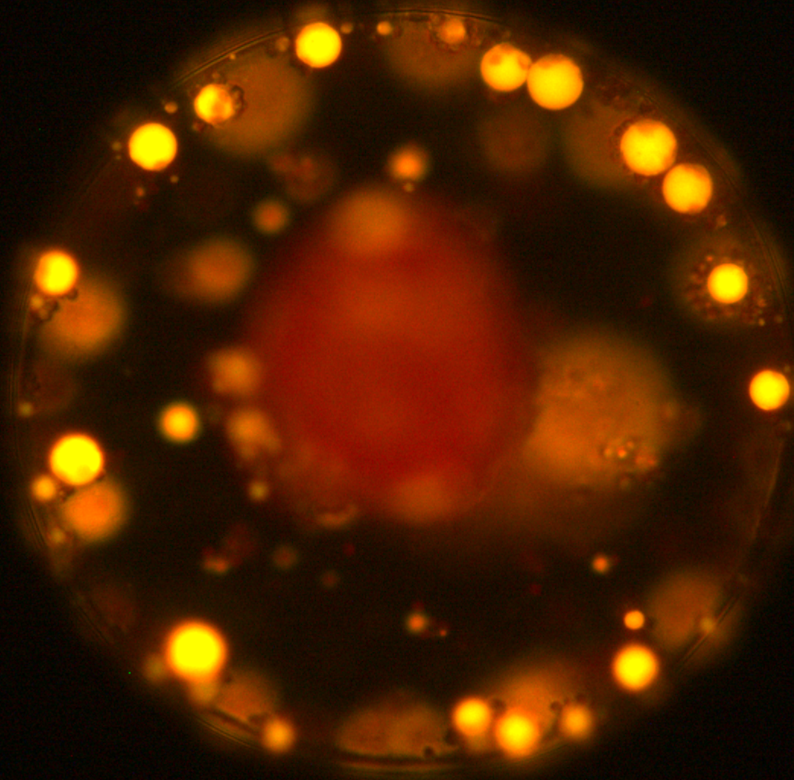
UiT The Arctic University of Norway

The Faculty of Biosciences, Fisheries and Economics

Towards mass cultivation of diatoms as a source of marine lipids

Jon Brage Svenning

A dissertation for the degree of Philosophiae Doctor – December 2020



Front page: Fluorescence imaging of *Coscinodiscus* sp. stained with nile red. Photo credit: Jon Brage Svenning.

Table of contents

Acknowledgements ... 1

Sammendrag ... 2

Summary ... 3

List of papers ... 4

Abbreviations ... 5

1 Introduction ... 6

2 Aims ... 8

3 Background ... 9

3.1 Diatoms ... 9

3.2 Diatom lipids ... 12

3.3 Microalgae in science and industry ... 16

4 Current commercial production of photoautotrophic microalgae – cultivation, harvesting and processing ... 19

4.1 Cultivation ... 19

4.2 Harvesting ... 21

4.3 Processing ... 22

5 Main results and general discussion ... 24

5.1 PUFA production in *P. glacialis* ... 24

5.2 Storage stability of lipids in fresh biomass of *P. glacialis* ... 27

5.3 Lipid availability in *P. glacialis* ... 29

6 Conclusion ... 31

7 Future prospects ... 32

8 References ... 33

Acknowledgements

This work was carried out at the Norwegian School of Fishery Science at UiT The Arctic University of Norway. The position was funded by UiT The Arctic University of Norway.

To my skipper and former main supervisor, Hans Christian Eilertsen, I cannot exaggerate my gratitude to you for the opportunity work on this subject, and for teaching me the intricate details of cod fishing. Your compassion, humor and enthusiasm are qualities that I will strive to achieve.

To my current main supervisor, Ragnar Ludvig Olsen. From the moment you signed my supervisor contract I knew I was in good hands. You have vast knowledge about all things lipids, combined with a disarming personality that instills a sense of security in those around you. Thank you so much for dissecting my work down to the last comma, and for your incredible ability to spot even the smallest errors.

To my co-supervisor Terje Vasskog, thank you for all the kindness and guidance you have given me both before and during my project period. Your technical knowledge has been vital to the success of our experiments. I also want to thank you for giving me the opportunity to co-supervise several of your master students, which I'm sure I benefited more from than the students themselves.

For my research group leader, Hans Christopher Bernstein, thank you for teaching me invaluable methods to improve my own work, and for always taking the time to help. You have managed to create a research group that combines a high level of professionalism with an inclusive and friendly environment.

To Lars, thank you for a very successful collaboration and the friendly atmosphere we have maintained in the office. I hope we can continue to work together in the future. The same goes for all my friends and colleagues from UiT and the Finnfjord project, Gunilla, Richard, Renate, Nerea, Ingeborg, Thea, Linn, Jo, John Steinar, Daniela, Hannah, Dennis and Stina.

And finally, Mathilde, thank you for all your love and support. Let's go surfing!

Sammendrag

Den globale etterspørselen etter de flerumettede omega-3 fettsyrene EPA og DHA er økende, og tilgangen på fiskeolje, som er den tradisjonelle kilden til disse fettsyrene, er ikke lenger tilstrekkelig for å dekke behovet. Dette er primært forårsaket av at de globale fiskeriene ikke har økt nevneverdig de siste 40 år, samtidig som at etterspørselen etter marine fettsyrer som ingredienser i fiskefôr og kosttilskudd er mangedoblet i den samme perioden. I tillegg har humant konsum av de små, pelagiske fiskeslagene som tradisjonelt har gått til produksjon av fiskeolje økt i takt med økt fangstkvalitet og etterspørsel. Det er derfor nødvendig å finne nye kilder til marine oljer som ikke øker beskatningen av fisk, og forskere leter i økende grad blant de lavere trofiske nivåene. Hovedprodusentene av flerumettede fettsyrer i havet er små, encellede kiselalger, som bruker CO_2 og sollys til å vokse gjennom fotosyntese. Disse organismene kan utnyttes i en kommersiell massedyrking, der CO_2 fra industri omdannes til en verdifull kiselalge-biomasse. Dette kan bidra til å senke behovet for fiskeolje dersom det er mulig å etablere en økonomisk bærekraftig produksjon.

Hensikten med dette arbeidet har vært å forbedre dagens dyrknings- og prosesseringsteknologi gjennom tre studier rettet mot produksjon av kiselalger fra kalde farvann som en kilde til langkjedede omega-3 fettsyrer. Det første studiet undersøkte hvordan dyrkingstemperatur påvirker vekst og sammensetningen av fettsyrer i en kaldtvanns-kiselalge. Temperatur påvirket ikke innholdet av EPA og DHA i kiselalgen mellom 2-12°C, dermed reduseres behovet for temperaturregulering i en massedyrking fokusert mot produksjon av langkjedede omega-3 fettsyrer. Det neste studiet sammenlignet vanlige konserveringsmetoder på kiselalgebiomasse, og viste at en moderat varmebehandling effektivt forsinket kvalitetsforringelse under lagring av fersk biomasse. Dermed kan varmebehandling fungere som en billig erstatning for de vanlige konserveringsmetodene som ellers brukes på marin biomasse. Det siste studiet undersøkte i hvilken grad knusing av cellevegger, valg av løsemiddel og antall ekstraksjoner påvirket utbytte i lipidekstraksjon fra kiselalgebiomasse. I motsetning til andre typer mikroalger som massedyrkes, viste det seg å være valg av løsemiddel og antall ekstraksjoner som hadde størst innflytelse på ekstraksjonsutbyttet i kiselalger. Disse funnene vil føre oss nærmere en industriell massedyrking av kiselalgebiomasse, både gjennom å senke kostnadene knyttet til dyrking og prosessering, og gjennom økt produktverdi.

Summary

The global demand for the long-chain omega-3 fatty acids EPA and DHA is increasing, and the traditional sources of these fatty acids, fish oils, are no longer able to sustain the demand due to the natural cap on wild fish stocks. This shortage is mainly a result of the surging requirement of EPA and DHA as ingredients in fish feed for aquaculture, and as dietary supplements for human consumption. As a result, there is an increased interest towards lower trophic levels as novel sources of EPA and DHA in an effort to reduce the dependence on wild fish. The main producers of long-chained omega-3 fatty acids in cold oceans are the single celled diatoms, which use CO_2 and sunlight to grow in the process of photosynthesis. Diatoms can be utilized as targets of a commercial mass cultivation, where CO_2 from industrial processes is converted to a valuable diatom biomass. This may in turn supplement fish-derived biomass for the production of EPA and DHA and thereby alleviate the pressure on wild fish stocks, if an economically viable production can be established.

The purpose of this work was to improve the current cultivation and processing technology directed towards the production of diatoms as a source of marine long-chain omega-3 fatty acids. The first study presented in this thesis investigated the effect of cultivation temperature on the growth and composition of fatty acids on a cold-water diatom. Temperature did not affect the content of EPA and DHA in the diatom at temperatures between 2-12°C, which reduces the necessity of temperature regulation in an industrial cultivation targeted towards marine omega-3 fatty acids. The second study compared common preservative treatments on diatom biomass, and showed that a moderate heat treatment effectively reduced quality loss during storage of fresh biomass. Heat treatment can therefore function as an inexpensive alternative to preserve diatom biomass. The final study examined to what extent cell wall disruption, choice of solvent and number of consecutive extractions affected the product yield in lipid extraction from diatoms. In contrast to other commonly mass cultivated microalgae, product yield was determined by choice of solvent and number of extractions, independent of cell disruption. These findings will bring us closer to an industrial cultivation of diatom biomass, by lowering production costs and increasing product value

List of papers

I Svenning, J. B., Dalheim, L., Eilertsen, H. C. & Vasskog, T. Temperature dependent growth rate, lipid content and fatty acid composition of the marine cold-water diatom Porosira glacialis. *Algal Research*, **37**, 11-16 (2019). doi: 10.1016/j.algal.2018.10.009.

II Dalheim, L., Svenning, J. B., Eilertsen, H. C., Vasskog, T. & Olsen, R. L. Stability of lipids during wet storage of the marine diatom *Porosira glacialis* under semi-preserved conditions at 4 and 20 degrees C. *Journal of Applied Phycology* (2020). doi:10.1007/s10811-020-02292-0.

III Svenning, J.B., Dalheim, L., Vasskog, T., Matricon, L., Vang, B., Olsen, R.L. Lipid yield from the diatom *Porosira glacialis* is determined by solvent choice and number of extractions, independent of cell disruption. *Scientific Reports,* **10**, 22229 (2020). doi: https://doi.org/10.1038/s41598-020-79269-z.

Abbreviations

DGCC	Diacylglyceryl-carboxyhydroxymethylcholine
DGDG	Dagalactosyldiacylglycerol
DGTA	Diacylglyceryl-hydroxymethyl-N,N,N-trimethyl-beta-alanine
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
FA	Fatty acid
FCP	Fucoxanthin chlorophyll protein-complex
FFA	Free fatty acid
LC-PUFA	Long-chain polyunsaturated fatty acid
MGDG	Monogalactosyldiacylglycerol
OTU	Operational taxonomic unit
PBR	Photobioreactor
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acid
SDV	Si deposition vesicle
SQDG	Sulfoquinovosyldiacylglycerol
TAG	Triacylglycerol

1 Introduction

The global demand for marine lipophilic components as ingredients in feed, dietary supplements and pharmaceuticals is increasing. The traditional sources of these lipids, fish oils, are no longer able to sustain the large demand from industry due to the natural cap on fish stocks, which prevents increased production by reduction fisheries. This shortage is mainly a result of the surging requirement of the long-chain polyunsaturated fatty acids (LC-PUFA) eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) as ingredients in fish feed for aquaculture: From 1990 to 2018, the global capture fisheries production increased by just 14%, while aquaculture production increased by more than 500% and is now our main source of fish for human consumption, with a total production of 82.1 million tonnes in 2018[1]. Fed aquaculture is responsible for the largest increase in fish production, and this sector alone consumes 75% of the annual fish oil production of approximately 1 million tonnes[2]. The consumer demand and production quality of conventional fisheries has also improved with the advancement of preservation technologies. Consequently, the amount of fish utilized for human consumption has risen from 67% in the 1960s to 88% in 2018, and species which have traditionally been used to produce fish oil are now increasingly consumed by humans directly[1]. In addition, commonly used species such anchovies, sardines and menhaden, are keystone species in marine food webs, and the continued reliance on these species in the production of fish oil and fishmeal has raised concerns over the sustainability of fish-fed aquaculture. Currently, no substitutes for fish oil exist in large enough quantities to cover the demand, and industries are to an increasing degree managing the shortage of marine biomass by increasing their inclusion of oils from terrestrial products such as soy or rapeseed.

As a result, there is an increased interest towards lower trophic levels as novel sources of marine lipids. In this respect, photoautotrophic diatoms from the marine environment are obvious candidates for mass cultivation focused on production of marine lipids, due to their ability to rapidly accumulate biomass through photosynthesis without consuming organic carbon or competing with conventional food crops for land. Compared to land plants, diatoms achieve higher growth rates and area efficiencies[3], and their lipid composition more closely resembles that of marine fish, with high abundances of LC-PUFAs[4,5]. Diatom biomass may therefore supplement conventional fish-derived biomass for human or animal consumption and thereby alleviate the pressure on wild fish stocks. Furthermore, the diatom lipidome displays a high degree of plasticity in response to variations in growth conditions such as temperature[6,7], irradiance[8,9] and inorganic nutrients[10,11], and by controlling the growth environment, cultivation

can be targeted toward specific lipids, pigments or fatty acids. If coupled with fossil-based industries, cultivation of diatoms can also convert waste CO_2 from industrial processes into a valuable biomass, thereby increasing the sustainability of food and feed production.

Despite the beneficial attributes of diatoms as targets for mass cultivation of bulk biomass, a number of challenges remain before an economically feasible production can be established. These are primarily associated with the high capital costs of establishing necessary infrastructure, as well as the upkeep required to maintain large-scale cultivations. In addition, once cultivation has been established, the dilute nature of microalgal cultures in general necessitates extensive downstream processing associated with harvesting and dewatering the biomass. Ultimately, diatom biomass cannot compete as a replacement for low-cost marine products in e.g. fish feed at the current technological level. The successful commercialization of diatom biomass, either as a substitute for other marine components or as a novel product, will require lowering production costs while also increasing the product value by tailoring production towards specific market requirements. This thesis seeks to bring us closer to an industrial production of diatom biomass as a source of marine LC-PUFA.

2 Aims

This thesis is centered around lipids in the marine diatom *Porosira glacialis*, and addresses specific aspects of cultivation, preservation and processing of diatom biomass. The overarching goal of this work was to bring us closer to an industrial cultivation of *P. glacialis* as a source of marine lipids, by decreasing production costs and increasing product value in mass cultivation of diatoms.

First, the effect of temperature on growth, total lipid content and fatty acid composition of *P. glacialis* was studied, with the aim of identifying the specific fatty acids responsible for maintaining membrane thermostability in *P. glacialis,* and optimizing cultivation towards the production of LC-PUFA (**Paper I**). Second, three preservative treatments were tested on wet biomass of *P. glacialis*, including a novel heat treatment, with the aim of developing an inexpensive method for preserving fresh biomass that ensures high and consistent quality of lipids during prolonged storage (**Paper II**). Finally, in an effort to establish a method for lipid extraction tailored towards diatoms, the effect of cell disruption, solvent choice and number of extractions were tested on the lipid yield from biomass of the two diatoms *P. glacialis* and *Odontella aurita*, and the green algae *Chlorella vulgaris* (**Paper III**).

3 Background

3.1 Diatoms

Diatoms are primary producers in all aquatic environments and represent the largest group within the microalgae. More than 12000 species have been described, and estimations place the total number of species between 20,000-100,000[12,13]. Diatoms vary in size from 2 µm to 2 mm, and while they are single celled organisms, many species form colonies in which the cells associate with one another in chains. Diatoms are characterized by their cell wall, which is comprised of a bipartite frustule consisting of two valves, or thecae. The shape of the thecae varies, but they are almost always heterovalvate; i.e. one valve is larger than the other. The largest of these, the epitheca, partially overlaps with the smaller hypotheca like the lid of a shoebox. During vegetative reproduction (binary fission), the frustules of the parent cell becomes the new epitheca of two daughter cells. As the cell prepares to divide, new hypotheca are created in segments within a specialized compartment known as the Silica deposition vesicle (SDV) of the parent cell[14]. This asexual mode of reproduction reduces the cell size of one cell for each generation, and as a result binary fission can typically only occur until the cell size is about one third of the original size. At this point diatoms may employ auxospore formation, either vegetatively or as a result of sexual reproduction by gametogenesis, to produce a new cell of the original size.

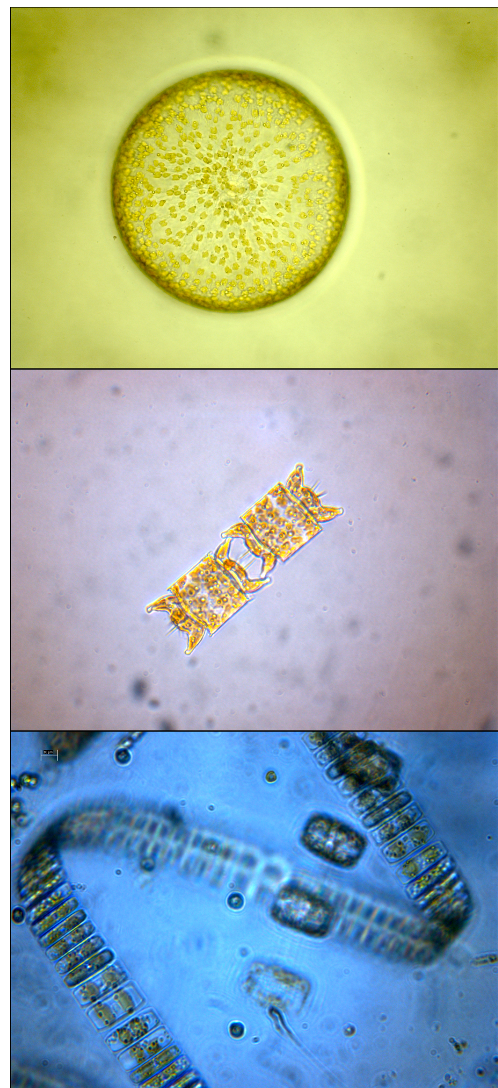


Figure 1: The radial centric diatom *Coscinodiscus* sp. (top), the polar centric diatom *Odontella aurita* (middle) and a chain of the pennate diatom *Navicula vanhoeffeni* (bottom). Photo credit: Jon B. Svenning (top), Richard A. Ingebrigtsen (middle) and Gunilla K. Eriksen (bottom).

All algae and higher plants are believed to have originated from cyanobacteria in a process called endosymbiosis, in which a non-photosynthetic eukaryote (the exosymbiont, or host cell), engulfed a cyanobacterium (the endosymbiont), thereby acquiring the ability to

photosynthesize[15]. Diatoms are members of the eukaryotic line named Heterokonts or Stramenopiles, which is defined by a motile life cycle stage in which the flagellated cells possess two unequal flagella. The group primarily includes other algae such as the golden algae (Chrysophyta) and the brown seaweeds (Phaeophyta), but also a multitude of heterotrophic microorganisms. The genomic background of Stramenopiles indicates a red algal-derived chloroplast, driven by green algal genes encoded in the nucleus. The Stramenopiles are therefore thought to be the result of a secondary endosymbiosis involving a red algal ancestor approximately 700-1200 million years ago, preceded by a green algal endosymbiont. The exact emergence of diatoms into the fossil record from this group is still debated, however, their rise to prominence occurred after the fragmentation of the supercontinent Pangea, in the Cretaceous period that began around 250 million years ago[16]. As a result of their complex evolutionary history, the nuclear genome of diatoms is a mix of genes from animal and green/red algal origin, and also includes a significant number of bacterial genes probably obtained through horizontal gene transfer[11]. Consequently, diatoms are incredibly diverse despite being one of the youngest algal groups, and marine planktonic diatoms alone represent an estimated 4748 operational taxonomic units (OTUs)[17].

The traditional taxonomic classification of diatoms separates between the bilaterally symmetrical *pennate* diatoms, and the radially symmetrical *centric* diatoms. These definitions are still in use as structural descriptions, however, phylogenetic methods have revealed that neither of these morphological characteristics define monophyletic groups. New classifications based on phylogenetics have been suggested, such as the "CMB-hypothesis" based on the nuclear-encoded SSU rDNA gene[18]. This system further separates the centric diatoms into two classes, resulting in the radial centrics Coscinodiscophyceae, the polar centrics Mediophyceae and the bilaterally symmetrical (pennate) diatoms Bacillariophyceae, see Figure 1. The CMB hypothesis is used by AlgaeBase and is probably the most commonly used system to classify diatoms today, however, it is not universally accepted; analyses of similar data sets and reanalysis of data in support of the CMB-hypothesis has returned incongruent results[19]. The relationship between the major diatom groups is therefore still uncertain, due to the limitations of morphology-based phylogeny and single-gene phylogenetics for determining monophylogeny[20]. Future research based on complete genome phylogeny, such as the JGI-project "100 diatom genomes" which aims to sequence the genome of 100 diatom species[21], will hopefully result in a more conclusive phylogeny.

The ecological success of diatoms is evident from their presence in virtually all wet environments, including oceans, lakes, hypersaline lakes[22], hot springs[23], wetlands and soil[24].

Their ability to thrive in such highly diverging habitats has been attributed to their unique physiology among the microalgae. First, diatoms, unlike other phytoplankton, are equipped with large intracellular vacuoles that they use to concentrate and store inorganic nutrients[25]. This characteristic gives diatoms a benefit in environments where inorganic nutrients enter the epipelagic zone periodically, and they are the main primary producers and carbon exporters in highly productive areas such as well mixed-coastal and upwelling regions. Secondly, the diatom frustule is not only energetically cheap to synthesize compared to organic cell walls[26], it also allows for efficient CO_2-concentrating mechanisms (CCMs). These function by coupling the highly permeable membranes with active transport of CO_2 across the cytoplasm into the chloroplasts, thereby accumulating inorganic carbon to concentrations much higher than those found in the surrounding medium[27] and saturating CO_2 fixation at the site of RuBisCO[28]. This mechanism is probably highly energetically demanding[29], and studies have found that increasing the CO_2 levels in both natural and artificial systems can increase primary production independently of other growth factors. This implies that CO_2 is a limiting factor at ambient concentrations (10 μM), even in nutrient-poor waters, and that the rate of atmospheric CO_2 diffusion to water is insufficient to maintain high productivity[30-32].

Estimations using satellite measurements of surface chlorophyll have placed the contribution of microphytoplankton, which is primarily composed of diatoms, at 50% of the net photosynthesis in temperate and sub-polar regions, and 70% of the net primary production in coastal upwelling systems during the spring-summer season[33]. The majority of the annual production of diatoms in these areas occurs during spring, in a rapid growth event known as the spring bloom[34]. This phenomenon is a culmination of three factors; Firstly, during winter, the water is mixed by the stormy weather, which regenerates the inorganic nutrients in the water column. Secondly, the arriving spring calms the weather that in combination with the warming temperature leads to a stratification in which the water column is divided into layers that do not mix. Finally, with the retuning light, stratification traps the phytoplankton in the euphotic zone, where net production (photosynthesis) exceeds loss by respiration. The scale of the spring bloom can be measured by the autofluorescent emissions from chlorophyll. The largest of these blooms, the North Atlantic spring bloom, propagates northwards at speeds of 20 km day^{-1} and

extends for distances of more than 2000 km during its relatively short lifetime[35], making it one of the largest greening events observed on planet earth.

Once initiated, the spring bloom increases exponentially until the inorganic nutrients in the water have been exhausted. In diatoms, cell division is driven by the availability of light, dissolved CO_2, and the inorganic nutrients nitrogen (NO_3^- and NH_4^+), phosphate (PO_4^{3-}), silicic acid ($Si(OH)_4$) and iron (Fe^{3+})[36]. Trace metals such as Zn, Mn, Cu, Ni and Co may also influence cellular processes and thereby growth, but less directly[37]. Temperature is not a dominating drive of phytoplankton growth, although different species display different optimum growth temperatures[38,39]. The limiting nutrient for diatoms in the Arctic is usually nitrogen, and the concentration of nitrate is approximately 1 µmol when the spring bloom ends[40]. As the spring progresses into summer and fall, nutrient regeneration in the water column by e.g. fresh water runoff and the microbial loop leads to more blooms, where diatoms reemerge whenever conditions become favorable[41]. Figure 2 shows a typical summer bloom of a calcareous Coccolithophore, most likely *Emiliania huxleyi*, off the coast of Northern Norway.

Figure 2: A bloom of Coccolithophores off the coast of Northern Norway on July 6, 2016. Photo credit: NASA Earth Observatory.

3.2 Diatom lipids

The lipochemistry of diatoms has been extensively studied due to their importance as primary producers of organic carbon in marine ecosystems, and for their potential for biotechnology, primarily as a source of neutral lipids for the production of biodiesel. The structural

lipochemistry of diatoms is characterized by polar glycerolipids that regulate the structure and function of intracellular membranes. The composition and organization of these lipids is differentially allocated among the subcellular compartments, which underlines their importance as both structural and functional molecules. The non-photosynthetic, or extra-chloroplastic membranes of diatoms primarily incorporate phospholipids, with phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI) as major constituents. The structure and function of these membranes is highly conserved across the eukaryote kingdoms, however, diatoms and other microalgae may substitute structural phospholipids with non-phosphorous betaine lipids during phosphate limitation[42]. These lipids are found naturally in lower eukaryotic organisms such as algae, cyanobacteria and fungi, but not in higher plants. Betaine lipids are similar to phospholipids in structure, but instead of an ester-bound phosphate head group, betaine lipids bind an ether-linked quaternary amine alcohol moiety at the sn-3 position. Both diacylglyceryl-hydroxymethyl-N,N,N-trimethyl-beta-alanine (DGTA) and diacylglyceryl-carboxyhydroxymethylcholine (DGCC) have been identified in diatoms[43]. In addition to reducing the phosphate quota of diatoms, recent studies from the heterokont *Nannochloropsis oceanica* also indicate that betaine lipids have important functions in adaptations to low temperatures[44]. Diatoms also synthesize a large variety of sterols with important functions as structural components and precursors of signaling molecules[45]. These include phytosterols such as brassicasterol and 24-methylene cholesterol, but also sterols commonly associated with both fungi and animals such as clionasterol, cholesterol and desmosterol[46].

The lipid composition of the thylakoid membranes within the chloroplasts is highly conserved among oxygenic phototrophs, and distinguished from non-photosynthetic membranes by incorporating a large ratio of the glycolipids monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), accounting for approximately 80 mol% of the total thylakoid lipids[47]. The remainder is made up of the sulfolipid sulfoquinovosyldiacylglycerol (SQDG), and the phospholipid phosphatidylglycerol (PG), each accounting for approximately 10 mol%. MGDG and DGDG are both neutral lipids with polar head groups, however, while DGDG tends to form a bilayer in aqueous phase, MGDG is a non-bilayer forming lipid[48]. The ratio of each therefore strongly affects the phase behavior of the thylakoid membrane, and the stability of the embedded protein-pigment complexes. The thylakoid lipids also function as structural molecules in both photosystem I and II, as well as

the light harvesting complexes, where they contribute to the electron transport processes[47]. The function of these lipids is therefore closely interlinked with the photosynthetic capability of the chloroplasts, which rely on lipophilic pigments to capture light energy. The principal light-harvesting pigment profile of diatoms consists of chlorophyll *a*, chlorophyll *c* and fucoxanthin, that combine to form the fucoxanthin chlorophyll protein-complexes (FCP) within the thylakoid membranes. These are present in both photosystem I and II in different forms unique to diatoms[49]. Besides fucoxanthin, the main carotenoids in diatoms are β-carotene and the xanothophylls (oxygenated carotenes) diatoxanthin, diadinoxanthin, zeaxanthin, violaxanthin and antheraxanthin[50].

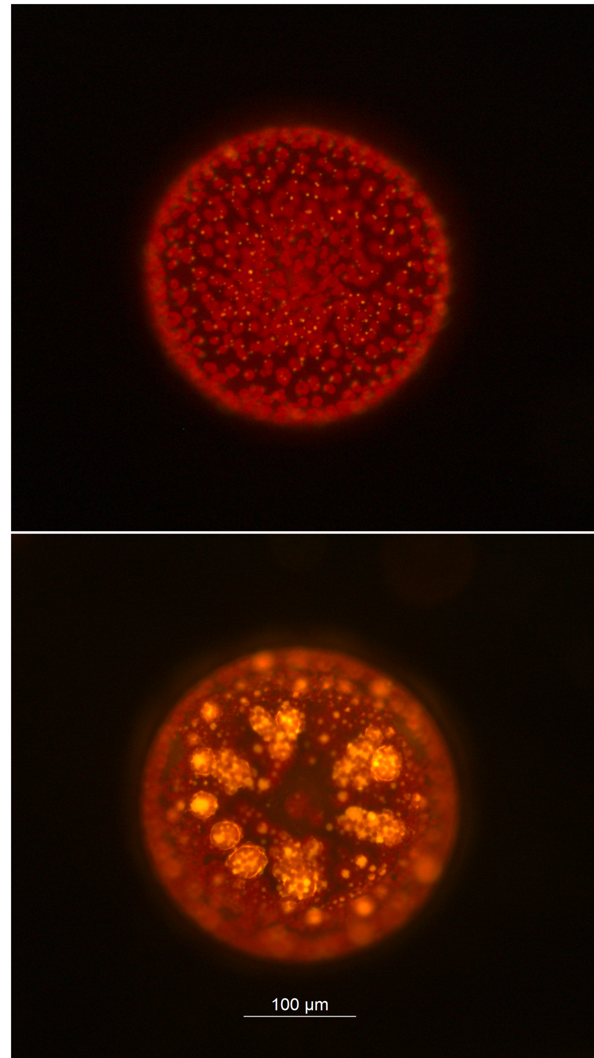


Figure 3: Lipid accumulation visualized by Nile Red staining of *Coscinodiscus* sp., showing chloroplasts (red) and lipid droplets (yellow) in cells incubated for 1 week (top) and 8 weeks (bottom) in Guillard's F/10 growth medium. Modified from Svenning (2014)[52].

The main energy storage lipid in diatoms are triacylglycerols (TAG), that aggregate in numerous spherical lipid droplets or oil bodies within the cytoplasm of the cell[51,52]. These droplets are dynamic organelles that function as a reversible storage of energy in response to the carbon allocation of the cell (See Figure 3). Diatoms also store energy as the carbohydrate chrysolaminarin, however, production of TAG is favored during nutrient limitation. Thus, during growth limitation, carbon is primarily allocated as neutral storage lipid within the lipid droplets, accompanied by a decrease in polar membrane lipids[53]. This shift in carbon allocation has two main functions. Initially, the shift towards storage energy functions as an energy sink when irradiance can no longer be used to produce biomolecules containing the limited nutrients, in order to dissipate the energy generated through photosynthesis[54]. In addition, as the algae enters the stationary phase, the photosynthetic apparatus is converted to storage lipid in order to

preserve energy for periods when inorganic nutrients become available again, and binary fission can continue.

The fatty acid composition of diatoms is distinguished from other microalgal groups and characterized by a predominance of 14:0, 16:0, 16:1n-7 and the polyunsaturated fatty acids (PUFA) 16:2n-4, 16:3n-3, 16:4n-1 and 20:5n-3[55,56]. Some diatoms may also incorporate substantial amounts of 18:4n-3 and 22:6n-3, although these fatty acids are not considered reliable markers for diatoms in general, and listed as non-diatom fatty acids in earlier studies of natural phytoplankton communities[4,57]. Other fatty acids such as 18:0, 18:1n-7, 18:2n-6, 18:3n-3, 20:4n-6 are frequently present, but usually in low concentrations[4,55]. Biosynthesis of fatty acids in diatoms via the substrate malonyl-CoA (Carboxylated acetyl-CoA) produces palmitic acid (16:0) through a series of enzyme catalyzed reactions[58]. Further conversion of palmitic acid occurs via the action of desaturases, that catalyze the addition of double bonds at specific positions of the acyl chain, and elongases, that catalyze the extension of the carbon chain, both of which may be specific for the species and cellular compartments in which they are localized[59,60] (See Figure 4). In diatoms and other microalgae, LC-PUFA biosynthesis is initiated by Δ12 desaturation of 18:1n-9 to 18:2n-6, commonly known as linoleic acid, and its subsequent desaturation to 18:3n-3, or α-Linolenic acid, by Δ15 desaturase.

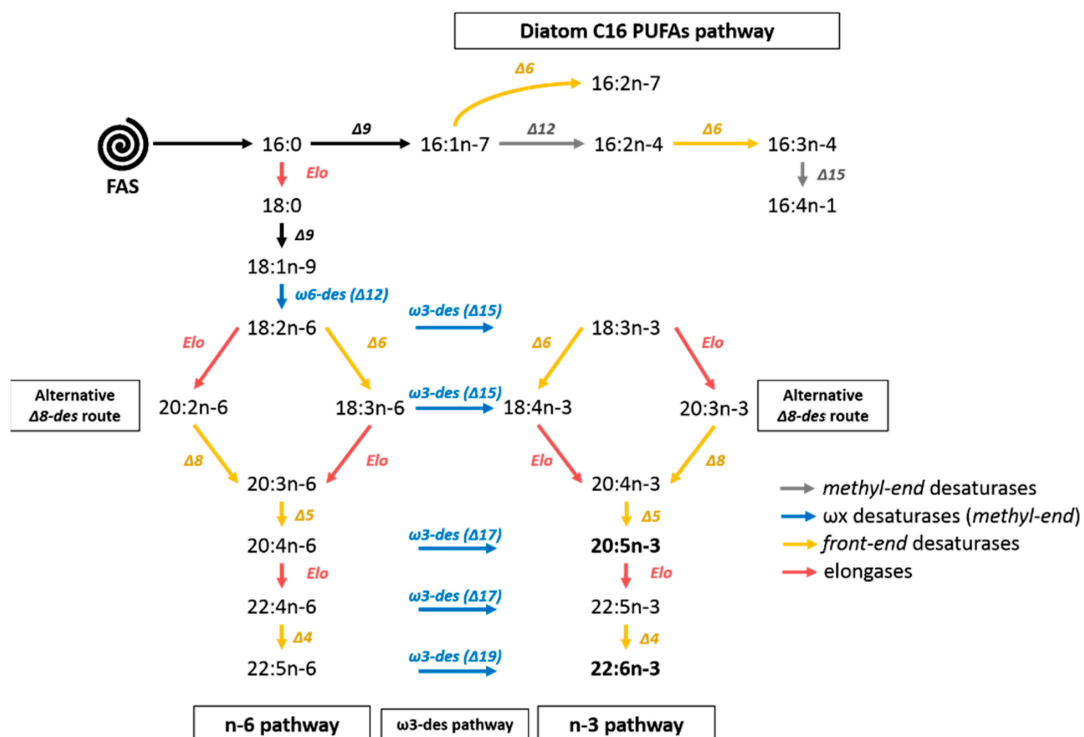


Figure 4: Fatty acid synthesis in diatoms, retrieved from Remize et al., 2020[58].

Linoleic acid and α-Linolenic acid act as precursors for n-6 or n-3 LC-PUFAs by further desaturation and elongation. The pathways for EPA and DHA synthesis displayed in Figure 4 were until recently believed distinct to marine phytoplankton as well as a limited selection of heterotrophic marine microbes, however, a recent functional characterization of desaturates from marine invertebrates have revealed that *de novo* synthesis of n-3 LC-PUFA is also prevalent in marine invertebrates[61].

3.3 Microalgae in science and industry

The first credited observation of microalgae was by Antonie van Leeuwemhoek in the 1670's, who observed "animacules" in his home-made microscope from water samples that he had collected. It would take more than 200 years before Martinus Beijerinck became the first person to successfully cultivate a unialgal culture of microalgae (*C. vulgaris*) in 1890, which set off future studies on microalgal physiology as model organisms for plants[62]. Spurred by findings such as high growth rates and the possibility of manipulating their nutritional composition[63], serious efforts to mass cultivate microalgae began in the 1940's in Stanford, Essen and Tokyo. Initially, mass culturing microalgae were studied as a potential source of food, due to their high protein content and digestibility[64]. Scientists saw the need for alternative food sources for the long-term planning of an ever-expanding world population, and as a source of protein in areas where food production was limited by the large area requirement of conventional farms. The first pilot plant was constructed in 1951 for the Carnegie institution, which produced approximately 50 kgs of dry *Chlorella* biomass and showed that a large-scale production was possible. The 60s, 70s and 80s saw the rise of commercial scale plants for production of *Chlorella, Arthrospira* and *Dunaliella salina,* respectively, and cultivation of diatoms as live feed for the rearing of invertebrates[62]. Modern cultivation techniques can be traced back to those early days of microalgal cultivation, with pioneers such as Warburg, Burlew and Guillard laying the foundation for today's commercial production. Scientific studies have since then proposed numerous uses for microalgae and their metabolites, for example gas exchangers for space travel[65]; food and feed for human and animal consumption[66,67]; lipids for production of biodiesel[68,69]; production of the marine omega-3 fatty acids EPA and DHA as dietary supplements[70] or for aquaculture feed[71]; polysaccharides for the pharmaceutical industry[72] and for production of bioethanol[73]; production of phycobiliproteins, carotenoids and other pigments[74], and production of recombinant proteins[75]. Although thousands of species have been screened for these and other uses in scientific studies and projects, such as the Aquatic Species

Program in the US which screened more than 3000 strains of microalgae for the production of biodiesel[3], very few species have been produced commercially. This is primarily a result of the high costs associated with cultivation and processing of the algal biomass, which prevents the production of low-cost secondary products[76].

By the early 2000s microalgae were cultivated as food for human and feed for animal consumption, as well as the production of valuable biomolecules for pharmaceuticals and cosmetics, and the total annual production exceeded 5000 tonnes of dry matter per year[77]. The following years mark the beginning of a period nicknamed the "biofuel bubble", in which the interest in microalgae as a feedstock for biofuels was renewed. Motivated by the increased focus on climate change and rising prices of crude oil, biofuels from microalgae were projected to replace fossil fuels. Despite massive efforts and investments to find suitable strains and culturing techniques, develop efficient bioreactors and lower the processing costs, no company has yet been able to produce microalgal-derived biofuels at competitive costs. The majority of these companies no longer exist, and those that still operate have adapted to other markets such as nutraceuticals. A notable example is the American based company Aurora algae, that after spending an approximate $100 million on R&D with a test plant located in North-Western Australia, ended up auctioning away their entire harvest of 40 tonnes of *Nannochloropsis* on Ebay at an asking price of $250[78]. In the shadow of biofuels, however, other markets for microalgae have continued to expand, and companies such as Cyanotech in Hawaii and Fitoplancton Marino in Spain have established strong footholds in aquaculture, cosmetics and nutraceuticals.

The interest in commercial production of photoautotrophic microalgae primarily stems from their potentially very high growth rates and area efficiencies, which can be exploited to rapidly produce large amounts of biomass, combined with their potential to convert CO_2 and other waste products into valuable forms of organic carbon through photosynthesis. The main bulk of today's microalgal production is limited to the green algae (Chlorophyta) *C. vulgaris*, *Dunaliella salina* and *Haematococcus pluvialis*. *C. vulgaris* is primarily marketed and sold as a nutraceutical for human consumption, and the total production of this algae alone surpasses 80,000 tonnes of dry weight/year[1]. *D. salina* is a halophile microalga with a large capacity to produce photoprotective pigments, and is cultivated commercially for the production of β-carotene for the food and pharmaceutical industry[79]. *H. pluvialis* is cultivated for its high content of the red pigment astaxanthin, which is used in aquaculture feed and cosmetics[80]. In addition to these species, some microalgae such as *Phaeodactylum tricornutum, Nitzschia*

closterium, Chaetoceros muelleri, Isochrysis galbana and Platymonas helgolandica are cultivated as live feed for shrimp and bivavle larvae in aquaculture[81].

4 Current commercial production of photoautotrophic microalgae – cultivation, harvesting and processing

4.1 Cultivation

Modern production of photoautotrophic microalgae relies on small-celled species, cultivated in photobioreactors with large surface area to volume ratios. The different types of photobioreactors (PBRs) that have been developed to cultivate microalgae vary greatly in shape and volume, and can be either open to the environment (open PBRs) or closed to the environment (closed PBRs). The simplest form of open PBRs are modified lakes or open ponds, such as the Hutt Lagoon in Western Australia used to cultivate *D. salina*. The advantage of the open PBRs is the low costs associated with upkeep, however, as these systems are exposed to the environment, they are limited to species that prefer highly selective growth conditions in order to avoid contamination by other microorganisms. Due to the severely limited control of the growth environment when cultivating in this way, growth rates are often low compared to theoretical maxima[82]. The raceway pond, which is a shallow artificial pond in the shape of an oval, adds a layer of control through one or more paddle wheels that provide continuous circulation of the algal culture to increase production (See Figure 5). Raceway ponds are primarily used to cultivate the cyanobacteria *Arthrospira* sp. (also known as spirulina) for human consumption and *H. pluvialis* for the production of astaxanthin.

Figure 5: Cultivation of microalgae in race-way ponds. Photo credit: Cyanotech corporation. Reproduced with permission.

The closed PBRs enclose the microalgal culture within a container made of a transparent material such as glass, plexiglass, acrylic PVC or polyethylene and are usually smaller in volume compared to open reactors[82]. This results in a higher degree of control of the growth environment, which enables optimization of the abiotic growth factors within the culture. Closed PBRs can therefore facilitate higher growth rates and cell densities compared to the open PBRs[83]. In addition, because the culture is isolated from the surrounding environment, closed PBRs can be used to cultivate species that do not grow in highly selective environments, and are vulnerable to contaminations from other microorganisms or variations in abiotic factors. However, the inherently low volume of closed PBRs means that high productivity per unit area can only be achieved by increasing cell densities and growth rates to much higher levels than those found in natural systems. This is cost intensive due to the large requirements of inorganic nutrients, CO_2 and irradiance when cultivating in this way. Numerous types of closed PBRs have been developed, with different strategies for culture mixing, illumination, up-scaling, and harvesting, all of which can be loosely grouped by their reactor design[62,82,84]. The main types of closed PBRs are the tubular, column, flat panel and plastic bag PBRs. The tubular PBRs consists of cylindrical vessels such as glass tubes where irradiance is absorbed, and a degasser-unit in which oxygen is removed (See Figure 6). Tubular PBRs are usually organized horizontally and illuminated by natural light, and water circulation is achieved by means of an impulsion device (such as a mechanical pump). Although the diameter of the tube is limited by the light depth of the culture, the total volume of a reactor can be increased by adding units together in repeating loops in which the water flow is interlinked between units. The world's largest tubular photobioreactor is probably the one operated by Roquette Klötze GmbH & Co. KG in Germany, which consists of 20 individual modules with a total volume of 700 m^3 and a total length of 500 km.

The column, flat panel and plastic bag PBRs are organized as single unit reactors in which all cell processes as well as heat and gas exchange occur in the same unit. Mixing is usually achieved by bubbling air enriched with CO_2 into the culture. The volume of these units is limited by of the necessity of maintaining a large surface area to volume ratio to ensure sufficient light availability, as well as the water-pressure tolerance of the materials which sets a maximum threshold for the height of the reactors. The main limitation to the closed single-unit PBRs is therefore upscaling, and the high costs associated with infrastructure, cleaning, water filtration, labor and energy have largely prevented the large-scale industrial application of closed PBRs for autotrophic cultivation[76,85]. Today, closed single-unit PBRs in the form of column- and plastic bag reactors are used to cultivate microalgae as live feed in shrimp

hatcheries and in the raising of bivalves[86], while tubular PBRs are used to produce *C. vulgaris* for human consumption.

Figure 6: Cultivation of microalgae in glass tubes. Photo credit: Ecoduna AG. Reproduced with permission.

4.2 Harvesting

Harvesting or dewatering microalgae from a culture involves separating the microalgal cells from their growth medium. There are several technical challenges associated with dewatering microalgal biomass, originating in the physical properties of the microalgal culture. These include the small size of microalgal cells, the small density difference between cells and culture medium, the low biomass concentration necessary for sufficient light penetration, the necessity for frequent harvesting due to high growth rates, and the negatively charged surface potential of microalgal cells which inhibits aggregation[87]. In its simplest form, harvesting can be performed by gravity filtration through a semipermeable membrane that collects the algal biomass. The process can be sped up by applying pressure on either side of the filter, however, filtration is generally considered a slow and laborious method for collecting microalgal biomass, due to the requirement of finely meshed filters that clog rapidly. Harvesting can be greatly sped up in a centrifuge. These function by separating the biomass from the surrounding

medium by centrifugal force. Examples of commonly used centrifuges in microalgal cultivation are decanter centrifuges, which decants the liquid media from the solid microalgae in a horizontal bowl, and disc stack centrifuges, which uses stacks of thin metal discs to split the mixture into layers and thereby reduce the sedimentation path of the cells. Centrifuges can be designed for either continuous or batch operation. In continuous centrifuges, both the liquid stream and product are discharged during operation so that the centrifuge can be operated non-stop. In batch centrifuges, the product accumulates inside the bowl of the centrifuge, and can only be collected by stopping the centrifuge.

Harvesting by centrifugation is characterized by high recovery rates; however, operational costs such as maintenance and energy consumption are generally high due to the relative diluteness of microalgal cultures[88]. A major factor in lowering harvesting costs is therefore to decrease the total volume that needs to be centrifuged, without sacrificing yield. For example, consider a culture of *C. vulgaris* with a dry-weight content of 0.1%, which corresponds to 1 g of dry algal biomass per L of seawater. During harvesting, conventional centrifugation can remove 99% of this water, to produce a microalgal slurry with a dry weight content of 10%. In other words, for every 1000 liters harvested from this culture, 10 liters of microalgal slurry containing 1 kg of dry microalgal biomass is produced. This slurry still has a water content of 90%, most of which must be removed for further processing into e.g. dietary supplements. Methods such as filtration, flocculation, sedimentation and flotation can contribute to this end in two-stage processes by drastically decreasing the total volume to be centrifuged, however, these methods have other disadvantages such as clogging, limited throughput, low recovery rates and are generally time consuming. Large scale harvesting of *H. pluvialis* for the production of astaxanthin normally use passive sedimentation in combination with centrifugation to collect the biomass before it is processed further[89]. Harvesting remains one of the most challenging issues in microalgal cultivation and represents a major cost in industrial production, and scientific studies regularly report harvesting costs at 20-30% of the total costs of cultivation[90].

4.3 Processing

Once harvested, microalgal biomass is subjected to different treatments depending on the end product. Initially, the biomass is usually subjected to a drying step where most of the water is removed from the biomass. Common drying methods for microalgae include freeze drying, spray drying, drum drying and air drying, and the ultimate choice of drying method depends

strongly on its cost and effect on the desired product[91]. Dehydrating biomass has several benefits, such as greatly increasing the shelf-life of the product by inhibiting the action of the microorganisms and enzymes that cause spoilage, as well as facilitating product extractions by weakening the integrity of the individual cells[92]. Dry biomass is also less energy-intensive to store and transport.

The main bulk of industrially produced microalgae is either spray- or freeze-dried, and sold as whole biomass for human consumption, or as a wet paste for inclusion in animal feeds. Freeze drying removes water by sublimation at low temperatures without affecting non-volatile compounds, and is therefore the optimal method in the objective of preserving the nutritional quality of the end product[93]. Spray drying, which applies liquid atomization in a heated chamber to rapidly dehydrate biomass, offers higher throughput but may damage heat-labile compounds. Both of these methods are energetically costly and are primarily used in laboratory settings and for high-cost products such as dietary supplements. In cultivations of green algae for the extraction of valuable biomolecules, the presence of polysaccharides such as sporopollenin in the cell walls creates an effective barrier that prevent the release of e.g. pigments[94,95]. For this reason, cell disruption prior to extraction is frequently performed in order to increase the product recovery from commonly mass-cultivated species[96]. Current commercial scale cultivations of *H. pluvialis* for the production of astaxanthin employ mechanical processes such as bead milling or expeller pressing for this purpose, due to their reliability and simple operation[89]. More sophisticated methods such as microwave- or ultrasound-assisted extraction can be more effective[97], however, the increased effect is largely countermanded by their increased energy expenditure and capital costs. Following cell disruption, pigments are extracted as oleoresins (a mixture of different compounds with shared solubility), either by traditional solvent extraction using a suitable organic solvent such as acetone, or by supercritical fluid extraction using CO_2 as the solvent[98]. While initially more expensive due to its high capital costs and energy expenditure, supercritical fluid extraction offers several benefits over conventional solvent extractions such as higher selectivity and throughput, as well as lower solvent toxicity and environmental impact[99].

5 Main results and general discussion

This thesis is centered around different aspects of lipid chemistry in a mass-cultivated psychrophilic diatom, and includes three papers addressing the aims presented in the introduction. **Paper I** presents a study on the effect of temperature on growth, total lipid content and fatty acid composition of *Porosira glacialis* cultivated in column photobioreactors, with the intention of evaluating the potential of this diatom as a source of marine LC-PUFA. In **Paper II,** three preservative treatments for increasing the storage stability of lipids in biomass of *P. glacialis* were compared and evaluated as inexpensive alternatives in an industrial diatom production. Finally, **Paper III** presents a study on the effect of cell disruption and choice of organic solvent on the lipid yield in conventional solvent extractions, on biomass from the diatoms *P. glacialis* and *Odontella aurita*, and the green algae *C. vulgaris.*

5.1 PUFA production in *P. glacialis*

The commercial interest in diatoms from cold areas as a novel source of marine lipids primarily stems from their consistently high content of LC-PUFAs, as demonstrated by[45,57,100]. The Arctic diatom *Porosira glacialis* is no exception, and the studies presented in this thesis collectively demonstrate its high PUFA content, primarily in the form of EPA, when harvested in the exponential growth phase. **Paper II** and **III** also include analyses of lipid classes in *P. glacialis*, which show a preference for complex structural lipids (MDGDG, DGDG, PG, PC) when harvested in the exponential growth phase. EPA and other LC-PUFAs are differentially allocated among lipid classes in living organisms. As a general rule among diatoms, LC-PUFAs are associated with the glycolipids of the thylakoid membrane, where they are thought to contribute to the photosynthetic function of the chloroplasts by stabilizing membrane proteins, as well as membrane phospholipids and betaine lipids[6,58,101]. TAGs show a preference for short- or medium chain fatty acids, however, LC-PUFA can also be directed to TAG under nutrient stress[58]. There are many exceptions to this rule, and the results from **Paper III** indicate a homogenous distribution of fatty acids among the lipid classes of *P. glacialis,* as the large differences observed in the composition of lipid classes depending on solvent were not reflected in the fatty acid compositions. The temporal requirement of specific fatty acids in diatom cells is regulated by environmental factors, of which temperature is of central importance due to its effect on the phase transition of cellular membranes. In an industrial cultivation of diatoms, it is therefore important to determine to what extent temperature regulates the composition of

fatty acids, in order to optimize cultivation towards the production of LC-PUFA. In **Paper I**, *P. glacialis* was cultivated at 5 different temperatures, from -2 to 12°C, to study the effect of temperature on fatty acid allocation. Diatoms maintain membrane function in response to variations in temperature by regulating the composition of fatty acids bound to structural lipids, and both the total lipid content and degree of unsaturation of these lipids is typically negatively correlated with temperature[6,102,103]. A similar trend was observed in our results as an increase in total lipid and the PUFAs 18:4 and 16:4 with decreasing temperature, and a decrease in the contribution of 16:3 (Table 1). Our results therefore indicate that C16 and C18 polyunsaturates modulate membrane thermostability in response to temperature changes in *P. glacialis*. In diatoms, 16:4 biosynthesis is driven by a methyl-end Δ15 desaturase, which converts the precursor 16:3n-4 to 16:4n-1 as the final product of a C16-PUFA pathway[60]. It seems likely that the expression of Δ15 desaturase is negatively correlated with temperature in *P. glacialis*, resulting in the opposite response of 16:3 and 16:4 to changes in temperature. This may also explain the increased contribution of 18:4 at lower temperatures, as Δ15-desaturase is also active in the synthesis of 18:4n-3 via 18:3n-6 and 18:3n-3. Neither of these precursors make significant contributions to the fatty acid composition of *P. glacialis,* nor do they display any clear dependency of temperature in our results. However, their low abundance also at higher temperatures could be a result of their conversion to LC-PUFA through the n-6 pathway, which is independent of Δ15-desaturase.

Table 1: The relative fatty acid composition (%) of *Porosira glacialis* at 5 different cultivation temperatures. Data shown are averages of replicates ± SD, n=3. TR=Trace values. Adapted from Paper I.

Fatty acid	**-2°C**	**2°C**	**4°C**	**8°C**	**12°C**
14:0	3.7	4.4	5.7	7.3	7.8
16:0	5.3	7.3	8.0	7.2	7.4
16:1	8.7	12.8	10.3	11.6	11.6
16:2	2.1	2.7	2.7	4.0	4.7
16:3	8.6	8.3	16.5	19.8	16.3
16:4	14.4	14.8	10.5	4.9	4.8
18:0	0.2	0.7	0.9	0.7	1.0
18:1	0.2	0.3	0.4	0.4	0.3
18:2	0.3	0.1	0.1	0.3	0.3
18:3	1.2	1.8	1.8	1.7	1.8
18:4	10.1	9.6	6.5	5.3	3.9
20:5	40.4	32.6	31.4	31.0	33.8
22:6	4.9	4.5	4.9	5.5	6.0
24:0	TR	0.2	0.3	0.3	0.4

The observed temperature effect in *P. glacialis* did not extend to the LC-PUFAs at temperatures above -2°C. Instead, the relative content of both 20:5n-3 (EPA) and 22:6n-3 (DHA) increased slightly from 2 to 12°C, indicating that these fatty acids have critical cellular functions beyond thermostability in *P. glacialis*. The strong reliance on PUFA as a characteristic of cold-adapted diatoms is clearly demonstrated in **Paper III**, which includes the fatty acid composition of *P. glacialis* and commercially acquired biomass of two mesophilic species, the polar centric diatom *O. aurita* and the green algae *C. vulgaris.* The fatty acid composition of the two mesophiles were characteristic of their respective classes; *O. aurita* contained primarily 14:0, 16:0, 16:1n-7 and 20:5n-3, and *C. vulgaris* contained primarily 16:0, 16:3n-4, 18:2n-6 and 18:3n-3 (Table 2). Although not an aim of the study itself, the data demonstrates the large differences in fatty acid allocation between different taxa of microalgae, and the preference for polyunsaturated fatty acids in *P. glacialis.*

Table 2: The relative fatty acid composition (%) of *Porosira glacialis, Odontella aurita* and *Chlorella vulgaris.* Data shown are averages of replicates ± SD, n=5. Fatty acids were included at a threshold of >1%. Adapted from Paper III.

Fatty acid	***P. glacialis***	***O. aurita***	***C. vulgaris***
14:0	4.9	12.5	-
16:0	5.9	26.1	23.3
16:1n-7	11.9	35.7	-
16:2n-4	3.5	3.0	-
16:3n-4	6.9	2.3	10.8
16:4n-1	29.5	-	-
18:0	-	1.3	1.5
18:1n-9	-	3.0	4.0
18:1n-7	-	1.8	-
18:2n-6	-	1.3	37.5
18:3n-3		-	21.9
18:4n-3	4.9	-	-
20:5n-3	30.7	11.6	-
22:6n-3	1.7	1.5	-
Σ SFA	10.8	39.8	24.7
Σ MUFA	11.9	40.5	5.0
Σ PUFA	78.2	19.7	70.3

In fact, the total PUFA content in *P. glacialis* is high even when compared to other psychrophilic diatoms, both in studies of diatom-dominated natural communities[57,104,105], and monocultures from laboratory cultivations[45,106,107]. This trait not only enables production of LC-PUFA rich biomass at a wide range of temperatures, it also reduces the need for temperature

regulation in industrial cultivations of cold-water diatoms targeted towards marine LC-PUFAs, thereby lowering cultivation costs.

5.2 Storage stability of lipids in fresh biomass of *P. glacialis*

The LC-PUFAs present in *P. glacialis* are vulnerable to lipid autoxidation in the presence of oxygen, the rate of which is positively correlated with the degree of unsaturation and free fatty acid content[108-110], as well as microbial digestion by bacteria and fungi. Lipid peroxidation can be accelerated further by the presence of pigments, which can act as photosensitizers that form singlet oxygen in the presence of light[111]. In diatom biomass, endogenous lipases released as a consequence of cell rupture during harvesting may also result in the rapid conversion of LC-PUFAs to oxylipins[112,113]. Cell lysis and lipase release may also occur as a result of freezing and subsequent thawing of the biomass. Preventing these processes in biomass of *P. glacialis* is vital to maintain its value as a marine feedstock, however, conventional methods for lipid preservation in marine biomass may add significant costs to the production. Typical methods to reduce microbial growth and lipid degradation in marine biomass generally involves a combination of treatments depending on the type of product. For example, wet products such as fish silage from whole fish or fish byproducts is stabilized either by the addition of an organic acid (e.g. formic acid) to pH<4 to prevent microbial growth, or by fermentation using lactic acid bacteria, and further addition of antioxidants (e.g. BHA, BHT) to prevent lipid oxidation[114]. Crude fish oil for inclusion in feeds is added antioxidants in the form of tocopherols, critic acid, ascorbyl palmiate or propyl gallate to protect the oil against oxidation, and fish oil for human consumption require subsequent refining steps such as bleaching, winterization and deodorization to remove impurities resulting from oxidation and hydrolyzation during processing and storage[115,116] (Jacobsen et al., 2013; Oterhals and Vogt, 2013). Commercially available microalgal biomass for human consumption are preserved by drying (e.g. lyophilization, spray drying) following harvesting, which effectively removes the need for adding antimicrobial preservatives and antioxidants but adds significant costs to the production.

In a mass cultivation of diatoms, circumventing these treatments without sacrificing the product quality would greatly benefit the industrial potential of diatom biomass as a low-cost product to supplement fish-derived biomass. With this goal in mind, **paper II** investigated three inexpensive preservative methods (formic acid, benzoic acid and heat treatment) on the stability of lipids in wet biomass (90% water content) of *Porosira glacialis* during storage at 4 and 20°C

for 14 days. Samples were taken after 1, 3, 7 and 14 days, and analyzed for formation of free fatty acids, changes in lipid classes and fatty acid composition. Not surprisingly, storage at 20°C resulted in the highest rate of lipid deterioration in all samples compared to 4°C. Benzoic acid did not improve the lipid stability during storage compared to the control samples, and while formic acid effectively reduced the formation of free fatty acids, it did not halt PUFA degradation. Heat treatment at 70°C for 30 minutes and subsequent storage at 4°C resulted in the lowest formation of free fatty acids and reduction in total PUFA, and was the most effective method of those tested for increasing the storage stability of wet *P. glacialis* biomass (Figure 7).

In a follow-up experiment where biomass was stored for 7 days, heating the biomass to 90°C proved to be more effective than both 70 and 80°C. The preservative effect of heat is two-fold; first, heat inactivates the lipases responsible for lipid hydrolysis and oxylipin synthesis by denaturing the protein structure[117]. Second, the heat treatment pasteurizes the algae, thereby slowing the rate of microbial digestion in wet biomass. The preservative effect of heat on microalgal lipids has been demonstrated in other studies[118-120], and our results also show that a moderate heat treatment can effectively preserve fresh diatom biomass during short-term (days) storage. Its relative inexpensiveness compared to other methods depends on its source, however, and heat as a preservative treatment may not offer a reduction in costs compared to e.g. freezing if produced for the sole purpose of heating large amounts of diatom biomass. In the study presented here, the biomass was cultivated at a smelting plant where heat is produced in vast amounts as a byproduct of the smelting process. An integrated heat treatment in the production line of diatom biomass would therefore not represent significant costs beyond the initial capital cost. In this or similar settings, our results indicate that heat treatment can offer an inexpensive alternative to conventional preservation methods that ensures consistent product quality.

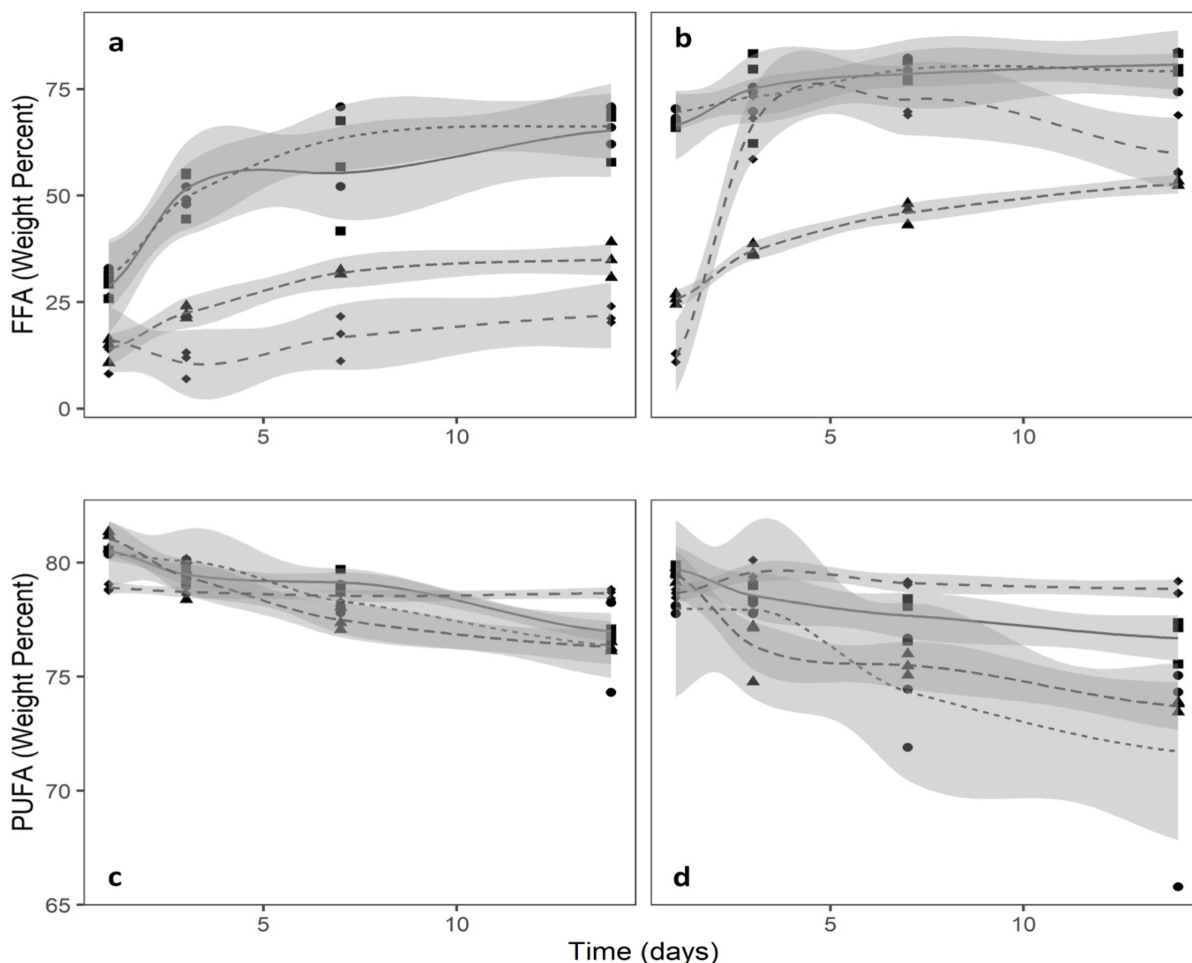


Figure 7: Contribution of free fatty acids (FFA, top) and polyunsaturated fatty acids (PUFA, bottom) in wet biomass of *Porosira glacialis* from day 1 to 14 for each treatment (n=3) at 4°C (a and c) and 20°C (b and d). Benzoic acid (0.1% (w/w), square), control (no pre-treatment, circle), formic acid (pH 3.0, triangle), and heat treatment (70 °C for 30 min, diamond). Adapted from **Paper II**.

5.3 Lipid availability in *P. glacialis*

Cell disruption prior to lipid extraction is a prerequisite to maximize product recovery in commonly mass-cultivated microalgae such as *Chlorella* sp. or *Nannochloropsis* sp., due to resilient cell walls that prevent the release of intracellular compounds[96,121,122]. The porous cell wall of diatoms is silica based and lacks the recalcitrant polysaccharide/glycoprotein matrixes characteristic of other mass cultivates[123], and an efficient extraction may be comparatively less problematic. This was the premise for **Paper III**, which investigated the effect of cell wall disruption, solvent choice and number of consecutive extractions on the diatoms *P. glacialis* and *O. aurita,* and the green algae *C. vulgaris*. The results showed that while both solvent choice and number of extractions had a significant effect on the lipid yield in *P. glacialis* and

O. aurita, cell wall disruption by sonication did not increase the maximum lipid yield in either diatom compared to physical stirring. In *P. glacialis*, neither sonication nor physical stirring resulted in a large increase in lipid yield compared to the control method (no treatment). This indicates that the majority of cellular lipids are readily available for direct extraction in lyophilized biomass of *P. glacialis,* provided that a solvent of sufficient polarity is used. On the other hand, maximum lipid yield in *C. vulgaris* was dependent on cell disruption and number of extractions, not solvent choice. This may indicate that the cell disruption applied was incapable of releasing the most polar lipids from *C. vulgaris*, or that the lipid profile of the biomass used was less polar compared to the two diatoms, allowing an efficient extraction with a solvent of lower polarity.

In addition to facilitating lipid extraction, the fragility of diatom cell walls also has implications for other aspects of diatom mass cultivation. First, the high probability of cells breaking during harvesting or subsequent processing increases the necessity of an effective preservation method to maintain lipid quality during storage, due to 1) The chemical self-defense that diatoms employ against grazing, which converts LC-PUFAs to oxylipins by the release of phospholipases following cell disruption, 2) accelerated lipid oxidation following cell lysis by increased exposure of intracellular lipids to prooxidants, and 3) the release of cell exudates which may promote microbial growth in stored biomass. These factors contribute to the rapid decline of lipid quality in fresh biomass of *P. glacialis* during storage, as demonstrated in **Paper II**. Second, the tough cell walls of some microalgae negatively influence the bioavailability of microalgae when used as ingredients in fish feed, as demonstrated with *C. vulgaris*[124] and *Nannochloropsis oceanica*[125]. The reduced need for cell disruption observed in *P. glacialis* in **Paper III** could signify an increased bioavailability of lipids in untreated biomass compared to recalcitrant species.

6 Conclusion

This thesis has demonstrated the large capacity for PUFA production in *P. glacialis*, primarily in the form of EPA, when harvested in the exponential growth phase. Temperature was shown to affect the cellular requirement of specific fatty acids and their degree of unsaturation in *P. glacialis*, however, the relative contribution of LC-PUFAs remained stable at temperatures between 2-12°C. This reduces the need for temperature regulation in an industrial cultivation targeted towards production of LC-PUFA. Following harvesting, heating *P. glacialis* biomass to 90°C for 30 minutes resulted in a large reduction in the formation of free fatty acids and PUFA degradation during storage of fresh biomass. Heat treatment can therefore be used as an alternative to conventional preservative methods on *P. glacialis* biomass, ideally in settings where heat is an inexpensive commodity. Finally, the cell walls of diatoms did not significantly impede lipid release in conventional solvent extractions, in contrast to the recalcitrant cell walls of commonly mass-cultivated microalgae. In summary, LC-PUFA rich biomass of *P. glacialis* can be cultivated at a wide range of temperatures and is readily available for lipid extraction independent of cell disruption, and effectively preserved using an inexpensive heat treatment. These findings will contribute to the commercialization of *P. glacialis* as a source of marine lipids.

7 Future prospects

Future research on *P. glacialis* as a target of mass cultivation should focus on the continued improvement of cultivation technology, coupled with a deeper understanding of the fundamental biology of cold-water diatoms. Identifying the key genes responsible for fatty acid synthesis in *P. glacialis,* and their expression as a function of temperature, will uncover the mechanisms that allow rapid changes in C16 and C18 polyunsaturates in response to temperature changes without sacrificing LC-PUFA biosynthesis. In the long term, a solid foundation in metabolic pathways can lead to a comprehensive overview of LC-PUFA function as functional components of lipids and precursors of metabolites in *P. glacialis* and other psychrophilic diatoms, when coupled with methods such as lipidomics and transcriptomics. These studies should also include other lipophilic molecules with potential value in industry, such as pigments, and explore the effect of the growth environment as a whole on the diatom lipidome. This knowledge is important in a cultivation optimized towards specific fatty acids or other lipophilic molecules as a means to increase product value. It also has potential ecological relevance by uncovering the adaptations that enables diatoms to dominate primary production in cold, productive areas, and in predicting the potential ramifications of climate change in areas where diatoms form the main nutritional basis for higher trophic levels.

Expanding on the potential utility of heat treatment as a conventional preservative for diatom biomass requires further studies to determine the optimum time and temperature to achieve sufficient storage stability of lipids in fresh biomass. In addition, the potential for an integrated heat treatment as part of the harvesting process in an industrial mass cultivation of diatom biomass should be examined, in order to halt lipid degradation as early as possible and further reduce processing costs by increasing cultivation efficiency. In this context, increased knowledge on the effects of cell wall disruption on enzymatic lipid degradation may also contribute to improved product quality, either through targeted lipase inactivation prior to harvesting or by modifying harvesting methods to prevent cell lysis. Finally, further studies on the cell wall morphology of *P. glacialis* and its effect on nutrient bioavailability are needed to establish diatom biomass as a viable ingredient in food and feed.

8 References

1 FAO. The State of World Fisheries and Aquaculture 2020. Sustainability in Action., (Rome, 2020).

2 Tocher, D. R. Omega-3 long-chain polyunsaturated fatty acids and aquaculture in perspective. *Aquaculture* **449**, 94-107, doi:10.1016/j.aquaculture.2015.01.010 (2015).

3 Sheehan, J., Dunahay, T., Benemann, J. & Roessler, P. A look back at the US Department of Energy’s Aquatic Species Program: Biodiesel from Algae. (National Renewable Energy Laboratory, Golden, CO, 1998).

4 Dunstan, G. A., Volkman, J. K., Barrett, S. M., Leroi, J. M. & Jeffrey, S. W. Essential polyunsaturated fatty-acids from 14 species of diatom (Bacillariophyceae). *Phytochemistry* **35**, 155-161 (1994).

5 Kelly, P. B., Reiser, R. & Hood, D. W. The origin of the marine polyunsaturated fatty acids - composition of some marine plankton. *Journal of the American Oil Chemists Society* **36**, 104-106, doi:10.1007/bf02639979 (1959).

6 Dodson, V. J., Mouget, J. L., Dahmen, J. L. & Leblond, J. D. The long and short of it: temperature-dependent modifications of fatty acid chain length and unsaturation in the galactolipid profiles of the diatoms Haslea ostrearia and Phaeodactylum tricornutum. *Hydrobiologia* **727**, 95-107, doi:10.1007/s10750-013-1790-4 (2014).

7 Pasquet, V. *et al.* Fatty acids profile and temperature in the cultured marine diatom Odontella aurita. *Journal of Applied Phycology* **26**, 2265-2271, doi:10.1007/s10811-014-0252-3 (2014).

8 Lepetit, B., Goss, R., Jakob, T. & Wilhelm, C. Molecular dynamics of the diatom thylakoid membrane under different light conditions. *Photosynthesis Research* **111**, 245-257, doi:10.1007/s11120-011-9633-5 (2012).

9 Su, Y. Y. The effect of different light regimes on pigments in Coscinodiscus granii. *Photosynthesis Research* **140**, 301-310, doi:10.1007/s11120-018-0608-7 (2019).

10 Mock, T. & Kroon, B. M. Photosynthetic energy conversion under extreme conditions--I: important role of lipids as structural modulators and energy sink under N-limited growth in Antarctic sea ice diatoms. *Phytochemistry* **61**, 41-51 (2002).

11 Brembu, T., Muhlroth, A., Alipanah, L. & Bones, A. M. The effects of phosphorus limitation on carbon metabolism in diatoms. *Philosophical Transactions of the Royal Society B-Biological Sciences* **372**, doi:10.1098/rstb.2016.0406 (2017).

12 Mann, D. G. & Vanormelingen, P. An Inordinate Fondness? The Number, Distributions, and Origins of Diatom Species. *Journal of Eukaryotic Microbiology* **60**, 414-420, doi:10.1111/jeu.12047 (2013).

13 Guiry, M. D. How Many Species of Algae Are There? *Journal of Phycology* **48**, 1057-1063, doi:10.1111/j.1529-8817.2012.01222.x (2012).

14 Shimizu, K., Del Amo, Y., Brzezinski, M. A., Stucky, G. D. & Morse, D. E. A novel fluorescent silica tracer for biological silicification studies. *Chemistry & Biology* **8**, 1051-1060, doi:Doi 10.1016/S1074-5521(01)00072-2 (2001).

15 Prihoda, J. *et al.* Chloroplast-mitochondria cross-talk in diatoms. *Journal of Experimental Botany* **63**, 1543-1557, doi:10.1093/jxb/err441 (2012).

16 Benoiston, A. S. *et al.* The evolution of diatoms and their biogeochemical functions. *Philosophical Transactions of the Royal Society B-Biological Sciences* **372**, doi: 10.1098/rstb.2016.0397 (2017).

17 Malviya, S. *et al.* Insights into global diatom distribution and diversity in the world's ocean. *Proceedings of the National Academy of Sciences of the United States of America* **113**, E1516-E1525, doi:10.1073/pnas.1509523113 (2016).

18 Medlin, L. K. & Kaczmarska, I. Evolution of the diatoms: V. Morphological and cytological support for the major clades and a taxonomic revision. *Phycologia* **43**, 245-270, doi:DOI 10.2216/i0031-8884-43-3-245.1 (2004).

19 Theriot, E. C., Cannone, J. J., Gutell, R. R. & Alverson, A. J. The limits of nuclear-encoded SSU rDNA for resolving the diatom phylogeny. *European Journal of Phycology* **44**, 277-290, doi:10.1080/09670260902749159 (2009).

20 Yu, M. J. *et al.* Evolution of the Plastid Genomes in Diatoms. *Advances in Botanical Research* **85**, 129-155, doi:10.1016/bs.abr.2017.11.009 (2018).

21 JGI. *100 Diatom Genomes*, <https://jgi.doe.gov/csp-2021-100-diatom-genomes/> (2020).

22 Maidana, N. I. & Romero, O. E. Diatoms from the Hypersaline La Amarga Lake (La Pampa, Argentina). *Cryptogamie, Algologie* **16**, 173-188 (1995).

23 Owen, R. B., Renaut, R. W. & Jones, B. Geothermal diatoms: a comparative study of floras in hot spring systems of Iceland, New Zealand, and Kenya. *Hydrobiologia* **610**, 175-192, doi:10.1007/s10750-008-9432-y (2008).

24 Lund, J. W. G. Observations on Soil Algae .1. The Ecology, Size and Taxonomy of British Soil Diatoms. *New Phytologist* **45**, 56-110, doi:DOI 10.1111/j.1469-8137.1946.tb05047.x (1946).

25 Raven, J. A. The Role of Vacuoles. *New Phytologist* **106**, 357-422, doi:DOI 10.1111/j.1469-8137.1987.tb00149.x (1987).

26 Raven, J. A. The Transport and Function of Silicon in Plants. *Biological Reviews of the Cambridge Philosophical Society* **58**, 179-207, doi:DOI 10.1111/j.1469-185X.1983.tb00385.x (1983).

27 Colman, B. & Rotatore, C. Photosynthetic Inorganic Carbon Uptake and Accumulation in 2 Marine Diatoms. *Plant Cell and Environment* **18**, 919-924, doi:DOI 10.1111/j.1365-3040.1995.tb00601.x (1995).

28 Hopkinson, B. M., Dupont, C. L., Allen, A. E. & Morel, F. M. M. Efficiency of the CO2-concentrating mechanism of diatoms. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 3830-3837, doi:10.1073/pnas.1018062108 (2011).

29 Raven, J. A. Physiology of Inorganic C Acquisition and Implications for Resource Use Efficiency by Marine-Phytoplankton - Relation to Increased Co2 and Temperature. *Plant Cell and Environment* **14**, 779-794, doi:DOI 10.1111/j.1365-3040.1991.tb01442.x (1991).

30 Hein, M. & SandJensen, K. CO2 increases oceanic primary production. *Nature* **388**, 526-527, doi:Doi 10.1038/41457 (1997).

31 Jansson, M., Karlsson, J. & Jonsson, A. Carbon dioxide supersaturation promotes primary production in lakes. *Ecology Letters* **15**, 527-532, doi:10.1111/j.1461-0248.2012.01762.x (2012).

32 McGinn, P. J. *et al.* Integration of microalgae cultivation with industrial waste remediation for biofuel and bioenergy production: opportunities and limitations. *Photosynthesis Research* **109**, 231-247, doi:10.1007/s11120-011-9638-0 (2011).

33 Uitz, J., Claustre, H., Gentili, B. & Stramski, D. Phytoplankton class-specific primary production in the world's oceans: Seasonal and interannual variability from satellite observations. *Global Biogeochemical Cycles* **24**, doi:10.1029/2009GB003680 (2010).

34 Sverdrup, H. U. On Conditions for the Vernal Blooming of Phytoplankton. *Ices Journal of Marine Science* **18**, 287-295, doi:https://doi.org/10.1093/icesjms/18.3.287 (1953).

35 Siegel, D. A., Doney, S. C. & Yoder, J. A. The North Atlantic spring phytoplankton bloom and Sverdrup's critical depth hypothesis. *Science* **296**, 730-733, doi:DOI 10.1126/science.1069174 (2002).

36 Debaar, H. J. W. Vonliebig Law of the Minimum and Plankton Ecology (1899-1991). *Progress in Oceanography* **33**, 347-386, doi:Doi 10.1016/0079-6611(94)90022-1 (1994).
37 Twining, B. S. & Baines, S. B. The Trace Metal Composition of Marine Phytoplankton. *Annual Review of Marine Science,* **5**, 191-215, doi:10.1146/annurev-marine-121211-172322 (2013).
38 Eppley, R. W. Temperature and Phytoplankton Growth in Sea. *Fishery Bulletin* **70**, 1063-1085 (1972).
39 Banse, K. Rates of Phytoplankton Cell-Division in the Field and in Iron Enrichment Experiments. *Limnology and Oceanography* **36**, 1886-1898, doi:DOI 10.4319/lo.1991.36.8.1886 (1991).
40 Kristiansen, S. & Lund, B. A. Nitrogen Cycling in the Barents Sea .1. Uptake of Nitrogen in the Water Column. *Deep-Sea Research* **36**, 255-268, doi:Doi 10.1016/0198-0149(89)90137-4 (1989).
41 Degerlund, M. & Eilertsen, H. C. Main Species Characteristics of Phytoplankton Spring Blooms in NE Atlantic and Arctic Waters (68-80A degrees N). *Estuaries and Coasts* **33**, 242-269, doi:10.1007/s12237-009-9167-7 (2010).
42 Martin, P., Van Mooy, B. A. S., Heithoff, A. & Dyhrman, S. T. Phosphorus supply drives rapid turnover of membrane phospholipids in the diatom Thalassiosira pseudonana. *Isme Journal* **5**, 1057-1060, doi:10.1038/ismej.2010.192 (2011).
43 Canavate, J. P., Armada, I., Rios, J. L. & Hachero-Cruzado, I. Exploring occurrence and molecular diversity of betaine lipids across taxonomy of marine microalgae. *Phytochemistry* **124**, 68-78, doi:10.1016/j.phytochem.2016.02.007 (2016).
44 Murakami, H., Nobusawa, T., Hori, K., Shimojima, M. & Ohta, H. Betaine Lipid Is Crucial for Adapting to Low Temperature and Phosphate Deficiency in Nannochloropsis. *Plant Physiology* **177**, 181-193, doi:10.1104/pp.17.01573 (2018).
45 Gillan, F. T., Mcfadden, G. I., Wetherbee, R. & Johns, R. B. Sterols and Fatty-Acids of an Antarctic Sea Ice Diatom, Stauroneis-Amphioxys. *Phytochemistry* **20**, 1935-1937, doi:Doi 10.1016/0031-9422(81)84038-1 (1981).
46 Gallo, C. *et al.* Diatoms synthesize sterols by inclusion of animal and fungal genes in the plant pathway. *Scientific Reports* **10**, doi:10.1038/s41598-020-60993-5 (2020).
47 Kobayashi, K., Endo, K. & Wada, H. Specific Distribution of Phosphatidylglycerol to Photosystem Complexes in the Thylakoid Membrane. *Frontiers in Plant Science* **8**, doi: 10.3389/fpls.2017.01991 (2017).
48 Shipley, G. G., Green, J. P. & Nichols, B. W. Phase Behavior of Monogalactosyl, Digalactosyl, and Sulfoquinovosyl Diglycerides. *Biochimica Et Biophysica Acta* **311**, 531-544 (1973).
49 Nagao, R., Yokono, M., Teshigahara, A., Akimoto, S. & Tomo, T. Light-Harvesting Ability of the Fucoxanthin Chlorophyll a/c-Binding Protein Associated with Photosystem II from the Diatom Chaetoceros gracilis As Revealed by Picosecond Time-Resolved Fluorescence Spectroscopy. *Journal of Physical Chemistry B* **118**, 5093-5100, doi:10.1021/jp502035y (2014).
50 Kuczynska, P., Jemiola-Rzeminska, M. & Strzalka, K. Photosynthetic Pigments in Diatoms. *Marine Drugs* **13**, 5847-5881, doi:10.3390/md13095847 (2015).
51 Maeda, Y., Nojima, D., Yoshino, T. & Tanaka, T. Structure and properties of oil bodies in diatoms. *Philosophical Transactions of the Royal Society B-Biological Sciences* **372**, doi:10.1098/rstb.2016.0408 (2017).
52 Svenning, J. B. Lipid content, composition and dynamics in northern marine diatoms Master thesis, UiT The Arctic University of Norway, (2014).

53 Liang, Y. *et al.* Dynamic oil body generation in the marine oleaginous diatom Fistulifera solaris in response to nutrient limitation as revealed by morphological and lipidomic analysis. *Algal Research-Biomass Biofuels and Bioproducts* **12**, 359-367, doi:10.1016/j.algal.2015.09.017 (2015).
54 Klok, A. J., Martens, D. E., Wijffels, R. H. & Lamers, P. P. Simultaneous growth and neutral lipid accumulation in microalgae. *Bioresource Technology* **134**, 233-243, doi:10.1016/j.biortech.2013.02.006 (2013).
55 Viso, A. C. & Marty, J. C. Fatty-Acids from 28 Marine Microalgae. *Phytochemistry* **34**, 1521-1533, doi:Doi 10.1016/S0031-9422(00)90839-2 (1993).
56 Ying, L., Mai, K. S. & Sun, S. C. Total lipid and fatty acid composition of eight strains of marine diatoms. *Chinese Journal of Oceanology and Limnology* **18**, 345-349 (2000).
57 Fahl, K. & Kattner, G. Lipid-content and fatty-acid composition of algal communities in sea-ice and water from the Weddel Sea (Antarctica). *Polar Biology* **13**, 405-409, doi:10.1007/bf01681982 (1993).
58 Zulu, N. N., Zienkiewicz, K., Vollheyde, K. & Feussner, I. Current trends to comprehend lipid metabolism in diatoms. *Progress in Lipid Research* **70**, 1-16, doi:10.1016/j.plipres.2018.03.001 (2018).
59 Muhlroth, A. *et al.* Pathways of Lipid Metabolism in Marine Algae, Co-Expression Network, Bottlenecks and Candidate Genes for Enhanced Production of EPA and DHA in Species of Chromista. *Marine Drugs* **11**, 4662-4697, doi:10.3390/md11114662 (2013).
60 Remize, M. *et al.* Study of Synthesis Pathways of the Essential Polyunsaturated Fatty Acid 20:5n-3 in the Diatom Chaetoceros Muelleri Using C-13-Isotope Labeling. *Biomolecules* **10**, doi: 10.3390/biom10050797 (2020).
61 Kabeya, N. *et al.* Genes for de novo biosynthesis of omega-3 polyunsaturated fatty acids are widespread in animals. *Science Advances* **4**, doi: 10.1126/sciadv.aar6849 (2018).
62 Borowitzka, M. A. Commercial production of microalgae: ponds, tanks, tubes and fermenters. *Journal of Biotechnology* **70**, 313-321, doi:Doi 10.1016/S0168-1656(99)00083-8 (1999).
63 Spoehr, H. A. & Milner, H. W. The Chemical Composition of Chlorella - Effect of Environmental Conditions. *Plant Physiol* **24**, 120-149, doi:DOI 10.1104/pp.24.1.120 (1949).
64 Burlew, J. S. *Algal Culture: From Laboratory to Pilot Plant*. (Carnegie Institution of Washington, 1953).
65 Ammann, E. C. B. & Frasersm.A. Gas Exchange of Algae .4. Reliability of Chlorella Pyrenoidosa. *Applied Microbiology* **16**, 669-&, doi:Doi 10.1128/Aem.16.5.669-672.1968 (1968).
66 Kay, R. A. Microalgae as Food and Supplement. *Critical Reviews in Food Science and Nutrition* **30**, 555-573, doi:Doi 10.1080/10408399109527556 (1991).
67 Madeira, M. S. *et al.* Microalgae as feed ingredients for livestock production and meat quality: A review. *Livestock Science* **205**, 111-121, doi:10.1016/j.livsci.2017.09.020 (2017).
68 Benemann, J. R., Weissman, J. C., Koopman, B. L. & Oswald, W. J. Energy-Production by Microbial Photosynthesis. *Nature* **268**, 19-23, doi:DOI 10.1038/268019a0 (1977).
69 Chisti, Y. Biodiesel from microalgae. *Biotechnology Advances* **25**, 294-306, doi:10.1016/j.biotechadv.2007.02.001 (2007).
70 Ryckebosch, E. *et al.* Nutritional evaluation of microalgae oils rich in omega-3 long chain polyunsaturated fatty acids as an alternative for fish oil. *Food Chemistry* **160**, 393-400, doi:10.1016/j.foodchem.2014.03.087 (2014).

71 Sprague, M., Betancor, M. B. & Tocher, D. R. Microbial and genetically engineered oils as replacements for fish oil in aquaculture feeds. *Biotechnology Letters* **39**, 1599-1609, doi:10.1007/s10529-017-2402-6 (2017).
72 Huleihel, M., Ishanu, V., Tal, J. & Arad, S. Antiviral effect of red microalgal polysaccharides on Herpes simplex and Varicella zoster viruses. *Journal of Applied Phycology* **13**, 127-134, doi:Doi 10.1023/A:1011178225912 (2001).
73 Goo, B. G. *et al.* Characterization of a renewable extracellular polysaccharide from defatted microalgae Dunaliella tertiolecta. *Bioresource Technology* **129**, 343-350, doi:10.1016/j.biortech.2012.11.077 (2013).
74 Cuellar-Bermudez, S. P. *et al.* Extraction and purification of high-value metabolites from microalgae: essential lipids, astaxanthin and phycobiliproteins. *Microbial Biotechnology* **8**, 190-209, doi:10.1111/1751-7915.12167 (2015).
75 Gimpel, J. A., Hyun, J. S., Schoepp, N. G. & Mayfield, S. P. Production of Recombinant Proteins in Microalgae at Pilot Greenhouse Scale. *Biotechnology and Bioengineering* **112**, 339-345, doi:10.1002/bit.25357 (2015).
76 Ratledge, C. & Cohen, Z. Microbial and algal oils: Do they have a future for biodiesel or as commodity oils? *Lipid Technology* **20**, 155-160 (2008).
77 Pulz, O. & Gross, W. Valuable products from biotechnology of microalgae. *Applied Microbiology and Biotechnology* **65**, 635-648, doi:10.1007/s00253-004-1647-x (2004).
78 Lane, J. *RIP, Aurora Algae: Algae and the Never-Never*, <http://www.biofuelsdigest.com/bdigest/2015/07/22/rip-aurora-algae-algae-and-the-never-never/> (2015).
79 Bogacz-Radomska, L. & Harasym, J. beta-Carotene-properties and production methods. *Food Quality and Safety* **2**, 69-74, doi:10.1093/fqsafe/fyy004 (2018).
80 Benedetti, M., Vecchi, V., Barera, S. & Dall'Osto, L. Biomass from microalgae: the potential of domestication towards sustainable biofactories. *Microbial Cell Factories* **17**, doi: 10.1186/s12934-018-1019-3 (2018).
81 Spolaore, P., Joannis-Cassan, C., Duran, E. & Isambert, A. Commercial applications of microalgae. *Journal of Bioscience and Bioengineering* **101**, 87-96, doi:10.1263/jbb.101.87 (2006).
82 Wang, B., Lan, C. Q. & Horsman, M. Closed photobioreactors for production of microalgal biomasses. *Biotechnology Advances* **30**, 904-912, doi:10.1016/j.biotechadv.2012.01.019 (2012).
83 Grobbelaar, J. U. Factors governing algal growth in photobioreactors: the "open" versus "closed" debate. *Journal of Applied Phycology* **21**, 489-492, doi:10.1007/s10811-008-9365-x (2009).
84 Chen, C. Y., Yeh, K. L., Aisyah, R., Lee, D. J. & Chang, J. S. Cultivation, photobioreactor design and harvesting of microalgae for biodiesel production: A critical review. *Bioresource Technology* **102**, 71-81, doi:10.1016/j.biortech.2010.06.159 (2011).
85 Acien, F. G., Fernandez, J. M., Magan, J. J. & Molina, E. Production cost of a real microalgae production plant and strategies to reduce it. *Biotechnology Advances* **30**, 1344-1353, doi:10.1016/j.biotechadv.2012.02.005 (2012).
86 Hemaiswarya, S., Raja, R., Kumar, R. R., Ganesan, V. & Anbazhagan, C. Microalgae: a sustainable feed source for aquaculture. *World Journal of Microbiology and Biotechnology* **27**, 1737-1746, doi:10.1007/s11274-010-0632-z (2011).
87 Singh, G. & Patidar, S. K. Microalgae harvesting techniques: A review. *Journal of Environmental Management* **217**, 499-508, doi:10.1016/j.jenvman.2018.04.010 (2018).
88 Dassey, A. J. & Theegala, C. S. Harvesting economics and strategies using centrifugation for cost effective separation of microalgae cells for biodiesel

applications. *Bioresource Technology* **128**, 241-245, doi:10.1016/j.biortech.2012.10.061 (2013).
89 Shah, M. M. R., Liang, Y. M., Cheng, J. J. & Daroch, M. Astaxanthin-Producing Green Microalga Haematococcus pluvialis: From Single Cellto High Value Commercia Products. *Frontiers in Plant Science* **7**, doi: 10.3389/fpls.2016.00531 (2016).
90 Fasaei, F., Bitter, J. H., Slegers, P. M. & van Boxtel, A. J. B. Techno-economic evaluation of microalgae harvesting and dewatering systems. *Algal Research-Biomass Biofuels and Bioproducts* **31**, 347-362, doi:10.1016/j.algal.2017.11.038 (2018).
91 Grima, E. M., Belarbi, E. H., Fernandez, F. G. A., Medina, A. R. & Chisti, Y. Recovery of microalgal biomass and metabolites: process options and economics. *Biotechnology Advances* **20**, 491-515 (2003).
92 Show, K. Y., Lee, D. J., Tay, J. H., Lee, T. M. & Chang, J. S. Microalgal drying and cell disruption - Recent advances. *Bioresource Technology* **184**, 258-266, doi:10.1016/j.biortech.2014.10.139 (2015).
93 Ratti, C. Hot air and freeze-drying of high-value foods: a review. *Journal of Food Engineering* **49**, 311-319, doi:Doi 10.1016/S0260-8774(00)00228-4 (2001).
94 Atkinson, A. W., John, P. C. L. & Gunning, B. E. S. Sporopollenin in Cell-Wall of Chlorella and Other Algae - Ultrastructure, Chemistry, and Incorporation of C-14-Acetate, Studied in Synchronous Cultures. *Planta* **107**, 1-&, doi:Doi 10.1007/Bf00398011 (1972).
95 Balduyck, L., Bruneel, C., Goiris, K., Dejonghe, C. & Foubert, I. Influence of High Pressure Homogenization on Free Fatty Acid Formation in Nannochloropsis sp. *European Journal of Lipid Science and Technology* **120**, doi: 10.1002/ejlt.201700436 (2018).
96 Lee, J. Y., Yoo, C., Jun, S. Y., Ahn, C. Y. & Oh, H. M. Comparison of several methods for effective lipid extraction from microalgae. *Bioresource Technology* **101**, S75-S77, doi:10.1016/j.biortech.2009.03.058 (2010).
97 Mercer, P. & Armenta, R. E. Developments in oil extraction from microalgae. *European Journal of Lipid Science and Technology* **113**, 539-547, doi:10.1002/ejlt.201000455 (2011).
98 Hosseini, S. R. P., Tavakoli, O. & Sarrafzadeh, M. H. Experimental optimization of SC-CO2 extraction of carotenoids from Dunaliella sauna. *Journal of Supercritical Fluids* **121**, 89-95, doi:10.1016/j.supflu.2016.11.006 (2017).
99 Herrero, M., Cifuentes, A. & Ibanez, E. Sub- and supercritical fluid extraction of functional ingredients from different natural sources: Plants, food-by-products, algae and microalgae - A review. *Food Chemistry* **98**, 136-148, doi:10.1016/j.foodchem.2005.05.058 (2006).
100 Henderson, R. J., Hegseth, E. N. & Park, R. T. Seasonal variation in lipid and fatty acid composition of ice algae from the Barents Sea. *Polar Biology* **20**, 48-55, doi:10.1007/s003000050275 (1998).
101 Dodson, V. J., Dahmen, J. L., Mouget, J. L. & Leblond, J. D. Mono- and digalactosyldiacylglycerol composition of the marennine-producing diatom, Haslea ostrearia: Comparison to a selection of pennate and centric diatoms. *Phycological Research* **61**, 199-207, doi:10.1111/pre.12015 (2013).
102 Mortensen, S. H., Borsheim, K. Y., Rainuzzo, J. R. & Knutsen, G. Fatty-Acid and Elemental Composition of the Marine Diatom Chaetoceros-Gracilis Schutt - Effects of Silicate Deprivation, Temperature and Light-Intensity. *Journal of Experimental Marine Biology and Ecology* **122**, 173-185, doi:Doi 10.1016/0022-0981(88)90183-9 (1988).

103 Teoh, M. L., Phang, S. M. & Chu, W. L. Response of Antarctic, temperate, and tropical microalgae to temperature stress. *Journal of Applied Phycology* **25**, 285-297, doi:10.1007/s10811-012-9863-8 (2013).

104 Nichols, D. S., Nichols, P. D. & Sullivan, C. W. Fatty-acid, sterol and hydrocarbon composition of Antarctic sea-ice diatom communities during the spring bloom in McMurdo Sound. *Antarctic Science* **5**, 271-278 (1993).

105 Whitaker, T. M. & Richardson, M. G. Morphology and Chemical-Composition of a Natural-Population of an Ice-Associated Antarctic Diatom Navicula-Glaciei. *Journal of Phycology* **16**, 250-257 (1980).

106 Artamonova, E. Y., Vasskog, T. & Eilertsen, H. C. Lipid content and fatty acid composition of Porosira glacialis and Attheya longicornis in response to carbon dioxide (CO2) aeration. *Plos One* **12**, doi:10.1371/journal.pone.0177703 (2017).

107 Boelen, P., van Dijk, R., Damste, J. S. S., Rijpstra, W. I. C. & Buma, A. G. J. On the potential application of polar and temperate marine microalgae for EPA and DHA production. *AMB Express* **3**, 9, doi:10.1186/2191-0855-3-26 (2013).

108 Fuller, I. D. *et al.* Free Fatty Acids in Commercial Krill Oils: Concentrations, Compositions, and Implications for Oxidative Stability. *Journal of the American Oil Chemists Society*, doi:10.1002/aocs.12368 (2020).

109 Miyashita, K. & Takagi, T. Study on the Oxidative Rate and Prooxidant Activity of Free Fatty-Acids. *Journal of the American Oil Chemists Society* **63**, 1380-1384, doi:Doi 10.1007/Bf02679607 (1986).

110 Richard, D., Kefi, K., Barbe, U., Bausero, P. & Visioli, F. Polyunsaturated fatty acids as antioxidants. *Pharmacological Research* **57**, 451-455, doi:10.1016/j.phrs.2008.05.002 (2008).

111 Kim, T. S., Decker, E. A. & Lee, J. Effects of chlorophyll photosensitisation on the oxidative stability in oil-in-water emulsions. *Food Chemistry* **133**, 1449-1455, doi:10.1016/j.foodchem.2012.02.033 (2012).

112 Pohnert, G. Phospholipase A(2) activity triggers the wound-activated chemical defense in the diatom Thalassiosira rotula. *Plant Physiology* **129**, 103-111, doi:10.1104/pp.010974 (2002).

113 Wichard, T. *et al.* Lipid and fatty acid composition of diatoms revisited: Rapid wound-activated change of food quality parameters influences herbivorous copepod reproductive success. *Chembiochem* **8**, 1146-1153, doi:10.1002/cbic.200700053 (2007).

114 Olsen, R. L. & Toppe, J. Fish silage hydrolysates: Not only a feed nutrient, but also a useful feed additive. *Trends in Food Science and Technology* **66**, 93-97, doi:10.1016/j.tifs.2017.06.003 (2017).

115 Jacobsen, C., Nielsen, N. S., Horn, A. F. & Sørensen, A. M. in *Food enrichment with omega-3 fatty acids* Ch. Stabilization of omega-3 oils and enriched foods using antioxidants, (Woodhead Publishing Limited, 2013).

116 Oterhals, Å. & Vogt, G. in *Food enrichment with omega-3 fatty acids* Ch. Impact of extraction, refining and concentration stages on the stability of fish oil, (Woodhead Publishing Limited, 2013).

117 Owusu, R. K., Makhzoum, A. & Knapp, J. S. Heat Inactivation of Lipase from Psychrotrophic Pseudomonas-Fluorescens P38 - Activation Parameters and Enzyme Stability at Low or Ultra-High Temperatures. *Food Chemistry* **44**, 261-268, doi:Doi 10.1016/0308-8146(92)90048-7 (1992).

118 Balduyck, L. *et al.* Inhibition of lipolytic reactions during wet storage of T-Isochrysis lutea biomass by heat treatment. *Algal Research-Biomass Biofuels and Bioproducts* **38**, doi: 10.1016/j.algal.2018.101388 (2019).

119 Berge, J. P., Gouygou, J. P., Dubacq, J. P. & Durand, P. Reassessment of Lipid-Composition of the Diatom, Skeletonema-Costatum. *Phytochemistry* **39**, 1017-1021, doi:Doi 10.1016/0031-9422(94)00156-N (1995).
120 Budge, S. M. & Parrish, C. C. Lipid class and fatty acid composition of Pseudo-nitzschia multiseries and Pseudo-nitzschia pungens and effects of lipolytic enzyme deactivation. *Phytochemistry* **52**, 561-566, doi:10.1016/s0031-9422(99)00241-1 (1999).
121 Alhattab, M., Kermanshahi-Pour, A. & Brooks, M. S. L. Microalgae disruption techniques for product recovery: influence of cell wall composition. *Journal of Applied Phycology* **31**, 61-88, doi:10.1007/s10811-018-1560-9 (2019).
122 Ranjan, A., Patil, C. & Moholkar, V. S. Mechanistic Assessment of Microalgal Lipid Extraction. *Industrial & Engineering Chemistry Research* **49**, 2979-2985, doi:10.1021/ie9016557 (2010).
123 Hildebrand, M., Lerch, S. J. L. & Shrestha, R. P. Understanding Diatom Cell Wall Silicification - Moving Forward. *Frontiers in Marine Science* **5**, doi: 10.3389/fmars.2018.00125 (2018).
124 Tibbetts, S. M., Mann, J. & Dumas, A. Apparent digestibility of nutrients, energy, essential amino acids and fatty acids of juvenile Atlantic salmon (Salmo salar L.) diets containing whole-cell or cell-ruptured Chlorella vulgaris meals at five dietary inclusion levels. *Aquaculture* **481**, 25-39, doi:10.1016/j.aquaculture.2017.08.018 (2017).
125 Gong, Y. Y. *et al.* Approaches to improve utilization of Nannochloropsis oceanica in plant-based feeds for Atlantic salmon. *Aquaculture* **522**, doi: 10.1016/j.aquaculture.2020.735122 (2020).

Paper I

Svenning, J.B., Dalheim, L., Eilertsen, H.C. & Vasskog, T. (2019).

Temperature dependent growth rate, lipid content and fatty acid composition of the marine cold-water diatom *Porosira glacialis*.

Algal Research, 37, 11-16.

Algal Research 37 (2019) 11–16

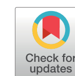
Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Short communication

Temperature dependent growth rate, lipid content and fatty acid composition of the marine cold-water diatom *Porosira glacialis*



Jon Brage Svenning[a,*], Lars Dalheim[a], Hans Christian Eilertsen[a], Terje Vasskog[b]

[a] Norwegian College of Fishery Science, UiT the Arctic University of Norway, 9037 Tromsø, Norway
[b] Department of Pharmacy, Faculty of Health Sciences, UiT the Arctic University of Norway, 9037 Tromsø, Norway

ARTICLE INFO

Keywords:
Diatom
Lipid
Desaturation
Psychrophilic

ABSTRACT

In this study, the northern cold-water marine diatom *Porosira glacialis* was cultivated in a pilot-scale mass cultivation system at 5 different temperatures (−2 to 12 °C), in order to evaluate temperature-dependent growth rate (in vitro Chl *a*), lipid content (Folch's method) and fatty acid (FA) composition (GC–MS) in the exponential growth phase. We found that *P. glacialis* has a wide temperature range, with maximum growth at 12 °C and positive growth even at sub-zero water temperatures. The lipid content was inversely correlated with temperature, peaking at 33.4 ± 4.0% at 2 °C, and was highly desaturated independently of temperature; PUFA content varied from 71.50 ± 0.88% at 12 °C to 82 ± 0.64% at −2 °C. EPA was the main FA at all temperatures (31.0 ± 0.7–40.4 ± 1.2% of total FAs).

1. Introduction

Microalgae are the main primary producers of the world's aquatic environments. Present in all habitats containing water, they display high physiological, chemical and morphological diversity, and are the fastest growing photoautotrophic organisms on the planet. Diatoms are the largest group of microalgae with an estimated 100,000 species worldwide [1]. They are the dominant primary producers in temperate & cold areas [2]. While their structural lipochemistry is similar to that of green algae and higher plants, they are distinguished by their ability to synthesize highly unsaturated fatty acids of > 18 carbons [3], such as EPA and DHA to serve as structural components in membranes or as precursors for metabolites, which regulate biological functions [4]. Generally referred to as long-chained polyunsaturated fatty acids (LC-PUFAs), these fatty acids are in high-demand by aquatic and terrestrial animals and are preserved as they pass through the food chain [5,6]. In humans, studies have demonstrated the positive health-effects of LC-PUFA intake [7–9], and different agencies recommend an intake of approximately 500 mg EPA + DHA day^{-1} [10].

Many studies have investigated diatom lipid allocation as a function of temperature. As a general rule, there is an inverse relationship between temperature and degree of desaturation [11–18], as the main functions of fatty acids is to regulate membrane fluidity in response to changing temperatures. However, as species are inherently different in their environmental adaptations, environmental effects on fatty acid allocation must be explored for each species independently. In later years, much of the research on diatom lipids has moved from an ecological focus to an industrial one, seeking to uncover oleaginous species with potential for production of biodiesels and/or valuable PUFAs [19–22]. These studies regularly focus on batch cultivated warm-water strains of small Chlorophyta and Cyanophyta species with low iodine values. There is very little research on psychrophilic diatoms cultivated in very cold environments, or the technology required to perform large scale industrial cultivations in areas with pronounced seasons. Nevertheless, studies from polar regions have revealed highly unsaturated fatty acid compositions in diatom-dominated microalgal communities [23–26], making them excellent candidates for LC-PUFA production.

Today, our main sources of EPA and DHA are marine fish, of which approximately 1 million tonnes of fish oil are produced annually. Of these, the aquaculture sector uses about 75% [27]. The annual capture of wild fish has, however, stagnated between 80 and 100 million tonnes in the last 30 years, while the aquaculture production has grown from near negligibility to contributing > 40% of the world's total fish production in 2014. This has had obvious implications for the composition of aquaculture feed; In 2013, Norwegian salmon producers were substituting up to 82% of the fish meal and 89% of the fish oil normally used in feed with terrestrial products [28]. Although this has no apparent negative effect for the growth of the salmon, it reduces the nutritional value for consumers by lowering the amount of n-3 LC PUFAs in the fillet [29,30], and requires large areas of arable land that

* Corresponding author.
E-mail addresses: jon.svenning@uit.no (J.B. Svenning), Lars.dalheim@uit.no (L. Dalheim), Hans.c.eilertsen@uit.no (H.C. Eilertsen), Terje.vasskog@uit.no (T. Vasskog).

https://doi.org/10.1016/j.algal.2018.10.009
Received 3 May 2018; Received in revised form 9 October 2018; Accepted 9 October 2018
Available online 27 November 2018
2211-9264/ © 2018 Published by Elsevier B.V.

otherwise could be utilized for human food. Diatoms have an immense potential as feed for the salmon industry [31,32], and could be the substitute for conventional sources if competitive large-scale production of LC-PUFA rich species can be achieved. Naturally, a potential salmon feed from diatoms should resemble (or improve upon) the nutrient content of the fish which it is meant to replace, and we believe the simplest way to achieve this is to harvest the diatom in the exponential growth phase. In this study, a large cold-water centric diatom, *Porosira glacialis*, was cultivated in a nutrient replete environment at 5 different temperatures (−2, 2, 4, 8 and 12 °C), in order to investigate the effect of temperature on the growth rate, total lipid and fatty acid composition during the exponential growth phase.

2. Materials and methods

2.1. Chemicals

All chemicals were obtained from Sigma Aldrich (Sigma Aldrich, St. Louis, Mo, USA) unless otherwise stated. Guillard's F2 Marine water enrichment solution (50×) was used for cell cultivation. Ethanol (96%) and 10% hydrochloric acid (Merck KgaA, 64271 Darmstadt, Germany) were used in Chlorophyll *a* (Chl *a*) extractions and quantification. Dichloromethane (99.9%), methanol (99.8%), sodium chloride (Merck KgaA) and sulfuric acid (95–97%) were used in lipid extractions and fatty acid derivation prior to GC–MS analysis.

Hexane (99%) pro analysis was used to dissolve the fatty acid methyl esters (FAMEs) before GC–MS analysis. Standards of the fatty acids 10:0, 12:0, 14:0, 16:0, 16:1n-7, 18:0, 18:1n-9, 18:1n-12, 18:2n-6, 18:3n-3, 20:0, 20:1n-9, 20:3n-6, 20:4n-6, 20:5n-3, 22:1n-9, 22:6n-3 and 24:1n-9 with purity > 99% were purchased from Sigma Aldrich, while standards of the fatty acids 16:2n-4, 16:3n-3, 18:4n-3 with purity > 98% were purchased from LGC Standards (Teddington, UK). The internal standards used for quantification were 14-methylhexadecanoic acid and 19-methylarachidic acid (> 99%, Sigma Aldrich).

2.2. Species

The monoculture of *Porosira glacialis* used in this experiment was originally isolated from a sediment sample collected in the Barents Sea (N 76° 27.54′, E 033° 03.54′) during a 2014 cruise and identified using light microscopy and the diatom key from Tomas [33].

2.3. Cultivation & Harvesting

All cultures were cultivated in filtered seawater (32 PSU) added 4 mL L^{-1} Guillard's F2 Marine water enrichment solution (50×) and 12.32 μM sodium metasilicate nonahydrate (≥98%). To ensure sufficient CO_2 supply, all cultures were mixed by aeration (2–3 L min^{-1}) for the entirety of the experiment. To obtain samples cultivated at 2, 4, 8 and 12 °C, monocultures of *P. glacialis* were cultivated in 100 L clear plexiglass columns, placed in a light- and temperature-controlled room set to each temperature consecutively, beginning with 12 °C, then 8, 4 and 2 °C, respectively. The cultures received 14 h of daylight per day using three North Light LED-strips (12 V) placed at regular intervals around the column, at a scalar irradiance of ca. 66 μmol m^{-2} s^{-1}. To obtain a sample cultivated at −2 °C, a 600 L plexiglass column was placed outside during February of 2016, using two LED work lights to achieve comparable irradiance conditions (measured each day). A larger volume was necessary to prevent sudden changes in the culture temperature as a result of the large variations in ambient air temperature during this time of year. The temperature was maintained by leading warm water (60 °C) through a silicone tube wrapped around the base of the column whenever necessary. All cultures' growth was monitored daily, both by cell-counts in 2 mL Nunc-chambers (Nunc A/S, Roskilde, Denmark) and by in vitro Chl *a* - extraction and quantification using the method described by Holm-Hansen and Riemann [34], using ethanol instead of methanol as the extractant. In vitro Chl *a* was used as a proxy for biomass. It is well known that Chl *a* may vary with cultivation conditions, especially irradiance, and the culture's growth phase. In the present experiment this error is assumed to be of minor importance, as we applied the same species throughout the experiment, and the cultures were never allowed to exit the exponential growth phase. Experiments prior to the present one also examined correlations between Chl *a* and cell concentrations, and this yielded the following results: Pearson $r = 0.67$ at $p < 0.05$, Min = 39 μg Chl *a* L^{-1} and Max 886 μg Chl *a* L^{-1}, $n = 594$. The in vitro Chl *a* measurements were used to calculate growth rates, visually represented both as the daily change of Chl *a*, and as doublings day^{-1} (μ) calculated from the increase in Chl *a* from the formula:

$$\mu = (\mathrm{Log2}(t_x) - \mathrm{Log2}(t_0))/x, \quad (1)$$

Where t equals the Chl *a* content and x equals the total number of days for each cultivation.

At each designated cultivation temperature, the photobioreactor was initiated with 20 L of stock culture and diluted every 1–2 days (determined by the cell counts; the culture density was never diluted below 1 million cells L^{-1} and never allowed to surpass 4 million cells L^{-1}) and harvested after 3–4 days of exponential growth at 100 L volume. The harvesting was performed by passing 80 L of culture through a plankton net (Sefar Nytal®), and collecting the biomass with a rubber spatula in 50 mL Falcon® tubes, which were subsequently placed in the freezer at −80 °C. The remaining 20 L of culture in the plexiglass-column was used as the new stock culture for the next cultivation temperature. With this set-up, the culture was given a minimum of 72 h to adapt to each temperature.

2.4. Lipid extraction & derivatization

The extraction procedure was adapted from Folch, Lees [35], using dichlormethane:methanol as the extractant [36]. Briefly, samples were freeze-dried and divided into triplicates of approximately 100 mg in 15 mL centrifuge tubes (Falcon). Each tube was added 2 mL dichloromethane: methanol (2:1 v/v) and 2 mL 5% (w/v) NaCl in MiliQ water. The tubes were gently shaken for 30 s by hand and then centrifuged for 4 min at 2000 G (Heraus Multifuge 1S-R, Germany). Following centrifugation, the organic phase was transferred to a preweighed and marked dram glass. The extraction procedure was repeated twice for each sample in order to increase the yield. Following extraction, the organic phase was evaporated under nitrogen and the total lipid was determined gravimetrically, as percentage of ash-free dry weight (AFDW). Due to lack of material, AFDW-calculations were based on samples from the same species harvested in the exponential growth phase, determined by combustion in a muffle furnace (AFDW = 46.04 ± 0.33% of dry weight, $n = 3$). Finally, the extracted lipids were dissolved in dichloromethane:methanol (2:1 v/v) to a concentration of 10 mg mL^{-1} and esterified using a method adapted from Stoffel, Chu [37] using sulfuric acid as the catalyst:

Triplicate dissolved extracts from each cultivation temperature (3 × 100 μL) was transferred to a test tube (Duran®) and added 100 μL internal standard (0.1 mg mL^{-1}) and 800 μL dichloromethane. The samples were then added 2 mL 10% H_2SO_4 (v/v) in methanol and placed at 100 °C for 1 h, before 3 mL hexane and 3 mL 5% (w/v) NaCl in MiliQ-water was added and the mixture shaken thoroughly. The resulting organic phase containing the fatty acid methyl esters (FAMEs) was transferred to and evaporated in GC–MS tubes (Waters TruView ™ LCMS Certified Vials), before being redissolved in 500 μL of hexane.

2.5. GC–MS

The method used here was originally described in Artamonova et al. [38]. The GC–MS analyses were performed on a Waters Quattro Premier GC (Waters, Milford, MA, USA) equipped with a 30-meter-long

fused silica Restek FameWax 0.25 mm column with 0.25 µm film thickness. The injector temperature was set to 250 °C, the injection was in splitless mode and He 6.0 (Aga, Oslo, Norway) was used as carrier gas with a 1.0 mL min^{-1} constant flow. One µl of the sample was injected, and the initial temperature on the column was 50 °C. The initial temperature was maintained for 3 min, and then increased by 2 °C per minute until the final temperature of 250 °C was reached. The final temperature was maintained for 10 min and the total runtime was 113 min. The GC–MS interface was kept at 250 °C, and the mass spectrometer was equipped with an EI ionization source operated at 70 eV. The MS source temperature was 210 °C and the trap current was 200 µA. The MS was run in full scan mode scanning *m/z* 150–400 with a scan time of 0.5 s. Each replicate was injected thrice, so that the final results are averages of 9 individual measurements for each cultivation temperature (triplicates measured three times each).

The quantification was based on relative peak area between the different analytes and the two internal standards. The choice of internal standard was based on retention time, and the FAs 10:0, 12:0, 14:0, 16:0, 16:1, 16:2, 16:3, 16:4, 18:0 and 18:1 were quantified with 14-methylhexadecanoic acid as internal standard, while the remaining longer chained FAs were quantified with 19-methylarichidic acid as internal standard. Standard curves were set up in the concentration range 10–1000 ng mL^{-1}. The quantification of 16:4 was based on the standard curve for 16:3 and gives an approximate value (while the relative amount between different samples is correct), as it was not possible to find a commercial supplier of 16:4 during the project period. The method does not distinguish between the position of the double bonds in mono-, di-, tri- and tetraenes where there is more than one possible configuration, e.g. 18:1n-9 will not be separated from 18:1n-12. All standards for the standard curve and the algae samples went through the same derivatization method to obtain FAMEs before analysis.

2.6. Statistical analyses

All data was presented as means and their standard deviations, either in tables or as figures with error bars representing one standard deviation. All data groups were subjected to normality tests (Shapiro Wilk). Normally distributed data was analysed with ANOVA, while data not normally distributed was analysed with a Mann-Whitney test, and post hoc Tukey's or Game-Howell tests, according to the error variance determined by a Levene's test,. Groups were determined homogenous at a significance level of > 0.05. All analyses were performed using IBM SPSS v24 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Growth rate & total lipid

The algal culture displayed positive growth at all temperatures, and temperature had a significant effect on the total lipid content, see Table 1 for growth rates and lipid content, and Fig. 1 for the daily increase in Chl *a*. A Levene's test revealed high error variance between all groups of Chl *a*-measurements ($p = 0.013$). The highest average growth rate was measured at 12 °C (0.41 µ), but this measurement was not statistically different from the growth rates at 2, 4 and 8 °C (p = 0.46, 0.652, 0.221, respectively). The lowest growth rate was measured at −2 °C (0.17 µ). The biomass measurements at −2 °C was influenced by water freezing; ice formation in the bioreactor trapped cells, resulting in an apparent reduction of biomass on day 1 and 2 (see Fig. 1). On day 3, however, the culture was thoroughly mixed and the sample collected for Chl *a* measurement left to thaw before being filtered, thereby revealing the true average growth from day 0–3. The highest lipid content was measured in the algae cultivated at 2 °C (33.4%), but this measurement was not statistically different from the total lipid in algae cultivated at −2 °C (28.4%). The lowest lipid content was measured in the algae cultivated at 12 °C (19.5%), but this measurement was not significantly different from 8 °C (22.0%) or 4 °C (22.8%).

3.2. Desaturation

Temperature had an effect on the degree of fatty acid desaturation. Table 2 lists the relative amounts and standard deviation of all fatty acids measured. A total of 14 fatty acids were detected in the cultured diatom at all temperatures.

Trends among individual fatty acids were observed; The amount of 14:0, 16:2 and 16:3 approximately halved from 12 to −2 °C. 14:0 decreased in increments as the temperature decreased (all changes were statistically significant). 16:2 decreased gradually, but more strongly at the transition from 8 to 4 °C, while for 16:3 the decrease was sudden during the transition from 4 to 2 °C. DHA (22:6) was also more abundant at 8 and 12 °C compared to the lower temperatures ($p < 0.05$).

From 12 to 2 °C, 16:4 and 18:4 increased by 208% and 148%, respectively. At −2 °C, results were similar to those found at 2 °C and did not display the same increasing trend. For 16:4, the increase occurred between 8 and 2 °C, forming three significantly different subgroups; −2 and 2 °C (14.4–14.8%), 4 °C (10.5%), and 8 and 12 °C (4.9–4.8%). For 18:4, the decrease occurred in increments with increasing temperature (all measurements were statistically significant). The relative EPA content was highest in the algae cultivated at −2 °C (40.4%), while all other measurements fluctuated between 31.0 and 33.8%. The relative contents of EPA at 2 and 12 °C were not statistically different ($p > 0.05$).

3.3. SFA, MUFA & PUFA

Total values of, and trends among saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids are displayed in Fig. 2. The SFA content (Fig. 2a) was lowest in the algae cultivated at −2 °C (10.43% of total FAs), and highest at 12 °C (16.48%). The SFA contents were statistically significant at all temperatures with the exception of 4 and 8 °C ($p = 0.061$).

The MUFA content (Fig. 2b), dominated by 16:1, was lowest in the algae cultivated at −2 °C (8.84%) and highest at 2 °C (13.31%). All MUFA contents were statistically significant at all temperatures apart from 8 and 12 °C ($p = 0.995$).

The PUFA content (Fig. 2c) was lowest in the algae cultivated at 12 °C (71.50%), and highest at −2 °C (82.0%). From −2 to 2 °C, the PUFA content was reduced by almost 8 percentage points, caused almost exclusively by the change observed in EPA. Two statistically similar groups were observed; 12 & 8 °C ($p = 0.71$) and 4 & 0 °C ($p = 1.0$).

Table 1
Growth rates and total lipid content (arithmetic mean ± SD, $n = 3$) for *Porosira glacialis* (P.g.) at each cultivation temperature. The growth rate was calculated from the increase in chlorophyll *a* (Chl a) from the start of cultivation to the point of harvest, while total lipids were measured from samples taken at the time of harvest.

Temperature (°C)	−2	2	4	8	12
Growth rate (µ)	0.17 ± 0.01	0.36 ± 0.07	0.34 ± 0.01	0.33 ± 0.04	0.41 ± 0.07
Lipid content (% of AFDW)	28.4 ± 1.3	33.4 ± 4.0	22.8 ± 1.8	22.0 ± 1.1	19.5 ± 1.5

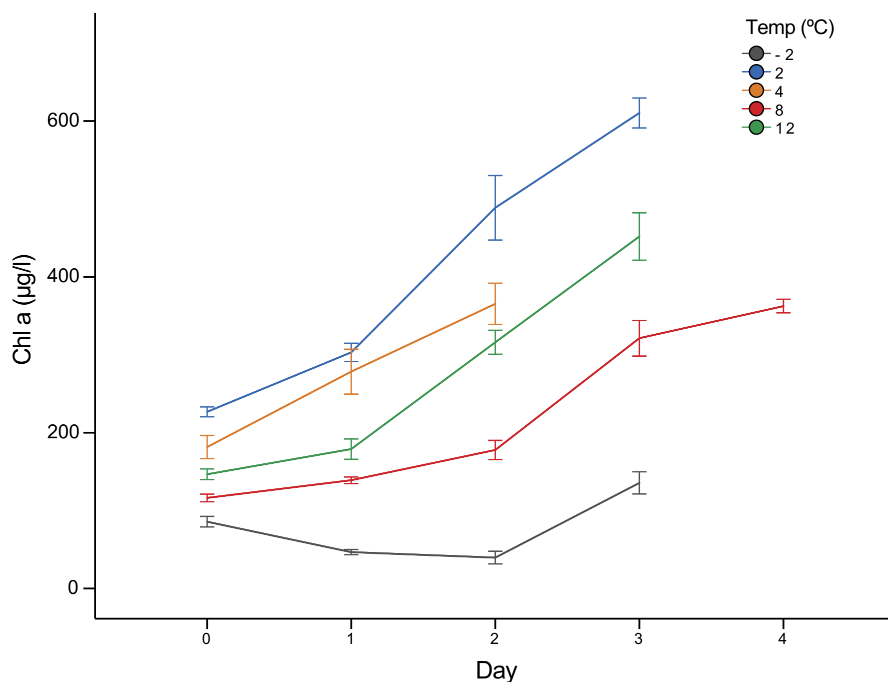


Fig. 1. In vitro chlorophyll *a* (Chl a) daily increase at each cultivation temperature. Data shown is the mean ± SD, *n* = 3. SDs are represented by T-bars.

Table 2
Relative fatty acid content of *Porosira glacialis* (P.g.) at all treatment temperatures. Data shown are averages of replicates ± SD, n = 3. TR = Trace values.

Fatty acid						
	14:0	3.7 ± 0.2	4.4 ± 0.2	5.7 ± 0.3	7.3 ± 0.2	7.8 ± 0.4
	16:0	5.3 ± 0.1	7.3 ± 0.3	8.0 ± 0.2	7.2 ± 0.3	7.4 ± 0.2
	16:1	8.7 ± 0.4	12.8 ± 0.4	10.3 ± 0.3	11.6 ± 0.2	11.6 ± 0.3
	16:2	2.09 ± 0.07	2.7 ± 0.1	2.68 ± 0.08	3.97 ± 0.05	4.7 ± 0.2
	16:3	8.6 ± 0.2	8.3 ± 0.3	16.5 ± 0.4	19.8 ± 0.2	16.3 ± 0.4
	16:4	14.4 ± 0.4	14.8 ± 0.5	10.5 ± 0.3	4.9 ± 0.2	4.8 ± 0.1
	18:0	0.23 ± 0.02	0.69 ± 0.09	0.90 ± 0.07	0.7 ± 0.1	0.95 ± 0.07
	18:1	0.18 ± 0.01	0.25 ± 0.02	0.44 ± 0.09	0.40 ± 0.03	0.39 ± 0.02
	18:2	0.25 ± 0.01	0.13 ± 0.01	0.09 ± 0.01	0.26 ± 0.01	0.31 ± 0.01
	18:3	1.18 ± 0.05	1.8 ± 0.1	1.83 ± 0.05	1.65 ± 0.04	1.75 ± 0.06
	18:4	10.1 ± 0.4	9.6 ± 0.4	6.5 ± 0.3	5.3 ± 0.2	3.9 ± 0.2
	20:5	40.4 ± 1.2	32.6 ± 1.2	31.4 ± 0.9	31.0 ± 0.7	33.8 ± 0.9
	22:6	4.9 ± 0.3	4.5 ± 0.3	4.9 ± 0.2	5.5 ± 0.3	6.0 ± 0.4
	24:0	*TR*	0.24 ± 0.05	0.31 ± 0.04	0.33 ± 0.09	0.4 ± 0.1
	Temperature (°C)	-2	2	4	8	12

4. Discussion

4.1. Temperature and growth

The algal culture displayed positive growth at all temperatures during the course of the experiment. The growth rates in Table 1 showed highest and lowest growth at 12 and − 2 °C, respectively, a result that follows the consensus of phytoplankton in general: Within a temperature range, diatom growth rates increase with temperature toward the species' optimum growth temperature [39–41]. A Tukey's test, however, revealed no statistical difference between growth rates at all temperatures other than −2 °C, indicating that from 2 to 12 °C the growth rate was independent of temperature. While this observation could be influenced by the large variation observed in the error variance of the measured growth rates, previous studies on cold-water diatom strains using comparable light regimes have also found that lowering temperatures within a species' natural temperature range do not necessarily slow growth: Teoh, Phang [12] found that the optimum cultivation temperature for an Antarctic strain of *Navicula* sp. was 4 °C (μ ≈ 0.35), with growth slowing drastically already at 6 °C. They did not try to cultivate at lower temperatures. Boelen, van Dijk [42] cultivated an Antarctic strain of *Chaetoceros brevis* and found higher growth rates at 3 °C compared to 7 °C (μ = 0.47 and 0.41, respectively). In these studies, the differences in the growth rates were more pronounced than in our data, indicating that *P. glacialis* has a wider temperature range than both *Navicula* sp. and *C. brevis,* with a potential for yearlong cultivation in areas with pronounced seasons.

4.2. Temperature and lipids

The total lipid content was highest ($p < 0.05$) in the samples harvested at the lowest temperatures. Other studies have found ambiguous effects of temperature on lipid content on diatoms [14,15,43], implying that responses to temperature are species specific and do not follow general trends. For the strain of *P. glacialis* studied here, the total lipid content was inversely correlated with cultivation temperature. The lipid content reported here is comparable to or higher than those found in other cultivated cold-water diatoms [12], but lower than those often reported for temperate and warm water cultivations [43,44]. However, it is important to keep in mind that this strain of *P. glacialis* was harvested while still in the exponential growth phase, while lipid accumulation as observed in other studies is a result of the algae entering the stationary phase. The high lipid content often observed in such batch cultures comes at the expense of FA chain length and desaturation

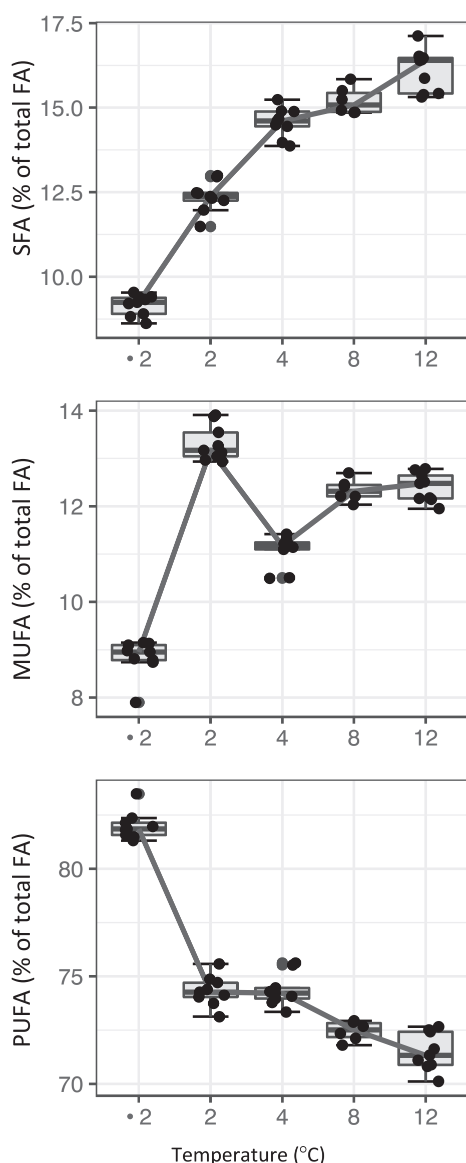


Fig. 2. Trends among saturated (SFA, a), monounsaturated (MUFA, b) and polyunsaturated fatty acids (PUFA, c). Data shown is the mean ± SD, $n = 9$. T-bars represent the SD for each sample.

[45] and protein content [46]. This has the unfortunate effect of reducing the versatility of the feed, as it would resemble those of terrestrial products such as soy- or rapeseed in FA composition, instead of offering a complete substitute for fish oil.

4.3. Desaturation

The degree of desaturation was also inversely correlated with temperature (see Fig. 2), with SFAs increasing and PUFAs decreasing with temperature. Although the inverse correlation of PUFAs with temperature is strongly exaggerated by the large EPA content in the sample cultivated at −2 °C, the composition of the FA fraction did change notably with temperature: At both −2 and 2 °C, the five most abundant fatty acids were 20:5, 16:4, 18:4, 16:3 and 16:1. At 8 and 12 °C, the five most abundant fatty acids were 20:5, 16:3, 16:1, 16:0 and 14:0. At all temperatures, the five most abundant fatty acids constituted > 75% of the total fatty acids. While this clearly shows that increasing the temperature increases saturation, it should be noted that the PUFA fraction dominated the fatty acids at all temperatures, constituting 71.49% of total FAs even at 12 °C. Furthermore, EPA was not observed to decrease with temperature from 2 to 12 °C, indicating that *P. glacialis* depends heavily on functional EPA during the growth phase, independently of temperature. These findings correlate well with a study by Gillan et al. [47], in which *Stauroneis amphioxys* was cultivated at 3 and 20 °C; while the ratio of the most desaturated fatty acids (16:4, 18:4, 20:5 and 22:6) to their equivalents with one less double bond was higher at 3 °C, there was no difference in the total amounts of PUFAs at the two growth temperatures.

Interestingly, the amount of 22:6 increased with temperature from −2 to 12 °C (4.9% at −2 °C to 6.0% at 12 °C, $p < 0,05$), a result that is in direct opposition with other findings on diatoms [16,48]. With a minimum content of 36.3% EPA + DHA at 4 °C, and a maximum content of 45.3% at −2 °C, this diatom contains far more LC-PUFA compared to most other autotrophic species of microalgae suggested for mass production [49]. õAlthough the PUFA content was highest at −2 °C, the low growth rate observed and the increased difficulty associated with cultivation at this temperature reduces the potential for industrial production of PUFAs. However, by displaying growth at sub-zero temperatures, this strain represents an exciting potential for production of cold-adapted bioactive molecules for e.g. the pharmaceutical industry. In comparison, both 2 °C and 12 °C displayed the highest growth rates, as well as high contents of EPA. Although there was some variation in the composition of the other PUFAs, the total PUFA content only varied by 2.92 percentage points from 2 to 12 °C. Based on these data, a high-quality feed especially rich in EPA can be produced at a large range of temperatures. Whether or not this production is economically feasible requires increased knowledge of the potential production and the associated costs in a large-scale production, which goes beyond the scope of this study. Therefore, future research should focus on optimizing growth or lipid content in an economically feasible manner, for example through increasing the relative concentration of CO_2 by addition of flue gas to the culture medium. While it would also have been interesting to investigate the growth and fatty acid composition of *P. glacialis* at higher temperatures, this specific strain has not been capable of maintaining growth at temperatures above 15 °C for a significant amount of time, also when temperatures have been gradually increased from lower temperatures.

4.4. Conclusion

To conclude, *P. glacialis* is a potential species for mass cultivation of diatoms. Its broad temperature range is well adapted for cultivation in cold areas with pronounced seasons and allows for yearlong cultivation at ambient sea-temperatures. The fatty acid composition was predominantly polyunsaturated, with EPA as the most abundant fatty acid at all temperatures. This makes *P. glacialis* an excellent source of marine fatty acids as a substitute for the conventional fish oil used in aquaculture feed, or as ingredients in other high-value products.

Acknowledgments

This research was funded by the Tromsø County Municipality (project TFK2013-262). The publishing costs were funded by UiT the Arctic University of Norway.

Authors contribution

Conception: Hans Christian Eilertsen.
Data collection: Jon Brage Svenning, Lars Dalheim, Terje Vasskog.
Data analysis and interpretation: Jon Brage Svenning, Lars Dalheim.
Drafting the article: Jon Brage Svenning, Lars Dalheim.
Critical revision of the article: Hans Christian Eilertsen, Terje Vasskog.
Final approval of the version to be published: Jon Brage Svenning, Lars Dalheim, Hans Christian Eilertsen, Terje Vasskog.

Declaration of authors' agreement

All authors agree to the authorship and submission of the manuscript for peer review.

Conflicts of interest

The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results. There are no conflicts, informed consent, human or animal rights applicable.

References

[1] D.G. Mann, P. Vanormelingen, An inordinate fondness? The number, distributions, and origins of diatom species, J. Eukaryot. Microbiol. 60 (4) (2013) 414–420.
[2] M. Degerlund, H.C. Eilertsen, Main species characteristics of phytoplankton spring blooms in NE Atlantic and Arctic waters (68-80A degrees N), Estuar. Coasts 33 (2) (2010) 242–269.
[3] P.B. Kelly, R. Reiser, D.W. Hood, The origin of the marine polyunsaturated fatty acids - composition of some marine plankton, J. Am. Oil Chem. Soc. 36 (3) (1959) 104–106.
[4] M. Certik, S. Shimizu, Biosynthesis and regulation of microbial polyunsaturated fatty acid production, J. Biosci. Bioeng. 87 (1) (1999) 1–14.
[5] T. Wichard, A. Gerecht, M. Boersma, S.A. Poulet, K. Wiltshire, G. Pohnert, Lipid and fatty acid composition of diatoms revisited: rapid wound-activated change of food quality parameters influences herbivorous copepod reproductive success, Chembiochem 8 (10) (2007) 1146–1153.
[6] J. Klungsoyr, S. Tilseth, S. Wilhelmsen, S. Falkpetersen, J.R. Sargent, Fatty-acid composition as an indicator of food-intake in cod larvae (*Gadus morhua*) from Lofoten, northern Norway, Mar. Biol. 102 (2) (1989) 183–188.
[7] L.A. Horrocks, Y.K. Yeo, Health benefits of docosahexaenoic acid (DHA), Pharmacol. Res. 40 (3) (1999) 211–225.
[8] S.M. Innis, Dietary (n-3) fatty acids and brain development, J. Nutr. 137 (4) (2007) 855–859.
[9] P.M. Kris-Etherton, W.S. Harris, L.J. Appel, C. Nutrition, Fish consumption, fish oil, omega-3 fatty acids, and cardiovascular disease, Circulation 106 (21) (2002) 2747–2757.
[10] P.M. Kris-Etherton, J.A. Grieger, T.D. Etherton, Dietary reference intakes for DHA and EPA, Prostaglandins Leukot. Essent. Fatty Acids 81 (2–3) (2009) 99–104.
[11] V. Pasquet, L. Ulmann, V. Mimouni, F. Guiheneuf, B. Jacquette, A. Morant-Manceau, G. Tremblin, Fatty acids profile and temperature in the cultured marine diatom *Odontella aurita*, J. Appl. Phycol. 26 (6) (2014) 2265–2271.
[12] M.L. Teoh, S.M. Phang, W.L. Chu, Response of Antarctic, temperate, and tropical microalgae to temperature stress, J. Appl. Phycol. 25 (1) (2013) 285–297.
[13] V.J. Dodson, J.L. Mouget, J.L. Dahmen, J.D. Leblond, The long and short of it: temperature-dependent modifications of fatty acid chain length and unsaturation in the galactolipid profiles of the diatoms *Haslea ostrearia* and *Phaeodactylum tricornutum*, Hydrobiologia 727 (1) (2014) 95–107.
[14] S.D. Araujo, V.M.T. Garcia, Growth and biochemical composition of the diatom *Chaetoceros* cf. *wighamii* Brightwell under different temperature, salinity and carbon dioxide levels. I. Protein, carbohydrates and lipids, Aquaculture 246 (1–4) (2005) 405–412.
[15] S.M. Renaud, H.C. Zhou, D.L. Parry, L.V. Thinh, K.C. Woo, Effect of temperature on the growth, total lipid content and fatty acid composition of recently isolated tropical microalgae *Isochrysis* sp, *Nitzschia closterium*, *Nitzschia paleacea*, and commercial species *Isochrysis* sp (clone T ISO), J. Appl. Phycol. 7 (6) (1995) 595–602.
[16] S.H. Mortensen, K.Y. Borsheim, J.R. Rainuzzo, G. Knutsen, Fatty-acid and elemental composition of the marine diatom *Chaetoceros gracilis* Schutt - effects of silicate deprivation, temperature and light-intensity, J. Exp. Mar. Biol. Ecol. 122 (2) (1988) 173–185.
[17] G.Q. Chen, Y. Jiang, F. Chen, Variation of lipid class composition in *Nitzschia laevis* as a response to growth temperature change, Food Chem. 109 (1) (2008) 88–94.
[18] H.M. Jiang, K.S. Gao, Effects of lowering temperature during culture on the production of polyunsaturated fatty acids in the marine diatom *Phaeodactylum tricornutum* (Bacillariophyceae), J. Phycol. 40 (4) (2004) 651–654.
[19] M.M. Joseph, K.R. Renjith, G. John, S.M. Nair, N. Chandramohanakumar, Biodiesel prospective of five diatom strains using growth parameters and fatty acid profiles, Biofuels U. K. 8 (1) (2017) 81–89.
[20] A. Ozkan, G.L. Rorrer, Lipid and chitin nanofiber production during cultivation of the marine diatom *Cyclotella* sp to high cell density with multistage addition of silicon and nitrate, J. Appl. Phycol. 29 (4) (2017) 1811–1818.
[21] L. Rodolfi, N. Biondi, A. Guccione, N. Bassi, M. D'Ottavio, G. Arganaraz, M.R. Tredici, Oil and eicosapentaenoic acid production by the diatom *Phaeodactylum tricornutum* cultivated outdoors in Green Wall Panel (GWP (R)) reactors, Biotechnol. Bioeng. 114 (10) (2017) 2204–2210.
[22] H. Chtourou, I. Dahmen, A. Jebali, F. Karray, I. Hassairi, S. Abdelkafi, H. Ayadi, S. Sayadi, A. Dhouib, Characterization of *Amphora* sp., a newly isolated diatom wild strain, potentially usable for biodiesel production, Bioprocess Biosyst. Eng. 38 (7) (2015) 1381–1392.
[23] K. Fahl, G. Kattner, Lipid-content and fatty-acid composition of algal communities in sea-ice and water from the Weddell Sea (Antarctica), Polar Biol. 13 (6) (1993) 405–409.
[24] J.H. Skerratt, P.D. Nichols, T.A. Mcmeekin, H. Burton, Seasonal and interannual changes in planktonic biomass and community structure in eastern Antarctica using signature lipids, Mar. Chem. 51 (2) (1995) 93–113.
[25] D.S. Nichols, P.D. Nichols, C.W. Sullivan, Fatty-acid, sterol and hydrocarbon composition of Antarctic Sea-ice diatom communities during the spring bloom in McMurdo Sound, Antarct. Sci. 5 (3) (1993) 271–278.
[26] R.J. Henderson, E.N. Hegseth, R.T. Park, Seasonal variation in lipid and fatty acid composition of ice algae from the Barents Sea, Polar Biol. 20 (1) (1998) 48–55.
[27] D.R. Tocher, Omega-3 long-chain polyunsaturated fatty acids and aquaculture in perspective, Aquaculture 449 (2015) 94–107.
[28] T. Ytrestoyl, T.S. Aas, T. Asgard, Utilisation of feed resources in production of Atlantic salmon (*Salmo salar*) in Norway, Aquaculture 448 (2015) 365–374.
[29] J.A. Emery, F. Norambuena, J. Trushenski, G.M. Turchini, Uncoupling EPA and DHA in fish nutrition: dietary demand is limited in Atlantic salmon and effectively met by DHA alone, Lipids 51 (4) (2016) 399–412.
[30] J.G. Bell, J. McEvoy, D.R. Tocher, F. McGhee, P.J. Campbell, J.R. Sargent, Replacement of fish oil with rapeseed oil in diets of Atlantic salmon (*Salmo salar*) affects tissue lipid compositions and hepatocyte fatty acid metabolism, J. Nutr. 131 (5) (2001) 1535–1543.
[31] M.A. Borowitzka, Microalgae for aquaculture: opportunities and constraints, J. Appl. Phycol. 9 (5) (1997) 393–401.
[32] J.K. Wang, M. Seibert, Prospects for commercial production of diatoms, Biotechnol. Biofuels 10 (2017).
[33] C.R. Tomas, G.R. Hasle, E.E. Syvertsen, K.A. Steidinger, K. Tangen, J. Throndsen, B.R. Heimdal, Marine diatoms, Identifying Marine Phytoplankton, Academic Press, 1997.
[34] O. Holm-Hansen, B. Riemann, Chlorophyll *a* determination - improvements in methodology, Oikos 30 (3) (1978) 438–447.
[35] J. Folch, M. Lees, G.H.S. Stanley, A simple method for the isolation and purification of total lipides from animal tissues, J. Biol. Chem. 226 (1) (1957) 497–509.
[36] E. Cequier-Sanchez, C. Rodriguez, A.G. Ravelo, R. Zarate, Dichloromethane as a solvent for lipid extraction and assessment of lipid classes and fatty acids from samples of different natures, J. Agric. Food Chem. 56 (12) (2008) 4297–4303.
[37] W. Stoffel, F. Chu, E.H. Ahrens, Analysis of long-chain fatty acids by gas-liquid chromatography - micromethod for preparation of methyl esters, Anal. Chem. 31 (2) (1959) 307–308.
[38] E.Y. Artamonova, T. Vasskog, H.C. Eilertsen, Lipid content and fatty acid composition of *Porosira glacialis* and *Attheya longicornis* in response to carbon dioxide (CO2) aeration, PLoS One 12 (5) (2017).
[39] R.W. Eppley, Temperature and phytoplankton growth in sea, Fish B-Noaa 70 (4) (1972) 1063–1085.
[40] J.C. Goldman, Ej Carpente, Kinetic approach to effect of temperature on algal growth, Limnol. Oceanogr. 19 (5) (1974) 756–766.
[41] D.J.S. Montagnes, D.J. Franklin, Effect of temperature on diatom volume, growth rate, and carbon and nitrogen content: reconsidering some paradigms, Limnol. Oceanogr. 46 (8) (2001) 2008–2018.
[42] P. Boelen, R. van Dijk, J.S.S. Damste, W.I.C. Rijpstra, A.G.J. Buma, On the potential application of polar and temperate marine microalgae for EPA and DHA production, AMB Express 3 (2013) 9.
[43] Y.C. Chen, The biomass and total lipid content and composition of twelve species of marine diatoms cultured under various environments, Food Chem. 131 (1) (2012) 211–219.
[44] G. D'Ippolito, A. Sardo, D. Paris, F.M. Vella, M.G. Adelfi, P. Botte, C. Gallo, A. Fontana, Potential of lipid metabolism in marine diatoms for biofuel production, Biotechnol. Biofuels 8 (2015).
[45] Y. Liang, K. Mai, Effect of growth phase on the fatty acid compositions of four species of marine diatoms, J. Ocean Univ. China 4 (2) (2005) 157–162.
[46] G.C. de Vicose, A. Porta, M.P. Viera, H. Fernandez-Palacios, M.S. Izquierdo, Effects of density on growth rates of four benthic diatoms and variations in biochemical composition associated with growth phase, J. Appl. Phycol. 24 (6) (2012) 1427–1437.
[47] F.T. Gillan, G.I. Mcfadden, R. Wetherbee, R.B. Johns, Sterols and fatty-acids of an Antarctic Sea ice diatom, *Stauroneis amphioxys*, Phytochemistry 20 (8) (1981) 1935–1937.
[48] P.A. Thompson, M.X. Guo, P.J. Harrison, J.N.C. Whyte, Effects of variation in temperature .2. On the fatty-acid composition of 8 species of marine-phytoplankton, J. Phycol. 28 (4) (1992) 488–497.
[49] E. Peltomaa, M.D. Johnson, S.J. Taipale, Marine cryptophytes are great sources of EPA and DHA, Mar. Drugs 16 (1) (2018).

Paper II

Dalheim, L., Svenning, J.B., Eilertsen, H.C., Vasskog, T. & Olsen, R.L. (2020).

Stability of lipids during wet storage of the marine diatom *Porosira glacialis* under semi-preserved conditions at 4 and 20 °C.

Journal of Applied Phycology, 33, 385-395.

Journal of Applied Phycology
https://doi.org/10.1007/s10811-020-02292-0

Stability of lipids during wet storage of the marine diatom *Porosira glacialis* under semi-preserved conditions at 4 and 20 °C

Lars Dalheim[1] · Jon B. Svenning[1] · Hans C. Eilertsen[1] · Terje Vasskog[2] · Ragnar L. Olsen[1]

Received: 2 July 2020 / Revised and accepted: 30 September 2020
© The Author(s) 2020

Abstract

Cultivation of diatoms may help alleviate the pressure on wild fish stocks for marine nutrient availability in aquaculture feed and for human consumption. However, the lipids in microalgae biomass are easily deteriorated, both trough lipolysis and degradation of polyunsaturated fatty acids (PUFA). Proper storage conditions are therefore necessary to maintain the lipid quality. Additionally, the storage conditions must have a low cost and facilitate further processing of the biomass. In this study, we investigated the formation of free fatty acids, changes in lipid classes, and fatty acid composition of the psychrophilic marine diatom *Porosira glacialis* under storage. The wet biomass was stored for 14 days at 4 and 20 °C with either heat treatment, formic acid, or benzoic acid addition, and a control sample. Heat-treated and formic acid samples had the lowest rate of free fatty acid formation during storage. Mainly, polar lipids were hydrolyzed to free fatty acids and this occurred fastest at 20 °C. The fatty acid composition remained stable in heat-treated samples during storage, whereas a loss of PUFA was observed in the other treatments. The lack of effect from benzoic acid indicates that the loss of lipid quality stems from endogenous enzymes rather than exogenous organisms. Heat treatment and formic acid appeared to effectively reduce lipase activity, and potentially lipoxygenase and similar enzymes that affect the fatty acids. The low pH of the formic acid samples seems to have a negative effect on the PUFA content, in particular at 20 °C.

Keywords Storage stability · Lipid · Omega-3 · Pre-treatment · Psychrophilic diatom · Microalgae

Introduction

The limit for sustainable utilization of wild fish stocks has been reached (FAO 2020), and there is a need for novel sources of marine nutritional components, especially the long-chain polyunsaturated omega-3 fatty acids (LC-PUFA n-3). Marine microalgae are the main de novo producers of the LC-PUFA n-3, eicosapentaenoic acid (EPA, 20:5n-3), and docosahexaenoic acid (DHA, 22:6n-3) (Khozin-Goldberg et al. 2011), which are important components in aquaculture feed (Tocher 2015; Sprague et al. 2016) and for human health (Kris-Etherton et al. 2009; Salem and Eggersdorfer 2015). In addition to omega-3 fatty acids, marine microalgae contain valuable pigments and other bioactive compounds (Cuellar-Bermudez et al. 2015; Kuczynska et al. 2015). Diatoms, the largest group of microalgae, have potentially high growth rates and can be cultivated in bioreactors using seawater, light, inorganic nutrients, and CO_2 at a wide range of temperatures (Artamonova et al. 2017b; Botte et al. 2018; Svenning et al. 2019). Large-scale industrial production of microalgae biomass could help alleviate the pressure on wild fish stocks by substituting some of the fishmeal and fish oil in the aquaculture feed (Shah et al. 2018) and also be used to produce health supplements for humans (Barkia et al. 2019).

One of the main challenges of large-scale utilization of diatoms is post-harvest storage. The diatom fatty acids are

✉ Lars Dalheim
lars.dalheim@uit.no

Jon B. Svenning
jon.b.svenning@uit.no

Hans C. Eilertsen
hans.c.eilertsen@uit.no

Terje Vasskog
terje.vasskog@uit.no

Ragnar L. Olsen
ragnar.olsen@uit.no

[1] Norwegian College of Fishery Science, UiT The Arctic University of Norway, N-9037 Tromsø, Norway

[2] Department of Pharmacy, UiT The Arctic University of Norway, N-9037 Tromsø, Norway

Published online: 17 October 2020

often highly unsaturated (Dunstan et al. 1994; Chen 2012; Svenning et al. 2019), which makes them prone to oxidation. In addition, endogenous enzymes in the biomass may deteriorate the lipids through lipolysis and oxidation (Fontana et al. 2007; Cutignano et al. 2011). The anti-grazer defense mechanism of diatoms, which is wound-activated, involves breaking down thylakoid lipids to produce volatile polyunsaturated aldehydes and other oxylipins (Pohnert 2002; Cutignano et al. 2006; Caldwell 2009). Cell rupture, which can occur during harvesting or because of autolytic reactions during storage, may initiate these processes. Another consequence of cell rupture is the formation of exudates, which may act as a growth medium for other microorganisms such as bacteria and fungi. Optimizing storage conditions are therefore essential for large-scale utilization of microalgae.

The storage conditions must be cost-effective for the microalgae to be a viable replacement for fish products in aquaculture feed. Large-scale freezing facilities are expensive and freezing and thawing the biomass for further processing may pose a challenge due to cell lysis. Pre-treatment of the biomass before storage in refrigerated rooms may be a more feasible solution to the storage problem. However, pre-treatments such as drying are costly (Hosseinizand et al. 2017; Wendt et al. 2019), and evaluation of cheaper alternatives are therefore important. Inhibition of enzyme activity could be a potential solution for storage of microalgae biomass. Cheap ways of denaturing and inhibiting enzymes are heat treatment or lowering of pH by adding an acid (Budge and Parrish 1999; Seyhan et al. 2002). Previous studies have found reduction in lipase activity by adding boiling water directly to the biomass (Berge et al. 1995; Budge and Parrish 1999) or by heating sample tubes in a water bath (Balduyck et al. 2019). Organic acids, such as formic acid and benzoic acid, may also inhibit bacterial and fungal growth (Heasman et al. 2000; Olsen and Toppe 2017; Cabezas-Pizarro et al. 2018).

In this study, the large centric psychrophilic diatom *Porosira glacialis* was cultivated using CO_2-containing flue gas from an industrial plant. The fatty acid profile of this diatom is highly unsaturated (Svenning et al. 2019) and could therefore be a valuable source of marine fatty acids for aquaculture feed and human consumption. The goal of this study was to investigate the best storage conditions, applicable for industrial use, to reduce lipid deterioration. The pre-treatments with formic acid (enzyme inhibiting and antimicrobial), benzoic acid (antimicrobial), and heat treatment (denaturing of endogenous enzymes) was compared with control samples without pre-treatment during storage for 14 days at 4 and 20 °C. The lipid quality was assessed in terms of lipolysis, lipid class, and fatty acid composition.

Materials and methods

Materials

Dichloromethane (DCM) (≥ 99.9%), hexane (≥ 99%), sulfuric acid (95–97%) were from Sigma-Aldrich (USA). LC-MS grade methanol (MeOH) was from Fisher Scientific (UK). Acetic acid (≥ 99.8%) was from Honeywell Fluka (USA). Sodium bicarbonate, sodium acetate, isooctane (LC-grade), ethyl acetate (LC-grade), acetone (LC-grade), and isopropanol (LC-grade) were obtained from Merck (Germany). Kristalon Flower® was obtained from Yara (Oslo, Norway). Sodium metasilicate pentahydrate was obtained from Permakem A/S (Norway).

Lipid standards for HPLC analysis DGTS (1,2-dipalmitoyl-sn-glycero-3-O-4′-(N,N,N-trimethyl)-homoserine), SQDG (sulfoquinovosyldiacylglycerol), and phosphatidylinositol were purchased from Sigma Aldrich. Phosphatidylcholine (PC; 1,2-dimyristoyl-sn-glycero-3-phosphatidylcholine), phosphatidylglycerol (PG; 1,2-dimyristoyl-sn-glycero-3-phosphatidylglycerol Na salt), phosphatidylserine (PS; 1,2-dipalmitoyl-sn-glycero-3-phosphatidylserine Na salt), phosphatidylethanolamine (PE; 1,2-dimyristoyl-sn-glycero-3-phosphatidylethanolamine), MGDG (hydrogenated monogalactosyldiacylglycerol), DGDG (hydrogenated digalactosyldiacylglycerol), ergosterol, triolein, diolein, and monoolein were purchased from Larodan AB. Ethyl elaidate, 11-eicosenol, and behenyl arachidate were purchased from Nu-Chek-Prep, Inc. Standards for the GC analysis, GLC-502 as free acids, were purchased from Nu-Chek-Prep, Inc.

Cultivation of *Porosira glacialis*

Biomass was harvested from a continuous culture maintained in the exponential growth phase on two occasions to obtain sufficient material for the experimental procedure. Briefly, the monocultures were cultivated in a 6000-L fiberglass vertical column photobioreactor using seawater collected at 25 m depth in the Indre Finnfjordbotn water reservoir. The water was filtered using 1 μm polypropylene filters (Model GX01-9 7/8, GE Power & Water, USA) and added inorganic nutrients in the form of 0.25 mL L^{-1} Substral and 1 mL L^{-1} dissolved silicate (3.5 g $Na_2O_3Si.9H_2O$ L^{-1} in miliQ water). The strain of *P. glacialis* used in this experiment was isolated from arctic waters and identified using SEM (Uradnikova 2020). The reactor, placed outdoors, was subjected to the natural environment of Finnfjordbotn (N 69° 13.76′, E 018° 05.02′), but illuminated with continuous artificial light (LED) at a scalar irradiance of 150–200 μmol quanta m^{-2} s^{-1}. The culture was aerated with either pressurized air or flue gas containing 6–12% CO_2, and maintained at pH 7.4–8.1. Temperature varied between 4 and 8 °C during the entire cultivation, reflecting the ambient seawater temperatures at Finnfjordbotn. The culture condition was monitored on a daily basis by cell counts in

2 mL Nunc-chambers (Nunc A/S, Denmark) and harvested when the density of the culture reached approximately 15 million cells L^{-1}. The harvesting was performed using a continuous solid bowl centrifuge (Model PTDC, Nanjing Kingreat Machinery Company, Jiangsu, China) operated at 835×*g*, the resulting biomass was collected using a spatula.

Storage conditions

Samples with 20% DM were given different pretreatments; a batch of microalgae biomass was added 0.1% benzoic acid (pH 6.9) and divided into 24 samples, another batch of microalgal biomass were added formic acid to a pH of 3.0 and divided into 24 samples; 24 samples were heated to 70 °C for 30 min and 24 samples were untreated (control). Triplicates of the samples were stored in capped 15 mL centrifuge tubes (VWR International, USA) at 4 and 20 °C for 1, 3, 7, and 14 days before lipid extraction and analysis.

In a follow-up study, higher temperature pre-treatments were investigated to see how it would affect lipolysis in the samples during storage. Frozen biomass was divided into 15 mL centrifuge tubes and heated for 30 min at 70, 80, or 90 °C. The samples were stored in triplicates for 7 and 14 days at 20 °C along with triplicates of untreated control samples.

Lipid extraction and analysis

The extraction procedure was adapted from Folch et al. (1957), using a mixture of dichloromethane and methanol as the solvent (Cequier-Sanchez et al. 2008). Briefly, samples were freeze-dried and divided into triplicates of 100 mg in 15 mL centrifuge tubes. Each tube was added 2 mL dichloromethane/methanol (2:1 v/v) and 2 mL 5% NaCl in MiliQ water. The tubes were gently shaken for 30 s by hand and then centrifuged for 10 min at 2000×*g* (Heraus Multifuge 1S-R, Germany). Following centrifugation, the organic phase was transferred to a pre-weighed vial. The extraction procedure was repeated twice and the organic phases were pooled before evaporation using nitrogen. Finally, the extracted lipids were dissolved in dichloromethane/methanol (2:1 v/v) to a concentration of 10 mg mL^{-1}, before methanolysis and methylation using a method adapted from Stoffel et al. (1959) with sulfuric acid as the catalyst. Dissolved extract (100 μL) was transferred to a test tube (Duran, Millville, USA) along with 100 μL internal standard (0.1 mg mL^{-1}) and 800 μL dichloromethane. The samples were then added 2 mL 10% H_2SO_4 in methanol and placed at 100 °C for 1 h. Finally, 3 mL hexane and 3 mL 5% NaCl in MiliQ water were added and the samples were shaken. The resulting organic phase containing the fatty acid methyl esters (FAMEs) was evaporated and dissolved in 100 μL hexane before GC-FID analysis.

The GC conditions were as follows: helium as carrier gas (1.6 mL min^{-1}), select FAME column (L 50 m, ID 0.25 mm, and FT 0.25 μm, Agilent J&W GC Columns), the inlet temperature was 240 °C (split 1:50), and the FID temperature was 250 °C. Initially, the GC oven was held at 60 °C for 1 min, then ramped up to 130 °C (30 °C min^{-1}), further up to 195 °C (1.3 °C min^{-1}) and finally up to 240 °C (30 °C min^{-1}) for 10 min. To quantify the fatty acids, calibration curves were made by analyzing the ratio between individual fatty acids at concentrations 7.8125–2000 μg mL^{-1} of GLC 502 free acids (Nu-Chek-Prep, USA) and heptadecanoic acid (Sigma-Aldrich, USA) as internal standard; every concentration was analyzed as triplicates.

Free fatty acids and other lipid classes were separated and quantified using HPLC-ELSD based on a method by Abreu et al. (2017). HPLC analyses were performed on a Waters e2795 separations module, using a SupelcosilLC-SI 5 μm (25 cm × 4.6 mm) column (Supelco, USA) set to a working temperature of 40 °C and 40 μL injection volume. The lipids were quantified using a Waters 2424 ELS detector with the following settings: gain 100, nebulizer 30% heating power level, drift tube 45 °C, and pressure 40 psi. The total run time was 41 min and the gradient profile can be seen in Table 1. Standard curves were made by analyzing 12.5–400 μg mL^{-1} of the lipid classes in triplicates. Both samples and standards were dissolved in mobile phase A/chloroform (4:1).

Table 1 Gradient profile for the HPLC program used for lipid class analysis. Mobile phase A = isooctane/ethyl acetate (99.8:0.2), mobile phase B = acetone/ethyl acetate (2:1) 0.15% acetic acid, and mobile phase C = isopropanol/H_2O (85:15)

Time (min)	Mobile phase A	Mobile phase B	Mobile phase C	Flow (mL/min)	Curve
0.0	100	0	0	1.5	1
1.5	100	0	0	1.5	6
1.6	97	3	0	1.5	6
6.0	94	6	0	1.5	6
8.0	50	50	0	1.5	6
8.1	46	39	15	1.5	6
14.0	43	30	27	1.5	6
14.1	43	30	27	1.5	6
18.0	40	0	60	1.5	6
23.0	40	0	60	1.5	6
24.0	0	100	0	1.5	6
25.0	0	100	0	2.0	6
27.0	0	100	0	2.0	6
27.1	100	0	0	2.0	6
36.0	100	0	0	2.0	6
36.1	100	0	0	1.5	6

Statistical analysis and data availability

All analyses were performed in triplicates. The data in tables are presented as means ± standard deviation. All analyses and figures were prepared using R v3.6.1. Means were considered statistically significant if $P < 0.05$ using the pairwise Tukey test. All data and the R markdown for this experiment are available from the Open Science Framework (OSF (https://osf.io/6nu9t/?view_only=a6a240b97c3c4d518a2e2222de901772)) under the name "Stability of lipids during wet storage of the marine diatom *Porosira glacialis* under semi-preserved conditions at 4 and 20 °C." Supplementary data containing heat maps of statistical test results can be found in the supplementary data folder using the OSF link. All changes within and differences between treatments presented in the text part of the "Results" section are statistically significant ($P < 0.05$).

Results

The lipid yield for each sample varied between 6.4 and 8.8% of dry weight (DW), see Table 2. There was no trend for loss of lipids during storage.

Figure 1 shows the evolution of formation of free fatty acids (FFA) content over time; day 1 represents 24 h of storage. At 4 °C day 1, the control samples contained 31% FFA, which increased to 66% after 14 days of storage. In the benzoic acid samples, the amount increased from 29 to 65% in the same time span at 4 °C. The formic acid and the heat-treated samples at 4 °C had the lowest relative amount of FFA at day 1 with 14 and 16%, which increased to 35% in formic acid samples after 14 days. After 24 h of storage at 20 °C, the control and benzoic acid samples contained as much as 70 and 65% FFA, which after 2 weeks increased to 79 and 81%, respectively. The FFA of the formic acid treated samples at 20 °C increased from 26 at day 1 to 53% after 14 days. The corresponding values in the heat-treated samples were 12 and 60%. In a separate experiment, *P. glacialis* biomass was heated to 70, 80, or 90 °C for 30 min (Table 3). The control samples contained 7% FFA at day 0, which increased to 84% after 7 days of storage at 20 °C. After storage at 20 °C for 7 days, the FFA content was 68% in lipids extracted from samples heated to 70 and 80 °C. The samples heated to 90 °C contained 25 and 40% FFA after 7 and 14 days of storage at 20 °C, respectively.

The lipid class composition for each time point and treatment is presented in Table 4 to study which lipid class was more prone to lipolysis (day 1 represents 24 h of storage). The polar lipid classes appear to be the main origin of the free fatty acids formed during storage. The control and the benzoic acid samples at 4 °C contained 28 and 31% MGDG after 1 day of storage, and this decreased to 4 and 5% after 14 days, respectively. The same two samples stored at 20 °C had a MGDG content of only 2 and 3% after 1 day of storage and was depleted at day 3 for the control and day 7 for the benzoic acid samples. The formic acid samples contained 25 and 22% MGDG after 1 day of storage at 4 and 20 °C, respectively. After 14 days of storage, these values had decreased to 11 and 4%. The amount of MGDG in heat-treated samples decreased from 30 to 10% at 20 °C during 14 days of storage. In both the control and the benzoic acid samples at 4 °C, PG decreased from 25 to 10% from day 1 to 14. Similarly, for the same samples at 20 °C, PG was reduced from 12 and 11% to depletion in both samples after 7 days of storage. Heat-treated samples contained 44% PG at 4 °C after 1 day of storage, which decreased to 40% after 14 days. At 20 °C, the content of PG changed from 38% to zero from day 1 to day 14. The amount of PG was lower in the formic acid samples compared with the other treatments. In formic acid samples, the amount of PG decreased from 4 to 1% during storage at 4 °C and from 3 to 0% at 20 °C.

PC was only detected in samples treated with formic acid. The content of PC at day 1 was 10%, which decreased to 6% during storage at 4 °C. At 20 °C, 4% PC was detected at day 1 with no change during the storage. Additionally, the amount of diacylglycerol (DAG) was much higher in the formic acid samples than the rest.

Table 2 Lipid yield (weight percentage) from day 1 to 14 for both temperatures (mean ± standard deviation, $n = 3$). Control samples had no pre-treatment, 0.1% (w/w) benzoic acid, formic acid to pH 3.0, and heat treatment 70 °C for 30 min

	Lipid yield (%)							
Temperature (° C)	4				20			
Days of storage	1	3	7	14	1	3	7	14
Control	8.6 ± 1.4^{a}	6.9 ± 0.5^{a}	7.6 ± 0.9^{a}	6.6 ± 0.6^{a}	7.4 ± 1.5^{ab}	7.4 ± 0.2^{a}	7.0 ± 0.9^{a}	6.9 ± 0.7^{ab}
Benzoic acid	7.7 ± 0.2^{a}	8.3 ± 1.2^{ab}	8.8 ± 2.0^{a}	7.0 ± 0.4^{a}	7.6 ± 0.8^{ab}	7.3 ± 0.1^{a}	7.0 ± 0.3^{a}	8.0 ± 0.3^{ab}
Formic acid	7.5 ± 0.6^{a}	7.5 ± 0.0^{ab}	7.1 ± 0.3^{a}	7.1 ± 0.4^{a}	6.4 ± 0.3^{a}	7.1 ± 0.1^{a}	7.4 ± 0.2^{a}	6.9 ± 0.4^{a}
Heat treatment	8.2 ± 0.7^{a}	8.7 ± 0.5^{b}	8.1 ± 0.6^{a}	8.8 ± 0.5^{b}	8.5 ± 0.3^{b}	7.6 ± 0.8^{a}	8.8 ± 0.9^{a}	8.1 ± 0.7^{b}

[a, b] Results in the same column sharing superscripted letters are not significantly different ($P \geq 0.05$)

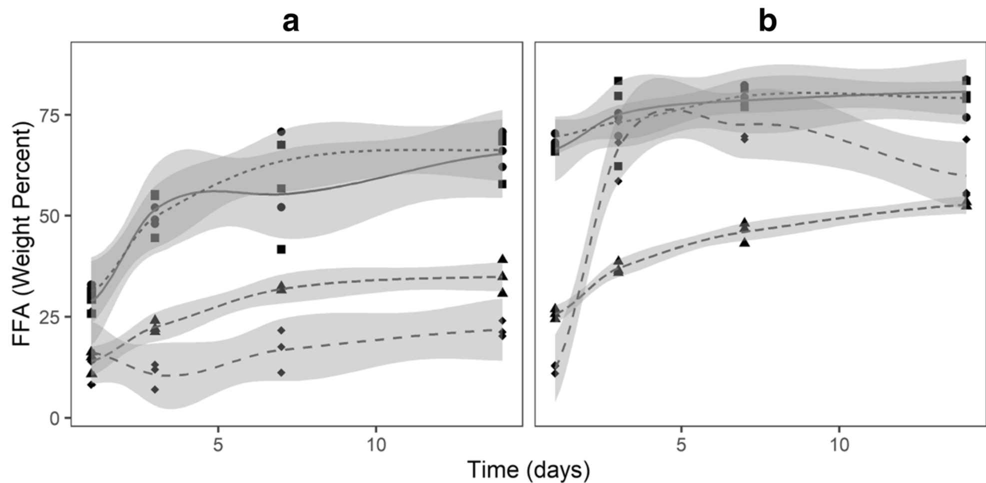


Fig. 1 Development of lipolysis (Free fatty acid (FFA) content, weight percentage) from day 1 to 14 for each treatment (n = 3) at 4 (A) and 20 °C (B). Benzoic acid (0.1% (w/w), square), control (no pre-treatment, circle), formic acid (pH 3.0, triangle), and heat treatment (70 °C for 30 min, diamond)

In general, the relative amount of saturated and monounsaturated fatty acids tended to increase, while the polyunsaturated fatty acids decreased in the control, benzoic acid, and formic acid samples during storage (Table 5). The changes appeared more prominent at 20 °C than at 4 °C. Smaller changes were observed in the heat-treated samples compared with the other three treatments.

For the individual polyunsaturated fatty acids (PUFA), minor changes were observed for C16:4n-1 (hexadecatetraenoic acid (HDTA)) and C20:5n-3 (EPA). At 4 °C, the only significant decrease in HDTA during 14 days of storage occurred in formic acid samples (28–25%). At 20 °C, HDTA decreased from 32 to 29%, 32 to 30%, and 27 to 24% in control, benzoic acid, and formic acid samples, respectively. The relative amount of this fatty acid was stable at both temperatures in heat-treated samples. A decrease from 28 to 26% and 27 to 24% were observed for EPA in control samples at 4 and 20 °C, respectively. For the benzoic acid sample, the decrease in EPA was 28 to 26% and 28 to 27% at 4 and 20 °C, respectively. At 20 °C, the relative amount of EPA in formic acid samples decreased from 32 to 30%. The amount of EPA in heat-treated samples at both temperatures and formic acid samples at 4 °C remained stable during storage.

Figure 2 presents the cumulative loss of PUFA where day 1 represents 24 h of storage. The control and benzoic acid samples contained 80 and 81% PUFA after 1 day of storage at 4 °C. The corresponding samples treated with formic acid contained 81% PUFA and the heat-treated samples contained 79% PUFA. On day 1 at 20 °C, the total amount of PUFA was 78% in the control samples, 80% in the benzoic acid and formic acid samples, and 79% in the heat-treated samples. From day 1 to day 14 of storage, the heat-treated samples were the most stable at both temperatures, with no marked decrease in total amount of PUFA. At 4 °C, the benzoic acid samples lost 3.5% points of PUFA. The formic acid samples had the highest loss at 4.8% points of PUFA from day 1 to 14 of storage at 4 °C. The benzoic acid samples stored at 20 °C lost 3.0% points from day 1 to day 14, whereas the formic acid samples and the control samples lost 5.8 and 6.6% points, respectively.

Discussion

We detected no apparent trends for the effect of pre-treatment or temperature on lipid yield. This is in accordance with previous studies on how storage affects lipid yield in microalgae biomass (Chen et al. 2012; Balduyck et al. 2016, 2017). A previous study (Ryckebosch et al. 2011) on *Phaeodactylum tricornutum* reported lower lipid yield in wet biomass than dried biomass after storage, which they ascribed to FFA formation during storage in wet biomass, however this was based on only one time point so no trends could be interpreted from this.

The amount of FFA increased with time in all samples with varying rate of lipolysis, and not surprisingly, the rate was generally faster at 20 °C than at 4 °C. This has also been reported by Balduyck et al. (2017). They found that loss of cell wall integrity affected the lipolytic processes in the eustigmatophyte *Nannochloropsis oculata* and the haptophyte *Tisochrysis lutea* when stored at 4 and 20 °C,

Table 3 Development of lipolysis (free fatty acid (FFA) content, weight percentage) in the second storage experiment from day 0 to 14 (mean ± standard deviation, $n = 3$) stored at 20 °C. Control samples had no pre-treatment, other samples were heated to 70, 80, or 90 °C

Day	0	7				14
Treatment	Control	Control	70 °C	80 °C	90 °C	90 °C
FFA (%)	7.2 ± 1.2	83.8 ± 2.0	68.3 ± 9.7	68.2 ± 2.8	24.6 ± 1.6	40.8 ± 3.5

Table 4 Lipid class composition (weight percentage) from day 1 to 14 for both temperatures (mean ± standard deviation, $n = 3$). Control samples had no pre-treatment, 0.1% (w/w) benzoic acid, formic acid to pH 3.0, and heat treatment 70 °C for 30 min. *ND*, not detected

	4 °C/20 °C			
	Day 1	Day 3	Day 7	Day 14
Triacylglycerol				
Control	1.9 ± 1.7/4.3 ± 1.0	3.9 ± 0.6/6.2 ± 0.3	2.6 ± 0.3/3.5 ± 0.6	3.6 ± 0.5/2.7 ± 0.5
Benzoic acid	2.5 ± 0.8/5.2 ± 1.0	3.6 ± 0.1/6.4 ± 3.0	3.2 ± 0.7/6.8 ± 1.9	2.9 ± 1.0/3.3 ± 0.7
Formic acid	1.9 ± 0.3/1.9 ± 0.0	1.9 ± 0.1/1.7 ± 0.2	1.7 ± 0.1/1.8 ± 0.1	1.5 ± 0.7/1.6 ± 0.1
Heat treatment	1.7 ± 0.3/2.8 ± 0.9	2.4 ± 0.3/2.0 ± 1.5	1.6 ± 0.2/1.9 ± 0.6	ND/3.2 ± 1.3
Diacylglycerol				
Control	3.2 ± 1.3[a]/2.2 ± 0.4[a]	3.2 ± 1.4[a]/5.8 ± 2.1[*a]	3.3 ± 1.1[a]/2.8 ± 1.3[a]	3.9 ± 0.6[a]/2.2 ± 1.3[a]
Benzoic acid	2.7 ± 1.6[a]/3.3 ± 1.2[ab]	3.9 ± 1.3[a]/6.1 ± 5.2[a]	7.7 ± 8.3[a]/3.2 ± 0.7[a]	3.3 ± 1.4[a]/2.2 ± 1.0[a]
Formic acid	38.4 ± 2.1[b]/36.3 ± 0.5[C]	39.0 ± 1.1[b]/36.8 ± 1.1[b]	37.4 ± 0.3[b]/34.3 ± 1.2[b]	38.3 ± 2.4[b]/32.0 ± 1.1[*b]
Heat treatment	3.7 ± 1.4[a]/5.8 ± 0.8[b]	6.5 ± 1.0[a]/3.1 ± 2.2[a]	5.4 ± 0.9[a]/8.3 ± 0.6[a]	3.7 ± 1.6[a]/15.0 ± 2.7[*C]
Monoacylglycerol				
Control	5.8 ± 0.7[a]/6.6 ± 0.5[a]	6.4 ± 0.1[a]/5.0 ± 0.6[a]	8.2 ± 0.5[a]/4.3 ± 0.5[a]	8.9 ± 1.2[*a]/5.1 ± 2.6[a]
Benzoic acid	4.9 ± 0.1[a]/6.8 ± 0.3[a]	6.1 ± 0.9[ab]/5.2 ± 1.3[a]	6.7 ± 0.4[a]/3.4 ± 0.7[*a]	9.7 ± 0.8[*a]/3.9 ± 1.6[ab]
Formic acid	1.4 ± 0.2[b]/1.4 ± 0.1[b]	1.4 ± 0.0[C]/1.6 ± 0.2[b]	1.6 ± 0.1[b]/1.3 ± 1.1[b]	1.7 ± 0.1[b]/2.0 ± 0.1[b]
Heat treatment	4.7 ± 1.2[a]/4.4 ± 0.6[a]	4.2 ± 0.8[b]/4.2 ± 1.3[a]	4.5 ± 0.4[C]/4.5 ± 0.8[a]	5.6 ± 0.1[C]/6.4 ± 0.2[*a]
Fatty acid esters				
Control	3.2 ± 1.4[a]/3.2 ± 0.4[a]	2.9 ± 1.3[ab]/8.0 ± 0.8[*a]	3.6 ± 1.2[a]/9.7 ± 1.1[*a]	3.4 ± 0.6[a]/10.9 ± 0.6[*a]
Benzoic acid	3.2 ± 1.3[a]/2.8 ± 0.7[ab]	3.5 ± 0.9[a]/5.8 ± 0.7[*b]	2.7 ± 0.2[a]/8.0 ± 0.7[*a]	3.1 ± 0.5[a]/9.9 ± 0.9[*a]
Formic acid	0.9 ± 0.1[b]/0.9 ± 0.0[b]	0.9 ± 0.1[b]/1.0 ± 0.0[C]	0.9 ± 0.0[b]/0.9 ± 0.0[b]	0.9 ± 0.0[b]/0.9 ± 0.0[b]
Heat treatment	2.4 ± 0.7[ab]/3.4 ± 0.3[a]	3.8 ± 0.9[a]/3.0 ± 1.5[C]	4.3 ± 0.5[a]/3.5 ± 0.9[C]	3.8 ± 0.9[a]/5.3 ± 0.7[C]
Monogalactosyldiacylglycerol				
Control	28.0 ± 7.4[a]/2.1 ± 0.5[a]	11.5 ± 0.3[*a]/ND[a]	4.8 ± 3.1[*a]/ND[a]	3.9 ± 1.4[*a]/ND[a]
Benzoic acid	31.3 ± 4.7[a]/2.9 ± 1.2[a]	12.8 ± 2.7[*a]/0.5 ± 0.7[a]	11.0 ± 6.6[*a]/ND[a]	5.1 ± 0.5[*a]/ND[a]
Formic acid	24.9 ± 4.8[a]/21.7 ± 1.0[b]	21.1 ± 1.4[b]/9.6 ± 0.7[*b]	12.2 ± 1.3[*a]/6.1 ± 1.2[*a]	10.5 ± 1.0[*b]/3.5 ± 0.5[*a]
Heat treatment	23.3 ± 7.3[a]/29.6 ± 1.5[b]	27.4 ± 7.1[b]/13.3 ± 1.2[*b]	25.2 ± 4.3[b]/7.5 ± 6.5[*a]	18.8 ± 2.6[C]/10.3 ± 4.0[*b]
Digalactosyldiacylglycerol				
Control	1.6 ± 1.4[a]/0.3 ± 0.6[a]	2.4 ± 0.6[a]/ND[a]	1.2 ± 1.1[a]/ND[a]	0.3 ± 0.5[a]/ND[a]
Benzoic acid	2.1 ± 0.4[a]/1.3 ± 0.6[a]	1.8 ± 1.7[a]/ND[a]	1.1 ± 0.9[a]/ND[a]	0.9 ± 0.8[a]/ND[a]
Formic acid	4.4 ± 0.8[b]/4.5 ± 0.1[b]	4.6 ± 0.8[b]/5.2 ± 0.5[b]	5.9 ± 0.6[*b]/4.4 ± 0.6[b]	5.2 ± 0.2[*b]/2.8 ± 0.1[b]
Heat treatment	4.4 ± 0.4[b]/4.1 ± 1.5[b]	3.2 ± 0.4[ab]/1.7 ± 0.5[*a]	4.0 ± 0.4[C]/0.7 ± 0.6[*a]	6.1 ± 1.5[*b]/ND[a]
Phosphatidylglycerol				
Control	25.2 ± 1.1[a]/11.6 ± 2.2[a]	20.0 ± 1.7[a]/1.8 ± 1.6[*a]	12.9 ± 5.6[*a]/ND[*a]	9.6 ± 1.6[*a]/ND[*a]
Benzoic acid	24.6 ± 1.5[a]/11.1 ± 1.4[a]	16.7 ± 3.3[*a]/1.0 ± 1.7[*a]	12.3 ± 1.1[*a]/ND[*a]	9.7 ± 3.0[*a]/ND[*a]
Formic acid	4.2 ± 0.2[b]/3.2 ± 0.5[a]	1.6 ± 0.4[*b]/1.9 ± 0.6[a]	2.0 ± 1.4[*b]/0.8 ± 0.1[*a]	1.3 ± 0.3[*b]/0.2 ± 0.4[*a]
Heat treatment	43.6 ± 10.8[C]/37.6 ± 1.9[b]	41.9 ± 9.7[C]/6.0 ± 5.6[*a]	38.2 ± 1.3[C]/1.0 ± 1.8[*a]	40.1 ± 6.1[C]/ND[*a]
Phosphatidylcholine				
Control	ND[a]	ND[a]	ND[a]	ND[a]
Benzoic acid	ND[a]	ND[a]	ND[a]	ND[a]
Formic acid	9.9 ± 1.0[b]/4.4 ± 0.2[b]	7.0 ± 0.3[*b]/5.1 ± 0.6[b]	6.3 ± 0.3[*b]/4.5 ± 0.1[b]	5.8 ± 0.4[*b]/4.2 ± 0.3[b]
Heat treatment	ND[a]	ND[a]	ND[a]	ND[a]

[a, b, c] Results in the same column and stored at the same temperature sharing superscripted letters are not significantly different ($P \geq 0.05$)

*Results significantly different from day 1 with the same pre-treatment and stored at the same temperature ($P < 0.05$)

and the lipolytic processes were faster at higher temperatures. Unfortunately, the samples from day 0 in our first study were lost, but it is known that the levels of FFA in microalgae may initially be high (Artamonova et al. 2017a). However, in our

Table 5 Fatty acid composition (weight percentage) from day 1 to 14 for both temperatures (mean ± standard deviation, $n = 3$). Control samples had no pre-treatment, 0.1% (w/w) benzoic acid, formic acid to pH 3.0, and heat treatment 70 °C for 30 min

	4 °C/20 °C			
	Day 1	Day 3	Day 7	Day 14
C14:0				
Control	3.9 ± 0.1[a]/5.7 ± 0.0[a]	4.4 ± 0.4[a]/5.6 ± 0.2[a]	5.2 ± 0.4[a]/6.8 ± 1.5[a]	5.9 ± 0.8[a]/7.4 ± 1.6[a]
Benzoic acid	4.4 ± 0.2[a]/5.1 ± 0.1[b]	4.6 ± 0.1[a]/5.5 ± 0.2[a]	4.7 ± 0.2[a]/5.6 ± 0.1*[ab]	5.4 ± 0.0*[a]/6.2 ± 0.3*[ab]
Formic acid	4.0 ± 0.1[a]/4.2 ± 0.1[c]	4.2 ± 0.2[a]/4.7 ± 0.3[b]	4.5 ± 0.2*[a]/4.8 ± 0.2*[b]	4.5 ± 0.1*[a]/5.3 ± 0.1*[bc]
Heat treatment	5.8 ± 0.4[b]/5.6 ± 0.1[D]	5.5 ± 0.4[b]/4.7 ± 0.3*[b]	5.6 ± 0.1[a]/4.5 ± 0.1[b]	5.5 ± 0.1[a]/4.2 ± 0.2*[c]
C16:0				
Control	4.2 ± 0.0[a]/4.3 ± 0.1[a]	4.1 ± 0.2[a]/4.3 ± 0.0[a]	4.3 ± 0.3[a]/5.5 ± 0.9[a]	5.0 ± 0.7[ab]/5.9 ± 1.2*[a]
Benzoic acid	4.0 ± 0.2[ab]/3.8 ± 0.1[b]	4.3 ± 0.2[a]/4.1 ± 0.2[a]	4.3 ± 0.3[a]/4.4 ± 0.4*[b]	4.7 ± 0.1*[ab]/4.5 ± 0.1*[b]
Formic acid	4.0 ± 0.1[ab]/4.3 ± 0.1[a]	4.3 ± 0.2[a]/5.4 ± 0.4*[b]	4.9 ± 0.1*[a]/5.8 ± 0.1*[a]	5.4 ± 0.1*[a]/6.2 ± 0.0*[a]
Heat treatment	3.8 ± 0.1[b]/4.1 ± 0.0[a]	4.1 ± 0.2[a]/4.0 ± 0.2[a]	4.1 ± 0.1[a]/4.3 ± 0.1[b]	4.1 ± 0.1[b]/4.5 ± 0.0*[b]
C18:0				
Control	1.3 ± 0.0[a]/1.2 ± 0.0[a]	1.2 ± 0.1[a]/1.1 ± 0.0[ab]	1.2 ± 0.1[a]/1.4 ± 0.2[a]	1.4 ± 0.3[a]/1.4 ± 0.3[a]
Benzoic acid	1.1 ± 0.2[b]/0.9 ± 0.1[b]	1.3 ± 0.2[a]/0.9 ± 0.1[a]	1.3 ± 0.2[a]/1.1 ± 0.1[b]	1.2 ± 0.0[a]/1.0 ± 0.1[b]
Formic acid	1.3 ± 0.1[a]/1.3 ± 0.0[a]	1.5 ± 0.1*[a]/1.3 ± 0.1[b]	1.4 ± 0.0[a]/1.2 ± 0.1[ab]	1.4 ± 0.1[a]/1.2 ± 0.0[ab]
Heat treatment	1.0 ± 0.1[b]/1.2 ± 0.0[a]	1.2 ± 0.2[a]/1.1 ± 0.2[ab]	1.2 ± 0.1[a]/1.1 ± 0.1[ab]	1.2 ± 0.0[a]/1.2 ± 0.0[ab]
C16:1n-7				
Control	10.1 ± 0.0[a]/10.8 ± 0.2[a]	10.2 ± 0.0[a]/11.1 ± 0.1[a]	11.0 ± 0.0[ab]/11.9 ± 0.8[a]	11.3 ± 0.3[a]/13.5 ± 2.2*[a]
Benzoic acid	10.1 ± 0.1[a]/10.4 ± 0.0[b]	10.3 ± 0.0[a]/10.9 ± 0.1[a]	10.6 ± 0.2[a]/11.1 ± 0.3*[ab]	11.7 ± 0.1*[a]/11.6 ± 0.7*[ab]
Formic acid	9.7 ± 0.3[b]/10.7 ± 0.1[ab]	10.5 ± 0.3*[a]/12.3 ± 0.6*[b]	11.7 ± 0.3*[b]/12.7 ± 0.2*[c]	12.4 ± 0.1*[a]/13.5 ± 0.1*[a]
Heat treatment	10.5 ± 0.1[c]/10.5 ± 0.0[ab]	10.5 ± 0.1[a]/10.6 ± 0.2[a]	10.5 ± 0.1[a]/11.0 ± 0.1[b]	10.5 ± 0.0[a]/11.2 ± 0.2*[b]
C16:2n-4				
Control	2.4 ± 0.0[a]/2.5 ± 0.1[a]	2.5 ± 0.0[a]/2.5 ± 0.0[a]	2.5 ± 0.0[a]/2.4 ± 0.1[a]	2.6 ± 0.0[a]/2.5 ± 0.1[a]
Benzoic acid	2.4 ± 0.0[a]/2.4 ± 0.0[a]	2.5 ± 0.0[a]/2.4 ± 0.0[a]	2.5 ± 0.0[a]/2.4 ± 0.0[a]	2.6 ± 0.0*[a]/2.5 ± 0.1*[ab]
Formic acid	5.3 ± 0.1[b]/5.2 ± 0.1[b]	5.2 ± 0.1[b]/5.0 ± 0.1[b]	5.1 ± 0.1*[b]/4.8 ± 0.1*[b]	4.9 ± 0.0*[b]/4.9 ± 0.0*[c]
Heat treatment	2.5 ± 0.0[a]/2.4 ± 0.0[a]	2.4 ± 0.0[a]/2.4 ± 0.0[a]	2.5 ± 0.0[a]/2.5 ± 0.0[a]	2.5 ± 0.0[c]/2.4 ± 0.0[b]
C16:3n-4				
Control	4.8 ± 0.1[ab]/4.7 ± 0.1[a]	4.8 ± 0.0[a]/4.6 ± 0.0[a]	4.8 ± 0.0[a]/4.5 ± 0.1[a]	4.8 ± 0.0[a]/4.4 ± 0.2*[a]
Benzoic acid	4.9 ± 0.0[a]/4.6 ± 0.0[a]	4.8 ± 0.0[a]/4.5 ± 0.0*[a]	4.7 ± 0.0*[a]/4.4 ± 0.1*[a]	4.8 ± 0.1[a]/4.5 ± 0.0*[a]
Formic acid	9.4 ± 0.1[c]/9.0 ± 0.0[b]	9.1 ± 0.1*[b]/8.5 ± 0.1*[b]	8.7 ± 0.0*[b]/8.2 ± 0.0*[b]	8.3 ± 0.0*[b]/8.0 ± 0.0*[b]
Heat treatment	4.7 ± 0.1[b]/4.6 ± 0.0[c]	4.6 ± 0.0[c]/4.7 ± 0.0[c]	4.6 ± 0.0[c]/4.7 ± 0.0[c]	4.6 ± 0.0[a]/4.7 ± 0.0[c]
C16:4n-1				
Control	31.9 ± 0.2[a]/31.6 ± 0.2[a]	32.2 ± 0.1[a]/31.1 ± 0.1[a]	31.5 ± 0.2[a]/29.5 ± 1.1[a]	31.0 ± 0.8[a]/28.9 ± 2.1*[a]
Benzoic acid	32.6 ± 0.1[b]/31.5 ± 0.1[a]	32.1 ± 0.4[a]/30.9 ± 0.0[a]	31.7 ± 0.3*[a]/29.9 ± 0.9*[a]	31.4 ± 0.4*[a]/30.2 ± 0.1*[ab]
Formic acid	28.0 ± 0.1[c]/26.7 ± 0.1[b]	26.9 ± 0.1*[b]/25.0 ± 0.2*[b]	25.6 ± 0.0*[b]/24.2 ± 0.1*[b]	24.6 ± 0.1*[b]/23.5 ± 0.1*[c]
Heat treatment	31.8 ± 0.6[a]/31.5 ± 0.2[a]	31.5 ± 0.1[c]/31.7 ± 0.3[c]	31.5 ± 0.0[a]/31.4 ± 0.1[c]	31.4 ± 0.1[a]/31.5 ± 0.2[b]
C18:4n-3				
Control	10.0 ± 0.0[a]/9.9 ± 0.1[a]	9.8 ± 0.0[ab]/9.3 ± 0.1[a]	9.7 ± 0.1[a]/9.0 ± 0.5[a]	9.5 ± 0.3[a]/9.0 ± 0.5*[a]
Benzoic acid	9.9 ± 0.0[b]/10.1 ± 0.0[b]	9.7 ± 0.1[a]/9.9 ± 0.1[b]	9.8 ± 0.0[a]/9.8 ± 0.4[b]	9.6 ± 0.1[ab]/10.0 ± 0.1[b]
Formic acid	3.7 ± 0.0[c]/3.5 ± 0.0[c]	3.5 ± 0.1[c]/3.6 ± 0.1[c]	3.6 ± 0.0[b]/3.7 ± 0.0*[c]	3.7 ± 0.1[c]/3.8 ± 0.0*[c]
Heat treatment	10.1 ± 0.1[a]/10.0 ± 0.0[b]	10.1 ± 0.0[b]/9.9 ± 0.2[b]	10.0 ± 0.0[a]/9.6 ± 0.0[b]	10.1 ± 0.0[b]/9.3 ± 0.1*[a]
C20:5n-3				
Control	28.3 ± 0.1[a]/26.6 ± 0.4[ab]	27.8 ± 0.1[a]/27.5 ± 0.2[a]	26.9 ± 0.4[a]/25.8 ± 1.2[a]	25.8 ± 1.1*[a]/24.2 ± 2.1*[a]
Benzoic acid	27.8 ± 0.1[ab]/28.0 ± 0.1[a]	27.5 ± 0.1[a]/27.7 ± 0.2[a]	27.6 ± 0.2[a]/27.7 ± 0.1[ab]	25.9 ± 0.2*[a]/26.7 ± 0.7*[ab]
Formic acid	31.5 ± 0.4[c]/32.3 ± 0.4[c]	31.7 ± 0.8[b]/31.3 ± 1.2[b]	31.6 ± 0.5[b]/31.2 ± 0.4[c]	31.7 ± 0.1[b]/30.0 ± 0.2*[c]
Heat treatment	27.1 ± 0.4[b]/27.1 ± 0.1[b]	27.2 ± 0.2[a]/27.9 ± 0.2*[a]	27.1 ± 0.1[a]/27.9 ± 0.1[b]	27.3 ± 0.1[a]/27.9 ± 0.3*[bc]
C22:6n-3				
Control	2.9 ± 0.1[ab]/2.7 ± 0.2[a]	2.9 ± 0.0[a]/3.0 ± 0.0[a]	2.8 ± 0.1[a]/3.1 ± 0.2[a]	2.8 ± 0.1*[ab]/2.8 ± 0.3[a]
Benzoic acid	2.9 ± 0.1[ab]/3.0 ± 0.0[a]	2.9 ± 0.0[a]/2.9 ± 0.0[a]	2.8 ± 0.0[a]/3.0 ± 0.1[a]	2.6 ± 0.0*[a]/2.9 ± 0.1[a]
Formic acid	3.2 ± 0.2[a]/2.9 ± 0.1[a]	2.9 ± 0.1*[a]/2.9 ± 0.2[a]	2.9 ± 0.0*[a]/3.4 ± 0.0*[b]	3.1 ± 0.1[b]/3.6 ± 0.0*[b]
Heat treatment	2.6 ± 0.4[b]/2.8 ± 0.0[a]	2.8 ± 0.0[a]/3.0 ± 0.1[a]	2.8 ± 0.0[a]/3.0 ± 0.0[a]	2.8 ± 0.0[ab]/3.0 ± 0.0[a]

[a, b, c] Results in the same column and stored at the same temperature sharing superscripted letters are not significantly different ($P \geq 0.05$)

*Results significantly different from day 1 with the same pre-treatment and stored at the same temperature ($P < 0.05$)

follow-up study of temperature treatment, the initial FFA content was 7%. One reason for high initial levels of FFA may be that the centrifugation process during harvesting damages the cells initiating enzymatic hydrolysis.

The rapid formation of FFA in diatoms has been attributed to grazer defense mechanisms, where the fatty acids are released from the glycerol backbones via hydrolytic enzymes to produce oxylipins downstream (d'Ippolito et al. 2004;

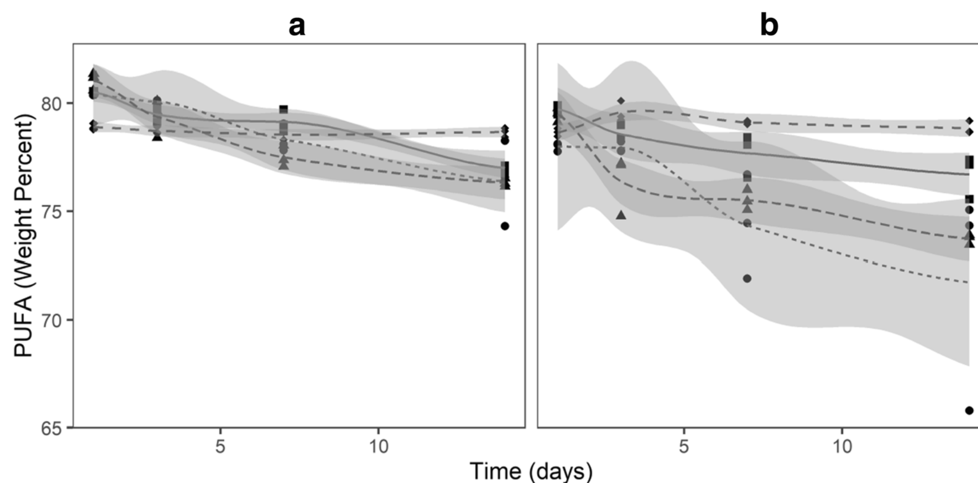


Fig. 2 Polyunsaturated fatty acids (PUFA, weight percentage) from day 1 to 14 for each treatment (n = 3) at 4 (A) and 20 °C (B). Benzoic acid (0.1% (w/w), square), control (no pre-treatment, circle), formic acid (pH 3.0, triangle), and heat treatment (70 °C for 30 min, diamond)

Wichard et al. 2007; Gerecht et al. 2011). The inhibiting effect of heat treatment on formation of FFA has previously been reported in other diatoms such as *Pseudo-nitzschia pungens* and *Pseudo-nitzschia multiseries* (Budge and Parrish 1999) and *Skeletonema costatum* (Berge et al. 1995). In both these studies, boiling water was poured over the biomass pellets to inactivate the lipolytic enzymes. In a recent study, Balduyck et al. (2019) found a heat treatment of 80 °C for 8 min in a water bath sufficient to inhibit the lipolytic reactions in *T. lutea* for 7 days of storage at 4 °C, whereas 65 °C was not enough. In formic acid samples, the formation of FFA was also slowed down and this indicates that a pH of 3.0 is enough to substantially inhibit the lipolytic enzymes. In hazelnut, lipase activity was inhibited at a pH of 3.5 (Seyhan et al. 2002), which is in accordance with our findings. In contrast, a pH of 4.0 was not enough to inhibit lipolytic reactions in *T. lutea* (Balduyck et al. 2016). It would clearly be of interest to study the combined use of heat treatment and lowering of pH on the formation of FFA in microalgae biomass.

d'Ippolito et al. (2004) found glycolipids to be the main sources for oxylipin production in *S. costatum*, whereas phospholipids played only a minor role. In this study, we found a large decrease in PG for most samples during storage, indicating lipolytic degradation of this class even though it may not be strictly involved in oxylipin formation. Another explanation may be that there is a difference in the oxylipin pathway in *P. glacialis* compared with *S. costatum*. The susceptibility of polar lipid classes to lipolysis during storage of microalgae biomass has also been reported for other species as well (Berge et al. 1995; Balduyck et al. 2016).

The samples used in formic acid treatment were harvested at a different time than the other samples, which probably explains the differences in initial lipid class composition. It is known that the lipid class composition of diatoms is affected by the culture age when harvesting microalgae from a continuous cultivation (Alonso et al. 2000).

Based on the formation of FFA, heating to 70 °C was apparently not enough to inactivate the lipolytic enzymes completely, but at a storage temperature of 4 °C, the activity of these enzymes appeared to be very low. For lipolysis to be slowed down at a 20 °C storage temperature, the samples had to be heated to 90 °C before storage, heating the samples to 80 °C was not enough. Some exogenous enzymes of microbial origin may have contributed to the formation of FFA during prolonged storage, in particular at 20 °C and neutral pH. It has previously been investigated whether the lipolysis in microalgae was caused by endogenous enzymes or by exogenous microbial lipases. Some have concluded that the origin was endogenous (Balduyck et al. 2016) whereas others have found indications of bacterial activity (Budge and Parrish 1999). The level of benzoic acid in our samples was based on the amount usually added to foodstuff to inhibit microbial growth and did not affect the pH of the samples. This was important to separate whether the effect from benzoic acid and formic acid was mainly a pH effect or an antimicrobial effect. Our results indicate an effect of pH rather than an antimicrobial effect.

Lipases from psychrophilic organisms are commonly inactivated at lower heating temperatures, however such enzymes have been reported to have high resistance to irreversible heat inactivation (Owusu et al. 1992; Makhzoum et al. 1993). Additionally, psychrophilic enzymes have a lower optimum temperature, usually around 20 °C, than enzymes from mesophilic organisms (Feller and Gerday 2003). The heat treatment applied in this study may have left some active lipases, therefore to avoid lipolysis during storage, it may be essential to apply lower storage temperatures. Storage of mesophilic microalgae has been studied much more extensively than psychrophilic microalgae, and these have reported lower development of free fatty acids (Balduyck et al. 2016, 2019). The much higher formation of free fatty acids in this study may be explained by the low optimum temperatures of enzymes in psychrophilic organisms compared with

mesophilic organisms during storage of biomass at refrigeration or room temperature.

The omega-3 content of diatoms stored at refrigerator temperatures has previously been found to decrease rapidly (Welladsen et al. 2014), but a heat treatment appears to make the omega-3 content more stable during storage of microalgae (Budge and Parrish 1999; Balduyck et al. 2019) which is similar to our results for the diatom *P. glacialis*.

The loss of PUFA may stem from endogenous enzyme activities, as production of oxylipins via lipoxygenase requires PUFA of a certain kind (Fontana et al. 2007). The other explanation for the loss of PUFA may be autoxidation, to which these fatty acids are very susceptible. Budge and Parrish (1999) found that heat treatment inhibited PUFA degradation. They speculated that the heat treatment might have inhibited some mechanism of PUFA degradation either by less PUFA available as FFA, which could be more exposed to autoxidation or lipoxygenase, or that the heat treatment inactivated the lipoxygenase enzymes. Previous studies have established that PUFA as FFA are more prone to autoxidation (Miyashita and Takagi 1986). However, the FFA content of the formic acid and heat-treated samples in our study was similar, yet the decrease of PUFA content was larger in the formic acid samples at 20 °C. It could well be that both formic acid and heat treatment are able to inhibit the enzymatic oxidation, and that the loss of PUFA in the formic acid samples is caused by the low pH. It has previously been proposed that low pH accelerates autoxidation of fish oil–enriched mayonnaise (Jacobsen et al. 2001) and in fish silage stabilized with formic acid (Ozyurt et al. 2018). However, commonly used methods for evaluating oxidation parameters are usually titrative (peroxide value) or spectrophotometric (peroxide value, anisidine value, conjugated dienes, and thiobarbituric acid reactive substances). The amount of pigments in microalgae biomass may interfere with the readouts from these methods (Safafar et al. 2017) and oxidation parameters were therefore not analyzed in this study.

Conclusions

Lipids were best preserved in heat-treated biomass stored at 4 °C, with regards to both the development of free fatty acids and reduction in PUFA level. Mainly, polar lipids were hydrolyzed to free fatty acids. Formic acid as pre-treatment reduced the amount of lipolysis during storage but was not able to inhibit PUFA deterioration to the same extent as heat treatment. Benzoic acid and control samples showed similar trends for lipid deterioration during storage and displayed high lipolytic activity. The effect of heat treatment on both lipid class and fatty acid composition, and the lack of effect from benzoic acid treatment, indicate that the degradation of lipids stems from endogenous enzymes of *P. glacialis* rather than exogenous enzymes from other microorganisms. The rate of lipid deterioration in this psychrophilic diatom was highest at 20 °C, so refrigeration seems prudent for storage over several days.

Acknowledgments Open Access funding provided by UiT The Arctic University of Norway. The authors wish to thank Linn Jakobsen Kristiansen, Therese Smelror Løkken, and Gunilla Eriksen for their help with algae cultivation and harvesting.

Authors' contributions Conception: Lars Dalheim and Jon Brage Svenning

Data collection: Lars Dalheim and Jon Brage Svenning

Data analysis and interpretation: Lars Dalheim and Jon Brage Svenning

Drafting of the article: Lars Dalheim, Ragnar Ludvig Olsen, and Jon Brage Svenning

Critical revision of the article: Ragnar Ludvig Olsen, Terje Vasskog, and Hans Christian Eilertsen

Final approval of the version to be published: Lars Dalheim, Jon Brage Svenning, Ragnar Ludvig Olsen, Terje Vasskog, and Hans Christian Eilertsen

Funding This project is funded by Troms County Regional Funds no. TFK2017-066, UiT The Arctic University of Norway, and Finnfjord AS.

Data availability Data for this experiment are available from the Open Science Framework (OSF (https://osf.io/6nu9t/?view_only=a6a240b97c3c4d518a2e2222de901772)) under the name "Stability of lipids during wet storage of the marine diatom *Porosira glacialis* under semi-preserved conditions at 4 and 20 °C."

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Code availability The R script for this experiment is available from the Open Science Framework (OSF (https://osf.io/6nu9t/?view_only=a6a240b97c3c4d518a2e2222de901772)) under the name "Stability of lipids during wet storage of the marine diatom *Porosira glacialis* under semi-preserved conditions at 4 and 20 °C".

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

References

Abreu S, Solgadi A, Chaminade P (2017) Optimization of normal phase chromatographic conditions for lipid analysis and comparison of associated detection techniques. J Chromatogr A 1514:54–71

Alonso DL, Belarbi EH, Fernandez-Sevilla JM, Rodriguez-Ruiz J, Grima EM (2000) Acyl lipid composition variation related to culture age and nitrogen concentration in continuous culture of the microalga *Phaeodactylum tricornutum*. Phytochemistry 54:461–471

Artamonova EY, Svenning JB, Vasskog T, Hansen E, Eilertsen HC (2017a) Analysis of phospholipids and neutral lipids in three common northern cold water diatoms: *Coscinodiscus concinnus, Porosira glacialis*, and *Chaetoceros socialis*, by ultra-high performance liquid chromatography-mass spectrometry. J Appl Phycol 29:1241–1249

Artamonova EY, Vasskog T, Eilertsen HC (2017b) Lipid content and fatty acid composition of *Porosira glacialis* and *Attheya longicornis* in response to carbon dioxide (CO_2) aeration. PLoS One 12:9

Balduyck L, Bijttebier S, Bruneel C, Jacobs G, Voorspoels S, Van Durme J, Muylaert K, Foubert I (2016) Lipolysis in T-*Isochrysis lutea* during wet storage at different temperatures. Algal Res 18:281–287

Balduyck L, Dejonghe C, Goos P, Jooken E, Muylaert K, Foubert I (2019) Inhibition of lipolytic reactions during wet storage of T-*Isochrysis lutea* biomass by heat treatment. Algal Res 38:10

Balduyck L, Stock T, Bijttebier S, Bruneel C, Jacobs G, Voorspoels S, Muylaert K, Foubert I (2017) Integrity of the microalgal cell plays a major role in the lipolytic stability during wet storage. Algal Res 25: 516–524

Barkia I, Saari N, Manning SR (2019) Microalgae for high-value products towards human health and nutrition. Mar Drugs 17:29

Berge JP, Gouygou JP, Dubacq JP, Durand P (1995) Reassessment of lipid-composition of the diatom, *Skeletonema costatum*. Phytochemistry 39:1017–1021

Botte P, d'Ippolito G, Gallo C, Sardo A, Fontana A (2018) Combined exploitation of CO_2 and nutrient replenishment for increasing biomass and lipid productivity of the marine diatoms *Thalassiosira weissflogii* and *Cyclotella cryptica*. J Appl Phycol 30:243–251

Budge SM, Parrish CC (1999) Lipid class and fatty acid composition of *Pseudo-nitzschia multiseries* and *Pseudo-nitzschia pungens* and effects of lipolytic enzyme deactivation. Phytochemistry 52:561–566

Cabezas-Pizarro J, Redondo-Solano M, Umana-Gamboa C, Arias-Echandi ML (2018) Antimicrobial activity of different sodium and potassium salts of carboxylic acid against some common foodborne pathogens and spoilage-associated bacteria. Rev Argent Microbiol 50:56–61

Caldwell GS (2009) The influence of bioactive oxylipins from marine diatoms on invertebrate reproduction and development. Mar Drugs 7:367–400

Cequier-Sanchez E, Rodriguez C, Ravelo AG, Zarate R (2008) Dichloromethane as a solvent for lipid extraction and assessment of lipid classes and fatty acids from samples of different natures. J Agric Food Chem 56:4297–4303

Chen L, Liu TZ, Zhang W, Chen XL, Wang JF (2012) Biodiesel production from algae oil high in free fatty acids by two-step catalytic conversion. Bioresour Technol 111:208–214

Chen YC (2012) The biomass and total lipid content and composition of twelve species of marine diatoms cultured under various environments. Food Chem 131:211–219

Cuellar-Bermudez SP, Aguilar-Hernandez I, Cardenas-Chavez DL, Ornelas-Soto N, Romero-Ogawa MA, Parra-Saldivar R (2015) Extraction and purification of high-value metabolites from microalgae: essential lipids, astaxanthin and phycobiliproteins. Microb Biotechnol 8:190–209

Cutignano A, d'Ippolito G, Romano G, Lamari N, Cimino G, Febbraio F, Fontana A (2006) Chloroplastic glycolipids fuel aldehyde biosynthesis in the marine diatom *Thalassiosira rotula*. Chembiochem 7: 450–456

Cutignano A, Lamari N, d'Ippolito G, Manzo E, Cimino G, Fontana A (2011) Lipoxygenase products in marine diatoms: a concise analytical method to explore the functional potential of oxylipins. J Phycol 47:233–243

d'Ippolito G, Tucci S, Cutignano A, Romano G, Cimino G, Miralto A, Fontana A (2004) The role of complex lipids in the synthesis of bioactive aldehydes of the marine diatom *Skeletonema costatum*. Biochim Biophys Acta Mol Cell Biol Lipids 1686:100–107

Dunstan GA, Volkman JK, Barrett SM, Leroi JM, Jeffrey SW (1994) Essential polyunsaturated fatty-acids from 14 species of diatom (Bacillariophyceae). Phytochemistry 35:155–161

FAO (2020) The state of world fisheries and aquaculture 2020. Sustainability in action. FAO, Rome, p 244

Feller G, Gerday C (2003) Psychrophilic enzymes: hot topics in cold adaptation. Nat Rev Microbiol 1:200–208

Folch J, Lees M, Stanley GHS (1957) A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 226:497–509

Fontana A, d'Ippolito G, Cutignano A, Miralto A, Ianora A, Romano G, Cimino G (2007) Chemistry of oxylipin pathways in marine diatoms. Pure Appl Chem 79:481–490

Gerecht A, Romano G, Ianora A, d'Ippolito G, Cutignano A, Fontana A (2011) Plasticity of oxylipin metabolism among clones of the marine diatom *Skeletonema marinoi* (Bacillariophyceae). J Phycol 47: 1050–1056

Heasman M, Diemar J, O'connor W, Sushames T, Foulkes L (2000) Development of extended shelf-life microalgae concentrate diets harvested by centrifugation for bivalve molluscs–a summary. Aquac Res 31:637–659

Hosseinizand H, Lim CJ, Webb E, Sokhansanj S (2017) Economic analysis of drying microalgae *Chlorella* in a conveyor belt dryer with recycled heat from a power plant. Appl Therm Eng 124:525–532

Jacobsen C, Timm M, Meyer AS (2001) Oxidation in fish oil enriched mayonnaise: ascorbic acid and low pH increase oxidative deterioration. J Agric Food Chem 49:3947–3956

Khozin-Goldberg I, Iskandarov U, Cohen Z (2011) LC-PUFA from photosynthetic microalgae: occurrence, biosynthesis, and prospects in biotechnology. Appl Microbiol Biotechnol 91:905–915

Kris-Etherton PM, Grieger JA, Etherton TD (2009) Dietary reference intakes for DHA and EPA. Prostaglandins Leukot Essent Fat Acids 81:99–104

Kuczynska P, Jemiola-Rzeminska M, Strzalka K (2015) Photosynthetic pigments in diatoms. Mar Drugs 13:5847–5881

Makhzoum A, Owusu RK, Knapp JS (1993) The conformational stability of a lipase from a psychrotrophic *Pseudomonas fluorescens*. Food Chem 46:355–359

Miyashita K, Takagi T (1986) Study on the oxidative rate and prooxidant activity of free fatty-acids. J Am Oil Chem Soc 63:1380–1384

Olsen RL, Toppe J (2017) Fish silage hydrolysates: not only a feed nutrient, but also a useful feed additive. Trends Food Sci Technol 66:93–97

Owusu RK, Makhzoum A, Knapp JS (1992) Heat inactivation of lipase from psychrotrophic *Pseudomonas fluorescens* p38—activation parameters and enzyme stability at low or ultra-high temperatures. Food Chem 44:261–268

Ozyurt G, Ozkutuk AS, Ucar Y, Durmus M, Ozogul Y (2018) Fatty acid composition and oxidative stability of oils recovered from acid silage and bacterial fermentation of fish (sea bass - *Dicentrarchus labrax*) by-products. Int J Food Sci Technol 53:1255–1261

Pohnert G (2002) Phospholipase a(2) activity triggers the wound-activated chemical defense in the diatom *Thalassiosira rotula*. Plant Physiol 129:103–111

Ryckebosch E, Muylaert K, Eeckhout M, Ruyssen T, Foubert I (2011) Influence of drying and storage on lipid and carotenoid stability of the microalga *Phaeodactylum tricornutum*. J Agric Food Chem 59: 11063–11069

Safafar H, Langvad S, Moller P, Jacobsen C (2017) Storage conditions affect oxidative stability and nutritional composition of freeze-dried *Nannochloropsis salina*. Eur J Lipid Sci Technol 119:10

Salem N, Eggersdorfer M (2015) Is the world supply of omega-3 fatty acids adequate for optimal human nutrition? Curr Opin Clin Nutr Metab Care 18:147–154

Seyhan F, Tijskens LMM, Evranuz O (2002) Modelling temperature and pH dependence of lipase and peroxidase activity in Turkish hazelnuts. J Food Eng 52:387–395

Shah MR, Lutzu GA, Alam A, Sarker P, Chowdhury MAK, Parsaeimehr A, Liang YM, Daroch M (2018) Microalgae in aquafeeds for a sustainable aquaculture industry. J Appl Phycol 30:197–213

Sprague M, Dick JR, Tocher DR (2016) Impact of sustainable feeds on omega-3 long-chain fatty acid levels in farmed Atlantic salmon, 2006-2015. Sci Rep 6:9

Stoffel W, Chu F, Ahrens EH (1959) Analysis of long-chain fatty acids by gas-liquid chromatography—micromethod for preparation of methyl esters. Anal Chem 31:307–308

Svenning JB, Dalheim L, Eilertsen HC, Vasskog T (2019) Temperature dependent growth rate, lipid content and fatty acid composition of the marine cold-water diatom *Porosira glacialis*. Algal Res 37:11–16

Tocher DR (2015) Omega-3 long-chain polyunsaturated fatty acids and aquaculture in perspective. Aquaculture 449:94–107

Uradnikova M (2020) Species validity of five common northern/Arctic spring bloom diatoms: a combined morphological and molecular study. UiT, the Arctic University of Norway,

Welladsen H, Kent M, Mangott A, Li Y (2014) Shelf-life assessment of microalgae concentrates: effect of cold preservation on microalgal nutrition profiles. Aquaculture 430:241–247

Wendt LM, Kinchin C, Wahlen BD, Davis R, Dempster TA, Gerken H (2019) Assessing the stability and techno-economic implications for wet storage of harvested microalgae to manage seasonal variability. Biotechnol Biofuels 12:14

Wichard T, Gerecht A, Boersma M, Poulet SA, Wiltshire K, Pohnert G (2007) Lipid and fatty acid composition of diatoms revisited: rapid wound-activated change of food quality parameters influences herbivorous copepod reproductive success. Chembiochem 8:1146–1153

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Paper III

Svenning, J.B., Dalheim, L., Vasskog, T., Matricon, L., Vang, B. & Olsen, R.L. (2020).

Lipid yield from the diatom *Porosira glacialis* is determined by solvent choice and number of extractions, independent of cell disruption.

Scientific Reports, 10, 22229.

scientific reports

OPEN

Lipid yield from the diatom *Porosira glacialis* is determined by solvent choice and number of extractions, independent of cell disruption

Jon Brage Svenning[1]✉, Lars Dalheim[1], Terje Vasskog[2], Lucie Matricon[3], Birthe Vang[3] & Ragnar Ludvig Olsen[1]

Cell wall disruption is necessary to maximize lipid extraction yields in conventional species of mass-cultivated microalgae. This study investigated the effect of sonication, solvent choice and number of extractions on the lipid yield, lipid class composition and fatty acid composition of the diatom *Porosira glacialis.* For comparison, the diatom *Odontella aurita* and green alga *Chlorella vulgaris* were included in the study. Sonication effectively disrupted *P. glacialis* cells, but did not increase the total lipid yield compared to physical stirring (mixing). In all three microalgae, the content of membrane-associated glyco- and phospholipids in the extracted lipids was strongly dependent on the solvent polarity. A second extraction resulted in higher yields from the microalgae only when polar solvents were used. In conclusion, choice of solvent and number of extractions were the main factors that determined lipid yield and lipid class composition in *P. glacialis.*

Marine photoautotrophic microalgae are a largely unexploited source of lipids with a wide range of possible uses, such as biodiesel production[1], fish oil substitution in aquaculture feed[2] or nutraceuticals for human consumption[3]. The high costs associated with cultivation and extraction of lipids have, however, prevented the production of low-cost products, and the current production of microalgae is limited to high-priced lipophilic products such as pigments, omega-3 supplements or other valuable biomolecules[4–6]. One of the main challenges to reduce the processing cost is to maximize the product recovery from microalgal biomass. In this respect, cell disruption prior to extraction is a prerequisite in frequently mass-cultivated microalgae due to tough cell walls that prevent lipid release[7,8]. Diatoms have silica-based cell walls, which are fragile compared to the tough cell walls associated with microalgae such as *Nannochloropsis* sp.[9] or *Chlorella* sp.[10]. As a consequence, an efficient extraction from diatom biomass may be less challenging. In addition, cell disruption has been shown to enzymatically release fatty acids from membrane lipids in diatoms. Polyunsaturated fatty acids may then be transformed and degraded by lipoxygenases and lyases to potentially harmful secondary oxidation products, collectively known as oxylipins[11–13]. Removing cell disruption from the oil extraction procedure may therefore avoid reduction in the PUFA content and the need for extensive refinement to remove free fatty acids and oxidation products from the oil.

Of the two most commonly used methods in laboratory settings, Folch's method[14] is preferentially used for lipid extraction of marine biomass, as the method of Bligh and Dyer[15] tends to underestimate the lipid content in lipid-rich marine organisms[16]. In its original design, the Folch method utilizes a mixture of chloroform and methanol as the organic phase. The less toxic dichloromethane functions equally well[17,18], and is now the preferred choice in most Folch extractions. However, as both methanol and dichloromethane pose potentially serious health hazards, alternative solvents of lower toxicity such as hexane/isopropanol have been suggested[19]. In large-scale industrial production of algal biomass, the traditional methods for lipid extraction become impractical due to the cost and health risks associated with organic solvents. As a result, studies have investigated the use of hexane alone to extract microalgal oils for biodiesel production[20,21]. Compared to the chlorinated solvents, hexane

[1]Norwegian College of Fishery Science, UiT The Arctic University of Norway, 9037 Tromsø, Norway. [2]Department of Pharmacy, Faculty of Health Sciences, UiT The Arctic University of Norway, 9037 Tromsø, Norway. [3]Nofima AS, Muninbakken 9-13, Breivika, 9019 Tromsø, Norway. ✉email: jon.b.svenning@uit.no

has a lower cost, higher specificity toward lipids of low polarity such as triglycerides, and is less problematic to dispose of. As the diatom studied in this experiment has a high content of polyunsaturated fatty acids bound to an abundance of complex membrane lipids[22,23], hexane alone is unlikely to provide an efficient extraction. Cell disruption prior to extraction may, however, increase the yield of the more polar lipids when extracting with hexane.

Although most laboratory procedures rely on a single extraction to isolate lipid from a given biomass, one study found that repeating the extraction twice independently of solvent choice significantly increased the lipid yield from the green microalgae *Chlorella* sp.[24]. However, while studies on other microalgae are helpful when developing new methods, the final choice of extraction method is probably species-dependent, due to the highly diverse membrane physiology, morphology and biochemistry within the phytoplankton[25]. *Porosira glacialis,* the diatom used in this study, is a large (Ø > 30 µm), cold-water strain with the potential to convert CO_2 from flue gas in large-scale photobioreactors into valuable products such as omega-3 fatty acids[26]. The aim of the present study was to investigate how different cell disruption methods affected the integrity of this diatom cultivated in a pilot scale. The lipid yield when using industrially relevant solvents after applying the most destructive disruption technique of the biomass was compared with a relatively gentle mixing more suitable for large scale processing. The lipid classes and the fatty acid composition of the extracted lipids were also determined. For comparison, the green algae *Chlorella vulgaris* and the diatom *Odontella aurita,* both commercially available, were included in the study.

Materials and methods

Materials. Lyophilized material of *C. vulgaris* (Midsona, Oslo, Norway) was purchased from a local health store. Lyophilized *O. aurita* was obtained from KissPlanet (Gembloux, Belgium). Kristalon Flower was purchased from Yara Norge as, Oslo, Norway. Sodium metasilicate pentahydrate was acquired from Skovly Engros as, Oslo, Norway. Kits for quantifying NO_3, NO_2, silicic acid, PO_4 and NO_4 were purchased from VWR, Radnor, Pennsylvania, USA. Dichloromethane (99.9%), methanol (99.8%), sulfuric acid (95–97%), hexane (99%), sodium metasilicate pentahydrate (≥ 95%), sodium chloride, isopropanol and lipid standards of diacylglyceryltrimethylhomoserine (1,2-dipalmitoyl-sn-glycero-3-O-4′-(N,N,N-trimethyl)-homoserine; DGTS), sulfoquinovosyldiacylglycerol (SQDG) and phosphatidylinositol (PI) were purchased from Sigma Aldrich, St. Louis, Missouri, USA. Lipid standards of phosphatidylcholine (1,2-Dimyristoyl-sn-Glycero-3-Phosphatidylcholine; PC), phosphatidylglycerol (1,2-Dimyristoyl-sn-Glycero-3-Phosphatidylglycerol Na Salt; PG), phosphatidylserine (1,2-Dipalmitoyl-sn-Glycero-3-Phosphatidylserine Na salt; PS), phosphatidylethanolamine (1,2-Dimyristoyl-sn-Glycero-3-Phosphatidylethanolamine; PE), hydrogenated monogalactosyl diglyceride (MGDG), hydrogenated digalactosyl diglyceride (DGDG), ergosterol, triolein (TAG), diolein (DAG) and monolein (MAG) were purchased from Larodan AB, Solna, Sweden.

Diatom strain cultivation and harvesting. The monoculture of *P. glacialis* used in this study was isolated from a sediment sample collected in the Barents Sea in 2014 and identified using light microscopy and SEM imaging[27]. The cultivation was performed in a 300,000-L vertical column photobioreactor placed outdoors, mixed by continuous aeration using pressurized air. The culture was illuminated with LEDs (VIS, PAR radiation) at a mean illumination of ca. 18 µmol m^{-2} s^{-1} with reference to a spherical PAR sensor (Biospherical, QSL-100). The seawater used in the cultivation was collected at 25-m depth, pre-filtered at 1 µm and disinfected using ultraviolet radiation. The cultivation temperature was 6 °C. Inorganic nutrients were added in the form of 0.1 g/l Kristalon flower (14% N, 3.9% P) and sodium metasilicate pentahydrate stock solution (0.1 g/l in H_2O). The concentration of inorganic nutrients was measured daily using the kits listed above. In order to maintain a nutrient replete environment, the concentrations of N and Si were maintained within 50–150 µM and 20–150 µM, respectively. The culture medium was also enriched with CO_2 by aerating the culture with flue gas (6–12% CO_2) to pH < 8.0 on a daily basis. The culture, as part of a longer period of sampling for various experimental work, was maintained in exponential growth at approximately 20 million cells/l by daily cell counts and dilutions. Harvesting was performed by passing the culture through a continuous solid bowl centrifuge (Model PTDC, Nanjing Kingreat Machinery Company, Jiangsu, China) at 835 G and collecting the resulting biomass with a spatula and placing the biomass at − 80 °C while awaiting analysis.

Evaluation of methods for cell disruption. Thawed biomass of *P. glacialis* was mixed in water (1 mg/ml) and exposed to the following cell disruption methods: Microwave (EV-880MD, Evalet) at 2450 MHz for 3 min and 45 s; sonication at 20 kHz for 3, 5, 10 and 15 min on ice (VC50, Sonics and Materials Inc.), ultrathurax (Polytron PT 1200 E) for 10 min at 25,000 RPM and manual grinding using a PTFE pestle. The effect of lyophilization was also evaluated by freeze-drying *P. glacialis* and re-dissolving 1 mg/ml wet-weight equivalent in water. Following treatment, each method was evaluated by the visual appearance of the biomass in a microscope (Zeiss Axio Vert.A1) at 100 × magnification, and the most efficient method chosen as the cell disruption method prior to lipid extraction.

Determination of ash-free dry weight. Following freeze-drying, five replicates of 300 mg dry weight (DW) were placed at 105 °C in pre-burned, open aluminum containers for 24 h to determine the DW, and then combusted in a muffle furnace to determine the ash-free dry weight (AFDW).

Physical treatment and lipid extraction:. The control lipid extraction method used in this experiment was based on the method developed by Folch et al.[14]. Lyophilized biomass was divided into five replicates of 150 mg in 15 ml centrifuge tubes and added 20 volumes (3 ml) of either dichloromethane/methanol (2:1 v/v, DCM/MeOH), hexane/isopropanol (2:1 v/v, hexane/IPA) or hexane. Following the addition of solvent, the sam-

ples were subjected to the following treatments: No treatment (control), stirring using a shaker (Heidolph Multireax) at 1000 RPM for 60 min at room temperature (mixing), and sonication at 20 kHz for 10 min (sonication) on ice. The samples were then added 3 ml MiliQ water added 5% NaCl and centrifuged for 5 min at 3000 G, before the organic phase was transferred to a 4 ml vial and evaporated under nitrogen. The extraction procedure was repeated once without physical treatment for each sample, and the yield was determined gravimetrically for each extraction respectively as percent of AFDW. Finally, the samples were dissolved (10 mg/ml) in DCM/MeOH (2:1 v/v) and stored at -80 °C.

Fatty acid methylation and GC analysis. Fatty acids were methylated using a method developed by Stoffel et al.[28] with modifications, for a detailed description of the derivatization procedure see Svenning et al.[23]. The fatty acid methyl esters (FAMEs) were analyzed on a GC-FID (Agilent Technologies) coupled to a Select FAME column (length 50 m, ID 0.25 mm and FT 0.25 µm, Agilent J&W Columns). The GC conditions were as follows: Helium was used as the carrier gas at a rate of 1.6 ml/min. The inlet temperature was set to 240 °C (split 1:50), and the FID was set to 250 °C. The oven temperature was programmed to 60 °C for one minute, then increased to 130 °C at a rate of 30 °C/min, then to 195 °C at a rate of 1.3 °C/min, before finally increasing to 240 °C at a rate of 30 °C/min for 10 min. The fatty acids were identified using fatty acids standards quantified by dividing the peak area of the chromatograms with the area of the internal standard (heptadecaenoic acid), and converted to absolute amounts using the slopes calculated from standard curves (triplicates of 7.8125–2000 µg/ml of GLC 502 Free Acids, Nu-Check-Prep, Elysian, MN, USA).

Lipid class analysis by HPLC. The composition of lipid classes was analyzed using a Waters e2795 separations module, coupled to a Supelcosil™ LC-SI 5 µm (25 cm × 4.6 mm) column (Supelco HPLC products, Bellefonte, PA, USA) set to a working temperature of 40 °C. The HPLC method used was developed by Abreu et al.[29]. Lipids were quantified using a Waters 2424 ELS detector set to gain 100, nebulizer heating level set to 30%, drift tube temperature set to 45 °C and pressure set to 40 PSI. The total run time was 41 min, using the gradient profile and mobile phases listed in Supplementary Table S1. Lipids were quantified based on the peak area in the chromatograms and converted to absolute amounts based on standard curves (triplicates of 12.5–400 µg/ml of the lipid classes listed in "Materials"). All samples and standards were dissolved in mobile phase A/Chloroform (4:1 v/v) prior to analysis.

Data presentation and statistics. All analyses were performed using 5 replicates and presented as means ± standard deviations, either in tables or as figures with error bars representing one standard deviation. All statistical analyses were prepared using R v3.6.1 ('Action of the Toes'), making use of the 'ggplot2' package, and a range of packages in the Tidyverse. Means of total lipid content, fatty acid and lipid class composition were compared with the pair-wise Tukey test, assuming a normal distribution. Means were determined different at a significance level of < 0.05. All numerical values and methods for hypothesis testing and descriptive statistical procedures are included in the R markdown supplied with this study, see Data availability.

Results and discussion

Evaluation of methods for cell disruption. Of the five treatments applied for cell disruption, lyophilization (Fig. 1d) was the least effective method compared to the control (Fig. 1a). Sonication (Fig. 1e) was the only method that effectively disrupted the cell wall of *P. glacialis*, and 10 min was sufficient to achieve complete lysis of the cells (the data for the other timepoints are not shown, but included in the OSF for this study). Neither manual grinding (Fig. 1b), microwave (Fig. 1c) or ultrathurax (Fig. 1f) was effective at disrupting the cell walls of *P. glacialis*. Sonication for 10 min was therefore chosen as the cell disruption method for lipid extraction.

Total lipid yields. In *P. glacialis,* the solvent DCM/MeOH gave higher total lipid yields than both hexane/IPA and hexane independently of treatment, and the differences were statistically significant for both extractions (Fig. 2). The highest lipid yields overall was achieved when extracting with DCM/MeOH using mixing and sonication, both for the first extraction (15.9% for both treatments) and for the total yield after two extractions (20.7% for both treatments). The lipid yield in the control sample was significantly lower ($p < 0.05$) compared to mixing and sonication for both extractions in DCM/MeOH (13.0% and 18.8%, respectively).

Hexane/IPA was almost as effective as DCM/MeOH in extracting lipids from *P. glacialis*. The total lipid yield was approximately 18.1% of AFDW after both mixing and sonication and as for DCM/MeOH, this was significantly higher than in the sample with no treatment (Fig. 2). Extracting with hexane resulted in much lower lipid yields independently of treatment compared to both DCM/MeOH and hexane/IPA, with a maximum yield of 5.3% in the sonicated samples. Hexane was also the only solvent in which sonication resulted in a significantly higher yield compared to mixing (3.9%).

Performing a second extraction had a significant effect on the total lipid yields with both DCM/MeOH and hexane/IPA, independently of treatment. In contrast, the effect of the second extraction in hexane was negligible.

Cell disruption by sonication did not increase the total lipid yields when using the polar solvents compared with mixing, despite the clear disruptive effect of the treatment (Fig. 1). This result is contrary to those found in studies on green algae and cyanobacteria[7,30,31] and *Nannochloropsis* sp.[9], and shows that lipids in *P. glacialis* are more accessible for extraction compared to other commonly mass-cultivated microalgae. Our results therefore indicate that solvent choice and number of extractions are the main factors that determine lipid yield in lyophilized material of *P. glacialis*.

The highest lipid yield in *O. aurita* was achieved with DCM/MeOH in combination with mixing (7.8%), although this result was not statistically different from the yield when extracted with DCM/MeOH in combination

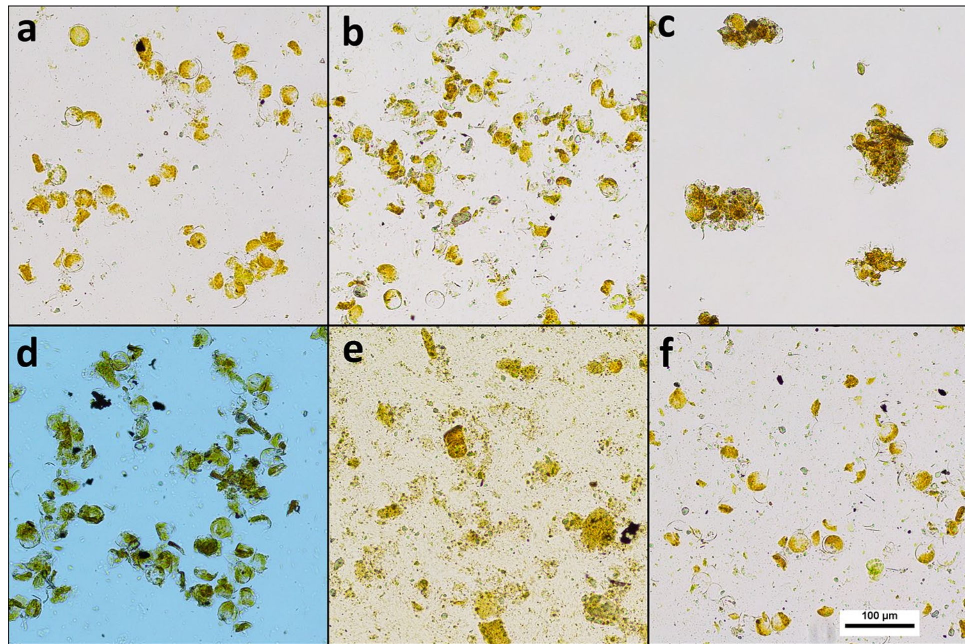


Figure 1. The effect of cell disruption on thawed biomass of *Porosira glacialis*. (**a**) Control, (**b**) manual grinding using a PTFE pestle, (**c**) microwave, (**d**) lyophilization, (**e**) sonication, (**f**) Ultrathurax. All images were captured at 100 × magnification.

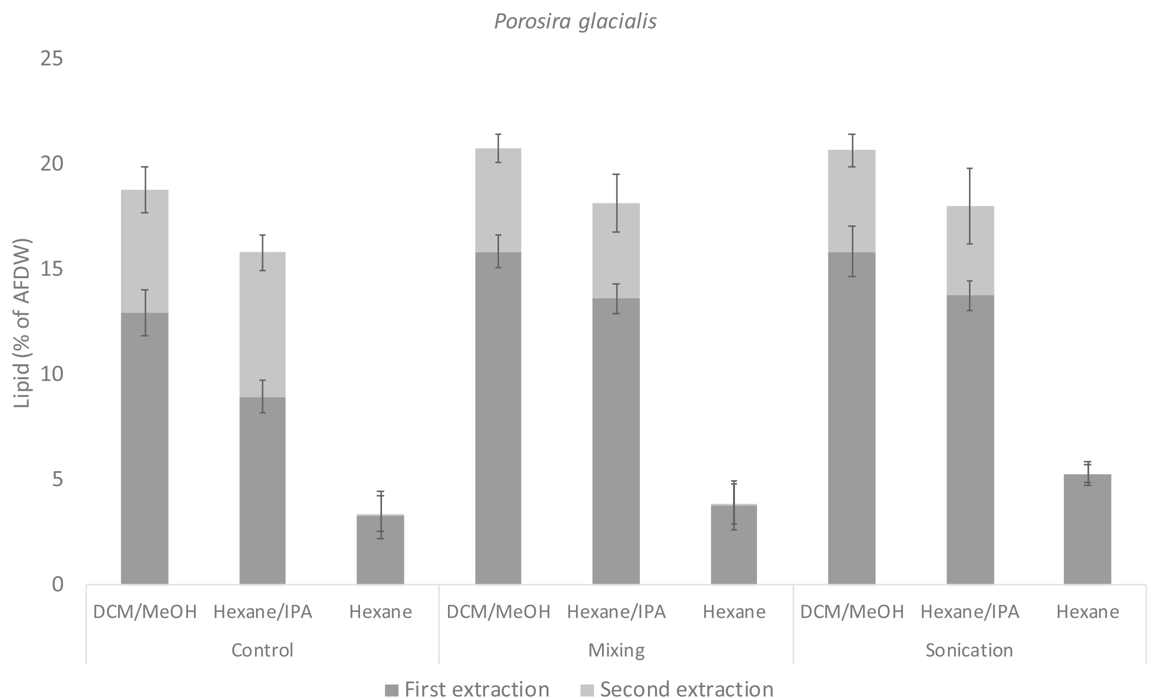


Figure 2. Lipid yields as percent of AFDW (Ash-free dry weight) from *Porosira glacialis* using three solvent systems; dichloromethane/methanol (2:1 v/v); DCM/MeOH), Hexane/isopropanol (2:1 v/v; Hexane/IPA) and hexane; and three cell disruption treatments; no treatment (control), shaking at 1000 RPM for 60 min (mixing) and sonication at 20 kHz for 10 min (sonication). Two consecutive extractions were performed, data shown is the arithmetic mean of each extraction, n = 5. The error bars represent the standard deviation of the mean for the first extraction (bottom bar) and for the total yield (top bar).

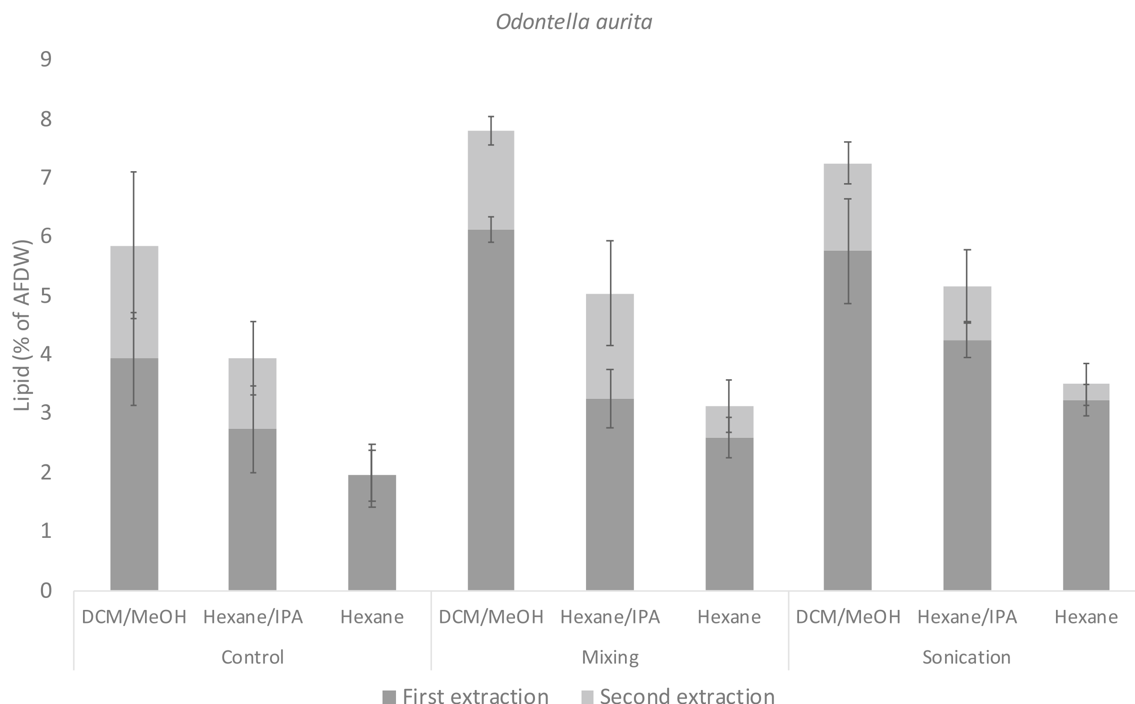


Figure 3. Lipid yields as percent of AFDW (Ash-free dry weight) from *Odontella aurita* using three solvent systems; dichloromethane/methanol (2:1 v/v) (DCM/MeOH), hexane/isopropanol (2:1 v/v) (Hexane/IPA) and hexane; and three cell disruption treatments; no treatment (control), mixing at 1000 RPM for 60 min (mixing) and sonication at 20 kHz for 10 min (sonication). Two consecutive extractions were performed, data shown is the arithmetic mean of each extraction, n = 5. The error bars represent the standard deviation of the mean for the first extraction (bottom bar) and for the total yield (top bar).

with sonication (7.3%) (Fig. 3). Hexane/IPA was less effective, resulting in a maximum yield of 5.2% in the sonicated samples. Hexane was the least effective solvent in *O. aurita*, with a maximum lipid yield of 3.5% in the sonicated samples. Performing a second extraction had a significant effect on the total lipid yields for all three solvents with the exception of hexane in combination with control.

DCM/MeOH was a more effective solvent than both hexane/IPA and hexane when extracting lipids from both *P. glacialis* and *O. aurita*. Other studies have found that hexane/IPA can replace chlorinated solvent mixtures when extracting specific lipids from bacteria[32] and rat brain[19]. However, a study on fish meal found that hexane/IPA gave lower lipid yields compared to chloroform-based methods, probably due the lower polarity of hexane/IPA[33]. Likewise, our results indicate that Hexane/IPA is not an ideal substitute for chlorinated solvents when extracting lipids from diatom biomass in laboratory-scale extractions. With that being said, the effect of replacing DCM/MeOH with hexane/IPA was less severe for *P. glacialis.* Extracting with hexane/IPA resulted in a 33.3% lower lipid yield in *O. aurita,* and only 12.1% in *P. glacialis* compared to the highest yield achieved with DCM/MeOH. Our results therefore indicate that hexane/IPA can be used as an alternative to chlorinated solvent mixtures in large-scale lipid extractions from *P. glacialis* with a minor loss of product recovery.

In contrast to the two diatoms, the highest yield in *C. vulgaris* was dependent on treatment, not solvent (Fig. 4). Sonication in combination with DCM/MeOH and hexane/IPA resulted in the highest yields (4.4% and 4.2%, respectively). Mixing did not increase the total yield significantly ($p > 0.05$) compared to the control with any of the three solvents. Based on the first extraction alone, the lipid yield when extracting with hexane was not statistically different ($p > 0.05$) to the yield when using DCM/MeOH and hexane/IPA in the sonicated samples. However, the second extraction increased the yield significantly with both DCM/MeOH and hexane/IPA independently of treatment. The effect of the second extraction was much lower in hexane. Our results indicate that hexane/IPA can replace DCM:MeOH when extracting lipids from *C. vulgaris,* if used in combination with sonication. Hexane was not an effective solvent for lipid extraction in any of the three microalgae tested in this experiment, despite applying sonication to disrupt the cell walls, and should be avoided with the objective of maximizing yield. All numerical values are provided in the Open Science Framework supplied with this study.

Lipid class composition. In *P. glacialis,* the distribution of lipid classes was highly dependent on solvent polarity (Table 1). The lipids extracted with DCM/MeOH and hexane/IPA were dominated by the polar membrane lipids MGDG, PG and PC, including some DGDG in the sonicated samples. The lipids extracted with hexane primarily contained the neutral lipids TAG, DAG and FFA. The content of FFA in *P. glacialis* was dependent on solvent, with a minimum of about 9% in the DCM/MeOH extracts and a maximum of 40% with hexane. The high content of FFA in the hexane extracts is because only the more hydrophobic lipids, not membrane lipids, are present.

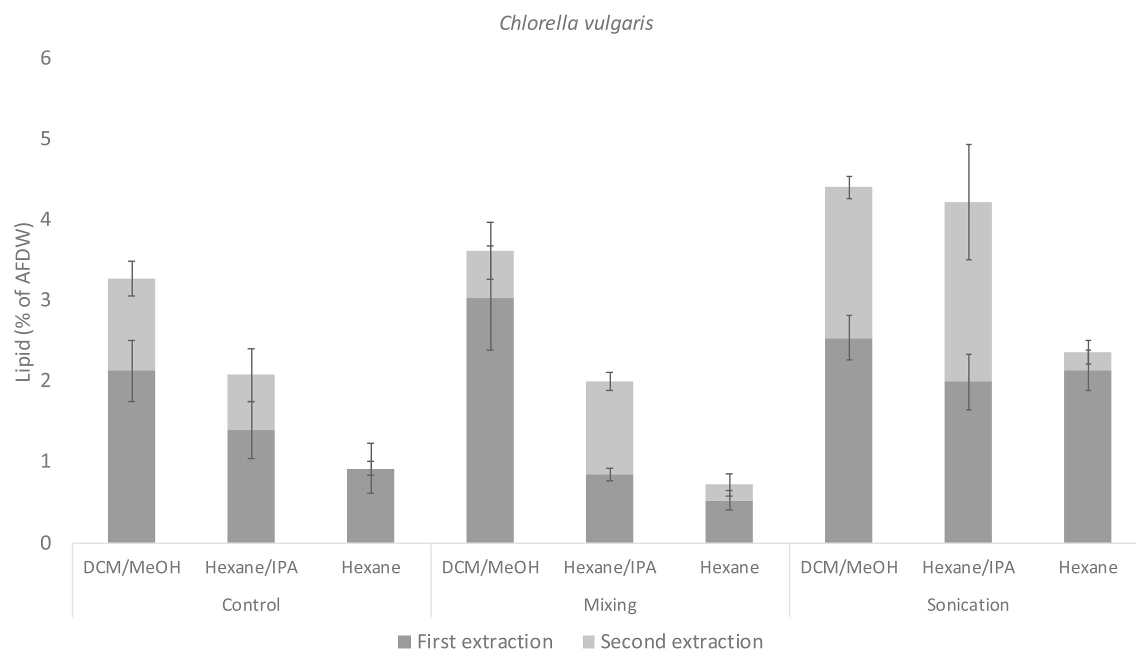


Figure 4. Lipid yields as percent of AFDW (Ash-free dry weight) from *Chlorella vulgaris* using three solvent systems; dichloromethane/methanol (2:1 v/v) (DCM/MeOH), Hexane/isopropanol (2:1 v/v) (Hexane/IPA) and hexane; and three cell disruption treatments; no treatment (control), mixing at 1000 RPM for 60 min (mixing and sonication at 20 kHz for 10 min (sonication). Two consecutive extractions were performed, data shown is the arithmetic mean of each extraction, n = 5. The error bars represent the standard deviation of the mean for the first extraction (bottom bar) and for the total yield (top bar).

Sonication of *P. glacialis* in combination with DCM/MeOH resulted in a reduction in the relative amount of MGDG and PG, and an increase in the relative amount of PCand DGDG compared to the control and mixing treatments. These 4 lipid classes are associated with the thylakoid membranes of the chloroplasts[34], and one might expect sonication to result in higher yields of all 4 classes if the membranes are effectively disrupted by the treatment. The observed reduction of PG and MGDG is therefore difficult to explain, but could tentatively be caused by enzymatic hydrolysis of these lipids and subsequent enzymatic oxidation of polyunsaturated fatty acids to aldehydes[12,35]. However, there was no increase in the free fatty acids (Table 1) or change in the amount of PUFA in the sonicated samples (see "Fatty acid composition").

The composition of the lipid classes in *O. aurita* and *C. vulgaris* was different from what was found in the *P. glacialis* samples. The most striking difference was the low levels of membrane lipids and the very high concentrations of free fatty acids in the extracts from the two commercially available microalgae. It is tempting to suggest that these values are interconnected, i.e. primarily the membrane lipids, with the exception of PC, have been hydrolyzed to free fatty acids. The rapid formation of FFA during storage has been well-documented in microalgae[36,37], and both phospho- and glycolipids are targets of lipase activity in the marine diatom *Skeletonema costatum*[38] and the haptophyte *T-Isochrysis lutea*[39]. Based on the reduction in specific lipid classes, our results indicate that enzymatic lipid hydrolysis is primarily targeted at the thylakoid-associated lipids (i.e. MGDG, DGDG, PG), and not PC, which is also associated with extra-chloroplastic membranes.

Studies have found that high levels of FFA reduces the oxidative stability of vegetable and marine oils by acting as a prooxidant[40,41]. The high levels of FFA may have resulted in a reduction in the observed amount of PUFA in our samples, as the rate of oxidation is positively correlated with the degree of unsaturation. These results demonstrate the importance of post-harvest preservation methods of microalgal biomass, which should be investigated further. The relative composition of the other lipid classes in *O. aurita* and *C. vulgaris* was dependent on both solvent and treatment. The relative content of TAG was in most cases highest in the sonicated samples, which is probably a result of increased lipid diffusion due to cell lysis. None of the polar lipids were detected in the samples extracted with hexane, with the exception of PC in *C. vulgaris*.

Fatty acid composition. The relative composition of fatty acids in the lipids extracted from *P. glacialis* was similar in all samples, both when comparing treatments and solvents (Table 2). The dominating fatty acids were C20:5n-3 and C16:4n-1, each contributing approximately 30% in all samples, while docosahexaenoic acid (C22:6n-3, DHA) contributed only 2–3%. Previous studies on this species found similar levels of EPA and DHA, but lower contributions of C16:4n-1[23,26]. The sum of SFA and PUFA was similar across all treatments and solvents. In general, there were no clear effects of treatment on the fatty acid composition of *P. glacialis* when comparing the extracts obtained with the polar solvents (DCM/MeOH and hexane/IPA) and the non-polar solvent (hexane), despite large differences in the lipid class composition. This indicates a homogenous distribution of fatty acids among the lipid classes in this diatom.

Class	DCM/MeOH			Hexane/IPA			Hexane		
	Control	Mixing	Sonication	Control	Mixing	Sonication	Control	Mixing	Sonication
P. glacialis									
TAG	5.66 ± 0.20	5.55 ± 0.18	5.97 ± 0.37	7.92 ± 0.75	7.12 ± 0.42	8.14 ± 0.55	52.61 ± 5.56	46.12 ± 4.38	43.29 ± 3.13
DAG	10.56 ± 0.54	12.25 ± 0.62	17.38 ± 0.77	14.84 ± 0.96	15.64 ± 1.11	19.86 ± 1.57	9.13 ± 0.78	12.19 ± 2.57	20.80 ± 3.33
FFA	9.81 ± 0.70	9.31 ± 2.43	8.67 ± 1.07	12.27 ± 0.75	12.71 ± 1.75	14.02 ± 1.72	37.15 ± 6.28	40.48 ± 6.31	33.72 ± 5.82
MGDG	40.06 ± 1.09	36.30 ± 2.62	26.92 ± 1.84	36.69 ± 1.58	35.56 ± 1.08	33.28 ± 5.07	1.11 ± 0.57	1.21 ± 0.48	2.18 ± 0.28
DGDG	0	0	8.52 ± 0.46	0	0	0	0	0	0
PG	19.10 ± 0.41	21.6 ± 2.32	0	15.95 ± 1.18	14.29 ± 0.86	0	0	0	0
PC	14.81 ± 0.68	14.95 ± 2.19	32.55 ± 1.21	12.33 ± 1.95	14.68 ± 1.17	24.70 ± 2.51	0	0	0
O. aurita									
TAG	4.39 ± 0.28	8.27 ± 0.93	7.45 ± 0.41	3.70 ± 0.77	4.23 ± 0.46	5.75 ± 0.93	3.92 ± 0.48	3.59 ± 0.56	5.93 ± 0.61
DAG	3.05 ± 0.14	4.24 ± 0.58	3.58 ± 0.15	3.98 ± 0.62	3.53 ± 0.43	3.78 ± 0.52	3.71 ± 0.43	2.57 ± 0.39	4.39 ± 0.26
FFA	70.81 ± 3.48	71.81 ± 3.79	74.67 ± 1.67	78.26 ± 3.63	80.70 ± 1.91	76.26 ± 4.35	92.37 ± 0.91	93.84 ± 0.92	89.68 ± 0.77
MAG	2.55 ± 0.31	3.14 ± 0.44	2.79 ± 0.31	2.49 ± 0.30	2.64 ± 0.27	2.78 ± 0.40	0	0	0
MGDG	0.39 ± 0.08	0.89 ± 0.16	1.06 ± 0.27	0.54 ± 0.36	0.47 ± 0.21	1.22 ± 0.48	0	0	0
PC	18.81 ± 3.33	11.64 ± 5.04	10.45 ± 1.64	11.03 ± 2.18	8.43 ± 1.13	10.21 ± 4.96	0	0	0
C. vulgaris									
TAG	17.42 ± 1.02	15.88 ± 1.49	19.11 ± 0.82	4.09 ± 0.33	9.00 ± 1.38	21.07 ± 2.04	20.87 ± 3.68	39.39 ± 2.08	45.84 ± 2.44
DAG	9.69 ± 0.45	9.38 ± 0.79	10.29 ± 0.51	5.72 ± 0.56	7.81 ± 0.34	11.67 ± 0.94	5.56 ± 1.13	10.18 ± 0.55	15.80 ± 0.72
FFA	39.31 ± 2.21	38.40 ± 2.32	26.33 ± 1.18	54.08 ± 2.53	45.52 ± 2.10	25.06 ± 2.93	58.42 ± 3.66	39.76 ± 2.48	12.40 ± 1.31
MGDG	15.06 ± 1.39	13.76 ± 0.75	19.54 ± 0.78	17.53 ± 2.67	21.27 ± 0.75	22.67 ± 2.10	0	0	3.23 ± 0.42
DGDG	0	0	3.08 ± 0.12	0	0	2.50 ± 0.28	0	0	0
PC	18.52 ± 1.23	22.58 ± 2.75	21.65 ± 1.95	18.57 ± 4.48	16.40 ± 1.72	17.04 ± 1.20	15.15 ± 5.56	10.67 ± 1.24	22.73 ± 3.97

Table 1. The relative composition of lipid classes in extracts of *Porosira glacialis*, *Odontella aurita* and *Chlorella vulgaris* extracted with dichloromethane/methanol (2:1 v/v; DCM/MeOH), hexane/isopropanol (2:1 v/v; Hexane/IPA) and hexane, using three pre-treatments; no treatment (control), shaking at 1000 RPM for 60 min, (mixing), or sonication at 20 kHz for 10 min (sonication). Data shown is averages of replicates ± SD, n = 5. *TAG* triacylglycerol, *DAG* diacylglycerol, *FFA* free fatty acid, *MAG* monoacylglycerol, *MGDG* monogalactosyldiacylglycerol, *DGDG* digalactosyldiacylglycerol, *PG* phosphatidylglycerol, *PC* phosphatidylcholine.

P. glacialis	DCM/MeOH			Hexane/IPA			Hexane		
FA	Control	Mixing	Sonication	Control	Mixing	Sonication	Control	Mixing	Sonication
C14:0	4.60 ± 0.42	4.63 ± 0.38	4.94 ± 0.32	4.45 ± 0.10	4.51 ± 0.35	5.30 ± 0.33	4.22 ± 0.12	4.54 ± 0.18	4.38 ± 0.15
C16:0	7.03 ± 3.81	7.33 ± 3.43	5.86 ± 0.57	5.48 ± 0.13	6.67 ± 2.38	5.91 ± 0.31	5.43 ± 0.17	5.70 ± 0.24	6.06 ± 0.12
C16:1n-7	11.16 ± 1.02	11.45 ± 0.84	11.89 ± 0.33	11.89 ± 0.16	11.44 ± 0.45	11.94 ± 0.23	13.43 ± 0.13	13.39 ± 0.42	13.23 ± 0.07
C16:2n-4	3.21 ± 0.56	3.24 ± 0.49	3.48 ± 0.05	3.36 ± 0.06	3.20 ± 0.27	3.40 ± 0.06	2.90 ± 0.02	3.95 ± 2.25	2.86 ± 0.02
C16:3n-4	6.32 ± 1.12	6.42 ± 0.96	6.91 ± 0.11	7.09 ± 0.15	6.87 ± 0.61	7.09 ± 0.15	5.87 ± 0.01	5.78 ± 0.17	5.97 ± 0.06
C16:4n-1	31.38 ± 4.62	30.79 ± 3.32	29.53 ± 0.46	30.32 ± 0.64	30.86 ± 1.75	29.87 ± 0.71	28.28 ± 0.11	27.72 ± 0.75	27.73 ± 0.31
C18:4n-3	4.69 ± 0.73	4.71 ± 0.63	4.93 ± 0.07	4.90 ± 0.10	4.63 ± 0.32	4.94 ± 0.07	5.37 ± 0.02	5.17 ± 0.14	5.09 ± 0.07
C20:5n-3	28.68 ± 5.24	29.18 ± 4.55	30.73 ± 0.51	30.31 ± 0.64	28.47 ± 2.58	29.43 ± 0.43	31.09 ± 0.21	30.99 ± 1.06	30.90 ± 0.15
C22:6n-3	2.93 ± 1.54	2.26 ± 1.81	1.73 ± 1.85	2.20 ± 1.73	3.34 ± 0.26	2.12 ± 1.64	3.41 ± 0.07	2.76 ± 1.40	3.79 ± 0.08
SFA	11.63 ± 4.21	11.96 ± 3.73	10.80 ± 0.88	9.93 ± 0.12	11.19 ± 2.72	11.20 ± 0.61	9.79 ± 0.32	10.24 ± 0.36	10.44 ± 0.25
PUFA	77.21 ± 3.24	76.59 ± 3.09	77.30 ± 1.18	78.19 ± 0.23	77.37 ± 2.28	76.86 ± 0.77	76.78 ± 0.36	76.37 ± 0.77	76.34 ± 0.32

Table 2. The relative content (%) of fatty acids from Porosira glacialis extracted in dichloromethane/methanol (2:1 v/v; DCM/MeOH), hexane/isopropanol (2:1 v/v; hexane/IPA) and hexane, using three pre-treatments; no treatment (control), shaking at 1000 RPM for 60 min, (mixing), or sonication at 20 kHz for 10 min (sonication). Data shown is averages of replicates ± SD, n = 5. *SFA* saturated fatty acids, *PUFA* polyunsaturated fatty acids.

O. aurita	DCM/MeOH			Hexane/IPA			Hexane		
FA	**Control**	**Mixing**	**Sonication**	**Control**	**Mixing**	**Sonication**	**Control**	**Mixing**	**Sonication**
C14:0	11.29 ± 0.45	12.15 ± 0.12	12.46 ± 0.29	12.09 ± 0.23	12.26 ± 0.16	12.64 ± 0.15	11.81 ± 0.32	12.12 ± 0.19	12.10 ± 0.12
C16:0	27.14 ± 0.33	26.18 ± 0.18	26.10 ± 0.20	27.49 ± 0.29	27.38 ± 0.27	26.88 ± 0.13	28.84 ± 0.25	28.25 ± 0.24	27.91 ± 0.25
C16:1n-7	36.51 ± 0.65	35.71 ± 0.30	35.68 ± 0.29	37.23 ± 0.14	37.11 ± 0.37	36.06 ± 0.40	37.86 ± 0.26	38.60 ± 0.10	37.31 ± 0.22
C16:2n-4	3.03 ± 0.09	3.10 ± 0.02	3.04 ± 0.03	2.96 ± 0.02	2.96 ± 0.02	2.96 ± 0.04	2.93 ± 0.02	2.83 ± 0.01	2.81 ± 0.04
C16:3n-4	2.28 ± 0.07	2.35 ± 0.01	2.30 ± 0.02	2.02 ± 0.04	2.07 ± 0.03	2.10 ± 0.04	1.80 ± 0.02	1.62 ± 0.01	1.67 ± 0.05
C18:0	1.11 ± 0.03	1.20 ± 0.02	1.25 ± 0.05	1.21 ± 0.12	1.41 ± 0.07	1.53 ± 0.13	1.22 ± 0.07	1.40 ± 0.04	1.62 ± 0.04
C18:1n-9	2.98 ± 0.06	3.11 ± 0.03	3.02 ± 0.02	2.70 ± 0.05	2.77 ± 0.05	2.82 ± 0.06	2.47 ± 0.02	2.29 ± 0.03	2.31 ± 0.08
C18:1n-7	1.60 ± 0.02	1.94 ± 0.02	1.81 ± 0.02	1.63 ± 0.09	1.57 ± 0.10	1.58 ± 0.12	1.13 ± 0.02	1.17 ± 0.01	1.15 ± 0.01
C18:2n-6	1.21 ± 0.05	1.27 ± 0.05	1.25 ± 0.01	1.13 ± 0.01	1.20 ± 0.03	1.20 ± 0.03	1.04 ± 0.04	1.12 ± 0.05	1.13 ± 0.03
C20:5n-3	10.96 ± 0.17	12.35 ± 0.15	11.56 ± 0.12	10.03 ± 0.15	10.17 ± 0.15	11.14 ± 0.09	9.77 ± 0.07	10.11 ± 0.11	10.67 ± 0.20
C22:6n-3	1.89 ± 1.25	0.65 ± 0.72	1.52 ± 0.70	1.52 ± 0.03	1.08 ± 0.64	1.09 ± 0.80	1.14 ± 0.52	0.47 ± 0.45	1.32 ± 0.62
SFA	39.54 ± 0.29	39.53 ± 0.31	39.82 ± 0.34	40.79 ± 0.17	41.05 ± 0.29	41.05 ± 0.27	41.86 ± 0.31	41.77 ± 0.34	41.63 ± 0.39
MUFA	41.09 ± 0.73	40.76 ± 0.31	40.51 ± 0.31	41.56 ± 0.14	41.46 ± 0.31	40.46 ± 0.49	41.46 ± 0.29	42.06 ± 0.11	40.77 ± 0.28
PUFA	19.37 ± 0.99	19.72 ± 0.61	19.67 ± 0.64	17.65 ± 0.19	17.49 ± 0.54	18.50 ± 0.64	16.68 ± 0.54	16.17 ± 0.38	17.61 ± 0.58

Table 3. The relative content (%) of fatty acids from Odontella aurita extracted in dichloromethane/methanol (2:1 v/v; DCM/MeOH), hexane/isopropanol (2:1 v/v; hexane/IPA) and hexane, using three pre-treatments; no treatment (control), shaking at 1000 RPM for 60 min, (mixing), or sonication at 20 kHz for 10 min (sonication). Data shown is averages of replicates ± SD, n = 5. *SFA* saturated fatty acids, *MUFA* monounsaturated fatty acids, *PUFA* polyunsaturated fatty acids.

C. vulgaris	DCM/MeOH			Hexane/IPA			Hexane		
FA	**Control**	**Mixing**	**Sonication**	**Control**	**Mixing**	**Sonication**	**Control**	**Mixing**	**Sonication**
C16:0	24.79 ± 0.55	24.91 ± 0.17	23.25 ± 0.08	30.03 ± 5.95	25.42 ± 0.03	27.41 ± 2.75	24.63 ± 0.6	26.69 ± 0.2	29.29 ± 5.05
C16:3n-4	10.40 ± 0.21	10.61 ± 0.07	10.80 ± 0.08	9.93 ± 1.32	10.62 ± 0.25	9.79 ± 0.65	12.18 ± 0.21	10.80 ± 0.24	8.95 ± 0.99
C18:0	1.49 ± 0.09	2.01 ± 0.10	1.49 ± 0.12	2.47 ± 0.56	2.86 ± 0.28	1.95 ± 0.15	6.11 ± 0.13	8.30 ± 0.11	2.80 ± 0.61
C18:1n-9	4.01 ± 0.12	3.96 ± 0.06	3.96 ± 0.04	3.47 ± 0.07	3.74 ± 0.08	4.02 ± 0.05	3.63 ± 0.01	4.46 ± 0.08	4.85 ± 0.15
C18:1n-7	1.10 ± 0.01	1.09 ± 0.01	1.04 ± 0.01	1.00 ± 0.03	1.10 ± 0.02	1.05 ± 0.01	0	0.92 ± 0.01	1.07 ± 0.04
C18:2n-6	37.34 ± 0.19	36.51 ± 0.02	37.53 ± 0.07	33.89 ± 3.02	35.29 ± 0.2	35.63 ± 1.27	32.93 ± 0.22	30.00 ± 0.04	33.88 ± 2.7
C18:3n-3	20.87 ± 0.25	20.90 ± 0.09	21.93 ± 0.03	19.21 ± 2.15	20.97 ± 0.25	20.15 ± 1.02	20.52 ± 0.31	18.83 ± 0.04	19.16 ± 1.65
SFA	26.28 ± 0.60	26.92 ± 0.27	24.74 ± 0.16	32.50 ± 6.50	28.28 ± 0.57	29.36 ± 2.88	30.74 ± 0.73	34.99 ± 0.32	32.10 ± 5.52
MUFA	5.11 ± 0.13	5.06 ± 0.05	5.00 ± 0.05	4.47 ± 0.09	4.84 ± 0.10	5.07 ± 0.06	3.63 ± 0.01	5.38 ± 0.10	5.92 ± 0.19
PUFA	68.61 ± 0.61	68.02 ± 0.25	70.27 ± 0.14	63.03 ± 6.47	66.88 ± 0.65	65.57 ± 2.92	65.63 ± 0.74	59.63 ± 0.23	61.99 ± 5.34

Table 4. The relative content (%) of fatty acids from Chlorella vulgaris extracted in dichloromethane/methanol (2:1 v/v; DCM/MeOH), hexane/isopropanol (2:1 v/v; hexane/IPA) and hexane, using three pre-treatments; no treatment (control), shaking at 1000 RPM for 60 min, (mixing), or sonication at 20 kHz for 10 min (sonication). Data shown is averages of replicates ± SD, n = 5. *SFA* saturated fatty acids, *MUFA* monounsaturated fatty acids, *PUFA* polyunsaturated fatty acids.

The dominating fatty acids in *O. aurita* were C16:0 and C16:1n-7, with a combined contribution of more than 60% in all samples (Table 3). In total, saturated and monounsaturated fatty acid made up about 80% of all fatty acids in this diatom. In contrast to *P. glacialis*, eicosapentaenoic acid (C20:5n-3, EPA) contributed only 11% of the fatty acids in all extracts of *O. aurita,* but the content of DHA was similar (2–3%). A previous study on *O. aurita* found a comparable fatty acid composition when the algae was cultivated at 24 °C, but not at lower temperatures[42].

The relative composition of the individual fatty acids and the total amount of SFA, MUFA and PUFA showed little variation across both solvent and treatment (Table 3). The dominating fatty acids in *C. vulgaris* were C16:0, C18:2n-6 and C18:3n-3, totaling approximately 80% of the fatty acids in all samples (Table 4). In contrast to *P. glacialis* and *O. aurita*, *C. vulgaris* did not contain any fatty acids of more than 18 carbons, which agrees with other studies on *C. vulgaris*[43,44]. The changes in lipid class composition of *O. aurita* and *C. vulgaris* as a result of solvent and treatment were accompanied by changes in the fatty acid composition; the relative content of PUFA was higher in DCM/MeOH compared to hexane. This indicates that the relative content of PUFAs is higher in the membrane-associated lipids in *O. aurita* and *C. vulgaris*.

Conclusions

DCM/MeOH is a better solvent than hexane and hexane/IPA for extracting lipids from *P. glacialis*. However, hexane/IPA also works well and is a better alternative in large-scale extractions. Sonication did not increase the lipid yield or influence the fatty acid composition in *P. glacialis* and *O. aurita* compared to mixing. Cell wall disruption is therefore not a prerequisite to obtain high product yields in *P. glacialis* and probably diatoms in general, in contrast to other mass-cultivated microalgae. In conclusion, choice of solvent and number of extractions were the main factors that determined lipid yield and composition in *P. glacialis*.

Data availability

The raw data obtained in this study along with the R scripts used for analysis and graphing are available from the Open Science Framework (OSF) under the name "Choice of solvent and number of extractions are the main factors that determine lipid yield in a marine centric diatom" at https://osf.io/sxrvz/?view_only=aa4342d55dd348768ed77ac06aee7c97.

Received: 3 July 2020; Accepted: 30 September 2020
Published online: 17 December 2020

References

1. Chisti, Y. Biodiesel from microalgae. *Biotechnol. Adv.* **25**, 294–306. https://doi.org/10.1016/j.biotechadv.2007.02.001 (2007).
2. Sprague, M., Betancor, M. B. & Tocher, D. R. Microbial and genetically engineered oils as replacements for fish oil in aquaculture feeds. *Biotechnol. Lett.* **39**, 1599–1609. https://doi.org/10.1007/s10529-017-2402-6 (2017).
3. Ryckebosch, E. *et al.* Nutritional evaluation of microalgae oils rich in omega-3 long chain polyunsaturated fatty acids as an alternative for fish oil. *Food Chem.* **160**, 393–400. https://doi.org/10.1016/j.foodchem.2014.03.087 (2014).
4. Benedetti, M., Vecchi, V., Barera, S. & Dall'Osto, L. Biomass from microalgae: the potential of domestication towards sustainable biofactories. *Microb. Cell Fact* **17**, 1. https://doi.org/10.1186/s12934-018-1019-3 (2018).
5. Borowitzka, M. A. High-value products from microalgae-their development and commercialisation. *J. Appl. Phycol.* **25**, 743–756. https://doi.org/10.1007/s10811-013-9983-9 (2013).
6. Ratledge, C. Are algal oils realistic options for biofuels?. *Eur. J. Lipid Sci. Tech.* **113**, 135–136. https://doi.org/10.1002/ejlt.201100034 (2011).
7. Lee, J. Y., Yoo, C., Jun, S. Y., Ahn, C. Y. & Oh, H. M. Comparison of several methods for effective lipid extraction from microalgae. *Biores. Technol.* **101**, S75–S77. https://doi.org/10.1016/j.biortech.2009.03.058 (2010).
8. Ranjan, A., Patil, C. & Moholkar, V. S. Mechanistic assessment of microalgal lipid extraction. *Ind. Eng. Chem. Res.* **49**, 2979–2985. https://doi.org/10.1021/ie9016557 (2010).
9. Balduyck, L., Bruneel, C., Goiris, K., Dejonghe, C. & Foubert, I. Influence of high pressure homogenization on free fatty acid formation in *Nannochloropsis* sp.. *Eur. J. Lipid Sci. Tech.* **120**, 1. https://doi.org/10.1002/ejlt.201700436 (2018).
10. Atkinson, A. W., John, P. C. L. & Gunning, B. E. S. Sporopollenin in cell-wall of Chlorella and other algae: Ultrastructure, chemistry, and incorporation of C-14-acetate studied in synchronous cultures. *Planta* **107**, 1–000. https://doi.org/10.1007/Bf00398011 (1972).
11. Pezzolesi, L., Pichierri, S., Samori, C., Totti, C. & Pistocchi, R. PUFAs and PUAs production in three benthic diatoms from the northern Adriatic Sea. *Phytochemistry* **142**, 85–91. https://doi.org/10.1016/j.phytochem.2017.06.018 (2017).
12. Pohnert, G. Phospholipase A(2) activity triggers the wound-activated chemical defense in the diatom Thalassiosira rotula. *Plant Physiol.* **129**, 103–111. https://doi.org/10.1104/pp.010974 (2002).
13. Wichard, T. *et al.* Lipid and fatty acid composition of diatoms revisited: Rapid wound-activated change of food quality parameters influences herbivorous copepod reproductive success. *ChemBioChem* **8**, 1146–1153. https://doi.org/10.1002/cbic.200700053 (2007).
14. Folch, J., Lees, M. & Stanley, G. H. S. A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* **226**, 497–509 (1957).
15. Bligh, E. G. & Dyer, W. J. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**, 911–917 (1959).
16. Iverson, S. J., Lang, S. L. C. & Cooper, M. H. Comparison of the Bligh and Dyer and Folch methods for total lipid determination in a broad range of marine tissue. *Lipids* **36**, 1283–1287. https://doi.org/10.1007/s11745-001-0843-0 (2001).
17. Cequier-Sanchez, E., Rodriguez, C., Ravelo, A. G. & Zarate, R. Dichloromethane as a solvent for lipid extraction and assessment of lipid classes and fatty acids from samples of different natures. *J. Agric. Food Chem.* **56**, 4297–4303. https://doi.org/10.1021/jf073471e (2008).
18. Chen, I. S., Shen, C. S. J. & Sheppard, A. J. Comparison of methylene-chloride and chloroform for the extraction of fats from food-Products. *J. Am. Oil Chem. Soc.* **58**, 599–601. https://doi.org/10.1007/Bf02672373 (1981).
19. Hara, A. & Radin, N. S. Lipid extraction of tissues with a low-toxicity solvent. *Anal. Biochem.* **90**, 420–426. https://doi.org/10.1016/0003-2697(78)90046-5 (1978).
20. Cheng, J., Huang, R., Li, T., Zhou, J. H. & Cen, K. F. Biodiesel from wet microalgae: Extraction with hexane after the microwave-assisted transesterification of lipids. *Biores. Technol.* **170**, 69–75. https://doi.org/10.1016/j.biortech.2014.07.089 (2014).
21. Shin, H. Y., Ryu, J. H., Bae, S. Y., Crofcheck, C. & Crocker, M. Lipid extraction from Scenedesmus sp microalgae for biodiesel production using hot compressed hexane. *Fuel* **130**, 66–69. https://doi.org/10.1016/j.fuel.2014.04.023 (2014).
22. Artamonova, E. Y., Svenning, J. B., Vasskog, T., Hansen, E. & Eilertsen, H. C. Analysis of phospholipids and neutral lipids in three common northern cold water diatoms: Coscinodiscus concinnus, Porosira glacialis, and Chaetoceros socialis, by ultra-high performance liquid chromatography-mass spectrometry. *J. Appl. Phycol.* **29**, 1241–1249. https://doi.org/10.1007/s10811-017-1055-0 (2017).
23. Svenning, J. B., Dalheim, L., Eilertsen, H. C. & Vasskog, T. Temperature dependent growth rate, lipid content and fatty acid composition of the marine cold-water diatom Porosira glacialis. *Algal Res. Biomass Biofuels Bioprod.* **37**, 11–16. https://doi.org/10.1016/j.algal.2018.10.009 (2019).
24. Jones, J., Manning, S., Montoya, M., Keller, K. & Poenie, M. Extraction of algal lipids and their analysis by HPLC and mass spectrometry. *J. Am. Oil Chem. Soc.* **89**, 1371–1381. https://doi.org/10.1007/s11746-012-2044-8 (2012).
25. Metting, F. B. Biodiversity and application of microalgae. *J. Ind. Microbiol. Biot* **17**, 477–489. https://doi.org/10.1007/Bf01574779 (1996).
26. Artamonova, E. Y., Vasskog, T. & Eilertsen, H. C. Lipid content and fatty acid composition of Porosira glacialis and Attheya longicornis in response to carbon dioxide (CO2) aeration. *PLoS ONE* **12**, 1. https://doi.org/10.1371/journal.pone.0177703 (2017).
27. Uradnikova, M. *Species validity of five common northern/Arctic spring bloom diatoms: a combined morphological and molecular study* Master's thesis thesis, UiT The Arctic University of Norway, (2020).
28. Stoffel, W., Chu, F. & Ahrens, E. H. Analysis of long-chain fatty acids by gas-liquid chromatography: micromethod for preparation of methyl esters. *Anal. Chem.* **31**, 307–308. https://doi.org/10.1021/ac60146a047 (1959).

29. Abreu, S., Solgadi, A. & Chaminade, P. Optimization of normal phase chromatographic conditions for lipid analysis and comparison of associated detection techniques. *J. Chromatogr. A* **1514**, 54–71. https://doi.org/10.1016/j.chroma.2017.07.063 (2017).
30. Lee, S. J., Yoon, B. D. & Oh, H. M. Rapid method for the determination of lipid from the green alga Botryococcus braunii. *Biotechnol. Tech.* **12**, 553–556. https://doi.org/10.1023/A:1008811716448 (1998).
31. Prabakaran, P. & Ravindran, A. D. A comparative study on effective cell disruption methods for lipid extraction from microalgae. *Lett. Appl. Microbiol.* **53**, 150–154. https://doi.org/10.1111/j.1472-765X.2011.03082.x (2011).
32. Guckert, J. B. & White, D. C. Evaluation of a hexane isopropanol lipid solvent system for analysis of bacterial phospholipids and application to chloroform-soluble nuclepore (polycarbonate) membranes with retained bacteria. *J. Microbiol. Methods* **8**, 131–137. https://doi.org/10.1016/0167-7012(88)90014-0 (1988).
33. Gunnlaugsdottir, H. & Ackman, R. G. 3 Extraction methods for determination of lipids in fish-meal - evaluation of a hexane isopropanol method as an alternative to chloroform-based methods. *J. Sci. Food Agr.* **61**, 235–240. https://doi.org/10.1002/jsfa.2740610216 (1993).
34. Lepetit, B., Goss, R., Jakob, T. & Wilhelm, C. Molecular dynamics of the diatom thylakoid membrane under different light conditions. *Photosynth. Res.* **111**, 245–257. https://doi.org/10.1007/s11120-011-9633-5 (2012).
35. Cutignano, A. *et al.* Chloroplastic glycolipids fuel aldehyde biosynthesis in the marine diatom Thalassiosira rotula. *ChemBioChem* **7**, 450–456. https://doi.org/10.1002/cbic.200500343 (2006).
36. Berge, J. P., Gouygou, J. P., Dubacq, J. P. & Durand, P. Reassessment of lipid-composition of the diatom skeletonema-costatum. *Phytochemistry* **39**, 1017–1021. https://doi.org/10.1016/0031-9422(94)00156-N (1995).
37. Ryckebosch, E., Muylaert, K., Eeckhout, M., Ruyssen, T. & Foubert, I. Influence of drying and storage on lipid and carotenoid stability of the microalga phaeodactylum tricornutum. *J. Agric. Food Chem.* **59**, 11063–11069. https://doi.org/10.1021/jf2025456 (2011).
38. d'Ippolito, G. *et al.* The role of complex lipids in the synthesis of bioactive aldehydes of the marine diatom Skeletonema costatum. *Biochim. Biophys. Acta Mol. Cell Biol. Lipids* **1686**, 100–107. https://doi.org/10.1016/j.bbalip.2004.09.002 (2004).
39. Balduyck, L. *et al.* Lipolysis in T-Isochrysis lutea during wet storage at different temperatures. *Algal Res. Biomass Biofuels Bioprod.* **18**, 281–287. https://doi.org/10.1016/j.algal.2016.07.003 (2016).
40. Fuller, I. D. *et al.* Free fatty acids in commercial krill oils: concentrations, compositions, and implications for oxidative stability. *J. Am. Oil Chem. Soc.* https://doi.org/10.1002/aocs.12368 (2020).
41. Miyashita, K. & Takagi, T. Study on the oxidative rate and prooxidant activity of free fatty-acids. *J. Am. Oil Chem. Soc.* **63**, 1380–1384. https://doi.org/10.1007/Bf02679607 (1986).
42. Pasquet, V. *et al.* Fatty acids profile and temperature in the cultured marine diatom Odontella aurita. *J. Appl. Phycol.* **26**, 2265–2271. https://doi.org/10.1007/s10811-014-0252-3 (2014).
43. Hultberg, M., Jonsson, H. L., Bergstrand, K. J. & Carlsson, A. S. Impact of light quality on biomass production and fatty acid content in the microalga Chlorella vulgaris. *Biores. Technol.* **159**, 465–467. https://doi.org/10.1016/j.biortech.2014.03.092 (2014).
44. Petkov, G. & Garcia, G. Which are fatty acids of the green alga Chlorella?. *Biochem. Syst. Ecol.* **35**, 281–285. https://doi.org/10.1016/j.bse.2006.10.017 (2007).

Acknowledgements
The authors wish to thank Hans Christian Eilertsen, Linn Jakobsen Kristiansen, Therese Smelror Løkken and Gunilla Eriksen for their help with algae cultivation, harvesting and processing.

Author contributions
J.B.S., L.D., T.V., B.V. and R.O. planned the experiment. J.B.S., L.D. and L.M. collected, analysed and interpreted the data. J.B.S. and L.D. wrote the manuscript and prepared the tables and Figs. 2, 3 and 4, B.V. and L.M. prepared Fig. 1. R.O. and T.V. provided critical revisions of the manuscript. All authors reviewed the final version of the manuscript to be published.

Funding
The project was financed by Troms County Regional Funds no. TFK2017-066, UiT The Arctic University of Norway and Finnfjord AS.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41598-020-79269-z.

Correspondence and requests for materials should be addressed to J.B.S.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2020