds and lipids. Addition of cholesterol, ergosterol, brassicasterol (common sterols of marine protists) and phosphatidylcholine (constituent of biological membranes) mitigated lytic activity of supernatant. Cholesterol mitigated the lytic activity more efficiently than other lipids, showing a higher affinity of cholesterol for the lytic compound. Lytic compounds are thus likely to disrupt plasmic membranes and form pores with sterols and especially cholesterol as a potential target. Cells of *A. tamarense* contain sterols with cholesterol accounting for 55 to 75 % of total sterols (Ma et al., 2011a), suggesting that *A. tamarense* have protective mechanisms or that another molecular target with more affinity for the toxic compounds is involved. Also, dinoflagellates possess thecates with multiple layers (Domozych, 2016) and their specific

biochemical compositions are difficult to define. Membrane sterol composition may thus not be representative from the outer layer that would be in contact with lytic compounds.

4.5 Environmental regulation of *Alexandrium* spp. allelochemical potency

Production of secondary metabolites (e.g. toxins and allelochemicals) is regulated by environmental factors. For instance production of paralytic shellfish toxins by *Alexandrium* spp. can be modulated with cell cycle (Persson et al., 2012; Smith et al., 2011), light (Taroncher-Oldenburg et al., 1999), nutrients (Anderson et al., 1990b, 1990a; Taroncher-Oldenburg et al., 1999), temperature (Anderson et al., 1990a), presence/absence of grazers (Selander et al., 2006) etc. Very little is known about the regulation of allelochemical activity of *Alexandrium* spp. Although *Alexandrium* spp. allelochemicals seem to be continually released in the surrounding environment, allelopathy was shown to be higher during stationary growth phase compared to exponential growth phase (Arzul et al., 1999; Lelong et al., 2011). This may be the result of allelochemicals accumulation in the culturing media or an increase in allelochemical production by *Alexandrium* spp. Variability was also observed over a 6 month period in controlled laboratory conditions: concentration of cells needed to be three times more concentrated to reach a similar allelochemical activity achieved 6 month earlier (Lyczkowski and Karp-Boss, 2014). Similar variations allelochemical potency were observed with *A. minutum* in laboratory conditions (Unpublished data). Very little is known about the environmental regulation of allelochemical activity. Nutrient starvation (nitrate and phosphate) was found to only slightly modify the allelochemical potency of *A. tamarense* (Zhu and Tillmann, 2012), and to have no effect on the allelochemical potency of *A. minutum* (Yang et al., 2011). Low salinities were shown to increase the lytic potency of *A. ostenfeldii* (Martens et al., 2016), while changes in culturing temperature (14 – 20°C) did not affect *A. tamarense* allelochemical activity (Fistarol et al., 2004). Chemical composition of solvent was also shown to modify allelochemical potency of *A. tamarense* (Ma et al., 2009), a decrease in potency was observed when extracts were suspended in deionized water as compared to natural seawater. Environmental parameters regulating allelochemical potency remain largely unknown and need to be further studied.

Among environmental parameters which deserves to be investigated, metal concentration appears to be an important factor. The genus *Alexandrium* is found in environments with high levels of metals (Jean et al., 2006, 2005; Tessier et al., 2011; Triki et al., 2017). (Arzul et al., 1999) hypothesized that allelochemicals released by the genus *Alexandrium* could chelate metals. In

terrestrial plants, some phycotoxins including allelochemicals were reported to have chelating properties (Inderjit et al., 2011; Tharayil et al., 2009). Thus, production of allelochemicals by *Alexandrium* spp. may be modulated by metals. Exposure of *A. catenella* to toxic concentrations of copper or cadmium increased exudation of dissolved organic carbon (DOC), probably to chelate metals (Herzi et al., 2013). While the nature of DOC was roughly characterized by 3D-fluorescence spectroscopy, the allelochemical potency was not studied. Enhanced exudation of DOC may have modulated allelochemical activity by either decreasing allelochemical release, mitigating allelopathy through the adsorption on DOC or increasing allelochemical release. Moreover some dinoflagellates are known to produce some metal-containing phycotoxins being toxic through the production of free radical mediated by the complexed metal (Moeller et al., 2007). Blooms of *Alexandrium* spp. occur in coastal areas that are prone to metal pollutions, this highlight the need to assess the effects of Cu on *Alexandrium* spp. as it may exacerbate allelochemical potency.

4.6 Chemical characterization of *Alexandrium* spp. allelochemicals

Compounds responsible for the allelochemical potency of *Alexandrium* spp. exudates remain uncharacterized. Allelochemical potency is unrelated to the known PST (Flores et al., 2012; Hakanen et al., 2014; Tillmann and John, 2002) or spiroclides (Tillmann et al., 2007) produced by the genus *Alexandrium*. Even though the precise nature of allelochemicals remains unknown, attempts were made to extract, isolate and preliminary characterize allelochemicals produced by *A. tamarensis* (Flores et al., 2012; Ma et al., 2011b, 2009).

(Ma et al., 2011b, 2009) extracted the allelochemicals from the supernatant and used a bioassay-guided approach with *Rhodomonas salina* to isolate allelochemicals from *A. tamarensis*. The general properties and stability of the biological activity were first characterized (Ma et al., 2009). The activity was labile unless stored in glass containers under ambient conditions (e.g. room temperature, ambient light). When stored in glass flasks, an apparent “loss” of activity was still observed but was easily recovered by vigorous shaking this was in contrast with other materials (e.g. plastics). The loss of biological activities was attributed to a binding to the container rather than a degradation of the product. The allelochemicals were resistant to freezing (- 20°C – 3 months), moderate heating (60 °C – 15 min) but did lose most of their potency when heated (95 °C – 15 min). Allelochemicals were also resistant when temporarily exposed to pH changes, activity was maximum at pH = 8 and decreased with increasing or decreasing pH but never totally disappeared ($\approx 40\%$ of initial activity at pH = 3 and $\approx 10\%$ of initial activity at pH = 2). Moreover

allelochemicals were not subjected to bacterial degradation (e.g. microbial communities from *A. tamarensis* cultures) as supernatant containing bacteria did not lose allelochemical activity.

Controversial hypothesis were made on the potential proteinaceous nature of allelochemicals from *A. tamarensis* and *A. taylori*. In their first study (Ma et al., 2009), Ma and collaborators suggested that the allelochemicals were not related to an enzyme since the allelochemical extracts were resistant to heat. Furthermore, (Ma et al., 2011b) found that the allelochemical extract contained some proteins but that the toxicity was not related to the proteinaceous structure as the potency of the extract could not be mitigated by trypsin. On the other hand, (Flores et al., 2012) could not confirm this hypothesis. Addition of trypsin mitigated the allelochemical potency of *A. tamarensis* cultures against the ciliate *Tiarina fusus* and the dinoflagellate (Flores et al., 2012), suggesting that allelochemicals from this strain were proteinaceous compounds. These contrasting results suggest that different allelochemicals, some proteinaceous and some non-proteinaceous, are produced by *A. tamarensis*.

Sugars were found in allelochemical extracts from *A. tamarensis* (Ma et al., 2011b). The sugar content was higher in the allelochemical strain as compared to a non-allelochemical strain, although, they were at a residual level. This highlighted that if the allelochemicals are complexes containing polysaccharides, the polysaccharides would only be a small fraction of the allelochemicals.

Allelochemicals from *A. tamarensis* were hypothesized to be amphiphatic (Ma et al., 2011b, 2009), i.e. compounds with a polar head and a hydrophobic part. The amphiphatic nature was highlighted by the allelochemical potency found in emulsion at the interface of aqueous and n-hexane interface during liquid-liquid partitioning. The loss of activity through container wall adsorption or after filtration using some specific filters membranes corroborate the hypothesis of amphiphatic compounds. Ultrafiltration with membranes of different cut-off sizes suggested that the molecular size of allelochemicals was comprised between 5 and 15 kDa. These large “compounds” corroborates the amphiphatic nature of allelochemicals as amphiphatic compounds (e.g. surfactants, detergents, lipids). However, fatty acids as potential allelochemicals because the n-hexane phase from liquid/liquid partitioning did not exhibit allelochemical activity (Ma et al., 2011b, 2009).

Allelochemicals were extracted from *A. tamarensis* supernatant through solid phase extraction (LC-18 SPE) allowing separation, one hydrophilic fraction (eluted with 20% methanol) and one less

polar fraction (eluted with 80% of methanol) that was much more potent (Ma et al., 2011b). In order to simplify analysis, a focus was made on the most potent fraction (80% methanol). In advanced steps to characterize allelochemicals, the authors compared signals obtained from an allelochemical producing and a non-producing strain of *A. tamarensis*, and decided to focus on peaks that were only present with the strain releasing allelochemicals. They compared different separation methods (reversed phase chromatography and hydrophilic interaction ion-chromatography) to isolate a molecular candidate. Eventually, the authors concluded that the purified allelochemicals from *A. tamarensis* are large (7 - 15 kDa) non-proteinaceous and probably non-polysaccharidic molecules but they could not isolate precise mass candidates. In another study by (Zheng et al., 2016), 11 compound candidates with lower molecular masses (< 1600 Da) were detected in *A. tamarensis* supernatant. However, the potency of each candidate needs to be confirmed and further characterized.

Discrepancies in the nature of allelochemicals between studies, isolation of several fraction with allelochemical potencies and different molecular candidates suggest that the *A. tamarensis* does not produce only one extracellular toxin but several toxins responsible for allelochemical activities. Moreover, these variations of allelochemical activity depend on donor/target combinations. This enhances the hypothesis that the genus *Alexandrium* produces many different allelochemicals (Tillmann et al., 2008). To our knowledge, characterization of allelochemical has only been investigated in the species *A. tamarensis*.

4.7 *Alexandrium* spp. exudates

In addition to allelochemical potency, exudates from *Alexandrium* spp. were found to have other biological activities (e.g. hemolytic, cytotoxic, ichthyotoxic) unrelated to PST. Some strains show biological activities to different targets at the same time. For instance the strain *A. minutum* AM89BM was found to have hemolytic activity (Arzul et al., 1999), allelochemical potency (Arzul et al., 1999; Lelong et al., 2011), ichthyotoxicity to bivalves (Borcier et al., 2017; Castrec et al., 2018) and cytotoxicity to oyster oocytes or hemocytes (Hégaret et al., 2011; Le Goïc et al., 2014). Some activities were positively correlated to each other, such as hemolytic/allelochemical activities (Arzul et al., 1999) and allelochemical/ichthyotoxic activities (Castrec et al., 2018). Even though correlations were not studied, a hemolytic strain of *A. taylori* was also toxic to human hepithelial carcinoma cells and brine shrimp *Artemia salina* (Emura et al., 2004). In another study, (Chen et al., 2015) observed that *A. tamarensis* allelochemical activity did correlate with hemolytic activity

extracted from *A. tamarense* with some solvent methods but did not correlate to the hemolytic activity of an extract obtained by dialysis. Altogether those results highlight that (1) *Alexandrium* genus may produce different toxins, and (2) some of the toxic compounds may be connected and have various target cells. The multi-target potency of allelochemicals has already been highlighted with other HAB species (Fistarol et al., 2004). Cells of *Prymnesium parvum* (Fistarol et al., 2003; Granéli and Johansson, 2003), *Karenia mikimotoi* (Arzul et al., 1993) and *Chrysodinium polylepis* (Edvardsen and Paasche, 1998; Schmidt and Hansen, 2001) can exhibit both allelochemical and ichthyotoxic potencies. This shows the need to not only look at allelochemical potency but also other biological activities of exudates that could give valuable information on the mode of action and nature of *Alexandrium* spp. allelochemicals.

5 Biological activities of *Alexandrium* spp. exudates

5.1 Hemolytic and cytotoxic activities

Among all the potential targets for cytotoxic compounds, blood cells are the most common cells used in cytotoxicity assays. In hemolytic bioassays, red blood cells are exposed to the potential hemolytic compounds and lysis of erythrocytes (hemolysis) is quantified. Hemolytic activity of exudates has been shown with different species from the genus *Alexandrium* (*A. catenella*, *A. minutum*, *A. tamarense*) on mammalian blood cells (Arzul et al., 1999; Chen et al., 2015; Emura et al., 2004; Eschbach et al., 2001; Ogata and Kodama, 1986; Yamasaki et al., 2008; Yang et al., 2010). Like allelopathy, hemolytic activity was not correlated to PST content (Chen et al., 2015; Simonsen et al., 1995; Yang et al., 2010). Hemolytic activity was positively correlated to *Alexandrium* spp. culture age and concentration (Arzul et al., 1999). Moreover hemolytic activity of *A. taylori* was dependent on temperature (Emura et al., 2004), potency increased positively with the temperature and was totally inhibited at 4°C. The hemolytic extract isolated from *A. tamarense*, was also responsible for the apoptosis of mammalian cell lines (Vero Cells) (Yamasaki et al., 2008).

5.2 Toxicity to bivalves

Hemocytes from bivalves are also impaired in presence of *Alexandrium* spp. extracellular compounds. The adherence and phagocytosis of hemocytes from the clams *Ruditapes philippinarium* and *Mya arenaria* was inhibited by *A. tamarense* filtrate (Ford et al., 2010). Similar immuno-suppressive effects were observed on hemocytes from the northern quahog *Mercenaria mercenaria* and the eastern oyster *Crassostrea virginica* when exposed to *A. fundyense* supernatant

(Hégaret et al., 2011). Gametes from the pacific oyster *Crassostrea gigas* saw their production of reactive oxygen species increasing in presence of a strain of *A. minutum* releasing allelochemicals (Le Goïc et al., 2014).

The physiology of bivalves is usually impaired when they feed on *Alexandrium* spp. cells. It is, however, difficult to differentiate deleterious effects of paralytic shellfish toxins (PST) vs extracellular compounds, especially as the information on allelochemical potency of strains is often lacking. The allelochemical and PST producing strain *A. minutum* AM89BM was used in *in vivo* for experiments with the european periwinkle *Littorina littorea* (Neves et al., 2015) and the pacific oyster *C. gigas* (Haberhorn, 2009). Feeding on the toxic dinoflagellate induced deleterious effects to both mollusks, however it was not possible to attribute effects to extracellular toxins or PST.

In recent studies, more attention was given to extracellular compounds to disentangle *A. minutum* toxic effects from PST and other compounds. (Borcier et al., 2017) observed harmful effects of extracellular compounds when juveniles of great scallop *Pecten maximus* were fed with a mix of microalgae containing a strain of *A. minutum* strictly producing allelochemicals (no PST). Daily shell growth was delayed, tissues in direct contact with *A. minutum* cells were altered and reaction time when exposed to predators was slowed. Similarly, distinct effects were visible when *C. gigas* was exposed to different strains of *A. minutum* exhibiting either allelochemical activity or PST production (Castrec et al., 2018). This later study demonstrated that the majority of the physiological effects (e.g. modification of valve-activity, hemocyte mobilization, harmful effects on the gills) of *A. minutum* on oyster were attributed to extra-cellular compounds.

Brine shrimp *Artemia salina* were first immobilized then died when exposed to the whole culture of a hemolytic strain of *A. taylori* at high cell concentration. Supernatant of *A. taylori* culture also induced shrimp death but to a smaller extent. Nevertheless, not all marine organisms seem to be affected by allelochemicals. Indeed, the feeding behaviour of the copepod *Temora longicornis* was not affected when fed with allelochemical strain of *A. tamarensis* (Xu et al., 2016).

5.3 Toxicity to fish

Toxicity to fish was first shown with *Gonyaulax monilata* (= *A. monilatum*) on the guppy *Lebistes reticulatus* in 1967 (Aldrich et al., 1967). Later (Ogata and Kodama, 1986) observed signs of hypoxia of surf smelt *Hypomesus pretiosus japonicus* exposed to *A. catenella* and *A. tamarensis* culture filtrate. Toxic activity was attributed to some extracellular compounds as the filtrate was free of cells and free of PST. Extracellular toxins were found to swell and exfoliate fish gills as

well as decreasing membrane elasticity of rainbow trout eggs. Toxicity from supernatants of different strains of *A. catenella* was also observed on rainbow trout cell lines (RTgill-W1) (Mardones et al., 2015). Toxic activity to gill cells was variable between strains and nature (whole culture, lysed cells or supernatant) of algal extracts. Even though not all supernatant from different strains were ichthyotoxic, all the whole or lysed algal extracts were ichthyotoxic. Toxicity was positively correlated to cell concentration but not correlated to PST content of cells. Synergistic effects of reactive oxygen species (ROS) and free fatty acids (FFA) was hypothesized to be responsible for the ichthyotoxic activity of *Chatonella marina* on damselfish *Acanthochromis polycaanthus* (Marshall et al., 2003). When microalgal cells are in contact with gills, the breakdown of cells release FFA and superoxide anion, that results in lipid peroxidation and gill damage (Figure 10). Eventually it induces reduced respiratory capacity, decreased blood pH through absorption of FFA and superoxide anion, as well as reduced osmoregulatory capacity (Marshall et al., 2003). A similar hypothesis was made by (Mardones et al., 2015) for *A. catenella* that produces great amounts of superoxide. Superoxides were found to enhance toxicity (9 fold) of some *A. catenella* purified polyunsaturated fatty acids (PUFA). However, presence of FFA and peroxidation products were not measured in the supernatant that shows ichthyotoxic activity. Thus, other extracellular toxins could be involved in the toxic effects. Synergistic effects of FFA, superoxide anion, peroxide products and extracellular compounds may be responsible for the overall ichthyotoxic activity observed in fish kills. Such hypothesis should be further studied to disentangle the effects of different toxicities to fish.

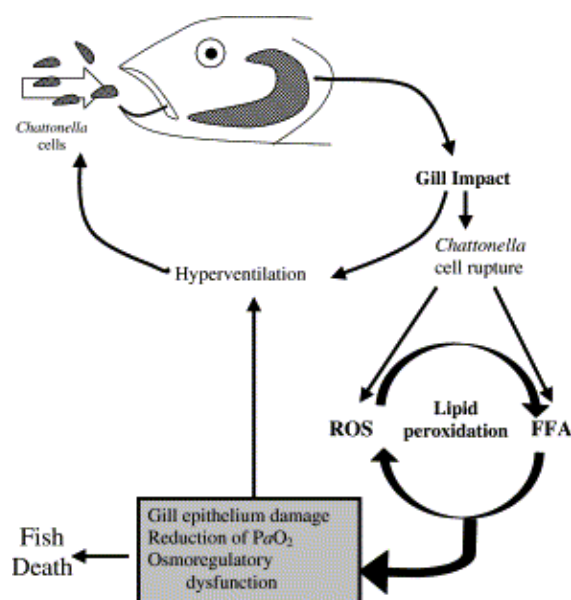


Figure 10: Diagram summarising possible mechanisms of ichthyotoxicity when exposed to free fatty acids (FFA) in combination with the ROS superoxide: *Chattonella* cells are brought into contact with the fish gill lamellae through ventilation. Cell contents released from broken *Chattonella* cells hydrolyse in high ROS environment to produce higher levels of FFA and ROS. Lipid peroxidation occurs on gill and other membranes resulting in reduced respiratory and osmoregulatory capacity and allowing the transfer of FFA and O₂⁻ into the blood stream. Damage to the chloride cells of the gills can also lead to reduced osmoregulatory capacity. Toxic mechanisms may occur in isolation or combination. Ichthyotoxic activity (molluscs). From (Marshall et al., 2003).

6 Chemical nature of *Alexandrium* spp. exudates

As mentioned above, literature on the nature of allelochemicals is scarce. Thus, it is interesting to have an overview of the literature covering all extracellular compounds characterization regardless of the biological activity. The following paragraphs will thus focus on the characterization of *Alexandrium* spp. exudates and extracellular toxins.

6.1 Characterization of *Alexandrium* spp. exudates

Even though toxicity of exudates was not assessed, extracellular particulate (> 3.5 kDa) algal organic matter (AOM) from *A. tamarense* have already been characterized through several techniques (Li et al., 2015; Villacorte et al., 2015a, 2015b). (Passow and Alldredge, 1995) identified transparent exopolymer particles (TEPs; mainly acidic polysaccharides) that accounted for 70 - 75 % of biopolymers, as measured with alcian blue coloration. TEPs increased in stationary growth phase, and their origin was hypothesized to come from cell lysis rather than exudates. TEPs precursors that are high-molecular-weight were also found. Acid and neutral compounds of smaller molecular weight, as well as biopolymers, were found containing fucose and sulphated functional

groups. Allelochemical activity and fine structures of the different compounds were not assessed. Similar patterns between *A. tamarensis* and *Chaetoceros affinis* (non-toxic algae) in nuclear magnetic resonance (NMR), fluorescence excitation-emission matrix (FEEM) and fourier transform infrared spectroscopy (FTIR) spectra (Figure 11) suggest that many of the components identified are non-toxic organic matter (Li et al., 2015; Villacorte et al., 2015a). However, by providing an overview of the main components from *A. tamarensis* filtrates (e.g. polyssacharides, protein, humic-like compounds), these studies highlight that exudates are complex and that bioassay-guided purification is needed for their characterization. Nevertheless, once allelochemicals are purified, these tools (e.g. FTIR, NMR, FEEM, LC-OCD) will be helpful to determine the chemical nature of allelochemicals.

6.2 Characterization of hemolytic and cytotoxic compounds

Chemical characterization of hemolytic compounds was performed by (Emura et al., 2004) on *A. taylori* cell-free supernatant. Hemolytic compounds were identified as large compounds (> 10 kDa) by ultrafiltration. Loss of hemolytic activity when the supernatant was treated with trypsin, loss of potency with heat treatment (100 °C) or activity of ammonium sulfate precipitate are several indicators suggesting a proteinaceous origin of the compounds (Emura et al., 2004). Non-proteinaceous candidates with hemolytic activities were extracted from strains of *A. tamarensis* (Chen et al., 2015; Yamasaki et al., 2008). Hemolytic compounds of approximately 1 kDa were extracted in *A. tamarensis* with HPLC-MALDI-MS (Chen et al., 2015). Even though the structure of these compounds remains unknown, the hemolytic activity was resistant to acids, bases and heating (75 °C) suggesting the non-proteinaceous nature of hemolytic compounds. The non-proteinaceous nature of hemolytic/cytotoxic extracellular compounds from *A. tamarensis* was also shown by (Yamasaki et al., 2008). A candidate (AT-toxin) with a high molecular weight estimated at 1000 kDa was found by electrophoresis. Similarly to the allelochemicals isolated by (Ma et al., 2011b), the AT-toxin fraction did not contain much protein. In contrast to (Ma et al., 2011b), the AT-toxin fraction contained sugars. The sugar composition was thoroughly described by the authors who hypothesized that an extracellular polysaccharide-based toxin was responsible for the hemolytic activity and the induction of apoptosis. The contrasting results suggest that the genus *Alexandrium* releases at least two cytotoxic compounds with different nature: one non-proteinaceous (Chen et al., 2015; Yamasaki et al., 2008) and one protein-like compound (Emura et al., 2004).

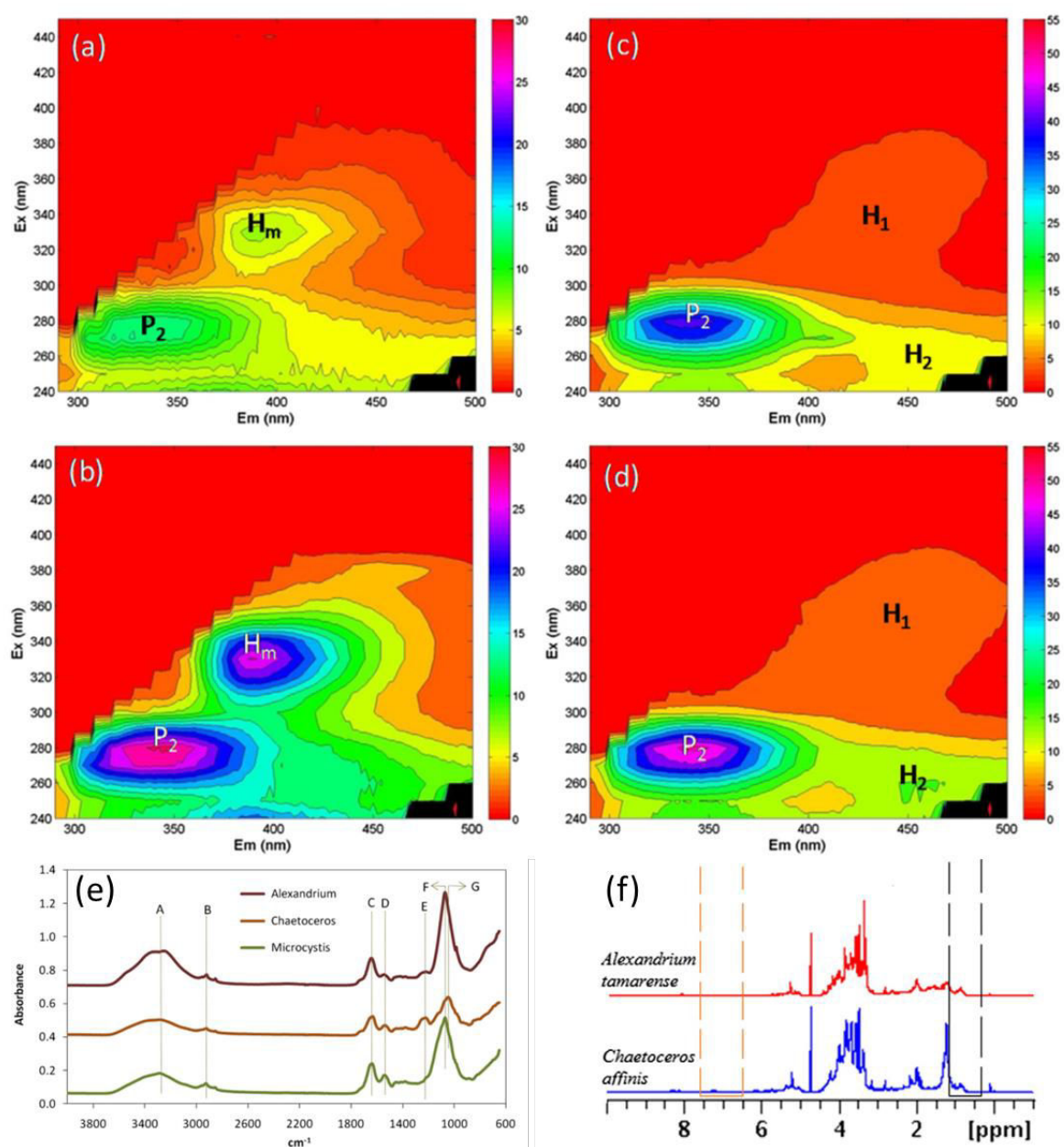


Figure 11: (a, b, c, d) Typical excitation emission matrix spectra of free (detached from cells) organic matter samples from *A. tamarense* (a, b), *Chaetoceros affinis* (c, d) collected during the exponential (a, c) and stationary phases (b, d). Legend: H1 primary humic-like peak; H2 secondary humic-like peak; Hm marine humic-like peak; P1 tyrosine-like peak; P2 tryptophan-like peak. (e) FTIR spectra of freeze dried organic matter samples from the three species of algae. Offsets in absorbance spectra were intentionally made for clarity. The functional groups identified by letters and the original figure. (f) ¹Hsolution-state NMR spectrum at 700MHz of isolated TEP substances from different bacteria and algae. Modified from (Villacorte et al., 2015a)

6.3 Characterization of ichthyotoxic compounds

Stability of ichthyotoxic toxins from Chilean *A. catenella* was assessed on supernatant and lysed cell extracts (Mardones et al., 2015). The extract activities were reduced over several days of storage and the loss of activity was enhanced by light. The light-sensitive extracts were found to be resistant to heat (80°C, 5min) and not sensitive in the range of pH 7 to 9. Some ichthyotoxic compounds from *A. catenella* are hydrophilic as shown by the toxicity of aqueous, acetic acid and HCl fractions (Dorantes-Aranda et al., 2015).

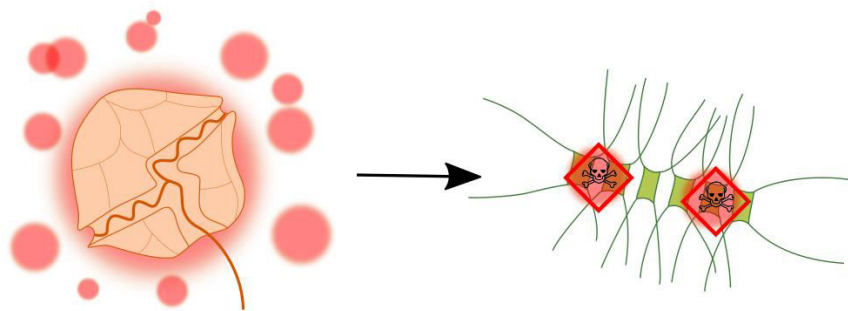
In contrast to previous studies on allelochemicals (Ma et al., 2011b, 2009), no bio-guided extraction was performed to characterize ichthyotoxic chemicals. Based on the hypothesis of superoxide anion and fatty acids synergistic effect (Marshall et al., 2003), (Mardones et al., 2015) described the ichthyotoxicity of *A. catenella* FFA and superoxide. Analysis of the lipid composition of *A. catenella* strains revealed that polyunsaturated fatty acids (PUFA) accounted for more than 50% of total fatty acids (FA) including docosahexaenoic acid (DHA; 22:6n-3), that counted for 16 to 20% of total FA. FFA were hypothesized to be ichthyotoxic and biologic activity of the molecules was tested. DHA was highlighted to be the more ichthyotoxic to fish larvae ($LC_{50} = 1.5 \mu\text{M}$) than 19 other FAs (Henrikson et al., 2010). Toxicity of DHA ($LC_{50} = 0.15 \mu\text{g mL}$) was greatly enhanced when coupled to superoxide anions, which production by *A. catenella* is high ($8.7 \pm 0.1 \text{ pmol O}_2 \text{ cell}^{-1} \text{ h}^{-1}$), compared to the fatty acids alone ($LC_{50} = 1.3 \mu\text{g mL}$). Though DHA solely might not be the ichthyotoxic compounds, FA (e.g. DHA) oxidation products are more likely to play that role. In (Mardones et al., 2015), the supernatants from different strains exhibits different ichthyotoxic potency, however the FA composition of the two *A. catenella* strains is similar. This suggests the presence of at least two types of toxins with ichthyotoxic activities: cells bound PUFAs (e.g. DHA) that are mainly released during cell lysis (coupled with superoxide anions) and potentially other extracellular toxins released by viable cells in the surrounding environment. Also it can be hypothesized that some “enzymatic tools”, present only with the toxic species, could biotransform FA in toxic compounds.

Objectives and organization of the thesis

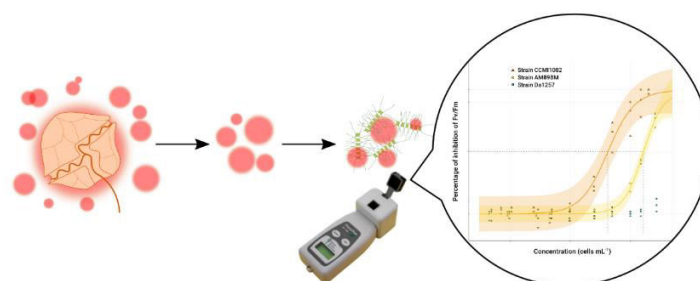
This research project focuses on the marine microalgal species, *Alexandrium minutum*, which has been reported worldwide in temperate oceans. Microalgae from the genus *Alexandrium* are dinoflagellate species responsible for “red tides” that produce paralytic shellfish toxins (PST) responsible for paralytic shellfish poisoning (PSP) syndrome. In parallel with this production of PST by *Alexandrium* spp., studies revealed the production of unknown extracellular compounds with toxicity to marine organisms. Some of the extracellular toxins are allelochemicals and induce negative effects on competing protists, bacteria or predators. Allelochemicals may have an important role in establishment of *Alexandrium* spp. blooms and toxicity of marine organisms. The mode of action and the nature of allelochemicals between *A. minutum* and other protists remain unknown. The general objective of this PhD project is to shed light on the allelochemical interactions from *A. minutum* with other protists. To disentangle allelochemical mechanisms we especially focus on the interactions between *A. minutum* and the diatom *C. muelleri*, a diatom naturally co-occurring with *A. minutum*.

The present chapter of this PhD is a comprehensive review of the state of our current knowledge on allelochemical interactions in the genus *Alexandrium*. This chapter allow to highlight the knowledge gaps in *Alexandrium* spp. allelochemical interaction that frame the objectives of this project.

The **chapter 2** aim is to understand the mechanisms of allelochemical interactions. This 2nd chapter is constituted of two articles, entitled “*Allelochemicals from Alexandrium minutum induce rapid inhibition of metabolism and modify the membranes from Chaetoceros muelleri*” published in *Algal Research* and “*Allelochemical interactions between Alexandrium minutum and Chaetoceros muelleri: insights into photosynthesis and membrane integrity*” currently under preparation for submission in *HarmfulAlgae*.

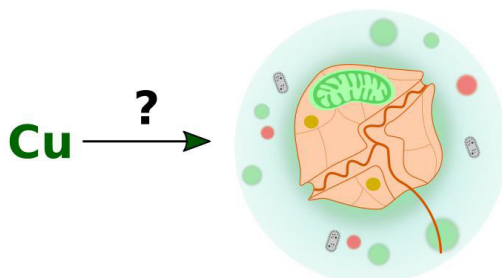


Building on results from the 2nd chapter, in the **3rd chapter** we highlighted a useful biomarker that allowed us to develop a bioassay to study allelopathy. This research entitled “*A rapid quantitative fluorescence-based bioassay to study allelochemical interactions from Alexandrium minutum*” which has been published in *Environmental Pollution* constitutes the 3rd chapter of this thesis. The bioassay developed in the 3rd chapter allow to further study allelochemical interactions and the environmental factors that can modulate allelochemical interactions.

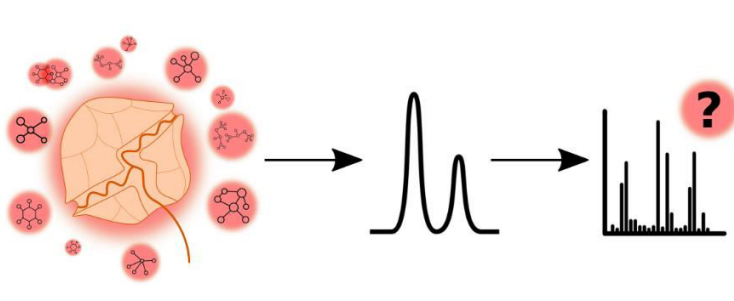


Chapter 1 – State of the art

In the **chapter 4**, we studied the effects of environmental parameter on allelochemical potency that are largely unknown. A focus was made on the effects of copper based on the observations that toxic copper concentration modifies exudates and that some allelochemicals can chelate metals. This research lead to a paper “*A multi-traits approach reveals Cu effects on allelopathic Alexandrium minutum physiology*” submitted to *Aquatic Toxicology*.



The **chapter 5** presents the last part of this research which aimed to extract, purify and partially characterize allelochemicals from *A. minutum*. This purification was based on the bioassay developed in the 3rd chapter.



Finally, the **chapter 6** is a discussion that describe the contribution of this thesis to the discipline and define what needs to be done in the field.

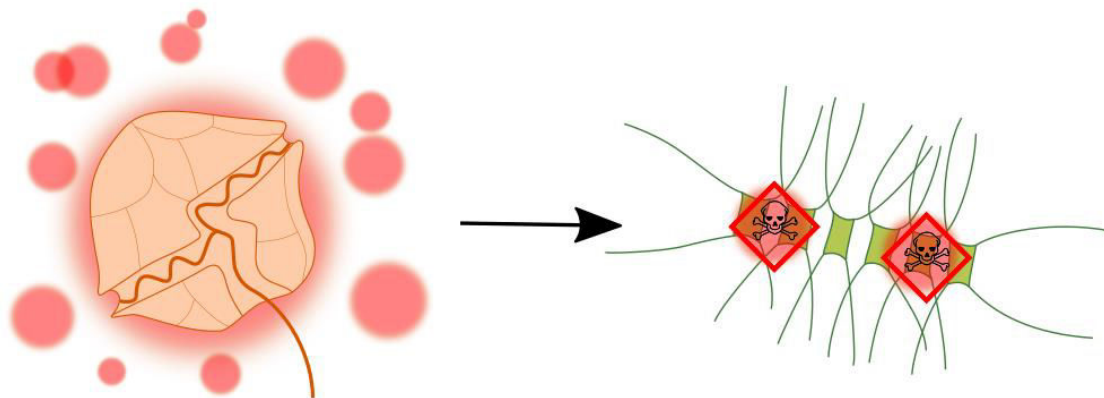
Chapter 2

Unraveling the mechanisms of allelochemical interactions

In marine systems, our understanding of the ecological role of allelochemical interactions and how they shape plankton dynamics is hindered by the poor knowledge of the modes of action of allelochemicals. In the literature, allelochemical interactions are described through growth inhibition, photosynthesis inhibition, cell lysis but the actual molecular targets and mode of action are usually unknown. The species *Alexandrium minutum* is not an exception to the rule as biological effects are described but the mode of action and the cellular target remain unidentified. The objective of this chapter is to unravel the mechanisms of allelochemical interactions between the dinoflagellate *A. minutum* and a phytoplankton target, the diatom *Chaetoceros muelleri*. This chapter consists of two articles, the first article is already published in *Algal Research* and the second article is a manuscript in preparation for submission to *Harmful Algae*.

Article 1: Long, M., Tallec, K., Soudant, P., Le Grand, Donval, A., Lambert, C., F., Sarthou, G., Jolley, D., Hégaret, H. Allelochemicals from *Alexandrium minutum* induce rapid inhibition of metabolism and modify the membranes from *Chaetoceros muelleri*. *Algal Research* (2018).

Article 2: Long, M., Peltekis, A., González-Fernández, C., Sarthou, G., Jolley, D., Hégaret, H., Bailleul, B. Allelochemical interactions between *Alexandrium minutum* and *Chaetoceros muelleri*: insights into photosynthesis and membrane integrity. In preparation for *Harmful Algae*.



Article 1

Allelochemicals from *Alexandrium minutum* induce rapid inhibition of metabolism and modify the membranes from *Chaetoceros muelleri*

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The article in its original format can be found in annexe 1.

Abstract

Allelochemical interactions are likely to be a contributing factor explaining the success of large blooms of the harmful marine dinoflagellates *Alexandrium* spp., however, the physiological mechanisms of allelochemical interactions remain poorly described. Here we investigated the sub-lethal effects (on an hourly scale) of a filtrate containing allelochemicals from *Alexandrium minutum* on the physiology of the common diatom *Chaetoceros muelleri*. The filtrate induced deleterious effects to the diatom physiology within only 30 min of exposure. Esterase activity and photosynthesis were drastically inhibited, with up to 34% of the population being metabolically inactive and up to 30% reduction in photosystem II quantum yield when exposed to the filtrate. In addition, intracellular reactive oxygen species increased by 26% in response to allelochemical exposure. *C. muelleri* pigment and lipid analyses indicated that the photosystem II was inhibited, with photoinhibition-like responses (activation of xanthophyll cycles, and changes in associated lipids) upregulated to mitigate the toxic effects of allelochemicals. Changes in the proportions of membrane lipid classes and increased membrane fatty acids saturation by 9% may be an attempt to maintain membrane integrity and associated enzyme activity, or could be the result of deleterious effects on membranes. An 8% decrease in cellular storage lipids (triglycerides) revealed a mobilization of energy suggesting an energetic cost for the diatom to counteract the allelochemical effects. We hypothesize that the rapid alteration of physiological functions such as photosynthesis and some enzymatic activities may result from direct damage on external membranes. Overall this study describes the sub-lethal mechanisms and provides useful biomarkers to understand the role of allelochemical interactions and associated ecological processes in structuring plankton communities.

1 Introduction

The structure of plankton communities is shaped by complex interactions with their environment. The influence of abiotic factors, such as light, nutrients, seawater or stratification are well studied and known to have a strong role in community structure. The roles of biotic factors however are less well understood despite the increasing evidence that biotic factors are shaping plankton communities (Lima-Mendez et al., 2015; Worden et al., 2015). Chemical interactions between organisms (i.e. chemical ecology) are influencing the structure of marine communities. Chemical compounds can mediate communication between microbes, can play a role in sexual reproduction and competition between species or can be a chemical defense against grazers and pathogens (Ianora et al., 2006; Pohnert, 2010; Puglisi et al., 2014). Within this field, allelochemical interactions refer to the beneficial or adverse effects of chemicals (allelochemicals) released by a protist on the biology of co-occurring protists, predators or bacterial cells. The release of toxic allelochemicals gives the donor species a competitive advantage that could result in the formation of large blooms (Granéli et al., 2006; Legrand et al., 2003).

The genus *Alexandrium* is known to produce uncharacterized extracellular compounds with potent allelochemical activity. It is hypothesized that the allelochemical potency of this genus may enhance the establishment of large *Alexandrium* spp. blooms (Tillmann and Hansen, 2009). The allelochemical potency is widespread among the genus *Alexandrium* as this activity was shown in at least 9 species: *A. minutum*, *A. fundyense*, *A. lusitanicum*, *A. affine*, *A. ostenfeldii*, *A. pseudogonyaulax*, *A. tamarense*, *A. taylori* and *A. tamutum* (Blanco and Campos, 1988; Lelong et al., 2011; Lyczkowski and Karp-Boss, 2014; Tillmann et al., 2016; Tillmann and John, 2002). Allelochemicals are released by cells into the surrounding environment and within minutes to hours induce cell lysis, cell immobilization and cyst formation (Tillmann et al., 2007). Furthermore some of these allelochemicals may be responsible for other toxic effects on mammalian erythrocytes (Arzul et al., 1999), and bivalve gametes (Le Goïc et al., 2014). They may also be responsible for reduced growth and behavior modification of the great scallop *Pecten maximus* (Borcier et al., 2017) or deleterious effects on the physiology of the oyster *Crassostrea gigas* (Castrec et al., 2018). Nevertheless the nature and mode of action of those extracellular compounds remain unknown.

Researchers studying allelochemical interactions often quantify their effects on microalgal growth rate, membrane permeability and cell lysis in target cells however very few studies precisely elucidate the mode of action. For example, studies exploring allelochemical mechanisms from the genus *Alexandrium* have investigated their effects on photosynthesis (Lelong et al., 2011), membranes (Ma et al., 2011) and transcriptomic (Zheng et al., 2016), however the story is still incomplete. While the long-term (i.e. mainly lethal) effects are fairly well described, the short-term (i.e. sub-lethal) effects and cellular processes leading to cell death remain unclear. More specifically, the effects of allelochemicals on the biochemical composition of membranes, that seem to have a central role in allelochemical sensitivity (Ma et al., 2011) are unknown.

The aim of this study was to elucidate the mechanistic effects (at a subcellular level) of allelochemicals released by *Alexandrium minutum* on the common diatom *Chaetoceros muelleri* a diatom co-occurring with *A. minutum* (Chapelle et al., 2014). The study focused on the photosynthetic apparatus and biochemistry of membranes. Allelochemicals were separated from *A. minutum* cells by filtration to specifically focus on allelochemical interactions and avoid interference by cell-cell interactions. A pulse of allelochemicals was performed to study the short-term sub-lethal effects of allelochemicals on *C. muelleri*, measuring the effects on photosynthesis, intracellular reactive oxygen species production and esterase activity, a proxy of primary metabolism (Brookes et al., 2000; Dorsey et al., 1989). To further explore the allelochemical interactions at the membrane level, we analyzed the membrane composition of the diatom (polar lipid classes, fatty acids composition of polar lipids, and sterol content) and pigments after 30 and 60 min of exposure to the filtrate. Neutral lipids classes were also analyzed to detect free fatty acid increases as a cell lysis indicator or a decrease of storage lipids (triacylglycerols) as a stress response.

2 Materials and methods

2.1 Microalgal cultures

A strain of *Alexandrium minutum* (strain CCMI1002, isolated from a bloom in Gearhies, Bantry Bay, Ireland; Touzet et al., 2007), not producing paralytic shellfish toxins (PST), was selected according to its high allelochemical potency (Borcier et al., 2017). The dinoflagellates were cultivated in triplicate for 10 days in 1 L of autoclaved L1 media (Guillard, 1975) prepared with

natural filtered (0.2 μm) seawater from Argenton, France (Salinity = 34 psu, pH = 8.4) a pristine site for *Alexandrium* spp. Cultures of *A. minutum* were maintained in exponential growth phase to obtain a concentration of 9.6×10^4 cells mL^{-1} on the experimental day (growth rate: 0.18 day^{-1}). A strain of *Chaetoceros muelleri* (strain CCAP 1010-3 obtained from the CCAP culture collection, formerly listed as *Chaetoceros neogracile* or *Chaetoceros* sp.) was selected because of its sensitivity to *A. minutum* allelochemicals (Borcier et al., 2017; Lelong et al., 2011) and because of the ubiquity of its genus in phytoplankton communities (Dalsgaard et al., 2003). Cultures of the diatom were grown for 10 days (2.12×10^6 cells mL^{-1}) in 6 L of autoclaved synthetic ocean seawater (Morel et al., 1979) enriched with L1 media (salinity = 35 psu, pH = 8.4) and silicate (1.06×10^{-4} M). Cultures of *C. muelleri* were in late exponential growth phase on the day of the experiment (growth rate: 0.15 day^{-1}). All cultures were maintained at $18 \text{ }^\circ\text{C}$ under a continuous light intensity of $100 - 110 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$. Cultures were not axenic but were handled under sterile conditions to minimize additional bacterial contamination.

2.2 Exposure to allelochemicals

Allelochemicals were separated from cultures by centrifugation (10 min, 800 g, $18 \text{ }^\circ\text{C}$) followed by a filtration (0.2 μm , Minisart syringe filter, acetate cellulose membrane, 16534-K, Sartorius) of supernatant (Lelong et al., 2011). The filtrate concentrations were expressed as cell concentration (cells mL^{-1}) based on the initial culture concentration prior to filtration. Cultures of *C. muelleri* in late exponential growth phase were diluted to 1.38×10^6 cells mL^{-1} with filtered seawater and distributed to six 2 L glass balloon flasks. The diatom *C. muelleri* was then exposed in triplicate to two different conditions for 90 min: i) 12 mL of *A. minutum* filtrate (final concentration from 1200 *A. minutum* cells mL^{-1}), ii) the same volume (12 mL) of filtered seawater as a control (final volume in each condition 1 L). During the exposure (maximum 90 min), the flasks were kept under ambient laboratory light and temperature conditions (not measured).

2.3 Photosynthetic parameters

Photosynthetic measurements were performed by pulse amplitude modulation (PAM) fluorometry using an AquaPen-C AP-C 100 with a blue light (455 nm). Sub-samples of *C. muelleri* were dark-adapted for at least 10 min, allowing the full oxidation of photosystem II

(PSII) reaction centers and electron chain transport, before the measurement of fluorescence variables and PSII maximum quantum yield ($F_v/F_m = (F_m - F_0) / F_m$). F_0 is the initial fluorescence intensity, and F_m the maximal intensity under saturating light (Strasser et al., 2000). Measurement of fluorescence variables were performed every 10 min for 100 min following the exposure.

Non-photochemical quenching (NPQ) measurements were performed in a separate experiment. Six replicates of the diatom *C. muelleri* (2×10^5 cells mL⁻¹) in exponential growth phase were exposed for 45 min to *A. minutum* filtrate (final *A. minutum* concentration of 6×10^3 cells mL⁻¹). The cells of *C. muelleri* were exposed to three successive flashes of 60 s (saturated light, $3\,000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) and 3 dark phases of 80 s for NPQ measurements, in the presence of filtered seawater or filtrate.

2.4 Flow-cytometry measurements

Population growth counts and measurements of phytoplanktonic cell variables were performed using a FACScalibur (BD Biosciences, San Jose, CA, USA) flow cytometer with a 488 nm argon laser. Counts were estimated according to flowrate (Marie et al., 1999). Cell variables, e.g. forward scatter (Forward scatter, FSC), side scatter (Side scatter, SSC) and red autofluorescence (F13, red emission filter long pass, 670 nm) were used to select diatom population. All flow cytometry measurements on *C. muelleri* (cell variables, esterase activity and reactive oxygen species production) were performed after 30, 60 and 90 min of exposure to *A. minutum* filtrate or filtered seawater.

Microalgal esterase activity, a proxy of primary metabolism was assessed with fluorescein diacetate staining (FDA; Invitrogen # F1303; at a final concentration of $6 \mu\text{M}$) (Brookes et al., 2000; Dorsey et al., 1989; Franklin et al., 2001; Garvey et al., 2007). FDA is a nonpolar compound that can permeate into the cells. Once inside the cells, FDA is cleaved by esterases into acetate and fluorescein molecules (emission wavelength 525 nm), which are retained within the cells. Intracellular concentrations increase with both metabolic activity and time. Samples were incubated with the stain for precisely 10 min in the dark prior to flow-cytometry measurements. Intracellular fluorescence intensity, which is proportional to the amount of FDA cleaved by esterases within the cells, was measured with F11 emission filter (green emission filter band pass, 530/30 nm). Cell populations of *C. muelleri* could be divided into 3 sub-

populations: cells with highest F11 fluorescence corresponding to high esterase activity (metabolically active), cells with a low F11 fluorescence corresponding to cells with reduced esterase activity (reduced metabolism), and cells which F11 fluorescence equivalent to microalgal auto-fluorescence from cells with inactive esterases (metabolically inactive). Results were expressed as the percentages of cells with high esterase activity, cells with low reduced esterase activity and cells with inactive esterases.

Intracellular reactive oxygen species (ROS) production was measured using 2',7'-dichlorofluoresceindiacetate (DCFH-DA; Sigma-Aldrich, D6883; at a final concentration of 10 μ M). DCFH-DA is a cell-membrane permeable fluorescent stain that is a proxy of ROS production. Once inside the cells, DCFH-DA is hydrolyzed by esterase to form the non-fluorescent DCFH retained within the cell. DCFH can thus be oxidized by H₂O₂ and other oxidants to produce fluorescent 2',7'-dichlorofluorescein (DCF). Intracellular oxidation levels were correlated to DCF fluorescence within the cells and measurable by flow cytometry with F11 emission filter (green emission filter band pass, 530/30 nm). Samples were incubated with the stain for 20 min in the dark prior to flow-cytometry measurements. The relative ROS production is expressed as the mean F11 fluorescence per cell.

2.5 Pigments extraction and composition

For pigment analysis, 3.4×10^7 cells (25 mL of culture) of *C. muelleri* exposed to the filtrate or seawater were filtered over glass fiber filters (0.2 μ m, Whatman GF/F) after 30 and 60 min of exposure. Pigments were extracted from cells using methanol and analyzed by high-performance liquid chromatography (HPLC) according to the method described by (Ras et al., 2008), adapted from (Van Heukelem and Thomas, 2001). All the pigment standards were purchased from DHI (HØRSBOLM, Denmark).

2.6 Lipid analysis

For lipid class and fatty acid analyses, 3.4×10^8 cells (250 mL of culture) of the diatom were filtered over glass fiber filters (0.2 μ m Whatman GF/F filters were heated for 6 h at 450 °C prior to use) after 30 and 60 min of exposure. Boiling water was immediately passed through the filter after filtration to inhibit lipase activity. Lipids were then extracted with chloroform:methanol (2:1; v:v). Lipid extracts were stored at -20°C under nitrogen (N_{2(g)}) until analysis.

Lipid class composition analyses were performed by high-performance thin layer chromatography (HPTLC) using a CAMAG auto-sampler to spot the sample on HPTLC glass plates pre-coated with silica gel (Merck & Co., Ltd., Darmstadt, Germany). Neutral and polar lipid classes were analyzed according to (Moutel et al., 2016). For polar and neutral lipid fatty acid (FA) analysis, an aliquot of the chloroform: methanol (2:1, v:v) extract was dried under $N_{2(g)}$ and then resuspended in chloroform:methanol (98:2, v:v) prior to neutral and polar lipid separation. Separation of neutral and polar lipids was realized by solid phase extraction (Le Grand et al., 2014). Each fraction (polar and neutral lipids) was transesterified and the resulting fatty acid methyl esters (FAME) were analyzed and quantified by gas chromatography (GC-FID) according to the method from (Le Grand et al., 2014). Lipid class and fatty acid standards were the same standards used in (Le Grand et al., 2014). FA were expressed as percentage of total FA in polar and neutral lipids. The level of unsaturation was calculated from the % FA derived from the gas chromatographic data according to the equation:

$$Unsaturation\ level = \sum [\% \text{ of fatty acid} \times \text{number of double bond}]$$

2.7 Statistics

All statistical analyses were performed using R software (R Foundation for Statistical Computing, Vienna, 2011). A generalized linear model (Glm) was performed to evaluate the influence of time, *A. minutum* filtrate and their interaction on the maximum photosystem II quantum yield (F_v/F_m) of *C. muelleri*. The effects of filtrate on the different physiological and biochemical parameters (NPQ, flow-cytometry measurements, pigments, lipid classes and fatty acid composition) were assessed at each time. Statistical differences between seawater and filtrate were analyzed by a Student t-test after checking homoscedasticity assumption with a Bartlett test. When not meeting homoscedasticity assumption, metabolic activity (as a percentage of cells) was transformed with square root transformations. When homoscedasticity could still not be met, a non-parametric Wilcoxon test was applied. The Bray-Curtis similarity matrix was used for a one-way analysis of similarities (ANOSIM) to test whether samples within polar and neutral lipid fractions were clustered by condition (after 30 or 60 min exposures). ANOSIM calculated a global R statistic that weighs the differences between groups, with $R = 1$, $R = 0.5$ and $R = 0$ indicating a perfect, satisfactory, and poor separation of the

clusters, respectively (da Costa et al., 2017; González-Fernández et al., 2016). Principal component analyses (PCA) were performed to investigate the variation in fatty acid profile in *C. muelleri* exposed to seawater or to the allelochemicals after 30 or 60 min. Fatty acid (FA) data was analyzed with a similarity percentage analysis (SIMPER) performed on the relative FA content to identify the FAs accounting for 80% of dissimilarity between the groups and were shown on the PCA. All results are presented as mean \pm standard deviation (n = 3). Graphs were performed with the R package ggplot.

3 Results

3.1 Pulse Amplitude Modulated fluorescence

Overall, F_v/F_m strongly decreased during the exposure time in presence of the filtrate (Figure 1). The filtrate of *A. minutum* rapidly (< 20 min) modified *C. muelleri* maximum photosystem II quantum yield. Allelochemicals in the filtrate induced a temporary increase in F_v/F_m in the first 10 min of exposure compared to the controls. Following this, F_v/F_m steadily decreased to 0.41 ± 0.02 (27% inhibition) at 60 min, then increased to 0.45 ± 0.03 (20 % inhibition) after 100 min. In parallel, F_v/F_m from the seawater control culture remained stable between 0.58 and 0.56. After 45 min of exposure to *A. minutum* filtrate, non-photochemical quenching increased two-fold in filtrate exposures compared to controls (Figure 2).

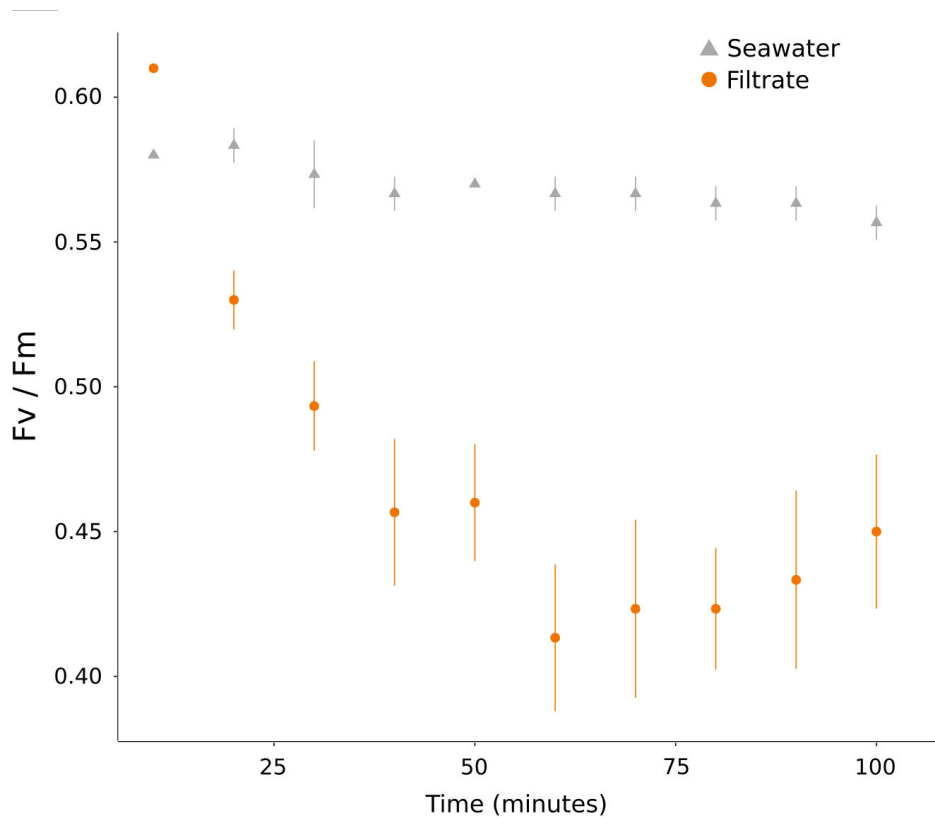


Figure 1: Maximum photosystem II quantum yield (F_v/F_m) of *C. muelleri* exposed to filtered seawater (grey triangles) and to *A. minutum* filtrate (orange dots). F_v/F_m significantly decreased over time (Glm t value = 7.469, p-value < 0.001) and a significant influence of the interaction between time and filtrate (Glm t value = -5.281, p-value < 0.001). Error bars represent the standard deviation (n = 3).

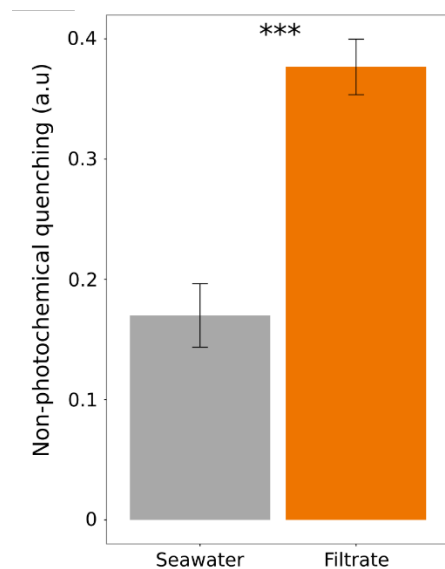


Figure 2: Non-photochemical quenching (NPQ; arbitrary unit) of *C. muelleri* after 45 min exposure to filtered seawater (control, grey bar) or *A. minutum* filtrate (orange bar) at a theoretical cell concentration equivalent to 6 x

10^3 cells mL^{-1} . Error bars represent the standard deviation ($n = 3$). ***refers to a significant difference (p -value < 0.001) between the control (seawater) and the filtrate.

3.2 Flow-cytometry parameters

Forward scatter (FSC; Figure 3A) of *C. muelleri* exposed to filtrate significantly decreased from 74.7 ± 0.6 to 67.2 ± 0.5 (arbitrary unit; a.u.) and 67.7 ± 8.7 a.u. to 43.8 ± 1.2 a.u. as compared to control after 60 and 90 min, respectively. Side scatter (SSC; Figure 3B) significantly decreased from 60.6 ± 8.3 a.u. (seawater) to 45.8 ± 0.5 a.u. (filtrate) after 90 min of exposure. Chlorophyll auto-fluorescence, as estimated by F13 fluorescence (Figure 3 C), steadily and significantly decreased in the cultures exposed to the filtrate compared the control cultures. After 30 min, F13 decreased from 1430 ± 9 a.u. in the control (seawater) to 1298 ± 19 a.u. with the filtrate (9% reduction). F13 fluorescence further decreased after 60 and 90 min when the inhibition reached 21 and 35%, respectively (1285 ± 122 a.u. for the control and 837 ± 44 a.u. when exposed to filtrate). A significant increase in intracellular ROS production (i.e. F11 fluorescence; Figure 3D) in the diatom cells was observed once exposed to filtrate. ROS production increased from 6.1 ± 0.3 a.u. in the control to 7.7 ± 0.5 a.u. when exposed to the filtrate for 30 min (26 % increase). ROS production kept increasing until 60 min to 9.5 ± 0.2 a.u. (63 % increase compared to the control at 5.8 ± 0.1 a.u. for the control), but by 90 min exposure, the ROS production from the exposed cells returned to control levels, 5.0 ± 0.1 a.u. and 5.2 ± 0.7 a.u., respectively.

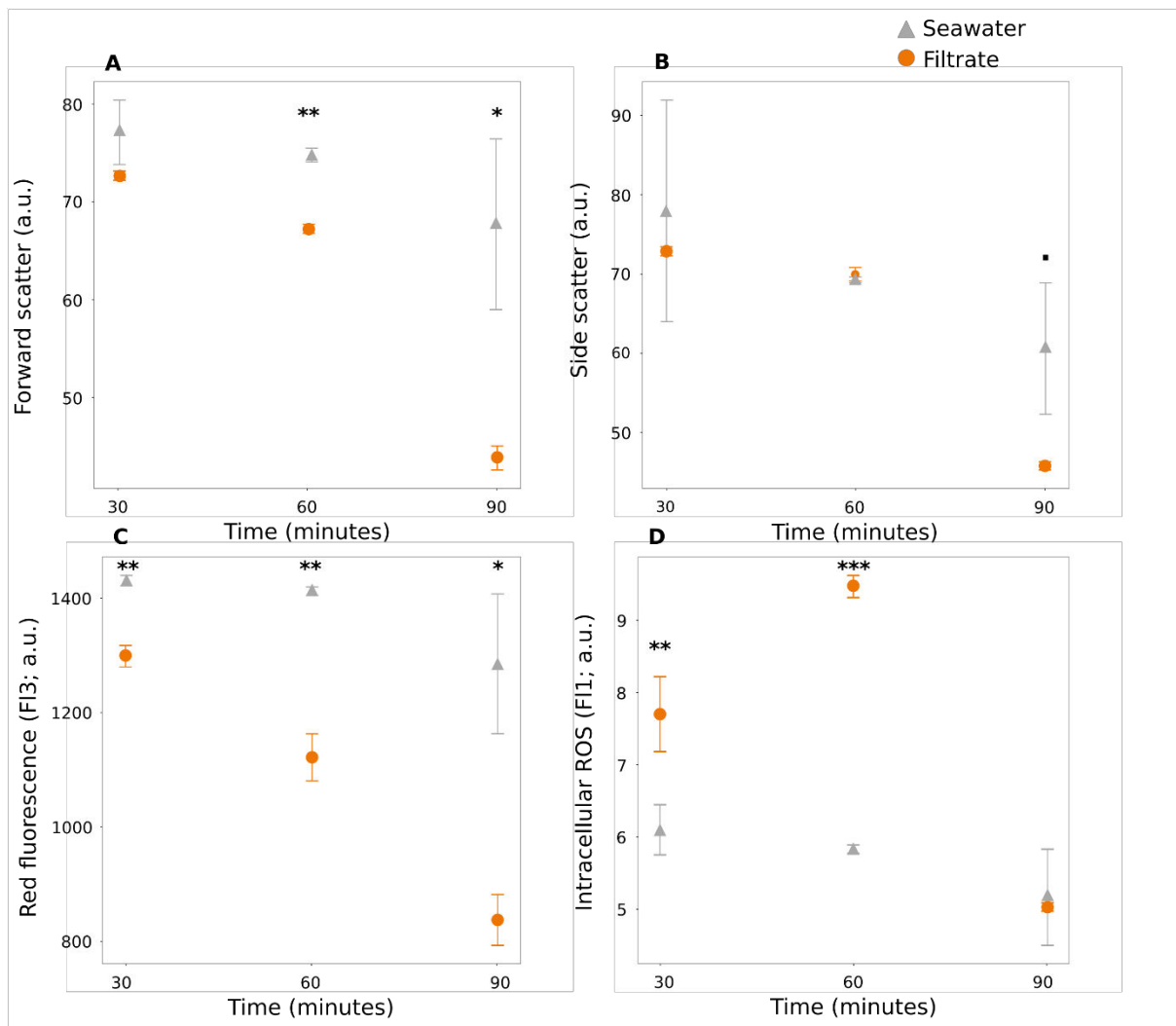


Figure 3 : Flow-cytometry parameters of *C. muelleri* for controls (seawater; grey triangles) and after exposure to *A. minutum* filtrate (orange dots) for 30, 60 and 90 min. A: Forward scatter (FSC), B: Side scatter (SSC), C: F13: red fluorescence, D: Reactive Oxygen Species (ROS) as measured by F11 fluorescence after DCFH-DA staining. Results are expressed as mean \pm standard deviation (n = 3). Statistical differences between seawater and filtrate at each different time are indicated as followed “.” 0.05 < p-value < 0.1, “*” 0.01 < p-value < 0.05, “**” 0.001 < p-value < 0.01, “***” p-value < 0.001.

Filtrate of *A. minutum* significantly increased the proportion of diatom cells with a reduced esterase activity (i.e. reduced metabolic activity) and cells with inactive esterases (i.e. no metabolic activity) compared to controls (Figure 4). The cells with a reduced esterase activity represented 25 ± 4 % of the population at 30 min and reached a plateau of 34 ± 5 % after 60 min of exposure. In the control culture, this population stayed below 4 ± 1 % throughout the 90 min bioassay. Some cells had no esterase activity in cultures exposed to *A. minutum* filtrate,

while in controls all cells presented esterase activity. The proportion of cells with no esterase activity increased from $3 \pm 1\%$ to $10 \pm 2\%$ and $13 \pm 1\%$ at 30, 60 and 90 min, respectively.

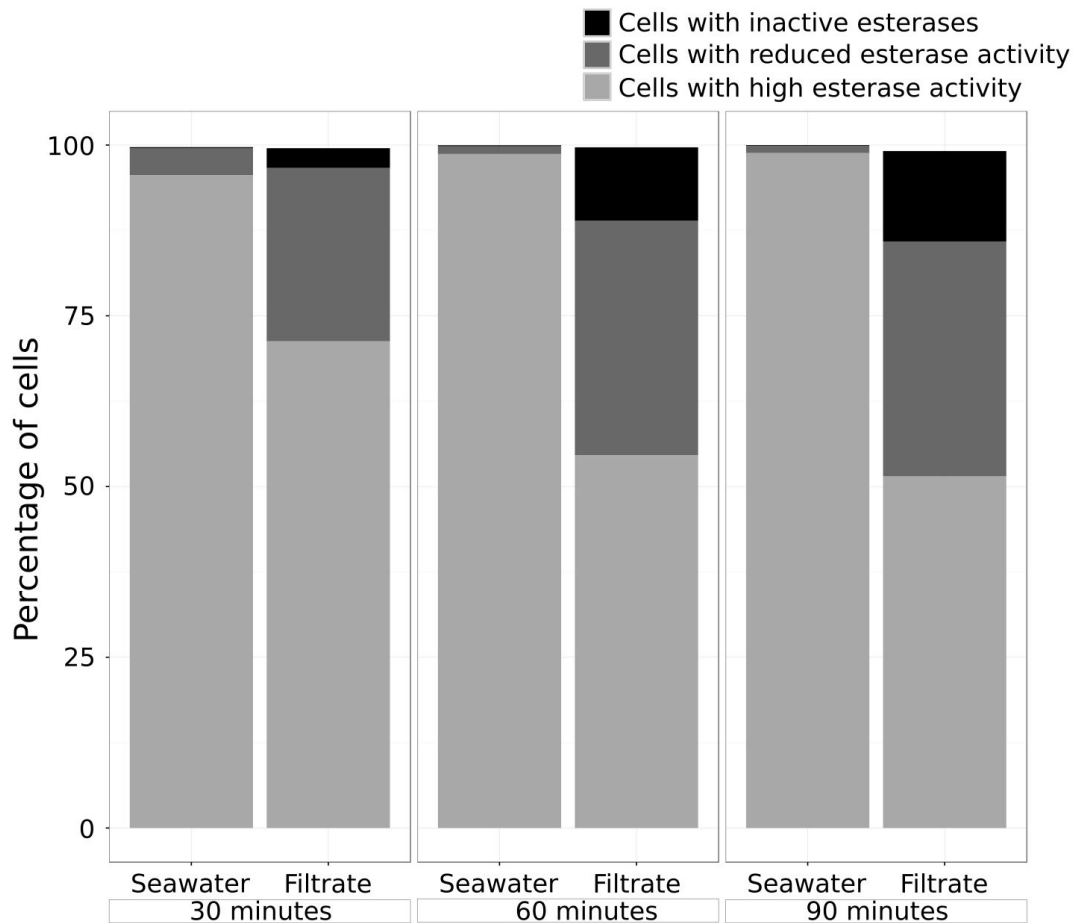


Figure 4: Metabolic activity of *C. muelleri* following exposure to control filtered seawater (seawater) or *A. minutum* filtrate (Filtrate) after 30, 60 and 90 min. Cells of *C. muelleri* are classified as cells with high esterase activity (light grey), reduced esterase activity (dark grey) and h inactive esterase (black).

3.3 Pigment composition

Among the 12 pigments identified, the amount (expressed as peak area) of 7 pigments significantly changed in the diatoms exposed to *A. minutum* filtrate (Table 1). Chlorophyll *a* (Chl *a*), chlorophyll C2 (Chl C2), diadinoxanthin (Ddx), and β -carotene decreased while allo-chlorophyll *a*, diatoxanthin (Dtx) and zeaxanthin (Zx) increased within 60 min of exposure. Changes occurred in pigments involved in xantophyll cycles with significant increases in zeaxanthin/violaxanthin (Zx/Vx) and diatoxanthin/diadinoxanthin (Dtx/Ddx) ratios. It is

noteworthy that among six minor pigments that were quantified but not identified, three of them decreased significantly in the diatom exposed to filtrate (data not shown).

Table 1: Pigment composition expressed as peak area (peak area corresponding to 7×10^5 cells). Peak areas are expressed as mean \pm standard deviation ($n = 3$). Statistical differences between seawater and filtrate are indicated as followed “•” $0.05 < p\text{-value} < 0.1$, “*” $0.01 < p\text{-value} < 0.05$, “**” $0.001 < p\text{-value} < 0.01$, “***” $p\text{-value} < 0.001$.

	30 min exposure		p-value	60 min exposure		p-value
	Seawater	Filtrate		Seawater	Filtrate	
Chl. α	2444 \pm 138	2399.4 \pm 102		2565 \pm 101	2296 \pm 27	*
Epi Chl. α	76 \pm 27	70 \pm 9		58 \pm 9	48 \pm 3	
Allo Chl. α	31 \pm 11	39 \pm 12		19 \pm 0	47 \pm 4	**
Chl. C1	781 \pm 42	743 \pm 18		736 \pm 25	735 \pm 6	
Chl. C2	834 \pm 54	799 \pm 53		893 \pm 37	747 \pm 10	*
Fucoxanthin	2781 \pm 71	2771 \pm 12		2801 \pm 105	2600 \pm 9	•
Diadinoxanthin	740 \pm 3	499 \pm 20	**	738.1 \pm 22.5	479.1 \pm 21.9	***
Diatoxanthin	94.6 \pm 15.6	326.0 \pm 22.3	***	102 \pm 12	263 \pm 11	***
Violaxanthin	12.5 \pm 0.5	13 \pm 1		14 \pm 1	13 \pm 1	
Zeaxanthin	20.4 \pm 0.9	33 \pm 1	***	21 \pm 1	28.8 \pm 0.9	***
Pheophytin α	6.7 \pm 0.6	7.0 \pm 0.4		7.2 \pm 0.2	6.8 \pm 0.1	•
β-carotene	263 \pm 10	266 \pm 4		272 \pm 11	241 \pm 5	*

Chl. α : Chlorophyll α , Allo Chl. α : Allo chlorophyll α , Chl. C1: Chlorophyll C1, Chl.C2: Chlorophyll C2.

3.4 Lipid class composition

After 30 min of filtrate exposure, there was no significant effect on the content of sterol esters, free sterols, triglycerides, alcohols and free fatty acids (FFA) (Table 2). After 60 min, the alcohol, sterol ester and free sterol content followed an increasing trend ($0.1 < p\text{-value} < 0.05$) while triglyceride (storage lipids) content followed a decreasing trend ($0.1 < p\text{-value} < 0.05$) in presence of filtrate. Phospholipids (phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylcholine (PC) levels significantly decreased after 30 min ($p\text{-value} < 0.01$). After 60 min, the cellular PC remained lower in cells exposed to the filtrate than in control cells ($0.1 < p\text{-value} < 0.05$). Monogalactosyl-diacylglycerol (MGDG) was the only glycolipid effected by the filtrate (decreased after 30 min exposure; $p\text{-value} < 0.05$), whereas sulfoquinovosyl-diacylglycerol (SQDG) and digalactosyl-diacylglycerol (DGDG) were unaffected.

Table 2: Total (polar plus neutral) lipid classes composition (pg cell⁻¹) of *C. muelleri* exposed to seawater (control) or to *A. minutum* filtrate (Filtrate). Results are expressed as mean \pm standard deviation. N = 3. Statistical differences between seawater and filtrate are indicated as followed “•” 0.05 < p-value < 0.1, “*” 0.01 < p-value < 0.05, “***” 0.001 < p-value < 0.01.

	30 min exposure			60 min exposure		
	Seawater	Filtrate	p-value	Seawater	Filtrate	p-value
Sterol ester	0.08 \pm 0.01	0.08 \pm 0.02		0.08 \pm 0.01	0.10 \pm 0.01	•
Trigly.	10 \pm 1	11.1 \pm 0.8		12 \pm 1	11.1 \pm 0.8	•
FFA	0.29 \pm 0.08	0.26 \pm 0.04		0.4 \pm 0.2	0.4 \pm 0.2	
Alcohols	0.08 \pm 0.01	0.08 \pm 0.02		0.08 \pm 0.01	0.10 \pm 0.01	•
Free Sterols	0.26 \pm 0.02	0.31 \pm 0.03		0.29 \pm 0.01	0.40 \pm 0.06	•
PI	0.03 \pm 0.00	0.05 \pm 0.01	•	0.06 \pm 0.02	0.05 \pm 0.03	
PE	0.43 \pm 0.06	0.38 \pm 0.03		0.43 \pm 0.03	0.41 \pm 0.01	
PC	1.12 \pm 0.02	0.61 \pm 0.06	**	0.80 \pm 0.09	0.6 \pm 0.1	•
MGDG	2.2 \pm 0.3	1.7 \pm 0.1	*	2.0 \pm 0.2	1.65 \pm 0.09	
SQDG	1.2 \pm 0.3	1.23 \pm 0.08		1.38 \pm 0.08	1.18 \pm 0.09	
DGDG	0.25 \pm 0.06	0.25 \pm 0.02		0.28 \pm 0.01	0.26 \pm 0.02	
Σ lipids	16 \pm 2	16 \pm 1		17.9 \pm 0.7	16.2 \pm 0.9	

Trigly.: Triglyceride, FFA: Free fatty acids, PI: Phosphatidylinositol, PE: Phosphatidylethanolamine, PC: Phosphatidylcholine, MGDG: Monogalactosyl-diacylglycerol, SQDG, Sulfoquinovosyl-diacylglycerol, DGDG: Digalactosyl-diacylglycerol.

3.5 Fatty acid composition

Polar fatty acid composition was differed greatly when *C. muelleri* were exposed to seawater or to the allelochemicals after 30 min (global R: 1, significance = 0.09, ANOSIM) and 60 min (global R: 0.7, significance = 0.09, ANOSIM), with changes in the proportion of different fatty acids. The composition of neutral fatty acids was also differed greatly between seawater and filtrate exposures after 30 min (global R: 0.7, significance = 0.12, ANOSIM) and to a smaller extent after 60 min (global R: 0.48, significance = 0.09, ANOSIM). Overall, the presence of allelochemicals induced a decrease in the unsaturation index of polar lipids (Table 3) and in neutral FA content (Table 4). Polar FA composition change was observed within 30 min of filtrate exposure to the filtrate, while it took 60 min of exposure to the filtrate to modify the neutral FA composition. There were 8 fatty acids primarily effected by the allelochemicals, which accounted for 80% of differencies (Simper analysis) with controls. Filtrate exposure

decreased the proportions of 22:6n-3, 20:4n-6, 20:5n-3, 16:3n-4, whereas the proportions of 18:1n-7, 16:1n-7, 16:0 and 14:0 were increased in presence of *A. minutum* filtrate (Figure 5).

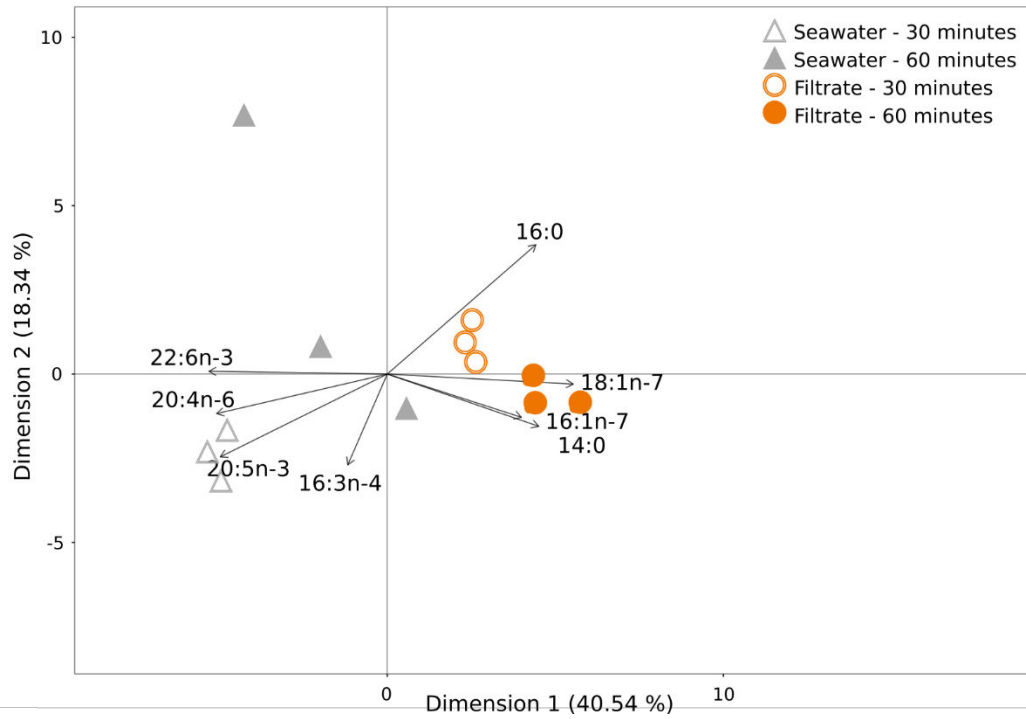


Figure 5: Principal component analysis (PCA) of polar fatty acid (FA) composition between *C. muelleri* cultures in presence of filtered seawater (grey triangles) or *A. minutum* filtrate (orange dots). Grey triangles represent samples exposed to filtered seawater after 30 min (empty triangles) and 60 min (filled triangles) of exposure. Orange dots represent samples exposed to *A. minutum* filtrate after 30 min (empty dots) and 60 min (filled dots). Dimension 1 represents 40.54% of variance and dimension 2 represents 18.34% of variance. FA shown explain 80% of differences (Simpser analysis).

Table 3: Polar fatty acids (FA) composition (mass % of total polar fatty acids). The total mass (μg) correspond to the mass of 10^6 cells. Results are expressed as mean \pm standard deviation ($n = 3$). P-value from the statistical tests between seawater and filtrate at each different time are indicated as followed “•” $0.05 < p\text{-value} < 0.1$, “*” $0.01 < p\text{-value} < 0.05$, “**” $0.001 < p\text{-value} < 0.01$, “***” $p\text{-value} < 0.001$.

	30 min exposure			60 min exposure		
	Seawater	Filtrate	p-value	Seawater	Filtrate	p-value
iso15:0	0.44 \pm 0.02	0.68 \pm 0.02	***	0.5 \pm 0.1	0.75 \pm 0.05	*
ant15:0	0.21 \pm 0.02	0.26 \pm 0.01	•	0.25 \pm 0.07	0.31 \pm 0.03	
iso17:0	0.64 \pm 0.01	0.73 \pm 0.00	**	0.75 \pm 0.05	0.77 \pm 0.05	
14:0	8.3 \pm 0.3	8.8 \pm 0.2	•	8.6 \pm 0.8	9.0 \pm 0.2	
15:0	0.58 \pm 0.01	0.62 \pm 0.01	**	0.61 \pm 0.02	0.63 \pm 0.03	
16:0	10.2 \pm 0.1	11.1 \pm 0.1	***	10.8 \pm 0.2	11.02 \pm 0.03	
18:0	1.22 \pm 0.01	1.30 \pm 0.01	**	1.5 \pm 0.3	1.5 \pm 0.2	
22:0	0.21 \pm 0.01	0.23 \pm 0.03		0.20 \pm 0.06	0.21 \pm 0.01	
24:0	0.2 \pm 0.0	0.2 \pm 0.2		0.17 \pm 0.00	0.16 \pm 0.01	•
Σ SFA	22.1 \pm 0.3	24.1 \pm 0.2	**	23.6 \pm 0.6	24.5 \pm 0.2	
16:1n-7	14.00 \pm 0.2	14.7 \pm 0.2	*	14.2 \pm 0.6	14.4 \pm 0.5	
16:1n-5	0.21 \pm 0.01	0.22 \pm 0.00	•	0.22 \pm 0.00	0.23 \pm 0.01	
18:1n-9	2.0 \pm 0.1	1.93 \pm 0.04		2.12 \pm 0.05	1.90 \pm 0.08	*
18:1n-7	2.98 \pm 0.02	3.7 \pm 0.1	*	3.2 \pm 0.2	4.1 \pm 0.3	**
18:1n-5	0.28 \pm 0.03	0.34 \pm 0.04	•	0.28 \pm 0.05	0.37 \pm 0.03	*
Σ MUFA	19.7 \pm 0.2	21.24 \pm 0.04	**	20.2 \pm 0.7	21.3 \pm 0.8	
16:2n-7	1.64 \pm 0.02	1.73 \pm 0.05	•	1.77 \pm 0.03	1.78 \pm 0.08	
16:2n-4	2.09 \pm 0.04	2.15 \pm 0.03		2.10 \pm 0.05	2.2 \pm 0.1	
16:3n-4	8.9 \pm 0.2	8.4 \pm 0.2	•	8.9 \pm 0.2	8.9 \pm 0.2	
16:4n-1	0.31 \pm 0.01	0.27 \pm 0.01	**	0.30 \pm 0.01	0.30 \pm 0.01	
18:2n-7	0.16 \pm 0.00	0.18 \pm 0.00	**	0.18 \pm 0.00	0.18 \pm 0.01	
18:2n-6	1.98 \pm 0.02	1.91 \pm 0.05		2.05 \pm 0.03	1.79 \pm 0.11	*
18:3n-6	6.14 \pm 0.04	6.09 \pm 0.16		6.3 \pm 0.1	5.7 \pm 0.4	•
18:4n-3	2.90 \pm 0.00	2.78 \pm 0.04	•	2.88 \pm 0.08	2.74 \pm 0.06	•
20:3n-6	0.22 \pm 0.01	0.17 \pm 0.00	***	0.22 \pm 0.02	0.16 \pm 0.01	*
20:4n-6	9.2 \pm 0.2	8.2 \pm 0.2	**	8.5 \pm 0.1	7.5 \pm 0.5	•
20:4n-3	0.18 \pm 0.00	0.16 \pm 0.01	*	0.15 \pm 0.02	0.16 \pm 0.00	
20:5n-3	20.2 \pm 0.1	17.9 \pm 0.2	***	18.0 \pm 0.3	17.7 \pm 0.1	
22:5n-6	0.18 \pm 0.01	0.20 \pm 0.00	*	0.20 \pm 0.02	0.17 \pm 0.03	
22:5n-3	0.14 \pm 0.00	0.12 \pm 0.00	**	0.14 \pm 0.01	0.12 \pm 0.00	
22:6n-3	3.06 \pm 0.04	2.71 \pm 0.12	*	2.9 \pm 0.2	2.4 \pm 0.2	*
Σ PUFA	57.5 \pm 0.3	53.2 \pm 0.4	***	54.8 \pm 0.6	52.8 \pm 0.4	*
Unsaturation index	230 \pm 1	210 \pm 2	***	216 \pm 3	206 \pm 2	*
Mass (μg)	490 \pm 10	410 \pm 50	.	446 \pm 3	426 \pm 86	

SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids.

Table 4: Neutral fatty acids (FA) composition (mass % of total neutral fatty acids). The total mass (μg) correspond to the mass of 10^6 cells. Results are expressed as mean \pm standard deviation ($n = 3$). P-value from the statistical tests (student t test or wilcoxon test) between the conditions at each different time are indicated as followed “•” $0.05 < p\text{-value} < 0.1$, “*” $0.01 < p\text{-value} < 0.05$, “**” $0.001 < p\text{-value} < 0.01$.

	30 min exposure			60 min exposure		
	Seawater	Filtrate	p-value	Seawater	Filtrate	p-value
iso15:0	0.17 \pm 0.02	0.17 \pm 0.02		0.14 \pm 0.04	0.21 \pm 0.02	•
14:0	12.6 \pm 0.2	12.6 \pm 0.1		12.8 \pm 0.2	12.8 \pm 0.1	
15:0	0.98 \pm 0.01	0.98 \pm 0.00		0.97 \pm 0.01	1.0 \pm 0.1	
16:0	18.8 \pm 0.2	18.5 \pm 0.1		18.6 \pm 0.2	18.3 \pm 0.2	
18:0	1.69 \pm 0.06	1.67 \pm 0.06		1.68 \pm 0.03	1.65 \pm 0.04	
20:0	0.12 \pm 0.01	0.11 \pm 0.00		0.11 \pm 0.00	0.11 \pm 0.00	*
22:0	0.20 \pm 0.00	0.19 \pm 0.01		0.19 \pm 0.01	0.17 \pm 0.00	
24:0	0.21 \pm 0.02	0.19 \pm 0.01		0.21 \pm 0.01	0.19 \pm 0.02	
Σ SFA	35.0 \pm 0.1	34.65 \pm 0.09	*	34.9 \pm 0.1	34.6 \pm 0.3	
15:1n-5	0.19 \pm 0.02	0.20 \pm 0.02		0.16 \pm 0.01	0.22 \pm 0.00	**
16:1n-7	35.4 \pm 0.2	35.01 \pm 0.03	•	35.06 \pm 0.05	34.82 \pm 0.40	
16:1n-5	0.34 \pm 0.00	0.35 \pm 0.00		0.33 \pm 0.00	0.34 \pm 0.00	•
18:1n-9	0.81 \pm 0.02	0.81 \pm 0.01		0.79 \pm 0.01	0.79 \pm 0.01	
18:1n-7	0.36 \pm 0.01	0.37 \pm 0.01		0.35 \pm 0.00	0.36 \pm 0.00	
Σ MUFA	37.3 \pm 0.2	36.89 \pm 0.02	•	36.85 \pm 0.08	36.7 \pm 0.4	
16:2n-7	1.43 \pm 0.02	1.46 \pm 0.01		1.42 \pm 0.02	1.47 \pm 0.01	*
16:2n-4	0.92 \pm 0.01	1.00 \pm 0.02	**	0.95 \pm 0.00	0.7 \pm 0.6	
16:3n-4	1.14 \pm 0.03	1.6 \pm 0.1	*	1.29 \pm 0.02	1.6 \pm 0.1	*
16:3n-1	0.40 \pm 0.03	0.42 \pm 0.04		0.39 \pm 0.02	0.48 \pm 0.01	**
18:2n-6	0.87 \pm 0.02	0.86 \pm 0.00		0.86 \pm 0.00	0.8 \pm 0.1	
18:3n-6	3.17 \pm 0.04	3.21 \pm 0.01		3.12 \pm 0.01	3.11 \pm 0.05	
18:4n-3	2.02 \pm 0.02	2.03 \pm 0.02		1.99 \pm 0.02	2.08 \pm 0.02	**
20:3n-6	0.39 \pm 0.01	0.38 \pm 0.01		0.38 \pm 0.00	0.37 \pm 0.02	*
20:4n-6	4.86 \pm 0.09	4.83 \pm 0.09		5.03 \pm 0.04	4.77 \pm 0.09	*
20:4n-3	0.31 \pm 0.01	0.30 \pm 0.01		0.30 \pm 0.01	0.30 \pm 0.00	
20:5n-3	9.5 \pm 0.2	9.61 \pm 0.05		9.78 \pm 0.04	9.7 \pm 0.3	
22:5n-6	0.02 \pm 0.00	0.02 \pm 0.00		0.02 \pm 0.00	0.02 \pm 0.00	
22:5n-3	0.13 \pm 0.01	0.12 \pm 0.01		0.13 \pm 0.00	0.12 \pm 0.00	*
22:6n-3	0.58 \pm 0.02	0.55 \pm 0.02		0.56 \pm 0.02	0.55 \pm 0.01	
Σ PUFA	21.9 \pm 0.3	21.9 \pm 0.1		22.25 \pm 0.03	22.0 \pm 0.4	
Unsaturation index	107 \pm 1	109.3 \pm 0.2	*	109.6 \pm 0.2	107 \pm 2	
Mass (μg)	1121 \pm 111	899 \pm 47	•	1111 \pm 142	746 \pm 171	*

SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids.

4 Discussion

4.1 *Alexandrium minutum* allelochemicals are fast acting

This study demonstrated several significant effects (Figure 6) of *A. minutum* allelochemicals on the metabolism (decrease in esterase activity and increase in ROS production), photosynthesis (decrease in maximum PSII quantum yield and increase of non-photochemical quenching) and membrane composition (changes in membrane lipids and pigments) in the diatom *C. muelleri* as compared to the controls. Overall, allelochemicals induced a decrease in metabolism within an hour, as highlighted by the inhibition of esterase activity and photosynthesis of the diatom *C. muelleri*, thus confirming that *A. minutum* allelochemicals are fast acting (Lelong et al., 2011; Tillmann et al., 2007). Moreover, this study highlights that photosynthesis was affected within a short time (< 30 min), as was the primary metabolism, as estimated by the esterase activity decrease after only 30 min. Indeed, 60 min was sufficient to almost completely cease esterase activity, down to 10% of the unexposed seawater control cells. This highlights the usefulness of analyzing cellular effects at a minute scale to elucidate the allelochemicals mode of action, which eventually lead to cell lysis. The combination of fluorescence, flow-cytometry and biochemical analysis of diatom physiology provided new insights on the short-term (within 30 min) cellular and biochemical mechanisms involved in allelochemical effects previously reported in *A. minutum* (Lelong et al., 2011) and *A. tamarense* (Ma et al., 2011; Zheng et al., 2016).

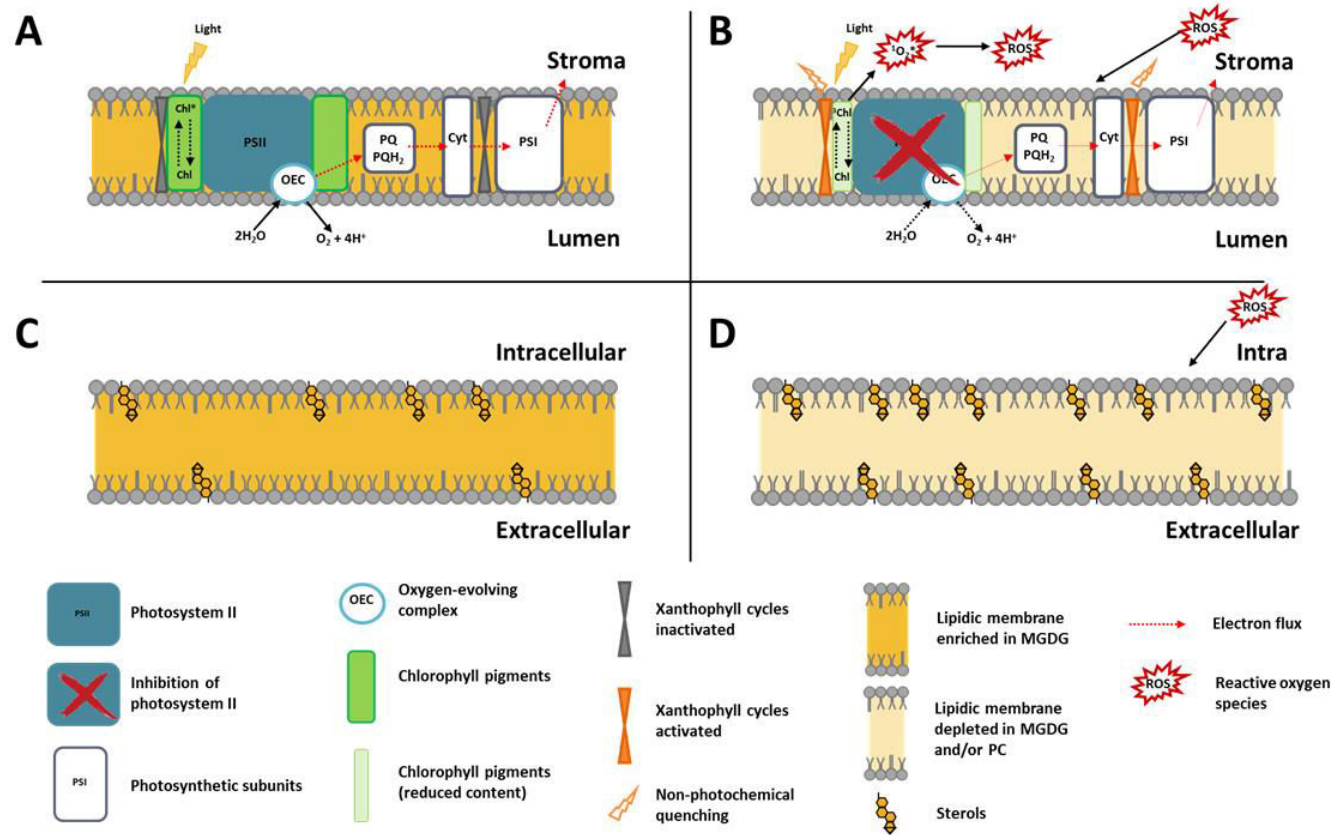


Figure 6 : Diagram of the mechanisms of *A. minutum* allelochemicals on photosynthetic (A and B) and cytoplasmic (C and D) membranes of *C. muelleri* in presence of seawater (A and C) and allelochemicals (B and D). Mechanisms are described in the last “general view” paragraph of discussion. PSII: Photosystem II, PSI: Photosystem I, PQ: Plastoquinone, Cyt, Cytochrome, OEC: Oxygen-evolving complex, Chl: Chlorophyll, PC: Phosphatidylcholine, MGDG: Monogalactosyl-diacylglycerol, ROS: Reactive oxygen species.

4.2 Effects of allelochemicals on photosystem II

A decrease in the maximum photosystem II (PSII) quantum yield (F_v/F_m) of *C. muelleri* was observed within 20 min of exposure to *A. minutum* filtrate, highlighting rapid deleterious effects to the PSII and supporting previous studies (Lelong et al., 2011; Tillmann et al., 2007). (Lelong et al., 2011) highlighted that the decrease of photosynthetic efficiency resulted from an inactivation of PSII reaction centers (RCs) and a decrease of electron transport through PSII. Inhibition of photosynthesis was also reported through a transcriptomic analysis of the diatom *Phaeodactylum tricorutum* when co-cultured for 24 h with an allelochemical strain of *A. tamarense* (Zheng et al., 2016). The authors hypothesized that the oxygen evolving complex (OEC) that catalyzes the oxidation of water to molecular oxygen was inhibited. This damage to PSII would result in an electron flux reduction through PSII and in the decreased quantum yield measured in our study. Effects of allelochemicals on the PSII of the diatom mimic the deleterious effects reported under excessive light intensity (i.e. photoinhibition). During photoinhibition, PSII are deactivated via two main steps: first OEC are damaged by light and deactivated, then PSII RCs are deactivated due to excess light absorption (Hakala et al., 2005; Nishiyama et al., 2006; Ohnishi et al., 2005). With less active RCs, the energy absorbed by chlorophyll pigments cannot be utilized for photochemistry (i.e. photosynthetic reactions) and accumulates, becoming an excess. Our results highlighted the deactivation of photosynthesis, however regardless of PSII inhibition, in presence of light, chlorophyll (Chl) pigments absorb photons and move to an excited state ($^1\text{Chl}^*$). Excess light absorption can induce the production of toxic $^3\text{Chl}^*$ that reacts with O_2 to produce the superoxide ion ($^1\text{O}_2^*$), a highly reactive oxygen species (ROS) (Müller et al., 2001). Similarly, during our study a transitory increase of ROS production was observed upon exposure to *A. minutum* allelochemicals. The excess energy can be “safely” dissipated through fluorescence emission or heat dissipation (non-photochemical quenching; NPQ) as observed in our study and described below.

4.3 Effects of allelochemicals on pigments

Significant decreases in chlorophyll *a* and chlorophyll C2 content and red fluorescence (F13; 630 nm), in parallel with an increase in the chlorophyll (Chl) degradation product (allo-chlorophyll *a*) was observed after only 60 min of filtrate exposure (Figure 6 A and B). Bleaching (i.e. loss of cell pigmentation) of microalgal cells exposed to *Alexandrium* spp. filtrate has been

previously reported (Lelong et al., 2011; Lyczkowski and Karp-Boss, 2014), and this phenomenon is likely related to a decrease of Chl content as reported here. Induction of Chl degradation during allelochemical interactions has been recently shown (Yang et al., 2017; Zhou and Yu, 2006) and is a common photoacclimation process (Deblois et al., 2013; Dubinsky and Stambler, 2009; Sui and Han, 2014). We speculate that the decrease of Chl pigments results in a decrease in light harvesting antenna size. By decreasing the number of photons absorbed, the excess energy received by PSII would be decreased, mitigating photoinhibition and subsequent associated energy cost (Raven, 2011) as well as excessive ROS production and cell damages.

4.4 Effects of allelochemicals on non-photochemical quenching

Upon allelochemical exposure, the increase in non-photochemical quenching (NPQ) of *C. muelleri* is hypothesized to dissipate excess energy from damaged PSII through heat. Allelochemicals also induced a decrease in β -carotene and changes in xanthophyll pigments ratios (diadinoxanthin/diatoxanthin and violaxanthin/zeaxanthin; Figure 6B) within photosynthetic membranes. Heat dissipation is mediated in thylakoid membranes by carotenoids and particularly xanthophyll pigment cycles (Goss and Jakob, 2010). The diadinoxanthin (Ddx) and violaxanthin (Vx) can be de-epoxidated in diatoxanthin (Dx) and zeaxanthin (Zx), respectively, to dissipate excess energy through heat emission (non-photochemical quenching; (Behrenfeld and Milligan, 2013; Goss and Jakob, 2010)). In the current study, activation of xanthophyll cycles, as demonstrated by increases in Dx/Ddx and Zx/Vx ratios, in the presence of allelochemicals was observed after both 30 and 60 min of filtrate exposure. While the increase in Dx was linked to a decrease in Ddx, Vx content did not change along with the increase in Zx content. *De novo* synthesis of Vx from β -carotene, a Vx precursor (Fraser and Bramley, 2004), may explain the homeostasis of Vx.

4.5 Effects of allelochemicals on lipids

The lipid composition of photosynthetic membranes, which physicochemical properties greatly influence NPQ and indeed diadinoxanthin (Ddx) and violaxanthin (Vx) de-epoxidation reactions (Latowski et al., 2011). These lipids were significantly affected by the presence of allelochemicals (Fig 6A and 6B). Monogalactosyldiacylglycerol (MGDG), a non-bilayer glycolipids exclusively found in thylakoid membranes, decreased in the presence of

allelochemicals. Previous studies highlighted that MGDG allows xanthophyll solubilization and promotes de-epoxidation (Goss et al., 2009, 2005; Wilhelm et al., 2014). In parallel, a decrease in phosphatidylcholine (PC) content, a significant constituent of thylakoid membranes (Kates, 1987; Lepetit et al., 2010; Vieler et al., 2007), was also observed in the presence of allelochemicals. Similar decreases of glycolipids along with a decrease in PC were observed during the photoinhibition process (Lepetit et al., 2010; Wilhelm et al., 2014). The modifications in the biochemical composition of photosynthetic membranes are another change similar to photoinhibition processes.

Changes in lipid class composition occurred alongside significant modifications in the fatty acid (FA) composition of both polar and neutral lipids. Filtrate of *A. minutum* reduced the cellular content of neutral fatty acids, mainly bound to storage lipids (i.e. triglyceride that represents more than 90% of neutral lipids), by more than 20% of its mass. This decrease in neutral FAs may be linked to the decrease in triglyceride and result from an energy mobilization to cope with the energy cost induced by allelochemicals such as lipid synthesis and de-epoxidation reactions, or from the mobilization of FAs for the synthesis of membrane FAs (Muhlroth et al., 2013). Exposure to *A. minutum* allelochemicals also reduced the unsaturation levels of polar lipid fatty acids which are the constituents of biological membranes. The decrease of membrane lipid unsaturation mainly results from the decreases of eicosapentaenoic acid (EPA) 20:5n-3 and 16:3n-4, accounting for 38% of total polar lipid FA. This decrease in polyunsaturated fatty acids (PUFA) paralleled the decrease in PC and MGDG, mainly constituted in 20:5n-3 and 16:3n-4, respectively (Da Costa et al., 2016; Guella et al., 2003; Li et al., 2014). In the diatom *Phaeodactylum tricornutum*, 20:4n-6 was only found associated to MGDG and PC and to a smaller extent to DGDG (Arao et al., 1987). Its decrease also occurred in parallel with the general decrease in PC and MGDG observed in the presence of allelochemicals. Concomitantly, proportions of other major FA (14:0, 16:0, 16:1n-7, 18:1n-7) increased.

Sterols are predominant constituents of plasma membranes (Hartmann and Benveniste, 1987; Hartmann, 1998) with many important roles including the regulation of membrane fluidity and permeability (Dufourc, 2008), signal transduction, modulation of enzyme activity and precursors of secondary metabolites (Hartmann, 1998). In microalgae, sterol can increase under increasing light (Gordillo et al., 1998; Piepho et al., 2010), indeed sterols may have a role under

photoinhibition. The slight increase of sterols in the presence of filtrate, as observed under photoinhibition-like conditions, may confirm such a hypothesis. At the same time, some sterol classes are known to potentially act against antimicrobial compounds with membrane-disruptive activity (Dufourc, 2008; Mason et al., 2007). Authors have hypothesized that *A. tamarense* allelochemicals may be membrane-disruptive lytic compounds with a high affinity for sterols and that the molecular targets involve sterol components (Ma et al., 2011). In the same study, the authors showed that allelochemicals had different affinities to different phytosterols and speculated that the composition of the outermost cellular membrane plays a role in tolerance to allelochemicals. Yet, in our study, sterols increased in presence of allelochemicals. While this may reflect a protective role of sterols, the true role of sterols in allelochemical interactions should be addressed in future studies as it could be a membrane feature driving allelochemical tolerance.

4.6 Effects of allelochemicals on ROS

Exposure of the diatom to *A. minutum* filtrate resulted in a relative increase in intracellular reactive oxygen species (ROS) during the first hour. Photosynthesis is one of the processes inducing ROS production that can damage photosystem II (Asada, 1999; Müller et al., 2001; Nishiyama et al., 2006). However, it is unlikely that the diatoms' photosystem II is the only source of ROS as a ROS increase was also reported in non-photosynthetic cells exposed to *A. minutum* exudates (Flores et al., 2012; Le Goïc et al., 2014). The two-fold ROS increase in *C. muelleri* exposed to allelochemicals may have led to some lipid peroxidation (yet to be measured). Lipid peroxides can act as a signal inducing programmed cell death (Yang et al., 2017) and cell cycle blockage (Farmer and Mueller, 2013) which was shown in *Phaeodactylum tricornutum* co-cultured with *A. tamarense* (Zheng et al., 2016), where ROS production returned to levels similar to control conditions after 90 min. While this decrease may be linked to the decrease in esterase activity, it is also hypothesized that diatoms may cope with ROS stress through activation of antioxidant systems (Li et al., 2015; Yang et al., 2017) such as xanthophyll pigments (Lepetit et al., 2012, 2010; Wilhelm et al., 2014), or through saturation of membranes. Xanthophyll pigments not only play a role in dissipation of energy, as mentioned above, but can also act as antioxidants, especially against ROS produced at PSII oxygen

evolving complex (Wilhelm et al., 2014). Indeed, xanthophyll cycle may scavenge intracellular ROS induced by the presence of allelochemicals.

4.7 General view

Based on our results and previous studies (Lelong et al., 2011; Ma et al., 2011; Zheng et al., 2016) we propose a conceptual model to describe the mechanisms of action of *Alexandrium* spp. allelochemicals (Figure 6). Alteration of physiological functions such as photosynthesis and primary metabolism may result from direct damage to external membranes. Such damage may lead to the loss of cell integrity as shown by (Ma et al., 2011). Here we propose that the membrane disruption observed by (Ma et al., 2011; Zheng et al., 2016) leads to an inhibition of photosynthesis. Membrane disruption was previously reported to lead to inhibition of *Fv/Fm* and electron transport (Yang et al., 2017). Following the inactivation of photosystem II, the energy trapped by chlorophyll pigments cannot be used for photochemistry. Thus photons become excessive within the cells and may induce an increase in ROS production by the cell. Photoinhibition-like processes appeared to be activated by the cells to mitigate the stress. The observed reduction of chlorophyll pigments is hypothesized to limit the energy trapped by the cell, together with the xanthophyll cycles to eliminate excess energy through heat (non-photochemical quenching) and to scavenge ROS. These changes of pigment content and ratio are accompanied by a decrease in MGDG and saturation of photosynthetic membranes. In parallel, plasma membranes were also modified as demonstrated by the increase in free sterols. While the role of sterols has yet to be investigated, they may help the cell to maintain membrane integrity and associated enzyme activity or act as protection mechanisms against membrane disruption. ROS increase is likely to be a key factor in understanding allelochemical interactions. Their origin, effects and role remain unclear, and the mechanisms involving ROS should be further studied. The main pending issues are to understand how the allelochemicals interact with cell membranes, and how it is related to the sensitivity of some species to better understand their ecological implications. Overall, the mode of action of allelochemicals has to be further investigated from the minute scale effects to fully understand the mechanisms leading to microalgal cell death.

5 Conclusion

In conclusion, our results bring new insights on allelochemical interaction mechanisms and indicate that several biochemical pathways and structures were rapidly impaired resulting in photoinhibition-like responses. Even though allelopathy does not automatically lead to cell death, sub-lethal effects, such as photoinhibition, ROS production, decreased metabolism or decreased energy absorption, would have a significant energy cost. For example, the cost of photoinhibition can lead to a decreased growth rate. Overall the allelochemical interactions would also impair the ability of the exposed cells to cope with other environmental stress, thus decreasing competitiveness and fitness. The sub-lethal markers reported here are valuable tools in assessing the importance and ecological roles of allelopathy in structuring plankton communities.

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Article 2

Allelochemical interactions between *Alexandrium minutum* and *Chaetoceros muelleri*: insights into photosynthesis and membrane integrity

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Abstract

Allelochemicals from the toxic dinoflagellate *Alexandrium minutum* have deleterious effects on diatoms, inhibiting metabolism and photosynthesis. The precise mechanisms of allelochemical interactions and the molecular target of allelochemicals remain unknown. In this study, we investigated the short-term effects of *A. minutum* allelochemicals simultaneously on the cytoplasmic membrane integrity (depolarization and permeability) of the diatom *Chaetoceros muelleri* using flow-cytometry and on the different components of the photosynthetic electron transfer chain using fluorescence and absorption spectroscopy. The allelochemicals first induced a depolarization (associated with an increasing permeabilization) of the cytoplasmic membranes and an inhibition of the photosynthetic electron transfer between photosystem 2 and photosystem 1, impairing the photochemical rate of the diatom. Within 30 minutes of exposure, the cytoplasmic membranes of 50% of the microalgal cells were permeable and the efficiencies of the photosystems I and II were diminished. The relationship of cytoplasmic membranes disruption with the inhibition between the two photosystems still eludes us and warrants further investigation.

1 Introduction

Harmful algal blooms (HAB) refer to the proliferation of microalgae with negative consequences such as ecological, public health or economic issues (contamination of seafood, human poisoning). Toxic dinoflagellates from the genus *Alexandrium* produce paralytic shellfish toxins and unknown extracellular secondary metabolites with various biological activities (Arzul et al., 1999; Castrec et al., 2018; Dorantes-Aranda et al., 2015) including allelochemical activity (Long et al., 2018a; Tillmann and Hansen, 2009; Tillmann and John, 2002). In marine environments, allelochemical interactions can be defined as the negative effects of a protist on competing protists, bacteria or predators through the release of allelochemicals in their surrounding environment. Allelochemical interactions can influence the structure of plankton communities (Fistarol et al., 2003; Inderjit et al., 2011) by inhibiting or favoring different species (Cembella, 2003; Legrand et al., 2003).

In aquatic systems, our understanding of the ecological role of allelochemical interactions on plankton dynamics is hindered by the poor knowledge of the chemical nature and modes of action of allelochemicals. The reported allelochemical effects include growth inhibition (Chen et al., 2015; Paul et al., 2009; Pushparaj et al., 1998; Suikkanen et al., 2011; Ternon et al., 2018), damage to cell membrane or cell lysis (Hakanen et al., 2014; Ma et al., 2009; Poulin et al., 2018; Prince et al., 2008; Tillmann et al., 2007), and inhibition of photosystem II (Gantar et al., 2008;

Long et al., 2018b; Poulin et al., 2018; Prince et al., 2008; Ternon et al., 2018; Tillmann et al., 2007). There are also cases where the allelochemical interactions between the dinoflagellates *Alexandrium* spp. (probably the most studied one within phytoplankton) and other protists, lead to the disruption of membranes, modification of membrane biochemistry, inhibition of photosynthesis and esterase activity, increased production of reactive oxygen species (ROS) (Flores et al., 2012; Lelong et al., 2011; Long et al., 2018a and b; Ma et al., 2011; Tillmann et al., 2007). However, the sequence of physiologic events occurring is unknown, in part because of the use of different algal couples (*A. minutum*/*Chaetoceros muelleri*; *A. tamarensis*/*Rhodomonas salina*; *A. tamarensis*/*Tiarina fusus*; *A. tamarensis*/*Polykrikos kofoidii*). For instance, disruption of membranes was shown for *A. tamarensis* (Ma et al., 2011) but was not investigated for *A. minutum*.

The inhibition of PSII is probed in a quasi-routine manner simply because PSII activity is easy to measure with the very sensitive and widespread fluorescence techniques. A modification of the integrity of the PSII has been reported for diatoms in the presence of *A. minutum* filtrate (Lelong et al., 2011; Long et al., 2018b), and in other allelochemical interactions (Hagmann and Jiittner, 1996; Prince et al., 2008; Tillmann et al., 2007). However, the inhibition of PSII can be the direct consequence of an allelochemical targeting PSII specifically, but can also be the indirect consequence of an inhibition elsewhere in the photosynthetic apparatus or even outside of the plastid. Given these previous results, different scenarios remain possible: (1) allelochemicals target cell membranes and induce indirect effects on photosynthesis; (2) allelochemicals target a photosynthetic subunit; (3) allelochemicals target both cell membranes and a photosynthetic subunit; and (4) allelochemicals neither target membranes nor photosynthetic subunit, but eventually have deleterious effects acting on the whole cell.

We therefore investigated the specific effects of allelochemicals produced by *A. minutum* on photosystems, photosynthesis activity and cytoplasmic membranes of the common diatom *Chaetoceros muelleri*, a diatom co-occurring with *A. minutum* (Chapelle et al., 2014). Allelochemicals were separated from *A. minutum* cells by filtration to specifically focus on allelochemical interactions and avoid interference of cell-cell interactions. The short-term sub-lethal effects of a pulse of allelochemicals on *C. muelleri* was investigated. Effects of allelochemicals on photosystems and photosynthetic electron flows was assessed by absorption and fluorescence spectroscopy and effects on membranes was assessed by flow-cytometry.

2 Materials and methods

2.1 Microalgal cultures

A strain of *Alexandrium minutum* (strain CCMI1002, isolated from a bloom in Gearhies, Bantry Bay, Ireland; Touzet et al., 2007), not producing paralytic shellfish toxins (PST), was selected according to its high allelochemical potency (Long et al., 2018a). A strain of *Chaetoceros muelleri* (strain CCAP 1010-3 obtained from the CCAP culture collection, formerly listed as *Chaetoceros neogracile* or *Chaetoceros* sp.) was selected because of its sensitivity to *A. minutum* allelochemicals (Borcier et al., 2017; Lelong et al., 2011; Long et al., 2018a) and of its co-occurrence in the field with *A. minutum* (Chapelle et al., 2014). Cells of *A. minutum* were grown in autoclaved synthetic ocean seawater (Morel et al., 1979) supplemented with L1 (Guillard, 1975) media (salinity = 35 psu, pH = 8.4). Cultures of the diatom *C. muelleri* were grown in filtered (0.2 μm) natural seawater supplemented with L1 media (salinity = 35 psu, pH = 8.4) and silicate (1.06×10^{-4} M). The natural seawater used for culturing *C. muelleri* was filtered over a carbon filter to retain organic compounds that could possibly inhibit microalgal growth and interfere with *A. minutum* allelochemicals in this study. All cultures were maintained under exponential growth at 18°C under a 12/12 h light/dark cycle at a light intensity of $30 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ (Neons LUMILUX T8 L 36W/965 BIOLUX). Supplementary replicates were performed in another laboratory where the same culture were maintained at 18°C under a 12/12 h light/dark cycle at a light intensity of $38 \mu\text{E m}^{-2} \text{s}^{-1}$ (OSRAM L 30W/640). Cultures were not axenic but were handled under sterile conditions to minimize additional bacterial contamination.

2.2 Counts of microalgal cells and preparation of filtrate

Counts of microalgal cells were performed using a Coulter (Beckman Coulter z2) or a FACScalibur (BD Biosciences, San Jose, CA, USA) flow cytometer with a 488 nm argon laser. Counts with the flow cytometer were estimated according to flowrate (Marie et al., 1999). Cell variables, e.g. forward scatter (Forward scatter, FSC), side scatter (Side scatter, SSC) and red auto-fluorescence (F13, red emission filter long pass, 670 nm) were used to select microalgal populations. Filtrates were prepared with 0.2 μm syringe filters (Minisart, 16534-K, Sartorius) with an acetate cellulose membrane to avoid any loss of allelochemicals on the membrane (Lelong et al., 2011). The filtrate concentrations were expressed as cell concentration (cells ml^{-1}) based on the initial culture concentration prior to filtration.

2.3 Photosynthesis measurements

For measurements of photosynthetic parameters, we used a Joliot-type spectrophotometer (JTS-10, Biologic, Grenoble, France) equipped with a white probing LED (Luxeon; Lumileds) and a set of interference filters (3–8 nm bandwidth). The device combines absorbance and fluorescence spectroscopy measurements (Figure 1), allowing us to study in the same conditions, on the same sample, the activities of the two photosystems independently, as well as the photochemical rate (which reflects the additive activities of both photosystems, see below). The actinic light was provided by a crown of red light emitting diodes (LED) (619 nm, intensities used in this study: 56, 135, 340 and 800 $\mu\text{mol photons/m}^2/\text{s}$). For photosynthesis measurements, cultures of *C. muelleri* in exponential growth phase were concentrated 10 times by centrifugation (4500 rpm, 4 min) and resuspended in its own supernatant to reach a final concentration in the range of 5-10 x 10⁶ cells.mL⁻¹. The centrifuged samples were then left ~30 minutes to allow the cells to recover from centrifugation. The filtrate of *A. minutum* was obtained from an initial culture in the range of 18 000 to 22 000 cells.mL⁻¹. After adding the filtrate of *A. minutum* (or *C. muelleri* for the control), flasks were left for 10 minutes before starting measurements.

Photosynthesis and flow cytometry measurements were made concomitantly on the same samples in UMR 6539. However, for photosynthesis measurements, additional replicates have been performed in UMR 7141 laboratory. The effect of the supernatant of *A. minutum* was the same in the two laboratories, but the initial physiology of the *C. muelleri* cultures was slightly different in the two laboratories. For this reason, and because we are mostly interested in the changes in photosynthetic physiology due to the supernatant of *A. minutum*, all electron transfer rates measurements were normalized to their maximal values in the control, to correct for the variability in the initial physiology of *C. muelleri*.

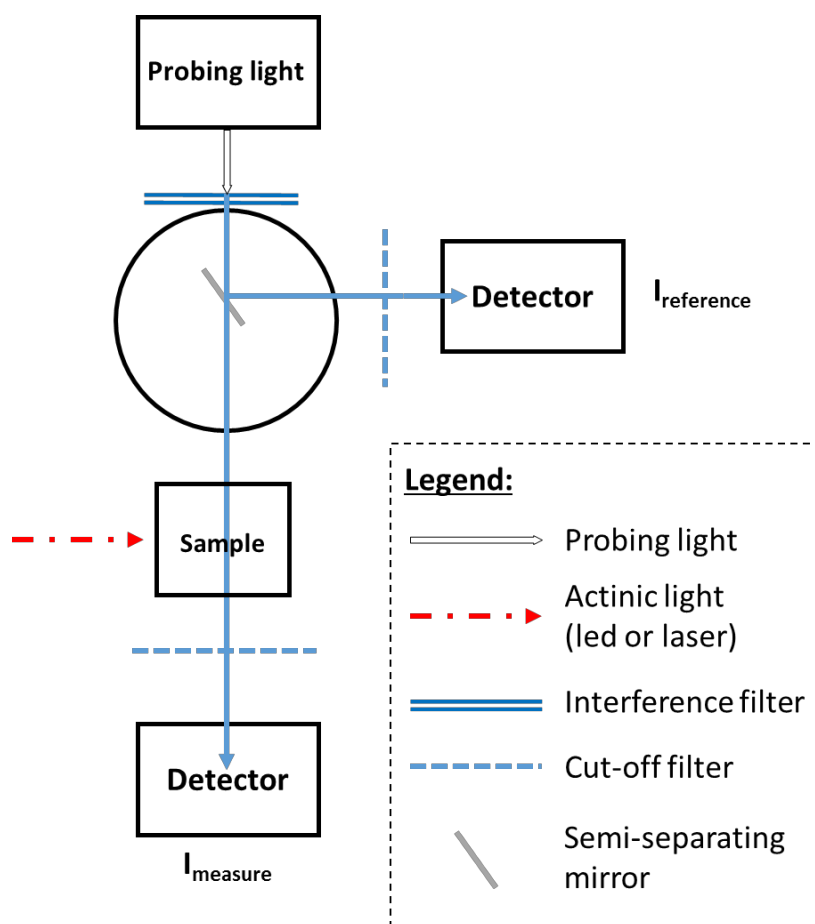


Figure 1: Schematic figure of Joliot type spectrophotometer JTS-10. Detecting pulses are provided by a white probing LED (Luxeon; Lumileds) and a set of interference filters (3–8 nm bandwidth). A crown of red leds (619 nm) or a laser flash is used for the actinic light. In the fluorescence mode, a blue filter (470nm) was used to preferentially excite chlorophyll a, the cut-off filter on the measuring photodiode is a LPF650 + RG 665 from Schott (Mainz, Germany) and on the reference side is a BG39 filter from Schott. And then the output is $I_{\text{measure}}/I_{\text{reference}}$. In the absorption mode, the cut-off filters were the same on measure and reference photodiodes (BG39 for ECS measurements and a high pass RG695 for P700 measurements). In absorption mode, the output is $\Delta I/I = (I_{\text{measure}} - I_{\text{reference}})/I_{\text{reference}}$.

2.3.1 PSI photochemistry

The redox state of the PSI primary donor (P_{700}) was calculated as the difference between 705 nm and 735 nm, to eliminate spectrally flat contributions due to diffusion. Quantum yields of PSI were calculated from the measurements of the absorption changes (Figure 2 and figure 5B) at 700 nm – 735 nm, in the dark (P_0 ; with or without PSII inhibitor DCMU), in the light-adapted condition (P_{stat} ; without DCMU) and after a saturating pulse (P_{sp} ; without DCMU). P_{max} , corresponding to 100% oxidized P_{700} , was measured as the light-minus-dark absorption difference in the presence of the PSII inhibitor 3-(3,4-Dichlorophenyl)-1,1-dimethylurea (DCMU; final concentration of $10\mu\text{M}$ in ethanol; Sigma-Aldrich). Once normalized to P_{max} , the percent of oxidized P_{700} in the different light conditions was measured.

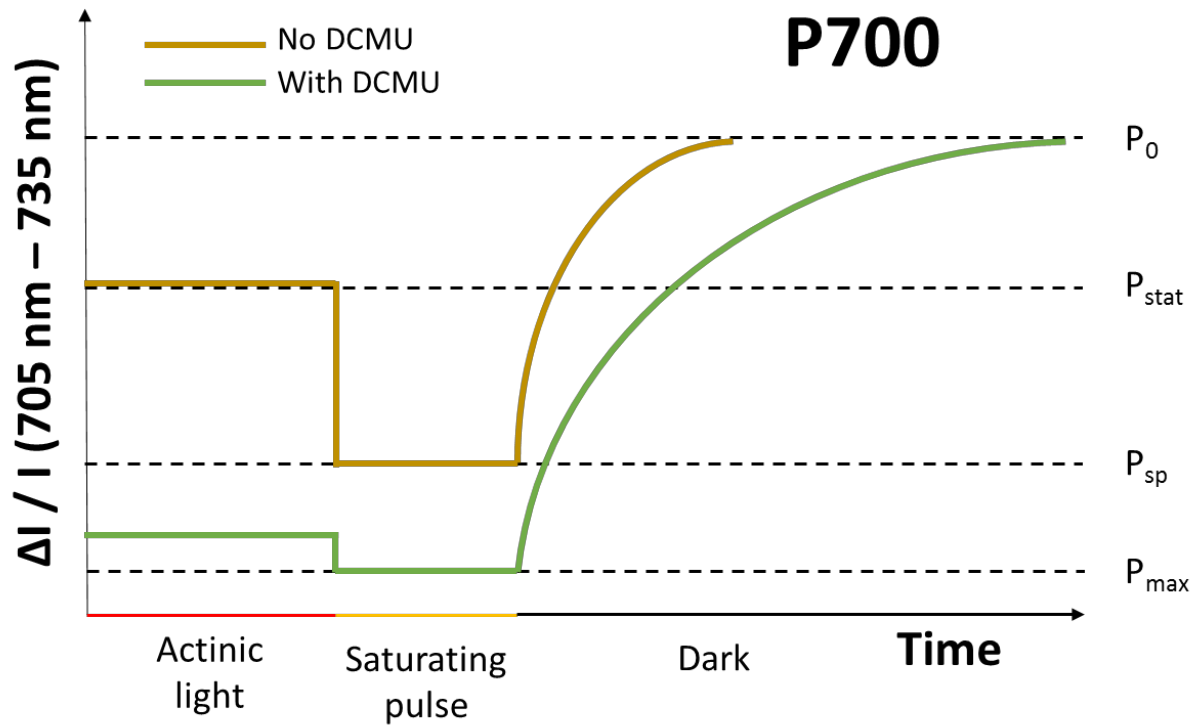


Figure 2: Principle of the measurement of the PSI parameters according to (Klughammer and Schreiber, 2008). P_0 corresponds to the situation where P_{700} is fully reduced (in the dark). P_{stat} corresponds to the redox state of the P_{700} under steady state illumination without DCMU. P_{sp} is the level of oxidation of P_{700} attained during a saturating light pulse (without DCMU) and P_{max} is the situation where 100% of P_{700} is oxidized (during the saturating pulse in the presence of DCMU).

The quantum yield of PSI (Φ_{PSI}), the donor side (Y_{ND}) and acceptor side (Y_{NA}) limitations were calculated according to (Klughammer and Schreiber, 2008).

$$\Phi_{PSI} = (P_{sp} - P_{stat}) / (P_{max} - P_0)$$

$$Y_{ND} = (P_{stat} - P_0) / (P_{max} - P_0)$$

$$Y_{NA} = (P_{max} - P_{sp}) / (P_{max} - P_0)$$

The relative electron transport rate through PSI (rETR_{PSI}) was calculated as:

$$rETR_{PSI} = \Phi_{PSI} * I, \text{ where } I \text{ is the actinic light irradiance.}$$

2.3.2 ECS spectrum and determination of the appropriate wavelengths for ECS measurements

To obtain the spectra of the linear and quadratic Electro-Chromic Shift (ECS) signals in *Chaetoceros muelleri*, we generated a strong electric field by shining a 10 ms saturating pulse (red light, 639 nm, $6000 \mu E m^{-2} s^{-1}$), then used the approach described in (Bailleul et al., 2015). We followed the kinetics of the absorption changes following the pulse, removing the first 50 ms after the pulse (additional signals like the ones associated to the redox changes of the c-type

cytochromes are then relaxed). Based on the spectra of the linear and quadratic components, we used the same deconvolution procedure used by (Bailleul et al., 2015) for *Phaeodactylum tricornutum* to calculate the ECS signals. We used three wavelengths, to eliminate contribution from c-type cytochromes (Cyt c). The latter was calculated as:

$$\text{Cyt c} = [554] - 0.4 * [520] - 0.4 * [566],$$

where [554], [520] and [566] are the absorption difference signals at 554 nm, 520 nm and 566 nm, respectively.

Then, the linear ECS (ECSlin) was calculated as:

$$\text{ECSlin} = [520] - 0.25 * \text{Cyt c}.$$

2.3.3. Photochemical rates.

The photochemical rate (rate at which PSI and PSII perform charge separations) was calculated from ECS measurements as described in (Bailleul et al., 2010). The slope of the initial linear ECS decay (first 5 ms after light offset) was measured and this slope (Figure 3A) was normalized by the ECS increase after a saturating laser flash. Indeed, the ECS signal obtained shortly (130 μs) after a saturating flash is proportional to the number of flash-induced charge separations performed by photosystems, i.e., the total number of PSs in the sample. Therefore, once normalized, the photochemical rate was expressed in charge separation per photosystem per second. Actinic flashes were provided by a dye laser at 690 nm. Data were normalized to the maximal value at $800 \mu\text{E m}^{-2} \text{s}^{-1}$.

2.3.4 Fluorescence spectroscopy

In the fluorescence spectroscopy mode (Figure 1), the JTS-10 was equipped with a white probing LED (Luxeon; Lumileds) and a blue filter for detecting pulses. PSII parameters were calculated as described in (Genty et al., 1989).

Maximum quantum yield of PSII (F_v/F_m) was calculated as:

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

where F_0 is the fluorescence of the dark-adapted sample and F_m the fluorescence when a saturating pulse is applied on dark-adapted sample (Figure 5A).

Quantum yields in light-adapted samples of PSII (Φ_{PSII}) was calculated as :

$$\Phi_{\text{PSII}} = (F_m' - F_{\text{stat}})/F_m',$$

where F_{stat} is the fluorescence of the sample adapted to the actinic light and F_m' the fluorescence when a saturating pulse is applied on light-adapted sample.

The relative electron transport rate through PSII (rETRPSII) was calculated as:

$$\text{rETRPSII} = \Phi_{\text{PSII}} * I,$$

where I is the actinic light irradiance, and all data were normalized to the maximal value in the control at $800 \mu\text{E m}^{-2} \text{s}^{-1}$.

2.5 Flow cytometric measurements

The same *A. minutum* filtrate (from a culture at $20\,000 \pm 2\,000 \text{ cells.ml}^{-1}$) was used for flow cytometry and for photosynthesis measurements. For flow cytometric measurements, cultures of *C. muelleri* were diluted 10 times with their own filtrate to reach a final concentration around 200 000 cells/mL.

2.5.1 Cytoplasmic membrane potential

Modifications of the cytoplasmic membrane potential were assessed using an EasyCyte Plus cytometer (Guava Technologies, Millipore, Billerica, MA) equipped with a 488-nm argon laser and three fluorescence detectors: green (525/30 nm), yellow (583/26 nm), and red (680/30 nm). To estimate the relative depolarization of cytoplasmic membranes, cells were stained with Bis-(1,3-Dibutylbarbituric Acid) Trimethine Oxonol (DiBAC₄(3): (Molecular Probes, Eugene, OR, USA) for 10 min at a final concentration of 0.97 μM . DiBAC₄(3) is a fluorescent probe emitting at 516 nm that can enter cells with depolarized cytoplasmic membranes (Seoane et al., 2017). Once inside the cells, DiBAC₄(3) binds to intracellular proteins and membranes. The amount of protein-bound DiBAC₄(3) is dependent on the cytoplasmic membrane potential (Bräuner et al., 1984). Cells with an increased depolarization (increase in the membrane potential toward 0) of their cytoplasmic membrane exhibit an increase in their internal fluorescence (i.e. proportional to the accumulation of dye within the cell). The relative depolarization of membranes was expressed as the mean fluorescence value of the cell population. After checking the homoscedasticity and the normality of data, the significant differences in the DiBAC₄(3) fluorescence between *C. muelleri* from the control or exposed to the filtrate were compared with a Student t-test.

2.5.2 Membrane permeability

Measurements of membrane permeability were performed on the FACSCalibur flow cytometer. To assess the membrane permeability, samples were stained with SYTOXGreen (Molecular Probes, Eugene, OR, USA) for at least 10 min at a final concentration of 0.05 μM according to

(Koppel et al., 2017). SYTOXGreen is a fluorescent probe (523 nm) that cannot cross cell membranes unless the membranes are compromised. Once inside the cell, the probe binds to DNA and forms a fluorescent dye. Thus, only cells that lost their membrane integrity (i.e. became permeable) become fluorescent and the permeabilization of membranes was expressed as the percentage of fluorescent cells. After checking the homoscedasticity and the normality of data, the significant differences in the percentage of cells permeable to SYTOXGreen between *C. muelleri* from the control or exposed to the filtrate, after 10 min or 30 min were compared with a Student t-test.

3 Results and discussion

3.1 Photochemical rate

In order to assess the effect of *A. minutum* filtrate on the photosynthetic activity of *Chaetoceros muelleri*, we first measured the overall activity of photosynthesis at different irradiances. This was done by using the electro-chromic shift (ECS) of photosynthetic pigments (Witt, 1979). In *C. muelleri*, like in other diatoms (Bailleul et al., 2015) and pinguiphytes (Berne et al., 2018), the ECS is the sum of a linear and a quadratic component, which have been deconvoluted as described in the Methods section. The linear ECS is proportional to the electric field generated by the photosynthetic process and can be used to measure the overall photosynthetic activity (Bailleul et al., 2010). Under steady-state illumination, all photosynthetic complexes participate in the generation or consumption of the electric field across the thylakoid. Briefly, under steady-state illumination, the ECS signal results from positive contributions to the transmembrane potential from PSII, the cytochrome b6f complex and PSI (which translocate protons from the stroma to the lumen, increasing the electric field) as well as the negative one by the chloroplastic ATP synthase (moving protons out of the lumen). Because only photosystem I (PSI) and photosystem II (PSII) use light as a substrate, the change of slope of the ECS at the offset of a steady-state light (Figure 3A) is a direct measurement of the photochemical rate, i.e. the rate at which PSI and PSII were performing charge separations under illumination. In the control, the photochemical rate increased with light irradiance (Figure 3B), as expected, and did not reach saturation at $800 \mu\text{E m}^{-2} \text{s}^{-1}$. The fact that photosynthesis was not yet fully saturated at such a high photon flux stems from the wavelength of the red actinic light used, chosen to correspond to the minimum of absorption in diatoms to ensure an homogeneous light condition in the cuvette. In the presence of *A. minutum* filtrate, the slope of the ECS at the offset of a steady-state light was drastically reduced (Figure 3A), resulting in an almost full inhibition of *C. muelleri* photochemical rates at all light intensities (Figure 3B).

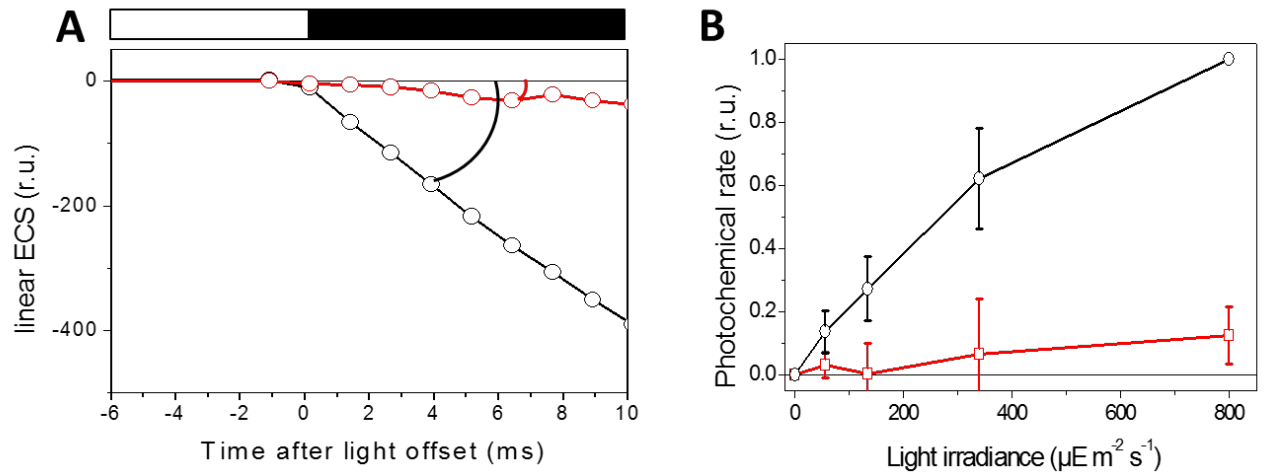


Figure 3: Effect of *A. minutum* supernatant on the overall photosynthesis activity. A. ECS changes at the offset of steady state illumination in the absence (black dots/lines) and presence (red dots/lines) of *A. minutum* filtrate. White bar represents the light phase (here, $800 \mu\text{E m}^{-2} \text{s}^{-1}$), dark bar the period of darkness. The arc of circles represent the change of slope at the light offset. B. Light dependency of photochemical rates of *C. muelleri* with (red dots/lines) or without (black dots/lines) *A. minutum* filtrate. Data were normalized to the maximal value at $800 \mu\text{E m}^{-2} \text{s}^{-1}$ (see Methods). Results are expressed in relative unit (r.u.). Error bars represent standard deviation ($n = 3-4$).

3.2 PSI and PSII activities

The very strong inhibition of the photochemical rate by the filtrate of *A. minutum* suggests that both the activities of the linear electron flow (from water to PSI acceptors; Figure 4) and cyclic electron flow around PSI are suppressed by the allelochemicals of *A. minutum*.

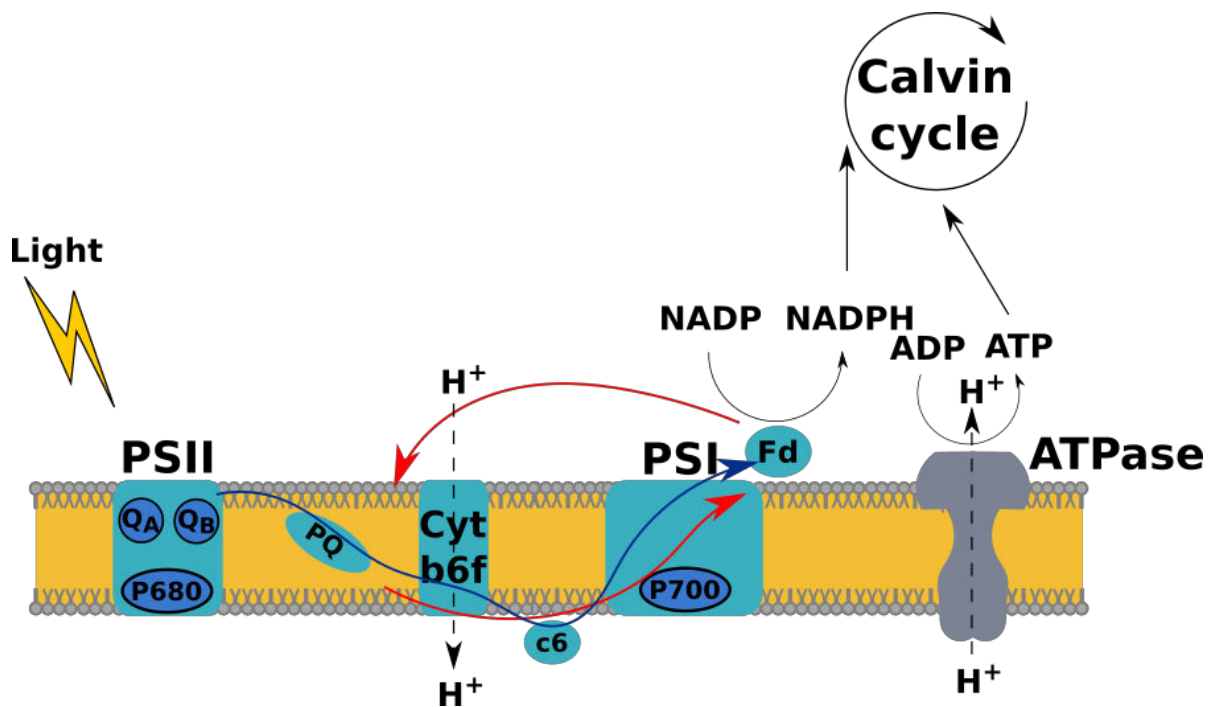


Figure 4: Diagram of a diatom photosynthetic chain and electron fluxes. The blue arrow represents the linear electron flow and red arrows represent the cyclic electron flow. PSI: Photosystem I, PSII: Photosystem II, QA and QB: Quinones A and B, PQ: pool of plastoquinones, Cyt b6f: Cytochrome b6f, c6: Cytochrome c6, Fd: Ferredoxine.

To further confirm this, we measured the fluorescences of PSII and PSI (Figures 5A and 5B) in the same conditions, and calculated their activities in the diatom, under the same illumination conditions. The activity of PSII should reflect the efficiency of the linear process only, whereas PSI activity is the sum of the linear and cyclic pathways. The quantum yield of PSII (Φ_{PSII}) was measured with fluorescence spectroscopy and the quantum yield of PSI (Φ_{PSI}) with P₇₀₀ absorption measurements. As expected, both the quantum yields of PSII (Figure 5C) and PSI (Figure 5D) decreased with light irradiance in the control sample. However, at all light irradiances, the quantum yields of both photosystems were significantly lower in the presence of *A. minutum* filtrate (Figures 5E and 5F). In the control, the dependence of the relative electron transfer rate through PSII ($\text{rETR}_{\text{PSII}}$) upon light irradiance was very similar to the one of the photochemical rate (Figure 5 E). The filtrate of *A. minutum* almost fully inhibited $\text{rETR}_{\text{PSII}}$, which confirms that it hampers the linear electron flow. We wanted to measure if cyclic electron flow was still present in cells in contact with *A. minutum* filtrate by measuring the electron transfer rate through PSI (rETR_{PSI}), involving both linear and cyclic electron flows. Again, PSI electron flow rates (rETR_{PSI}) were almost fully suppressed in the presence of filtrate of *A. minutum* allelochemicals (Figure 5F). This confirms that both linear and cyclic electron flow are suppressed by *A. minutum* filtrate.

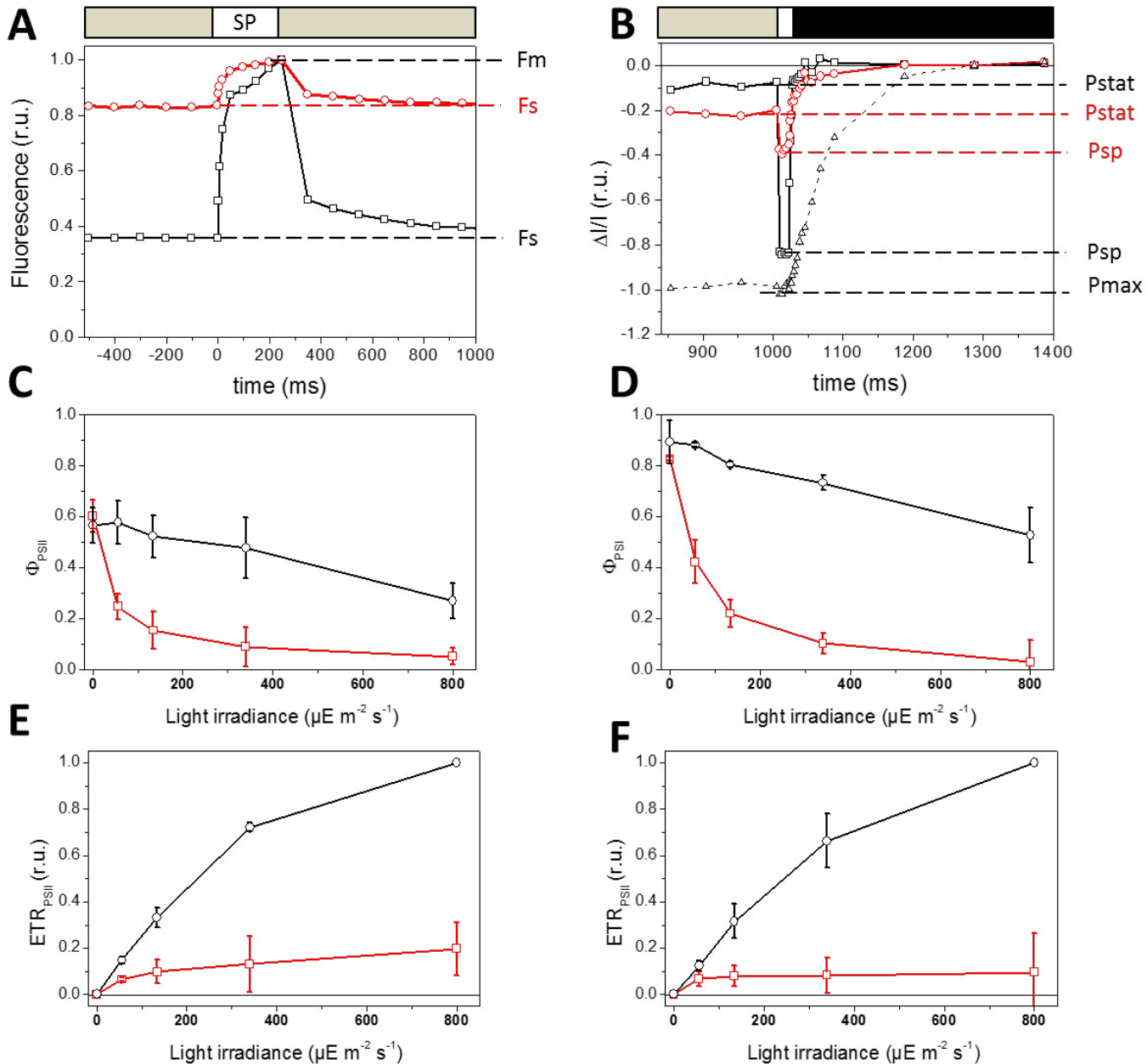


Figure 5: Effects of *A. minutum* filtrate on activity of *C. muelleri* PSII (A, C, E) and PSI (B, D, F) in the control (dark) and in the presence of *A. minutum* filtrate (red). The fluorescence measurements for the calculation of PSII and PSI quantum yields are shown in (A) and (B) respectively (see Methods for explanations of the different parameters). The dashed lines and rectangles show the $\Delta I/I$ in the control with DCMU. Data in panels A and B were obtained under a $340 \mu E m^{-2} s^{-1}$ actinic light. C/D: Light dependencies of the quantum yields of PSII (C) and PSI (D). (E) Electron transfer rates through PSII (rETR_{PSII}) under increasing light irradiance. (F) Electron transfer rates through PSI (rETR_{PSI}, right) under increasing light irradiance. Results are expressed in relative unit (r.u.). Error bars represent standard deviation (n = 3 for the right panel, n = 6 for the left panel).

3.3 An inhibition of photochemical or non-photochemical reaction?

Such a complete suppression of photosynthetic activity could be due to the specific inhibition of any of the photosynthetic complexes, as well as of the Calvin_Benson-Bassham cycle. One

could imagine that the photosystems themselves are inhibited by the allelochemicals. Indeed, an inhibition of the maximal quantum yield of PSII (Fv/Fm) was shown to occur within 30 minutes in these conditions (Long et al., 2018b) and this inhibition was even proposed as a bioassay for these allelopathic interactions (Long et al., 2018a). In agreement with that, we observed a strong inhibition of both PSII (Figure 6A) and PSI (Figure 6B) at the end of the measurements, in the exposed samples only. The Fv/Fm decreased by ~50 % (Figure 6A) and the total amount of photo-oxidizable P₇₀₀ (P_{max}) decreased by ~75 % at the end of the experiment (Figure 6B). However, neither PSI nor PSII were inhibited at the beginning of the experiment (highlighted by the similar Φ_{PSII} and Φ_{PSI} in the dark in absence or presence of *A. minutum* filtrate; Figure 5C and 5D), whereas the inhibition of the photosynthetic activity in the light occurred immediately, from the first light step, as observed with for the photochemical rate ETRPSII or ETRPSI .

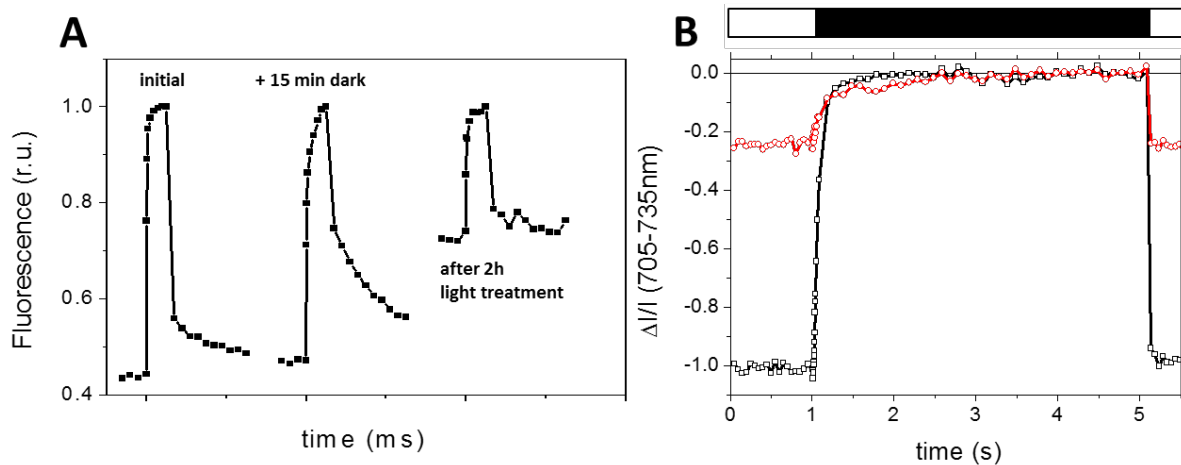


Figure 6: (A) Fluorescence of *C. muelleri* exposed to *A. minutum* filtrate where a saturating pulse is applied immediately after exposure, after 15 min of dark exposure and after 2 hours of light treatments. (B) Relative amount of P₇₀₀ photo-oxidizable in absence (black dots/lines) and presence (red dots/lines) of *A. minutum* filtrate given by the change in $\Delta I/I$ under different light conditions. Results are expressed in relative unit (r.u.).

In order to test if the suppression of the photosynthetic activity was due to photosystems inhibition or not, we performed another experiment to measure the quantum yield of both PSI and PSII in the dark and in moderate light, with the two measurements performed in close succession. This experiment showed that the activities of both PSI and PSII in the presence of light were already suppressed in conditions where PSI and PSII integrity (maximal efficiencies) were unaffected, demonstrating that the *A. minutum* filtrate inhibits photosynthetic activity by targeting a non-photochemical step first.

3.4 Which photosynthetic target?

Our results lead to two possibilities: the allelochemicals affect (i) the electron transfer from the Quinone B (Q_B) pocket of PSII to P_{700} at the PSI level (this part of the photosynthetic chain is usually referred to as the donor side of PSI), or (ii) the electron transfer downhill PSI (acceptor side of PSI). To distinguish between those two possibilities, we first measured the acceptor (Y_{NA}) and donor (Y_{ND}) side limitation of PSI (see Methods) under the same illumination. The data clearly show that the decrease of PSI quantum yield (Φ_{PSI}) is paralleled by an increase of the donor side (Y_{ND}) limitation in the treated sample (Figure 7A). This strongly suggests that the effect occurs between PSII and PSI, and not on the acceptor side of PSI. To further confirm this, we used the artificial PSI electron acceptor, methylviologen (MV, or paraquat), which releases acceptor side (Y_{NA}) limitation by providing an efficient sink to electrons from PSI. In agreement with the Y_{ND} and Y_{NA} measurements, the addition of MV did not modify the activities of PSII or PSI, neither in the control nor in the treated sample (Figure 7A). Together, these results indicate that the first site of inhibition of the photosynthetic electron transfer chain takes place between Q_B and P_{700} . This is further confirmed by the slowing down of the fluorescence recovery after a saturating pulse (Figure 7B), which indicates that the treatment with *A. minutum* supernatant tends to impair the reoxidation of Q_A , i.e. the electron transfer from the quinone pockets in PSII to the plastoquinone pool or cytochrome b6f. The decrease of the efficiency of PSII (Figure 6A) and PSI only comes at longer times of incubation, possibly as a side-effect of the inhibition of the electron transfer between the two photosystems.

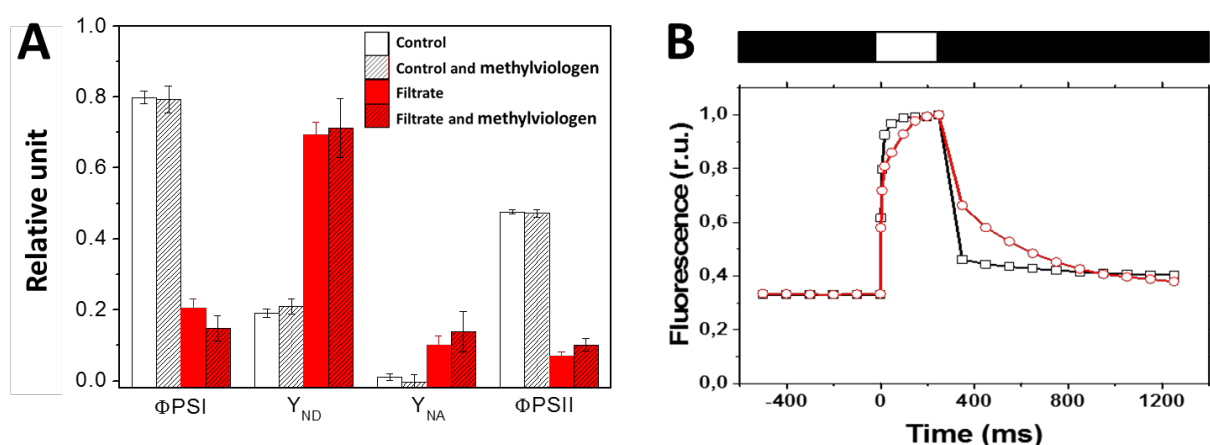


Figure 7: (A) Short-term effect of *A. minutum* supernatant on *C. muelleri* PSI and PSII activities. The quantum yields of PSII and PSI, as well as the donor-side (Y_{ND}) and acceptor-side (Y_{NA}) limitations are shown for the control (white bars) and in the presence of *A. minutum* filtrate, with (hatched) and without (plain) the PSI acceptor methylviologen. All experiments were performed at $340 \mu E m^{-2} s^{-1}$ actinic light. Error bars represent standard deviation ($n = 3$). F_v/F_m for the control and filtrate exposed samples were 0.66 ± 0.02 and 0.69 ± 0.03 , respectively.

(B) Fluorescence of *C. muelleri* in the absence (black dots/lines) or in the presence of *A. minutum* filtrate (red dots/lines) where a saturating pulse (white rectangle) is applied. Results are expressed in relative unit (r.u.).

3.5 A modification of membranes ?

The inhibition of the electron transfer chain can be due to a problem in the diffusion of the quinones between Q_B to the quinone-binding sites in the cytochrome b6f or to an alteration of the function of the cytochrome b6f itself. Our data did not allow us to distinguish between those two possibilities. However, we can conclude that the problem stems somewhere in the thylakoid membrane. Given that allelochemicals from *A. tamarensis* cause the disruption of membranes that leads to lytic activity (Ma et al., 2011), we wondered if such a disruption of membranes could take place with *A. minutum* allelochemicals. Flow-cytometry analysis revealed that *A. minutum* filtrated induced a strong depolarization of membranes from *C. muelleri* within only 10 min (Figures 8 and 9A). Only metabolically active cells with no damage to membranes can maintain a normal membrane potential (Jepras et al., 1997; Prado et al., 2012). Depolarization of membranes can highlight an inhibition of the energy metabolism or can also be an early signal of membrane disruption as the uncoupling agent eliminates the proton gradient.

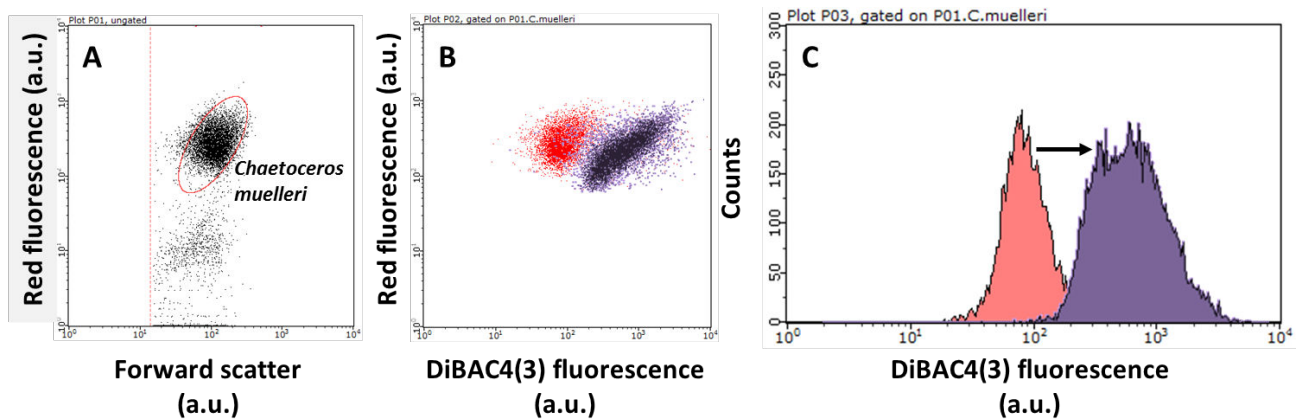


Figure 8: Depolarization of cytoplasmic membranes from *C. muelleri* in presence of *A. minutum* filtrate. The red population represent *C. muelleri* in the control, the purple population represent *C. muelleri* exposed to *A. minutum* filtrate for 10 min. (A) *C. muelleri* population as a function of chlorophyll autofluorescence (Red fluorescence; in arbitrary unit) and forward scatter (FSC; in arbitrary unit), (B) *C. muelleri* population as a function of chlorophyll autofluorescence (Red fluorescence; in arbitrary unit) and DiBAC(3) fluorescence (green fluorescence; in arbitrary unit), (C) Distribution of *C. muelleri* cells as a function of their DiBAC(3) fluorescence (green fluorescence; in arbitrary unit).

Allelochemicals from *A. minutum* alter the energy metabolism from *C. muelleri* as shown with the decrease in esterase activity in presence of filtrate (Long et al., 2018b). To test whether allelochemicals could disrupt membranes and induce side effects on photosynthesis, we measured the permeability of cytoplasmic membranes with SytoxGreen dye. Allelochemicals disrupted the membranes of *C. muelleri* as the percentage of cells stained with SytoxGreen greatly increased with the filtrate of *A. minutum* (Figure 9B).

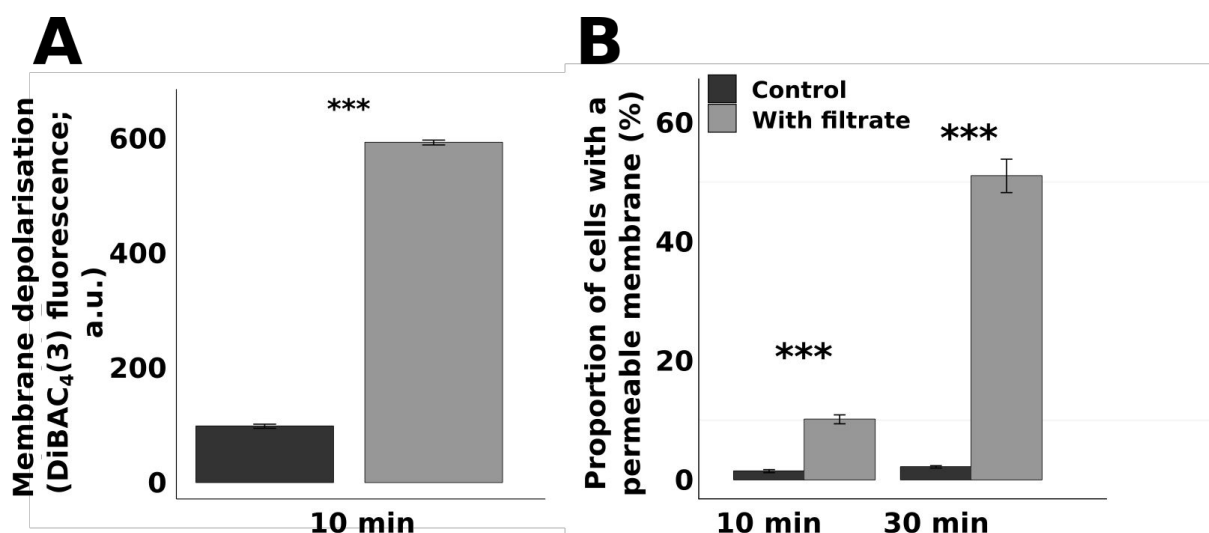


Figure 9: Effect of *A. minutum* filtrate on *C. muelleri* cytoplasmic membranes. A) Depolarization of cytoplasmic membranes from *C. muelleri* as shown by the increase of green fluorescence (a.u.) of DiBAC₄(3). B) Variation in the proportion of cells with a permeable membrane (labelled with SytoxGreen) in the control (Dark grey) and in the presence of *A. minutum* filtrate (Light grey) after 10 min and 30 min of exposure. *** indicate a level of significance < 0.001. Results are expressed as the mean \pm standard error. N = 3.

In agreement with the allelochemical mode of action of *A. tamarense* on other protists membranes (Ma et al., 2011), allelochemicals from *A. minutum* induce a disruption of membrane of the diatom *C. muelleri*. It is hypothesized that allelochemicals disrupt the cytoplasmic membranes, inducing depolarization that eventually leads to a permeabilization of cytoplasmic membrane (Figure 9B). Such a disruption and permeabilization of membranes would have side-effects on photosynthesis.

3.6 Kinetic of allelochemicals on membranes

The present study demonstrated the short-term allelopathic effects of *A. minutum* filtrate on the cytoplasmic membrane integrity and photosynthetic chain. Results highlighted both a loss of cytoplasmic membrane integrity and an alteration in the e- transfer between Q_B and b6f and degradation of PSI, PSII. The study of kinetics allowed us to identify “primary” targets from the side effects of filtrate exposure and allowed us to hypothesize the cascade of events occurring within the different membranes of *C. muelleri* (Figure 10). Within the first 10 min of exposure, the allelochemicals induced a depolarization of the cytoplasmic membranes of the diatom *C. muelleri*, associated with a slight increasing membrane permeabilization. Simultaneously, photosynthetic membranes were also impaired as observed by a decrease of the overall photosynthetic activity. This decrease can be attributable to an alteration of the diffusion of the quinones between Q_B and PSI or of the function of the cytochrome b6f. The exact site where photosynthesis is impaired could be investigated by measuring the b6f turnover rate by following the b phase of the flash -induced ECS kinetics.

After 30 minutes of exposure, the membranes of *C. muelleri* seemed to be facing more severe effects from allelochemical interactions. Cytoplasmic membranes appeared more permeable, probably because of the generation of pores in the membranes. The photosynthetic chain also appeared more affected, as shown by the strong inhibitions of PSI and PSII. These inhibitions of PSI and PSII may be a consequence of the dysfunction of the diffusion of the quinones between Q_B and cytochrome b6f or of the cytochrome b6f observed at an earlier stage. This dysfunction led to a suppression of the electron flow in the photosystems and to the suppression of the overall photochemical rate. The overall deleterious effects on the photosynthetic apparatus induced photoprotection mechanisms previously measured in *C. muelleri* exposed to *A. minutum* filtrate (Long et al., 2018b).

While the cascade of events in the membranes are now better understood, the link between cytoplasmic and photosynthetic membranes still has to be elucidated. Few hypotheses can be made to understand how allelochemicals interact with membranes. Allelochemicals that disrupt cell membranes might enter the cells and impair chloroplast membranes in a similar way. Nevertheless, it is unclear how allelochemicals cross the membrane as they may remain trapped within the membrane leaflets. Once inside the cells, allelochemicals may also selectively target a photosynthetic subunit between Q_B and the cytochrome b6f. In another scenario, the lesion of the cytoplasmic membranes and the depolarization of membranes may trigger some stress responses in the cells that would impair photosynthesis. The depolarization and permeabilization of membranes may also lead to a loss of homeostasis (e.g. pH gradient, ion gradient) within the cells that might impair the overall cell physiology. All of these scenarios are non-exclusive and may explain the cascade of events, which will be further discussed in general discussion from chapter 6.

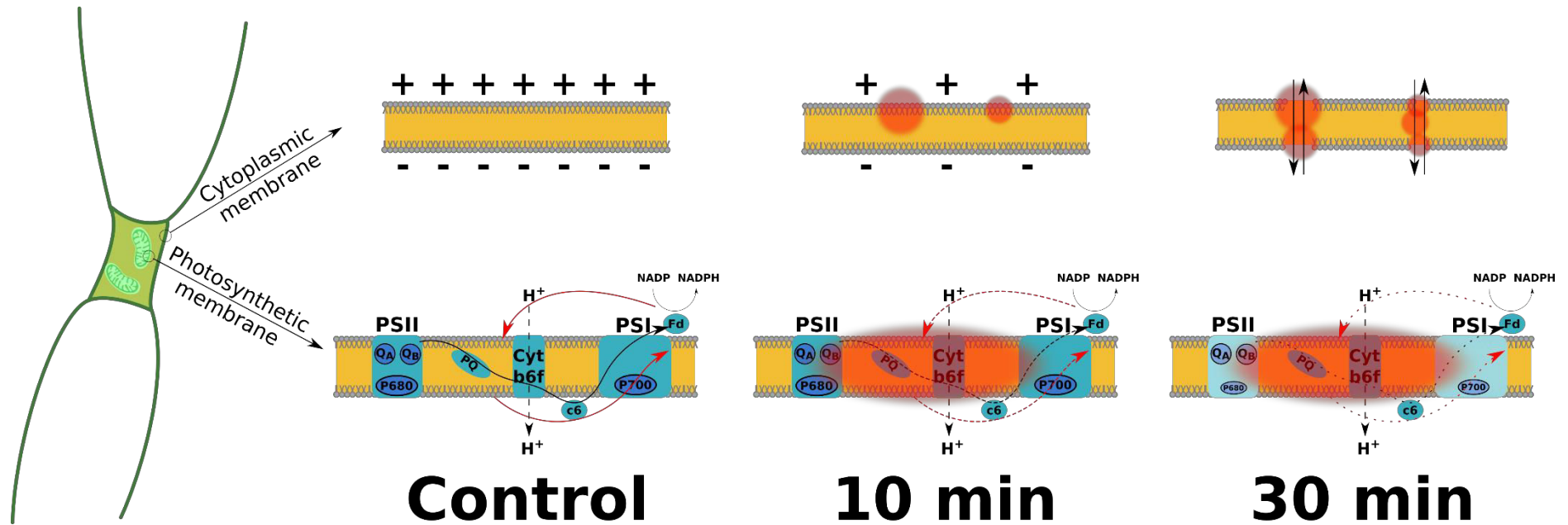


Figure 10: Diagram summarizing the effects of *A. minutum* filtrate on *C. muelleri* after 10 and 30 min of exposure. The red oval highlight the molecular sites where allelochemicals impair membranes. Blue and red dashed lines highlight a decrease in the electron fluxes. After 30 minutes, the pale blue highlight an inhibition of PSI and PSII as measured in this study. PSI: Photosystem I, PSII: Photosystem II, Q_A and Q_B: Quinones A and B, PQ: pool of plastoquinones, Cyt b6f: Cytochrome b6f, c6: Cytochrome c6, Fd: Ferredoxine.

4 Conclusion

This study reveals the first physiological steps of allelochemical interactions between the toxic dinoflagellate *A. minutum* and the diatom *C. muelleri*. The filtrate of *A. minutum* first induced a depolarization of cytoplasmic membranes and an inhibition of electron flow between the two photosystems. The exact target site has to be further investigated. This primary damage to the cytoplasmic membranes and photosynthetic chain, lead to wider effects on membranes such as permeabilization of cytoplasmic membranes and damages to the photosynthetic chain on the donor side of PSII causing a larger inhibition of PSI and PSII. This study demonstrated that allelochemicals from *A. minutum* first impair the cells by disrupting the cytoplasmic membranes, similarly to *A. tamarense*. It also highlights that, while PSII can be probed to measure allelochemical potency, it is not a specific target. The mechanisms identified in this study highlight several molecular targets that might be useful in understanding the variability of cell responses to *Alexandrium spp.* allelochemicals. Overall, this will help us understand how allelochemical interactions shape the plankton community.

Acknowledgments

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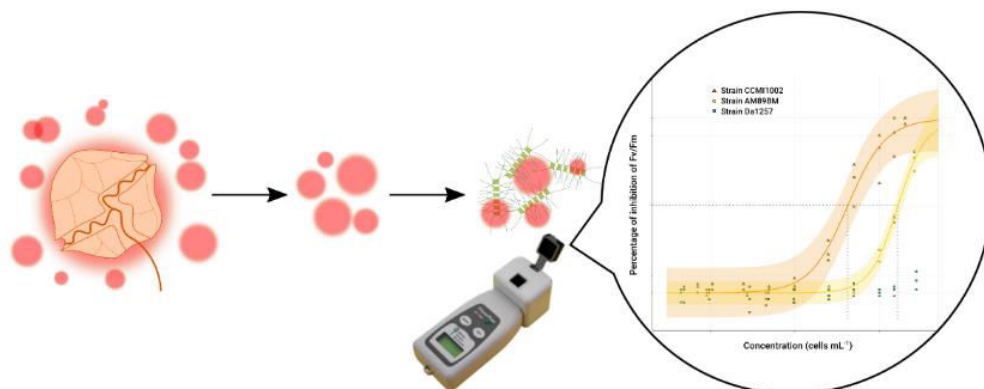
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Chapter 3

Development of a bioassay to quantify allelochemical interactions from *Alexandrium minutum*

In the Chapter 2, we described the mechanisms of allelochemical interactions between the dinoflagellate *A. minutum* and diatom *C. muelleri*. We showed that allelochemicals from *A. minutum* induce a rapid disruption of membranes in *C. muelleri*. This uncoupling of cytoplasmic membranes is likely to be responsible for the inhibition of photosynthesis and other physiological effects observed in *C. muelleri* in presence of *A. minutum* filtrate. In addition to unraveling the mechanisms of allelochemicals interactions, this chapter allowed us to find a new sensitive biomarker to study these interactions. The research on allelochemical interactions in marine environment is hampered by analytical detection limits. Detection is so far based on lethal parameters and bioassays that require relatively long incubation times. In this chapter, we propose to quantify allelochemical interaction with a rapid fluorescence-based bioassay. This chapter consists of one article (Article 3) that has been published in *Environmental Pollution*.

Article 3: Long, M., Tallec, K., Soudant, P., Lambert, C., Le Grand, F., Sarthou, G., Jolley, D., Hégaret, H. A rapid quantitative fluorescence-based bioassay to study allelochemical interactions from *Alexandrium minutum*. *Environmental Pollution* (2018). Doi:10.1016/j.envpol.2018.07.119



Article 3

A rapid quantitative fluorescence-based bioassay to study allelochemical interactions from *Alexandrium minutum*

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The article in its original format can be found in annexe 2.

Abstract

Harmful microalgal blooms are a threat to aquatic organisms, ecosystems and human health. Toxic dinoflagellates of the genus *Alexandrium* are known to produce paralytic shellfish toxins and to release bioactive extracellular compounds (BECs) with potent cytotoxic, hemolytic, ichthyotoxic and allelopathic activity. Negative allelochemical interactions refer to the chemicals that are released by the genus *Alexandrium* and that induce adverse effects on the physiology of co-occurring protists and predators. Releasing BECs gives the donor a competitive advantage that may help to form dense toxic blooms of phytoplankton. However BECs released by *Alexandrium minutum* are uncharacterized and it is impossible to quantify them using classical chemical methods. Allelochemical interactions are usually quantified through population growth inhibition or lytic-activity based bioassays using a secondary target organism. However these bioassays require time (for growth or microalgal counts) and/or are based on lethal effects. The use of pulse amplitude modulation (PAM) fluorometry has been widely used to assess the impact of environmental stressors on phytoplankton but rarely for allelochemical interactions. Here we evaluated the use of PAM and propose a rapid chlorophyll fluorescence based bioassay to quantify allelochemical BECs released from *A. minutum*. We used the ubiquitous diatom *Chaetoceros muelleri* as a target species. The bioassay, based on sub-lethal effects, quantifies allelochemical activity from different samples (filtrates, extracts in seawater) within a short period of time (2 hours). This rapid bioassay will help investigate the role of allelochemical interactions in *A. minutum* bloom establishment. It will also further our understanding of the potential relationship between allelochemical activities and other cytotoxic activities from BECs. While this bioassay was developed for the species *A. minutum*, it may be applicable to other species producing allelochemicals and may provide further insights into the role and impact of allelochemical interactions in forming dense algal blooms and structuring marine ecosystems.

1 Introduction

Proliferation of microalgae is recognized as a major threat for marine ecosystems. Harmful algal blooms can be responsible for toxic impacts on marine organisms and ecosystems and can also be toxic to human. This involves public health risks (e.g. intoxications) and socio-economics issues (e.g. loss of fisheries stock, fisheries closure). Dinoflagellates from the genus *Alexandrium* have the potential to produce paralytic shellfish toxins but also to release uncharacterized bioactive extracellular compounds (BECs) exhibiting allelochemical (Arzul et al., 1999; Lelong et al., 2011; Tillmann et al., 2007), cytotoxic (Le Goïc et al., 2014), hemolytic (Arzul et al., 1999; Emura et al., 2004), and ichthyotoxic activities (Borcier et al., 2017; Castrec et al., 2018; Mardones et al., 2015). BECs from the genus *Alexandrium* affect a large range of marine protists including autotrophic and heterotrophic organisms (Fistarol et al., 2004; Tillmann et al., 2008, 2007; Tillmann and Hansen, 2009) by inducing cell lysis within hours.

Negative allelochemical interactions can be defined as the chemical release of compounds by a protists that causes adverse effects on the physiology of other competing protists and predators, thus giving the allelopathic species a competitive advantage. These negative interactions allow protists to outcompete for resources (e.g. nutrient, light), induce prey immobilization, defend against grazers and therefore are a key factor structuring plankton ecosystems (Legrand et al., 2003). Allelochemical interactions are mediated by the production of BECs, named as allelochemicals, released into the surrounding environment. BECs are poorly described (e.g. chemical nature, mode of action) because of methodological and analytical difficulties, but are mainly characterized by their activity spectra and effects on target cells: growth inhibition, death, lysis, paralysis, inhibition of photosystem II etc. (Legrand et al., 2003). Short-term (minutes to hour) deleterious effects were observed on the photosystem II of several phytoplankton species exposed to the genus *Alexandrium* (Tillmann et al., 2007) including the globally distributed diatom *Chaetoceros muelleri* (Lelong et al., 2011, formerly named as *Chaetoceros neogracile*).

No standard and rapid methodology is available for the study of allelochemical interactions in marine environments (Legrand et al., 2003). Development of bioassays would benefit the allelochemical interactions research field by enabling the rapid confirmation and quantification of

allelochemical interactions. This would help to assess the role and relevance of these interactions in plankton ecology. It would also greatly ease the isolation and characterization of unknown BECs through bio-guided purification. Population growth inhibition (Chan et al., 1980; Paul et al., 2009; Pushparaj et al., 1998) or enumeration of lysed cells (Hakanen et al., 2014; Ma et al., 2009; Tillmann et al., 2007) are the most common parameters used in bioassays to demonstrate allelochemical interactions but such techniques are time-consuming. According to the OECD (2011) guidelines for testing chemicals, a growth inhibition test should be long enough to obtain a 16-fold population growth in control treatments (from few days to weeks depending on the target species growth rate). Moreover, microscopic observations cannot be performed in every field situation and distinction between viable and non-viable cells can be subjective because of a lack of consensus on a microalgal death definition (Garvey et al., 2007). This is compounded by the fact that the enumeration of lysed cells is based on lethal effects, although allelochemical interactions in marine environments may not automatically imply cell lysis. Therefore, new rapid, quantitative and sensitive methods are worth exploring to study allelochemical interactions.

Pulse amplitude modulatory (PAM) fluorometry is a method based on chlorophyll *a* fluorescence that allows studying photosynthetic processes. PAM is frequently used to assess the impact of environmental stressors (Barranguet et al., 2002; Juneau et al., 2003; Lippemeier et al., 1999; Miao et al., 2005), pollutants (see review from Ralph et al., 2007) or algicidal compounds (Yang et al., 2017) on phytoplankton physiology, as it provides insights into phytoplankton “health”. Maximum photosystem II quantum yield (F_v/F_m) is the optimal photosynthetic efficiency and is one of the most popular and easiest parameter to interpret. PAM has the advantage of being a rapid non-invasive and non-destructive method that can detect sub-lethal effects on photosystems. Some devices can be portable, allowing rapid measurements useful in many situations. However, only few studies report the measurement of chlorophyll *a* fluorescence to demonstrate allelochemical interactions (Blossom et al., 2014a; Borcier et al., 2017; Tillmann et al., 2007). The lack of homogeneity in methods and summary parameters (e.g. single concentration measurement, EC10, EC50: the effect concentrations inhibiting 10 or 50 % of a physiologic parameter) prevent comparisons between studies, highlighting the need of standardized bioassays.

The aim for this study was to assess the use of pulse amplitude modulation (PAM) fluorometry in detecting allelochemical activity from *A. minutum* and to establish a rapid and reliable

fluorescence-based bioassay to quantify allelochemical activity from *A. minutum* samples. The genus *Chaetoceros* was selected as a target species based on its known sensitivity to *Alexandrium* spp. BECs (Arzul et al., 1999; Lelong et al., 2011; Weissbach et al., 2010), its ubiquity in phytoplankton communities (Dalsgaard et al., 2003) and its co-occurrence with *A. minutum* blooms (Chapelle et al., 2014; Klein et al., in prep). Moreover the genus *Chaetoceros* is a microalgal model commonly used in ecotoxicological studies (Desai et al., 2006; Hii et al., 2009; Hourmant et al., 2009). The bioassay was conceived to be applied to microalgal cultures, extracts or field samples.

2 Materials and methods

2.1 Algal culture

Three strains of the toxic dinoflagellate *Alexandrium minutum* were selected based upon their different allelochemical activity. The strains AM89BM (isolated from a bloom in the Bay of Morlaix, France) and CCMI1002 (isolated from a bloom in Ireland; Borcier et al., 2017) were chosen according to their cytotoxic potency (Arzul et al., 1999; Borcier et al., 2017; Lelong et al., 2011). The strain Da1257 (isolated from a bloom in the Bay of Daoulas, France; Pousse et al., 2017) was selected according to its low allelochemical potency. The cultures were grown in natural filtered (0.2 μm) seawater (collected in Argenton, France) supplemented with L1 medium (Guillard and Hargraves, 1993) and maintained in exponential growth phase. The diatom *Chaetoceros muelleri* (strain CCAP 1010-3, formerly named *Chaetoceros neogracile* or *Chaetoceros* sp.) was cultured in filtered (0.2 μm) artificial seawater (synthetic ocean water: Morel et al., 1979; Price et al., 1989) supplemented with L1 medium (Guillard and Hargraves, 1993) and silica (1.06×10^{-4} M final concentration). Both media were autoclaved. Microalgal cultures were maintained at $17 \pm 1^\circ\text{C}$ under continuous light ($100 - 110 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$).

For each experiment, cultures of *C. muelleri* in exponential growth phase were used. Diatoms were diluted with filtered artificial seawater to achieve 2×10^5 cells ml^{-1} (Lelong et al., 2011) in the experiments and bioassays. Microalgal cells were counted and algal growth was monitored using a FACSCalibur flow cytometer (Becton Dickinson, San Diego, CA, USA) equipped with a blue laser (excitation 488 nm). Concentrations (cells mL^{-1}) were calculated from the number of events

per unit of time and the estimate of the FACSCalibur flow rate measured according to Marie et al. (1999).

2.2 BECs extraction

BECs were separated from culture by filtration (0.2 μm , acetate cellulose membrane, 16534-K, Sartorius) according to Lelong et al. (2011). Filtrates were prepared fresh in glass tubes to avoid any loss to plastic during storage (Ma et al., 2009). The initial *A. minutum* culture concentration, prior to filtration, was used to describe the potential allelochemical concentration of each filtrate. Serial dilutions of this filtrate with filtered natural seawater were performed to obtain various concentrations. In the following text, the theoretical allelochemical concentration of each filtrate is given by the concentration in *A. minutum* (cell mL^{-1}) of the culture used to obtain the filtrate.

2.3 Pulse amplitude modulatory (PAM) fluorometry

Maximum photosystem II (PSII) quantum yield (F_v/F_m), which is a proxy of PSII photochemical efficiency, was measured by pulse amplitude modulation (PAM) fluorometry with a handheld AquaPen-C AP-C 100 (Photon Systems Instruments, Drassov, Czech Republic) equipped with a blue light (455 nm). In each experiment, algal samples were dark-adapted for at least 20 minutes before measurement of fluorescence variables (F_0 , F_m). F_0 is the initial fluorescence intensity, F_m the maximal intensity under saturating light conditions (0.5 to 1 s at $1500 \mu\text{mol photon m}^{-2} \text{s}^{-1}$), and $F_v = F_m - F_0$. Maximum quantum yield of photosystem II was then calculated as followed: F_v/F_m (Strasser et al., 2000).

2.4 Optimization of protocol

2.4.1. Sensitivity of F_v/F_m to filtrate

In a first experiment, the kinetic impact of BECs upon the diatom's photosystem was studied. Cells of *C. muelleri* ($2 \times 10^5 \text{ cells mL}^{-1}$) were exposed to *A. minutum* (strain CCM11002) filtrate dilutions in filtered natural seawater, with final filtrate concentrations equivalent to 0, 50, 500, 5000 and 50 000 cells mL^{-1} . F_v/F_m was then measured every 30 min for the first 120 min of exposure then every 60 min until 360 min of exposure. Results are expressed as the percentage of F_v/F_m inhibition compared to seawater control (see section 2.6).

2.4.2 Fv/Fm and FDA inhibition assay

The second experiment was performed to confirm that Fv/Fm is a good proxy of microalgal metabolism in the bioassay. Fv/Fm was compared to another proxy of primary metabolism, the fluorescein diacetate-activity assay (Brookes et al., 2000; Franklin et al., 2001; Garvey et al., 2007). Fluorescein diacetate (FDA, Molecular probes, Invitrogen, Eugene, OR, USA) is a hydrophobic fluorescent dye that permeates and stains cells exhibiting esterase activity, i.e. viable cells. Inside the cells, FDA is cleaved by esterases and releases a fluorescent by-product (fluorescein; emission 525 nm) which is retained within the cells. Cells that are not stained in the presence of FDA do not exhibit esterase activity and are considered as metabolically inactive (Supplementary figure 1). FDA fluorescence in the diatoms was measured by flow cytometry (FACSCalibur) equipped with a blue laser (excitation 488 nm) through the detector of fluorescence FL1 (green emission filter band pass, 530/30 nm). FDA staining protocol was optimized (Supplementary figures 2 and 3) for *C. muelleri* and samples were stained (final concentration = 1.5 μM) for 10 min in dark prior to flow-cytometric measurement, while samples for Fv/Fm measurement were put in the dark 20 min before measurement. *C. muelleri* (2×10^5 cells mL^{-1}) was exposed to *A. minutum* exudate filtrate (strain CCM11002) at concentrations (theoretical concentrations) equivalent to 500, 5000 and 50 000 cells mL^{-1} . Fv/Fm and FDA fluorescence were measured after 120 min of exposure.

2.5 Bioassay procedure: comparison of *A. minutum* strains

2.5.1 Algal culturing

To compare allelochemical activity from three different *A. minutum* strains, cultures in exponential growth phase were centrifuged (280 g, 5 min, 17°C) and re-suspended in new L1 medium to reach final cell concentrations of 3×10^4 cells mL^{-1} . These cultures were left to equilibrate (release BECs) for 24 h prior to the commencement of bioassays.

2.5.2 Exposure

Triplicate cultures of *C. muelleri* in exponential growth phase (7 days old) were diluted with filtered (0.2 μm) synthetic ocean water to reach 2×10^5 cells mL^{-1} in the bioassay. Diatoms were then exposed to dilutions of *A. minutum* culture filtrate (concentration 3×10^4 cells mL^{-1}) dilutions (in artificial seawater) in flow cytometry tubes (3 mL final volume), with filtrate concentrations

equivalent to 0, 50, 100, 300, 500, 1000, 2500, 5000, 10 000, 15 000 and 25 000 cells mL⁻¹. Bioassay tubes were then incubated for 120 min: the first 100 min under light and the last 20 min in the dark, before Fv/Fm measurements. General bioassays procedures and test conditions are summarized in Table 1.

2.6 Calculations and graphics

The percentage of inhibition of maximum quantum yield was calculated as follow:

$$Inhibition = \frac{Fv/Fm_{control} - Fv/Fm_{filtrate}}{Fv/Fm_{control}} \times 100$$

Where Fv/Fm_{control} and Fv/Fm_{filtrate} were the maximum quantum yield of the diatom in artificial seawater and exposed to *A. minutum* filtrate, respectively. All graphics were performed using R software (R Foundation for Statistical Computing, Vienna, 2011). The effective concentration inhibiting 10 % (EC₁₀), 50 % (EC₅₀) and 90 % (EC₉₀) of the Fv/Fm as compared to the control, were calculated from the dose-response curve based on the required *A. minutum* cell density to achieve a filtrate toxicity to inhibit Fv/Fm. To calculate the effective concentrations, the “Dose-Response Curve” package of R statistical analysis software was used (Gerhard et al., 2014). The “Akaike’s Information Criterion” was used to determine model suitability where multiple models were tested (Koppel et al., 2017; Pinheiro and Bates, 2000). In all cases, a log-logistic model with 3 parameters was favored. While inhibition data will be given in the manuscript, the Fv/Fm data are available in the supplementary files.

Table 1: Culturing and allelochemical interaction test conditions

Allelopathic sample	
Type of sample	Culture filtrates (0.2µm, acetate cellulose)
Storage	Glass containers, no plastic
Concentration	Series of dilutions
Target cells	
Species	<i>C. muelleri</i>
Strain	CCAP 1010-3
Culture media	L1 media supplemented with silica in synthetic ocean water
Culturing temperature	17 ± 1 °C
Culturing light condition	100 – 110 µmol photon m ⁻² s ⁻¹
Culturing light cycle	Continuous light
Physiological state	Exponential growth rate
Concentration in bioassay	2 x 10 ⁵ cells mL ⁻¹
Allelopathic test condition	
Test conditions	Static, well homogenized
Test duration	120 minutes
Test temperature	Ambient temperature (≈ 20 °C)
Test light condition	100 minutes under light then 20 minutes in dark (dark-adapted sample)
Test chamber	Flow-cytometric tubes (Polystyrene), cuvettes (Polymethyl methacrylate)
Test solution volume	2-3 mL
Endpoint	Maximum photosystem II quantum yield (Fv/Fm)

3 Results and discussion

3.1 Sensitivity of Fv/Fm to filtrate

When *C. muelleri* was exposed to the lowest concentration of *A. minutum* filtrate (50 cells mL⁻¹), Fv/Fm was not affected (Figure 1). For *C. muelleri* exposed to higher filtrate concentrations, Fv/Fm was inhibited within the first 30 min corroborating the rapid toxicity mechanisms reported by Lelong et al. (2011) and Tillmann et al. (2007). At a concentration of 500 cells mL⁻¹, *C. muelleri* Fv/Fm inhibition varied between 6 ± 2 % and 24 ± 6 % during the six hours exposure. For filtrate concentrations above 500 cells mL⁻¹, Fv/Fm inhibition in *C. muelleri* increased quickly within the first 120 min then reached a plateau. When exposed to a theoretical cell concentration of 5 000 cells mL⁻¹, Fv/Fm inhibition was 21 ± 3 % at 30 min and increased to a 100 ± 0 % inhibition at 120 min. With the highest filtrate concentration tested (50 000 cells mL⁻¹), Fv/Fm inhibition was as high as 62 ± 4 % after only 30 min of exposure and reached 100 ± 0 % inhibition within 90 min. For all tested concentration, the maximum inhibition and a plateau were reached within the first 120 minutes. For the two highest concentrations, the complete inhibition was maintained for the duration of the experiment (6 hours). Thus the following experiments (and bioassays) were performed with an incubation time of 120 minutes. The measurement of an EC₅₀ after only 120 minutes is of interest because the bioassay should be sufficiently short to avoid any loss of allelochemical activity which can occur within a short time.

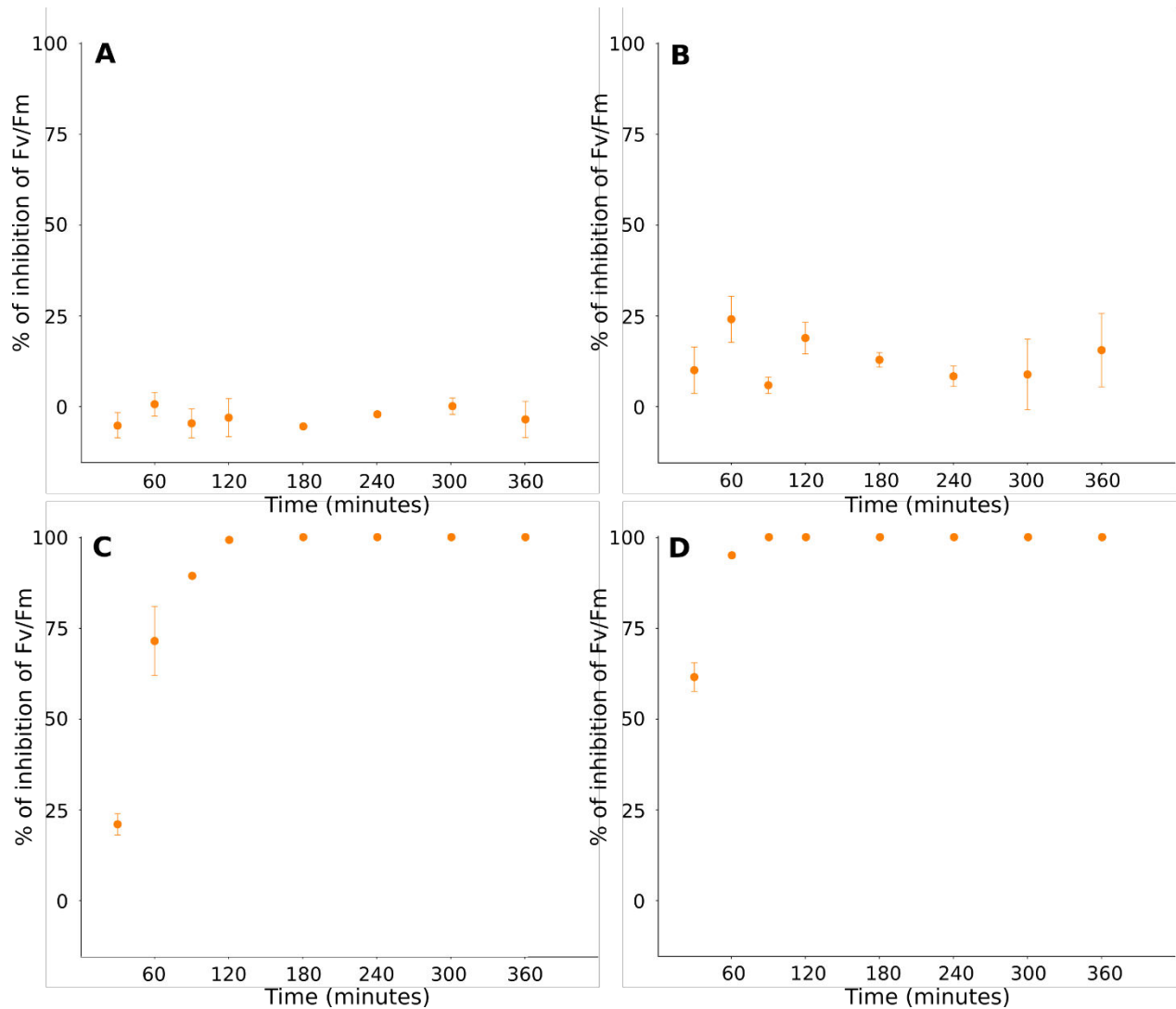


Figure 1: Kinetics of *C. muelleri* Fv/Fm inhibition when exposed to *A. minutum* (CCMI1002) filtrate at different theoretical cell concentrations of A: 50 cells mL⁻¹, B: 500 cells mL⁻¹, C: 5000 cells mL⁻¹ and D: 50 000 cells mL⁻¹. Results are expressed as mean \pm standard deviation.

3.2 Fv/Fm and FDA inhibition assay

In a second experiment, the filtrate-induced inhibition of Fv/Fm was compared to another proxy of metabolic activity. The fluorescein diacetate (FDA) assay measures esterase activity, is commonly used to determine phytoplankton viability (Agusti and Sánchez, 2002; Gentien et al., 2007) and has been proven to be efficient with the genus *Chaetoceros* (Garvey et al., 2007). Results showed that

C. muelleri esterase activity was inhibited by the allelochemical filtrate, as the proportion of metabolically inactive cells increased. This was observed by flow cytometry with a decrease in the green (FL1) fluorescence (Supplementary figure 1). Three of the filtrate concentrations (500, 5 000 and 50 000 cells mL⁻¹) induced a loss in metabolic activity as measured by both FDA staining and inhibition of Fv/Fm (Figure 2).

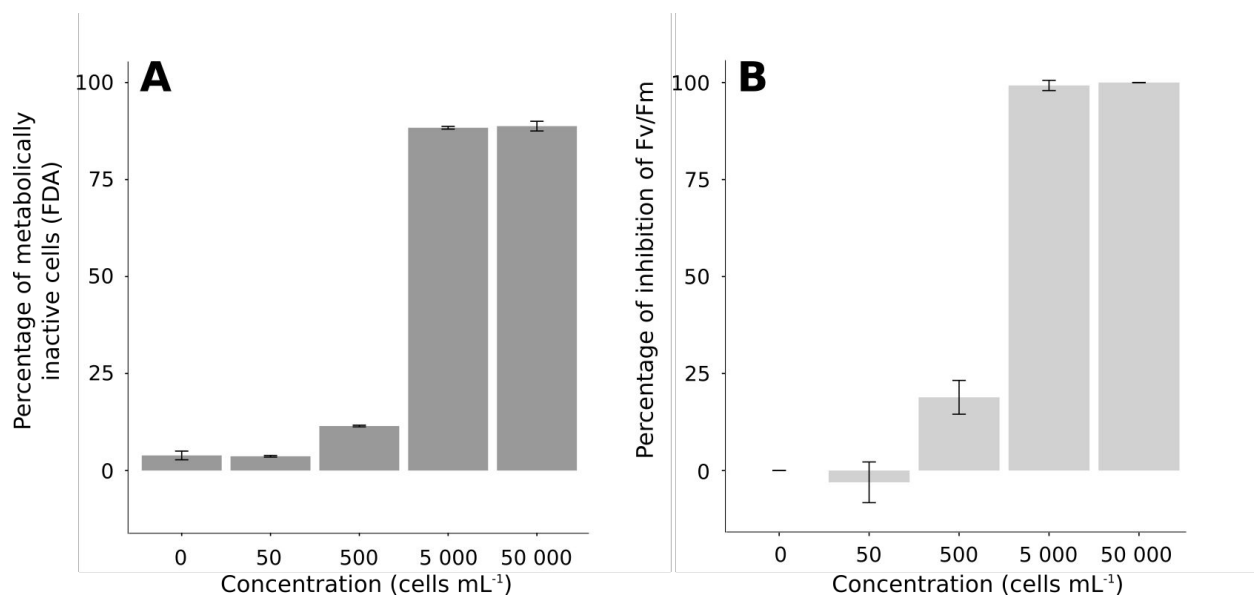


Figure 2: Effects of *A. minutum* (CCMI1002) filtrate on A) the percentage of metabolically inactive cells (FDA) and B) the percentage of inhibition of Fv/Fm of the diatom *C. muelleri* at different theoretical cell concentrations (0, 50, 500, 5 000 and 50 000 cells mL⁻¹) after 120 min of exposure. Results are expressed as mean \pm standard deviation. N=3.

No increase in the proportion of metabolically inactive cells or decrease in Fv/Fm (compared to control) was observed when the diatom was exposed to the filtrate concentration of 50 cells mL⁻¹. When exposed to higher filtrate concentrations, the inhibition of Fv/Fm was always higher than the percentage of metabolically inactive cells measured with FDA staining. At a filtrate concentration of 500 cells mL⁻¹, the percentage of metabolically inactive cells was 11.5 ± 0.2 %, while inhibition of Fv/Fm was of 19 ± 4 %. Once exposed to filtrate concentrations of 5 000 and 50 000 cells mL⁻¹, the proportion of metabolically inactive cells and inhibition of Fv/Fm were close to 88 and 100 %, respectively. While the use of FDA allows an estimation of the percentage of metabolically active cells, Fv/Fm is a measurement of the maximum photosystem II quantum yield (proxy of

photosynthetic metabolism), and both are good proxies as metabolic responses to *A. minutum* BECs with similar dose-response behaviour (dynamic/trend).

3.3 Bioassay: comparison of *A. minutum* strains

Inhibition of Fv/Fm in *C. muelleri* exposed to *A. minutum* culture filtrates was highly dependent on the *A. minutum* strain. In addition Fv/Fm inhibition was also related to theoretical cell concentration for the strains CCMI1002 and AM89BM. For those two strains, the relationship between Fv/Fm inhibition and the (logarithmic) theoretical cell concentration was a sigmoidal dose-response pattern (Figure 3). This dose-response curve enabled the calculation of the effective concentrations inhibiting Fv/Fm by 10 % (EC₁₀), 50 % (EC₅₀) and 90 % (EC₉₀) in the *C. muelleri* populations (Table 2). The strain CCMI1002 induced the most adverse effects with an EC₁₀ = 1350 ± 210 cells mL⁻¹ and an EC₅₀ = 4220 ± 480 cells mL⁻¹. In comparison, to obtain a similar inhibition of Fv/Fm of the marine diatom *Skeletonema costatum*, a concentration of 13 µg mL⁻¹ of the herbicide bentazon is necessary (Macedo et al., 2008). The strain AM89BM was four times less toxic than the strain CCMI1002, with an EC₁₀ = 7710 ± 349 and an EC₅₀ = 16500 ± 1700 cells mL⁻¹. These cell concentrations are well within the range of environmental *A. minutum* bloom concentrations, with reported densities as high as 41 000 cells mL⁻¹ in the bay of Brest, France (Chapelle et al., 2015) highlighting that *A. minutum* blooms may impact plankton communities. The results confirmed the difference in allelochemical potency of the strains observed by Borcier et al. (2017) in a single-concentration bioassay, and also allowed us to precisely quantify the allelochemical potency of each strain. The strain Da1257 had minimal effects on maximum photosystem II quantum yield, with the highest cell concentration tested (27 400 cells mL⁻¹) only inducing an Fv/Fm inhibition of 7 ± 3 % in *C. muelleri*., therefore effective concentrations (EC₁₀, EC₅₀, EC₉₀) could not be calculated. While this study validate the used of PAM fluorometry to quantify allelopathic potency from *A. minutum*, the proposed bioassay may be applied to other species of the genus *Alexandrium*. Many species from the genus *Alexandrium* induce lytic effects and therefore could inhibit photosynthesis. For instance the species *A. ostenfeldii* inhibits Fv/Fm (Tillmann et al., 2007). This bioassay could also be applied to other genus producing allelochemicals such as the dinoflagellate *Karlodinium venificum* which BECs also inhibits

photosystem II quantum yield (Sheng et al., 2010). Moreover the variability in the sensitivity of target species that has already been highlighted (Tillmann et al., 2008) could precisely be quantified and compared with this bioassay.

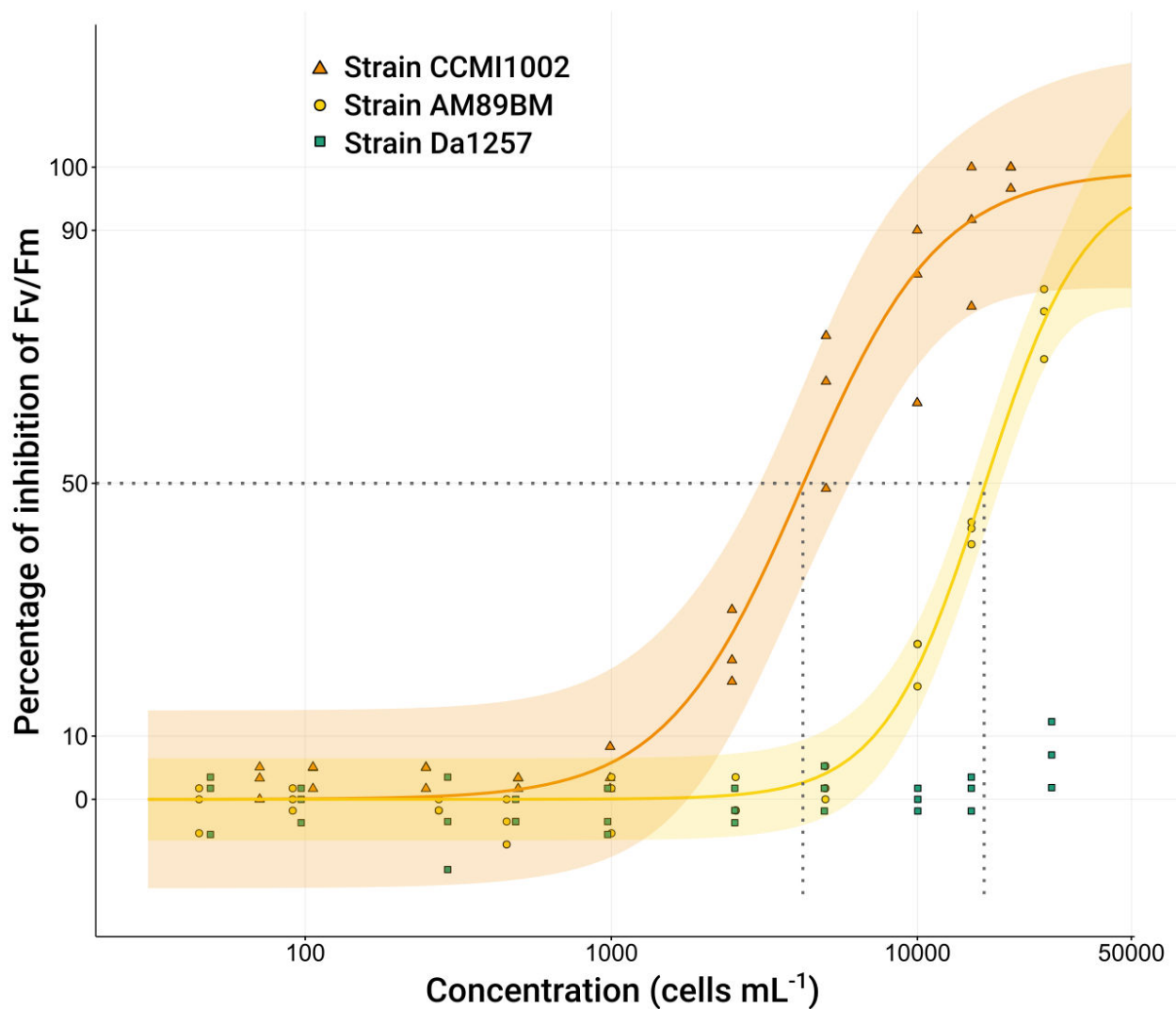


Figure 3: Inhibition of *C. muelleri* maximum photosystem II quantum yield (Fv/Fm) by *A. minutum* filtrates from different strains. Dots represent the percentage of inhibition of Fv/Fm as compared to controls, as a function of filtrate dilution. The diatoms were exposed to strain CCMI1002 (red triangle), strain AM89BM (yellow circle) and strain Da1257 (green squares) (n=3). For both curves, the ribbon represents the 95 % confidence interval from the log-logistic model. EC₅₀ are indicated by the dotted lines.

3.4 Precautions

This study provides important information on how to properly assess a microalgal bioassay using PAM fluorometry. F_v/F_m is a sensitive physiologic parameter that responds to many different environmental parameters (e.g. physiological state, light, temperature, various stress; Ralph et al., 2007) that can modify bioassay outcomes. Indeed, it is essential to be consistent in the protocol (Table 1) and to perform controls to ensure that the inhibition is attributable to allelochemical interactions. *Alexandrium* spp. BECs chemical features were partially described in previous studies and special care must be taken as the test conditions may greatly influence the outcomes of the bioassay. To maintain biological activity of the BECs over time, plastics should be avoided, especially for filtrate storage (Ma et al., 2009). When stored in glassware at a cool temperature ($\approx 5^\circ\text{C}$), allelochemical activity can be maintained for several weeks (Supplementary figure 4), and up to 4 months when stored at -30°C (Martens et al., 2016). Similarly, the choice of the filter composition is important, because most filter membranes retained allelochemical activity (unpublished data). Here we recommend the use of acetate cellulose or asymmetric polyethersulfone (aPES) membrane filters.

3.5 Bioassay applications

The ease of use and the rapid response of this bioassay makes it convenient for various experiments. PAM techniques have many logistical advantages as they only require small sample volumes (2 to 3 mL), they are cheap, they can be used with environmentally relevant chlorophyll concentrations and they are rapid (a few seconds per sample) compared to a classic growth inhibition test or a lytic bioassay. Moreover the handled devices are practical, and allow the bioassays to be performed *in-situ*. This enables a fast quantification of allelochemical potency from various *A. minutum* samples: cultures, bio-guided purification of BECs, and field samples.

Table 2: Effective filtrate concentrations (cells mL⁻¹) inhibiting 10 % (EC10), 50 % (EC50) and 90 % (EC90) of *C. muelleri* Fv/Fm for three different *A. minutum* strains. “NC” indicate that no value could be calculated. Results are expressed as mean ± standard error (n=3).

Strain	EC10	EC50	EC90
CCMI1002	1350 ± 210	4220 ± 480	13160 ± 3980
AM89BM	7710 ± 340	16510 ± 1690	38020 ± 7780
Da1257	NC	NC	NC

This bioassay can also be used for the purification of uncharacterized BECs (in prep.) produced by the genus *A. minutum*. The choice of the bioassay used to identify BECs during fraction purification is essential. During characterization of allelochemicals from *A. catenella*, Ma et al. (2011, 2009) preferred a phytoplankton (*Rhodomonas salina*) based bioassay rather than the usual bioassay with brine shrimp and red blood cells (Arzul et al., 1999; Emura et al., 2004). Ma et al. (2011, 2009) quantified allelochemical activity through microscopy counts of lysed *R. salina* cells, however the present study highlights an easier and faster bioassay to follow bioactivity during purification of these chemicals.

3.6 A unique bioassay for other cytotoxic activities ?

Exudates of *A. minutum* with allelochemical activity can have other toxic features. In a recent study, Borcier et al. (2017) were able to distinguish between the contrasting effects of paralytic shellfish toxins versus allelochemical exudates from *A. minutum* cultures over the great scallop *Pecten maximus* thanks to a similar but single-concentration bioassay. Delayed shell growth, reduced gill filtration, tissue damages and impaired escape behaviour when exposed to a predator were specifically attributed to the allelochemical exudates. This research highlights the importance of screening allelochemicals and studying the relationship between allelochemical interactions and other cytotoxic activities. Other studies have revealed that allelochemical activity may be related to other toxins (Arzul et al., 1999; Castrec et al., 2018; Le Goïc et al., 2014). This bioassay could be used to investigate the links between allelochemical activity and other cytotoxic (e.g. haemolytic, ichthyotoxic) activities through dose-response curves as performed by Blossom et al. (2014b) to, for example, establish the relationship between allelochemical activity and ichthyotoxicity from the prymnesiophyceae *Prymnesium parvum*.

3.7 Potential for field application

Allelochemical interactions research has been mainly based on laboratory experiments and lacks field reports to evaluate its role in plankton ecology. The bioassay developed here would be a useful tool during *A. minutum* blooms to measure/quantify allelochemical activity within an environmental matrix on a selected microalgal sample. The bioassay could be performed on *in-situ* samples when monitoring a bloom directly or under laboratory conditions following an appropriate sample storage (cooled glass container) to avoid any loss of activity. Moreover, measurement of a non-lethal parameter, such as photosystem II quantum yield, would allow the detection of mild effects. Indeed, non-lethal effects may reduce a cells ability to cope with other stress and indeed to compete with other species.

4 Conclusion

This study has demonstrated that the fluorescence-based bioassay can provide a fast and sensitive measure of allelochemical activity from various samples: culture filtrates and extracts resuspended in seawater. The developed bioassay successfully used the diatom *C. muelleri* as a target cell and enabled the quantification of allelochemical activity from three different *A. minutum* strains. Accurate quantification of allelochemical activity revealed a high variability in allelochemical potency of different strains from the same species. This bioassay may facilitate further research to broaden our understanding of allelochemical interactions from *Alexandrium* genus (purification and characterization, toxicity mechanisms, ecologic role). The information in this study also provides protocols to applying this technique to the study of allelochemical potency from other microalgal models.

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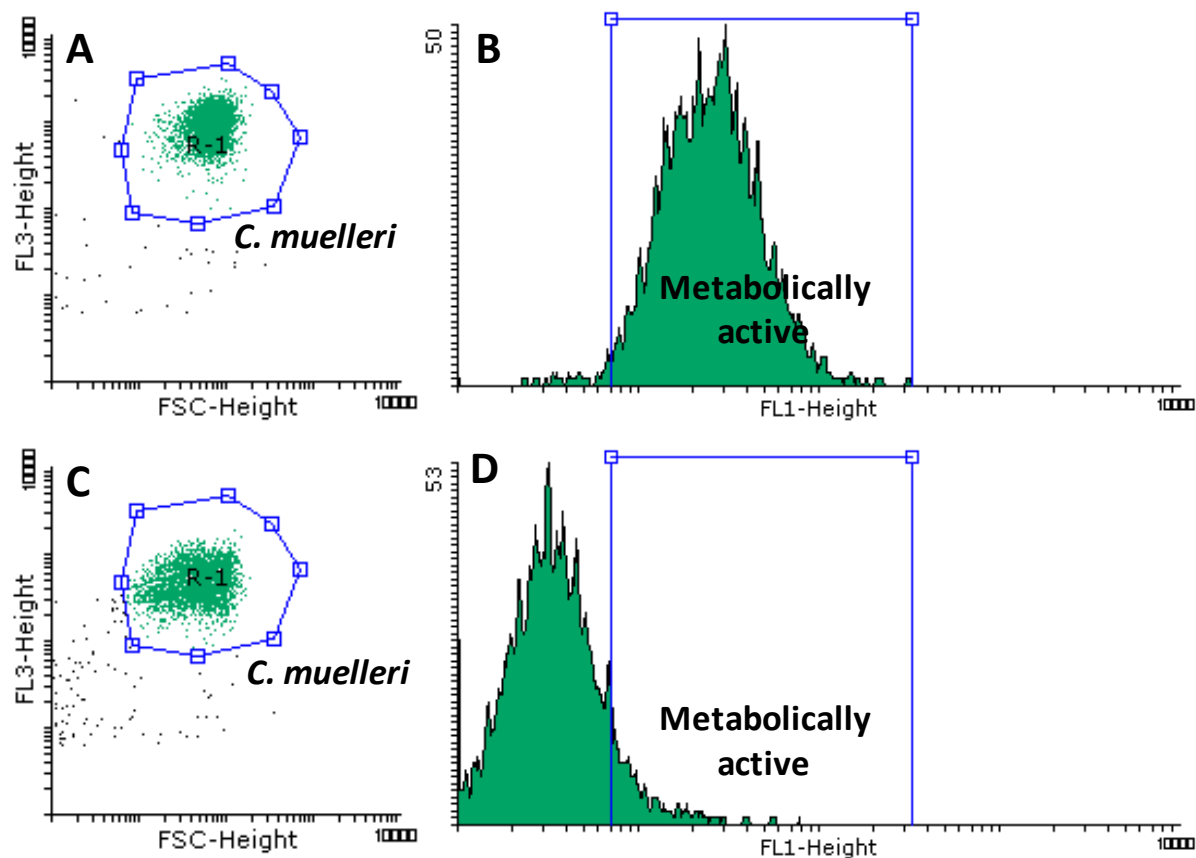
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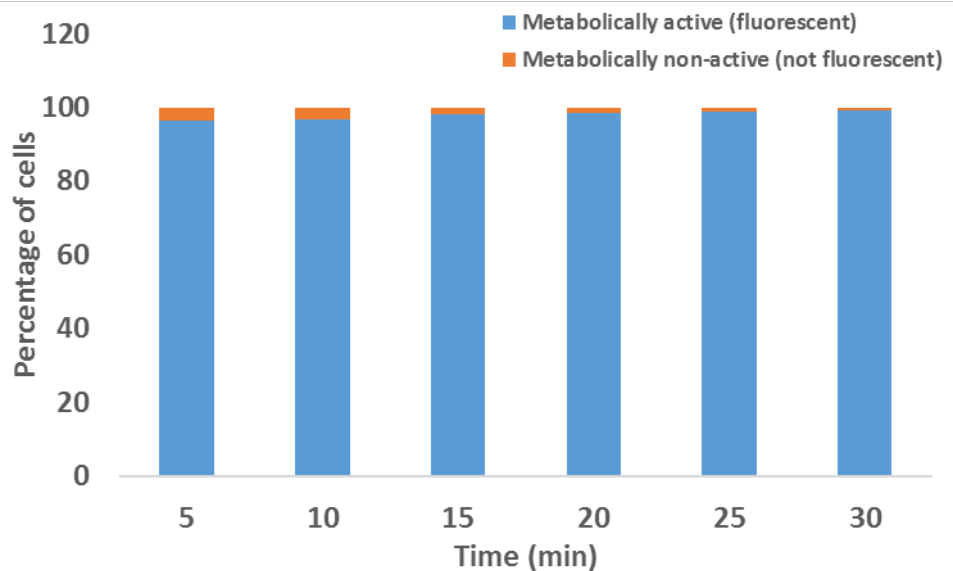
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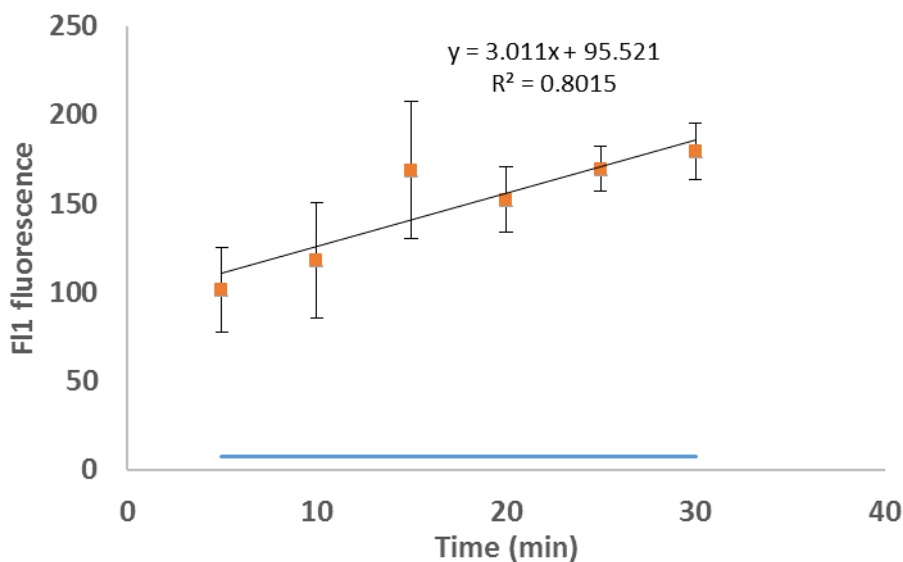
Supplementary files



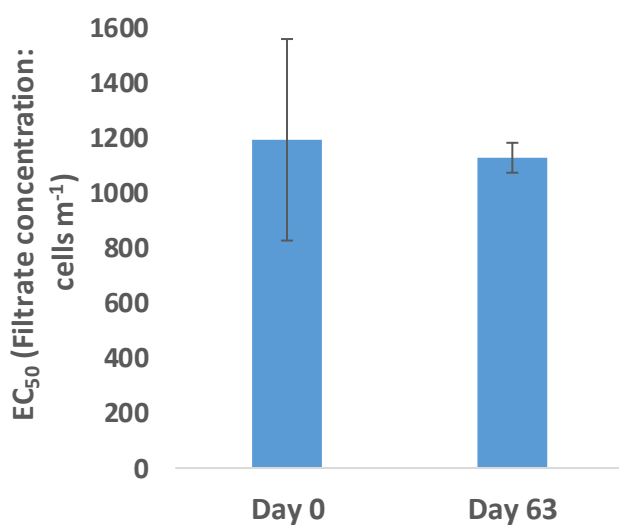
Supplementary figure 1: Cytograms of *Chaetoceros muelleri* culture: health cells (A and B) and cells exposed to *Alexandrium minutum* filtrate (C and D). The microalgal population (R-1) is illustrated in green on dot plots A and C representing red fluorescence (FL3-Height) vs. forward scatter (FSC-Height). The histograms B and D represent the count of cells vs. their green fluorescence (FL1-Height) after FDA staining. Metabolically active cells (high green fluorescence) are within the blue rectangle while metabolically inactive cells (low green fluorescence) are on the left of the blue rectangle.



Supplementary figure 2: Percentage of metabolically active (fluorescent cells; in blue) and metabolically inactive (not fluorescent; in orange) *C. muelleri* cells stained with FDA for 5, 10, 15, 20, 25 and 30 min (in seawater).



Supplementary figure 3: Mean internal F11 fluorescence of *C. muelleri* stained with FDA for 5, 10, 15, 20, 25 and 30 min (in seawater at room temperature). The black line represents the linear model of *C. muelleri* F11 fluorescence as a function of time of incubation. The Blue line represents the F11 fluorescence levels of *C. muelleri* (not stained).



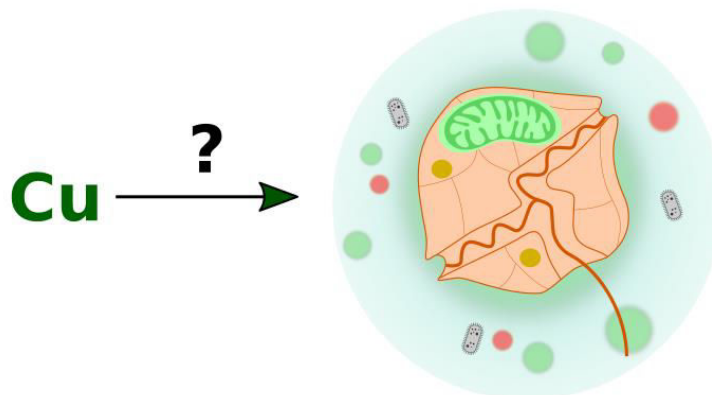
Supplementary figure 4: Example of the effect of storage on the allelopathic potency of filtrate of *A. minutum* CCMI1002. The allelopathic potency (EC₅₀) of the filtrate was measured on the fresh filtrate (Day 0) and on the filtrate stored for 63 days in a glass tube in the dark at 5°C. The culture used for the preparation of the filtrate was grown in L1 media in artificial seawater. The bioassay was performed according to the standard bioassay procedure given in the manuscript. Error bars represent standard error of the EC₅₀.

Chapter 4

Effect of abiotic factors on allelochemical potency

The environmental influences on *Alexandrium minutum* has been poorly investigated. In this chapter we use the bioassay developed in Chapter 2 to study the effect of abiotic factors on the allelochemical potency of *A. minutum* exudates. We chose to focus on the effect of Cu, a metal that contaminates coastal environments where the genus *Alexandrium* is distributed. Cu is a metal of interest as toxic concentrations increase the release of dissolved organic carbon by *A. tamarense* and modify the quality of DOC, regardless of allelochemical potency. Moreover, Cu is known to increase the toxicity from other microalgal genus. In this chapter, we tested whether a toxic Cu exposure could modify the release of allelochemicals by *A. minutum*. The effects of Cu on *A. minutum* allelochemical potency were related to other physiological parameters. This chapter consists of one article (Article 4) that is under review in *Aquatic Toxicology*.

Article 4: Long, M., Holland, A., Planquette, H., González Santana, D., Whitby, H., Soudant, P., Sarthou, G., Hégaret, H., Jolley, D. A multi-trait approach reveals the effects of Cu on the physiology of an allelochemical-producing strain of *Alexandrium minutum*. Under review in *Aquatic Toxicology*.



Article 4

A multi-traits approach reveals the effects of Cu on the physiology of an allelochemical-producing strain of *Alexandrium minutum*

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Abstract

The dinoflagellate *Alexandrium minutum* produces toxic compounds, including paralytic shellfish toxins, but also some unknown extracellular toxins. Copper (Cu) can inhibit microalgal physiology but may also increase their toxic potency. This study investigated the effect of different concentrations of dissolved Cu (7 nM, 79 nM and 164 nM) on the physiology of a strain of allelochemical-producing *A. minutum*. The effects of Cu were assessed on *A. minutum* growth, stress responses (ecdysis and reactive oxygen species), photosynthesis proxies, lipid metabolism, exudation of dissolved organic compounds, allelochemical potency and on the associate bacterial community of *A. minutum*. Only the highest Cu exposure (164 nM) inhibited and delayed the growth of *A. minutum*. Within the first 7 days of the high Cu treatment, the physiology of *A. minutum* was severely impaired with decreased growth and photosynthesis, and increased stress responses and bacterial density per algal cell. After 15 days, *A. minutum* partially recovered from Cu stress as highlighted by the growth rate, reactive oxygen species and photosystem II yields. This recovery could be attributed to the apparent decrease in background dissolved Cu concentration to a non-toxic level, suggesting that the release of exudates may have partially decreased the bioavailable Cu fraction. The allelochemical potency of exudates significantly increased after 7 days of exposure, when the dissolved Cu concentration was still toxic and when *A. minutum* cells were stressed. Overall, *A. minutum* appeared quite tolerant to Cu, and this work suggests that the modifications in the physiology and in the exudates help the algae to cope with Cu exposure. Modulation in allelochemical potency may have ecological implications with an increased competitiveness of *A. minutum* in contaminated environments.

1 Introduction

Harmful algal blooms (HAB) refer to the proliferation of microalgae with negative consequences such as ecological and economic issues, contamination of seafood and human poisoning. Species responsible for HAB sometimes bloom without producing any harmful effects; however, they can become toxic under specific environmental conditions. Dinoflagellates account for 75% of marine HAB (Smayda, 1997). One of the most well-known genus responsible for HAB is the genus *Alexandrium*, which is responsible for Paralytic Shellfish Poisoning, due to the production of saxitoxin and its derivatives, which affect human health. Because of this toxicity, dinoflagellates of the genus *Alexandrium* have thus been extensively studied, and most of the effects of *Alexandrium* spp. on marine organisms have been attributed to these toxins. Several studies have however highlighted the existence of some other extracellular toxins, which can impair marine organisms such as bivalves (Castrec et al., 2018), fishes (Mardones et al., 2015) or protists (Tillmann and John, 2002).

Studies have intended to understand the parameters controlling *Alexandrium* spp. blooms and their toxicity, however the environmental parameters responsible for the proliferation and toxicity of these microalgae remain poorly understood. Physicochemical parameters such as temperature, hydrodynamism (Guallar et al., 2017), nutrient availability (Hwang and Lu, 2000; Vila et al., 2005), presence of organic compounds (Gagnon et al., 2005) or metals (Herzi et al., 2013) are known to influence growth and modulate blooms. However, biological interactions such as parasitism (Chambouvet et al., 2008; Garcés et al., 2005), predation (Calbet et al., 2003; Collos et al., 2007) and allelochemical interactions (Legrand et al., 2003) are also known to influence phytoplankton assemblages. It is indeed very difficult to predict HAB at the interface of all the physicochemical and biological processes, especially as these factors may influence each other.

Copper (Cu) has been shown to affect microalgal physiology by affecting processes such as microalgal growth, photosynthesis or exudation (Herzi et al., 2013; Juneau et al., 2002). In the case of HAB, Cu can modulate the toxicity of some species (Maldonado et al., 2002; Moeller et al., 2007). Proliferations of *Alexandrium* spp. occur in coastal and estuarine environments (Anderson et al., 2012; Guallar et al., 2017). Within this genus, the species *A. minutum* is predominant and is present worldwide (Lassus et al., 2016) in coastal areas that can be subject to anthropogenic inputs including Cu contamination (Herzi, 2013; Melwani et al., 2014). For instance, *A. minutum* bloom

in the bay of Brest, France (Chapelle et al., 2015; Guallar et al., 2017) or in the Ria de Vigo, Spain (Frangópulos et al., 2004), which are areas that are subject to Cu contamination (García et al., 2013; Guillou et al., 2000; Lacroix, 2014; Michel and Averty, 1997; Prego and Cobelo-García, 2003; Quiniou et al., 1997; Rodriguez, 2018). To our knowledge, only one study has investigated the effects of Cu on *A. catenella* (Herzi et al., 2013). This research showed that toxic Cu concentrations increased the exudation of the dinoflagellate and modified the quality of the exudates but no attention was given to phycotoxins. No studies have investigated the effects of dissolved Cu on the physiology and allelochemical potency of *A. minutum*.

This study intends to (1) provide an overview of the main physiologic functions altered by toxic Cu concentrations, and (2) evaluate the effects of Cu on allelochemical potency to better understand the responses of the toxic dinoflagellate *Alexandrium minutum* in response to Cu stress. A strain of *A. minutum* was exposed to toxic but environmentally relevant concentrations of dissolved Cu for 15 days. The effects of Cu on many different physiological parameters were then studied over the 15 days of exposure to understand how *A. minutum* responds to a metallic stress. The physiological parameters included growth, cell stress responses (ecdysis and reactive oxygen species), proxies of photosynthesis, lipid metabolism, the exudation and bacterial community of *A. minutum*. This study specifically focused on one strain of *A. minutum* that produces allelochemicals in order to study the effects of Cu stress on its allelopathic potency.

2 Materials and methods

2.1 General material

All culturing glassware used in toxicity tests was coated in a silanizing solution (Coatasil; Ajax Chemicals) to prevent Cu losses due to adsorption to flasks during toxicity tests. Culturing glassware used in Cu exposure was nitric acid washed (10%, v/v HNO₃ AR grade, Merck) for at least 24 hours. Consumables used in culturing and Cu sampling/measurements were new or nitric acid washed as previously described. A Cu stock solution (15.7 mM) was prepared in ultrapure water (MERCK Millipore, 18M Ω) from CuSO₄.5H₂O (analytical reagent grade; Ajax Chemicals) in acidified ultrapure water (HCl, Tracepur; Merck; 0.2% v/v). An intermediate Cu solution of 157 μ M used for spiking was prepared by diluting the stock solution in ultrapure water. Seawater for Cu exposures was collected from Oak Park, Cronulla, New South Wales, Australia (Salinity = 35.9, pH = 8.16). Seawater was filtered (0.2 μ m, aPES, Rapidflow, Nalgene) and autoclaved.

2.2 Microalgal stock culture

Microalgal cultures and glassware were not axenic, however culture handling was performed under a laminar flow hood to avoid any additional bacterial or metal contamination. Cultures of *A. minutum* were grown in natural seawater (Cronulla, Australia) supplemented with F/2 media (Guillard, 1975). The *A. minutum* strain CCM11002, isolated from a bloom in Gearhies (Bantry Bay in Ireland), was selected according to its allelochemical potency (Borcier et al., 2017; Castrec et al., 2018; Long et al., 2018; Long et al., In press). Volume of the *A. minutum* ($5560 \pm 2350 \mu\text{m}^3$) was considered to resemble to the volume of an ellipsoid (Hillebrand et al., 1999) and calculated according to its length ($24.1 \pm 3.1 \mu\text{m}$), diameter ($20.5 \pm 3.0 \mu\text{m}$) and width ($0.4 \pm 0.1 \mu\text{m}$) measured on 55 cells. Cultures of *A. minutum* were kept under exponential growth phase through weekly culturing and were maintained at $17 \pm 1^\circ\text{C}$ under a 12/12 day/night light cycle ($150 - 210 \mu\text{mol photon m}^{-2} \text{s}^{-1}$).

2.3 Experimental procedure

The chronic toxicity of Cu on the physiology of *A. minutum* was studied over exponential growth phase for 15 days. One day before the start of the exposure, cells in exponential growth phase were used to inoculate the treatment flasks after centrifugation (280 g, 6 min, 19°C) and one wash in filtered seawater to remove residual culture medium. Cells of *A. minutum* were inoculated in the test media of natural seawater (Cronulla, Australia) supplemented with NO_3^- (15 mg L^{-1}), PO_4^{3-} (1.5 mg L^{-1}) and vitamins (1/5 of the F/2 vitamin concentration). Test medium were inoculated at $5\,000 \text{ cells mL}^{-1}$ one day before (day -1) the Cu spike (day 0) in order to let the culture decyst from centrifugation. Cultures were carefully handled and pipetted to mitigate cyst formation due to mechanic stress. Cultures were exposed to three different treatments with different initial Cu concentrations (measured concentrations): a control with no Cu and a “natural” dissolved Cu concentration of $7 \pm 1 \text{ nM}$ added (5 replicates), a “low” Cu treatment [Cu_1] with a dissolved Cu concentration of $79 \pm 6 \text{ nM}$ (5 replicates) and a “high” Cu treatment [Cu_2] with a dissolved Cu concentration of $164 \pm 6 \text{ nM}$ (6 replicates). Samples for dissolved Cu analysis were collected 2 hours after the Cu spike and after 7 and 15 days of exposure. Microalgal concentration, percentage of ecdysal cysts, and bacterial population were monitored every 2 days for 20 days. Lipid content and intracellular reactive oxygen species (ROS) were monitored after 1, 7 and 15 days of exposure. The photosynthetic parameters, Photosystem II quantum yields (maximum: F_v/F_m and effective:

Φ_{PSII}) and non-photochemical quenching (NPQ), and the allelochemical potency of the filtrate were monitored after 7 and 15 days of exposure. The chromophoric dissolved organic carbon (cDOC) was characterized after 15 days.

2.4 Copper analyses

Samples were filtered (0.45 μm , Sartobran P sterile midicap; Sartorius) and acidified to 0.2 % with HNO_3 (Suprapur, Merck Millipore) before metal analysis. Dissolved Cu was measured with a Sector Field Inductive Coupled Plasma Mass Spectrometry (SF-ICP-MS, Element XR) combined with the seaFAST-pico (Elemental Scientific Inc., ESI, Omaha, NE, USA; <http://www.icpms.com/PDF/seaFAST-pico-open-ocean%20seawater.pdf>) (Lagerström et al., 2013) introduction system and a 4DX autosampler (Elemental Scientific Inc.). This allowed for the automated online pre-concentration of samples (pre-concentration factor of 50). Every 10 samples, a replicate was run. In house standard and SAFe D2 standard reference seawater was run to check for accuracy. The dissolved Cu concentrations were expressed in nM of Cu. The theoretical cellular Cu (Cu adsorbed on cell membranes and internalized by cells, expressed in fmol Cu cell⁻¹) was calculated as the difference in dissolved Cu between day 1 and day 15, divided by the cell concentration. To compare the obtained results with literature, the theoretical cellular Cu could also be divided by *A. minutum* biovolume (expressed in $\mu\text{mol Cu L}^{-1}$ of *A. minutum* cells, also named μM biovolume).

2.5 Flow cytometry

2.5.1 General and cells enumeration

Flow cytometric analyses were performed on a flow cytometer Becton Dickinson LSRII. Cell variables, e.g. forward scatter (Forward scatter, FSC), side scatter (Side scatter, SSC), red autofluorescence (FL3, red emission filter long pass, 670 nm) and green fluorescence (FL1, green emission filter band pass, 530/30 nm) were used to select *A. minutum* population. Microalgal cells and bacteria (after SYBR Green I staining; see paragraph 2.4.3) were counted by flow cytometry according to flow rate (Marie et al., 1999).

2.5.2 Cysts

Culture samples were stained for 30 minutes in the dark with SYBR Green I (final concentration in tube) according to (Haberkorn et al., 2011). SYBR Green I is a fluorescent (520 nm) dye that

binds double-stranded DNA molecules. SYBR Green I permeates vegetative cells of *A. minutum* and bacteria, however the probe does not permeate cysts thus allowing the distinction between vegetative cells (highly fluorescent on FL1 detector) and temporary cysts (not or very little fluorescent on FL1 detector).

2.5.3 Bacteria

Bacteria in *A. minutum* cultures were stained with SYBR Green I (da Costa et al., 2017). Sub-samples of SYBR Green I stained culture samples were diluted (10 to 50 times) in 0.2 µm filtered seawater for bacterial enumeration by flow-cytometry.

2.5.4 Lipid content

To assess changes in intracellular neutral lipid content, the BODIPY probe (BODIPY 493/503, Invitrogen, D3922; at a final concentration of 10 µM) was used. Samples were stained for 30 minutes in the dark prior to flow-cytometric measurement. Intracellular fluorescence intensity was measured with FL1 emission filter (green emission filter band pass, 530/30 nm). The relative neutral lipid content is expressed as the mean FL1 fluorescence (green fluorescence expressed in arbitrary unit; a.u.) per cell. The lipid production (a.u. cell⁻¹ day⁻¹) rate was calculated according to (Lelong et al., 2013) by multiplying the relative lipid content by the growth rate (day⁻¹).

2.5.5 Intracellular Reactive Oxygen Species

Intracellular reactive oxygen species (ROS) production was measured using 2',7'-dichlorofluoresceindiacetate (DCFH-DA; Sigma-Aldrich, D6883; at a final concentration of 10 µM). DCFH-DA is a cell permeable fluorescent indicator of ROS production. Once inside the cells, DCFH-DA is hydrolyzed by esterase to form the non-fluorescent DCFH retained within the cell. DCFH can thus be oxidized by H₂O₂ and other oxidants to produce fluorescent 2',7'-dichlorofluorescein (DCF). Intracellular oxidation level is correlated to DCF fluorescence within the cells and measurable by flow cytometry with FL1 emission filter (green emission filter band pass, 530/30 nm). Samples were stained for 30 minutes in the dark prior to flow-cytometry measurements. The relative ROS production is expressed as the mean FL1 fluorescence per cell.

2.6 Pulse amplitude modulation fluorometry

Fluorescence measurements were performed with an AquaPen-C AP-C 100 with a blue light (455 nm). For the measurement of the maximum PSII quantum yield (F_v/F_m), algal samples were dark-

adapted for 15 minutes before measurement of fluorescence variables (F_0 , F_m). F_0 is the initial fluorescence intensity, F_m is the maximal intensity under saturating light conditions (0.5 to 1 s at $1500 \mu\text{mol photon m}^{-2} \text{s}^{-1}$), and $F_v = F_m - F_0$ (Strasser et al., 2000). For the measurement of effective quantum yield (Φ_{PSII} ; the proportion of absorbed energy being used in photochemistry (Maxwell et al., 2000)) and the non-photochemical quenching (NPQ), a short saturating flash of light ($1500 \mu\text{mol photon m}^{-2} \text{s}^{-1}$) was applied to measure the maximum fluorescence in the dark adapted state, F_m . After a short dark relaxation, the sample was exposed to actinic irradiance ($300 \mu\text{mol photon m}^{-2} \text{s}^{-1}$) for 60 s and a sequence of 5 saturating flashes ($1500 \mu\text{mol photon m}^{-2} \text{s}^{-1}$) was applied on top of the actinic light to probe the NPQ and Φ_{PSII} in light-adapted state. After exposure to continuous illumination, the relaxation of non-photochemical quenching was determined by means of 3 saturating pulses applied in dark (dark period of 88 s).

2.7 Characterization of exudates

Samples for the characterization of exudates (dissolved organic carbon or allelochemicals) were filtered with an acetate cellulose filter ($0.2 \mu\text{m}$) to keep allelochemical activity (Long et al., 2018). Filtrates were stored in glass tubes before characterization to avoid any binding of allelochemicals or dissolved organic carbon (DOC) on plastic. Filtrates were sampled at days 7 and 15 for allelopathy and at day 15 for the quantification of DOC (mg L^{-1}) and characterization of chromophoric DOC (cDOC).

2.7.1 Allelochemical potency of *A. minutum* filtrates

The allelochemical potency of *A. minutum* filtrates was assessed according to the bioassay developed by Long et al. (2018). Briefly, dilutions of the filtrate were added to cultures of the diatom *Chaetoceros muelleri* and the F_v/F_m of the diatom was measured after 2 hours. Dose-response curves (inhibition of F_v/F_m as a response of filtrate dilution) allowed the quantification of allelochemical potency by quantifying the effective concentration inhibiting 50% of the F_v/F_m of the diatom. Cultures of the diatom *C. muelleri* (CCAP 1010/3, formerly described as *Chaetoceros* sp. or *Chaetoceros neogracile*) were grown in natural seawater (Argenton, France; Salinity = 34, pH = 8.4) supplemented with L1 media (Guillard and Hargraves, 1993). Cultures of *C. muelleri* were kept under exponential growth phase through weekly culturing. Cultures were maintained at 18°C under a continuous light intensity of $100 - 110 \mu\text{mol photon m}^{-2} \text{s}^{-1}$. Cultures

were not axenic but were handled under sterile treatments to minimize additional bacterial contamination.

2.7.2 Dissolved organic carbon and quality of chromophoric dissolved organic carbon

Dissolved organic carbon in the 0.2 μm filtrate was measured using a Shimadzu TOC-VCSH/CSN, TOC/TN analyser with an auto-sampler (recommended in US EPA method 415.3). Chromophoric DOC was analyzed by fluorescence excitation emission (FEEM) scans according to (Holland et al., 2018). FEEM allows for the characterization of the fluorescent fraction of DOC (Stubbins et al., 2014). FEEM scans along with simultaneous absorbance measurements were conducted on filtrates. Excitation wavelengths were performed in 3-nm steps between 240 and 450 nm, and emission wavelengths at 2 nm between 210-620 nm. Parallel factor analysis (PARAFAC, PLS-toolbox in MATLAB: Eigenvectors Research Inc, WA, USA) was used to determine the number of components present within extracts. The model was validated using split-half analysis following recommendations from Murphy et al. (2013). Our PARAFAC model was then compared to others in the literature using the Openfluor database (Murphy et al., 2014).

2.8 Statistical analyses

All statistical analyses were performed using R software (R Foundation for Statistical Computing, Vienna, 2011). Significant differences in the dissolved Cu concentrations, the flow-cytometry (Lipids, ROS) and photosystem II (F_v/F_m , Φ_{PSII} , NPQ) parameters were assessed with a one-way ANOVA followed by a post-hoc Tukey HSD (ANOVA-HSD) when meeting the homoscedasticity with a Bartlett test and normality with a Shapiro-Wilk test. When homoscedasticity or normality were not met, a non-parametric Kruskal-Wallis test followed by a post-hoc Conover with a bonferroni adjust was applied (KW-bf). All tests were performed with a significance level of $p\text{-value} = 0.05$. To calculate the effective filtrate concentrations inhibiting F_v/F_m of the diatom in bioassays, the “Dose-Response Curve” package of R statistical analysis software was used (Gerhard et al., 2014). The “Akaike’s Information Criterion” was used to determine model suitability where multiple models were tested (Koppel et al., 2017; Pinheiro and Bates, 2000). Significant differences in effective concentrations (EC10, EC50) were analysed in pairs according to (Wheeler et al., 2006). If the confidence intervals gave an overlap of '1' then it was not considered significant.

3 Results

3.1 Copper

Cultures of *A. minutum* were exposed to different concentrations of Cu (Figure 1). The control was exposed to the natural dissolved Cu concentration of seawater (7 ± 1 nM). Cu was added to the two other treatments, [Cu₁] and [Cu₂], to reach initial concentrations of 79 ± 6 nM and 164 ± 6 nM respectively. While the dissolved concentration of Cu stayed stable in the control, in the Cu treatments [Cu₁] and [Cu₂] the concentration significantly (ANOVA-HSD, p-values < 0.001) decreased 2.5 – 3 times as compared to the initial value over the test period. The calculated theoretical cellular Cu concentration taken up between days 1 and 15 was comprised of between $0.6 \text{ fmol cell}^{-1}$ ($110 \mu\text{M}$ biovolume) and $3.8 \text{ fmol cell}^{-1}$ ($680 \mu\text{M}$ biovolume) for [Cu₁] and [Cu₂], respectively.

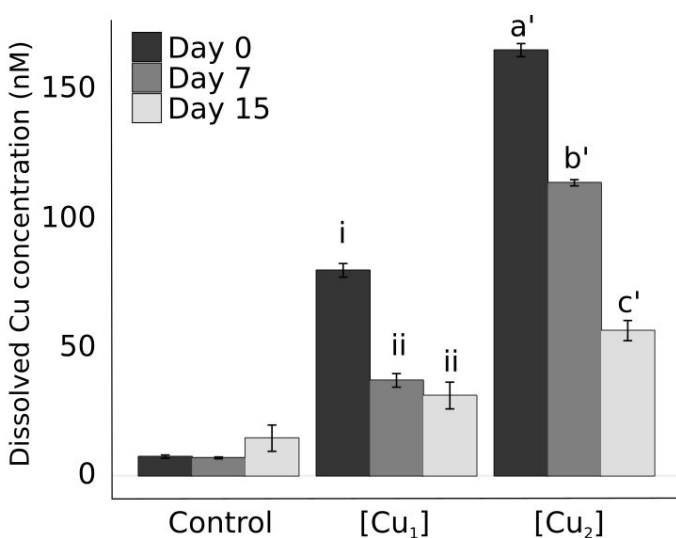


Figure 1: Dissolved copper concentrations (nM) in the Control, [Cu₁] and [Cu₂] treatments at day 0 (black bars), and after 7 days (dark grey bars) and 15 days (light grey bars) of exposure. Different letters indicate significant differences between the sampling times. Results are expressed as the mean \pm standard error ($5 < n < 6$).

3.2 Microalgal growth

Microalgal growth was maintained in exponential growth rate, in all the treatments, throughout the experiment (Figure 2 **Erreur ! Source du renvoi introuvable.**A). Although the lower Cu concentration applied (79 ± 6 nM) did not significantly affect population growth, *A. minutum* growth was decreased in [Cu₂] as shown by a significant (ANOVA-HSD, p-value < 0.001) decrease in growth rate and a significant (KW-bf, p-value < 0.001) lower cell concentration at 20 days.

3.3 Cysts

Exposure of *A. minutum* to dissolved Cu induced a significant (ANOVA-HSD, p-value < 0.05) increase in the formation of cysts in [Cu₂] after one day of exposure, as compared to the control and [Cu₁] (Figure 2B). In the control and [Cu₁], the percentage of cysts increased to reach a maximum after 7 days of exposure (15 % and 17 %, respectively) before decreasing. A similar, but delayed trend was observed for [Cu₂] with a maximum percentage of cysts (19 %) reached after 15 days of exposure.

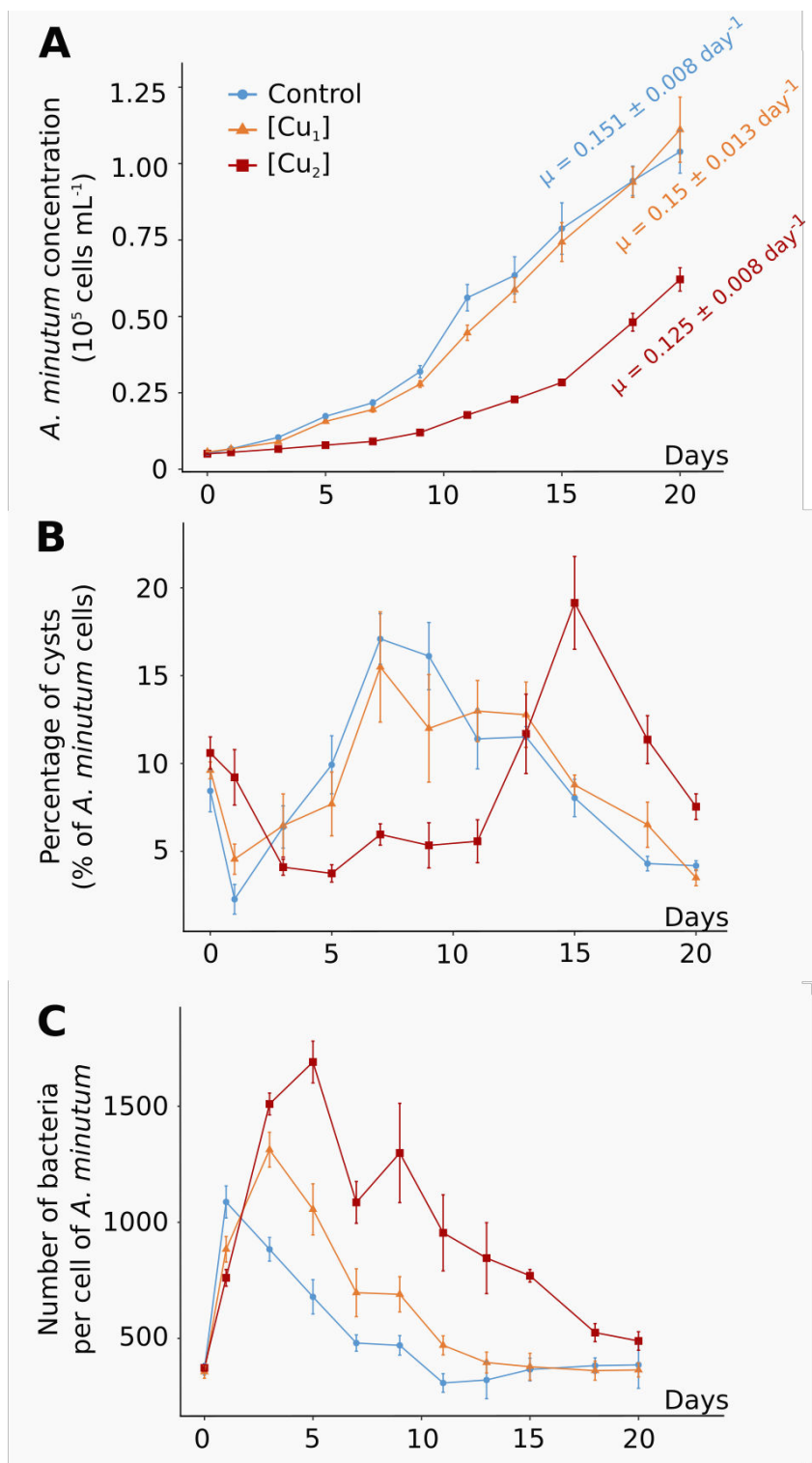


Figure 2: Effect of Cu treatments on growth of *A. minutum* and its associated bacterial community. A: Cell concentration (cells mL^{-1}) of *A. minutum*, B: Proportion (%) of *A. minutum* cysts in cultures, C: Ratio bacteria per cell of *A. minutum*. Blue lines and circles represent the control, orange lines and triangles represent the $[\text{Cu}_1]$, red lines and squares represent $[\text{Cu}_2]$. Results are expressed as the mean \pm standard error ($5 < n < 6$).

3.4 Bacterial-like community

The bacterial concentration followed an exponential increase throughout the duration of the experiment. Exposure to Cu significantly (ANOVA-HSD, p -values < 0.001) decreased the bacterial concentration and the number of bacteria per algal cell in [Cu₁] and [Cu₂] during the first day following the exposure (Figure 2C). Then, from day 3, the number of bacteria per *A. minutum* cell was higher in cells exposed to Cu (up to 3 times the control ratio for [Cu₂] at day 9).

3.5 Flow cytometry parameters

The lipid content of the vegetative cells decreased in each of the treatments following the first day of experiment (Figure 3A; in a.u. cell⁻¹). However, there was less of a decrease in [Cu₂], where the lipid content was significantly (ANOVA-HSD, p -value < 0.001) higher than the control after 1 day of exposure. After 7 days, the lipid content of *A. minutum* from [Cu₂] treatment was significantly (ANOVA-HSD, p -value < 0.01) higher than cells from [Cu₁] treatment but not from the control (ANOVA-HSD, p -value < 0.07). After 15 days of exposure, the lipid content in the control and [Cu₁] increased and was significantly (ANOVA-HSD, p -value < 0.05) higher than [Cu₂]. After 7 days, the lipid production (Figure 3B; in a.u. cell⁻¹ day⁻¹) was significantly lower for [Cu₁] (ANOVA-HSD, p -value < 0.05) and [Cu₂] (ANOVA-HSD, p -value < 0.001) as compared to the control. The lipid production rate in [Cu₂] was significantly lower than the control after 15 days (ANOVA-HSD, p -value < 0.01).

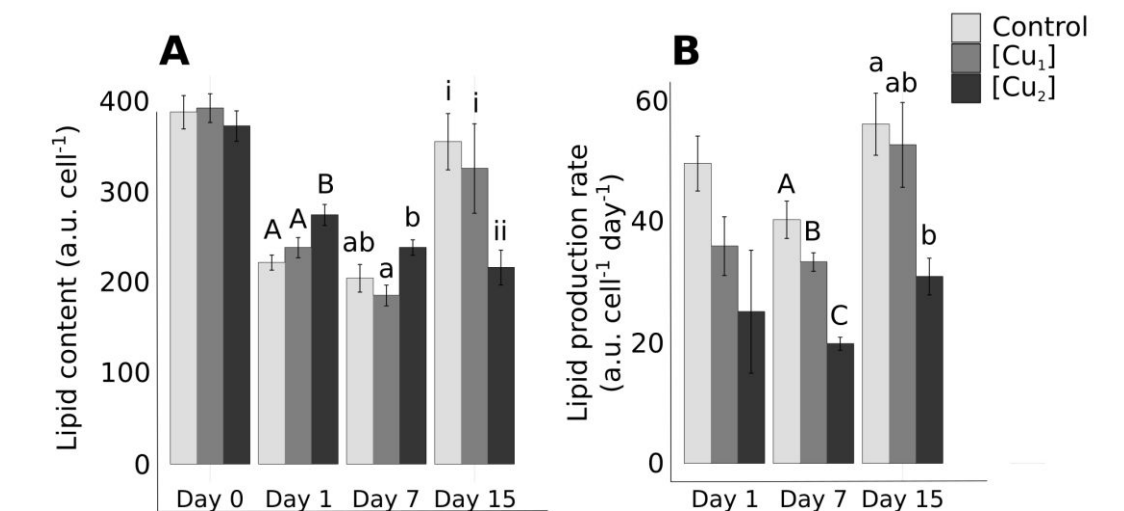


Figure 3: Lipid metabolism of *A. minutum* over the 15 days of exposure to the different Cu treatments. A: Lipid content per cell, B: Daily lipid production rate per cell. Results are expressed as the mean \pm standard error ($5 < n < 6$). Different letters indicate significant differences between the treatments at each day of exposure.

In all the treatments, intracellular ROS production by vegetative cells varied between day 1 and 15, with the highest values at day 7 (Figure 4). The intracellular ROS production was consistently higher in [Cu₂], however this difference was only significant (ANOVA-HSD, p-value < 0.05) at day 7. The lower Cu exposure [Cu₁] did not affect the ROS production of the vegetative cells.

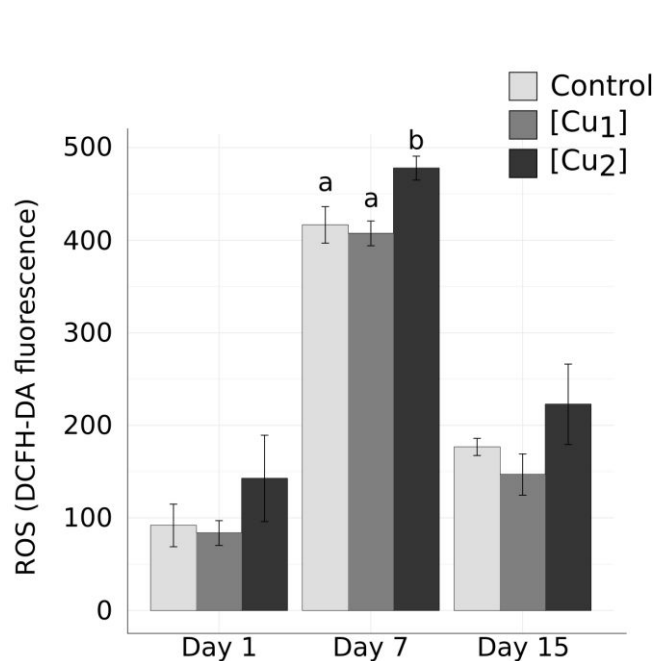


Figure 4: Intracellular reactive oxygen species (ROS) production of *A. minutum* in the control or after 1, 7 and 15 days of exposure to both copper concentrations measured by flow cytometry. Results are expressed as the mean \pm standard error ($5 < n < 6$). Different letters indicate significant differences between the treatments at each day of exposure.

3.6 Photosynthesis

Exposure to the highest Cu concentration [Cu₂] induced slight, but significant (ANOVA-HSD, p-value < 0.05) decreases in the maximum PSII quantum yield (F_v/F_m) as compared to the control after 7 and 15 days (Figure 5A). The effective quantum yield of PSII (Φ_{PSII}) was more sensitive to Cu with a significant (ANOVA-HSD, p-value < 0.01) decrease of 35% after 7 days of exposure (Figure 5B). After 15 days of exposure, this trend reversed, with a Φ_{PSII} significantly (ANOVA-HSD, p-value < 0.01) higher in [Cu₂] than in the control. Despite the high variability at day 7, NPQ appeared negative/close to 0 in the control and [Cu₁] but positive in [Cu₂] (Figure 5C). After 15 days of exposure, the NPQ was positive in all treatments but no significant difference was observed between the treatments.

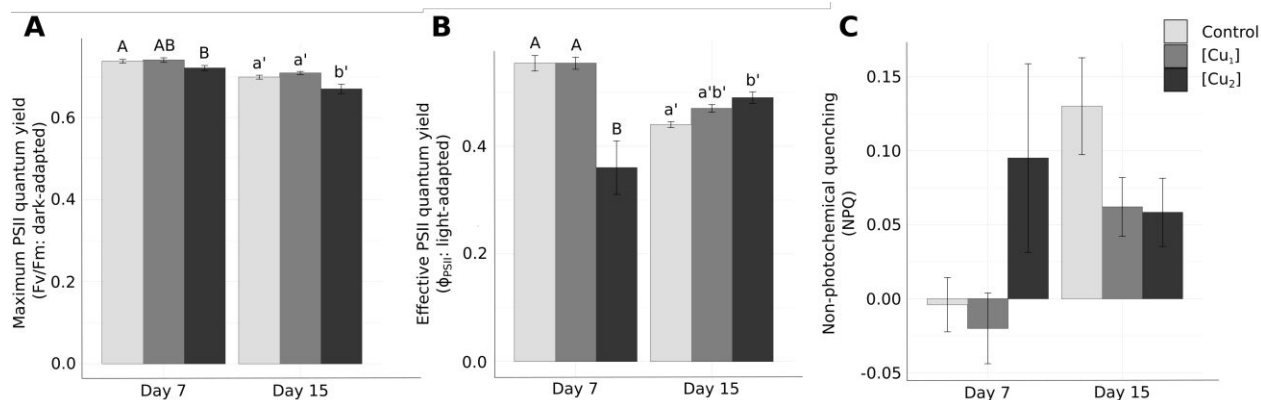


Figure 5: Effect of Cu on photosystem II of *A. minutum* after 7 and 15 days of exposure. A: Maximum PSII quantum yield (Fv/Fm; dark adapted), B: Effective PSII quantum yield (Φ_{PSII} ; light adapted), C: Non-photochemical quenching (NPQ). Different letters indicate significant differences between the treatments for each day of exposure. Results are expressed as the mean \pm standard error ($5 < n < 6$).

3.7 Allelochemical potency of *A. minutum* filtrates

Exposure of *C. muelleri* to *A. minutum* filtrates induced inhibition of maximum photosystem II quantum yield (Fv/Fm) with filtrates from all treatments (Figure 6). At day 7, allelochemical potency of filtrates from the control and [Cu₁] were not significantly different (Wheeler ratio approach) with respective EC₅₀ of 13270 ± 1900 cells mL⁻¹ and 14760 ± 2680 cells mL⁻¹. The filtrate from *A. minutum* exposed to [Cu₂] was significantly more potent with an EC₅₀ of 2940 ± 330 cells mL⁻¹. After 15 days, EC₅₀ from the control was not significantly different from [Cu₁] or [Cu₂]. Filtrate from [Cu₁] was the least toxic with an EC₅₀ of 10050 ± 1210 cell mL⁻¹ and was significantly different from the filtrate of [Cu₂] with an EC₅₀ of 6320 ± 580 cells mL⁻¹.

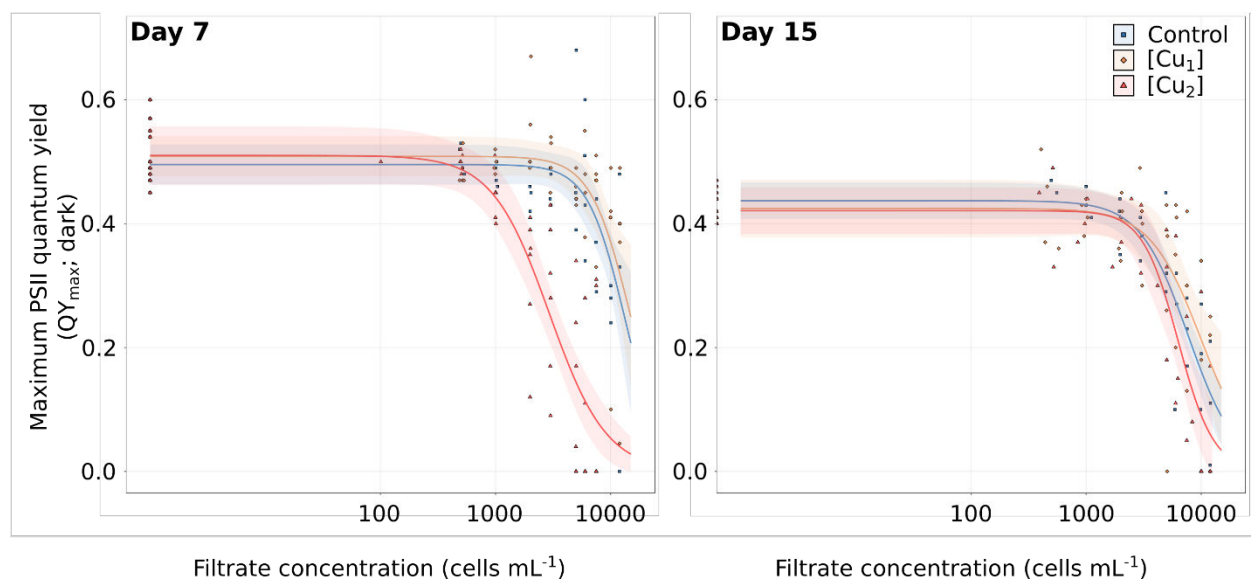


Figure 6: Inhibition of photosystem II maximum quantum yield (F_v/F_m) of *C. muelleri* in presence of filtrates of *A. minutum* exposed to different Cu concentrations for 7 and 15 days. Log-logistic models with 3 parameters were used to model the inhibition. Dots represent the maximum quantum yield as measured in the bioassay in presence of filtrate from the control (blue squares), [Cu₁] (orange diamonds), [Cu₂] (red triangles). For both curves, the ribbon represents the 95% confidence interval from the log-logistic model.

3.8 Dissolved organic carbon and characterization of chromophoric dissolved organic carbon

Only exposure to the highest Cu concentration [Cu₂] modified the exudation of *A. minutum* (Figure 7). [Cu₂] induced a 2.5 fold increase in the release of DOC per cell as compared to the control and [Cu₁] (KW-bf, p-values < 0.01). Moreover, this increase in exudation came along with changes in the nature of the chromophoric DOC. PARAFAC analysis determined the presence of four components: two humic-like (C1 and C4) and two protein-like (C2 and C3). The four components explained 98.3% of the variation between treatments and comparison with the Openfluor database revealed our model showed $\geq 95\%$ similarity to 19/70 models in the database. Component C1 significantly (ANOVA-HSD, p-values < 0.01) increased in presence of [Cu₂] while component C2 significantly (ANOVA-HSD, p-values < 0.001) decreased. The abundance of C3 in [Cu₂] was, however, not different from the control or [Cu₁]. Modification in the nature of chromophoric DOC was also observed in [Cu₁] as compared to the control. The abundance of the component C3 was significantly (ANOVA-HSD, p-value < 0.05) lower in [Cu₁] than the control. The component C4 was not different between the treatments.

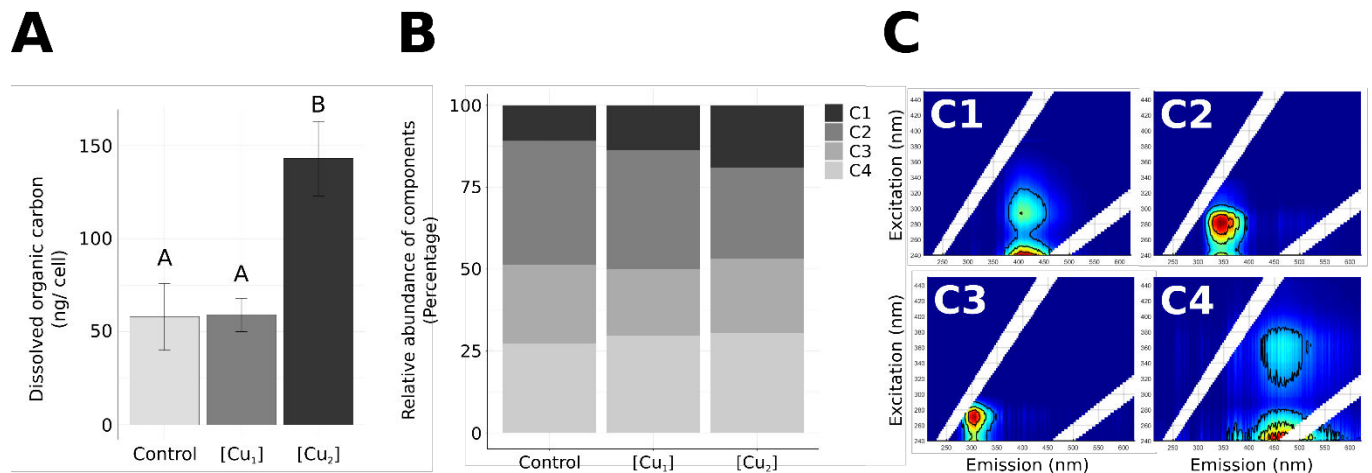


Figure 7: Characterization of the dissolved organic carbon after 15 days of exposure. A: Total dissolved organic carbon (ng cell⁻¹), B: Relative abundance of the chromophoric components C1, C2, C3 and C4, C: Fluorescence excitation emission spectra of the components C1 (Fulvic-like), C2 (Tryptophan-like), C3 (Tyrosine-like), C4 (Humic-like). In graph A, results are expressed as the mean \pm standard error ($5 < n < 6$). Different letters indicate significant differences between the treatments for each day of exposure.

4 Discussion

4.1 Copper toxicity

Growth occurred in all the cultures of *A. minutum* in the presence or absence of added Cu. Only the highest concentration of Cu induced a significant decrease of population growth rate. Overall, toxic effects in the [Cu₂] treatment were observed from day 1 to day 7. After 7 days, *A. minutum* seemed to recover from Cu exposure (highlighted by the different physiological measurements). Biological effects of Cu exposure on microalgae have been frequently related to the free metal ion concentration (Koppel et al., 2017; Morel et al., 1978; Sunda, 1975). The decrease in toxicity may be linked to the decrease in dissolved Cu concentrations from 164 nM to 56 nM in [Cu₂] after 15 days. Indeed, the lowest-observable-effect concentration (LOEC) that inhibited growth of *A. minutum* (strain CCM11002) is between 79 nM ([Cu₁] day 0) and 113 nM ([Cu₂] at day 7) of dissolved Cu. This result fits in the range of phytoplankton copper sensitivity (Levy et al., 2007; Pistocchi et al., 2000). This strain of *A. minutum* can be considered as tolerant when compared to the Australasian marine water quality guideline (ANZECC, 2000), and to other phytoplankton genus (Levy et al., 2007). It is nevertheless difficult to compare *A. minutum* results with *A. catenella* (strain ACT03) that had LOEC of between 6 μ M and 12 μ M added Cu, respectively 2 nM and 7 nM of free Cu²⁺ (Herzi et al., 2013), as exposure were performed in the presence of EDTA.

4.2 Fate of copper within the flasks

The fate of Cu in the experimental flasks was not experimentally confirmed, however three hypotheses are proposed to explain the decrease of Cu over time in the Cu treatments. (1) Cu can bind to glass, however, flasks were silanized to avoid Cu binding to glassware and no loss of Cu was observed between the theoretical concentrations of added Cu and the measured dissolved concentration at day 0. (2) Binding of Cu to algal cell surfaces and/or internalization of Cu (named as cellular Cu) by algal cells may partially explain the decrease of dissolved Cu along the experiment. To test this hypothesis, we estimated the cellular Cu uptaken by *A. minutum* (Cu adsorbed on cell membranes and internalized by cells). The predicted cellular Cu of *A. minutum* fits in the range of concentrations found in literature for other genus (Debelius et al., 2009; Rijstenbil et al., 1998; Walsh and Ahner, 2014) when taking into account the biovolume of microalgae and could therefore partially explain the decrease in dissolved Cu. (3) Complexation of Cu by strong microalgal ligands may have occurred during the exposure. Dissolved Cu is partitioned between the Cu free ion in solution and Cu complexed by organic ligands. In this study, samples were acidified to dissociate organically bound Cu before dissolved Cu analysis. Some studies showed that acidification may not be enough to dissociate all the Cu from organic ligands and that UV treatment is required to dissociate Cu from strong ligands (Posacka et al., 2017). Cells of *A. minutum* released exudates that may strongly complex Cu and acidification without UV-treatment may have not been sufficient to dissociate Cu from these ligands. In this study, the Cu measurements may not be representative of total dissolved Cu but only represents the free and weakly complexed Cu. Overall, the combination of loss on the glassware, binding and internalization of Cu by the cells and increase in complexation of Cu by strong microalgal exudates, may explain the apparent loss of dissolved Cu in the flasks. Quantification of cellular Cu following the protocol given by Levy et al. (2008) and measurement of Cu complexation after UV-treatment of samples would help understand the fate of Cu in the flasks in future.

4.3 Toxic effects of copper on the physiology of *A. minutum*

Decreased growth rates in [Cu₂] indicated drastic toxic effects on microalgal physiology (Figure 8). Toxic Cu exposures inhibit the growth of many phytoplankton species (Debelius et al., 2009; Franklin et al., 2001; Koppel et al., 2017; Lelong et al., 2012; Levy et al., 2007; Rocchetta and Küpper, 2009), including the species *A. catenella* (Herzi et al., 2013). An absence of growth

inhibition does not necessarily mean an absence of physiological effects. While the overall growth rate in the [Cu₁] was not affected, significant modifications were measured in the cultures, with changes in the formation of cysts at 24 hours, in the lipid production rate at 7 days and in the microbial community over the whole exposure, consistently appearing intermediate between the high Cu concentration and the control.

Exposure to Cu induced an ecdysis of *A. minutum* cells within the first 24 hours of exposure. Ecdysis is a stress response to protect dinoflagellates from unfavorable conditions (Haber Korn et al., 2011; Jauzein and Erdner, 2013). Ecdysis in response to Cu was already reported for *A. catenella* (Herzi et al., 2013) and may partially explain the environmental abundance of cysts in sediments from contaminated areas (Triki et al., 2017). Ecdysis may have partially decreased the dissolved Cu concentration by increasing the “biological” surface available to bind Cu (surfaces of vegetative cells versus surface of both cells, cysts and theca) by approximately 10 % as compared to the control. It is however unlikely to be an effective environmental strategy to decrease bioavailable Cu in open waters. Ecdysis may be a strategy to protect the most sensitive cells from Cu and save a pool of cells that would excyst under more favorable conditions.

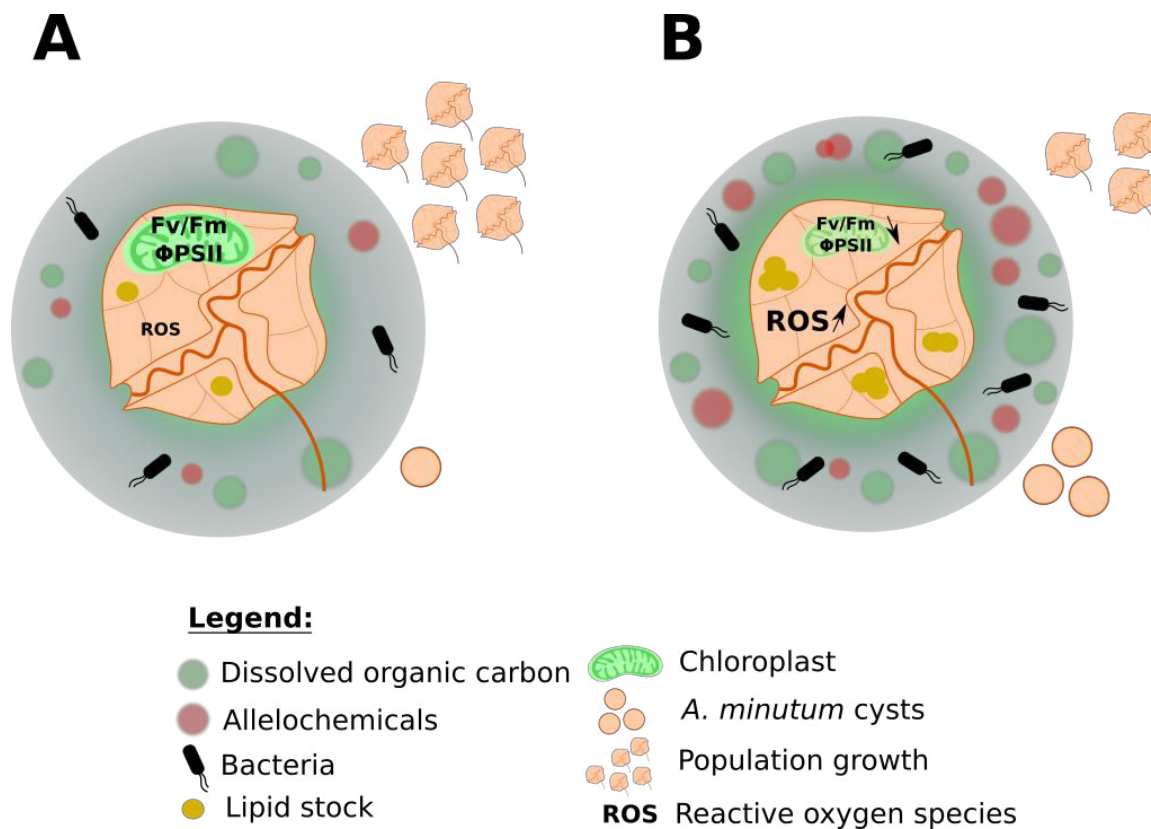


Figure 8: Physiological responses of *A. minutum* to an acute Cu stress. A: Cell of *A. minutum* in the control conditions, B cells of *A. minutum* exposed to toxic Cu concentrations.

Under stress or reduced growth, microalgae can modify their allocation of energy. Lipid storage plays an essential role in the reallocation of energy. When microalgae are not able to divide, they can store their energy in the form of lipids (Giordano et al., 2001). This has been reported for the diatom *Pseudo-nitzschia* spp. exposed to Cu (Lelong et al., 2012). In the case of *A. minutum*, lipid metabolism in presence of Cu appears to be slightly different. The higher lipid content per cell in the presence of Cu after 7 and 15 days suggests a storage of energy. However, the decrease of lipid content between day 0 and day 1 reveals a lowered lipid mobilization in the presence of Cu rather than a storage. This is supported by the decreased lipid production rate in the presence of Cu. Under Cu exposure, cells of *A. minutum* do not store extra energy, thus decreasing their lipid production rate and slowing their growth rates. The decrease in growth rates must lead to a decrease of lipid mobilization and to the apparent increase in cell lipid content.

Inhibition of photosynthesis is a common feature under Cu stress reported in many species (Franklin et al., 2001; Juneau et al., 2002; Lelong et al., 2012; Rocchetta and Küpper, 2009), and is a marker of Cu toxicity for *A. minutum*. Copper inhibits the PSII-PSI electron transport by

targeting the PSII (Juneau et al., 2002; Ralph et al., 2007) explaining the decrease in F_v/F_m and Φ_{PSII} observed for *A. minutum* or other species (e.g. Juneau et al., 2002). When photosystems are not properly functioning, the excess energy that cannot be used for photochemistry is dissipated through fluorescence or through heat as non-photochemical quenching (Behrenfeld and Milligan, 2013; Müller et al., 2001; Rocchetta and Küpper, 2009). While Pulse-Amplitude-Modulation fluorescence is a rapid and convenient tool to investigate damage to photosynthesis, it only provides a partial picture of damage to PSII. Other complementary measurements are needed to evaluate the effect of Cu on the overall photosynthesis and on the carbon fixation by the dinoflagellate. This potential decrease in carbon fixation may decrease the growth of the dinoflagellate or limit the mobilization of carbon allocated to organic compounds such as lipids. ROS have a central role in stress-related pathways and trigger stress responses, including for the genus *Alexandrium* (Jauzein and Erdner, 2013). An increase in the production of ROS in microalgae has been reported in presence of toxic concentrations of Cu (Knauert and Knauer, 2008; Rocchetta and Küpper, 2009) and ROS are reported to play a primary role in Cu toxicity to microalgae. Accordingly, the ROS production by *A. minutum* was reported to increase in presence of Cu as an indicator of stress. Microalgae have detoxifying mechanisms (e.g. exudation, vacuolization, production of intracellular copper binding-ligands) in response to Cu (Knauert and Knauer, 2008; Levy et al., 2008; Smith, 2016) and excessive ROS production (e.g. release of ROS out of the cell, ROS scavengers). This may have helped mitigate stress between days 7 and 15. Moreover, the defensive mechanisms in response to ROS and more generally Cu stress must have had an energy cost. Some lipid energy may have been reallocated to these mechanisms and may have partially decreased the lipid production rate.

4.4 Recovery from copper exposure

All the physiological parameters measured after 7 days were affected by Cu exposure (cell concentration, cysts percentage, ratio bacteria/*A. minutum*, lipid metabolism, ROS production, F_v/F_m , Φ_{PSII} , NPQ, allelochemical potency). After 15 days, fewer physiological parameters were different from the control (cell concentration, percentage of cysts, bacteria/*A. minutum* ratio, lipid metabolism, F_v/F_m , Φ_{PSII}), suggesting a partial recovery of *A. minutum* from Cu exposure. Moreover, it is hypothesized that some of the significant differences between the control and [Cu₂] observed after 15 days of experiment were not toxic effects, strictly speaking, but only consequences of a delayed growth. Morel et al. (1978) highlighted that Cu induced a lag period by

blocking initial divisions in a culture until the algae are adapted or the chemistry of Cu in the media is modified and becomes less-toxic. This scenario may have occurred here and would explain the delayed growth of *A. minutum* exposed to [Cu₂]. The microalgal cell concentrations after 7 days were always lower in the presence of Cu; however, 2-day growth rates (data not shown) between day 7 to 15 were similar or higher in [Cu₂] than in the control. While the control is reaching late exponential growth phase at day 15, [Cu₂] is still under exponential growth phase. This is also highlighted by the percentage of cysts, which reaches a peak at the beginning of exponential growth phase in the control and [Cu₁] treatment. This peak is also present for [Cu₂] treatment but later in the experiment, as if it was delayed together with the growth. This delay in growth is corroborated by the mean lipid content that did not increase in [Cu₂] while it increased in the control and [Cu₁] treatment. Lipid content increase in microalgae when cells reach stationary phase (Brown et al., 1993; da Costa et al., 2017). No more differences in NPQ were apparent after 15 days: another sign of recovery from Cu toxicity. Differences in the bacteria/*A. minutum* ratio, Fv/Fm, and Φ_{PSII} could however still be effects of Cu stress. Microalgae have several mechanisms to face Cu stress (e.g. antioxidant, phytochelatin production (Li et al., 2006; Morelli and Scarano, 2004; Sabatini et al., 2009; Smith et al., 2014)) or the production of Cu-complexing exudates, as a protective response, that may have been used by *A. minutum*.

4.5 Copper increases *A. minutum* exudates

Increase of exudates in the presence of toxic Cu concentrations has been shown for various species of phytoplankton (Croot et al., 2000; Levy et al., 2008; Pistocchi et al., 2000) including the species *A. catenella* (Herzi et al., 2013). It is widely known that increases in DOC concentration decrease toxicity of Cu to variety of different algal species (Apte et al., 2005; De Schamphelaere et al., 2003; Heijerick et al., 2005; Ma et al., 2003). The release of exudates protect the cells by either complexing metals and decreasing metal bioavailability in the dissolved phase (Fisher and Fabris, 1982; Koukal et al., 2007) or by exporting the metals out of the cells (Croot et al., 2000). Indeed, the increased release of DOC by *A. minutum* may be a response to decrease metal toxicity. Not all microalgal exudates have the same ability to chelate copper (Croot et al., 2000; Levy et al., 2008) and that complexation depends on the quality of the DOC (Al-Reasi et al., 2012, 2011; Croot et al., 2000).

The chromophoric DOC from *A. minutum* is similar to the chromophoric DOC produced by *A. tamarensis* and *A. catenella* that is constituted of protein-like and humic-like substances (Herzi et

al., 2013; Li et al., 2015; Villacorte et al., 2015). Humic-like DOC has been shown to decrease Cu toxicity more greatly than proteinous DOC (Al-Reasi et al., 2012, 2011). In this study, higher amounts of the C1 humic-like component was produced in the [Cu₂] treatment, with decreases in the C2 protein-like component also occurring. Given that humic-like DOC has been shown to bind metals and decrease toxicity more greatly than proteinous DOC, this may indicate a purposeful shift in type of DOC produced to optimize protection against metal stress. Herzi et al. (2013) also reported an increase in a humic-like component and a decrease in a protein-like component similar to our C1 and C2 by *A. catenella* in response to Cu, Zn and Pb contamination. Thus supporting a possible shift in type of DOC produced under metal stress. Future studies should include analysis of Cu complexation within exudates in order to understand the role of *A. minutum* exudates on copper toxicity.

When considering toxic algae, metal concentrations were shown to modify the release of toxins. Maldonado et al. (2002) observed an increase in the domoic acid toxin by two species of *Pseudo-nitzschia* in the presence of toxic concentrations of Cu and hypothesized that domoic acid chelate Cu. In this study, *A. minutum* allelochemical potency increased in the presence of Cu and could also have chelating properties. The dinoflagellate *Pfiesteria piscicida* produces metal-containing toxins where the toxicity originates from the metal-mediated free radical production of complex Cu ions (Moeller et al., 2007). Indeed the toxicity of *P. piscicida* is influenced by the environmental concentrations of metal. This may be the case of *A. minutum*, with allelochemicals which may complex and concentrate Cu, thus increasing the toxicity of allelochemical agents. This is corroborated by Ma et al. (2009) who observed a decrease in allelochemical potency when allelochemical extracts were suspended in deionized water as compared to natural seawater. In such a scenario, allelochemicals would have a dual purpose: they would protect the cell from metal protection and additionally have an allelochemical role. Other toxins such domoic acid (Maldonado et al., 2002) or the terrestrial phytotoxin 8-hydroxyquoline (Inderjit et al., 2011; Tharayil et al., 2009) were observed to have a dual purpose. It is noteworthy that the allelochemical potency of exudates significantly increased only when the dissolved Cu concentrations were the highest and when the physiology of the dinoflagellate was severely impaired (Day 7). Thus, it appears that increase of allelochemical activity is linked to Cu concentration or to the stress state of *A. minutum*.

4.6 Copper affects *A. minutum* microbial community

Similarly to *A. minutum* cells, bacteria-like cells in the bioassay were affected by Cu exposure. While Cu first caused a decrease in bacteria concentration as well as the ratio per algal cell after 3 days, cultures exposed to Cu had more bacteria per algal cell. Stressed microalgae are more densely surrounded by bacteria than healthy cells (Grossart, 1999; Levy et al., 2009). While cell lysis is hypothesized to fuel bacterial growth, no signs of cell lysis were observed in this study (no decrease in microalgal concentration was observed after Cu spike as compared to initial cell concentration). Nevertheless, the DOC in the bioassay media was higher in the [Cu₂] treatment than the control and [Cu₁]. It is difficult to conclude whether the DOC had a microalgal or a bacterial origin, but the component C1 which showed a significant increase in [Cu₂] has been shown to be produced by marine phytoplankton rather than be bacterial in nature (Romera-Castillo et al., 2011). The release of some microalgal chemicals such as dimethylsulfoniopropionate (DMSP) by some dinoflagellates can be chemical hotspots for microbes (Seymour et al., 2010). Tryptophan been hypothesized to attract bacteria (Amin et al., 2015). There were more bacteria per microalgae in [Cu₂] treatment, however the exudates in [Cu₂] contain relatively less tryptophan-like compounds (Component C2). This may be due to the increased bacterial load consuming more of the C2 component in this treatment or changes in the composition of exudates to protect against metal toxicity. The component C1 may have been attracting bacteria as studies have shown it can also be consumed by bacteria (Romera-Castillo et al., 2011). Bacteria may have been mitigating Cu toxicity to *A. minutum* as observed for *Chlorella* sp. (Levy et al., 2009). Bacteria could mitigate toxicity by producing ligands (Bhaskar and Bhosle, 2006; Gordon et al., 2000), internalizing some Cu and increasing the biological surface available for Cu binding.

4.7 Adaptation of *A. minutum* to coastal environments

In the light of other studies, *A. minutum* appeared to be quite tolerant to Cu, providing this species with a competitive advantage to grow in metal-contaminated areas. In polluted aquatic environments, Cu concentration can be reported in high concentrations, as high as 3 µM in estuaries (Beiras et al., 2003) or 47 µM in rivers (Juneau et al., 2002; MENVIQ, 2003). Within this concentration range, the *A. minutum* strain CCM11002 could indeed significantly increase its allelochemical potency. The increased release of allelochemicals under Cu contamination would thus increase the ability of this strain to outcompete other species and would ease bloom formation.

Overall, different ecotypes (Kobiyama et al., 2010) may have different sensitivities to Cu and different responses. A high variability in the tolerance of *Emiliana huxleyi* to Cu was observed and appeared to be dependent on the strain, the ecotype and annual Cu inputs (Echeveste et al., 2018). A high variability can be observed in the physiological traits of the genus *Alexandrium* vary according to their life history (Brandenburg et al., 2018), highlighting the need to further explore the variability of *A. minutum* sensitivity to Cu and allelochemical potency.

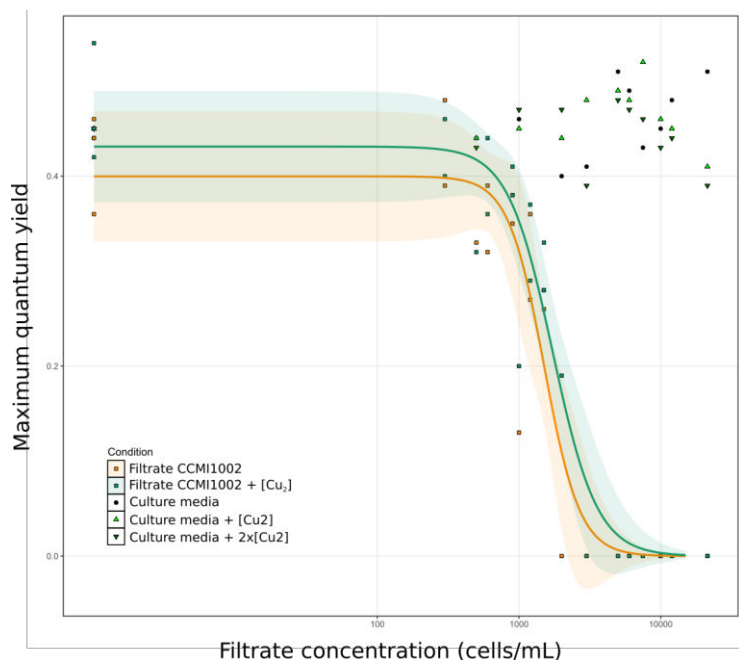
5 Conclusion

The multi-trait approach used in this study provides an overview of the main physiological effects of Cu on the toxic dinoflagellate *A. minutum*. Within the first 7 days of exposure, under [Cu₂] treatment, physiology of the cells was impaired as highlighted by the stress responses. After 15 days of exposure, cells of *A. minutum* partially coped with Cu stress. Within the remarkable stress responses, *A. minutum* substantially increased its allelochemical potency. It is hypothesized that allelochemicals may chelate dissolved Cu and this deserves further investigation. The growth in this treatment was delayed when compared to the control and the moderate [Cu₁] treatment. We hypothesized that concomitant increase in cysts, production of ROS, production of exudates, reallocation of energy and modifications of bacterial community may have helped *A. minutum* to cope with Cu stress by decreasing its toxicity and/or availability. Lastly, the tolerance of *A. minutum* to the high Cu concentration in parallel to its increased allelopathic potency may be one out of the features explaining the success of *A. minutum* blooms in metal contaminated areas.

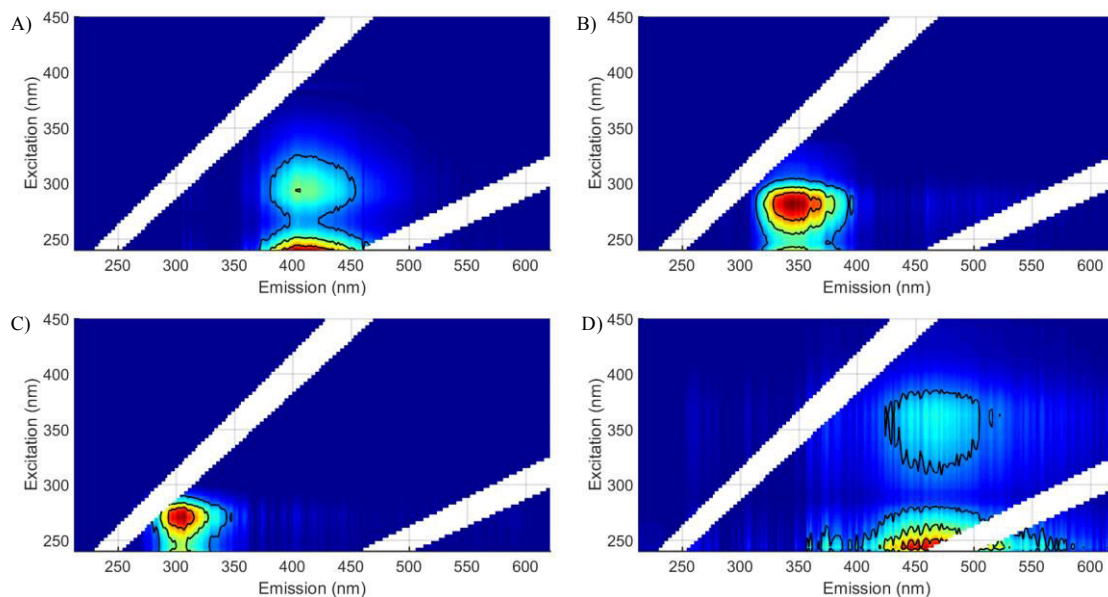
Acknowledgments

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Supplementary files:



Supplementary figure 1: Inhibition of *C. muelleri* photosystem II maximum quantum yield in presence of culture media, culture media spiked with [Cu₂], spiked with two times [Cu₂], A. minutum CCM1002 filtrate and CCM1002 filtrate spiked with [Cu₂]. Log-logistic models with 3 parameters were used to model the inhibition. Dots represent the maximum quantum yield as measured in the bioassay in presence of filtrate from the control (blue squares), [Cu₁] (orange diamonds), [Cu₂] (red triangles). For both curves, the ribbon represents the 95% confidence interval from the log-logistic model.



Supplementary figure 2: Spectra of the four main components determined via PARAFAC analysis, used to describe the organic matter present A) Fulvic-like (*Humic-like*); B) Protein-like 1 (similar to Tryptophan); C) Protein-like 2 (similar to Tyrosine); D) Humic-like. These three components explained 98.3% of the variation between samples, with a core consistency of 42%.

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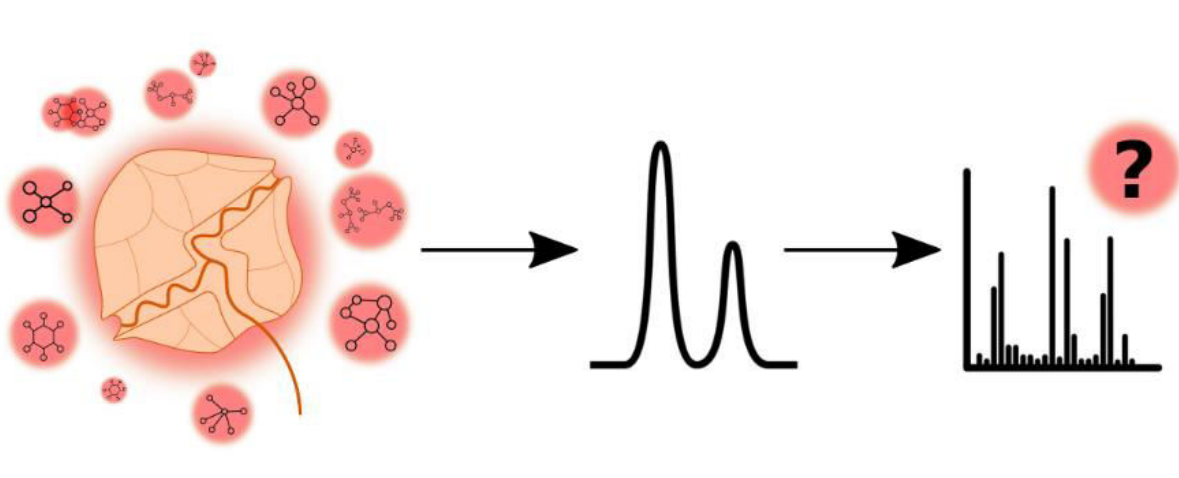
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Chapter 5

What is the chemical nature of *A. minutum* allelochemicals ?

One of the main bolts to unlock before gaining a better understanding of the effects of allelochemical interactions is the characterization of the allelochemical compounds. Since the chemicals are unknown, it is difficult to assess the prevalence of allelochemical interactions in the environment. In previous chapters, we highlighted that it is possible to evaluate the allelochemical potency with a bioassay and to quantify modifications in the exudation of allelochemicals. However, knowledge of the compounds is necessary to fully understand allelochemical interactions. In the previous chapter, we also highlighted an increase in the allelochemical potency of exudates from *A. minutum* in the presence of Cu. We emitted several hypothesis to explain this increase (1) the exudation of allelochemicals was increased and/or qualitatively modified, (2) the chelation of Cu by allelochemicals made the exudates more toxic. The identification of allelochemicals is required to validate the different hypotheses. The objective of this chapter is to isolate the compounds responsible for *A. minutum* allelochemical interactions. The partial isolation of allelochemicals was possible thanks to the bioassay developed in chapter 3 through a bioassay-guided purification.



Partial purification of *Alexandrium minutum* allelochemicals

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Abstract

The toxic dinoflagellate *Alexandrium minutum* is known for its' so called “paralytic shellfish toxins (PST)”, a well-studied series of toxins, which have been related to fish and shellfish contamination and human poisoning. Besides from intracellular PST, *A. minutum* also produces uncharacterized exudates which have been implicated in ichthyotoxic, hemolytic and allelochemical (toxic to competing protists or bacteria) activities, but the isolation and characterization of these extracellular toxins remain to be accomplished. The aim of this work was to isolate and characterize *A. minutum* extracellular compounds (i.e. allelochemicals) responsible for allelochemical potency. To identify and purify these allelochemicals, monocultures of *A. minutum* and a bioassay-guided fractionation were used. Allelochemicals were extracted from culture media and concentrated by solid-phase extractions. Multiple steps of chromatographic techniques were used to isolate the chemicals. The semi-purified fractions that were found to be active were analyzed by mass-spectrometry, candidate compounds were identified and their molecular masses were compared to databases. Our results indicate that positively charged hydrophobic compounds with molecular sizes between 200 and 700 Da may be responsible for the allelochemical potency of *A. minutum*. Further studies are required to isolate candidates and confirm their allelochemical potency before being able to characterize their nature.

1 Introduction

In his *article*, Georg Pohnert depicts a noisy version of the oceans in contrast with the widespread picture of “silent oceans” often described in literature (Pohnert, 2010). In fact, the author talks about the chemical noise of marine organisms that “communicate” through the release of numerous chemical signals in the marine environment. Marine plankton, including protists, release a wide range of signaling molecules in the surrounding environment. These cues control the coexistence and coevolution of species according to their various functions: they can be sexual signals (i.e. pheromones) (Gillard et al., 2013), influence resource research (Bondoc et al., 2016; Steinke et al., 2006), trigger cell death (Gallo et al., 2017), mediate grazer-prey interactions (Amato et al., 2018), and some signals can even change the toxic potency of a toxic algae (Selander et al., 2006). There is growing evidence that chemical interactions are a driving force shaping plankton communities (Amato et al., 2018; Amin et al., 2015; Wohlrab et al., 2016). However the plankton “chemical language” is largely unspoken as only a few compounds have so far been identified.

The genus *Alexandrium* releases chemical cues in the surrounding environment, with several biological targets. Exudates from *A. minutum* can reduce feeding activity of bivalves (Borcier et al., 2017; Castrec et al., 2018), might be responsible for deleterious effects on copepods (Ianora et al., 2004; Xu et al., 2016). Therefore they might decrease grazing pressure on the dinoflagellate community. Cell free filtrates or supernatants can also be responsible for deleterious effects on fish (Mardones et al., 2015; Ogata and Kodama, 1986), ovocytes (Le Goïc et al., 2014) or hemocytes (Arzul et al., 1999; Ford et al., 2010; Simonsen et al., 1995). Cells of *Alexandrium* spp. also release chemicals responsible for negative effects on protists (Hakanen et al., 2014a; Lelong et al., 2011; Tillmann et al., 2008; Tillmann and Hansen, 2009; Tillmann and John, 2002), they are so-called allelochemicals. These allelochemicals may be significant in the dynamics of blooms by giving a competitive advantage to the dinoflagellate.

Allelochemical activity has been unrelated to the known paralytic shellfish toxins (Flores et al., 2012; Hakanen et al., 2014b; Tillmann and John, 2002) or to spirolides (Tillmann et al., 2007). The compounds responsible for the allelochemical potency of *A. tamarense* have been partly characterized (Ma et al., 2009, 2011b). In these studies, the authors isolated the compound through a bioassay-guided fractionation and by comparing one lytic strain versus a non-lytic strain of *A. tamarense*. A group of large molecules, with a molecular size between 7 kDa and 15 kDa, was partially isolated and could be responsible for the allelochemical potency of *A. tamarense*. The

molecules were non-proteinaceous and probably non-polysaccharidic compounds. The precise masses and chemical structure of allelochemicals, however, could not be determined. To our knowledge, no similar work was performed on the allelochemicals of *A. minutum* that remain uncharacterized. The identification of *A. minutum* allelochemicals would bring significant advances to the field. The characterization would help to monitor their concentration in the environment and better assess their role in shaping biological communities. Isolation of allelochemicals would also be valuable in evaluating their toxicities over other marine organisms and humans.

In this study, we focused on the purification and characterization of allelochemicals from the species *A. minutum* that have never been investigated. The strain CCMI1002 of *A. minutum* was selected according to its potent allelochemical activity (Long et al., 2018). Extraction of allelochemicals were performed by solid-phase extraction on cell-free filtrates. The allelochemicals were purified from non-toxic exudates through bioassay-guided fractionation coupled with chromatographic methods. Allelochemical potency of the different fractions were tested on the maximum photosystem II quantum yield of the diatom *Chaetoceros muelleri*, a diatom sensitive to *A. minutum* allelochemicals. Semi-purified fractions were analyzed by mass-spectrometric methods and compared to compounds in databases.

2 Materials and methods

2.1 Chemicals and glassware

All water used in the experiment is ultrapure water (MERCK Millipore, 18M Ω). Methanol (MeOH; for HPLC, gradient grade $\geq 99.9\%$), chloroform (CHCl₃; for HPLC, gradient grade $\geq 99.8\%$), and acetonitrile (AcN; for HPLC, gradient grade $\geq 99.9\%$) were purchased from Sigma (Aldrich, Germany). For HPLC separations, solvents were acidified to 0.1% with formic acid (HCOOH; Analytical grade $> 99\%$). To minimize the loss of allelochemicals, filtrate and fractions were sampled and stored in glassware and glass pipettes were used where possible. All glassware was acid-washed (HCl 10% v/v) and rinse ultrapure water before use.

2.2 Cultures

The strain CCMI1002 of *Alexandrium minutum* (isolated from a bloom in Gearhies, Bantry Bay, Ireland) was selected according to its high allelopathic potency (Long et al., 2018) and because this strain does not produce paralytic shellfish toxins (Castrec et al., 2018). Cells of *A. minutum* were

grown under controlled conditions in 2 L glass balloon with aeration. Cultures were performed in artificial seawater (synthetic ocean water: Morel et al., 1979; Price et al., 1989) supplemented with L1 media (Guillard and Hargraves, 1993). Cultures of the strain CCM11002 were kept in exponential growth phase. Cultures of the diatom *Chaetoceros muelleri* (strain CCAP 1010-3, formerly named *Chaetoceros neogracile* or *Chaetoceros* sp.) were maintained under exponential growth phase in filtered (0.2 µm) natural seawater (collected in Argenton, France) supplemented with L1 media (Guillard and Hargraves, 1993) and silica (1.06×10^{-4} M final concentration). All microalgal cultures were maintained at $18 \pm 1^\circ\text{C}$ under continuous light ($100 - 110 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$). Dinoflagellate and diatom cultures were not axenic but were handled under sterile conditions to minimize additional bacterial contamination.

2.3 Microalgal and bacterial counts

Population growth counts and measurements were performed using a FACScalibur (BD Biosciences, San Jose, CA, USA) flow cytometer with a 488 nm argon laser. Cell variables, e.g. forward scatter (Forward scatter, FSC), side scatter (Side scatter, SSC) and red autofluorescence (FL3, red emission filter long pass, 670 nm) were used to select the *A. minutum* population. The free living bacteria in cultures were also monitored. Samples for bacterial enumeration were diluted in 0.2µm filtered seawater then SYBR Green I (Molecular probes, Invitrogen, Eugene, OR, USA) stained (da Costa et al., 2017). Bacteria with stained DNA, were detected by FL1 and discriminated from *A. minutum* cells according to their side-scatter (SSC) and forward scatter (FSC). Counts of bacteria and algae were estimated according to flowrate (Marie et al., 1999).

2.4 Preparation of filtrates

Cultures were filtered through 0.2 µm asymmetric polyethersulfone membrane (Nalgene, Rapidflow) to separate exudates from microalgal cells, bacteria and cell debris. Filtrate was stored in glass bottles at 6°C until solid phase extraction (SPE) extraction to avoid loss on plastics. The pH of *A. minutum* filtrate was measured before each filtration and was within the range of 8.1 – 8.6.

2.5 General procedure: Bioassay guided purification

Allelochemicals from *A. minutum* were isolated and partially purified through bioassay-guided purification (Figure 1). In short, compounds were extracted from their original matrix (culture filtrate), the crude extract was then fractionated into smaller fractions (i.e. less complex fractions)

with different chromatographic techniques based on their chemical characteristics (e.g. charge, polarity, size). The smaller fractions were screened for their allelochemical potency. We used the bioassay developed previously in this PhD (Long et al., 2018; Chapter 3) to screen/assess the allelochemical potency of the fractions. The inactive fractions were discarded as they probably did not contain the allelochemicals, while the active fractions were stored at -20°C. Further purification was then performed on active fractions following the same procedure (i.e. fractionation followed by a bioassay) with another purification protocol/method. Performing multiple purification steps allows reducing the sample complexity and eventually, isolate a single compound that could be chemically characterized/identified. When a suitable fractionation protocol was developed, the purification steps were performed several times and the fractions from the different experiments were pooled to concentrate the samples.

Once the purification protocol was established, a control extract was generated (see paragraph 2.11) in parallel with the culturing media (artificial seawater supplemented with L1 media). This extraction was performed to i) ensure that the toxicity of fractions was not coming from the media, and ii) enable comparative metabolomics to delete noise coming from the media or from the purification steps.

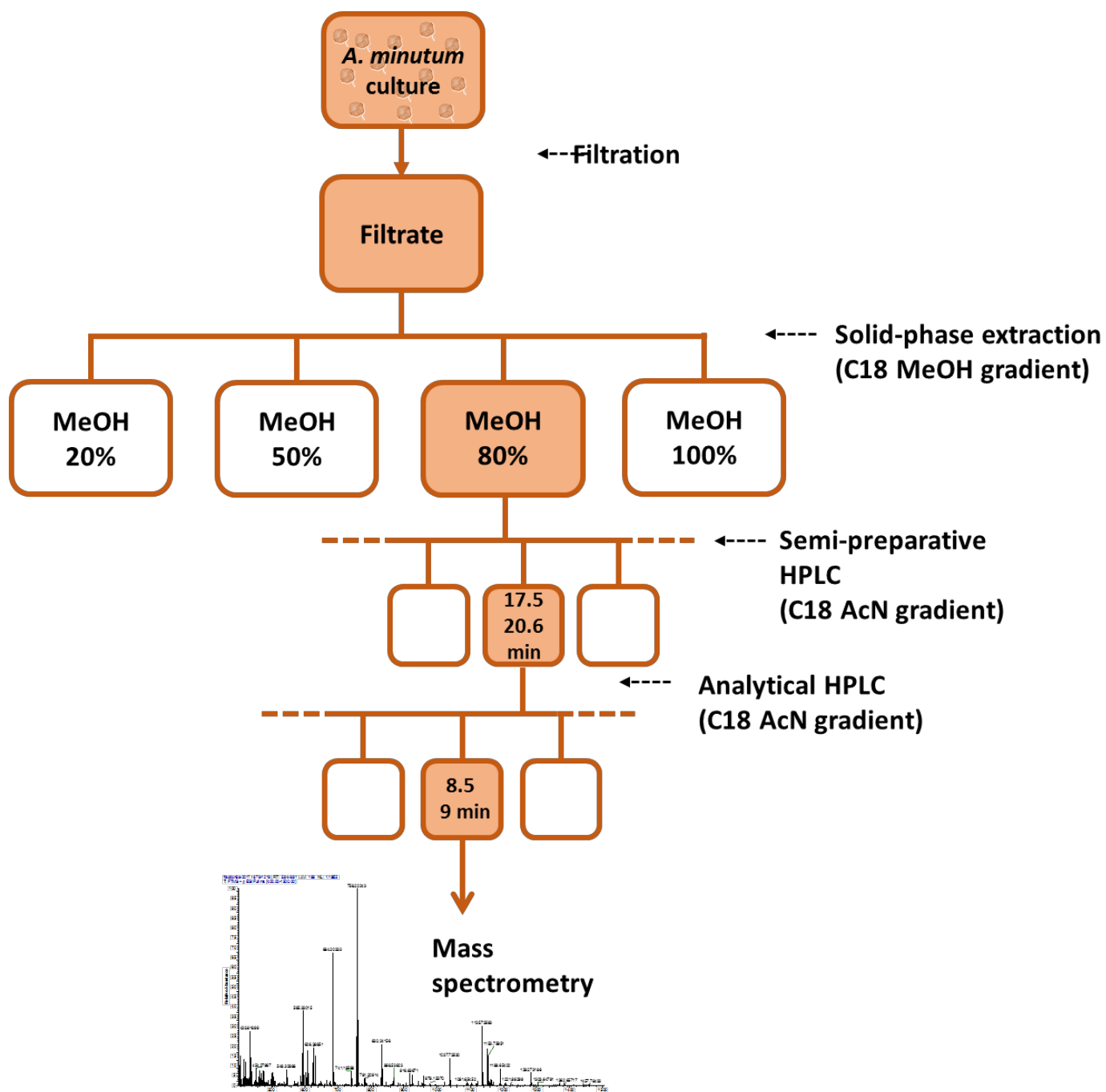


Figure 1: Diagram of the bioassay guided purification steps. Extracts were fractionated according to their chemical properties through different analytical methods and protocol. After each extraction/purification steps, allelochemical potency was searched in the fraction with a bioassay. Only the most active fractions (orange boxes) were kept for further purification. The non-active or less active fractions were stored for later analysis. The more purified fractions, the less complex. Scheme adapted from (Selander, 2007).

2.6 Bioassay

The bioassay measured the inhibition of the diatoms *C. muelleri* maximum photosystem II quantum yield (Fv/Fm) according to previously established protocol (Long et al., 2018). The bioassay was

performed in two different ways: i) a single-concentration bioassay expressed as the inhibition of Fv/Fm was performed to qualitatively compare activity from different fractions. and ii) *C. muelleri* was exposed to several dilutions of the filtrate or fractions to obtain dose-response curves. The initial *A. minutum* culture concentration, prior to filtration and extraction, was used to describe the potential allelochemical concentration in each filtrate or fraction (in cell mL⁻¹). The dose-responses curves allowed a detailed quantification of allelochemical potency by calculating the effective concentration of filtrate or fraction inhibiting 50 % of *C. muelleri* Fv/Fm (EC₅₀). In order to perform the bioassays, solvents were evaporated under reduced pressure and the resultant fractions suspended in methanol: artificial seawater (1: 120, v: v) in glass vials. Fractions were sonicated (sonication bath, Fisherbrand, FB15048) for 2 min to solubilize the compounds coated on glass vials. Final concentrations of MeOH (<1%) alone in the bioassays did not affect the Fv/Fm of *C. muelleri* (data not shown).

2.7 Extraction and first fractionation by solid-phase extraction

Allelochemicals were first extracted from the filtrate and concentrated using C18 solid-phase extraction (SPE) cartridges (Supelclean, LC-18 500 mg/ 3 mL). The cartridges were conditioned with 20 mL of MeOH followed by 20 mL of ultrapure water (10 times the bed weight). Approximately 300 mL of filtrate (or L1 medium in artificial seawater as a negative control) was loaded on each SPE cartridge using a peristaltic pump. Secondly, a fractionation was performed with water/methanol eluting mixtures to selectively desorb the compounds according to their polarity as followed: 20 ml ultrapure water to desalt the column then, 30 ml MeOH 20%, 30 ml MeOH 50%, 30 ml MeOH 80%, 20 ml MeOH 100%, using a vacuum manifold (Visiprep SPE Vacuum Manifold). Based on the hypothesis that the compounds were potent at low concentrations, SPE fractions were pooled (5 liters of filtrate) to concentrate allelochemicals and improve detected signal. Pools of extracts were dried under N_{2(g)} before storage at -20°C. The allelochemical potency of the filtrates were measured before and after extraction on SPE to ensure that extraction worked. The activity of each separate fraction was tested. The activity of all the fractions were pooled together was tested to test the synergistic activity of fractions. The pools of the most potent fraction were kept for further purification.

2.8 Analytical HPLC

Before injection, SPE fractions were filtered through 0.45 μm (PTFE/GF membrane; Interchim). The optimization of chromatographic conditions was performed using a Thermofisher UHPLC U3000SD equipped with a quaternary pump (UHPLC+ LPG-3400SD), autosampler WPS-3000TSL, temperature-controlled column compartment TCC-3000SD, diode array detector (DAD) DAD-3000 and evaporative light-scattering detector (ELSD) 85 LT-ELSD (Sedex). Compounds were separated on a C18 reverse phase column (Phenomenex Kinetex Evo C18 column, 150 x 4.6 mm; i.d. 5.6 μm) at 25°C, using a gradient AcN/H₂O with 0,1% of formic acid. Two different types of detectors were used to allow the detection of a wide range of compounds based on their properties. The DAD allowed detection of compounds absorbing in the UV-visible spectra, and gave information concerning the characteristic wavelengths absorbed by each compound. The ELSD allowed the detection of a wide range of compounds independently of their spectroscopic properties and this detector gives relative quantification of the compounds. After optimization steps, the following gradient (Figure 2) was applied (flow rate = 1 mL.min⁻¹): 5/95 AcN/ H₂O held for 2 min followed by a linear decrease over 20 min to 45 % AcN. It was followed by a sharper linear decrease over 5 min to 100 % AcN. The 100 % AcN was held for 10 min before returning to initial flow conditions within 3 min, which was then held for 5 min.



Figure 2 : Acetonitrile/water (AcN/ H₂O) gradient used in the analytical and semi-preparative HPLC separations.

2.9 Semi-preparative HPLC

Before injections, SPE fractions were filtered (PTFE/GF; 0.45 μm ; Interchim). Allelochemical fractions were collected after a semi-preparative HPLC purification. The purification was performed using the same UHPLC system described above. Compounds were separated on a C18 reverse phase column (Phenomenex Kinetex Evo C18 column 150 x 10 mm; i.d. 5.6 μm) at 25°C, using a gradient AcN/H₂O with 0,1% of formic acid. Compounds were detected with the DAD and the ELSD, by derivation of 10% of the flow rate. Flow conditions were 5 mL.min⁻¹ and followed the same solvent gradient program described above in analytical HPLC (Figure 2). The

chromatogram was first divided into 12 fractions according to ELSD profile to identify the potent fraction(s). Upon this first screening, a new fractionation protocol was applied to only collect the potent fractions. Six runs with injections of 500 μl of the 80 % MeOH fraction were performed with the second fractionation protocol. Active fractions from the runs were pooled and dried under $\text{N}_{2(\text{g})}$ before storage at -20°C .

2.10 LC-MS analysis

Optimization of chromatographic conditions was performed using a UHPLC U3000SD system (ThermoFisher) equipped with a HP6-3400SD pump, autosampler HP6-3400SD and temperature-controlled column compartment TCC-3000SD. Compounds were separated on a C18 reverse phase column (Phenomenex Kinetex Evo C18 column 150 x 2.1 mm; i.d. 2.6 μm) at 25°C using a gradient AcN/H₂O with 0.1% of formic acid. Injection volume was 100 μL . Flow conditions were 0.3 $\text{mL}\cdot\text{min}^{-1}$ and the following gradient was applied: 30/70 AcN/ H₂O held for 2 min followed by a linear decrease over 20 min to 100 % AcN. The 100 % AcN was held for 10 min before increasing back to initial flow conditions within 3 min, which was held for 5 min (Figure 3). According to the chromatogram, fractions were sampled and allelochemical potency was screened. Three runs with injections of 100 μl were performed, active fractions from the runs were pooled and dried under $\text{N}_{2(\text{g})}$ before storage at -20°C . The active LC-MS fraction resuspended in 80% MeOH was injected (100 μL) in mass spectrometer without preliminary chromatographic separation. Samples were infused by syringe at 4.5 $\mu\text{l}\cdot\text{min}^{-1}$ into a LTQ XL Orbitrap (Thermo Fisher Scientific, Villebon sur Yvette, France) interfaced with an electrospray ionization (ESI) source. The ESI spray voltage was set to |4.0| kV with the flow rate of sheath gas, auxiliary gas and sweep gas set to 3, 1 and 0, respectively (Nitrogen, arbitrary units). The capillary temperature was maintained at 300°C , the capillary and the tube lens voltage were set at 35 and 110 V, respectively. All data were collected using the Thermo Fisher Xcalibur software with full-scan mass analysis at 60 000 resolution in the

range of 400–1500 m/z. All samples were run both in positive and negative ionization mode, with the same conditions.

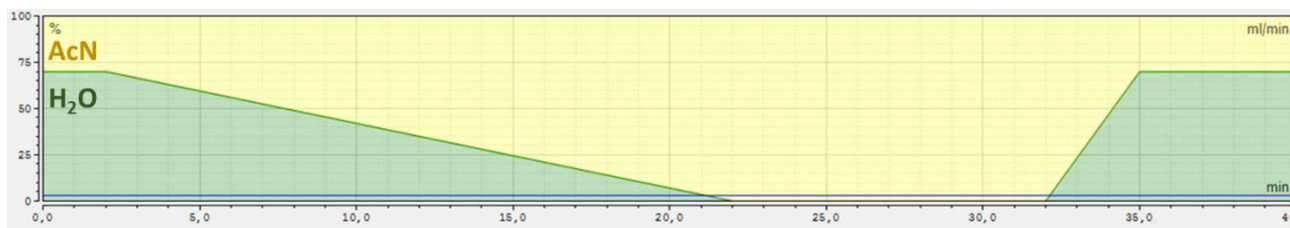


Figure 3: Acetonitrile/water (AcN/ H₂O) gradient used in the LC-MS separation.

2.11 Extraction blank

An extraction blank was performed with L1 media in artificial seawater to control the innocuity of the culturing media by itself after fractionation. This blank was also used to subtract the compounds present in the media and the possible contaminants extracted during fractionation. Ten liters of L1 media in artificial seawater were extracted on C18 solid-phase extraction (SPE) cartridges, and the extract was fractionated accordingly with the protocol for *A. minutum* filtrate (see section 2.7). The 80% MeOH fraction of media was tested for its toxicity with the bioassay. The composition of the extraction blank was analyzed by LC-MS following the protocol previously given in section 2.10.

2.12 Data analysis

Significant peaks were selected with the algorithm ICIS (Baseline width = 40; Area noise factor = 5; Peak noise factor = 200) from the software Thermo Xcalibur Qual Browser. Exact mass (in Da) were determined by subtracting 1 from the measured m/z, as the isotopic profiles reveals that all candidates ions were monoprotonated. The mass was then used to generate the most likely compounds formulae, using a module from the software Thermo Xcalibur Qual Browser. The mass and formulae were compared with the database MarinLit (Royal Society of Chemistry).

3 Results and discussion

3.1 Filtrate allelochemicals extraction

Solid phase extraction (SPE) successfully isolated allelochemicals from culture media on hydrophobic C18 silica column as eluted filtrate lost most of its activity (Figure 4). Allelochemicals were eluted with 80 % methanol, suggesting that the compounds are highly lipophilic, similarly to the allelochemicals extracted from *A. tamarense* and eluted with the same solvent mixture (Ma et

al., 2011a). There was, however, a substantial loss of activity in the 80% MeOH fraction (EC_{50} of the 80% MeOH fraction was 10 times higher as compared to the filtrate). Indeed, allelochemical interactions may involve more than one allelochemical as different extracellular compounds may have synergistically effects (Pohnert et al., 2007). To test this hypothesis SPE fractions were combined together before quantifying their potency together. The combination of all SPE fractions (i.e. pool MeOH fraction containing both 20, 50, 80 and 100% fractions) did not increase allelochemical potency as compared to the 80% fraction. This shows that the combination of the SPE methanolic fractions has no synergistic effect. Similarly, the lytic activity of *A. tamarensis* (Ma et al., 2009) was not recovered when recombining fractions. Losses of compounds may have occurred either for highly hydrophilic compounds not retained on the SPE column (low activity was still measured in the filtrate after SPE extraction) or for highly hydrophobic compounds that would not eluted from the SPE column. However, no synergistic effect of dissolving the potent fraction into the hydrophilic fraction (not retained on SPE) was shown for *A. tamarensis* (Ma et al., 2009). Deterioration during purification, evaporation or loss of solubility of the fractions may also partially explain the loss of potency.

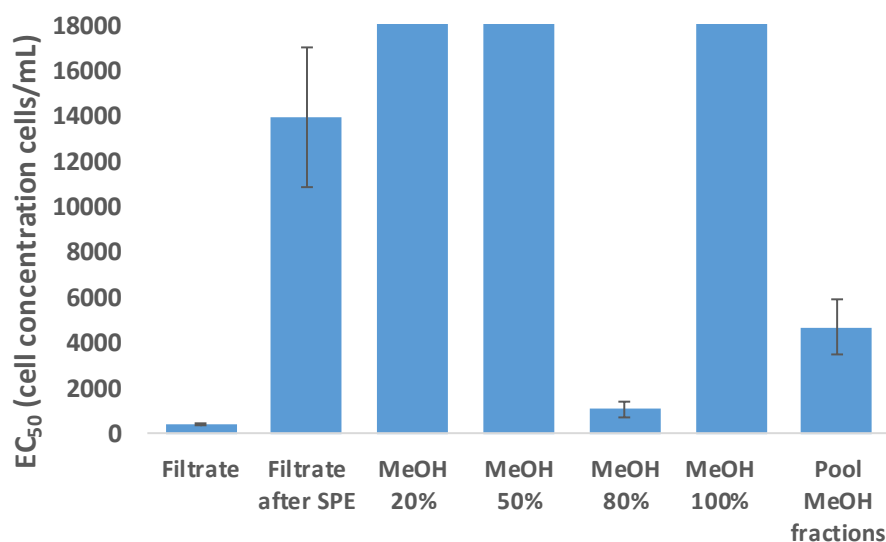


Figure 4: Allelochemical potency (Effective concentrations inhibiting 50% of *C. muelleri* Fv/Fm) of one *A. minutum* filtrate before and after elution from SPE, of the pool of methanolic fractions separately or recombined (Pool MeOH fractions). EC_{50} of “MeOH 20%”, “MeOH 50%” and “MeOH 100%” fractions could not be calculated as their allelochemical potency was extremely low. Results are indicated as the mean \pm standard error.

3.2 Analytical HPLC

The HPLC separation was initially optimized to properly separate the different compounds. Several peaks were detected in ELSD (Figure 5A), at UV wavelengths of 197 nm (Figure 5B) and 228 nm (Figure 5C), however, only a few peaks were observed at 280 nm (Figure 5D) and 330 nm (Figure 5E). ELSD and UV absorbance at 197 nm were favored to monitor the elution of compounds in the subsequent chromatographic steps. Compounds began to elute from the column 13 min after injection (20 % AcN).

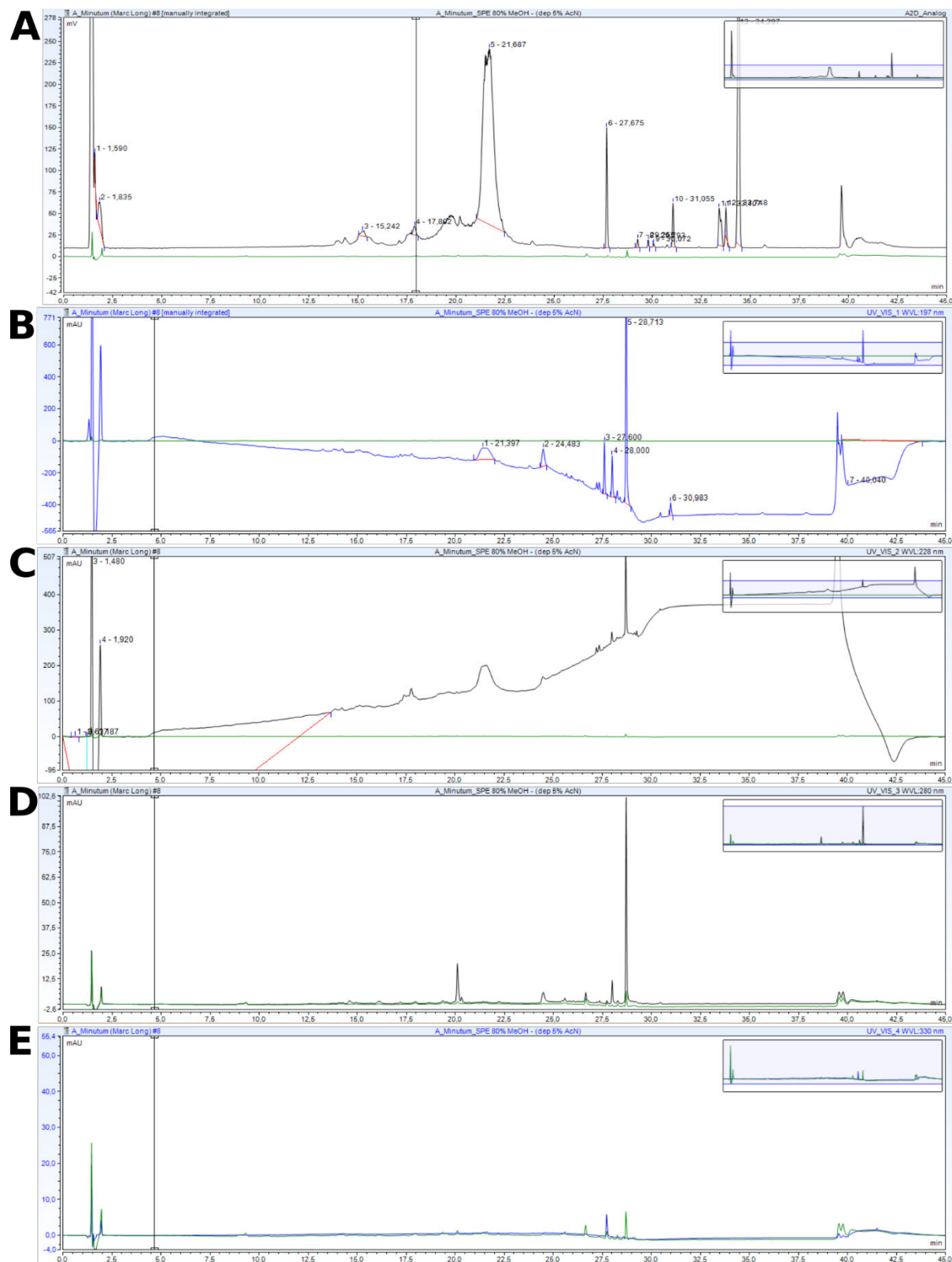


Figure 5: HPLC spectra of the fraction SPE 80% in (A) ELSD, and DAD-UV at (B) 197 nm, (C) 228 nm, (D) 280 nm, (E) 330 nm.

3.3 Preliminary screening of allelochemical potency

Semi-preparative fractionation of the 80% MeOH fraction resulted in three fractions that inhibited the Fv/Fm of *C. muelleri* (impaired photosynthesis) and nine fractions that did not (Figure 6). The allelochemical activities were recovered between 17.8 – 25.1 min and between 39.1 – 42.6 min. The three active HPLC fractions suggest that there is at least one (possibly several) active compound(s) responsible for the inhibition of the diatom Fv/Fm.

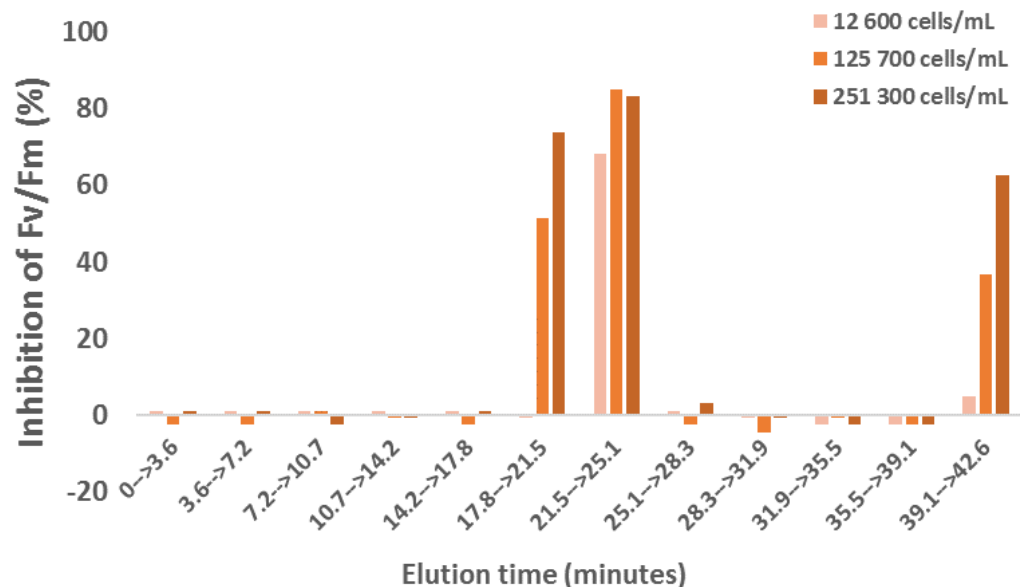


Figure 6: Allelochemical potency (inhibition of Fv/Fm) of the semi-preparative HPLC fractions.

3.4 Semi-preparative (SP) HPLC

Based on the primary fractionation and the ELSD profile, the SP chromatogram was divided into six fractions and three active fractions were collected (Figure 7A): between 17.5 and 20.5 min (35 to 45 % AcN), 20.5 and 23.6 min (45 to 60 % AcN), and finally between 38 and 41.3 min (70 to 95% AcN). The rest of the SP chromatogram did not show significant allelochemical potency. The fraction collected between 20.5 and 23.6 min (45 % can to 60 % AcN) had the highest allelopathic potency (Figure 7B). The active fraction from *A. minutum* did not absorb in UV at 280 nm, which was in agreement with active fraction isolated from *A. tamarense* (Ma et al., 2011b). For further purification, we focused on the SP fraction eluted between 20.5 and 23.6 min believed to contain the most allelochemicals.

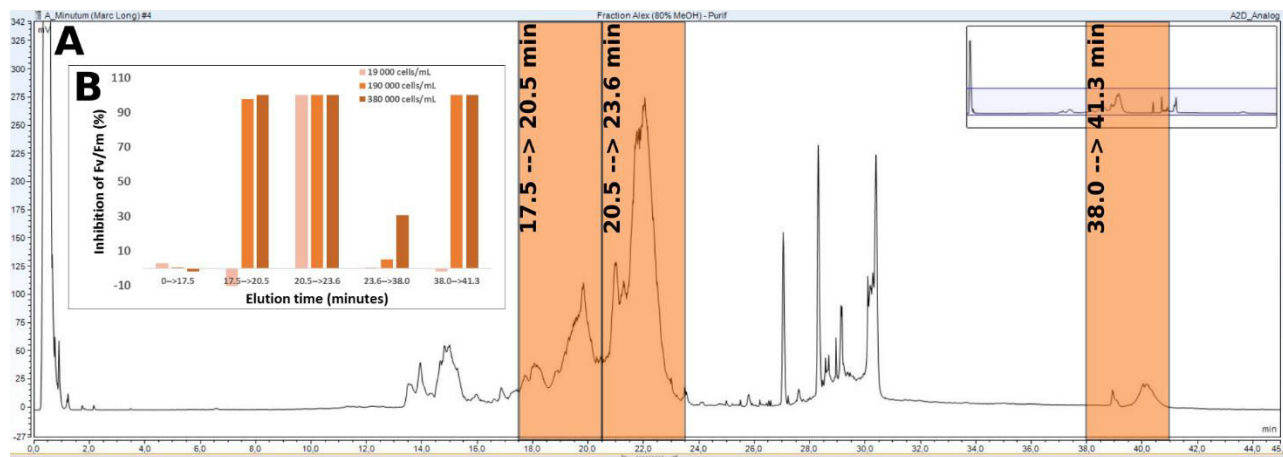


Figure 7: (A) Semi-preparative HPLC-ELSD spectra of the fraction SPE 80%. The orange boxes represent the active fractions of the spectra. (B) Allelochemical potency (Inhibition of Fv/Fm) of the HPLC-MS fractions.

3.5 HPLC-MS

The pool of SP fractions between 20.5 and 23.6 min (45 to 60 % AcN) from the SP-HPLC was analysed by HPLC-MS (Figure 8). Peaks were detected in both positive (Figure 8A) and negative mode (Figure 8B). Most of the peaks eluted between 5 and 11 min (40 to 60 % AcN) which is in agreement with the SP fraction collected between 45 % and 60 % AcN. The separation on the analytical column coupled to mass analysis allowed a much better resolution as compared to SP column. Fractions were collected based on the peaks detected by mass-spectrometry and allelochemical potency was measured (Figure 8C). Only one fraction, between 8.5 and 9 min (50 to 55 % AcN), exhibited a significant allelochemical potency. This fraction showed one major peak in positive mode (Figure 8A) but no significant peak could be observed in negative mode (Figure 8B). The active fraction (50 to 55 % AcN) was then injected into the mass spectrometer without preliminary chromatography and was analysed in positive mode.

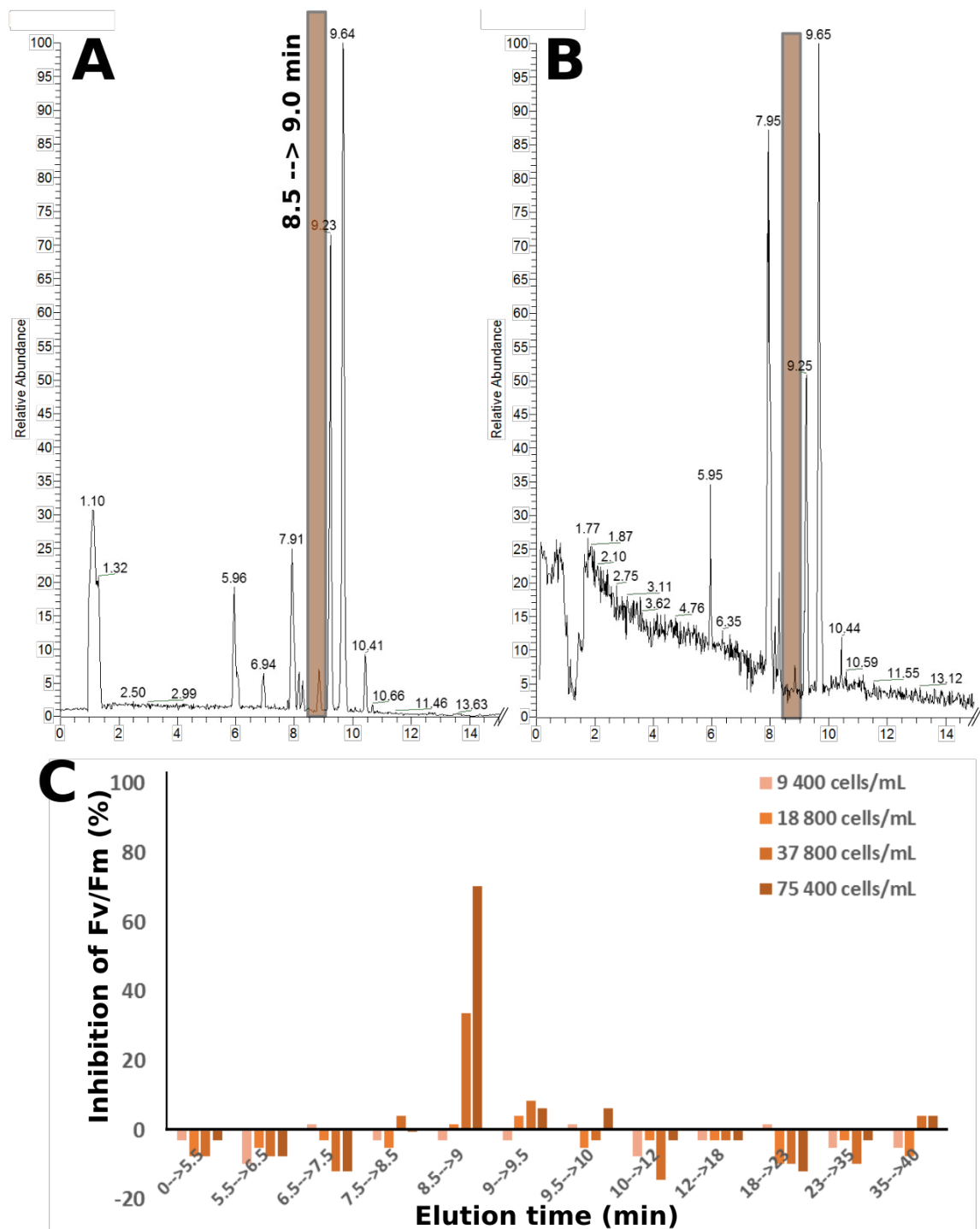


Figure 8: HPLC-MS spectra from the active semi-preparative fraction (between 20.5 and 23.6 min) in positive mode (A) and negative mode (B). The orange box in (A) and (B) represents the active fraction (between 8.5 and 9 min) of the spectra. (C) Allelochemical potency of the HPLC-MS fractions.

3.6 Mass-spectrometry of fractions

Mass-spectrometry of the purified fraction (8.5 – 9 min) revealed presence of 20 predominant compounds in the positive mode (Figure 9). The signal in negative mode was low, similar to the instrument noise and did not allow the identification of candidates.

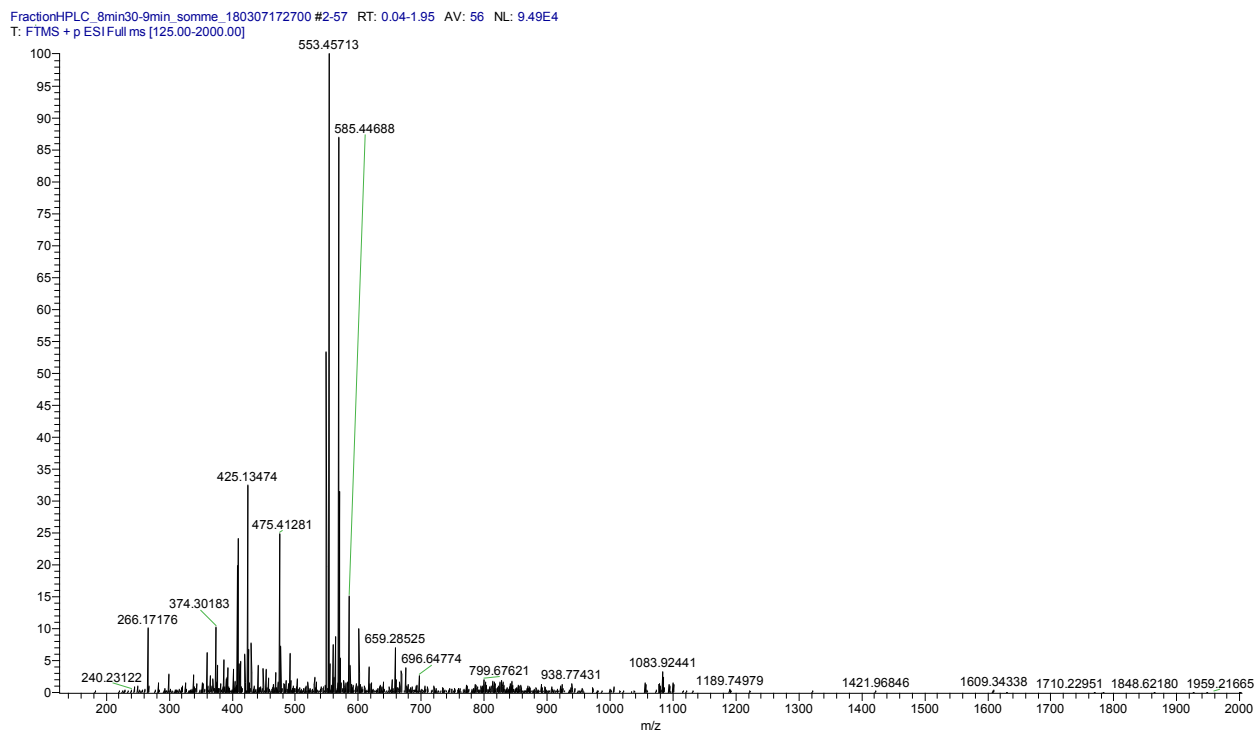


Figure 9: Mass spectrum of the 8.5-9 min analytical fraction in positive mode.

The composition of the active fraction in positive mode was compared to the composition of non-active fractions (HPLC-MS fraction) and to the composition of the L1 media fraction (SPE 80 % MeOH fraction of the culturing media). Compounds present in both active, inactive fractions and L1 were excluded as possible allelochemical candidates as they must originate from L1 media or contamination. Once these compounds excluded it remained nine candidate compounds with molecular weights comprised between 266 and 659 m/z (Table 1).

Table 1: Candidates detected in positive mode in the 8.5 – 9 min fraction.

Mode	m/z	Delta	Mass (Da)	Elementary formulae
+	266.1719	-0.575	265.17188	C11 H19 O N7
+	374.3020	0.307	373.30197	C19 H39 O4 N3
+	408.3074	0.108	407.3074	C19 H41 O6 N3
+	409.1611	0.098	408.1611	C19 H24 O8 N2
+	424.2813	0.252	423.28128	C22 H37 O5 N3
+	425.1349	0.193	424.13492	C22 H20 O7 N2
+	601.4213	2.709	600.42133	C30 H56 O8 N4
+	659.2857	0.001	658.28571	C22 H42 O15 N8

3.7 Molecular size of the candidates

Relatively small molecular weight candidates (< 1 kDa, Table 1) were isolated from *A. minutum*. In comparison, large molecular candidates (> 1 kDa) with allelochemical potency (Ma et al., 2011b; Zheng et al., 2016) and hemolytic activity (Chen et al., 2015; Emura et al., 2004; Yamasaki et al., 2008) were isolated from *A. tamarensis*. Discrepancies between candidates from *A. tamarensis* and *A. minutum* could reveal the production of different allelochemicals between *Alexandrium* species. It is, however, important to remember that these masses are only candidates and the activity of each candidate needs to be confirmed following further purification. Moreover, the different techniques used induce a major bias in the comparison between studies. The mass spectrometer used for the identification of candidate from *A. minutum* covers a range of mass comprised between 200 and 1500 m/z. This technic prevents the identification of “large” (over 1500 m/z). Therefore, it cannot be excluded that the compounds identified by Ma et al (2011b) in *A. tamarensis* using a MALDI-TOF-TOF could be present but not detected in our samples. Further analysis by another type of mass spectrometer (such as MALDI-TOF) are required to confirm this. The size of *A. minutum* allelochemical compounds could also be estimated through dialysis with different cut-off sizes, or size-exclusion chromatography. For the size estimation of allelochemicals from *A. minutum* we do not recommend the use of AMICON ultrafiltration as allelochemical losses were observed using these ultrafiltration units (*i.e.* 60% of the activity is lost; data not shown). The low molecular masses found for *A. minutum* are, however, consistent with masses of some unidentified (Prince et al., 2010) or known allelochemicals such as fischerellin A (Hagmann and Jiittner, 1996), cyanobacterin (Mason et al., 1982), polyunsaturated aldehydes (Franzè et al., 2017; Pichierri et al., 2016) or cholesterol sulfates (Gallo et al., 2017). While reversed phase chromatography allow a partial separation of allelochemicals, used alone it is not suitable for the complete isolation of

allelochemicals (Ma et al., 2011b, 2009). Reversed phase chromatography must be coupled to other separation methods.

3.8 Chemical nature of allelochemicals

Usually, the choice of methods for purification is guided by the chemical nature of the compounds of interest, however chemical nature of allelochemicals of *A. minutum* is still unknown. Even for *A. tamarense*, which allelochemicals were partially characterized, the nature is unknown and proteinaceous nature is in debate (Emura et al., 2004; Flores et al., 2012; Ma et al., 2011b; Yamasaki et al., 2008). The presence of one peak at 280 nm around 20 min in the active semi-preparative fraction (Figure 5D) indicates that there may be proteins in the fractions and a proteinaceous origin of *A. minutum* allelochemicals cannot be excluded. The proteinaceous nature of *A. minutum* allelochemicals could be further investigated by adding protease to the fraction or by quantifying the protein content of the fraction. Overall further purification is required to isolate the allelochemical(s) in the active fraction before the characterization.

3.9 Challenges in the identification of *A. minutum* allelochemicals

Several constraints slowed down the identification of *A. minutum* or *A. tamarense* allelochemicals i) the low quantity of allelochemicals, ii) the low yields of extraction and purification and iii) the complexity/chemical noise of the exometabolome.

Firstly, the main limitation comes from the limited amount of allelochemicals, thus the possible number of purifications and analyses. Allelochemicals are diluted in the surrounding environment and that are potent at low concentrations. Liters of cultures are necessary to collect only small amounts (below mg) of non-purified extracts, revealing the potency of *A. minutum* at such low concentrations. Working with cell extract could help increasing the amount of allelochemicals available for analysis but many inconvenient exist. It would increase the chemical noise, fraction complexity and the risk of identifying false positives (compounds inhibiting Fv/Fm but not related to allelochemical interactions).

Secondly, the substantial losses of allelochemical potency during the purification process does not ease their identification. The allelochemicals of the genus *Alexandrium* are particularly prone to adsorption on plastics (Ma et al., 2011b, 2009) that cannot always be avoided. Pre-filtration of fractions is one more step that induce a substantial loss of compounds. Optimization of the protocol (e.g. investigating different filters or limiting the number of filtration steps) is necessary to

minimize the loss. In addition, the allelochemicals appeared difficult to solubilize following extraction steps resulting in white deposit after fraction evaporation steps.

Thirdly, the chemical noise is a major barrier for the identification of allelochemicals. While comparative metabolomics (between allelochemical and non-allelochemical strains) is a way to identify candidates, allelochemicals still have to be isolated for further characterization. As allelochemicals are potent at low concentrations, their signal could be below the chemical noise (detection limit) of the fraction. In an attempt to decrease the chemical noise, cultures were grown in media prepared with artificial seawater. This diminishes the chemical noise that can originate from many marine organisms (Pohnert, 2010). In another attempt to decrease the chemical noise, we were unable to ensure that our cultures were axenic. The bacterial concentration in *A. minutum* cultures was $7.6 \cdot 10^7 \pm 3 \cdot 10^7$ bacteria mL⁻¹ (700 ± 400 bacteria per *A. minutum* cell). While there is no evidence for a bacterial origin of allelochemicals (Fistarol et al., 2004; John et al., 2015; Tillmann et al., 2008), bacteria might release exudates that make the fraction even more complex. In (Ma et al., 2011b), two non-allelopathic compounds with respective masses of 1291.6 Da and 1061.6 Da were isolated in supernatant from an allelopathic strain of *A. tamarense*. Further research on MarinLit (a database of the marine natural product literature), allowed us to identify potential candidates related to these masses, some of them having a bacterial origin. For instance Tausalarin C (1261.7 Da) could originate from sponges bacterial community (Bishara et al., 2009). The molecule with a mass of 1061.6 Da had three known candidates: the cyclodysidins A-D (1061.6 Da) from *Streptomyces* (Schmitz, 2011), thalassospiramides A and B (1061.6 Da) from *Thalassospira* sp. (Oh et al., 2007) and 8-carboxy-iso-iantheran A from bacterial community (Greve et al., 2007). In future studies, work with axenic cultures could ease the purification process by decreasing fraction complexity. Another solution to cope with the low allelochemical concentration, could be the optimization of culturing conditions. In the chapter 4, we reported an increase of allelochemicals upon exposure to toxic concentration of Cu. In future extraction attempts, one may expose *A. minutum* to toxic concentrations of Cu or try to increase the production of allelochemicals by modulating other culturing factors.

3.10 What is next?

In the chapter 4, allelochemicals concentrations was shown to increase with toxic concentration of Cu, suggesting a potential Cu complexation capacity of allelochemicals. One may take advantage of this potential feature to isolate allelochemicals and try immobilized metal affinity chromatography. This technique is used for the isolation of proteins exhibiting affinity to metals (Gaberc-porekar and Menart, 2001; Smith et al., 2014) and may be pertinent for *A. minutum* allelochemicals. Additionally, protein-like compounds may be separated using SDS-PAGE while low MW lipophilic compounds (e.g. fatty acids, oxylipin, sterols) may be identified by gas-chromatography coupled to mass spectrometry. Moreover, allelochemicals could be complex compounds, e.g. glycolipids (Gentien et al., 2007), polyketides (Adolf et al., 2007, 2006), lipopeptides (Kaya et al., 2002), polysaccharide-protein complexes (Yamasaki et al., 2009), that would make them even more difficult to isolate and characterize.

4 Conclusions

Bioassay-guided fractionation showed that several allelochemicals may be responsible for allelochemical interactions as several fractions were active. Most of the activity was found in one fraction analyzed by mass spectrometry. Our results indicated that allelochemicals of *A. minutum* are hydrophobic compounds similarly to allelochemicals of *A. tamarense*. We isolated an active fraction that contained candidates with low molecular weight ranging from 200 to 700 Da although their individual activity remains to be confirmed upon their further purification. Our results showed that allelochemicals from *A. minutum* are potent at really low concentrations which makes them difficult to characterize because of the low quantity within purified fractions. Additional purification steps are required to further isolate candidates and confirm their allelochemical potency and elucidate their chemical nature. Chemical identification of allelochemicals is essential to understand their role in shaping plankton communities as well as their influence on marine organisms and human health.

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Chapter 5 – Partial purification of *A. minutum* allelochemicals

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Chapter 5 – Partial purification of *A. minutum* allelochemicals

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Chapter 6

General discussion

1 Chemical nature of allelochemicals

Despite attempts to characterize allelochemicals, the compounds mediating allelochemical interactions of the genus *Alexandrium* remain unknown. Although we did not totally purify allelochemicals, in the 5th chapter we isolated hydrophobic candidates that were in agreement with the hydrophobic nature of *A. tamarensis* allelochemicals (Ma et al., 2011b, 2009). Isolated candidates have a molecular weight ranging from 200 to 700 Da, while allelochemicals from *A. tamarensis* might have a larger molecular weight (between 7 kDa and 15 kDa). Nevertheless, candidates with larger molecular weights cannot be excluded from *A. minutum* and this warrants further investigations with further purification required to characterize allelochemicals from both *A. minutum* and *A. tamarensis*. However, hypotheses regarding the nature of these compounds are proposed, with two “groups” of compounds with a common evolutionary origin containing most of the toxins produced by dinoflagellates: fatty acids (FA) related compounds and the polyketides (PK) (Kohli et al., 2016).

Fatty acids are primary metabolites that can, in some cases, exhibit cytotoxic activities. Docosahexaenoic acid (22:6n-3; DHA) was shown to contribute to the ichthyotoxic effects of *A. catenella* when combined with reactive oxygen species (ROS) (Mardones et al., 2018, 2015). A study by (Flores et al., 2012) showed that ROS were implicated in the allelochemical potency of *A. tamarensis* to protists. The authors speculated that ROS-mediated oxidation of lipids was responsible for allelochemical potency. The toxicity of octadecapentaenoic acid (18:5n-3; as a free fatty acid or associated to glycerolipids) has been shown for the dinoflagellate *Karenia mikimotoi* (Gentien et al., 2007). The production of 18:5n-3 exhibited allelochemical potency to *Karenia mikimotoi* itself and is speculated to inhibit algal competitors or other biological targets. The allelochemical potency of FA was reported in other microalgal groups such as Chlorophyte or Cyanobacteria (Wu et al., 2006). So far, the role of FA in *Alexandrium* spp. allelochemical potency is yet to be elucidated. While 18:5n-3 and 22:6n-3 are present in membrane lipids of *A. minutum* (unpublished data), compounds of m/z of 288 or 327 corresponding to 18:5n-3 (Svetashev and Imbs, 2014) and 22:6n-3 (Yamamoto et al., 2017) respectively were not detected in the active fraction. It is noteworthy that the protocol for mass spectrometry used in this project may not be optimised for FA detection. The ionization may have been too strong (and may have fragmented FA) as compared to ionization used for lipidomic approach with a similar spectrometer (Schuhmann et al., 2012).

Oxylipins are metabolites derived from oxidative transformation of fatty acids (Fontana et al., 2007; Mosblech et al., 2009). Oxilipins constitute another group of compounds of interest regarding their cytotoxicity to a wide range of organisms including microalgae (Adolph et al., 2004). In the marine environment, oxylipins production by diatoms has been widely documented (Adolph et al., 2004; Fontana et al., 2007; Gerecht et al., 2013; Pohnert et al., 2002), however, less is known about their production by dinoflagellates.

The majority of dinoflagellate toxins are polyketides (Rein and Snyder, 2006), a diverse class of secondary metabolites that are produced by polyketide synthases (Kellmann et al., 2010; Tidgewell et al., 2010). Polyketides diversity stems from their structures (Figure 1) and biological activities (Rein and Borrone, 1999) including cytotoxic, ichthyotoxic, and hemolytic activities (García-Altare, 2017). The role of some polyketides (Figure 1) in allelochemical interactions has been demonstrated for the karlotoxins of *Karlodinium spp.* (Adolf et al., 2007, 2006; Deeds and Place, 2006; Ozbay et al., 2014; Sheng et al., 2010) and is suspected for the prymnesins of *Prymnesium parvum* (Manning and La Claire, 2010), the amphidinols from *Amphidinium spp* (Granéli et al., 2006; Paul et al., 1995) and the ovatoxins from *Ostreopsis cf. ovata* (Ternon et al., 2018). Other microalgal polyketides exhibit hemolytic or ichthyotoxic activity but their allelochemical activity has not been shown, this is the case of maitotoxin. While polyketides could be a component of allelochemicals produced by *Alexandrium spp.*, they are highly diverse in terms of structure (Figure 1) and their specific structures make them difficult to characterize.

An interesting analogy between allelochemicals from *A. minutum* and *A. tamarense* and fatty acid or polyketide resides in their interactions with membranes (discussed in the following paragraph). We can however, not exclude the hypothesis that *A. minutum* are neither FA nor PK and instead could be other type of toxin. For instance, antimicrobial peptides can form amphiphatic structures that disrupt membranes of bacteria (Sperstad et al., 2011), however we could not find any reference of antimicrobial peptides production by dinoflagellates. While the compounds of *A. minutum* mediating allelochemical interactions has yet to be found, understanding the mode of action may provide new insight that would help characterizing them.

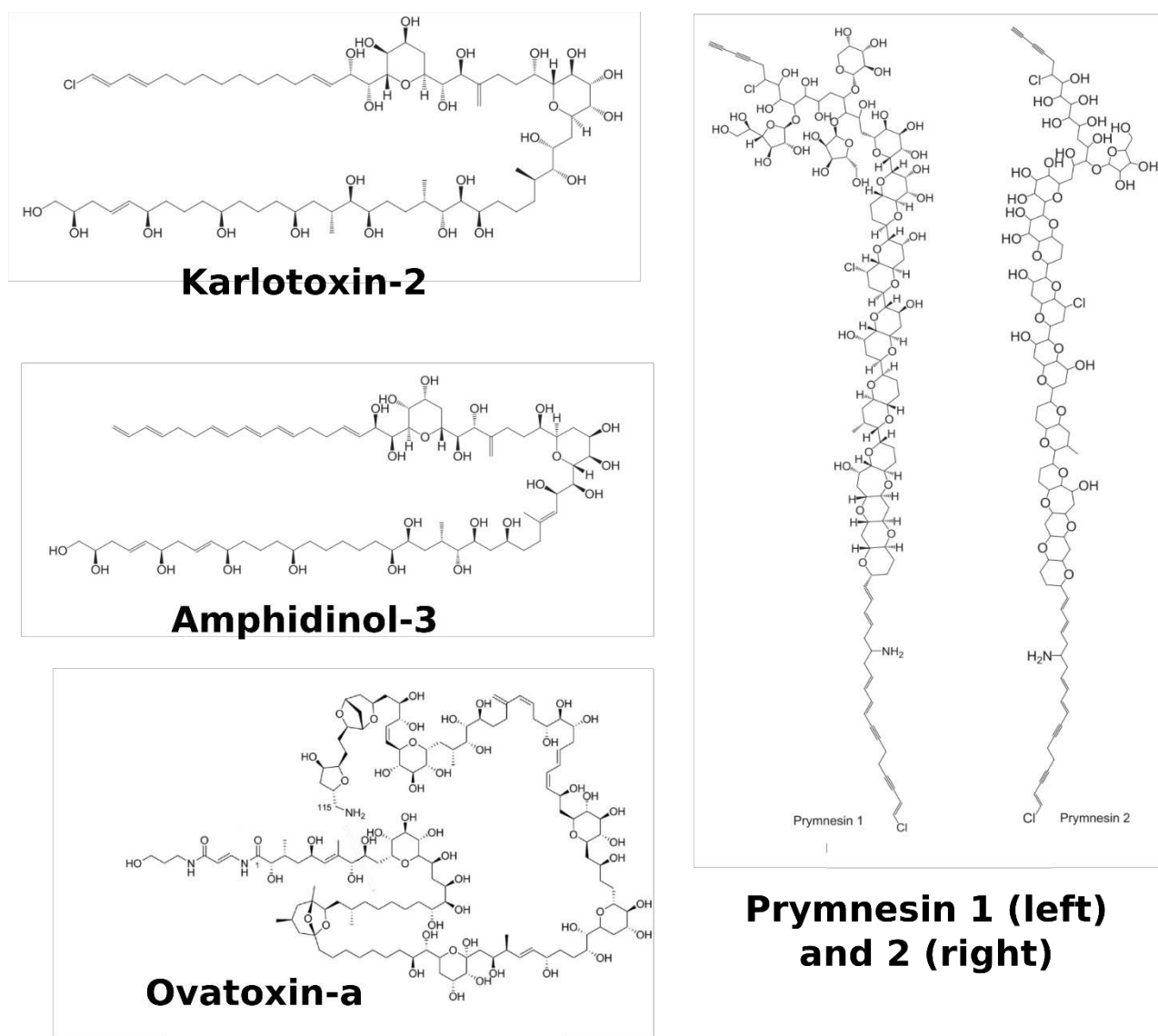


Figure 1: Chemical structures of polyketides with allelochemical potency. (García-Altare, 2017; Rein and Borrone, 1999; Rein and Snyder, 2006; Ternon et al., 2018)

2 Allelochemicals mode of action

While the nature of *A. minutum* allelochemicals remains unknown, the mode of action of *A. minutum* is now partially described. The cellular target and the cascade of physiologic events following exposure to *A. minutum* allelochemicals have been studied in the 2nd chapter of this thesis. First, allelochemicals disrupt the cytoplasmic membranes and induce their depolarization. In the meantime, the photosynthetic membranes are also impaired with an inhibition of the electron flow between the two photosystems. Second, the disruption of cytoplasmic membranes leads to larger pores and their permeabilization. The inhibition of the e- flux leads to larger effects on the photosynthetic chain with inhibitions of PSI and PSII. These effects on cytoplasmic and photosynthetic membranes occur simultaneously with the increased

production of intracellular reactive oxygen species (ROS) and inhibition of primary metabolism. Third, the cells respond to photosynthesis inhibition and ROS production with protective mechanisms (i.e. non-photochemical quenching) that induce modifications in the biochemical composition (lipid and pigments) of photosynthetic (thylakoids) membranes. Eventually, the deleterious effects lead to the death of the microalgal cells.

Some mechanisms of *A. minutum* allelochemicals are similar to the mechanisms of karlotoxins produced by *Karlodinium veneficum* (Sheng et al., 2010) and to the unknown allelochemical produced by *Karenia brevis* (Remington X Poulin et al., 2018). The dinoflagellate *K. veneficum* generates pores in membrane that result in membrane depolarization, disruption of cellular functions and eventually cell lysis (Sheng et al., 2010). Another dinoflagellate, *K. brevis* permeabilises membranes of competitors, inhibits photosynthesis and modifies the lipid composition of membranes (Remington X Poulin et al., 2018), however, the deleterious effects appear on longer timescale (days) as compared to *A. minutum*. The allelochemical interactions between *A. minutum* and *C. muelleri* is, however, far from being totally characterized. The precise mechanisms of interaction with membranes as well as the link between cytoplasmic membranes and side effects (e.g. photosynthesis, esterase) remain unclear and should be further investigated.

3 Interactions between allelochemicals and cytoplasmic membranes

In the 2nd chapter, we suggest that allelochemicals first interact with the cytoplasmic membrane of the cells by disrupting them. Therefore, this disruption might be responsible for the depolarization and permeabilization of cytoplasmic membranes. Membranes are constituted of different types of molecular components that can be targeted by toxins (e.g. lipids, proteins, polysaccharides; figure 2). Cholesterol is a component that is particularly sensitive to membrane-permeabilizing agents. Karlotoxins from *Karlodinium micrum* and amphidinols from *Amphidinium klebsii* permeabilize cytoplasmic membranes with a specific affinity to cholesterol and ergosterol (Adolf et al., 2007; Deeds and Place, 2006; Morsy et al., 2008a; Paul et al., 1997). The producers of toxins protect themselves from autotoxicity with some specific sterols in their membranes. For instance, the presence of gymnodinosterol in *K. micrum* membranes might protect them from their own allelochemical polyketides. Similarly, allelochemicals from *A. tamarense* were shown to have some specific affinity to sterols (i.e. cholesterol, ergosterol, brassicasterol) and phosphatidylcholine (Ma et al., 2011a). The authors suggested that allelochemicals damage membranes at some specific sites containing cholesterol, ergosterol, brassicasterol or phosphatidylcholine. Simultaneously, the authors

speculated that dinosterol (mainly present in dinoflagellates) self-protect *A. tamarensis* from its own allelochemicals. Although the affinity of *A. minutum* allelochemicals to different lipids and sterols was not assessed, overall sterol content of *C. muelleri* increased during allelochemical interactions (chapter 2). We speculated that the diatom might try to adapt their sterol composition to mitigate the potency of allelochemicals. Investigating the affinity of *A. minutum* allelochemicals for lipids and phytosterols may provide new insights in the molecular target of these allelochemicals.

Studies revealing the affinity of allelochemicals/toxins to some sterols did so by using artificial liposomes or quantifying the mitigation of toxicity with the addition of different lipid classes (Deeds and Place, 2006; Ma et al., 2011a; Morsy et al., 2008a). While, these approaches are very useful they do not take into consideration the biochemical complexity of cytoplasmic membranes that also contain proteins and polysaccharides, for instance. The role of other membrane constituents may influence toxicity. Proteins were reported to play a role in the permeabilization of membranes by amphidinols (Morsy et al., 2008b). The authors highlighted that amphidinols have a high affinity for some proteins and that the lytic activity was enhanced on liposomes that contained protein as compared to 100% lipid-based liposomes.

The potential influence of sterols and proteins on the sensitivity of cytoplasmic membranes may be attributed to the alteration of the membrane structure (Knopik-Skrocka and Bielawski, 2002) or to interactions with molecular complexes such as ion channels (Andreoli, 1974; De Kruijff and Demel, 1974). Both interactions might result in the permeabilization and depolarization of membranes. Interactions with ion-channels may be investigated with the use of ion-channel blockers. For instance, (Ma et al., 2011a) highlighted that Ca^{2+} channels were not the target of *A. tamarensis* allelochemicals. Further studies should focus on the fine interactions of *A. minutum* and membrane components.

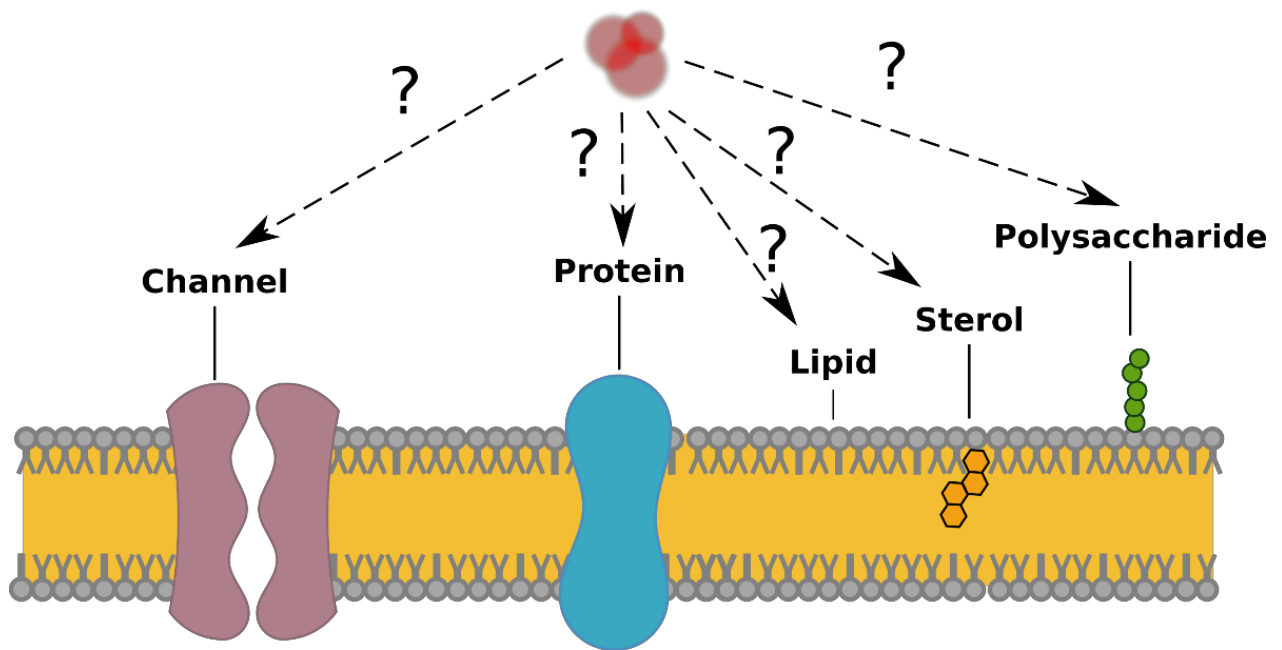


Figure 2: Molecular component of cytoplasmic membranes susceptible to interact with *A. minutum* allelochemicals

4 Interactions between allelochemicals and photosynthetic membranes

Photosynthetic membranes were also impaired by allelochemicals, however the link between cytoplasmic and photosynthetic membranes remains unknown. We proposed three non-exclusive scenarios of cellular cascade (Figure 3) in order to make this link:

- (1) In the first scenario, we propose that allelochemicals produced by *A. minutum* disrupt the cytoplasmic membranes then enter the cells and induce the same disruptive action on thylakoid membranes. This disruption of membranes might be responsible for the inhibition of photosynthesis. This first hypothesis could be tested by isolating chloroplasts and incubating them with allelochemical extracts. The effects on the photosynthetic chain could be measured with the protocol used in Chapter 2. In the hypothesis that allelochemicals enter the cells and can diffuse through the cytoplasm to impair organelles, it may be interesting to investigate the effects of allelochemicals on mitochondrial membranes. This could be investigated by measuring the mitochondrial membrane potential with the JC10 dye for instance (the JC-10 dye is capable of selectively entering mitochondria, and reversibly changes its color from green to orange as membrane potentials increase). Such tests could also be performed on other organisms; such as bivalve or fish mitochondria.
- (2) In the second scenario, ROS play a central role. ROS may increase in response to membrane depolarization and induce some damage to the cell. ROS could therefore be solely responsible for the inhibition of PSII, observed under photoinhibition. This

hypothesis is corroborated by the increase in ROS production observed in the presence of allelochemicals (c.f. Chapter 2) and the mitigation of potency previously observed with the addition of antioxidants (Flores et al., 2012). During oxidative stress in photosynthetic organisms, ROS are usually reported to first impair PSII at the acceptor side of PSII (i.e. Oxygen Evolving Complex; (Ali et al., 2006; Nishiyama et al., 2006)). In chapter 2, Article 2, we showed that PSII was inhibited at the donor side between the two photosystems. Nevertheless, ROS may impair photosynthesis (and other metabolic processes) by peroxidation products, issued from the reaction between ROS and FA (Farmer and Mueller, 2013). Lipid peroxidation of membranes can result in a conformational modification of structural lipids and thereby alter membrane permeability and fluidity (Farmer and Mueller, 2013). Peroxidation of membranes might therefore lead to the permeabilization of membranes measured in chapter 2, and alter their overall fluidity. These modifications of membrane physical characteristics may explain the alteration of membrane-bound enzymes related activities (i.e. photosynthesis between the two photosystems and esterase activity) as the physical characteristics of membranes are essential for their function (Parrish, 2013). Reaction between FA and ROS can also lead to the formation of oxylipins and could be related to the decrease of neutral FA (Chapter 2, Article 1). Oxylipins are lipophilic signaling molecules (Fontana et al., 2007; Mosblech et al., 2009) derived from the oxidation of polyunsaturated FA (Mosblech et al., 2009). They are known to be produced by diatom including the genus *Chaetoceros* (Fontana et al., 2007), and can also be toxic (Adolph et al., 2004). An excess production of oxylipins may be responsible for the inhibition of PSII as shown in *Arabidopsis* (Améras et al., 2003). Production of reactive electrophile species (e.g. peroxidation products, oxylipins) should be quantified to evaluate their implication in allelochemical interactions.

- (3) In the third scenario, the overall deleterious effects would come from permeabilization of the cytoplasmic membranes. Perforation of the membrane might induce a leak of ions, metabolites essential for the osmotic gradient and homeostasis of microalgal cells. Without homeostasis, essential functions (e.g. enzyme activity, photosynthesis) are impaired.

Finally, all (or some) of the processes described above could be happening at the same time or subsequently, and eventually result in the overall toxic effects and death of the cells.

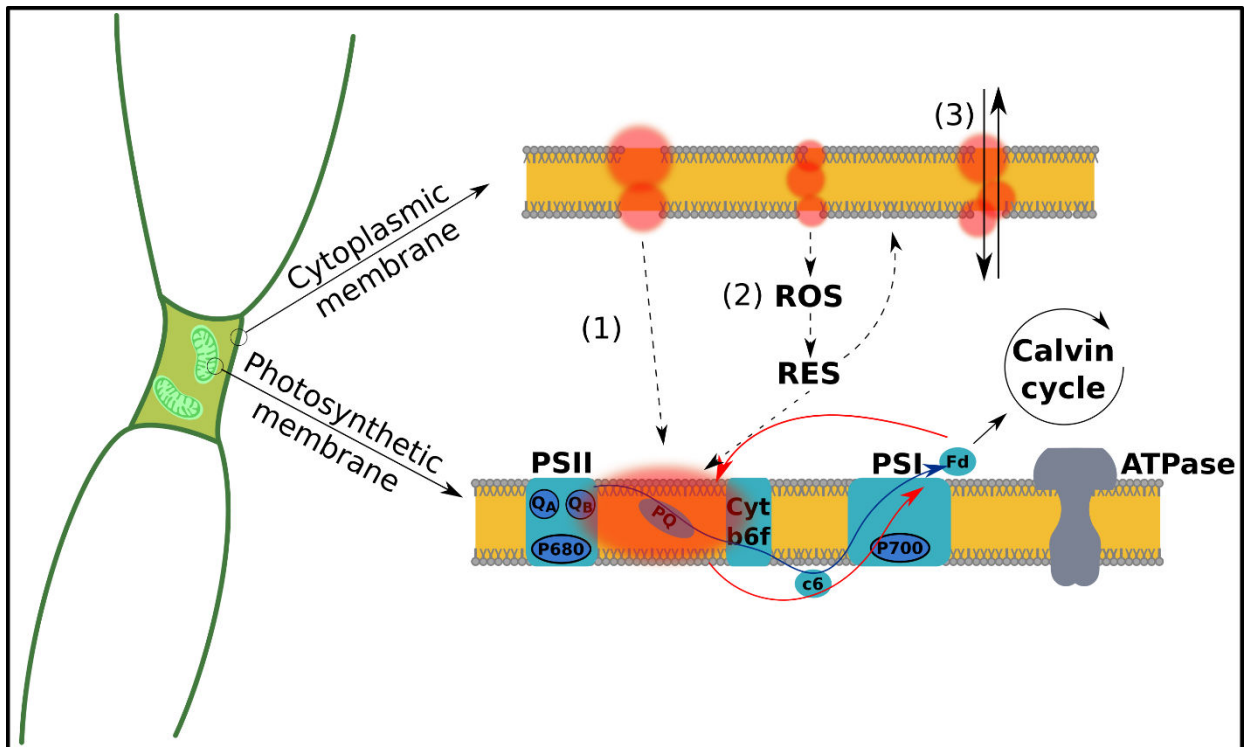


Figure 3: Hypothetical scenarios linking between the cytoplasmic membranes and photosynthetic membranes in the diatom *C. muelleri*. RES: Reactive electrophile species, ROS: Reactive oxygen species, PSI: Photosystem I, PSII: Photosystem II, Q_A and Q_B : Quinones A and B, PQ: pool of plastoquinones, Cyt b6f: Cytochrome b6f, c6: Cytochrome c6, Fd: Ferredoxine.

Answering these two questions is essential: 1) What is the molecular target of allelochemicals on cytoplasmic membranes? and 2) What is the link between cytoplasmic and photosynthetic membranes? Further understanding the mode of action may provide some valuable insights into the variability of organism sensitivity and how allelochemicals shape plankton communities.

5 Understanding the mode of action to explain the variability in the sensitivity to allelochemicals

The release of allelochemicals by *Alexandrium* spp. enables the toxic dinoflagellate to outcompete other protists by impairing or killing them. Allelochemicals released by *Alexandrium* spp. have a wide inhibition spectra of action as they impair the physiology of diverse protists (Arzul et al., 1999; Hansen et al., 1992; Tillmann et al., 2008, 2007). Despite the wide inhibition spectra, there is great variability in the sensitivity of target cells to allelochemicals from a same *Alexandrium* strain (Tillmann et al., 2008). In this same study, the authors suggested that diatoms were particularly resistant to *Alexandrium* spp. as the diatom *T. weissflogii* was particularly insensitive. In other studies, athecate dinoflagellate were highly

sensitive to *A. ostenfeldii* (Tillmann et al., 2007), while in another study thecate flagellates were more sensitive to *A. tamarensis* than athecate flagellates (Tillmann and John, 2002). Finally, in another study by (Hattenrath-Lehmann and Gobler, 2011), autotrophic nanoflagellates and diatoms were particularly sensitive to *A. fundyense* while dinoflagellates were favored. These contrasting results show that the donor/target combination is essential in allelochemical interactions.

Our study shows that the species *A. minutum* is not an exception to the rule, as target cell responses exhibit great variability when exposed to *A. minutum* filtrate (Figure 4). The results from the screening of sensitivity of protists isolated from *A. minutum* biotope (Rance estuary, Brittany, France) showed a great variability in protist sensitivity (Figure 4). However, we could not relate sensitivity/resistance to a specific algal group as genus from all phylum were inhibited when screened (Chlorophyta, Bacillariophyta, Cryptophyta, Miozoa). Dinoflagellates (i.e. Miozoa) might have been slightly less sensitive as only one of the seven strains tested was impaired by the filtrate. Our results (Figure 4) also highlight the variability in sensitivity of different strains from the same genus/species (i.e. *Thalassiosira* sp., *Rhodomonas* sp. *Heterocapsa rotundata*). It highlights that conclusions cannot be made on a target species based on the response of only one strain because of intraspecific trait diversity. Several strains from a same species can exhibit high trait variations. A recent paper (Brandenburg et al., 2018) highlighted the high trait variation (including allelochemical potency) of *A. ostenfeldii* populations and suggested that this variation would facilitate the development and success of HAB under changing environmental conditions. The same hypothesis can be made for co-occurring species and would explain the variability in their sensitivity during allelochemical interactions.

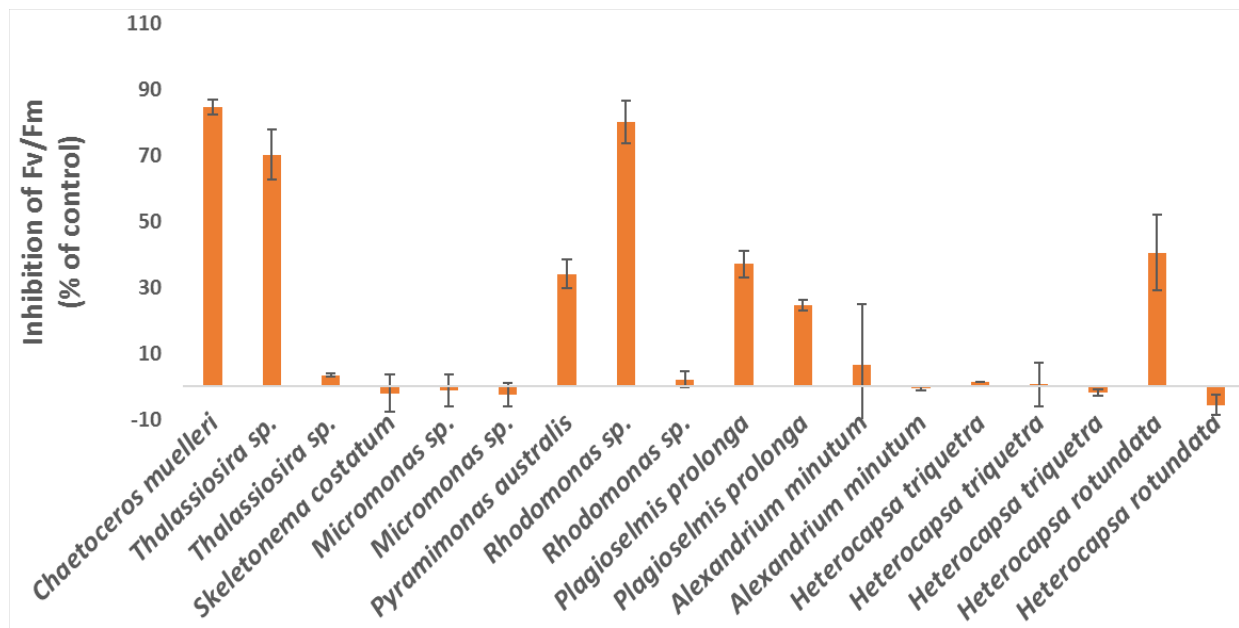


Figure 4: Sensitivity of different microalgal strains to *A. minutum* (strain CCMI1002) filtrate (3000 cells mL⁻¹) according to the bioassay developed in the 3rd chapter. All the strains screened here are different strains. Results are expressed as the mean of inhibition \pm standard error. N = 3.

Despite this research, the factors triggering sensitivity/resistance of co-occurring protists to some allelochemical species remain unclear. In the 2nd chapter, we highlighted that *A. minutum* allelochemicals interact with biological membranes. The biochemical composition of cytoplasmic membranes vary greatly among eukaryotes, we can thus hypothesize that the biochemical composition of membranes might drive, at least partially, the tolerance of cells in *A. minutum* allelochemical interactions. Two molecular components of membranes, the lipids and proteins, were already shown to be important during allelochemical interactions (Morsy et al., 2008a, 2008b). While the role of proteins has not been investigated in allelochemical sensitivity, the potential importance of lipids interactions was shown by the affinity of *A. tamarense* allelochemicals to some sterols (Ma et al., 2011a) and modifications in the lipid composition of a target cell during allelochemical interaction. The relatively low sensitivity of dinoflagellates to *Alexandrium* spp. allelochemicals (Hattenrath-Lehmann and Gobler, 2011; Figure 4) might be explained by the predominance of dinosterols in their lipid composition as compared to other microalgae (Kaku and Hiraga, 2003; Volkman, 2003; Volkman et al., 1998, 1993). Dinosterols were suggested to self-protect *A. tamarense* from the release of its own allelochemicals (Ma et al., 2011a). We can suggest that the ratio of lipid classes with affinity to allelochemicals (e.g. cholesterol, ergosterol, phosphatidylcholine) over protective lipids (e.g. dinosterol) may partially trigger the response of a species during allelochemical interactions. Comparative lipidomics and proteomics between resistant and sensitive strains/communities,

as well as the use of artificial liposomes, may help unravel the role of biochemical composition of membranes in allelochemical interactions.

However, intraspecific differences in the sensitivity to allelochemicals as observed with *H. rotundata* (Figure 4) partially rule out this theory and highlight that other components must be taken into account. The two different strains of *H. rotundata* were inoculated on the same day, however they may have not been in the exact same physiological state. It has been shown that the lipid and sterol composition of microalgae is modified with their physiological state (Mansour et al., 2003; Zhukova, 2004), and may partially explain differences in the sensitivity. Cytoplasmic membranes are complex structures with other molecular components that may play a role in the sensitivity (e.g. proteins). Reactive oxygen species were shown to be involved in the allelochemical potency of *Alexandrium* spp. (Flores et al., 2012; Long et al., 2018b), indeed variability in the antioxidant system of *H. rotundata* may have contributed to its sensitivity variability to allelochemicals. Other protective mechanisms may have to be studied, such as transport of allelochemicals outside of the cells or the release of mucus that may have trapped allelochemicals, preventing them making contact with cell membranes. Finally, we suggest that potential different bacterial communities of *H. rotundata* may have explained the contrasting results between the two strains. Bacterial communities can modify the physiology and the production of secondary metabolites by diatoms (Lelong et al., 2014). Bacteria might have been mitigating the allelochemical potency by changing the physiology of *H. rotundata* or they may have sequestered allelochemicals.

6 Variability of allelochemical potency from *Alexandrium* strains

The variability in allelochemical potency of *Alexandrium* species and strains has been largely documented. However, on top of that intraspecific variability, the knowledge on the variability induced by the environment is still scarce. While the allelochemical potency of *Alexandrium* spp. seems independent from temperature (Fistarol et al., 2004) or nutrient (Yang et al., 2011; Zhu and Tillmann, 2012), low salinity and toxic Cu concentrations greatly increased the potency of *A. ostenfeldii* (Martens et al., 2016) and *A. minutum* (Chapter 4). These results highlight that the production of allelochemicals is under environmental control and that other biotic or abiotic factors may modulate the allelochemical potency of *Alexandrium* spp. It is therefore difficult, to extrapolate the allelochemical potency observed in laboratory studies to environmental conditions.

Other abiotic factors may influence the potency of allelochemicals, such as the presence of aquatic humic and fulvic acids that can increase the permeability of biologic membranes to

lipophilic solutes (Vigneault et al., 2000), potentially allelochemicals. The main role of allelochemicals is to outcompete co-occurring protists, therefore it may be interesting to test if the presence of competing species (or their culture filtrate) may increase the release of allelochemicals. Overall, a better knowledge of the factors triggering this variability is needed to better evaluate and predict the effects of *Alexandrium* spp. blooms on plankton communities.

7 The relevance of laboratory studies in an environmental context

In the natural environment, the situation is more complex than the simple donor/target combination. In the marine environment, *Alexandrium* spp. populations show a high intraspecific variation in allelochemical potency (Brandenburg et al., 2018; Tillmann et al., 2016) and are in competition with complex plankton communities that also vary within a season. Moreover, microalgae communicate not only through the release of allelochemicals but also through cell-cell contact. The use of supernatant/ filtrate is useful to characterize the allelochemical interactions at their strict definition (interaction through the release of allelochemicals in the surrounding environment) but their environmental relevance is limited. It is necessary to include allelochemical interactions in a wider context and to study allelochemical interactions with co-cultures (that will include grazing and cell-cell interactions) and environmental studies.

So far the study of allelochemical interactions in co-cultures or in environmental samples has been limited mainly by technical issues. For instance, it is difficult to separate cells for metabolomic (e.g. lipidomic, proteomic) studies or for photosynthesis measurements due to overlapping signals in co-culture. Some new protocols already offer promising results in the study of interactions in co-cultures, this is the case for transcriptomic studies (Zheng 2016) but it can only be performed on species which the genome is known. Some techniques used during this PhD offer very promising perspectives for future research with co-cultures or with environmental samples. Flow-cytometric analyses allow the differentiation of groups of algae based on their morphology and pigments (Estrada et al., 2004) and the use of specific dyes to allow the measurement of physiologic parameters. When coupled to a cell-sorter, flow cytometry could allow the separation of cells for further analysis (e.g. proteomic, lipidomic, transcriptomic). Photosynthesis was shown to be a good probe to study allelochemical interactions (Long et al., 2018a; Remington X. Poulin et al., 2018; Prince, 2008; Ternon et al., 2018). However the photosynthetic probes used so far (i.e. Fv/Fm) do not work with mixtures of microalgae as it is not yet possible to separate the contribution of different algae to the signal. The phenomenon of electrochromism (reviewed in (Bailleul et al., 2010)) allows us to study

the effects of allelochemical interactions on photosynthesis in a mixture of algae. Briefly, electrochromism is a physical phenomenon based on a spectral shift of some photosynthetic pigments under an electric field, the so-called electrochromic shift (ECS). ECS could be compared to a spectroscopic voltmeter that allows us to probe photosynthesis. Species with pronounced differences in their pigment composition (diatoms and dinoflagellates for instance) have specific ECS spectra that allow them to be distinguished in a mixture. Such measurements can be applied to study interactions between microalgae or populations of microalgae that have different ECS. During the course of this PhD, the ECS was successfully applied to study the couple *A. minutum*/*C. muelleri* in co-culture (Work in preparation).

8 Beyond allelochemical interactions

The release of allelochemicals by *Alexandrium* spp. provides a competitive advantage over competing microalgae but these compounds may also target other organisms. The activity spectra of allelochemicals seem to be wider than strict allelochemical interactions.

It is suggested that allelochemicals might favor species producing allelochemicals by reducing the grazing pressure (Cembella, 2003). There is a plethora of evidence that *Alexandrium* spp. produces toxins that impair grazers (Emura et al., 2004; Ianora et al., 2004; John et al., 2015; Lasley-Rasher et al., 2016; Roncalli et al., 2016; Tillmann et al., 2008, 2007; Xu et al., 2016) and may decrease the grazing pressure from the water column/pelagic compartment. In some studies, *Alexandrium* anti-grazing activity was concomitant with their allelochemical potency (Emura et al., 2004; John et al., 2015; Tillmann et al., 2008, 2007). However, it is not always related to allelochemicals (Xu et al., 2016). We can propose several hypotheses to explain these contrasted results. Inter and intraspecific variability in sensitivity may explain the differences. We can also hypothesize that *Alexandrium* spp. do not produce only one allelochemical compound but a cocktail of compounds, some being allelochemicals and other having anti-grazing activity. The use of a bioassay-guided fractionation (Chapter 5) might have failed in identifying all active fractions if *C. muelleri* is resistant to some allelochemicals. The potency of the fractions should be tested on different target cells. Finally, if the compounds with anti-grazing activity are the allelochemicals, grazer sensitivity might also be related to their lipid/sterol. Their lipid/sterol composition is indeed modified depending on what grazers have been feeding on (Ederington et al., 1995; Klein Breteler et al., 1999), differences in the diets of grazers might therefore partially explain their variability of response to allelochemicals.

The effects of strains of *A. minutum* producing allelochemicals was shown to impair the physiology of several filter-feeders (Borcier et al., 2017; Castrec et al., 2018; Ford et al., 2010;

Le Goïc et al., 2014a), suggesting that allelochemicals are also involved in the toxic effects on filter-feeders. The toxic effects consisted of lower adherence and phagocytosis of clam hemocytes (Ford et al., 2010), increased production of ROS in oyster oocytes (Le Goïc et al., 2014b), modification of oyster valve-activity and mobilization of hemocytes in the gills (Castrec et al., 2018), reduced growth, and decreased filtration rate and an increase in reaction time against predators (Borcier et al., 2017). Gills were particularly impaired by *A. minutum* strains producing allelochemicals (Borcier et al., 2017; Castrec et al., 2018), the same authors related this to the fact that gills are the first organ in contact with allelochemicals. It is noteworthy that gill lipids consist mainly in sterols and phospholipids (Pernet et al., 2007), which may have played a role in their sensitivity. Overall, allelochemicals were suggested to mitigate the grazing pressure from the pelagic compartment (e.g. copepods, heterotrophic flagellates) but the effect of allelochemicals on the grazing pressure from the benthic compartment has received less attention. The benthic compartment can, however, influence and even mitigate microalgal blooms with benthic organisms (e.g. filter feeders) feeding on microalgae (Lindahl et al., 2005), particularly in coastal/shallow environments where *Alexandrium* spp. blooms occur. Here, we suggest that allelochemicals might favor *Alexandrium* spp. blooms by decreasing the grazing pressure from the benthic compartment. Finally, this PhD highlights that allelochemical interactions are complex and result from the interactions of abiotic and biotic factors. The conclusion from laboratory experiments under controlled conditions are difficult to extrapolate to environmental conditions, however, they provide valuable information and new insights on how allelochemicals may influence ecosystems. As an example, (Haberkorn et al., 2014) highlighted that the oyster *Crassostrea gigas*, simultaneously exposed to an allelochemical strain of *A. minutum* (a strain producing allelochemicals) and a metal mixture (Cu-Cd), contained much less PST in the presence of the metal mixture. In different studies, (Borcier et al., 2017; Castrec et al., 2018) observed that PST accumulation in great scallops and oysters decreased with an increasing allelochemical potency. These authors suggested that this decrease might be explained by a decrease in the feeding rate with allelochemicals. We therefore suggest that the decrease in the PST content from oyster exposed to *A. minutum* and the metal mixture (Haberkorn et al., 2014) might result from an increased allelochemical potency of the dinoflagellate in presence of toxic concentrations of Cu. These results highlight the need to further our knowledge on allelochemicals interactions and to include them in an environmental context.

Conclusions and perspectives

The main objective of this PhD thesis was to better understand the allelochemical interactions between *A. minutum* and competing species. To do so we focused on the interactions between *A. minutum* and the diatom *C. muelleri*, a microalgal species naturally co-occurring with *A. minutum*. This PhD brings new insights on allelochemical interactions by shading some light on their physiological effects and unraveling their mode of action. This approach allowed us to better understand how it disturbs microalgal physiology and will help predicting how it may affect plankton communities. Our results, also revealed the complexity of allelochemical interactions that are influenced by many parameters (e.g. environmental Cu concentration, donor/target couple). This approach also allowed us to identify new techniques to study allelochemical interactions in an environmental context. Overall, the research performed during this PhD raised promising perspectives to better unravel allelochemicals interactions in marine environment.

Allelochemicals of *A. minutum* are toxic to competing microalgae by disrupting the biological membranes of the target cells.

In the 2nd chapter, we highlighted the essential role of membranes in the mode of action of *A. minutum* allelochemicals. We demonstrated that allelochemicals are rapid acting compounds that impair plasmic and photosynthetic membranes within minutes. Allelochemicals from *A. minutum* induced a disruption of plasmic membrane of *C. muelleri*, first inducing a depolarization, followed by a permeabilisation of these membranes. In the meantime, allelochemicals impaired the photosynthetic apparatus by inhibiting the photosynthetic chain between Qb and P700. These deleterious effects resulted in an increase of ROS, as well as larger effects on photosynthesis (inhibition of PSI and PSII), inhibition of primary metabolism, and biochemical modifications (i.e. lipids and pigments) of membranes.

Future studies should focus on the fine interactions between allelochemicals and plasmic membranes, especially as this interaction may determine allelochemical sensitivity. This could be done by comparing the biochemical composition of resistant versus sensitive species and quantifying the affinity of allelochemicals to different membranes constituents with artificial liposomes. The several techniques (i.e. flow cytometry and fluorescence and absorption spectroscopy) used in this chapter highlighted new biomarkers to study allelochemical interactions in an environmental context.

The inhibition of photosystem II of the diatom *C. muelleri* can be used to probe and quantify the allelochemical potency of *Alexandrium* spp. in a bioassay.

In the third chapter, we took advantage of both the sensitivity of *C. muelleri* and the several biomarkers of allelochemical interactions identified in the second chapter. We highlighted that the inhibition of Fv/Fm was a good probe to quantify the deleterious effects of a cell free extract and was thus developed as a specific bioassay to precisely quantify the intraspecific variability in allelochemical potency from three *A. minutum* strains.

In future studies, this bioassay will allow a rapid quantification of intra and interspecific variability in the allelochemical potency of *Alexandrium* spp. This bioassay could also be used in to investigate allelochemical interactions *in situ* and to study the links between allelochemical potency and other toxicities (e.g. ichthyotoxicity, cytotoxicity, anti-grazing activity).

Environmental Cu contamination influences allelochemical interactions

In the 4th chapter, we highlighted that exposure to toxic concentrations of Cu significantly increased the allelochemical potency of *A. minutum* and the overall exudation of dissolved organic carbon. Simultaneously, the physiology of *A. minutum* was severely impaired but microalgal cells coped with Cu stress by decreasing its dissolved Cu concentration. Additionally, the increase in dissolved organic carbon exuded and the release of allelochemical simultaneously with an increase in the bacterial population might have led to the decrease of dissolved Cu concentrations.

Future studies should investigate the inter and intraspecific variability of *Alexandrium* spp. in response to Cu toxicity. The evaluation of the chelating properties of allelochemicals that may give new insights into their mode of action and may also ease their purification by separating them on their chelating properties.

Allelochemicals from *A. minutum* were partially isolated

Allelochemicals of *A. minutum* were partially isolated with a bioassay-guided fractionation. We isolated a potent fraction following three different purification steps by HPLC. Hydrophobic candidates with molecular weights between 200 and 700 Da were detected by mass-spectrometry in the active fraction.

Additional purification steps are required to isolate candidates, confirm their allelochemical potency, and elucidate their chemical nature. Further studies may also consider complementary detection methods to screen a wider range of compounds.

PhD outcomes

Published (2)

- Long, M., Tallec, K., Soudant, P., Lambert, C., Le Grand, F., Sarthou, G., Jolley, D., Hégaret, H. (2018) A rapid quantitative fluorescence-based bioassay to study allelochemical interactions from *Alexandrium minutum*. *Environmental Pollution*. Doi:10.1016/j.envpol.2018.07.119
- Long, M., Tallec, K., Soudant, P., Lambert, C., Le Grand, F., Donval, A., Sarthou, G., Jolley, D., Hégaret, H. (In proof) Allelochemicals from *Alexandrium minutum* induce rapid inhibition of metabolism and modify the membranes from *Chaetoceros muelleri*. *Algal Research*. Doi:10.1016/j.algal.2018.09.023

Submitted (1)

- Long, M., Holland, A., Soudant, P., Planquette, H., González Santana, D., Whitby, A., Sarthou, G., Hégaret, H., Jolley, D. A multi-traits approach reveals Cu effects on allelopathic *A. minutum* physiology. Under review in *Aquatic Toxicology*.

In preparation (1)

- Long, M., Peltekis, A., González-Fernández, C., Sarthou, G., Jolley, D., Hégaret, H., Bailleul, B. Allelochemical interactions between *Alexandrium minutum* and *Chaetoceros muelleri*: insights into photosynthesis and membrane integrity (In prep).

Publications outside the frame of the PhD (4)

- Pousse, E., Flye-Sainte-Marie, J., Alunno-Bruscia, M., Hégaret, H., Rannou, E., Pecquerie, L., Marques, G., Thomas, Y., Castrec, J., Fabioux, C., **Long, M.**, Lassudrie, M., Hermabessière, L., Amzil, Z., Soudant, P., Jean, F. (2018). Paralytic shellfish toxin accumulation in *Crassostrea gigas* : modeling through Dynamic Energy Budget (DEB) theory. *Journal of Sea Research*. Doi: 10.1016/j.seares.2018.09.002
- Long, M.**, Paul-Pont, I., Hégaret, H., Moriceau, B., Lambert, C., Huvet, A., Soudant, P. (2017). Interactions between polystyrene microplastics and marine phytoplankton lead to species-specific hetero-aggregation. *Environmental pollution*. Doi: 10.1016/j.envpol.2017.05.047
- Moutel, B., Goncalves, O., Legrand, F., **Long, M.**, Soudant, P., Grizeau, J., Legrand, J., Pruvost, J., (2016). Development of a screening procedure for the characterization of *Botryococcus braunii* strains for biofuel application. *Process Biochemistry*. 51(11), 1855-1865. Doi: 10.1016/j.procbio.2016.05.002
- Hermabessiere, L., Fabioux, C., Lassudrie, M., Boullot, F., **Long, M.**, Lambert, C., Le Goïc, N., Gouriou, J., Le Gac, M., Chapelle, A., Soudant, P., Hégaret, H. (2016). Influence of gametogenesis pattern and sex on paralytic shellfish toxin levels in triploid Pacific oyster *Crassostrea gigas* exposed to a natural bloom of *Alexandrium minutum*. *Aquaculture*. 455: 118-124. Doi: 10.1016/j.aquaculture.2016.01.001

Thesis presentations

Oral (7)

- Oct. 2018 A multi-traits approach reveals physiologic effects of Cu on *Alexandrium minutum* and their exudates. Long, M.*, Holland, A., Lambert, C., Soudant, P., Planquette, H., Sarthou, G., Hégaret, H., Jolley, D. 18th International Conference on Harmful Algae (ICHA), Nantes, France.
- Dec. 2017 A rapid and quantitative fluorescence-based bioassay to study allelopathy from *Alexandrium* sp. Long, M.*, Tallec, K., Soudant, P., Lambert, C., Le Grand, F., Sarthou, G., Jolley, D., Hégaret, H. AQUAFLUO II, Sydney, Australia.
- Oct. 2017 Production of allelopathic compounds by *Alexandrium* sp. Seminar, invited by Dr. Shauna Murray at the Climate Change Cluster from University of Technology Sydney, Australia.
- Sept. 2017 Chemical interactions between the toxic dinoflagellate *Alexandrium minutum* and phytoplankton: specificity and mode of action. Long, M.*, Guillou, L., Tallec, K., Le Grand, F., Lambert, C., Soudant, P., Sarthou, G., Jolley, D., Hégaret, H. 10th European Conference on Marine Natural Products, Kolymbari, Greece.
- March 2017 Short-term effects of the allelochemicals released by the toxic dinoflagellate *Alexandrium minutum* upon the diatom *Chaetoceros* sp.. Long, M.*, Tallec, K., Soudant, P., Le Grand, F., Sarthou, G., Hégaret, H., Jolley, D. Conférence GDR PHYCOTOX 2017, Gif / Yvettes, France.
- Oct. 2016 Allelochemicals released by the toxic dinoflagellate *Alexandrium minutum* impact *Chaetoceros neogracile* photosystem and viability. Long, M.*, Tallec, K., Soudant, P., Le Grand, F., Sarthou, G., Hégaret, H., Jolley, D. 17th International Conference on Harmful Algae (ICHA) 2016, Florianopolis, Brazil.
- Mar. 2016 Kinetics of the allelopathic activity of the toxic dinoflagellate *Alexandrium minutum* upon the photosystem II of the diatom *Chaetoceros neogracile*. Long, M.*, Tallec, K., Guilloneau, R., Soudant, P., Le Grand, F., Sarthou, G., Jolley, D., Hégaret, H. Conférence GDR PHYCOTOX 2016, Villefranche Sur Mer, France.

Thesis presentations

Posters (3)

- Sept. 2017 From marine chemical ecology to drug discovery: Allelopathic *Alexandrium minutum* exudates, a new source of bioactive metabolites ? Long, M.*, Serive, B., Bach, S., Le Grand, F., Petek, S., Soudant, P., Sarthou, G., Jolley, D., Hégaret, H. 10th European Conference on Marine Natural Products, Kolymbari, Greece.
- June 2016 First insights in the characterization of allelopathic and cytotoxic extracellular compounds from the toxic dinoflagellate *Alexandrium minutum*. Long, M.*, Wikfors, G. H., Soudant, P., Le Grand, F., Jolley, D., Sarthou, G., Hégaret, H. Ecole Thématique Ecologie Chimique (ETEC) 2016, Roscoff, France.

May 2016 First insights in the characterization of allelopathic and cytotoxic extracellular compounds from the toxic dinoflagellate *Alexandrium minutum*. Long, M.*, Wikfors, G. H., Soudant, P., Le Grand, F., Jolley, D., Sarthou, G., Hégaret, H. Society of Environmental Toxicology and Chemistry (SETAC) Europe 2016, Nantes, France.

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Allelochemicals from *Alexandrium minutum* induce rapid inhibition of metabolism and modify the membranes from *Chaetoceros muelleri*

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ABSTRACT

Allelochemical interactions are likely to be a contributing factor explaining the success of large blooms of the harmful marine dinoflagellate *Alexandrium*, however, the physiological mechanisms of allelochemical interactions remain poorly described. Here we investigated the sub-lethal effects (on an hourly scale) of a filtrate containing allelochemicals from *Alexandrium minutum* on the physiology of the common diatom *Chaetoceros muelleri*. The filtrate induced deleterious effects to the diatom physiology within only 30 min of exposure. Esterase activity and photosynthesis were drastically inhibited, with up to 34% of the population being metabolically inactive and up to 30% reduction in photosystem II quantum yield when exposed to the filtrate. In addition, intracellular reactive oxygen species increased by 26% in response to allelochemical exposure. *C. muelleri* pigment and lipid analyses indicated that the photosystem II was inhibited, with photoinhibition-like responses (activation of xanthophyll cycles, and changes in associated lipids) upregulated to mitigate the toxic effects of allelochemicals. Changes in the proportions of membrane lipid classes and increased membrane fatty acids saturation by 9% may be an attempt to maintain membrane integrity and associated enzyme activity, or could be the result of deleterious effects on membranes. An 8% decrease in cellular storage lipids (triglycerides) revealed a mobilization of energy suggesting an energetic cost for the diatom to counteract the allelochemical effects. We hypothesize that the rapid alteration of physiological functions such as photosynthesis and some enzymatic activities may result from direct damage on external membranes. Overall this study describes the sub-lethal mechanisms and provides useful biomarkers to understand the role of allelochemical interactions and associated ecological processes in structuring plankton communities.

1. Introduction

The structure of plankton communities is shaped by complex interactions with their environment. The influence of abiotic factors, such as light, nutrients, seawater or stratification are well studied and known to have a strong role in community structure. The roles of biotic factors however are less well understood despite the increasing evidence that biotic factors are shaping plankton communities [1,2]. Chemical interactions between organisms (i.e. chemical ecology) are influencing the structure of marine communities. Chemical compounds can mediate communication between microbes, can play a role in sexual reproduction and competition between species or can be a chemical defense

against grazers and pathogens [3–5]. Within this field, allelochemical interactions refer to the beneficial or adverse effects of chemicals (allelochemicals) released by a protist on the biology of co-occurring protists, predators or bacterial cells. The release of toxic allelochemicals gives the donor species a competitive advantage that could result in the formation of large blooms [6,7].

The genus *Alexandrium* is known to produce uncharacterized extracellular compounds with potent allelochemical activity. It is hypothesized that the allelochemical potency of this genus may enhance the establishment of large *Alexandrium* blooms [8]. The allelochemical potency is widespread among the genus *Alexandrium* as this activity was shown in at least 9 species: *A. minutum*, *A. fundyense*, *A. lusitanicum*, *A.*

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affine, *A. ostenfeldii*, *A. pseudogonyaulax*, *A. tamarensis*, *A. taylora* and *A. tamutum* [9–13]. Allelochemicals are released by cells into the surrounding environment and within minutes to hours induce cell lysis, cell immobilization and cyst formation [14]. Furthermore some of these allelochemicals may be responsible for other toxic effects on mammalian erythrocytes [15], and bivalve gametes [16]. They may also be responsible for reduced growth and behavior modification of the great scallop *Pecten maximus* [17] or deleterious effects on the physiology of the oyster *Crassostrea gigas* [18]. Nevertheless the nature and mode of action of those extracellular compounds remain unknown.

Researchers studying allelochemical interactions often quantify their effects on microalgal growth rate, membrane permeability and cell lysis in target cells however very few studies precisely elucidate the mode of action. For example, studies exploring allelochemical mechanisms from the genus *Alexandrium* have investigated their effects on photosynthesis [10], membranes [19] and transcriptomic [20], however the story is still incomplete. While the long-term (i.e. mainly lethal) effects are fairly well described, the short-term (i.e. sub-lethal) effects and cellular processes leading to cell death remain unclear. More specifically, the effects of allelochemicals on the biochemical composition of membranes, that seem to have a central role in allelochemical sensitivity [19] are unknown.

The aim of this study was to elucidate the mechanistic effects (at a subcellular level) of allelochemicals released by *Alexandrium minutum* on the common diatom *Chaetoceros muelleri* a diatom co-occurring with *A. minutum* [21]. The study focused on the photosynthetic apparatus and biochemistry of membranes. Allelochemicals were separated from *A. minutum* cells by filtration to specifically focus on allelochemical interactions and avoid interference by cell-cell interactions. A pulse of allelochemicals was performed to study the short-term sub-lethal effects of allelochemicals on *C. muelleri*, measuring the effects on photosynthesis, intracellular reactive oxygen species production and esterase activity, a proxy of primary metabolism [22,23]. To further explore the allelochemical interactions at the membrane level, we analyzed the membrane composition of the diatom (polar lipid classes, fatty acids composition of polar lipids, and sterol content) and pigments after 30 and 60 min of exposure to the filtrate. Neutral lipids classes were also analyzed to detect free fatty acid increases as a cell lysis indicator or a decrease of storage lipids (triacylglycerols) as a stress response.

2. Materials and methods

2.1. Microalgal cultures

A strain of *Alexandrium minutum* (strain CCM11002, isolated from a bloom in Gearhies, Bantry Bay, Ireland [24]), not producing paralytic shellfish toxins (PST), was selected according to its high allelochemical potency [17]. The dinoflagellates were cultivated in triplicate for 10 days in 1 L of autoclaved L1 media [25] prepared with natural filtered (0.2 µm) seawater from Argenton, France (salinity = 34 psu, pH = 8.4) a pristine site for *Alexandrium* sp. Cultures of *A. minutum* were maintained in exponential growth phase to obtain a concentration of 9.6×10^4 cells mL⁻¹ on the experimental day (growth rate: 0.18 day⁻¹). A strain of *Chaetoceros muelleri* (strain CCAP 1010-3 obtained from the CCAP culture collection, formerly listed as *Chaetoceros neogracile* or *Chaetoceros* sp.) was selected because of its sensitivity to *A. minutum* allelochemicals [10,17] and because of the ubiquity of its genus in phytoplankton communities [26]. Cultures of the diatom were grown for 10 days (2.12×10^6 cells mL⁻¹) in 6 L of autoclaved synthetic ocean seawater [27] enriched with L1 media (salinity = 35 psu, pH = 8.4) and silicate (1.06×10^{-4} M). Cultures of *C. muelleri* were in late exponential growth phase on the day of the experiment (growth rate: 0.15 day⁻¹). All cultures were maintained at 18 °C under a continuous light intensity of 100–110 µmol photons m⁻²s⁻¹. Cultures were not axenic but were handled under sterile conditions to minimize additional bacterial contamination.

2.2. Exposure to allelochemicals

Allelochemicals were separated from cultures by centrifugation (10 min, 800g, 18 °C) followed by a filtration (0.2 µm, Minisart syringe filter, acetate cellulose membrane, 16534-K, Sartorius) of supernatant [10]. The filtrate concentrations were expressed as cell concentration (cells mL⁻¹) based on the initial culture concentration prior to filtration. Cultures of *C. muelleri* in late exponential growth phase were diluted to 1.38×10^6 cells mL⁻¹ with filtered seawater and distributed to six 2 L glass balloon flasks. The diatom *C. muelleri* was then exposed in triplicate to two different conditions for 90 min: i) 12 mL of *A. minutum* filtrate (final concentration from 1200 *A. minutum* cells mL⁻¹), ii) the same volume (12 mL) of filtered seawater as a control (final volume in each condition 1 L). During the exposure (maximum 90 min), the flasks were kept under ambient laboratory light and temperature conditions (not measured).

2.3. Photosynthetic parameters

Photosynthetic measurements were performed by pulse amplitude modulation (PAM) fluorometry using an AquaPen-C AP-C 100 with a blue light (455 nm). Sub-samples of *C. muelleri* were dark-adapted for at least 10 min, allowing the full oxidation of photosystem II (PSII) reaction centers and electron chain transport, before the measurement of fluorescence variables and PSII maximum quantum yield ($F_v/F_m = (F_m - F_0)/F_m$). F_0 is the initial fluorescence intensity, and F_m the maximal intensity under saturating light [28]. Measurement of fluorescence variables were performed every 10 min for 100 min following the exposure.

Non-photochemical quenching (NPQ) measurements were performed in a separate experiment. Six replicates of the diatom *C. muelleri* (2×10^5 cells mL⁻¹) in exponential growth phase were exposed for 45 min to *A. minutum* filtrate (final *A. minutum* concentration of 6×10^3 cells mL⁻¹). The cells of *C. muelleri* were exposed to three successive flashes of 60 s (saturated light, 3000 µmol photons m⁻²s⁻¹) and 3 dark phases of 80 s for NPQ measurements, in the presence of filtered seawater or filtrate.

2.4. Flow-cytometry measurements

Population growth counts and measurements of phytoplanktonic cell variables were performed using a FACScalibur (BD Biosciences, San Jose, CA, USA) flow cytometer with a 488 nm argon laser. Counts were estimated according to flowrate [29]. Cell variables, e.g. forward scatter (forward scatter, FSC), side scatter (side scatter, SSC) and red autofluorescence (FL3, red emission filter long pass, 670 nm) were used to select diatom population. All flow cytometry measurements on *C. muelleri* (cell variables, esterase activity and reactive oxygen species production) were performed after 30, 60 and 90 min of exposure to *A. minutum* filtrate or filtered seawater.

Microalgal esterase activity, a proxy of primary metabolism was assessed with fluorescein diacetate staining (FDA; Invitrogen # F1303; at a final concentration of 6 µM) [22,23,30,31]. FDA is a nonpolar compound that can permeate into the cells. Once inside the cells, FDA is cleaved by esterases into acetate and fluorescein molecules (emission wavelength 525 nm), which are retained within the cells. Intracellular concentrations increase with both metabolic activity and time. Samples were incubated with the stain for precisely 10 min in the dark prior to flow-cytometry measurements. Intracellular fluorescence intensity, which is proportional to the amount of FDA cleaved by esterases within the cells, was measured with FL1 emission filter (green emission filter band pass, 530/30 nm). Cell populations of *C. muelleri* could be divided into 3 sub-populations: cells with highest FL1 fluorescence corresponding to high esterase activity (metabolically active), cells with a low FL1 fluorescence corresponding to cells with reduced esterase activity (reduced metabolism), and cells which FL1 fluorescence

equivalent to microalgal auto-fluorescence from cells with inactive esterases (metabolically inactive). Results were expressed as the percentages of cells with high esterase activity, cells with low reduced esterase activity and cells with inactive esterases.

Intracellular reactive oxygen species (ROS) production was measured using 2',7'-dichlorofluoresceindiacetate (DCFH-DA; Sigma-Aldrich, D6883; at a final concentration of 10 μ M). DCFH-DA is a cell-membrane permeable fluorescent stain that is a proxy of ROS production. Once inside the cells, DCFH-DA is hydrolyzed by esterase to form the non-fluorescent DCFH retained within the cell. DCFH can thus be oxidized by H₂O₂ and other oxidants to produce fluorescent 2',7'-dichlorofluorescein (DCF). Intracellular oxidation levels were correlated to DCF fluorescence within the cells and measurable by flow cytometry with F11 emission filter (green emission filter band pass, 530/30 nm). Samples were incubated with the stain for 20 min in the dark prior to flow-cytometry measurements. The relative ROS production is expressed as the mean F11 fluorescence per cell.

2.5. Pigments extraction and composition

For pigment analysis, 3.4×10^7 cells (25 mL of culture) of *C. muelleri* exposed to the filtrate or seawater were filtered over glass fiber filters (0.2 μ m, Whatman GF/F) after 30 and 60 min of exposure. Pigments were extracted from cells using methanol and analyzed by high-performance liquid chromatography (HPLC) according to the method described by [32], adapted from [33]. All the pigment standards were purchased from DHI (HØRSBOLM, Denmark).

2.6. Lipid analysis

For lipid class and fatty acid analyses, 3.4×10^8 cells (250 mL of culture) of the diatom were filtered over glass fiber filters (0.2 μ m Whatman GF/F filters were heated for 6 h at 450 °C prior to use) after 30 and 60 min of exposure. Boiling water was immediately passed through the filter after filtration to inhibit lipase activity. Lipids were then extracted with chloroform: methanol (2:1; v:v). Lipid extracts were stored at -20 °C under nitrogen (N_{2(g)}) until analysis. Lipid class composition analyses were performed by high-performance thin layer chromatography (HPTLC) using a CAMAG auto-sampler to spot the sample on HPTLC glass plates pre-coated with silica gel (Merck & Co., Ltd., Darmstadt, Germany). Neutral and polar lipid classes were analyzed according to [34]. For polar and neutral lipid fatty acid (FA) analysis, an aliquot of the chloroform: methanol (2:1; v:v) extract was dried under N_{2(g)} and then resuspended in chloroform:methanol (98:2; v:v) prior to neutral and polar lipid separation. Separation of neutral and polar lipids was realized by solid phase extraction [35]. Each fraction (polar and neutral lipids) was transesterified and the resulting fatty acid methyl esters (FAME) were analyzed and quantified by gas chromatography (GC-FID) according to the method from [35]. Lipid class and fatty acid standards were the same standards used in [35]. FAs were expressed as percentage of total FAs in polar and neutral lipids. The level of unsaturation was calculated from the % FAs derived from the gas chromatographic data according to the equation:

$$\text{Unsaturation level} = \sum [\% \text{ of fatty acid} \times \text{number of double bond}]$$

2.7. Statistics

All statistical analyses were performed using R software [36]. A generalized linear model (Glm) was performed to evaluate the influence of time, *A. minutum* filtrate and their interaction on the maximum photosystem II quantum yield (*Fv/Fm*) of *C. muelleri*. The effects of filtrate on the different physiological and biochemical parameters (NPQ, flow-cytometry measurements, pigments, lipid classes and fatty acid composition) were assessed at each time. Statistical differences

between seawater and filtrate were analyzed by a Student *t*-test after checking homoscedasticity assumption with a Bartlett test. When not meeting homoscedasticity assumption, metabolic activity (as a percentage of cells) was transformed with square root transformations. When homoscedasticity could still not be met, a non-parametric Wilcoxon test was applied. The Bray-Curtis similarity matrix was used for a one-way analysis of similarities (ANOSIM) to test whether samples within polar and neutral lipid fractions were clustered by condition (after 30 or 60 min exposures). ANOSIM calculated a global R statistic that weighs the differences between groups, with $R = 1$, $R = 0.5$ and $R = 0$ indicating a perfect, satisfactory, and poor separation of the clusters, respectively [37,38]. Principal component analyses (PCA) were performed to investigate the variation in fatty acid profile in *C. muelleri* exposed to seawater or to the allelochemicals after 30 or 60 min. Fatty acid (FA) data was analyzed with a similarity percentage analysis (SIMPER) performed on the relative FA content to identify the FAs accounting for 80% of dissimilarity between the groups and were shown on the PCA. All results are presented as mean \pm standard deviation ($n = 3$). Graphs were performed with the R package ggplot.

3. Results

3.1. Pulse amplitude modulated fluorescence

Overall, *Fv/Fm* strongly decreased during the exposure time in presence of the filtrate (Fig. 1). The filtrate of *A. minutum* rapidly (< 20 min) modified *C. muelleri* maximum photosystem II quantum yield. Allelochemicals in the filtrate induced a temporary increase in *Fv/Fm* in the first 10 min of exposure compared to the controls. Following this, *Fv/Fm* steadily decreased to 0.41 ± 0.02 (27% inhibition) at 60 min, then increased to 0.45 ± 0.03 (20% inhibition) after 100 min. In parallel, *Fv/Fm* from the seawater control culture remained stable between 0.58 and 0.56. After 45 min of exposure to *A. minutum* filtrate, non-photochemical quenching increased two-fold in filtrate exposures compared to controls (Fig. 2).

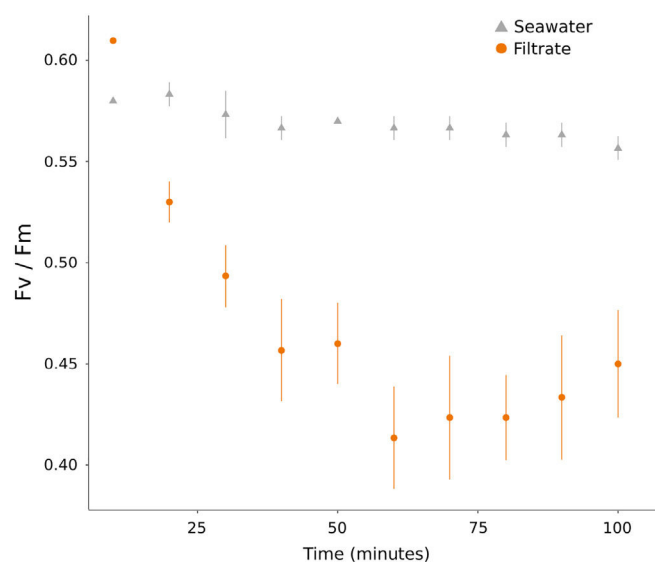


Fig. 1. Maximum photosystem II quantum yield (*Fv/Fm*) of *C. muelleri* exposed to filtered seawater (grey triangles) and to *A. minutum* filtrate (orange dots). *Fv/Fm* significantly decreased over time (Glm *t* value = 7.469, *p*-value < 0.001) and a significant influence of the interaction between time and filtrate (Glm *t* value = -5.281, *p*-value < 0.001). Error bars represent the standard deviation ($n = 3$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

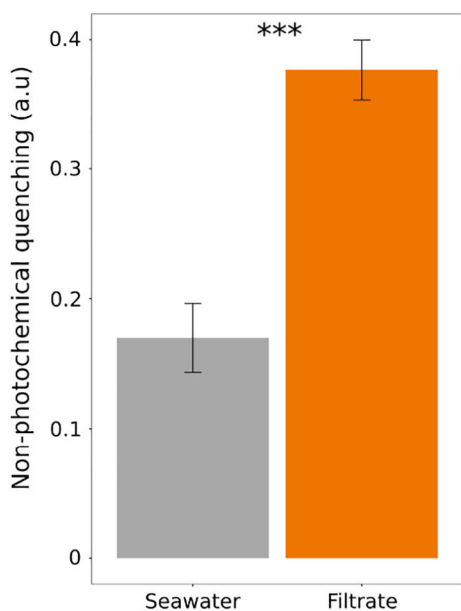


Fig. 2. Non-photochemical quenching (NPQ; arbitrary unit) of *C. muelleri* after 45 min exposure to filtered seawater (control, grey bar) or *A. minutum* filtrate (orange bar) at a theoretical cell concentration equivalent to 6×10^3 cells mL^{-1} . Error bars represent the standard deviation ($n = 3$). *** refers to a significant difference (p -value < 0.001) between the control (seawater) and the filtrate. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. Flow-cytometry parameters

Forward scatter (FSC; Fig. 3A) of *C. muelleri* exposed to filtrate significantly decreased from 74.7 ± 0.6 to 67.2 ± 0.5 (arbitrary unit; a.u.) and 67.7 ± 8.7 a.u. to 43.8 ± 1.2 a.u. as compared to control after 60 and 90 min, respectively. Side scatter (SSC; Fig. 3B) significantly decreased from 60.6 ± 8.3 a.u. (seawater) to 45.8 ± 0.5 a.u. (filtrate) after 90 min of exposure. Chlorophyll auto-fluorescence, as estimated by F13 fluorescence (Fig. 3C), steadily and significantly decreased in the cultures exposed to the filtrate compared the control cultures. After 30 min, F13 decreased from 1430 ± 9 a.u. in the control (seawater) to 1298 ± 19 a.u. with the filtrate (9% reduction). F13 fluorescence further decreased after 60 and 90 min when the inhibition reached 21 and 35%, respectively (1285 ± 122 a.u. for the control and 837 ± 44 a.u. when exposed to filtrate). A significant increase in intracellular ROS production (i.e. F11 fluorescence; Fig. 3D) in the diatom cells was observed once exposed to filtrate. ROS production increased from 6.1 ± 0.3 a.u. in the control to 7.7 ± 0.5 a.u. when exposed to the filtrate for 30 min (26% increase). ROS production kept increasing until 60 min to 9.5 ± 0.2 a.u. (63% increase compared to the control at 5.8 ± 0.1 a.u. for the control), but by 90 min exposure, the ROS production from the exposed cells returned to control levels, 5.0 ± 0.1 a.u. and 5.2 ± 0.7 a.u., respectively.

Filtrate of *A. minutum* significantly increased the proportion of diatom cells with a reduced esterase activity (i.e. reduced metabolic activity) and cells with inactive esterases (i.e. no metabolic activity) compared to controls (Fig. 4). The cells with a reduced esterase activity represented $25 \pm 4\%$ of the population at 30 min and reached a plateau of $34 \pm 5\%$ after 60 min of exposure. In the control culture, this population stayed below $4 \pm 1\%$ throughout the 90 min bioassay. Some cells had no esterase activity in cultures exposed to *A. minutum* filtrate, while in controls all cells presented esterase activity. The proportion of cells with no esterase activity increased from $3 \pm 1\%$ to $10 \pm 2\%$ and $13 \pm 1\%$ at 30, 60 and 90 min, respectively.

3.3. Pigment composition

Among the 12 pigments identified, the amount (expressed as peak area) of 7 pigments significantly changed in the diatoms exposed to *A. minutum* filtrate (Table 1). Chlorophyll *a* (Chl *a*), chlorophyll *c2* (Chl *c2*), diadinoxanthin (Ddx), and β -carotene decreased while allo-chlorophyll *a*, diatoxanthin (Dtx) and zeaxanthin (Zx) increased within 60 min of exposure. Changes occurred in pigments involved in xanthophyll cycles with significant increases in zeaxanthin/violaxanthin (Zx/Vx) and diatoxanthin/diadinoxanthin (Dtx/Ddx) ratios. It is noteworthy that among six minor pigments that were quantified but not identified, three of them decreased significantly in the diatom exposed to filtrate (data not shown).

3.4. Lipid class composition

After 30 min of filtrate exposure, there was no significant effect on the content of sterol esters, free sterols, triglycerides, alcohols and free fatty acids (FFA) (Table 2). After 60 min, the alcohol, sterol ester and free sterol content followed an increasing trend ($0.1 < p$ -value < 0.05) while triglyceride (storage lipids) content followed a decreasing trend ($0.1 < p$ -value < 0.05) in presence of filtrate. Phospholipids (phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylcholine (PC)) levels significantly decreased after 30 min (p -value < 0.01). After 60 min, the cellular PC remained lower in cells exposed to the filtrate than in control cells ($0.1 < p$ -value < 0.05). Monogalactosyl-diacylglycerol (MGDG) was the only glycolipid effected by the filtrate (decreased after 30 min exposure; p -value < 0.05), whereas sulfoquinovosyl-diacylglycerol (SQDG) and digalactosyl-diacylglycerol (DGDG) were unaffected.

3.5. Fatty acid composition

Polar fatty acid composition was differed greatly when *C. muelleri* were exposed to seawater or to the allelochemicals after 30 min (global R: 1, significance = 0.09, ANOSIM) and 60 min (global R: 0.7, significance = 0.09, ANOSIM), with changes in the proportion of different fatty acids. The composition of neutral fatty acids was also differed greatly between seawater and filtrate exposures after 30 min (global R: 0.7, significance = 0.12, ANOSIM) and to a smaller extent after 60 min (global R: 0.48, significance = 0.09, ANOSIM). Overall, the presence of allelochemicals induced a decrease in the unsaturation index of polar lipids (Table 3) and in neutral FA content (Table 4). Polar FA composition change was observed within 30 min of filtrate exposure to the filtrate, while it took 60 min of exposure to the filtrate to modify the neutral FA composition. There were 8 fatty acids primarily effected by the allelochemicals, which accounted for 80% of differences (Simpser analysis) with controls. Filtrate exposure decreased the proportions of 22:6n-3, 20:4n-6, 20:5n-3, 16:3n-4, whereas the proportions of 18:1n-7, 16:1n-7, 16:0 and 14:0 were increased in presence of *A. minutum* filtrate (Fig. 5).

4. Discussion

4.1. *Alexandrium minutum* allelochemicals are fast acting

This study demonstrated several significant effects (Fig. 6) of *A. minutum* allelochemicals on the metabolism (decrease in esterase activity and increase in ROS production), photosynthesis (decrease in maximum PSII quantum yield and increase of non-photochemical quenching) and membrane composition (changes in membrane lipids and pigments) in the diatom *C. muelleri* as compared to the controls. Overall, allelochemicals induced a decrease in metabolism within an hour, as highlighted by the inhibition of esterase activity and photosynthesis of the diatom *C. muelleri*, thus confirming that *A. minutum* allelochemicals are fast acting [10,14]. Moreover, this study highlights

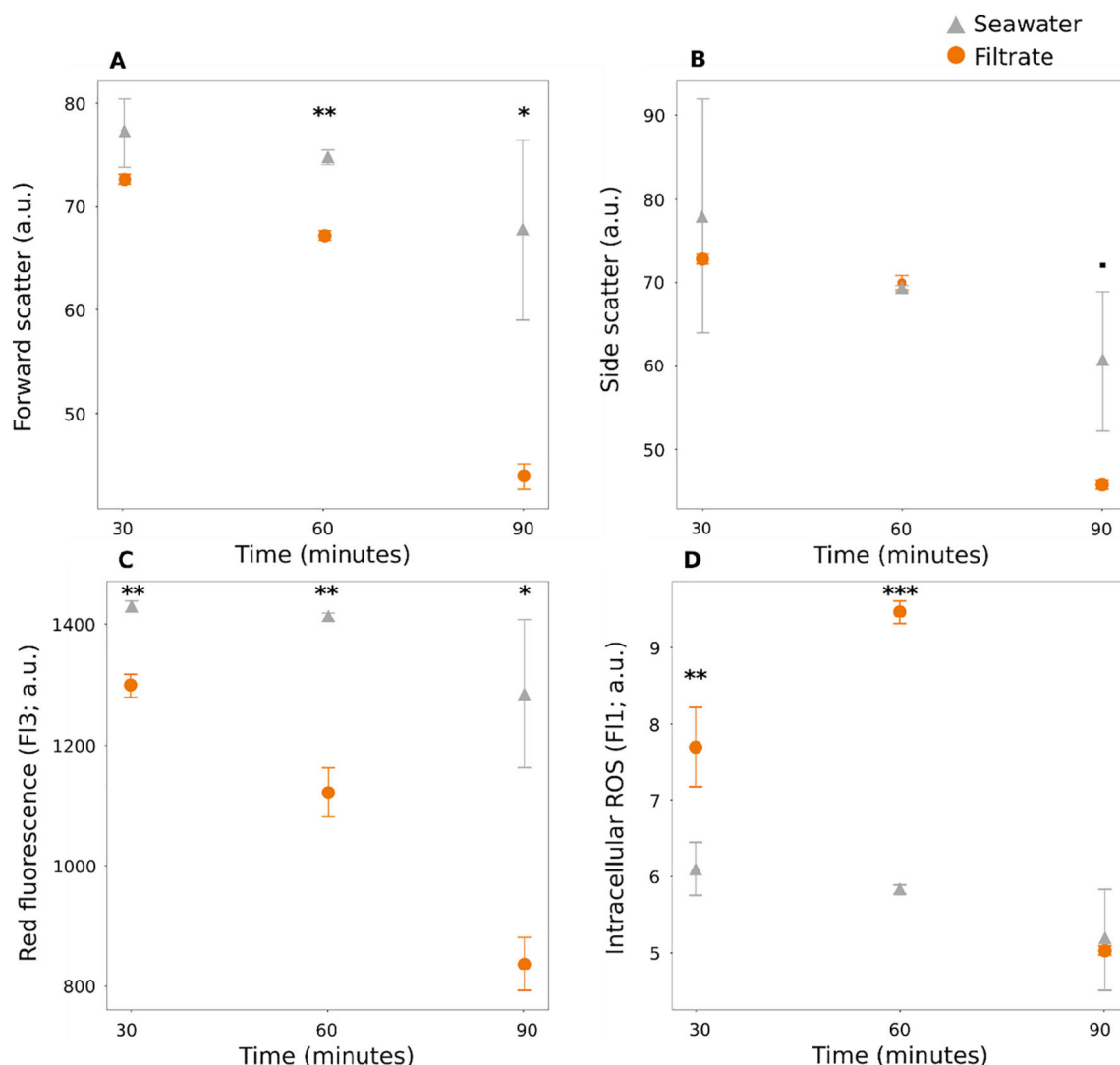


Fig. 3. Flow-cytometry parameters of *C. muelleri* for controls (seawater; grey triangles) and after exposure to *A. minutum* filtrate (orange dots) for 30, 60 and 90 min. A: Forward scatter (FSC), B: side scatter (SSC), C: F13: red fluorescence, D: reactive oxygen species (ROS) as measured by F11 fluorescence after DCFH-DA staining. Results are expressed as mean \pm standard deviation (n = 3). Statistical differences between seawater and filtrate at each different time are indicated as followed “.” 0.05 < p-value < 0.1, “*” 0.01 < p-value < 0.05, “**” 0.001 < p-value < 0.01, “***” p-value < 0.001. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

that photosynthesis was affected within a short time (< 30 min), as was the primary metabolism, as estimated by the esterase activity decrease after only 30 min. Indeed, 60 min was sufficient to almost completely cease esterase activity, down to 10% of the unexposed seawater control cells. This highlights the usefulness of analyzing cellular effects at a minute scale to elucidate the allelochemicals mode of action, which eventually lead to cell lysis. The combination of fluorescence, flow-cytometry and biochemical analysis of diatom physiology provided new insights on the short-term (within 30 min) cellular and biochemical mechanisms involved in allelochemical effects previously reported in *A. minutum* [10] and *A. tamarensis* [19,20].

4.2. Effects of allelochemicals on photosystem II

A decrease in the maximum photosystem II (PSII) quantum yield (F_v/F_m) of *C. muelleri* was observed within 20 min of exposure to *A. minutum* filtrate, highlighting rapid deleterious effects to the PSII and supporting previous studies [10,14]. [10] highlighted that the decrease of photosynthetic efficiency resulted from an inactivation of PSII reaction centers (RCs) and a decrease of electron transport through PSII. Inhibition of photosynthesis was also reported through a transcriptomic

analysis of the diatom *Phaeodactylum tricoratum* when co-cultured for 24 h with an allelochemical strain of *A. tamarensis* [20]. The authors hypothesized that the oxygen evolving complex (OEC) that catalyzes the oxidation of water to molecular oxygen was inhibited. This damage to PSII would result in an electron flux reduction through PSII and in the decreased quantum yield measured in our study. Effects of allelochemicals on the PSII of the diatom mimic the deleterious effects reported under excessive light intensity (i.e. photoinhibition). During photoinhibition, PSII are deactivated via two main steps: first OEC are damaged by light and deactivated, then PSII RCs are deactivated due to excess light absorption [39–41]. With less active RCs, the energy absorbed by chlorophyll pigments cannot be utilized for photochemistry (i.e. photosynthetic reactions) and accumulates, becoming an excess. Our results highlighted the deactivation of photosynthesis, however regardless of PSII inhibition, in presence of light, chlorophyll (Chl) pigments absorb photons and move to an excited state ($^1\text{Chl}^*$). Excess light absorption can induce the production of toxic $^3\text{Chl}^*$ that reacts with O_2 to produce the superoxide ion ($^{\cdot}\text{O}_2^-$), a highly reactive oxygen species (ROS) [42]. Similarly, during our study a transitory increase of ROS production was observed upon exposure to *A. minutum* allelochemicals. The excess energy can be “safely” dissipated through

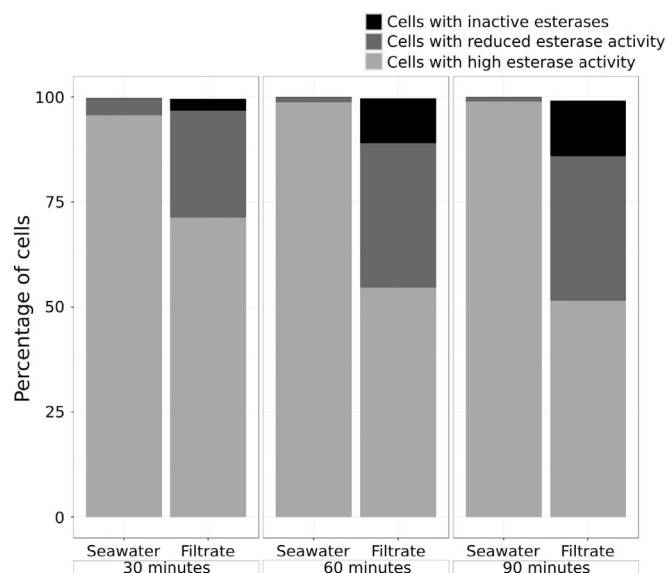


Fig. 4. Metabolic activity of *C. muelleri* following exposure to control filtered seawater (seawater) or *A. minutum* filtrate (filtrate) after 30, 60 and 90 min. Cells of *C. muelleri* are classified as cells with high esterase activity (light grey), reduced esterase activity (dark grey) and inactive esterase (black).

fluorescence emission or heat dissipation (non-photochemical quenching; NPQ) as observed in our study and described below.

4.3. Effects of allelochemicals on pigments

Significant decreases in chlorophyll *a* and chlorophyll *c2* content and red fluorescence (F13; 630 nm), in parallel with an increase in the chlorophyll (Chl) degradation product (allo-chlorophyll *a*) was observed after only 60 min of filtrate exposure (Fig. 6A and B). Bleaching (i.e. loss of cell pigmentation) of microalgal cells exposed to *Alexandrium* spp. filtrate has been previously reported [10,11], and this phenomenon is likely related to a decrease of Chl content as reported here. Induction of Chl degradation during allelochemical interactions has been recently shown [43,44] and is a common photoacclimation process [45–47]. We speculate that the decrease of Chl pigments results in a decrease in light harvesting antenna size. By decreasing the number of photons absorbed, the excess energy received by PSII would be decreased, mitigating photoinhibition and subsequent associated energy cost [48] as well as excessive ROS production and cell damages.

Table 1

Pigment composition expressed as peak area (peak area corresponding to 7×10^5 cells). Peak areas are expressed as mean \pm standard deviation ($n = 3$). Statistical differences between seawater and filtrate are indicated as followed “*” $0.05 < p\text{-value} < 0.1$, “**” $0.01 < p\text{-value} < 0.05$, “***” $0.001 < p\text{-value} < 0.01$, “****” $p\text{-value} < 0.001$.

	30 min exposure			60 min exposure		
	Seawater	Filtrate	p-Value	Seawater	Filtrate	p-Value
Chl <i>a</i>	2444 \pm 138	2399.4 \pm 102		2565 \pm 101	2296 \pm 27	*
Epi Chl <i>a</i>	76 \pm 27	70 \pm 9		58 \pm 9	48 \pm 3	
Allo Chl <i>a</i>	31 \pm 11	39 \pm 12		19 \pm 0	47 \pm 4	**
Chl <i>c1</i>	781 \pm 42	743 \pm 18		736 \pm 25	735 \pm 6	
Chl <i>c2</i>	834 \pm 54	799 \pm 53		893 \pm 37	747 \pm 10	*
Fucoxanthin	2781 \pm 71	2771 \pm 12		2801 \pm 105	2600 \pm 9	*
Diadinoxanthin	740 \pm 3	499 \pm 20	**	738.1 \pm 22.5	479.1 \pm 21.9	****
Diatoxanthin	94.6 \pm 15.6	326.0 \pm 22.3	***	102 \pm 12	263 \pm 11	***
Violaxanthin	12.5 \pm 0.5	13 \pm 1		14 \pm 1	13 \pm 1	
Zeaxanthin	20.4 \pm 0.9	33 \pm 1	***	21 \pm 1	28.8 \pm 0.9	***
Pheophytin <i>a</i>	6.7 \pm 0.6	7.0 \pm 0.4		7.2 \pm 0.2	6.8 \pm 0.1	*
β -Carotene	263 \pm 10	266 \pm 4		272 \pm 11	241 \pm 5	*

Chl *a*: chlorophyll *a*, Allo Chl *a*: allo chlorophyll *a*, Chl *c1*: chlorophyll *c1*, Chl *c2*: chlorophyll *c2*.

4.4. Effects of allelochemicals on non-photochemical quenching

Upon allelochemical exposure, the increase in non-photochemical quenching (NPQ) of *C. muelleri* is hypothesized to dissipate excess energy from damaged PSII through heat. Allelochemicals also induced a decrease in β -carotene and changes in xanthophyll pigments ratios (diadinoxanthin/diatoxanthin and violaxanthin/zeaxanthin; Fig. 6B) within photosynthetic membranes. Heat dissipation is mediated in thylakoid membranes by carotenoids and particularly xanthophyll pigment cycles [49]. The diadinoxanthin (Ddx) and violaxanthin (Vx) can be de-epoxidated in diatoxanthin (Dx) and zeaxanthin (Zx), respectively, to dissipate excess energy through heat emission (non-photochemical quenching; [49,50]). In the current study, activation of xanthophyll cycles, as demonstrated by increases in Dx/Ddx and Zx/Vx ratios, in the presence of allelochemicals was observed after both 30 and 60 min of filtrate exposure. While the increase in Dx was linked to a decrease in Ddx, Vx content did not change along with the increase in Zx content. De novo synthesis of Vx from β -carotene, a Vx precursor [51], may explain the homeostasis of Vx.

4.5. Effects of allelochemicals on lipids

The lipid composition of photosynthetic membranes, which physicochemical properties greatly influence NPQ and indeed diadinoxanthin (Ddx) and violaxanthin (Vx) de-epoxidation reactions [52]. These lipids were significantly affected by the presence of allelochemicals (Fig. 6A and B). Monogalactosyldiacylglycerol (MGDG), a non-bilayer glycolipids exclusively found in thylakoid membranes, decreased in the presence of allelochemicals. Previous studies highlighted that MGDG allows xanthophyll solubilization and promotes de-epoxidation [53–55]. In parallel, a decrease in phosphatidylcholine (PC) content, a significant constituent of thylakoid membranes [56–58], was also observed in the presence of allelochemicals. Similar decreases of glycolipids along with a decrease in PC were observed during the photoinhibition process [54,58]. The modifications in the biochemical composition of photosynthetic membranes are another change similar to photoinhibition processes.

Changes in lipid class composition occurred alongside significant modifications in the fatty acid (FA) composition of both polar and neutral lipids. Filtrate of *A. minutum* reduced the cellular content of neutral fatty acids, mainly bound to storage lipids (i.e. triglyceride that represents > 90% of neutral lipids), by > 20% of its mass. This decrease in neutral FAs may be linked to the decrease in triglyceride and result from an energy mobilization to cope with the energy cost induced by allelochemicals such as lipid synthesis and de-epoxidation reactions,

Table 2

Total (polar plus neutral) lipid classes composition (pg cell⁻¹) of *C. muelleri* exposed to seawater (control) or to *A. minutum* filtrate (Filtrate). Results are expressed as mean ± standard deviation. N = 3. Statistical differences between seawater and filtrate are indicated as followed “*” 0.05 < p-value < 0.1, “**” 0.01 < p-value < 0.05, “***” 0.001 < p-value < 0.01.

	30 min exposure			60 min exposure		
	Seawater	Filtrate	p-Value	Seawater	Filtrate	p-Value
Sterol ester	0.08 ± 0.01	0.08 ± 0.02		0.08 ± 0.01	0.10 ± 0.01	•
Trigly.	10 ± 1	11.1 ± 0.8		12 ± 1	11.1 ± 0.8	•
FFA	0.29 ± 0.08	0.26 ± 0.04		0.4 ± 0.2	0.4 ± 0.2	
Alcohols	0.08 ± 0.01	0.08 ± 0.02		0.08 ± 0.01	0.10 ± 0.01	•
Free sterols	0.26 ± 0.02	0.31 ± 0.03		0.29 ± 0.01	0.40 ± 0.06	•
PI	0.03 ± 0.00	0.05 ± 0.01	•	0.06 ± 0.02	0.05 ± 0.03	
PE	0.43 ± 0.06	0.38 ± 0.03		0.43 ± 0.03	0.41 ± 0.01	
PC	1.12 ± 0.02	0.61 ± 0.06	**	0.80 ± 0.09	0.6 ± 0.1	•
MGDG	2.2 ± 0.3	1.7 ± 0.1	*	2.0 ± 0.2	1.65 ± 0.09	
SQDG	1.2 ± 0.3	1.23 ± 0.08		1.38 ± 0.08	1.18 ± 0.09	
DGDG	0.25 ± 0.06	0.25 ± 0.02		0.28 ± 0.01	0.26 ± 0.02	
Σlipids	16 ± 2	16 ± 1		17.9 ± 0.7	16.2 ± 0.9	

Trigly.: triglyceride, FFA: free fatty acids, PI: phosphatidylinositol, PE: phosphatidylethanolamine, PC: phosphatidylcholine, MGDG: monogalactosyl-diacylglycerol, SQDG, sulfoquinovosyl-diacylglycerol, DGDG: digalactosyl-diacylglycerol.

or from the mobilization of FAs for the synthesis of membrane FAs [59]. Exposure to *A. minutum* allelochemicals also reduced the unsaturation levels of polar lipid fatty acids which are the constituents of biological membranes. The decrease of membrane lipid unsaturation mainly results from the decreases of eicosapentaenoic acid (EPA) 20:5n-3 and 16:3n-4, accounting for 38% of total polar lipid FAs. This decrease in polyunsaturated fatty acids (PUFA) paralleled the decrease in PC and

MGDG, mainly constituted in 20:5n-3 and 16:3n-4, respectively [60–62]. In the diatom *Phaeodactylum tricorutum*, 20:4n-6 was only found associated to MGDG and PC and to a smaller extent to DGDG [63]. Its decrease also occurred in parallel with the general decrease in PC and MGDG observed in the presence of allelochemicals. Concomitantly, proportions of other major FAs (14:0, 16:0, 16:1n-7, 18:1n-7) increased.

Table 3

Polar fatty acid (FA) composition (mass % of total polar fatty acids). The total mass (μg) corresponds to the mass of 10⁶ cells. Results are expressed as mean ± standard deviation (n = 3). p-Value from the statistical tests between seawater and filtrate at each different time are indicated as followed “•” 0.05 < p-value < 0.1, “*” 0.01 < p-value < 0.05, “**” 0.001 < p-value < 0.01, “***” p-value < 0.001.

	30 min exposure			60 min exposure		
	Seawater	Filtrate	p-Value	Seawater	Filtrate	p-Value
iso15:0	0.44 ± 0.02	0.68 ± 0.02	***	0.5 ± 0.1	0.75 ± 0.05	*
ant15:0	0.21 ± 0.02	0.26 ± 0.01	•	0.25 ± 0.07	0.31 ± 0.03	
iso17:0	0.64 ± 0.01	0.73 ± 0.00	**	0.75 ± 0.05	0.77 ± 0.05	
14:0	8.3 ± 0.3	8.8 ± 0.2	•	8.6 ± 0.8	9.0 ± 0.2	
15:0	0.58 ± 0.01	0.62 ± 0.01	**	0.61 ± 0.02	0.63 ± 0.03	
16:0	10.2 ± 0.1	11.1 ± 0.1	***	10.8 ± 0.2	11.02 ± 0.03	
18:0	1.22 ± 0.01	1.30 ± 0.01	**	1.5 ± 0.3	1.5 ± 0.2	
22:0	0.21 ± 0.01	0.23 ± 0.03		0.20 ± 0.06	0.21 ± 0.01	
24:0	0.2 ± 0.0	0.2 ± 0.2		0.17 ± 0.00	0.16 ± 0.01	•
ΣSFA	22.1 ± 0.3	24.1 ± 0.2	**	23.6 ± 0.6	24.5 ± 0.2	
16:1n-7	14.00 ± 0.2	14.7 ± 0.2	*	14.2 ± 0.6	14.4 ± 0.5	
16:1n-5	0.21 ± 0.01	0.22 ± 0.00	•	0.22 ± 0.00	0.23 ± 0.01	
18:1n-9	2.0 ± 0.1	1.93 ± 0.04		2.12 ± 0.05	1.90 ± 0.08	*
18:1n-7	2.98 ± 0.02	3.7 ± 0.1	*	3.2 ± 0.2	4.1 ± 0.3	**
18:1n-5	0.28 ± 0.03	0.34 ± 0.04	•	0.28 ± 0.05	0.37 ± 0.03	*
Σ MUFA	19.7 ± 0.2	21.24 ± 0.04	**	20.2 ± 0.7	21.3 ± 0.8	
16:2n-7	1.64 ± 0.02	1.73 ± 0.05	•	1.77 ± 0.03	1.78 ± 0.08	
16:2n-4	2.09 ± 0.04	2.15 ± 0.03		2.10 ± 0.05	2.2 ± 0.1	
16:3n-4	8.9 ± 0.2	8.4 ± 0.2	•	8.9 ± 0.2	8.9 ± 0.2	
16:4n-1	0.31 ± 0.01	0.27 ± 0.01	**	0.30 ± 0.01	0.30 ± 0.01	
18:2n-7	0.16 ± 0.00	0.18 ± 0.00	**	0.18 ± 0.00	0.18 ± 0.01	
18:2n-6	1.98 ± 0.02	1.91 ± 0.05		2.05 ± 0.03	1.79 ± 0.11	*
18:3n-6	6.14 ± 0.04	6.09 ± 0.16		6.3 ± 0.1	5.7 ± 0.4	•
18:4n-3	2.90 ± 0.00	2.78 ± 0.04	•	2.88 ± 0.08	2.74 ± 0.06	•
20:3n-6	0.22 ± 0.01	0.17 ± 0.00	***	0.22 ± 0.02	0.16 ± 0.01	*
20:4n-6	9.2 ± 0.2	8.2 ± 0.2	**	8.5 ± 0.1	7.5 ± 0.5	•
20:4n-3	0.18 ± 0.00	0.16 ± 0.01	*	0.15 ± 0.02	0.16 ± 0.00	
20:5n-3	20.2 ± 0.1	17.9 ± 0.2	***	18.0 ± 0.3	17.7 ± 0.1	
22:5n-6	0.18 ± 0.01	0.20 ± 0.00	*	0.20 ± 0.02	0.17 ± 0.03	
22:5n-3	0.14 ± 0.00	0.12 ± 0.00	**	0.14 ± 0.01	0.12 ± 0.00	
22:6n-3	3.06 ± 0.04	2.71 ± 0.12	*	2.9 ± 0.2	2.4 ± 0.2	*
ΣPUFA	57.5 ± 0.3	53.2 ± 0.4	***	54.8 ± 0.6	52.8 ± 0.4	*
Unsaturation index	230 ± 1	210 ± 2	***	216 ± 3	206 ± 2	*
Mass (μg)	490 ± 10	410 ± 50	.	446 ± 3	426 ± 86	

SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids.

Table 4

Neutral fatty acid (FA) composition (mass % of total neutral fatty acids). The total mass (μg) corresponds to the mass of 10^6 cells. Results are expressed as mean \pm standard deviation ($n = 3$). p-Value from the statistical tests (Student *t*-test or Wilcoxon test) between the conditions at each different time are indicated as followed “•” $0.05 < p\text{-value} < 0.1$, “*” $0.01 < p\text{-value} < 0.05$, “**” $0.001 < p\text{-value} < 0.01$.

	30 min exposure			60 min exposure		
	Seawater	Filtrate	p-Value	Seawater	Filtrate	p-Value
iso15:0	0.17 \pm 0.02	0.17 \pm 0.02		0.14 \pm 0.04	0.21 \pm 0.02	•
14:0	12.6 \pm 0.2	12.6 \pm 0.1		12.8 \pm 0.2	12.8 \pm 0.1	
15:0	0.98 \pm 0.01	0.98 \pm 0.00		0.97 \pm 0.01	1.0 \pm 0.1	
16:0	18.8 \pm 0.2	18.5 \pm 0.1		18.6 \pm 0.2	18.3 \pm 0.2	
18:0	1.69 \pm 0.06	1.67 \pm 0.06		1.68 \pm 0.03	1.65 \pm 0.04	
20:0	0.12 \pm 0.01	0.11 \pm 0.00		0.11 \pm 0.00	0.11 \pm 0.00	*
22:0	0.20 \pm 0.00	0.19 \pm 0.01		0.19 \pm 0.01	0.17 \pm 0.00	
24:0	0.21 \pm 0.02	0.19 \pm 0.01		0.21 \pm 0.01	0.19 \pm 0.02	
ESFA	35.0 \pm 0.1	34.65 \pm 0.09	*	34.9 \pm 0.1	34.6 \pm 0.3	
15:1n-5	0.19 \pm 0.02	0.20 \pm 0.02		0.16 \pm 0.01	0.22 \pm 0.00	**
16:1n-7	35.4 \pm 0.2	35.01 \pm 0.03	•	35.06 \pm 0.05	34.82 \pm 0.40	
16:1n-5	0.34 \pm 0.00	0.35 \pm 0.00		0.33 \pm 0.00	0.34 \pm 0.00	•
18:1n-9	0.81 \pm 0.02	0.81 \pm 0.01		0.79 \pm 0.01	0.79 \pm 0.01	
18:1n-7	0.36 \pm 0.01	0.37 \pm 0.01		0.35 \pm 0.00	0.36 \pm 0.00	
Σ MUFA	37.3 \pm 0.2	36.89 \pm 0.02	•	36.85 \pm 0.08	36.7 \pm 0.4	
16:2n-7	1.43 \pm 0.02	1.46 \pm 0.01		1.42 \pm 0.02	1.47 \pm 0.01	*
16:2n-4	0.92 \pm 0.01	1.00 \pm 0.02	**	0.95 \pm 0.00	0.7 \pm 0.6	
16:3n-4	1.14 \pm 0.03	1.6 \pm 0.1	*	1.29 \pm 0.02	1.6 \pm 0.1	*
16:3n-1	0.40 \pm 0.03	0.42 \pm 0.04		0.39 \pm 0.02	0.48 \pm 0.01	**
18:2n-6	0.87 \pm 0.02	0.86 \pm 0.00		0.86 \pm 0.00	0.8 \pm 0.1	
18:3n-6	3.17 \pm 0.04	3.21 \pm 0.01		3.12 \pm 0.01	3.11 \pm 0.05	
18:4n-3	2.02 \pm 0.02	2.03 \pm 0.02		1.99 \pm 0.02	2.08 \pm 0.02	**
20:3n-6	0.39 \pm 0.01	0.38 \pm 0.01		0.38 \pm 0.00	0.37 \pm 0.02	*
20:4n-6	4.86 \pm 0.09	4.83 \pm 0.09		5.03 \pm 0.04	4.77 \pm 0.09	*
20:4n-3	0.31 \pm 0.01	0.30 \pm 0.01		0.30 \pm 0.01	0.30 \pm 0.00	
20:5n-3	9.5 \pm 0.2	9.61 \pm 0.05		9.78 \pm 0.04	9.7 \pm 0.3	
22:5n-6	0.02 \pm 0.00	0.02 \pm 0.00		0.02 \pm 0.00	0.02 \pm 0.00	
22:5n-3	0.13 \pm 0.01	0.12 \pm 0.01		0.13 \pm 0.00	0.12 \pm 0.00	*
22:6n-3	0.58 \pm 0.02	0.55 \pm 0.02		0.56 \pm 0.02	0.55 \pm 0.01	
Σ PUFA	21.9 \pm 0.3	21.9 \pm 0.1		22.25 \pm 0.03	22.0 \pm 0.4	
Unsaturation index	107 \pm 1	109.3 \pm 0.2	*	109.6 \pm 0.2	107 \pm 2	
Mass (μg)	1121 \pm 111	899 \pm 47	•	1111 \pm 142	746 \pm 171	*

SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids.

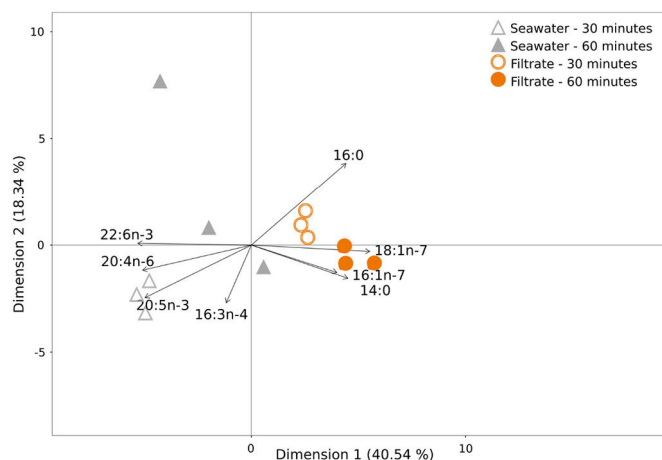


Fig. 5. Principal component analysis (PCA) of polar fatty acid (FA) composition between *C. muelleri* cultures in presence of filtered seawater (grey triangles) or *A. minutum* filtrate (orange dots). Grey triangles represent samples exposed to filtered seawater after 30 min (empty triangles) and 60 min (filled triangles) of exposure. Orange dots represent samples exposed to *A. minutum* filtrate after 30 min (empty dots) and 60 min (filled dots). Dimension 1 represents 40.54% of variance and dimension 2 represents 18.34% of variance. FAs shown explain 80% of differences (Simpser analysis). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Sterols are predominant constituents of plasma membranes [64,65] with many important roles including the regulation of membrane fluidity and permeability [66], signal transduction, modulation of enzyme activity and precursors of secondary metabolites [65]. In microalgae, sterol can increase under increasing light [67,68], indeed sterols may have a role under photoinhibition. The slight increase of sterols in the presence of filtrate, as observed under photoinhibition-like conditions, may confirm such a hypothesis. At the same time, some sterols classes are known to potentially act against antimicrobial compounds with membrane-disruptive activity [66,69]. Authors have hypothesized that *A. tamarensis* allelochemicals may be membrane-disruptive lytic compounds with a high affinity for sterols and that the molecular targets involve sterol components [19]. In the same study, the authors showed that allelochemicals had different affinities to different phytosterols and speculated that the composition of the outermost cellular membrane plays a role in tolerance to allelochemicals. Yet, in our study, sterols increased in presence of allelochemicals. While this may reflect a protective role of sterols, the true role of sterols in allelochemical interactions should be addressed in future studies as it could be a membrane feature driving allelochemical tolerance.

4.6. Effects of allelochemicals on ROS

Exposure of the diatom to *A. minutum* filtrate resulted in a relative increase in intracellular reactive oxygen species (ROS) during the first hour. Photosynthesis is one of the processes inducing ROS production that can damage photosystem II [41,42,70]. However, it is unlikely that the diatoms' photosystem II is the only source of ROS as a ROS increase was also reported in non-photosynthetic cells exposed to *A. minutum*

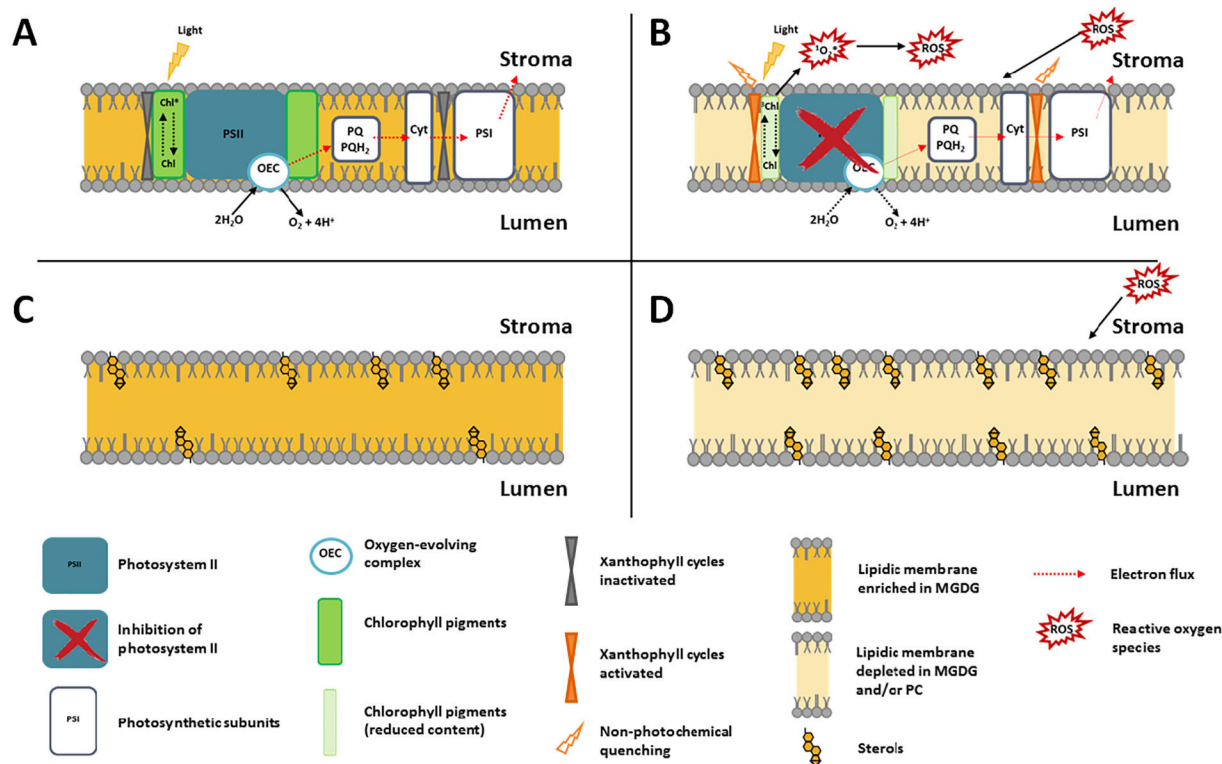


Fig. 6. Schematic depicting the mechanisms of *A. minutum* allelochemicals on photosynthetic (A and B) and plasma (C and D) membranes of *C. muelleri* in presence of seawater (A and C) and allelochemicals (B and D). Mechanisms are described in the last “general view” paragraph of discussion. PSII: photosystem II, PSI: photosystem I, PQ: plastoquinone, Cyt, cytochrome, OEC: oxygen-evolving complex, Chl: chlorophyll, PC: phosphatidylcholine, MGDG: monogalactosyl-diacylglycerol, ROS: reactive oxygen species.

exudates [16,71]. The two-fold ROS increase in *C. muelleri* exposed to allelochemicals may have led to some lipid peroxidation (yet to be measured). Lipid peroxides can act as a signal inducing programmed cell death [43] and cell cycle blockage [72] which was shown in *Phaeodactylum tricornutum* co-cultured with *A. tamarense* [20], where ROS production returned to levels similar to control conditions after 90 min. While this decrease may be linked to the decrease in esterase activity, it is also hypothesized that diatoms may cope with ROS stress through activation of antioxidant systems [43,73] such as xanthophyll pigments [54,58,74], or through saturation of membranes. Xanthophyll pigments not only play a role in dissipation of energy, as mentioned above, but can also act as antioxidants, especially against ROS produced at PSII oxygen evolving complex [54]. Indeed, xanthophyll cycle may scavenge intracellular ROS induced by the presence of allelochemicals.

4.7. General view

Based on our results and previous studies [10,19,20] we propose a conceptual model to describe the mechanisms of action of *Alexandrium* allelochemicals (Fig. 6). Alteration of physiological functions such as photosynthesis and primary metabolism may result from direct damage to external membranes. Such damage may lead to the loss of cell integrity as shown by [19]. Here we propose that the membrane disruption observed by [19,20] leads to an inhibition of photosynthesis. Membrane disruption was previously reported to lead to inhibition of *Fv/Fm* and electron transport [43]. Following the inactivation of photosystem II, the energy trapped by chlorophyll pigments cannot be used for photochemistry. Thus photons become excessive within the cells and may induce an increase in ROS production by the cell. Photo-inhibition-like processes appeared to be activated by the cells to mitigate the stress. The observed reduction of chlorophyll pigments is hypothesized to limit the energy trapped by the cell, together with the xanthophyll cycles to eliminate excess energy through heat (non-

photochemical quenching) and to scavenge ROS. These changes of pigment content and ratio are accompanied by a decrease in MGDG and saturation of photosynthetic membranes. In parallel, plasma membranes were also modified as demonstrated by the increase in free sterols. While the role of sterols has yet to be investigated, they may help the cell to maintain membrane integrity and associated enzyme activity or act as protection mechanisms against membrane disruption. ROS increase is likely to be a key factor in understanding allelochemical interactions. Their origin, effects and role remain unclear, and the mechanisms involving ROS should be further studied. The main pending issues are to understand how the allelochemicals interact with cell membranes, and how it is related to the sensitivity of some species to better understand their ecological implications. Overall, the mode of action of allelochemicals has to be further investigated from the minute scale effects to fully understand the mechanisms leading to microalgal cell death.

5. Conclusion

In conclusion, our results bring new insights on allelochemical interaction mechanisms and indicate that several biochemical pathways and structures were rapidly impaired resulting in photoinhibition-like responses. Even though allelopathy does not automatically lead to cell death, sub-lethal effects, such as photoinhibition, ROS production, decreased metabolism or decreased energy absorption, would have a significant energy cost. For example, the cost of photoinhibition can lead to a decreased growth rate. Overall the allelochemical interactions would also impair the ability of the exposed cells to cope with other environmental stress, thus decreasing competitiveness and fitness. The sub-lethal markers reported here are valuable tools in assessing the importance and ecological roles of allelopathy in structuring plankton communities.

Author contribution

M.L., K.T., P.S., F.L.G., and H.H. designed the study. M.L., K.T., C.L., F.L.G., P.S., H.H., and A.D. performed the experimental analysis. M.L. coordinated and wrote the manuscript. G.S. and D.F.J. contributed to the text of the paper. D.F.J., H.H. and G.S. obtained the fundings. All authors discussed the results and commented on the manuscript.

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A rapid quantitative fluorescence-based bioassay to study allelochemical interactions from *Alexandrium minutum*[☆]

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ABSTRACT

Harmful microalgal blooms are a threat to aquatic organisms, ecosystems and human health. Toxic dinoflagellates of the genus *Alexandrium* are known to produce paralytic shellfish toxins and to release bioactive extracellular compounds (BECs) with potent cytotoxic, hemolytic, ichthyotoxic and allelopathic activity. Negative allelochemical interactions refer to the chemicals that are released by the genus *Alexandrium* and that induce adverse effects on the physiology of co-occurring protists and predators. Releasing BECs gives the donor a competitive advantage that may help to form dense toxic blooms of phytoplankton. However BECs released by *Alexandrium minutum* are uncharacterized and it is impossible to quantify them using classical chemical methods. Allelochemical interactions are usually quantified through population growth inhibition or lytic-activity based bioassays using a secondary target organism. However these bioassays require time (for growth or microalgal counts) and/or are based on lethal effects. The use of pulse amplitude modulation (PAM) fluorometry has been widely used to assess the impact of environmental stressors on phytoplankton but rarely for allelochemical interactions. Here we evaluated the use of PAM and propose a rapid chlorophyll fluorescence based bioassay to quantify allelochemical BECs released from *Alexandrium minutum*. We used the ubiquitous diatom *Chaetoceros muelleri* as a target species. The bioassay, based on sub-lethal effects, quantifies allelochemical activity from different samples (filtrates, extracts in seawater) within a short period of time (2 h). This rapid bioassay will help investigate the role of allelochemical interactions in *Alexandrium* bloom establishment. It will also further our understanding of the potential relationship between allelochemical activities and other cytotoxic activities from BECs. While this bioassay was developed for the species *A. minutum*, it may be applicable to other species producing allelochemicals and may provide further insights into the role and impact of allelochemical interactions in forming dense algal blooms and structuring marine ecosystems.

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1. Introduction

Proliferation of microalgae is recognized as a major threat for marine ecosystems. Harmful algal blooms can be responsible for toxic impacts on marine organisms and ecosystems and can also be

toxic to human. This involves public health risks (e.g. intoxications) and socio-economics issues (e.g. loss of fisheries stock, fisheries closure). Dinoflagellates from the genus *Alexandrium* have the potential to produce paralytic shellfish toxins but also to release uncharacterized bioactive extracellular compounds (BECs) exhibiting allelochemical (Arzul et al., 1999; Lelong et al., 2011; Tillmann et al., 2007), cytotoxic (Le Goïc et al., 2014), hemolytic (Arzul et al., 1999; Emura et al., 2004), and ichthyotoxic activities (Borcier et al., 2017; Castrec et al., 2018; Mardones et al., 2015). BECs from the genus *Alexandrium* affect a large range of marine protists including autotrophic and heterotrophic organisms (Fistarol et al., 2004;

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Tillmann et al., 2008, 2007; Tillmann and Hansen, 2009) by inducing cell lysis within hours.

Negative allelochemical interactions can be defined as the chemical release of compounds by a protists that causes adverse effects on the physiology of other competing protists and predators, thus giving the allelopathic species a competitive advantage. These negative interactions allow protists to outcompete for resources (e.g. nutrient, light), induce prey immobilization, defend against grazers and therefore are a key factor structuring plankton ecosystems (Legrand et al., 2003). Allelochemical interactions are mediated by the production of BECs, named as allelochemicals, released into the surrounding environment. BECs are poorly described (e.g. chemical nature, mode of action) because of methodological and analytical difficulties, but are mainly characterized by their activity spectra and effects on target cells: growth inhibition, death, lysis, paralysis, inhibition of photosystem II etc. (Legrand et al., 2003). Short-term (minutes to hour) deleterious effects were observed on the photosystem II of several phytoplankton species exposed to the genus *Alexandrium* (Tillmann et al., 2007) including the globally distributed diatom *Chaetoceros muelleri* (Lelong et al., 2011, formerly named as *Chaetoceros neogracile*).

No standard and rapid methodology is available for the study of allelochemical interactions in marine environments (Legrand et al., 2003). Development of bioassays would benefit the allelochemical interactions research field by enabling the rapid confirmation and quantification of allelochemical interactions. This would help to assess the role and relevance of these interactions in plankton ecology. It would also greatly ease the isolation and characterization of unknown BECs through bio-guided purification. Population growth inhibition (Chan et al., 1980; Paul et al., 2009; Pushparaj et al., 1998) or enumeration of lysed cells (Hakanen et al., 2014; Ma et al., 2009; Tillmann et al., 2007) are the most common parameters used in bioassays to demonstrate allelochemical interactions but such techniques are time-consuming. According to the OECD (2011) guidelines for testing chemicals, a growth inhibition test should be long enough to obtain a 16-fold population growth in control treatments (from few days to weeks depending on the target species growth rate). Moreover, microscopic observations cannot be performed in every field situation and distinction between viable and non-viable cells can be subjective because of a lack of consensus on a microalgal death definition (Garvey et al., 2007). This is compounded by the fact that the enumeration of lysed cells is based on lethal effects, although allelochemical interactions in marine environments may not automatically imply cell lysis. Therefore, new rapid, quantitative and sensitive methods are worth exploring to study allelochemical interactions.

Pulse amplitude modulatory (PAM) fluorometry is a method based on chlorophyll *a* fluorescence that allows studying photosynthetic processes. PAM is frequently used to assess the impact of environmental stressors (Barranguet et al., 2002; Juneau et al., 2003; Lippemeier et al., 1999; Miao et al., 2005), pollutants (see review from Ralph et al., 2007) or algicidal compounds (Yang et al., 2017) on phytoplankton physiology, as it provides insights into phytoplankton “health”. Maximum photosystem II quantum yield (Fv/Fm) is the optimal photosynthetic efficiency and is one of the most popular and easiest parameter to interpret. PAM has the advantage of being a rapid non-invasive and non-destructive method that can detect sub-lethal effects on photosystems. Some devices can be portable, allowing rapid measurements useful in many situations. However, only few studies report the measurement of chlorophyll *a* fluorescence to demonstrate allelochemical interactions (Blossom et al., 2014a; Borcier et al., 2017; Tillmann et al., 2007). The lack of homogeneity in methods and summary parameters (e.g. single concentration measurement, EC10, EC50: the effect concentrations inhibiting 10 or 50% of a physiologic

parameter) prevent comparisons between studies, highlighting the need of standardized bioassays.

The aim for this study was to assess the use of pulse amplitude modulation (PAM) fluorometry in detecting allelochemical activity from *A. minutum* and to establish a rapid and reliable fluorescence-based bioassay to quantify allelochemical activity from *A. minutum* samples. The genus *Chaetoceros* was selected as a target species based on its known sensitivity to *Alexandrium* spp. BECs (Arzul et al., 1999; Lelong et al., 2011; Weissbach et al., 2010), its ubiquity in phytoplankton communities (Dalsgaard et al., 2003) and its co-occurrence with *A. minutum* blooms (Chapelle et al., 2014; Klein et al., in prep). Moreover the genus *Chaetoceros* is a microalgal model commonly used in ecotoxicological studies (Desai et al., 2006; Hii et al., 2009; Hourmant et al., 2009). The bioassay was conceived to be applied to microalgal cultures, extracts or field samples.

2. Materials and methods

2.1. Algal culture

Three strains of the toxic dinoflagellate *Alexandrium minutum* were selected based upon their different allelochemical activity. The strains AM89BM (isolated from a bloom in the Bay of Morlaix, France) and CCMI1002 (isolated from a bloom in Gearhies, Bantry Bay, Ireland; Borcier et al., 2017) were chosen according to their cytotoxic potency (Arzul et al., 1999; Borcier et al., 2017; Lelong et al., 2011). The strain Da1257 (isolated from a bloom in the Bay of Daoulas, France; Pousse et al., 2017) was selected according to its low allelochemical potency. The cultures were grown in natural filtered (0.2 µm) seawater (collected in Argenton, France) supplemented with L1 medium (Guillard and Hargraves, 1993) and maintained in exponential growth phase. The diatom *Chaetoceros muelleri* (strain CCAP 1010-3, formerly named *Chaetoceros neogracile* or *Chaetoceros* sp.) was cultured in filtered (0.2 µm) artificial seawater (synthetic ocean water: Morel et al., 1979; Price et al., 1989) supplemented with L1 medium (Guillard and Hargraves, 1993) and silica (1.06×10^{-4} M final concentration). Both media were autoclaved. Microalgal cultures were maintained at 17 ± 1 °C under continuous light ($100\text{--}110$ µmol photons $\text{m}^{-2} \text{s}^{-1}$).

For each experiment, cultures of *C. muelleri* in exponential growth phase were used. Diatoms were diluted with filtered artificial seawater to achieve 2×10^5 cells mL^{-1} (Lelong et al., 2011) in the experiments and bioassays. Microalgal cells were counted and algal growth was monitored using a FACSCalibur flow cytometer (Becton Dickinson, San Diego, CA, USA) equipped with a blue laser (excitation 488 nm). Concentrations (cells mL^{-1}) were calculated from the number of events per unit of time and the estimate of the FACSCalibur flow rate measured according to Marie et al. (1999).

2.2. BECs extraction

BECs were separated from culture by filtration (0.2 µm, acetate cellulose membrane, 16534-K, Sartorius) according to Lelong et al. (2011). Filtrates were prepared fresh in glass tubes to avoid any loss to plastic during storage (Ma et al., 2009). The initial *A. minutum* culture concentration, prior to filtration, was used to describe the potential allelochemical concentration of each filtrate. Serial dilutions of this filtrate with filtered natural seawater were performed to obtain various concentrations. In the following text, the theoretical allelochemical concentration of each filtrate is given by the concentration in *A. minutum* (cell mL^{-1}) of the culture used to obtain the filtrate.

2.3. Pulse amplitude modulatory (PAM) fluorometry

Maximum photosystem II (PSII) quantum yield (Fv/Fm), which is a proxy of PSII photochemical efficiency, was measured by pulse amplitude modulation (PAM) fluorometry with a handheld AquaPen-C AP-C 100 (Photon Systems Instruments, Drassov, Czech Republic) equipped with a blue light (455 nm). In each experiment, algal samples were dark-adapted for at least 20 min before measurement of fluorescence variables (F0, Fm). F0 is the initial fluorescence intensity, Fm the maximal intensity under saturating light conditions (0.5–1 s at 1500 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$), and $F_v = F_m - F_0$. Maximum quantum yield of photosystem II was then calculated as followed: F_v/F_m (Strasser et al., 2000).

2.4. Optimization of protocol

2.4.1. Sensitivity of Fv/Fm to filtrate

In a first experiment, the kinetic impact of BECs upon the diatom's photosystem was studied. Cells of *C. muelleri* (2×10^5 cells mL^{-1}) were exposed to *A. minutum* (strain CCMI1002) filtrate dilutions in filtered natural seawater, with final filtrate concentrations equivalent to 0, 50, 500, 5000 and 50 000 cells mL^{-1} . Fv/Fm was then measured every 30 min for the first 120 min of exposure then every 60 min until 360 min of exposure. Results are expressed as the percentage of Fv/Fm inhibition compared to seawater control (see section 2.6).

2.4.2. Fv/Fm and FDA inhibition assay

The second experiment was performed to confirm that Fv/Fm is a good proxy of microalgal metabolism in the bioassay. Fv/Fm was compared to another proxy of primary metabolism, the fluorescein diacetate-activity assay (Brookes et al., 2000; Franklin et al., 2001; Garvey et al., 2007). Fluorescein diacetate (FDA, Molecular probes, Invitrogen, Eugene, OR, USA) is a hydrophobic fluorescent dye that permeates and stains cells exhibiting esterase activity, i.e. viable cells. Inside the cells, FDA is cleaved by esterases and releases a fluorescent by-product (fluorescein; emission 525 nm) which is retained within the cells. Cells that are not stained in the presence of FDA do not exhibit esterase activity and are considered as metabolically inactive (Supplementary figure 1). FDA fluorescence in the diatoms was measured by flow cytometry (FACSCalibur) equipped with a blue laser (excitation 488 nm) through the detector of fluorescence FL1 (green emission filter band pass, 530/30 nm). FDA staining protocol was optimized (Supplementary figures 2 and 3) for *C. muelleri* and samples were stained (final concentration = 1.5 μM) for 10 min in dark prior to flow-cytometric measurement, while samples for Fv/Fm measurement were put in the dark 20 min before measurement. *C. muelleri* (2×10^5 cells mL^{-1}) was exposed to *A. minutum* exudate filtrate (strain CCMI1002) at concentrations (theoretical concentrations) equivalent to 500, 5000 and 50 000 cells mL^{-1} . Fv/Fm and FDA fluorescence were measured after 120 min of exposure.

2.5. Bioassay procedure: comparison of *A. minutum* strains

2.5.1. Algal culturing

To compare allelochemical activity from three different *A. minutum* strains, cultures in exponential growth phase were centrifuged (280 g, 5 min, 17 °C) and re-suspended in new L1 medium to reach final cell concentrations of 3×10^4 cells mL^{-1} . These cultures were left to equilibrate (release BECs) for 24 h prior to the commencement of bioassays.

2.5.2. Exposure

Triplicate cultures of *C. muelleri* in exponential growth phase (7

days old) were diluted with filtered (0.2 μm) synthetic ocean water to reach 2×10^5 cells mL^{-1} in the bioassay. Diatoms were then exposed to dilutions of *A. minutum* culture filtrate (concentration 3×10^4 cells mL^{-1}) dilutions (in artificial seawater) in flow cytometry tubes (3 mL final volume), with filtrate concentrations equivalent to 0, 50, 100, 300, 500, 1000, 2500, 5000, 10 000, 15 000 and 25 000 cells mL^{-1} . Bioassay tubes were then incubated for 120 min: the first 100 min under light and the last 20 min in the dark, before Fv/Fm measurements. General bioassays procedures and test conditions are summarized in Table 1.

2.6. Calculations and graphics

The percentage of inhibition of maximum quantum yield was calculated as follow:

$$\text{Inhibition} = \frac{F_v/F_{m_{\text{control}}} - F_v/F_{m_{\text{filtrate}}}}{F_v/F_{m_{\text{control}}}} \times 100$$

Where $F_v/F_{m_{\text{control}}}$ and $F_v/F_{m_{\text{filtrate}}}$ were the maximum quantum yield of the diatom in artificial seawater and exposed to *A. minutum* filtrate, respectively. All graphics were performed using R software (R Foundation for Statistical Computing, Vienna, 2011). The effective concentration inhibiting 10% (EC₁₀), 50% (EC₅₀) and 90% (EC₉₀) of the Fv/Fm as compared to the control, were calculated from the dose-response curve based on the required *A. minutum* cell density to achieve a filtrate toxicity to inhibit Fv/Fm. To calculate the effective concentrations, the "Dose-Response Curve" package of R statistical analysis software was used (Gerhard et al., 2014). The "Akaike's Information Criterion" was used to determine model suitability where multiple models were tested (Koppel et al., 2017; Pinheiro and Bates, 2000). In all cases, a log-logistic model with 3 parameters was favored. While inhibition data will be given in the manuscript, the Fv/Fm data are available in the supplementary files.

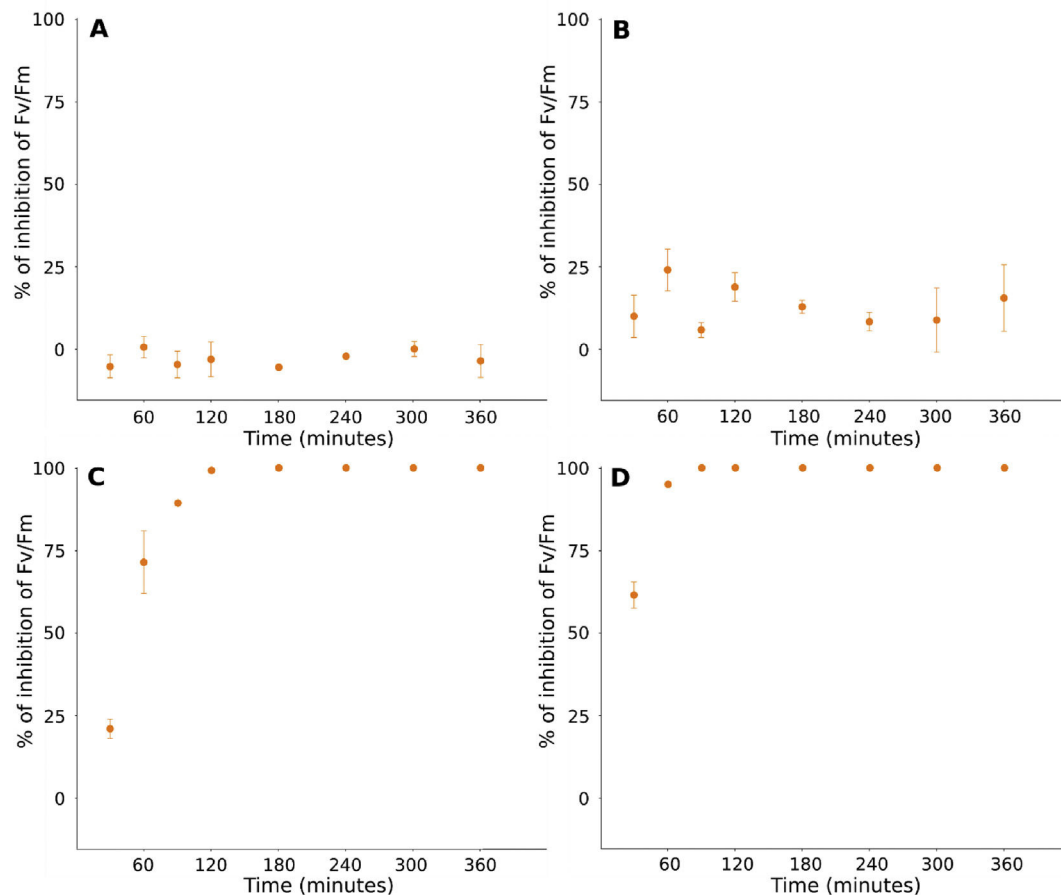
3. Results and discussion

3.1. Sensitivity of Fv/Fm to filtrate

When *C. muelleri* was exposed to the lowest concentration of *A. minutum* filtrate (50 cells mL^{-1}), Fv/Fm was not affected (Fig. 1). For *C. muelleri* exposed to higher filtrate concentrations, Fv/Fm was inhibited within the first 30 min corroborating the rapid toxicity mechanisms reported by Lelong et al. (2011) and Tillmann et al. (2007). At a concentration of 500 cells mL^{-1} , *C. muelleri* Fv/Fm inhibition varied between $6 \pm 2\%$ and $24 \pm 6\%$ during the six hours exposure. For filtrate concentrations above 500 cells mL^{-1} , Fv/Fm inhibition in *C. muelleri* increased quickly within the first 120 min then reached a plateau. When exposed to a theoretical cell concentration of 5000 cells mL^{-1} , Fv/Fm inhibition was $21 \pm 3\%$ at 30 min and increased to a $100 \pm 0\%$ inhibition at 120 min. With the highest filtrate concentration tested (50 000 cells mL^{-1}), Fv/Fm inhibition was as high as $62 \pm 4\%$ after only 30 min of exposure and reached $100 \pm 0\%$ inhibition within 90 min. For all tested concentration, the maximum inhibition and a plateau were reached within the first 120 min. For the two highest concentrations, the complete inhibition was maintained for the duration of the experiment (6 h). Thus the following experiments (and bioassays) were performed with an incubation time of 120 min. The measurement of an EC₅₀ after only 120 min is of interest because the bioassay should be sufficiently short to avoid any loss of allelochemical activity which can occur within a short time.

Table 1
Culturing and allelochemical interaction test conditions.

Allelopathic sample	
Type of sample	Culture filtrates (0.2 μm , acetate cellulose)
Storage	Glass containers, no plastic
Concentration	Series of dilutions
Target cells	
Species	<i>Chaetoceros muelleri</i>
Strain	CCAP 1010-3
Culture media	L1 media supplemented with silica in synthetic ocean water
Culturing temperature	17 ± 1 °C
Culturing light condition	$100\text{--}110$ $\mu\text{mol photon m}^{-2} \text{s}^{-1}$
Culturing light cycle	Continuous light
Physiological state	Exponential growth rate
Concentration in bioassay	2×10^5 cells mL^{-1}
Allelopathic test condition	
Test conditions	Static, well homogenized
Test duration	120 min
Test temperature	Ambient temperature (≈ 20 °C)
Test light condition	100 min under light then 20 min in dark (dark-adapted sample)
Test chamber	Flow-cytometric tubes (Polystyrene), cuvettes (Polymethyl methacrylate)
Test solution volume	2–3 mL
Endpoint	Maximum photosystem II quantum yield (Fv/Fm)

**Fig. 1.** Kinetics of *C. muelleri* Fv/Fm inhibition when exposed to *A. minutum* (CCMI1002) filtrate at different theoretical cell concentrations of A: 50 cells mL^{-1} , B: 500 cells mL^{-1} , C: 5000 cells mL^{-1} and D: 50 000 cells mL^{-1} . Results are expressed as mean \pm standard deviation.

3.2. Fv/Fm and FDA inhibition assay

In a second experiment, the filtrate-induced inhibition of Fv/Fm was compared to another proxy of metabolic activity. The fluorescein diacetate (FDA) assay measures esterase activity, is commonly

used to determine phytoplankton viability (Agusti and Sánchez, 2002; Gentien et al., 2007) and has been proven to be efficient with the genus *Chaetoceros* (Garvey et al., 2007). Results showed that *C. muelleri* esterase activity was inhibited by the allelochemical filtrate, as the proportion of metabolically inactive cells increased.

This was observed by flow cytometry with a decrease in the green (FL1) fluorescence (Supplementary figure 1). Three of the filtrate concentrations (500, 5000 and 50 000 cells mL⁻¹) induced a loss in metabolic activity as measured by both FDA staining and inhibition of Fv/Fm (Fig. 2). No increase in the proportion of metabolically inactive cells or decrease in Fv/Fm (compared to control) was observed when the diatom was exposed to the filtrate concentration of 50 cells mL⁻¹. When exposed to higher filtrate concentrations, the inhibition of Fv/Fm was always higher than the percentage of metabolically inactive cells measured with FDA staining. At a filtrate concentration of 500 cells mL⁻¹, the percentage of metabolically inactive cells was 11.5 ± 0.2%, while inhibition of Fv/Fm was of 19 ± 4%. Once exposed to filtrate concentrations of 5000 and 50 000 cells mL⁻¹, the proportion of metabolically inactive cells and inhibition of Fv/Fm were close to 88 and 100%, respectively. While the use of FDA allows an estimation of the percentage of metabolically active cells, Fv/Fm is a measurement of the maximum photosystem II quantum yield (proxy of photosynthetic metabolism), and both are good proxies as metabolic responses to *A. minutum* BECs with similar dose-response behaviour (dynamic/trend).

3.3. Bioassay: comparison of *A. minutum* strains

Inhibition of Fv/Fm in *C. muelleri* exposed to *A. minutum* culture filtrates was highly dependent on the *A. minutum* strain. In addition Fv/Fm inhibition was also related to theoretical cell concentration for the strains CCM11002 and AM89BM. For those two strains, the relationship between Fv/Fm inhibition and the (logarithmic) theoretical cell concentration was a sigmoidal dose-response pattern (Fig. 3). This dose-response curve enabled the calculation of the effective concentrations inhibiting Fv/Fm by 10% (EC₁₀), 50% (EC₅₀) and 90% (EC₉₀) in the *C. muelleri* populations (Table 2). The strain CCM11002 induced the most adverse effects with an EC₁₀ = 1350 ± 210 cells mL⁻¹ and an EC₅₀ = 4220 ± 480 cells mL⁻¹. In comparison, to obtain a similar inhibition of Fv/Fm of the marine diatom *Skeletonema costatum*, a concentration of 13 µg mL⁻¹ of the herbicide bentazon is necessary (Macedo et al., 2008). The strain AM89BM was four times less toxic than the strain CCM11002, with an EC₁₀ = 7710 ± 349 and an EC₅₀ = 16500 ± 1700 cells mL⁻¹. These cell concentrations are well within the range of environmental

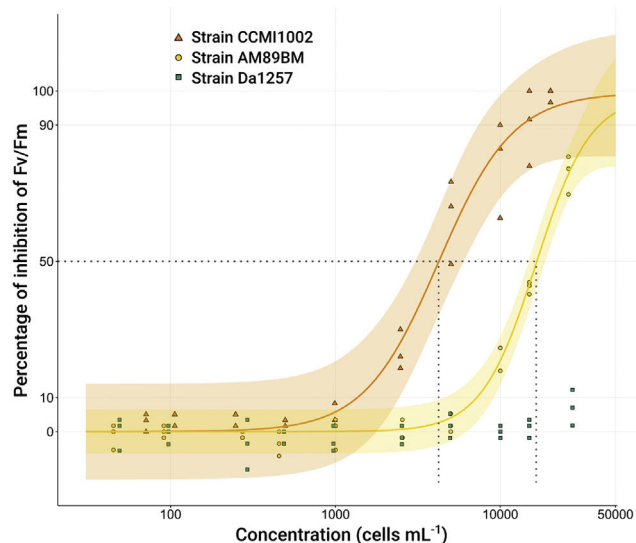


Fig. 3. Inhibition of *C. muelleri* maximum photosystem II quantum yield (Fv/Fm) by *A. minutum* filtrates from different strains. Dots represent the percentage of inhibition of Fv/Fm as compared to controls, as a function of filtrate dilution. The diatoms were exposed to strain CCM11002 (red triangle), strain AM89BM (yellow circle) and strain Da1257 (green squares) (n = 3). For both curves, the ribbon represents the 95% confidence interval from the log-logistic model. EC₅₀ are indicated by the dotted lines. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 2

Effective filtrate concentrations (cells mL⁻¹) inhibiting 10% (EC₁₀), 50% (EC₅₀) and 90% (EC₉₀) of *C. muelleri* Fv/Fm for three different *A. minutum* strains. "NC" indicate that no value could be calculated. Results are expressed as mean ± standard error (n = 3).

Strain	EC ₁₀	EC ₅₀	EC ₉₀
CCM11002	1350 ± 210	4220 ± 480	13160 ± 3980
AM89BM	7710 ± 340	16510 ± 1690	38020 ± 7780
Da1257	NC	NC	NC

A. minutum bloom concentrations, with reported densities as high as 41 000 cells mL⁻¹ in the bay of Brest, France (Chapelle et al., 2015) highlighting that *A. minutum* blooms may impact plankton

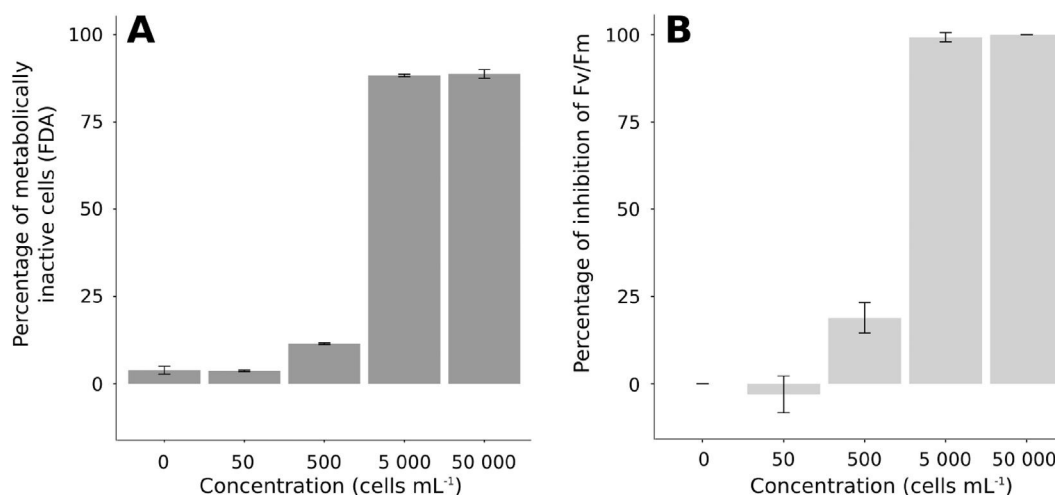


Fig. 2. Effects of *A. minutum* (CCM11002) filtrate on A) the percentage of metabolically inactive cells (FDA) and B) the percentage of inhibition of Fv/Fm of the diatom *C. muelleri* at different theoretical cell concentrations (0, 50, 500, 5000 and 50 000 cells mL⁻¹) after 120 min of exposure. Results are expressed as mean ± standard deviation. N = 3.

communities. The results confirmed the difference in allelochemical potency of the strains observed by Borcier et al. (2017) in a single-concentration bioassay, and also allowed us to precisely quantify the allelochemical potency of each strain. The strain Da1257 had minimal effects on maximum photosystem II quantum yield, with the highest cell concentration tested ($27\,400\text{ cells mL}^{-1}$) only inducing an Fv/Fm inhibition of $7 \pm 3\%$ in *C. muelleri*, therefore effective concentrations (EC10, EC50, EC90) could not be calculated. While this study validates the use of PAM fluorometry to quantify allelopathic potency from *A. minutum*, the proposed bioassay may be applied to other species of the genus *Alexandrium*. Many species from the genus *Alexandrium* induce lytic effects and therefore could inhibit photosynthesis. For instance the species *A. ostensfeldii* inhibits Fv/Fm (Tillmann et al., 2007). This bioassay could also be applied to other genus producing allelochemicals such as the dinoflagellate *Karlodinium venificum* which BECs also inhibits photosystem II quantum yield (Sheng et al., 2010). Moreover the variability in the sensitivity of target species that has already been highlighted (Tillmann et al., 2008) could precisely be quantified and compared with this bioassay.

3.4. Precautions

This study provides important information on how to properly assess a microalgal bioassay using PAM fluorometry. Fv/Fm is a sensitive physiologic parameter that responds to many different environmental parameters (e.g. physiological state, light, temperature, various stress; Ralph et al., 2007) that can modify bioassay outcomes. Indeed, it is essential to be consistent in the protocol (Table 1) and to perform controls to ensure that the inhibition is attributable to allelochemical interactions. *Alexandrium* spp. BECs chemical features were partially described in previous studies and special care must be taken as the test conditions may greatly influence the outcomes of the bioassay. To maintain biological activity of the BECs over time, plastics should be avoided, especially for filtrate storage (Ma et al., 2009). When stored in glassware at a cool temperature ($\approx 5\text{ }^\circ\text{C}$), allelochemical activity can be maintained for several weeks (Supplementary figure 4), and up to 4 months when stored at $-30\text{ }^\circ\text{C}$ (Martens et al., 2016). Similarly, the choice of the filter composition is important, because most filter membranes retained allelochemical activity (unpublished data). Here we recommend the use of acetate cellulose or asymmetric polyethersulfone (aPES) membrane filters.

3.5. Bioassay applications

The ease of use and the rapid response of this bioassay makes it convenient for various experiments. PAM techniques have many logistical advantages as they only require small sample volumes (2–3 mL), they are cheap, they can be used with environmentally relevant chlorophyll concentrations and they are rapid (a few seconds per sample) compared to a classic growth inhibition test or a lytic bioassay. Moreover the handled devices are practical, and allow the bioassays to be performed *in-situ*. This enables a fast quantification of allelochemical potency from various *A. minutum* samples: cultures, bio-guided purification of BECs, and field samples.

This bioassay can also be used for the purification of uncharacterized BECs (in prep.) produced by the genus *A. minutum*. The choice of the bioassay used to identify BECs during fraction purification is essential. During characterization of allelochemicals from *A. catenella*, Ma et al. (2011, 2009) preferred a phytoplankton (*Rhodomonas salina*) based bioassay rather than the usual bioassay with brine shrimp and red blood cells (Arzul et al., 1999; Emura et al., 2004). Ma et al. (2011, 2009) quantified allelochemical

activity through microscopy counts of lysed *R. salina* cells, however the present study highlights an easier and faster bioassay to follow bioactivity during purification of these chemicals.

3.6. A unique bioassay for other cytotoxic activities?

Exudates of *A. minutum* with allelochemical activity can have other toxic features. In a recent study, Borcier et al. (2017) were able to distinguish between the contrasting effects of paralytic shellfish toxins versus allelochemical exudates from *A. minutum* cultures over the great scallop *Pecten maximus* thanks to a similar but single-concentration bioassay. Delayed shell growth, reduced gill filtration, tissue damages and impaired escape behaviour when exposed to a predator were specifically attributed to the allelochemical exudates. This research highlights the importance of screening allelochemicals and studying the relationship between allelochemical interactions and other cytotoxic activities. Other studies have revealed that allelochemical activity may be related to other toxins (Arzul et al., 1999; Castrec et al., 2018; Le Goïc et al., 2014). This bioassay could be used to investigate the links between allelochemical activity and other cytotoxic (e.g. haemolytic, ichthyotoxic) activities through dose-response curves as performed by Blossom et al. (2014b) to, for example, establish the relationship between allelochemical activity and ichthyotoxicity from the prymnesiophyceae *Prymnesium parvum*.

3.7. Potential for field application

Allelochemical interactions research has been mainly based on laboratory experiments and lacks field reports to evaluate its role in plankton ecology. The bioassay developed here would be a useful tool during *A. minutum* blooms to measure/quantify allelochemical activity within an environmental matrix on a selected microalgal sample. The bioassay could be performed on *in-situ* samples when monitoring a bloom directly or under laboratory conditions following an appropriate sample storage (cooled glass container) to avoid any loss of activity. Moreover, measurement of a non-lethal parameter, such as photosystem II quantum yield, would allow the detection of mild effects. Indeed, non-lethal effects may reduce a cells ability to cope with other stress and indeed to compete with other species.

4. Conclusion

This study has demonstrated that the fluorescence-based bioassay can provide a fast and sensitive measure of allelochemical activity from various samples: culture filtrates and extracts resuspended in seawater. The developed bioassay successfully used the diatom *C. muelleri* as a target cell and enabled the quantification of allelochemical activity from three different *A. minutum* strains. Accurate quantification of allelochemical activity revealed a high variability in allelochemical potency of different strains from the same species. This bioassay may facilitate further research to broaden our understanding of allelochemical interactions from *Alexandrium* genus (purification and characterization, toxicity mechanisms, ecologic role). The information in this study also provides protocols to applying this technique to the study of allelochemical potency from other microalgal models.

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Titre : Interactions allélopathiques entre le dinoflagellé *Alexandrium minutum* et la diatomée *Chaetoceros muelleri*

Mots clés : *Alexandrium minutum* ; *Chaetoceros muelleri* ; Allélopathie ; Ecologie chimique ; Physiologie

Résumé : Comprendre les facteurs favorables à l'établissement et au maintien de proliférations d'algues toxiques et nuisibles est essentiel afin de mieux les prévoir. L'allélopathie, c'est-à-dire la libération de composés qui inhibent les compétiteurs, favorise l'espèce allélopathique et contribue ainsi à modifier la communauté planctonique. Les dinoflagellés toxiques du genre *Alexandrium* produisent des composés allélopathiques, cependant les interactions allélopathiques sont encore mal comprises. L'objectif de cette thèse était de contribuer à la compréhension des mécanismes d'allélopathie en étudiant l'interaction allélopathique entre *A. minutum* et la diatomée *Chaetoceros muelleri*. Les résultats de ces travaux ont mis en évidence que les composés allélopathiques d'*A. minutum* perturbaient le fonctionnement des membranes des diatomées en quelques minutes.

La cascade d'événements physiologiques suivant l'interaction allélopathique comprend des effets tels que la perméabilisation de membranes, l'inhibition de la photosynthèse, la production d'espèces réactives de l'oxygène et la modification de la composition des membranes. Ce projet a mis en évidence le fait que l'allélopathie d'*A. minutum* est sous contrôle environnemental, son activité s'est accrue d'un facteur 4 en présence de concentrations toxiques de Cu. Enfin, le développement d'un bioessai a facilité la purification des composés allélopathiques par fractionnement guidé. Nous avons isolé un groupe de candidats hydrophobes dont l'activité doit être testée après isolement. Cette thèse propose de nouveaux outils et ouvre de nouvelles perspectives pour étudier les conséquences de ces interactions dans les écosystèmes marins.

Title : Allelochemical interactions between the dinoflagellate *Alexandrium minutum* and the diatom *Chaetoceros muelleri*

Keywords : *Alexandrium minutum* ; *Chaetoceros muelleri*; Allelopathy; Chemical Ecology; Physiology

Abstract : Understanding the factors that favor the establishment and the persistence of harmful algal blooms is essential for predicting them. Allelochemical potency, i.e. the release of compounds that inhibit competitors, is hypothesized to favor the organisms that produce them and shape plankton community. The toxic dinoflagellates from the genus *Alexandrium* produce allelochemicals, however, associated interactions are poorly understood. The objective of this thesis is to contribute to our understanding by studying the mechanisms behind the allelochemical interactions between the *A. minutum* and the diatom *Chaetoceros muelleri*. The results of this work have highlighted that *A. minutum* allelochemicals disrupted membrane functioning of the diatoms within minutes. The cascade of physiological events following the allelochemical interaction that was described here,

included permeabilisation of membranes, inhibition of photosynthesis, production of reactive oxygen species and modifications of the biochemical composition of membranes. This project has also highlighted that the allelochemical potency of *A. minutum* can be significantly modulated by environmental parameters, as allelochemical potency increased 4 times when exposed to toxic concentrations of Cu. Finally, a bioassay developed during this PhD eased the partial isolation of the allelochemicals through fractionation. We isolated a hydrophobic candidates whose activity have to be investigated following their isolation. This PhD provides a better understanding of the cellular mechanisms underlying allelochemical interactions and offers new tools and raises new perspectives to study the consequences of these interactions on marine ecosystems.