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

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

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

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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 Dichtyochophyceae \( Apedinella radians \) (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...
dihydroxy fatty acids (Gelin et al., 1997b). Because of the high similarities in their chemical
structures, LCAs and LCDs in *Nannochloropsis* species have been suggested to originate
from the reduction of the carboxylic groups of the LCHFAs (Gelin et al., 1997b).

Factors controlling the composition and abundance of LCDs in algae have rarely been
studied in detail. Rampen et al. (2014a) showed that LCDs increase in chain length and in the
proportion of saturated diols with increasing temperatures. However, it is not clear whether
other environmental conditions can affect LCD abundance and composition. Since the
Eustigmatophyceae comprises both freshwater and marine species (Hibberd, 1981) and these
occur in estuarine and hypersaline environments (Vinogradova and Darienko, 2008; Samanta
and Bhadury, 2014; Balzano et al., 2015), so they possess the metabolic plasticity to adapt to
different salinities. Estuarine phytoplankton can also experience rapid shifts in nutrients
because of the interactions between nutrient-rich freshwater and nutrient-poor seawater as
well as shifts in light irradiance caused by water turbidity. The reliability of proxies based on
LCDs might be compromised if the cellular levels and composition of LCDs change with
these varying conditions of light, salinity and nutrients, which typically occur in the
environment where LCD-producers occur. Indeed, culture experiments with *Nannochloropsis*
spps. show that their fatty acid composition may vary strongly during algal growth (Dunstan et
al., 1993) or under different salinity, light irradiance and nitrate regimes (Pal et al., 2011;
Martinez-Roldan et al., 2014).

In the present study, we analysed the concentrations of LCDs in three species
(*Nannochloropsis gaditana, Nannochloropsis oceanica and Nannochloropsis oculata*) during
different stages of their growth. We incubated the species yielding the highest levels of LCDs,
LCHFAs and LCAs (*N. oceanica*) under different culturing (light irradiance, salinity) or stress
(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/(m²s), 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
An aliquot of 50 mL containing about $1.5 \times 10^7$ cells was inoculated in 9 replicate 2.5 L Erlenmeyer flasks containing 1.45 L of f/2 medium. Cells were enumerated daily and the growth rate subsequently calculated as described previously (Balzano et al., 2011). The cells were harvested for the first time (early exponential phase) from triplicate flasks, when their abundance reached $10^6$ cells/mL (Supplementary Fig. S1). When a decline in the growth rate was observed (late exponential phase) three further flasks from each strain were filtered and cells were harvested from the last three flasks when no further growth was observed for two consecutive days (stationary phase, Supplementary Fig. S1).

2.3. Light irradiance

Six replicate volumes of 10 mL, containing about $2 \times 10^7$ cells each from *N. oceanica*, were transferred into six 1.5 L flasks containing 740 mL of f/2 medium and were incubated at 20 °C. Three replicate flasks were exposed to an irradiance of about 300 μE/(m²s) and used as high light (HL) treatment, whereas the other three flasks were covered by several shading nets (irradiance 25 μE/(m²s)) and were considered to reflect low light (LL) conditions. Cells grew faster in the HL treatment and were harvested from the cultures when their concentration exceeded $5 \times 10^6$ cells/mL, on Day 6 for the HL treatment and Day 7 for the LL treatment (Supplementary Fig. S2).

2.4. Osmotic stress

To assess the impact of salinity on LCD abundance and composition, cells from *N. oceanica* were pre-adapted to grow at both brackish (below seawater salinity) and hypersaline (above seawater salinity) conditions until reaching the highest and the lowest salinities allowing growth (i.e. 10 and 50 ppt, respectively). Brackish conditions were achieved by 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 × 10⁶ 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\textsubscript{14:0} and C\textsubscript{16:0}, whereas C\textsubscript{16:1} and C\textsubscript{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
c. 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\textsubscript{30:0} 1,13 and 1,15 diols,
C\textsubscript{32:1} 1,15 diol, C\textsubscript{32:0} 1,15 diol, C\textsubscript{30:0} 13-hydroxy and C\textsubscript{32:0} 15-hydroxy fatty acids and C\textsubscript{32:1} and
C\textsubscript{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
with growth phase of LCAs, LCDs and LCHFAs. The total LCD abundance ranged from 14 ± 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 to 12 ± 3.8 in *N. oculata* (Table 1). *N. oceanica* showed also the highest concentrations of 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 the other two species (Table 1). During the growth of *N. oceanica*, C32:0 and C32:1 diols as well as LCAs increased towards the stationary phase, whereas the C30:0 13-hydroxy fatty acid decreased, while no significant changes were observed for the C30:0 diol and the C32:0 15-hydroxy fatty acid (Fig. 1A). In contrast, LCHFA concentration decreased during the growth of *N. gaditana*, while concentrations of LCDs and LCAs did not change significantly (Fig. 1B) whereas for *N. oculata* LCA, LCD and LCHFA concentrations were fairly constant (Fig. 1C).

Overall, we did not find a consistent trend with growth phase for LCAs, LCDs and LCHFAs across the three species and thus the distribution and abundance of these compounds does not seem to be affected by growth stage. Because of the higher concentrations of LCAs, LCDs and LCHFAs in *N. oceanica* (Table 1), we used this species for the subsequent experiments.

### 3.2. Impact of cultivation conditions on lipid abundance and composition

#### 3.2.1. Effect of light intensity

Higher abundance of saturated fatty acids occurred in cells incubated under HL conditions (Fig. 2D), which agrees with previous studies where *Nannochloropsis* sp. stored higher amounts of triacylglycerols (TAGs), mostly consisting of C16:0 and C18:0 fatty acids, under HL conditions (Sukenik and Carmeli, 1990; Fabregas et al., 2004; Pal et al., 2011). As for LCDs and LCD-related compounds, cells of *N. oceanica* cultured at HL conditions exhibited 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\textsubscript{14}-C\textsubscript{20} saturated and unsaturated fatty acids (Fig. 2F). The cellular concentration of LCHFAs also increased by almost eight-fold (Fig. 2C). However, the C\textsubscript{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\textsubscript{14}-C\textsubscript{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
resting stages, potentially leading to major changes in its lipid composition, in particular in the outer cell wall. For example *Chlorella kessleri* and *Chlamydomonas reinhardtii* substantially increased their fatty acid content upon desiccation (Shiratake et al., 2013).

Resting cells often have a thicker cell wall to better resist degradation and spores with a thicker cell wall have been observed in *Nannochloropsis limnetica* (Fietz et al., 2005), as well as in other eustigmatophytes (Pribyl et al., 2012).

Two days after the experiment started, about 90% of the medium volume evaporated in the desiccation treatment while no evaporation occurred in the control. No visible difference was noted between cells from desiccation and the control treatment under light microscopy and no significant changes in lipids, fatty acids, LCDs and LCD-derivatives were observed between the desiccated culture and the positive control (Table 1, Fig. 3A). LCAs, LCDs and LCHFAs are thus not affected by desiccation stress.

### 3.2.5. Cold shock

Another factor, which can potentially affect outer cell walls and lipid composition, is cold shock. Rapid exposure of phytoplankton to a temperature lower than that of their standard culturing conditions has been observed to promote desaturation of fatty acids in diatoms (Jiang and Gao, 2004) and dinoflagellates (Jiang and Chen, 2000). Therefore, cultures of *N. oceanica* were rapidly transferred from their standard temperature for growth (20 °C) to 10 °C and lipids were analysed after 6 h and 12 h. The abundance of fatty acids, LCAs, LCDs and LCHFAs did not change as a response to cold shock over the relatively short incubation times used here (Fig. 3B and E). Compositional changes in LCDs with growth temperature have been previously reported by Rampen et al. (2014a) for *N. gaditana*. However, in that study the cultures were actively growing at different temperatures. Apparently, such changes do not
occur when a strain is exposed to a different culturing temperature over the short incubation times applied here.

### 3.2.6. Oxidative stress

Stress from radicals can induce defense mechanisms in phytoplankton affecting the abundance and composition of lipids and potentially also their outer cell wall. Hydrogen peroxide is a known source of radicals and \textit{N. oculata} has been previously found to grow in the presence of up to 50 μM hydrogen peroxide (Taylor et al., 2012).

In the present study, \textit{N. oceanica} was able to grow with hydrogen peroxide daily added to the cultures at concentrations up to 100 μM (Supplementary Fig. S2). We evaluated the composition of lipids in cultures amended with 100 μM H\textsubscript{2}O\textsubscript{2}: the hydrogen peroxide-amended cultures were significantly depleted in unsaturated fatty acids (38% decrease), LCHFAs (37%), C\textsubscript{32:2} alkenol (36%) and C\textsubscript{32:1} diol (48%) compared to the unamended control (Fig. 3C) suggesting that some of these compounds were either partially degraded by hydrogen peroxide or synthesized at a lower rate. Interestingly, most of the compounds that decreased in the presence of hydrogen peroxide, are unsaturated and their double bonds are likely to be highly reactive towards hydrogen peroxide. Similarly, in the presence of radicals, the degradation rates of alkenones increases with increasing number of double bonds (Rontani et al., 2006). The decrease in unsaturated fatty acids, LCHFAs, C\textsubscript{32:2} alkenol and C\textsubscript{32:1} diol is thus unlikely the result of changes in their biosynthetic pathways but these compounds may have been directly chemically oxidized by hydrogen peroxide. In any case, our results suggest that radicals do not affect LCD synthesis rates.

### 3.3. Role of LCAs, LCDs and LCHFAs in \textit{Nannochloropsis}

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Principal Component Analysis (PCA) of the fractional abundances of the major compounds in the differently grown cultures reveals that $C_{30:0}$ 13-hydroxy and the $C_{32:0}$ 15-hydroxy fatty acids cluster with $C_{16:0}$ and $C_{14:0}$ fatty acids (Fig. 4). This suggests that LCHFAs may originate from $C_{14:0}$ and $C_{16:0}$ fatty acids via hydroxylation and chain elongation. It is not clear at which point of the elongation process the hydroxylation occurs. LCHFAs were previously suggested to share the same biosynthetic pathways as the LCAs and the LCDs in *Nannochloropsis* spp., because compounds from these three classes exhibiting the same carbon number are functionalised at the same position (Volkman et al., 1992; Gelin et al., 1997b). However, LCHFAs, $C_{14:0}$ and $C_{16:0}$ fatty acids do not cluster with LCDs as well as LCAs abundance in our PCA, suggesting that not all the LCHFAs biosynthesised by *Nannochloropsis* spp. were converted to LCDs and LCAs.

In *Nannochloropsis* species, LCAs, LCDs and LCHFAs are thought to be bound together via ether or ester bonds to form algaenans (Gelin et al., 1996, 1997a, 1999; Volkman et al., 1998; Zhang and Volkman, 2017). Algaenans have also been described in several genera of Chlorophyta (Derenne et al., 1988; Gelin et al., 1997a; Blokker et al., 1998, 1999; Allard and Templier, 2001) and the dinoflagellate *Gymnodinium catenatum* (Gelin et al., 1999). Algaenans are thought to be highly resistant to degradation (Tegelaar et al., 1989; Volkman et al., 1998) and are likely present in the outer layers of the cell walls, forming a trilaminar structure (Gelin et al., 1999). Fourier transform infrared spectroscopy on the cell wall of *N. gaditana* confirmed the ether linked aliphatic structures of algaenans and revealed also the presence of C=O bonds (Scholz et al., 2014), which likely correspond to ketone or carboxylic functional groups of long chain keto-ols, LCHFAs and dihydroxy fatty acids, respectively, which were previously reported to occur in *Nannochloropsis* spp. (Volkman et al., 1992; Gelin et al., 1997b; Versteegh et al., 1997). As algaenans are situated in the outer cell wall of *Nannochloropsis* spp. they might play a role in the protection of cells from chemical attack.
This is evidenced in our study as *N. oceanica* was able to grow in the presence of high (100 μM) concentrations of hydrogen peroxide. In fact, phytoplankton can usually tolerate hydrogen peroxide concentrations higher than cyanobacteria (Drabkova et al., 2007; Barrington and Ghadouani, 2008) and heterotrophic bacteria (Xenopoulos and Bird, 1997). However, to the best of our knowledge, no phytoplankton species has been shown to grow, for three consecutive days, under a daily amendment of 100 μM hydrogen peroxide. We hypothesize that algaenans in *Nannochloropsis* spp. might then play a key role against oxidative stress making *Nannochloropsis* species highly resistant to radicals.

A protective role of some algaenan-associated compounds is also suggested by the higher proportions of LCHFAs, C\(_{32:2}\) alkenol and C\(_{32:1}\) diol found at HL conditions and by the increase in C\(_{30:0}\) 13-hydroxy fatty acid and C\(_{32:0}\) diol in nitrogen depleted medium (Fig. 2A).

Both the exposure to excessive light (i.e. HL treatment) and the nutrient deficiency (i.e. N-) can stimulate the production of reactive oxygen species, although to a much lesser extent than hydrogen peroxide (McKersie and Leshem, 1995). While the hydrogen peroxide amendment likely implied the degradation of part of the outer cell wall, leading to the lower proportions of LCAs, LCDs and LCHFAs measured, the exposure to radicals under HL or N- conditions was likely to be less aggressive and might have stimulated a cellular response to biosynthesize higher amounts of algaenan-associated compounds in order to protect the cell.

### 3.4. LCDs as tracers for biomass of eustigmatophytes

Overall, the cellular levels of LCAs and LCDs found here in three different *Nannochloropsis* species, as well as those measured for *N. oceanica* under different culturing conditions and manipulation did not vary greatly, i.e. over all experiments and species, the concentrations were 12 ± 8.0 fg/cell for LCAs and 23 ± 15 fg/cell for LCDs. In contrast a greater variability was found for LCHFAs (8.2 ± 10 fg/cell) and fatty acids (541 ± 570...
fg/cell). This suggests that the cellular levels of LCAs and LCDs are fairly constant within the
eustigmatophytes investigated here. These consistent levels may be due to the fact that they
are not storage lipids, but are associated with the outer cell wall. In turn, it suggests that if cell
size and cell wall thickness are fairly constant across the Eustigmatophyceae, the
concentration of LCDs may be a good proxy for the abundance of eustigmatophytes in aquatic
environments. Furthermore, sedimentary records of LCDs may be used to constrain relative
changes in abundance, at least in regions not affected by variable past oxygen levels in the
water column as diols are known to degrade at different rates between oxic and anoxic
sediments (Rodrigo-Gamiz et al., 2016).

4. Conclusions

Culturing conditions that typically promote TAG accumulation in Nannochloropsis
lead to a slight increase in LCHFAs but do not enhance the concentration of LCAs and LCDs.
LCHFAs are likely to derive from $C_{14:0}$ and $C_{16:0}$ fatty acids, whereby $C_{14:0}$ and $C_{16:0}$ fatty
acids might undergo one hydroxylation and several elongation steps to form LCHFAs which
in turn can be precursors of LCAs and LCDs.

Our study indicates that LCAs, LCDs and LCHFAs do not serve as storage lipids but
likely function as structural lipids present in the outer cell wall playing a protective function
from oxidative stress. The nearly constant concentrations of LCAs and LCDs, along with their
specific chain length and position of functionalization for Nannochloropsis spp., suggest that
these compounds might be considered as proxies for eustigmatophyte biomass in recent
settings. A deeper understanding of the genetic mechanisms controlling the biosynthetic
pathways of LCAs, LCDs and LCHFAs can contribute, in the future, to a better understanding
of the controls on LCD synthesis.
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production of phytoplankton and bacterioplankton in a mesohumic lake.


Figure legends

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

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

Fig. 3. Impact of different stress conditions on the concentration of: (A–C) LCAs, LCDs and LCHFAs, and (D–F) sum of saturated and unsaturated fatty acids and cholesterol on *Nannochloropsis oceanica* CCMP1779. Error bars represent standard deviation of triplicate cultures. (A, D) Effect of desiccation, the cultures were poured into Petri plates and exposed to the air for 48 h without the lid to promote the evaporation of the medium, the positive
control consist in Petri plates with the lid. (B, E) Effect of cold shock: the cultures were rapidly transferred to a colder (10 °C) temperature and incubated for 6 or 12 h. (C, F) Effect of daily amendment of hydrogen peroxide (radical stress). The level of significance of the differences between the concentration at a growth stage and the previous one is indicated (*, p-value < 0.05; **, p-value < 0.01).

Fig. 4. Ordination of the different LCDs and LCD-derivatives as well as the major fatty acids in the space defined by principal component analysis: PCA1 on the x axis and PCA2 on the y axis. The values in brackets indicate the percentage of variance explained by each axis.
Figure 1

(A) N. oceanica Abundance (fg per cell) for Early, Late, and Stationary experiments.

(B) N. gaditana Abundance (fg per cell) for Early, Late, and Stationary experiments.

(C) N. oculata Abundance (fg per cell) for Early, Late, and Stationary experiments.

(D) N. oceanica Abundance (fg per cell) for Early, Late, and Stationary experiments.

(E) N. gaditana Abundance (fg per cell) for Early, Late, and Stationary experiments.

(F) N. oculata Abundance (fg per cell) for Early, Late, and Stationary experiments.

Legend:
- Saturated FA
- Unsaturated FA
- Cholesterol

Key:
- 13-OH-C\(_{30:0}\) FA
- C\(_{32:1}\) alkenol
- 15-OH-C\(_{32:0}\) FA
- C\(_{32:0}\) diols
- C\(_{32:2}\) alkenol
- C\(_{30:0}\) diols
- C\(_{32:1}\) diols
Figure 4
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.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Species/strain</th>
<th>Treatment</th>
<th>Replicates</th>
<th>Duration (days)</th>
<th>Abundance (cells/mL)</th>
<th>Lipid content (pg/cell)</th>
<th>LCAs (fg/cell)</th>
<th>LCDs (fg/cell)</th>
<th>LCHFAs (fg/cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth rate</td>
<td><em>N. oceanica</em> CCMP1779</td>
<td>Early</td>
<td>3</td>
<td>7</td>
<td>$1.8 \pm 0.2 \times 10^6$</td>
<td>$2.5 \pm 0.2$</td>
<td>$12 \pm 1$</td>
<td>$9.4 \pm 2.9$</td>
<td>$16 \pm 5.2$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Late</td>
<td>3</td>
<td>10</td>
<td>$3.1 \pm 0.5 \times 10^6$</td>
<td>$4.7 \pm 1.9$</td>
<td>n.a.</td>
<td>$8.6 \pm 1.0$</td>
<td>$16 \pm 4$</td>
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<tr>
<td></td>
<td></td>
<td>Stationary</td>
<td>3</td>
<td>11</td>
<td>$5.6 \pm 1.1 \times 10^6$</td>
<td>$2.2 \pm 0.4$</td>
<td>$11 \pm 2$</td>
<td>$27 \pm 11$</td>
<td>$34 \pm 5.8$</td>
</tr>
<tr>
<td>N. gaditana</td>
<td><em>N. oceanica</em> CCMP526</td>
<td>Early</td>
<td>3</td>
<td>9</td>
<td>$1.5 \pm 0.1 \times 10^6$</td>
<td>$2.2 \pm 0.4$</td>
<td>$14 \pm 3$</td>
<td>$2.9 \pm 0.6$</td>
<td>$20 \pm 4.2$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Late</td>
<td>3</td>
<td>12</td>
<td>$7.4 \pm 0.5 \times 10^6$</td>
<td>$1.0 \pm 0.1$</td>
<td>$11 \pm 3$</td>
<td>$2.1 \pm 0.9$</td>
<td>$17 \pm 8.2$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Stationary</td>
<td>3</td>
<td>19</td>
<td>$1.3 \pm 0.2 \times 10^7$</td>
<td>$1.0 \pm 0.2$</td>
<td>$11 \pm 2$</td>
<td>$1.8 \pm 0.5$</td>
<td>$14 \pm 2.5$</td>
</tr>
<tr>
<td>N. oculata</td>
<td><em>N. oceanica</em> CCMP2195</td>
<td>Early</td>
<td>3</td>
<td>7</td>
<td>$1.4 \pm 0.2 \times 10^6$</td>
<td>$2.5 \pm 0.9$</td>
<td>$13 \pm 6$</td>
<td>$7.8 \pm 0.8$</td>
<td>$12 \pm 1.3$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Late</td>
<td>3</td>
<td>10</td>
<td>$6.5 \pm 0.6 \times 10^6$</td>
<td>$1.4 \pm 0.5$</td>
<td>$16 \pm 9$</td>
<td>$9.1 \pm 2.2$</td>
<td>$12 \pm 3.8$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Stationary</td>
<td>3</td>
<td>15</td>
<td>$1.4 \pm 0.1 \times 10^7$</td>
<td>$1.4 \pm 0.4$</td>
<td>$21 \pm 9$</td>
<td>$12 \pm 2.1$</td>
<td>$11 \pm 2.1$</td>
</tr>
<tr>
<td>Light</td>
<td><em>N. oceanica</em> CCMP1779</td>
<td>HL</td>
<td>3</td>
<td>7</td>
<td>$5.6 \pm 0.1 \times 10^6$</td>
<td>$2.3 \pm 0.5$</td>
<td>$17 \pm 4$</td>
<td>$9.4 \pm 2.9$</td>
<td>$20 \pm 3.6$</td>
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<tr>
<td></td>
<td></td>
<td>LL</td>
<td>3</td>
<td>9</td>
<td>$4.3 \pm 0.3 \times 10^5$</td>
<td>$5.5 \pm 0.4$</td>
<td>$3.5 \pm 0.3$</td>
<td>$8.6 \pm 1.0$</td>
<td>$20 \pm 2.8$</td>
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<tr>
<td>Salinity</td>
<td><em>N. oceanica</em></td>
<td>10 ppt</td>
<td>3</td>
<td>7</td>
<td>$2.6 \pm 0.1 \times 10^6$</td>
<td>$3.9 \pm 0.6$</td>
<td>$15 \pm 3$</td>
<td>$16 \pm 4.2$</td>
<td>$47 \pm 14$</td>
</tr>
<tr>
<td>Treatment</td>
<td>Condition</td>
<td>CCMP1779</td>
<td>35 ppt</td>
<td>7</td>
<td>2.9 ± 0.1x 10^6</td>
<td>3.7 ± 1.3</td>
<td>11 ± 4</td>
<td>18 ± 7.7</td>
<td>55 ± 18</td>
</tr>
<tr>
<td>---------------------------</td>
<td>-----------</td>
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<tr>
<td></td>
<td>50 ppt</td>
<td>7</td>
<td>9.8 ± 0.4 x 10^6</td>
<td>8.5 ± 2.7</td>
<td>8 ± 2</td>
<td>22 ± 10</td>
<td>43 ± 19</td>
<td>25 ± 11</td>
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<tr>
<td>Nitrogen depletion</td>
<td>N. oceanica</td>
<td>CCMP1779</td>
<td>3</td>
<td>6</td>
<td>2.7 ± 0.4 x 10^6</td>
<td>5.7 ± 1.2</td>
<td>18 ± 5</td>
<td>16 ± 7</td>
<td>33 ± 14</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>3</td>
<td>6</td>
<td>5.2 ± 0.8 x 10^6</td>
<td>2.3 ± 0.1</td>
<td>16 ± 4</td>
<td>6.8 ± 0.5</td>
<td>17 ± 5.5</td>
<td>3.4 ± 0.7</td>
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<tr>
<td>Desiccation</td>
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<td>CCMP1779</td>
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<td>1.3 ± 0.2 x 10^7</td>
<td>3.6 ± 0.9</td>
<td>6 ± 4</td>
<td>7.5 ± 2.8</td>
<td>21 ± 5.6</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>3</td>
<td>2</td>
<td>1.3 ± 0.2 x 10^7</td>
<td>2.6 ± 0.5</td>
<td>6 ± 1</td>
<td>8.6 ± 0.5</td>
<td>22 ± 5.4</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>Cold shock</td>
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<td>CCMP1779</td>
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<td>0.25</td>
<td>3.4 ± 0.2 x 10^6</td>
<td>2.5 ± 0.5</td>
<td>15 ± 5</td>
<td>12 ± 0.3</td>
<td>23 ± 0.3</td>
</tr>
<tr>
<td>Cold 6h</td>
<td></td>
<td>2</td>
<td>0.25</td>
<td>4.0 ± 0.1 x 10^6</td>
<td>2.5 ± 0.1</td>
<td>13 ± 1</td>
<td>11 ± 1.0</td>
<td>19 ± 3.0</td>
<td>4.6 ± 0.4</td>
</tr>
<tr>
<td>Control 6h</td>
<td></td>
<td>2</td>
<td>0.25</td>
<td>3.4 ± 0.2 x 10^6</td>
<td>2.5 ± 0.6</td>
<td>11 ± 3</td>
<td>14 ± 5.6</td>
<td>26 ± 7.4</td>
<td>5.6 ± 1.6</td>
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<tr>
<td>Cold 12h</td>
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<td>0.5</td>
<td>4.0 ± 0.2 x 10^6</td>
<td>2.3 ± 0.2</td>
<td>12 ± 1</td>
<td>12 ± 1.3</td>
<td>23 ± 5.7</td>
<td>8.3 ± 1.3</td>
</tr>
<tr>
<td>Control 12h</td>
<td></td>
<td>2</td>
<td>0.5</td>
<td></td>
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<tr>
<td>Oxidative stress</td>
<td>N. oceanica</td>
<td>CCMP1779</td>
<td>4</td>
<td>3</td>
<td>5.5 ± 0.8 x 10^6</td>
<td>2.3 ± 0.1</td>
<td>11 ± 1</td>
<td>10 ± 1.0</td>
<td>17 ± 1.5</td>
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<tr>
<td>H_2O_2</td>
<td></td>
<td>4</td>
<td>3</td>
<td>2.9 ± 0.7 x 10^6</td>
<td>2.2 ± 0.8</td>
<td>17 ± 5</td>
<td>14 ± 2.1</td>
<td>24 ± 5.4</td>
<td>13 ± 2.7</td>
</tr>
</tbody>
</table>

a Early exponential phase
b Late exponential phase
c Stationary phase
d 25 μE/(m²s)
e 300 μE/(m²s)
Culture incubated in f/2 medium without sodium nitrate. No addition nitrogen sources were present in the medium.

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