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1 **Allelochemicals of *Alexandrium minutum*: kinetics of membrane**
2 **disruption and photosynthesis inhibition in a co-occurring diatom**

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21

22 **Abstract**

23 Allelopathy is an efficient strategy by which some microalgae can outcompete other species.
24 Allelochemicals from the toxic dinoflagellate *Alexandrium minutum* have deleterious effects
25 on diatoms, inhibiting metabolism and photosynthesis and therefore giving a competitive
26 advantage to the dinoflagellate. The precise mechanisms of allelochemical interactions and the
27 molecular target of allelochemicals remain however unknown. To understand the mechanisms,
28 the short-term effects of *A. minutum* allelochemicals on the physiology of the diatom
29 *Chaetoceros muelleri* were investigated. The effects of a culture filtrate were measured on the
30 diatom cytoplasmic membrane integrity (polarity and permeability) using flow-cytometry and
31 the photosynthetic performance using fluorescence and absorption spectroscopy. Within 10
32 minutes, the unknown allelochemicals induced a depolarization of the cytoplasmic membranes
33 and an impairment of photosynthesis through the inhibition of the plastoquinone-mediated
34 electron transfer between photosystem II and cytochrome b_6/f . At longer time of exposure, the
35 cytoplasmic membranes were permeable and the integrity of photosystems I, II and cytochrome
36 b_6/f was compromised. Our demonstration of the essential role of membranes in this
37 allelochemical interaction provides new insights for the elucidation of the nature of the
38 allelochemicals. The relationship between cytoplasmic membranes and the inhibition of the
39 photosynthetic electron transfer remains however unclear and warrants further investigation.

40

41

42 **Keywords:** allelochemicals, allelopathy, *Alexandrium minutum*, *Chaetoceros*, photosynthesis,
43 membrane

44

45

46 **1 Introduction**

47

48 Phytoplankton is under constant biotic pressures (e.g. grazing, competition, parasitism,
49 virus), however some species can form dense monospecific blooms, indicating the involvement
50 of defensive strategies. Some species are capable of releasing secondary metabolites that
51 directly affect the physiology of co-occurring protists with which they compete for nutrients
52 and thus allow them to proliferate freely. This competitive strategy is called allelopathy (Granéli
53 and Hansen, 2006) and the mediating agents of these interactions are called allelochemicals.
54 Allelochemical interactions not only have the potential of inhibiting competitors (Xu et al.,
55 2015 ; Driscoll et al., 2016) but can also facilitate mixotrophic behavior (Tillmann, 2003 ;
56 Blossom et al., 2012) and mitigate grazing by heterotrophic dinoflagellates (John et al., 2015).
57 Allelochemicals are produced by various microalgal groups including cyanobacteria,
58 dinoflagellates, prymnesiophytes, rapidophytes or diatoms (Legrand et al., 2003), but they have
59 mainly been reported in toxic species responsible for harmful algal blooms. The species
60 responsible for these harmful blooms are particularly studied according to their negative effects
61 on ecosystems, fisheries or human health. The harmful effects usually come from the
62 production of paralytic, amnesic or diarrheic toxins. Understanding the factors that favor the
63 establishment and the persistence of these harmful algal blooms is essential to predict them,
64 and mitigate their consequences on the environment.

65 The understanding of allelochemical interactions is hindered by the poor knowledge of
66 the chemical natures and modes of action of allelochemicals. Most allelochemicals remain
67 uncharacterized. If some of the phycotoxins such as karlotoxins (Place et al., 2012) for example,
68 can mediate allelochemical interactions, it is now accepted that the allelochemicals are not
69 necessarily the phycotoxins produced by harmful species. The known phycotoxins are rarely
70 responsible for the allelochemical potencies (Granéli and Hansen, 2006). Allelochemical
71 interactions are usually reported by their negative effects, but the understanding of their
72 mechanisms remains vague. The physiological outcomes of these interactions are reviewed in
73 (Granéli and Hansen, 2006), and include growth inhibition (Pushparaj et al., 1998; Paul et al.,
74 2009; Suikkanen et al., 2011; Chen et al., 2015; Wang et al., 2017 ; Ternon et al., 2018),
75 damages to cell membranes and cell lysis (Tillmann et al., 2007 ; Prince et al., 2008; Ma et al.,
76 2009; Hakanen et al., 2014; Poulin et al., 2018) and inhibition of photosystem II (PSII)

77 (Tillmann et al., 2007 ; Gantar et al., 2008; Long et al., 2018a; Poulin et al., 2018; Prince et al.,
78 2008; Ternon et al., 2018). Allelochemical interactions are not restricted to a producer – donor
79 interaction, they can alter species composition of the whole community (Hattenrath-Lehmann
80 and Gobler, 2011).

81 The genus *Alexandrium* is well known for its potential to form dense blooms reaching
82 more than 10^7 cells L^{-1} (Garcés et al., 2004 ; Chapelle et al., 2015) with deleterious effects on
83 marine wildlife, resources or humans especially through the production of paralytic shellfish
84 toxins (Cembella et al., 2002 ; Anderson et al., 2012;Álvarez et al., 2019). This genus also
85 displays allelochemical potency, which is probably amongst the most studied one within marine
86 phytoplankton. Allelochemical interactions are not mediated by these phycotoxins nor
87 cycloimines but by some unknown extracellular compounds (Arzul et al., 1999; Tillmann and
88 John, 2002 ; Fistarol et al., 2004; Tillmann et al., 2007, Long et al 2018a, 2018b) that can also
89 affect marine resource such as fish or shellfish (Borcier et al., 2017; Bianchi et al., 2019; Castrec
90 et al., 2020, 2019, 2018). The nature of allelochemicals remains mostly unknown. To date, only
91 one putative compound has been identified (Satake et al., 2019) but its activity still has to be
92 confirmed.

93 Allelochemicals produced by *Alexandrium* spp. induced membrane disruption,
94 modification of membrane biochemistry, inhibition of photosynthesis and esterase activity,
95 increased production of reactive oxygen species (Tillmann et al., 2007 ; Lelong et al., 2011a;
96 Ma et al., 2011; Flores et al., 2012; Long et al., 2018a) in co-occurring protists. However, the
97 sequence of these physiological responses remains unknown, in part because of the use of
98 different algal couples: *Alexandrium minutum*/*Chaetoceros muelleri* (Lelong et al., 2011a;
99 Long et al., 2018a, 2018b), *Alexandrium catenella* (formerly group I of the *A.*
100 *tamarense/fundyense/catenella*)/*Rhodomonas salina* (Ma et al., 2011), *Alexandrium tamarense*
101 *complex*/*Tiarina fusus*; *Alexandrium tamarense* *complex*/*Polykrikos kofoidii* (Flores et al.,
102 2012). For instance, disruption of membranes was shown for *A. catenella* on a cryptophyte (Ma
103 et al., 2011), but was not investigated for *A. minutum*. The inhibition of PSII in allelochemical
104 interactions is probed in a quasi-routine manner simply because PSII activity is easy to measure
105 with the very sensitive and widespread fluorescence techniques. A modification of the integrity
106 of the PSII has been reported for diatoms in the presence of *A. minutum* filtrate (Lelong et al.,
107 2011a; Long et al., 2018b), and in other allelochemical interactions (Hagmann and Jiittner,
108 1996; Tillmann et al., 2007 ; Prince et al., 2008). Allelochemicals could specifically target and
109 inhibit PSII. Alternatively, the inhibition could be an indirect effect resulting from an inhibition

110 elsewhere in the photosynthetic electron transfer chain (ETC) or outside of the plastid. Indeed,
111 the inhibition of any step in the ETC leads to a more reduced ETC, which in turn increases the
112 probability of PSII photo-inhibition.

113 From these observations, several questions arise regarding the mechanism and cellular
114 targets of *A. minutum* allelochemicals. In this frame, this work aims at answering two questions:
115 Do those allelochemicals target first and/or specifically thylakoid membranes and
116 photosynthetic activity? Is the inhibition of photosynthesis a collateral damage following the
117 disruption of cellular membranes including cytoplasmic ones? Therefore, the specific effects of
118 allelochemicals produced by *A. minutum* were investigated on photosystems, photosynthesis
119 activity and cytoplasmic membranes of the common diatom *Chaetoceros muelleri*, co-
120 occurring in the field with *A. minutum* (Chapelle et al., 2014). Allelochemicals were isolated
121 from *A. minutum* cultures by filtration to specifically focus on allelochemical interactions and
122 avoid interference of cell-cell interactions. The short-term effects of a pulse of allelochemicals
123 on photosynthetic and cytoplasmic membranes of *C. muelleri* were investigated by absorption
124 and fluorescence spectroscopy and flow-cytometry, respectively.

125

126 **2 Materials and methods**

127

128 **2.1 Microalgal cultures and reproducibility between laboratories**

129 A strain of *Alexandrium minutum* (strain CCM11002, isolated in Ireland and obtained from the
130 Culture Collection of the Marine Institute of Galway), not producing paralytic shellfish toxins
131 (Castrec et al., 2018), was selected according to its high allelochemical potency (Long et al.,
132 2018a). A strain of *Chaetoceros muelleri* (strain CCAP 1010-3 obtained from the CCAP culture
133 collection, formerly listed as *Chaetoceros neogracile* or *Chaetoceros* sp.) was selected because
134 of its sensitivity to *A. minutum* allelochemicals (Borcier et al., 2017; Lelong et al., 2011a; Long
135 et al., 2018a) and its co-occurrence in the field with *A. minutum* (Chapelle et al., 2014). Cells
136 of *A. minutum* were grown in autoclaved synthetic ocean seawater (Morel et al., 1979)
137 supplemented with L1 (Guillard and Hargraves, 1993) medium (salinity = 35 psu, pH = 8.4).
138 Cultures of the diatom *C. muelleri* were grown in filtered (0.2 μm) natural seawater
139 supplemented with L1 medium (salinity = 35 psu, pH = 8.4) and silicate (1.06×10^{-4} M). The
140 natural seawater used for culturing *C. muelleri* was filtered over a carbon filter to retain organic
141 compounds that could possibly inhibit microalgal growth and interfere with *A. minutum*
142 allelochemicals in this study. All cultures were maintained under exponential growth under a

143 12/12 h light/dark cycle in similar conditions: at 18°C at a light intensity of 30 $\mu\text{mol photon m}^{-2}$
144 s^{-1} (LUMILUX T8 L 36W/965 BIOLUX) in Brest laboratory, and at 19°C at a light intensity
145 of 38 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (OSRAM L 30W/640) in Paris laboratory. Cultures were not axenic
146 but were handled under sterile conditions to minimize additional bacterial contamination.

147 Despite slight differences in the growth conditions, the reproducibility of the photosynthesis
148 performances between the two laboratories was verified. Indeed, the light dependency of
149 photosynthesis, with and without *A. minutum* filtrate, was measured both in Paris and in Brest.
150 No significant difference was observed, confirming that the effect of the supernatant of *A.*
151 *minutum* on the photosynthetic physiology of *C. muelleri* was the same in both laboratories.
152 The photosynthesis (ETR_{PSI} , Φ_{PSI} , ETR_{PSII} , Φ_{PSII} , photochemical rate) versus intensity data
153 presented in the manuscript are averages of measurements made in the two laboratories. In-
154 depth photosynthesis analysis and flow cytometry investigation of cytoplasmic membranes
155 were then performed in Paris and Brest, respectively. All the raw data used in this manuscript
156 are available at <http://www.ibpc.fr/UMR7141/>

157

158 **2.2 Counts of microalgal cells and preparation of filtrate**

159 Counts of microalgal cells were performed using a cell counter (Beckman Coulter, Z2 Cell and
160 Particle Counter) or a FACScalibur (BD Biosciences, San Jose, CA, USA) flow-cytometer
161 equipped with a 488 nm argon laser, red (red emission filter long pass, 670 nm) and green
162 (green emission filter band pass, 530/30nm) fluorescence detectors. Counts with the flow-
163 cytometer were estimated according to flowrate (Marie et al., 1999). Cell variables, e.g. forward
164 scatter (Forward scatter, FSC), side scatter (Side scatter, SSC) and red auto-fluorescence were
165 used to select microalgal populations.

166 Filtrates were prepared with 0.2 μm syringe filters (Minisart, 16534-K, Sartorius) with an
167 acetate cellulose membrane to avoid any loss of allelochemicals on the membrane (Lelong et
168 al., 2011a). The filtrate concentrations were expressed as cell concentration (cells ml^{-1}) based
169 on the initial culture concentration prior to filtration.

170

171 **2.3 Inhibitors**

172 To study the photosynthetic transient, several inhibitors were used. 3-(3,4-dichlorophenyl)-1,1-
173 dimethylurea (DCMU) is an inhibitor of the electron transfer to plastoquinone in the Q_B pocket
174 of PSII, 2,5-dibromo-3-methyl-6-isopropyl benzoquinone (DBMIB) is a quinone analogue,

175 which inhibits the cytochrome *b₆f* by competitive inhibition at the Q₀ site and carbonyl cyanide
176 m-chlorophenyl hydrazone (CCCP) is an uncoupler that inhibit that suppress trans-thylakoidal
177 proton motive force (pmf). DCMU, DBMIB and CCCP were obtained from Sigma-Aldrich and
178 dissolved in ethanol. Hydroxylamine (HA) is an artificial electron donor to PSII, whereas
179 methylviologen (MV) is an artificial PSI electron acceptor. Both HA and MV were dissolved
180 in water. DCMU, DBMIB, MV and CCCP were used at a final concentration of 10 μM, 0.5
181 μM, 1 mM and 100 μM, respectively. The combination of 15 μM DCMU and 150 μM HA were
182 used to fully suppress PSII activity (Bailleul et al., 2010), because those concentrations
183 suppressed all the variable fluorescence.

184

185 **2.4 Photosynthesis measurements**

186 For photosynthesis measurements, cultures of *C. muelleri* in exponential growth phase, at 1-2
187 10⁶ cells mL⁻¹, were concentrated by centrifugation (4500 rpm, 4 min) and resuspended in its
188 own supernatant to reach a final concentration in the range of 5 10⁶ to 10⁷ cells mL⁻¹. The
189 centrifuged samples were then left ~30 minutes to allow the cells to recover from centrifugation.
190 The filtrate of *A. minutum* was obtained from an initial culture within the range of 18 10³ to 22
191 10³ cells mL⁻¹. After adding the filtrate of *A. minutum* (or the filtrate of *C. muelleri* for the
192 control), flasks were incubated for 10 minutes prior to measurements (otherwise stated).

193

194 For measurements of photosynthetic parameters, a Joliot-type spectrophotometer (JTS-10,
195 Biologic, Grenoble, France) equipped with a white probing LED (Luxeon; Lumileds) and a set
196 of interference filters (3–8 nm bandwidth) was used. The device combines absorbance and
197 fluorescence spectroscopy measurements, allowing us to study in the same conditions, on the
198 same sample, the activities of the two photosystems independently, as well as the
199 photochemical rate (which reflects the additive activities of both photosystems, see below), the
200 redox changes of the electron carriers or the pmf. A schematic view of the photosynthetic
201 measurements performed in this work are presented in (Fig. 1). The actinic light was provided
202 by a crown of red-light emitting diodes (LED) (619 nm) or by a dye laser at 690 nm. The red
203 actinic light was chosen to correspond to the minimum of absorption in diatoms and ensure
204 homogeneous light condition in the cuvette. In the fluorescence spectroscopy mode, the JTS-
205 10 was equipped with a white probing LED (Luxeon; Lumileds) and a blue filter (470 nm) for
206 detecting pulses. The cut-off filter on the measuring photodiode is a LPF650 + RG 665 from
207 Schott (Mainz, Germany) and on the reference side is a BG39 filter from Schott. In the

208 absorption mode, the cut-off filters were the same on measure and reference photodiodes (BG39
209 for ECS measurements and a high pass RG695 for P700 measurements). In absorption mode,
210 the absorption changes induced by the measuring flashes alone (in the absence of actinic light)
211 were subtracted from those recorded in presence of actinic light for every trace to eliminate
212 small artifacts due to measuring flashes.

213

214 *Fluorescence spectroscopy.* PSII parameters were calculated as described in (Genty et al.,
215 1989). Maximum quantum yield of PSII was calculated as $F_v / F_m = (F_m - F_0) / F_m$, where F_0 is
216 the fluorescence of the dark-adapted (1 minute) sample and F_m the fluorescence when a
217 saturating pulse is applied on dark-adapted sample. Quantum yield in light-adapted samples of
218 PSII (Φ_{PSII}) was calculated as $\Phi_{PSII} = (F_m' - F_{stat}) / F_m'$, where F_{stat} is the fluorescence of the sample
219 adapted to the actinic light and F_m' the fluorescence when a saturating pulse is applied on light-
220 adapted sample. The electron transport rate through PSII (ETR_{PSII}) was normalized to the
221 maximal value in the control at $800 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ to focus on filtrate-induced changes
222 and corrected for variations in the initial photosynthetic physiology of *C. muelleri*. It was
223 calculated as $ETR_{PSII} = \Phi_{PSII} * I / (\Phi_{PSII-800} * 800)$, where I is the actinic light irradiance.

224 Fluorescence induction kinetic was performed at room temperature using a DeepGreen
225 Fluorometer (JbeamBio, France) with samples that were dark-adapted for 1 minute before
226 measurements (Rappaport et al., 2007).

227

228 *P700 measurements.* For photosystem I (PSI) parameters, the redox state of the PSI primary
229 donor (P_{700}) was calculated as the difference between the absorption changes kinetic at 705 nm
230 and 735 nm, to eliminate spectrally flat contributions due to diffusion. Properties of PSI were
231 calculated from the measurements of the absorption changes in the dark (P_0), in the light-
232 adapted condition (P_{stat}) and after a saturating pulse (P_{sp}). The quantum yield of PSI (Φ_{PSI}), the
233 donor side ($Y(ND)$) and acceptor side ($Y(NA)$) limitations were then calculated according to
234 (Klughammer and Schreiber, 2008).

235
$$\Phi_{PSI} = (P_{sp} - P_{stat}) / (P_{max} - P_0)$$

236
$$Y(ND) = (P_{stat} - P_0) / (P_{max} - P_0)$$

237
$$Y(NA) = (P_{max} - P_{sp}) / (P_{max} - P_0)$$

238 All data were normalized to $P_{max} - P_0$ (corresponding to 100% oxidized P_{700}), P_{max} being the
239 absorption change after a saturating pulse in the presence of the PSII inhibitor DCMU. The

240 electron transport rate through PSI (ETR_{PSI}) was normalized to the maximal value of the control
241 at $800 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ (like for ETR_{PSII}) and calculated as $ETR_{PSI} = \Phi_{PSI} * I / (\Phi_{PSI-800} * 800)$,
242 where I is the actinic light irradiance.

243

244 *Measurement of linear and quadratic ECS spectra.* To obtain the spectra of the linear and
245 quadratic Electro-Chromic Shift (ECS) signals in *C. muelleri*, a strong electric field was
246 generated by shining a 10 ms saturating pulse (red light, 619 nm, $6000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$).
247 Then the approach described in (Bailleul et al., 2015) was used to deconvolute the two ECS
248 components during the relaxation of the electric field. In brief, the kinetics of the absorption
249 changes following the pulse was followed, removing the first 50 ms after the pulse (additional
250 signals like the ones associated to the redox changes of the c-type cytochromes are then
251 relaxed). The ECS decay kinetics was then fitted as a sum of a linear and a quadratic component,
252 which allowed to reconstruct the two spectra. Cells were treated with $100 \mu\text{M}$ of the uncoupler
253 CCCP to ensure that no trans-thylakoidal electric field was present in the dark-adapted sample,
254 prior to the pulse.

255 *Determination of the appropriate wavelengths for measurements.* The linear and quadratic ECS
256 spectra revealed that 520 nm and 564 nm are convenient wavelengths, where only linear and
257 quadratic signals contribute to ECS, respectively. On that basis, a three wavelengths
258 deconvolution was performed to obtain contributions from linear ECS, quadratic ECS and c-
259 type cytochromes (Cyt c). The latter was calculated as:

260 $\text{Cyt c} = [554] - 0.35 * [520] - 0.5 * [564]$, where [554], [520] and [564] are the absorption
261 difference signals at 554 nm, 520 nm and 564 nm, respectively.

262 Then, the linear ECS (ECS_{lin}) was calculated as $ECS_{lin} = [520] - 0.25 * \text{Cyt c}$, and the
263 quadratic ECS (ECS_{quad}) was calculated as $ECS_{quad} = [554] + 0.15 * \text{Cyt c}$.

264

265 *Flash-induced ECS kinetics.* The ECS kinetics following a single turnover of both photosystems
266 were measured using a 6 ns saturating laser flash provided by a dye laser at 690 nm. Then the
267 kinetics of flash-induced ECS and cytochromes redox changes were calculated using the
268 deconvolution procedure described above. The linear ECS signal at the first point (measured
269 shortly -160 μs - after the flash) provides the contribution to the electric field of 1 charge
270 separation per photosystem (used for normalization, see below). The rate of re-reduction of c-

271 type cytochromes and the rate of ECS decay were calculated as the time constant of the mono-
272 exponential extrapolation of the c-type cytochromes and ECS relaxation kinetics, respectively.

273

274 *Photochemical rates.* The photochemical rate (rate at which PSI and PSII perform charge
275 separations) was calculated from ECS measurements as described in (Bailleul et al., 2010).
276 Briefly, under steady-state illumination, the ECS signal results from positive contributions to
277 the transmembrane potential from PSII, the cytochrome *b₆f* complex and PSI (which contribute
278 to the net movement of positive charges from the stroma to the lumen, increasing the electric
279 field), as well as the negative one by the chloroplastic ATP synthase (moving protons out of the
280 lumen). Because only PSI and PSII use light as a substrate, the change of slope of the ECS
281 immediately after turning off the light is a direct measurement of the photochemical rate, i.e.
282 the rate at which PSI and PSII were performing charge separations under illumination. The
283 slope of ECS was measured for the first 5 ms after light offset and then normalized by the laser
284 flash induced ECS increase, providing an expression of the photochemical rate in charge
285 separations per photosystem per second (charge sep. PS⁻¹ s⁻¹). Like for ETR_{PSII} and ETR_{PSI}, data
286 were normalized to the maximal value in the control at 800 μmol photons m⁻² s⁻¹.

287

288 **2.5 Flow-cytometric (FCM) measurements**

289 The *A. minutum* filtrate (from a culture at 20 000 ± 2 000 cells mL⁻¹) used for flow-cytometry
290 was checked for some photosynthesis measurements in parallel, to ensure that those
291 experiments were made in the same conditions of inhibition than the ones focusing on the
292 mechanism of photosynthetic inhibition. For FCM measurements, cultures of *C. muelleri* were
293 diluted 10 times with their own filtrate to reach a final concentration around 200 000 cells mL⁻¹.
294

295 *Cytoplasmic membrane potential.* Modifications of the cytoplasmic membrane potential were
296 assessed using an EasyCyte Plus cytometer (Guava Technologies, Millipore, Billerica, MA,
297 USA) equipped with a 488-nm argon laser and red (680, 30 nm bandwidth) and green (525 nm,
298 30 nm bandwidth) fluorescence detectors. To estimate the relative depolarization of
299 cytoplasmic membranes, cells were stained with Bis-(1,3-Dibutylbarbituric Acid) Trimethine
300 Oxonol, DiBAC₄(3) (Molecular Probes, Eugene, OR, USA) for 10 min at a final concentration
301 of 0.97 μM. DiBAC₄(3) is a fluorescent probe emitting at 516 nm that can enter cells with
302 depolarized cytoplasmic membranes (Seoane et al., 2017). Once inside the cells, DiBAC₄(3)

303 binds to intracellular proteins and membranes. The amount of protein-bound DiBAC₄(3) is
304 dependent on the cytoplasmic membrane potential (Bräuner et al., 1984). Cells with an
305 increased depolarization (increase in the membrane potential) of their cytoplasmic membrane
306 exhibit an increase in their internal fluorescence (proportional to the accumulation of dye within
307 the cell). To obtain a positive control (cells with depolarized membranes), *C. muelleri* cell
308 membranes were depolarized with digitonin (30 min incubation, final concentration of 40 µg
309 mL⁻¹), a weak non-ionic detergent (Prado et al., 2012). The relative depolarization of
310 membranes was expressed as the mean fluorescence value of the cell population. After checking
311 the homoscedasticity and the normality of data, the significant differences in the DiBAC₄(3)
312 fluorescence between *C. muelleri* from the control or exposed to the filtrate, for 10 min or 30
313 min, were compared with a Student t-test.

314

315 *Membrane permeability.* Measurements of membrane permeability were performed on a
316 FACSCalibur flow-cytometer. The membrane permeability was assessed with SYTOXGreen
317 (Molecular Probes, Eugene, OR, USA), a fluorescent probe (523 nm) that cannot cross cell
318 membranes unless membrane integrity is compromised according to manufacturer and several
319 studies (Brussaard et al., 2001; Lelong et al., 2011b; Naghdi et al., 2016). Once inside the cell,
320 the probe binds to DNA and forms a fluorescent dye. Thus, only cells that lost their membrane
321 integrity (i.e. became permeable) become fluorescent. Samples were stained with
322 SYTOXGreen for at least 10 min at a final concentration of 0.05 µM (Koppel et al., 2017).
323 Membrane permeabilization was expressed as the percentage of fluorescent cells. After
324 checking for homoscedasticity and normality of data, the significant differences in the
325 percentage of cells permeable to SYTOXGreen between *C. muelleri* from the control or
326 exposed to the filtrate were compared with a Student t-test.

327 **3 Results**

328

329 ***3.1 A strong inhibition of photosynthetic activity by A. minutum filtrate***

330 In order to assess the effect of *A. minutum* filtrate on the photosynthetic activity of *C. muelleri*,
331 the overall activity of photosynthesis was first measured at different irradiances. This was
332 performed using the electro-chromic shift (ECS) of photosynthetic pigments (Witt, 1979). In
333 *C. muelleri*, like in other diatoms (Bailleul et al., 2015) and pinguiphytes (Berne et al., 2018),
334 the ECS is the sum of a linear and a quadratic component, which have been deconvoluted as

335 described in the Methods section (Fig. 2A and B). The deconvolution allowed the extraction of
336 pure linear and quadratic components, as demonstrated in (Fig. 2C).

337 The linear ECS is proportional to the electric field generated by the photosynthetic process and
338 can be used to measure the overall photosynthetic activity (Bailleul et al., 2010). Indeed, the
339 change of slope of the ECS immediately after turning off the light (Fig. 3A) was a direct
340 measurement of the photochemical rate (see Material and Methods, 2.4). In the control, the
341 photochemical rate increased with light irradiance (Fig. 3B), as expected, and did not reach
342 saturation at $800 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ in agreement with the spectral characteristics of the
343 illumination (low light absorption by diatoms, see Material and Methods, 2.4). In the presence
344 of *A. minutum* filtrate, the slope of the ECS after turning off the light was drastically reduced
345 (Fig. 3A), resulting in an almost full inhibition of *C. muelleri* photochemical rates at all light
346 intensities (Fig. 3B).

347 The very strong inhibition of the photochemical rate by the filtrate of *A. minutum* suggested
348 that both the activities of the linear electron flow (from water to PSI acceptors) and the cyclic
349 electron flow around PSI ((Shikanai, 2007) and see Fig. 1), a process whose extent is still
350 debated in diatoms (Falciatore et al., 2020), were suppressed by the allelochemicals of *A.*
351 *minutum*. To further confirm this hypothesis, the activities of PSII and PSI in the diatom (Fig.
352 4) were measured under the same illumination conditions. The activity of PSII reflects the
353 efficiency of the linear process only, whereas PSI activity is the sum of the linear and cyclic
354 pathways. The quantum yield of PSII (Φ_{PSII}) was measured with fluorescence spectroscopy and
355 the quantum yield of PSI (Φ_{PSI}) with P700 absorption measurements (Fig. 4A and B, see
356 Methods). As expected, both the quantum yields of PSII (Fig. 4C) and PSI (Fig. 4D) decreased
357 with light irradiance in the control sample, because of the progressive reduction of PSII
358 acceptors and oxidation of PSI donors with increasing light (Falkowski and Raven, 2007).
359 However, at all light irradiances, the quantum yields of both photosystems were significantly
360 lower in the presence of *A. minutum* filtrate (Fig. 4C and D). In the control, the light dependence
361 of the electron transfer rate through PSII (ETR_{PSII} , Fig. 4E) and photochemical rate (Fig. 3B)
362 were very similar. The filtrate of *A. minutum* almost fully inhibited ETR_{PSII} , which confirmed
363 that it hampered the linear electron flow. PSI electron flow rate (ETR_{PSI}) was also almost fully
364 suppressed in the presence of filtrate of *A. minutum* allelochemicals (Fig. 4F). This confirmed
365 that both linear and cyclic electron flow rates were very low in the presence of *A. minutum*
366 filtrate.

367

368 **3.2 The first target of *A. minutum* was a non-photochemical step of photosynthesis.**

369 Such a complete suppression of photosynthetic activity under steady state illumination could
370 be due to the specific inhibition of any of the photosynthetic steps, i.e. at the level of
371 photosynthetic complexes (PSII, PSI, cytochrome b_6/f , ATPase), or an impairment of the
372 diffusion of the electron carriers transferring electrons from one photosynthetic complex to
373 another (e.g. plastoquinones or cytochrome c_6) or an inhibition of the Calvin-Benson-Bassham
374 cycle. One could imagine that the photosystems themselves were inhibited by the
375 allelochemicals. Indeed, an inhibition of the maximal quantum yield of PSII (F_v/F_m) was shown
376 to occur within 30 minutes in these conditions (Long et al., 2018b) and this inhibition was even
377 proposed as a bioassay for these allelopathic interactions (Long et al., 2018a). In agreement
378 with that, a strong inhibition of both PSII and PSI activities was observed at the end of the
379 measurements, in the exposed samples only. The F_v/F_m decreased by ~50 % and the total
380 amount of photo-oxidizable P700 (P_{max}) decreased by ~75 % at the end of the experiment (Sup
381 Fig. 1). However, neither PSI nor PSII activities were inhibited at the beginning of the
382 experiment (highlighted by the similar yields of PSII and PSI in the dark in absence or presence
383 of *A. minutum* filtrate; Fig. 4C and D), whereas the inhibition of the photosynthetic activity in
384 the light occurred immediately, from the first light step, as observed for the photochemical rate,
385 ETR_{PSII} or ETR_{PSI} (Fig. 4E and F). In order to confirm that the suppression of the photosynthetic
386 activity was not due to photosystems inhibition, another experiment was performed to measure
387 the quantum yield of both PSI and PSII in the dark and in moderate light, with the two
388 measurements performed in close succession. This experiment showed that the activities of
389 both PSI and PSII in the presence of light were already suppressed in the presence of *A. minutum*
390 filtrate (Fig. 5A) in conditions where PSI and PSII integrity (maximal efficiencies) were
391 unaffected (Fig 5B), demonstrating that the *A. minutum* filtrate inhibited photosynthetic activity
392 by targeting a non-photochemical step first.

393

394 **3.3 The photosynthetic inhibition occurred between PSII and PSI**

395 Our results lead to two possibilities. The allelochemicals could have affected the electron
396 transfer from Q_A , the first quinone acceptor in PSII, to P700 at the PSI level - this part of the
397 photosynthetic chain provides electrons to PSI and is therefore usually referred to as the donor
398 side of PSI. Alternatively, they could have modified the electron transfer from ferredoxins to
399 the carbon fixation in the Calvin-Benson-Bassham cycle and other alternative pathways - this
400 part of the photosynthetic chain is fueled by electrons from PSI and is therefore usually referred

401 to as the acceptor side of PSI. To distinguish between these two possibilities, the acceptor
402 (Y(NA)) and donor (Y(ND)) side limitation of PSI (see Methods) were measured under the
403 same illumination. The data clearly showed that the decrease of PSI quantum yield (Φ_{PSI}) was
404 due to an increase in the donor side limitation in the treated sample (Fig. 5A). In other terms, a
405 lower proportion of PSI centers was open (and participating to electron transfer) because of a
406 higher percentage of oxidized P700 in the light (P700⁺, ~70% in the treated sample vs ~20% in
407 the control at the highest intensity). This indicated that the inhibition occurred between PSII
408 and PSI, and not on the acceptor side of PSI. To further confirm this, the artificial PSI electron
409 acceptor, methylviologen (MV, or paraquat), was used to release acceptor side limitation by
410 providing an efficient sink to electrons from PSI. In agreement with the Y(ND) and Y(NA)
411 measurements, the addition of 1 mM MV did not modify the activities of PSII or PSI, neither
412 in the control nor in the treated sample (Fig. 5A). Together, these results indicated that the first
413 site of inhibition of the photosynthetic electron transfer chain took place between Q_A and P700.
414

415 ***3.4 The first photosynthetic target of *A. minutum* was the thylakoid membrane (electron*** 416 ***transfer from PSII to b₆f)***

417 To determine the primary target of the unknown secondary metabolite(s) produced by *A.*
418 *minutum*, the ECS kinetics following a saturating laser flash (Fig. 6) was used to probe the
419 activity of each photosynthetic complex (PSI, PSII, b₆f, ATPase). Indeed, the photosystems and
420 cyt b₆f participate in the generation of the electric field, by moving protons (positive charges)
421 inside the lumen, whereas the ATPase neutralizes this electric field by moving the protons back
422 to the stroma (Bailleul et al., 2010). The redox changes of the c-type cytochromes (cytochrome
423 *f* and cytochrome c₆) were also measured as described in Methods. The results are shown in
424 (Fig. 6) for diatom cells alone or 10 min after adding *A. minutum* filtrate. After a saturating
425 laser flash, three distinct phases are typically observed (Joliot and Delosme, 1974).

426 i) Right after the flash (here 160μs) all photosystems I and II perform one (and only
427 one) charge separation (Fig. 6A), increasing the electric field by as much (“a
428 phase”). The similar “a phase” in control and exposed cells confirmed that the
429 photochemical events at PSII and PSI level were not the direct targets of the
430 allelopathic compound(s) (first time point after the flash, panel A). These charge
431 separations corresponded to the electron transfer from water to the Q_A pocket in PSII
432 and from P700 to ferredoxins at the PSI level. At the same time point, a similar

433 oxidation of the c-type cytochromes (cytochromes c_6 in the lumen and cytochrome
434 f) was observed in control and exposed cells (panel B). This means that regardless
435 of the exposure to the filtrate or not, the transfer from c-type cytochromes to P700
436 was faster than 160 μ s.

437 ii) The second phase (“b phase”, ~10 ms) corresponds to the electron transfer from
438 plastoquinols to c-type cytochromes catalyzed by the cytochrome b_6f (Fig. 6B). The
439 filtrate did not affect this phase, since the re-reduction of c-type cytochromes was
440 not significantly modified by the filtrate. The proton pumping activity of the
441 cytochrome b_6f , the “b phase” should have given a second rise of the electric field
442 (Joliot and Delosme, 1974) but here this phase was partly hidden by the fast activity
443 of the ATPase.

444 iii) The last phase of ECS kinetics (“c phase”) corresponds to the consumption of the
445 flash-induced proton motive force (pmf) by the ATPase, resulting in the relaxation
446 of the ECS (Fig. 6C). This phase did not seem to be affected by *A. minutum* filtrate.

447

448 The quadratic ECS increase right after the flash was significantly reduced in the exposed cells,
449 suggesting that the pmf in the dark was affected by *A. minutum* filtrate. This aspect will be
450 discussed later (see Discussion). The unaffected oxidation and reduction of c-type cytochromes
451 revealed together a normal electron transfer from the plastoquinol in the Q_O pocket of the
452 cytochrome b_6f to P700. These results, together with the previous results indicating that the
453 limitation occurred between Q_A and P700, pointed to a limitation of the electron flow between
454 the Q_A pocket in PSII and the Q_O pocket in b_6f .

455 To confirm this and get further insight into the mechanism of inhibition, the fluorescence
456 induction and relaxation kinetics were measured (see Methods). The protocol consisted in the
457 measurement of the F_0 parameter in the dark, followed by 500 ms of low light illumination
458 (fluorescence reaches F_{stat}). Then a saturating pulse was applied to reduce all plastoquinones
459 and quinone pockets (F_m is measured) and the kinetics of relaxation of the fluorescence is
460 measured in subsequent darkness. The latter phase of fluorescence relaxation (from F_m to F_0)
461 reflected the re-oxidation of Q_A , i.e. the electron transfer from this quinone pocket in PSII to
462 the oxidized components of the ETC at the end of the saturating pulse, i.e. cofactors in the
463 cytochrome b_6f , cytochromes c_6 or P700. The slowing down of this phase after exposition to
464 the filtrate therefore confirmed that the treatment with *A. minutum* supernatant impaired the
465 electron transfer from Q_A to PSI donors (Fig. 7A). The phase of fluorescence increase was more

466 informative. When the light was turned on in the control, fluorescence increased in <100 ms
467 and reached a steady state value before the saturating pulse was applied. In the presence of the
468 filtrate, after a first increase, which was similar to the one observed in the control, a second
469 increase of fluorescence occurred in the 100 to 400 ms time range, which reflected an over-
470 saturation of the ETC (Fig. 7A). The increase kinetics could be compared to the one of the
471 control with DCMU (10 μ M), an inhibitor of Q_B pocket of PSII, and DBMIB (0.5 μ M), an
472 inhibitor of the cytochrome b_6f . In both cases, a strong increase of the stationary fluorescence
473 was observed, but the kinetics were different: in DCMU conditions, PSII can perform only one
474 charge separation before Q_A is fully reduced and reaches F_m very fast, whereas in DBMIB
475 conditions, Q_B and the whole plastoquinol pool must be reduced before Q_A becomes fully
476 reduced and fluorescence reaches F_m with a slower kinetics. The comparison between the
477 fluorescence induction curve in the presence of the filtrate and in the presence of DCMU
478 indicated that the inhibition of photosynthesis was not at the Q_B level; the effect of the filtrate
479 is indeed closer to the one of DBMIB (Fig. 7B). This experiment ruled out the possibility that
480 the inhibition step is in vicinity to PSII and therefore points towards an inhibition at the level
481 of the PQ pool and/or its interaction with the cytochrome b_6f .

482 Altogether, at short time of exposure (10 min) with *A. minutum* filtrate, PSII and PSI were fully
483 functional (Fig. 4 and Fig. 5) and the electron transfer from Q_0 in the b_6f to P_{700} was unaffected
484 (Fig. 5). Simultaneously, the overall photosynthetic activity (measured via three different
485 techniques: fluorescence P700 or ECS measurements) was decreased by 80-90%. These results
486 pointed out a primary target of *A. minutum* on the photosynthetic apparatus of *C. muelleri* at
487 the level of the electron transfer between Q_B in PSII and Q_0 in b_6f , i.e. the diffusion of the
488 plastoquinol/plastoquinone in the thylakoid membrane or its docking to/release from the
489 cytochrome b_6f . This conclusion is in agreement with the rise of fluorescence kinetics at the
490 onset of light, as well as the re-oxidation of Q_A kinetics.

491

492 ***3.5 A degradation of the whole photosynthetic apparatus was observed at longer incubation*** 493 ***times***

494 To get a more complete view of the time response of the photosynthetic apparatus to the
495 exposure to *A. minutum* filtrate, the flash-induced ECS and c-type cytochrome redox kinetics
496 were measured at different exposure times (10, 40 and 90 minutes), in parallel to photochemical
497 rate measurements (Fig. 8). In the control cells, the flash-induced ECS kinetics and the

498 photochemical rate were the same at the three time points, which indicated that the overall
499 photosynthetic characteristics and performance were constant over time. Again, 10 min
500 exposure did not affect the photosystem capacities (« a phase » of ECS, Fig. 8E), the oxidation
501 and reduction of c-type cytochromes (Fig. 8B and C) or the activity of the ATPase (ECS
502 relaxation, Fig. 8F). Only the photochemical rate (Fig. 8A) and the quadratic-to-linear ratio
503 (Fig. 8D, see Discussion) were strongly reduced. At longer time of exposure (40 and 90 min),
504 the photochemical rate (Fig. 8A) and quadratic-to-linear ratio (Fig. 8D) remained lower in the
505 treated cells, whereas a decrease of PSI and PSII efficiencies (Fig. 8C and E) appeared, in
506 agreement with previous results based on fluorescence and P700 measurements (Sup Fig. 1).
507 The rate of re-reduction of c-type cytochromes, which reflected the rate of b_6f turnover, also
508 decreased after 40 min of exposure to *A. minutum* filtrate (Fig. 8C). These results highlighted
509 an overall degradation of the photosynthetic apparatus (PSI, PSII, b_6f) as time of exposure
510 increases.

511

512 **3.6 A modification of membranes**

513 The inhibition of the electron transfer chain was due to a problem in the quinone-mediated
514 electron transfer between Q_B to the quinone-binding site Q_O in the cytochrome b_6f in the
515 thylakoid membrane. Given that allelochemicals from *A. catenella* caused the disruption of
516 membranes that led to lytic activity (Ma et al., 2011), a disruption of membranes could also
517 take place with *A. minutum* allelochemicals. In other words, are *A. minutum* allelochemicals
518 disrupting all cellular membranes? Flow-cytometry analysis revealed that *A. minutum* filtrate
519 induced a strong depolarization of cytoplasmic membranes of more than 95% of *C. muelleri*
520 cells within only 10 min (Fig. 9A). Only metabolically active cells with no damages to
521 membranes can maintain a normal membrane potential (Jepras et al., 1997; Prado et al., 2012).
522 Depolarization of membranes can originate from membrane permeabilization or from an
523 inhibition of the energy metabolism. This strongly suggests that the depolarization of
524 membranes represents an early signal of membrane disruption as the uncoupling agent
525 eliminated the proton gradient. Simultaneously, a significant, but small increase in the
526 percentage of cells with permeable cytoplasmic membranes as compared to the control could
527 be observed at 10 min (Fig. 9B). This was later confirmed by the strong increase in the
528 percentage of cells with permeable membranes after 30 minutes of exposure (Fig. 9B).

529

530 **4 Discussion**

531 This study reveals that the (thylakoid and cytoplasmic) membranes are amongst the first
532 physiological targets of allelochemicals from *A. minutum*. Two distinct phases were observed,
533 allowing us to identify “primary” targets from the side effects of filtrate exposure, and to
534 hypothesize the cascades of events occurring within the different membranes of *C. muelleri*.

535 In the first phase (within the first 10 min), a general depolarization of cytoplasmic membranes
536 and permeabilization of external membranes from few cells were induced in agreement with
537 the mode of action of *A. catenella* allelochemicals on other protists (Ma et al., 2011). A direct
538 effect of *A. minutum* filtrate was also observed on membranes of oyster hemocytes causing
539 membrane permeability (Hégaret et al., 2011). Concomitantly, an inhibition of electron flow
540 between PSII and b_6/f was measured. This resulted in a quasi-complete arrest of the
541 photosynthetic electron transfer chain, illustrated by the strong inhibition of the activities of
542 PSII and PSI in the light. It is to note that the inhibition of PSI seemed slightly stronger than
543 the one of PSII. This could reflect the presence of some cyclic electron flow in the control
544 conditions and its full suppression in the presence of *A. minutum* filtrate. Another fast response
545 of photosynthesis to the allelochemicals was the suppression of the quadratic ECS increase right
546 after the flash. This parameter is difficult to interpret, as it depends both on the electric field
547 generated by PSII and PSI charge separations and on the pre-existing electric field (Joliot and
548 Joliot, 1979 ; Bailleul et al., 2010). In contrast, the linear ECS increase only depends on the
549 electric field generated by PSII and PSI after the saturating laser flash. Theoretically, the ratio
550 of the quadratic-to-linear ECS is a direct measurement of the pre-existing electric field, the
551 electric component of the pmf (Bailleul et al., 2010, 2015; Dow et al., 2020). This ratio was
552 significantly reduced in the exposed cells, suggesting that the pmf in the dark was affected by
553 *A. minutum* filtrate. This can be due to an inhibition of mitochondrial respiration or to an
554 uncoupling effect of the secondary metabolites that affect mitochondria, the chloroplast, or both
555 (Bailleul et al., 2015). A partial inhibition of *C. muelleri* respiratory activity in response to *A.*
556 *minutum* filtrate was observed before (Lelong et al., 2011a), but this inhibition developed
557 slowly, compared to the fast suppression of the pmf measured in this work. For this reason, the
558 hypothesis of an uncoupler effect seems more plausible and should be tested in future
559 investigations.

560 After longer times of exposure, the membranes of *C. muelleri* seemed to become much more
561 affected by *A. minutum* allelochemicals. Cytoplasmic membranes appeared more permeable,

562 suggesting that the loss of membrane polarization could be attributed to pores in the membranes
563 that became larger or more numerous with longer exposure time. Meanwhile, in the
564 photosynthetic membranes, strong inhibitions of PSI and PSII, and the reduced turnover of the
565 cytochrome b_6f occurred. Results also highlighted that, while PSII can be probed to measure
566 allelochemical potency (Long et al., 2018a), it is not a specific target. These alterations of
567 photosynthetic complexes may be a consequence of the dysfunction of the diffusion of the
568 quinones between Q_B and cytochrome b_6f , leading to the suppression of the photosynthetic
569 electron transport. The overall deleterious effects on the photosynthetic apparatus induced
570 photoprotection mechanisms previously measured in *C. muelleri* exposed to *A. minutum* filtrate
571 (Long et al., 2018b). These defense mechanisms are, however, likely insufficient to counteract
572 the severe damages to the membranes at the *A. minutum* concentration we chose for this study.

573 The nature of allelochemicals of *A. minutum* still eludes us, but our results provide new insights
574 into the physico-chemical properties expected for candidate molecules. Indeed, the three main
575 consequences of the allelochemicals here (Fig. 10) were 1) the disruption of cytoplasmic
576 membranes, 2) the decrease of the plastoquinone diffusion and 3) the suppression of the electric
577 component of the pmf in the dark-adapted cells. Overall, the mode of action of *A. minutum*
578 allelochemicals is very similar to that of *Karenia brevis*, which alter the membrane integrity,
579 modify the lipidome and compromise photosynthesis of the competing species (Poulin et al.,
580 2018). Membrane biochemical composition varies greatly among eukaryotes. As membranes
581 are amongst the first targets for *A. minutum* allelochemicals, it can be hypothesized that their
582 biochemical composition might drive, at least partially, the sensitivity of species to *A. minutum*.

583 The composition of membranes was already suggested to be an important factor in some
584 allelochemical interactions (Deeds and Place, 2006; Morsy et al., 2008a, 2008b; Ma et al.,
585 2011).

586 Our study demonstrates that allelochemicals from *A. minutum* have the potential to inhibit
587 photosynthesis and quickly lyse co-occurring protists at environmentally relevant cell densities.
588 Indeed, the concentration of *A. minutum* used in this study ($\sim 20\,000$ cells mL^{-1}) was higher than
589 the half maximal effective concentration inhibiting Fv/Fm of a culture by 50 % measured in
590 previous study ($\sim 4\,000$ cells mL^{-1} ; Long et al., 2018) but lower than the maximal concentrations
591 of *A. minutum* measured in the field, which can reach $40\,000$ cells mL^{-1} (Chapelle et al., 2015)
592 or more (Garcés et al., 2004). The allelochemical potency would favor the establishment of *A.*
593 *minutum* monospecific bloom by eliminating other microalgae competing for nutrients, by
594 facilitating mixotrophic behavior (already evidenced in this species by (Meng et al., 2019)) and

595 potentially decreasing the grazing by other dinoflagellates as observed for *A. fundyense* (John
596 et al., 2015). A better description of the biochemistry of planktonic membranes, the chemical
597 elucidation of allelochemicals and a precise identification of their specific targets is essential to
598 identify the potential marine species (not only planktonic) affected by these metabolites.
599 Eventually, these results will help predicting the potential structuring effects of allelochemical
600 interactions on planktonic communities.

601

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612 **Contributions**

613 ML, AP, HH and BB designed the research. ML, AP, CGF, HH and BB performed the research
614 and analyzed the data. ML, AP, HH and BB wrote the first version of the manuscript and all
615 co-authors revised the manuscript. ML and AP contributed equally to this work.

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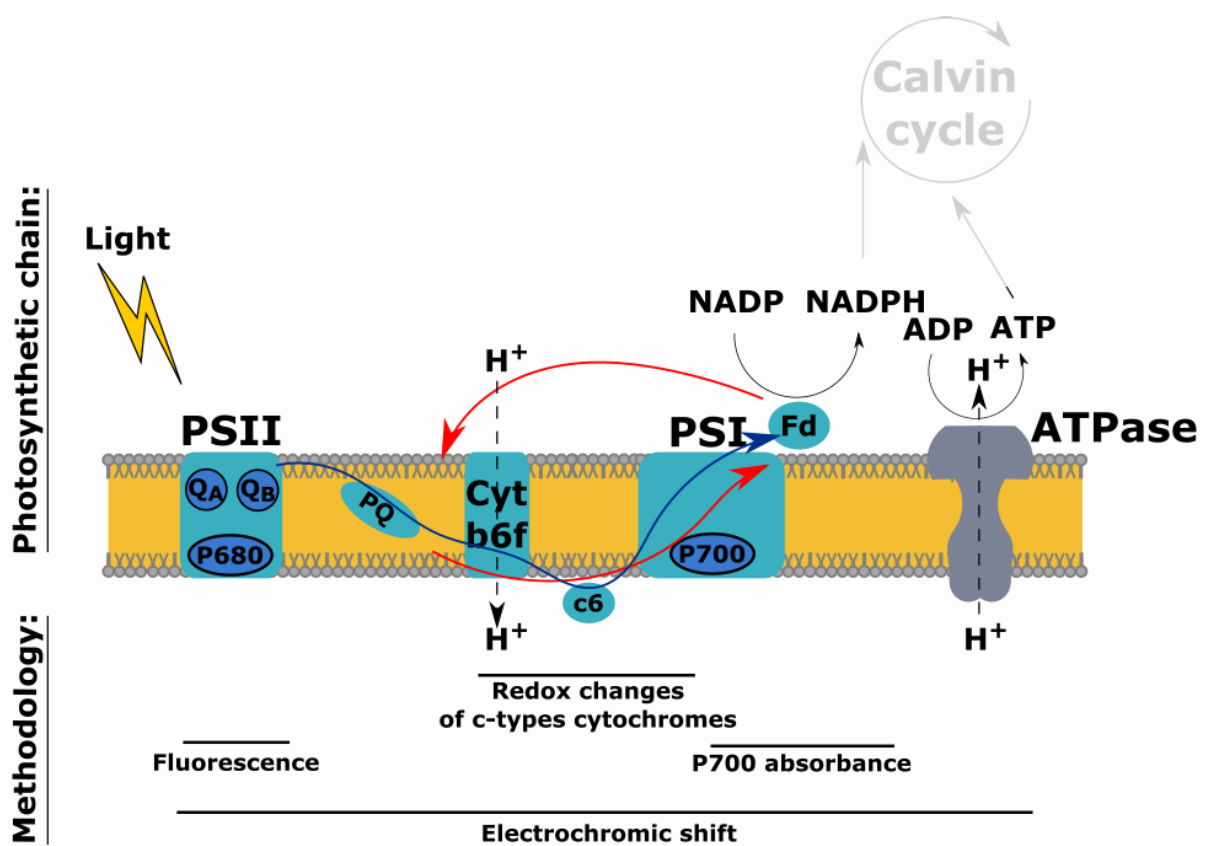
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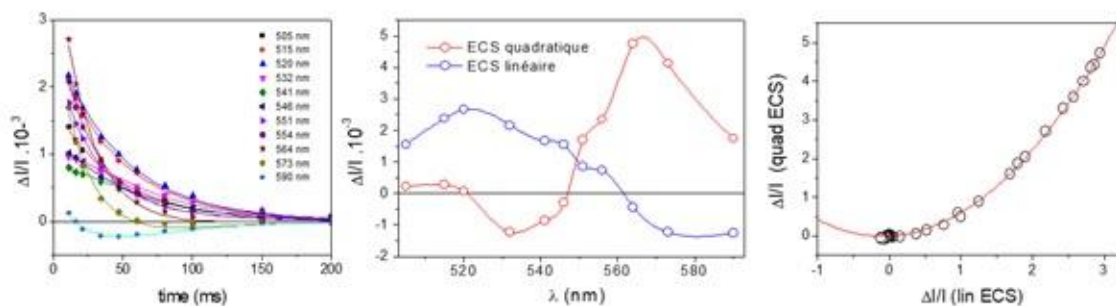
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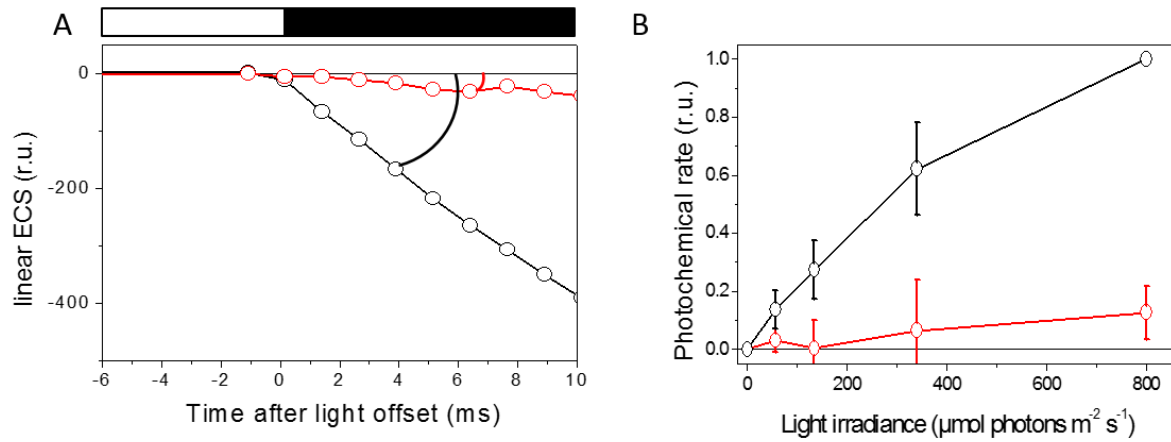
860 *Figure 1: Schematic figure of the photosynthetic chain and methodology used for measuring the different*
 861 *photosynthetic components. The blue arrow represents the linear electron flow; the red arrows represent the cyclic*
 862 *electron flow around the PSI. PSI and PSII: Photosystems I and II, Q_A and Q_B : Quinones A and B, PQ :*
 863 *Plastoquinone, Cyt b6f: Cytochrome b6f, c6 : Cytochrome 6, P700: PSI reaction center, Fd: Ferredoxin*



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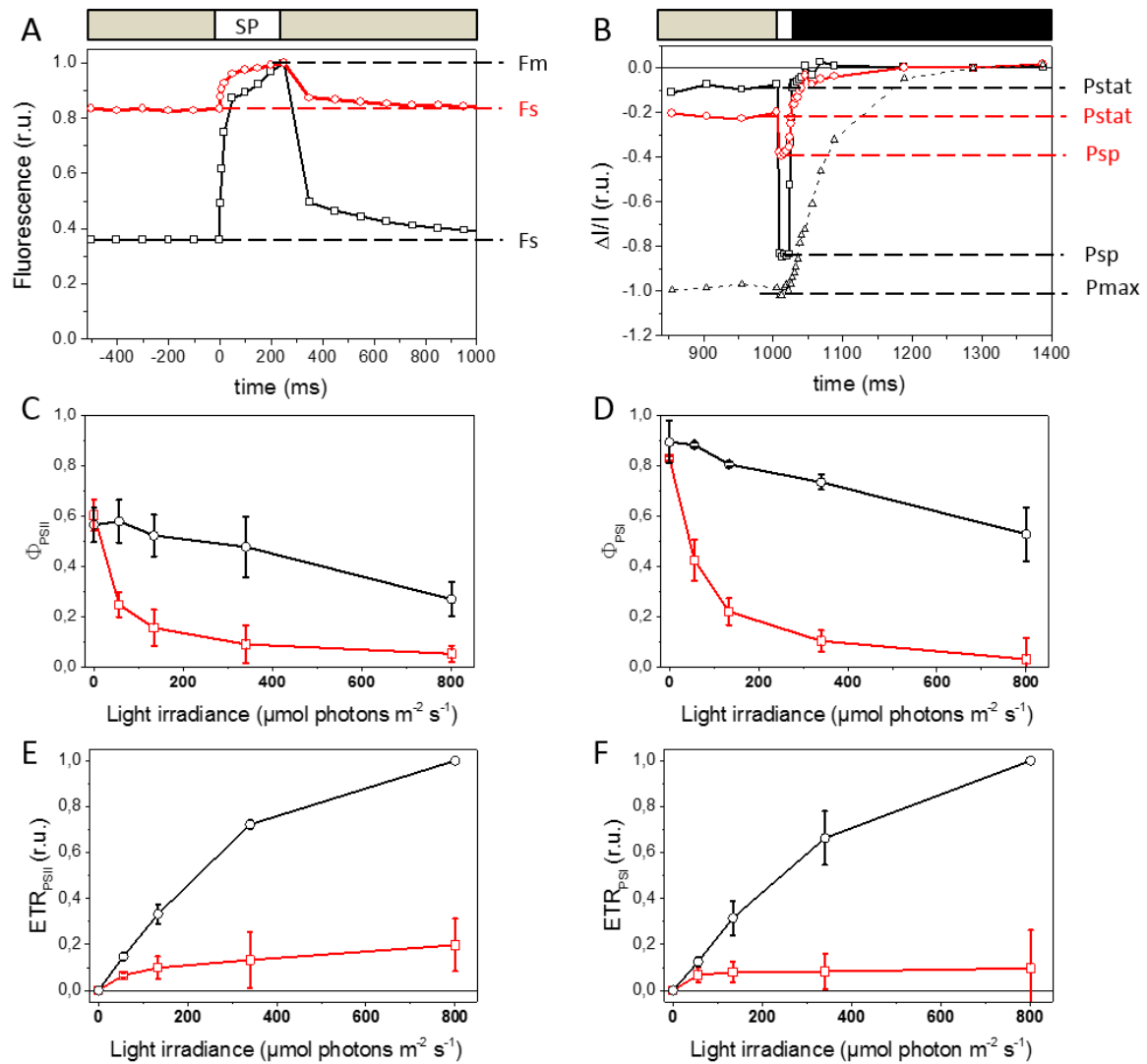
865 *Figure 2: Deconvolution of the linear and quadratic ECS contributions in Chaetoceros muelleri, treated with*
 866 *100 μ M CCCP (see Methods). Panel A: Relaxation of the absorption changes ($\Delta I/I$) induced by a saturating pulse*
 867 *of light, at different wavelengths. Lines are extrapolation of the experimental points by a sum of two exponentials*
 868 *(see Methods). Panel B: the linear and quadratic ECS spectra are obtained from the amplitudes of the two*
 869 *exponentials in the extrapolation shown in the left panel (see Methods). Panel C: Absorption changes at 564 nm*
 870 *(where only quadratic ECS contributes, see panel B) is plotted as a function of the absorption change at 520 nm*

871 (where only linear ECS contributes, see panel B). The experimental points are fitted by a quadratic function (red
872 line), which validates the deconvolution procedure.



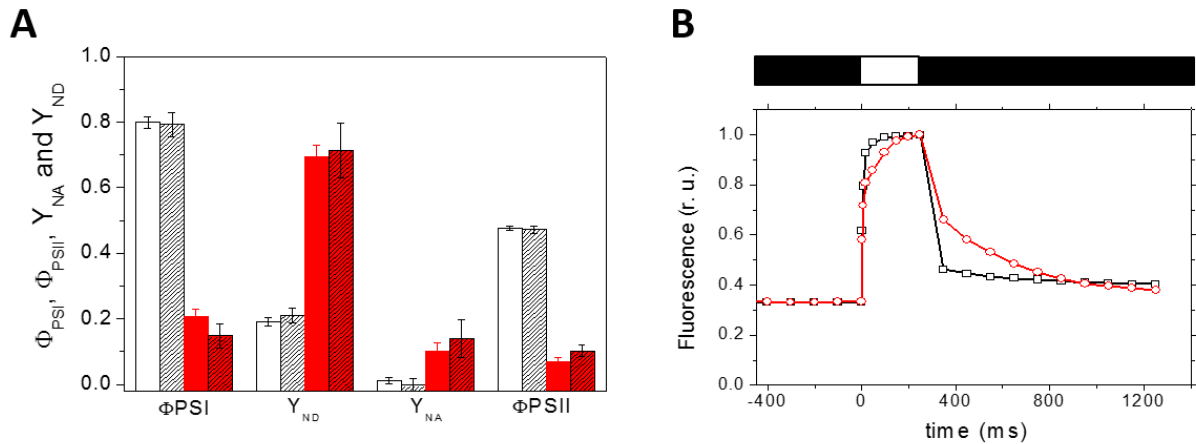
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874 *Figure 3: Effect of A. minutum supernatant on the overall photosynthesis activity. A) Linear ECS changes after*
875 *turning off the light in the absence (black dots/lines) and presence (red dots/lines) of A. minutum filtrate. White*
876 *bar represents the light phase (here, 800 μmol photons m⁻² s⁻¹), dark bar the period of darkness. The arc of circles*
877 *represents the change of slope at the light offset. B. Light dependency of photochemical rates of C. muelleri with*
878 *(red dots/lines) or without (black dots/lines) A. minutum filtrate. Data were normalized to the maximal value at*
879 *800 μmol photons m⁻² s⁻¹ in the untreated sample (see Methods). Results are expressed in relative unit (r.u.). Error*
880 *bars represent standard deviation (n = 4).*



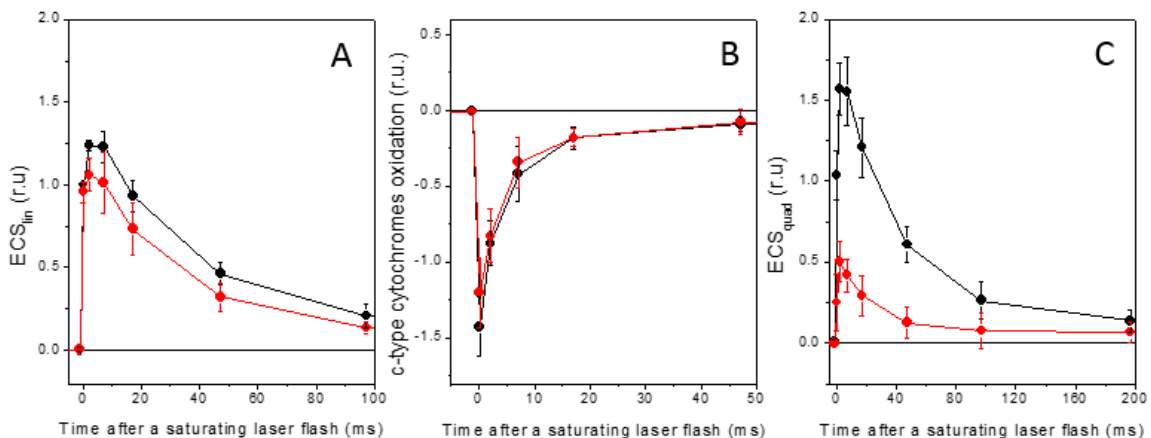
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882 *Figure 4: Effects of A. minutum filtrate on the activity of PSII (A, C, E) and PSI (B, D, F) of C. muelleri. Dark:*
 883 *control, red: in the presence of A. minutum filtrate. A/B: The fluorescence and P700 measurements for the*
 884 *calculation of PSII (A) and PSI (B) quantum yields (see Methods for explanations of the different parameters). In*
 885 *panel B, the dashed lines and rectangles show the absorption changes in the control with DCMU. Grey bars above*
 886 *the panels represent the light phase (here, 340 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$), dark bar is the period of darkness and white*
 887 *"SP" phase shows the saturating pulse phase. Data in panels A and B were obtained under 340 $\mu\text{mol photons m}^{-2}$*
 888 *s^{-1} actinic light. C/D: Light dependencies of the quantum yields of PSII (C) and PSI (D). E/F: Light dependency*
 889 *of the electron transfer rates through PSII (ETR_{PSII} , E) and through PSI (ETR_{PSI} , F). Results are normalized to the*
 890 *value at the highest intensity (see Methods). Error bars represent standard deviation ($n = 3$ for the right panels*
 891 *(B, D, F), $n=6$ for the left panels (A, C, E)).*



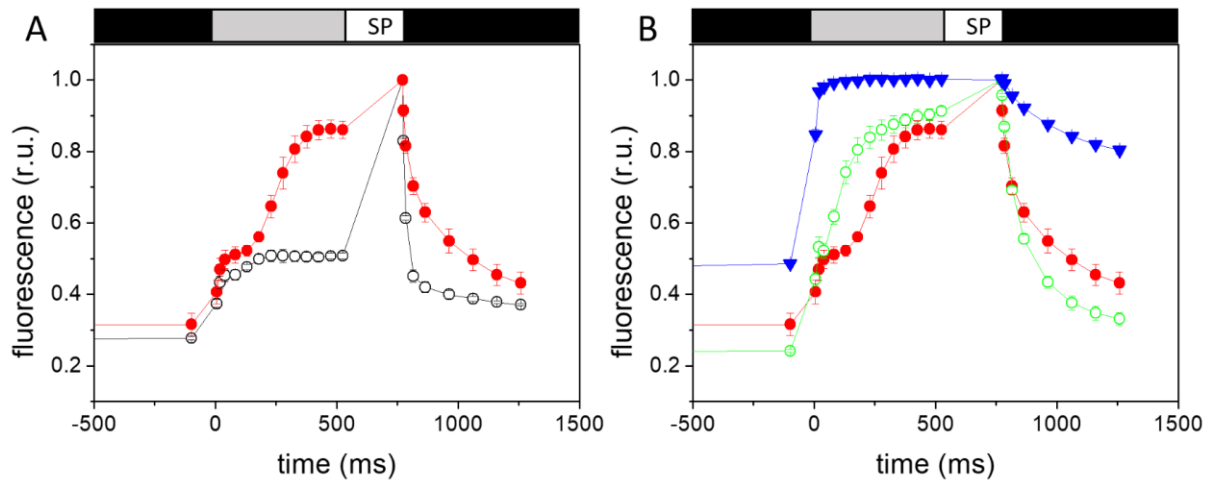
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893 *Figure 5 Short-term effect of A. minutum supernatant on the PSI and PSII activities of C. muelleri. A: The quantum*
 894 *yields of PSII and PSI, as well as the donor-side (Y_{ND}) and acceptor-side (Y_{NA}) limitations are shown for the*
 895 *control (white bars) and in the presence of A. minutum filtrate (red bars), with (hatched) and without (plain) the*
 896 *PSI acceptor methylviologen (1mM). All experiments were performed at $340 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ actinic light.*
 897 *Error bars represent standard deviation ($n = 3$). B: F_v/F_m for the control (black dots/lines) and filtrate (red*
 898 *dots/lines) exposed samples were 0.66 ± 0.02 and 0.69 ± 0.03 , respectively. A saturating pulse (white rectangle)*
 899 *is applied on dark-adapted (black rectangle) samples to measure F_v/F_m . Results are normalized to the F_m .*



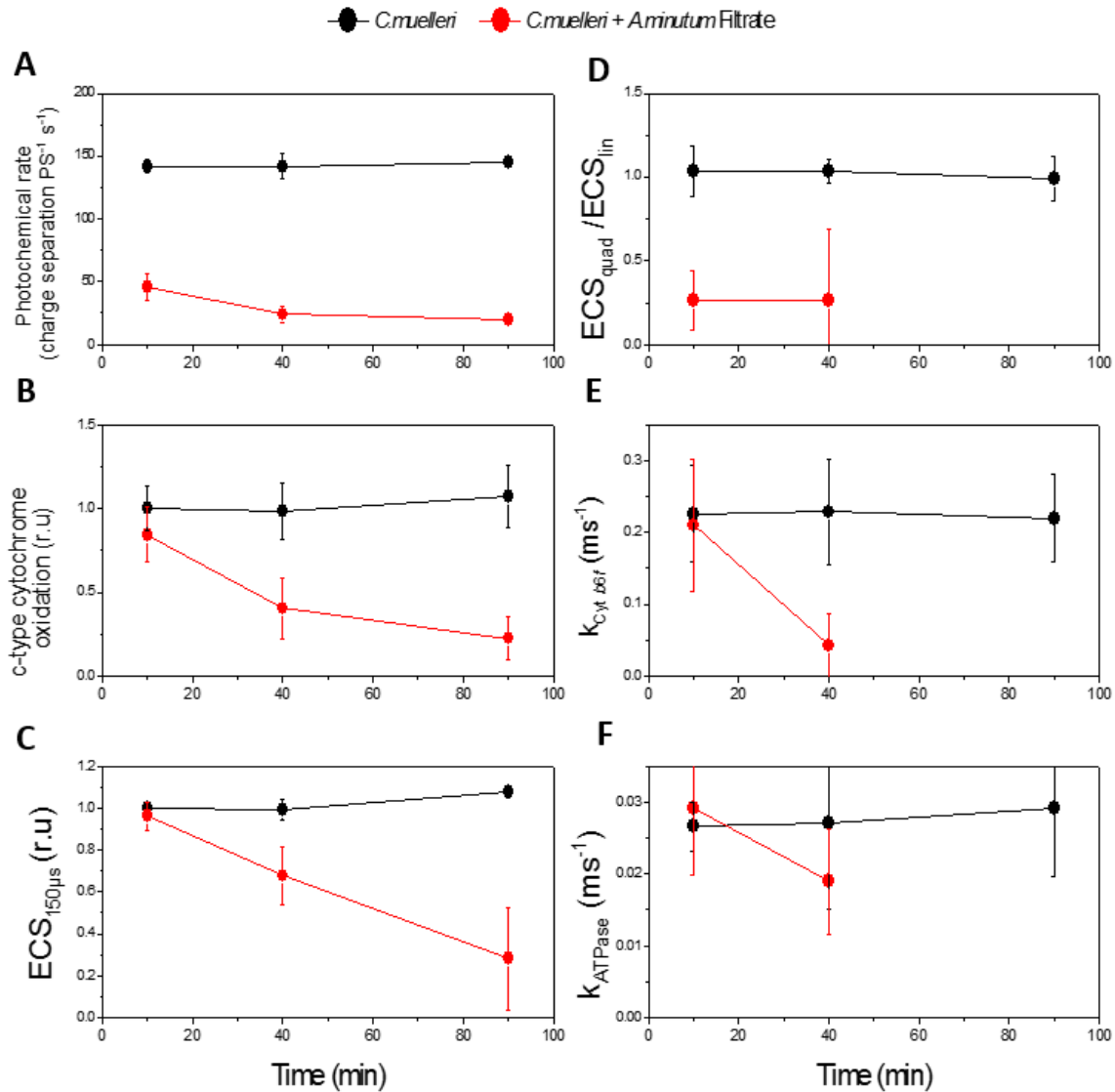
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901 *Figure 6. Kinetic of the linear ECS.(A), c-type cytochromes oxidation (B) and quadratic ECS (C) after a saturating*
 902 *laser flash. Dark: control diatom cells. Red: After 10 min of incubation with the filtrate of A. minutum. The linear*
 903 *and quadratic ECS and c-type cytochrome are calculated from absorption changes at 520, 554, 564 nm (see*
 904 *Methods). Error bars represent standard deviation ($n = 4$).*



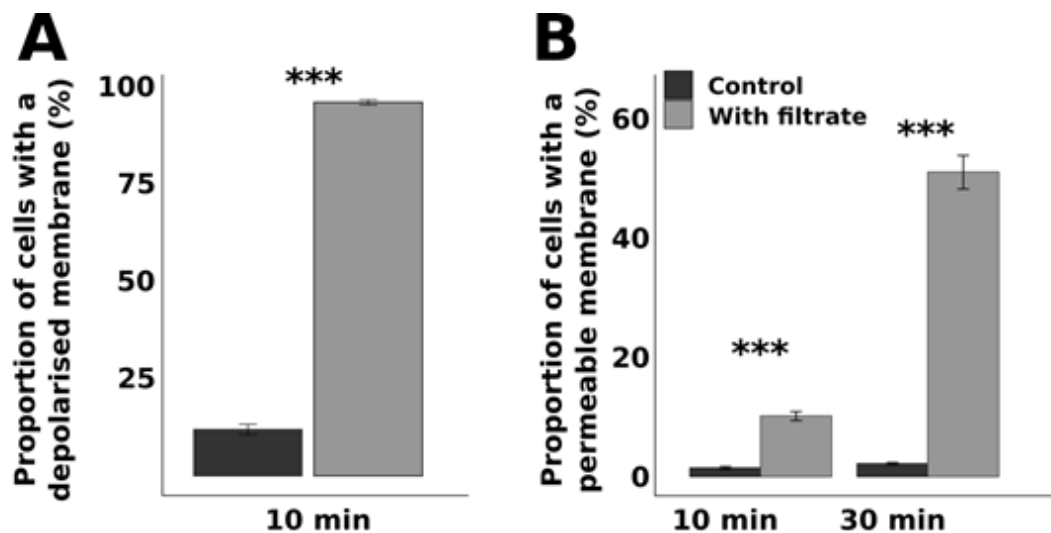
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906 *Figure 7. Effect of A. minutum filtrate on C. muelleri fluorescence induction and relaxation curves. A:*
 907 *Fluorescence induction curve of C. muelleri control (black dots/lines) and in presence of A. minutum filtrate (red*
 908 *dots/lines). B: Fluorescence induction curve of C. muelleri in the presence of A. minutum filtrate (red dots/lines),*
 909 *with DCMU (10 μ M) (blue dots/lines) and DBMIB (0.5 μ M) (green dots/lines). Error bars correspond to the S.D*
 910 *(3 independent replicates). White and black bars above the panels represent the light and dark periods,*
 911 *respectively.*



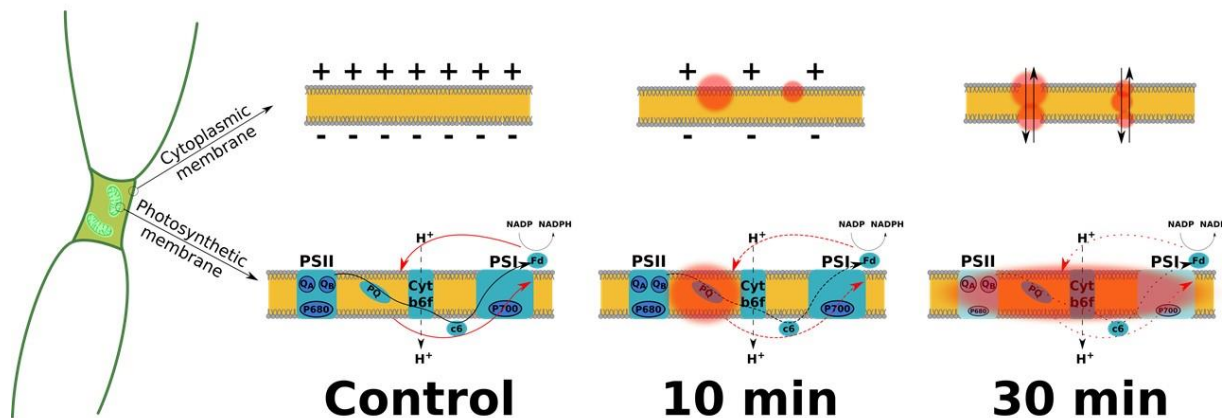
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913 *Figure 8. Effect of the filtrate of A. minutum on photosynthetic complexes and overall photosynthesis at different*
 914 *times of exposure (10, 40 and 90 minutes). Black: control. Red: cells treated with the filtrate of A. minutum. A:*
 915 *photochemical rate measured after 3 minutes illumination at 800 μmol photons m⁻² s⁻¹. B: ratio of the quadratic-*
 916 *to-linear ECS increase 160 μs after the saturating laser flash. C/D: Extent of oxidation (C) and rate of re-reduction*
 917 *(D) of c-type cytochromes. E/F: fast rise (a phase, 160 μs after the saturating laser flash, E) and rate of decay of*
 918 *linear ECS (F). The linear and quadratic ECS and c-type cytochrome oxidation are calculated from absorption*
 919 *changes at 520, 554, 564 nm (see Methods). The ECS and c-type cytochromes signals become too small after 90*
 920 *minutes exposure to allow measurements of the quadratic-to-linear ECS, or b_{of} and ATPase rate constants. Error*
 921 *bars represent standard deviation (n = 3-5).*



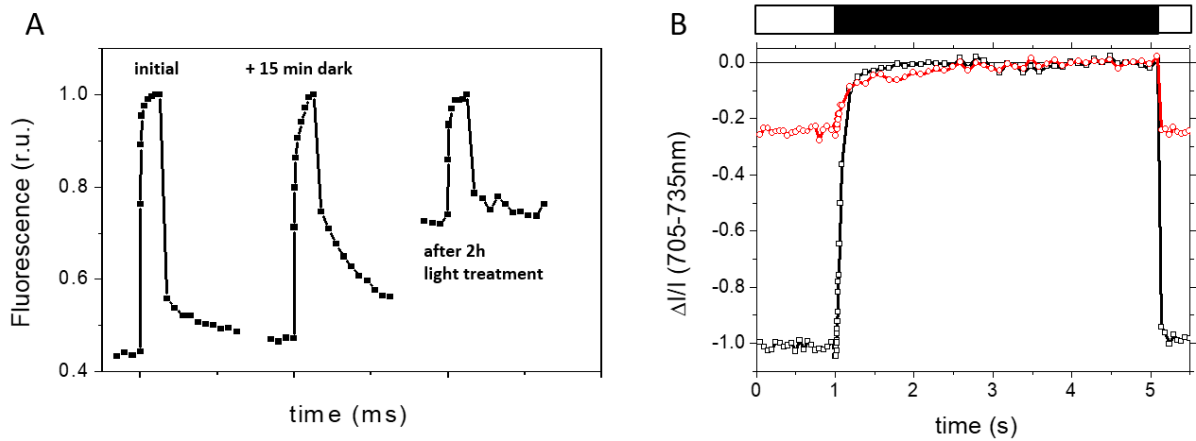
922

923 *Figure 9: Effect of A. minutum filtrate on C. muelleri cytoplasmic membranes. A) Variation in the proportion of*
 924 *cells with a depolarized membrane after 10 min of exposure to the filtrate (labelled with DiBAC₄(3)). B) Variation*
 925 *in the proportion of cells with a permeable membrane after 10 and 30 min of exposure to the filtrate (labelled with*
 926 *SytoxGreen). Cells in the control are shown in dark grey and cells in the presence of A. minutum filtrate are shown*
 927 *in light grey. *** indicate a level of significance < 0.001. Results are expressed as the mean ± standard error (n*
 928 *= 3).*



929

930 *Figure 10: Schematic figure of the cascade of events occurring to the cytoplasmic (top) and photosynthetic*
 931 *membranes (bottom) following the exposure to A. minutum filtrate. The red circles represent the membrane*
 932 *components affected by allelochemicals. The blue arrow represents the linear electron flow and the red arrows*
 933 *represent the cyclic electron flow around PSI. PSI and PSII: Photosystems I and II, Q_A and Q_B: Quinones A and*
 934 *B, PQ : Plastoquinone, Cyt b₆f : Cytochrome b₆f, c₆ : Cytochrome 6, P700: PSI reaction center, Fd: Ferredoxin.*
 935



937

938 **Fig. S1** Effect of *Alexandrium*'s allelochemicals on fluorescence induction and relaxation curves

939 (A) Fluorescence of *C. muelleri* exposed to *A. minutum* filtrate where a saturating pulse is applied immediately

940 after exposure, after 15 min of dark exposure and after 2 hours of light treatments. (B) Relative amount of P700

941 photo-oxidizable in absence (black dots/lines) and presence (red dots/lines) of *A. minutum* filtrate given by the

942 change in $\Delta I/I$ under different light conditions. Results are expressed in relative unit (r.u.).

943

944