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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 LCD production. LCD abundances were relatively stable suggesting that they are not used as storage lipids, and supporting 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.

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1. Introduction

Long chain alkyl diols (LCDs) are lipids that contain an *n*-alkyl chain with 28–32 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 a 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 lipids and proxies, 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.,

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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. Eustigmatophyceae comprise both freshwater and marine species (Hibberd, 1981) that occur in estuarine and hypersaline environments (Vinogradova and Darienko, 2008; Samanta and Bhadury, 2014; Balzano et al., 2015), they thus 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* spp. 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 analyzed 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 cycle. *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

An aliquot of 50 mL containing about 1.5×10^7 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 2×10^7 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/(m^2s) and used as a high light (HL) treatment, whereas the other three flasks were covered by several shading nets (irradiance 25 μE/(m^2s)) and were considered to reflect low light (LL) conditions. Cells grew faster in the HL treatment and were harvested from the cultures 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 (SD $\leq$ 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 from exponentially growing cultures 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 Fig. 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

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