Impact of culturing conditions on the abundance and composition of long chain alkyl diols in species of the genus *Nannochloropsis*

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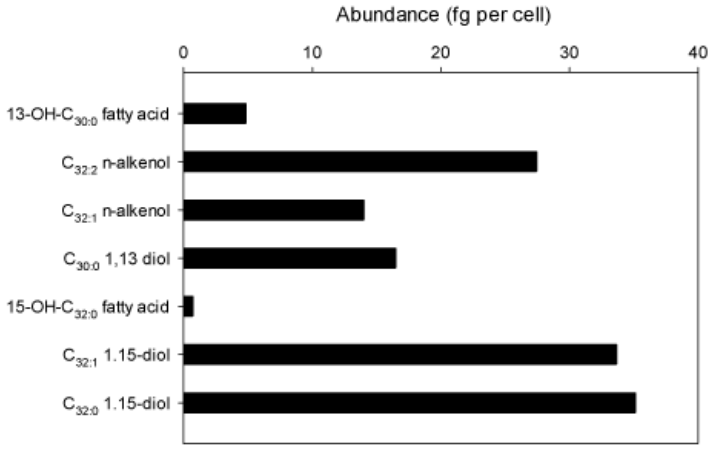
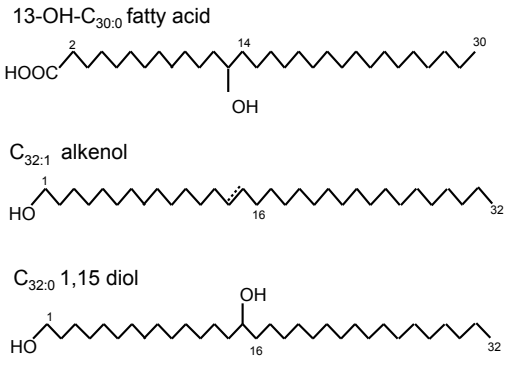
Nannochloropsis species contain lipids with C_{28}-C_{32} alkane carbon skeleton with one terminal and one mid-chain (13-15) functional group; their abundance under different culturing conditions was investigated and found to be remarkably stable.





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Sergio Balzano [a*], Laura Villanueva [a], Marijke de Bar [a], Jaap S. Sinninghe Damsté [a,b], Stefan Schouten [a,b]

[a] *NIOZ, Royal Netherlands Institute for Sea Research, Department of Marine Microbiology and Biogeochemistry, and Utrecht University. P.O. Box 59, NL-1790 AB Den Burg, The Netherlands*

[b] *Utrecht University, Faculty of Geosciences, Department of Earth Sciences, P.O. Box 80.021, 3508 TA Utrecht, The Netherlands*

* Corresponding author. Tel.: +31 222 369 526

E-mail address: sergio.balzano@nioz.nl

ABSTRACT

Long chain alkyl diols (LCDs) are widespread in sediments and are synthesized, among others, by microalgae of the genus *Nannochloropsis*. The factors regulating the synthesis of LCDs and their biological function are, however, unclear. We investigated the changes in abundance of free + ester-bound LCDs, extracted by saponification and acid hydrolysis, during the growth of three *Nannochloropsis* species and incubated the species having the highest LCD abundance (*Nannochloropsis oceanica*) under different conditions known to affect the fatty acid content (i.e. light irradiance, salinity, nitrogen depletion, desiccation, cold shock) in order to evaluate their impact on LCDs production. LCD abundances were relatively stable suggesting that they are not used as storage lipids, and support the assumption that LCDs are building blocks of an aliphatic biopolymer located in the outer cell wall (algaenan). Oxidative stress caused by hydrogen peroxide led to a decrease in the $C_{32:1}$ diol, as well as other algaenan-associated compounds such as 15-OH-$C_{32:0}$ fatty acid and $C_{32:2}$ alkenol suggesting that algaenans can play a role in the protection of *Nannochloropsis* cells. The relatively constant amount of LCDs per cell suggests that the abundance of LCDs in aquatic environments may be used as an indicator for the abundance of diol-producing algae. Interestingly, the abundance of $C_{30:0}$ 13-hydroxy and $C_{32:0}$ 15-hydroxy fatty acids, potential precursors for LCDs, correlate with those of the major $C_{14:0}$ and $C_{16:0}$ fatty acids. This supports the idea that the biosynthesis of LCHFAs might proceed by hydroxylation and elongation of shorter C_{14}–C_{16} fatty acids.

Keywords: Nannochloropsis gaditana, *Nannochloropsis oceanica, Nannochloropsis oculata*, salinity, light irradiance, nitrogen depletion, growth rate, oxidative stress

1. Introduction

Long chain alkyl diols (LCDs) are lipids that contain an *n*-alkyl chain with 28 to 32 carbons, hydroxyl groups at C-1 and a mid-chain position (predominantly at C-13, C-14, or C-15), and can be saturated or monounsaturated. LCDs have been identified in several *Nannochloropsis* species (Volkman et al., 1992; Gelin et al., 1997b; Rampen et al., 2014a), as well as other eustigmatophytes (Volkman et al., 1999a; Rampen et al., 2014a), the Dichtyochophycea *Apedinella radians* (Rampen et al., 2011), and diatoms from the genus *Proboscia* (Sinninghe Damsté et al., 2003; Rampen et al., 2009). In spite of their presence in only a few algal taxa, which typically account for minor proportions of the phytoplankton biomass in seawater (de Vargas et al., 2015), LCDs are widespread in both sediment and suspended particulate matter from freshwater (Xu et al., 2007; Zhang et al., 2011; Atwood et al., 2014; Villanueva et al., 2014) and marine environments (Versteegh et al., 1997; Rampen et al., 2007; 2008, 2014b; Volkman et al., 2008). The distribution of LCDs has also been explored as potential proxy for ancient water temperatures (Rampen et al., 2008, 2012, 2014b; de Bar et al., 2016; Rodrigo-Gamiz et al., 2016) and nutrient conditions (Rampen et al., 2014b).

Despite their potential as biomarker lipid and proxy, relatively little is known about LCDs, in terms of both biological function and biosynthetic pathways. LCDs are thought to be the precursors of algaenans, aliphatic biopolymers occurring in the cell wall of algae and consisting of long-chain *n*-alkyl units linked by ether and ester bonds (de Leeuw et al., 1981; Tegelaar et al., 1989; Gelin et al., 1997a; Volkman et al., 1998; Scholz et al., 2014; Zhang and Volkman, 2017). Algaenans in *Nannochloropsis* cell wall are likely to contain also several compounds with similar carbon number and often functionalized at the same positions as the LCDs, such as long-chain alkenols (LCAs, Volkman et al., 1992, 1999a), keto-ols (Mejanelle et al., 2003), long-chain hydroxy fatty acids (LCHFAs, Volkman et al. 1999b), and long-chain

66 dihydroxy fatty acids (Gelin et al., 1997b). Because of the high similarities in their chemical
67 structures, LCAs and LCDs in *Nannochloropsis* species have been suggested to originate
68 from the reduction of the carboxylic groups of the LCHFAs (Gelin et al., 1997b).
69 Factors controlling the composition and abundance of LCDs in algae have rarely been
70 studied in detail. Rampen et al. (2014a) showed that LCDs increase in chain length and in the
71 proportion of saturated diols with increasing temperatures. However, it is not clear whether
72 other environmental conditions can affect LCD abundance and composition. Since the
73 Eustigmatophyceae comprises both freshwater and marine species (Hibberd, 1981) and these
74 occur in estuarine and hypersaline environments (Vinogradova and Darienko, 2008; Samanta
75 and Bhadury, 2014; Balzano et al., 2015), so they possess the metabolic plasticity to adapt to
76 different salinities. Estuarine phytoplankton can also experience rapid shifts in nutrients
77 because of the interactions between nutrient-rich freshwater and nutrient-poor seawater as
78 well as shifts in light irradiance caused by water turbidity. The reliability of proxies based on
79 LCDs might be compromised if the cellular levels and composition of LCDs change with
80 these varying conditions of light, salinity and nutrients, which typically occur in the
81 environment where LCD-producers occur. Indeed, culture experiments with *Nannochloropsis*
82 spp. show that their fatty acid composition may vary strongly during algal growth (Dunstan et
83 al., 1993) or under different salinity, light irradiance and nitrate regimes (Pal et al., 2011;
84 Martinez-Roldan et al., 2014).
85 In the present study, we analysed the concentrations of LCDs in three species
86 (*Nannochloropsis gaditana, Nannochloropsis oceanica and Nannochloropsis oculata*) during
87 different stages of their growth. We incubated the species yielding the highest levels of LCDs,
88 LCHFAs and LCAs (*N. oceanica*) under different culturing (light irradiance, salinity) or stress
89 (nitrogen depletion, desiccation, cold shock, oxidative stress) conditions, which are known to

enhance the fatty acid content or in general to affect the lipid composition, in order to evaluate the impact of such conditions on the production of LCDs and on LCD-related compounds.

2. Materials and methods

2.1. General culturing conditions

For the growth experiments, three marine strains from the genus *Nannochloropsis* were used in this study: *N. gaditana* strain CCMP526, *N. oceanica* strain CCMP1779, and *N. oculata* strain CCMP2195. For all subsequent experiments only *N. oceanica* was used.

The strains were cultured in f/2 medium (Guillard, 1975) and maintained in sterile conditions under a 16:8 light: dark. *N. oculata* was cultured at 15 °C, whereas *N. oceanica* and *N. gaditana* were grown at 20 °C, as recommended by the supplier (ncma.bigelow.org). The strains were grown in 0.8 L volumes within 1.5 L glass Erlenmeyer flasks. Except for the growth rate experiments, where cells were harvested at different stages of their growth, cells were sampled for lipid analyses from exponentially growing cultures. For all the experiments, except those related to the impact of different light regimes on LCDs, the flasks were exposed to an irradiance of 100 μE/(m^2s), which was measured using a Universal Light Meter-500 (Walz, Germany). The strains were cultured in batch for all the experiments, except those related to the impact of different salinities, where they were cultured in semi-continuous mode.

Algal growth was regularly monitored by flow cytometry (Marie et al., 2001) and, at the end of each experiment, cells were harvested by filtration through 0.7 μm GF/F filters (Whatman, Maidstone, UK).

2.2. Growth rate experiments

115 An aliquot of 50 mL containing about 1.5×10^7 cells was inoculated in 9 replicate 2.5 L
116 Erlenmeyer flasks containing 1.45 L of f/2 medium. Cells were enumerated daily and the
117 growth rate subsequently calculated as described previously (Balzano et al., 2011). The cells
118 were harvested for the first time (early exponential phase) from triplicate flasks, when their
119 abundance reached 10^6 cells/mL (Supplementary Fig. S1). When a decline in the growth rate
120 was observed (late exponential phase) three further flasks from each strain were filtered and
121 cells were harvested from the last three flasks when no further growth was observed for two
122 consecutive days (stationary phase, Supplementary Fig. S1).
123
124 *2.3. Light irradiance*
125 Six replicate volumes of 10 mL, containing about 2×10^7 cells each from *N. oceanica*,
126 were transferred into six 1.5 L flasks containing 740 mL of f/2 medium and were incubated at
127 20 °C. Three replicate flasks were exposed to an irradiance of about 300 μE/(m^2s) and used as
128 high light (HL) treatment, whereas the other three flasks were covered by several shading nets
129 (irradiance 25 μE/(m^2s)) and were considered to reflect low light (LL) conditions. Cells grew
130 faster in the HL treatment and were harvested from the cultures when their concentration
131 exceeded 5×10^6 cells/mL, on Day 6 for the HL treatment and Day 7 for the LL treatment
132 (Supplementary Fig. S2).
133
134 *2.4. Osmotic stress*
135 To assess the impact of salinity on LCD abundance and composition, cells from *N.*
136 *oceanica* were pre-adapted to grow at both brackish (below seawater salinity) and hypersaline
137 (above seawater salinity) conditions until reaching the highest and the lowest salinities
138 allowing growth (i.e. 10 and 50 ppt, respectively). Brackish conditions were achieved by
139 mixing 0.2 μm filtered seawater with deionized water, whereas sodium chloride was added to

seawater to reach hypersaline conditions. The media were then autoclaved and 0.2 μm filtered nutrient solutions were added to reach typical f/2 concentrations. Cells were then inoculated in triplicate in 1.5 L flasks containing 800 mL of medium at the appropriate salinity under semi-continuous growth conditions. The growth rates achieved were lower at 50 ppt (0.56 doublings per day) compared to 10 and 35 ppt (0.73 and 0.80 doublings per day, respectively) and cells were harvested after 5 days of constant growth rate (≤ 0.1 doublings per day).

2.5. Nitrogen depletion

Cells from *N. oceanica* were added to a medium containing all the nutrients except sodium nitrate at typical f/2 concentrations (N-); f/2 medium (N+) was used as control. Cells were initially harvested by centrifugation at 3500 **g** for 5 min and two mL of pellet containing 1.6×10^8 cells were added in each N+ treatment whereas 20 mL from the same pellet were added to the N- treatment. All the flasks were incubated at 20 °C and harvested after 6 days (Supplementary Table S2).

2.6. Desiccation

Cells were harvested by centrifugation as described above and a volume of 20 mL containing about 2×10^8 cells was poured into six Petri plates which were incubated at 20 °C under laminar flow. The lids were removed from three replicate Petri plates to allow water evaporation, whereas the other three Petri plates served as control and the lids were kept closed to prevent evaporation. After 48 h of incubation about 90% of the water had evaporated from the desiccation treatments and cells were harvested from all the Petri plates.

2.7. Cold shock

We evaluated the impact of immediate exposure to a lower temperature on LCDs, i.e. a so-called cold shock. Cells of *N. oceanica* were incubated in eight 1.5 L flasks at 20 °C under a dark/light regime. After three days, four flasks were transferred at 10 °C under the same conditions of light irradiance whereas the others were kept at 20 °C as control treatment. Cells were then harvested from two replicate flasks of each treatment after 6 h and 12 h.

2.8. Oxidative stress (hydrogen peroxide treatment)

We assessed the impact of radical stress on LCDs by exposing *N. oceanica* to hydrogen peroxide. Hydrogen peroxide reacts with dissolved iron and copper, which are present in the f/2 medium, forming hydroxyl radicals that can cause several damage to cells (Gutteridge and Wilkins, 1983). We hypothesized that such damage mostly affects the algaenan-rich outer cell wall of *N. oceanica* with potential consequences on the abundance of LCDs. Cells from *N. oceanica* were incubated in eight replicate 1.5 L flasks at the same culturing conditions used above and an initial density of 1.3×10^6 cells/mL. Four flasks were then amended with 100 µM hydrogen peroxide every morning at the same time, whereas the other flasks were used as control and cells were harvested after three days. Algal growth in the control treatment was slightly faster than those of the amended treatment (Supplementary Fig. S2).

2.9. Lipid extraction and analysis

Frozen filters were dried using a LyoQuest (Telstart, Life Sciences) freeze-drier. The freeze-dried filters were then saponified and subsequently acid hydrolyzed according to Rodrigo-Gámiz et al. (2015). The total lipid extracts (TLEs) were methylated by the addition of diazomethane and subsequently cleaned over a small silica gel 60 column by eluting with ethyl acetate. The TLEs were then silylated and dissolved in ethyl acetate as described before (de Bar et al., 2016) and analysed by gas chromatography (GC) and GC–mass spectrometry.

GC analysis was done on a HP 6890 Series GC System, mounted with a 25 m fused silica column with 0.32 mm diameter, coated with CB Sil-5 (thickness = 0.12 µm) and flame ionization detection. The carrier gas was helium at a constant pressure of 70 kPa. The oven temperature was 70 °C at injection and was then increased by 20 °C/min to 130 °C and subsequently by 4 °C/min until 320 °C, at which it was held for 10 min. A selection of the samples was analysed on an Agilent 7890B GC interfaced to a 7000C GC–MS Triple Quad, for identification. The applied temperature program and column were identical to those described for the GC system. Helium was used as carrier gas at a constant flow of 2 mL/min. The mass spectrometer operated with an ionization energy of 70 eV. The LCAs, LCDs and LCHFAs were identified by means of the characteristic mass spectra obtained in full scan mode, using a *m*/*z* range of 50–800 (Versteegh et al., 1997). The abundances of the compounds were quantified by integration of the peak areas of the compounds and the internal standard in the GC-FID using Atlas 8.2 software (Thermo Electron). For the co-eluting diols we determined the relative contributions of each diol to the total peak area in the GC-FID by integration of the characteristic fragment ions of each compound (Versteegh et al., 1997) using MassHunter software (Agilent Technologies).

2.10. Statistical analysis

In order to evaluate whether the lipid changes observed were statistically significant, a 2-sample T testing using Welch's test (Welch, 1947) was carried out using R software (https://www.r-project.org). We also performed a principal component analysis (PCA). The PCA on the distribution of the most abundant short chain fatty acids ($C_{14:0}$, $C_{16:0}$, $C_{16:1}$, $C_{20:5}$), LCDs, LCHFAs and LCAs was computed using R software for a total of 11 compounds across 70 data points (24 samples in 2, 3 or 4 replicates). The cellular concentrations of these

lipids were square-transformed and PCA was performed using the function prcomp and displayed using the function biplot.

3. Results and discussion

3.1. Impact of growth phase on lipid abundance and composition of Nannochloropsis species

Analysis of lipids of the three *Nannochloropsis* species investigated here revealed that the saturated fatty acids were dominated by $C_{14:0}$ and $C_{16:0}$, whereas $C_{16:1}$ and $C_{20:5}$ were the most abundant unsaturated fatty acids, consistent with previous studies on *Nannochloropsis* spp. (Volkman et al., 1993; Olofsson et al., 2012; Xiao et al., 2013; Mitra et al., 2015) as well as other eustigmatophytes (Volkman et al., 1999b). *N. oceanica* exhibited higher concentrations of total lipids (p-value < 0.05), saturated (p-value < 0.01) and unsaturated (p-value < 0.01) fatty acids than the other two species (Fig. 1D–F). The concentrations of LCAs, LCDs and LCHFAs were an order of magnitude lower than those of the fatty acids (i.e. ca. 30 fg/cell vs ca. 500 fg/cell for the fatty acids; Fig. 1A–C). Greater concentrations of LCAs, LCDs and LCHFAs were also found in *N. oceanica* (Fig. 1A) compared to *N. gaditana* (p-value < 0.01, Fig. 1B) and *N. oculata* (p-value < 0.05; Fig. 1C). The composition of LCDs, LCAs and LCHFAs is consistent with that reported in literature for these three species (Volkman et al., 1992; Mejanelle et al., 2003; Rampen et al., 2014a) and other eustigmatophytes (Volkman et al., 1992, 1999a; Gelin et al., 1997b; Rampen et al., 2014a), i.e.. $C_{30:0}$ 1,13 and 1,15 diols, $C_{32:1}$ 1,15 diol, $C_{32:0}$ 1,15 diol, $C_{30:0}$ 13-hydroxy and $C_{32:0}$ 15-hydroxy fatty acids and $C_{32:1}$ and $C_{32:2}$ alkenols.

We examined the lipid composition of *N. gaditana*, *N. oceanica* and *N. oculata*, harvested during the early exponential phase, the late exponential phase and the stationary phase (Table 1). *N. oceanica* differed from *N. gaditana* and *N. oculata* in both the abundance and changes

238 with growth phase of LCAs, LCDs and LCHFAs. The total LCD abundance ranged from 14 ±
239 2.5 to 20 ± 4.2 fg/cell in *N. gaditana*, 16 ± 5.2 to 34 ± 5.8 fg/cell in *N. oceanica* and 11 ± 2.1
240 to 12 ± 3.8 in *N. oculata* (Table 1). *N. oceanica* showed also the highest concentrations of
241 LCAs (9.4 ± 2.9 to 27 ± 11 fg/cell) and LCHFAs (2.3 ± 0.5 to 7.3 ± 2.9 fg/cell) compared to
242 the other two species (Table 1). During the growth of *N. oceanica*, $C_{32:0}$ and $C_{32:1}$ diols as well
243 as LCAs increased towards the stationary phase, whereas the $C_{30:0}$ 13-hydroxy fatty acid
244 decreased, while no significant changes were observed for the $C_{30:0}$ diol and the $C_{32:0}$ 15-
245 hydroxy fatty acid (Fig. 1A). In contrast, LCHFA concentration decreased during the growth
246 of *N. gaditana*, while concentrations of LCDs and LCAs did not change significantly (Fig.
247 1B) whereas for *N. oculata* LCA, LCD and LCHFA concentrations were fairly constant (Fig.
248 1C).
249 Overall, we did not find a consistent trend with growth phase for LCAs, LCDs and
250 LCHFAs across the three species and thus the distribution and abundance of these compounds
251 does not seem to be affected by growth stage. Because of the higher concentrations of LCAs,
252 LCDs and LCHFAs in *N. oceanica* (Table 1), we used this species for the subsequent
253 experiments.
254
255 *3.2. Impact of cultivation conditions on lipid abundance and composition*
256 *3.2.1. Effect of light intensity*
257 Higher abundance of saturated fatty acids occurred in cells incubated under HL conditions
258 (Fig. 2D), which agrees with previous studies where *Nannochloropsis* sp. stored higher
259 amounts of triacylglycerols (TAGs), mostly consisting of $C_{16:0}$ and $C_{18:0}$ fatty acids, under HL
260 conditions (Sukenik and Carmeli, 1990; Fabregas et al., 2004; Pal et al., 2011). As for LCDs
261 and LCD-related compounds, cells of *N. oceanica* cultured at HL conditions exhibited
262 significantly higher concentrations of LCHFAs (3.4 ± 0.7 vs 0.3 ± 0.05 pg/cell at LL

conditions), $C_{32:2}$ alkenol (6.3 ± 1.1 vs 2.7 ± 0.7 pg/cell) and $C_{32:1}$ diol (8.0 ± 0.9 vs 3.9 ± 0.7), whereas the concentration of $C_{32:1}$ alkenol and that of other LCDs did not change significantly (Fig. 2A). Thus, there is no consistent pattern in changes of LCDs and only the LCHFAs seemed to substantially increase in abundance although the cause for this is not clear as LCHFAs are unlikely to be part of TAGs.

3.2.2. Effect of salinity

N. oceanica was cultured at salinities of 10, 35 and 50 ppt. Cells at 50 ppt exhibited the highest contents in lipids (p-value < 0.05) and saturated fatty acids (Table 1, Fig. 2E), which is 2–3 fold higher than at lower salinities, confirming previous findings (Pal et al., 2011). The concentration of $C_{30:0}$ 13-hydroxy fatty acid at 10 ppt was only ca. 25% of that measured at both 35 and 50 ppt, whereas the cellular content in $C_{32:0}$ 15-hydroxy fatty acid, LCAs and LCDs did not change significantly with salinity (Fig. 2B). Since the fractional and absolute abundance of most compounds analysed here did not change significantly at changing salinities (Fig. 2B) the osmotic pressure does not seem to affect the composition and abundance of LCAs and LCDs. The algaenan layer present in the outer cell wall of *Nannochloropsis* spp. is therefore unlikely to undergo compositional changes as a consequence of increased or decreased salinity. This suggests that algaenans do not play a role in protecting the cell from osmotic pressure.

3.2.3. Nitrogen limitation

N. oceanica was incubated into N+ and N- medium to evaluate the impact of nitrogen starvation on LCAs, LCDs and LCHFAs. The cells grew under N+ conditions much faster than in the N- treatment (Supplementary Fig. S2). Cells incubated at N- conditions exhibited lower concentrations of total lipids (Table 1), unsaturated and saturated fatty acids (Fig. 2F).

Under nitrogen-deficient conditions phytoplankton cells typically accumulate storage lipids (Dunstan et al., 1993; Li et al., 2014; Jia et al., 2015), and we observed indeed, in the N- treatment, a substantial increase in C_{14}-C_{20} saturated and unsaturated fatty acids (Fig. 2F). The cellular concentration of LCHFAs also increased by almost eight-fold (Fig. 2C). However, the $C_{32:0}$ diols only doubled in concentration at N- conditions while the concentrations of the other LCDs and of LCAs did not change significantly (Fig. 2C). It has been suggested that the lack of nitrogen in the medium promotes a preferential synthesis of lipids over proteins leading to an increase in the fatty acid content (Vieler et al., 2012). Under nitrogen deprivation, *Nannochloropsis* spp. typically accumulate TAGs and to a lesser extent other glycerolipids (Tonon et al., 2002; Jia et al., 2015), in agreement with our observation that C_{14}–C_{20} saturated and unsaturated fatty acids in the N- treatments increased substantially (Fig. 2F). This accumulation seems also to enhance the production of LCHFAs but not that of LCAs and LCDs (Fig. 2C). Since under conditions which typically stimulate lipid accumulation, i.e. increased light intensity, salinity and nutrient deficiency, LCDs and LCAs did not systematically increase like the fatty acids, these compounds are unlikely to serve as storage lipids within *Nannochloropsis* cells. Since they do not accumulate under culturing conditions which promote energy storage, LCDs might thus serve as structural lipids present in the outer cell wall as building blocks of algaenans, as has been previously hypothesized (de Leeuw et al., 1981; Tegelaar et al., 1989; Gelin et al., 1997a; Volkman et al., 1998; Scholz et al., 2014; Zhang and Volkman, 2017).

3.2.4. Desiccation

We evaluated the impact of desiccation on LCAs, LCDs and LCHFAs in *N. oceanica* by incubating our cultures in open Petri plates for two days. We hypothesized that desiccation stress may create an unfavorable environment inducing *Nannochloropsis* cells to elapse into

313 resting stages, potentially leading to major changes in its lipid composition, in particular in
314 the outer cell wall. For example *Chlorella kessleri* and *Chlamydomonas reinhardtii*
315 substantially increased their fatty acid content upon desiccation (Shiratake et al., 2013).
316 Resting cells often have a thicker cell wall to better resist degradation and spores with a
317 thicker cell wall have been observed in *Nannochloropsis limnetica* (Fietz et al., 2005), as well
318 as in other eustigmatophytes (Pribyl et al., 2012).
319 Two days after the experiment started, about 90% of the medium volume evaporated in the
320 desiccation treatment while no evaporation occurred in the control. No visible difference was
321 noted between cells from desiccation and the control treatment under light microscopy and no
322 significant changes in lipids, fatty acids, LCDs and LCD-derivatives were observed between
323 the desiccated culture and the positive control (Table 1, Fig. 3A). LCAs, LCDs and LCHFAs
324 are thus not affected by desiccation stress.
325
326 *3.2.5. Cold shock*
327 Another factor, which can potentially affect outer cell walls and lipid composition, is cold
328 shock. Rapid exposure of phytoplankton to a temperature lower than that of their standard
329 culturing conditions has been observed to promote desaturation of fatty acids in diatoms
330 (Jiang and Gao, 2004) and dinoflagellates (Jiang and Chen, 2000). Therefore, cultures of *N.*
331 *oceanica* were rapidly transferred from their standard temperature for growth (20 °C) to 10 °C
332 and lipids were analysed after 6 h and 12 h. The abundance of fatty acids, LCAs, LCDs and
333 LCHFAs did not change as a response to cold shock over the relatively short incubation times
334 used here (Fig. 3B and E). Compositional changes in LCDs with growth temperature have
335 been previously reported by Rampen et al. (2014a) for *N. gaditana*. However, in that study the
336 cultures were actively growing at different temperatures. Apparently, such changes do not

337 occur when a strain is exposed to a different culturing temperature over the short incubation
338 times applied here.
339
340 *3.2.6. Oxidative stress*
341 Stress from radicals can induce defense mechanisms in phytoplankton affecting the
342 abundance and composition of lipids and potentially also their outer cell wall. Hydrogen
343 peroxide is a known source of radicals and *N. oculata* has been previously found to grow in
344 the presence of up to 50 μM hydrogen peroxide (Taylor et al., 2012).
345 In the present study, *N. oceanica* was able to grow with hydrogen peroxide daily added to
346 the cultures at concentrations up to 100 μM (Supplementary Fig. S2). We evaluated the
347 composition of lipids in cultures amended with 100 μM H_2O_2: the hydrogen peroxide-
348 amended cultures were significantly depleted in unsaturated fatty acids (38% decrease),
349 LCHFAs (37%), $C_{32:2}$ alkenol (36%) and $C_{32:1}$ diol (48%) compared to the unamended control
350 (Fig. 3C) suggesting that some of these compounds were either partially degraded by
351 hydrogen peroxide or synthesized at a lower rate. Interestingly, most of the compounds that
352 decreased in the presence of hydrogen peroxide, are unsaturated and their double bonds are
353 likely to be highly reactive towards hydrogen peroxide. Similarly, in the presence of radicals,
354 the degradation rates of alkenones increases with increasing number of double bonds (Rontani
355 et al., 2006). The decrease in unsaturated fatty acids, LCHFAs, $C_{32:2}$ alkenol and $C_{32:1}$ diol is
356 thus unlikely the result of changes in their biosynthetic pathways but these compounds may
357 have been directly chemically oxidized by hydrogen peroxide. In any case, our results suggest
358 that radicals do not affect LCD synthesis rates.
359
360 *3.3. Role of LCAs, LCDs and LCHFAs in Nannochloropsis*

361 Principal Component Analysis (PCA) of the fractional abundances of the major
362 compounds in the differently grown cultures reveals that $C_{30:0}$ 13-hydroxy and the $C_{32:0}$ 15-
363 hydroxy fatty acids cluster with $C_{16:0}$ and $C_{14:0}$ fatty acids (Fig. 4). This suggests that LCHFAs
364 may originate from $C_{14:0}$ and $C_{16:0}$ fatty acids via hydroxylation and chain elongation. It is not
365 clear at which point of the elongation process the hydroxylation occurs. LCHFAs were
366 previously suggested to share the same biosynthetic pathways as the LCAs and the LCDs in
367 *Nannochloropsis* spp., because compounds from these three classes exhibiting the same
368 carbon number are functionalised at the same position (Volkman et al., 1992; Gelin et al.,
369 1997b). However, LCHFAs, $C_{14:0}$ and $C_{16:0}$ fatty acids do not cluster with LCDs as well as
370 LCAs abundance in our PCA, suggesting that not all the LCHFAs biosynthesised by
371 *Nannochloropsis spp.* were converted to LCDs and LCAs.
372 In *Nannochloropsis* species, LCAs, LCDs and LCHFAs are thought to be bound together
373 via ether or ester bonds to form algaenans (Gelin et al., 1996, 1997a, 1999; Volkman et al.,
374 1998; Zhang and Volkman, 2017). Algaenans have also been described in several genera of
375 Chlorophyta (Derenne et al., 1988; Gelin et al., 1997a; Blokker et al., 1998, 1999; Allard and
376 Templier, 2001) and the dinoflagellate *Gymnodinium catenatum* (Gelin et al., 1999).
377 Algaenans are thought to be highly resistant to degradation (Tegelaar et al., 1989; Volkman et
378 al., 1998) and are likely present in the outer layers of the cell walls, forming a trilaminar
379 structure (Gelin et al., 1999). Fourier transform infrared spectroscopy on the cell wall of *N.*
380 *gaditana* confirmed the ether linked aliphatic structures of algaenans and revealed also the
381 presence of C=O bonds (Scholz et al., 2014), which likely correspond to ketone or carboxylic
382 functional groups of long chain keto-ols, LCHFAs and dihydroxy fatty acids, respectively,
383 which were previously reported to occur in *Nannochloropsis* spp. (Volkman et al., 1992;
384 Gelin et al., 1997b; Versteegh et al., 1997). As algaenans are situated in the outer cell wall of
385 *Nannochloropsis* spp. they might play a role in the protection of cells from chemical attack.

386 This is evidenced in our study as *N. oceanica* was able to grow in the presence of high (100
387 μM) concentrations of hydrogen peroxide. In fact, phytoplankton can usually tolerate
388 hydrogen peroxide concentrations higher than cyanobacteria (Drabkova et al., 2007;
389 Barrington and Ghadouani, 2008) and heterotrophic bacteria (Xenopoulos and Bird, 1997).
390 However, to the best of our knowledge, no phytoplankton species has been shown to grow,
391 for three consecutive days, under a daily amendment of 100 μM hydrogen peroxide. We
392 hypothesize that algaenans in *Nannochloropsis* spp. might then play a key role against
393 oxidative stress making *Nannochloropsis* species highly resistant to radicals.
394 A protective role of some algaenan-associated compounds is also suggested by the higher
395 proportions of LCHFAs, $C_{32:2}$ alkenol and $C_{32:1}$ diol found at HL conditions and by the
396 increase in $C_{30:0}$ 13-hydroxy fatty acid and $C_{32:0}$ diol in nitrogen depleted medium (Fig. 2A).
397 Both the exposure to excessive light (i.e. HL treatment) and the nutrient deficiency (i.e. N-)
398 can stimulate the production of reactive oxygen species, although to a much lesser extent than
399 hydrogen peroxide (McKersie and Leshem, 1995). While the hydrogen peroxide amendment
400 likely implied the degradation of part of the outer cell wall, leading to the lower proportions
401 of LCAs, LCDs and LCHFAs measured, the exposure to radicals under HL or N- conditions
402 was likely to be less aggressive and might have stimulated a cellular response to biosynthesize
403 higher amounts of algaenan-associated compounds in order to protect the cell.
404
405 *3.4. LCDs as tracers for biomass of eustigmatophytes*
406 Overall, the cellular levels of LCAs and LCDs found here in three different
407 *Nannochloropsis* species, as well as those measured for *N. oceanica* under different culturing
408 conditions and manipulation did not vary greatly, i.e. over all experiments and species, the
409 concentrations were 12 ± 8.0 fg/cell for LCAs and 23 ± 15 fg/cell for LCDs. In contrast a
410 greater variability was found for LCHFAs (8.2 ± 10 fg/cell) and fatty acids (541 ± 570

411 fg/cell). This suggests that the cellular levels of LCAs and LCDs are fairly constant within the
412 eustigmatophytes investigated here. These consistent levels may be due to the fact that they
413 are not storage lipids, but are associated with the outer cell wall. In turn, it suggests that if cell
414 size and cell wall thickness are fairly constant across the Eustigmatophyceae, the
415 concentration of LCDs may be a good proxy for the abundance of eustigmatophytes in aquatic
416 environments. Furthermore, sedimentary records of LCDs may be used to constrain relative
417 changes in abundance, at least in regions not affected by variable past oxygen levels in the
418 water column as diols are known to degrade at different rates between oxic and anoxic
419 sediments (Rodrigo-Gamiz et al., 2016).
420
421 **4. Conclusions**
422 Culturing conditions that typically promote TAG accumulation in *Nannochloropsis*
423 lead to a slight increase in LCHFAs but do not enhance the concentration of LCAs and LCDs.
424 LCHFAs are likely to derive from $C_{14:0}$ and $C_{16:0}$ fatty acids, whereby $C_{14:0}$ and $C_{16:0}$ fatty
425 acids might undergo one hydroxylation and several elongation steps to form LCHFAs which
426 in turn can be precursors of LCAs and LCDs.
427 Our study indicates that LCAs, LCDs and LCHFAs do not serve as storage lipids but
428 likely function as structural lipids present in the outer cell wall playing a protective function
429 from oxidative stress. The nearly constant concentrations of LCAs and LCDs, along with their
430 specific chain length and position of functionalization for *Nannochloropsis* spp., suggest that
431 these compounds might be considered as proxies for eustigmatophyte biomass in recent
432 settings. A deeper understanding of the genetic mechanisms controlling the biosynthetic
433 pathways of LCAs, LCDs and LCHFAs can contribute, in the future, to a better understanding
434 of the controls on LCD synthesis.
435

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667 **Figure legends**

668

669 **Fig. 1.** Compositional changes in the cellular abundances of: (A–C) LCDs, LCAs and
670 LCHFAs, and (D–F) sum of saturated and unsaturated fatty acids and cholesterol during
671 different stages of the growth of the strains investigated in the present study. Error bars
672 represent standard deviation of triplicate cultures. Experiments were performed with (A, D)
673 *Nannochloropsis oceanica* CCMP1779, (B, E) *Nannochloropsis gaditana* CCMP526, and (C,
674 F) *Nannochloropsis oculata* CCMP2195. Early exp. = early exponential phase; late exp. = late
675 exponential phase. The level of significance of the differences between the concentration at a
676 growth stage and the previous one is indicated (*, p-value < 0.05; **, p-value < 0.01).

677

678 **Fig. 2.** Impact of different culturing conditions on the cellular levels of: (A–C) LCAs, LCDs
679 and LCHFAs, and (D–F) sum of saturated and unsaturated fatty acids and cholesterol on
680 *Nannochloropsis oceanica* CCMP1779. Error bars represent standard deviation of triplicate
681 cultures. Effect of (A, D) different levels of light irradiance, (B, E) different salinities of the
682 culturing medium, and (C, F) depletion of nitrogen from the medium. Please note that the
683 culturing medium used here, f/2, typically contains 900 µM nitrate. Refer to Fig. 1 for
684 abbreviations. The level of significance of the differences between the concentration at a
685 growth stage and the previous one is indicated (*, p-value < 0.05; **, p-value < 0.01).

686

687 **Fig. 3.** Impact of different stress conditions on the concentration of: (A-–C) LCAs, LCDs and
688 LCHFAs, and (D–F) sum of saturated and unsaturated fatty acids and cholesterol on
689 *Nannochloropsis oceanica* CCMP1779. Error bars represent standard deviation of triplicate
690 cultures. (A, D) Effect of desiccation, the cultures were poured into Petri plates and exposed
691 to the air for 48 h without the lid to promote the evaporation of the medium, the positive

692 control consist in Petri plates with the lid. (B, E) Effect of cold shock: the cultures were
693 rapidly transferred to a colder (10 °C) temperature and incubated for 6 or 12 h. (C, F) Effect
694 of daily amendment of hydrogen peroxide (radical stress). The level of significance of the
695 differences between the concentration at a growth stage and the previous one is indicated (*,
696 p-value < 0.05; **, p-value < 0.01).
697
698 **Fig. 4.** Ordination of the different LCDs and LCD-derivatives as well as the major fatty acids
699 in the space defined by principal component analysis: PCA1 on the x axis and PCA2 on the y
700 axis. The values in brackets indicate the percentage of variance explained by each axis.
701
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703

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706

Figure 1

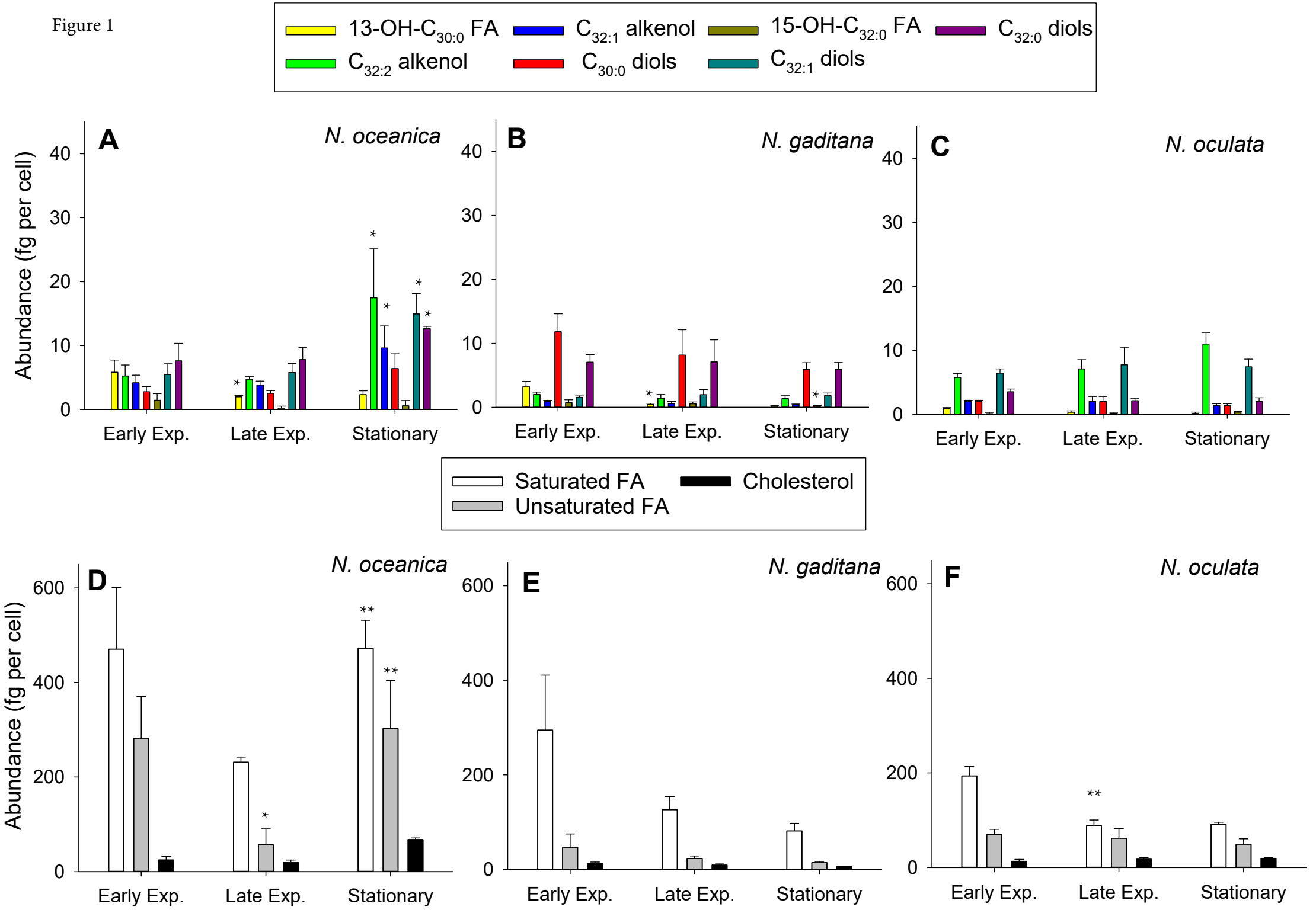

13-OH-C30:0 FA
C32:1 alkenol
15-OH-C32:0 FA
C32:0 diols
C32:2 alkenol
C30:0 diols
C32:1 diols
A
N. oceanica
B
N. gaditana
C
N. oculata
Abundance (fg per cell)
0
10
20
30
40
Early Exp.
Late Exp.
Stationary
Saturated FA
Unsaturated FA
Cholesterol
D
N. oceanica
E
N. gaditana
F
N. oculata
0
200
400
600

Figure 2

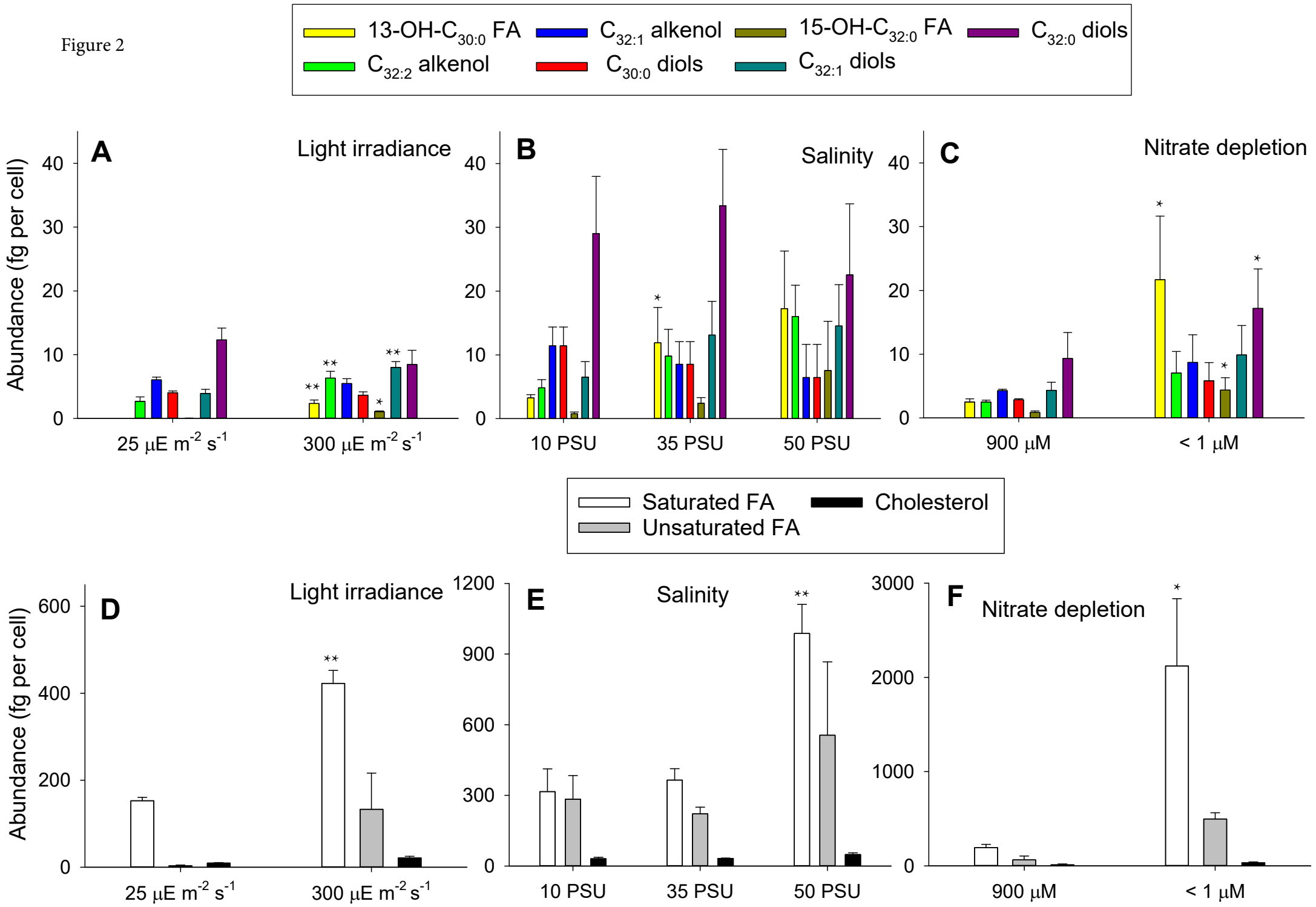

13-OH-C30:0 FA
C32:1 alkenol
15-OH-C32:0 FA
C32:0 diols
C32:2 alkenol
C30:0 diols
C32:1 diols
A
Light irradiance
B
Salinity
C
Nitrate depletion
Abundance (fg per cell)
25 µE m-2 s-1
300 µE m-2 s-1
10 PSU
35 PSU
50 PSU
900 µM
< 1 µM
Saturated FA
Unsaturated FA
Cholesterol
D
Light irradiance
E
Salinity
F
Nitrate depletion

Figure 3

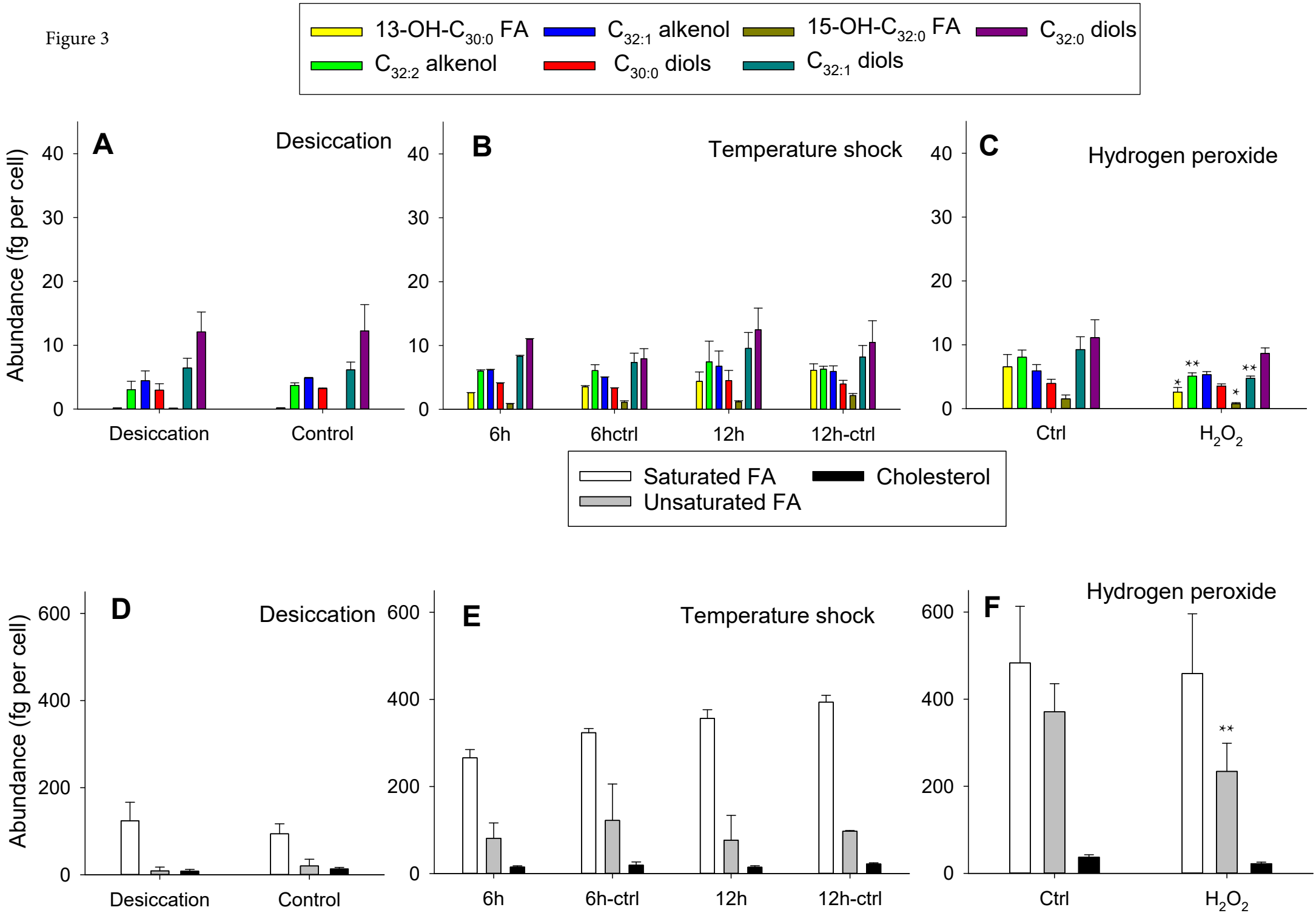

13-OH-C30:0 FA
C32:1 alkenol
15-OH-C32:0 FA
C32:0 diols
C32:2 alkenol
C30:0 diols
C32:1 diols
A
Desiccation
B
Temperature shock
C
Hydrogen peroxide
Abundance (fg per cell)
0
10
20
30
40
Desiccation
Control
6h
6hctrl
12h
12h-ctrl
Ctrl
H2O2
*
**
Saturated FA
Unsaturated FA
Cholesterol
D
Desiccation
E
Temperature shock
F
Hydrogen peroxide
0
200
400
600
6h
6h-ctrl
12h
12h-ctrl

Figure 4

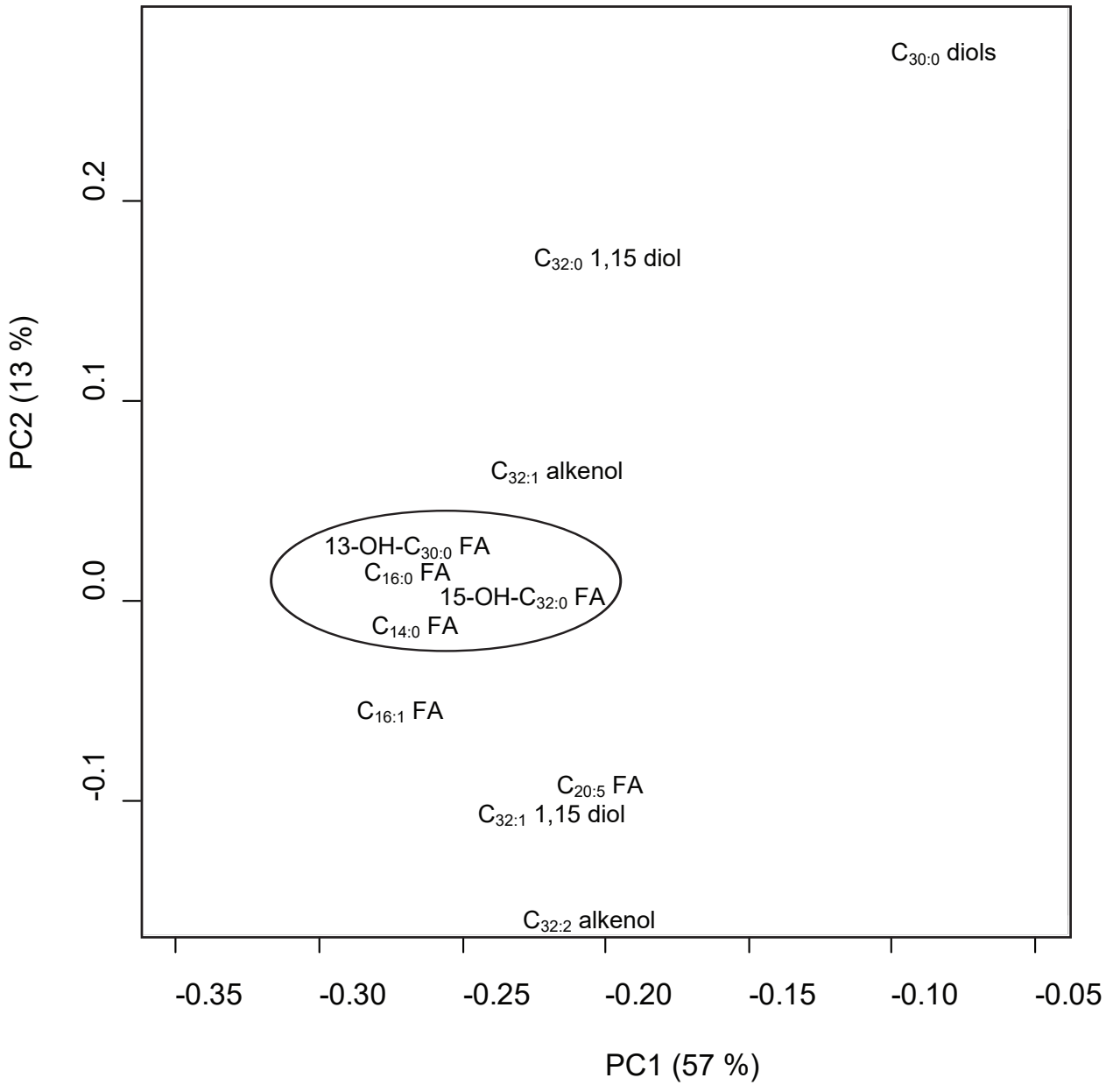

C30:0 diols
C32:0 1,15 diol
C32:1 alkenol
13-OH-C30:0 FA
C16:0 FA
15-OH-C32:0 FA
C14:0 FA
C16:1 FA
C20:5 FA
C32:1 1,15 diol
C32:2 alkenol
0.2
0.1
0.0
-0.1
PC2 (13 %)
-0.35
-0.30
-0.25
-0.20
-0.15
-0.10
-0.05
PC1 (57 %)

Table 1. Cell abundance, and concentrations of total lipids and the sum of long chain alkenols (LCAs), long chain alkyl diols (LCDs), and long chain hydroxy fatty acids (LCHFAs) for the different experiments carried out during this study.

Experiment	Species/strain	Treatment	Replicates	Duration (days)	Abundance (cells/mL)	Lipid content (pg/cell)	(% w/w)	LCAs (fg/cell)	LCDs (fg/cell)	LCHFAs (fg/cell)
Growth rate	*N. oceanica* CCMP1779	Early [a]	3	7	$1.8 \pm 0.2 \times 10^6$	2.5 ± 0.2	12 ± 1	9.4 ± 2.9	16 ± 5.2	7.3 ± 2.9
		Late [b]	3	10	$3.1 \pm 0.5 \times 10^6$	4.7 ± 1.9	n.a.	8.6 ± 1.0	16 ± 4	2.3 ± 0.5
		Stationary [c]	3	11	$5.6 \pm 1.1 \times 10^6$	2.2 ± 0.4	11 ± 2	27 ± 11	34 ± 5.8	2.9 ± 1.4
	N. gaditana CCMP526	Early	3	9	$1.5 \pm 0.1 \times 10^6$	2.2 ± 0.4	14 ± 3	2.9 ± 0.6	20 ± 4.2	4.1 ± 1.2
		Late	3	12	$7.4 \pm 0.5 \times 10^6$	1.0 ± 0.1	11 ± 3	2.1 ± 0.9	17 ± 8.2	1.0 ± 0.4
		Stationary	3	19	$1.3 \pm 0.2 \times 10^7$	1.0 ± 0.2	11 ± 2	1.8 ± 0.5	14 ± 2.5	0.5 ± 0.1
	N. oculata CCMP2195	Early	3	7	$1.4 \pm 0.2 \times 10^6$	2.5 ± 0.9	13 ± 6	7.8 ± 0.8	12 ± 1.3	1.1 ± 0.3
		Late	3	10	$6.5 \pm 0.6 \times 10^6$	1.4 ± 0.5	16 ± 9	9.1 ± 2.2	12 ± 3.8	0.5 ± 0.2
		Stationary	3	15	$1.4 \pm 0.1 \times 10^7$	1.4 ± 0.4	21 ± 9	12 ± 2.1	11 ± 2.1	0.5 ± 0.2
Light	*N. oceanica* CCMP1779	HL[d]	3	7	$5.6 \pm 0.1 \times 10^6$	2.3 ± 0.5	17 ± 4	9.4 ± 2.9	20 ± 3.6	3.4 ± 0.7
		LL[e]	3	9	$4.3 \pm 0.3 \times 10^5$	5.5 ± 0.4	3.5 ± 0.3	8.6 ± 1.0	20 ± 2.8	0.3 ± 0.05
Salinity	*N. oceanica*	10 ppt	3	7	$2.6 \pm 0.1 \times 10^6$	3.9 ± 0.6	15 ± 3	16 ± 4.2	47 ± 14	4.1 ± 0.8

	CCMP1779									
		35 ppt	3	7	$2.9 \pm 0.1 \times 10^6$	3.7 ± 1.3	11 ± 4	18 ± 7.7	55 ± 18	14 ± 6.4
		50 ppt	3	7	$9.8 \pm 0.4 \times 10^5$	8.5 ± 2.7	8 ± 2	22 ± 10	43 ± 19	25 ± 11
Nitrogen depletion	*N. oceanica* CCMP1779	N- [f]	3	6	$2.7 \pm 0.4 \times 10^6$	5.7 ± 1.2	18 ± 5	16 ± 7	33 ± 14	26 ± 12
		Control	3	6	$5.2 \pm 0.8 \times 10^6$	2.3 ± 0.1	16 ± 4	6.8 ± 0.5	17 ± 5.5	3.4 ± 0.7
Desiccation	*N. oceanica* CCMP1779	Desiccated	3	2	$1.3 \pm 0.2 \times 10^7$	3.6 ± 0.9	6 ± 4	7.5 ± 2.8	21 ± 5.6	0.3 ± 0.2
		Control	3	2	$1.3 \pm 0.2 \times 10^7$	2.6 ± 0.5	6 ± 1	8.6 ± 0.5	22 ± 5.4	0.2 ± 0.1
Cold shock	*N. oceanica* CCMP1779	Cold 6h	2	0.25	$3.4 \pm 0.2 \times 10^6$	2.5 ± 0.5	15 ± 5	12 ± 0.3	23 ± 0.3	3.5 ± 0.1
		Control 6h	2	0.25	$4.0 \pm 0.1 \times 10^6$	2.5 ± 0.1	13 ± 1	11 ± 1.0	19 ± 3.0	4.6 ± 0.4
		Cold 12h	2	0.5	$3.4 \pm 0.2 \times 10^6$	2.5 ± 0.6	11 ± 3	14 ± 5.6	26 ± 7.4	5.6 ± 1.6
		Control 12h	2	0.5	$4.0 \pm 0.2 \times 10^6$	2.3 ± 0.2	12 ± 1	12 ± 1.3	23 ± 5.7	8.3 ± 1.3
Oxidative stress	*N. oceanica* CCMP1779	H_2O_2 [g]	4	3	$5.5 \pm 0.8 \times 10^6$	2.3 ± 0.1	11 ± 1	10 ± 1.0	17 ± 1.5	8.3 ± 0.7
		Control	4	3	$2.9 \pm 0.7 \times 10^6$	2.2 ± 0.8	17 ± 5	14 ± 2.1	24 ± 5.4	13 ± 2.7

[a] Early exponential phase
[b] Late exponential phase
[c] Stationary phase
[d] 25 μE/(m^2s)
[e] 300 μE/(m^2s)

[f] Culture incubated in f/2 medium without sodium nitrate. No addition nitrogen sources were present in the medium.
[g] Treatment amended daily with 100 μM hydrogen peroxide.

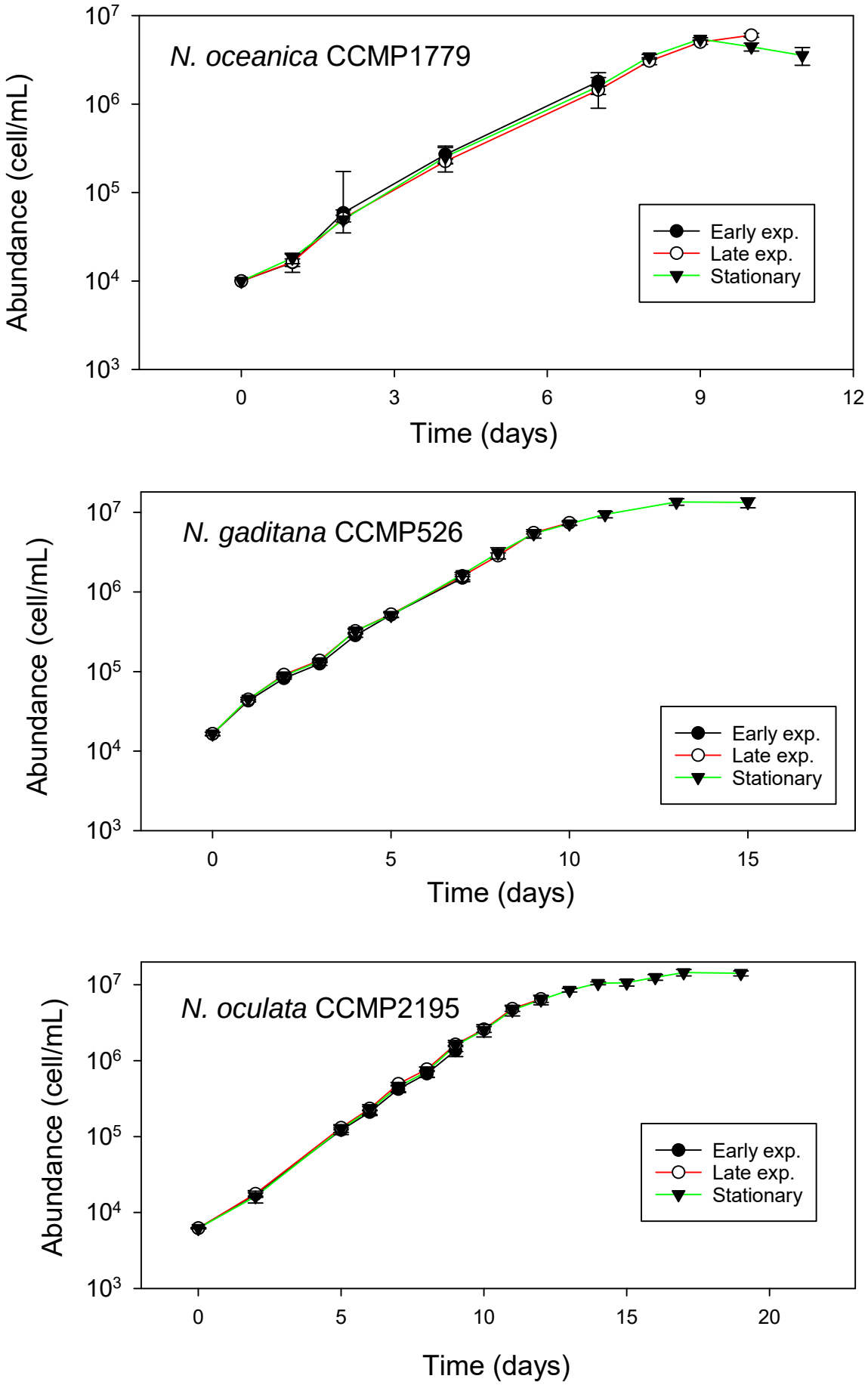
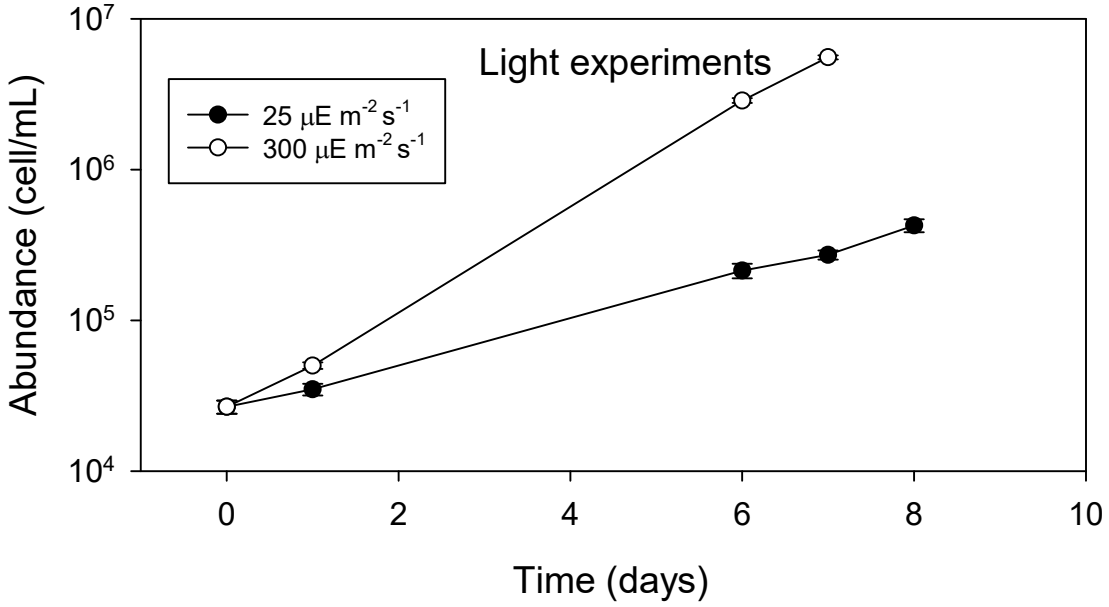
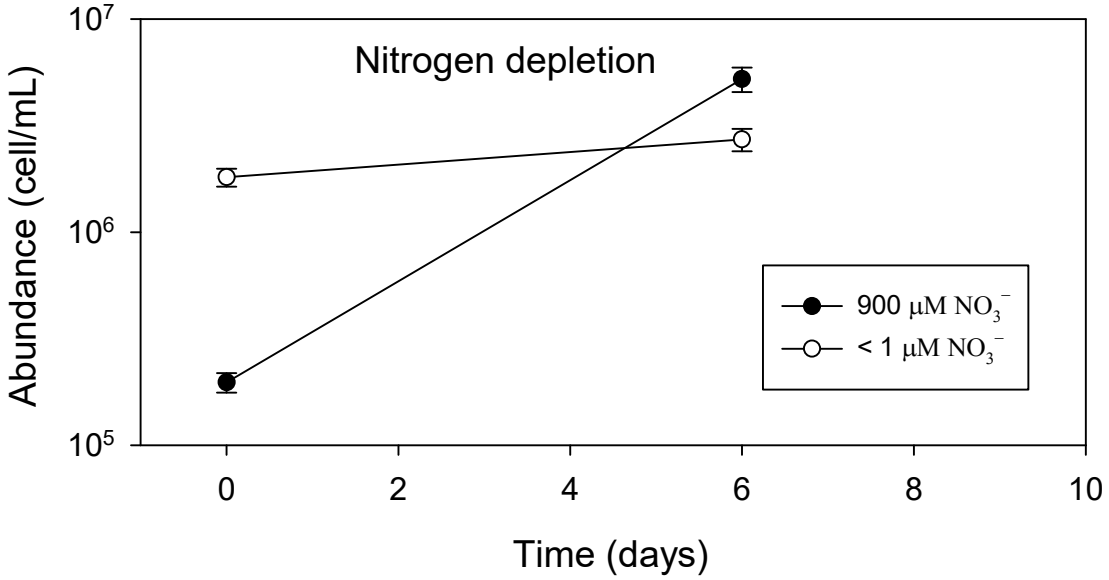


Fig. S1. Growth curves of (A) Nannochloropsis oceanica CCMP1779, (B) Nannochloropsis gaditana CCMP526, and (C) Nannochloropsis oculata CCMP2195. The different colours refer to the replicate samples harvested during the early exponential phase and the late exponential phase of their growth as well as the stationary phase.





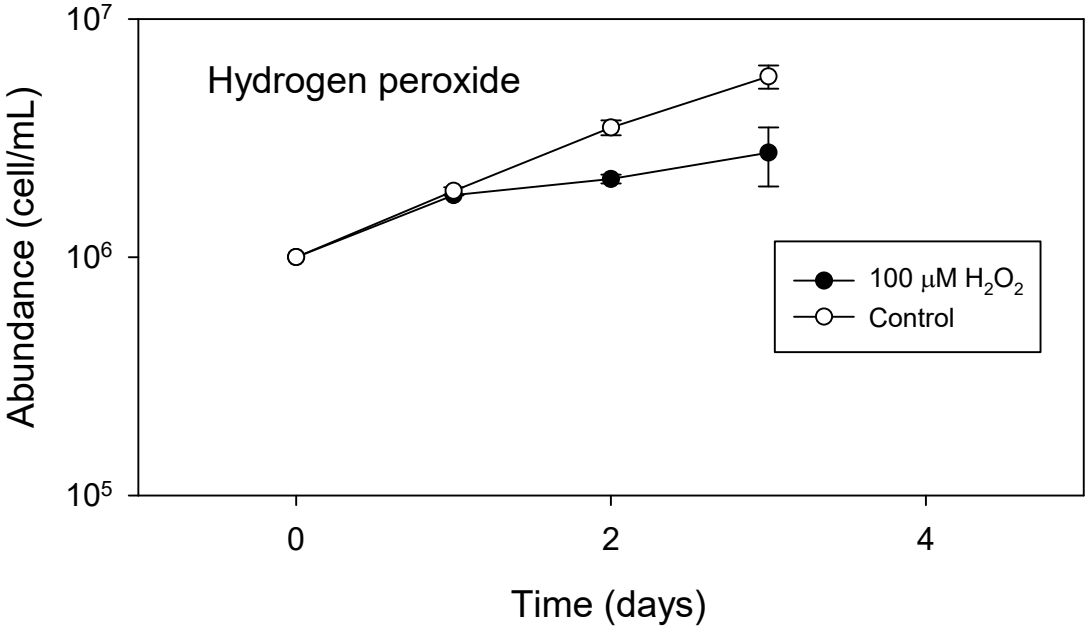


Fig. S2. Growth curve of Nannochloropsis oceanica CCMP1779 under different conditions of (A) light, (B) nitrate availability, and (C) when spiked with hydrogen peroxide.

Highlights

- We investigated long chain alkyl diol (LCD) abundance in *Nannochloropsis* spp.
- LCD abundances are stable during growth and under different culturing conditions
- LCDs are likely to play a protective role in *Nannochloropsis* cells wall
- LCDs concentrations in seawater and freshwater may reflect algal abundance