
Effects of copper on the dinoflagellate *Alexandrium minutum* and its allelochemical potency

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Abstract :

The dinoflagellate *Alexandrium minutum* produces toxic compounds, including paralytic shellfish toxins, but also some unknown extracellular toxins. Although copper (Cu) is an essential element, it can impair microalgal physiology and increase their toxic potency. This study investigated the effect of different concentrations of dissolved Cu (7 nM, 79 nM and 164 nM) on *A. minutum* allelochemical potency, here defined as negative effects of a protist on competing protists through the release of chemicals. This was studied in relation to its physiology. The effects of Cu were assessed on *A. minutum* growth, reactive oxygen species level, photosynthesis proxies, lipid metabolism, exudation of dissolved organic compounds, allelochemical potency and on the associate free bacterial community of *A. minutum*. Only the highest Cu exposure (164 nM) inhibited and delayed the growth of *A. minutum*, and only in this treatment did the allelochemical potency significantly increase, when the dissolved Cu concentration was still toxic. 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 free bacterial density per algal cell. After 15 days, *A. minutum* partially recovered from Cu stress as highlighted by the growth rate, reactive oxygen species level 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. 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. Moreover, this study shows the complex interplay between abiotic and biotic factors that can influence the dynamic of *A. minutum* blooms. Modulation in allelochemical potency of *A. minutum* by Cu may have ecological implications with an increased competitiveness of this species in environments contaminated with Cu.

Highlights

► Cu stress significantly increased allelochemical potency of *A. minutum*. ► The increase in allelochemical potency was linked to a physiological stress. ► Cu stress modified *A. minutum* growth, photosynthesis, ROS, lipids and bacteria. ► Cells of *A. minutum* coped with Cu stress after 7 days.

Keywords : Alexandrium, Copper, Toxicity, Allelopathy, Physiology, Exudate

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 been extensively studied, and most of the effects of *Alexandrium* sp. 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* 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) can also 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.

The genus *Alexandrium* including the species *A. minutum* exhibits allelochemical potency, here defined as the negative effects of a protist on competing protists, bacteria or predators through the release of chemicals, named allelochemicals, in their surrounding environment. The release of allelochemicals is hypothesized to benefit *Alexandrium* spp. against competitors (Tillmann and Hansen, 2009) and enhance monospecific blooms of the genus *Alexandrium* (Hakanen et al., 2014). While the nature of allelochemicals remains unknown (Ma et al., 2011b, 2009), they disrupt biological membranes of target cells (Long et al., 2018b; Ma et al., 2011a). The environmental modulation of *Alexandrium* spp. allelochemical potency is also poorly studied. Low salinities increased 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). Nutrient starvation (nitrate and phosphate) did slightly modify the allelochemical potency of *A. tamarense* (Zhu and Tillmann, 2012), and had no effect on the allelochemical potency of *A. minutum* (Yang et al., 2011).

Copper (Cu) is an essential element that, under high concentrations, impairs physiological 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 HAB species (Maldonado et al., 2002; Moeller et al., 2007). Cu stress is also hypothesized to increase the allelochemical potency of the cyanobacteria *Synechocystis* sp. (Cheloni et al., 2019). Proliferations of *Alexandrium* occur in coastal and estuarine environments (Anderson et al., 2012; Guallar et al., 2017). Within this genus, the species *A. minutum* is predominant and distributed worldwide (Lassus et al., 2016) in coastal areas potentially subjected to anthropogenic inputs including Cu contamination (Herzi, 2013; Melwani et al., 2014). For instance, *A. minutum* blooms regularly occur 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 subjected 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 the genus *Alexandrium* (*A. catenella*; Herzi et al., 2013) and none on the species *A. minutum*. This research showed that toxic Cu concentrations increased the exudation by the dinoflagellate and modified the quality of the exudates but no specific attention was given to allelochemicals. No studies have so far investigated the effects of dissolved Cu on allelochemical potency of dinoflagellates.

This study intends to gain better understanding of the allelochemical and physiological responses of the toxic dinoflagellate *Alexandrium minutum* to Cu stress. A strain of *A. minutum* was exposed to toxic but environmentally relevant concentrations of dissolved Cu for 20 days. The effects of Cu on many different physiological parameters were then studied over 15 days of exposure to understand how *A. minutum* responds to a metallic stress. The physiological parameters included growth, intracellular reactive oxygen species, photosynthesis, lipid metabolism, the exudation and free 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 allelochemical 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, 18MΩ) 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 μM 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 μm, 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; Long et al., 2018b, 2018a). Cultures of *A. minutum* were kept under exponential growth phase through weekly culturing and were maintained at $17 \pm 1^\circ\text{C}$ under white light (Sylvania premium extra F18W/840 cool white and Lumilux OSRAM L 36W/840 cool white) with a 12/12 day/night light cycle ($150 - 210 \mu\text{mol photon m}^{-2} \text{s}^{-1}$).

2.3 Experimental procedure

The copper exposure was performed once with 5 to 6 replicates per condition. The physiology of *A. minutum* was studied over exponential growth phase for 20 days, at the University of Wollongong. 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 (pH = 8.25) 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 of $7 \pm 1 \text{ nM}$ (5 replicates), a “low” Cu treatment [Cu_1] with a dissolved Cu of $79 \pm 6 \text{ nM}$ (5 replicates) and a “high” Cu treatment [Cu_2] with a dissolved Cu of $164 \pm 6 \text{ nM}$ (6 replicates). Daily mixing avoided CO_2 limitation in cultures, in all the treatments the pH was between 8.07 and 8.25 along the exposure. Samples for dissolved Cu analysis were collected 2 hours after the Cu spike and after 7 and 15 days of exposure. Microalgal concentration and free bacteria were monitored every 2 days for 20 days. Lipid content and intracellular reactive oxygen species (ROS) were monitored at days 1, 7 and 15. The effective Photosystem II quantum yield (Φ_{PSII}), and the allelochemical potency of the filtrate were monitored after 7 and 15 days of exposure. The chromophoric dissolved organic carbon (cDOC) was characterized at day 15.

2.4 Preparation of filtrates

Samples for Cu analysis were filtered over 0.45 μm (Sartobran P sterile midicap; Sartorius) and acidified to pH 1.8 with HNO_3 (Suprapur, Merck Millipore, 0.2 % v/v). Acidified filtrates were stored for two months in plastic (polypropylene tubes with polyethylene cap) tubes before analysis at LEMAR. Samples for the characterization of exudates (dissolved organic carbon or allelochemicals) were filtered with acetate cellulose filters (0.2 μm) to maintain allelochemical activity (Long et al., 2018). Filtrates were stored for 2 months at cool temperature ($< 5^\circ\text{C}$) in glass tubes before characterization at LEMAR. Glass tubes were used to avoid any binding of allelochemicals or dissolved organic carbon (DOC) on plastic.

2.5 Copper analyses

Dissolved Cu in the acidified filtrates was measured at LEMAR (within the Pôle de Spectrométrie Océan) using 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.

2.6 Flow cytometry

2.6.1 General and cell 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 free bacteria (after SYBR Green I staining; see paragraph 2.4.3) were counted by flow cytometry according to flow rate (Marie et al., 1999). A performance check of the flow-cytometer was performed weekly with BD FACSDiva™ CS&T Research Beads.

2.6.2 Bacteria

Free bacteria (bacteria free in the culturing media) in *A. minutum* cultures were stained with SYBR Green I (da Costa et al., 2017). SYBR Green I is a fluorescent (520 nm) dye that binds double-stranded DNA molecules. The stock solution (Concentration of 10,000 X) was diluted to 100 X in ultrapure water. Culture samples were stained for 30 minutes in the dark with the intermediate solution of SYBR Green I (final concentration in tube = 2 X). Culture samples stained with SYBR Green I were diluted (10 to 50 times) in 0.2 μm filtered seawater for enumeration of free bacteria by flow-cytometry.

2.6.3 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. $\text{cell}^{-1} \text{day}^{-1}$) rate was calculated according to (Lelong et al., 2013) by multiplying the relative lipid content by the growth rate (day^{-1}).

2.6.4 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 H_2O_2 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.

2.7 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 effective quantum yield (Φ_{PSII} ; the proportion of absorbed energy being used in photochemistry (Maxwell et al., 2000)), a short saturating flash of light (1500 μmol

photon $\text{m}^{-2} \text{s}^{-1}$) was applied to measure the maximum fluorescence in the dark adapted state. 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 Φ_{PSII} in light-adapted state. Effective quantum yield in light-adapted samples of PSII (Φ_{PSII}) was calculated as :

$$\Phi_{\text{PSII}} = (\text{Fm}' - \text{Fstat}) / \text{Fm}' ,$$

where Fstat is the fluorescence of the sample adapted to the actinic light and Fm' the fluorescence when a saturating pulse is applied on light-adapted sample.

2.8 Characterization of exudates

2.8.1 Allelochemical potency of *A. minutum* filtrates

The allelochemical potency of *A. minutum* filtrates was assessed in the Laboratory of environmental marine sciences (LEMAR, France) according to the bioassay developed by Long et al. (2018). Dilutions of the filtrate were added to cultures of the diatom *Chaetoceros muelleri* and the Fv/Fm of the diatom was measured after 2 hours. The effect of the filtrate on the diatoms Fv/Fm was compared to the Fv/Fm of the diatom exposed to *A. minutum* culturing media (i.e. seawater supplemented with nitrate, phosphate and vitamins solely) without algae. To disentangle the effects of allelochemicals, Cu and the eventual combined effects, controls were performed with the addition of Cu to the culture media and to the filtrate of *A. minutum* grown in the control conditions. Dose-response curves (inhibition of Fv/Fm as a response of filtrate dilution) allowed the quantification of allelochemical potency by quantifying the effective concentration inhibiting 50% of the Fv/Fm of the diatom. Filtrate concentrations were expressed as the theoretical cell concentration based upon the culture concentration before filtration. Cultures of the diatom *C. muelleri* (CCAP 1010/3, formerly described as *Chaetoceros* sp. or *Chaeteroceros 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.

2.8.2 Dissolved organic carbon and quality of chromophoric dissolved organic carbon

DOC 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).

2.9 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 growth rates, the flow-cytometry (Lipids, ROS) and photosynthesis (Φ_{PSII}) 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 adjustment 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 the 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.

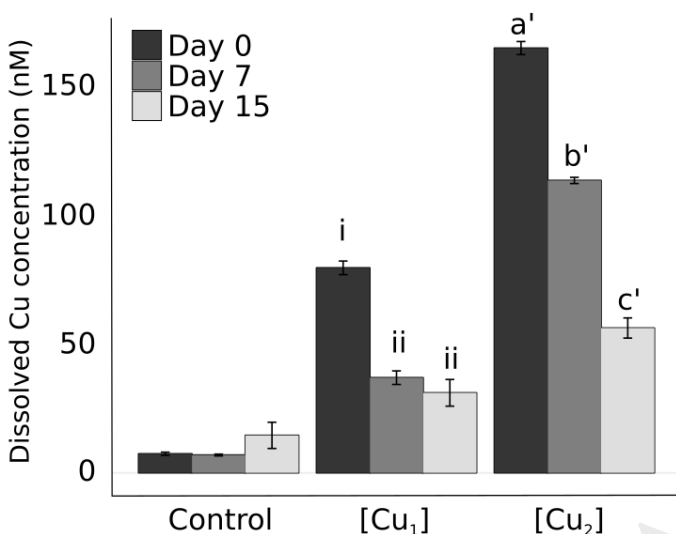


Figure 1: Dissolved copper concentrations (nM) in the Control, [Cu₁] and [Cu₂] treatments at day 0 (black bars), at 7 days (dark grey bars) and 15 days (light grey bars) of exposure. Different letters indicate significant differences between the sampling times. Results are expressed as the mean \pm standard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

3.2 Microalgal growth

Microalgal growth was maintained in exponential growth rate, in all the treatments, throughout the experiment (Figure 2A). The growth rate of the control was of 0.20 ± 0.02 day⁻¹ (N = 5), similar to the value routinely measured for this strain in the same culturing media (0.18 ± 0.05 day⁻¹, N = 9). 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. The growth rate of *A. minutum* was significantly inhibited within the first 7 days in the [Cu₂] flasks (Figure 2B). Between days 7 and 15, no significant differences were measured between the different treatments. During the last 5 days of the exposure, the growth rate in [Cu₂] was significantly higher than in the two other treatments.

3.3 Free bacterial-like community

The free bacterial concentration followed an exponential increase throughout the duration of the experiment. Exposure to Cu significantly (ANOVA-HSD, p -values < 0.001) decreased the free bacterial concentration and the number of free bacteria per algal cell in $[Cu_1]$ and $[Cu_2]$ during the first day following the exposure (Figure 2C). Then, from day 3, the number of free bacteria per *A. minutum* cell was higher in cells exposed to Cu (up to 3 times the control ratio for $[Cu_2]$ at day 9).

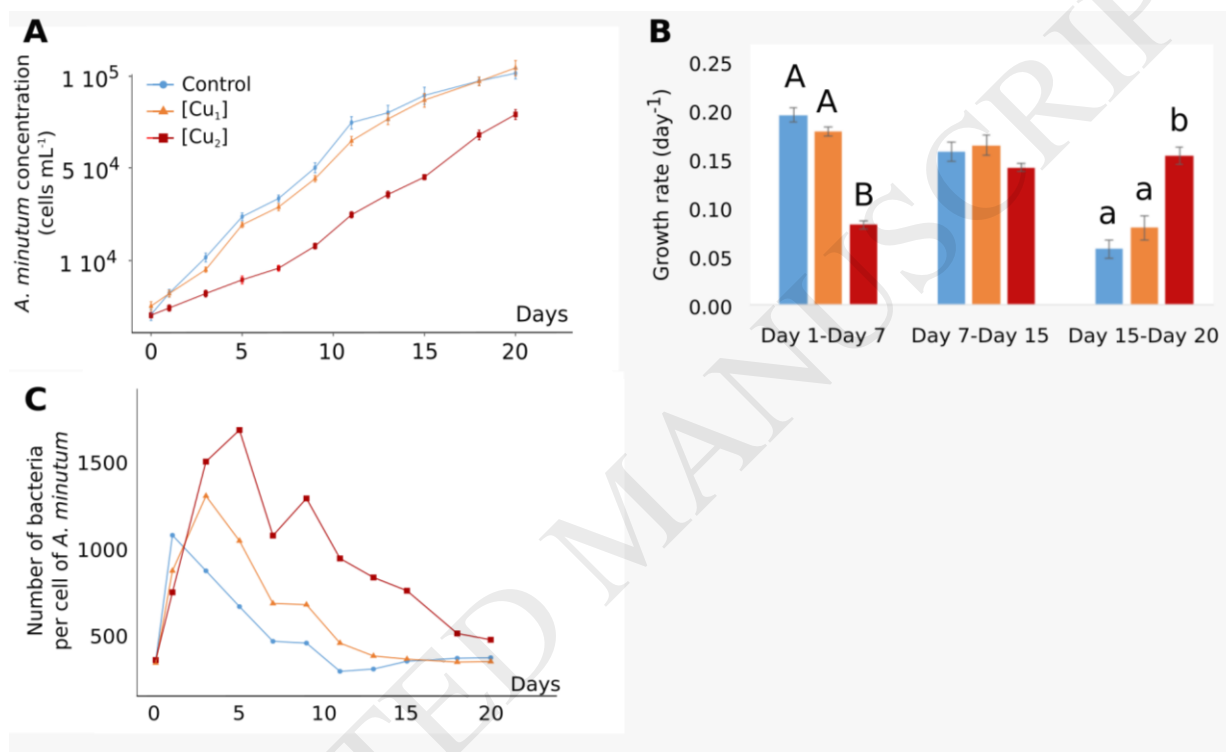


Figure 2: Effect of Cu treatments on growth of *A. minutum* and its associated free bacterial community. A: Cell concentration (cells mL⁻¹) of *A. minutum*, B: Growth rates of *A. minutum* between the sampling points, C: Ratio of free bacteria per cell of *A. minutum*. Blue lines and circles represent the control, orange lines and triangles represent the $[Cu_1]$, red lines and squares represent $[Cu_2]$. Results are expressed as the mean \pm standard error ($5 < N < 6$). Different letters indicate significant differences between the growth rates at each period of time. No letters indicate no significant differences.

3.4 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 $[Cu_2]$, where the lipid content was significantly (ANOVA-HSD, p -value < 0.001) higher than the control after 1 day of exposure. At 7 days, the lipid content of *A. minutum* from $[Cu_2]$ treatment was significantly (ANOVA-HSD, p -value < 0.01) higher than cells from $[Cu_1]$ treatment but not from the control (ANOVA-HSD, p -value < 0.07). At 15 days of exposure, the lipid content in the control and $[Cu_1]$

increased and was significantly (ANOVA-HSD, p -value < 0.05) higher than [Cu₂]. At 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 at 15 days (ANOVA-HSD, p -value < 0.01).

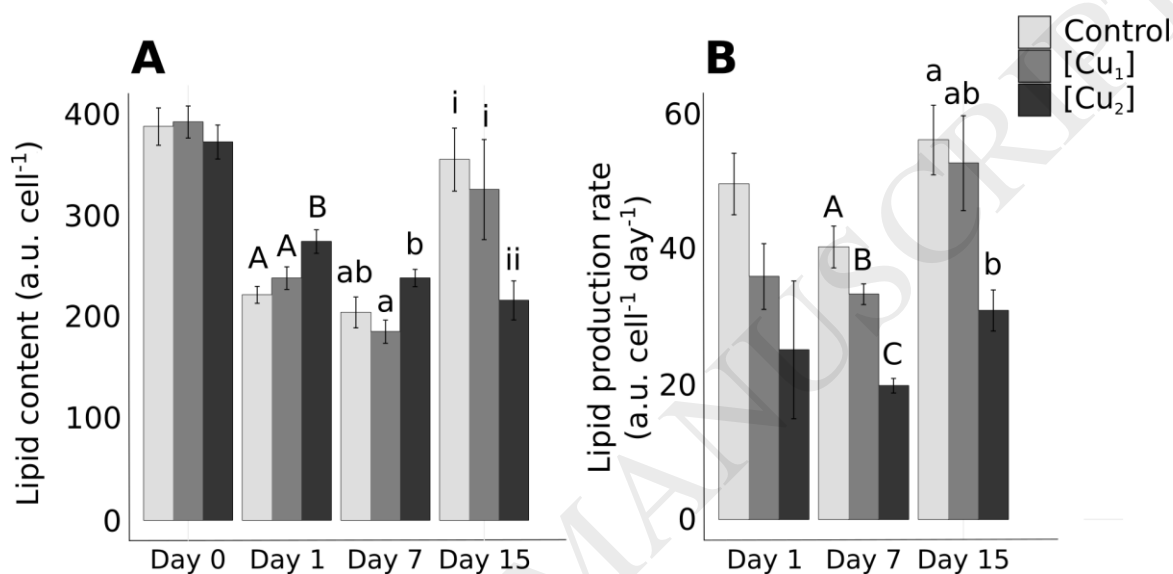


Figure 3: Lipid metabolism over the 15 days of exposure to the different Cu treatments. A: Lipid content per cell, B: Daily lipid production rate per cell. Results are expressed as the mean \pm standard error ($5 < N < 6$). Different letters indicate significant differences between the treatments at each day of exposure. No letters indicate no significant differences.

In all the treatments, intracellular ROS production by vegetative cells varied between day 1 and 15, with the highest values at day 7 (Figure 4A). 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.

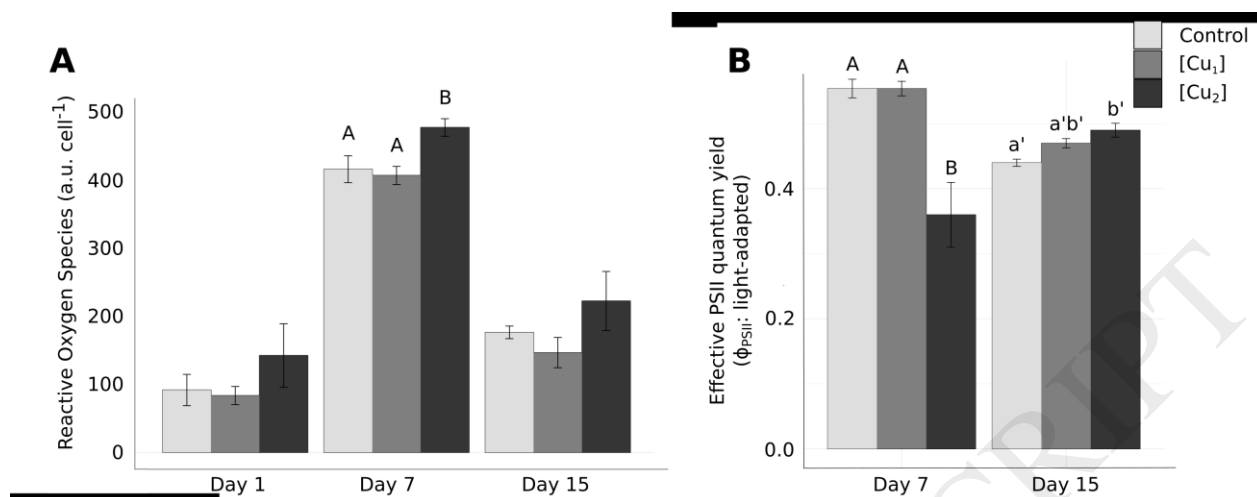


Figure 4: (A) Intracellular reactive oxygen species (ROS) production of *A. minutum* in the control or at 1, 7 and 15 days of exposure to both copper concentrations measured by flow cytometry. B: Effective photosystem II (PSII) quantum yield (Φ_{PSII} ; light adapted) at 7 and 15 days. Results are expressed as the mean \pm standard error ($5 < N < 6$). Different letters indicate significant differences between the treatments at each day of exposure. No letters indicate no significant differences.

3.5 Photosynthesis

Exposure to the highest Cu concentration [Cu₂] induced a significant decrease (ANOVA-HSD, p -value < 0.01) of the effective quantum yield of PSII (Φ_{PSII}) with a decrease of 35% at 7 days of exposure (Figure 4B). At day 15 of exposure, this trend reversed, with a Φ_{PSII} significantly (ANOVA-HSD, p -value < 0.01) higher in [Cu₂] than in the control.

3.6 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 5). 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. The presence of Cu in the filtrate was not responsible for the increased toxicity as addition of Cu to culturing media or to a filtrate of *A. minutum* did not increase the inhibition of Fv/Fm (Figure 6). At day 15, 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.

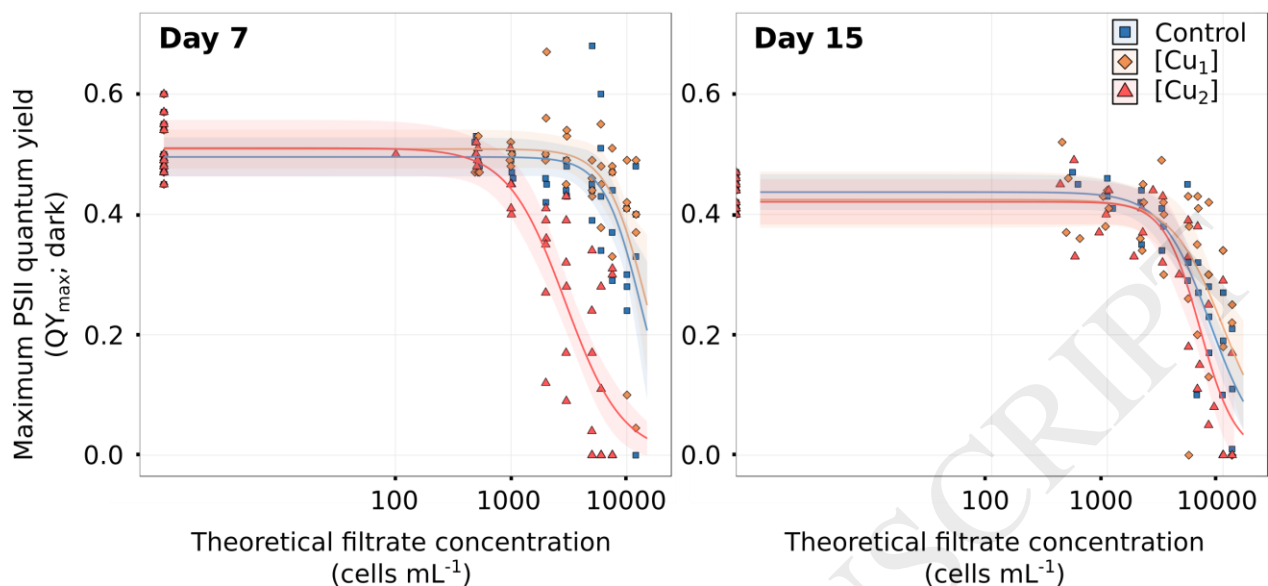


Figure 5: Inhibition of photosystem II maximum quantum yield (F_v/F_m) of *C. muelleri* in the presence of filtrates of *A. minutum* exposed to different Cu concentrations for 7 and 15 days. Log-logistic models with 3 parameters were used to model the inhibition. Dots represent the maximum quantum yield as measured in the bioassay in the presence of filtrate from the control (blue squares), $[Cu_1]$ (orange diamonds), $[Cu_2]$ (red triangles). For both curves, the ribbon represents the 95% confidence interval from the log-logistic model.

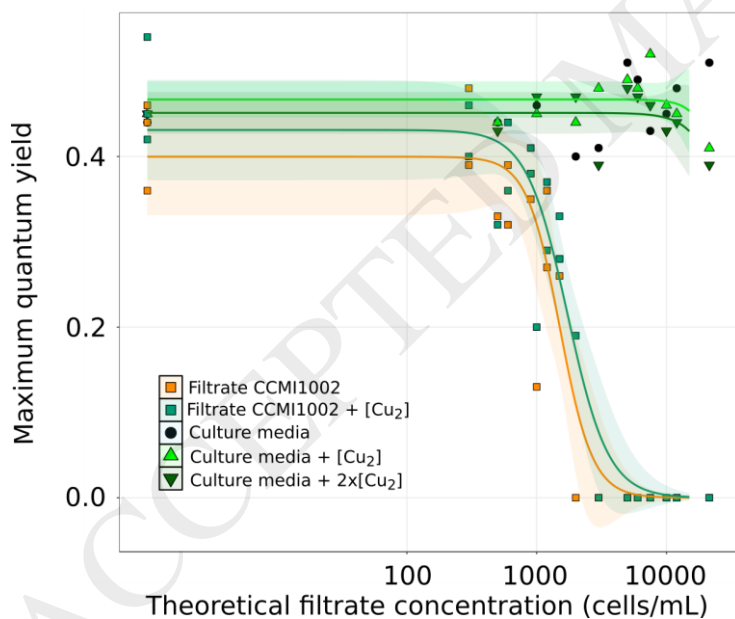


Figure 6: Inhibition of photosystem II maximum quantum yield in the presence of culture media, culture media spiked with $[Cu_2]$, spiked with two times the initial dissolved Cu concentration of $[Cu_2]$, *A. minutum* CCM11002 filtrate and CCM11002 filtrate spiked with the initial dissolved Cu concentration of $[Cu_2]$. Log-logistic models with 3 parameters were used to model the inhibition. Dots represent the maximum quantum yield as measured in the bioassay in the presence of filtrate from the control (blue squares), $[Cu_1]$ (orange diamonds), $[Cu_2]$ (red triangles). For both curves, the ribbon represents the 95% confidence interval from the log-logistic model.

3.7 Dissolved organic carbon and characterization of chromophoric dissolved organic carbon

Only exposure to the highest Cu concentration [Cu₂] modified the exudation by *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 cDOC. 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 ≥95% similarity to 19/70 models in the database. Component C1 significantly (ANOVA-HSD, p-values < 0.01) increased in the 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.

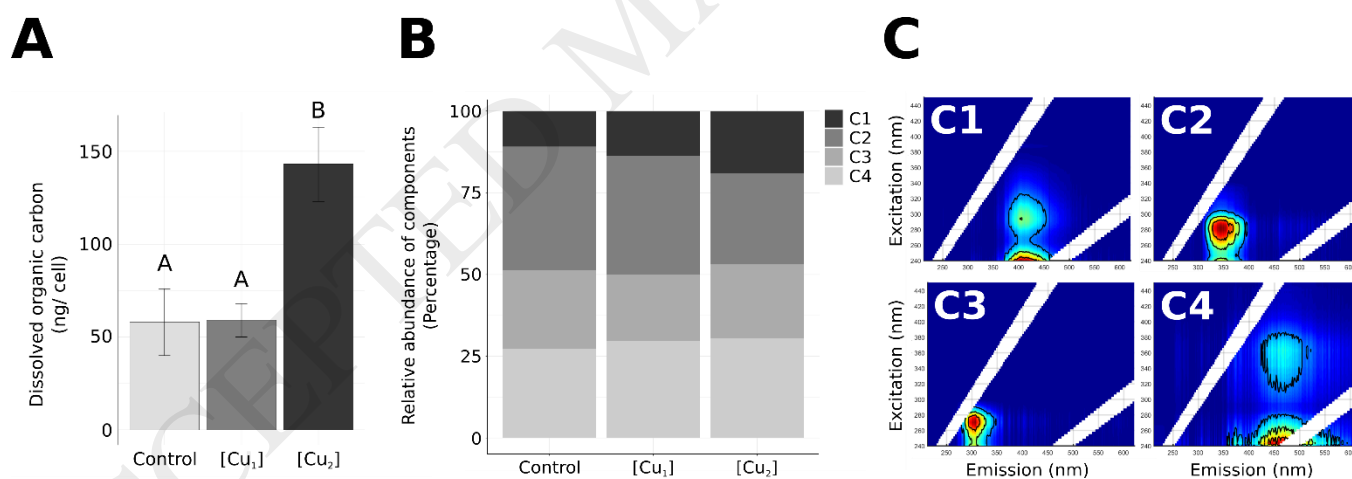


Figure 7: Characterization of the dissolved organic carbon at 15 days of exposure. A: Total dissolved organic carbon (ng cell⁻¹), B: Relative abundance of the chromophoric components C1, C2, C3 and C4, C: Fluorescence excitation emission spectra of the components C1 (Fulvic-like), C2 (Tryptophan-like),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

4 Discussion

4.1 Copper increases the allelochemical potency of *A. minutum*

Exposure to the highest Cu concentration [Cu₂] lead to a significant increase in the allelochemical potency of *A. minutum* exudates. Relevant controls highlighted that the increase in

the allelochemical potency to the exposed diatom was not related to a synergistic effect of Cu and allelochemicals. Instead, an increased exudation and/or a modification of allelochemicals in response to Cu may have been responsible for this higher potency. Similarly, the cyanobacteria *Synechocystis* sp. was hypothesized to increase its allelochemical potency under a combined copper and high light intensity stress (Cheloni et al., 2019). Maldonado et al. (2002) observed that two species of *Pseudo-nitzschia* increased their release of domoic acid, a phycotoxin, in the presence of toxic concentrations of Cu. The same authors hypothesized that domoic acid could protect the cells by chelating Cu. Increase in the allelochemical potency of the genus *Alexandrium* has been observed in response to other abiotic factors (i.e. low salinity, phosphate starvation) and was related with a physiological stress (Martens et al., 2016; Zhu and Tillmann, 2012). It is noteworthy that the increase in the allelochemical potency of exudates was only observed for [Cu₂] at 7 days. The temporary increase in allelochemical potency may be related to a temporary physiological stress of *A. minutum*.

4.2 Temporary toxicity of Cu

Growth occurred in all the cultures of *A. minutum* in the presence or absence of added Cu, however, the highest concentration of Cu induced a significant decrease of microalgal growth rate for the first 7 days of exposure. 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 genus *Alexandrium* (Herzi et al., 2013). After 7 days, the similar growth rates between treatments suggest that *A. minutum* recovered from Cu exposure. 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 highest growth rate in [Cu₂] between days 15 and 20. The control reached late exponential growth phase while [Cu₂] is still under exponential growth phase. Overall, decreased growth rates in [Cu₂] indicated toxic effects on microalgal physiology (Figure 8) that was confirmed with several physiological parameters.

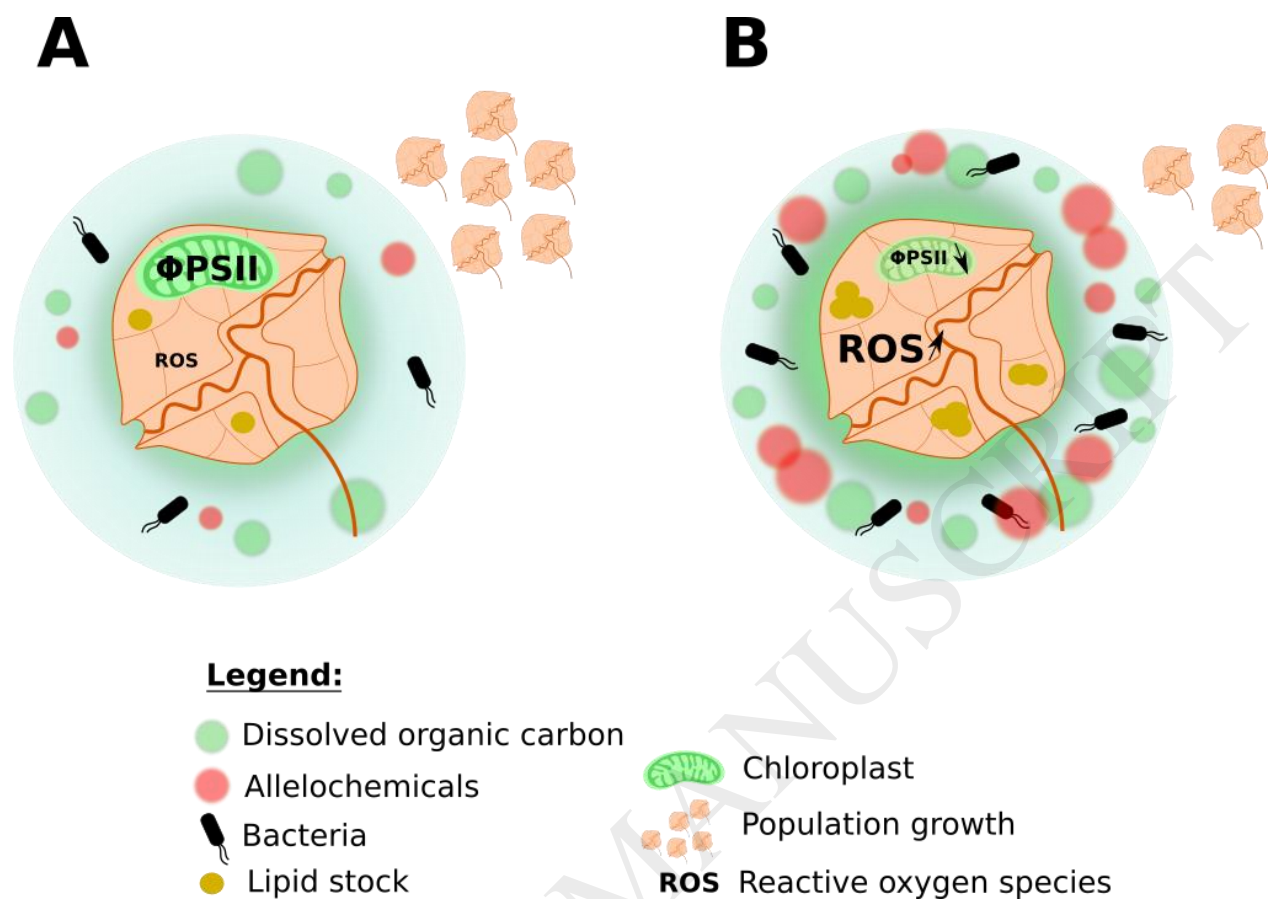


Figure 8: Physiological responses of *A. minutum* to an acute Cu stress. A: Cell of *A. minutum* in the control conditions, B cells of *A. minutum* exposed to toxic Cu concentrations.

ROS production by *A. minutum* increased in the presence of $[Cu_2]$ and highlighted an acute stress for *A. minutum* at day 7 only. 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 the presence of toxic concentrations of Cu (Knauert and Knauer, 2008; Rocchetta and Küpper, 2009) and were shown to play a primary role in Cu toxicity to microalgae. Free Cu ions can catalyze the production of hydroxyl radical *via* the Haber-Weiss reaction (Morelli and Scarano, 2004; Nikookar et al., 2005). These radicals can oxidize biological molecules such as lipids or proteins that can lead to other cellular damages (e.g. inhibition of photosynthesis, damages to membranes). Nevertheless, microalgae have protective mechanisms (Li et al., 2006; Morelli and Scarano, 2004; Sabatini et al., 2009; Smith, 2016; Smith et al., 2014) that may have mitigated ROS stress in *A. minutum*. Our data highlight the need to

further study the potential damages of ROS on cellular compartments of *A. minutum* in the presence of Cu.

The photosynthesis of *A. minutum* was negatively affected by [Cu₂] at day 7 as shown by the inhibition of the effective PSII quantum yield. 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). Copper inhibits the PSII-PSI electron transport by targeting the PSII (Juneau et al., 2002; Ralph et al., 2007) explaining the decrease in Φ_{PSII} observed for *A. minutum* or other species (e.g. Juneau et al., 2002). This decrease in photosynthesis may decrease the growth of the dinoflagellate or limit the mobilization of carbon allocated to organic compounds such as lipids. Pulse-Amplitude-Modulation fluorescence only provides a partial picture of the photosynthetic chain, complementary measurements are required to evaluate the effects of Cu on the overall photosynthesis and on the carbon fixation by the dinoflagellate.

Cu concentrations also affected the lipid metabolism, as shown by the modifications in lipid contents and production rates. Microalgal cells can store energy as carbohydrates or as lipids (da Costa et al., 2017; Li et al., 2011). 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 toxic concentrations of Cu (Lelong et al., 2012). The decrease of lipid content of *A. minutum* between day 0 and day 1 reveals a lipid mobilization rather than a storage. This lipid mobilization was lower for the cells in the presence of [Cu₂] as the cells divided less. This highlighted that cells were saving their reserves in response to Cu. In the meantime, the daily lipid production rates were lower in [Cu₂], this may result from a higher energy requirement for detoxication mechanisms (before day 7) or from the energy required to undergo their exponential growth phase (after day 7). At 15 days, cells in the in the control and [Cu₁] accumulated lipids as they reached stationary phase (Brown et al., 1993; da Costa et al., 2017) on contrary to [Cu₂]. It would be valuable to investigate the overall energy metabolism (e.g. production and consumption of ATP and carbohydrates) to confirm these hypotheses.

An absence of growth inhibition does not necessarily mean an absence of physiological effects. While the growth rate in the [Cu₁] was not affected, significant modifications were measured in the cultures, with changes in the lipid production rate at day 7 and in the microbial

community over the whole exposure, consistently appearing intermediate between the high Cu concentration and the control. While the treatment [Cu₁] only slightly stressed *A. minutum*, it did not increase its allelochemical potency. Therefore, we suggest that the increase in allelochemical potency is linked to an acute copper stress.

4.3 Copper affects *A. minutum* microbial community

Similarly to *A. minutum* cells, free bacteria-like cells in the bioassay were affected by Cu exposure. While the free bacteria concentration as well as the ratio per algal cell were first lower in the presence of Cu as compared to the control, after 3 days, cultures exposed to Cu had more free bacteria per algal cell than the control. This last observation is another indicator of physiological stress as 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 media was higher in the [Cu₂] treatment than the control and [Cu₁] and could have enhanced bacterial growth.

4.4 Decrease and fate of dissolved Cu in the flasks

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) and it is likely to have triggered toxicity in our experimental design. The decrease in toxicity, and subsequently the decrease in allelochemical potency between day 7 and day 15 may be linked to the decrease in dissolved Cu concentrations from 164 nM to 56 nM. While we cannot calculate the lowest-observable-effect concentration (LOEC) that inhibited growth of *A. minutum* (strain CCMI1002), we can estimate it 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). The strain CCMI1002 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 µM and 12 µM added Cu, respectively 2 nM and 7 nM of free Cu²⁺ (Herzi et al., 2013), as exposure were performed in the presence of EDTA.

While the dissolved Cu concentration decreased over the exposure, the fate of Cu in the flasks remain to be elucidated. Some of the Cu spiked must have bound to flask walls even if flasks were silanized. This proportion, however, is likely insignificant as compared to the losses of Cu over 15 days. A study by (Levy et al., 2008) performed in similar conditions highlighted that the Cu losses to the flasks were really low and not significant as compared to the proportion of Cu binding to/internalized by microalgal cells. Cu can interact with cells (i.e. microalgae and bacteria) by binding to cell surfaces or by being internalized by microalgae (Debelius et al., 2009; Levy et al., 2008). The increase in cell concentrations lead to an increase in the biomass available for Cu to bind or be internalised. This eventually lead to a decrease in the amount of Cu per cell. Bacteria, which density per microalgal cells increased with Cu might also mitigate toxicity by increasing the biomass that could internalize Cu and increase the biological surface available for Cu binding. Cu can also be 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* and their associated bacteria 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 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 organic ligands would help understand the fate of Cu in the flasks.

4.5 Copper increases *A. minutum* exudates

The [Cu₂] increased the exudation of DOC per microalgal cells as measured after 15 days of exposure. 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 Schampelaere 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). 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 cDOC from *A. minutum* is similar to the cDOC produced by *A. tamarense* 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). 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 bind metals and decrease toxicity more efficiently than proteinous DOC (Al-Reasi et al., 2012, 2011), 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. 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 component C1 may have been attracting bacteria as studies have shown it can also be consumed by bacteria (Romera-Castillo et al., 2011). Overall, bacteria may have been participating in the mitigation of Cu toxicity (Levy et al., 2009) by increasing the biomass available for Cu to bind.

The allelochemical potency of *A. minutum* increased in the presence of Cu and could also have chelating properties. In such a scenario, allelochemicals would have a dual purpose: they would complex metal and additionally have an allelochemical role. Other toxins such domoic acid (Maldonado et al., 2002), *Pfiesteria piscida* toxins (Moeller et al., 2007) or the terrestrial phytotoxin 8-hydroxyquoline (Inderjit et al., 2011; Tharayil et al., 2009) were observed to have a dual purpose. Future studies should include analysis of Cu complexation within exudates and allelochemicals in order to understand their role on copper toxicity.

4.6 Adaptation of *A. minutum* to coastal environments

In the light of other studies, *A. minutum* appeared to be quite tolerant to Cu. This tolerance may provide this species a competitive advantage to grow in metal-contaminated areas. In polluted

aquatic environments, Cu concentration can be reported in high concentrations, in estuaries and in rivers (Beiras et al., 2003; Juneau et al., 2002; MENVIQ, 2003). Within this concentration range, the *A. minutum* strain CCMI1002 could 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. However, in areas with high Cu concentrations, Cu is unlikely to be the only metal or contaminant at high concentrations. Future studies should therefore focus on other metals alone but also in mixtures with relevant ratio metal concentrations (Koppel et al., 2018). 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). The sensitivity of *A. minutum* to Cu and its allelochemical potential may vary according to their life history as previously highlighted for their physiological traits (Brandenburg et al., 2018). This highlight the need to further explore the variability of *A. minutum* sensitivity to Cu and its allelochemical potency.

5 Conclusion

This study provides an overview of the main physiological effects of Cu on the toxic dinoflagellate *A. minutum*. The growth under $[Cu_2]$ was delayed when compared to the control and the moderate $[Cu_1]$ treatment. The effects of Cu on *A. minutum* physiology were observed during the first 7 days of exposure. We hypothesized that concomitant increase in the 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 availability. Within the responses of *A. minutum* to Cu, the allelochemical potency against the diatom was significantly increased at day 7 when *A. minutum* cells were stressed. It is however unclear if this increased potency is a non-specific response and could be induced by any acute stress or if this is a specific response to Cu. Furthermore, 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.

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