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

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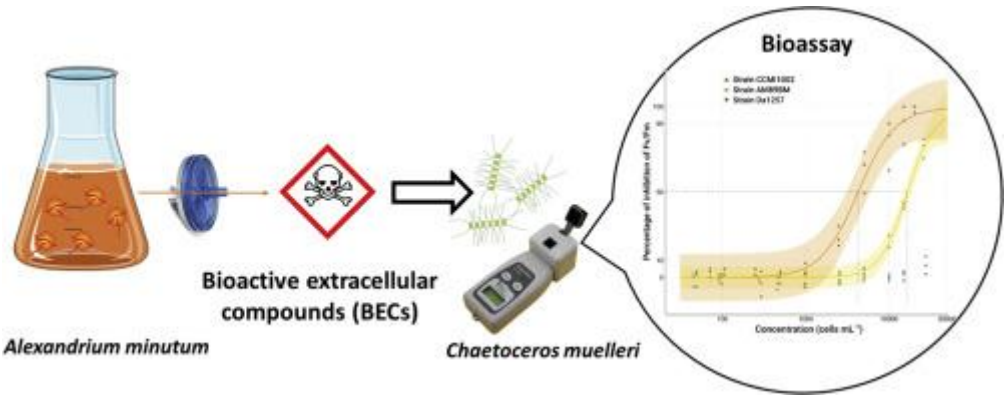
Abstract :

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, ichtyotoxic 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.

Graphical abstract



Highlights

► We developed a fast bioassay to screen Alexandrium bioactive extracellular compounds. ► The rapid quantification of allelopathic BECs is based on PAM fluorometry. ► This will help investigating links between allelo-, ichtyo-, hemo- and cytotoxicity. ► The bioassay will ease chemical identification of unknown bioactive compounds. ► The bioassay could be applied to algal cultures, extracts and environmental samples.

Keywords : Harmful algal bloom, *Alexandrium*, Allelopathy, Cytotoxicity, PAM fluorometry, Bioassay

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 ichtyotoxic 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

82 exposed to the genus *Alexandrium* (Tillmann et al., 2007) including the globally distributed diatom
83 *Chaetoceros muelleri* (Lelong et al., 2011, formerly named as *Chaetoceros neogracile*).
84 No standard and rapid methodology is available for the study of allelochemical interactions
85 in marine environments (Legrand et al., 2003). Development of bioassays would benefit the
86 allelochemical interactions research field by enabling the rapid confirmation and quantification of
87 allelochemical interactions. This would help to assess the role and relevance of these interactions
88 in plankton ecology. It would also greatly ease the isolation and characterization of unknown BECs
89 through bio-guided purification. Population growth inhibition (Chan et al., 1980; Paul et al., 2009;
90 Pushparaj et al., 1998) or enumeration of lysed cells (Hakanen et al., 2014; Ma et al., 2009;
91 Tillmann et al., 2007) are the most common parameters used in bioassays to demonstrate
92 allelochemical interactions but such techniques are time-consuming. According to the OECD
93 (2011) guidelines for testing chemicals, a growth inhibition test should be long enough to obtain a
94 16-fold population growth in control treatments (from few days to weeks depending on the target
95 species growth rate). Moreover, microscopic observations cannot be performed in every field
96 situation and distinction between viable and non-viable cells can be subjective because of a lack of
97 consensus on a microalgal death definition (Garvey et al., 2007). This is compounded by the fact
98 that the enumeration of lysed cells is based on lethal effects, although allelochemical interactions
99 in marine environments may not automatically imply cell lysis. Therefore, new rapid, quantitative
100 and sensitive methods are worth exploring to study allelochemical interactions.
101 Pulse amplitude modulatory (PAM) fluorometry is a method based on chlorophyll *a*
102 fluorescence that allows studying photosynthetic processes. PAM is frequently used to assess the
103 impact of environmental stressors (Barranguet et al., 2002; Juneau et al., 2003; Lippemeier et al.,
104 1999; Miao et al., 2005), pollutants (see review from Ralph et al., 2007) or algicidal compounds
105 (Yang et al., 2017) on phytoplankton physiology, as it provides insights into phytoplankton

106 "health". Maximum photosystem II quantum yield (Fv/Fm) is the optimal photosynthetic efficiency
107 and is one of the most popular and easiest parameter to interpret. PAM has the advantage of being
108 a rapid non-invasive and non-destructive method that can detect sub-lethal effects on photosystems.
109 Some devices can be portable, allowing rapid measurements useful in many situations. However,
110 only few studies report the measurement of chlorophyll *a* fluorescence to demonstrate
111 allelochemical interactions (Blossom et al., 2014a; Borcier et al., 2017; Tillmann et al., 2007). The
112 lack of homogeneity in methods and summary parameters (e.g. single concentration measurement,
113 EC10, EC50: the effect concentrations inhibiting 10 or 50 % of a physiologic parameter) prevent
114 comparisons between studies, highlighting the need of standardized bioassays.

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

125 2. MATERIALS AND METHODS

126 *2.1 Algal culture*

127 Three strains of the toxic dinoflagellate *Alexandrium minutum* were selected based upon
128 their different allelochemical activity. The strains AM89BM (isolated from a bloom in the Bay of
129 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 ± 1°C under continuous light (100 – 110 µmol photons m^{-2} 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 minutes before measurement of fluorescence variables (F0, Fm). F0 is the initial fluorescence intensity, Fm the maximal intensity under saturating light conditions (0.5 to 1s at 1500 µmol photon m^{-2} s^{-1}), and Fv = Fm - F0. Maximum quantum yield of photosystem II was then calculated as followed: Fv/Fm (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 x 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).

177 Fluorescein diacetate (FDA, Molecular probes, Invitrogen, Eugene, OR, USA) is a hydrophobic
178 fluorescent dye that permeates and stains cells exhibiting esterase activity, i.e. viable cells. Inside
179 the cells, FDA is cleaved by esterases and releases a fluorescent by-product (fluorescein; emission
180 525 nm) which is retained within the cells. Cells that are not stained in the presence of FDA do not
181 exhibit esterase activity and are considered as metabolically inactive (Supplementary figure 1).
182 FDA fluorescence in the diatoms was measured by flow cytometry (FACSCalibur) equipped with
183 a blue laser (excitation 488 nm) through the detector of fluorescence FL1 (green emission filter
184 band pass, 530/30 nm). FDA staining protocol was optimized (Supplementary figures 2 and 3) for
185 *C. muelleri* and samples were stained (final concentration = 1.5 µM) for 10 min in dark prior to
186 flow-cytometric measurement, while samples for Fv/Fm measurement were put in the dark 20 min
187 before measurement. *C. muelleri* (2×10^5 cells mL^{-1}) was exposed to *A. minutum* exudate filtrate
188 (strain CCMI1002) at concentrations (theoretical concentrations) equivalent to 500, 5000 and 50
189 000 cells mL^{-1}. Fv/Fm and FDA fluorescence were measured after 120 min of exposure.

190 *2.5 Bioassay procedure: comparison of A. minutum strains*

191 *2.5.1 Algal culturing*

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

196 *2.5.2 Exposure*

197 Triplicate cultures of *C. muelleri* in exponential growth phase (7 days old) were diluted
198 with filtered (0.2 µm) synthetic ocean water to reach 2×10^5 cells mL^{-1} in the bioassay. Diatoms
199 were then exposed to dilutions of *A. minutum* culture filtrate (concentration 3×10^4 cells mL^{-1})
200 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 % (EC_{10}), 50 % (EC_{50}) and 90 % (EC_{90}) 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 (Figure 1). For *C. muelleri* exposed to higher filtrate concentrations,

226 Fv/Fm was inhibited within the first 30 min corroborating the rapid toxicity mechanisms reported
227 by Lelong et al. (2011) and Tillmann et al. (2007). At a concentration of 500 cells mL^{-1}, *C. muelleri*
228 Fv/Fm inhibition varied between 6 ± 2 % and 24 ± 6 % during the six hours exposure. For filtrate
229 concentrations above 500 cells mL^{-1}, Fv/Fm inhibition in *C. muelleri* increased quickly within the
230 first 120 min then reached a plateau. When exposed to a theoretical cell concentration of 5 000
231 cells mL^{-1}, Fv/Fm inhibition was 21 ± 3 % at 30 min and increased to a 100 ± 0 % inhibition at 120
232 min. With the highest filtrate concentration tested (50 000 cells mL^{-1}), Fv/Fm inhibition was as
233 high as 62 ± 4 % after only 30 min of exposure and reached 100 ± 0 % inhibition within 90 min.
234 For all tested concentration, the maximum inhibition and a plateau were reached within the first
235 120 minutes. For the two highest concentrations, the complete inhibition was maintained for the
236 duration of the experiment (6 hours). Thus the following experiments (and bioassays) were
237 performed with an incubation time of 120 minutes. The measurement of an EC_{50} after only 120
238 minutes is of interest because the bioassay should be sufficiently short to avoid any loss of
239 allelochemical activity which can occur within a short time.
240
241 *3.2 Fv/Fm and FDA inhibition assay*
242 In a second experiment, the filtrate-induced inhibition of Fv/Fm was compared to another
243 proxy of metabolic activity. The fluorescein diacetate (FDA) assay measures esterase activity, is
244 commonly used to determine phytoplankton viability (Agusti and Sánchez, 2002; Gentien et al.,
245 2007) and has been proven to be efficient with the genus *Chaetoceros* (Garvey et al., 2007). Results
246 showed that *C. muelleri* esterase activity was inhibited by the allelochemical filtrate, as the
247 proportion of metabolically inactive cells increased. This was observed by flow cytometry with a
248 decrease in the green (FL1) fluorescence (Supplementary figure 1). Three of the filtrate
249 concentrations (500, 5 000 and 50 000 cells mL^{-1}) induced a loss in metabolic activity as measured
250 by both FDA staining and inhibition of Fv/Fm (Figure 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 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 % (EC_{10}), 50 % (EC_{50}) and 90 % (EC_{90}) in the *C. muelleri* populations (Table 2). The strain CCMI1002 induced the most adverse effects with an EC_{10} = 1350 ± 210 cells mL^{-1} and an EC_{50} = 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 EC_{10} = 7710 ± 349 and an EC_{50} = 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.* m*uelleri.*, 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.

3.4 Precautions

This study provides important information on how to properly assess a microagal 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 (≈ 5°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.

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, ichtyotoxic) activities through dose-response curves as performed by Blossom et al. (2014b) to, for example, establish the relationship between allelochemical activity and ichtyotoxicity 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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FIGURES

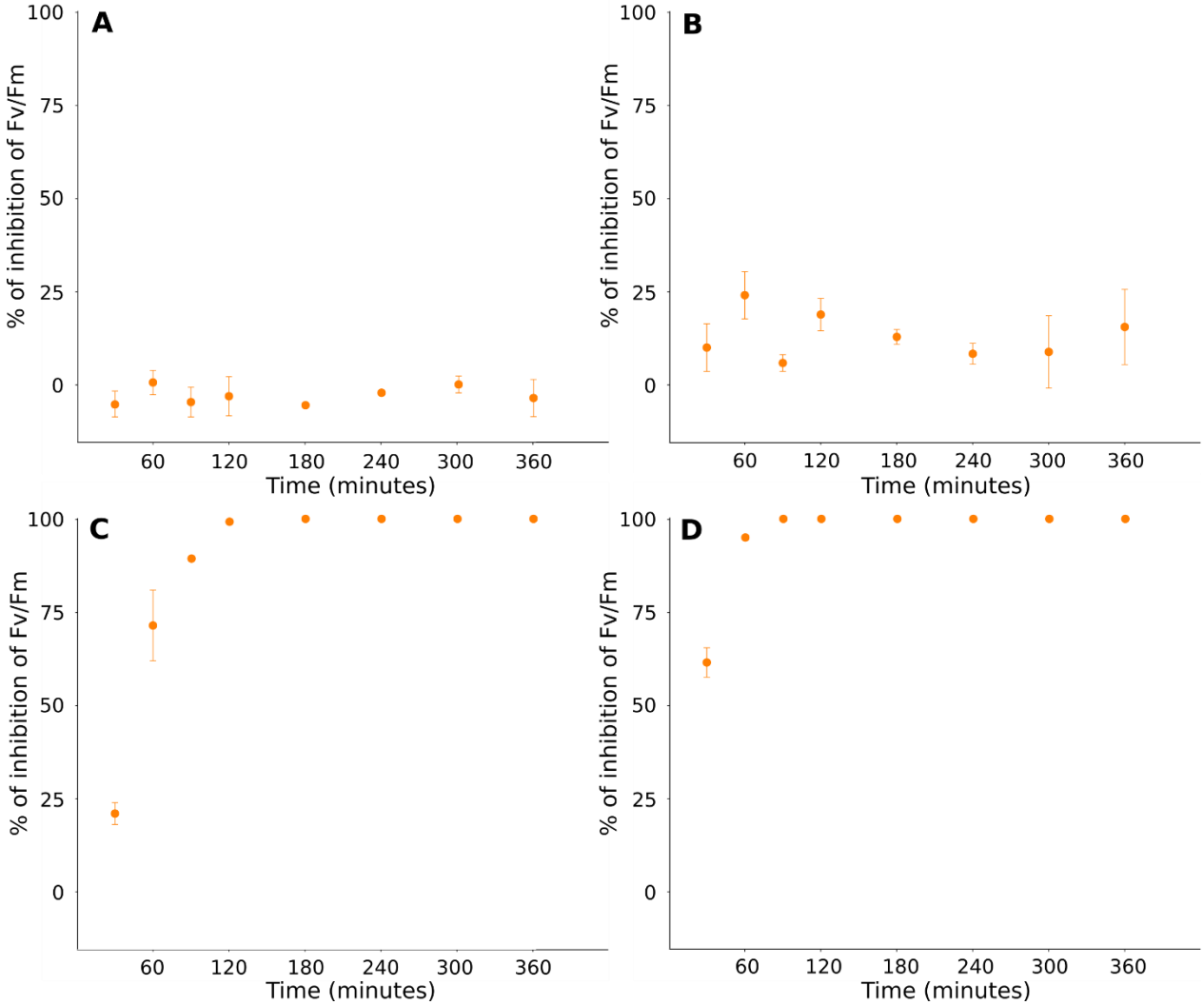


Figure 1: Kinetics of *C. muelleri* Fv/Fm inhibition when exposed to *A. minutum* (CCMI1002) filtrate at different theoretical cell concentrations of A: 50 cells mL^{-1}, B: 500 cells mL^{-1}, C: 5000 cells mL^{-1} and D: 50 000 cells mL^{-1}. Results are expressed as mean ± standard deviation.

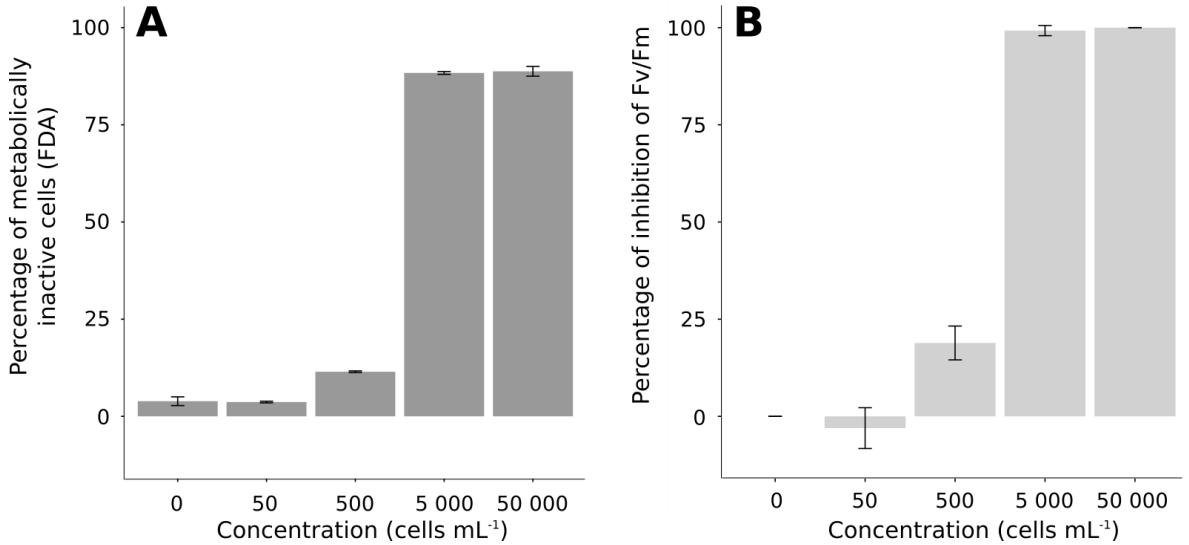


Figure 2: Effects of *A. minutum* (CCMI1002) filtrate on A) the percentage of metabolically inactive cells (FDA) and B) the percentage of inhibition of Fv/Fm of the diatom *C. muelleri* at different theoretical cell concentrations (0, 50, 500, 5 000 and 50 000 cells mL^{-1}) after 120 min of exposure.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

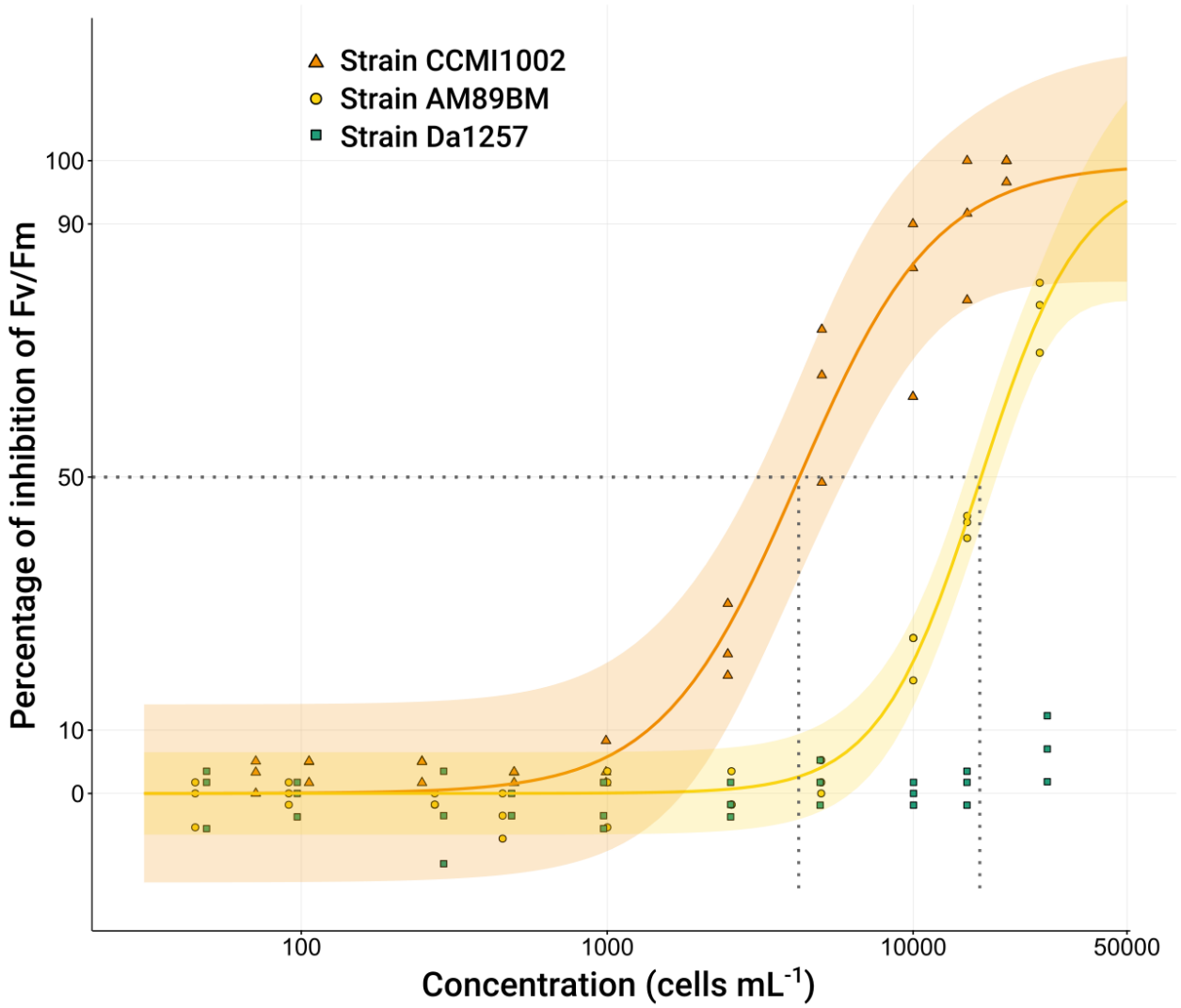


Figure 3: Inhibition of *C. muelleri* maximum photosystem II quantum yield (Fv/Fm) by *A. minutum* filtrates from different strains. Dots represent the percentage of inhibition of Fv/Fm as compared to controls, as a function of filtrate dilution. The diatoms were exposed to strain CCMI1002 (red triangle), 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. EC_{50} are indicated by the dotted lines.

TABLES

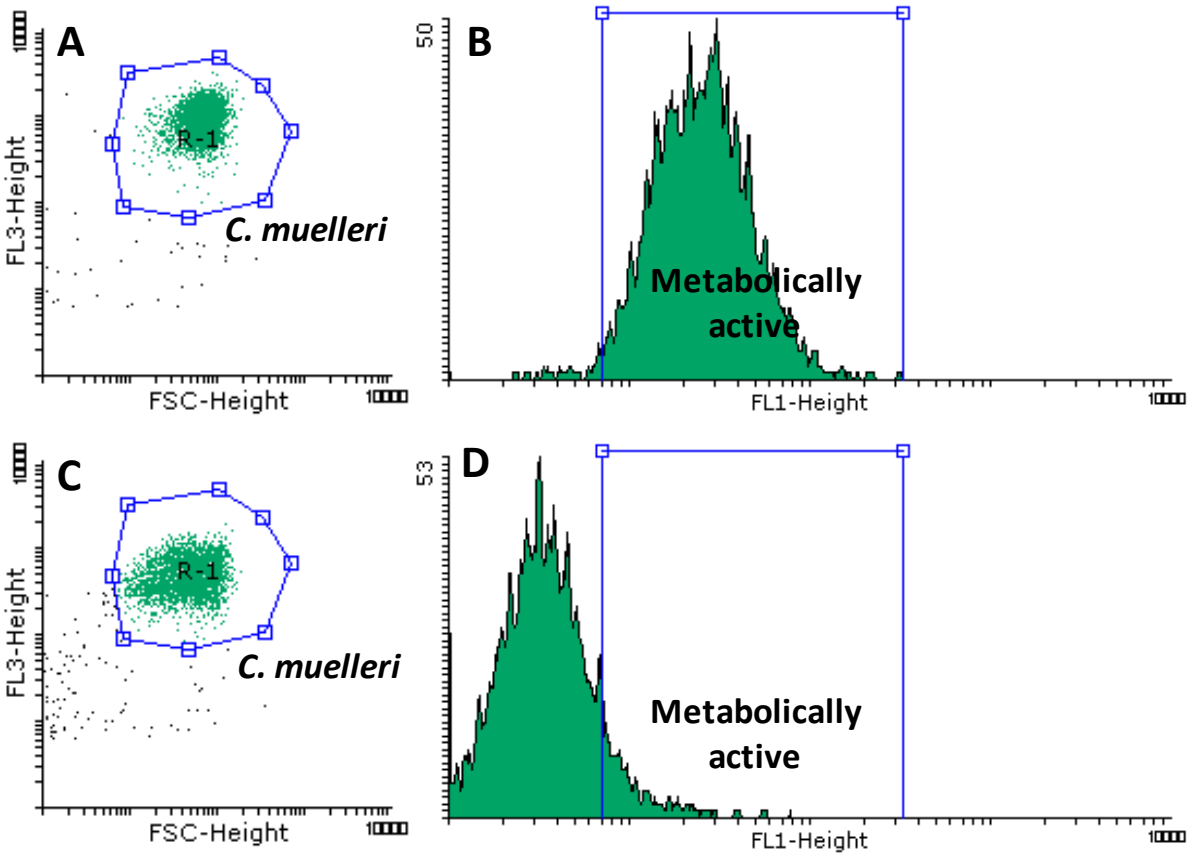
Table 1: Culturing and allelochemical interaction test conditions

Allelopathic sample	
Type of sample	Culture filtrates (0.2µm, acetate cellulose)
Storage	Glass containers, no plastic
Concentration	Series of dilutions
Target cells	
Species	*C. muelleri*
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)

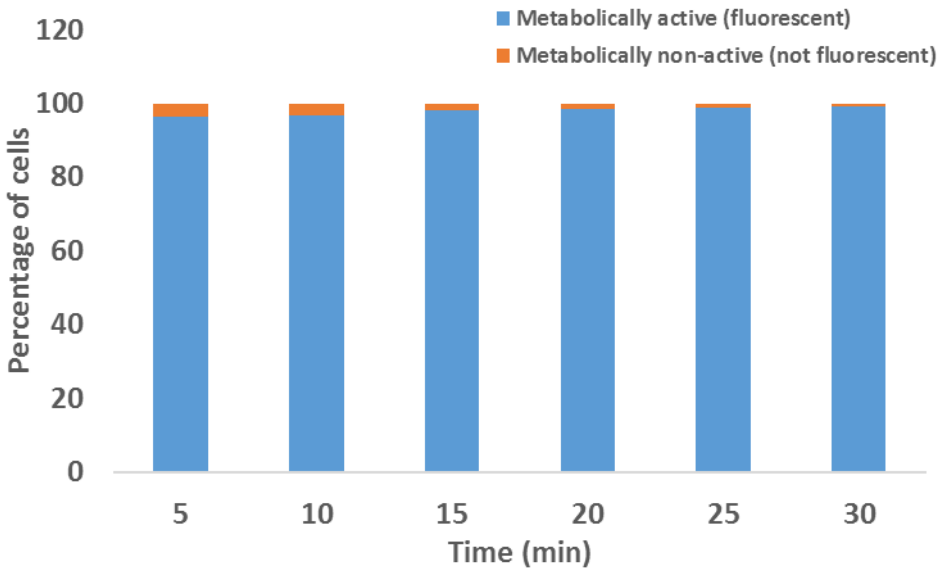
Table 2: Effective filtrate concentrations (cells mL-1) inhibiting 10 % (EC10), 50 % (EC50) and 90 % (EC90) of *C. muelleri* Fv/Fm for three different *A. minutum* strains. "NC" indicate that no value could be calculated. Results are expressed as mean ± standard error (n=3).

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

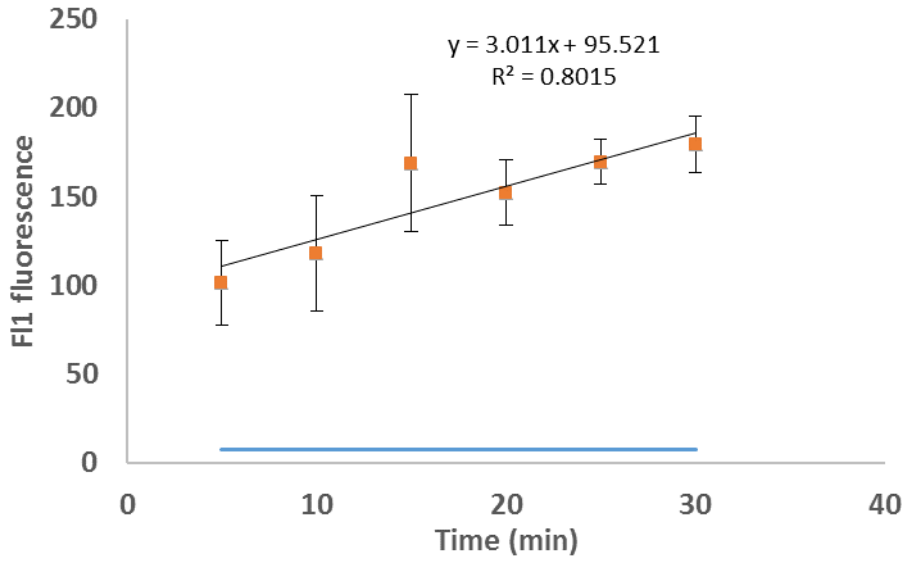
Supplementary/supporting files



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.*

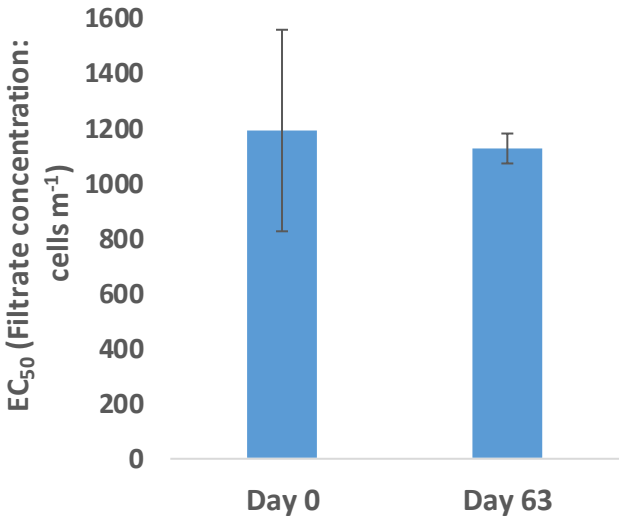


565
566 *Supplementary figure 2: Percentage of metabolically active (fluorescent cells; in blue) and metabolically inactive (not fluorescent;*
567 *in orange) C. muelleri cells stained with FDA for 5, 10, 15, 20, 25 and 30 min (in seawater).*



568
569 *Supplementary figure 3: Mean internal Fl1 fluorescence of C. muelleri stained with FDA for 5, 10, 15, 20, 25 and 30 min (in*
570 *seawater at room temperature). The black line represents the linear model of C. muelleri Fl1 fluorescence as a function of time of*
571 *incubation. The Blue line represents the Fl1 fluorescence levels of C. muelleri (not stained).*

572



573

574 *Supplementary figure 4: Example of the effect of storage on the allelopathic potency of filtrate of A. minutum CCMI1002. The*
575 *allelopathic potency (EC_{50}) of the filtrate was measured on the fresh filtrate (Day 0) and on the filtrate stored for 63 days in a glass*
576 *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*
577 *was performed according to the standard bioassay procedure given in the manuscript. Error bars represent standard error of the*
578 *EC_{50}.*

579