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1 **Flow cytometric measurements of cellular responses in a toxic dinoflagellate,**
2 ***Alexandrium minutum* upon exposure to thermal, chemical and mechanical stresses.**

3

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6

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12

12 **Abstract**

13 The purpose of the present study was to provide a flow-cytometric (FCM) approach
14 evaluating *A. minutum* cellular responses to mechanical, thermal and chemical stresses.
15 Coupling SYBR-Green I and SYTOX-Green staining, FCM analysis and sorting, and
16 microscopic observations permitted identification and characterization of five cellular
17 states/forms of *A. minutum*; 1) vegetative cells, 2) pellicle cysts, 3) degraded cells, 4) empty
18 theca and 5) dead cells. Storage on ice resulted in the transformation of a portion of vegetative
19 cells into dead cells, pellicle cysts, and “degraded” cells; however, centrifugation resulted
20 mostly in pellicle cysts and a few degraded cells. After these thermal and mechanical
21 treatments, control and stressed cultures were monitored for 14 days. Stressed *A. minutum*
22 cultures appeared to grow at the same rates as control cultures during the first seven days.
23 During the last week of monitoring, however, cell densities of stressed cultures reached their
24 stationary phase earlier than control cultures, suggesting incomplete recovery. Additional
25 experiment assessing kinetics of excystment indicated that it can occur less than 9 hours
26 following mechanical stress (centrifugation) and that 75% of the culture can excyst within 24
27 hours. Upon 30 min of exposure to chemical stressors (saponine and H₂O₂), only vegetative
28 cells, pellicle cysts, and dead cells were detected. For both chemicals, encystment was dose-
29 dependent. Counts of pellicle cysts increased with increasing saponine or H₂O₂ concentration.
30 Pellicle cysts were the most-predominant cell type at chemical treatments above 0.05 g.L⁻¹
31 saponine and above 0.015% H₂O₂. Occurrence of dead cells appeared to follow an all-or-none
32 response as dead-cell percentage increased from 3% at 0.015% H₂O₂ to 81% at 0.03% H₂O₂
33 without pellicle cyst formation. Overall, encystment-excystment of *A. minutum* upon changes
34 of environmental conditions can occur very rapidly but can be monitored using FCM and
35 SYBR-Green I staining.

36

36 **1 Introduction**

37

38 The microalgal genus *Alexandrium* (Dinophyceae, Gonyaulacales) includes 29 described
39 species (Figueroa et al., 2008). Nine *Alexandrium* species (including *A. catenella*, *A.*
40 *minutum*, *A. fundyense*, *A. tamarense*) are known to produce paralytic shellfish toxins (PSTs)
41 (Moestrup et al., 2002) and represent a risk for public health and a source of economic loss
42 for aquaculture and fisheries in many coastal waters (Hoagland et al., 2002).

43 In the life cycle of dinoflagellates there are two forms of cysts: the sexual resting cysts and
44 the asexual pellicle cysts. Sexual resting cysts (hypnozygotic) possess a double-layered wall
45 and require a mandatory dormancy period prior to germination. These resting cysts are
46 thought to remain viable over extended exposures to adverse environmental conditions
47 (Doucette et al., 1989). The pellicle cysts, also referred as temporary or ecdysal cysts, have a
48 thin-layered wall and can germinate without a mandatory dormancy period (Anderson and
49 Wall, 1978; Pfiester and Anderson, 1987; Fistarol et al., 2004; Bravo et al., 2010). These
50 temporary cysts are non-motile, surrounded by a pellicle, and are produced by vegetative cells
51 shedding the theca (ecdysis) in response to short-term or sudden adverse conditions (Olli,
52 2004; Kremp and Parrow, 2006; Bravo et al., 2010).

53

54 At least 48 species of dinoflagellates have been reported to form thin-walled cysts as part of
55 the life cycle, both in culture and in nature (Bravo et al., 2010). Formation of pellicle cysts
56 can result from various physical, chemical or biological stresses or unfavorable conditions,
57 including: changes in temperature (Grzebyk and Berland, 1996), ageing of cultures (Jensen
58 and Moestrup, 1997), nutrient stress (Anderson and Wall, 1978; Doucette et al., 1989),
59 bacterial attack (Nagasaki et al., 2000), or allelopathic interactions (Fistarol et al., 2004).
60 Pellicle cysts are thought to play a major role in dinoflagellate life cycles and population
61 dynamics (Bolli et al., 2007). Formation of pellicle cysts is an effective strategy for surviving
62 short-term environmental fluctuations, as pellicle cysts can quickly re-establish a vegetative,
63 motile existence when conditions become favourable again (Anderson, 1998; Olli, 2004;
64 Fistarol et al., 2004). Garcés et al. (1999) argued that a decrease in the motility of a
65 population during a bloom, caused by physical disturbances (e.g. a wind event), could be
66 compensated for by the revival of benthic, temporary cysts within 24h. Pellicle cysts can also
67 be formed during cell transit in the digestive tract of mussels and oysters (Laabir and Gentien,
68 1999; Laabir et al., 2007, Hégaret et al., 2008). It is believed that revival of temporary cysts
69 can serve as a potential seed source for subsequent dinoflagellate blooms. Persson et al.

70 (2006) demonstrated that eastern oysters, *C. virginica*, fed vegetative cells of toxic *A.*
71 *fundyense* digested 72% of ingested cells, and 28% survived gut passage by forming
72 temporary cysts. Hégaret et al. (2008) even demonstrated that culture of *A. fundyense* can be
73 re-established from vegetative cells or temporary cysts, which survived bivalve gut passage.

74

75 Cell-counter and microscopic observations are the most widely-used techniques to monitor
76 morphology and growth of a microalgal culture. Several assays using flow cytometry (FCM)
77 are now available to measure cell morphology and physiology of phytoplankton (Dubelaar
78 and Jonker, 2000; Veldhuis and Kraay, 2000). FCM has been recognized to be useful for
79 studies involving phytoplankton, such as assessing chemical toxicity to phytoplankton in the
80 laboratory (Howlett and Avery, 1999; Franqueira et al., 2000; Lage et al., 2001; Stauber et al.,
81 2002), *in-situ* microcosm experiments (Stachowski-Haberkorn et al., 2008, 2009), or to
82 monitor natural populations in the field (Marie et al., 1997; Rutten et al., 2005). Coupling
83 FCM with the use of specific fluorescent dyes allowed access to specific cellular parameters
84 and functions in phytoplankton. SYTOX-Green, a membrane impermeable DNA-binding dye,
85 was shown to stain phytoplankton cells that have lost membrane integrity, thus assumed to be
86 dead (Veldhuis et al., 1997; Marie et al., 2005). This dye was also used to evaluate
87 phytoplankton viability following viral infection in laboratory experiments (Bussaard et al.,
88 2001). More recently, SYTOX-Green was applied to assessment of the viability of resting
89 cysts in dinoflagellate species (Binet and Stauber, 2006).

90

91 The objective of the present study was to characterize the different cell forms and viability of
92 *A. minutum*, combining flow cytometry, microscopic observations, and fluorescent dyes
93 (SYBR-Green I and SYTOX-Green). Various types of stresses (thermal, mechanical, and
94 chemical) of varied intensities were also applied to *A. minutum* cultures to establish ranges of
95 morphological and physiological responses. Cultures of *A. minutum* were exposed to i)
96 freezing, ice cooling and centrifugation and ii) a gradient of saponine and H₂O₂. Thermal and
97 mechanical treatments were designed to simulate various stresses that *A. minutum* could
98 encountered during culture handling and/or bivalve filtration. The two tested chemicals aimed
99 to “simulate” environmental chemical stresses (pollutant or natural) as both could
100 compromise cell membrane integrity by two different mechanisms. The saponine is a
101 surfactant that can disorganize the lipid bilayer membrane and solubilize proteins. Many
102 detergents, natural or human activity related, can be encountered in coastal environment. The
103 H₂O₂ is an oxidizing agent which can compromise cell membranes by oxidizing lipids.

104 Oxidant agents can be naturally present in seawater or can be produced by other organisms
105 upon interaction with *Alexandrium* cells.

106 Although the methods presented was intended to compare *A. minutum* status in different
107 experimental manipulations used to expose filter-feeders to this toxic dinoflagellate, they can
108 also be used to assess the impact of various environmental factors (including pollutants) or
109 common protocols used during laboratory studies (such as centrifugation, dilution,
110 homogenization) on phytoplankton status.

111

112 **2 Materials and Methods**

113

114 **2.1 Algal culture**

115

116 Non-axenic cultures of *Alexandrium minutum* (strain AM89BM) were grown in 250-ml (for
117 *in-vitro* experiment) or 10-L (for simulation of experimental conditions) batch culture in L1
118 medium made with autoclaved, 1- μ m-filtered seawater (Guillard and Hargraves, 1993), for 12
119 days (exponential growth phase in our conditions) at 16 ± 1 °C and $100 \mu\text{mol photon.m}^2.\text{s}^{-1}$,
120 with a dark:light cycle of 12:12h.

121

122 **2.2 Flow-cytometric analyses**

123

124 **2.2.1 SYTOX-Green and SYBR-Green I staining procedures**

125 For each treatment and sampling time, two 250- μ L sub-samples were transferred into flow
126 cytometer tubes and stained with two fluorescent DNA/RNA specific dyes. SYBR-Green I
127 (Molecular probes, Eugene, Oregon, USA), that permeates both dead and live cells, was
128 diluted in DMSO 10% and used at 1/1,000 of the commercial solution. SYTOX-Green
129 (Molecular probes, Eugene, Oregon, USA), that permeates membranes of dead cells only, was
130 used at 0.05 μ M final concentration. Frozen cells (24h at -20°C) were used as positive
131 controls for SYTOX-Green staining (frozen cells were confirmed to be dead as no growth was
132 observed after 14 days of culture). Thus, by counting the cells stained by SYTOX-Green it
133 was possible to estimate the percentage of dead cells in each sample (Veldhuis et al., 2001).
134 For both dyes, tubes were incubated in the dark at 16°C for 30 minutes before flow-
135 cytometric analyses. SYBR-Green I and SYTOX-Green fluorescence were measured at 500-
136 530 nm (green, FL1 detector) by flow-cytometry. Presence of chlorophyll pigments in *A.*
137 *minutum* cells provided a “natural staining”. Their red chlorophyll fluorescence was measured

138 at >650 nm (red, FL3 detector) by flow-cytometry and was used to discriminate particles
139 containing chlorophyll pigments.

140

141 **2.2.2 Measurement of *A. minutum* cell variables by flow cytometry**

142 Counting and characterization of *A. minutum* forms and states were performed using a
143 FACScalibur (BD Biosciences, San Jose, CA USA) flow cytometer (FCM) equipped with a
144 488 nm argon laser. The FL3 detector (red fluorescence, >650 nm) was set with a threshold
145 allowing only the detection of particles containing chlorophyll, thus assumed to be
146 phytoplankton cells. Dinoflagellate cells were visualized on a Forward Scatter (FSC) and Side
147 Scatter (SSC) cytogram, as well as on a red fluorescence (FL3, chlorophyll related) and green
148 fluorescence (FL1, SYBR green I or SYTOX Green related) cytogram. These “relative”
149 morphometric and fluorometric variables (expressed in arbitrary units, AU) were used to
150 characterize the different *A. minutum* forms and states. Counts were estimated from the flow-
151 rate measurement of the flow-cytometer (Marie et al., 1999) as all samples were run for 1
152 min. Results were expressed as number of cells per ml.

153

154 **2.2.3 Identification of *A. minutum* subpopulations**

155 A cell suspension of *A. minutum* was centrifuged three times for 5 min at 2,000 rpm (= 800 x
156 g) and 16°C. Twenty-four hours later, the suspension was used to identify *A. minutum*
157 subpopulations. The suspension was stained with SYBR-Green I as described above (2.2.1).
158 Subpopulations of interest were gated and sorted using a sorting flow cytometer (FACS Aria -
159 Becton Dickinson Biosciences, San Jose, CA USA) for microscopic identification and
160 photography.

161

162 **2.3 Microscopy**

163

164 An epi-fluorescent, inverted microscope was used to identify and describe *A. minutum* cells,
165 previously sorted with FCM. The inverted microscope (Leica DM IRB) was equipped with a
166 mercury vapor lamp, two excitation filters (bandpass 450-490 nm > red and green
167 fluorescence; bandpass 515-560 nm > red fluorescence), and a Q-IMAGING (RETIGA
168 2000R FAST 1394) camera plugged into a Personal Computer with Image-Pro 6.0 software
169 (Media Cybernetic).

170

171 **2.4 Experimental conditions**

172

173 **2.4.1 Thermal and mechanical stresses**

174 Three cultures of *A. minutum* (12 days old) were diluted to 5,000 cells.mL⁻¹ in sterile-filtered
175 sea water (FSSW) enriched with L1 medium and distributed into twelve 15-mL plastic tubes
176 (four treatments in triplicate). Treatments were: i) control, maintained at 16°C; ii) centrifuged
177 three times 5 min at 2,000 rpm (= 800 x g) and 16°C (cells were re-suspended after each
178 centrifugation); iii) cooled by setting tubes into an ice-bath for 1h; iv) frozen for 24h at –
179 20°C.

180 After treatments, culture tubes were returned to previous culture conditions (16 ± 1°C and 100
181 μmol photon.m².s⁻¹, with a dark:light cycle of 12:12h). Two 250-μL samples were taken from
182 each tube just after treatments and after 1, 4, 7 and 14 days and analyzed as previously
183 described (2.2).

184

185 **2.4.2 Chemical stress**

186 Three cultures of *A. minutum* (12 days old) were diluted to 15,000 cells.mL⁻¹ with FSSW into
187 33 15-mL plastic tubes (eleven treatments in triplicate). Three tubes were used as controls and
188 the thirty remaining tubes were exposed to increasing concentrations of saponine (0.001,
189 0.005, 0.01, 0.05 and 0.1 g.L⁻¹ as final concentrations) and H₂O₂ (0.0015%, 0.003%, 0.015%,
190 0.03% and 0.15% as final concentrations, % v/v). Previous experiments were performed (data
191 not shown) to determine the chemical concentrations allowing the observation of dose-
192 dependent responses. All tubes were then maintained for 30 min in the dark at 16°C. After
193 incubation, two samples of 250 μL were taken from each tube and analyzed as described
194 previously (2.2).

195

196 **2.4.3 Time for excystment after mechanical stress**

197 Ten-mL samples of cultures of *A. minutum* at a concentration of 85,000 cells mL⁻¹ were
198 distributed into twelve 15-mL plastic tubes (6 replicates of two treatments). Treatments were:
199 i) control, maintained at 16°C; ii) centrifuged three times 5 min at 2,000 rpm (= 800 x g) and
200 16°C (cells were re-suspended after each centrifugation).

201 After treatments, culture tubes were returned to previous culture conditions (16 ± 1 °C and
202 100 μmol photon.m².s⁻¹, with a dark:light cycle of 12:12h). Every 3 hours, from 0h to 24h
203 after the centrifugation, 300-μL samples were taken from each tube and incubated for 30 min
204 in the dark at 16°C with SybrGreen I and analyzed with the flow cytometer to determine the
205 percentage of temporary cysts.

206

207 **2.5 Statistical analysis**

208

209 In all experiments (thermal, mechanical and chemical stresses), statistical differences between
210 conditions were analyzed by one way ANOVA after checking assumptions (normality and
211 homoscedasticity of the error term). In thermal and mechanical stress experiments, time effect
212 after exposure was also tested by one-way ANOVA. In the chemical-stress experiments, a
213 linear model was used to correlate pellicle cysts and empty thecae. The fit between predicted
214 and measured values was statistically tested using ANOVA. For all statistical results, a
215 probability of $p < 0.05$ was considered significant. Statistical analyses were performed using
216 Statgraphics Plus (Manugistics, Inc, Rockville, MD, USA).

217 **3 Results**

218

219 **3.1 Identification and characterization of *A. minutum* cell states using SYBR Green I** 220 **staining**

221

222 Five *A. minutum* forms/states were identified and characterized by coupling SYBR-Green I
223 staining, FCM analysis and sorting, and microscopic observations. Figure 1 presents FSC-
224 SCC and FL1-FL3 characteristics of *A. minutum* cells one day after: centrifugation (Fig 1A
225 and B; Fig. 2), ice cooling (Fig 1C and D), and freezing at -20°C (Fig. 1E and F; Fig. 3). Five
226 regions (Fig 1B, D, and F) were designated, identified with different colors, and used to sort
227 *A. minutum* forms/states. Dead cells (R5, light blue dots, Fig. 1E and F) were not sorted
228 because they constituted 100% of analyzed cells after freezing and were all positive with
229 SYTOX-Green staining (data not shown).

230 Vegetative cells (R1, green dots in Fig. 1A-D) were characterized by high chlorophyll-
231 pigment red fluorescence (FL3) and high SYBR-Green I fluorescence (FL1). Upon sorting,
232 vegetative cells were still motile with normal shape and morphological characteristics
233 (presence of theca and chloroplasts) (Fig. 2A1, 2A3). The nucleus was intensely stained by
234 SYBR-Green I (Fig. 2A2 and 2A4), revealing a characteristic U-shaped nucleus in these cells
235 (Fig. 2A4).

236 Pellicle cysts (R2, red dots in Fig. 1A-D) had very low SYBR-Green I fluorescence,
237 suggesting that the dye did not penetrate the cells. Chlorophyll (red fluorescence, FL3) was
238 high but lower (about 35% less) than in vegetative cells (Fig 1B, 1D). FSC and SCC values of
239 pellicle cysts were higher than those of vegetative cells (Fig 1B, 1D). Identification of pellicle
240 cysts was further confirmed after FCM sorting and microscopic observations (Fig. 2B). Cells
241 were immotile (loss of flagella), athecate (absence of theca), and round with chloroplasts (Fig.
242 2B1). The nucleus was not stained by SYBR-Green I (Fig. 2B2), even after several hours of
243 incubation (data not shown).

244 Degraded cells (R3, dark blue dots in Fig. 1A-D) were characterized by low chlorophyll
245 fluorescence (FL3) and low SYBR-Green I fluorescence (FL1). Mean FSC was about twice
246 that of vegetative cells, but SCC was only slightly higher. Degraded cells were found to be
247 immotile upon FCM sorting. Thecae were still present but appeared damaged (Fig. 2C1).
248 Microscopic observations (Fig. 2C) revealed that chloroplasts or chlorophyll pigments
249 migrated towards the center of the cell, resulting into a star-shaped red fluorescence pattern.
250 Green fluorescence of SYBR-Green I appeared to be diffuse with some green dots in the cells,

251 and did not show the characteristic U-shaped nucleus (Fig. 2C2). Green rods and cocci,
252 revealed by SYBR-Green I staining (white arrows), were likely bacteria associated with
253 thecae.

254 Empty thecae (R4, pink-purple dots in Fig. 1A-D) were characterized by very low FL3
255 fluorescence and low FL1 fluorescence. After sorting, microscopic observations revealed a
256 transparent, empty theca (Fig. 2D1). Bacteria, revealed by SYBR-Green I staining, were
257 found stuck to the surface of thecae (white arrows, Fig. 2D2).

258 Freezing (24h at -20°C) the culture resulted in 100% dead cells, all found to be permeable to
259 SYTOX-Green. No growth in frozen cultures was observed for the following 14 days (Fig.
260 4A). Dead cells (R5, light blue dots in Fig. 1C-F), were characterized by high chlorophyll (red
261 fluorescence, FL3), although half that of live, vegetative cells. Mean SYBR-Green I
262 fluorescence (FL1) was about five times lower than that of live vegetative cells. Microscopic
263 observations (Fig. 3) of dead *A. minutum* cells revealed that SYBR-Green I fluorescence was
264 diffused throughout the entire cell (very different from the U-shaped nucleus observed in
265 vegetative cells, Fig. 3C) and that red fluorescence (chlorophyll) appeared to be diffuse (Fig.
266 3B). Thus, nucleus and chloroplast structures were not visible in dead cells (Fig. 3). The
267 presence of green fluorescent particles (white arrows) surrounding dead cells (Fig 2C)
268 incubated with SYBR Green I revealed the presence of bacteria (detected by the FL1 detector
269 of the flow cytometer).

270

271 **3.2 Monitoring of cultures exposed to short term mechanical and thermal stresses**

272

273 Cellular modifications of *A. minutum* observed upon mechanical and thermal stresses were
274 monitored for 14 days after the stress.

275 Figure 4 presents counts of live cells (A), percentages of dead cells (B), and red chlorophyll
276 fluorescence (C) in cultures subjected to i) 24h -20°C freezing, ii), 1h ice cooling and iii),
277 centrifugation three times for 5 min at 800 g and vi) control culture.

278 Ice cooling killed approximately 30% of *A. minutum* cells. Dead cells were still detected one
279 day after stress but had reduced chlorophyll fluorescence (Fig 4C). After four days, the mean
280 percentage of dead cells decreased steadily to 3% at day 14. Percentages of dead cells in
281 centrifuged and control cultures were less than 1% from 0 to 7 days after the stress (Fig 4B).
282 On day 14, however, control and centrifuged treatments showed significant increases in
283 percentages of dead cells (2.3% and 4.8%, respectively) as compared to previous days.

284 Four days after the stress, cell division was observed in centrifuged, ice cooled, and control
285 cultures, reaching 16.10^3 , 10.10^3 and 15.10^3 cells.ml⁻¹. Cell counts in ice-cooled cultures were
286 lower than those of centrifuged and control cultures until day 4 ($p < 0.05$). After 7 and 14
287 days of monitoring, cell densities in centrifuged and ice cooled cultures were similar, but
288 significantly lower than those of control cultures (about -20% at day 14, $p < 0.05$). After 7
289 days, centrifuged culture had a higher percentage of cysts (53%) than ice-cooled and control
290 cultures (35% and 36%, respectively; ANOVA, $p < 0.05$). Percentage of cysts remained quite
291 stable in centrifuged and ice-cooled cultures after 14 days of culture (58% and 32%
292 respectively) but more than in control culture (less than 19%). As all *A. minutum* cells were
293 dead in the frozen treatment, presence and occurrence of cysts were not monitored.
294 After freeze-killing and ice-cooling *A. minutum* cells, chlorophyll fluorescence in dead cells
295 decreased steadily during the 14 days (Fig. 4C). In the other conditions (ice cooled,
296 centrifuged, control), the red fluorescence in live cells increased from day 0 until day 4 and
297 then returned to initial values by day 14 (Fig. 4C).

298

299 **3.3 Cellular responses to chemical treatments**

300

301 In controls, there was a large majority of vegetative cells (91%), 9% of pellicle cysts, and no
302 dead cells (Fig. 5).

303 Upon increasing the concentration of saponine, counts of vegetative cells gradually decreased
304 while pellicle cysts concomitantly increased (Fig. 5A). All vegetative cells were transformed
305 in pellicle cysts when saponine concentration was above 0.05 g.L^{-1} . However, even at the
306 highest concentration of saponine, no dead cells were detected using SYTOX-Green staining
307 (Fig. 5C).

308 Counts of vegetative cells steadily decreased when H_2O_2 concentration increased (Fig. 5B).

309 All vegetative cells were replaced by pellicle cysts when the H_2O_2 concentration reached
310 0.015%. Above this concentration, the percentage of dead cells increased rapidly to reach
311 82% and 99% of total cells when H_2O_2 concentrations were 0.03% and 0.15%, respectively
312 (Fig. 5D).

313 In both experiments, counts of empty thecae increased concomitantly along with
314 concentration of pellicle cysts. Very good correlations ($R^2 = 0.97$, $p < 0.0001$) were obtained
315 between pellicle cysts and empty thecae when combining the saponine and H_2O_2 experiments.
316 Empty thecae resulting from cell death were also found at highest H_2O_2 concentrations.

317

318 **3.4 Excystment of *A. minutum* after mechanical stress**

319

320 Results of this experiment indicated that pellicle cysts of *A. minutum* were able to excyst in
321 less than 24h. No significant difference in the percentage of pellicle cysts was observed from
322 0 to 6 hours after centrifugation. After 9h, the percentage of pellicle cysts decreased
323 significantly every 3h for the rest of the experiment, down to 24.5%, 24h after centrifugation
324 (Fig. 6). The total cell counts did not increase, thereby identifying that excystment, and not
325 growth of the remaining vegetative cells was responsible for the increasing percentage of
326 vegetative cells.

327 **4 Discussion**

328

329 *Cysts formation in A. minutum and detection by flow cytometry*

330 To survive through unfavorable environmental conditions, dinoflagellates can form resting
331 and pellicle cysts. The resting cysts (or hypnocyts) are formed through a complex sexual
332 process. They are surrounded by persistent cell wall, and require a mandatory dormancy
333 period prior to germination (Garcés et al., 1998). Pellicle cysts, also called ecdysal or
334 temporary cysts, are formed from vegetative cells by shedding theca (ecdysis) and do not
335 require a mandatory dormancy period (Olli, 2004; Figueroa and Bravo, 2005; Figueroa et al.,
336 2005, 2008; Bravo et al., 2010).

337 In the present study, pellicle cysts were shown to be impermeable to SYBR-Green I, a cell
338 permeant dye. During formation of pellicle cysts, armored dinoflagellate cells undergo a
339 process termed ecdysis during which the cell covering (including plasma membranes, outer
340 amphiesmal vesicle membranes and thecal plates) is shed and immediately followed by the
341 transformation of the cell into a non-motile cyst covered by a single-layered wall or pellicle
342 (Sekida et al., 2001). The low permeability of this pellicle was previously demonstrated
343 (Montresor, 1995; Garcés et al., 1998), thus explaining why pellicle cysts of *A. minutum* were
344 impermeable to SYBR-Green I in our conditions.

345 Red fluorescence measured by flow cytometry of pellicle cysts was only reduced by about 20-
346 30% compared to vegetative cells, suggesting that chlorophyll pigments remained fairly
347 intact. Microscopic observations of pellicle cysts confirmed that chloroplasts and chlorophyll
348 pigments were not degraded and little modified upon encystment. Indeed, such pellicle cysts
349 are known to remain fully metabolically active (Taylor, 1987).

350 Concomitant with the formation of pellicle cysts, empty thecae were found in the culture.
351 Empty thecae were detected by flow cytometry because of SYBR-Green I stained bacteria
352 present on the surface of thecae. Microscopic observations confirmed the presence of bacteria
353 associated with empty thecae of *A. minutum*. SYBR-Green I stained bacteria were also
354 observed on the surface of degraded and dead cells. Biegala et al. (2002) also observed
355 bacteria associated with empty thecae of *Alexandrium* sp.

356 As pellicle cysts generally appear in culture upon short-term or sudden adverse conditions
357 (Bravo et al., 2010), shear stress caused by pipette handling was tested as a potential factor
358 inducing encystment prior running to our stress experiments. In our experimental conditions,
359 however, pipette handling did not cause the formation of pellicle cysts if cultures were
360 handled carefully (data not shown). Age of culture may also influence the propensity of cells

361 to transform into pellicle cysts. Pellicle cysts are “naturally” produced in *A. minutum* cultures
362 under normal conditions ranging from 2-3% to 24-25% of total cells, respectively, during the
363 exponential and stationary phases (Bolli et al., 2007). In our experimental conditions, the
364 culture grew steadily until day 14; however, pellicle cysts appeared after 7 days of culture.
365 Occurrence of these pellicle cysts likely resulted from culture ageing and nutrient stress, as
366 previously demonstrated in other *Alexandrium* species (Anderson and Wall, 1978; Jensen and
367 Moestrup, 1997).

368

369 ***Impact of thermal and mechanical treatments on A. minutum cellular responses***

370 Storage on ice resulted in mainly the transformation of vegetative cells into dead cells,
371 pellicle cysts, and “degraded” cells and a few empty thecae (immediately following
372 treatment); whereas, centrifugation resulted mostly in pellicle cysts and a few degraded cells.
373 Vegetative cells re-appeared rapidly (one day after stress) and multiplied while pellicle cysts
374 decreased. This confirms that *A. minutum* asexual encystment is a protective process, that is
375 rapidly reversible (Doucette et al., 1989; Figueroa and Bravo, 2005).

376 Presence of degraded cells was clearly related to both experimental treatments as counts
377 remained low in the control culture. Degraded cells were revealed by both reduced
378 chlorophyll fluorescence and SYBR-Green I fluorescence, as compared to vegetative cells.
379 Reduction in red fluorescence likely resulted from degradation and/or modification of
380 chlorophyll pigments. Chloroplasts or pigments seemed to migrate and fuse toward the center
381 of the cells, resulting in a star-like shape of red fluorescence. Reduction in SYBR-Green I
382 fluorescence intensity may reveal some DNA degradation or nuclear re-arrangement or both.
383 Within the cell, green fluorescence of SYBR-Green I appeared to be diffuse and did not show
384 the characteristic U-shaped nucleus, common to the genus *Alexandrium* and described in
385 detail for *A. minutum* by Figueroa et al. (2007).

386 Appearance of degraded cells is probably an irreversible process in contrast to that of pellicle
387 cyst formation. The morphological and fluorescent characteristics of degraded cells were very
388 different from those of dead cells, suggesting that these two forms resulted from independent
389 cellular mechanisms. We speculated that degraded cells possibly resulted from failed asexual
390 encystments, while dead cells were obtained from fast killing (thermal shock) prior to
391 encystment. Thermal and mechanical stresses may directly impair vegetative cell division or
392 interfere with pellicle or even sexual cyst formation. The fact that all these stages could exist
393 simultaneously in asynchronous cultures (Olli, 2004) would explain the variety of observed

394 cellular responses to stress. Thecae of degraded cells were clearly damaged, supporting the
395 hypothesis of a failed/aborted encystment.

396

397 The main difference between mechanical and thermal stresses was the presence of dead cells
398 in ice-cooled culture (absent in centrifuged culture); 30% of *A. minutum* cells were found
399 dead after the stress, but counts decreased during the following days of culture.

400 After these stresses, growth of *A. minutum* during the first seven days was similar to that
401 observed in control culture. During the last week of monitoring, however, cell counts of
402 stressed cultures reached a plateau earlier compared to control cultures. Stressed cultures also
403 contained a higher percentage of cysts than control culture. This suggests that a stress
404 encountered early in the culture may have physiological and growth consequences several
405 days after this stress.

406

407 ***Impact of chemical stresses on A. minutum cellular responses***

408 We further explored cellular responses of *A. minutum* cultures by exposing them to chemical
409 stresses in order to determine if formation of pellicle cysts appears in a dose-dependent
410 manner. Thus, different *A. minutum* cultures were exposed to gradients of saponine and H₂O₂.
411 Indeed, formation of pellicle cysts could be used as a defense mechanism to resist or tolerate
412 allochemicals produced by other microalgal species including dinoflagellates (Fistarol et al.,
413 2004) or even digestive enzymes when ingested by bivalves. For both chemicals (saponine or
414 H₂O₂), encystment was dose-dependent. Pellicle cysts were the predominant cell type above
415 0.05 g.L⁻¹ saponine and above 0.015% H₂O₂. Responses to chemical stress observed here are
416 similar to those observed in the dinoflagellate *S. trochoidea* when exposed to allelochemicals
417 produced by competing microalgae (Fistarol et al., 2004). Some researchers also observed
418 that strong allelopathic effects resulted in cell death with no pellicle cyst formation. Fistarol et
419 al. (2004) hypothesized that strong allelopathic effects caused permanent damage to cell
420 membranes prior to encystment. Similarly, in our study, occurrence of dead cells appeared to
421 follow an all-or-none pattern as dead cell percentage increased from 3% at 0.015% H₂O₂ to
422 81% at 0.03% H₂O₂ without pellicle cyst formation. Present method can be used, for example,
423 to assess the impact of pollution or in the evaluation of treatment efficiency of ballast waters.
424 Indeed, surfactants are produced for multiple applications all over the world and are
425 responsible of marked environmental pollution, especially in aquatic environment (Cserhádi et
426 al., 2002). Some studies evaluated efficiency of oxidants, such as hydrogen peroxide, in

427 eradication of phytoplankton from ballast waters to avoid dissemination of invasive/non-
428 endemic species (Gavand et al., 2007).

429 In both experiments, counts of empty thecae were very well correlated to counts of pellicle
430 cysts (excluding highest doses of H₂O₂ at which pellicle cysts were not detected).

431 Surprisingly, when exposed to the highest doses of H₂O₂, empty thecae were detected
432 suggesting that encystment was initiated. The loss of thecae seemed to be an extremely rapid
433 process as it occurred before cells were killed. When stress is too brutal and sudden,
434 formation of complete pellicle cysts (pellicle formation) may not be fast or efficient enough to
435 avoid cell death, or the pellicles produced were not strong enough to withstand high H₂O₂
436 concentration.

437

438 ***Encystment and excystment dynamics***

439 As demonstrated with other *Alexandrium* species (Doucette et al., 1989; Figueroa and Bravo,
440 2005), encystment and excystment are very fast phenomena in *A. minutum*. The present study
441 demonstrated that encystment could be triggered very rapidly by mechanical stresses (*i.e.*
442 centrifugation) and caused the encystment of nearly the totality of all vegetative cells.

443 Similarly, excystment of *A. minutum* also occurs quite rapidly. The first cysts of *A. minutum*
444 started to excyst after 6 to 9 hours after mechanical stress, to reach about 75% of excystment
445 after only 24 hours in good culture conditions (16°C, light, no handling). These results
446 therefore suggest that encystment of *A. minutum* represent a very good and efficient strategy
447 for this algal species to overcome any short term adverse environmental condition.

448

449 ***Towards a better understanding of the role of encystment***

450 Overall, these experiments confirmed that formation of pellicle cyst is a cellular process
451 allowing protection of *A. minutum* against mechanical, thermal, or chemical stresses. Pellicle
452 encystment is a reversible process as excystment (production of vegetative cells from pellicle
453 cysts) can occur rapidly within a few hours after a stress and be followed by culture growth.

454 Our data indicate, however, that recovery from stress was not absolute, as cell counts of
455 stressed cultures were lower than control cultures after 7 and 14 days of culture. The ability of
456 *A. minutum* to quickly produce a resistant form (cyst) can have implications for the study of
457 interactions between harmful dinoflagellates and other cells or aquatic organisms. Formation
458 of pellicle cysts in experimental tanks could, for example, modulate toxin uptake upon
459 bivalve feeding on these pellicle cysts. The fact that pellicle cysts are immotile could also lead
460 to faster sedimentation of these cells compared to vegetative cells, making them more or less

461 accessible for bivalve feeding. Temporary cysts were observed following cell transit in the
462 digestive tract of *Crassostrea gigas* (Laabir and Gentien, 1999; Laabir et al., 2007; Haberkorn
463 et al., 2010). Recent studies showed the presence of temporary cysts of *A. fundyense* and *A.*
464 *minutum* in digestive systems of bivalves (*Mytilus edulis* and *C. gigas*) (Galimany et al.,
465 2008; Haberkorn et al., 2010). Laabir and Gentien (1999) reported that 50–90% of the fecal
466 pellets produced by the Pacific Oyster *C. gigas* fed *A. minutum* and *A. tamarense* were
467 composed of intact, immobile *Alexandrium* sp. cells. Similarly, Persson et al. (2006)
468 demonstrated that 28% of *A. fundyense* survived gut passage by forming pellicle cysts in
469 oysters (*C. virginica*) fed toxic vegetative cells. Moreover, *A. fundyense* was able to resume
470 vegetative growth after gut passage (Hégaret et al., 2008). Formation of pellicle cysts could
471 also therefore appear as a way to rapidly protect cells against digestion upon ingestion by
472 bivalves thus favoring development of *Alexandrium* sp. blooms despite benthic grazing.
473 The present study provides a new approach to quantify life-cycle changes and viability in *A.*
474 *minutum* cells. This new and rapid method can be used to improve our understanding of
475 encystment/excystment, cell viability, and subsequent growth of *Alexandrium* populations,
476 under real or simulated environmental conditions.
477

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481

482

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621

622

622 **Figure captions:**

623

624 Figure 1: SSC (Side SCatter)-FSC (Forward SCatter) cytogram (A, C and E) and FL3
625 (chlorophyll-related pigment red fluorescence)-FL1 (SYBR-Green I fluorescence) cytogram
626 (B, D and F) of *A. minutum* cells after centrifugation (A and B), incubation one hour on ice (C
627 and D), freezing 24h at -20°C (E and F) and staining with SYBR-Green I. R1: vegetative
628 cells; R2: cysts; R3: degraded cells; R4: theca; R5: dead cells.

629

630 Figure 2: *A. minutum* cells (A, B, C and D) under white light (A1, B1, C1 and D1) as well as
631 red and green epi-fluorescent light (A2, B2, C2 and D2) after three centrifugations at 800 x g
632 during 5 min. A: vegetative cells; B: pellicle cysts; C: degraded cells; D: theca. Scale bar = 10
633 μm .

634

635 Figure 3: Dead *A. minutum* cells (A, B and C) under white light (A), red epi-fluorescent light
636 (B) as well as red and green epi-fluorescent light (C) after freezing (24h at -20°C). Scale bar =
637 10 μm .

638

639 Figure 4: A: Changes in *A. minutum* cell concentration during 14 days in control, freezing,
640 centrifugation and ice-cooling conditions after SYTOX-Green staining. B: Changes in *A.*
641 *minutum* dead cell concentration during 14 days in control, freezing, centrifugation and ice-
642 cooling conditions after SYTOX-Green staining. C: Changes in *A. minutum* red fluorescence
643 intensity during 14 days in control live cells, centrifuged live cells, ice-cooled live cells and
644 frozen dead cells after SYTOX-Green staining. Results are expressed as mean \pm CI with n=3.

645

646 Figure 5: Effect of 1h incubation with increasing concentrations of saponine (A, C) and H₂O₂
647 (B, D) on *A. minutum* cyst and vegetative cell concentrations after SYBR-Green I staining (A,
648 B) and on the percentage of dead cells and red fluorescence intensity of *A. minutum* cells after
649 SYTOX-Green staining (C, D). Results are expressed as mean \pm CI with n=3.

650

651 Figure 6: Percentages of *A. minutum* cysts and vegetative cells after SYBR-Green I staining
652 over a 24h time period, after three centrifugations at 800 x g, compared to a non-centrifuged
653 control. Results are expressed as mean \pm CI with n=6.

654

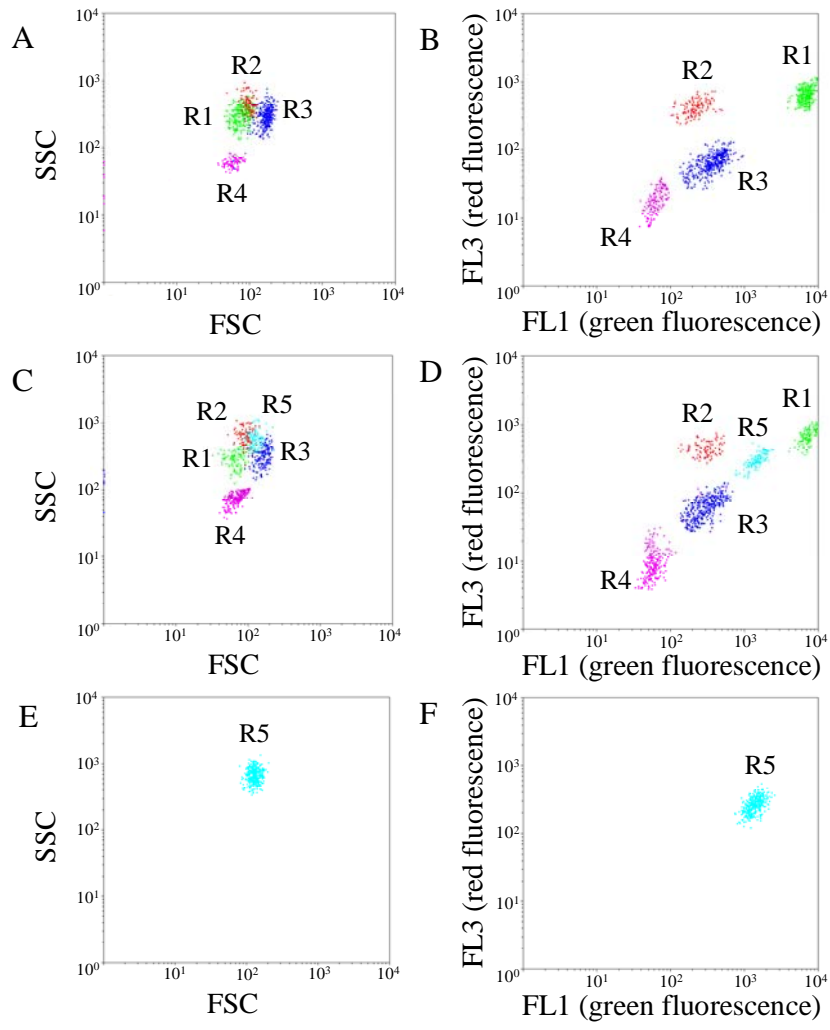


Figure 1

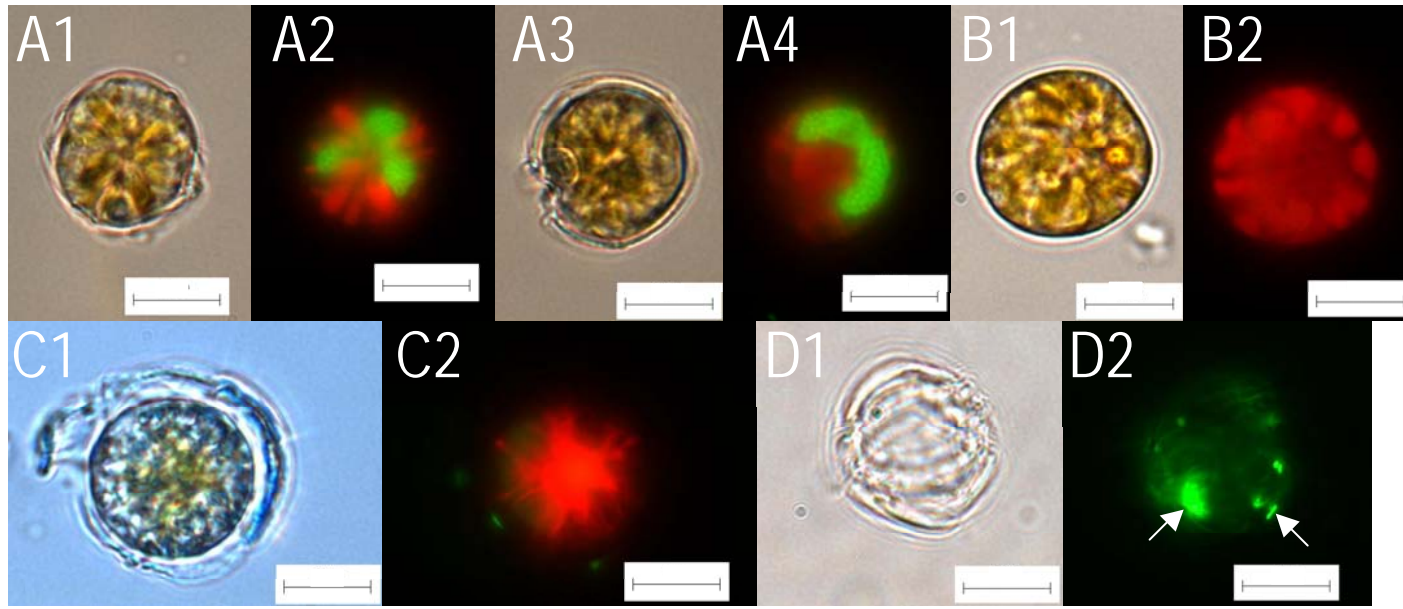


Figure 2

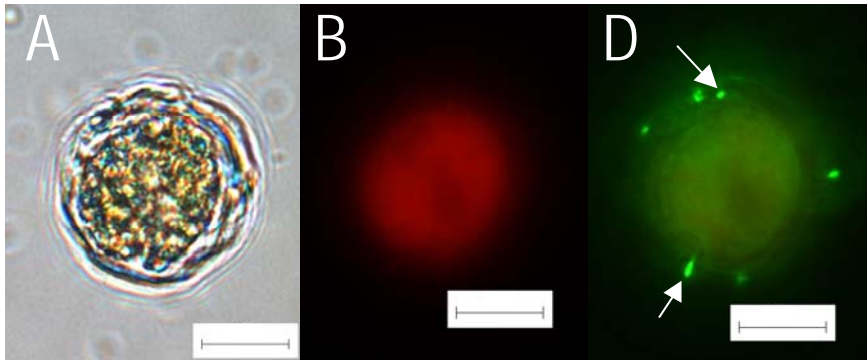


Figure 3

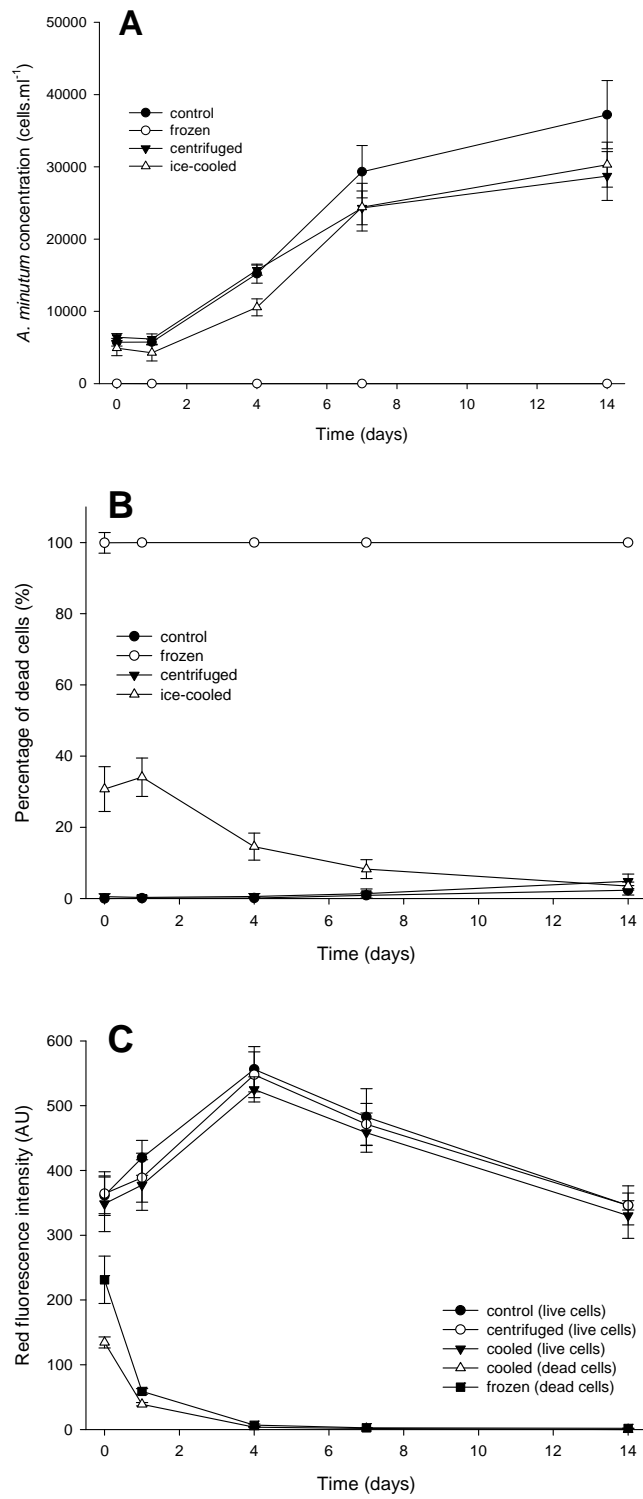


Figure 4

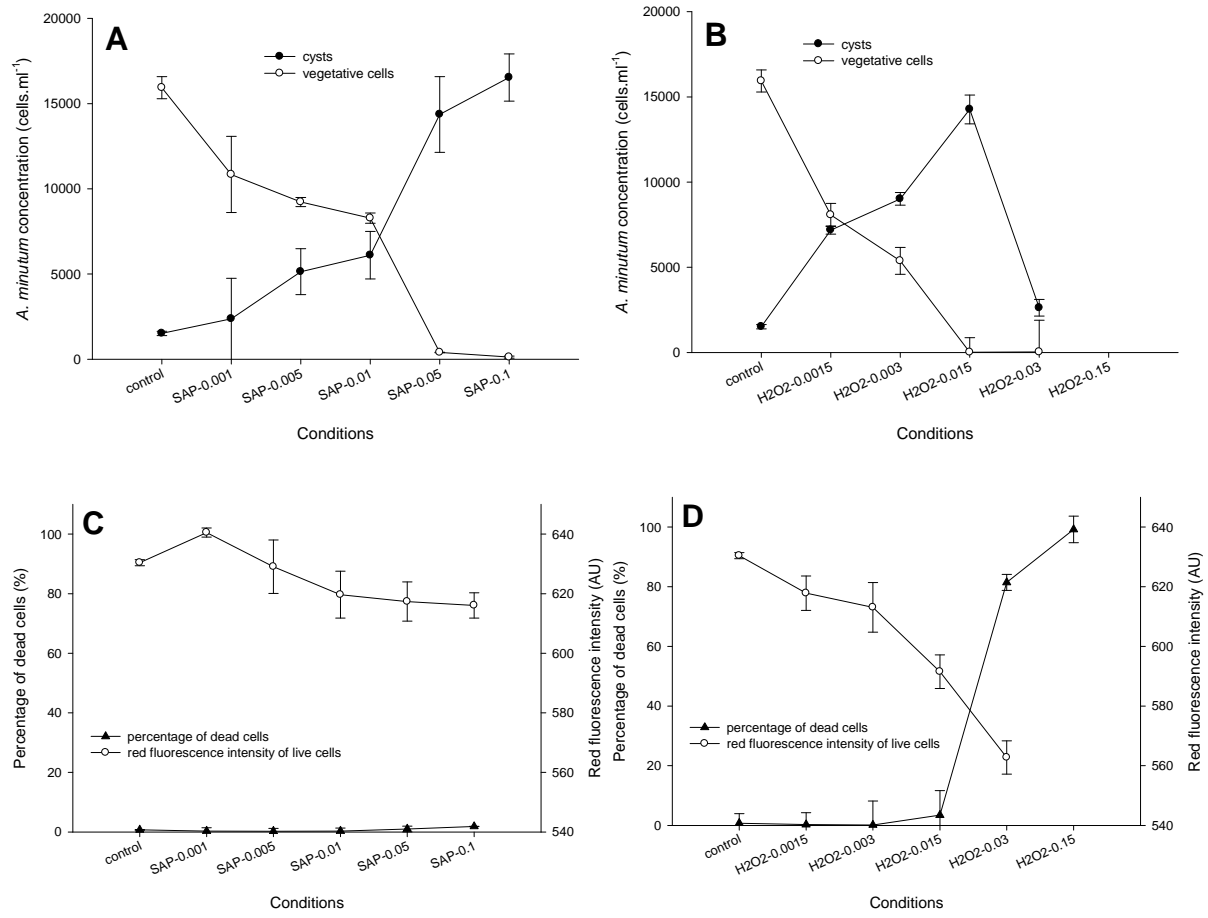


Figure 5

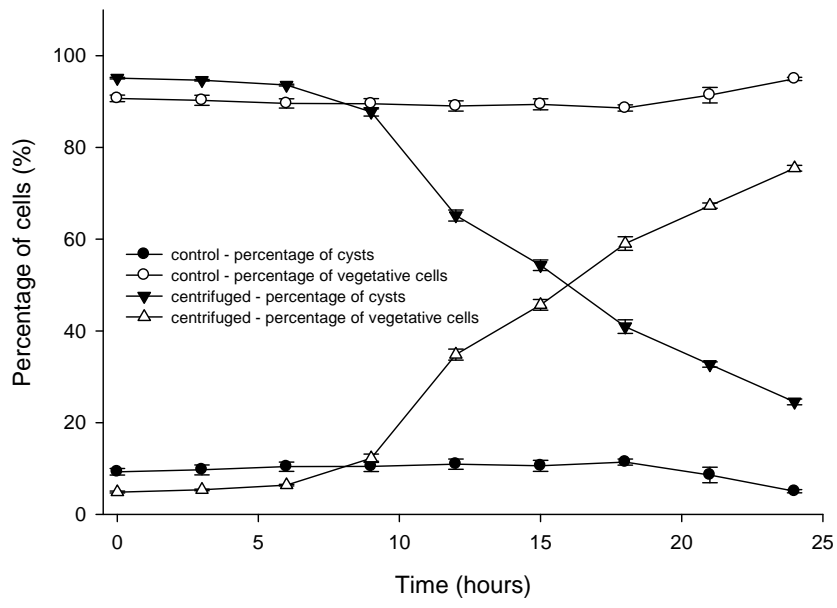


Figure 6