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**Abstract**

Diatoms of the genus *Pseudo-nitzschia* are potentially toxic microalgae, which blooms can trigger Amnesic Shellfish Poisoning. The purpose of this study was to test and adapt different probes and procedures to assess the physiological status of *Pseudo-nitzschia multiseries* at the cell-level, using flow cytometry. To perform these analyses, probes and procedures were first optimized for concentration and incubation time. The percentage of dead *Pseudo-nitzschia* cells, the metabolic activity of live cells and their intracellular lipid content were then measured following a complete growth cycle. Additionally, chlorophyll autofluorescence and efficiency of photosynthesis (quantum yield) were also monitored. The concentration and viability of bacteria present in the medium were also assessed. Domoic acid (DA) was quantified as well. Just before the exponential phase, cells exhibited a high metabolic activity, but a low DA content. DA content per cell became most important at the beginning of the exponential phase, when lipid storage was high, which provided a metabolic energy source, and when they were surrounded with a high number of bacteria (high bacteria/*P. multiseries* ratio). These physiological measurements tended to decrease during exponential phase and until stationary phase, where *P. multiseries* cells did not content any DA nor stored any lipids and started to die.

Keywords: flow cytometry; cell physiology; domoic acid; *Pseudo-nitzschia multiseries*; bacteria; fluorescent probes

## 1. Introduction

*Pseudo-nitzschia* is a potentially toxic diatom genus with a worldwide distribution. Some species are able to produce domoic acid (DA), an amnesic shellfish toxin leading to food poisoning (Sierra-Beltrán et al., 1998) with a few cases of mortality to humans (Wright et al., 1989), plus hundreds of sea bird (Sierra-Beltrán et al., 1997, Work et al., 1993) or marine mammal mortalities (Scholin et al., 2000, Fire et al., 2009, de la Riva et al., 2009). These poisonings often occurred following a bloom of *Pseudo-nitzschia* spp. The reasons why these blooms occurred are poorly known. Some studies tried to create models to predict there occurrence (Anderson et al., 2009, Lane et al., 2009), but the determinism of each bloom seems different. Although factors enhancing or decreasing *Pseudo-nitzschia* cell toxicity have been intensively studied, they still remain unclear. The study of *Pseudo-nitzschia* spp. physiology may help to understand why a bloom appears and becomes toxic. Tools to assess the physiological status of microalgae are still fairly scarce. Photosynthetic capacities of *Pseudo-nitzschia* spp. have been studied under different conditions (Ilyash et al., 2007, El-Sabaawi and Harrison, 2006) but do not provide enough information to assess cell physiological status. Besides photosynthetic parameters and chlorophyll content, others parameters have sometimes been studied in diatoms, e.g. silicification (Leblanc et al., 2005, Kroger and Poulsen, 2008) or carbohydrate levels (De Philippis et al., 2002, Magaletti et al., 2004), but these are also insufficient to characterize the physiological processes occurring inside the cell. It is therefore important to develop and then simultaneously measure several different physiological parameters that may help to better understand the factors or status associated with toxin production.

Assessment of cell physiology using fluorescent probes is a well-known subject in medicine (Greenspan et al., 1985, Knot et al., 2005). Among the numerous fluorescent probes

56 available to assess cell physiology, some can be adapted to cultures of unicellular organisms.
57 They allow measurements of different physiological parameters such as metabolic activity
58 (with fluorescein diacetate, FDA), intracellular lipid content (Nile Red and BODIPY), total
59 DNA (SYBR Green) or mortality (SYTOX Green). Some of these probes have been used in
60 microalgal studies for several years but are often limited to microscopic observations or
61 spectrofluorimetric methods (Dempster and Sommerfeld, 1998, Okochi et al., 1999). The
62 latter allow measurement of an entire population, but differences between cells can not be
63 observed. Microscopic observations allow cell-by-cell analyses but are time consuming and
64 fluorescence quantification is difficult. On the other hand, flow cytometry (FCM) allows a
65 rapid analysis of the morphological and fluorescence characteristics of unicellular organisms
66 or individual cells. Even though FCM has a long history of routine use in medical analyses,
67 the first experiments to use FCM on microalgae were run only about thirty years ago (Olson
68 et al., 1983, Yentsch et al., 1983) and the approach still remains only a minor component for
69 measuring the physiology of phytoplankton. Some probes have already been tested on
70 microalgae using FCM, such as FDA (Dorsey et al., 1989, Brookes et al., 2000, Jochem,
71 1999), SYTOX Green (Veldhuis et al., 1997) or SYBR Green (Marie et al., 1997). Each of
72 these probes provides new insights for understanding how cells react under different
73 conditions, e.g. dark adaptation (Jochem, 1999), but they have never been applied
74 simultaneously to assess physiological status in a more comprehensive manner.
75
76 This study aims to assess the physiological status of *Pseudo-nitzschia multiseries*
77 using a set of cell-based measurements. To reach this objective, different measurements were
78 developed and adapted to this species (i) to better understand its physiology under culture
79 conditions and (ii) to seek the relationship between the production of DA and cell
80 physiological status. The morpho-functional characteristics of *P. multiseries* cells were

assessed by FCM, using different fluorescent probes (FDA, BODIPY 493/503, Nile Red, SYTOX Green, SYBR Green and propidium iodide) and the measurement of chlorophyll autofluorescence. Quantum yield (QY), which is a measurement of the efficiency of photosynthesis, was measured using a pulse amplitude modulated (PAM) fluorometer. Dissolved and particulate DA were measured on each culture using an ELISA assay. DA is a secondary metabolite, thus supposed to be produced when cells have more energy than necessary for the primary metabolism. Thus primary metabolism was assessed using FDA and esterases activity. The availability of energy was assessed by measuring storage lipids, as extra-energy is stored by microalgae under lipid form. The concentration of bacteria may influence DA production by *P. multiseries*, as they are known to enhance DA production (Bates et al., 1995). Chlorophyll and QY measurements allow knowing if the culture is healthy and were completed by the measure of dead cells percentage.

## 2. **Materials and methods**

### *2.1. Cultures*

Strain CCAP 1061/32 of *Pseudo-nitzschia multiseries* (isolated in 2007 in England) was used for the experiments,. Cultures (n=6) were grown in sterilized f/2 medium (Guillard and Hargraves, 1993) at 15.6°C (±0.2°C), and 131 ± 16 µmol photons $m^{-2}$ $s^{-1}$ (light:dark photoperiod of 12:12 h). Seawater used for f/2 medium was first filtered at 0.22µm, to eliminate any remaining bacteria (which was confirmed by flow cytometric measurements, as described below) and then autoclaved. Cultures were xenic and grown without antibiotics. Before each sampling, cultures were homogenized by gentle manual stirring. Almost all the cells were present as single cells in our cultures; sometimes cells were forming 2 cells chains. For the flow cytometry analysis, they were all considered as single cells.

### *2.2. Physiological measurements*

Measurements were made with a FACScalibur flow cytometer (BD Biosciences, San Jose, CA USA), using an argon blue laser (488 nm). Three fluorescence signals can be detected by the flow cytometer: FL1 (green, 530 nm), FL2 (orange, 585 nm) and FL3 (red, 670 nm). Red fluorescence is linearly linked to the chlorophyll content of the cells and was used as a discriminating characteristic to detect the microalgae (Fig. 1). Bacteria were detected on the FL1 channel (Fig. 2), with different settings to those used for microalgae analysis. Cell counts were estimated from the flow-rate measurement of the flow cytometer (Marie et al., 1999) as all samples were run for 45 s. The flow rate from the FCM was controlled every two days. Forward Scatter (FSC, light scattered less than 10 degrees) and Side Scatter (SSC, light scattered at a 90 degree angle) were also measured. FSC is commonly related to cell size and SSC to cell complexity. The same instrument settings were used for the entire duration of the experiment to allow comparison between days.

*Bacteria*. Quantification of free-living bacteria in the *P. multiseries* culture and the percentage of dead bacteria in the culture were assessed by adding SYBR Green I (Molecular probes, Invitrogen, Eugene, Oregon, USA) at a final concentration of 1/10000 of the commercial solution, and propidium iodide (PI, Sigma, St. Louis, MO, USA) at 10 µg $ml^{-1}$ to each sample. During analyses, aggregates of bacteria were taken into account with correction according to aggregate size (Fig. 2). Bacterial counts were estimated as described for *Pseudo-nitzschia* cells, using FL1 as a discriminating characteristic (due to SYBR Green fluorescence staining).

*Mortality.* To assess *Pseudo-nitzschia* cell mortality, we used a cell membrane-impermeable dye, SYTOX Green (Molecular probes, Invitrogen, Eugene, Oregon, USA) prepared at a working solution of 5 µM. A mix of live/dead cells was prepared to confirm that SYTOX Green stained only dead cells (Veldhuis et al., 2001) and to calibrate the measurement. Cells from a dead culture (killed by heating for 15 min at 100°C) were mixed with those from a live culture to give a range of 0 to 100% dead cells (increments of 10%) and stained with SYTOX Green at 0.1 µM (final concentration) for 30 min. The percentage of measured dead cells (those stained with SYTOX Green) was then compared to the theoretical percentage of dead cells present in the mixture.

*Metabolic activity.* To assess metabolic activity, esterase activity was measured using fluorescein diacetate (FDA, Molecular probes, Invitrogen, Eugene, Oregon, USA). FDA is a probe that is cleaved by esterases inside the cells, resulting in fluorescein accumulation over time (Jochem, 1999). A 5 mg $ml^{-1}$ stock solution of FDA was prepared by diluting the commercial powder in DMSO. A fresh 300 µM working solution was prepared before each

143 experiment by adding stock solution directly into distilled water cooled on ice. The working
144 solution was kept in darkness and on ice during the experiment and was agitated to prevent
145 the formation of aggregates.
146
147 *Lipids*. To assess the intracellular lipid content, two probes were tested on *P.*
148 *multiseries*. A 10 mM stock solution of BODIPY 493/503 (4,4-difluoro-1,3,5,7,8-
149 pentamethyl-4-bora-3a,4a-diaza-s-indacene, Molecular probes, Invitrogen, Eugene, Oregon,
150 USA) was made by diluting the commercial powder in DMSO. A 1 mM working solution
151 was then prepared by a 10-fold dilution of the stock solution in distilled water. A 1 mg $ml^{-1}$
152 stock solution of Nile Red (NR, Sigma, St. Louis, MO, USA) was prepared by diluting 100-
153 fold the commercial powder in acetone and then 10-fold in distilled water to obtain a working
154 solution of 0.1 mg $ml^{-1}$.
155
156 Each of these measurements had to be optimized for *P. multiseries*. Thus, final probe
157 concentrations and incubation times were chosen following two rules: (i) the concentration
158 had to be as low as possible to avoid toxic effects of the probe itself (and of the DMSO
159 contained in stock solutions of the probes) and (ii) the staining had to be homogeneous (all of
160 the cells had to be stained or only the dead cells for SYTOX Green), relatively stable over
161 time and reproducible between analytical replicates. For each probe, fluorescence
162 measurements were performed every 5 min during 1 h on the FL1 (or FL2 for NR) channel of
163 the flow cytometer. Concentrations of 0.5, 1.0, 2.5 and 5.0 µg $ml^{-1}$ were tested for NR; 1.0,
164 2.5, 5.0 and 10 µM for BODIPY; 0.75, 1.50 and 3.00 µM for FDA and 0.025, 0.05, 0.1 and
165 0.2 µM for SYTOX Green.
166

Quantum yield (QY), a measurement of the efficiency of photosynthesis, was measured using an AquaPen-C AP-C 100 (Photo Systems Instruments, Czech Republic) pulse amplitude modulated (PAM) fluorometer. $QY = (F_m-F_0)/F_m$, where $F_0$ and $F_m$ are the minimum and maximum fluorescence of cells, respectively, after 30 min of dark adaptation. To ensure that there was no background fluorescence, *P. multiseries* supernatant and f/2 medium were used as blanks.

Domoic acid (DA) was quantified using the ASP ELISA kit (Biosense Laboratories, Bergen, Norway), according to the manufacturer's protocol. Cultures (cells and supernatant) were sonicated and filtered at 0.22 µm to measure total DA. Supernatant (culture filtered at 0.22 µm) was used to measure dissolved DA. Intracellular DA was measured by subtracting dissolved DA to total DA.

### *2.3. Monitoring the physiology of* P. multiseries *over a growth cycle*

Six *P. multiseries* cultures of the same strain were sampled every day from day 4 to 21. The following were assessed on each sampling day: *P. multiseries* morphology, concentration and mortality, bacterial concentration and mortality, total and dissolved DA concentrations, quantum yield, chlorophyll fluorescence, intracellular lipid content and metabolic activity. Growth rate was measured during exponential phase following the formula: $\mu\ (d^{-1}) = \ln(N1/N0)/\Delta t$ (in days). Fluorescence measurements were performed using the optimal concentrations obtained in the previous experiments: 0.1 µM SYTOX Green, 3 µM FDA, 1 µg $ml^{-1}$ Nile Red and 10 µM BODIPY. FDA measurements were performed precisely after 6 min of incubation, and SYTOX Green and BODIPY measurements after 30 min. Bacteria were stained with SYBR Green and PI for 15 minutes.

192 *2.4. Statistics*

193 Results were analyzed statistically with simple regressions, One-Way ANOVA with
194 time as the main factor, as well as with principal component analysis (PCA) followed by a
195 factorial plan. For all statistical results, a probability of $p<0.05$ was considered significant.
196 Statistical analyses were performed using StatGraphics Plus (Manugistics, Inc, Rockville,
197 MD, USA).

198

## 3. Results

### *3.1. Optimization of probe concentration*

*Mortality.* A 0.1 µM final concentration of SYTOX Green allowed a good distinction between dead and live cells (Fig. 1). Incubation time was optimal at 30 min. The best correlation ($y=0.95x$, $R^2 = 0.99$, $P<0.01$) between the measured and theoretical percentages of dead cells in the mixtures of dead/live *P. multiseries* was established at a SYTOX Green concentration of 0.1 µM, which was therefore applied for the further analyses.

*Bacteria*. Free-living bacteria are able to form aggregates that can be distinguished after SYBR Green staining (Fig. 2A). Each aggregates exhibited a fluorescence that was equivalent to the fluorescence of one bacteria x number of bacteria in the aggregate. The number of total free-living bacteria can thus be deduced and measurements of FSC and SSC can be done for each aggregate size (Fig. 2B).

*Metabolic activity.* Concentrations of FDA lower than 3.0 µM exhibited a low fluorescence, indicating that there was little accumulation of the probe (data not shown). At 3.0 µM, fluorescein accumulated within the cells (Fig. 3I), in a linear manner during the 15 first min ($y=35.9x+133.1$, $R^2 = 0.9964$, $p<0.001$), and then reached a plateau (Fig. 4). For the further analyses, fluorescein accumulation was measured after 6 min of staining within the linear part of the curve.

*Lipids.* A final concentration of 10 µM BODIPY 493/503 and 1 µg $ml^{-1}$ of Nile Red (NR) allowed the best staining of all cells (one distinct population of cells, not a diffuse cloud

of cells, can be seen on the cytograms, data not shown). After 30 min, all cells were well stained and the fluorescence was stable (Fig. 3B, C, E, F).

*3.2. Monitoring of morpho-functional characteristics during* P. multiseries *growth*

The exponential growth phase of *P. multiseries* started after a 7-d lag phase, giving a growth rate of 0.24 ± 0.01 $d^{-1}$, and then the stationary phase was reached after day 17 (Fig. 5). A maximal concentration of ~8 x $10^4$ cells $ml^{-1}$ was observed at days 17 and 19, after which the cell concentration rapidly declined.

Bacteria within the *P. multiseries* culture started their exponential growth phase on day 7 and were still growing steadily until the end of the experiment (Table 1), exhibiting a growth rate of 0.07±0.01 $d^{-1}$. The bacteria/*P. multiseries* cell ratio decreased during the exponential phase of *P. multiseries*, remained stable between days 14 to 20, and then increased again on the last day of the experiment, when *P. multiseries* numbers declined (Fig. 5, Table 1). Proportions of bacteria in aggregates of one, two or more cells did not change with growth phases. The percentage of dead bacteria decreased between days 4 and 12 (from 5.8% ± 0.6 to 2.0% ± 0.2) and then remained stable between 1.9 and 2.5% until day 21 (Table 1). Values of FSC and SSC for the bacterial community (free-living bacteria that were not forming aggregates) decreased steadily during the course of *P. multiseries* culture (Table 1).

The percentage of dead *P. multiseries* cells averaged 28% throughout the entire experiment (Table 1) and decreased from 30.3% on day 12 to 18.9% at day 16, after which it increased to 43.8% on day 20 (stationary phase). FSC values for *P. multiseries* continuously decreased during the experiment, almost linearly with culture age ($R^2$=0.76, $p<0.01$). SSC

values decreased until day 13 (mid-exponential phase) and became stable between day 13 and the end of the experiment (Table 1).

Total DA in the *P. multiseries* culture, expressed as pg $ml^{-1}$, increased steadily from day 7 (200±21 pg $ml^{-1}$) until day 14 (798±164 pg $ml^{-1}$) during exponential growth. Total DA in the culture then decreased sharply, reaching a concentration below 100 pg $ml^{-1}$ on day 21. Total DA content was highest on days 13 and 14, during the mid-exponential phase, and decreased steadily after day 14, when it reached late-exponential phase and stationary phase (Fig. 5). The amount of dissolved DA was low and remained constant throughout the culture, from 41.3 (±2.9) pg $ml^{-1}$ on day 6 to 103.0 (±7.3) pg $ml^{-1}$ on day 12, representing 11 to 40 % of total DA.

FL3 values (related to chlorophyll content) were measured on live cells, discriminated from dead cells using SYTOX Green staining. FL3 values sharply decreased from day 4 to 6, remained stable between days 6 (590±12) and 9 (593±10), and then slightly decreased from day 9 to 20 (506±12), stationary phase, Fig. 6). Quantum yield (QY) values increased between days 5 (0.46±0.01) and 8 (0.59±0.01), became relatively stable until day 14 (0.62±0.00), and then decreased in mid-exponential phase after day 14 (Fig. 6). Day 11 exhibited a significant decrease of both FL3 and QY. Supernatant and media did not exhibit QY values or were below the detection threshold of the fluorometer.

The metabolic activity of the *P. multiseries* cells, as measured with the FDA assay after 6 min of incubation, increased rapidly from day 6 to 7 and then just as rapidly decreased after day 7, to values just below the initial level, on day 9 (Fig. 7). The percentage of stained cells after 6 min of incubation increased between day 8 (72.5% ± 1.6) and 16 (87.9% ± 1.0)

and decreased on day 20 (74.7% ± 5.4). The percentages of live cells, as measured with the SYTOX Green and FDA assays, were significantly correlated, even though the correlation remained quite weak ($R^2$=0.67 $p<0.001$, Fig. 7).

The amount of intracellular lipids, interpreted from BODIPY fluorescence, increased between days 4 and 6, decreased from days 6 to 14 (during the exponential phase), stayed stable until day 16, and finally increased during the stationary phase (Fig. 8). NR fluorescence, the traditional indicator of lipid content, decreased between days 9 and 20, with one higher fluorescence value on day 11 (Fig. 8).

PCA showed that DA content of cells (total DA) had coordinates really close to those of Nile Red, SSC and bacteria/*Pseudo-nitzschia* ratio, knowing that components 1 and 2 explained 74 % of the variability (Fig. 9). FSC and BOPIDY uptake were also closely correlated with these previous parameters, indicating that increased DA production was associated with a higher intracellular lipid content. A factorial plan (Fig. 10) was developed from the previous PCA, plotting the age of the *P. multiseries* culture, in exponential and stationary phases from day 9 to 20. Days follow a consistent trend, from high component 1 and low component 2 (i.e. high lipid concentration, high DA content, high cell/bacteria ratio, low esterases activity, ....) , towards lower component 1 and higher component 2 (i.e. high esterases activity, low DA content and low lipid concentration, …). Day 20 was the only days which did not follow this trend, on the extremities of the factorial plan (extremely low components 1 and 2, i.e. driven mainly by the high *P. multiseries* mortality and high number of bacteria).

## 4. Discussion

The first aim of this study was to test and optimize several methods and probes to assess *Pseudo-nitzschia* physiological status. The percentage of cell mortality in the cultures was determined using SYTOX Green, which only penetrates cells that have lost their membrane integrity, and are thus considered as dead cells (Veldhuis et al., 1997). A final concentration of 0.1 µM was optimal for staining *P. multiseries* dead cells and is in good agreement with those found in the literature for other phytoplankton species (Veldhuis et al., 2001, Binet and Stauber, 2006, Ribalet et al., 2007, Miller-Morey and Van Dolah, 2004, Lawrence et al., 2006).

Fluorescein diacetate (FDA) has previously been used to measure metabolic activity (Jochem, 1999, Regel et al., 2002, Brookes et al., 2000) as well as viability of microalgae (Lawrence et al., 2006, Dorsey et al., 1989, Jansen and Bathmann, 2007). It penetrates the cells passively and once within the cell is hydrolyzed by non-specific esterases into fluorescein and two acetate molecules. The more metabolically active the cells are, the more esterases they produce, resulting in a greater amount of fluorescein accumulation within the cells. The probe will not be cleaved within dead cells, as esterases are inactive. Moreover, if the probe is hydrolyzed by any remaining esterases, the fluorescein will leak out of the cells, as the membranes are permeable. Thus, unstained cells are considered as dead cells. In the literature, measurement of fluorescein released from FDA inside the cells most often occurs between 5 and 20 min of incubation (Jochem, 1999, Regel et al., 2002, Dorsey et al., 1989, Jamers et al., 2009). FDA was only accumulated linearly during the first 15 to 20 min, as previously observed by Gilbert et al. (1992). Accordingly, based on our results and supported by the above publications, measurements were performed after 6 min of incubation. A final concentration of 3 µM was optimal for this assay and is consistent with some publications

320 (Dorsey et al., 1989, Gilbert et al., 1992) but lower than others (Regel et al., 2002, Jamers et
321 al., 2009). Higher concentrations of FDA were not tested, as 3 µM provided satisfactory
322 staining and higher concentrations of FDA and DMSO may become toxic to the cells.
323
324 BODIPY 493/503 and Nile Red (NR) were tested to localize and quantify intracellular
325 lipids in *P. multiseries* cells. NR has been used traditionally to stain lipids of microalgae
326 (Cooksey et al., 1987), whereas this is the first time that BODIPY 493/503 has been used to
327 study microalgal lipids. NR fluorescence of microalgal lipids, measured by FCM, has been
328 shown to be linearly correlated to the lipid content of cells (de la Jara et al., 2003). Lipids of
329 *P. multiseries*, revealed by BODIPY and NR, were observed to form vacuoles inside the cells
330 (Fig. 3), and NR gave a lower fluorescence intensity than BODIPY. These vacuoles are likely
331 to contain reserve lipids, as BODIPY and NR are reported to stain neutral lipids (Gocze and
332 Freeman, 1994). Such vacuoles have previously been described within microalgae (Eltgroth
333 et al., 2005, Liu and Lin, 2001, Remias et al., 2009, Cooper et al., 2010), although lipid-
334 staining BODIPY and NR did not reveal a specific distribution of these vesicles. Both
335 BODIPY and NR were used to quantify intracellular lipid contents by FCM, in the green
336 (FL1) and orange (FL2) channels respectively. In the present study, NR was used at a final
337 concentration of 1 µg $ml^{-1}$, which is the same as used in previous studies on microalgae
338 (Chen et al., 2009, Chen et al., 2010, Liu et al., 2008, Huang et al., 2009, McGinnis et al.,
339 1997). BODIPY was used at a final concentration of 10 µM. This concentration allowed the
340 detection of subtle variations in the intracellular lipid content of *P. multiseries* grown, for
341 example, in culture media with or without nitrate (data not shown), whereas lower
342 concentrations did not. Higher concentrations were not tested, as 10 µM provided satisfying
343 staining and higher concentrations of BODIPY and DMSO may become toxic to the cells.
344 The concentration used was 100 times higher than the one used for fungus (Saito et al., 2004)

345 but in agreement with those on human muscle (Wolins et al., 2001) and lower than the one
346 used on amoeba (Kosta et al., 2004).
347
348 The development of these methods allowed the physiological status of *P. multiseries*
349 cells to be monitored over a complete growth cycle. The lag phase of *P. multiseries* lasted 7
350 days, which is long compared to other studies on the same species, but not the same strain
351 (Thessen et al., 2009, Lundholm et al., 2004, Kudela et al., 2003, Kotaki et al., 1999, Bates et
352 al., 2000). The *P. multiseries* growth rate ($0.24 \pm 0.01$ $d^{-1}$) was lower than those previously
353 reported in the literature (Thessen et al., 2009, Lundholm et al., 2004, Kudela et al., 2003,
354 Kotaki et al., 1999, Bates et al., 2000). This might be explained by the age of the isolate
355 (isolated in 2007, more than 2 years ago) and the short cell length (~20 µm); Amato et al.
356 (2005) reported a slight decrease in the growth rate of *P. delicatissima* with a decrease in
357 apical cell length. Culture conditions were the same or close to studies using *Pseudo-*
358 *nitzschia* cultures (media, irradiance and temperature) and thus could not explain differences
359 in growth rates.
360
361 FSC and SSC values of *P. multiseries* decreased during the entire experiment, by 17%
362 and 22%, respectively. FSC and SSC result from the diffraction of the laser by the cell
363 surface. Their decrease in *P. multiseries* may be related to changes in external morphology,
364 cell size and internal cell complexity. During growth, cells undergo asexual reproduction and
365 thus decrease in cell length. FSC and SSC values were, however, similar to values measured
366 over the last year (data not shown) at both the beginning (during the lag phase) and end of
367 experiments. This indicates that FSC and SSC values changed very little over the last year,
368 possibly because this strain isolated in 2007 was already quite old. Inoculation of *P.*
369 *multiseries* into a new medium resulted in a return to high FSC and SSC values. Because

diatoms cannot increase their cell size, the changes in FSC and SSC values are more likely related to both surface membrane and cytoplasmic modifications than cell size modifications, thus modifying the diffraction of the laser. This hypothesis is based on the correlation between SSC and both BODIPY and NR fluorescences ($R^2$ = 0.77 and 0.64, respectively, at $p<0.01$). It may possible that when cells had a lot of lipid vesicles within their cytoplasm, this increased cell complexity was reflected by the changes in FSC and SSC values.

Bacterial community counts and morphological changes within *Pseudo-nitzschia* cultures were estimated for the first time by FCM. In this microalgal culture, the growth rate of the bacteria was 0.07 $d^{-1}$, which remained constant over the course of the experiment; the bacteria did not reach stationary phase during the 20 days of the experiment. This growth rate is in the lower range of bacteria grown in adapted culture media, that can grow from 0.01 $h^{-1}$ (Kemp et al., 1993) to 1.5 $h^{-1}$ (Makino et al., 2003). These differences may be due to the competition with *P. multiseries* for some nutrients or the fact that they may not have all the nutrients they need and that are usually added in agar plates. The highest bacteria/*P. multiseries* ratios were measured during the lag phase (day 4 to 7) and at the beginning of the exponential phase (day 7 and 8). Bacteria measured are the free-living bacteria contained in the medium; however, some bacteria can also be attached directly to *P. multiseries* cells (Kaczmarska et al., 2005), these attached bacteria were not taken into account (their signal was confounded within these of *P. multiseries*). The decrease in the number of bacteria per *P. multiseries* cell during the exponential phase of *P. multiseries* (from 922 to 180) is explained by a faster growth rate of *P. multiseries* compared to bacteria. The increase in the bacteria/*P. multiseries* ratio during the senescent phase of *P. multiseries* may be a result of bacteria taking advantage of organic materials released from dead *P. multiseries* cells (Kaczmarska et al., 2005). Stewart et al. (1997) found between 7 and 10 bacteria per *P. multiseries* cell,

which is about 20 to 80 times lower than our values. This difference may be explained by (i) a high residual percentage of dead *P. multiseries* cells present during the entire experiment, or (ii) the age of our isolate, which provided sufficient time (two years) for the bacterial community to adapt to the culture conditions of *P. multiseries*. Differences found in bacterial communities over time in culture, for non-toxic *Pseudo-nitzschia pungens* support this possibility (Sapp et al., 2007), but Wrabel and Rocap (2007) found no shifts in bacterial assemblages in a *Pseudo-nitzschia* culture over its initial nine months (Wrabel and Rocap, 2007). Nevertheless, shift in the bacterial community may appear after 9 months in culture. FSC and SSC values of the bacterial community decreased during the experiment. These values are related to size and complexity of bacterial cells. This may reflect a shift in species composition of the bacterial community to smaller bacteria or a decrease in bacterial cell size. Between 1.9 and 5.8% of the bacteria in our cultures were dead, with the highest percentage at day 4. The percentage of dead bacteria remained quite low (1.9-2.7%) until the end of the experiment, as they were still in exponential phase.

Values of FL3 (related to the chlorophyll content) decreased slightly during the entire experiment, with a greatest decrease between days 4 and 6. The chlorophyll content of *P. multiseries* decreased only slightly during the exponential phase. Nevertheless, cells with more chlorophyll may not necessarily have the most efficient photosynthesis. Indeed, QY, a measure of the efficiency of photosynthesis, was not well correlated to FL3 values, as QY decreased during the stationary phase when FL3 remained high. QY increased at the beginning of the exponential phase and remained high during the remaining exponential phase, with cells having an efficient photosynthesis with a lot of energy produced. Such an increase of QY during the exponential phase has been shown for other microalgal species, e.g. *Symbiodinium* sp. (Rodriguez-Roman and Iglesias-Prieto, 2005), and is currently used as

a measure of algal culture health. As the QY value is not affected by the percentage of dead cells in the cultures (Franklin et al., 2009), it can be speculated that at the end of the stationary phase, live *P. multiseries* cells still contained high amounts of chlorophyll, but with a poor photosynthetic efficiency.

During the entire experiment, the percentage of dead *P. multiseries* cells was relatively high, ranging from 19% to 54%. Nevertheless, our cultures reached a maximum cell concentration of $8 \times 10^4$ cells $ml^{-1}$, which is consistent with some previous studies (Mengelt and Prézelin, 2002, Bates and Richard, 1996, Lewis et al., 1993, Kotaki et al., 1999) but lower than the results of the majority of the studies (Bates and Richard, 1996, Kotaki et al., 1999, Mengelt and Prézelin, 2002), suggesting that our cultures were not in good health, which also explains the low growth rate and the high percentage of dead cells. Generally, in healthy and young cultures of *Pseudo-nitzschia* sp., the percentage of dead cells has been described under 5% (Mengelt and Prézelin, 2002). The increase in dead cells at the end of the experiment may be due to the limitations in nutrients and associated with the beginning of the stationary phase. Such a consistently high percentage of dead cells in the culture may be explained by the age of the isolate. The percentage of dead cells assessed with FDA was significantly but not perfectly correlated ($R^2 = 0.67$, $p<0.01$) to those obtained with SYTOX Green and appeared slightly lower than when measured with SYTOX Green. Cells can have a compromised cell membrane, and be considered as dead when assessed with SYTOX Green, but they may still have active esterases. These false-positive cells (dead but stained with FDA) have been shown to represent 1.6% of total cells of *Chlamydomonas reinhardtii* (Jamers et al., 2009). Such differences between SYTOX Green and FDA have also been previously observed in *Heterosigma akashiwo* (Lawrence et al., 2006). Using these two probes not only provides the percentage of dead versus live cells but also provides an

445 indication of the way cells are dying. In our cultures, cells most likely died by loss of
446 membrane integrity prior to inactivation of esterases, which was also observed by Lawrence
447 et al. (2006) in cultures of *H. akashiwo*. Thus, SYTOX Green and FDA provide useful
448 information and could both be used in physiological measurements.
449
450 Lipid-related fluorescence assessed with BODIPY was high during the lag phase,
451 indicating that the cells contained energy stored as neutral lipids. BODIPY fluorescence
452 decreased during the entire exponential phase, suggesting that cells were using these stored
453 lipids to grow, in addition to the energy produced by photosynthesis. Cells stopped growing
454 at the stationary phase, and energy was once again stored as lipids, as evidenced by the
455 increase in BODIPY fluorescence. Although no data are available between days 4 and 6, NR
456 fluorescence decreased during the remainder of the experiment, with the exception of a high
457 value on day 11. There was a weak correlation between BODIPY and NR fluorescence
458 during the exponential phase (between days 7 and 18; $R^2 = 0.65$, $p<0.01$). During the
459 stationary phase, however, BODIPY fluorescence is higher than that of NR, which confirms
460 that these two probes may not actually stain the same compounds during that period. This
461 emphasises the importance of using both lipid probes. These differences may be explained by
462 the chemical properties of the two probes. BODIPY 493/503 stains intracellular lipids more
463 effectively than NR, with a higher sensitivity and lower background (Kacmar et al., 2006).
464 BODIPY 493/503 also stains intracellular lipid droplets more specifically than does NR
465 (Gocze and Freeman, 1994). NR is an uncharged hydrophobic molecule whose fluorescence
466 is strongly influenced by the polarity of its environment. As well as lipids, NR interacts with
467 many, but not all, native proteins (Sackett and Wolff, 1987) and can undergo changes in
468 fluorescence intensity when it binds to certain proteins (Brown et al., 1995). The fact that NR
469 binds proteins may explain its lower sensitivity to small variations in lipid content, as

measured by BODIPY. This is especially evident during the stationary phase, when differences in lipid staining were observed between the two probes. Thus, the combined use of BODIPY and NR probes is of interest as they may reflect different physiological changes.

The maximum of total DA per cell was observed on days 9 and 10, in early exponential phase, and it decreased during the remainder of the exponential phase, and the stationary phase. The same pattern of DA production has been observed for *Pseudo-nitzschia calliantha* (Besiktepe et al., 2008) and *Pseudo-nitzschia pseudodelicatissima* (Pan et al., 2001), where the maximum DA production was observed during the early exponential phase. All studies of *P. multiseries*, however, have found a maximum DA production during the stationary phase (Kotaki et al., 1999, Bates et al., 2000, Lewis et al., 1993, Osada and Stewart, 1997). Maybe old cultures of *P. multiseries* exhibit a shift of DA production from stationary phase to early exponential phase, which is difficult to prove, as one strain has never been studied throughout its lifetime in laboratory. Moreover, strains exhibiting DA production during early exponential phase seem to have a lower DA content per cell (Besiktepe et al., 2008, Pan et al., 2001). In our study, total cellular DA varied between 0 and 192 fg $cell^{-1}$, which is low compared to previous studies on *P. multiseries*, where DA attained 1.2 to 45 pg $cell^{-1}$ (Bates et al., 2000, Thessen et al., 2009). Our values are more consistent with those of *P. calliantha* (Álvarez et al., 2009) or *P. pseudodelicatissima* (Pan et al., 2001), which had a maximum toxicity of 10 and 36 fg $cell^{-1}$, respectively, but these species have a smaller cell volume. Our strain of *P. multiseries* was really short (around 20 µm length here, whereas cells can be 100 µm long just after sexual reproduction, which may explain the low values of DA it produced. DA intracellular content started to decrease from day 11 to the end of the stationary phase. This decrease of DA may also coincide with a physiological stress. Unfortunately, bacteria were not measured that day. Nevertheless, day 11 exhibited surprising values of NR, FL3 and QY (i.e. out of the trend). Cells might have undergone a stress, with

loss of chlorophyll and thus decreased QY, thus energy was stored under lipid form and DA production was stopped. Dissolved DA was particularly low but remained constant over time, with cells excreting 11 to 40 % of their total DA. This low DA release may be due to the age of the strain, isolated in 2007, and its consequent smaller size.

DA is a secondary metabolite and is thus believed to be produced when cells have excess energy that is not used for primary metabolism (Bates, 1998). Meanwhile, extra energy is stored as lipids when cells are not able to use it for primary metabolism. In this study, the measure of FDA provided information regarding primary metabolism and QY (photosynthetic efficiency) was measured to estimate the production of energy. There was no clear relationship between DA production (total or dissolved) and QY or FDA hydrolysis. Conversely, a positive correlation was observed between total DA content and NR after PCA analysis (Fig. 9). Cells of *P. multiseries* seemed to produce more DA when they had more lipids, thus more available stored energy, which is in agreement with some studies (Whyte et al., 1995) but not all (Pan et al., 1996). Indeed, Pan et al. (1996) made the hypothesis that DA and lipid synthesis shared some precursors as Acetyl-CoA, so when DA is produced, lipids can not be stored. Bacteria are also known to play a role in DA production, by enhancing DA production through unknown mechanisms (Bates et al., 1995, Stewart et al., 1997). In this study, the ratio of bacteria per *P. multiseries* cell was also weakly correlated to DA content, as the variable coordinates are quite close in the PCA analysis ($R^2$=0.49, $p<0.001$, Fig. 9). Cells seemed to produce more DA when more bacteria per *P. multiseries* cell were present in the culture, possibly indicating that more DA was produced either when competition with bacteria was greater or if bacteria produced toxin-enhancing compounds. FSC and BOPIDY uptake were also closely correlated with DA content, SSC, NR and ratio bacteria/*Pseudo-nitzschia*, indicating that increased DA content is associated with a higher intracellular lipid

content. This lipid increase can cause an increase in the amount and/or the size of lipid vacuoles within the cells, which could also explain the increase observed in FSC and SSC of the *P. multiseries* cells. A factorial plan (Fig. 10) was developed from the previous PCA, which plots the incubation time of the *P. multiseries* culture, from day 9 to 20 (values included in the previous PCA). The position of the days included on this factorial plan clearly demonstrates and summarizes our findings: the gradual and continuous shift of the culture from low algal concentration, high bacteria/algal ratio, large SSC, high lipid and DA content in early stationary phase, towards increasing concentrations, reaching a maximum at the end of the exponential phase, to finally showing a high percentage of dead algal cells and bacteria in late stationary phase.

FCM has been previously used on microalgae, mainly to cell count or measure of only one physiological parameter per experiment. Here, we developed a set of physiological measurements, which provides a more complete description of the physiological status of the microalgae. This technique has been applied to one species of *Pseudo-nitzschia* but can be broadened to other microalgal species, whether or not they are toxic or diatoms. Developing cell-based physiological measurements with FCM will help to further our understanding of phytoplankton physiology and its responses to environmental changes, both biotic and abiotic.

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784
785

## Legends to figures

Figure 1. Cytograms of 50/50 dead/live cells of *Pseudo-nitzschia multiseries* stained with SYTOX Green. A. Cytogram of FSC and SSC (morphological parameters, expressed in arbitrary units, AU) of *P. multiseries*. B. Cytogram of FL1 and FL3 fluorescence of *P. multiseries*. FL1 is the green fluorescence due to SYTOX Green, FL3 is the red fluorescence due to chlorophyll (AU). R1 are unstained cells (considered as live cells, in red) and R2 are stained cells (considered as dead cells, in green).

Figure 2. Bacteria stained with SYBR Green and propidium iodide. A. Histogram of FL1 (green) fluorescence of bacteria; 1 to 7 representing aggregates of 1 to 7 or more bacteria. FL1 is the green fluorescence due to SYBR Green. B. Cytograms of morphological parameters of bacteria (FSC and SSC, expressed in arbitrary units, AU). Each colour represents one aggregate size (light green=one bacteria, dark blue=2 bacteria, pink=3 bacteria, light blue=4 bacteria, yellow=5 bacteria, red=6 bacteria, dark green=7 or more bacteria).

Figure 3. Photomicrographs of *Pseudo-nitzschia multiseries* cells in white light (A, D, G), epifluorescence light with filter "BP 515/560 / BS 580 / LP 590" (B, E, H), and filter "BP 450-490 / BS 510 / LP 515" (C, F, I). A, B, C) Cells stained with BODIPY. D, E, F) Cells stained with Nile Red. G, H, I) Cells stained with FDA. Scale bar=10 µm.

Figure 4. Green fluorescence of *Pseudo-nitzschia multiseries* cells (in arbitrary units, AU) stained with 3.0 µM of fluorescein diacetate (FDA) and measured on FL1 detector of a flow cytometer (n=3, mean ± SD).

Figure 5. A- *Pseudo-nitzschia multiseries* growth curve (y-axis) and bacteria/*P. multiseries* ratio (z-axis, n=6, mean ± SE). The exponential growth phase of *P. multiseries* is framed with a black-lined rectangle. B- Concentration of total and dissolved domoic acid (DA) in the whole culture (y-axis, pg $ml^{-1}$) and cellular DA in fg $cell^{-1}$ (z-axis, n=6, mean ± SE). Exponential growth phase of *P. multiseries* is framed with a black-lined rectangle.

Figure 6. Chlorophyll fluorescence (FL3, in arbitrary units, AU, y-axis) and Quantum Yield (QY, z-axis) of live *Pseudo-nitzschia multiseries* cells as a function of culture age. FL3 was measured using flow cytometry on live cells, as determined by SYTOX Green staining (n=6, mean ± SE). The exponential growth phase of *P. multiseries* is framed with a black-lined rectangle.

Figure 7. Fluorescein diacetate (FDA) uptake (FL1 fluorescence of live cells, y-axis) and percentage of *Pseudo-nitzschia multiseries* live cells stained by FDA (z-axis) and detected using flow cytometer FL1 detector (n=6, mean ± SE). Exponential growth phase of *P. multiseries* is framed with a black lined rectangle. Correlation between the percentages of live cells measured with the SYTOX Green and FDA assays is indicated in the small graph (in arbitrary units, AU).

Figure 8. Green and orange fluorescences of *Pseudo-nitzschia multiseries* cells stained with BODIPY 493/503 and Nile Red (indicators of lipid content) and detected by the FL1 (y-axis) and FL2 (z-axis) detectors, respectively, on a flow cytometer, in arbitrary units (n=6, mean ± SE). Exponential growth phase of *P. multiseries* is framed with a black lined rectangle.

Figure 9. Principal Component Analysis (PCA) plot of all physiological measurements between days 9 and 20 (D9 to D20) of the *P. multiseries* culture (n=52).

Figure 10. Factorial plan issued from the previous PCA and plotting days of culture of *P. multiseries*, from day 9 to day 20 (D9 to D20, n=52).

832 Table 1. *Pseudo-nitzschia multiseries* and associated bacteria concentration (of live cells),
833 morphological parameters (FSC and SSC, in arbitrary units), percentage of dead *P.*
834 *multiseries* measured using SYTOX Green and percentage of dead bacteria assessed using
835 SYBR Green - propidium iodide double staining (n=6, mean ± SE).

| Day | *P. multiseries* | | | | | | | | Bacteria | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | FSC (AU) | | SSC (AU) | | % dead cells | | Concentration (cell $ml^{-1}$) | | FSC (AU) | | SSC (AU) | | % dead cells | | Concentration ($10^6$ bact $ml^{-1}$) | |
| | mean | ±SE | mean | ±SE | mean | ±SE | mean | ±SE | mean | ±SE | mean | ±SE | mean | ±SE | mean | ±SE |
| 4 | 196.3 | 2.6 | 76.8 | 0.7 | 25.0 | 1.2 | 4 526 | 190 | 226.4 | 10.8 | 23.5 | 0.7 | 5.8 | 0.6 | 3.01 | 0.07 |
| 5 | 203.5 | 0.7 | 76.9 | 1.4 | 27.9 | 1.7 | 4 574 | 255 | 207.3 | 9.1 | 23.1 | 0.5 | 4.2 | 0.3 | 4.14 | 0.15 |
| 6 | 186.0 | 1.3 | 80.0 | 1.8 | 22.6 | 0.9 | 5 452 | 214 | 179.1 | 5.2 | 22.9 | 0.4 | 4.0 | 0.3 | 3.80 | 0.13 |
| 7 | 197.6 | 2.7 | 71.2 | 1.6 | 32.7 | 0.7 | 3 533 | 93 | | | | | | | | |
| 8 | 183.7 | 2.9 | 72.6 | 1.5 | 30.6 | 0.8 | 6 226 | 275 | 177.4 | 2.8 | 23.0 | 0.2 | 3.5 | 0.3 | 5.61 | 0.04 |
| 9 | 185.2 | 1.5 | 72.1 | 1.0 | 32.6 | 2.0 | 7 711 | 677 | 179.1 | 4.6 | 24.5 | 0.3 | 2.6 | 0.3 | 5.33 | 0.23 |
| 10 | 190.5 | 2.0 | 67.5 | 1.3 | 28.5 | 2.7 | 9 059 | 1 179 | 168.9 | 7.5 | 24.3 | 0.4 | 2.7 | 0.2 | 5.15 | 0.20 |
| 11 | 190.5 | 1.6 | 62.9 | 0.7 | 54.5 | 9.4 | 12 393 | 1 730 | | | | | | | | [2] |
| 12 | 186.3 | 1.4 | 63.3 | 0.9 | 30.3 | 3.8 | 15 863 | 2 716 | 127.6 | 5.0 | 23.2 | 0.4 | 2.0 | 0.2 | 6.09 | 0.21 |
| 13 | 180.4 | 1.3 | 59.1 | 0.8 | 22.8 | 3.0 | 23 837 | 5 343 | 186.5 | 18.7 | 23.0 | 1.5 | 1.9 | 0.1 | 6.60 | 0.12 |
| 14 | 177.4 | 2.0 | 58.7 | 1.1 | 22.9 | 2.9 | 30 726 | 6 223 | 171.4 | 26.7 | 20.2 | 0.6 | 2.2 | 0.2 | 6.80 | 0.21 |
| 15 | 173.5 | 0.9 | 59.7 | 1.3 | 19.1 | 0.7 | 33 796 | 4 967 | 193.3 | 7.7 | 21.1 | 0.7 | 2.2 | 0.1 | 7.71 | 0.23 |
| 16 | 171.1 | 1.9 | 60.0 | 1.3 | 18.9 | 1.6 | 36 148 | 2 064 | 225.9 | 18.1 | 22.7 | 1.4 | 2.2 | 0.1 | 8.19 | 0.16 |
| 17 | 185.4 | 0.9 | 57.3 | 1.2 | | | 77 322 | 3 718 | | | | | | | | |
| 18 | 173.9 | 3.1 | 60.2 | 1.3 | 27.4 | 4.2 | 49 222 | 4 818 | 99.2 | 5.9 | 19.3 | 0.7 | 1.9 | 0.1 | 8.72 | 0.55 |
| 19 | 174.9 | 2.1 | 56.3 | 0.7 | | | 78 730 | 10 135 | | | | | | | | |
| 20 | 168.8 | 1.3 | 60.3 | 0.3 | 43.8 | 5.5 | 55 889 | 8 682 | 177.9 | 8.6 | 19.0 | 0.7 | 2.0 | 0.1 | 11.31 | 0.97 |
| 21 | 163.7 | 2.3 | 59.7 | 0.5 | | | 40 163 | 2 226 | 25.1 | 0.7 | 17.3 | 0.4 | 2.1 | 0.2 | 13.30 | 0.81 |

836

# Figures

Fig. 1

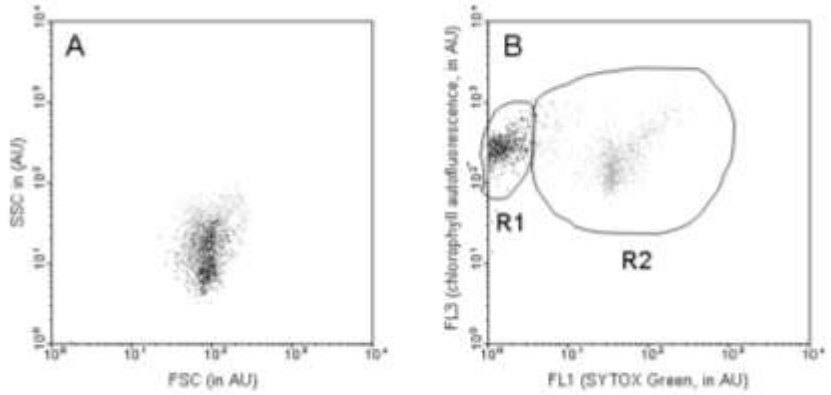
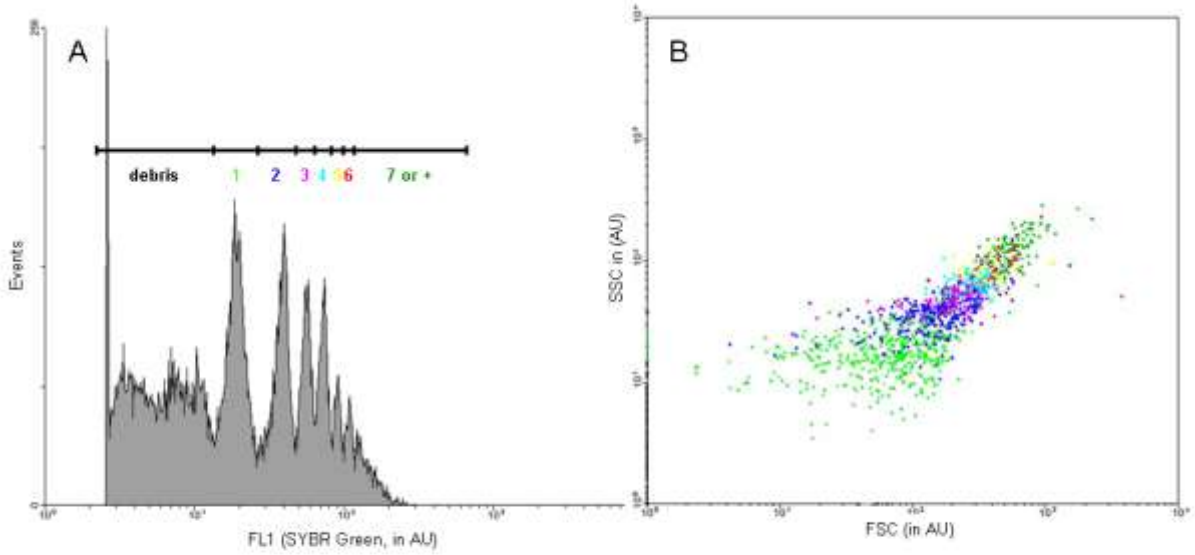
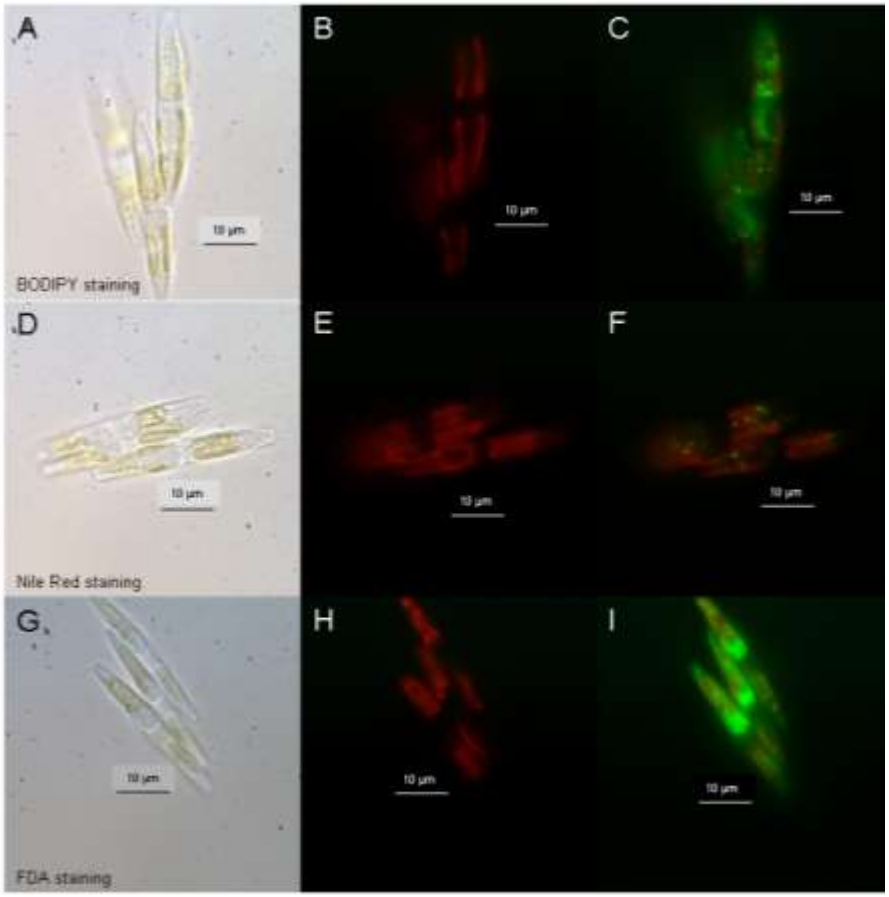



Fig. 2



843 Fig. 3

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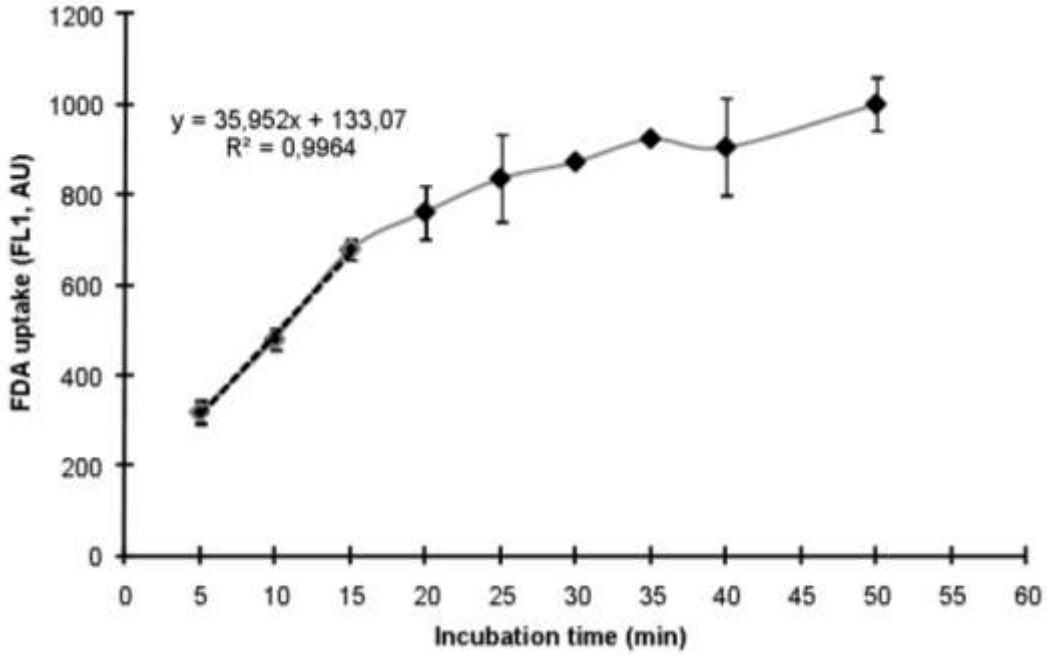
846 Fig. 4

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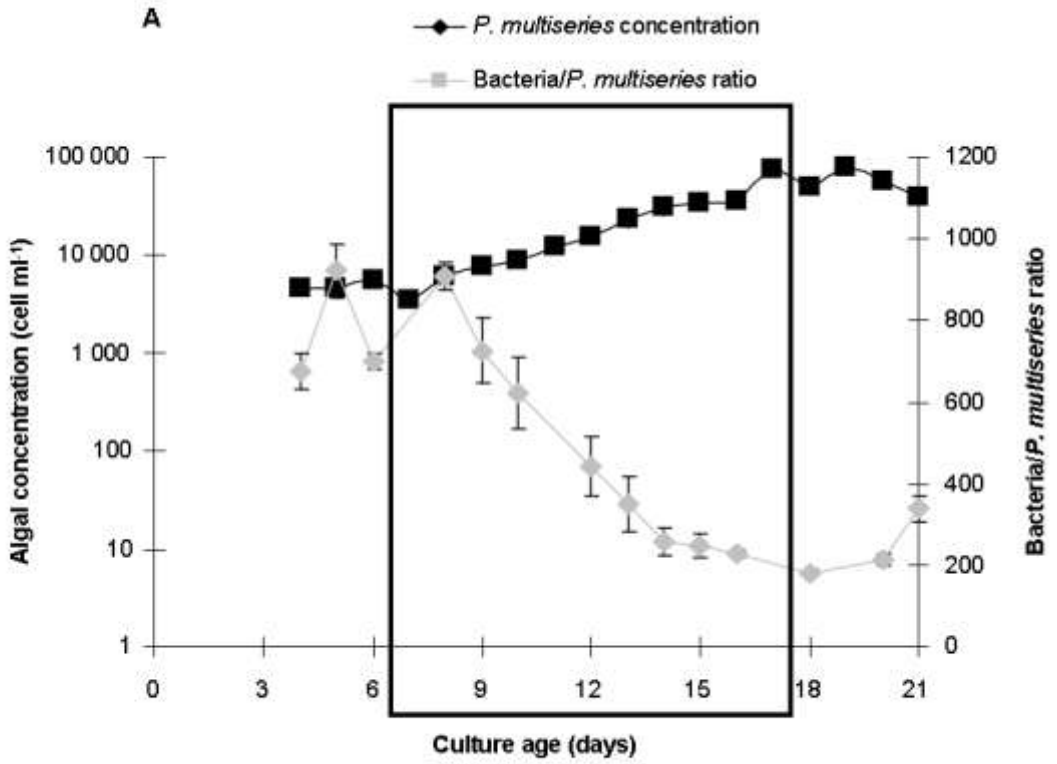


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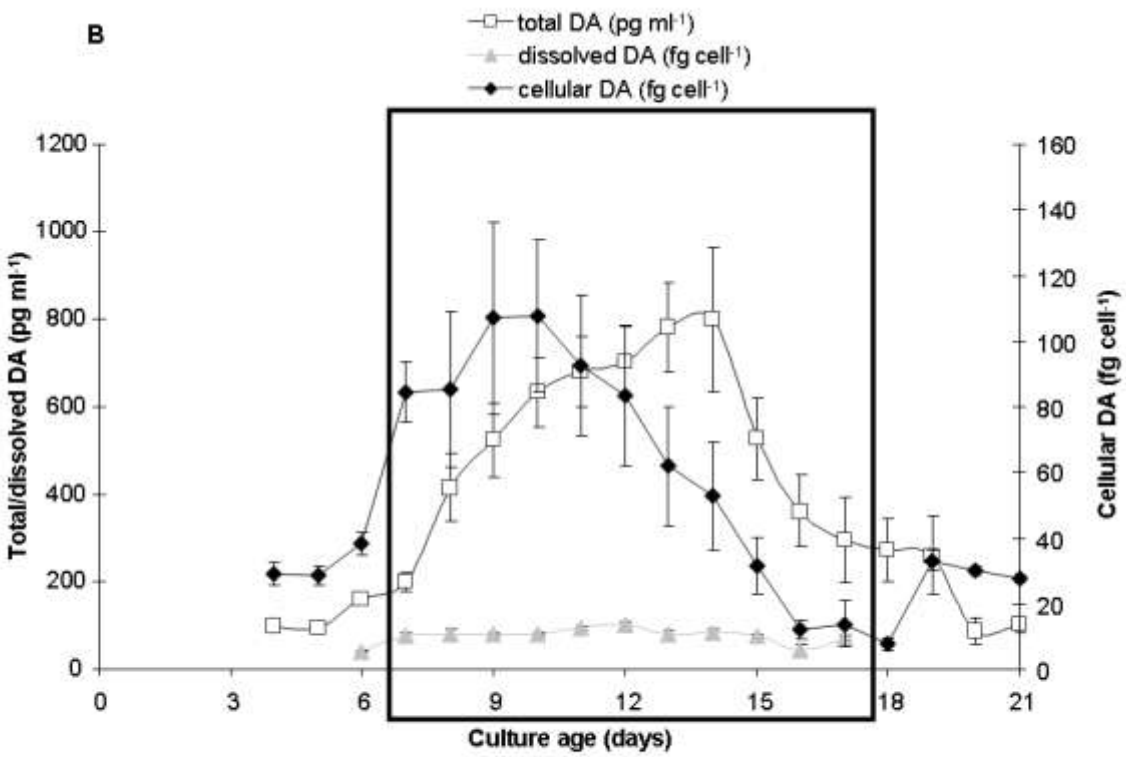
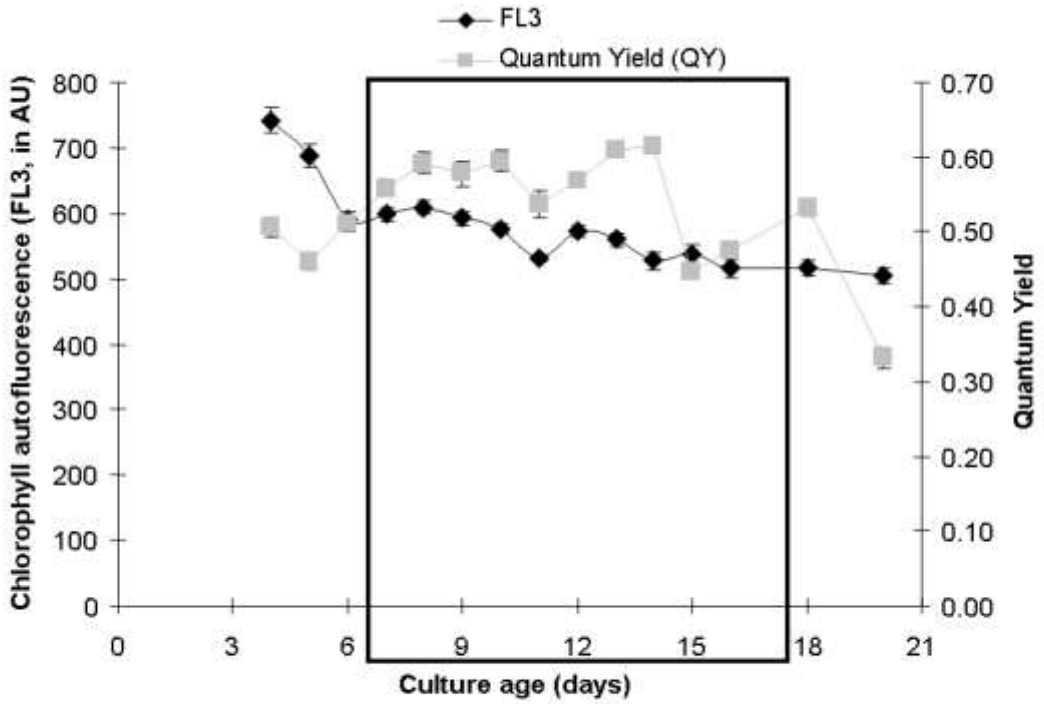

B
total DA (pg ml-1)
dissolved DA (fg cell-1)
cellular DA (fg cell-1)
Total/dissolved DA (pg ml-1)
Cellular DA (fg cell-1)
Culture age (days)
0
200
400
600
800
1000
1200
0
20
40
60
80
100
120
140
160
0
3
6
9
12
15
18
21

850

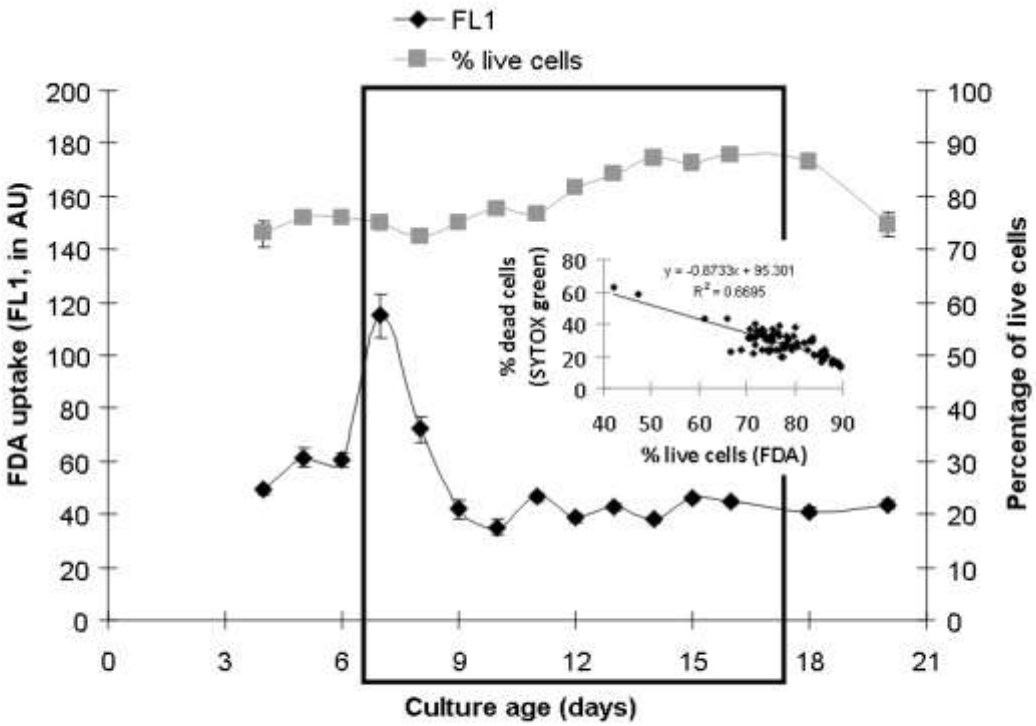
851 Fig. 6


FL3
Quantum Yield (QY)
Chlorophyll autofluorescence (FL3, in AU)
Quantum Yield
Culture age (days)
800
700
600
500
400
300
200
100
0
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
3
6
9
12
15
18
21

852

853

854 Fig. 7

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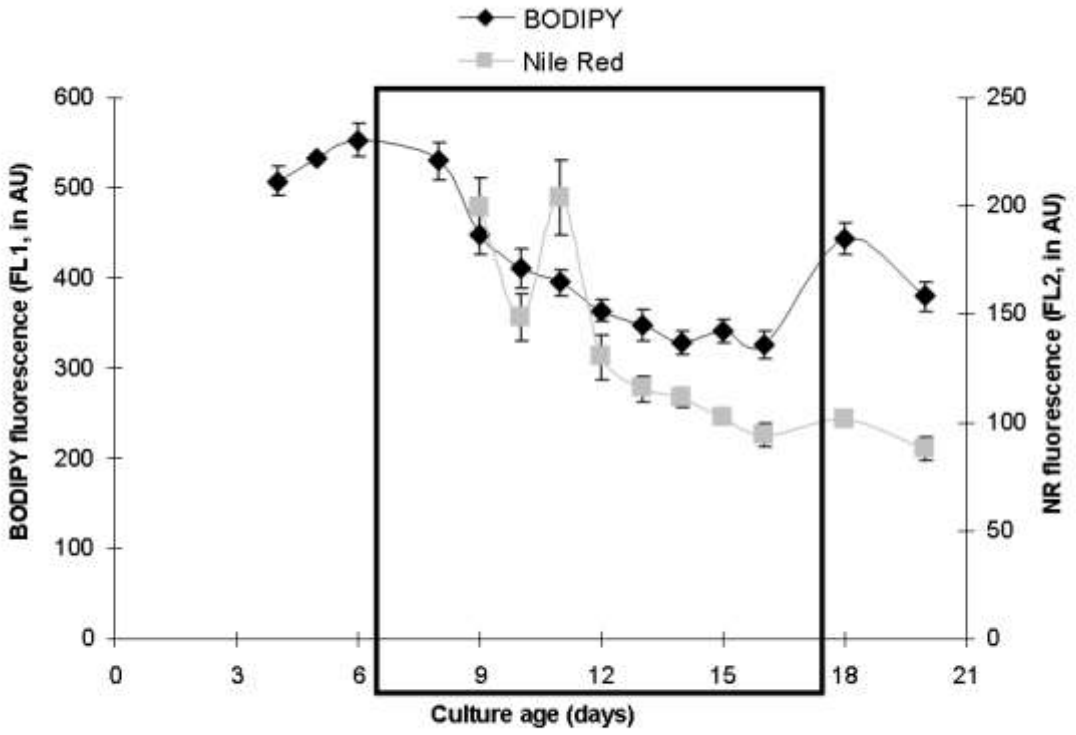
856 Fig. 8


BODIPY
Nile Red
BODIPY fluorescence (FL1, in AU)
NR fluorescence (FL2, in AU)
Culture age (days)
600
500
400
300
200
100
0
250
200
150
100
50
0
0
3
6
9
12
15
18
21

857

858 Fig. 9

859

860

861 Fig. 10

862