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Abstract

The purpose of the present study was to provide a flow-cytometric (FCM) approach evaluating *A. minutum* cellular responses to mechanical, thermal and chemical stresses. Coupling SYBR-Green I and SYTOX-Green staining, FCM analysis and sorting, and microscopic observations permitted identification and characterization of five cellular states/forms of *A. minutum*; 1) vegetative cells, 2) pellicle cysts, 3) degraded cells, 4) empty theca and 5) dead cells. Storage on ice resulted in the transformation of a portion of vegetative cells into dead cells, pellicle cysts, and "degraded" cells; however, centrifugation resulted mostly in pellicle cysts and a few degraded cells. After these thermal and mechanical treatments, control and stressed cultures were monitored for 14 days. Stressed *A. minutum* cultures appeared to grow at the same rates as control cultures during the first seven days. During the last week of monitoring, however, cell densities of stressed cultures reached their stationary phase earlier than control cultures, suggesting incomplete recovery. Additional experiment assessing kinetics of excystment indicated that it can occur less than 9 hours following mechanical stress (centrifugation) and that 75% of the culture can excyst within 24 hours. Upon 30 min of exposure to chemical stressors (saponine and H_2O_2), only vegetative cells, pellicle cysts, and dead cells were detected. For both chemicals, encystment was dose-dependent. Counts of pellicle cysts increased with increasing saponine or H_2O_2 concentration. Pellicle cysts were the most-predominant cell type at chemical treatments above 0.05 $g.L^{-1}$ saponine and above 0.015% H_2O_2. Occurrence of dead cells appeared to follow an all-or-none response as dead-cell percentage increased from 3% at 0.015% H_2O_2 to 81% at 0.03% H_2O_2 without pellicle cyst formation. Overall, encystment-excystment of *A. minutum* upon changes of environmental conditions can occur very rapidly but can be monitored using FCM and SYBR-Green I staining.

1 Introduction

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

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

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

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

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

The objective of the present study was to characterize the different cell forms and viability of *A. minutum*, combining flow cytometry, microscopic observations, and fluorescent dyes (SYBR-Green I and SYTOX-Green). Various types of stresses (thermal, mechanical, and chemical) of varied intensities were also applied to *A. minutum* cultures to establish ranges of morphological and physiological responses. Cultures of *A. minutum* were exposed to i) freezing, ice cooling and centrifugation and ii) a gradient of saponine and H_2O_2. Thermal and mechanical treatments were designed to simulate various stresses that *A. minutum* could encountered during culture handling and/or bivalve filtration. The two tested chemicals aimed to “simulate” environmental chemical stresses (pollutant or natural) as both could compromise cell membrane integrity by two different mechanisms. The saponine is a surfactant that can disorganize the lipid bilayer membrane and solubilize proteins. Many detergents, natural or human activity related, can be encountered in coastal environment. The H_2O_2 is an oxidizing agent which can compromise cell membranes by oxidizing lipids.

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

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

2 Materials and Methods

2.1 Algal culture

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

2.2 Flow-cytometric analyses

2.2.1 SYTOX-Green and SYBR-Green I staining procedures

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

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

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

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

2.2.3 Identification of *A. minutum* subpopulations

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

2.3 Microscopy

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

2.4 Experimental conditions

2.4.1 Thermal and mechanical stresses

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

After treatments, culture tubes were returned to previous culture conditions (16 ± 1°C and 100 µmol photon.m^2.s^{-1}, with a dark:light cycle of 12:12h). Two 250-µL samples were taken from each tube just after treatments and after 1, 4, 7 and 14 days and analyzed as previously described (2.2).

2.4.2 Chemical stress

Three cultures of *A. minutum* (12 days old) were diluted to 15,000 cells.mL^{-1} with FSSW into 33 15-mL plastic tubes (eleven treatments in triplicate). Three tubes were used as controls and the thirty remaining tubes were exposed to increasing concentrations of saponine (0.001, 0.005, 0.01, 0.05 and 0.1 g.L^{-1} as final concentrations) and H_2O_2 (0.0015%, 0.003%, 0.015%, 0.03% and 0.15% as final concentrations, % v/v). Previous experiments were performed (data not shown) to determine the chemical concentrations allowing the observation of dose-dependent responses. All tubes were then maintained for 30 min in the dark at 16°C. After incubation, two samples of 250 µL were taken from each tube and analyzed as described previously (2.2).

2.4.3 Time for excystment after mechanical stress

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

After treatments, culture tubes were returned to previous culture conditions (16 ± 1 °C and 100 µmol photon.m^2.s^{-1}, with a dark:light cycle of 12:12h). Every 3 hours, from 0h to 24h after the centrifugation, 300-µL samples were taken from each tube and incubated for 30 min in the dark at 16°C with SybrGreen I and analyzed with the flow cytometer to determine the percentage of temporary cysts.

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207 **2.5 Statistical analysis**

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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).

3 Results

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

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

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

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

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

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

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

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

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

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

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

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

Four days after the stress, cell division was observed in centrifuged, ice cooled, and control cultures, reaching 16.10^3, 10.10^3 and 15.10^3 cells.ml^{-1}. Cell counts in ice-cooled cultures were lower than those of centrifuged and control cultures until day 4 ($p < 0.05$). After 7 and 14 days of monitoring, cell densities in centrifuged and ice cooled cultures were similar, but significantly lower than those of control cultures (about -20% at day 14, $p < 0.05$). After 7 days, centrifuged culture had a higher percentage of cysts (53%) than ice-cooled and control cultures (35% and 36%, respectively; ANOVA, $p < 0.05$). Percentage of cysts remained quite stable in centrifuged and ice-cooled cultures after 14 days of culture (58% and 32% respectively) but more than in control culture (less than 19%). As all *A. minutum* cells were dead in the frozen treatment, presence and occurrence of cysts were not monitored.

After freeze-killing and ice-cooling *A. minutum* cells, chlorophyll fluorescence in dead cells decreased steadily during the 14 days (Fig. 4C). In the other conditions (ice cooled, centrifuged, control), the red fluorescence in live cells increased from day 0 until day 4 and then returned to initial values by day 14 (Fig. 4C).

3.3 Cellular responses to chemical treatments

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

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

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

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

3.4 Excystment of *A. minutum* after mechanical stress

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

4 Discussion

Cysts formation in A. minutum and detection by flow cytometry

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

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

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

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

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

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

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

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

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

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

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

The main difference between mechanical and thermal stresses was the presence of dead cells in ice-cooled culture (absent in centrifuged culture); 30% of *A. minutum* cells were found dead after the stress, but counts decreased during the following days of culture.
After these stresses, growth of *A. minutum* during the first seven days was similar to that observed in control culture. During the last week of monitoring, however, cell counts of stressed cultures reached a plateau earlier compared to control cultures. Stressed cultures also contained a higher percentage of cysts than control culture. This suggests that a stress encountered early in the culture may have physiological and growth consequences several days after this stress.

Impact of chemical stresses on A. minutum cellular responses

We further explored cellular responses of *A. minutum* cultures by exposing them to chemical stresses in order to determine if formation of pellicle cysts appears in a dose-dependent manner. Thus, different *A. minutum* cultures were exposed to gradients of saponine and H_2O_2. Indeed, formation of pellicle cysts could be used as a defense mechanism to resist or tolerate allochemicals produced by other microalgal species including dinoflagellates (Fistarol et al., 2004) or even digestive enzymes when ingested by bivalves. For both chemicals (saponine or H_2O_2), encystment was dose-dependent. Pellicle cysts were the predominant cell type above 0.05 $g.L^{-1}$ saponine and above 0.015% H_2O_2. Responses to chemical stress observed here are similar to those observed in the dinoflagellate *S. trochoidea* when exposed to allelochemicals produced by competing microalgae (Fistarol et al., 2004). Some researchers also observed that strong allelopathic effects resulted in cell death with no pellicle cyst formation. Fistarol et al. (2004) hypothesized that strong allelopathic effects caused permanent damage to cell membranes prior to encystment. Similarly, in our study, occurrence of dead cells appeared to follow an all-or-none pattern as dead cell percentage increased from 3% at 0.015% H_2O_2 to 81% at 0.03% H_2O_2 without pellicle cyst formation. Present method can be used, for example, to assess the impact of pollution or in the evaluation of treatment efficiency of ballast waters. Indeed, surfactants are produced for multiple applications all over the world and are responsible of marked environmental pollution, especially in aquatic environment (Cserháti et al., 2002). Some studies evaluated efficiency of oxidants, such as hydrogen peroxide, in

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

In both experiments, counts of empty thecae were very well correlated to counts of pellicle cysts (excluding highest doses of H_2O_2 at which pellicle cysts were not detected). Surprisingly, when exposed to the highest doses of H_2O_2, empty thecae were detected suggesting that encystment was initiated. The loss of thecae seemed to be an extremely rapid process as it occurred before cells were killed. When stress is too brutal and sudden, formation of complete pellicle cysts (pellicle formation) may not be fast or efficient enough to avoid cell death, or the pellicles produced were not strong enough to withstand high H_2O_2 concentration.

Encystment and excystment dynamics

As demonstrated with other *Alexandrium* species (Doucette et al., 1989; Figueroa and Bravo, 2005), encystment and excystment are very fast phenomena in *A. minutum*. The present study demonstrated that encystment could be triggered very rapidly by mechanical stresses (*i.e.* centrifugation) and caused the encystment of nearly the totality of all vegetative cells. Similarly, excystment of *A. minutum* also occurs quite rapidly. The first cysts of *A. minutum* started to excyst after 6 to 9 hours after mechanical stress, to reach about 75% of excystment after only 24 hours in good culture conditions (16°C, light, no handling). These results therefore suggest that encystement of *A. minutum* represent a very good and efficient strategy for this algal species to overcome any short term adverse environmental condition.

Towards a better understanding of the role of encystement

Overall, these experiments confirmed that formation of pellicle cyst is a cellular process allowing protection of *A. minutum* against mechanical, thermal, or chemical stresses. Pellicle encystment is a reversible process as excystement (production of vegetative cells from pellicle cysts) can occur rapidly within a few hours after a stress and be followed by culture growth. Our data indicate, however, that recovery from stress was not absolute, as cell counts of stressed cultures were lower than control cultures after 7 and 14 days of culture. The ability of *A. minutum* to quickly produce a resistant form (cyst) can have implications for the study of interactions between harmful dinoflagellates and other cells or aquatic organisms. Formation of pellicle cysts in experimental tanks could, for example, modulate toxin uptake upon bivalve feeding on these pellicle cysts. The fact that pellicle cysts are immotile could also lead to faster sedimentation of these cells compared to vegetative cells, making them more or less

accessible for bivalve feeding. Temporary cysts were observed following cell transit in the digestive tract of *Crassostrea gigas* (Laabir and Gentien, 1999; Laabir et al., 2007; Haberkorn et al., 2010). Recent studies showed the presence of temporary cysts of *A. fundyense* and *A. minutum* in digestive systems of bivalves (*Mytilus edulis* and *C. gigas*) (Galimany et al., 2008; Haberkorn et al., 2010). Laabir and Gentien (1999) reported that 50–90% of the fecal pellets produced by the Pacific Oyster *C. gigas* fed *A. minutum* and *A. tamarense* were composed of intact, immobile *Alexandrium* sp. cells. Similarly, Persson et al. (2006) demonstrated that 28% of *A. fundyense* survived gut passage by forming pellicle cysts in oysters (*C. virginica*) fed toxic vegetative cells. Moreover, *A. fundyense* was able to resume vegetative growth after gut passage (Hégaret et al., 2008). Formation of pellicle cysts could also therefore appear as a way to rapidly protect cells against digestion upon ingestion by bivalves thus favoring development of *Alexandrium* sp. blooms despite benthic grazing.

The present study provides a new approach to quantify life-cycle changes and viability in *A. minutum* cells. This new and rapid method can be used to improve our understanding of encystment/excystment, cell viability, and subsequent growth of *Alexandrium* populations, under real or simulated environmental conditions.

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Figure captions:

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

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

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

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

Figure 5: Effect of 1h incubation with increasing concentrations of saponine (A, C) and H_2O_2 (B, D) on *A. minutum* cyst and vegetative cell concentrations after SYBR-Green I staining (A, B) and on the percentage of dead cells and red fluorescence intensity of *A. minutum* cells after SYTOX-Green staining (C, D). Results are expressed as mean ± CI with n=3.

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

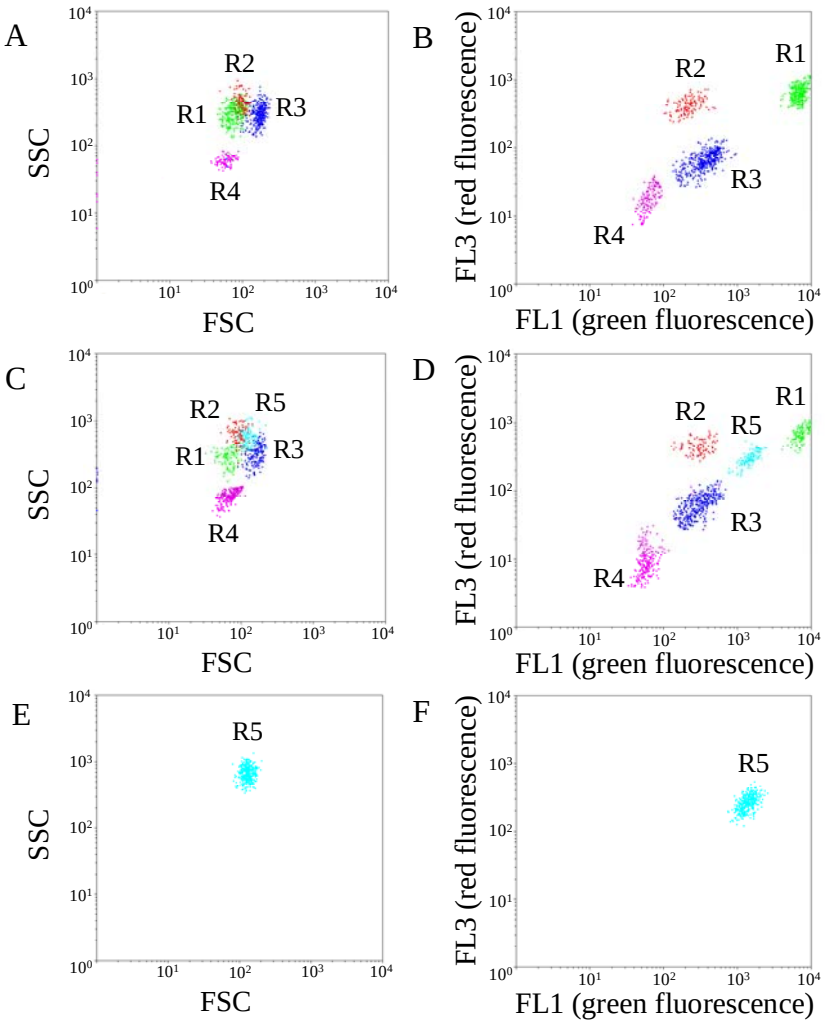
A
B
C
D
E
F
SSC
FSC
FL3 (red fluorescence)
FL1 (green fluorescence)
R1
R2
R3
R4
R5

Figure 1

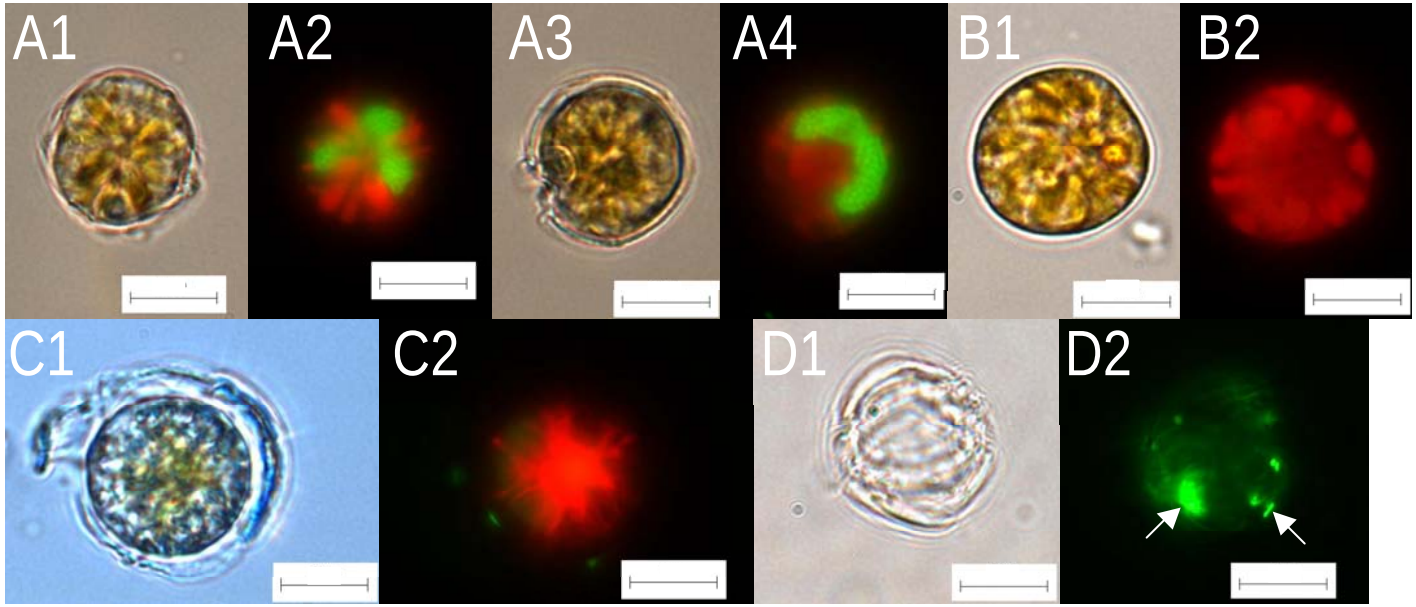

Figure 2

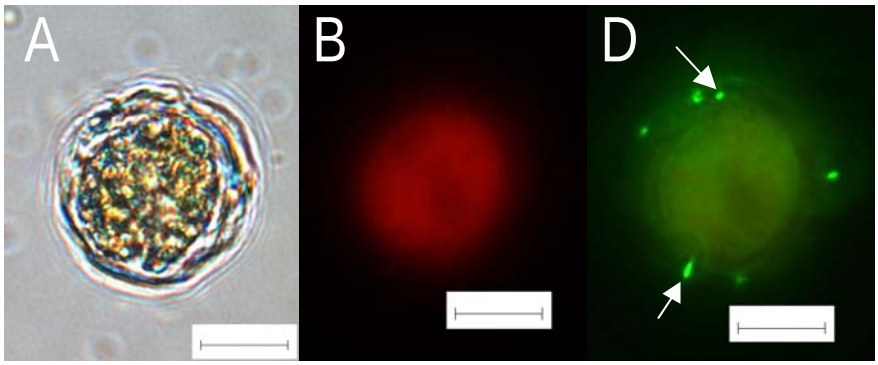

Figure 3

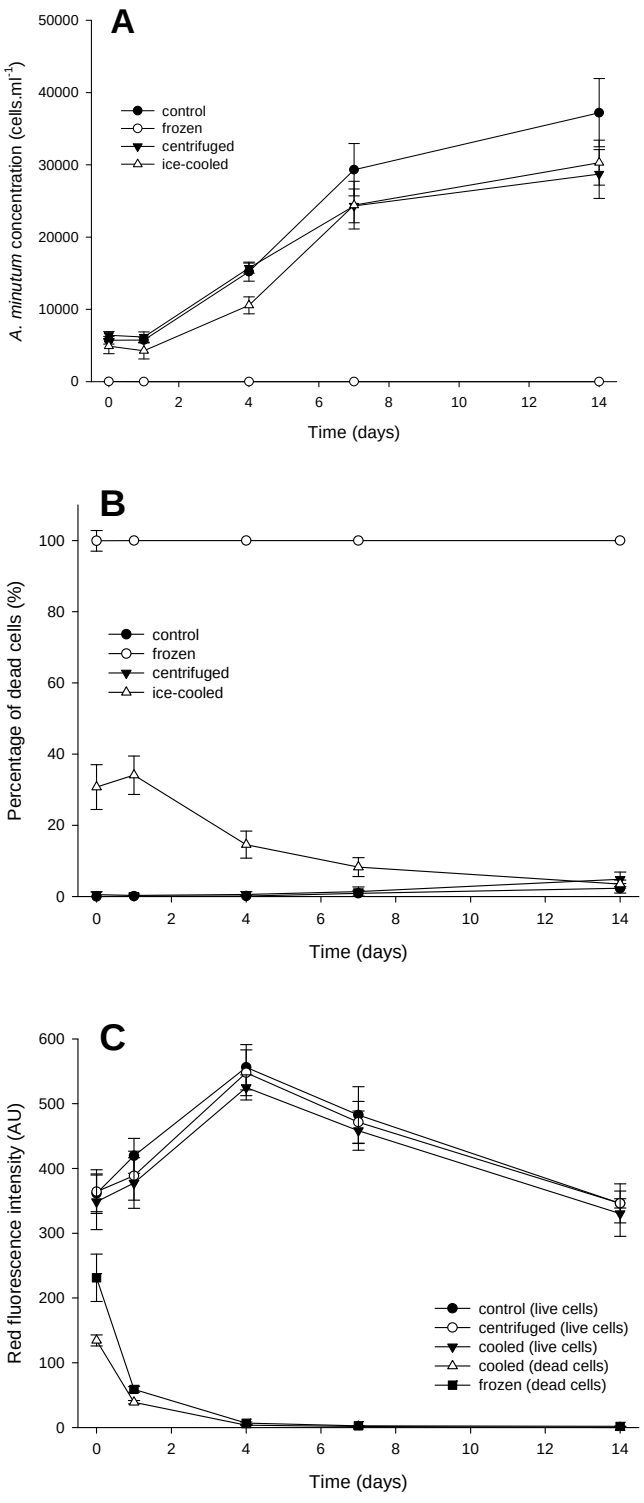
A
A. minutum concentration (cells.ml-1)
50000
40000
30000
20000
10000
0
control
frozen
centrifuged
ice-cooled
0
2
4
6
8
10
12
14
Time (days)
B
Percentage of dead cells (%)
100
80
60
40
20
0
control
frozen
centrifuged
ice-cooled
0
2
4
6
8
10
12
14
Time (days)
C
Red fluorescence intensity (AU)
600
500
400
300
200
100
0
control (live cells)
centrifuged (live cells)
cooled (live cells)
cooled (dead cells)
frozen (dead cells)
0
2
4
6
8
10
12
14
Time (days)

Figure 4

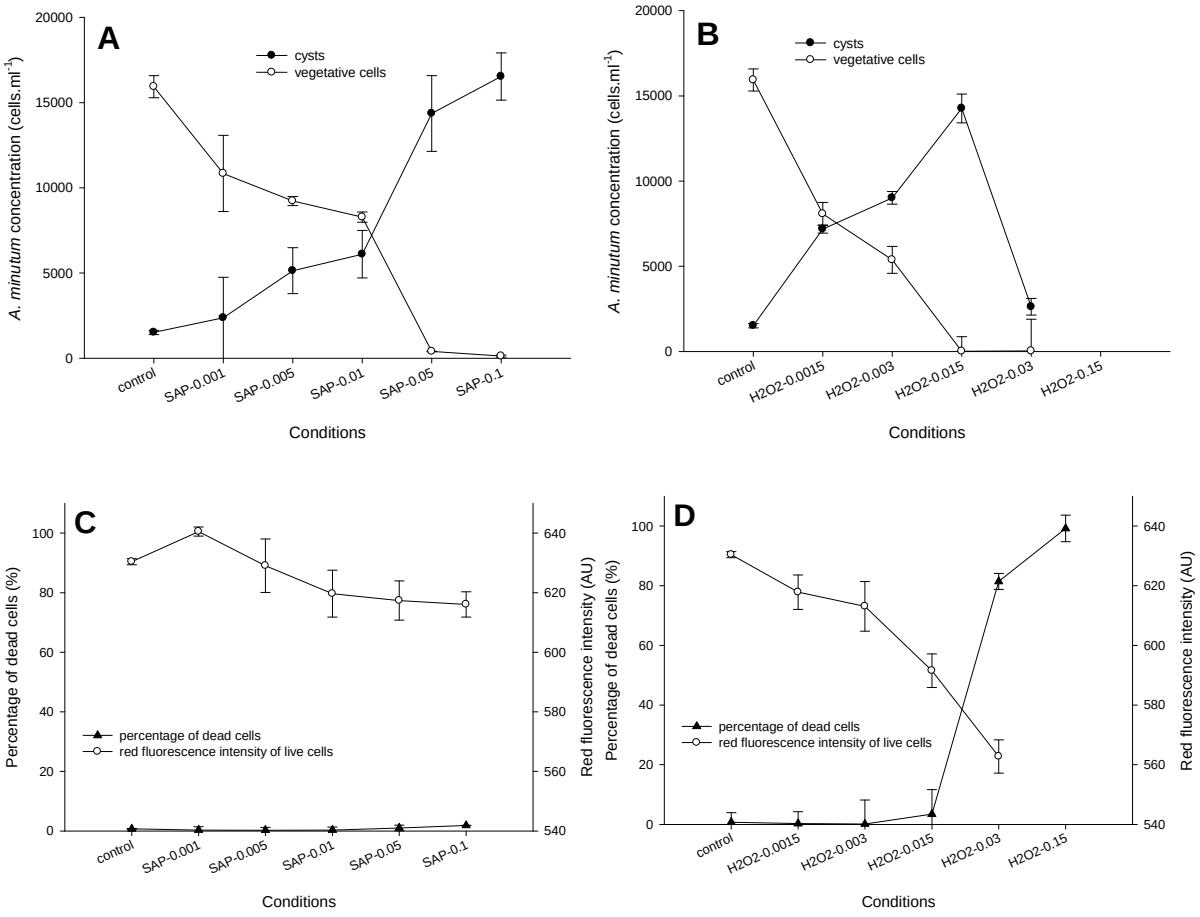
A
cysts
vegetative cells
A. minutum concentration (cells.ml-1)
20000
15000
10000
5000
0
control
SAP-0.001
SAP-0.005
SAP-0.01
SAP-0.05
SAP-0.1
Conditions
B
cysts
vegetative cells
A. minutum concentration (cells.ml-1)
control
H2O2-0.0015
H2O2-0.003
H2O2-0.015
H2O2-0.03
H2O2-0.15
Conditions
C
percentage of dead cells
red fluorescence intensity of live cells
Percentage of dead cells (%)
Red fluorescence intensity (AU)
100
80
60
40
20
0
640
620
600
580
560
540
control
SAP-0.001
SAP-0.005
SAP-0.01
SAP-0.05
SAP-0.1
Conditions
D
percentage of dead cells
red fluorescence intensity of live cells
Percentage of dead cells (%)
Red fluorescence intensity (AU)
control
H2O2-0.0015
H2O2-0.003
H2O2-0.015
H2O2-0.03
H2O2-0.15
Conditions

Figure 5

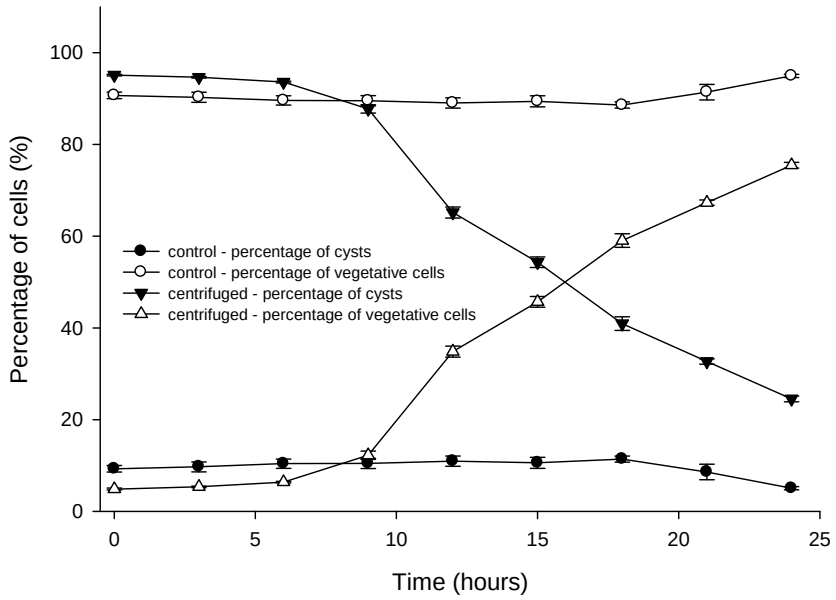
Percentage of cells (%)
100
80
60
40
20
0
0
5
10
15
20
25
Time (hours)
control - percentage of cysts
control - percentage of vegetative cells
centrifuged - percentage of cysts
centrifuged - percentage of vegetative cells

Figure 6