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Porosira glacialis as a possible source of lipids for human consumption and aquaculture feed

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Summary

The demand for marine long-chain omega-3 fatty acids for both human consumption and fish feed is large. The long-chain omega-3 fatty acids, EPA and DHA, have several health benefits for both humans and fish. The amount of wild caught fish has reached maximum exploitation over the last 40 years, while the production from aquaculture has increased drastically. The shortage of marine raw material has led to an increased inclusion of terrestrial raw material in the aquaculture feed, which has affected the nutritional composition of the farmed fish. Cultivation of marine microalgae may be part of the solution to these challenges, since these are the ocean's primary producers of EPA and DHA. Microalgae can be cultivated in water using only CO_2, light, and inorganic nutrients. Microalgae can also reduce the amount of direct CO_2 emissions from large industry by utilizing the gas for cultivation.

In this project, the cold-water adapted diatom *Porosira glacialis* was cultivated to investigate the lipid fraction's suitability for fish feed and/or human consumption. The specific aims were to i) investigate the storage stability of lipid in wet biomass, ii) compare the *in vitro* digestibility of lipids from *P. glacialis* with common sources of long-chain omega-3 fatty acids, iii) investigate the extractability of lipids from *P. glacialis* compared to other mass-cultivated microalgae.

P. glacialis contains large amounts of polyunsaturated fatty acids, of which EPA makes up a substantial part. Compared to the lipid fraction of the other oils that were investigated, the omega-3 content is high. The main fraction of lipid classes in *P. glacialis* is the polar galactolipids and phospholipids. These lipid classes differ from the usual fish oil products, like cod-liver oil and omega-3 concentrates. The lipids in *P. glacialis* are exposed to hydrolysis during storage, which results in increased amounts of free fatty acids that are more exposed to oxidation. Our results indicate that the storage stability of lipids in the wet biomass can be enhanced by a simple heat treatment and storage at 4 °C. The lipids in *P. glacialis* appear to be digested well compared to common omega-3 supplements. The only other oil with a higher degree of hydrolysis was cod-liver oil, which contains mostly triacylglycerol. The extraction of lipids from *P. glacialis* does not require any pre-treatment. However, like other species with large amounts of polar lipid classes, a partially polar solvent is needed for complete extraction. *P. glacialis* grows well using CO_2 from the flue gas of a smelting plant and could therefore help limit the emission of greenhouse gases. The prospect of investigating this diatom directly for fish feed or human consumption is interesting.

Sammendrag

Det er et stort behov for marine langkjedede omega-3 fettsyrer både til humant konsum og til fiskefôr. De langkjedede omega-3 fettsyrene EPA og DHA har gunstige helseeffekter for både mennesker og fisk. Mengden villfanget fisk har de siste 40 årene nådd maksimal utnyttelse mens volumet av oppdrettsfisk har økt betraktelig. Mangelen på marint råstoff har ført til en økt andel terrestrisk råstoff i fiskefôret, noe som påvirker næringssammensetningen i oppdrettsfisken. Dyrking av marine mikroalger kan være med på å løse utfordringene knyttet til mangel på marine langkjedede omega-3 fettsyrer, da de er havets primærprodusenter av EPA og DHA. Mikroalger dyrkes i vann og trenger CO_2, lys og uorganiske næringsstoffer for å vokse. Mikroalger kan også være med på å redusere direkte utslipp av CO_2 fra store utslippspunkter ved å benytte denne gassen til slik dyrking.

I dette prosjektet ble den kaldtvannstilpassede kiselalgen *Porosira glacialis* dyrket for å undersøke om lipidfraksjonen er egnet som tilskudd i fiskefôr og/eller til menneskelig konsum. De spesifikke delmålene var i) å undersøke lagringsstabiliteten til lipidene i våt biomasse, ii) sammenligne *in vitro* fordøyelighet av lipidene i *P. glacialis* med vanlige kilder til langkjedede omega-3 fettsyrer, iii) undersøke utbytte av lipider med ulike fettekstraksjonsmetoder sammenlignet med andre massedyrkede mikroalger.

P. glacialis inneholder store mengder flerumettet fett, hvorav fettsyren EPA utgjør en stor andel. Sammenlignet med lipidfraksjonen i de andre artene og oljene som ble undersøkt, er omega-3-innholdet høyt. Størstedelen av lipidklassene i *P. glacialis* er polare, blant annet galaktolipider og fosfolipider. Disse lipidklassene skiller seg ut fra vanlige fiskeoljeprodukter som tran og omega-3-konsentrater. Lipidene i *P. glacialis* er svært utsatt for hydrolyse under lagring, noe som fører til økte mengder frie fettsyrer samt at oljen er mer utsatt for oksidasjon. Våre resultater viser at lagringsstabiliteten til lipidene i våt biomasse kan økes betraktelig ved en enkel varmebehandling før lagring ved 4 °C. Det ser også ut til at lipidene i *P. glacialis* er godt fordøyelige sammenlignet med vanlige omega-3 kosttilskudd. Den eneste oljen som ble hydrolysert til en høyere grad under *in vitro* fordøyelse var tran, som i hovedsak inneholder triacylglycerol. Ekstraksjon av lipider fra *P. glacialis* krever ingen forbehandling. Men som andre arter med høyt innhold av polare lipidklasser trengs et delvis polart løsemiddel for ekstraksjon. Den har god vekst på CO_2 fra røykgassen til et smelteverk og kan derfor være med på å begrense direkte utslipp av drivhusgasser. Det vil være spennende å undersøke om denne kiselalgen kan benyttes direkte til fiskefôr eller humant konsum.

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List of papers

Paper I:
Dalheim L, Svenning JB, Eilertsen HC, Vasskog T, Olsen RL (2020) 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*:11 doi:10.1007/s10811-020-02292-0

Paper II:

Dalheim, L, Svenning JB, Olsen RL (2021) *In vitro* digestion of lipids from the marine diatom *Porosira glacialis* compared to commercial omega-3 products (manuscript submitted to *Plos One*)

Paper III:

Svenning JB, Dalheim L, Vasskog T, Matricon L, Vang B, Olsen RL (2020). "Lipid yield from the diatom Porosira glacialis is determined by solvent choice and number of extractions, independent of cell disruption." *Scientific Reports* **10**(1): 22229.

Abbreviations and acronyms

ALA	α-linolenic acid
CCU	Carbon capture and utilization
CEL	Carboxyl ester lipase
CoA	Coenzyme A
DAG	Diacylglycerol
DCM	Dichloromethane
DGDG	Digalactosyldiacylglycerol
DHA	Docosahexaenoic acid
EFA	Essential fatty acids
ELSD	Evaporative light scattering detector
EPA	Eicosapentaenoic acid
FFA	Free fatty acids
FID	Flame ionization detector
GC	Gas chromatography
HPLC	High performance liquid chromatography
IPA	Isopropanol
IS	Internal standard
LPL	Lysophospholipid
MAG	Monoacylglycerol
MeOH	Methanol
MGDG	Monogalactosyldiacylglycerol
MS	Mass spectrometry
MUFA	Monounsaturated fatty acids
PG	Phosphatidylglycerol
PLRP2	Pancreatic lipase-related protein 2
PS I	Photosystem I
PS II	Photosystem II
PUFA	Polyunsaturated fatty acids
SFA	Saturated fatty acids
SFC	Supercritical fluid chromatography
SPE	Solid phase extraction
TAG	Triacylglycerol
UPLC	Ultra-precision liquid chromatography
WE	Wax ester

1 Introduction

Earth is a perpetually changing place. The human population has been growing from 1 billion early in the 19th century to almost 8 billion today, and is estimated to be 9.7 billion by the year 2050 (DESA 2019). In addition to the growing population, more people are moving out of poverty and into the middle class (Kharas 2017). With an increasing number of people inhabiting this planet and a change in social structure comes a growing demand for food and essential nutrients. To provide food security for the estimated population size in the year 2050, global food production must increase, and to meet the increased demand for food we are reliant on technological advances and novel food sources. One of the important factors of an optimal diet is omega-3 fatty acids, especially eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), which have several potential health benefits for humans (Salem and Eggersdorfer 2015). The most widely recognized benefits are related to inflammation (Calder 2015) and protection against cardiovascular disease (Watanabe and Tatsuno 2017, Abdelhamid et al. 2020, Bernasconi et al. 2020). In addition to cardiovascular disease, there are indications that EPA and DHA may have positive effects on other diseases such as diabetes, cancer, depression, Alzheimer's, and dementia (Shahidi and Ambigaipalan 2018). Omega-3 fatty acids are also important for the proper development of visual and neurological tissue (Mallick et al. 2019). Vertebrates cannot *de novo* synthesize omega-3 fatty acids, and these are therefore essential in the diet. There is evidence that humans evolved on a diet containing approximately equal amounts of omega-6 and omega-3 fatty acids (Simopoulos 2006). For optimal function and health in humans, several different authorities recommend a daily intake of EPA and DHA of 250-500 mg day^{-1} (Kris-Etherton et al. 2009). The most common sources of EPA and DHA in a normal diet are fish or fish oil supplements.

There are many benefits of consuming fish rather than fish oils. Seafood is an important source of high quality proteins and essential nutrients like vitamin D, taurine, selenium, and iodine in addition to the long-chain omega-3 fatty acids (Lund 2013). However, many of the wild fish stocks are overexploited and the capture of wild fish has not increased since it reached a plateau in the 1980's (FAO 2020). The same period of time have seen a rapid expansion of the aquaculture industry, in which the production reached 82 million metric tons in 2018, contributing almost half of the global fish production that year (FAO 2020). To produce enough fish for the growing world population we are dependent on the continuing growth of the

aquaculture industry. However, the growth in aquaculture production has led to increased demand for marine ingredients, such as fish oil, for fish feed. Approximately 70% of the globally produced fishmeal and fish oil are utilized by the aquaculture industry (Tacon and Metian 2015). Because of the high price and low availability of marine feedstuff, there has been a transition from feed containing mostly ingredients of marine origin to a feed where ingredients of terrestrial origin are the major components (Tacon and Metian 2015, Aas et al. 2019). However, the lack of nutrients from marine sources may be an obstacle to further growth of volume in the aquaculture industry. Terrestrial plants can only synthesize the omega-3 fatty acid α-linolenic acid (ALA, 18:3n-3), and not the long chain omega-3 fatty acids EPA and DHA. Marine fish, unlike other vertebrates, are unable to elongate and desaturate ALA into EPA and DHA (Sargent et al. 2003). In fact, there is evidence that anadromous fish also require certain amounts of EPA and DHA to ensure animal welfare and optimal growth (Glencross 2009, Rosenlund et al. 2016, Sissener et al. 2016). In addition, there is a need for EPA and DHA in the fish feed for the fish fillet to be a good provider of these fatty acids to the human consumer (Tocher 2015).

Because of the limited supply of wild caught fish for food, feed, and oil production, there is a need for novel sources of marine nutrients (Salem and Eggersdorfer 2015). As an alternative to fish, several other sources of EPA and DHA have been investigated (Klinger and Naylor 2012, Adarme-Vega et al. 2014). Oil from transgenic plants, in which genes for elongase and desaturase enzymes needed to synthesize EPA and DHA from ALA have been inserted, have been suggested to be a novel source of these fatty acids (West et al. 2021). In these gene modified plant oils, EPA and DHA may make up 12 to 28% of the total fatty acids (Tocher et al. 2019). Lower trophic level organisms from the marine environment, such as Antarctic krill (*Euphausia superba*) and *Calanus finmarchicus*, are also being utilized for EPA and DHA production (Pedersen et al. 2014, Yurko-Mauro et al. 2015). Even if the biomasses of these organisms are large, the ecological effects of harvesting these from the marine environment must be taken into account (Nicol et al. 2012). Additionally, the high cost of producing oil from crustaceans may limit the use as a bulk ingredient in fish feed (Pedersen et al. 2014).

Another potential source of EPA and DHA are the ocean's primary producers of these fatty acids, the microalgae. Microalgae can be cultivated using seawater, light, CO_2, and inorganic nutrients. Different types of microalgae usually contain high amounts of EPA and

low amounts of DHA or vice versa (Patil et al. 2007, Ryckebosch et al. 2014). The diatoms, which differ from other microalgae by having a silica cell wall, usually contain large amounts of EPA and a high proportion of polyunsaturated fatty acids (PUFA) (Sayanova et al. 2017). Microalgae oil may be a supplement to fish oil for EPA and DHA in aquaculture feed, and several promising trials have been performed (Sprague et al. 2017, Tocher et al. 2019). The microalgae oil or biomass could also be used as a health food supplement for omega-3 delivery to humans (Haimeur et al. 2016, Barkia et al. 2019). Cultivation and processing of microalgae are expensive, and the costs needs to be reduced for it to become a profitable and viable source of omega-3 for different uses (Chauton et al. 2015, Wang and Seibert 2017). Harvesting and dewatering of the biomass represent a large part of the cost, but can be reduced by some levels of automation (Fasaei et al. 2018). If oil is to be extracted from the biomass, this could also represent a major part of the cost, especially if cell disruption is necessary (Demuez et al. 2015). However, the cultivation of microalgae in the vicinity of high CO_2 emission sources mitigates the effect of these sources on the environment while providing the microalgae with a source of CO_2 for photosynthesis, a process known as carbon capture and utilization (CCU) (Yen et al. 2015). Industries commonly have to pay taxes based on the amount of CO_2 emitted and CCU could therefore reduce the costs for these industries as well.

1.1 Aims

The overall aim of this thesis was to characterize important features of the lipid fraction of industrially cultivated *Porosira glacialis* biomass.

The primary aim of **Paper I** was to investigate the storage stability of lipids from *P. glacialis* using different pre-treatments. The primary aim of **Paper II** was to study the *in vitro* digestibility of lipids from *P. glacialis* compared to commercially available omega-3 rich oils (cod-liver oil, krill oil, Calanus oil, and an ethyl ester concentrate). In **Paper III,** the primary aim was to investigate the extractability of lipids from *P. glacialis* compared to *Odontella aurita* and *Chlorella vulgaris.*

2 Background

2.1 Diatoms

Microalgae are phylogenetically diverse, photosynthetic, small unicellular organisms found in most environments around the world. They are some of the world's most important primary producers, forming the base of food webs in oceans and other waters. The microalgae evolved through an endosymbiotic event around 1 billion years ago, when a cyanobacteria-like prokaryote was absorbed by a heterotrophic eukaryotic cell to form a chloroplast (McFadden 2001, Shih and Matzke 2013). Diatoms are a class of microalgae that evolved through a secondary endosymbiotic event involving a eukaryotic host and a eukaryotic phototrophic endosymbiont in addition to horizontal gene transfer from bacteria (Moustafa et al. 2009, Bowler et al. 2010). The diatoms are a diverse group, with estimates of up to 100,000-200,000 species, making them the most species-rich microalgae (Armbrust 2009, Mann and Vanormelingen 2013). The diatoms contribute more than 40% of the primary production in the oceans and play an important part in the biogeochemical cycles around the world, affecting biogenic silica production and generating large amounts of oxygen (Nelson et al. 1995). The diatoms can be separated broadly into two groups, the centric diatoms and the pennate diatoms (Figure 1). The centric diatoms are radially symmetrical, and appear to have become a prevalent species during the cretaceous era, whereas the pennate diatoms are bilaterally symmetrical and evolved from the centric diatoms at a later stage (Medlin et al. 1993).

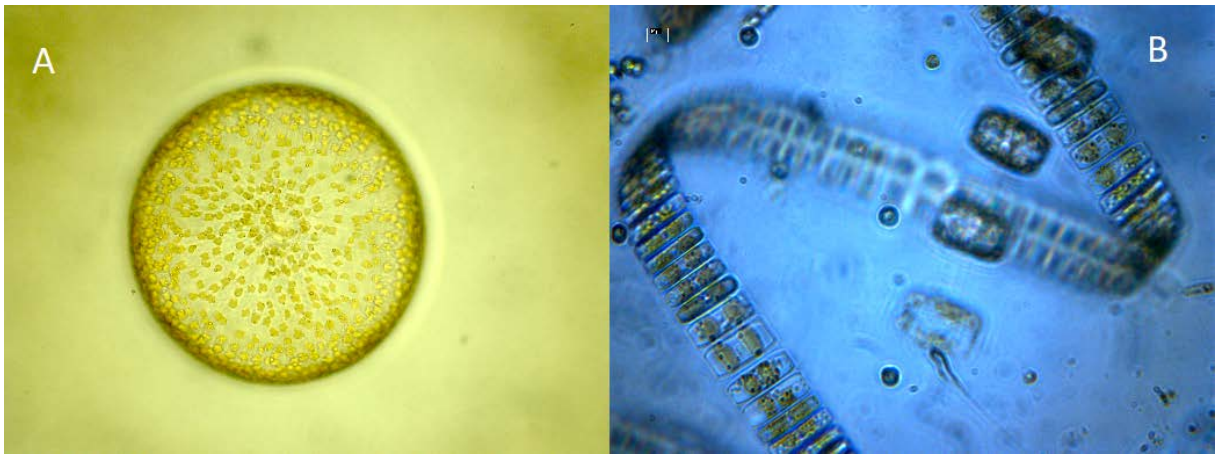

Figure 1 A: Coscinodiscus sp.*, a centric diatom (photo credit Jon Brage Svenning). B:* Navicula vanhoeffeni*, a pennate diatom (photo credit Gunilla Eriksen).*

The diatom cell range in sizes from 5-200 µm in diameter or length, and is distinguished from other microalgae, such as green algae, by having a silica cell wall, called a frustule. The frustule is made up of two theca that fit each other like a petri dish, where epitheca is the top half (outer, slightly larger) and hypotheca the lower half (inner, slightly smaller). The silica cell wall is covered with pores that facilitate the exchange of molecules between the cytoplasm and the environment (Hale and Mitchell 2001). Diatoms require several nutrients to grow, such as nitrogen, phosphorus, iron, and silicon as building blocks. However, carbon make up the bulk of the biomass, and this carbon is available from CO_2 processed into organic carbon through photosynthesis.

When diatoms reproduce through binary fission, two new individuals are formed within the parent cell (Figure 2). The thecas of the parent cell become the epitheca of the two newly formed cells, and the cell division ends with the formation of a new hypotheca. Because one of the daughter cells receives the parent hypotheca as their epitheca, there is an inevitable reduction in size of the cells after a series of fission events. For the diatom cell to reach its maximum size again an auxospore is formed. An auxospore is a diatom cell that has shed its theca and formed an organic membrane in which it can grow to maximum size before forming a new frustule. The formation of auxospore cells depend on size and usually happens when the cell reach 1/3 of its maximum size (Hasle and Syvertsen 1997). The formation of auxospore cells may be vegetative or sexual.

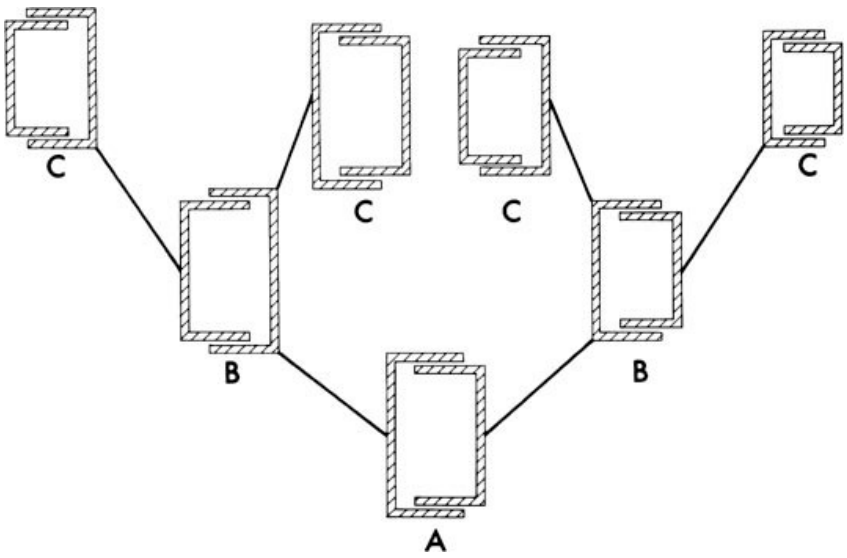

Figure 2 Binary fission of a diatom cell. One part of the parent cell theca becomes the epitheca of the daughter cell. Illustration taken from Burckle (1979).

2.1.1 Photosynthesis

Photosynthesis is the process in which inorganic matter and energy from light are converted into organic matter, i.e. chemical energy. The process of photosynthesis occurred for the first time 3.2 to 3.5 billion years ago in several different prokaryotes, however this photosynthesis was not oxygen generating, but producing e.g. sulfur (Blankenship 2010). Before or during the great oxidation event, which took place around 2.4 billion years ago, cyanobacteria started to produce oxygen through photosynthesis (Lyons et al. 2014). Oxygenic photosynthesis requires H_2O, CO_2 and light to produce O_2 and organic compounds. The free oxygen concentration did not increase immediately after the great oxidation event, probably because of a buffer capacity of other gases and minerals present in the atmosphere and the ground at the time. Therefore, it took almost 2 billion years to reach the oxygen concentrations of today (Holland 2006), and at this point eukaryotic organisms had become photosynthetic, through the aforementioned endosymbiosis of prokaryotes and eukaryotes. The total primary production of the oceans are approximately 60 billion metric tons carbon per year, of which the diatoms contribute 40 % (Nelson et al. 1995).

The photosynthetic processes of diatoms are similar to those occurring in most other photosynthetic eukaryotes. The process itself takes place within the chloroplast of the diatom. Within the chloroplast are thylakoid membranes, which are built from the lipids monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), and other polar lipids interspersed with proteins and pigments. The intrathylokoidal space is named lumen and the area outside the thylakoid membrane is named stroma, and the various reactions of photosynthesis takes place either within the thylakoid membrane, the lumen or the stroma. The light reactions, in which light energy is converted to ATP and $NADPH_2$, occur in the thylakoid membrane. The light energy is captured by photosystem I (PS I) and photosystem II (PS II), in concert with light harvesting antennae I and II (LHC I and LHC II). The LHC of diatoms contain chlorophyll *a* and *b*, and the carotenoid fucoxanthin (Büchel 2020), and capture light and transfer the energy to the photosynthetic reaction centers of PS II and PS I. In the oxygen evolving complex of photosystem II, two electrons are extracted from H_2O and O_2 is formed. The electrons are then transferred from PS II, via plastoquinones, the cytochrome b6/f complex, and plastocyanin, into PS I, where the reductant $NADPH_2$ is formed. The membrane bound enzyme ATP synthase utilize a pH gradient over the membrane, which is formed by protons being carried from the stroma to the lumen, to form the energy molecule ATP, which is needed

downstream for carbon fixation. The whole process, named photophosphorylation, is summed up in equation 1:

$$(1) 2NADP + 2H_2O + 3\,ADP + 3P_i \xrightarrow{Light\ energy} 2NADPH_2 + 3ATP + O_2$$

The dark reaction, named the Calvin-Benson cycle (Figure 3), in which carbon is fixated, occurs in the stroma. The dark reaction use the $NADPH_2$ and ATP formed in the light reaction. This process can be divided into four phases, namely; the carboxylation phase (i), the reduction phase (ii), the regeneration phase (iii), and the production phase (iv). In phase i, CO_2 is added to the 5-carbon sugar ribulose bisphosphate (Ribulose-bis-P) to form two molecules of phosphoglycerate (Glycerate-P), a reaction catalyzed by the enzyme ribulose bisphosphate carboxylase/oxygenase (Rubisco), this intermediate product can be used to synthesize lipids and amino acids (Li-Beisson et al. 2019). The second phase (ii) is a two-step process; first ATP donate phosphorus to phosphoglycerate to form diphosphoglycerate (Glycerate-bis-P), before $NADPH_2$ reduces it to phosphoglyceraldehyde (Glyceraldehyde-P). Both ATP and $NADPH_2$ is produced in the light reaction. Phase III of the Calvin-Benson cycle involves regeneration of ribulose phosphate (Ribulose-P) to repeat the dark reaction, and phase IV is the production of carbohydrates for energy storage.

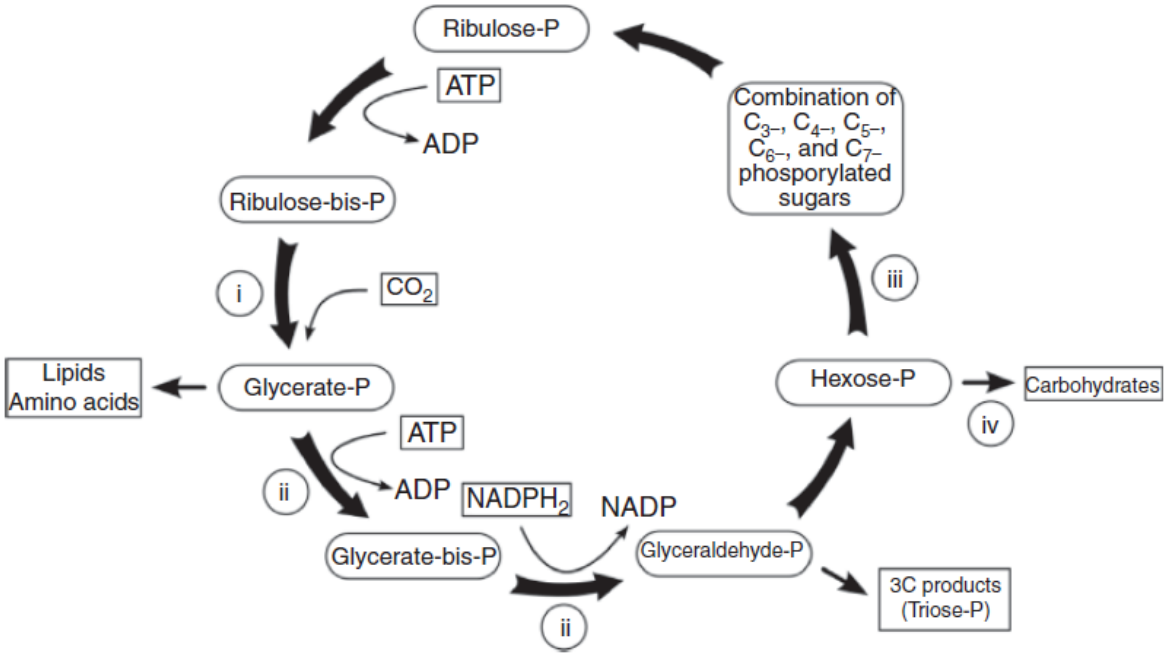

Figure 3 The Calvin-Benson cycle or dark reaction, where carbon is fixated. The figure is taken from Masojídek et al. (2013).

A competing process to carboxylation in the Calvin-Benson cycle is oxidation. This process is named photorespiration, and occurs due to the dual nature of Rubisco. When Rubisco functions as an oxygenase, organic carbon is converted into CO_2. Photorespiration is dependent on the relative concentration of O_2 and CO_2. High irradiance, high levels of O_2, and low levels of CO_2 stimulate oxidation of organic carbon in photosynthetic organisms.

2.2 Lipids in diatoms

Lipids are a highly diverse group of molecules. Fahy et al. (2005) defines lipids as hydrophobic or amphipathic small molecules that may originate entirely or in part by carbocation-based condensations of thioesters and/or carbocation-based condensations of isoprene units. Lipids serve several different functions, from acting as energy storage and structural cell membrane components to signaling pathways. Lipids are often divided into two different subtypes, namely simple and complex lipids. Simple lipids (e.g. acylglycerols) will generate a maximum of two types of products after hydrolysis, while the complex lipids (e.g. galactolipids and phospholipids) will form at least three types of products after hydrolysis. Fatty acids are made up of a carbon chain with a carboxyl group at one end. The carbon chain may have no, one or several double bonds along the chain. The fatty acids without double bonds are saturated fatty acids (SFA), one double bond are monounsaturated fatty acids (MUFA), and the ones containing two or more double bonds are polyunsaturated fatty acids (PUFA). Many fatty acids have common names, but are usually denoted by number of carbons, double bonds, and position of double bonds. For example 20:5n-3 signifies a fatty acid with 20 carbons, 5 double bonds, where the last double bond is situated at the third carbon from the methyl end of the molecule. The fatty acids are often esterified to a glycerol molecule. When fatty acids are the only molecules esterified to glycerol they form acylglycerols, which belong to the simple lipids. If for example a phosphate group and fatty acids are linked to the glycerol molecule it is a phospholipid, which is a complex lipid. Most lipids are hydrophobic. However, some lipids, such as phospholipids and galactolipids, have polar “heads” which make them amphipathic. The amphipathic lipids are important for cell wall structure, as the hydrophilic heads will face the intra- and extracellular space while the hydrophobic fatty acid chains face inwards towards each other, creating a barrier. Lipids are key components in maintaining cell wall structure and fluidity.

2.2.1 Diatom lipid composition and synthesis

The total lipid content of diatoms is usually between 5-40% of dry weight (DW), depending on species and culture conditions (Fields and Kociolek 2015). One important factor controlling lipid content is nutrient status during cultivation. In nutrient deplete situations, diatoms tend to increase lipid content in their cells, while at the same time growth is decreased (Fields and Kociolek 2015, Levitan et al. 2015, Lin et al. 2018, Sahin et al. 2019). Additionally, lipid content can be increased by increasing CO_2 supplementation (Wang et al. 2014), decreasing light concentration (Liang et al. 2001), and lowering temperatures (Araujo and Garcia 2005). The predominant fatty acids in diatoms are 14:0, 16:0, 16:1, and 20:5n-3 (Dunstan et al. 1994, Ishida et al. 2000, Lang et al. 2011). The most valuable of these fatty acids is 20:5n-3 (eicosapentaenoic acid, EPA), which is an omega-3 fatty acid. Diatoms also contain small amount of the valuable omega-3 fatty acid 22:6n-3 (docosahexaenoic, DHA), usually around 1-4% (Sayanova et al. 2017). The production of EPA in diatoms, like total lipids, is dependent on abiotic factors. For example, low temperatures, higher osmotic pressure, or increased CO_2 may give rise to higher concentrations of EPA, whereas nutrient limitation reduce the amount of EPA (Mortensen et al. 1988, Jiang and Gao 2004, Sayanova et al. 2017). EPA plays an important role in cell membrane fluidity and structure as well as being a vital part of the anti-grazer defense mechanism in diatoms. In response to cell rupture, lipase and lipoxygenase pathways are initiated and oxylipins are formed in the diatom cell (Fontana et al. 2007). These oxylipins have detrimental effect on copepod reproductive and developmental success (Caldwell 2009). Omega-3 is highly valuable because of its importance for vertebrates, such as humans and fish.

De novo synthesis of fatty acids occur either in the endoplasmic reticulum or in the envelope of plastid membranes, the latter is the most common in diatoms (Zulu et al. 2018). Firstly, acetyl-CoA is converted to malonyl-CoA, catalyzed by acetyl-CoA carboxylase, which is then transformed to malonyl-ACP by a malonyl-CoA:acyl carrier protein, malonyltransferase. In sequential steps, two-carbon units are added from malonyl-ACP to acetyl-CoA, to form longer acyl chains, this reaction is catalyzed by the fatty acid synthase (FAS) complex (Figure 4), which involve condensation, reduction, dehydration, and another reduction (Li-Beisson et al. 2013). The newly formed 16:0 can be transported into the endoplasmic reticulum for desaturation and further elongation. The elongase enzymes adds a two-carbon unit to the fatty acid carbon chain. Between elongation steps the desaturase

enzymes may add double bonds onto specific locations of the fatty acid (Dolch and Marechal 2015). The specificity of desaturase enzymes is denoted by Δ and a number, indicating the carbon atom from the carboxyl end of the fatty acid on which the double bond is inserted. Desaturase enzymes with Δ4, 5, 6, 8, 9, 12, 15, 17, and 19 have been found or indicated in diatoms (Dolch and Marechal 2015, Zulu et al. 2018, Remize et al. 2020). The elongation and desaturation of fatty acids usually follow the omega-6 (n-6) or omega-3 (n-3) pathway after the formation of 18:2n-6 and 18:3n-3, but there are indications of fatty acids crossing from the n-6 pathway to the n-3 pathway (Remize et al. 2020).

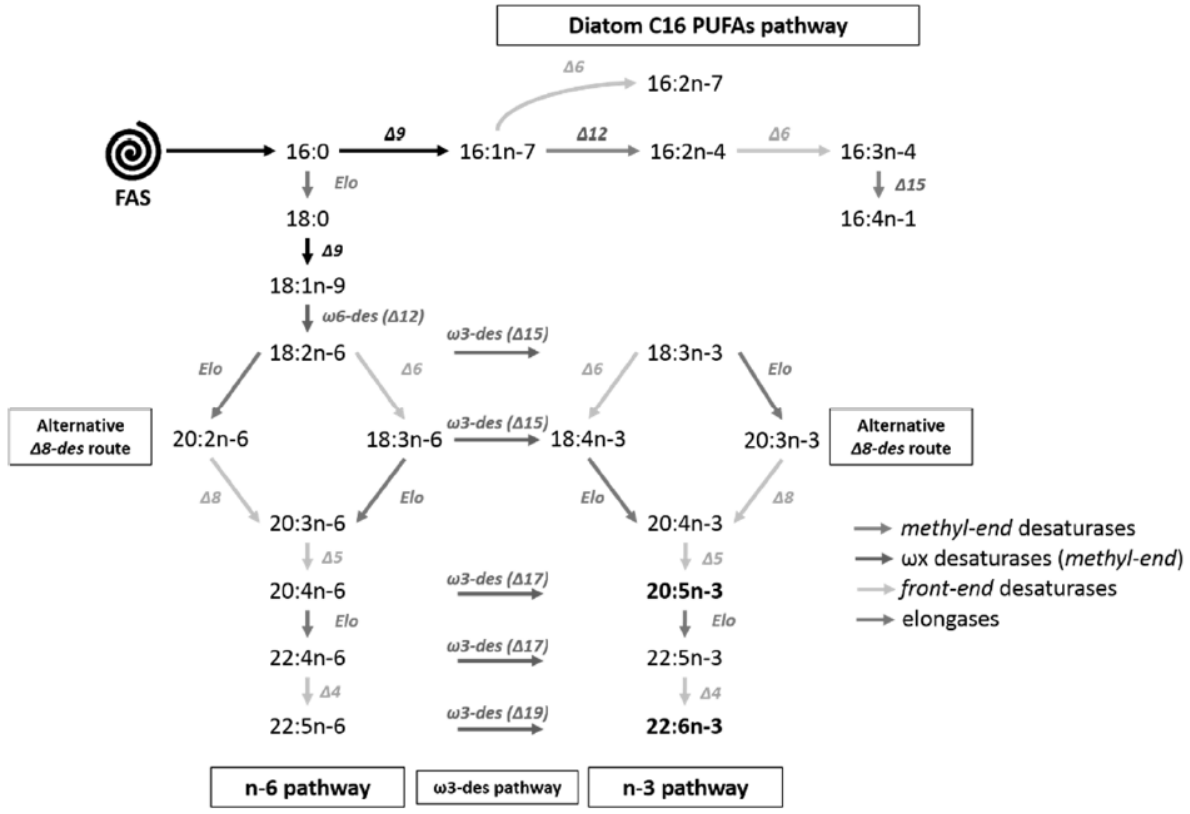

Figure 4 The synthesis pathway of PUFA in diatoms. Δ indicates the location of insertion of double bonds by desaturases, counting from the carboxyl end of the carbon chain. FAS: fatty acid synthase, des: desaturase, elo: elongase. The figure was adapted from Remize et al. (2020).

The lipid class composition of diatoms is complicated, as the different types of lipids have unique functions. The polar lipids, such as phospholipids and galactolipids, play essential roles in the membrane structure and function. The galactolipids, monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), and the charged lipid classes, phosphatidylglycerol (PG) and sulfoquinovosyldiacylglycerol (SQDG), are the major lipid

classes in the thylakoid membranes. These lipid classes play important roles for the function and structure of the thylakoid membrane for optimal photosynthesis (Boudiere et al. 2014) and often contain large amount of PUFA (Sayanova et al. 2017). MGDG, the most abundant lipid class in thylakoid membranes, does not form lipid bilayers in contrast to DGDG, PG, and SQDG (Deme et al. 2014). However, MGDG are structurally important in membranes where large protein complexes occur, because MGDG allows for more curvature in the membrane due to the ability to form reverse micelles (Boudiere et al. 2014). SQDG and PG, the two least abundant of the four lipid classes in thylakoid membranes, have similar functions and are often closely related to membrane proteins. In fact, during limited phosphorus availability, lipid remodeling occurs and SQDG tend to be upregulated to free up phosphorus from PG (Van Mooy et al. 2009). On the other hand, nitrogen limitation tends to reduce growth in diatoms and during the stationary phase diatoms tend to increase their concentration of neutral lipids with a concomitant reduction in polar lipids (Abida et al. 2015, Yoneda et al. 2018). These neutral lipids act as storage energy and are often packed in lipid droplets, which consists of a neutral lipid core (usually triacylglycerol (TAG)) surrounded by a monolayer of polar lipids (Goold et al. 2015).

2.3 Cultivation, harvesting and processing of diatoms

There are several ways to cultivate and harvest microalgae. In this subsection some of the different methods will be explained. The focus will be placed on phototrophic cultivation rather than heterotrophic cultivation. While most methods are feasible in a laboratory scale, there are several considerations to be made when increasing cultivation to an industrial scale, e.g. robustness of the system, construction cost, and operating cost. The industrial systems for microalgae cultivation are often categorized as open or closed systems.

The open pond cultivation systems have the longest history of microalgae production. These systems are exposed to the natural environment, and have a low cost of construction and operation (Xu et al. 2009). Open cultivation systems may be built around natural structures, such as ponds or lagoons, or artificial structures, such as circular central pivot ponds or raceway ponds (Figure 5). The most common practice for commercial production of microalgae are the raceway ponds (Borowitzka and Moheimani 2013). Because the open systems are exposed to the natural environment, the temperature and light will vary according to season and day/night. In periods, the temperature and light will increase, resulting in increased temperature and lower

solubility of CO_2, and water evaporation may also occur. On the other hand, low temperatures may be outside the optimum range of the cultivated strain, and reduce the growth of the microalgae. The open systems are shallow to ensure maximum light distribution (Jorquera et al. 2010), which leads to a rapid impact of ambient temperatures. The shallowness of the ponds also requires large areas of land for large-scale production, meaning open pond systems are very area intensive. In addition to shallow waters for maximum light distribution, proper water circulation must be ensured to provide mixing of nutrients, algae, and CO_2. Mixing of nutrients and algae, and effective light distribution and gas composition, are all important factors of algae growth. Another challenge for the open systems is contaminating organisms, which could out-compete the cultivated microalgae (Xu et al. 2009).

Figure 5 The open raceway ponds of Cyanotech, located in Hawaii. Picture used with permission, photo credit Cyanotech.

In closed cultivation systems, the growing biomass is contained within a photobioreactor with control of environmental parameters such as light, temperature, nutrients, and mixing (Zittelli et al. 2013). The most common structures of these photobioreactors are tubular (Figure 6) or flat panels, placed horizontally or vertically. The systems are constructed in a way to ensure an optimized surface to volume ratio for optimal light distribution and growth

of the cultivated algae. The complete control of mixing facilitates optimal distribution of gas and nutrients. The productivity of photobioreactors are higher than open pond systems (Jorquera et al. 2010). The closed systems are less likely to be exposed to contaminating organisms compared with open pond systems. The biggest drawbacks of the closed photobioreactors are the high construction and operating costs (Gupta et al. 2015).

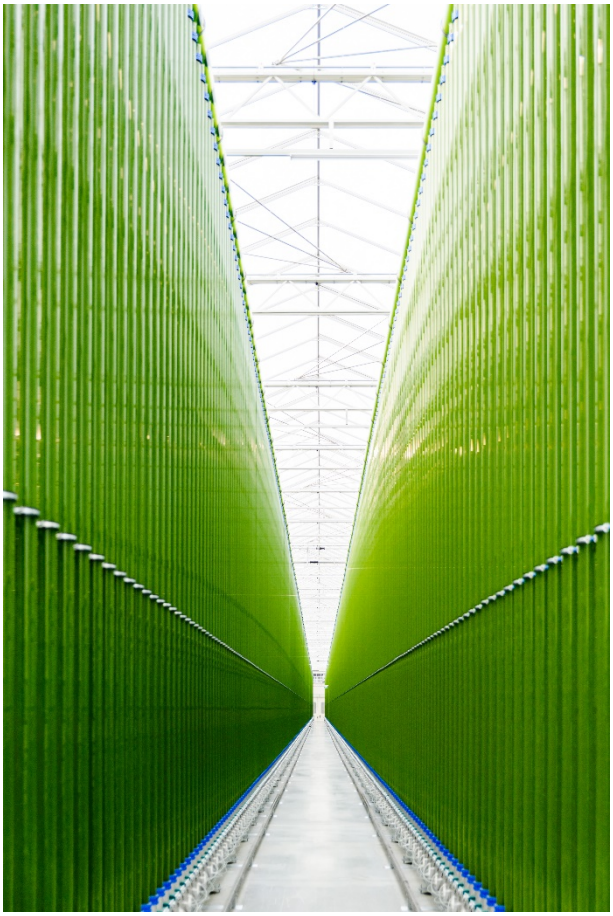

Figure 6 A vertical tubular closed photobioreactor used at Ecoduna. Picture used with permission (photo credit @jongerius_ecoduna_GmbH).

The concentration of biomass in the media during cultivation is low, water removal is therefore necessary for further processing of the microalgae biomass. Several ways of harvesting the microalgae biomass from the cultivation media have been explored. Dewatering processes include centrifugation, filtration, flocculation, flotation, and sedimentation (Pragya et al. 2013). Each method has benefits and drawbacks, and the most important factor is to reduce production costs while maintaining the quality of the nutritional components of the biomass. For example, centrifugation may crush the cells and induce enzymatic reactions that reduce the quality of the lipids; these reactions will be discussed further in section 2.5. The efficiency of

the different procedures varies, but it is possible to combine several methods, for example filtration and centrifugation, to enhance the harvesting process (Singh and Patidar 2018).

2.4 Extraction of lipids from microalgae

The extraction of lipids from microalgae may be challenging, due to the tough cell walls and the complexity of lipid composition present in many species. The extraction method applied must be able to extract the desired lipids, especially the PUFA, which in many cases are esterified to both neutral and polar lipids in the microalgae biomass. The combination of chlorinated organic solvents (chloroform or dichloromethane (DCM)) and methanol was developed several years ago (Folch et al. 1957, Bligh and Dyer 1959) and have become the benchmark methods for lipid extraction in the laboratory. In recent years, DCM has replaced chloroform because the former is less toxic and equally able to extract the complete spectrum of lipids (Cequier-Sanchez et al. 2008). In large-scale operations, such as industrial scale production of microalgae, the use of chlorinated organic solvents may not be applicable (Herrero et al. 2015). To extract lipids for the production of health food supplements or feed, food grade or "green" solvents should be applied (Gallego et al. 2018). However, replacing the chlorinated organic solvents may require different pre-treatments to achieve similar yields.

Several different solvents, extraction techniques, and pre-treatments have been investigated for extraction of lipids from various microalgae. Proposed solvents and extraction techniques, other than chlorinated organic solvents, include ethanol (Fajardo et al. 2007), isopropanol (Yao et al. 2012), dimethyl carbonate (Tommasi et al. 2017), methyl tert-butyl ether (Angles et al. 2017), pressurized fluid extraction (Pieber et al. 2012), and supercritical CO_2 extraction (Baumgardt et al. 2016, Millao and Uquiche 2016). There are clear indications that neutral solvents, such as hexane, perform worse than polar solvents or combinations of neutral and polar solvents (Balasubramanian et al. 2013, Li et al. 2014). Most of the investigated extraction solvents have lower yield than the traditional extraction methods, but the yield can be improved by pre-treatment of the biomass. The different pre-treatments of microalgae biomass include drying, mechanical disruption (Zheng et al. 2011), sonication, and use of microwaves (Menendez et al. 2014), or enzymes (Liang et al. 2012, Zuorro et al. 2016).

Different groups and species of microalgae are differently affected by the various extraction procedures, and it is therefore necessary to identify the optimal method for the

biomass that is produced (Yao et al. 2013, Ryckebosch et al. 2014). Additionally, the cost of extraction and preservation of lipid quality must be considered for large-scale operations.

2.5 Lipid stability during storage and handling

A challenge when working with lipids, especially the highly unsaturated marine fatty acids, is oxidation. Oxidation reduces both the nutritional value and sensory quality of an oil by breaking down unsaturated fatty acids and generating compounds with bad taste and odor (Marsili and Laskonis 2014). The three major types of lipid oxidation are autoxidation, photooxidation, and enzymatic oxidation (Schaich et al. 2013). The rate of oxidation depends on the number of double bonds present in the fatty acids, because highly unsaturated fatty acids have more doubly allylic hydrogen atoms, which are more easily abstracted from fatty acids to form radicals (Cosgrove et al. 1987). In addition, factors like heat, light, oxygen, and transition metals will strongly affect the rate of lipid oxidation.

There are three stages of lipid autoxidation, namely initiation, propagation, and termination (Figure 7). Common initiators are the transition metals (e.g. iron and copper), which can be found almost anywhere. Oxidized transition metals are able to form lipid radicals by withdrawing an electron from the double bonds in unsaturated fatty acids (LH). Preformed free radicals (e.g. hydroxyl (•OH) and peroxyl (•OOH)) may also react with LH to initiate autoxidation, these free radicals are formed from H_2O_2 by reactions with enzymes or metals. Photosensitizers (photooxidation), such as chlorophyll, can produce free radicals or singlet oxygen ($^1O_2$), which also react readily with the double bonds in LH, forming a fatty acid peroxide (LOOH) (Terao and Matsushita 1977). The initiation process is required because normal molecular oxygen is in a triplet state ($^3O_2$) and does not react directly with the double bond of lipids (Schaich et al. 2013). Therefore, as an alternative to photooxidation, the formation of a radical is required for lipid autoxidation to occur. However, when the chain reaction of autoxidation starts, the process is autocatalytic, and the radicals will react with normal molecular oxygen. In the initiation step, removal of a doubly allylic hydrogen from the unsaturated fatty acid produces highly reactive lipid alkyl radicals (fatty acid radical, L•).

In the propagation step of autoxidation, the previously formed lipid alkyl radical goes on to react with molecular oxygen ($^3O_2$), and forms a peroxyl radical (LOO•), which establishes the cascading free radical chain reaction. This LOO• abstracts hydrogen from LH and give rise

to lipid hydroperoxides (LOOH) and L•, the latter will react with molecular oxygen and the process repeats itself. The first part of the propagation is slow, but the rate increase when propagation enters the branching stage. In branching, accumulated lipid hydroperoxides are decomposed to alkoxyl radicals (LO•), LOO•, and •OH by transition metals, UV light or heat. LO• and •OH are more reactive than LOO• and increase the reaction rate of autoxidation (Schaich 2013). LOOH can also be formed by lipoxygenase enzymes (Glickman and Klinman 1996). Since LOOH is not a radical it does not drive the chain reaction further, but LOOH can be decomposed to its radical constituents by UV light, heat, or transition metals. UV light, for example, contain the energy to react with hydroperoxides and break O—O bonds, thus creating LO• and •OH.

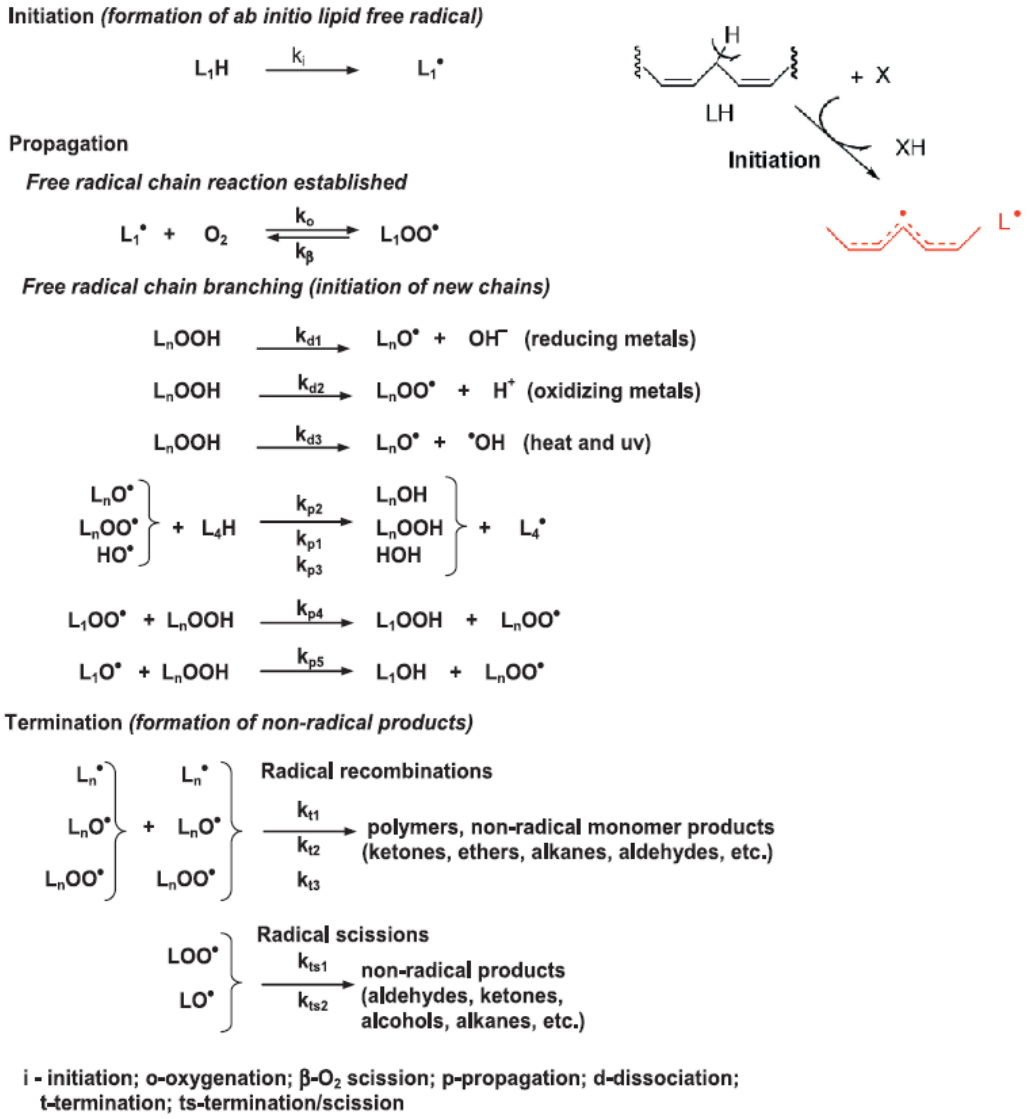

Figure 7 Lipid autoxidation pathway adapted from Schneider (2009) and Schaich et al. (2013). LH: unsaturated fatty acid, L•: lipid radical, LOO•: lipid peroxyl radical, LOOH: lipid peroxide, LO•: lipid alkoxyl radical, LOO•: lipid peroxyl radical, •OH: hydroxyl radical, i: initiation, o: oxygenation, β: β-O_2 scission, d: dissociation, p: propagation, t: termination, ts: termination/scission.

The propagation step is followed by the termination step, in which formation of stable molecules occurs through several different reaction pathways. In radical recombination, two radicals react and produce dimers or ketones through fragmentation. β scission give rise to the products with bad flavor and smell, such as ketones, aldehydes, and other volatile compounds (Frankel 1982, Marsili and Laskonis 2014). Radical scavenging (type 2) antioxidants may also terminate the autoxidation cycle, by donating a hydrogen to LOO• or LO•. The antioxidant radicals are much more stable than lipid radicals are because these often have phenolic structures that are stabilized by resonance delocalization of the free electron over the aromatic

ring (Leopoldini et al. 2004). Type 1 antioxidants, are scavengers of reactive oxygen species (e.g. carotenoids) (Stahl and Sies 2003), or metal chelators and complexers (e.g. EDTA) (Celus et al. 2020), which prevent formation of free radicals.

Another challenge of storing lipids, especially in intact biomass, is the formation of free fatty acids through lipolysis. Endogenous enzymes, called lipases and phospholipases, may be present in stored feed, food, or biomass. These enzymes hydrolyze lipid classes and increase the amount of free fatty acids (O'Connor et al. 1992, Dierick and Decuypere 2002). The enzymes responsible for lipid deterioration may also stem from exogenous microbial sources (Machado et al. 2017). Free fatty acids in themselves may give rise to soapy flavors (Lindsay 2017). More importantly, free fatty acids are very good substrates for lipoxygenase enzymes, which fuel lipid oxidation and reduce the quality of lipids (Hayward et al. 2017).

Diatoms may contain high amounts of PUFA, as discussed in section 2.2.1. Because diatoms are photosynthetic organisms, they also contain large concentrations of the photosensitizer chlorophyll (Stauber and Jeffrey 1988). Additionally, the anti-grazer lipolytic and lipoxygenase enzymes of diatoms are activated by cell rupture, and may present challenges during storage of diatom biomass (Wichard et al. 2007, Balduyck et al. 2017). All these factors make the diatom lipids highly susceptible to lipid oxidation during storage. Refrigeration may not be enough to avoid lipid deterioration in cold-water adapted diatoms, since their enzymes have high activities at lower temperatures (Feller and Gerday 2003, Zheng et al. 2011, Siddiqui 2015). In addition to chlorophyll, diatoms contain pigments such as carotenoids along with several phenolic compounds, and these may help prevent lipid oxidation (Goiris et al. 2012).

2.6 Digestibility of lipids

Lipids serve both as an energy source and as vital components for optimal function of the body, through essential fatty acids, membrane components, and lipid soluble vitamins (Shahidi 2009, Shahidi and Ambigaipalan 2018). The human a diet consists mainly of TAG and small amounts of other lipid classes such as cholesterol and PL (Iqbal and Hussain 2009). The human digestive system is quite efficient at absorbing and utilizing dietary lipids, depending on the delivery form. Most of the lipid digestion occurs in the small intestine; however, it is helped in some part by lingual lipase and gastric lipase. Gastric lipase breaks down some of the ingested TAG

in the stomach (Armand et al. 1996). The most important contribution of gastric lipase to digestion of lipids is most likely the formation of small lipid droplets, through breakdown of lipids and peristaltic movements, which facilitates further hydrolysis in the small intestine.

When the lipids enter the small intestine, bile and pancreatic juice is excreted to form emulsions and break down the ingested lipids (Figure 8). The lipid classes must be broken down to free fatty acids (FFA), monoacylglycerol (MAG) or lysophospholipids (LPL) to be absorbed by the enterocytes. The formation of small emulsion droplets by bile salts (mainly from cholic acid) is necessary to create a large surface area with an interfacial composition that favors anchoring of the hydrolyzing enzymes (Bauer et al. 2005).

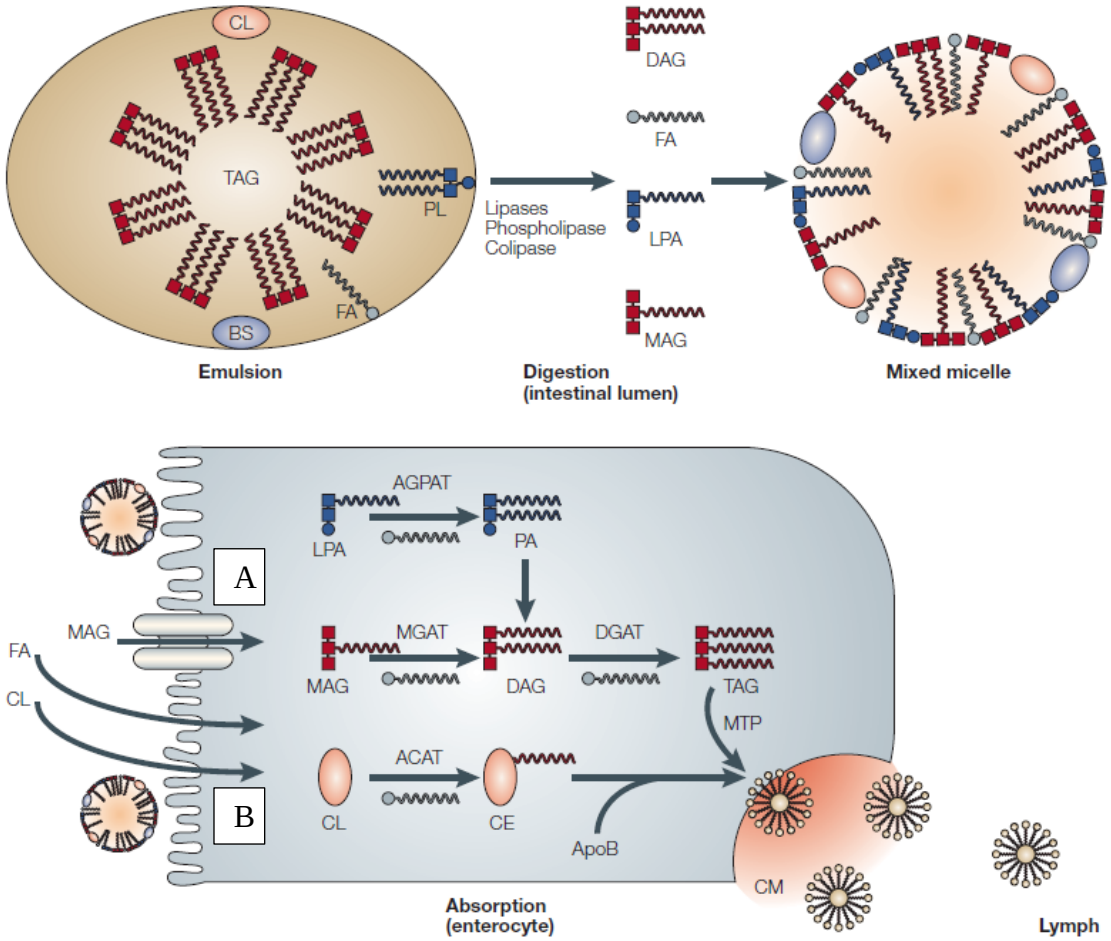

Figure 8 Intestinal lipid digestion and absorption. CL – cholesterol, PL – phospholipid, LPA – lysophosphatidic acid, FA – fatty acid, TAG – triacylglycerol, DAG – diacylglycerol, MAG – monoacylglycerol, CE – cholesterol ester, BS – bile salt, AGPAT – 1-acyl-glycerol-3-phosphate acyltransferase, MGAT – monoacylglycerol acyltransferase, DGAT – diacylglycerol acyltransferase, ACAT – acyl-CoA:cholesterol acyltransferase, MTP – microsomal triglyceride transfer protein, CM – chylomicron. A is active transport and B is passive diffusion. The illustration is adapted from Shi and Burn (2004).

The hydrolyzing enzymes, i.e. lipases, usually have activity towards specific substrates (lipid classes). Pancreatic triacylglycerol lipase is the most abundant lipolytic enzyme in the small intestine, and has activity towards triacylglycerol (TAG) and diacylglycerol (DAG) and cleaves of the fatty acid at position *sn*-1(3) on the glycerol backbone (Shi and Burn 2004). Pancreatic phospholipase A2 is responsible for hydrolyzing phospholipids, and attacks on the *sn*-2 position of the molecule (Yuan and Tsai 1999). Contrary to the two previously mentioned enzymes, carboxyl ester lipase (CEL) and pancreatic lipase-related protein2 (PLRP2) are non-specific lipolytic enzymes with activity towards TAG, DAG, MAG, phospholipids, lysophospholipids, cholesterol esters, MGDG, DGDG, and ceramides (Whitcomb and Lowe 2007). Another important component of lipid digestion in the small intestine is the non-enzymatic protein colipase. Colipase is secreted into the small intestine as its precursor molecule procolipase, which is cleaved to its active state. Colipase helps the activity of pancreatic triacylglycerol lipase in the presence of inhibitory substances by anchoring the enzyme to the substrate and keeping it in the active conformation (Whitcomb and Lowe 2007).

Once the lipids have been hydrolyzed to MAG, FFA, and LPL they must be transported to and absorbed by the enterocytes. The cleaved products form mixed micelles along with bile salts, which can travel through the aqueous media of the intestine. Once the micelles reach the epithelial surface FFA, MAG, and LPL are absorbed across the membrane of the enterocytes. This absorption can happen through passive diffusion of by active transport. The active transport of fatty acids across the enterocyte membrane is facilitated by the proteins cluster determinant 36 (CD36), plasma membrane associated fatty acid-binding protein (FABP), and a family of fatty acid transport proteins (FATP) (Wang et al. 2013). Once the digestion products have been absorbed into the enterocytes, they are metabolized into TAG or phospholipids.

As was discussed in section 2.2.1, the diatom lipid composition is complex and contains a plethora of lipid classes and fatty acids. The ability of the digestive system to hydrolyze the various lipid classes is essential to the bioavailability of the important fatty acids of the diatoms. Diatoms contain large amounts of galactolipids, which are not among the most common lipid classes in the human diet. The lipase enzymes responsible for breaking galactolipids down, CEL and PLRP2, must be present in high enough concentrations for effective hydrolysis.

2.7 Diatoms for feed or food

All vertebrate species require a specific amount of certain polyunsaturated fatty acids (PUFA) in their diet to survive and function optimally. These specific PUFA are referred to as essential fatty acids (EFA), and include both omega-3 and omega-6 fatty acids. Vertebrates cannot synthesize omega-3 and omega-6 fatty acids because they lack Δ12 and Δ15 fatty acid desaturases required to insert a double bond at the omega-6 and omega-3 carbon of 18:1n-9, respectively (Hastings et al. 2001).

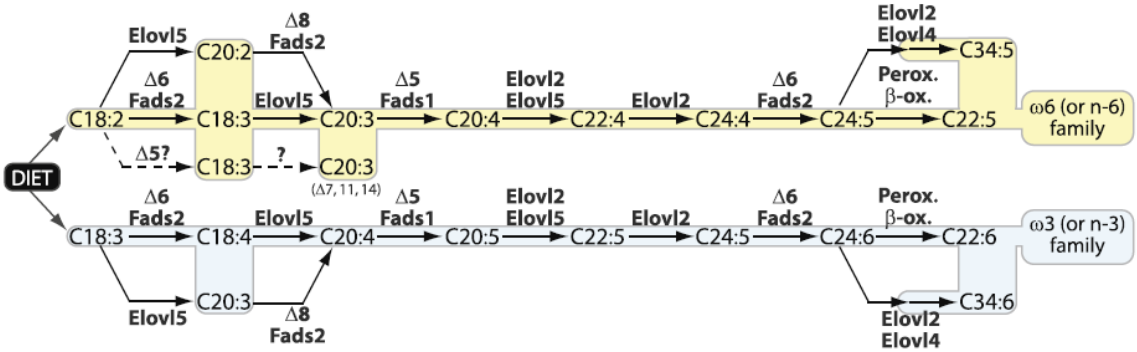

Figure 9 The vertebrate LC-PUFA synthesis pathway. Adapted from Guillou et al. (2010). Fads: fatty acid desaturase, Elovl: elongation of very-long-chain fatty acids, Δn: location of double bond insertion on carbon chain.

However, most vertebrates except marine fish are able to synthesize the long-chain highly unsaturated fatty acids (HUFA) arachidonic acid (C20:4n-6, ARA), eicosapentaenoic acid (C20:5n-3, EPA), and docosahexaenoic acid (C22:6n-3, DHA), using linoleic acid (C18:2n-6, LA) and α-linolenic acid (C18:3n-3, ALA) from a dietary source as substrate. The process described in Figure 9 occurs in the endoplasmic reticulum of the cell. In vertebrates, elongase enzymes, referred to as Elongation of very-long-chain fatty acids (Elovl), catalyze the elongation of fatty acids (Guillou et al. 2010). The elongation of fatty acids involves the addition of two carbon units to a fatty acyl-CoA, using malonyl-CoA as a donor and NADPH as a reducing agent. Briefly, Elovl condense malonyl-CoA and fatty acyl-CoA to yield 3-ketoacyl-CoA, which is then reduced to form 3-hydroxylacyl-CoA, which in turn is dehydrated to form trans-2-enoyl-CoA, and finally reduced to form an elongated fatty acyl-CoA. Desaturase enzymes, referred to as Fatty acyl desaturases (Fads), introduce a double bond at a specific position in long-chain fatty acids. Fads1 is a Δ5-desaturase and Fads2 is a Δ6-desaturase, where the delta number refers to the location where the enzyme introduces the double bond on the carbon chain, counting from the carboxylic end of the fatty acid. The

desaturation of a single bond to a double bond requires O_2, NADH, an electron donor, and cytochrome b5 reductase, to remove two hydrogen atoms from the bond. Although humans have the ability to produce EPA and DHA from ALA, the process is slow (Burdge and Calder 2006); therefore, dietary sources of EPA and DHA are recommended (Kris-Etherton et al. 2009).

3 Methodological considerations

3.1 General considerations regarding chromatography and detection methods

The method applied for analyzing lipid classes in this thesis utilizes normal phase liquid chromatography (HPLC) and an evaporative light scattering detector (ELSD). Normal phase refers to a polar packing material in the chromatographic column, in this case silica. It is a useful method because it enables detection and quantification of neutral and polar lipid classes in a single run. However, the large difference in polarity of lipid classes is also a challenge, since neutral lipid classes will not bind to the stationary phase of the column and the polar lipid classes will bind tightly. The tight bond of polar lipid classes, such as phospholipids, results in band broadening. This band broadening may make detection of low concentration polar analytes more difficult, because of the signal-to-noise ratio. A possible solution to band broadening may be to separate the lipid classes into neutral and polar lipid classes using solid phase extraction (SPE) prior to HPLC analysis, and then have one method optimized for neutral lipids and one for polar lipids on the HPLC. However, this would probably require the use of internal standards (IS) to calibrate the quantitation for eventual losses during SPE, and the IS must be optimized so as not to coelute with any analytes of interest. We decided to solve this problem using two different injection concentrations (1 and 0.1 mg ml^{-1}) for each sample, and pooling the results during analysis. Another challenge with this HPLC method is the volume of mobile phase used for each single run. Several modern types of liquid chromatography have been developed over the last decades, and methods such as ultra-precision liquid chromatography (UPLC) require much lower volumes of mobile phase than HPLC, whereas supercritical fluid chromatography (SFC) requires CO_2 gas and small amount of additives.

ELSD is a common detector used for lipid analysis. However, the sensitivity of the ELSD is also a thing to consider. The response of ELSD to analytes is non-linear, and this requires fitting a second degree polynomial equation to the calibration curves for quantitation. Mass spectrometry (MS) is a more modern approach in analyte detection, and the response of these detectors are usually linear. The sensitivity of MS detection is also greater than ELSD, making detection of lower concentration analytes possible. Additionally, MS methods in tandem could be used to ascertain the analyte structure based on the molecular ion and fragments, such that one would not be reliant only on retention time to determine the identity

of the analyte and could also solve some problems of coelution. The more modern types of liquid chromatography, in conjunction with mass spectrometry, are also being used in the expanding field of lipidomics.

Gas chromatography (GC) is an established method for fatty acid separation. The fatty acids are made separable and more volatile by hydrolyzing the fatty acids from the glycerol backbone (or the fatty alcohol in the case of wax esters) and esterifying it to a methyl group. In our papers, we used a GC with flame ionization detection (FID). FID is an excellent way to quantify fatty acids. However, to identify the fatty acids with this detector one is reliant solely on the retention time of external standard fatty acids. This makes coelution of fatty acids, for example 18:1n-9 and 16:4n-1 in our samples, a challenge during analyte peak identification. Here too, the use of mass spectrometry could be useful to ascertain the identity of a fatty acid within a peak.

3.2 Stability of lipids in *P. glacialis* biomass

When studying the stability of lipids in *P. glacialis* during storage we analyzed changes in lipid class composition and fatty acid profile as determinants of lipid deterioration. Lipid class analysis by HPLC is a better alternative than the common method of titrating for FFA values, because it enables one to see which lipid classes are more vulnerable to lipolysis during storage. The change in fatty acid profile, especially loss of PUFA, gave an indication of quality changes. Measuring lipid quality changes, especially in the presence of non-lipid components, can be challenging. The quality of the oil may change during extraction, e.g. oxidation could occur or the volatile components could be lost (Schaich 2013). Additionally, the reactions in common methods for analyzing lipid deterioration may not be specific and could react with other components of the biomass, leading to an overestimation of lipid deterioration (Jardine et al. 2002).

Analysis of oxidation parameters such as peroxides and aldehydes, along with FFA content, are the most common ways of determining lipid deterioration and quality during storage. These parameters are often determined by titration or spectrometry, such as the TBARS, *p*-anisidine value, and peroxide value methods (Barriuso et al. 2013). The amount of pigments in *P. glacialis* and other microalgae biomass may interfere with the read-outs from these analyses (Safafar et al. 2017). However, a method which utilize HPLC with a UV detector

after reaction of the oxidized fatty acids with triphenylphosphine has been proposed for peroxide analysis for colored lipids (Gotoh et al. 2011). The analysis of volatile components formed during lipid oxidation can be performed using headspace GC-MS (Iglesias and Medina 2008), and would not be affected by the pigments present in our samples.

3.3 *In vitro* digestion of lipids from *P. glacialis*

For our investigations of digestibility of lipid from *P. glacialis* and other omega-3 containing oils, we measured changes in FFA and other lipid classes by normal phase HPLC along with changes in fatty acid profile of neutral, polar, and free fatty acid fractions after solid phase extraction. Porcine pancreatin was used as the *in vitro* digestion system, because it is fairly similar to humans. Using human duodenal juice, could have been a possible solution. There are indications that some fatty acids are hydrolyzed differently with porcine or human pancreatic enzymes (Aarak et al. 2013). It could also have been interesting to include the gastric phase, especially the low pH, to see how this would have affected the digestion of the different lipid classes.

A common method for analyzing lipid digestion is the pH-stat method (Li et al. 2011, Minekus et al. 2014). The pH-stat method is based on addition of alkali to maintain a pH during digestion as free fatty acids are released. This is probably a good method when working with samples containing only TAG, but in a sample with a more complex lipid class composition it would provide no information on which lipid class is digested and to what extent. Another possible solution is to include an IS in FFA, neutral and polar form, separate the lipids using SPE and analyzing the fractions on GC-FID (Aarak et al. 2013). Using this method it is possible to investigate if the polar and/or neutral fraction of the lipids is being hydrolyzed, but the hydrolysis of specific lipid classes cannot be identified.

3.4 Extraction of lipids from *P. glacialis*

To extract our lipids we investigated sonication, ultra-turrax, manual grinding, freeze drying, and microwave treatment for cell disruption. Sonication was the only cell disruption treatment that worked for *P. glacialis*. The extraction solvents were dichloromethane/methanol

(DCM/MeOH, 2:1 v/v), hexane/isopropanol (hexane/IPA, 2:1 v/v), and pure hexane. It could have been interesting to include other solvents as well. Ethanol, for example, have been found to be a good extraction solvent for lipids from microalgae, and this could have been tested. However, ethanol extraction would require a neutral organic solvent and water to create two separable phases, since ethanol is able to extract some non-lipid impurities as well (Fajardo et al. 2007). It could also have been interesting to test different ratios of hexane/IPA. Hexane is less polar than DCM and IPA is less polar than MeOH. Therefore, it could be of interest to increase the amount of IPA to match the polarity between DCM/MeOH and hexane/IPA to see if this would affect the extraction yield.

4 Main results and general discussion

The aim of this thesis was to investigate and characterize the lipid fraction of the industrially cultivated *P. glacialis* biomass. To produce feed or food lipids from large-scale cultivation of diatoms it is important to examine how stable the lipid fraction of the biomass is during storage, and to investigate if cheap methods of conservation could be applied to increase the storage stability of the biomass (**Paper I)**. A second important aspect of the diatom biomass for feed or food is the digestibility of the lipids. This was examined using a porcine *in vitro* digestion system and the digestibility was compared to other commercially available omega-3 rich oils (**Paper II**). The extraction of lipids is important, for both laboratory and industrial purposes. Many studies have examined cell disruption and extraction of lipids from various microalgae; however, the main body of work have focused on a small number of species. In **Paper III**, cell disruption and lipid extraction from the large centric diatom *P. glacialis* was compared to another diatom (*Odontella aurita*) and a green microalgae (*Chlorella vulgaris*).

4.1 Fatty acid and lipid class composition of *P. glacialis* compared to other species

The fatty acid composition of *P. glacialis* is highly unsaturated, and differs from other current and potential sources of long-chain omega-3 that was investigated in our studies (Table 1). Nearly 80% of the fatty acids in the *P. glacialis* biomass have two or more double bonds. In our studies, the only similar amounts of PUFA were found in the fish oil ethyl ester concentrate (EEC) that had been concentrated using short path distillation, and in the *Chlorella vulgaris* biomass. In contrast, most of the other species investigated in **Paper II** and **III** contained between 20-40% PUFA. Importantly, the high PUFA content of *C. vulgaris* was made up of fatty acids with chain length 18 or shorter, common to freshwater microalgae and similar to terrestrial plants. The PUFA of highest interest to aquaculture feed and health food supplements are the omega-3 fatty acids, especially EPA and DHA (Tocher 2015, Shahidi and Ambigaipalan 2018). Omega-3 fatty made up more than 35% of the fatty acid profile of *P. glacialis*, which is slightly less than what was found in the Calanus oil, but more than cod-liver oil, krill oil, *Odontella aurita*, and *C. vulgaris*. The EEC, which uses fish oil as a starting point, contained almost twice the amount of omega-3, but this oil has been processed to contain high amounts of these fatty acids specifically (Breivik 2012). Approximately 30% of the fatty acids in *P. glacialis* are EPA, with low amounts of DHA present. This distribution of EPA and DHA is

common in diatoms, and the amount of EPA in *P. glacialis* is in the high range of what is usually found in diatoms (Dunstan et al. 1994, Sayanova et al. 2017). The EPA content of *P. glacialis* was slightly lower than in the EEC. A large fraction of the PUFA content in *P. glacialis* was made up of C16:4n-1, which is a fatty acid common to diatoms (Leu et al. 2007), but relatively rare or uncommon in other biomass (Li et al. 2012). The bioactivity of C16:4n-1 has not been studied in humans and fish, but this fatty acid is part of the diatoms anti-grazer defense (Pohnert 2005). The high content of PUFA in the cold-adapted diatom *P. glacialis* is likely due to cell membrane function (Dodson et al. 2014, Menard et al. 2017, Svenning et al. 2019).

Table 1 Summation of polyunsaturated fatty acid profile as percent of total fatty acids of Porosira glacialis (PGla), Odontella aurita (OA), Chlorella vulgaris (CV), Calanus oil (CO), Cod-liver oil (CLO), Ethyl ester concentrate (EEC), and Krill oil (KO). ND: not detected.

	PGla *	OA *	CV *	PGla ^	CO ^	CLO ^	EEC ^	KO ^	PGla §
C16:2n-4	3.2	3.1	ND	2.3	ND	ND	ND	0.9	2.4
C16:3n-4	6.4	2.4	10.6	4.4	ND	ND	ND	ND	4.8
C18:1n-9/ C16:4n-1*	30.8	3.1	4.0	35.2	5.6	15.6	7.3	11.5	31.9
C18:2n-6	ND	1.3	36.5	ND	1.2	2.6	1.3	1.9	ND
C18:3n-3	ND	ND	20.9	ND	1.5	1.0	1.0	0.6	ND
C18:4n-3	4.7	ND	ND	4.1	15.5	4.4	2.4	1.7	10.0
C20:2n-6	ND	ND	ND	ND	ND	ND	0.5	ND	ND
C20:4n-6	ND	ND	ND	ND	0.8	ND	2.4	ND	ND
C20:5n-3	29.2	12.4	ND	28.9	17.0	9.1	37.2	21.2	28.3
C22:4n-6	ND	ND	ND	ND	0.5	ND	ND	0.7	ND
C22:5n-3	ND	ND	ND	ND	0.9	1.3	5.2	ND	ND
C22:6n-3	2.3	0.7	ND	4.9	12.5	13.7	27.5	10.0	2.9
∑ PUFA	77.2	19.7	68.0	79.8	49.9	32.1	77.4	37.0	ND
∑ omega-3	36.2	13.0	20.9	37.8	47.4	29.5	73.2	33.5	ND

*: data from **Paper III** (Svenning et al. 2020) samples mixed and extracted with DCM/MeOH
^: data from **Paper II** "*In vitro* digestion of lipids from the marine diatom *Porosira glacialis* compared to commercial omega-3 products" (manuscript)
§: data from **Paper I** (Dalheim et al. 2020) control samples stored at 4 °C, day 1

The *P. glacialis* biomass contains a complex lipid class composition (Table 2), commonly found in microalgae (Chen et al. 2007, Yang et al. 2017). There were some variations in the lipid class composition of *P. glacialis* between **Paper I**, **II**, and **III**. For example, the amount of FFA was lower in **Paper II** than in **Paper I** and **III**. The amount of the polar lipids PG and MGDG was similar in **Paper II** and **III**, whereas in **Paper I** PG was much

higher and MGDG lower. These differences in lipid class composition within the same species may be attributed to differences in growth phase, harvesting, and/or storage conditions (Miller et al. 2014, Balduyck et al. 2016). The biomass in **Paper I** had been stored in a refrigerator for 24 hours before analysis. The other microalgae investigated in **Paper III**, *O. aurita* and *C. vulgaris*, contained large amounts of FFA, and the MGDG content in the former was almost depleted. Calanus oil contained mainly wax esters (WE) whereas cod-liver oil contain mainly TAG, both these biomasses may have contained some polar lipids that were not extracted or lost in processing (Bimbo 2012, Pedersen et al. 2014). The ethyl ester concentrate, derived from fish oil, contains mainly fatty acid ethyl esters because the fatty acids must be transformed from TAG to EE to distill the omega-3 fraction of the oil (Breivik 2012). However, some producers of omega-3 concentrates re-esterify the fatty acids back to TAG after concentrating the omega-3 fatty acids. Other than microalgae oils, krill oil was the only one that contained polar lipids, with approximately 40% phospholipids.

Table 2 Summation of lipid class composition (weight percent of lipid class) from Porosira glacialis (PGla), Odontella aurita (OA), Chlorella vulgaris (CV), Calanus oil (CO), Cod-liver oil (CLO), Ethyl ester concentrate (EEC), and Krill oil (KO). ND: not detected.

	PGla *	OA *	CV *	PGla ^	CO ^	CLO ^	EEC ^	KO ^	PGla §
WE	ND	ND	ND	ND	84.7	ND	ND	ND	ND
EE	ND	ND	ND	ND	ND	ND	87.3	ND	2.4
TAG	5.6	8.3	15.9	4.9	1.4	99.7	11.9	42.8	1.7
FAlc	ND	ND	ND	ND	2.5	0.3	ND	ND	ND
DAG	12.3	4.2	9.4	14.2	1.2	ND	0.8	9.8	3.7
FFA	9.3	71.8	38.4	4.8	10.2	ND	ND	4.2	16.2
MAG	ND	3.1	ND	3.8	ND	ND	ND	ND	4.7
MGDG	36.3	0.9	13.8	36.3	ND	ND	ND	ND	23.3
DGDG	ND	ND	ND	4.1	ND	ND	ND	ND	4.4
PG	21.6	ND	ND	23.5	ND	ND	ND	ND	43.6
PC	15.0	11.6	22.6	8.5	ND	ND	ND	43.3	ND

*: data from **Paper III** (Svenning et al. 2020) samples mixed and extracted with DCM/MeOH
^: data from **Paper II** "*In vitro* digestion of lipids from the marine diatom *Porosira glacialis* compared to commercial omega-3 products" (manuscript)
§: data from **Paper I** (Dalheim et al. 2020) heat-treated samples stored at 4 °C, day 1

4.2 Storage stability of lipids in *P. glacialis*

The high amount of PUFA in *P. glacialis* may affect the quality of the lipids during storage, since highly unsaturated fatty acids have lower oxidative stability (Choe and Min 2006). In addition, these fatty acids are the substrate for oxylipin synthesis in diatoms (Rettner et al. 2018). In **Paper I**, we wanted to investigate cost-effective and simple methods to increase the stability of lipids during storage that would enable further processing of the biomass. Freezing and thawing the biomass could lead to lipid deterioration and make the biomass less suitable for further handling, due to cell lysis. Therefore, refrigerator temperature was considered a better alternative for storage. The pre-treatments selected for the biomass was 0.1% benzoic acid (antimicrobial), addition of formic acid to pH 3 (antimicrobial and enzyme inactivation), 70 °C heat-treatment for 30 minutes (enzyme inactivation), and control samples. These samples were stored at 4 and 20 °C for 14 days, and the rate of lipid deterioration was measured as changes in lipid class and fatty acid composition. In a follow-up experiment, the biomass was warmed to 70, 80, or 90 °C for 30 minutes before storage for 7 and 14 days at 20 °C.

The most notable change in the lipids during storage was the formation of FFA (Figure 10). At 4 °C, the FFA content of benzoic acid and control samples increased gradually to 65% during 14 days of storage. At 20 °C, however, the FFA content in benzoic acid and control samples had reached 65-70% after only 24 hours of storage, and after 14 days the FFA content had reached 80% of total lipid classes. At 4 °C, the FFA content of heat-treated samples were relatively stable, whereas a slight increase was observed for samples stored with formic acid. In contrast, heat-treated and formic acid samples stored at 20 °C showed an increase in FFA content to 50-60%. The main substrate for hydrolysis appears to be the polar lipids MGDG and PG (PC in the case of formic acid samples). A less pronounced change, but still important, was the decrease in PUFA content in control, formic acid, and benzoic acid samples. As would be expected, the loss of PUFA was higher when the samples were stored at 20 °C than at 4 °C.

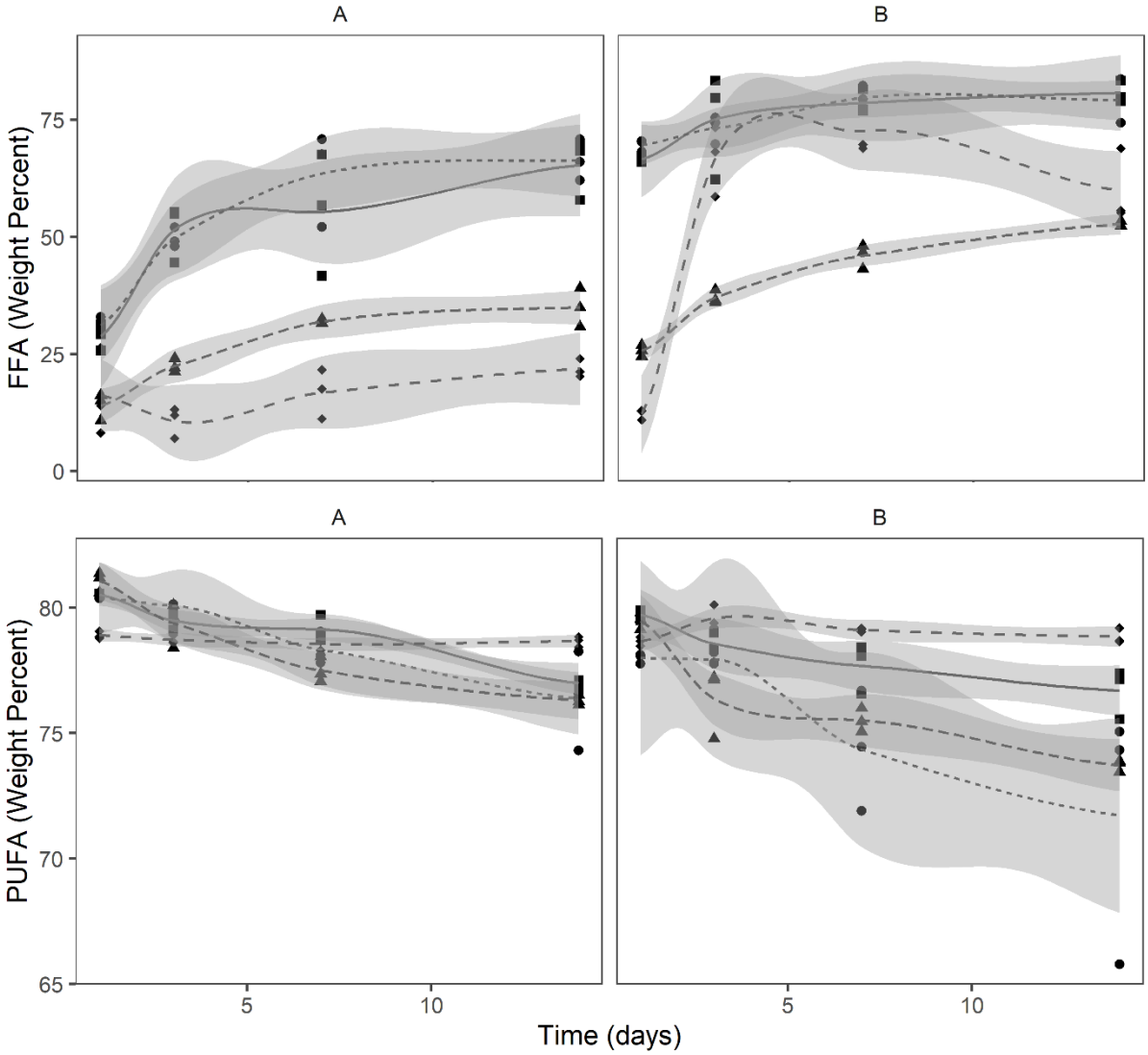

Figure 10 Development of FFA (top) and PUFA (bottom) content in Porosira glacialis biomass during storage at 4 °C (A) and 20 °C (B). The pre-treatments were benzoic acid (0.1% (W/W), square), control (no pre-treatment, circle), formic acid (pH 3.0, triangle), and heat-treatment (70 °C for 30 minutes, diamond). Adapted from ***Paper I*** *(Dalheim et al. 2020).*

The high formation of FFA during storage is likely due to endogenous lipolytic enzymes. These enzymes could have been triggered during harvesting, as the cells are likely to rupture at this stage. Cell rupture induces the anti-grazer defense mechanism of diatoms, which include formation of free fatty acids by lipolytic enzymes and oxygenation of fatty acids by lipoxygenase enzymes to form oxylipins (Pohnert 2002). The loss of PUFA in the samples may also be attributed to the oxylipin pathway, since PUFA with several double bonds, such as 16:4n-1 and 20:5n-3, are turned into volatile polyunsaturated short-chain aldehydes (d'Ippolito et al. 2004). Heating the samples to 70 °C and storing these at 4 °C appears to have inactivated

lipases and lipoxygenases in the biomass. Contrastingly, when the same samples were stored at 20 °C lipolysis occurred, which suggests that the enzymes had not been completely inactivated or the inactivation had been reversed. The diatom *P. glacialis* is a cold-water organism, which means the enzymes have lower optimum temperatures (Feller and Gerday 2003). It has also been observed that the heat inactivation of lipase enzymes in psychrotrophic organisms could be reversible (Owusu et al. 1992, Makhzoum et al. 1993). This may explain why the rate of lipolysis was high, even in heat-treated samples, when the biomass was stored at 20 °C. In the follow-up experiment, we found a reduction of lipolysis when the samples were heated to 90 °C when stored at 20 °C. It may appear that 90 °C was high enough for some irreversible inactivation of the lipolytic enzymes. The deterioration of lipids may not only arise from enzymatic activity. The loss of PUFA, even in samples stored with formic acid at a pH where enzyme activity is suspected to be lower, could potentially be explained by autoxidation. It has been shown that omega-3 containing oils exposed to lower pH are more susceptible to autoxidation (Jacobsen et al. 2001, Ozyurt et al. 2018).

As was discussed in the background section, diatoms contain several antioxidants, such as pigments and phenolic compounds (Goiris et al. 2012). The antioxidative capacity of the pigments of *P. glacialis* has not been analyzed yet, but it has been studied in several other species of microalgae, including diatoms, and some species have high antioxidant activity (Li et al. 2007, Goiris et al. 2012, Xia et al. 2013). *P.* glacialis may have a potentially high antioxidant content, which may have prevented some autoxidation and photooxidation in **Paper I**. However, the diatom *O. aurita* analyzed in **Paper III**, contained only 12% EPA and less than 1% DHA in our study, whereas it in other studies have been shown to contain 20-40% EPA and 2-4% DHA (Guihéneuf et al. 2010, Pasquet et al. 2014), which is similar to *P. glacialis*. Additionally, *O. aurita* contains fucoxanthin with strong antioxidant properties (Xia et al. 2013). If the low amounts of EPA and DHA in *O. aurita* detected in **Paper III** stems from lipid deterioration this may suggest an enzymatic effect rather than autoxidation or photooxidation because of the proven antioxidant capacity of this species. Similarly, we detected large amounts (72%) of FFA in *O. aurita*, which indicates a high lipolytic activity post-harvest.

4.3 The digestibility of lipids from *P. glacialis*

The complex lipid class composition and the large amounts of PUFA in *P. glacialis* biomass may affect the digestibility. The most common lipid class in human diets and fish feed is TAG, which diatoms contain only small amounts of when grown in a continuous culture in the exponential growth phase (Brown et al. 1996, Miller et al. 2014). In **Paper II**, we wanted to investigate how well lipids extracted from *P. glacialis* was digested compared to commercially available omega-3 products, using a porcine *in vitro* digestion system. Calanus oil, cod-liver oil, ethyl ester concentrate (EEC), krill oil, and *P. glacialis* oil were hydrolyzed for 0, 30, 60, and 180 minutes to assess the rate of hydrolysis for each supplement. The formation of FFA was used as a measure of digestibility, because TAG, DAG, phospholipids, and galactolipids must be hydrolyzed to a certain degree before they can be absorbed from the intestine. However, MAG and lyso-phospholipids may also cross the membrane of enterocytes, and the fatty acid composition of these specific lipid classes were not analyzed so the complete bioavailability of the different fatty acids was not mapped for the different oils.

The study found that SFA and MUFA were more easily released from the glycerol backbone than PUFA during *in vitro* digestion with porcine pancreatin (Table 3). This was evident in all the different oils analyzed, no matter what the initial lipid class composition was. This has been reported earlier for oils containing only TAG (Zhu et al. 2013, Giang et al. 2016). PUFA may be attached to the sn-2 position of the glycerol backbone of the various lipid classes, a position which some lipases cannot hydrolyze. However, MAG containing PUFA in the *sn*-2 position would also be absorbed in the intestine (Schuchardt and Hahn 2013). The initial FFA content of the oils varied to some extent. The "natural" oils from *P. glacialis, Calanus finmarchicus,* and krill contained FFA before hydrolysis, whereas the processed oils, cod-liver oil and the ethyl ester concentrate, did not. Crude fish oil is known to contain FFA, but these are removed during refining (Bimbo 2012). The FFA formation during *in vitro* digestion varied according to lipid class composition. Cod-liver oil was hydrolyzed to a larger extent than the other oils. *P. glacialis* and krill oils had the second and third highest formation of FFA during digestion, respectively (Table 3). The fact that *P. glacialis* and krill oil had the second highest formation indicates that the polar lipids in these oils are digestible. The FFA increase in ethyl ester concentrates and Calanus oil was low. However, the initially high FFA content of Calanus oil resulted in 14% FFA after 180 minutes of *in vitro* digestion. The lipid class most susceptible

to hydrolysis was TAG, followed by the phospholipids. The neutral lipid classes EE and WE were much more slowly hydrolyzed than other lipid classes. Even though cod-liver oil was hydrolyzed more readily than the other oils, the amount of bioavailable PUFA in free form would be low. The low digestibility of EEC also resulted in lower amounts PUFA in the bioavailable form FFA. Calanus oil, Krill oil, and *P. glacialis* oil all contained large amounts of PUFA as FFA after *in vitro* digestion. The initially high FFA content of an unprocessed oil may be a benefit, since it would not require enzymatic activity for absorption, however, FFA may be more exposed to oxidation and may impact the taste of the oil (Hayward et al. 2017, Lindsay 2017).

*Table 3 Summary of important findings adapted from **Paper II**. FFA (%) in the different oils after 0 and 180 minutes and SFA, MUFA, and PUFA (%) in the free fatty acid fraction after 0 and 180 minutes of in vitro digestion. FFA: free fatty acids, SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, CO: Calanus oil, CLO: cod-liver oil, EEC: ethyl ester concentrate, KO: krill oil, PGO: Porosira glacialis oil.*

	FFA (%)		SFA in FFA fraction (%)		MUFA in FFA fraction (%)		PUFA in FFA fraction (%)	
Min	0	180	0	180	0	180	0	180
CO	10.2	14.2	25.1	27.2	9.4	13.6	65.5	59.2
CLO	0	28.8	16.8	18.7	51.0	58.2	32.1	23.1
EEC	0	4.6	18.5	29.4	25.4	25,7	56.1	44.9
KO	4.2	18.9	22.3	29.7	19.3	18.6	58.4	51.7
PGO	4.8	21.8	11.6	17.1	14.6	16.3	73.8	66.6

4.4 The extractability of lipids from *P. glacialis*

The cell wall of many mass cultivated microalga, such as *C. vulgaris* and *Nannochloropsis sp.*, is made up of polysaccharides and glycoproteins, which make cell disruption necessary for maximum yield during extraction of lipids (Lee et al. 2010, Zheng et al. 2011). Common cell rupture methods like sonication, microwaves, bead milling, or grinding could be expensive to implement in the production line, since both equipment, energy, and operating cost would increase (Lee et al. 2012). Diatoms have a silica cell wall, which is not as tough as the polysaccharide/glycoprotein cell walls. In **Paper III**, we wanted to investigate how the lipid yield, lipid class and fatty acid composition of the diatoms *P. glacialis* and *O. aurita*, and the green microalgae *C. vulgaris* was affected by cell disruption, number of extractions, and solvent choice. Microwave, ultra-turrax, manual grinding, freeze drying, and sonication were evaluated for cell disruption of *P. glacialis*, and the latter appeared to be the only effective method of cell disruption. Further, the combination of no treatment (control), mixing for 60 minutes (mixing),

or sonication at 20 kHz for 10 minutes on ice were combined with the solvents dichloromethane/methanol (DCM/MeOH, 2:1 (v/v)), hexane/isopropanol (hexane/IPA, 2:1 (v/v)), or pure hexane. After the first extraction, the addition of extraction solvents was repeated to investigate the effect on lipid yield.

The polarity of the extraction solvents and repetition of extraction was the most important factors for the two diatoms, along with mixing or sonication. The solvents containing the alcohols methanol or isopropanol were able to extract the polar lipids associated with the thylakoid membranes (MGDG, DGDG, and PG), whereas pure hexane was not. In *P. glacialis*, hexane/IPA was not as effective as DCM/MeOH, but resulted in quite high yields. *C. vulgaris* on the other hand was more dependent on pre-treatment than the diatoms, and the extraction yield was improved by sonication. The fatty acid composition of *P. glacialis* was largely unaffected by solvents and treatments, indicating an even distribution of fatty acids between the different lipid classes. PUFA appeared to be more closely related with the polar lipid classes in the other two species. The findings in this study may indicate that the cost of lipid extraction on an industrial scale may be cheaper for *P. glacialis* than for other species. Extraction of lipids does not require cell disruption and the less toxic solvent combination hexane/isopropanol (Hara and Radin 1978) could be used with a low loss of lipid yield. However, the vulnerability of the diatom cell wall introduces some problems during harvesting. As was discussed in **Paper I**, cell disruption may lead to lipid deterioration (Balduyck et al. 2017), and therefore proper storage conditions or treatments must be applied to maintain lipid quality.

5 Conclusions and further work

The diatoms *P. glacialis* appears to be a good candidate for feed or food production, based on its lipid composition. It contains high amounts of PUFA and omega-3 fatty acids, which may be easily extracted compared to other microalgae. The reduced need for cell disruption for this diatom will reduce the cost of oil production compared to other mass-cultivated species. The digestibility of the lipids from *P. glacialis* is comparable to krill oil, which is currently being sold as a health supplement. Further investigations into the bioavailability of lipids from *P. glacialis* should be performed, for both the oil fraction and the whole biomass, which contain other valuable nutrients, such as proteins. *In vitro* digestibility studies using human gastrointestinal enzymes would clearly be of interests, since these may differ from enzymes of porcine origin. It would be interesting to include the gastric phase with pepsins and low pH to investigate if this has an effect on lipid digestion in the intestinal phase with pancreatin. Large scale *in vivo* studies should also be performed on fish to assess the potential of *P. glacialis* as an aquaculture feed ingredient. The challenge of lipid stability during biomass storage could be overcome by heat-treating the biomass before storage at refrigerator temperatures. The possibility of incorporating a heat treatment into the harvesting process, preferably after filtration and before the centrifugation step, to reduce enzymatic activity during storage should also be investigated. However, heat treatment of microalgae during harvesting may present high energy requirements.

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Paper I

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

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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

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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 ($P \geq 0.05$)

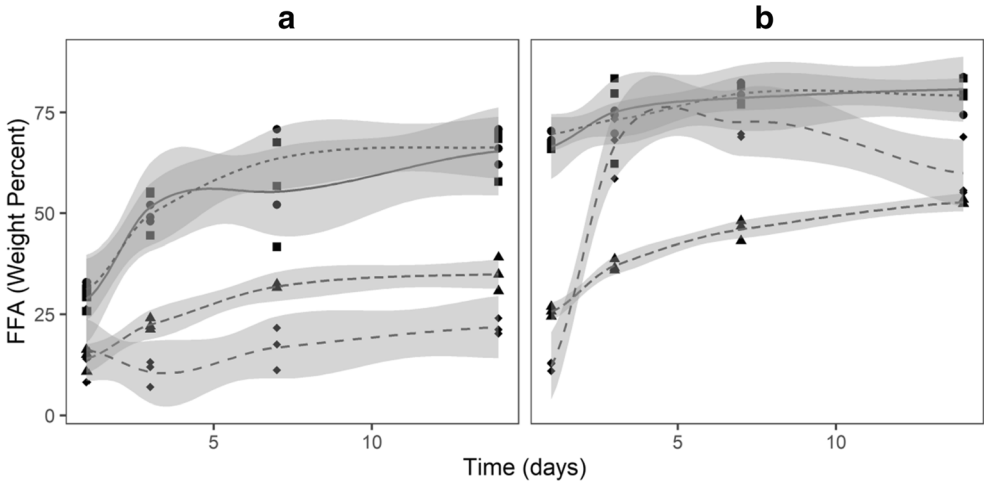

Fig. 1 Development of lipolysis (Free fatty acid (FFA) content, weight percentage) from day 1 to 14 for each treatment (n = 3) at 4 (A) and 20 °C (B). Benzoic acid (0.1% (w/w), square), control (no pre-treatment, circle), formic acid (pH 3.0, triangle), and heat treatment (70 °C for 30 min, diamond)

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	$3.2 \pm 1.3^{a}/2.2 \pm 0.4^{a}$	$3.2 \pm 1.4^{a}/5.8 \pm 2.1^{*a}$	$3.3 \pm 1.1^{a}/2.8 \pm 1.3^{a}$	$3.9 \pm 0.6^{a}/2.2 \pm 1.3^{a}$
Benzoic acid	$2.7 \pm 1.6^{a}/3.3 \pm 1.2^{ab}$	$3.9 \pm 1.3^{a}/6.1 \pm 5.2^{a}$	$7.7 \pm 8.3^{a}/3.2 \pm 0.7^{a}$	$3.3 \pm 1.4^{a}/2.2 \pm 1.0^{a}$
Formic acid	$38.4 \pm 2.1^{b}/36.3 \pm 0.5^{C}$	$39.0 \pm 1.1^{b}/36.8 \pm 1.1^{b}$	$37.4 \pm 0.3^{b}/34.3 \pm 1.2^{b}$	$38.3 \pm 2.4^{b}/32.0 \pm 1.1^{*b}$
Heat treatment	$3.7 \pm 1.4^{a}/5.8 \pm 0.8^{b}$	$6.5 \pm 1.0^{a}/3.1 \pm 2.2^{a}$	$5.4 \pm 0.9^{a}/8.3 \pm 0.6^{a}$	$3.7 \pm 1.6^{a}/15.0 \pm 2.7^{*C}$
Monoacylglycerol				
Control	$5.8 \pm 0.7^{a}/6.6 \pm 0.5^{a}$	$6.4 \pm 0.1^{a}/5.0 \pm 0.6^{a}$	$8.2 \pm 0.5^{a}/4.3 \pm 0.5^{a}$	$8.9 \pm 1.2^{*a}/5.1 \pm 2.6^{a}$
Benzoic acid	$4.9 \pm 0.1^{a}/6.8 \pm 0.3^{a}$	$6.1 \pm 0.9^{ab}/5.2 \pm 1.3^{a}$	$6.7 \pm 0.4^{a}/3.4 \pm 0.7^{*a}$	$9.7 \pm 0.8^{*a}/3.9 \pm 1.6^{ab}$
Formic acid	$1.4 \pm 0.2^{b}/1.4 \pm 0.1^{b}$	$1.4 \pm 0.0^{C}/1.6 \pm 0.2^{b}$	$1.6 \pm 0.1^{b}/1.3 \pm 1.1^{b}$	$1.7 \pm 0.1^{b}/2.0 \pm 0.1^{b}$
Heat treatment	$4.7 \pm 1.2^{a}/4.4 \pm 0.6^{a}$	$4.2 \pm 0.8^{b}/4.2 \pm 1.3^{a}$	$4.5 \pm 0.4^{C}/4.5 \pm 0.8^{a}$	$5.6 \pm 0.1^{C}/6.4 \pm 0.2^{*a}$
Fatty acid esters				
Control	$3.2 \pm 1.4^{a}/3.2 \pm 0.4^{a}$	$2.9 \pm 1.3^{ab}/8.0 \pm 0.8^{*a}$	$3.6 \pm 1.2^{a}/9.7 \pm 1.1^{*a}$	$3.4 \pm 0.6^{a}/10.9 \pm 0.6^{*a}$
Benzoic acid	$3.2 \pm 1.3^{a}/2.8 \pm 0.7^{ab}$	$3.5 \pm 0.9^{a}/5.8 \pm 0.7^{*b}$	$2.7 \pm 0.2^{a}/8.0 \pm 0.7^{*a}$	$3.1 \pm 0.5^{a}/9.9 \pm 0.9^{*a}$
Formic acid	$0.9 \pm 0.1^{b}/0.9 \pm 0.0^{b}$	$0.9 \pm 0.1^{b}/1.0 \pm 0.0^{C}$	$0.9 \pm 0.0^{b}/0.9 \pm 0.0^{b}$	$0.9 \pm 0.0^{b}/0.9 \pm 0.0^{b}$
Heat treatment	$2.4 \pm 0.7^{ab}/3.4 \pm 0.3^{a}$	$3.8 \pm 0.9^{a}/3.0 \pm 1.5^{C}$	$4.3 \pm 0.5^{a}/3.5 \pm 0.9^{C}$	$3.8 \pm 0.9^{a}/5.3 \pm 0.7^{C}$
Monogalactosyldiacylglycerol				
Control	$28.0 \pm 7.4^{a}/2.1 \pm 0.5^{a}$	$11.5 \pm 0.3^{*a}/ND^{a}$	$4.8 \pm 3.1^{*a}/ND^{a}$	$3.9 \pm 1.4^{*a}/ND^{a}$
Benzoic acid	$31.3 \pm 4.7^{a}/2.9 \pm 1.2^{a}$	$12.8 \pm 2.7^{*a}/0.5 \pm 0.7^{a}$	$11.0 \pm 6.6^{*a}/ND^{a}$	$5.1 \pm 0.5^{*a}/ND^{a}$
Formic acid	$24.9 \pm 4.8^{a}/21.7 \pm 1.0^{b}$	$21.1 \pm 1.4^{b}/9.6 \pm 0.7^{*b}$	$12.2 \pm 1.3^{*a}/6.1 \pm 1.2^{*a}$	$10.5 \pm 1.0^{*b}/3.5 \pm 0.5^{*a}$
Heat treatment	$23.3 \pm 7.3^{a}/29.6 \pm 1.5^{b}$	$27.4 \pm 7.1^{b}/13.3 \pm 1.2^{*b}$	$25.2 \pm 4.3^{b}/7.5 \pm 6.5^{*a}$	$18.8 \pm 2.6^{C}/10.3 \pm 4.0^{*b}$
Digalactosyldiacylglycerol				
Control	$1.6 \pm 1.4^{a}/0.3 \pm 0.6^{a}$	$2.4 \pm 0.6^{a}/ND^{a}$	$1.2 \pm 1.1^{a}/ND^{a}$	$0.3 \pm 0.5^{a}/ND^{a}$
Benzoic acid	$2.1 \pm 0.4^{a}/1.3 \pm 0.6^{a}$	$1.8 \pm 1.7^{a}/ND^{a}$	$1.1 \pm 0.9^{a}/ND^{a}$	$0.9 \pm 0.8^{a}/ND^{a}$
Formic acid	$4.4 \pm 0.8^{b}/4.5 \pm 0.1^{b}$	$4.6 \pm 0.8^{b}/5.2 \pm 0.5^{b}$	$5.9 \pm 0.6^{*b}/4.4 \pm 0.6^{b}$	$5.2 \pm 0.2^{*b}/2.8 \pm 0.1^{b}$
Heat treatment	$4.4 \pm 0.4^{b}/4.1 \pm 1.5^{b}$	$3.2 \pm 0.4^{ab}/1.7 \pm 0.5^{*a}$	$4.0 \pm 0.4^{C}/0.7 \pm 0.6^{*a}$	$6.1 \pm 1.5^{*b}/ND^{a}$
Phosphatidylglycerol				
Control	$25.2 \pm 1.1^{a}/11.6 \pm 2.2^{a}$	$20.0 \pm 1.7^{a}/1.8 \pm 1.6^{*a}$	$12.9 \pm 5.6^{*a}/ND^{*a}$	$9.6 \pm 1.6^{*a}/ND^{*a}$
Benzoic acid	$24.6 \pm 1.5^{a}/11.1 \pm 1.4^{a}$	$16.7 \pm 3.3^{*a}/1.0 \pm 1.7^{*a}$	$12.3 \pm 1.1^{*a}/ND^{*a}$	$9.7 \pm 3.0^{*a}/ND^{*a}$
Formic acid	$4.2 \pm 0.2^{b}/3.2 \pm 0.5^{a}$	$1.6 \pm 0.4^{*b}/1.9 \pm 0.6^{a}$	$2.0 \pm 1.4^{*b}/0.8 \pm 0.1^{*a}$	$1.3 \pm 0.3^{*b}/0.2 \pm 0.4^{*a}$
Heat treatment	$43.6 \pm 10.8^{C}/37.6 \pm 1.9^{b}$	$41.9 \pm 9.7^{C}/6.0 \pm 5.6^{*a}$	$38.2 \pm 1.3^{C}/1.0 \pm 1.8^{*a}$	$40.1 \pm 6.1^{C}/ND^{*a}$
Phosphatidylcholine				
Control	ND^{a}	ND^{a}	ND^{a}	ND^{a}
Benzoic acid	ND^{a}	ND^{a}	ND^{a}	ND^{a}
Formic acid	$9.9 \pm 1.0^{b}/4.4 \pm 0.2^{b}$	$7.0 \pm 0.3^{*b}/5.1 \pm 0.6^{b}$	$6.3 \pm 0.3^{*b}/4.5 \pm 0.1^{b}$	$5.8 \pm 0.4^{*b}/4.2 \pm 0.3^{b}$
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	$3.9 \pm 0.1^{a}/5.7 \pm 0.0^{a}$	$4.4 \pm 0.4^{a}/5.6 \pm 0.2^{a}$	$5.2 \pm 0.4^{a}/6.8 \pm 1.5^{a}$	$5.9 \pm 0.8^{a}/7.4 \pm 1.6^{a}$
Benzoic acid	$4.4 \pm 0.2^{a}/5.1 \pm 0.1^{b}$	$4.6 \pm 0.1^{a}/5.5 \pm 0.2^{a}$	$4.7 \pm 0.2^{a}/5.6 \pm 0.1^{*ab}$	$5.4 \pm 0.0^{*a}/6.2 \pm 0.3^{*ab}$
Formic acid	$4.0 \pm 0.1^{a}/4.2 \pm 0.1^{c}$	$4.2 \pm 0.2^{a}/4.7 \pm 0.3^{b}$	$4.5 \pm 0.2^{*a}/4.8 \pm 0.2^{*b}$	$4.5 \pm 0.1^{*a}/5.3 \pm 0.1^{*bc}$
Heat treatment	$5.8 \pm 0.4^{b}/5.6 \pm 0.1^{D}$	$5.5 \pm 0.4^{b}/4.7 \pm 0.3^{*b}$	$5.6 \pm 0.1^{a}/4.5 \pm 0.1^{b}$	$5.5 \pm 0.1^{a}/4.2 \pm 0.2^{*c}$
C16:0				
Control	$4.2 \pm 0.0^{a}/4.3 \pm 0.1^{a}$	$4.1 \pm 0.2^{a}/4.3 \pm 0.0^{a}$	$4.3 \pm 0.3^{a}/5.5 \pm 0.9^{a}$	$5.0 \pm 0.7^{ab}/5.9 \pm 1.2^{*a}$
Benzoic acid	$4.0 \pm 0.2^{ab}/3.8 \pm 0.1^{b}$	$4.3 \pm 0.2^{a}/4.1 \pm 0.2^{a}$	$4.3 \pm 0.3^{a}/4.4 \pm 0.4^{*b}$	$4.7 \pm 0.1^{*ab}/4.5 \pm 0.1^{*b}$
Formic acid	$4.0 \pm 0.1^{ab}/4.3 \pm 0.1^{a}$	$4.3 \pm 0.2^{a}/5.4 \pm 0.4^{*b}$	$4.9 \pm 0.1^{*a}/5.8 \pm 0.1^{*a}$	$5.4 \pm 0.1^{*a}/6.2 \pm 0.0^{*a}$
Heat treatment	$3.8 \pm 0.1^{b}/4.1 \pm 0.0^{a}$	$4.1 \pm 0.2^{a}/4.0 \pm 0.2^{a}$	$4.1 \pm 0.1^{a}/4.3 \pm 0.1^{b}$	$4.1 \pm 0.1^{b}/4.5 \pm 0.0^{*b}$
C18:0				
Control	$1.3 \pm 0.0^{a}/1.2 \pm 0.0^{a}$	$1.2 \pm 0.1^{a}/1.1 \pm 0.0^{ab}$	$1.2 \pm 0.1^{a}/1.4 \pm 0.2^{a}$	$1.4 \pm 0.3^{a}/1.4 \pm 0.3^{a}$
Benzoic acid	$1.1 \pm 0.2^{b}/0.9 \pm 0.1^{b}$	$1.3 \pm 0.2^{a}/0.9 \pm 0.1^{a}$	$1.3 \pm 0.2^{a}/1.1 \pm 0.1^{b}$	$1.2 \pm 0.0^{a}/1.0 \pm 0.1^{b}$
Formic acid	$1.3 \pm 0.1^{a}/1.3 \pm 0.0^{a}$	$1.5 \pm 0.1^{*a}/1.3 \pm 0.1^{b}$	$1.4 \pm 0.0^{a}/1.2 \pm 0.1^{ab}$	$1.4 \pm 0.1^{a}/1.2 \pm 0.0^{ab}$
Heat treatment	$1.0 \pm 0.1^{b}/1.2 \pm 0.0^{a}$	$1.2 \pm 0.2^{a}/1.1 \pm 0.2^{ab}$	$1.2 \pm 0.1^{a}/1.1 \pm 0.1^{ab}$	$1.2 \pm 0.0^{a}/1.2 \pm 0.0^{ab}$
C16:1n-7				
Control	$10.1 \pm 0.0^{a}/10.8 \pm 0.2^{a}$	$10.2 \pm 0.0^{a}/11.1 \pm 0.1^{a}$	$11.0 \pm 0.0^{ab}/11.9 \pm 0.8^{a}$	$11.3 \pm 0.3^{a}/13.5 \pm 2.2^{*a}$
Benzoic acid	$10.1 \pm 0.1^{a}/10.4 \pm 0.0^{b}$	$10.3 \pm 0.0^{a}/10.9 \pm 0.1^{a}$	$10.6 \pm 0.2^{a}/11.1 \pm 0.3^{*ab}$	$11.7 \pm 0.1^{*a}/11.6 \pm 0.7^{*ab}$
Formic acid	$9.7 \pm 0.3^{b}/10.7 \pm 0.1^{ab}$	$10.5 \pm 0.3^{*a}/12.3 \pm 0.6^{*b}$	$11.7 \pm 0.3^{*b}/12.7 \pm 0.2^{*c}$	$12.4 \pm 0.1^{*a}/13.5 \pm 0.1^{*a}$
Heat treatment	$10.5 \pm 0.1^{c}/10.5 \pm 0.0^{ab}$	$10.5 \pm 0.1^{a}/10.6 \pm 0.2^{a}$	$10.5 \pm 0.1^{a}/11.0 \pm 0.1^{b}$	$10.5 \pm 0.0^{a}/11.2 \pm 0.2^{*b}$
C16:2n-4				
Control	$2.4 \pm 0.0^{a}/2.5 \pm 0.1^{a}$	$2.5 \pm 0.0^{a}/2.5 \pm 0.0^{a}$	$2.5 \pm 0.0^{a}/2.4 \pm 0.1^{a}$	$2.6 \pm 0.0^{a}/2.5 \pm 0.1^{a}$
Benzoic acid	$2.4 \pm 0.0^{a}/2.4 \pm 0.0^{a}$	$2.5 \pm 0.0^{a}/2.4 \pm 0.0^{a}$	$2.5 \pm 0.0^{a}/2.4 \pm 0.0^{a}$	$2.6 \pm 0.0^{*a}/2.5 \pm 0.1^{*ab}$
Formic acid	$5.3 \pm 0.1^{b}/5.2 \pm 0.1^{b}$	$5.2 \pm 0.1^{b}/5.0 \pm 0.1^{b}$	$5.1 \pm 0.1^{*b}/4.8 \pm 0.1^{*b}$	$4.9 \pm 0.0^{*b}/4.9 \pm 0.0^{*c}$
Heat treatment	$2.5 \pm 0.0^{a}/2.4 \pm 0.0^{a}$	$2.4 \pm 0.0^{a}/2.4 \pm 0.0^{a}$	$2.5 \pm 0.0^{a}/2.5 \pm 0.0^{a}$	$2.5 \pm 0.0^{c}/2.4 \pm 0.0^{b}$
C16:3n-4				
Control	$4.8 \pm 0.1^{ab}/4.7 \pm 0.1^{a}$	$4.8 \pm 0.0^{a}/4.6 \pm 0.0^{a}$	$4.8 \pm 0.0^{a}/4.5 \pm 0.1^{a}$	$4.8 \pm 0.0^{a}/4.4 \pm 0.2^{*a}$
Benzoic acid	$4.9 \pm 0.0^{a}/4.6 \pm 0.0^{a}$	$4.8 \pm 0.0^{a}/4.5 \pm 0.0^{*a}$	$4.7 \pm 0.0^{*a}/4.4 \pm 0.1^{*a}$	$4.8 \pm 0.1^{a}/4.5 \pm 0.0^{*a}$
Formic acid	$9.4 \pm 0.1^{c}/9.0 \pm 0.0^{b}$	$9.1 \pm 0.1^{*b}/8.5 \pm 0.1^{*b}$	$8.7 \pm 0.0^{*b}/8.2 \pm 0.0^{*b}$	$8.3 \pm 0.0^{*b}/8.0 \pm 0.0^{*b}$
Heat treatment	$4.7 \pm 0.1^{b}/4.6 \pm 0.0^{c}$	$4.6 \pm 0.0^{c}/4.7 \pm 0.0^{c}$	$4.6 \pm 0.0^{c}/4.7 \pm 0.0^{c}$	$4.6 \pm 0.0^{a}/4.7 \pm 0.0^{c}$
C16:4n-1				
Control	$31.9 \pm 0.2^{a}/31.6 \pm 0.2^{a}$	$32.2 \pm 0.1^{a}/31.1 \pm 0.1^{a}$	$31.5 \pm 0.2^{a}/29.5 \pm 1.1^{a}$	$31.0 \pm 0.8^{a}/28.9 \pm 2.1^{*a}$
Benzoic acid	$32.6 \pm 0.1^{b}/31.5 \pm 0.1^{a}$	$32.1 \pm 0.4^{a}/30.9 \pm 0.0^{a}$	$31.7 \pm 0.3^{*a}/29.9 \pm 0.9^{*a}$	$31.4 \pm 0.4^{*a}/30.2 \pm 0.1^{*ab}$
Formic acid	$28.0 \pm 0.1^{c}/26.7 \pm 0.1^{b}$	$26.9 \pm 0.1^{*b}/25.0 \pm 0.2^{*b}$	$25.6 \pm 0.0^{*b}/24.2 \pm 0.1^{*b}$	$24.6 \pm 0.1^{*b}/23.5 \pm 0.1^{*c}$
Heat treatment	$31.8 \pm 0.6^{a}/31.5 \pm 0.2^{a}$	$31.5 \pm 0.1^{c}/31.7 \pm 0.3^{c}$	$31.5 \pm 0.0^{a}/31.4 \pm 0.1^{c}$	$31.4 \pm 0.1^{a}/31.5 \pm 0.2^{b}$
C18:4n-3				
Control	$10.0 \pm 0.0^{a}/9.9 \pm 0.1^{a}$	$9.8 \pm 0.0^{ab}/9.3 \pm 0.1^{a}$	$9.7 \pm 0.1^{a}/9.0 \pm 0.5^{a}$	$9.5 \pm 0.3^{a}/9.0 \pm 0.5^{*a}$
Benzoic acid	$9.9 \pm 0.0^{b}/10.1 \pm 0.0^{b}$	$9.7 \pm 0.1^{a}/9.9 \pm 0.1^{b}$	$9.8 \pm 0.0^{a}/9.8 \pm 0.4^{b}$	$9.6 \pm 0.1^{ab}/10.0 \pm 0.1^{b}$
Formic acid	$3.7 \pm 0.0^{c}/3.5 \pm 0.0^{c}$	$3.5 \pm 0.1^{c}/3.6 \pm 0.1^{c}$	$3.6 \pm 0.0^{b}/3.7 \pm 0.0^{*c}$	$3.7 \pm 0.1^{c}/3.8 \pm 0.0^{*c}$
Heat treatment	$10.1 \pm 0.1^{a}/10.0 \pm 0.0^{b}$	$10.1 \pm 0.0^{b}/9.9 \pm 0.2^{b}$	$10.0 \pm 0.0^{a}/9.6 \pm 0.0^{b}$	$10.1 \pm 0.0^{b}/9.3 \pm 0.1^{*a}$
C20:5n-3				
Control	$28.3 \pm 0.1^{a}/26.6 \pm 0.4^{ab}$	$27.8 \pm 0.1^{a}/27.5 \pm 0.2^{a}$	$26.9 \pm 0.4^{a}/25.8 \pm 1.2^{a}$	$25.8 \pm 1.1^{*a}/24.2 \pm 2.1^{*a}$
Benzoic acid	$27.8 \pm 0.1^{ab}/28.0 \pm 0.1^{a}$	$27.5 \pm 0.1^{a}/27.7 \pm 0.2^{a}$	$27.6 \pm 0.2^{a}/27.7 \pm 0.1^{ab}$	$25.9 \pm 0.2^{*a}/26.7 \pm 0.7^{*ab}$
Formic acid	$31.5 \pm 0.4^{c}/32.3 \pm 0.4^{c}$	$31.7 \pm 0.8^{b}/31.3 \pm 1.2^{b}$	$31.6 \pm 0.5^{b}/31.2 \pm 0.4^{c}$	$31.7 \pm 0.1^{b}/30.0 \pm 0.2^{*c}$
Heat treatment	$27.1 \pm 0.4^{b}/27.1 \pm 0.1^{b}$	$27.2 \pm 0.2^{a}/27.9 \pm 0.2^{*a}$	$27.1 \pm 0.1^{a}/27.9 \pm 0.1^{b}$	$27.3 \pm 0.1^{a}/27.9 \pm 0.3^{*bc}$
C22:6n-3				
Control	$2.9 \pm 0.1^{ab}/2.7 \pm 0.2^{a}$	$2.9 \pm 0.0^{a}/3.0 \pm 0.0^{a}$	$2.8 \pm 0.1^{a}/3.1 \pm 0.2^{a}$	$2.8 \pm 0.1^{*ab}/2.8 \pm 0.3^{a}$
Benzoic acid	$2.9 \pm 0.1^{ab}/3.0 \pm 0.0^{a}$	$2.9 \pm 0.0^{a}/2.9 \pm 0.0^{a}$	$2.8 \pm 0.0^{a}/3.0 \pm 0.1^{a}$	$2.6 \pm 0.0^{*a}/2.9 \pm 0.1^{a}$
Formic acid	$3.2 \pm 0.2^{a}/2.9 \pm 0.1^{a}$	$2.9 \pm 0.1^{*a}/2.9 \pm 0.2^{a}$	$2.9 \pm 0.0^{*a}/3.4 \pm 0.0^{*b}$	$3.1 \pm 0.1^{b}/3.6 \pm 0.0^{*b}$
Heat treatment	$2.6 \pm 0.4^{b}/2.8 \pm 0.0^{a}$	$2.8 \pm 0.0^{a}/3.0 \pm 0.1^{a}$	$2.8 \pm 0.0^{a}/3.0 \pm 0.0^{a}$	$2.8 \pm 0.0^{ab}/3.0 \pm 0.0^{a}$

[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;

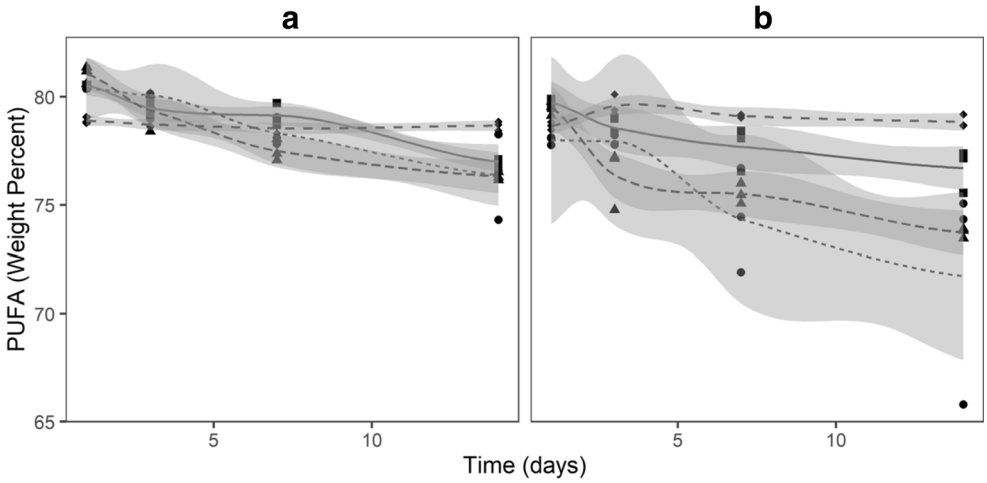

Fig. 2 Polyunsaturated fatty acids (PUFA, weight percentage) from day 1 to 14 for each treatment (n = 3) at 4 (A) and 20 °C (B). Benzoic acid (0.1% (w/w), square), control (no pre-treatment, circle), formic acid (pH 3.0, triangle), and heat treatment (70 °C for 30 min, diamond)

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.

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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".

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Paper II

Dalheim, L., Svenning, J.B. & Olsen, R.L.

In vitro digestion of lipids from the marine diatom *Porosira glacialis* compared to commercial omega-3 products.

Submitted manuscript.

In vitro digestion of lipids from the marine diatom *Porosira glacialis* compared to commercial omega-3 products

Short title: Digestion of diatom lipids

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Keywords: digestibility; omega-3; PUFA; lipid class; microalgae

Running title: Digestibility of diatom lipids and commercial omega-3

Abbreviations: CLO, cod-liver oil; CO, Calanus oil; DAG, diacylglycerol; DGDG, digalactosyldiacylglycerol; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; EE, ethyl esters; EEC, ethyl ester concentrate; FAlc, fatty alcohols; FFA, free fatty acids; KO, krill oil; LC-PUFA, long-chain polyunsaturated fatty acids; MAG, monoacylglycerol; MGDG, monogalactosyldiacylglycerol; MUFA, monounsaturated fatty acid; PC, phosphatidylcholine; PG, phosphatidylglycerol; PGO, Porosira glacialis oil; PL, phospholipids; SFA, saturated fatty acids; SPE, solid phase extraction; TAG, triacylglycerol; WE, wax esters

Abstract

Marine sources of omega-3 fatty acids are in high demand for use in health supplements. Mass cultivation of marine microalgae is a promising and sustainable source of omega-3 fatty acids, which relieves pressure on natural fish stocks. The lipid class profile from cultivated photosynthetic algae differ from the marine organisms currently used for the production of marine omega-3 fatty acids. The objective of this study was to compare *in vitro* enzymatic digestion of oil extracted from the cold-adapted marine diatom *Porosira glacialis* with commercially available marine omega-3 supplements; cod liver oil, krill oil, ethyl ester concentrate, and oil from the copepod *Calanus finmarchicus* (Calanus® oil). The changes in the free fatty acids and neutral and polar lipids during the enzymatic hydrolysis were characterized by liquid and gas chromatography. In Calanus® oil and the Ethyl ester concentrate, the free fatty acids increased very little (4.0 and 4.6%, respectively) during digestion. In comparison, free fatty acids in Krill oil and *P. glacialis* oil increased by 14.7 and 17.0 %, respectively. Cod liver oil had the highest increase (28.2%) in free fatty acids during digestion. Monounsaturated and saturated fatty acids were more easily released than polyunsaturated fatty acids in all five oils.

1 Introduction

The market for omega-3 supplements has seen substantial growth in the recent years, stimulated by scientific studies that demonstrate positive health effects of the long-chain polyunsaturated omega-3 fatty acids (LC-PUFA) eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) (1-5). The main sources of marine omega-3 fatty acids for supplements are oils from fish liver, crustaceans, and small pelagic fish species that are only to a limited degree used for direct human consumption. Due to the restrictions on conventional fisheries, the annual production of fish oil is capped at approximately 1 million metric tons (6), of which about 75% is used by the aquaculture industry to make fish feed (7). Therefore, the limited availability of fish oil and the increased demand from the aquaculture feed and nutraceutical industries has stimulated research and commercialization of alternative sources of marine omega-3 fatty acids.

In addition to nutraceutical oils extracted from lower trophic levels organisms in the marine food web like Antarctic krill (8) and the copepod *Calanus finmarchicus* (9), much focus has been on extracting oils from the main primary producers of LC-PUFA; the marine microalgae (10, 11). Microalgae can be grown in bioreactors under photosynthetic or heterotrophic conditions, thereby avoiding the challenges of overfishing and ecological impact which may occur when harvesting organisms from natural populations. However, the lipid class composition of oils from lower trophic levels, including microalgae, often differs from traditional fish oil supplements and ethyl ester fish oil concentrates (EEC) and this may affect the human bioavailability of the fatty acids in the oils. Ultimately, the bioavailability of any oil depends on the enzymes required to hydrolyze lipids. Some enzymes function on specific substrates, such as pancreatic triacylglycerol lipase or phospholipase which hydrolyze triacylglycerol (TAG) and phospholipids, respectively. Others may target a wider range of substrates, such as pancreatic lipase related protein 2 and carboxyl ester lipase, both of which

can hydrolyze TAG, cholesterol esters, phospholipids, galactolipids, and vitamin esters (12). However, the reaction rates of these enzymes vary according to their substrates, and the bioavailability of LC-PUFA therefore depend on the lipid classes they are bound in. For example, while fish oils mainly contain TAG, krill oil contains a large amount of phospholipids (PL) as well as TAG and TAG-derived molecules (8) and oil from *C. finmarchicus* contains a very high proportion of wax esters (WE) (9). Oils from phototrophic microalgae often contain substantial amounts of monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) originating from photosynthetic membranes, in addition to TAG, TAG-derived lipid classes and phospholipids (13, 14). It is therefore very important to measure the rate of enzymatic hydrolysis of novel oils when assessing their viability as dietary supplements.

In vivo studies of blood plasma uptake of EPA and DHA and digestion of TAG, EEC, PL, and WE have begun to reveal the complexity of marine omega-3 PUFA absorption, but due to heterogeneous study designs and mixed results it is difficult to establish conclusive bioavailabilities (15-21). Some *in vitro* studies have been carried out to investigate the digestibility of MGDG and DGDG (22, 23), whole microalga (24), TAG and EE (25), vegetable, fish and krill oils (26). These studies have all used different methodologies for analyzing the digestibility and this makes it difficult to compare the results. Therefore it has been suggested that the pH-stat method commonly used for determining release of fatty acids is not optimal, and that other methods should be considered (27).

The photoautotrophic diatom *Porosira glacialis* is a large psychrophilic centric diatom with a highly desaturated fatty acid profile (28) and may therefore represent a valuable source of LC-PUFA. To our knowledge, there are no previous studies in which the bioavailability of fatty acids from this or similar microalgae have been studied. In addition, *P. glacialis* can be cultivated using flue gases from high-emission industry, turning greenhouse gases into a resource, which in the long term may help to reduce the impact of heavy industry (29). The

objective of this study was to compare *in vitro* enzymatic digestion of oil extracted from *P. glacialis*, cultivated on CO_2 containing flue gases from an industrial plant, with commercially available marine omega-3 products. The changes in lipid classes in the oils during the enzymatic hydrolysis were analyzed by HPLC. Free fatty acids, polar and neutral lipids in the hydrolyzed oil samples were isolated by solid phase extraction (SPE) and the fatty acid composition determined by GC. The viability of each oil as a dietary supplement for human consumption was assessed based on their rate of hydrolysis.

2 Materials and methods

2.1 Materials

Oil from *Calanus finmarchicus* (Calanus® Oil) was commercially produced and provided by Calanus AS, Tromsø, Norway. Krill oil (Life AS), ethyl ester concentrate (Biopharma) and cod liver oil (Møller's) were bought at a local health store. Microalgae oil was extracted from *Porosira glacialis* cultivated as described later.

Dichloromethane; DCM (≥99.9%), hexane (≥99%), chloroform (99.0-99.4%), diethyl ether (≥99.8%), sulfuric acid (95-97%), porcine bile and porcine pancreatin were purchased from Sigma-Aldrich (St. Louis, MO, USA). LC-MS grade methanol; MeOH was from Fisher Scientific (Loughborough, UK). Acetic acid (≥99.8%) was purchased from Honeywell Fluka (Muskegon, MI, USA). Sodium bicarbonate, sodium acetate, isooctane (LC-grade), ethyl acetate (LC-grade), acetone (LC-grade) and isopropanol (LC-grade) were obtained from Merck (Darmstadt, Germany). Kristalon Flower® was obtained from Yara (Oslo, Norway). Sodium metasilicate pentahydrate was obtained from Permakem A/S (Lørenskog, 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.

2.2 Cultivation of *Porosira glacialis*

The microalgae strain cultivated in this study was originally isolated from sediment samples collected in the Barents Sea (N 76° 27.54', E 033° 03.54') during a cruise in 2014. The cultivation took place outdoors in January 2019 in a 6000 L vertical column glass fiber photobioreactor, with similar cultivation conditions to those applied by Svenning, Dalheim (28). Briefly, 100 g Kristalon Flower® (14% N, 3.9% P) and 1 L silicate stock solution (100 g sodium metasilicate pentahydrate L^{-1} fresh water) were added per 1000 L filtered seawater in the reactor. The pH was maintained below 8.0 by bubbling the tank with factory smoke containing CO_2. The CO_2 in the smoke was also used as the carbon source during the cultivation.

2.3 Lipid extraction

Lipids were extracted from *P. glacialis* using a modification of the method of Folch, Lees (30). Briefly, 9 grams of freeze-dried biomass were partitioned into six 50 ml FEP tubes (Thermo Scientific) and added 30 ml dichloromethane/methanol (2:1, v/v) and mixed by hand. The samples were then added 10 ml of 5 % NaCl, before the organic phase from all samples were pooled and evaporated to dryness.

2.4 *In vitro* digestion

Porcine pancreatin was used for the *in vitro* digestion of the oils basically as described by Aarak, Kirkhus (31).The individual oils were dissolved to 15 mg ml^{-1} in 0.15 M $NaHCO_3$ containing 0.9 % NaCl (total 8.0 ml) and added 11.8 mM porcine bile and 13.6 mg ml^{-1} of porcine pancreatin. The pH was adjusted to 7.0 with 0.1 M HCl. The samples were then incubated for 30, 60, and 180 minutes at 37 °C under constant mixing using a LABINCO LD79 digital test-tube rotator (Breda, Netherland). The reaction was stopped by extracting the lipids using DCM/MeOH (2:1, v/v).

2.4.1 Lipid class isolation and fatty acid analysis

Free fatty acids, polar and neutral lipids were isolated from the samples digested for 0, 30, 60 and 180 minutes by SPE using the method developed by Ruiz, Antequera (32). The aminopropyl minicolumns (500 mg, Agilent, USA) were activated using 7.5 ml hexane before 150 µl sample in DCM/MeOH (2:1, v/v) was added. The neutral lipids were eluted with 5 ml of chloroform, the free fatty acids with 5 ml diethyl ether/acetic acid (98:2, v/v) and the polar lipids using 2.5 ml methanol/chloroform (6:1, v/v) and subsequently 2.5 ml 0.05 M sodium acetate in methanol/chloroform (6:1, v/v) and pooled. The 3 collected fractions were evaporated to dryness and dissolved in DCM/MeOH (2:1, v/v) to 10 mg ml^{-1}.

A GC-FID (Agilent Technologies) was used to quantify the fatty acids as FAMEs in the oils and in the fractions separated by SPE. The lipids were methylated using a modified version of Stoffel, Chu (33). Briefly, 100 µl of lipid sample (10 mg ml^{-1}) was added 100 µl of internal standard (C17:0, 0.1 mg ml^{-1}), 800 µl DCM and 2 ml 10% H_2SO_4 in MeOH and kept at 100 °C for one hour and subsequently separated into lipid soluble and water soluble fractions. The lipid soluble fractions were evaporated to dryness using nitrogen gas and dissolved in 100 µl of 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 one minute, 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 minutes. 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, Elysian, MN, USA) and heptadecanoic acid (Sigma-Aldrich Co, St. Louis, MO, USA) as internal standard, every concentration was analyzed as triplicates.

2.4.2 Lipid class composition

The lipid class composition of the oils and hydrolyzed samples was investigated using an HPLC method based on Abreu, Solgadi (34). HPLC analyses were performed on a Waters e2795 separations module, using a Supelcosil™ LC-SI 5 µm (25 cm x 4.6 mm) column (Supelco HPLC products, Bellefonte, PA, 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 minutes and the gradient profile can be seen in the supporting information file. 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).

2.5 Statistical analysis and data availability

All the analyses were performed in triplicates. The data are presented as means ± standard deviation. All analyses and figures were prepared using R v3.6.1. Data were considered significantly different if $P<0.01$ using a pairwise Tukey test. All data and the R markdown for this experiment are available from the Open Science Framework (OSF) under the name “In

vitro digestion of lipids from the marine diatom *Porosira glacialis* compared to commercially available marine oils" (https://osf.io/rxnm9/?view_only=e4bec4deed91412aa45b1cb4833ac61a). This link also includes supplementary data containing tables of lipid classes and fatty acids, as well as heat maps of statistical tests.

3 Results

3.1 Fatty acid composition of the oils

The fatty acid composition of the oils are presented in Table 1, and show a diverse distribution with omega-3 fatty acids varying from a minimum of 29.5 in cod-liver oil (CLO) to a maximum of 73.2% in the ethyl ester concentrate (EEC). Of these, α-linolenic acid (18:3n-3) was present in minor amounts, from 0.6 to 1.5%. The main fatty acids in Calanus® Oil (CO) were 19.4% myristic acid (14:0), 15.5% stearidonic acid (SDA, 18:4n-3), 17% EPA and 12.5% DHA. CLO contained 15.6% oleic acid (18:1n-9), 14.5% eicosenoic acid (20:1 n-9), 9.1% EPA and 13.7% DHA. EEC contained 37.2% EPA and 27.5% DHA, while krill oil (KO) contained 21.6% palmitic acid (16:0), 11.0% palmitoleic acid (16:1 n-7), 11.5% oleic acid, 21.2% EPA and 10% DHA. Finally, *P. glacialis* oil (PGO) contained 12.7% palmitoleic acid, 35.2% hexadecatetraenoic acid (16:4 n-1), 28.9 % EPA, and 4.9% DHA.

Table 1 Fatty acid composition (weight percent) of Calanus oil (CO), Cod liver oil (CLO), Ethyl ester concentrate (EEC), Krill oil (KO), and oil from Porosira glacialis (PGO) (n=3). ND = not detected.

	CO	CLO	EEC	KO	PGO
C14:0	19.4 ± 0.23	3.6 ± 0.03	ND	9.2 ± 0.23	4.0 ± 0.07
C14:1	0.5 ± 0.00	ND	ND	ND	ND
C16:0	9.7 ± 0.03	8.9 ± 0.02	ND	21.6 ± 0.24	3.5 ± 0.04
C16:1n-7	3.9 ± 0.04	9.6 ± 0.03	ND	11.0 ± 0.03	12.7 ± 0.01
C16:2n-4	ND	ND	ND	0.9 ± 0.03	2.3 ± 0.01
C16:3n-4	ND	ND	ND	ND	4.4 ± 0.02
C18:0	0.8 ± 0.01	2.0 ± 0.01	4.3 ± 0.03	1.1 ± 0.01	ND
C18:1n-9/ C16:4n-1*	5.6 ± 0.06	15.6 ± 0.01	7.3 ± 0.04	11.5 ± 0.26	35.2 ± 0.02
C18:1n-7	ND	4.9 ± 0.04	3.0 ± 0.03	6.3 ± 0.02	ND
C18:2n-6	1.2 ± 0.02	2.6 ± 0.00	1.3 ± 0.02	1.9 ± 0.01	ND
C18:3n-3	1.5 ± 0.03	1.0 ± 0.01	1.0 ± 0.01	0.6 ± 0.01	ND
C20:0	ND	ND	1.0 ± 0.01	ND	ND
C18:4n-3	15.5 ± 0.19	4.4 ± 0.03	2.4 ± 0.03	1.7 ± 0.03	4.1 ± 0.02
C20:1n-9	3.0 ± 0.03	14.5 ± 0.01	2.4 ± 0.02	0.9 ± 0.00	ND
C20:2n-6	ND	ND	0.5 ± 0.01	ND	ND
C20:4n-6	0.8 ± 0.01	ND	2.4 ± 0.01	ND	ND
C22:1n-11	4.4 ± 0.05	7.0 ± 0.04	1.4 ± 0.01	ND	ND
C22:1n-9	1.8 ± 0.04	1.8 ± 0.02	2.2 ± 0.00	1.3 ± 0.01	ND
C20:5n-3	17.0 ± 0.24	9.1 ± 0.02	37.2 ± 0.18	21.2 ± 0.15	28.9 ± 0.10
C22:4n-6	0.5 ± 0.00	ND	ND	0.7 ± 0.01	ND
C24:1n-9	1.0 ± 0.03	ND	0.9 ± 0.01	ND	ND
C22:5n-3	0.9 ± 0.25	1.3 ± 0.02	5.2 ± 0.12	ND	ND
C22:6n-3	12.5 ± 0.59	13.7 ± 0.07	27.5 ± 0.16	10.0 ± 0.04	4.9 ± 0.07
∑ SFA	29.8 ± 0.24	14.5 ± 0.03	5.3 ± 0.04	32.0 ± 0.38	7.5 ± 0.05
∑ MUFA	20.3 ± 0.15	53.3 ± 0.07	17.3 ± 0.05	31.0 ± 0.24	12.7 ± 0.01
∑ PUFA	49.9 ± 0.39	32.1 ± 0.10	77.4 ± 0.04	37.0 ± 0.14	79.8 ± 0.06
∑ omega-3	47.4 ± 0.39	29.5 ± 0.09	73.2 ± 0.07	33.5 ± 0.13	37.8 ± 0.08

* The fatty acids C18:1n-9 and C16:4n-1 have similar retention times. Previously published GC-MS analysis on this strain showed that C16:4n-1 is one of the main fatty acids in *P. glacialis* oil, while C18:1n-9 was present in very low amounts [12].

3.2 Digestibility

Prior to digestion, the dominating lipid classes in CO were wax esters (84.7%) and free fatty acids (10.2%) while fatty alcohols represented only 2.5 % (Table 2). The amount of FFA in CO increased slowly but gradually from 10.2 % at the beginning of the experiment to 14.2% after

180 minutes. The amount of WE appeared to decrease over time, however, the differences were not statistically significant at the threshold of $p<0.01$. No change in the concentration of fatty alcohols was observed. In the neutral fraction of CO (WE, TAG, DAG, and FAlc), the changes in MUFA, PUFA, and SFA were not statistically significant (Figure 1). In the free fatty acid fraction, SFA increased from 25.1 to 27.2 % and PUFA decreased from 65.5 to 59.2 % during the digestion. No significant changes occurred in MUFA in this fraction.

Table 2 Lipid class composition (weight percent) of Calanus® oil (time 0) and changes during in vitro digestion (n=3). Wax esters (WE), triacylglycerol (TAG), fatty alcohols (FAlc), diacylglycerol (DAG), free fatty acids (FFA).

Time (min)	0	30	60	180
WE	84.7 ± 0.76^{ABC}	85.4 ± 1.44^{B}	83.6 ± 1.47^{ABC}	81.7 ± 1.48^{C}
TAG	1.4 ± 0.15^{A}	1.3 ± 0.15^{A}	0.9 ± 0.60^{A}	0.9 ± 0.08^{A}
FAlc	2.5 ± 1.16^{A}	2.0 ± 0.36^{A}	2.0 ± 0.16^{A}	1.9 ± 0.16^{A}
DAG	1.2 ± 0.09^{A}	1.2 ± 0.23^{A}	1.4 ± 0.19^{A}	1.4 ± 0.02^{A}
FFA	10.2 ± 2.16^{A}	10.1 ± 0.87^{A}	12.1 ± 1.35^{AB}	14.2 ± 1.47^{B}

^{A,B,C}(Results in the same row sharing superscripted letters are not significantly different ($P \geq 0.01$))

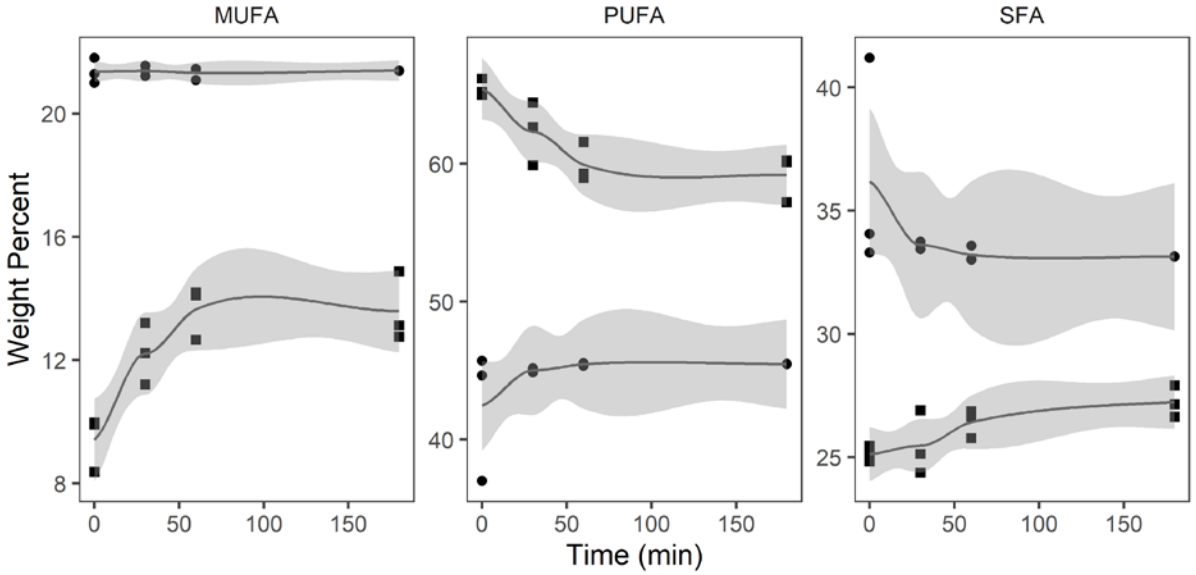

Figure 1 Monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), and saturated fatty acids (SFA) (weight percent) in free fatty acid (square) and neutral lipid (circle) fraction of Calanus® oil (n=3) during in vitro digestion.

CLO (Table 3) initially contained 99.7 % TAG, which decreased steadily to 64.4 % during the 180 min *in vitro* digestion. This was accompanied by an increase of FFA, and MAG from 0 to 28.2 and 4.5 %, respectively while DAG increased from 0.3 to 2.9 %. In the neutral fraction of CLO (TAG, DAG, and MAG), SFA was stable at approximately 15 % during the digestion (Figure 2). MUFA decreased from 51.8 to 49.1 % and PUFA increased from 32.5 to 35.7 % in the same fraction. In the free fatty acid fraction, SFA increased from 16.8 to 20.3 % during the first 30 minutes, and then decreased to 18.7 % at 180 minutes. During the digestion, PUFA decreased from 32.1 to 23.1 % and MUFA increased from 51.0 to 58.2% in the free fatty acid fraction.

Table 3 Lipid class composition (weight percent) of cod-liver oil (time 0) and changes during in vitro digestion (n=3). Triacylglycerol (TAG), diacylglycerol (DAG), free fatty acids (FFA), monoacylglycerol (MAG).

Time (min)	0	30	60	180
TAG	99.7 ± 0.07^{A}	90.1 ± 0.46^{B}	79.1 ± 2.24^{C}	64.4 ± 4.14^{D}
DAG	0.3 ± 0.07^{A}	0.7 ± 0.06^{A}	1.8 ± 0.31^{B}	2.9 ± 0.04^{C}
FFA	0.0 ± 0.00^{A}	8.7 ± 0.91^{B}	16.0 ± 1.67^{C}	28.2 ± 3.89^{D}
MAG	0.0 ± 0.00^{A}	0.5 ± 0.43^{A}	3.1 ± 0.31^{B}	4.5 ± 0.44^{C}

^{A,B,C,D}(Results in the same row sharing superscripted letters are not significantly different ($P \geq 0.01$))

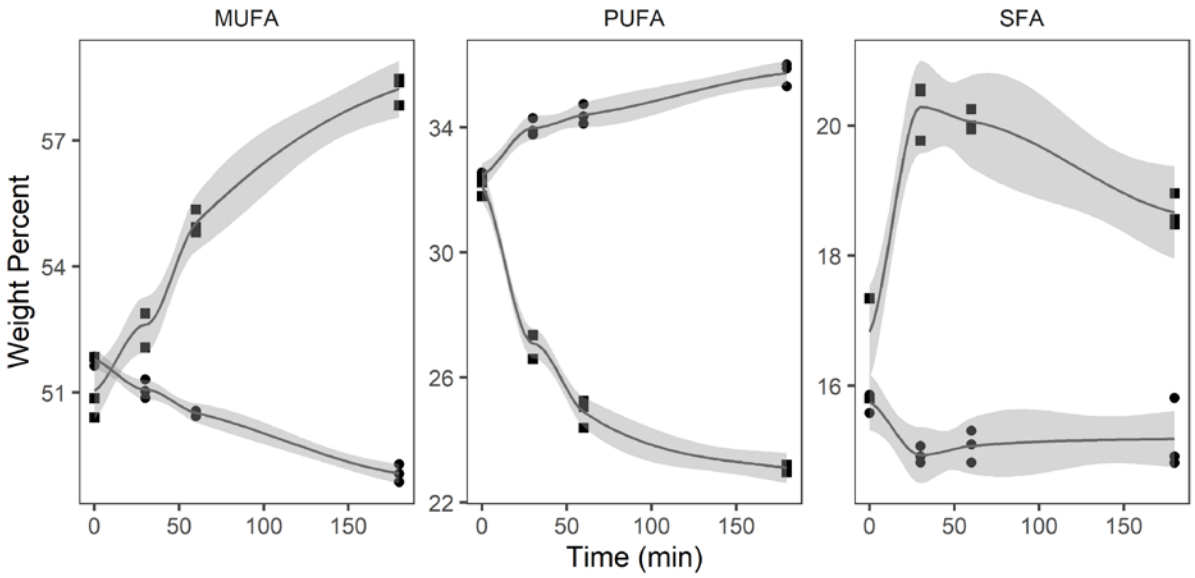

Figure 2 Monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), and saturated fatty acids (SFA) (weight percent) in free fatty acid (square) and neutral lipid (circle) fraction of cod-liver oil (n=3) during in vitro digestion.

In EEC, fatty acid ethyl esters (EE) were the most abundant lipid class (87.3 %) and a small, but not statistically significant, reduction appeared to occur during the first 30 min of the digestion (Table 4). The amount of FFA increased from 0 to 4.6% while small non-significant changes occurred in TAG and DAG during the digestion period. In the free fatty acid fraction, PUFA decreased from 56.1 to 44.9% and SFA increased from 18.5 to 29.4 during the digestion period, however, the standard deviations were large (Figure 3). In the neutral fraction (EE, TAG, and DAG), PUFA decreased from 78.3 to 75.1% and SFA increased from 6.0 to 7.6%.

Table 4 Lipid class composition (weight percent) of the ethyl ester concentrate (time 0) and changes during in vitro digestion (n=3). Ethyl esters (EE), triacylglycerol (TAG), diacylglycerol (DAG), free fatty acids (FFA)

Time (min)	0	30	60	180
EE	87.3 ± 1.5^{A}	83.8 ± 2.4^{A}	86.9 ± 0.6^{A}	84.1 ± 1.2^{A}
TAG	11.9 ± 1.5^{AB}	11.5 ± 1.6^{A}	8.4 ± 0.5^{B}	9.5 ± 0.6^{AB}
DAG	0.8 ± 0.0^{A}	1.4 ± 0.1^{B}	1.5 ± 0.1^{B}	1.8 ± 0.0^{B}
FFA	0.0 ± 0.0^{A}	3.3 ± 0.7^{BC}	3.2 ± 0.2^{B}	4.6 ± 0.6^{BC}

^{A,B,C}(Results in the same row sharing superscripted letters are not significantly different ($P \geq 0.01$))

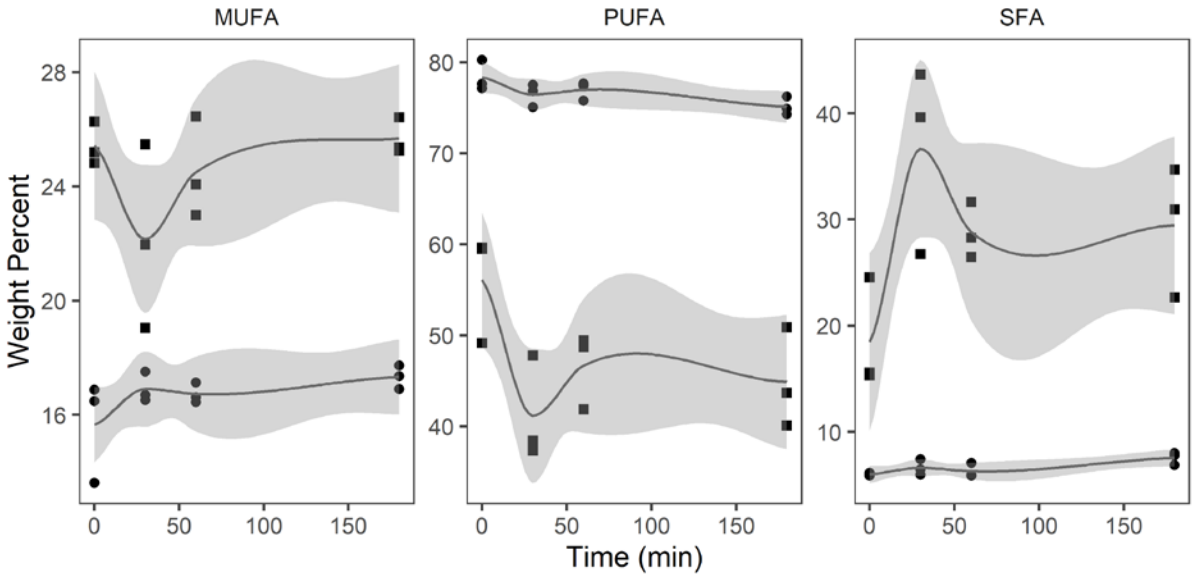

Figure 3 Monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), and saturated fatty acids (SFA) (weight percent) in free fatty acid (square) and neutral lipid (circle) fraction of the ethyl ester concentrate (n=3) during in vitro digestion.

Initially, krill oil contained primarily phosphatidylcholine (PC, 43.3%) and TAG (42.8%), however, free fatty acids (4.2%) were also present (Table 5). The FFA content increased from 4.2 to 18.9% during digestion with the fastest increase between 0 and 30 minutes. The conversion of TAG was also fastest between 0 and 30 minutes, and the total amount decreased from 42.8 to 35.4% during digestion. The amount of phosphatidylcholine (PC) decreased from 43.3 to 33.8% between 0 and 60 minutes and remained stable thereafter. During digestion, DAG decreased from 9.8 to 4.7%. MAG initially increased from 0 to 9.8% between 0 and 30 minutes, before decreasing to 6.8% at 180 minutes. In the neutral fraction (TAG, DAG, and MAG) of krill oil (Figure 4), MUFA and SFA did not show statistically significant changes with a content of approximately 40 and 38%, respectively. In the same fraction, PUFA increased from 19.5 to 21.8% during digestion. In the polar fraction (PC), the amount of MUFA, PUFA and SFA remained relatively unchanged during the digestion. In the

free fatty acid fraction, PUFA decreased from 58.4 to 51.7%. Additionally, the amount of SFA increased from 22.3 to 29.7% in this fraction.

Table 5 Lipid class composition (weight percent) of krill oil (time 0) and changes during in vitro digestion (n=3). Triacylglycerol (TAG), diacylglycerol (DAG), free fatty acids (FFA), monoacylglycerol (MAG), phosphatidylcholine (PC).

Time (min)	0	30	60	180
TAG	42.8 ± 1.06^{A}	29.9 ± 1.99^{B}	31.7 ± 4.86^{B}	35.4 ± 3.05^{AB}
DAG	9.8 ± 0.21^{A}	6.0 ± 1.44^{BC}	7.3 ± 0.20^{B}	4.7 ± 0.28^{C}
FFA	4.2 ± 0.07^{A}	14.7 ± 3.10^{B}	17.9 ± 2.25^{B}	18.9 ± 0.77^{B}
MAG	0.0 ± 0.00^{A}	9.8 ± 2.20^{B}	9.3 ± 1.47^{B}	6.8 ± 0.43^{B}
PC	43.3 ± 0.79^{A}	39.7 ± 4.61^{AB}	33.8 ± 1.07^{B}	34.2 ± 2.11^{B}

^{A,B,C}(Results in the same row sharing superscripted letters are not significantly different ($P \geq 0.01$))

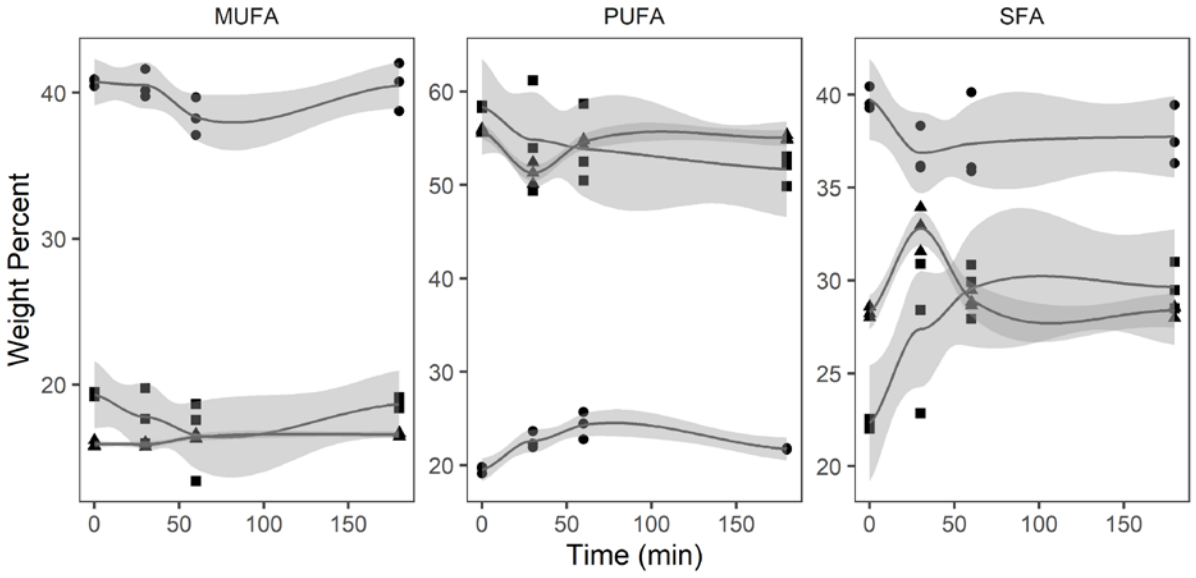

Figure 4 Monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), and saturated fatty acids (SFA) (weight percent) in free fatty acid (square), neutral lipid (circle) and polar lipid (triangle) fraction of krill oil (n=3) during in vitro digestion.

The oil extracted from *P. glacialis* displayed the most diverse lipid class composition (Table 6), differing from the other oils by containing monogalactosyldiacylglycerol (MGDG, 36.3%) and phosphatidylglycerol (PG, 23.5%). Digalactosyldiacylglycerol (DGDG, 4.1%) and free fatty acids (4.8%) were also present before digestion. PGO showed an increase in FFA

289 from 4.8 to 12.2% between 0 and 30 minutes, and from 14.0 to 21.8% between 60 and 180
290 minutes. Phosphatidylglycerol (PG) and TAG decreased from 23.5 and 4.9% to 15.3% and
291 1.3%, respectively, while DAG was reduced from 14.2 to 11.1%. MGDG was not affected
292 during the initial 60 minutes of digestion, but decreased from 36.6 to 32.0% between 60 and
293 180 minutes. No decrease of PC was observed during digestion of PGO. The relative amount
294 of MUFA decreased in the neutral (TAG, DAG, and MAG) and polar lipid (MGDG, DGDG,
295 PG, and PC) fraction over time, from 15.6 to 13.7% and 9.2 to 5.8%, respectively (Figure 5).
296 In the free fatty acid fraction, MUFA increased from 14.6 to 16.3%. PUFA remained unchanged
297 in the neutral fraction throughout the digestion period, whereas it increased from 84.8 to 89.2%
298 in the polar fraction and decreased from 73.8 to 66.6% in the free fatty acid fraction. SFA
299 increased from 11.6 to 17.1% in the free fatty acid fraction, remained unchanged in the polar
300 lipid fraction and increased from 10.3 to 11.0% in the neutral fraction.

301

302 Table 6 Lipid class composition (weight percent) of Porosira glacialis oil (time 0) and changes
303 during in vitro digestion (n=3). Triacylglycerol (TAG), diacylglycerol (DAG), free fatty acids
304 (FFA), monoacylglycerol (MAG), monogalactosyldiacylglycerol (MGDG),
305 digalactosyldiacylglycerol (DGDG), phosphatidylglycerol (PG), phosphatidylcholine (PC).

Time (min)	0	30	60	180
TAG	4.9 ± 0.64^{A}	3.4 ± 0.10^{B}	3.2 ± 0.54^{B}	1.3 ± 0.42^{C}
DAG	14.2 ± 0.17^{A}	9.7 ± 0.21^{B}	11.4 ± 1.53^{AB}	11.1 ± 2.22^{AB}
FFA	4.8 ± 0.48^{A}	12.2 ± 0.61^{B}	14.0 ± 2.18^{B}	21.8 ± 2.37^{C}
MAG	3.8 ± 0.21^{A}	2.9 ± 0.07^{B}	3.1 ± 0.24^{AB}	3.6 ± 0.47^{AB}
MGDG	36.3 ± 0.38^{A}	38.1 ± 0.42^{A}	36.6 ± 1.51^{A}	32.0 ± 1.62^{B}
DGDG	4.1 ± 0.12^{A}	5.8 ± 0.43^{B}	6.2 ± 0.53^{B}	5.7 ± 0.74^{B}
PG	23.5 ± 0.55^{A}	18.7 ± 0.13^{B}	17.3 ± 0.22^{B}	15.3 ± 1.09^{C}
PC	8.5 ± 0.50^{A}	9.2 ± 0.23^{A}	8.3 ± 0.26^{A}	9.4 ± 1.17^{A}

^{A,B,C}(Results in the same row sharing superscripted letters are not significantly different ($P \geq 0.01$))

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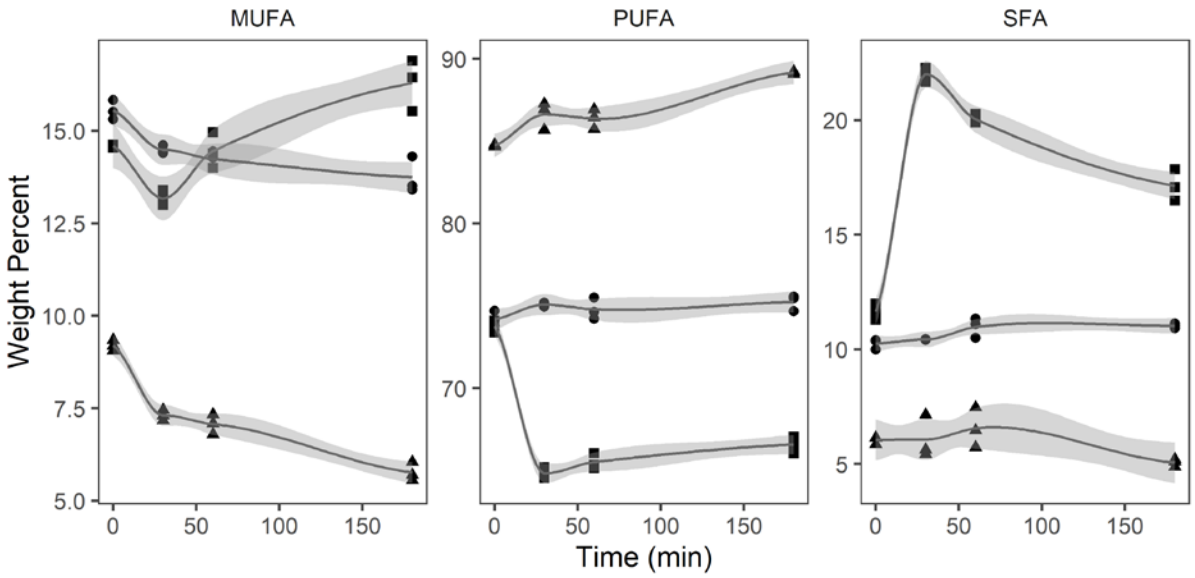

Figure 5 Monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), and saturated fatty acids (SFA) (weight percent) in free fatty acid (square), neutral lipid (circle) and polar lipid (triangle) fraction of oil from Porosira glacialis (n=3) during in vitro digestion.

There was a tendency towards a decrease for omega-3 fatty acids in the free fatty acid fraction of all samples: From 63.0 to 51.3% in CO, 27.5 to 15.2% in CLO, 43.9 to 29.7% in EEC, 54.0 to 46.6% in KO, and from 43.7 to 26.3% in PGO (Figure 6). The sum of omega-3 fatty acids in the neutral fraction of CO, EEC, and PGO was stable over time at approximately 41, 70, and 39%, respectively. In contrast, the relative amount of omega-3 fatty acids in the neutral fraction of CLO increased from 27.6 to 30.8% and from 15.0 to 16.0% in KO. The content of omega-3 fatty acids in the polar fraction in PGO decreased from 31.1 to 24.9%, whereas it remained unchanged in krill oil at approximately 50%.

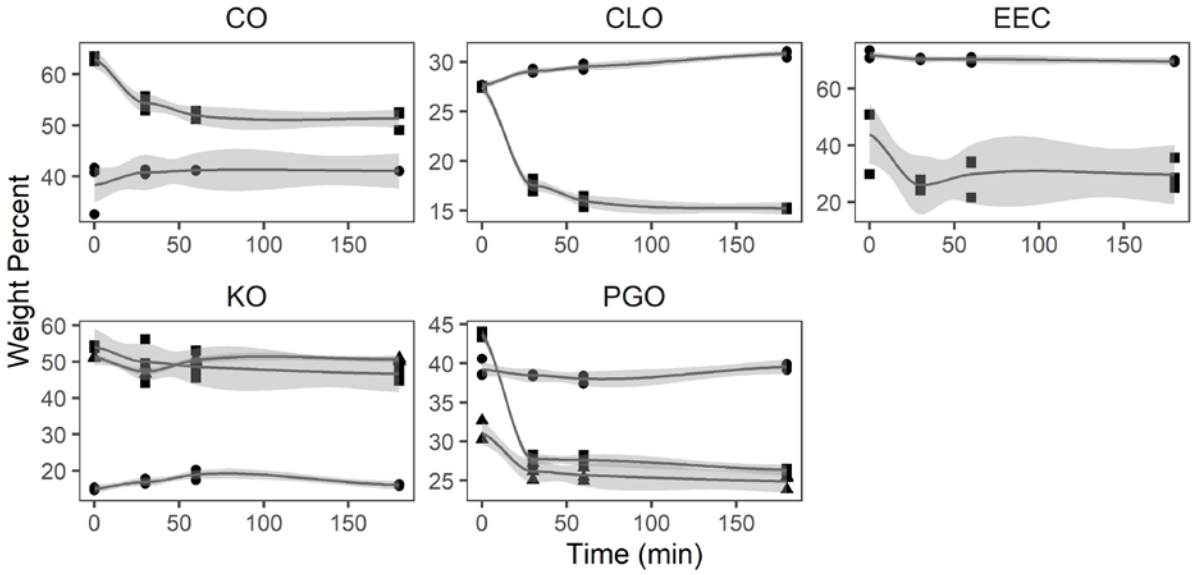

Figure 6 Amount of omega-3 fatty acids (weight percent) in free fatty acid (square), neutral lipid (circle) and polar lipid (triangle) fraction of samples from Calanus® oil (CO), cod-liver oil (CLO), ethyl ester concentrate (EEC), krill oil (KO) and oil from Porosira glacialis (PGO) (n=3) during in vitro digestion.

4 Discussion

The oils differed greatly in their fatty acid composition with varying amounts of SFA, MUFA and omega-3 fatty acids and were in general agreement with previously published results. However, except for the EEC, some variations in the content of the specific fatty acids will occur due to biological conditions and feeding habits of harvested organisms (8, 9) and cultivating conditions of the microalgae (28).

The lipid class composition of the different oils explored in this study ranged from the simple cod-liver oil, the non-polar lipid Calanus® oil and ethyl ester concentrate to the more complex krill oil and *P. glacialis* oil which contained substantial amount of polar lipids in addition to the neutral lipids. The cod liver oil and the ethyl ester concentrate had no detectable free fatty acids or other polar lipids because these products have been refined during the production. The relatively high content of free fatty acids in the unrefined crustacean oils have been attributed to high lipolytic activities degrading mainly phospholipids post mortem in these species (35,

36). This explains the lack of detectable PL in the Calanus® oil. The low content of FAlc compared to FFA initially in the CO also suggests that it is primarily PL that had been degraded in *C. finmarchicus*. Microalgae have also been reported to have high lipolytic activity post-harvest (37) which may explain the elevated FFA level in PGO prior to digestion. The presence of FFA in nutraceutical products has been reported to be an advantage since they are independent of digestive enzymes and fat for absorption (e.g. Lapointe, Harvey (38)). The main reason for this difference in processing of fish oil and oils from lower trophic level organisms is the low accumulation of organic pollutants in lower trophic level organisms.

As most lipid classes are too large to diffuse across the plasma membranes of the intestinal epithelial cells, they must first be broken down into FFA and e.g. MAG, lyso-phosphlipids, lyso-galactolipids or FAlc. Cod-liver oil, oil from *P. glacialis*, and krill oil were more easily digested than Calanus® oil and ethyl ester concentrate in this study. The formation of FFA was highest in cod-liver oil with an increase in FFA of 28.2 percent point during the digestion period. For the *P. glacialis* oil and the krill oil the change in FFA were 17 and 14.7 percent point, respectively. The increase of FFA in Calanus® oil and the ethyl ester concentrate was low, indicating that the lipid classes present are hydrolyzed more slowly. Cook, Larsen (19) showed that the *in vivo* bioavailability of Calanus® oil was similar to that of ethyl ester concentrates, and previous studies have found that the *in vitro* digestion of EEC is slow (25, 39). However, Krokan, Bjerve (40), observed that even though the *in vitro* digestion of EEC was slow compared to TAG, the *in vivo* bioavailability was the same. In humans, the median transit time of food through the small intestine is approximately four hours (41), i.e. one more than the digestion period in this study. As most of the hydrolysis of EE occurred within the first thirty minutes of the digestion, adding one more hour to the *in vitro* digestion is unlikely to have affected the total digestion of EEC. In contrast, the digestion of Calanus® oil began between thirty and sixty minutes, suggesting that longer time is necessary for a more complete

digestion of this oil. However, when evaluating the results from *in vitro* digestion of lipids product inhibition will probably occur resulting in reduced release of FFA.

TAG and PC which are present in approximately equal amounts in the krill oil, were hydrolyzed at a similar rate during the *in vitro* digestion. The phospholipids PC in krill oil and PG in *P. glacialis* oil were both digested in this study. However, both phospholipids were present in *P. glacialis*, but we observed hydrolytic activity only towards PG. Roussel, Yang (42) have demonstrated that rat pancreatic lipase related protein 2 preferred PG to PC. Our results also indicate that PG is hydrolyzed more readily than PC. The galactolipids (MGDG and DGDG) which constitute about 40 % of the lipid classes in the oil from *P. glacialis*, are also degraded by the same enzyme (43). We found that only the amount of MGDG was reduced during the digestion and the onset of the hydrolysis was later than that of phospholipids and TAG. A recent study (44) found that porcine pancreatin had lower phospholipase and galactolipase activities than human duodenal juice. Hence, the bioavailability for humans of free fatty acids from krill oil and oil from *P. glacialis* may be underestimated in this study.

MUFA and SFA were hydrolyzed more efficiently than PUFA in all the samples. In krill oil and the ethyl ester concentrate the relative amount of MUFA did not increase in the free fatty acid fraction during digestion. In the other oils, MUFA did increase in this fraction. SFA, on the other hand, increased in the free fatty acid fraction of all the oils during digestion. For all the oils in this study PUFA decreased in the free fatty acid fraction during *in vitro* digestion. This does not mean reduced absorption of these fatty acids since the co-products of the lipolysis, e.g. MAG, lyso-phosphlipids and lyso-galactolipids which will contain correspondingly higher concentrations of LC-PUFA, are absorbed by the intestinal cells. Our data on *in vitro* degradation of the lipids extracted from *P. glacialis* are in agreement with previous published results on other marine oils, which has shown that lipolysis are decreasing with increasing carbon chain length and number of double bonds of the fatty acids (45, 46).

5 Conclusions

In conclusion, the results showed that the lipids present in the oil extracted from the microalgae *Porosira glacialis* were hydrolyzed at a slightly higher rate than krill oil, but at a lower rate than the TAG present in cod liver oil. The slowest hydrolysis occurred with ethyl ester concentrate and in the oil from *Calanus finmarchicus*. As with the commercial oils, PUFA were released more slowly than MUFA and SFA from the lipid classes in the oil from *P. glacialis.*

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Conflict of interests

We declare no conflict of interests. There are no conflicts, informed consent, human or animal rights applicable.

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Paper III

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

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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 phosopholipids 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

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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

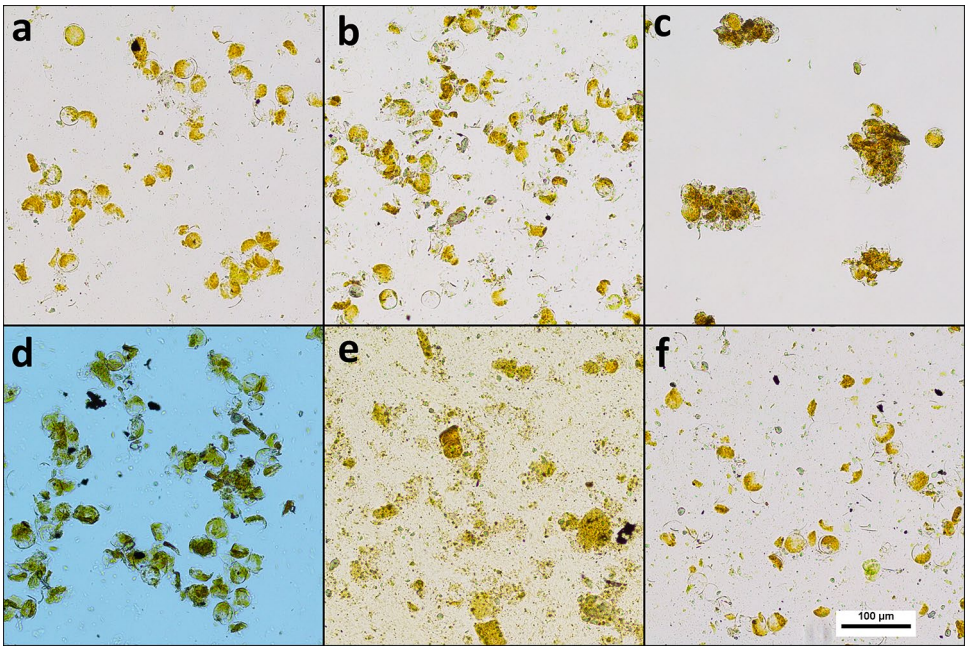

Figure 1. The effect of cell disruption on thawed biomass of *Porosira glacialis*. (**a**) Control, (**b**) manual grinding using a PTFE pestle, (**c**) microwave, (**d**) lyophilization, (**e**) sonication, (**f**) Ultrathurax. All images were captured at 100 × magnification.

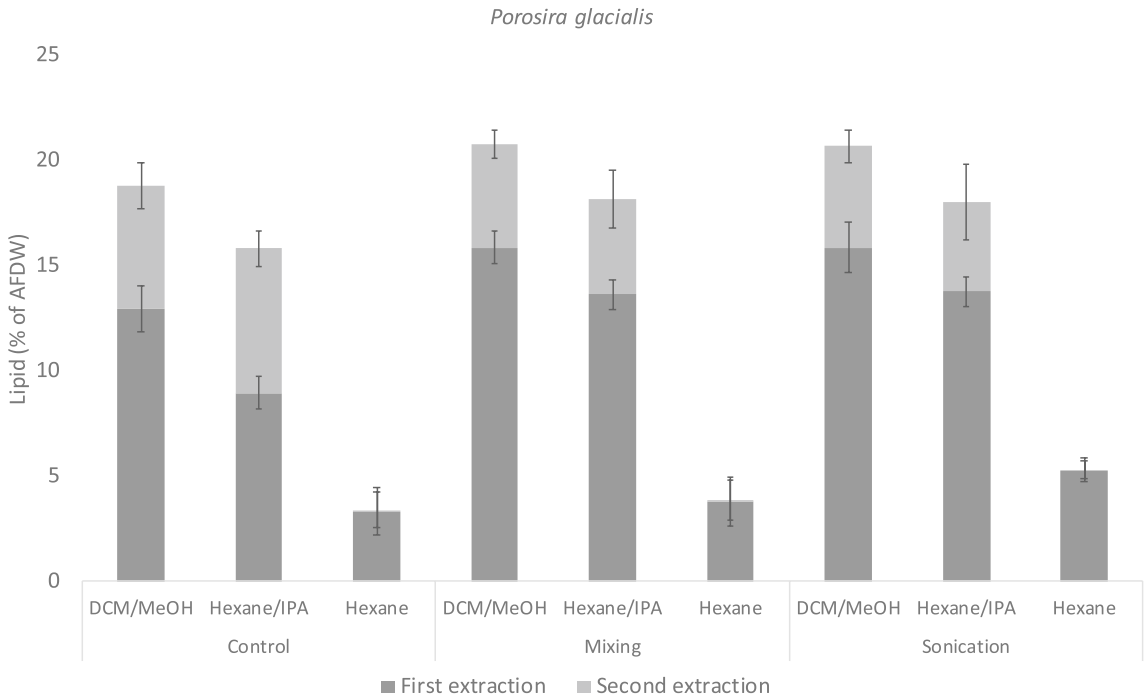

Figure 2. Lipid yields as percent of AFDW (Ash-free dry weight) from *Porosira glacialis* using three solvent systems; dichloromethane/methanol (2:1 v/v); DCM/MeOH), Hexane/isopropanol (2:1 v/v; Hexane/IPA) and hexane; and three cell disruption treatments; no treatment (control), shaking at 1000 RPM 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).

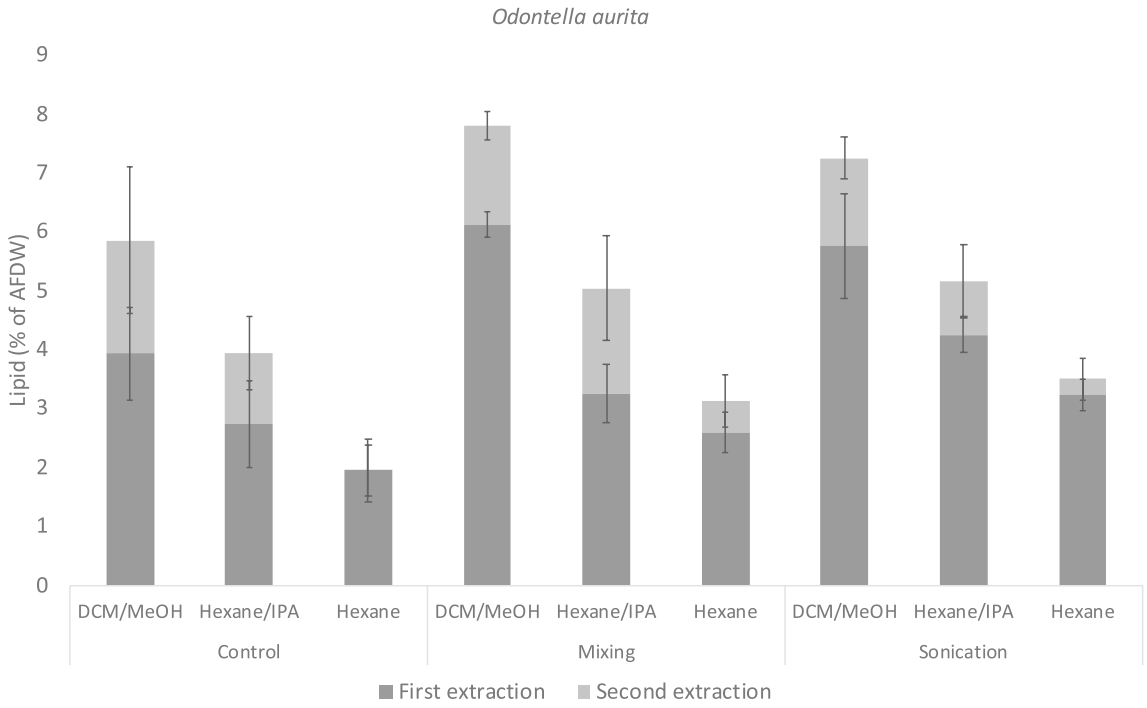

Figure 3. Lipid yields as percent of AFDW (Ash-free dry weight) from *Odontella aurita* using three solvent systems; dichloromethane/methanol (2:1 v/v) (DCM/MeOH), hexane/isopropanol (2:1 v/v) (Hexane/IPA) and hexane; and three cell disruption treatments; no treatment (control), mixing at 1000 RPM for 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.

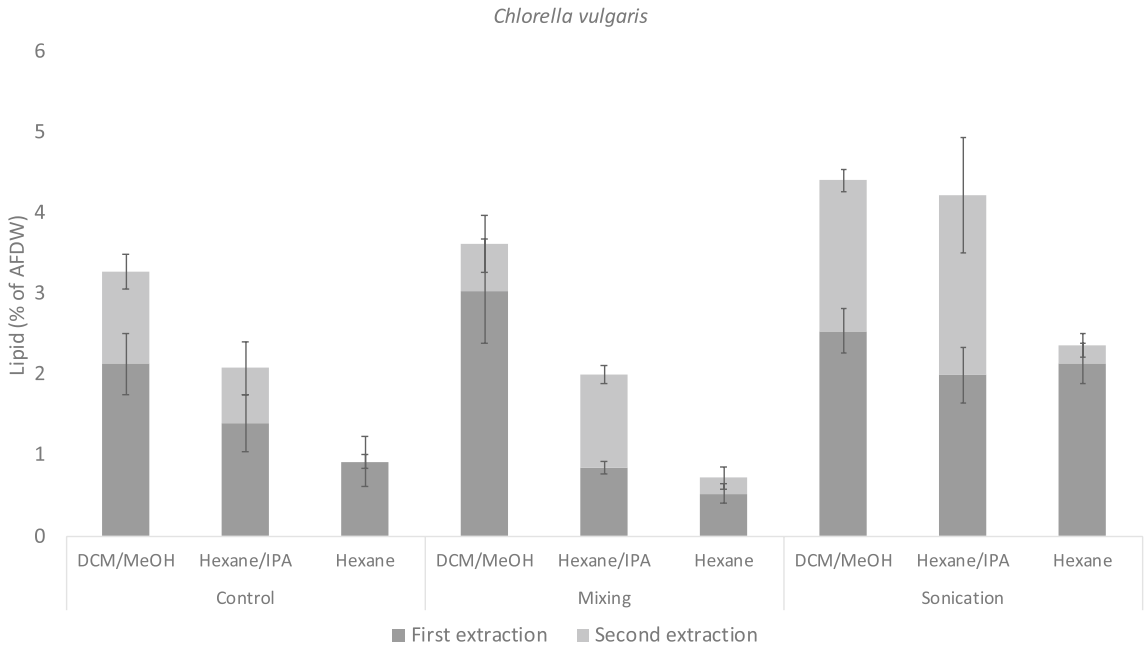

Figure 4. Lipid yields as percent of AFDW (Ash-free dry weight) from *Chlorella vulgaris* using three solvent systems; dichloromethane/methanol (2:1 v/v) (DCM/MeOH), Hexane/isopropanol (2:1 v/v) (Hexane/IPA) and hexane; and three cell disruption treatments; no treatment (control), mixing at 1000 RPM 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.

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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.

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Competing interests

The authors declare no competing interests.

Additional information

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