High-value fatty acids from microalgae

Bioprospecting and outdoor cultivation at northern latitudes

Pia Steinrücken

Thesis for the Degree of Philosophiae Doctor (PhD)
University of Bergen, Norway
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Abstract

Achieving a sustainable and cost-efficient production is imperative to establish microalgae as a new feedstock for aquaculture, where they can potentially replace the use of fish oil from wild catch as a source for the omega-3 polyunsaturated fatty acids (n-3 PUFAs) eicosapentaenoic (EPA) and docosahexaenoic acids (DHA). Both fatty acids are essential components for higher eukaryotes and are considered highly important for human health. Many marine microalgae naturally produce EPA and DHA and fit greatly as a new, natural and sustainable feedstock. They can be cultivated in large quantities in seawater, on non-arable land and using renewable resources such as sunlight, CO_2 and waste streams. However, the high production costs that are associated with large-scale microalgal cultivation and processing need to be reduced. Prospecting for new, robust and fast-growing strains with high n-3 PUFA content, optimizing microalgal strains and cultivation conditions, and improving large-scale productions are essential elements for progressing towards a cost-efficient commercial production, and for an improved development of new and more sustainable feed types for aquaculture.

This study focuses on the potential of microalgae from northern latitudes for production of these high value fatty acids. The aim was to find new microalgal strains from different North Atlantic habitats that meet the industrial demand in terms of high EPA and DHA content and growth rates, and to evaluate promising strains in laboratory experiments as well as under outdoor pilot-scale conditions. Microalgae were isolated from Arctic and Fjord-waters and 149 clonal cultures were established. In a first screening round, 20 strains were investigated for their growth rates and fatty acid content under laboratory conditions. Three strains (two strains of *Phaeodactylum tricornutum*, isolated from the fjord, and one strain *Attheya septentrionalis* isolated from the Artic) possessed both high growth rates (≥ 0.7 d^{-1}) and EPA content ($\geq 3\%$ of dry weight [DW]) (**Paper I**). The Arctic diatom *A. septentrionalis* was further investigated in a factorial-design experiment for the individual and interactive effects of irradiance, salinity and growth phase on the EPA content. Growth phase and salinity were identified to significantly affect EPA content in this diatom and the

highest EPA content (7.1% DW) was observed after Day 5 in the stationary phase and at low salinity of 22 (**Paper II**). Finally, one Spanish (Fito) and two local (M28 and B58) isolates of the diatom *P. tricornutum* were grown for six months in 35 L GWP-III flat-panel outdoor reactors in Bergen, western Norway, to evaluate strain specific productivities under northern climate conditions. Biomass productivities of all three strains were lower compared to results from southern latitudes, most probably due to the lower irradiances. Although the three strains possessed similar biomass productivities (average volumetric productivities of 0.20, 0.18, and 0.21 g L^{-1} d^{-1}, respectively), different EPA productivities (average volumetric productivities of 9.8, 5.7 and 6.9 mg L^{-1} d^{-1}, respectively) were observed. The Spanish strain possessed the highest EPA content with an average of 4.4% DW but only under outdoor, and not under laboratory conditions (**Paper III**). EPA productivities were strongly dependent on both, the strain chosen and the prevailing cultivation condition. The results highlight the great potential of North Atlantic diatoms for the production of high value fatty acids from microalgae.

List of publications

Paper I P. Steinrücken, S.R. Erga, S.A. Mjøs, H. Kleivdal, S.K. Prestegard (2017). Bioprospecting North Atlantic microalgae with fast growth and high polyunsaturated fatty acid (PUFA) content for microalgae-based technologies. *Algal Research* 26: 392–401.

Paper II P. Steinrücken, S.A. Mjøs, S.K. Prestegard, S.R Erga (2018). Enhancing EPA content in an Arctic diatom: A factorial-design study to evaluate interactive effects of growth factors. *Manuscript under review in Frontiers in Plant Science*

Paper III P. Steinrücken, S.K. Prestegard, J.H. De Vree, J. E. Storesund, B. Pree, S.A. Mjøs, S.R. Erga (2018). Comparing EPA production and fatty acid composition of three *Phaeodactylum tricornutum* strains under western Norwegian climate conditions. *Algal Research* 30: 11-22.

1. Introduction

Omega-3 polyunsaturated fatty acids (n-3 PUFAs) are essential components for higher eukaryotes with eicosapentaenoic (EPA, 20:5, n-3) and docosahexaenoic acids (DHA, 22:6, n-3) being nutritionally the most important (Adarme-Vega et al., 2012). Their regular intake has been recognized to reduce cardiovascular, nervous system and inflammatory diseases (Patil et al., 2005; Winwood, 2013). Human nutritional guidelines therefore recommend an increased intake of EPA and DHA (WHO, 2018; Williams and Burdge, 2006). The current major source for these PUFAs is fish oil from marine wild fish. The fish obtain and accumulate these PUFAs predominantly via the marine food chain from lower trophic levels; EPA- and DHA-synthesizing microalgae (Rubio-Rodríguez et al., 2010; Spolaore et al., 2006). As EPA and DHA are also essential for farmed fish, fish oil is an important additive in aquaculture feed. Almost 70% of the globally available fish oil is being used for aquaculture feed production (Chauton et al., 2015), and with the increasing public awareness about their important role in human health, other markets for direct human consumption of EPA and DHA-enriched products have emerged rapidly (Chauton et al., 2015).

Aquaculture might play an essential role in providing a healthy and sustainable diet for the growing world population, which is expected to increase towards 10 billion people in 2050 (UN DESA, 2017). In contrast to land-based agriculture, that already occupies 11% of the global land surface (FAO, 2003), the growing aquaculture sector has the capacity for further expansion. However, this expansion needs to be sustainable and demands new sources for aquaculture feed ingredients. Fish oil as supplement has raised economic, ethical and environmental concerns and has already reached maximum global production (Winwood, 2013). Besides, fatty fish have been associated with the risk of contamination with environmental pollutants such as mercury, polychlorinated biphenyls or organochlorine pesticides (Hong et al., 2015). As the annually available fish oil volumes are limited, and global aquaculture is continuing to grow, fish oil is increasingly being replaced by vegetable oils in modern aqua-feeds, which also have lower production costs (Olsen, 2011). However, terrestrial plants are absent of n-3 PUFAs above C_{18}, due to a lack of the required

elongation enzymes (Khozin-Goldberg et al., 2016). Furthermore, their production uses valuable and increasingly limited farmland and fresh water (Duarte et al., 2009). Thus, plant oils cannot currently be considered as a replacement for fish oil (Mozaffarian and Wu, 2011), and alternative supplies of EPA and DHA are required in order to meet the demands for the expanding markets (Patil et al., 2005).

Microalgae can be the solution when searching for an alternative, natural, and sustainable feedstock for these important n-3 fatty acids. Many marine species naturally produce EPA and DHA (Patil et al., 2005), can be cultivated on a large-scale in seawater and on non-arable land, and may therefore be grown in regions that are unsuitable for agriculture (Draaisma et al., 2013). Microalgae are also rich in proteins, carbohydrates, and other valuable compounds such as carotenoids, vitamins and minerals. Today, commercial DHA-production by fermentation of heterotrophic eukaryotes, such as the thraustochytrids *Thraustochytrium* spp. and *Schizochytrium* spp. is well established on an industrial-scale (Hamilton et al., 2016). Higher biomass production can be achieved with heterotrophic cultures compared to photoautotrophic cultures, as light requirements and self-shading effects are eliminated (Morales-Sánchez et al., 2016). However, phototrophic microalgae offer potential sustainability benefits. They can be cultivated on renewable resources such as sunlight, CO_2, and waste streams; while the fermentation based production of heterotrophic cultures produces CO_2 and requires an organic carbon source (mainly glucose) (Morales-Sánchez et al., 2013) which is produced from higher plants, thus competing with food production.

Although there has been intensive research in this field, the costs associated with phototrophic microalgal large-scale cultivation and processing for fatty acids are still greater than costs for fish and vegetable oil production (Olsen, 2011). Improvements at the different parts of the production chain are essential in order to reduce production costs and to achieve an economically feasible and sustainable commercial production. This includes searching for more effective production strains, and further optimizing microalgal performance at lab-, pilot- and large-scale production facilities.

2. Background

2.1 Microalgae—from natural habitats to laboratoy cultures

Photoautotrophic microalgae represent an extremely diverse group of microorganisms, comprising several different eukaryotic phyla and the prokaryotic phylum cyanobacteria, with over 40`000 species described (Hu et al., 2008). Microalgae are ubiquitous and colonize all aquatic habitats, such as marine-, brackish- and freshwaters, either pelagic as phytoplankton, benthic, epiphytic or symbiotic. They constitute the base of the aquatic food chain and play a tremendous role in ocean primary productivity by contributing to more than 40% of global photosynthesis (Andersen, 1992). Some specialized microalgae also colonize extreme habitats like high salinity, low temperature, or arid environments. Others are capable of growing at low pH and high temperatures, or as biofilms on tree barks, rocks, soils, and other surface environments (Hallmann, 2006).

Several microalgal species have been isolated from their natural habitats, to be cultivated in laboratories for research on their biochemistry and physiology. In a typical batch culture experiment, microalgae are grown at a defined temperature, pH and irradiance, and in nutrient- and CO_2-enriched media, that support growth for a limited time until one of the nutrients is depleted. In a well-mixed culture, growth usually follows three phases (Fig. 1). The lag phase (1) is an initial period of minimal growth where cells adjust to the new conditions after transfer. After this follows the exponential phase (2), characterized by an exponential increase of the biomass while nutrients, CO_2 and irradiance are supplied. The growth rate of individual microalgae during exponential phase is strongly dependent on the temperature, irradiance, pH and salinity. When a nutrient or CO_2 becomes depleted, growth declines and cultures enter the stationary phase (3). However, when the nutrient concentrations in the medium are high and irradiance low, culture density might increase to a level where mutual shading of the cells leads to reduced growth due to light limitation before nutrient depletion occurs (MacIntyre and Cullen, 2005). When microalgal growth is prevented by nutrient depletion during stationary phase, but sufficient light energy

and CO_2 is provided, many microalgae continue to assimilate CO_2, which is then channeled into the synthesis of storage lipids or polysaccharides, which accumulate in the cells (Ratledge 2004).

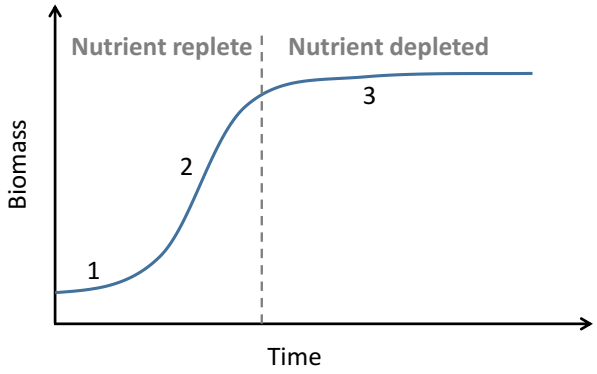

Fig. 1. Simplified growth curve for a light-sufficient microalgal culture with three growth phases. 1: lag phase, 2: exponential phase, 3: stationary phase.

2.2 Potential for industrial applications

Numerous microalgal species are rich in valuable compounds such as proteins, carotenoids, vitamins and n-3 PUFAs, and already before the age of algal biotechnology, cyanobacteria such as *Nostoc* and *Spirulina* have been used as a nutrient-rich food source in different countries in Asia and Africa and in Mexico (Hallmann, 2006). Furthermore, in fish and shellfish aquaculture, several microalgal species, especially diatoms, have been used as live feeds for all growth stages of molluscs, larval stages of crustaceans and certain fish species, and for production of zooplankton, which in turn is used as feed in aquaculture food chains. Hence, hatcheries have microalgae production systems included in parallel to their animal production (Guedes and Malcata, 2012; Zmora et al., 2013).

Interest in cultivating microalgae on an industrial-scale to be used as feedstock for commercial products such as food, feed, pharmaceuticals and health products developed in the middle of the last century. Large-scale production began with the green algae *Chlorella* in Japan in the 1960s, and in the following years plants producing different microalgal species were established in several countries in Asia,

and in Australia, USA and Israel (Hallmann, 2006). The potential in using microalgae for wastewater bioremediation or as renewable energy sources have accelerated and diversified the progress in microalgal biotechnology (Hallmann, 2006; Spolaore et al., 2006). Especially their potential as an alternative feedstock for biofuel production, due to their ability to accumulate high quantities of storage lipids under stressful conditions, has received increased attention in recent decades (Khozin-Goldberg et al., 2016). However, at the current stage, the feasibility to use microalgae for commercial biofuel production is highly questionable considering the competitive market, the low target price and the extensive energy input. The focus has shifted towards higher value products for feeds, food, and nutraceuticals (Chauton et al., 2015; Lam and Lee, 2011). Today, the most important commercially produced microalgae are the green algae *Chlorella vulgaris*, *Haematococcus pluvialis* and *Dunaliella salina,* and the cyanobacterium *Spirulina maxima*, which are primarily marketed as high value nutritional supplements for humans, animal feed additives and pharmaceutical products (Becker, 2013; Zittelli et al., 2013).

2.3 Large-scale cultivation systems

When considered for mass cultivation, high biomass concentrations of the culture are essential in order to yield high productivities, requiring a non-limiting nutrient and CO_2 supply. The increasing culture density consequently leads to mutual shading of the cells and an exponential decrease of light penetration into the culture. When temperature is maintained in an optimum range, irradiance becomes the major factor limiting microalgal growth (Richmond, 2004). By culture mixing, microalgal biomass is being circulated between the outer illuminated zone, where photosynthesis can take place, and an inner dark zone, where net photosynthesis is prevented. The frequency of this intermittent illumination is dependent on the optical path of the cultivation system, cell density and the extent of culture mixing (Richmond, 2013).

Several systems for microalgal large-scale cultivation have been established, comprising open pond systems and closed photobioreactors (PBR) which differ considerably in their design, operation, and production and maintenance costs (Zittelli et al., 2013). Open pond systems can be circular ponds or raceway ponds. Open

circular open ponds are shallow, round systems, up to 50 m in diameter that are mixed by a rotating arm attached to the center of the pond. They are one of the oldest systems for commercial microalgal cultivation and are widely used in Japan, Taiwan and Indonesia (Borowitzka and Moheimani, 2013). Open raceway ponds are shallow ring-channel systems, in which the culture is mixed and circulated by a paddle wheel. They currently represent the most applied and cheapest cultivation system for commercial microalgal production (de Vree et al., 2015). However, in the open systems, extreme cultivation conditions are used to prevent contamination and growth of undesired microorganisms and hence, only a few microalgal species can successfully be grown as monocultures. Particularly *Dunaliella*, *Spirulina* and *Chlorella* have been successfully cultivated on a commercial scale in high salinity, high alkalinity and high nutrient media, respectively (Lee, 2001).

Microalgal species that do not possess this selective advantage must be grown in closed systems (Guedes and Malcata, 2012). The enclosed PBRs allow an accurate control of the growth conditions and protect the culture from contaminations. They comprise two major types, tubular and flat panel PBRs. Tubular PBRs are the most common reactor types and are constructed of long transparent glass or plastic pipes (3–10 cm diameter), through which the microalgal culture is circulated by pumps (Zittelli et al., 2013). Nutrient addition and gas exchange normally occur in separate compartments. Tubular reactors can be arranged in various ways, but often, straight tubes are connected by U-bends to form a loop, which can be vertically or horizontally oriented (Zittelli et al., 2013). The flat panel PBR is a flat, transparent tank, which can often be tilted at different angles, in order to adjust the intensity of irradiation. Cultures are mixed with airflow and the optical path usually varies between one and 20 cm (de Vree et al., 2015). In the enclosed PBR, irradiance can be either natural sunlight, artificial light or a combination of both. The productivity under sunlight is less stable due to diurnal and seasonal variations in irradiation. Artificial light may increase the productivity, but will also cause higher energy costs (Solovchenko and Chekanov, 2014). Temperature-control systems are essential to maintain cultures at tolerable or favorable temperatures under outdoor conditions, but contribute significantly to high production costs (Huang et al., 2017).

Compared to the open pond systems, closed PBRs protect cultures from the environment and hence, provide a better quality of the microalgal biomass. Furthermore, the narrower optical path and the mixing of the culture allow for improved light exploitation and thus higher biomass concentrations (up to 20 g L^{-1}) and higher volumetric productivities (Lee, 2001). The main limitations are the high capital investment and energy costs during operation and the poor system scalability. In contrast, open pond systems have lower construction and operation costs, but can only reach biomass concentration of approximately 0.5 g L^{-1} (Lee, 2001), resulting in less effective production and higher harvesting costs. Besides, they are susceptible to contaminating microorganisms, evaporation, biomass dilution by rain, and temperature fluctuations. The ultimate reactor design, allowing maximal productivity at minimal operation costs in any situation does not exist yet, and the choice of the most optimal PBR is dependent on the location, microalgal species, and the final product of interest (Zittelli et al., 2013). However, improvements of the classical designs are ongoing, and new PBR-concepts have been proposed, such as the foam-bed PBR or the floating PBR. In the foam-bed PBR, microalgae grow on a thin liquid layer between foam bubbles, which reduces self-shading of the cells and supports higher biomass concentrations. Furthermore, energy requirements are expected to decrease considerably due to an improved mass transfer (Janoska et al., 2017). The concept of floating PBRs comprises PBRs that are deployed in surface waters of the ocean, which provides thermoregulation and culture mixing by means of wind and wave action. Although both technologies are highly appealing, they are still at an immature stage, and their large-scale feasibility and economy remain to be evaluated (Muller-Feuga, 2013; Zittelli et al., 2013).

2.4 The current challenges to cost-effective productions

Although phototrophic microalgae are considered a promising feedstock for various commercial applications, the industrial exploitation is still in its beginnings and not yet cost competitive. The high production costs are attributed to the high energy input required for water pumping, CO_2 transfer, culture mixing, heating and cooling, nutrient supply, as well as the algal biomass harvesting, drying and processing

(Jegathese and Farid, 2014; Rodolfi et al., 2009). To achieve an economically feasible commercial production, costs should be decreased ten-fold and the scale increased ten- to hundred-fold (Bosma et al., 2014; Ruiz et al., 2016). A recent study projected costs of 3.4 € kg^{-1} dry biomass, based on a 100-ha microalgae cultivation site in Spain (Ruiz et al., 2016). These costs are expected to decrease to 0.5 € kg^{-1} based on upcoming research and technology developments that will improve microalgal performance, cultivation process, and further processing into the final products (Münkel et al., 2013; Ruiz et al., 2016; Terashima et al., 2015).

2.5 Microalgal lipids

Microalgal lipids are valuable components, of particular commercial interest for the biofuel industry and the feed, food and health sector (Martins et al., 2013). Although there is no agreed definition and classification of the term lipid, it is generally referred to as hydrophobic or amphipathic molecules, that are readily soluble in organic solvents (Li-Beisson et al., 2016). Those include neutral lipids, polar lipids, wax esters, sterols and hydrocarbons, as well as the photosynthetic compounds tocopherols, carotenoids and chlorophylls (Guzman et al., 2012). However, in general microalgal lipids are grouped into polar and neutral lipids (Fig. 2A).

Polar lipids comprise the glycolipids and phospholipids, constituting the membranes of cell organelles (Li-Beisson et al., 2016). Phospholipids consist of a hydrophilic phosphate group and two hydrophobic fatty acid tails, connected by a glycerol molecule. Phosphatidylcholine and phosphatidylethanolamine are the main component of extra-chloroplast membranes, like plasma membranes and endoplasmatic membrane systems (Hu et al., 2008), while phosphatidylglycerol is typically present in chloroplast membranes (Li-Beisson et al., 2016). Glycolipids consist of a hydrophobic fatty acid tail linked by a glycosidic bond to one or more hydrophilic sugar groups. Typical algal glycolipids include monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG) and sulfoquinovosyldiacylglycerol (SQDG). MGDG and SQDG are predominantly restricted to photosynthetic membranes of the chloroplast, while DGDG is also found in extraplastidial membranes (Kumari et al., 2013). Neutral lipids in the form of

triacylglycerols (TAGs) are predominantly synthesized under unfavorable or stress conditions in many microalgae, and are deposited in lipid bodies in the cytoplasm of cells. TAGs serve as energy and carbon storage compounds, and can accumulate to 50% DW (Hu et al., 2008). Its formation is also assumed to prevent photo-oxidative damage to the cell by incorporating electrons from photosynthesis that, due to unfavorable conditions, no longer can be used for cell growth (Breuer et al., 2013).

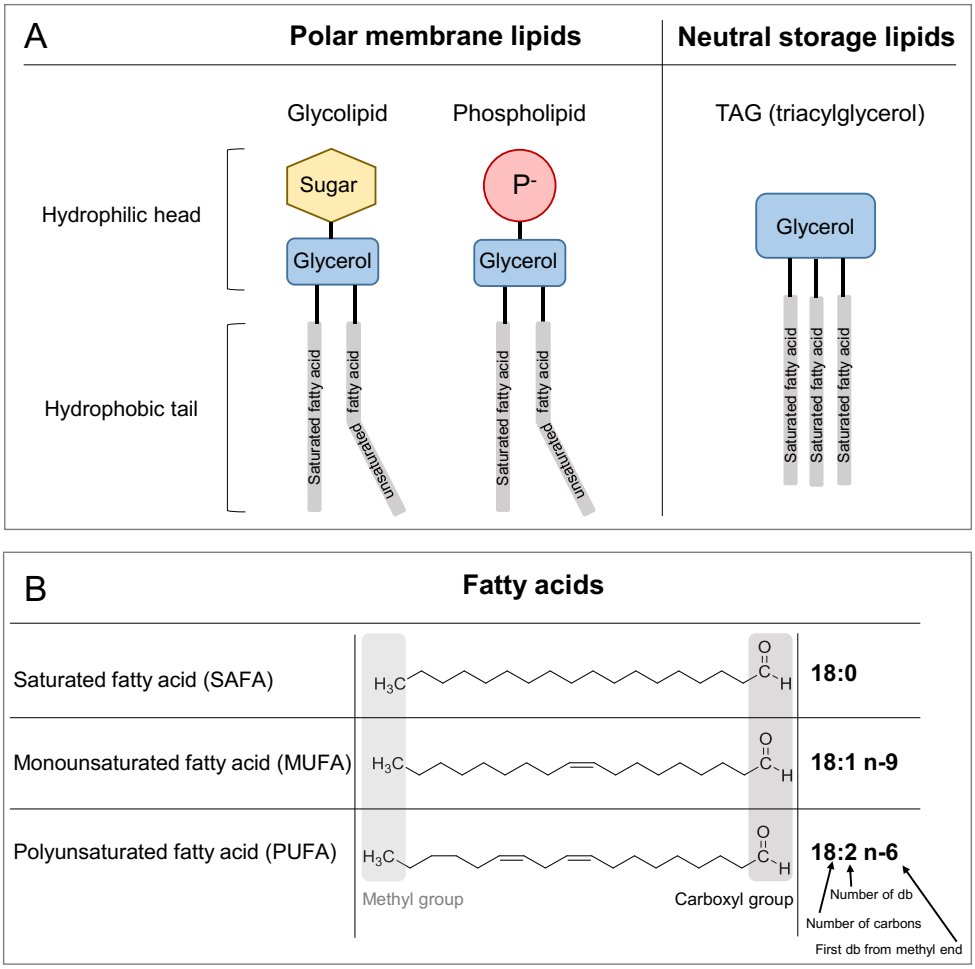

Fig. 2. **A**. Schematic representation of the main cellular lipids in microalgae, modified after Watson (2015). **B**. Examples of fatty acid molecule structures and designation. Db: double bond.

The building blocks of these cellular lipids are fatty acids. Fatty acids consist of a carboxyl group with a long hydrocarbon chain, which can either be saturated, monounsaturated or polyunsaturated, according to the number of double-bounds (Fig. 2B) (Hu et al., 2008; Rubio-Rodríguez et al., 2010). Fatty acids with a chain length of 20 carbons or more are termed as long-chain fatty acids (LC-FAs). The first number in fatty acid designation indicates the number of carbon atoms in the molecule and the second one the number of double bonds. PUFAs are further classified as n-3 or n-6 PUFAs, depending on the position of the first double bond proximal to the methyl-end (Kumari et al., 2013). Microalgae synthesize a variety of different fatty acids, mostly with an even number of carbon atoms (C_4–C_{28}); but also odd chain fatty acids occur. However, most carbon chains range from C_{12} to C_{24} and the mono- and polyunsaturated fatty acids are mainly derived from C_{16}, C_{18}, C_{20} or C_{22} (Kumari et al., 2013).

The fatty acid composition differs between lipid classes. Predominantly, neutral storage lipids (TAG) comprise shorter saturated and monounsaturated fatty acids. They are the target compound in biofuel production research. The n-3 PUFAs such as EPA and DHA are typically present in the polar membrane lipids and are of interest in the health, food and feed sectors (Olofsson et al., 2012).

2.6 EPA and DHA—the high value n-3 PUFAs

Biosynthesis of EPA and DHA in microalgae starts with the synthesis of stearic acid (18:0) in the chloroplast, followed by a series of alternating desaturation and chain elongation processes at the endoplasmatic reticulum, catalyzed by a set of highly specific fatty acid desaturases and elongases (Cagliari et al., 2011; Cook and Hildebrand, 2016). Desaturation adds a double bond to the molecule while chain elongation introduces two new carbon atoms to the molecule (Ratledge, 2004). After desaturation of stearic acid to oleic acid (18:1 n-9) and linoleic acid (LA, 18:2 n-6), fatty acid desaturation can go in two different metabolic directions, either the n-6 or the n-3 route. With the n-3 route, desaturation of LA produces α-linolenic acid (ALA, 18:3 n-3) by introducing the next double bond toward the methyl-end of the molecule. Further chain elongation and desaturation reactions yield EPA and DHA.

With the n-6 route, desaturation of LA produces ɤ-linolenic acid (18:3 n-6) by adding a double bond towards the carboxyl-end of the molecule. This pathway produces arachidonic acid (AA 20:4 n-6), that can further be desaturated to EPA. A basic overview of the conventional fatty acid synthesis pathway in microalgae leading to the biosynthesis of EPA and DHA is presented in Fig. 3. However, other alternative routes exist (Cook and Hildebrand, 2016; Khozin-Goldberg et al., 2016; Martins et al., 2013).

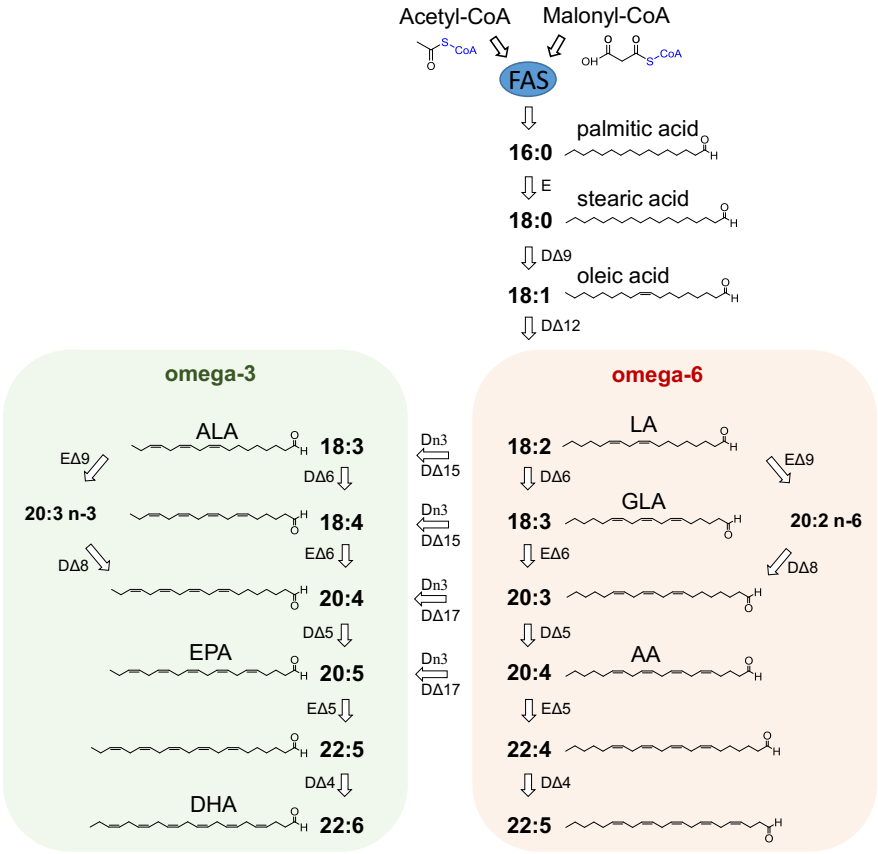

Fig. 3. Conventional pathway for the biosynthesis of LC-PUFAs in microalgae. AA: arachidonic acid, ALA: α-linolenic acid, CoA: Acetyl-Coenzyme A, D: desaturase, E: elongase, FAS: Fatty acid synthase, GLA: ɤ-linolenic acid, LA: linoleic acid, Δ: delta, indicating carbon position proximal to the carboxyl group, n: omega, indicating carbon position proximal to the methyl group. Modified after Khozin-Goldberg et al. (2016) and Martins et al. (2013).

Human and other vertebrates lack Δ12-desaturases, and thus cannot synthesize the LC-PUFA precursors LA and ALA from oleic acid (Nakamura and Nara, 2004). However, LA and ALA are present in different seeds and nuts of higher plants (Certik and Shimizu, 1999; Williams and Burdge, 2006) and if appropriate amounts are provided in the diet, these two fatty acids can be converted to LC-PUFAs such as EPA and DHA (Linder et al., 2010). Yet, these biochemical pathways are very limited. In humans, only up to 8% of ALA is converted to EPA, and 4% to DHA (Mozaffarian and Wu, 2011), making the direct dietary consumption of EPA and DHA highly essential (Li et al., 2010).

2.7 Bioprospecting and outdoor cultivation—why go North?

Many microalgal species are yet undiscovered. The northern latitudes are characterized by extreme environmental gradients, and comprise unique marine habitats and highly productive ecosystems (Lyon and Mock, 2014). Microalgae from these environments are exposed to cold temperatures and large variations in irradiance, salinity and nutrients. They are therefore considered tolerant to fluctuating growth conditions, which routinely occur in commercial production systems. Many species have been shown to cope with both high and low irradiances, with high photosynthetic efficiencies enabling growth at low irradiances, and non-photochemical quenching mechanisms, such as the xanthophyll-cycle, avoiding photoinhibition at high irradiances (Lyon and Mock, 2014). Furthermore, a high PUFA content of the membrane lipids is a well-documented mechanism to maintain membrane fluidity at low temperatures in different polar microalgal species. It promotes a looser packing of the lipids in the membranes, and therewith decreases the solidification temperature (Lyon and Mock, 2014). Both EPA and DHA are particularly found in taxa belonging to the Chromalveolata, such as diatoms, dinoflagellates and prymnesiophytes (Boelen et al., 2013), which are very abundant in North Atlantic waters. Based on this, a robust, cold-adapted strain with high n-3 PUFA content may have great potential as industrial production strain for high value microalgal biomass. However, the search for such strain candidates is challenging, and requires a systematic approach.

Evaluating microalgal performance also under pilot-scale conditions is imperative in order to assess the industrial potential of candidate strains from the laboratory. In fact, studies have shown that strains with improved performance under small-scale conditions did not give improved performance when grown under industrial-scale conditions (Huesemann et al., 2016). Furthermore, not all strains are suitable for upscaling to greater volumes and cell densities. The majority of studies on microalgal outdoor-productivity have been performed in temperate countries with high irradiance that promotes microalgal production. In contrast, research from northern latitudes is scarce, mainly due to reduced irradiances during winter and cold temperatures (Hulatt and Thomas, 2011). However, long days in spring and summer might promote microalgal productivity, and the relatively low electricity costs and the availability of cold water for cooling might provide lower production costs. Furthermore, the Nordic climate conditions, characterized by strong seasonal fluctuations in temperature, irradiance and photoperiod, might considerably affect the microalgal fatty acid composition and possibly reveal a valuable impact.

3. Aims

The overarching objective of this thesis is to explore the potential of northern latitudes for the production of high value fatty acids from microalgae that, in the long term, might contribute to an improved development of new and more sustainable feed types for aquaculture. The investigations covered three secondary objectives:

i. To design a systematic screening-pipeline to prospect for new, robust and fast growing microalgal strains from North Atlantic habitats with high EPA or DHA content (**Paper I**).

ii. To characterize promising isolates from the screening by investigating the influence of different cultivation conditions like irradiance, salinity and growth phase on the fatty acid composition, with special focus on the EPA and DHA content (**Paper II**).

iii. To investigate the potential for outdoor cultivation at northern latitude in 35 L flat-panel outdoor photobioreactors, and to examine the production potential of different high latitude isolates compared to a commercial strain (**Paper III**).

4. Summarizing results and discussion

4.1 Searching for a super-algae

Approximately 40`000 microalgal species are described worldwide but up to 400´000 or more are estimated to exist (Sharma and Rai, 2011). In an effort to explore the potential of the biodiversity and natural variation of microalgae, we developed a screening pipeline, which included the search, isolation and screening of new microalgal strains to find superior EPA and DHA producers.

"Where to look and how to look" (Bull et al., 2000)

In order to increase the success in discovering and commercializing exploitable products from natural resources, a well-elaborated and designed sampling approach is essential (Bull et al., 2000; Knight et al., 2003). Microalgae interact with their environment by responding and adapting to the ambient conditions and often, habitats with extreme growth conditions select for the evolution of unique metabolic pathways (Knight et al., 2003). Hence, sampling sites should be considered with respect to the product of interest. In this study, microalgal strains were sampled from two high latitude locations; the Atlantic waters North-West of Spitsbergen and four fjord systems on the South-West coast of Norway (**Paper I**). Both environments are characterized by harsh conditions, low temperatures and fluctuations in salinities and irradiances that were assumed to select for robust microalgae with high content of the n-3 PUFAs EPA and DHA. From the 75 samples taken and 7200 individual cells sorted, 149 isolates could be established as stock cultures, maintained at 10 °C (Arctic isolates) or 15 °C (fjord isolates). The fact that diatoms were the predominant group emerging from our screening pipeline (**Paper I**) might be related not only to their high abundance in the sampling areas, but also to their robustness, and therewith endurance throughout the sampling and isolation processes.

In a following batch experiment, strains that by visual observation grew most rapidly in stock culture were screened for their growth rates during the exponential phase and for their fatty acid contents in the exponential as well as in the stationary phase (**Paper I**). Irradiance (120 µmol photons m^{-2} s^{-1}) and CO_2 were non-limiting during

the batch experiment, and temperatures were maintained at 10 °C (Arctic isolates) or 15 °C (fjord isolates). Benchmark levels were set to ≥ 0.7 d^{-1} and 3% DW, for growth rates and for EPA or DHA contents, respectively, representing upper average values reported for production strains from literature. In a first screening round, 20 strains from the established stock cultures (10 Arctic and 10 fjord isolates) were examined (**Paper I**), followed by a second screening round with additional nine strains (five Arctic, four fjord isolates). Most of these 29 strains grew well under the applied experimental cultivation conditions. However, two diatom strains (M23 and M17) did not grow, and two green algae strains (M04 and M19) did not produce EPA or DHA (Fig. 4). The remaining 25 strains were all identified as diatoms, of which 18 (six fjord isolates and twelve Arctic isolates) reached the predetermined benchmark level for EPA, while all strains possessed low DHA contents (< 1% DW).

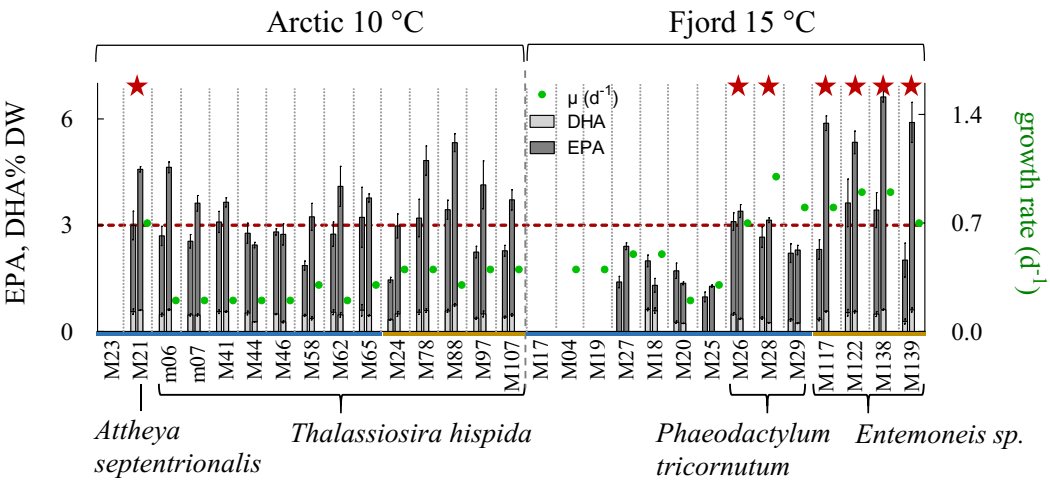

Fig. 4. Superimposed EPA and DHA contents (% DW) in the exponential (first bar) and the stationary growth phase (second bar) and growth rates (μ, green circles) of 29 strains during batch experiments. The red dotted line marks the benchmark level for EPA or DHA content (3% DW) and growth rate (0.7 d^{-1}). Red stars indicate selected strains that reached both benchmark levels. Blue lines indicate strains investigated in the first screening round (**Paper I**) and yellow lines strains examined in a second round. Species names are shown for the most interesting strains, the other names can be found in **Paper I**.

More Arctic than fjord strains attained the requested EPA content, but the growth rates were predominantly higher for the fjord strains (Fig. 4). Seven fjord strains compared to one Arctic strain (M21, *A. septentrionalis*) reached the predetermined growth rate of ≥ 0.7 d^{-1} or higher. Growth rates of the remaining Arctic strains, all identified as *Thalassiosira hispida*, were far below this benchmark level. Eventually, seven candidate strains with both high growth rates and EPA content were obtained from the screening pipeline (Fig. 4), representing 24% of the strains screened, which is a promising outcome. Three of these strains (M21 [*A.septentrionalis*], M26 and M28 [*P.tricornutum*]) derived from the first screening round (**Paper I**) and four strains from the second (M117, M122, M138 and M139 [*Entemoneis* sp.]).

The cultivation conditions in the experimental set-up differ considerably from those in large-scale outdoor cultivation systems. It is hence important to investigate the potential of these strains in detail also under pilot-scale conditions. In addition, new strains that are considered promising for commercial production will need to undergo detailed investigations for their approval and market acceptance. To comply with commercial food and feed regulations, their nutritional quality needs to be assessed and the presence of toxic compounds excluded (Becker, 2013).

4.2 The impact of growth conditions on strain performance

Growth rate, and the EPA and DHA contents are the two most important factors determining the economic potential of the targeted microalgal strains (Yongmanitchai and Ward, 1991). Several studies have revealed that both these factors are affected significantly by various cultivation parameters such as nutrient availability, temperature, irradiance, salinity and pH (Takagi et al. 2006; Araujo et al. 2011; Pal et al. 2011; Solovchenko 2012a).

4.2.1 Lab-scale cultivation—impact of defined growth conditions

A great challenge when searching for new microalgal strains is that the conditions that positively affect growth and the n-3 PUFA content are not easily defined. In this study, the isolated strains were screened for their growth, and EPA and DHA content at only one defined growth condition. Thus, applying different irradiances,

temperatures, salinities, pH or nutrient compositions, could probably have resulted in different EPA contents and growth rates in the individual strains, and hence to the selection of another set of candidate strains. However, fatty acid identification and quantification currently require fatty acids extraction and analysis by gas chromatography. This is a time and cost intensive analytical procedure, and was therefore the rate-limiting step in the screening pipeline (**Paper I**). Developing methods to quantify EPA and DHA content more rapidly, like for example with fluorescent dyes and cell sorting flow cytometry, would allow for a greater screening throughput and could help to better assess the potential of new strains. Yet, the fluorescent dyes currently available can only discriminate between polar and neutral lipids (Guzmán et al., 2011), which was not sufficient for the purpose of the study.

Although the performance of the strains in the screening was considered at only one defined temperature, salinity and irradiance, the impact of both nutrient replete and nutrient depleted conditions on the fatty acids were evaluated (**Paper I**). This revealed that in most diatoms, especially in the strains of the species *T. hispida*, *Entomoneis sp.* and *A. septentrionalis*, the relative EPA content (% DW) increased significantly from the exponential to the stationary phase (Fig. 4). In fact, most of the strains reached the EPA benchmark level of ≥ 3% DW in the stationary phase. This increase in the EPA content was concomitant with an increase in the total fatty acid content (**Paper I**). Interestingly, an increase in total fatty acids, but no significant increase in EPA content in the stationary phase was observed in the three *P. tricornutum* strains.

This impact of nutrient starvation on the EPA content was investigated in greater detail in the Arctic strain *A. septentrionalis* (M21), together with the impact of irradiance and salinity and their interactions using factorial design (**Paper II**). Therefore, batch cultures were grown using the same set-up as for the screening experiment, but with varying combinations of two salinities (22 and 35) and two irradiances (50 and 200 µmol photons m^{-2} s^{-1}), and samples were taken three times; on Day 1 of exponential phase, and Day 3 and 5 of stationary phase. Results revealed that both growth phase and salinity, alone and in combination, influenced the EPA

content significantly, while irradiance did not, under the experimental conditions used (**Paper II**). Growth phase had the greatest impact, with increasing nutrient starvation leading to a higher EPA content relative to DW. The effect of salinity and the combined effects of both salinity and growth phase were lower and negative. For the exponential phase, EPA values were higher for high salinity cultures, whereas in stationary phase, EPA values were higher in low salinity cultures. Maximum EPA values of 7.1% DW were obtained at low salinities of 22 and at Day 5 of the stationary phase. However, at the same time growth rates during exponential phase were reduced significantly (37%) at low salinities of 22, compared to high salinities of 35 (**Paper II**). In future large-scale cultivations, EPA productivity would be dependent on both the growth rates and the EPA content in the cells. Thus, calculating the EPA productivities from exponential phase until Day 5 of the stationary phase revealed higher productivities for the low salinity cultures (0.97 mg L^{-1} d^{-1}) compared to cultures grown at high salinities (0.72 mg L^{-1} d^{-1}). Yet, these productivities are much lower than seen in commercial productions due to the much lower biomass concentrations used in this experimental setup.

Although the limiting nutrient leading to progression into stationary phase could not be determined definitely, silicate was assumed the limiting nutrient for *A. septentrionalis* in the factorial-design experiment as well as for most diatoms in the screening. Nitrate, phosphate and silicate contents of the medium were measured during the factorial-design experiment. Although all three nutrients had been consumed in the stationary phase, silicate had been consumed the most after Day 1 of exponential phase (**Paper II**). Nitrate and phosphate are known to be accumulated in storage vacuoles during nutrient replete conditions in many diatoms, whereas more silicate than required for cell division is rarely taken up (Reynolds, 2006). Furthermore, *P. tricornutum*, a diatom that is known for its negligible silicate demand (Riedel and Nelson, 1985) and two green algae strains that do not require silicate, reached higher cell densities compared to the other diatom strains in the stationary phase of the screening batch experiment (**Paper I**). The key regulatory factors accounting for the observed increase of the EPA content following silicate starvation remain to be identified definitely. However, it might be related to (1) a decreased

silica content in the cells or (2) an accumulation of storage lipids containing EPA, or to a combination of both (**Paper II**). The effect of salinity on the EPA content might be related to reconstructions of membrane lipids as an adaptation to the changing osmotic conditions. Changes in EPA content or the upregulation of desaturase enzymes in response to salt stress has been reported for other microalgae (Gu et al., 2012; Lyon and Mock, 2014).

4.2.2 Outdoor cultivation—impact of environmental conditions

Changing environmental conditions during outdoor cultivation affected the EPA content considerably in three different *P. tricornutum* isolates. The three strains were Strain Fito; isolated from Cadiz Bay in Spain, and grown on a commercial scale by the company Fitoplancton Marino, Strain M28; a candidate strain from our screening and Strain B58; a local isolate, that has been maintained in stock culture in our laboratory since 1997 (**Paper III**). The three strains were grown as repeated batch cultures in separate 35 L flat-panel outdoor photobioreactors in Bergen, Norway, from end of April until end of October 2016. In contrast to the laboratory experiments **(Paper I and II**), the strains were grown as repeated batch cultures by regularly replacing defined culture volumes with fresh growth medium, to maintain biomass concentration between 0.5 and 3 g L^{-1}. Nutrients and CO_2 were provided in excess and temperatures were kept below 25 °C by cooling with tap water. EPA contents of the three strains varied considerably during the cultivation period with values between 2.6 and 5.6, 1.4 and 4.5, and 2.2 and 4.1% DW for strains Fito, M28 and B58, respectively (**Paper III**). No clear trend indicating which environmental factors were most responsible for the observed changes was evident. While the salinity (29) remained constant, the other environmental conditions varied greatly over the cultivation period. The irradiance available for the microalgal cells varied strongly diurnally and seasonally depending on the weather conditions, culture density and the changing day length. Culture temperatures changed considerably between night and day (5–25 °C), pH was fluctuating between 7.5 and 8.1 and nutrient concentrations although provided in excess, altered inversely to the biomass concentration.

Determining which, how, and in what combination the different environmental factors affect the EPA content in microalgal strains remains difficult and is likely to vary considerably between species or even strains. However, factorial-design experiments were proven a valuable method to investigate individual and interactive effects of different growth factors in lab-scale experiments (**Paper II**). Factorial designs considering even more factors as for example temperature and pH, could provide an even clearer picture on the interaction effects of growth factors on the EPA content in the individual strains. Especially the impact of changing temperatures should be assessed in future research. Low cultivation temperatures are one of the main environmental factors that have been reported to increase PUFA content of microalgal membrane lipids (Boelen et al., 2013). Yet, when considered for commercial productions, microalgal strains need to be able to grow reproducibly and keep a stable PUFA level at higher and varying temperatures as well.

4.3 The potential of northern latitudes

Various studies on microalgae inhabiting temperate waters have shown a general trend of increased PUFA content with decreasing water temperatures (Lyon and Mock, 2014). In accordance with this, several diatoms from our screening pipeline possessed a comparatively high EPA content and greater levels were found for Arctic than for fjord isolates. However, given the limited number of samples and the lack of comparable screenings of microalgae from other sampling sites, the observed high EPA values cannot definitely be related to the sampling origin. Diatoms in general have been reported to be highly enriched in EPA (Hildebrand et al., 2012). Furthermore, laboratory cultivation causes strains to acclimate or adapt to the prevailing conditions, which deviate noticeably from the original habitat conditions, and thus might lead to changes in their fatty acid composition.

Outdoor cultivation of the three *P. tricornutum* isolates under pilot-scale outdoor condition at the prevalent northern location was successful during six month of the year (**Paper III**). However, biomass productivities were lower when related to studies from Spain (Acién Fernández et al., 2003; Molina Grima et al., 1995; Sánchez Mirón et al., 2003) and Italy (Rodolfi et al., 2017). This was most probably due to

lower irradiances predominating at our cultivation site compared to those occurring at southern latitudes. Longer day lengths during spring and summer were assumed to promote productivity but could not compensate for the generally lower irradiances. Although the EPA contents of the three *P. tricornutum* strains varied greatly during outdoor cultivation (**Paper III**), the average EPA values of the strains Fito (Spanish isolate) and M28 (local isolate) were considerably higher during outdoor cultivation (4.4% and 3.2% DW, respectively) compared to laboratory cultivation under screening conditions (2.8% and 2.6% DW, respectively). In contrast, the EPA content in strain B58 (local isolate) was similar during outdoor and laboratory conditions (3.1% and 3.3% DW, respectively) (Fig. 5). This emphasizes the importance of evaluating strain performance during outdoor and up-scale production conditions, as the results can deviate considerably from the laboratory conditions. Furthermore, EPA contents of the three *P. tricornutum* strains during outdoor cultivation were higher than EPA contents of a *P. tricornutum* strain grown under outdoor conditions in Italy (2.1–2.4% DW) (Rodolfi et al., 2017). Hence, similar or higher EPA productivities were achieved for our strains, despite the lower biomass productivities compared to the strain grown in Italy **(Paper III).** The higher EPA content in the *P. tricornutum* strains observed under the prevalent outdoor condition might hence be connected to interactive effects to the prevailing environmental conditions.

It has been argued, that locally-isolated strains would be more suitable for cultivation in outdoor production systems at a respective location, as they might be better adapted to the prevailing environmental conditions (Lee et al., 2014). This could not be confirmed in our study. The biomass productivity of the Spanish strain (Fito), isolated from Cadiz Bay in Spain, was comparable to the productivity of the two local strains (M28 and B58), isolated from the immediate vicinity of the culturing facilities (**Paper III**). Moreover, the Spanish strain possessed significantly higher EPA contents during outdoor cultivation compared to the two local Norwegian fjord strains (Fig. 5) and thus, proved to be the most promising regarding EPA productivity at our location (**Paper III**). Actually, growth conditions in production systems deviate considerably from the environmental condition of natural habitats at a given site, and do therefore not necessarily provide beneficial conditions for locally isolated

strains. However, a benefit of using local strains when up-scaling culture volumes towards pilot- and commercial-scale might be less concern from governmental regulations about potential inadvertent introductions or release of introduced strains into natural waters (Knuckey et al., 2002).

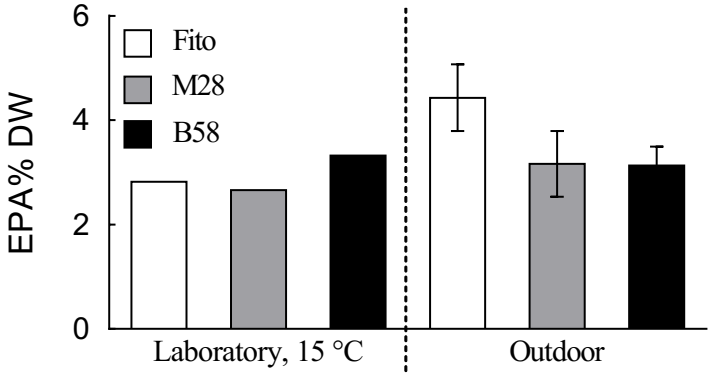

Fig. 5. EPA content relative to the dry weight (DW) for the two local (M28 and B58) and the Spanish (Fito) *Phaeodactylum tricornutum* strains under laboratory conditions and during six-months repeated-batch cultivation in flat panel outdoor bioreactors in Bergen, western Norway. Laboratory values are one biological replicate and outdoor values are average and standard deviation of 162 measurements.

Further research is needed to determine whether large-scale outdoor cultivation can be performed in a sustainable and economic way at northern latitudes. Biomass productivity might be further optimized by improving process design during photobioreactor operation. Although biomass productivities were lower compared to southern latitudes, the higher irradiances and temperatures in southern regions demand intensive cooling of cultures. Those demands are likely to be reduced at higher latitudes, as cooling was necessary only during days with high irradiance, and cost-efficient water could be used. Hence, further long-term investigations on outdoor productions, life-cycle assessments and techno-economic analyses could reveal the potential of northern latitudes for EPA or DHA production.

4.4 Diatoms—an untapped bioresource?

Our screening results revealed several diatoms as potential new EPA production strains (**Paper I**). In many strains, total fatty acid and EPA contents increased following silicate depletion (Fig 4), which might be due to a decrease of the cell silica content or to TAG accumulation comprising EPA (**Paper I and II**). In most diatoms, amorphous silica is an essential cell wall component making silicate availability a key factor, strictly controlling their growth in nature and cultivation (Javaheri et al., 2015; Martin-Jézéquel et al., 2000). Similar to nitrogen depletion, silicate depletion has been shown to induce the accumulation of TAG storage lipids. Although TAGs predominantly comprise saturated and monounsaturated FA, some microalgae have been reported to also accumulate PUFAs in their TAGs (Khozin-Goldberg et al., 2016; Solovchenko, 2012). Silicate depletion has been shown to be advantageous over nitrogen starvation to induce TAG accumulation, as it has little direct effect on the cellular metabolism apart from cell cycle prevention. In contrast, nitrogen limitation is associated with decreases in chlorophyll and protein contents (Hildebrand et al., 2012; Shrestha and Hildebrand, 2015). Enright et al. (1986) found similar protein levels for control and silicate limited cells, while in nitrogen limited cells protein content was reduced by 60%. These aspects are highly essential when considering microalgae for food and feed purposes as silicate depletion seems to increase the nutritional value of the cells.

Diatom cultivation has a long history in the hatchery industry as they are easy to grow and have high nutritional values (Brown, 2002; Wang and Seibert, 2017). Despite this, diatoms have been underrepresented in research on commercial production of high value compounds or biofuels (Hildebrand et al. 2012). However, they seem to hold a great potential for commercial exploitation, which is also reflected in their ecological success in natural environments. Diatoms represent the most diversified group of microalgae (Barra et al., 2014), are estimated to be responsible for 30 to 40% of marine primary production (Coesel et al., 2008), and play a superior role in the marine food chain due to their high nutritional value and edibility among the secondary producers (Martin-Jézéquel et al., 2000).

4.5 The alternatives—designing a super-algae?

An alternative approach to searching and investigating new strains is to improve the performance and marketability of available microalgae strains. Metabolic engineering through genetic manipulation can provide a targeted approach to create highly efficient microalgal strains. Several studies successfully increased the n-3 PUFA content in different species by overexpressing genes of different biosynthetic fatty acid elongase and desaturase enzymes (Cook and Hildebrand, 2016; Hamilton et al., 2014; Kaye et al., 2015). However, using transgenic algae for food or feed products is associated with concerns and controversy. Public objection, consumer acceptance and regulatory problems might challenge large-scale outdoor production of edible products from genetically modified microalgae in the near future (Larkum et al., 2012). Random mutation induced by chemicals or UV is another approach for strain improvement, with the advantage that the generated mutants are not considered genetically modified. UV-induced mutant strains of *Pavlova lutheri* (Haptophyceae) with increased EPA and DHA contents were reported by Meireles et al. (2003). Yet, this method does not provide a targeted approach (Chauton et al., 2015) and time intensive, in depth characterization of strains is required, to identify phenotypes with improved EPA and DHA productivity (Shrestha et al., 2013).

Breeding techniques have been used in plants for thousands of years to change specific traits in order to obtain the desired characteristics. In contrast to higher plants, most microalgae reproduce asexually under laboratory conditions or the conditions inducing sexual reproduction are not known for most species (Shrestha et al., 2013). However, similar to breeding techniques, strain improvement methods like adaptive laboratory evolution (ALE) and continuous selection pressure offer a great possibility to increase specific traits of microalgae. By cultivating promising candidate microalgae under clearly defined conditions for prolonged periods (months to years), the selection of improved phenotypes is achieved, which are associated with a certain growth environment that leads to the selection of traits (Wang et al., 2016). For example, temperature tolerance and optimum of a *Tisochrysis lutea* strain (Haptophyceae) has been expanded by slow and continuous adaptation to increasing temperatures (Bonnefond et al., 2017).

5. Conclusion and future perspectives

In the recent years, there has been growing interest of using microalgae-based technologies for a sustainable and commercial production of food, feed, chemicals and fuels and other application such as wastewater treatment (Barclay & Apt 2013). However, commercial applications have only been proven successful for the production of high-value nutritional ingredients from a few microalgal species, compassing DHA production via heterotrophically grown *Crypthecodinium cohnii*, *Thraustochytrium* sp. and *Schizochytrium* sp., and production of nutritional supplements by phototrophic cultivation of *Spirulina* spp., *Chlorella* spp., *Dunaliella* spp. and *Haematococcus pluvialis* (Barclay & Apt 2013). The fundamental technological problems preventing a cost-efficient production are low biomass and target compound productivity, and high production, harvesting and processing costs (Barclay & Apt 2013). In order to replace fish oil as ingredient in aquaculture feed, it is essential to make commercial production of the high value fatty acids EPA and DHA from microalgae more energy-efficient and cost-competitive.

In this work, we successfully established a screening pipeline and identified seven new diatom isolates with great potential as industrial production strains. We identified silicate availability and salinity to affect EPA content significantly in an Arctic diatom, and were able to increase its EPA content to 7.1% DW by varying growth conditions. Furthermore, we observed high EPA contents in different *P. tricornutum* strains when grown under outdoor conditions at high latitude (Bergen, Norway).

In further research, the impact of other growth factors such as temperature should be included to factorial-design experiments and the potential of other candidate strains from the screening pipeline need to be explored. In future fatty acid analyses, a discriminate between polar and neutral lipids could reveal in which lipid class the EPA accumulates during stationary phase, and thus give a better understanding on the mechanism behind the observed change in the EPA contents. Once these processes are better understood, it can guide the next steps on how to improve these strains

further. During outdoor or semi-outdoor cultivation in greenhouses, temperatures vary continuously during the day and over the seasons. Process control to keep temperatures within boundaries is energy expensive and costly. Hence, improving strains to perform well under varying temperatures could be a significant contribution to decrease production costs. The temperature tolerance and optimum of the selected strains could be expanded by slow and continuous adaptation to increasing daily temperature variations, as shown by Bonnefond et al. (2017). Adapted strains should then be further evaluated under outdoor conditions by comparing pilot-scale productivities of “improved” and “original” strains.

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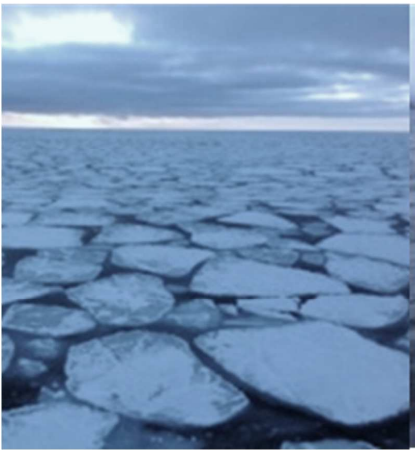

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Bioprospecting North Atlantic microalgae with fast growth and high polyunsaturated fatty acid (PUFA) content for microalgae-based technologies

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ABSTRACT

Microalgae are considered to be an important and sustainable alternative to fish oil as a source for the polyunsaturated fatty acids (PUFAs) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Due to their health benefits, there is an increasing interest in the commercial application of these fatty acids (FA) to health and dietary products, and to aquaculture feeds. However, FA from microalgae are still expensive to produce compared to fish or plant oils. With only a few microalgal strains being cultivated on a large scale for commercial PUFA production, prospecting for new, robust and fast-growing strains with increased PUFA content is essential in order to reduce production costs. Microalgae from northern high latitudes, exposed to cold temperatures, may be especially promising candidates as previous studies have shown increasing unsaturation of FA in response to decreasing growth temperatures in different microalgae, most likely to maintain membrane fluidity and function. We have designed a screening pipeline, targeting a focused search and selection for marine microalgal strains from extreme North Atlantic locations with high robustness and biomass production, and increased levels of EPA and DHA. The pipeline includes a rational sampling plan, isolation and cultivation of clonal strains, followed by a batch growth experiment designed to obtain information on robustness, growth characteristics, and the FA content of selected isolates during both nutrient replete exponential cultivation and nutrient limited stationary cultivation. A number of clonal cultures (N = 149) have been established, and twenty of these strains have been screened for growth and FA content and composition. Among those strains, three showed growth rates $\geq 0.7\ d^{-1}$ at temperatures of 15 °C or below, and high amounts of EPA (> 3% DW), suggesting their potential as candidates for large scale production.

1. Introduction

The omega-3 long-chained PUFAs (LC-PUFAs), eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6) have unique nutritional benefits for human health [1]. They appear to be essential in preventing cardiovascular disease, and most nutritional guidelines now include recommendations for increased intakes of these FA [2]. The current major source for these LC-PUFAs is marine fish oil. However, fish, like other animals, do not efficiently synthesize EPA and DHA themselves, but obtain and accumulate them via the marine food chain from PUFA-synthesizing microalgae [3–6]. Because of their high EPA and DHA levels, oily wild fish are considered a health food, and their oil is in much demand for use in aquaculture feed as a source of these PUFAs. Approximately 70% of the globally-available fish oil is currently consumed by aquaculture feed production [7]. Additionally, other applications and markets for direct human consumption of EPA and DHA rich fish oil are developing rapidly. This increasing demand for fish oil, and its commercial production from wild fish stocks has led to economic, ethical and environmental concerns. Due to increasing competition in the global market, fish oil is increasingly being substituted with vegetable alternatives in modern aquafeeds [8]. However, as plant oils lack omega-3 LC-PUFAs [1], this leads to a decrease in the relative EPA and DHA levels in farmed fish, and the production of plant oils also uses valuable and increasingly limited farmland. Questions concerning the relatively high content of omega-6 FA in land-plant based oils and their potentially negative effects on human health have also been raised in the literature [9]. Thus, there is a great demand for new sources of omega-3 FA, and microalgae are currently regarded a promising

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alternative, as many species naturally produce high levels of EPA and DHA.

In recent decades, there have been increasing efforts to apply microalgae-based technologies for a more sustainable production of many different compounds used in the biofuel, pharmaceutical, functional food and aquafeed industries [10]. In addition to their nutritious biomass and high areal productivity, microalgae can be cultivated in seawater and on non-arable land, and do not compete for resources with conventional agriculture [11]. The lipids of microalgae are of particular commercial interest; triacylglycerides (TAG), which are storage lipids comprising mostly saturated and monounsaturated FA [12], are in much demand in the biofuel industry, whereas the health, food and feed sectors target the LC-PUFAs [7] that are typically present in the polar membrane lipids of microalgae [13]. However, the many advantages derived from using microalgae as feedstocks, contrast with their cost-inefficient large-scale production [14]. Today, the production of FA from microalgae continues to be expensive when compared with current fish oil sources [15] and farmland plant oils, due to the high energy requirements of water pumping, CO_2 transfer, culture mixing, nutrient supply, biomass harvest and drying [16,17].

An essential requirement for the successful improvement and progression of microalgae-based technologies is the discovery of new and robust strains that grow fast and produce naturally high levels of the desired compounds [10]. A number of different strategies have been implemented in bioprospecting and screening for new strains to optimize FA production for biofuels [18–21], but so far, only a few studies have focused on the PUFA fraction [22,23]. In this study, we developed a screening pipeline, targeting microalgae from different North Atlantic habitats, with high growth rates and increased concentrations of the high value products EPA and DHA. If found suitable for process upscaling, candidate strains could contribute significantly to the development of a more cost efficient large-scale production of microalgae.

2. Materials and methods

2.1. Sampling sites and times

Microalgal samples were taken during 2014 at two different marine locations; the Atlantic waters around Spitsbergen and three fjord systems on the South-West coast of Norway (Fig. 1A). In the Arctic, samples were taken at a number of stations during four cruises in March, May, August, and November (Fig. 1B, sampling stations 5, 6, 7 and 8–11, respectively). The three fjord systems Store Lungegårdsvann, Puddefjorden and Raunefjorden (Fig. 1C, sampling stations 1, 2 and 3, respectively) were sampled four times during the year, in order to cover all four seasons. In addition to this, the re-isolation of natural samples obtained in 2012 from deep water masses at 1000 m in Sognefjorden (Fig. 1C, sampling station 4), was included. At all sampling sites, phytoplankton samples were taken by vertical net haul (mesh size 10 µm) at depths from 0 to 10 m in fjords and 0–50 m in Arctic waters, and Niskin water bottles were used for the deeper water layers. In the fjords, sediment and biofilm samples from the intertidal zone were also taken, with pre-sterilized equipment. Water and benthic samples were collected in 50 mL centrifuge tubes. The environmental samples were divided into two parts; one part was taken for single cell isolation directly, and the other part went through an enrichment phase, where 50% (v/v) Walne's medium [24], (prepared in 80% seawater [SW]), was added. The enrichments were initially incubated at 15 °C (fjord spring/summer samples) or 4–8 °C (fjord winter/spring samples and Arctic samples), and at a photon flux density (PFD) of 50 µmol photons $m^{-2} s^{-1}$, until visible development of microalgal biomass, followed by single cell isolation.

2.2. Single cell isolation

Single cell sorting of environmental and enrichment samples was performed with a Becton Dickinson FACS Aria™ Cell sorter (BD Biosciences, San Jose, CA, USA) at a flowrate of 300 events s^{-1}. The software BD FACS DIVA (Version 8.0; BD Biosciences, San Jose, CA, USA) was used for data analysis. The selection criteria were set after initial analysis of fluorescence signals of a pure *Phaeodactylum tricornutum* culture and environmental samples. Cells were excited with a red laser at 633 nm, and the resulting scattered light and fluorescence emission were recorded by a forward scatter detector, and a FL-4 detector (661/16 nm), respectively (see Fig. 2 for an example of a two-dimensional dot plot of an environmental sample). Events with > 1000 arbitrary units of auto fluorescence from chlorophyll *a* (chl *a*) were set as selection criteria for cell sorting. All samples were pre-filtered (BD Falcon™ USA, 40 µm Nylon) before sorting. A 100 µm nozzle dispensed one drop containing a single cell into each well of 96-well plates, containing 150 µL solid or liquid growth medium (80% SW enriched with Walne's nutrients). After sorting, the plates with cells were incubated at 10 °C (Fjord winter/spring samples and Arctic samples) or 15 °C (Fjord spring/summer samples) at approximately 50 µmol photons $m^{-2} s^{-1}$ until visible growth. In addition to this, cell isolation by serial dilution was performed with two enrichment samples from the

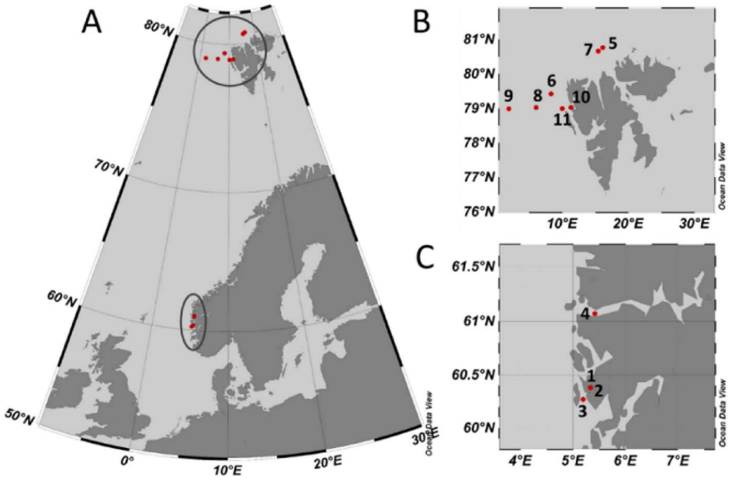

Fig. 1. Map illustrating the sampling sites (A) with seven different sampling stations to the north and west of Spitsbergen (B) and four different sampling stations at the Norwegian west coast (C). Names and coordinates of the sampling positions and sampling times can be found in Table 1.

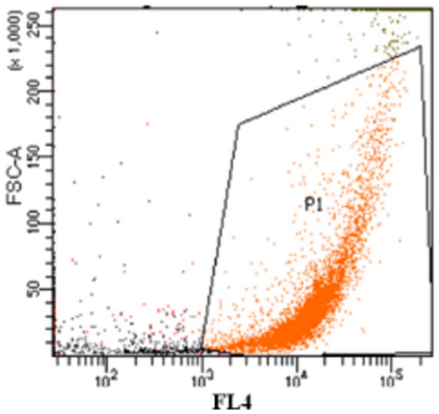

Fig. 2. Single cell isolation using FACS Aria™ Cell sorter. Example of a two-dimensional dot plot combining forward scattering (FSC-A) and fluorescence emission by chlorophyll *a* (FL-4). Events with > 1000 arbitrary units of auto fluorescence from chlorophyll *a* was set as selection criteria for cell sorting.

Arctic by monthly transfer and dilution of cells growing in 3 mL sterile Walnes's medium.

2.3. Upscaling and culturing of clonal cultures

Proliferating strains from the 96-well plates and isolates from serial dilution were transferred to glass tubes with 3 mL sterile Walne's medium [24] prepared in 80% SW, and incubated under conditions as described in Section 2.2. The proliferating clonal strains were sustained as stock cultures by sub-culturing every month. Cultures were not axenic, but were maintained as sterilely as possible. For preliminary identification, and to monitor contamination by other microalgae, the cultures were observed frequently under the microscope. Isolates showing highest growth rates in the stock culture (by visual observation) were selected for the determination of their FA profile and growth rates under controlled conditions.

2.4. Growth and FA profiling

A 280 mL batch culture was grown for twenty selected isolates to investigate their growth rate and FA content and composition in both the exponential and the stationary phase. For the inoculum, biomass of each strain was upscaled to 100 mL, harvested by centrifugation (2264*g*, 5 min), washed twice with fresh medium, recultured in 280 mL fresh medium (Walne's medium [24] prepared in 80% SW) and transferred to a 300 mL glass cylinder (3.5 cm inner diameter). The twenty glass cylinders were placed into temperature-controlled water tanks to mirror temperatures used in the previous cultivation step (10 or 15 °C). Continuous illumination of 120–150 μmol photons m^{-2} s^{-1} (measured with a 4π quantum scalar irradiance sensor [QSL-100, Biospherical Instruments, San Diego, CA, USA], inside the empty glass cylinder) was provided by banks of six white fluorescent tubes (Philips MASTER, TL-D 90 Graphica, 58W/95) in the back of the water tanks, running perpendicular to the glass cylinders. To ensure mixing and carbon supply, 0.2 μm-filtered and 1% CO_2-enriched air was bubbled through glass capillaries into the bottom of each 300 mL glass cylinder. The cultures were sampled every 24 h for optical density measurements at 750 nm (OD 750). The OD-based growth curves were used to determine a time point in the mid-exponential and the late stationary growth phase, for FA and biomass dry weight (DW) analyses. Due to variable growth rates, these sampling days were different for the individual strains. At the end of the experiment, 10 mL samples were taken for phylogenetic analysis of the isolates.

Optical density measurements were performed using a spectrophotometer (UV-1800, Shimadzu Corporation, Kyoto, Japan) at 750 nm wavelength. If the OD value exceeded 0.8, samples were diluted to give an attenuation between 0.2 and 0.8. The specific growth rate (μ_{avg} [d^{-1}]) was calculated for each culture by taking the average of the growth rates between two consecutive days (μ_x) during exponential phase. The growth rate between two consecutive days was calculated according to the changes in attenuation during 24 h with Eq. (1). N_{tx} and $N_{tx\ -\ 24\ h}$ are OD 750 at day x (t_x) and the previous day x − 24 h ($t_{x\ -\ 24\ h}$), respectively. Exponential phase was determined individually for each strain based on the logarithmic shape of its growth curve. Dry weights were determined in triplicate, as described in Zhu & Lee (1997) [25], using GF/F (47 mm) Whatman® filters and 0.5 M ammonium formate as a washing buffer, and are expressed as weight of the dried biomass per volume. For FA analysis, quadruplicate 10 mL microalgal cultures were sampled into 10 mL glass tubes (PYREX), centrifuged for 6 min at 2264*g*, and the supernatant was discarded. Pellets were stored in nitrogen atmosphere to prevent oxidation at − 20 °C prior to analysis.

$$\mu_x = \frac{\ln(N_{tx}) - \ln(N_{tx-24h})}{(t_x - t_{x-24h})} \quad (1)$$

2.5. FA extraction and FAME analysis

Total lipids were extracted and derivatized to fatty acid methyl esters (FAME) by direct esterification, according to Meier et al. (2006) [26]. The sample pellet was dried in the 10 mL glass tubes by evaporating water under a nitrogen stream, and the internal standard (23:0 FAME, dissolved in isooctane) was added. The solvent was then evaporated, and 0.5 mL of methylation reagent (2 M HCl in methanol) was added to each tube. Tubes were flushed with nitrogen, sealed and incubated in an oven at 90 °C for 2 h. After cooling to room temperature, half of the methylation reagent was evaporated, and 0.5 mL water was added. The samples were thereafter extracted twice with 1 mL isooctane. Before analysis by gas chromatography (GC), the combined extracts were further diluted with isooctane in order to yield a final internal standard concentration of approximately 20 μg mL^{-1}. FAMEs were analysed by GC as described in Prestegard et al. (2015) [27]. To aid identification of the FAMEs, selected samples were analysed by gas chromatography–mass spectrometry (GC–MS) as described in Wasta & Mjøs (2013) [28]. In order to calculate the FA concentrations appropriately, the internal standard content should be approximately 30% of the most abundant FA in the sample. Thus, an initial analysis of the FA content and composition for each strain at both growth phases was necessary to determine the correct amount of internal standard to be added to each sample. Therefore, 36 μg internal standard (460 μg for some stationary phase samples with a higher biomass content) was added to every first replicate of the quadruplicate samples and samples were analysed as described above. After the initial test, the correct amount of internal standard was calculated for each sample, and added to the remaining triplicates.

2.6. Phylogenetic analysis of isolates selected for screening

Molecular methods were used to give taxonomic information of the isolates. DNA was isolated from the pellet of 10 mL algal culture (harvested at 1663*g* for 6 min) using E.Z.N.A. SP Plant Kit (Omega Bio-tek, Inc., Norcross, GA, USA). Microalgal DNA was amplified by PCR using the HotStarTaq DNA Polymerase Kit (QIAGEN, Valencia, CA, USA), according to the manufacturer's instructions. Amplification of a region of the 28S ribosomal RNA (rRNA) gene (for the large subunit [LSU] of eukaryotic cytoplasmic ribosomes) was performed using the primers D1R-F [29] and D3B-R [30]. The reaction conditions were as follows: An initial activation of the enzyme at 95 °C for 15 min, followed by

30 cycles of denaturation (94 °C, 1 min), annealing (56 °C, 1 min) and extension (72 °C, 1 min), and a final extension at 72 °C for 10 min. Before sequencing, the PCR product was purified with GenElute™ PCR Clean-Up Kit (Sigma-Aldrich, St. Louis, MO, USA) and quantified with Qubit ® dsDNA BR Assay Kit and Qubit® 2.0 (Invitrogen, Eugene, Oregon, USA). Bi-directional sequencing of the PCR products was performed using the PCR forward and reverse primers with the BigDye v.3.1 Kit (ThermoFisher Scientific, Watham, MA, USA) at the sequencing facility at the University of Bergen (http://www.uib.no/en/seqlab). Sequences were edited and aligned manually in BioEdit [31], and Blastn [32] was used to search for similarities with previously published diatoms in GenBank (www.ncbi.nlm.nih.gov/blast/Blast/cgi).

2.7. Statistics

The batch growth experiments were run with one biological replicate for each strain. One measurement replicate was used for OD measurements of cultures to monitor the growth phase, whereas triplicate samples were taken for DW and FA analyses. One sample per culture was taken for phylogenetic analysis of the strains. As the FA content and biomass DW were not analysed from the same sample, the standard deviation for FA content relative to the biomass DW was calculated with Eq. (2) with SD: standard deviation, FA_{DW}: fatty acid dry weight (mg), BM_{DW}: biomass dry weight (g), FAc: fatty acid concentration (mg L^{-1}) and DWc: dry weight concentration (g L^{-1}).

$$\text{SD of } \frac{FA_{DW}}{BM_{DW}} = \frac{\sqrt{(\%\text{SD of FAc})^2 + (\%\text{SD of DWc})^2}}{100 * FA_{DW}} \quad (2)$$

Differences in the EPA and DHA content between exponential and stationary phases for the different strains was analysed by Student's *t*-test using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA), with $p < 0.01$ as a threshold for statistical significance.

3. Results and discussion

A screening-pipeline, including five different steps (Fig. 3) was developed for bioprospecting new, robust and fast growing microalgal strains from extreme North Atlantic habitats, with increased levels of the omega-3 fatty acids EPA and DHA, and thus with potential for future biotechnological applications. Selected habitats were sampled during different seasons of the year (step 1), and single cell isolation (step 2), upscaling and culturing of proliferating strains (step 3) led to the establishment of stock cultures. In a subsequent batch growth experiment (step 4), strains with high growth rates ($\geq 0.7\ d^{-1}$) and EPA or DHA levels of minimum 3% of biomass DW in either the exponential or the stationary phase were targeted. The selected benchmark levels represent an upper average level that is found for species that are already being applied in aquaculture and mariculture, e.g. *Nannochloropsis* sp. (Eustigmatophyceae) with a typical EPA content between 2.1 and 3.8% DW [33,34] and *P. tricornutum* (Bacillariophyceae) with values between 2.6 and 3.1% DW [4,35,36]. Isolates that were robust (to the mechanical shear forces from bubbling) and also fulfilled the desired requirements, were selected to be investigated in more detail (step 5).

3.1. Sampling site

Microalgal samples were obtained from two marine locations, the Atlantic waters around Spitsbergen and four fjord systems on the South-West coast of Norway (Fig. 1A). The fjord waters represent a wide range of environmental conditions, from the brackish water of the land-locked fjords Store Lungegårdsvann and Puddefjorden, with their frequent and strong fluctuations of environmental conditions (Salinity 11–32, temperature −0.5–20 °C), to the saline waters of the open coastal fjord

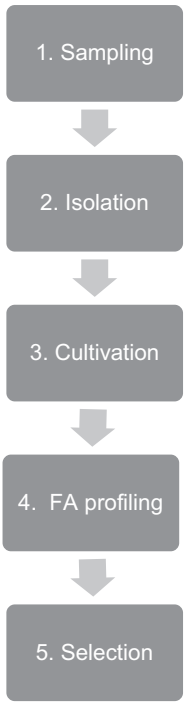

Fig. 3. Screening pipeline including five different steps.

Raunefjorden, and the world's deepest fjord (Sognefjorden) with annual variations in temperature and salinity in the surface layer of between 4 and 16 °C and 26–33, respectively. The photoperiod in Western Norway varies between 19:5 (light:dark) around mid-summer and 6:18 around winter solstice. Hence, microalgae isolated from these environments were exposed to large seasonal variations and fluctuations in salinity, temperature and irradiance, and were thus hypothesized to be robust and fast growing [37], and able to tolerate fluctuations in growth conditions which routinely occur in commercial production ponds. In contrast, the Arctic waters around Spitsbergen are characterized by lower variations in temperatures (−1.8–5.8 °C) and salinities (33.0–35.1) [38], but greater variations in photoperiod, typically ranging from 16:9 (light:dark) in early March to 24:0 after 25th of May, and complete darkness in winter. Microalgae found in these environments are expected to be promising candidates for EPA or DHA production, as the low temperature levels in the Arctic are assumed to cause increasing unsaturation of the FA in microalgae, most likely to maintain membrane fluidity and function [39].

3.2. Isolation and cultivation of clonal cultures

Altogether, 75 samples were sorted on 96-well plates (liquid and solid media) resulting in approximately 7200 individual cells (Table 1). In total, 147 clonal strains (strain designation "M"), constituting 2% of the sorted cells, were established successfully as clonal cultures under the conditions used. A higher fraction of clonal strains was established from Arctic samples (4.6% of sorted cells; M21–M24, M30–M116) compared with fjord samples (1.1% of the sorted cells; M01–M05, M08–M20, M25–M29, M117–M149) among which the least were from Raunefjorden (0.2% of sorted cells). In the fjord systems, isolates were established from spring and summer samples (March, May and August), but not from samples taken in November, possibly due to very low

Table 1
Results of the different steps in the screening pipeline. Strains designated with "M" are clonal strains obtained from single cell isolation by cell sorting flow cytometry. Strains designated with "m" were isolated by serial dilution.

Stat. no.	1. Sampling 2014			2. Isolation	3. Cultivation		4. FA profiling		5. Final selection
	Site	Coordinates	Month	No. of 96-well plates	No. of strains in culture	Strain designation	No. of clonal strains	Strain designation	
Norwegian fjord waters				54	56		10		2
1	Store Lungegårdsvann	N 60° 22.93733′ E 05° 20.17962′	March	4	–	–	–	–	
			May	2	2	M08, M09	–	–	
			Aug.	7	35	M26, M27, M117–M149	2	M26, M27	M26
			Nov.	2	–	–	–	–	
2	Puddefjorden	N 60° 22.86558′ E 05° 19.52838′	March	5	6	M02–M05, M28, M29	3	M04, M28, M29	M28
			May	2	6	M11–M16	–	–	
			Aug.	2	–	–	–	–	
			Nov.	2	–	–	–	–	
3	Raunefjorden	N 60° 16.265′ E 05° 11.456′	March	2	–	–	–	–	
			May	18	1	M10	–	–	
			Aug.	5	5	M01, M17–M20	4	M17,M18,M19,M20	
			Nov.	2	–	–	–	–	
4	Sognefjorden	N 61° 02.467′ E 05° 24.962′	2012 Aug.	1	1	M25	1	M25	
Arctic				21	93		10		1
5		N 80° 45.80′ E 16° 07.20′	March	2	–	–	–	–	
6		N 79° 25.14′ E 08° 18.84′	May	1	3	M21, M22, m06	2	M21, m06	M21
7		N 80° 39.72′ E 15° 26.55′	Aug.	4	3	M23, M24, m07	2	M23, m07	
8		N79° 01.46′ E 06° 02.98′	Nov.	4	–	–	–	–	
9		N 78° 59.33′ E 01° 54.94′	Nov.	2	–	–	–	–	
10		N 79° 01.64′ E 11° 19.49′	Nov.	4	7	M30–M36	–		
11		N 78° 59.66′ E 10° 00.17′	Nov.	4	80	M37–M116	6	M41, M44, M46, M58, M62, M65	
Total no.				75	149		20		3

phytoplankton abundance or fragility of strains. In the Arctic, strains were established from samples taken in May, August and from two stations sampled in November. Additionally, two Arctic strains were isolated by serial dilution of an enrichment culture (m06 and m07) and designated with "m" in order to distinguish them from the clonal isolates, as serial dilution is less accurate when it comes to establishment of cultures from one single cell. Microscopic observations of the isolates allowed for a preliminary morphological characterization of the stock cultures. All ninety-three isolates (Table 1) derived from the Arctic were identified as diatoms. Isolates from the fjords (56) were identified as diatoms (89.3%), Chlorophytes (8.9%) and Cyanobacteria (1.8%).

Diatoms were the main target in our screening pipeline, as they are known to be extremely robust and often comprise a high content of PUFAs like EPA [40]. They are one of the most important primary producers in marine environments, such as Arctic and temperate waters, and may constitute 25% of global primary production [41]. However, even though diatoms were the most highly represented group among the 149 isolated cultures, it was surprising that the diversity among the isolates was so low. Other microalgal groups, such as dinoflagellates [42] and prymnesiophytes (especially *Phaeocyctis pouchetii*) [43], frequently dominate the Atlantic and Arctic waters, but could not be isolated here. The mesh size of the net haul (10 μm) and the physical stress experienced by the cells during single cell isolation by the flow cytometry, are probable reasons for having not collected smaller algae and fragile strains, respectively. Using traditional isolation techniques like a micropipette, agar plating and serial dilutions could minimize cell damage during isolation. However, this was considered an important selection step, as robust strains (which could handle moderate mechanical stress) were preferred. Different types of medium and variation in the growth conditions might also have increased the diversity among the isolates. The conditions were selected so, as to provide a gentle transition of the sampled algae from their natural habitat to the laboratory conditions. Compared to the strains isolated from the fjords, which are expected to be highly tolerant to a rapidly changing environment, microalgae isolated from the Arctic could be more challenging candidates in terms of their decreased flexibility and tolerance to changing conditions, especially with regards to temperature. Culture conditions of 10 °C were higher than the ambient temperature Arctic strains experience in their natural habitat, but were deliberately chosen in order to acclimate the strains to slightly higher temperatures. For consideration for outdoor production, strains need to grow well at higher temperatures, as cooling is one of the main cost factors in large-scale algal production. The application of different isolation methods, media types, and growth conditions might have led to a higher diversity among our isolates, but would have been time- and cost-prohibitive. Thus, the potential risk of loss should be evaluated from a cost/benefit perspective in future bioprospecting studies.

3.3. Batch experiment—strain identification and growth rates

Initially, twenty isolates that (by visual observation) grew most rapidly in stock cultures were selected for further examination of growth rate and total FA (TFA), EPA and DHA contents during both the exponential and the stationary phase. Therefore, ten Fjord isolates (M04, M17–M20, M25–M29, 15 °C) and ten Arctic isolates (m06, m07, M21, M23, M41, M44, M46, M58, M62, M65, 10 °C), were grown in a

Table 2
Phylogenetic classification of the eighteen strains examined in the batch experiment by sequencing a region of the LSU. Asterisks indicate isolates which could not be characterized by molecular methods and were identified at the class level by microscopy only. Strains designated with "M" are clonal strains obtained from single cell isolation by cell sorting flow cytometry. Strains designated with "m" were isolated by serial dilution. ND: not detected.

Origin	Strain	Temp °C	Closest species (GenBank)	Class	Length (bp)	Coverage (%)	Similarity (%)
Fjord	M04	15	*Micractinium* sp. (HE861877.1)	Chlorophyceae	1021	83	88
	M18	15	*Nitzschia laevis* (AF417673.1)	Bacillariophyceae	767	98	100
	M19	15	ND	Chlorophyceae*	ND		
	M20	15	*Arcocellulus cornucervis* (JQ995445.1)	Bacillariophyceae	863	90	96
	M25	15	*Nanofrustulum shiloi* (AB430640.1)	Bacillariophyceae	765	67	99
	M26	15	*Phaeodactylum tricornutum* (EF553458.1)	Bacillariophyceae	830	100	99
	M27	15	ND	Bacillariophyceae*	ND		
	M28	15	*Phaeodactylum tricornutum* (EF553458.1)	Bacillariophyceae	832	100	99
	M29	15	*Phaeodactylum tricornutum* (EF553458.1)	Bacillariophyceae	842	100	99
Arctic	m06	10	*Thalassiosira hispida* (JQ995464.1)	Bacillariophyceae	865	87	100
	m07	10	*Thalassiosira hispida* (JQ995464.1)	Bacillariophyceae	863	88	100
	M21	10	*Attheya septentrionalis* (GQ219678.1)	Bacillariophyceae	896	96	99
	M41	10	*Thalassiosira hispida* (JQ995464.1)	Bacillariophyceae	545	100	100
	M44	10	*Thalassiosira hispida* (JQ995464.1)	Bacillariophyceae	882	86	100
	M46	10	*Thalassiosira hispida* (JQ995464.1)	Bacillariophyceae	863	88	99
	M58	10	*Thalassiosira hispida* (JQ995464.1)	Bacillariophyceae	870	87	100
	M62	10	*Thalassiosira hispida* (JQ995464.1)	Bacillariophyceae	874	87	100
	M65	10	*Thalassiosira hispida* (JQ995464.1)	Bacillariophyceae	871	87	99

controlled batch experiment at continuous irradiance (120–150 μmol photons $m^{-2} s^{-1}$), constant temperature (10 or 15 °C) and bubbled with 1% CO_2-enriched air.

In total eighteen isolates (9 Fjord and 9 Arctic) grew at the defined conditions. Sequencing of parts of the LSU rRNA gene revealed that 15 isolates belonged to the class Bacillariophyceae and one isolate (M04) to the Chlorophyceae. Two isolates (M19 and M27) could not be identified by molecular methods, but were recognized as type Chlorophyceae and Bacillariophyceae by microscopic examination, respectively. Eight of the Arctic strains were identified as *Thalassiosira hispida* (m06, m07, M41, M44, M46, M58, M62, M65) and one as *Attheya septentrionalis* (M21). The Fjord strains showed higher diversity with five different species: the green algae *Micractinium* sp. (M04) and the diatoms *Nitzschia laevi* (M18), *Arcocellulus cornucervis* (M20), *Nanofrustulum shiloi* (M25) and *P. tricornutum* (M26, M28 and M29) (see Table 2 for details).

Growth curves and growth rates differed between the eighteen strains (Fig. 4). Fjord strains (grown at 15 °C) generally grew faster and reached stationary phase earlier than the Arctic strains (grown at 10 °C). Except for strain M21 (*A. septentrionalis*), the Arctic strains (*T. hispida*) had low average growth rates (μ_{avg}) of between 0.2 and 0.3 d^{-1}. Four strains, the three *P. tricornutum* strains (Fjord) and *A. septentrionalis* (Arctic), reached the targeted average growth rate of ≥ 0.7 d^{-1}. Strain M28 had the overall highest growth rate of 1.0 d^{-1}, followed by M29 (0.8 d^{-1}) and M26 and M21, both with 0.7 d^{-1} (Table 3).

3.4. Batch experiment—DW and TFA content

The eighteen strains growing in the batch experiment were sampled for DW and FA analysis in the exponential and the stationary growth phase, with sampling days being chosen individually for each strain (Table 3). Large differences were observed in both DW and TFA content between strains, and between growth phases (Fig. 5A). Interestingly, the two *P. tricornutum* strains M28 and M29, and the green algae strain M04 reached much higher DW in stationary phase compared to the others, with DW of 0.90, 0.85 and 0.83 g L^{-1}, respectively. This response could be due to silicate becoming the limiting nutrient for most of the diatom strains. As green algae do not require silicate to grow and *P. tricornutum* requires only modest amounts [44], other nutrients such as nitrate or phosphate may have been limiting for them at a later stage, thus allowing them to grow to greater cell densities. To verify this, the nutrient composition during the growth experiment would need to be measured. However, the DW of the third *P. tricornutum* strain, M26, was lower in stationary phase (0.44 g L^{-1}) compared to strains M28 and M29. One explanation could be that strain M26 had not accumulated as much storage compound, as it was sampled earlier in the stationary phase (day 11) than M28 and M29 (sampled on day 15), resulting in a comparatively lower DW.

The TFA content relative to the biomass DW (w/w) varied between 5.0 (M19) and 24.1% (M46) in exponential phase, and between 5.0 (M19) and 42.9% (M28) in stationary phase (Table 3). All strains (except for M18 and M19) showed a significant increase in their TFA content relative to the DW from the exponential to the stationary phase ($p < 0.01$), indicating TAG accumulation (Fig. 5A). The sampling time

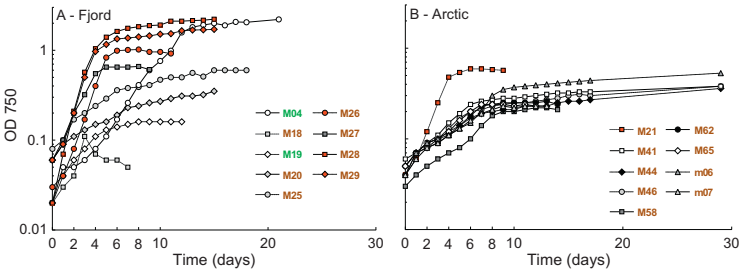

Fig. 4. Growth curves based on optical density measurements (n = 1) at 750 nm of 18 isolates in a batch growth experiment (one biological replicate each) for Fjord isolates (A) and Arctic isolates (B). Each strain was sampled for dry weight and fatty acid analysis during both, the exponential growth phase (day 2 for M27, M28 and M29, day 3 for M21, day 4 for M25 and M26, day 5 for M18 and day 7 for M04, M19, M20, m06, m07, M41, M44, M46, M58, M62 and M65) and the stationary growth phase (last point of the respective growth curve). Red colored growth curves indicate an average growth rate in exponential phase with ≥ 0.7 d^{-1}. Green strain designation highlight the Chlorophyceae and brown the Bacillariophyceae. Strains designated with "M" are clonal strains obtained from single cell isolation by cell sorting flow cytometry. Strains designated with "m" were isolated by serial dilution. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

Table 3
Summary of batch experimental data. Average and standard deviation (SD) of growth rates between two consecutive days during exponential phase based on changes in optical density (OD750, n = 1), sampling day, and average dry weight (DW) and fatty acid (FA) data with SD (n = 3), of the 20 isolates for both the exponential and the stationary phase. Average values and SD are from measurement replicates from one biological replicate. Strains designated with "M" are clonal strains obtained from single cell isolation by cell sorting flow cytometry. Strains designated with "m" were isolated by serial dilution.

No.	Growth rate	Exponential phase								Stationary phase							
		Day	DW	TFA		EPA		DHA		Day	DW	TFA		EPA		DHA	
	μ_{avg} d^{-1}		g L^{-1}	mg L^{-1}	% DW	% DW	% TFA	% DW	% TFA		g L^{-1}	mg L^{-1}	% DW	% DW	% TFA	% DW	% TFA
M04	0.4 ± 0.1	7	0.12 ± 0.02	12.8 ± 0.26	10.3 ± 1.55	0.0	0.0	0.0	0.0	21	0.83 ± 0.02	278.3 ± 6.80	33.5 ± 1.12	0.0	0.0	0.0	0.0
M17	No growth																
M18	0.5 ± 0.3	5	0.12 ± 0.01	15.3 ± 0.18	12.9 ± 0.77	2.00 ± 0.16	15.5 ± 0.75	0.62 ± 0.04	4.85 ± 0.08	7	0.18 ± 0.02	15.7 ± 0.44	8.9 ± 1.17	1.30 ± 0.20	14.6 ± 0.84	0.59 ± 0.08	6.60 ± 0.18
M19	0.4 ± 0.1	7	0.09 ± 0.01	4.6 ± 0.03	5.0 ± 0.49	0.0	0.0	0.0	0	12	0.09 ± 0.00	4.5 ± 0.13	5.0 ± 0.22	0.0	0.0	0.0	0.0
M20	0.2 ± 0.1	7	0.07 ± 0.01	8.03 ± 0.05	11.6 ± 1.43	1.72 ± 0.21	14.8 ± 0.20	0.26 ± 0.03	2.26 ± 0.05	15	0.13 ± 0.00	14.2 ± 0.40	11.3 ± 0.33	1.36 ± 0.04	12.0 ± 0.30	0.23 ± 0.01	2.02 ± 0.06
M25	0.3 ± 0.1	4	0.12 ± 0.01	14.5 ± 0.92	11.9 ± 1.24	0.98 ± 0.14	8.22 ± 0.43	0.0	0.0	18	0.40 ± 0.01	102.9 ± 4.00	25.8 ± 1.20	1.28 ± 0.03	4.98 ± 0.18	0.0	0.0
M26	0.7 ± 0.2	4	0.15 ± 0.01	19.4 ± 0.42	12.9 ± 1.01	3.10 ± 0.25	24.1 ± 0.08	0.49 ± 0.04	3.84 ± 0.02	11	0.44 ± 0.02	112.2 ± 0.62	25.5 ± 1.35	3.40 ± 0.18	13.3 ± 0.01	0.36 ± 0.02	1.41 ± 0.00
M27	0.5 ± 0.1	2	0.08 ± 0.01	14.5 ± 0.17	18.4 ± 2.08	1.40 ± 0.16	7.61 ± 0.07	0.0	0.0	9	0.23 ± 0.01	68.0 ± 0.92	29.3 ± 1.07	2.41 ± 0.10	8.23 ± 0.06	0.0	0.0
M28	1.0 ± 0.3	2	0.08 ± 0.01	10.2 ± 0.69	13.2 ± 1.42	2.66 ± 0.29	20.3 ± 0.08	0.38 ± 0.04	2.91 ± 0.10	15	0.90 ± 0.02	385.7 ± 7.48	42.9 ± 1.13	3.14 ± 0.07	7.33 ± 0.13	0.25 ± 0.01	0.58 ± 0.02
M29	0.8 ± 0.1	2	0.09 ± 0.01	9.7 ± 0.11	11.4 ± 1.37	2.21 ± 0.26	19.4 ± 0.05	0.33 ± 0.04	2.89 ± 0.05	15	0.85 ± 0.01	192.3 ± 17.-22	22.6 ± 2.04	2.30 ± 0.13	10.2 ± 0.33	0.24 ± 0.01	1.06 ± 0.04
m06	0.2 ± 0.2	7	0.10 ± 0.01	11.6 ± 0.08	12.2 ± 1.22	2.70 ± 0.27	22.2 ± 0.16	0.47 ± 0.05	3.87 ± 0.05	29	0.27 ± 0.01	87.3 ± 0.54	32.9 ± 1.09	4.63 ± 0.15	14.1 ± 0.07	0.61 ± 0.01	1.86 ± 0.02
m07	0.2 ± 0.1	7	0.11 ± 0.01	12.0 ± 0.17	11.0 ± 0.82	2.55 ± 0.19	23.0 ± 0.11	0.47 ± 0.04	4.27 ± 0.01	13	0.12 ± 0.01	41.8 ± 0.40	34.2 ± 2.04	3.62 ± 0.22	10.6 ± 0.02	0.47 ± 0.03	1.37 ± 0.01
M21	0.7 ± 0.0	3	0.09 ± 0.01	11.0 ± 0.31	11.8 ± 1.60	3.00 ± 0.40	25.4 ± 0.22	0.56 ± 0.08	4.78 ± 0.08	9	0.20 ± 0.00	37.5 ± 0.19	19.0 ± 0.29	4.58 ± 0.07	24.1 ± 0.15	0.60 ± 0.01	3.17 ± 0.04
M23	No growth																
M41	0.2 ± 0.0	7	0.16 ± 0.02	32.1 ± 0.47	20.2 ± 1.93	3.09 ± 0.30	15.3 ± 0.15	0.56 ± 0.05	2.78 ± 0.04	29	0.25 ± 0.01	75.6 ± 0.79	29.8 ± 1.10	3.65 ± 0.13	12.3 ± 0.11	0.56 ± 0.02	1.88 ± 0.02
M44	0.2 ± 0.1	7	0.12 ± 0.01	23.3 ± 0.63	20.2 ± 2.10	2.78 ± 0.28	13.7 ± 0.10	0.52 ± 0.05	2.59 ± 0.01	29	0.25 ± 0.01	77.2 ± 0.50	28.7 ± 0.91	2.44 ± 0.08	8.52 ± 0.05	0.28 ± 0.01	0.96 ± 0.01
M46	0.2 ± 0.1	7	0.16 ± 0.0	38.8 ± 0.34	24.1 ± 0.71	2.81 ± 0.09	11.7 ± 0.09	0.49 ± 0.02	2.04 ± 0.03	29	0.29 ± 0.03	96.0 ± 0.60	31.0 ± 3.38	2.74 ± 0.30	8.84 ± 0.03	0.27 ± 0.03	0.88 ± 0.01
M58	0.3 ± 0.0	7	0.10 ± 0.01	7.4 ± 0.09	7.7 ± 0.53	1.86 ± 0.13	24.3 ± 0.14	0.45 ± 0.03	5.94 ± 0.06	14	0.14 ± 0.01	47.3 ± 2.44	33.2 ± 3.90	3.24 ± 0.38	9.74 ± 0.13	0.37 ± 0.04	1.12 ± 0.02
M62	0.2 ± 0.1	7	0.15 ± 0.02	23.0 ± 0.38	15.5 ± 1.91	2.75 ± 0.34	17.7 ± 0.19	0.55 ± 0.07	3.52 ± 0.06	14	0.18 ± 0.02	62.9 ± 3.86	35.7 ± 5.19	4.10 ± 0.56	11.5 ± 0.24	0.47 ± 0.06	1.33 ± 0.05
M65	0.3 ± 0.1	7	0.14 ± 0.04	28.2 ± 0.28	20.8 ± 5.46	3.22 ± 0.85	15.5 ± 0.19	0.60 ± 0.16	2.90 ± 0.06	12	0.17 ± 0.01	63.3 ± 0.24	38.1 ± 1.18	3.77 ± 0.12	9.89 ± 0.08	0.45 ± 0.01	1.19 ± 0.00

point in exponential phase (day 7) was unfortunately chosen slightly too late for some *T. hispida* strains, as most had already entered early stationary phase except for strains M58, m06 and m07, as can be seen in Fig. 4. This was also reflected in the TFA content, which was much lower in exponential samples for strains M58, m06 and m07 (7.7, 12.2, and 11.0% DW, respectively), than for the others with TFA between 15.5 and 24.1% DW. However, the TFA content increased further in stationary phase (between 28.7 and 38.1% DW) in all *T. hispida* strains. The three *P. tricornutum* strains M26, M28 and M29 had a very similar TFA content in the exponential phase (between 11.4 and 13.2% DW), but showed differences in the stationary phase. M28 had with 42.9% DW a much higher TFA content compared to M26 (25.5%) and M29 (22.6%). As mentioned above, M26 was sampled earlier in stationary phase (day 11), thus its lower TFA content is probably due to lower level of TAG accumulation. However, M29 was sampled on the same day as M28 (day 15) and also reached a similar DW, and thus seems to differ in its physiological response to nutrient starvation.

3.5. Batch experiment—EPA and DHA content

EPA and DHA content in the exponential and the stationary phase of the eighteen strains grown in the batch experiment are presented in percentage (w/w) relative to biomass DW and to TFA (Fig. 5B, and C, respectively). Both EPA and DHA were present in all Arctic strains investigated, but not in all strains isolated from the fjords. EPA was not detected in the two chlorophytes M04 and M19. The absence (or only insignificant amounts) of EPA has been reported for some green algae previously, while high levels of EPA have been found in other green algae species [45]. All sixteen diatom strains possessed EPA, but in different concentrations. The EPA content relative to the biomass DW ranged between 1.0 (M25) and 3.2% (M65) in exponential, and between 1.3 (M25) and 4.6% (m06) in stationary phase. Strain M18 showed a significant decrease, whereas the five strains M27, m06, m07, M21 and M58 revealed a significant increase in their DW-based EPA content from exponential to stationary phase ($p < 0.01$). In contrast to this, the EPA content in percentage of TFA decreased significantly in all strains, except in M18 and M27 ($p < 0.01$), from exponential to stationary phase, possibly caused by accumulation of TAG. TAGs mostly comprise the saturated and monounsaturated FA and therefore TAG accumulation causes the relative amount of PUFAs like EPA to decrease in stationary phase [13]. Values varied between 7.6 (M27) and 25.4% TFA (M21) in exponential phase and between 5.0 (M25) and 24.1% TFA (M21) in stationary phase. Nine strains (7 Arctic, 2 fjord), comprising 50% of the isolates that grew in the experiment, obtained the targeted EPA content of 3% DW in either exponential or stationary phase or both.

DHA was found in fourteen isolates, and was not detected in the chlorophytes M04 and M19, and the two diatoms M25 and M27. The DHA content relative to DW was low, and varied between 0.26 (M20) and 0.62% (M18) in exponential, and 0.23 (M20) and 0.61% (m06) in stationary phase, and remained on the same level or decreased (M26, M28, M44, M46) from exponential to stationary phase. DHA content relative to the TFA content ranged between 2.0 (M46) and 5.9% (M58), and between 0.6 (M28) and 6.6% (M18) in exponential and stationary phase, respectively. A significant increase in M18 and a decrease in all other strains from exponential to stationary phase was observed. None of the strains reached the targeted DHA content relative to the DW.

Eight *T. hispida* strains and three *P. tricornutum* strains were investigated in the batch experiment. Interestingly, both the different *T. hispida* strains and the *P. tricornutum* strains showed differences in their growth, and TFA and EPA content that could only be explained to a certain degree by the different sampling times. This emphasizes that variations can be observed at the strain level, and it is therefore advisable to test multiple strains of specific species. This has also been reported before [20].

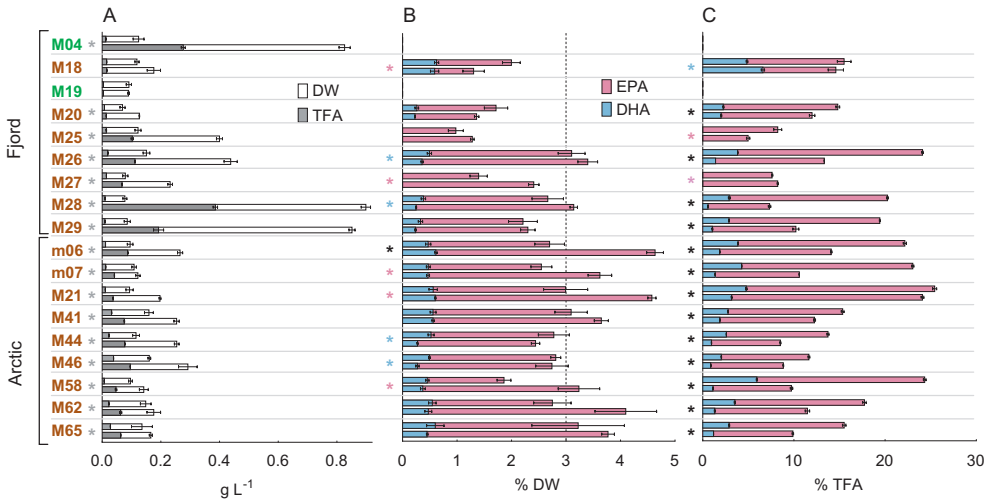

Fig. 5. Superimposed dry weight (DW) and total fatty acid (TFA), EPA and DHA content of eighteen strains in the exponential (upper bar) and the stationary growth phase (lower bar) during the batch experiment. Data are average values with standard deviation (n = 3) of measurement replicates from one biological replicate. A: Average DW and TFA concentration. B: Average EPA and DHA amount relative to DW. C: Average EPA and DHA amount relative to TFA. The dotted vertical line marks the particular benchmark level for EPA and DHA. Strains identified as Chlorophyceae are displayed in green and strains belonging to the class Bacillariophyceae in brown. Asterisks in front of the bars indicate significant difference between the exponential and the stationary phase (*t*-test, $p < 0.01$), in grey for TFA (relative to DW), red for EPA, blue for DHA and black for both EPA and DHA. Strains designated with "M" are clonal strains obtained from single cell isolation by cell sorting flow cytometry. Strains designated with "m" were isolated by serial dilution. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

3.6. Batch experiment—relative TFA composition

The relative TFA composition of microalgae is often used as a taxonomic indicator, as different microalgal classes are characterized by a specific FA profile [46]. Fig. 6 shows an overview on the FA composition as a percentage of TFA for the eighteen strains that grew in the batch experiment (FA with > 2% TFA in at least one strain are listed). The two chlorophytes, M04 and M19, clearly differed from the diatom strains. They had no, or only negligible amounts of FA with a carbon chain length higher than C_{18}, besides having C_{18} mono- and

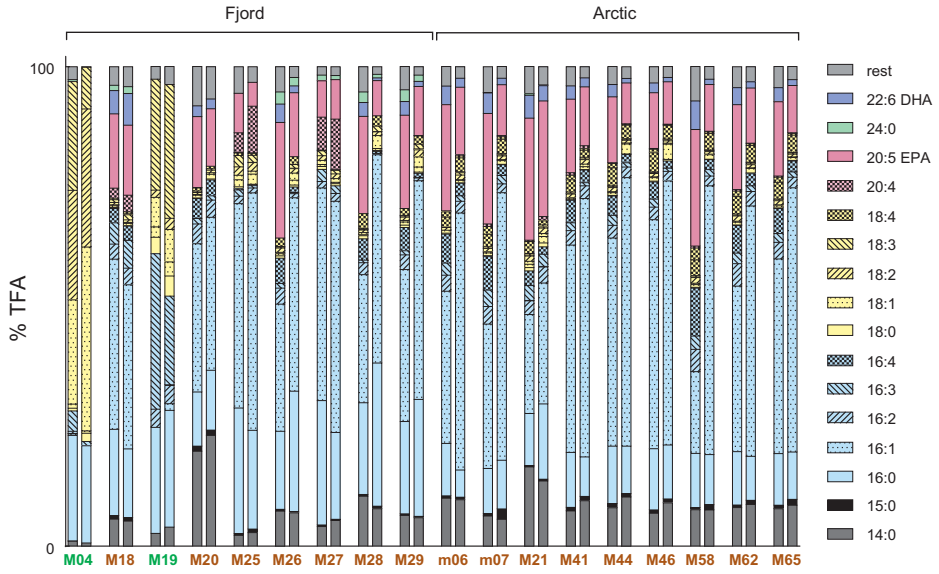

Fig. 6. Relative fatty acid composition of the 18 isolates for both the exponential (first bar) and the stationary (second bar) phase during the batch experiment. Data are average values (n = 3) of measurement replicates from one biological replicate. FA with amounts < 2% of total fatty acids (TFA) in all strains are summarized in rest. Strains identified as Chlorophyceae are displayed in green and strains belonging to the class Bacillariophyceae in brown. Strains designated with "M" are clonal strains obtained from single cell isolation by cell sorting flow cytometry. Strains designated with "m" were isolated by serial dilution. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

polyunsaturated FA as their major FA. All diatoms showed a similar overall picture of the FA composition, with C_{16} FA representing the most abundant, followed by LC-PUFAs (C > 20) and myristic acid (14:0), and low levels of C_{18} derivatives. Similar results were found by Zhukova & Aizdaicher (1995) [46], with a preference of palmitoleic acid (16:1) over palmitic acid (16:0), and high levels of myristic acid and EPA, together with an insignificant amount of C_{18} FA for diatoms. However, some differences among the diatom species were observed in our study. Only strains M18, M21, M25 and M27 possessed arachidonic acid (20:4) in amounts higher than 2% TFA, whereas only trace amounts were detected for all other diatoms. M18, M27 and the three *P. tricornutum* strains (M26, M28 and M29) were the only diatoms with lignoceric acid (24:0), and DHA was not detected for two diatoms (M25 and M27). In the future, when additional FA data from more strains are available, a cluster analysis of the FA composition data could reveal, if and to what degree the FA profile reflects the phylogenetic classification of the microalgae.

Most strains showed variations in the relative FA abundance between the exponential and the stationary phase, with palmitoleic acid (16:1) content predominantly increasing in stationary phase, and PUFAs such as EPA and DHA accounting for a higher fraction of TFA in the exponential phase for most, but not all strains. This correlates favorably with the aforementioned typical pattern of TAG accumulation, as TAGs comprise mostly saturated and monounsaturated FA, whereas PUFAs are typically present in membrane lipids [13]. However, in some microalgae, TAGs can be another, but much less common PUFA source [12,47]. TAG accumulation together with an incorporation of EPA and DHA into the TAGs have been observed, for example, in *Thalassiosira pseudonana* and *Pavlova lutheri* (Prymnesiophyceae) in the stationary growth phase [48].

3.7. Selection of candidate strains

Based on our findings and selection criteria for growth rate ($\geq 0.7\ d^{-1}$) and EPA/DHA content ($\geq 3\%$ DW), the three strains M21 (*A. septentrionalis*) and M26 and M28 (*P. tricornutum*) were selected, and recommended for further characterization with respect to upscaling and future utilization in microalgae-based technologies. Strain M21 seemed especially interesting and promising, as it had a high growth rate of $0.7\ d^{-1}$ at low temperatures (10 °C) and a high EPA content relative to biomass DW, which increased in stationary phase to 4.6%. Similar, but slightly lower growth rates ($0.6\ d^{-1}$) at 8.5 °C have been reported for another *Attheya* species, *A. longicornis*, isolated from northern Norwegian coastal waters [49]. Additional investigations might reveal if it is possible to increase the EPA levels further, suggesting sampling later in the stationary phase and varying growth conditions. As we did not discriminate between polar and non-polar lipids in this study, such analyses might provide information about which lipid fraction the EPA accumulates in, during the stationary phase. High growth rates and different EPA levels have been reported for *P. tricornutum* strains previously; Jiang & Gao (2004) [35] and Patil et al. (2006) [4] found an EPA content of 2.6 and 2.8% DW respectively, which is in a similar range as our findings. The two *P. tricornutum* strains selected in our study differed in their EPA content, with M26 reaching higher amounts (3.1% DW) in exponential phase than M28 (2.7% DW), but at the same time having a lower growth rate. Thus, further experiments will be needed to show which combination will result in a higher EPA productivity.

The selected strains are adapted to, and have so far been tested at only one growth condition, but in future upscaling and outdoor large-scale productions, growth conditions may differ from those used in our experiments. In this case higher media nutrient concentrations are necessary in order to reach higher biomass densities, and temperatures and irradiance will vary, both being important factors that regulate the relative content of FA and the absolute quantity of EPA [33]. Therefore, the influence of different growth factors (e.g. irradiance, temperature and nutrient content) on the growth rate and the EPA content, and thus EPA productivity, needs to be investigated in future studies.

3.8. Evaluation of the pipeline

The growth and FA profiling of the isolates was determined to be the rate-limiting step of our pipeline. Despite the experiment design being one of simplicity and speed, considerable time was needed in order to evaluate the FA content and composition for each isolate, as no rapid methods currently exist without conducting FA extraction and GC analysis. Furthermore, the fact that the strains were sampled at different time points during their exponential and stationary growth phase makes empirical comparisons difficult. However, the growth characteristics of the individual isolates were very different, and therefore appropriate sampling time points had to be estimated during the ongoing experiment.

Nevertheless, the first screening results obtained in this study are promising, and by continuing to investigate more isolates, additional information can be surmised from our data. Besides discovering new candidate strains with potential to be used in biotechnological applications, statistical analysis of a subsequent larger-scale dataset could reveal which of the factors (sampling location, time of the year, microalgae species or culture conditions) have the most beneficial effect on the bioprospecting success. This would help greatly improve future sampling strategies and also give a much more detailed insight into the ecophysiological properties of the microalgae.

4. Conclusion

A screening pipeline was developed in order to find new and promising North Atlantic microalgal strains to be used in biotechnological application targeting the production of the omega-3-fatty acids EPA and DHA. One hundred and forty nine different isolates (comprising mostly diatoms) were established as stock cultures, and twenty of these were investigated in terms of their growth rates and EPA/DHA content. Arctic strains generally had a higher EPA content than fjord strains, but lower growth rates. Promising data were found for three strains represented by the diatoms *Phaeodactylum tricornutum* (fjord) and *Attheya septentrionalis* (Artic), which showed high growth rates ($\geq 0.7\ d^{-1}$), together with an increased EPA content (> 3% DW), suggesting their potential use in microalgae-based technologies and EPA production. Similar findings have been described for *P. tricornutum* previously, and this species has widely been used in aquaculture [4,35,36]. Yet, to our knowledge, this is the first report on a high EPA content for *A. septentrionalis*; with an EPA content of 4.6% DW in stationary phase, this value is higher than those typically reported from industrially-applied microalgae (*Nannochloropsis* sp. 2.1–3.8%, *P. tricornutum* 2.6–3.1%). However, as those EPA values are generally derived from exponentially-grown algae, the EPA production potential of *A. septentrionalis* must be further evaluated, as growth rates in the stationary phase are low. This study highlights the value of establishing a screening pipeline with a targeted focus on productive microalgae strains from northern high latitude waters.

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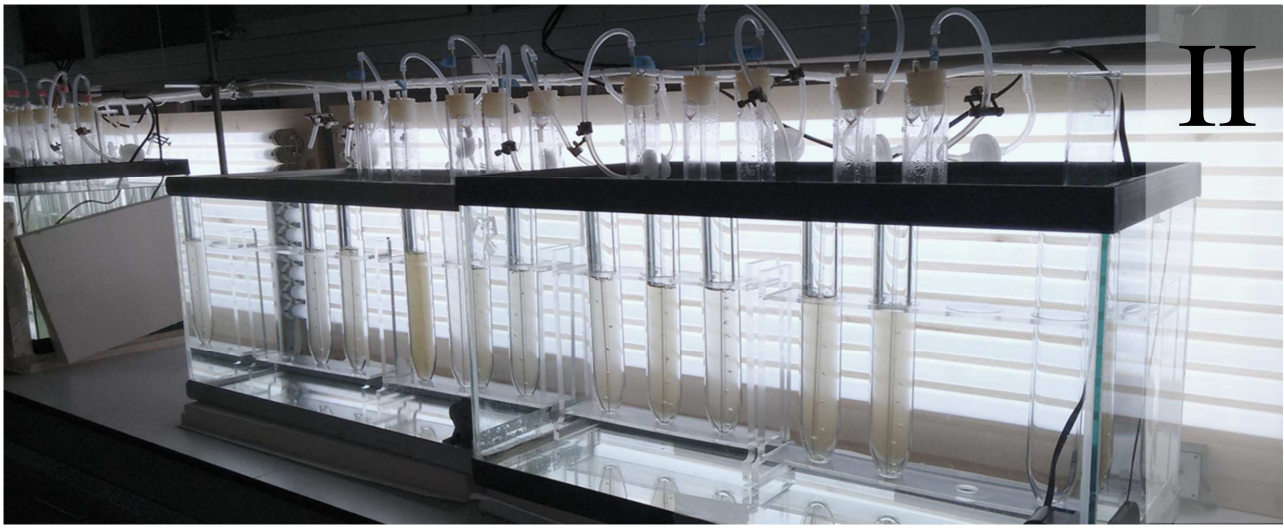

II

Enhancing EPA content in an Arctic diatom: A factorial design study to evaluate interactive effects of growth factors

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Abstract

Microalgae with a high content of the omega-3 polyunsaturated fatty acids (PUFAs), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are of great demand for microalgae-based technologies. An Arctic strain of the diatom *Attheya septentrionalis* was shown in previous experiments to increase its EPA content from 3.0% to 4.6% of dry weight (DW) in the nutrient-replete exponential phase and nutrient-depleted stationary phase, respectively. In the present study, a factorial-design experiment was used, to investigate this effect in more detail and in combination with varying salinities and irradiances. A mathematical model revealed that both growth phase and salinity, alone and in combination, influenced the EPA content significantly. Maximum EPA values of 7.1% DW were obtained at a salinity of 22 and after five days in stationary phase, and might be related to a decreased silica content, an accumulation of storage lipids containing EPA, or both. However, growth rates were lower for low salinity (0.54 and 0.57 d^{-1}) than high salinity (0.77 and 0.98 d^{-1}) cultures.

1 Introduction

The omega-3 polyunsaturated fatty acids (PUFAs), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are known to contribute significantly to human health (Martins et al., 2013). The current major source for the two fatty acids (FA) is fish oil from marine wild fish. The fish obtain and accumulate these PUFAs themselves predominantly via the marine food chain from EPA- and DHA-synthesizing microalgae (Spolaore et al., 2006). As EPA and DHA are also essential for farmed fish (Khozin-Goldberg et al., 2016), fish oil is an important additive in aquaculture feed. The increasing demand for EPA and DHA, particularly from the growing aquaculture industries, but also increasingly from the health and food sectors, necessitates other sustainable production sources. Many marine microalgae naturally produce EPA and DHA, and are therefore considered a promising alternative (Chauton et al., 2015; Patil et al., 2005). Although there has been intensive research in this field, the costs associated with microalgae large-scale cultivation and processing for FAs are still greater than in fish oil production. Improvements at the different parts of the production chain are therefore essential in order to reduce production costs. The selection of suitable species that are superior to existing cultures in terms of growth and EPA and DHA content, and the optimization of cultivation conditions are important contributions to the ongoing improvement of microalgae-based technologies (Adarme-Vega et al., 2012).

In previous research, we searched for new and fast growing microalgal strains from North Atlantic habitats with high growth rates and high EPA and DHA contents (Steinrücken et al., 2017). A strain of the diatom *Attheya septentrionalis*, isolated from Arctic Waters, demonstrated rapid growth at temperatures of 10 °C (0.7 d^{-1}) and a high EPA content, which increased from 3.0 to 4.6% of the dry weight (DW) from exponential phase to Day 3 of the stationary phase. This EPA content under nutrient-depleted conditions was higher than typically found in industrially applied microalgae and this strain was therefore suggested to be a potential candidate for future large-scale cultivation and EPA production. A high growth rate and EPA content in stationary phase in *A. septentrionalis* were also found by Knuckey et al. (2002) who suggested this diatom to be a promising feed source for oyster hatcheries. However, only few studies on *Attheya* species exist (Aizdaicher and Markina, 2011; Stonik et al., 2017), and more explicit investigations are needed in order to assess the potential of this diatom for microalgae-based technologies.

It is well known that microalgae modify their biochemical composition and FA content in response to environmental factors, including nutrient availability, irradiance, temperature and salinity (Boelen et al., 2013; Cepák et al., 2013; Dunstan et al., 1993; Renaud and Parry, 1994; Tatsuzawa and Takizawa, 1995; Van Wagenen et al., 2012; Xu and Beardall, 1997). To investigate the impact of the different factors, traditional methods vary one condition at a time, while keeping all other factors constant. However, FA composition and content in microalgae are dependent on synergistic and antagonistic interactions of cultivation conditions. Factorial designs are based on a multivariate approach, in which the variation in different factors are tested simultaneously (Duarte et al., 2001). These yield a predictive model which provides information on the magnitude of the effects of both

individual factors and combinations of factors, and on their statistical significance (Chen et al., 2012).

In this study, we aimed to elucidate additional information on the dynamics of the EPA content and relative FA composition in *A. septentrionalis*, and to determine the experimental conditions that might lead to a further increase in the EPA content. A factorial-design experiment was used to investigate the impact of nutrient starvation in greater detail, together with the effects of salinity and irradiance, and their respective interactions. Both salinity and irradiance are known to affect the FA composition in microalgae (Chen et al., 2008; Lu et al., 2001; Xu and Beardall, 1997), and are highly variable in Arctic environments due to melting and freezing sea ice, and strong variations in photoperiod. Hence, microalgae from these environments are expected to be promising in this context, and possess the necessary adaptations to cope with these changing conditions.

2 Methods

2.1 Stock cultures and inoculum

Attheya septentrionalis is a single celled diatom with four long setae, and is broadly distributed in Arctic and Temperate Waters (Rampen et al., 2009; Stonik et al., 2017). The strain used in this experiment was isolated from Arctic Waters north-west of Spitzbergen (N 79° 25.14` E 08° 18.84`) in May 2014 (3.5°C water temperature, salinity of 35, and 24 h daylight). For strain characterization, a region of the 28S ribosomal RNA (rRNA) gene was sequenced and compared with previously published sequences from diatoms at GenBank (Steinrücken et al., 2017). Partial sequence of the 28S rRNA gene have been deposited in GenBank (http://www.ncbi.nlm.nih.gov/) with accession number MH020639. Stock cultures were maintained in 50 mL Erlenmeyer flasks at 10 °C and 50 µmol photons m^{-2} s^{-1}, in nutrient-enriched (Walne, 1970) 80% seawater (SW) by monthly dilution. 80% SW was obtained by dilution of filtered and autoclaved fjord-SW (from 90 m depth, salinity of 35) with distilled water (80:20, v:v), giving a salinity of 29. For the inoculum, biomass was harvested from exponential phase stock cultures by centrifugation (2264 × g, 5 min), washed twice with fresh medium and re-inoculated into 10 mL fresh medium.

2.2 Experimental design

Factorial design was used to investigate the effects of salinity, irradiance and growth phase and their interactions on the EPA and total fatty acid (TFA) content, and the FA composition in the diatom *A. septentrionalis,* by growth of batch cultures at different conditions. The effects of salinity and irradiance were assessed at two levels (22 and 35, and 50 and 200 µmol photons m^{-2} s^{-1}, respectively), and the growth phase at three levels; exponential phase (e), Day 3 of stationary phase (first stationary phase, s1), and Day 5 of stationary phase (second stationary phase, s2), resulting in twelve treatment groups. Salinity and irradiance levels were selected as being representative of those occurring under natural conditions in the Arctic and additionally, so as to induce different responses but not to impair the cultures. Before the start of the batch experiment, pre-cultures (one

biological replicate for each condition) were grown semi-continuously for 14 d at either low or high salinity and low or high irradiance (LSLI, HSLI, LSHI and HSHI), to acclimate the cultures to their respective conditions. Two sterile and nutrient enriched media (Walne, 1970) were prepared with aged SW (salinity of 35) and respective dilutions with distilled water (salinity of 22). Pre-cultures were prepared in glass tubes (300 mL, 3.5 cm inner diameter), by adding 1 mL inoculum to 60 mL fresh medium of the respective salinity, and placed into temperature-controlled water tanks (10 °C). Continuous illumination with 50 or 200 µmol photons m^{-2} s^{-1} (measured with 4π quantum scalar irradiance sensor [QSL-100, Biospherical Instruments, San Diego, CA, USA], inside the empty glass cylinder) was provided by banks of six white fluorescent tubes (Philips MASTER, TL-D 90 Graphica, 58W/95) in the back of the water tanks, running perpendicular to the glass tubes. To ensure adequate mixing and carbon supply, 0.2 µm-filtered and 1% CO_2-enriched air was bubbled through glass capillaries into the bottom of each glass tube. Pre-cultures were kept in exponential phase by maintaining an optical density (OD_{750}) between 0.15 and 0.50, by the addition of fresh medium, successively added until volumes reached 260 mL. Thereafter, half of the culture volume was replaced with fresh medium daily or every other day. After the two-week acclimation period, the batch experiment was started. Biomass from each pre-culture was distributed into two sterile glass tubes to obtain two biological replicates, and each diluted with fresh medium to yield a starting OD and volume of approximately 0.15 and 260 mL, respectively. Cultures were then grown until Day 5 of the stationary phase, and all cultures were sampled daily for OD and maximum quantum yield (QY), and on Day 1 in exponential phase (e) and Days 3 and 5 in stationary phase (s1 and s2, respectively) for DW and FA analysis. Additionally, cultures were sampled for nutrient analysis of the media at the start of the experiment, and on the DW and FA sampling days.

2.3 Analytical procedures

Relative growth rates between repeated dilutions during pre-cultivation were calculated according to the changes in attenuation with Eq. [1]. N_{x0} and N_x are OD_{750} after dilution (t_{x0}) and before the subsequent dilution (t_x), respectively.

$$\mu\,(d^{-1}) = \frac{\ln(N_x) - \ln(N_{x0})}{(t_x - t_{x0})} \qquad [1]$$

Optical density measurements were performed using a spectrophotometer (UV1800, Shimadzu Corporation, Kyoto, Japan) at 750 nm and if required, samples were diluted to give an attenuation between 0.2 and 0.8. The QY was measured with AquaPen (AquaPen-C, AP-C 100, Photon System Instruments, Brno, Czech Republic) after initial dark incubation between 10 and 60 min. DWs (expressed as weight of the dried biomass [g] per volume [L]) were determined in triplicates as described by Zhu and Lee (1997), with 0.5 M ammonium formate as a washing buffer.

For FA analysis, triplicate 10 mL microalgal cultures were harvested by centrifugation (6 min at 2264×g) into glass tubes (PYREX), the supernatant discarded, and the pellet covered in nitrogen atmosphere and stored at −20 °C until analysis. FAs were extracted and derivatized to fatty acid

methyl esters (FAME) by direct esterification (Meier et al., 2006). The pellet was dried in the 10 mL tube by evaporating the remaining water under a nitrogen stream, and 18 or 34 µg internal standard (23:0 FAME dissolved in isooctane) was added to exponential or stationary phase samples, respectively. The solvent was evaporated under nitrogen stream and 0.5 mL methylation reagent (2M HCl in methanol) was added. Samples were covered with nitrogen gas, sealed and incubated at 90 °C for 2 h. After cooling to room temperature, half of the methylation reagent was evaporated, 0.5 mL water was added, and the samples were extracted twice with 1 mL isooctane. The combined extracts of stationary phase samples were diluted with isooctane (1:1, v:v) to yield a final internal standard concentration of approximately 18 µg mL^{-1} (Steinrücken et al. 2017). FAMEs were analyzed by GC (7890 gas chromatograph, Agilent, Santa Clara, CA, USA) equipped with an autosampler, split-splitless injector, flame ionization detector (FID) and a 60 m BPX70 capillary column (SGE, Ringwood, Australia) with internal diameter of 0.25 mm and film thickness of 0.25 µm. 1 µL sample volumes were injected splitless at 60 °C. This temperature was maintained for 3 min before raised by 40 °C min^{-1} to 150 °C and by 1.5 °C min^{-1} to 230 °C. Helium, with an estimated average velocity of 30 cm s^{-1} was used as carrier gas in constant flow mode. Injector and detector temperatures were 250 °C and 300 °C, respectively (Prestegard et al., 2015). The FAMEs were identified by analysis on gas chromatography coupled to mass spectrometry (GC-MS) as described in (Wasta and Mjøs, 2013), and by using libraries of mass spectra and retention indices available at www.chrombox.org/data.

For nutrient measurements of the media, 20 mL GF/F filtrates were collected in white plastic vials, 100 µl chloroform added and stored at 4 °C before analysis. Dissolved inorganic nitrate, nitrite, orthophosphate and silicate were analyzes at the Institute of Marine Research, Bergen, which offers accredited and standardized service for nutrient analyses, using colorimetric absorption measurements on an Alpkem-Lab analyzer (Alpkem Corporation, Oregon USA) according to Parsons et al. (1992).

2.4 Regression models

The model for growth rate as a function of salinity, irradiance and their interactions, and models for TFA and EPA content as functions of salinity, irradiance, growth phase and their interactions were calculated by multiple least squares regression. The models reported in the paper are based on the coded factor levels, where the low values are assigned -1 and the high values are assigned +1. For the growth phase, there are three levels; exponential phase, and first and second stationary phase. Exponential and second stationary phase were assigned the levels -1 and +1, respectively. The level for first stationary phase was set to 0.74. This level was found by iteratively testing values from -1 to 1 with increments of 0.01, and selecting the value that minimized the sum of squared residuals of the model. Models and model statistics were calculated by the fitlm function in the Statistics and Machine Learning Toolbox running under Matlab R2017a (Mathworks, Natick, MA, USA).

2.5 Statistics

The batch experiment was performed with two individual cultures (biological replicates) for each treatment, which is sufficient for solid statistics when using regression analysis and factorial design. One measurement replicate was taken for OD and QY measurements, whereas FA and DW were analyzed in triplicates for each biological replicate. The FA content and the DW were analyzed from individual subsamples, and the standard deviation (SD) for FA content relative to the DW was calculated using Eq. [2].

$$SD_{FA/DW} = \sqrt{\frac{\%SD_{FA}{}^2 + \%SD_{DW}{}^2}{100}} \times FA/DW \quad [2]$$

Euclidean dendrograms and Principal Component Analysis (PCA) of treatment groups and their FA composition were calculated using Sirius 10.0 (Pattern Recognition Systems AS, Bergen, Norway) and edited in GraphPad Prism 6.

3 Results

3.1 Pre-cultivation - growth rates

Pre-cultures were grown for two weeks to allow for cells to acclimate to the respective salinities and irradiances. After three dilutions, growth rates for each condition became more constant although they still varied slightly between the repeated dilutions. Only growth rates from the final seven dilutions prior to the batch experiment were used for analyses (Table S1). Average values, together with the estimates provided by the mathematical model, are shown in Fig. 1A. Lower growth rates (0.54 d^{-1} for low irradiance [LI] and 0.57 d^{-1} for high irradiance [HI]) were observed for low salinity (LS) cultures, compared to high salinity (HS) cultures (0.77 d^{-1} for LI and 0.98 d^{-1} for HI), together with increased values for HI cultures. High irradiance had a stronger positive effect on the growth rate at HS. The mathematical model representing the growth rate as a function of salinity (X1), irradiance (X2) and their combination (X1X2) in the experimental setup is expressed by Eq. [3]. Positive coefficients in the equations indicate that increasing values increase the growth rate and bold numbers indicate a high statistical significance (black: $p < 0.05$ and red: $p < 0.01$). According to the model, salinity had the strongest positive influence on the growth rate, with high significance (0.163, p-value $1.4*10^{-09}$), while irradiance (0.062, $p = 0.001$) and the combination of salinity and irradiance (0.043, $p = 0.016$) had lower, but still significant, impacts. A strong and significant correlation ($R^2 = 0.8484$) between the estimated values of the model and the measured values indicates a good fit between the model and the experimental data (Fig. 1B). Details on the measured and estimated growth rates can be found in the Supplementary section (Table S1).

$$\mu\,(d^{-1})_{est} = 0.715 + \mathbf{0.163} * \boldsymbol{X1} + \mathbf{0.062} * \boldsymbol{X2} + \mathbf{0.043} * \boldsymbol{X1X2} \quad [3]$$

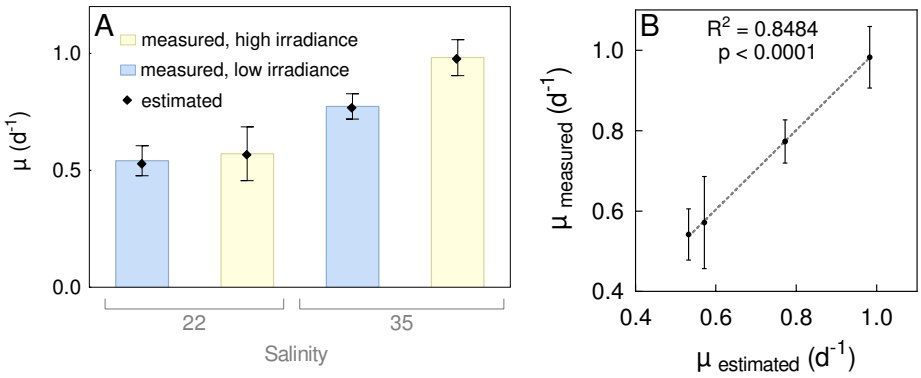

Fig. 1. Growth rates (μ) during pre-cultivation. A: Average growth rates of repeated dilutions (bars, n=7) and estimated growth rates by the model (dots) of the diatom *Attheya septentrionalis* grown at four different cultivation conditions (combinations of low or high salinity [22 and 35] and irradiance [50 and 200 μmol photons m^{-2} s^{-1}]. **B**: Average and standard deviation (n=7) of measured growth rates, plotted against the estimated growth rates by the model, with linear regression.

3.2 Batch experiment – growth and maximum quantum yield (QY)

Growth curves and QY during the batch experiment were very similar for the biological replicates, but there were differences between the cultivation conditions, especially between low and high salinities (Fig. 2A and B). Cultures grown at LS, revealed a declined slope of the growth curves and reached stationary phase one day later (Day four) than cultures grown at HS (Day three). During exponential growth, the QY was slightly lower for the LS cultures (replicate averages of 0.64 at Day one, both for low and high irradiances) than for the HS cultures (replicate averages of 0.69 and 0.68 at Day one, for low and high irradiance, respectively). During stationary phase, the QY decreased in all cultures. In HS cultures, QY dropped by 10% (LI) and 30% (HI) after entering stationary phase, and by 23% and 32%, respectively, at the end of cultivation period. A lower reduction was observed for the LS cultures, where QY was reduced by 6% and 8% (low and high irradiance, respectively) after entering stationary phase, and by 14% and 17% at the end of cultivation.

The nitrate, silicate and phosphate concentrations of the media decreased from the start of the experiment to the first day of exponential phase by 14%, 39% and 27% for low salinity cultures and by 23%, 45% and 38% for high salinity cultures, respectively (Fig. 2C and D). At the first stationary phase (Day 3), all nutrients had been consumed in all cultures (nitrate 99%, silicate 99% and phosphate 97%).

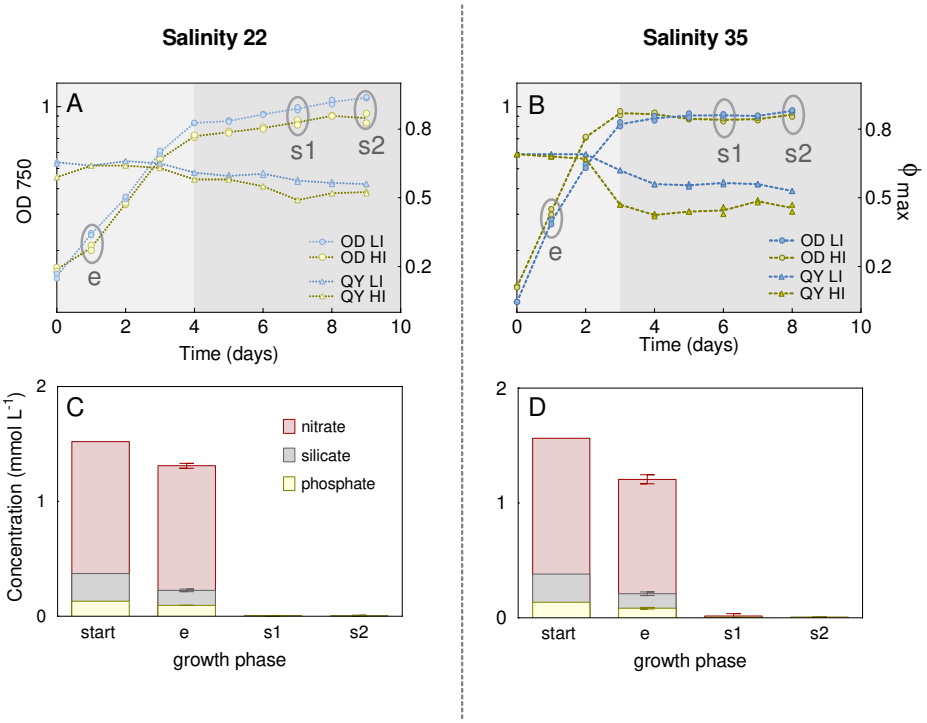

Fig. 2. Growth during batch experiment. Batch cultures of the diatom *Attheya septentrionalis* grown at four conditions (combinations of low and high salinity [22 and 35] and irradiance [50 and 200 µmol photons m^{-2} s^{-1}] with two biological replicates for each condition). **A** & **B**: Optical density (OD_{750}) based growth curves and maximum quantum yields (QY) for low and high salinities, respectively. Circles indicate sampling time points (e: exponential phase, s1: first stationary phase, s2: second stationary phase). LI: low irradiance HI: high irradiance. **C** & **D**: Superimposed nutrient concentration in the media at the start of the experiment and during exponential (e) and stationary (s1 and s2) sampling time points for low and high salinities, respectively. Values are the averages and standard deviations from samples of the respective salinity.

3.3 Batch experiment - dry weights (DW), total fatty acids (TFA) and EPA

DW together with TFA and EPA contents (% DW) for the twelve different treatment groups, each with two biological replicates are shown in Fig. 3. The DW (average of the biological replicates) increased from the exponential to the first stationary phase by 0.13, 0.12, 0.13 and 0.11 g L^{-1} and further by 0.04, 0.03, 0.01, 0.02 g L^{-1} to the second stationary phase, giving a total increase of 0.17, 0.15, 0.14 and 0.13, g L^{-1} for low salinity-low irradiance (LSLI), high salinity-low irradiance (HSLI), low salinity-high irradiance (LSHI) and high salinity-high irradiance (HSHI) conditions, respectively (Fig. 3A).

The TFA and EPA contents (Fig. 3B and C, respectively) increased from the exponential to the stationary phase at all growth conditions, but to different extents. In exponential phase, EPA contents (average of replicates) were 2.8, 3.2, 2.4 and 3.1% DW for LSLI, HSLI, LSHI and HSHI conditions, respectively, and increased to 6.8, 5.7, 5.8 and 5.5% DW, respectively, in the first stationary phase. For LSLI, LSHI and HSHI cultures, EPA content increased further to 7.2, 7.1 and 5.8% DW, respectively, in the second stationary phase, while it decreased in the HSLI conditions to 5.2% DW. A similar pattern with increase from the exponential phase to the first stationary phase in all cultures and further increase to the second stationary phase in LSLI, LSHI and HSHI cultures, and decrease from the first to the second stationary phase in HSLI cultures was found for the TFA content. Corresponding values with standard deviation can be found in the Supplementary section (Table S2). In exponential phase, TFA and EPA contents were higher in HS cultures, while after five days of stationary phase, levels were higher for LS cultures. EPA and TFA contents (% DW), estimated by the model, are shown in Fig. S1 in the Supplementary section.

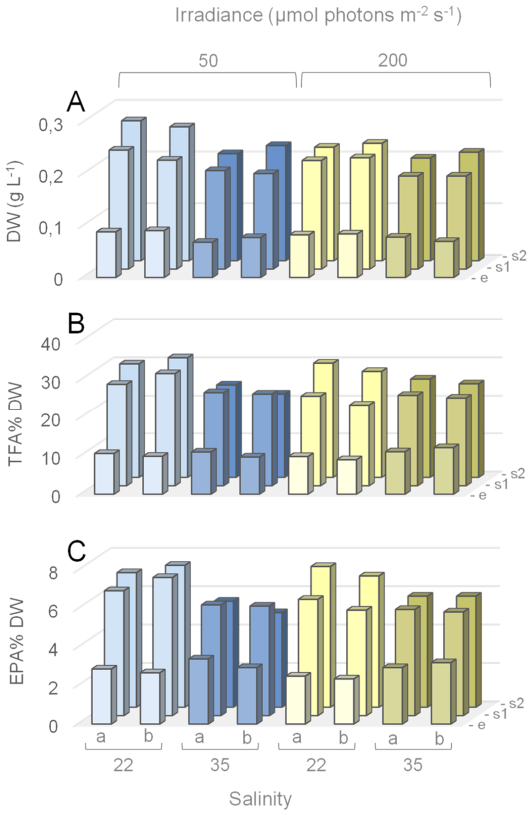

Fig. 3. Dry weights (DW) and fatty acid content during factorial-design batch experiment. Average DW (A), and total fatty acid (TFA) and EPA contents (B and C, respectively) of *Attheya septentrionalis* cultures at twelve different treatments (two biological replicates per treatment, a and b). Values are average of three measurement replicates. Detailed values with standard deviations can be found in Table S2. e: exponential phase, s1: first stationary phase (Day3), s2: second stationary phase (Day 5).

The mathematical models, representing the EPA and the TFA content as a function of salinity (X1), irradiance(X2), growth phase (X3) and their combinations (X1X2, X1X3, X2X3) are expressed by Eqs. [4] and [5], respectively. A positive term for the combinations in the equations indicates a synergistic effect (increasing values increase the content), whereas negative terms indicate an antagonistic effect (increasing values decrease the content) (Chen et al., 2012). Coefficients in bold indicate statistical significance for the corresponding coefficients (black: $p < 0.05$ and red $p < 0.01$). For the EPA content, coefficients for growth phase, salinity and the combination of salinity and growth phase were statistically significant whilst the other factors were not. The most significant variable with highest impact on EPA content was the growth phase with a positive estimated effect of 1.74 ($p = 2.0*10^{-13}$). The combined effect of salinity and growth phase was lower (- 0.48, $p = 0.00003$) and negative, while salinity had a less negative effect on EPA content (- 0.19, $p = 0.0241$). The TFA content was also significantly influenced by growth phase (8.34, $p = 1.1*10^{-15}$), salinity (- 0.77, $p = 0.0116$) and the combination of salinity and growth phase (- 1.43, $p = 0.0002$), and additionally, by the combination of salinity and irradiance (0.73 $p = 0.0126$). A high correlation ($R^2 = 0.9651$ and 0.9802 for EPA and TFA content, respectively) of estimated values by the model and measured values indicates a good fit between the model and the experimental data (Fig. S2). More details on measured and estimated values can be found in the Supplementary section (Table S2).

$$\text{EPA\% DW}_{\text{est}} = 4.61 - \mathbf{0.19} * \mathbf{X1} - 0.12 * \text{X2} + \mathbf{1.74} * \mathbf{X3} + 0.14 * \text{X1X2} - \mathbf{0.48} * \mathbf{X1X3} + 0.02 * \text{X2X3} \quad [4]$$

$$\text{TFA\% DW}_{\text{est}} = 18.74 - \mathbf{0.77} * \mathbf{X1} - 0.25 * \text{X2} + \mathbf{8.34} * \mathbf{X3} + \mathbf{0.73} * \mathbf{X1X2} - \mathbf{1.43} * \mathbf{X1X3} - 0.26 * \text{X2X3} \quad [5]$$

3.4 Batch experiment - relative fatty acid (FA) composition

In total thirty-six FAs were detected in the GC for *A. septentrionalis*, from which eleven (14:0, 16:0, 16:1 n-7, 16:2 n-4, 16:3 n-4, 16:4 n-1, 18:1 n-7, 18:4 n-3, 20:4 n-3, 20:5 n-3 and 22:6 n-3) constituted more than 1% TFA (Fig. 4). C_{16}-FA were the most abundant, whereas C_{18}-FA were present in only low amounts. In all treatments, palmitoleic acid (16:1 n-7) and EPA (20:5 n-3) were the two major FA, together accounting for between 45 and 52% TFA, followed by myristic acid (14:0) with 10-18% TFA, palmitic acid (16:0) with 6-16% TFA, and DHA (22:6 n-3) with 3-6% TFA. However, small variations in the relative FA content between the different treatment groups were apparent. These differences became more distinct by means of a principal component analysis (PCA, Fig 5A). The distribution of the twelve different treatment groups (objects) represents their similarities and differences in the relative FA composition (% TFA), and the distribution of the FAs (vectors) indicate their contribution to the grouping of the objects.

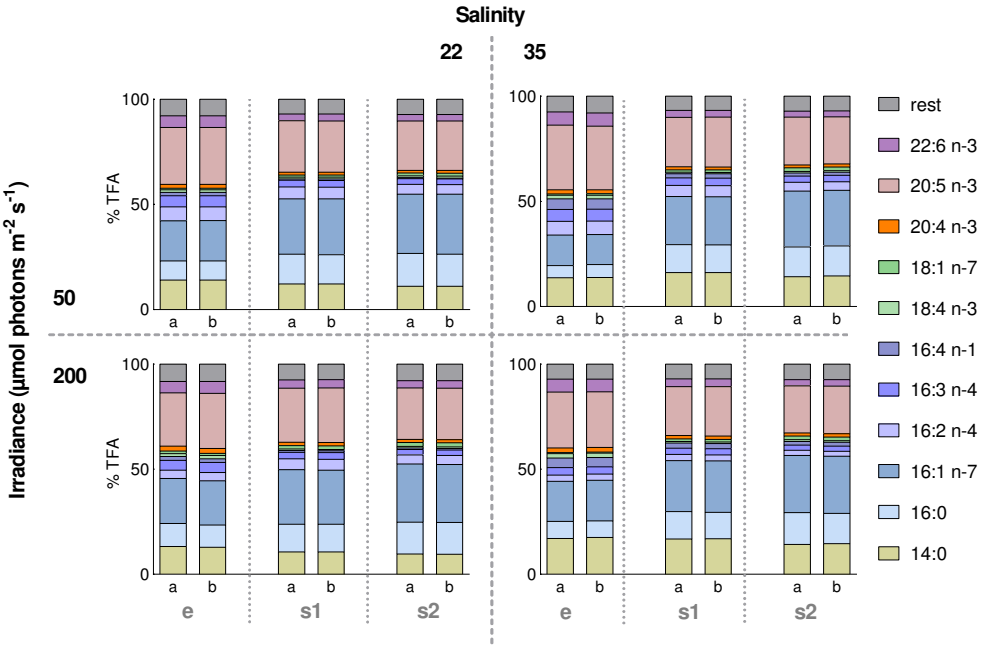

Fig 4. Relative fatty acid (FA) composition during factorial-design batch experiment. Effect of the twelve different treatments on the relative FA composition (% TFA) of the diatom *Attheya septentrionalis* (each with two biological replicates, a and b). Treatments were altering combinations of the three factors salinity (22 and 35), irradiance (50 and 200 µmol photons m^{-2} s^{-1}) and growth phase (exponential [e], three days stationary phase [s1], and five days stationary phase [s2]). Values are average of three measurement replicates.

Objects were arranged along component 1 and component 2 according to the growth phases and salinities, respectively. The exponential phase objects, grouping on the left side of component 1, were clearly separated from the stationary phase objects, which clustered on the right side, and objects were shifted further to the right along component 1 with increasing nutrient starvation (stationary phases). Palmitic and palmitoleic acids were correlated positively with stationary phase samples while the PUFAs were correlated with exponential phase samples. Except for the treatment HSLI_e (high-salinity, low-irradiance and exponential phase), treatments of the same salinity were grouped together, with LS samples on the upper region along component 2, and HS samples arranged on the lower part of component 2. Myristic acid, and to a lesser extent hexadecatetraenoic (16:4 n-1) acid, were correlated positively with HS treatments, while hexadecadienoic (16:2 n-4) was correlated positively with LS samples. Irradiance had only small effect on the FA composition.

The Euclidean Dendrogram illustrates the distinct grouping of the treatments (Fig. 5B). Exponential phase samples were separated from stationary phase samples and both groups were then further divided according to the salinity treatment. For stationary phase samples, all LS samples grouped separately from the HS samples, followed by grouping according to their growth

phase (s1 and s2) and finally by irradiance. Within exponential phase samples, HSHI samples grouped between HSLI and the LS samples, and separation was followed by irradiance, where replicates of LI were separated from the HI replicates. The two biological replicates of each treatment group were very similar.

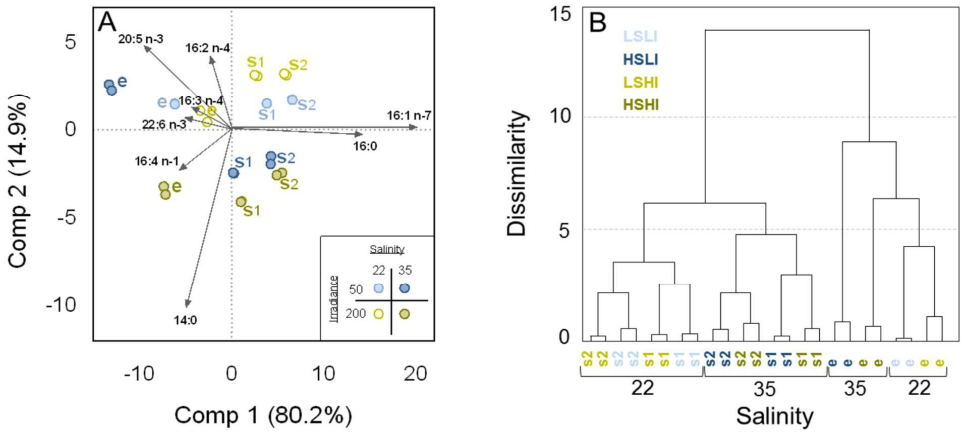

Fig. 5. Similarities and differences in relative fatty acids (FA) composition (% TFA) of *Attheya septentrionalis* cultures at twelve treatments during the factorial-design batch experiment (each with two biological replicates). Treatments were altering combinations of the three factors salinity (22 and 35), irradiance (50 and 200 µmol photons m^{-2} s^{-1}) and growth phase (e, s1, s2). **A**: Principal component analysis (PCA). Twenty-four objects representing the twelve treatment groups, and eight variables, representing the FAs with highest impact on the distributions. Values are average of three measurement replicates. e: exponential phase, s1: first stationary phase (Day 3), s2: second stationary phase (Day 5). **B**: Euclidean Dendrogram showing dissimilarities between the treatment groups. LSLI: low salinity and low irradiance, HSLI: high salinity and low irradiance, LSHI: low salinity and high irradiance, HSHI: high salinity and high irradiance.

4 Discussion

4.1 Impact of culture conditions on growth

Growth rates during the pre-cultivation were strongly dependent on the salinity, followed by irradiance and the interaction of both factors. Growth rates were 43% (LI) and 72% higher (HI) at HS compared to LS, and 5% (LS) and 27% (HS) higher when grown at HI compared to LI. The negligible effect of HI on the growth rate at LS, and the much stronger effect it caused at HS conditions, reveals the combined effect of salinity and irradiance and emphasizes the importance of investigating combinatory effects of different growth factors.

These growth characteristics during the pre-cultivation also became evident when considering the growth curves during the batch experiment. The transition from exponential to stationary phase was defined by the decline of the growth curve with a concomitant decrease in the QY and was one day earlier for HS than for LS cultures. The QY reflects the photosynthetic performance of photosystem II and is used as a vitality indicator for cultures, as decreasing values are associated with stressful growth conditions (Kräbs and Büchel, 2011; Maxwell and Johnson, 2000). During exponential growth, QYs were slightly lower for LS cultures, but decreased to a lesser extent in stationary phase than they did for HS cultures. The strongest decrease in QY was observed for cultures grown at HS and HI. Hence, while the combination HSHI appeared most advantageous during nutrient replete conditions as it caused the highest growth rates in exponential phase, it was also most stressful for the cultures during nutrient starvation.

After the transition from exponential to stationary phase, cell division was assumed to have stopped due to nutrient depletion. Although all nutrients had been consumed on Day 3 in stationary phase, silicate most likely became the major limiting nutrient, as almost half of the silicate was consumed after one day in exponential phase, whereas nitrate and phosphate were both consumed to a lesser extent. When one element becomes limiting, other elements, that are more abundant, may be accumulated in the cell (Reynolds, 2006). Therefore, nitrate and phosphate, whilst not necessarily limiting, might have been taken up by the microalgae cells after silicate was depleted. In contrast to other elements that are essential for survival, diatoms rarely take up more silicon than is required for cell division (Reynolds, 2006). When silicon becomes scarce, its uptake depends on special silicic transport proteins (SITs); however, when silicon is abundant, its uptake is by diffusion (Shrestha and Hildebrand, 2015).

4.2 EPA content during batch experiment

Within the range of experimental variables considered, three factors were identified by the model as having affected the EPA content in the present *A. septentrionalis* strain significantly; the growth phase, salinity and the interaction of both. Growth phase had the greatest impact, with increasing nutrient starvation leading to a higher EPA content relative to DW. The effect of salinity and the combined effects of both salinity and growth phase were lower and negative. Irradiances used in this experimental set-up did not affect the EPA content significantly. The mathematical model reflected the measured EPA values very accurately, with one exception: during the experiment, the EPA content decreased slightly from first to second stationary phase under HSLI conditions, while the model predicted a further increase, similar to under the other conditions. Both the measured data and the model emphasized, that combining LS with five days' nutrient starvation yielded a maximum EPA content of 7.1% DW on average. This DW-based EPA content is, to our knowledge, higher than previously reported for microalgae (Hu and Gao, 2006; Jiang and Gao, 2004; Lu et al., 2001; Patil et al., 2006; Sukenik et al., 1991).

The EPA dynamics revealed the same pattern as for the TFA, suggesting that they were triggered by the same processes. Following exponential growth, the DW continued to increase between 5%

and 20% from the first to the second stationary phase, although nutrients were depleted, and accordingly cell division inhibited. When cell division is hampered due to an insufficient nutrient supply, microalgae often produce carbonaceous storage compounds like carbohydrates and lipids. Many diatoms accumulate neutral storage lipids in the form of triacylglycerol (TAG), causing the lipid content to increase up to 50% of DW (Hu et al., 2008). Total FA content increased in all cultures from on average 10% in exponential phase, to 25% and 27% DW in the two stationary phases. Storage lipids consist predominantly of saturated and monounsaturated FAs, while PUFAs are generally present in polar membrane lipids (Olofsson et al., 2012). Therefore, TAG accumulation is typically accompanied by a noticeable increase of both palmitic (16:0) and palmitoleic acids (16:1 n-7), which often constitute the predominant FA in the TAG. Yet, PUFAs have also been reported to accumulate in TAG in different microalgae species (Sharma et al., 2012; Tonon et al., 2002). The relative FA compositions observed during the experiment revealed a slight increase of the palmitic and palmitoleic acid fractions, together with a weak decrease of PUFAs after cultures progressed from exponential to stationary phase in all conditions. However, these shifts towards palmitic and palmitoleic acids were lower than typically observed during TAG accumulation, and revealed a concurrent increase of all major FA. This might indicate that TAG accumulated in the cells during stationary phase, containing PUFAs such as EPA, alongside palmitic and palmitoleic acids.

Another reason for the increase of the FA fraction in stationary phase might be a decrease of silica in the cells. As a result of the silicified cell walls of diatoms, silicate availability in media is a key factor regulating their growth, as cells can only divide when new valves can be synthesized (Martin-Jézéquel et al., 2000). Studies have shown that in silicate-limited diatom cultures, uptake is restricted to the SITs (Shrestha and Hildebrand, 2015), and silicification is reduced, resulting in thinner cell walls and a decreased silica content per cell (Javaheri et al., 2015; Martin-Jézéquel et al., 2000). Knuckey et al. (2002) found comparable results to our findings in an *A. septentrionalis* isolate from coastal waters in Tasmania, with an EPA content increasing from 1.3% to 4.2% DW from exponential to stationary phase. Concomitantly, the ash content fell sharply from 26.1% to 8.8% DW resulting in a corresponding increase of the other major organic fractions; proteins, carbohydrates and lipids. The decreased ash content was most likely related to a diminished silica content of the DW, due to silicate limitation in stationary phase. The same effect might have contributed to the observed increase in TFA and EPA contents relative to DW in the stationary phase in our study. The typical ash content of microalgae contributes between 5% and 12% DW, but these values are higher in silicified diatoms, between 20% and 55% DW (Nalewajko, 1966; Renaud and Parry, 1994), where much of it is attributable to the extent of silicon in the cell walls. Hildebrand et al. (2012) stated that expressing the FA content as percentage of DW might underestimate the actual amount of FA in diatoms in terms of a per cell carbon basis, when compared with other microalgae, due to their high silica content. Expressing the FA content relative to AF-DW might preclude such an underestimation and furthermore might give a better understanding of the FA content and dynamics during silicate-replete and silicate-depleted conditions.

Salinity also affected the TFA and EPA content, especially in combination with the growth phase. Interestingly, TFA and EPA contents were higher for HS cultures in exponential phase, but in contrast were higher for LS cultures in stationary phase. Hence, a greater increase in TFA and EPA from exponential to stationary phase occurred in the LS cultures. These observations might also be linked to differences in the FA accumulation and silica content of cells grown at different salinities, but might also be related to the changing FA composition of membrane lipids, as an adaptation to variable salt concentrations and the resultant osmotic stress, as has been reported in several studies (Chen et al., 2008; Kumari et al., 2013). Microalgae grown at higher salinities might also reveal an increased ash content, due to an increased ion concentration (Renaud and Parry 1994).

Determining the ash and silicon contents of the cells and differentiating between polar (membrane) and neutral (storage) lipids, and their FA compositions in future experiments, might contribute to a better understanding of the reasons we observe different EPA and TFA contents relative to DW under different salinities and growth phases, and furthermore, might reveal in which lipid fraction the increased EPA levels are located.

4.3 Relative fatty acid (FA) composition during batch experiment

Interestingly, irradiance did not significantly affect the EPA content relative to DW, although irradiances have been shown to affect photosynthetic membranes. Generally, photosynthetic membranes increase at low irradiance and are reduced at high irradiances and hence, increasing irradiance has been reported to result in a decrease in EPA and other PUFAs in different microalgae species (Adlerstein et al., 1997; Fábregas et al., 2004). However, irradiance did affect the FA profile in this study, although only to a minor degree. The relative amounts of the main FAs (% TFA) were for the most part affected by nutrient availability primarily, followed by salinity, time of nutrient starvation (days in stationary phase) and irradiance. The differences between the growth phases were mainly due to palmitic and palmitoleic acids and therefore might be related to an accumulation of TAG in stationary phase. The effects of the growth conditions (salinity and irradiance) were less distinct and are more difficult to explain, but might be related to reconstructions of cellular membranes as an adaptation to the cultivation conditions.

4.4 Potential for microalgae-based technologies

The low salinity of 22 was more effective in increasing EPA content in the stationary phase in the prevalent *A. septentrionalis* strain, but at the same time, it considerably decreased growth rates compared to a HS of 35. In future large-scale cultivations, EPA productivity would be dependent on both the growth rates and the EPA content in the cells. Calculating the EPA productivities from exponential phase until Day 5 of the stationary phase revealed 0.97 mg L^{-1} d^{-1} for LS cultures and 0.72 mg L^{-1} d^{-1} for HS cultures. Thus under the prevailing conditions, higher productivities were obtained for the LS cultures, although these productivities are much lower than those seen in commercial production due to the much lower biomass concentrations used in this experimental setup. Whether our results can be successfully repeated in up-scaled systems needs to be evaluated

further. Higher nutrient concentrations would be necessary in order to achieve higher cell densities and productivities before cultures reach the stationary phase. Furthermore, other growth conditions such as irradiance, temperature and pH might change considerably when moving from small-scale to large-scale systems, and can thereby affect the EPA content of the cells. The strain used in the current study was isolated from an Arctic habitat and adapted to low temperatures, and therefore temperatures in the experiment were maintained at 10 °C. Several studies have shown that low temperatures can increase the PUFA content to maintain membrane fluidity (Boelen et al., 2013). EPA values in the present experiments were higher than values recorded for the *A. septentrionalis* strain by Knuckey et al. (2002) grown at 20 °C. However, at the same time, growth rates of their strain were twice as high as the ones observed in this study. Hence, changing temperatures could additionally affect both the EPA content and growth rates. This should also be evaluated with further work.

Knuckey et al. (2002) suggested *A. septentrionalis* to be an excellent feed species for juvenile bivalve molluscs and other filter feeders. Its cell size is within the range that is suitable for ingestion by filter feeders and its protein level (32% DW) remained stable from exponential to stationary phase, while carbohydrate and lipid fractions increased. As shown in our study, EPA contents can be further increased in stationary phase, by changing growth conditions. This could make this diatom strain a promising EPA source for the North Atlantic fish aquaculture industry or for other application areas, such as the health and food sectors.

5 Conclusion

The effect of growth phase, salinity and irradiance, and their interactions on the EPA content in an Arctic *A. septentrionalis* strain was investigated by means of a factorial design experiment. The highest EPA values of 7.1% DW were achieved at a salinity of 22 and Day 5 of the stationary phase. However, at the same time, growth rates during exponential phase were reduced considerably at low salinities. Mathematical models revealed interactive effects of salinity and irradiance on growth and of salinity and growth phase on the EPA content, emphasizing the importance of investigating the additive effects of different growth factors.

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Supplementary Material

Enhancing EPA content in an Arctic diatom: A factorial design study to evaluate interactive effects of growth factors

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1 Supplementary Figures

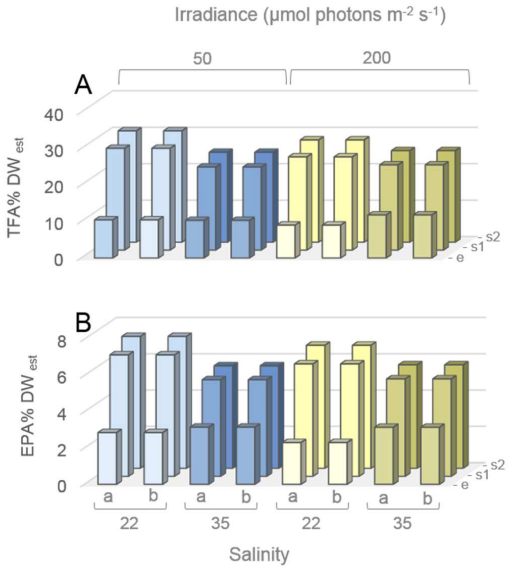


Fig. S1. Model-estimated total fatty acid (TFA) (**A**) and EPA contents (**B**) relative to DW of *Attheya septentrionalis,* at twelve different treatments (each with two biological replicates). e: exponential phase, s1: first stationary phase (Day3), s2: second stationary phase (Day 5).

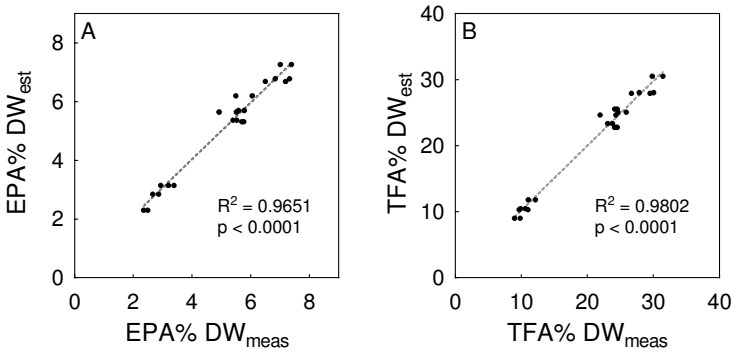


Fig. S2. Measured EPA (A) and TFA content (B) during the factorial-design experiment plotted against the estimates by the mathematical model with linear regression and R^2 value with high significance. **Meas**: measured values, **est**: values estimated by the model.

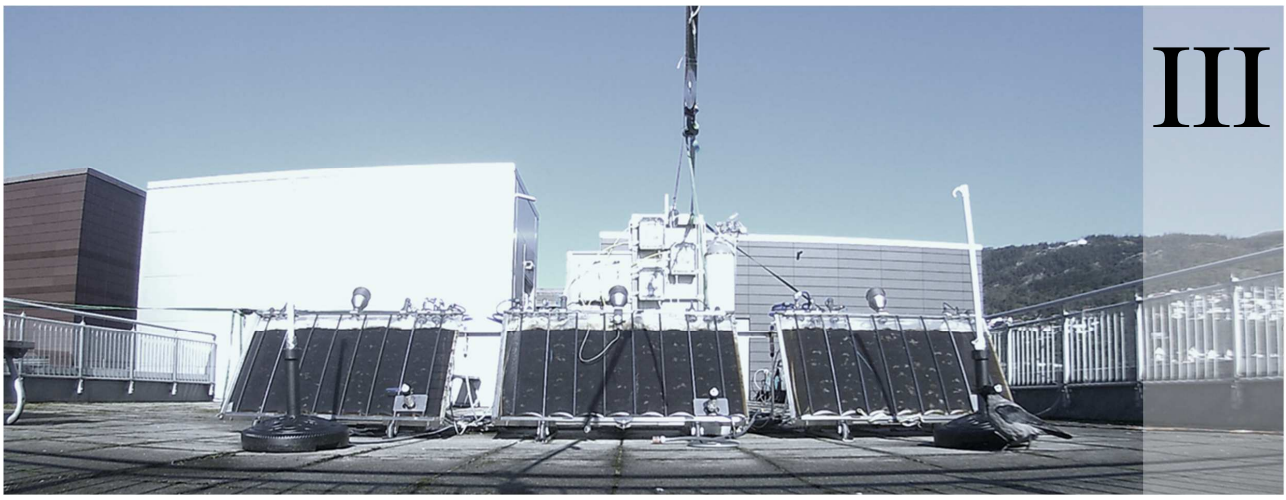
2 Supplementary Tables

Table S1. Growth rates (μ) during pre-cultivation. Growth rates of the last seven repeated dilutions and estimated growth rates by the mathematical model of the diatom *Attheya septentrionalis* grown at four different cultivation conditions. LSLI: Low salinity and low irradiance (μmol photons m^{-2} s^{-1}), HSLI: High salinity and low irradiance, LSHI: Low salinity and high irradiance, HSHI: High salinity and high irradiance.

	Factors			Measurements							Model		
No	Salinity X1	Irradiance X2	Condition	μ (d^{-1})							Coded levels		μ (d^{-1}) estimated
				1	2	3	4	5	6	7	X1	X2	
1	22	50	LSLI	0.55	0.62	0.60	0.56	0.46	0.56	0.45	-1	-1	0.53
2	35	50	HSLI	0.68	0.80	0.78	0.76	0.75	0.86	0.79	1	-1	0.77
3	22	200	LSHI	0.66	0.78	0.54	0.53	0.45	0.47	0.57	-1	1	0.57
4	35	200	HSHI	0.90	1.00	1.09	0.93	0.92	1.08	0.96	1	1	0.98

Table S2. Concept and results of factorial designed batch experiment. LSLI: low salinity and low irradiance (µmol photons m^{-2} s^{-1}), HSLI: high salinity and low irradiance, LSHI: low salinity and high irradiance, HSHI: High salinity and high irradiance, e: exponential phase, s1: fist stationary phase (Day 3), s2: second stationary phase (Day 5). DW: dry weight, TFA: total fatty acids, EPA: eicosapentaenoic acid, Av: average of three measurement replicates, SD: standard deviation of three measurement replicates.

	Factors				Measurements									Model				
No	Salinity X1	Irradiance X2	Growth phase X3	Treatment group	Quantum Yield	DW		TFA % DW		EPA % DW		EPA %TFA		Coded levels			TFA% DW estimated	EPA% DW estimated
						Av	SD	Av	SD	Av	SD	Av	SD	X1	X2	X3		
1a	22	50	exponential	LSLI_e	0.64	0.09	0.01	10.6	1.9	2.9	0.5	27.0	0.1	-1	-1	-1	10.46	2.85
1b					0.64	0.09	0.01	9.9	1.6	2.7	0.4	27.0	0.4					
2a	22	50	stationary 1	LSLI_s1	0.58	0.23	0.01	26.7	1.2	6.5	0.3	24.4	0.2	-1	-1	0.74	27.93	6.69
2b					0.57	0.21	0.01	29.5	2.2	7.2	0.6	24.4	0.2					
3a	22	50	stationary 2	LSLI_s2	0.56	0.27	0.01	29.8	1.6	7.0	0.4	23.5	0.1	-1	-1	1	30.54	7.27
3b					0.56	0.26	0.01	31.4	1.7	7.4	0.4	23.5	0.2					
4a	35	50	exponential	HSLI_e	0.69	0.07	0.00	11.0	0.5	3.4	0.2	30.8	0.6	1	-1	-1	10.32	3.15
4b					0.69	0.08	0.00	9.7	0.8	2.9	0.3	30.3	0.7					
5a	35	50	stationary 1	HSLI_s1	0.56	0.19	0.00	24.5	1.5	5.8	0.4	23.5	0.0	1	-1	0.74	22.79	5.32
5b					0.57	0.18	0.01	24.1	1.6	5.7	0.3	23.7	0.4					
6a	35	50	stationary 2	HSLI_s2	0.53	0.21	0.00	24.3	1.0	5.5	0.2	22.7	0.1	1	-1	1	24.65	5.65
6b					0.53	0.22	0.01	21.9	1.0	4.9	0.3	22.5	0.1					
7a	22	200	exponential	LSHI_e	0.64	0.08	0.02	9.8	2.5	2.5	0.7	25.4	0.4	-1	1	-1	9.02	2.30
7b					0.64	0.08	0.00	9.0	0.9	2.4	0.2	26.2	0.1					
8a	22	200	stationary 1	LSHI_s1	0.49	0.21	0.01	24.5	1.5	6.1	0.6	25.7	0.3	-1	1	0.74	25.57	6.20
8b					0.49	0.22	0.02	24.1	1.6	5.5	0.7	25.9	0.2					
9a	22	200	stationary 2	LSHI_s2	0.53	0.22	0.01	30.0	3.5	7.3	0.8	24.4	0.2	-1	1	1	28.04	6.78
6b					0.52	0.23	0.01	27.8	2.7	6.8	0.7	24.5	0.1					
10a	35	200	exponential	HSHI_e	0.68	0.08	0.00	11.0	0.7	2.9	0.2	26.6	0.4	1	1	-1	11.81	3.15
10b					0.68	0.07	0.01	12.1	2.0	3.2	0.5	26.4	0.5					
11a	35	200	stationary 1	HSHI_s1	0.46	0.18	0.00	23.8	0.6	5.5	0.2	23.2	0.0	1	1	0.74	23.37	5.37
11b					0.43	0.18	0.01	23.1	1.6	5.4	0.4	23.4	0.2					
12a	35	200	stationary 2	HSHI_s2	0.47	0.20	0.01	25.9	1.7	5.8	0.4	22.4	0.3	1	1	1	25.09	5.70
12b					0.44	0.21	0.01	24.6	1.0	5.8	0.2	22.7	0.1					



III

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Comparing EPA production and fatty acid profiles of three *Phaeodactylum tricornutum* strains under western Norwegian climate conditions

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ABSTRACT

Microalgae could provide a sustainable alternative to fish oil as a source for the omega-3 polyunsaturated fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). However, growing microalgae on a large-scale is still more cost-intensive than fish oil production, and outdoor productivities vary greatly with reactor type, geographic location, climate conditions and microalgae species or even strains. The diatom *Phaeodactylum tricornutum* has been intensively investigated for its potential in large-scale production, due to its robustness and comparatively high growth rates and EPA content. Yet, most research have been performed in southern countries and with a single commercial *P. tricornutum* strain, while information about productivities at higher latitudes and of local strains is scarce. We examined the potential of the climate conditions in Bergen, western Norway for outdoor cultivation of *P. tricornutum* in flat panel photobioreactors and cultivated three different strains simultaneously, one commercial strain from Spain (Fito) and two local isolates (M28 and B58), to assess and compare their biomass and EPA productivities, and fatty acid (FA) profiles. The three strains possessed similar biomass productivities (average volumetric productivities of 0.20, 0.18, and 0.21 $g\,L^{-1}\,d^{-1}$), that were lower compared to productivities reported from southern latitudes. However, EPA productivities differed between the strains (average volumetric productivities of 9.8, 5.7 and 6.9 $mg\,L^{-1}\,d^{-1}$), due to differing EPA contents (average of 4.4, 3.2 and 3.1% of dry weight), and were comparable to results from Italy. The EPA content of strain Fito of 4.4% is higher than earlier reported for *P. tricornutum* (2.6–3.1%) and was only apparent under outdoor conditions. A principal component analysis (PCA) of the relative FA composition revealed strain-specific profiles. However, including data from laboratory experiments, revealed more significant differences between outdoor and laboratory-grown cultures than between the strains, and higher EPA contents in outdoor grown cultures.

1. Introduction

Microalgae are suggested to be a promising and sustainable feedstock for various food and non-food products. They are fast growing, rich in oils, proteins and carbohydrates, and can be cultivated in seawater and on non-arable land, and may therefore be grown in regions unsuitable for agriculture [1]. Microalgae compounds of particular interest are the omega-3 long-chained polyunsaturated fatty acids (LC-PUFAs), eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6). Both are recognized as being essential for human health by helping prevent cardiovascular and inflammatory diseases [2]. Marine fish are the major EPA and DHA source, obtaining these PUFAs themselves predominantly via the marine food chain from EPA- and DHA-synthesizing microalgae. The market for marine fish oil has been increasing in recent years, dominated by the aquaculture industries that use fish oil as an ingredient in aqua-feeds in order to meet the desired EPA and DHA content in cultivated fish [3]. Additionally, there is also an increasing demand for using fish oil for EPA- and DHA-enriched products for direct human consumption [4,5]. As the fish oil derives from wild fish stocks, its production and application has raised economic, ethical and environmental concerns, together with considerations on its purity, taste and quality, as fatty fish have been associated with the risk of contamination with environmental pollutants [3,6].

Many marine microalgae species naturally produce EPA and DHA as components of the polar membrane lipids [7], and could, thus, provide a sustainable alternative source for the two PUFAs. However, growing

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microalgae on a large-scale is still more cost intensive compared to fish oil production [8]. Thus, in the past decades, efforts have been made to improve the microalgae large-scale production, and different microalgae species and photobioreactor systems have been investigated and evaluated [9]. The majority of studies on microalgal outdoor productivity have been performed in temperate countries like Spain or Australia with high irradiance, that promote microalgae production. In contrast to this, only limited studies are available from higher latitudes, where outdoor grown microalgae face strong seasonal fluctuations in temperature, irradiance and photoperiod [10]. As a result of the increased interest in microalgae-based products, long-term investigations from higher latitudes are considered important in order to evaluate the potential of different locations for microalgal outdoor production and for its impact on fatty acid (FA) content and composition.

The diatom *Phaeodactylum tricornutum* has been intensively studied for its potential for large-scale production, as it is easy to cultivate, fast growing and comprises a comparatively high EPA content. Moreover, its low silica requirement makes it more attractive in terms of costs of growth media compared to other diatoms [11]. As this species is common in coastal brackish and marine waters worldwide, numerous isolates exist in different culture collections [12]. However, many studies on large-scale outdoor productivity have been performed with the same commercial *P. tricornutum* strain (UTEX 640).

In this study, three different *P. tricornutum* strains were grown simultaneously as repeated batch cultures during a six-months period, in individual outdoor flat panel photobioreactors (35 L) at northern latitudes in Bergen, western Norway, to evaluate the potential of the local climate conditions for large-scale outdoor cultivation and EPA production. The increased photoperiod during spring and summer at higher latitudes (19:5 h light:dark [L:D] around mid-summer in Western Norway) might be expected to positively affect growth rates and, thus, productivities. By comparing three different strains (two local isolates and one commercial strain), we sought to assess and compare strain-specific responses to changing environmental conditions in terms of productivity and FA profile; which are important selection criteria for outdoor cultivation.

2. Methods

2.1. Strains, stock cultures and inoculum

Three different strains of *P. tricornutum* were used in the experiment. Two strains (B58, M28) were isolated from the fjord next to the cultivation site and have been maintained in the laboratory since 1997 (B58 as ND58 [13]) and 2014 [14], respectively. The third strain (Fito) was obtained from the Microalgae Culture collection of Fitoplancton Marino, SL Cadiz, Spain (strain CCFM 06, isolated from local marine habitat) where it is being grown on a commercial scale, and the strain has been maintained in our laboratory since 2014. Stock cultures of the three strains were kept in 50 mL Erlenmeyer flasks in sterile Walne's medium ([15], prepared with seawater [SW; from 90 m depth in the fjord] and distilled water [80:20, v:v], salinity 29), at 15 °C and an irradiance of 50 μmol $m^{-2} s^{-1}$ with a L:D cycle of 16:8 h. For inocula, biomass was up-scaled to 10 L flat-bottom glass flasks in modified F2 medium [16]. The media was prepared with SW and distilled water (80:20, v:v, salinity 29) and an increased macronutrient concentration to avoid nutrient limitation ($NaNO_3$, 29 mM, 2.5 g L^{-1}; $NaH_2PO_4{\cdot}H_2O$, 2.9 mM, 0.4 g L^{-1}; $Na_2SiO_3{\cdot}5H_2O$, 0.14 mM, 30 mg L^{-1}). Cultures were kept at room temperature (15–20 °C) and continuous illumination between 100 and 200 μmol $m^{-2} s^{-1}$. For carbon supply and culture mixing, 0.2 μm filtered air, enriched with 1% CO_2, was bubbled through glass capillaries into the bottom of each flat-bottom glass flask, and the cultures were additionally stirred with a magnet stirrer to prevent settling and biofilm formation. Cultures reached dry weights (DW) of approximately 1 g L^{-1} before they were inoculated into the outdoor reactors. Backup cultures were maintained during the whole experiment in 10 L flat-bottom glass flasks under the above-mentioned conditions by monthly dilution with fresh medium.

2.2. Photobioreactor

The photobioreactor used in this study comprised three individual modules of Green Wall Panels (GWP®-III, Fotosintetica & Microbiologica S.r.l, Florence, Italy) connected with a HMI (human machine interface) control unit. Each panel consisted of a metal frame (120 cm × 78 cm) encompassing a disposable bag (flexible and PAR transparent [> 90%] LDPE film), with a culture volume capacity of between 30 and 38 L (a volume of 35 L was used for calculations) and an optical path of approximately 3.5 cm. The panels were placed in parallel, and unshaded and south facing, on the roof of the Department of Biology in Bergen, Norway (60°22′49.7″N, 5°19′54.3″E). The inclination of each panel could be varied between 50 and 105° (panel back side with reference to the horizontal) and was adjusted to 50° during the day (04 am–24 pm) for maximum exploitation of solar irradiance and at 105° during the night (24 pm–04 am) for a better mixing of the cultures. At 50°, the ground area occupied by one panel of the photobioreactor was 1.2 m^2. Each panel was equipped with a pH- and temperature sensor, and a quantum irradiance sensor (LP PAR 03, Delta Ohm, Padova, Italy) was attached on top of the second panel, facing the same direction as the panel surface. Ambient air was pumped through perforated pipes into the bottom of the bags to ensure culture mixing, and carbon was supplied by pH-controlled injection of pure CO_2 into the cultures. Temperature was controlled by an internal stainless steel coil with circulating cold tap water. Temperature, pH and photon flux density (PFD, μmol $m^{-2} s^{-1}$) of photosynthetically active radiation (PAR, 400–700 nm) data were recorded and stored on a PC every thirty seconds.

2.3. Outdoor cultivation

Prior to inoculation, cultivation bags were filled with tap water and sterilized with 1 mL L^{-1} sodium hypochlorite (sodium hypochlorite solution, 10–15%, Sigma-Aldrich, St. Louis, MO, USA) for at least 24 h. The sodium hypochlorite was thereafter neutralized with 1 mL L^{-1} sodium thiosulfate (sodium thiosulfate pentahydrate 15%, Sigma-Aldrich, St. Louis, MO, USA), and the panels were emptied and refilled with fresh and filter-sterilized medium and the inoculum. Modified F2 medium with increased macronutrient concentration (as described in 2.1), was prepared in 100 L tanks with seawater and tap water (80:20, v:v, salinity 29), and was pumped with a peristaltic pump (Masterflex Easy-Load, Millipore, Billerica, Massachusetts, USA) through 0.45 μm filters (Durapore Membrane Filters, Merck Millipore Billerica, Massachusetts, USA) into the culture bags. The three *P. tricornutum* strains were grown as repeated-batch cultures for a period of six months (25.04.2016–28.10.2016) in the individual GWP®-III panels (35 L), by discharging a defined volume of culture that was backfilled with fresh medium every 7–14 d to maintain a biomass concentration between approximately 0.4 and 2.5 g L^{-1}. Every interval between two dilutions is referred to as a "batch" with consecutive numbering. Cultures were mixed by aeration at 15–20 L min^{-1}, and pure CO_2 was injected into the cultures when the pH exceeded 8.1 and until the pH reached 7.9 again. Temperatures were kept between 5 and 25 °C with cold tap water (~9 °C), circulating through the internal coil whenever the temperature settings were underrun or exceeded, respectively. Culture volumes were topped with ultra-pure water (Milli-Q) in the morning when required, to counter eventual evaporations. The position of the strains in the panels was rotated twice (06.07.16 and 13.09.16), allowing each strain to grow in each panel for a certain period, in order to exclude any influence of panel position or material on the performance of the cultures. To prevent mixing of the three strains, panels were cleaned and sterilized prior to position change as described above.

2.4. Sampling and analytical procedures

A sample volume between 50 and 100 mL was taken for each culture three times weekly between 9:00 and 10:00 am and daily for a period of two weeks (22.07.16–29.07.16 and 07.10.16–14.10.16) at 01:00 pm to measure DW, optical density (OD), and maximum quantum yield (QY) in triplicate, and take quadruplicate samples for FA analysis. To monitor strain morphology and contamination, cultures were regularly observed under the microscope. When cultures were diluted, samples were taken before and after the dilution, and a volume of 10 mL was stored at − 20 °C for phylogenetic analysis. Nitrate and phosphate concentrations were measured regularly using colour-indication-stripes (Quantofix, Macherey-Nagel GmbH & Co. KG, Düren, Germany). For batches one, two and three in the spring season, FA samples were only taken at the end of the batch, before dilution. In order to also calculate FA productivities for batches two and three, their FA starting concentrations were calculated from the former FA end-concentrations and their respective DW. From the second day of batch number four, FA were sampled at the same time intervals as the other parameters.

Optical densities were measured with a spectrophotometer (UV-2401 PC, Shimadzu Corporation, Kyoto, Japan) at 750 nm. If necessary, samples were diluted to give an attenuation of between 0.2 and 0.7. Dry weights were determined as described by Zhu and Lee [17] using 0.5 M ammonium formate as a washing buffer and are expressed as weight of the dried biomass (g) per volume (L). The QY was measured with Aqua Pen (AquaPen-C, AP-C 100, Photon System Instruments, Brno, Czech Republic) after initial dark incubation (10–60 min). For FA analysis, microalgae biomass was harvested by centrifugation (10 min at 2264 × *g*) into 10 mL glass tubes (PYREX), the supernatant discarded, and the pellet covered with N_2 gas and kept at − 20 °C until analysis. Fatty acid extraction, methylation and analysis on gas chromatograph (GC) was performed as described in Steinrücken et al. [14]. The concentration of internal standard (23:0 FAME dissolved in isooctane) was adjusted for the respective DW of the samples, with 100, 240, 380, and 480 μg for < 1, 1–2, 2–3 and > 3 g L^{-1}, respectively.

2.5. Phylogenetic identification of strains

For each strain, one sample from stock culture and four samples from different time points during the cultivation period were used for phylogenetic analyses. DNA was isolated from pelleted samples using the EZNA plant kit (stock cultures and samples from 25.04.16 and 17.08.16) or the MoBio PowerWater® DNA isolation kit (samples from 13.09.16 and 28.10.16) according to instructions from the manufacturers. PCR was performed using primers GF (5′-GGGATCCGTTTCC GTAGGTGAACCTGC-3′) and GR (5′-GGGATCCATATGCTTAAGTTCAG CGGGT-3′), which target ~820 bp of the variable region, ITS1-5.8S-ITS2, of the genome [18]. The PCR mastermix contained (per 50 μL reaction); 25 μL Hotstart PCR mix, 2 μL 10% BSA, 1.2 μL 100% DMSO, 1 μL 10 μM primer GF, 1 μL 10 μM primer GR, 17.8 μL dH_2O, and 2 μL template DNA. PCR conditions were: 15 min at 95 °C followed by 30 cycles of 1 min at 95 °C, 1 min at 55 °C and 1 min at 72 °C, and a final elongation at 72 °C for 7 min. Size of PCR products was confirmed using gel electrophoresis. PCR products were subsequently purified using ExoProStar™ (GE Healthcare, Chicago, Illinois, USA) according to the manufacturer's instructions, and prepared for sequencing using BigDye v.3.1 Kit (ThermoFisher Scientific, Watham, MA, USA) and the PCR GF/GR primer combination. Sanger sequencing was performed by the sequencing facility at the University of Bergen (http://www.uib.no/en/seqlab). Sequencing chromatograms were examined, and forward and reverse sequences were assembled and aligned using BioEdit (v.7.2.5) [19]. A minimal evolution tree of these sequences and their closest relatives obtained from Genbank was constructed using Mega6 [20].

2.6. Calculations

Average daily biomass, total fatty acid (TFA) and EPA productivities (per volume Eq. (1), panel surface-area Eq. (2) and panel ground-area Eq. (3)), and yields on light Eq. (4) were calculated for each batch by taking start and end points of its linear slope. A volume of 35 L and a surface area of 0.95 m^2, and a ground area of 1.2 m^2 were used for calculating the panel surface-areal and panel ground-areal productivities, respectively.

$$P_{b,vol} = \frac{c_{x1} - c_{x0}}{t_{x1} - t_{x0}} \quad (1)$$

$$P_{b,panel} = \frac{P_{b,vol} * 35}{0.95} \quad (2)$$

$$P_{b,ground} = \frac{P_{b,vol} * 35}{1.2} \quad (3)$$

$$Y_{b,light} = \frac{P_{b,panel}}{E_{av,b}} \quad (4)$$

$P_{b,vol}$: volumetric productivity during a batch (DW [g L^{-1} d^{-1}], TFA, EPA [mg L^{-1} d^{-1}]), c: concentration (DW [g L^{-1}], TFA, EPA [mg L^{-1}]), x_0 and x_1: defined start and end time point of the linear slope of a batch, t: time (day), $P_{b,panel}$: panel surface-areal productivity during a batch (DW [g m^{-2} d^{-1}], TFA, EPA [mg m^{-2} d^{-1}]), 35: panel volume (L), 0.95: panel surface area (m^2), $P_{b,ground}$: panel ground-areal productivity during a batch (DW [g m^{-2} d^{-1}], TFA, EPA [mg m^{-2} d^{-1}]), 1.2: panel ground area (m^2), $Y_{b,light}$: Yield on light during a batch (DW [g mol^{-1} photon], TFA, EPA [mg mol^{-1} photon]) and $E_{av,b}$: average daily PFD on the panel surface during a batch (mol m^{-2} d^{-1}). The average daily irradiance on the panel surface during a batch ($E_{av,b}$, mol m^{-2} d^{-1}) was calculated with Eq. [5]. The measured PFDs (μmol m^{-2} s^{-1}) between the start and end time point of the linear slope of a batch were multiplied by 30, summed up and divided by the amount of days. E: photon flux densities (μmol m^{-2} s^{-1}, measured every 30 s), x_0 and x_1: start and end time point of the linear slope of a batch, t: time (day).

$$E_{av,b} = \frac{\sum_{t_{x0}}^{t_{x1}} (E(t) * 30)}{t_{x1} - t_{x0}} \quad (5)$$

Average seasonal (spring, summer and autumn) productivities ($P_{av,s}$), yields ($Y_{av,s}$) and PFDs ($E_{av,s}$) were calculated by taking the average from of the corresponding batches.

2.7. Statistics

The three strains were grown as one biological replicate each. Optical density, QY and DW were measured in triplicates. Quadruplicate samples were taken for FA analysis, but eventually only duplicate samples were analysed due to the great amount of samples. The FA content and the biomass DW were analysed from individual subsamples. Thus the standard deviation for FA content relative to the biomass DW was calculated using the Eq. [6] with SD: standard deviation, FA: fatty acid and DW: biomass dry weight.

$$SD\ \frac{FA}{BM} = \frac{\sqrt{\%SD_{FA}{}^2 + \%SD_{DW}{}^2}}{100} * \frac{FA}{BM} \quad (6)$$

Differences in biomass and EPA productivities between the strains and between the seasons were analysed by 2-way ANOVA using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA), with $p < 0.05$ as a threshold for statistical significance. Principal Component Analyses (PCA) and Euclidean dendrograms of strains and their FA composition were calculated using Sirius 10.0 (Pattern Recognition Systems AS, Bergen, Norway) and edited using GraphPad Prism 6.

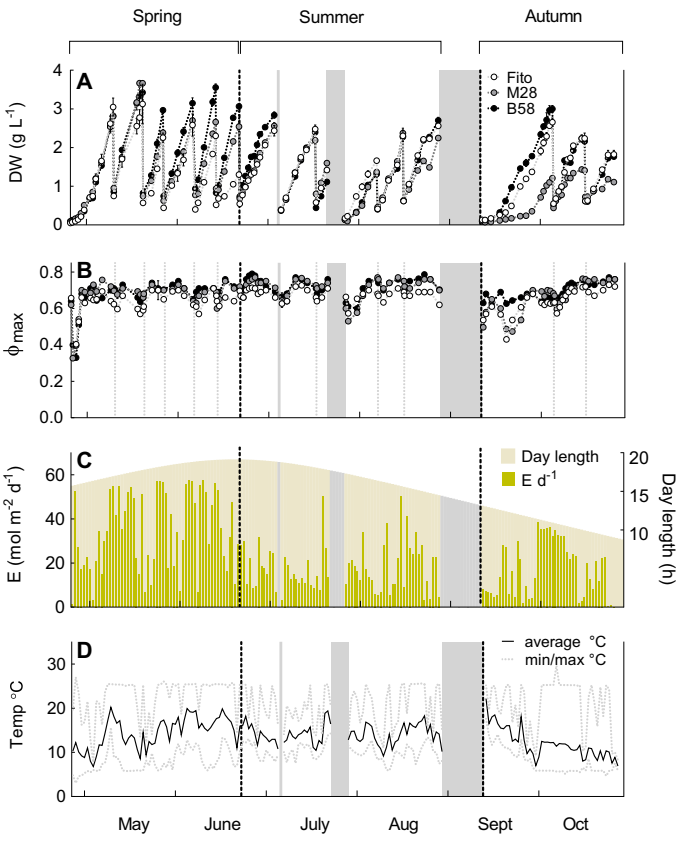

Fig. 1. Overview of culture parameters during six-months repeated-batch cultivation of three *Phaeodactylum tricornutum* strains (Fito, M28, B58) in flat panel outdoor bioreactors in Bergen, western Norway. A: Biomass dry weight (DW) and B: Photosystem II efficiency of dark-adapted samples (Φ_{max}, maximum quantum yield) during fifteen repeated batches. Data show average and standard deviation of measurement replicates (n = 3) from one biological batch. C: Daily-integrated irradiance (E) of photosynthetically active radiation (PAR) measured on the reactor surface, and day length. D: Daily average, minimum and maximum temperature inside the cultivation chambers. The three grey bars indicate interruptions of the cultivation process. After interruption (1) and (3), the position of the strains was rotated, and after (2) and (3), the panels were re-inoculated with backup cultures. Grey dotted vertical lines in (B) indicate dilution of the cultures with consequent decrease in biomass concentration (A). Black dotted lines mark the division of the cultivation period into three seasons; spring, summer and autumn.

3. Results

3.1. Cultivation conditions

Three *P. tricornutum* strains (local strains M28, B58, and Spanish strain Fito) were grown as repeated-batch cultures for six months (25.04.2016–28.10.2016) in separate flat panel outdoor reactors (Green Wall III Panels, each 35 L) in Bergen, Norway. In total, fifteen repeated batches were conducted with biomass DW, on average, ranging between 0.52 and 1.94, 0.67 and 1.88, and 0.63 and 2.33 g L^{-1} for strains Fito, M28 and B58, respectively, not including the starting concentrations after inoculation (Fig. 1A). The cultivation period was divided into three seasons; spring (25.04–22.06, comparatively high daily irradiance and increasing day length, batches 1–6), summer (22.06–29.08, decreasing day length and comparably low irradiance, batches 7–12) and autumn (13.09–28.10, short day length, but relatively high irradiance during the day, batches 13–15). The reactor panels had to be re-inoculated with backup cultures twice (July and September see Fig. 1), due to punctures in the cultivation bags (caused by birds) and an inappropriately connected cooling system (with a consequent lethal increase of culture temperature to > 40 °C), respectively. The starting biomass concentrations after inoculations in our study ranged between 0.05 and 0.17 g L^{-1}. Because of the high irradiance and low starting biomass concentration after the third inoculation, the cultures were covered with white plastic bags and a parasol until biomass concentrations reached approximately 0.2 g L^{-1} to avoid photoinhibition. During the last two batches of the cultivation period, all strains appeared light-limited at earlier DW concentrations than previously, and strain M28 possessed overall lower growth rates and did not reach DW > 1.43 g L^{-1} during autumn season. The maximum quantum yield was reasonably stable and varied between 0.57 and 0.73 (Fito), 0.60 and 0.77 (M28), and 0.63 and 0.79 (B58) during the cultivation period, and was generally higher for strains M28 and B58, with average value of 0.71, than for strain Fito (0.66). Only after inoculations, QY dropped below the average range with minimum values of 0.35 for strain Fito, and 0.33 for M28 and B58, but recovered after two to three days (Fig. 1B).

The daily irradiances during the cultivation period, together with the day lengths are illustrated in Fig. 1C. Day length increased from the start of the culture period (15 h 36 min) until summer solstice (20.06.2016, 19 h 01 min), and then decreased towards the end of the cultivation period (8 h 55 min). The daily-integrated irradiance varied strongly during the cultivation season depending on the weather conditions and day length, and ranged between 0.1 and 57 mol m^{-2} d^{-1}, whereas short-term maximum irradiance during a day varied between 41 and 2555 μmol m^{-2} s^{-1} (data not shown).

The culture temperature varied significantly between night and day during the whole cultivation period (Fig. 1D). It was connected to the irradiance with higher temperatures (up to 25 °C before being cooled) at high irradiance, and lower temperatures at low irradiance and during the nights. Daily average values fluctuated between 6.5 and 19.8 °C, daily minimum between 3 and 14 °C, and the daily maximum between 8.1 and 27.3 °C. Greatest observed fluctuations during 24 h were between 6 °C at night and 25 °C during the day. Average temperatures

during the three seasons were 15, 14, and 11 °C for spring, summer and autumn, respectively.

An average pH of approximately 7.8 was maintained in the cultures during the experiment (Appendix, Fig. 1). However, due to a time delay between CO_2 injection and pH registration by the sensor, the pH typically varied between 7.5 and 8.1 during a day, but was also found to drop down to pH of 6 for a short time. A defect in the CO_2 solenoid-valve during batch number six led to a continuous injection of CO_2 into panel 1 containing strain Fito culture, resulting in a significant decrease in the daily average pH (pH 6.2–7.5), and a consequent decreased growth rate (Fig. 1A) and biomass production ($0.09\ g\ L^{-1}\ d^{-1}$). Therefore, this batch was excluded for productivity calculations for strain Fito.

3.2. Strain morphology and discrimination

P. tricornutum is pleiomorphic and can exist in at least three different forms (oval, fusiform or triradiate) whereas only little information is available on transformation processes [21]. The cells of the three strains were all predominantly fusiform-shaped, but differed in their form and cell size, and could, thus, be distinguished under the microscope (Fig. 2). The cells of strain Fito were exclusively found to be fusiform, and were noticeably shorter than the cells of the other two strains with an average length of approximately 20 μm (Fig. 2A). Strain M28 cells were predominantly fusiform, elongated (20–40 μm) and sickle-shaped (Fig. 2B), but also triradiate cells were present in the culture and clusters of oval morphotypes were found occasionally. The cells of strain B58 possessed different morphologies under laboratory cultivations (oval or fusiform), but only the fusiform morphotypes (and very rarely triradiate forms) were observed during the outdoor experiment. They were on average 30 μm long, and straighter and thicker than strain M28 cells. During the outdoor cultivation period, no changes in either shape or size of the cells were observed for strains Fito and M28, but approximately 40% of the cells of strain B58 became smaller after half of the cultivation period, resembling the shape of strain Fito cells.

Sequencing the ITS1-5.8S-ITS2 region of the genomes confirmed the separation of the three strains and revealed that strain M28 diverged from strains Fito and B58 at several positions in the genome (Fig. 3). Strains Fito and B58 had identical sequences, with the exception of one bp (position 553, 5′-3′), where strain B58 displayed thymine, whereas the sequencing chromatograms indicated that strain Fito displayed equal amounts of cytosine and thymine. This observation was consistent throughout the experimental period, and the results, thus, exclude any mixing of the three strains.

3.3. Seasonal productivities of cultures

The average volumetric biomass and EPA productivities for the three strains and three seasons (spring [batches 1–6 for biomass and 2–6 for EPA], summer [batches 7–12] and autumn [batches 13–15]) are shown in Fig. 4 together with the average daily irradiance. Highest average irradiance occurred during spring season ($36.3 \pm 8.2\ mol\ m^{-2}\ d^{-1}$), whereas the irradiance was equally low during the summer and autumn seasons (19.2 ± 3.5 and $20.1 \pm 3.4\ mol\ m^{-2}\ d^{-1}$, respectively, Fig. 4A).

Corresponding to this, the volumetric biomass productivities for all three strains were significantly higher in spring ($p < 0.05$) with 0.25, 0.26 and $0.30\ g\ L^{-1}\ d^{-1}$ for Fito, M28 and B58, respectively, compared to summer (0.18, 0.18 and $0.18\ g\ L^{-1}\ d^{-1}$) and autumn (0.16, 0.09 and $0.16\ g\ L^{-1}\ d^{-1}$) (Fig. 4B). Strain M28 additionally revealed a significant decrease from summer to autumn. Differences in biomass productivity were also found between the three strains, but were not consistent during all three seasons. In spring, biomass productivity of strain B58 was significantly higher than biomass productivity of strain Fito, and in autumn strain M28 had a significant lower biomass productivity compared to strains Fito and B58.

The volumetric EPA productivities were also significantly higher during spring for all strains (11.8, 8.0 and $9.2\ mg\ L^{-1}\ d^{-1}$ for Fito, M28 and B58, respectively) than in summer (8.8, 6.2 and $6.0\ mg\ L^{-1}\ d^{-1}$) and autumn (8.7, 2.8 and $5.6\ mg\ L^{-1}\ d^{-1}$) (Fig. 4C). In contrast to the biomass productivities, EPA productivities were significantly higher for strain Fito compared to the other two strains in all three seasons. EPA productivities of strains M28 and B58 showed no significant differences in spring and summer, but strain M28 had a significantly lower EPA productivity in autumn. An overview for productivities (volumetric, panel surface-area and ground-area), and the yield of light for biomass, TFA and EPA is summarized in Table 1.

3.4. Total fatty acid (TFA) and eicosapentaenoic acid (EPA) content

The TFA and EPA content showed moderate variations during the cultivation period, but no obvious pattern was apparent (Fig. 5). TFA content was most of the time slightly higher for strain Fito, and varied between 10.0 and 17.4, 6.3 and 18.1, and 8.5 and 16.0% DW for strains Fito, M28 and B58, respectively (Fig. 5A). The EPA content relative to DW varied between 2.6 and 5.6, 1.4 and 4.5, and 2.2 and 4.1% (Fig. 5B) and relative to TFA between 23.0 and 36.4, 19.1 and 30.7, and 19.3 and 28.9% (Fig. 5C), for strains Fito, M28 and B58, respectively. EPA content was predominantly higher for strain Fito, both relative to DW and relative to TFA throughout the cultivation period. After re-inoculation, TFA and EPA contents were considerably lower, and increased to higher levels with cultivation time in all three strains; this was particularly noticeable for the EPA content in strain Fito.

3.5. Total fatty acid (TFA) composition

The same thirty-four FA were detected in the GC for the three stains in all samples analysed, from which 30 could be identified. In total ten FA (14:0, 16:0, 16:1 n-7, 16:2 n-4, 16:3 n-4, 16:4 n-1, 18:2 n-6, 20:4 n-6, 20:5 n-3, 24:0) accounted (on average) for > 2% TFA. EPA (20:5 n-3) and palmitoleic acid (16:1 n-7) were the two major FA, together accounting for approximately 50% TFA, followed by palmitic acid (16:0) with an average of 12% TFA and myristic acid (14:0) with on average 7% TFA. However, the relative TFA content differed between the strains and varied during the cultivation period within the strains (Table 2).

A PCA on the seasonal average of the relative FA content (% TFA) for the three strains revealed strain-specific grouping (Fig. 6A). All three seasons clustered closely together for each strain, whereas the three strains were clearly separated. The FA that predominantly contributed to the distribution of the three strains were EPA, palmitoleic

Fig. 2. Microscope photo of the three *Phaeodactylum tricornutum* strains Fito (A), M28 (B) and B58 (C). A Zeiss Axio Imager Z1 microscope (1000 × magnification with immersion oil) and an AxioCam MR3 (Carl Zeiss, Göttingen, Germany) were used, and photos were edited with GIMP 2.8 (www.gimp.org).

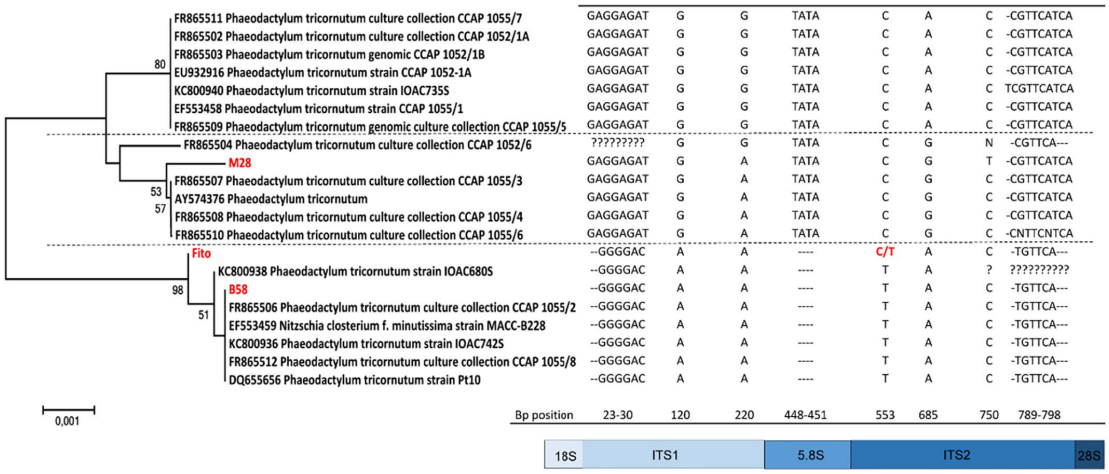

Fig. 3. Minimal evolution tree displaying the evolutionary relationships in the ITS1-5.8S-ITS2 region of the genome of the three *Phaeodactylum tricornutum* strains (Fito, M28 and B58) used in the experiment and closest related strains obtained from Genbank. The evolutionary history was inferred using the Minimum Evolution method [22]. The optimal tree with the sum of branch length = 0,01 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [23]. The evolutionary distances were computed using the Maximum Composite Likelihood method [24] and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA6 [20].

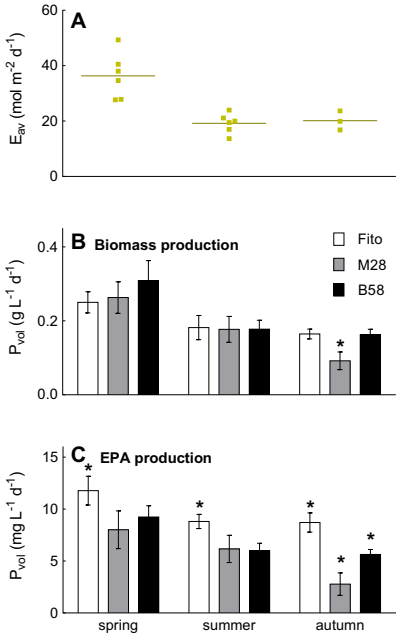

Fig. 4. Average daily irradiance (E_{av}) and average biomass and EPA productivities for three strains of *Phaeodactylum tricornutum* (Fito, M28 and B58) for three seasons (spring, summer and autumn), during six-months repeated-batch cultivation in flat panel outdoor bioreactors in Bergen, western Norway. A: Dots display average daily irradiance (mol m^{-2} d^{-1}) during a batch ($E_{av,b}$) and lines are the mean value of the seasons ($E_{av,s}$). Values for biomass (B) and EPA production (C) are average and standard deviation from the respective batches of each season (biomass: $n = 6$ [5 for Fito], 6 and 3, and EPA: $n = 5$ [4 for Fito], 6 and 3 for spring, summer and autumn, respectively). Asterisks indicate significant difference to the other two strains.

acid, arachidonic acid (20:4 n-6), hexadecatetraenoic acid (16:4 n-1), hexadecatrienoic acid (16:3 n-4) and hexadecadienoic acid (16:2 n-4), at which EPA correlated positively with strain Fito, hexadecatetraenoic acid with M28, and arachidonic acid with strain B58. Palmitoleic acid was negatively correlated to strain Fito, hexadecadienoic and hexadecatrienoic acids to strain M28, and hexadecatetraenoic acid to strain B58. Palmitic acid and myristic acid contributed to only a minor extent in the grouping.

Including FA composition data from the same strains from laboratory experiments into the PCA revealed strong differences in the relative FA composition between outdoor and laboratory cultures for each strain (Fig. 6B). Laboratory FA data derived from exponential growth phase during a batch experiment (15 °C, continuous irradiance of 120 µmol m^{-2} s^{-1} and aeration with 1% CO_2 enriched air. Experimental set up and data analysis are described for M28 in Steinrücken et al. [14]). The laboratory strains did not group together with their respective outdoor strains, but were clearly separated along Component 1. The FA EPA, palmitoleic, palmitic, hexadecatrienoic and arachidonic acid were primarily responsible for this separation. EPA, hexadecatrienoic acid and arachidonic acid correlated negatively with the laboratory strains, whereas palmitoleic acid and palmitic acid were positively correlated to the laboratory strains, and negatively to the outdoor grown ones. A Euclidean dendrogram (Fig. 6C) of the outdoor and laboratory data additionally illustrates the relationships between the strains, seasons and laboratory experiments in terms of similarities in their relative FA composition, and confirms that the laboratory strains group together, clearly separated from the outdoor cultures. The same clustering pattern was obtained in PCA analyses using FA content relative to DW. However, the contribution of FA to the distribution of the objects differed slightly (Appendix, Fig. 2).

4. Discussion

4.1. Production potential

Biomass productivities for strains Fito, M28 and B58 were maximal in spring when irradiance was highest (average of 39 mol m^{-2} d^{-1}) and lower during summer and autumn when irradiances, on average, decreased by 45%. Although the day lengths were comparable during

Table 1
Biomass, total fatty acid (TFA) and eicosapentaenoic acid (EPA) productivities per volume (P_{vol}), panel area ($P_{area,\ panel}$) and ground area ($P_{area,\ ground}$) and the yields on light for the three *Phaeodactylum tricornutum* strains (Fito, M28 and B58) for the three seasons (spring, summer and autumn) during a six-months repeated-batch cultivation in flat panel outdoor bioreactors in Bergen, western Norway. Values are average and standard deviation of the respective batch values of each season (biomass: $n = 6$ [5 for Fito], 6 and 3, TFA, EPA: $n = 5$ [4 for Fito], 6 and 3 for spring, summer and autumn, respectively).

	Spring			Summer			Autumn		
Biomass	Fito	M28	B58	Fito	M28	B58	Fito	M28	B58
P_{vol} ($g\ L^{-1}\ d^{-1}$)	0.25 ± 0.03	0.26 ± 0.04	0.30 ± 0.06	0.18 ± 0.03	0.18 ± 0.03	0.18 ± 0.02	0.16 ± 0.01	0.09 ± 0.02	0.16 ± 0.01
$P_{area,\ panel}$ ($g\ m^{-2}\ d^{-1}$)	9.3 ± 0.9	9.5 ± 1.5	10.9 ± 2.1	6.7 ± 1.2	6.5 ± 1.3	6.5 ± 0.9	6.1 ± 0.5	3.4 ± 0.9	6.0 ± 0.5
$P_{area,\ ground}$ ($g\ m^{-2}\ d^{-1}$)	7.3 ± 0.7	7.5 ± 1.2	8.7 ± 1.7	5.3 ± 1.0	5.2 ± 1.0	5.2 ± 0.7	4.8 ± 0.4	2.7 ± 0.7	4.7 ± 0.4
Yield $_{light}$ ($g\ mol^{-1}$ photon)	0.25 ± 0.05	0.27 ± 0.04	0.31 ± 0.05	0.36 ± 0.08	0.35 ± 0.09	0.35 ± 0.07	0.31 ± 0.06	0.16 ± 0.01	0.30 ± 0.04
TFA									
P_{vol} ($mg\ L^{-1}\ d^{-1}$)	38.7 ± 4.5	31.5 ± 5.0	43.8 ± 7.9	26.7 ± 2.7	21.5 ± 3.9	24.0 ± 3.0	25.7 ± 1.3	10.2 ± 3.3	22.3 ± 1.5
$P_{area,\ panel}$ ($mg\ m^{-2}\ d^{-1}$)	1426 ± 167	1162 ± 185	1613 ± 293	985 ± 99	791 ± 143	883 ± 109	947 ± 49	375 ± 123	821 ± 55
$P_{area,\ ground}$ ($mg\ m^{-2}\ d^{-1}$)	1129 ± 132	920 ± 146	1277 ± 232	780 ± 78	626 ± 113	699 ± 87	750 ± 38	297 ± 97	650 ± 44
Yield $_{light}$ ($mg\ mol^{-1}$ photon)	35.3 ± 2.9	31.3 ± 5.5	43.3 ± 7.7	52.7 ± 10.1	43.5 ± 13.2	47.9 ± 9.4	48.1 ± 9.6	17.3 ± 3.2	41.3 ± 5.2
EPA									
P_{vol} ($mg\ L^{-1}\ d^{-1}$)	11.8 ± 1.4	8.0 ± 1.8	9.2 ± 1.1	8.8 ± 0.7	6.2 ± 1.3	6.0 ± 0.7	8.7 ± 0.9	2.8 ± 1.1	5.6 ± 0.5
$P_{area,\ panel}$ ($mg\ m^{-2}\ d^{-1}$)	434 ± 51	295 ± 67	340 ± 40	325 ± 25	227 ± 48	221 ± 27	321 ± 34	102 ± 40	208 ± 17
$P_{area,\ ground}$ ($mg\ m^{-2}\ d^{-1}$)	344 ± 40	234 ± 53	269 ± 32	257 ± 20	180 ± 38	175 ± 21	254 ± 27	81 ± 32	164 ± 14
Yield $_{light}$ ($mg\ mol^{-1}$ photon)	10.7 ± 0.8	8.0 ± 2.2	9.2 ± 1.9	17.3 ± 2.7	12.5 ± 4.1	12.0 ± 2.5	16.2 ± 2.4	4.7 ± 1.2	10.5 ± 1.8

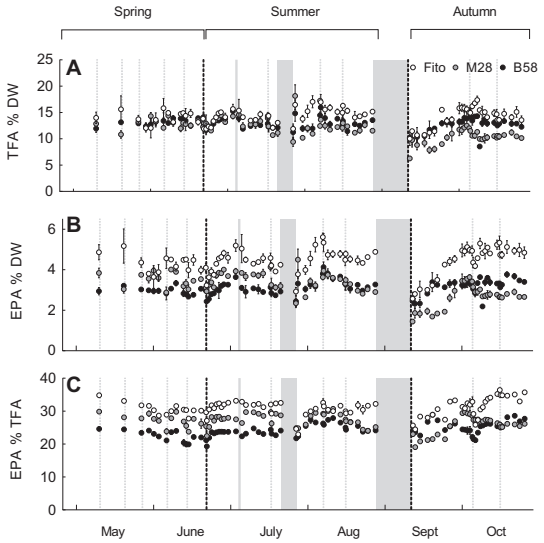

Fig. 5. Fatty acid values of three *Phaeodactylum tricornutum* strains during six-months repeated-batch cultivation in flat panel outdoor bioreactors in Bergen, western Norway, with total fatty acid (TFA) content relative to the dry weight (DW) (A), eicosapentaenoic acid (EPA) content relative to DW (B) and EPA content relative to TFA (C). Values are average and standard deviation of measurement replicates ($n = 2$ and 3 for FA and DW values respectively). The three grey bars indicate interruptions of the cultivation process. After interruption (1) and (3), the position of the strains was rotated, and after (2) and (3), the panels were re-inoculated with backup cultures. Grey dotted, vertical lines indicate dilution of the cultures and black dotted lines mark the division of the cultivation period into three seasons; spring, summer and autumn.

spring and summer seasons, the exceptionally bad weather conditions in summer of 2016 resulted in daily irradiances that were far below normal for the experimental site (ten years average around mid-summer is 42 mol $m^{-2}\ d^{-1}$ [own unpublished data]). Contrariwise, high irradiances during the day were measured in autumn, but concurrently day length was becoming progressively shorter and, thus, the average daily irradiances were similar to those found during summer. The results showed that outdoor production of *P. tricornutum* is certainly feasible during at least six months of the year at high latitude locations, even though weather conditions were inclement.

When compared with studies from southern latitudes, the average volumetric biomass productivities of the three strains were generally lower (Fig. 4B). Several studies on outdoor production of *P. tricornutum* in photobioreactors from Almería, Spain (36°50′N, 2°27′W) revealed up to five times higher biomass productivities with 1.5, 1.25 and 0.3 g $L^{-1}\ d^{-1}$ for cultures grown in a helical tubular PBR (3 cm internal diameter [i.d.]) [25], a horizontal tubular PBR (2.6 cm i.d.) [26] and vertical column PBRs (19 cm i.d.) [27], respectively. Additionally, a two to three times higher EPA productivity of 21 mg $L^{-1}\ d^{-1}$ was reported from the horizontal tubular PBR [26]. More similar to our findings (Table 1) were results from *P. tricornutum* cultures grown in a horizontal tubular PBR (4.85 cm i.d.) during summer in Florence, Italy (43°46′N, 11°15′E) with a mean areal biomass productivity of 13.1 g $m^{-2}\ d^{-1}$ (equivalent to a volumetric productivity of 0.26 g $L^{-1}\ d^{-1}$) [11]. Generally, highest productivities in these studies were achieved in PBRs with smaller optical paths. However, the different reactor types, operation modes and cultivation conditions used in these studies make empirical comparisons difficult. The same GWP®-III reactor panels were used by Rodolfi et al. [28] for cultivation of *P. tricornutum* during spring and summer in Florence, Italy. They reported higher panel-areal biomass productivities of 12.3 and 17.2–17.8 g $m^{-2}\ d^{-1}$ for nutrient replete cultures during spring and summer, respectively, but similar EPA productivities as found for our strains (0.29 and 0.35–0.38 g $m^{-2}\ d^{-1}$ in spring and summer, respectively) due to a lower EPA content (2.06–2.38% DW) of their *P. tricornutum* strain. When recalculating the panel-areal productivities for our strains with the volume (37–43 L) and panel-area (0.78 m^2) used in Rodolfi et al. [28], higher EPA productivities were achieved for strain Fito in all seasons (0.60, 0.45, and 0.45 g $m^{-2}\ d^{-1}$) and for strain M28 and B58 in spring (0.41 and 0.47 g $m^{-2}\ d^{-1}$, respectively).

Extrapolating the biomass that could be produced in one year (assuming 180 d of operation) per hectare (using eight GWP®-II1250 reactors, www.femonline.it/products) at our location, suggested an amount of 10.7 t (10.8, 9.7 and 11.7 t $ha^{-1}\ yr^{-1}$ on average for strains Fito, M28 and B58, and 14.8, 9.8 and 7.5 t $ha^{-1}\ yr^{-1}$ on average for spring, summer and autumn, respectively). Tredici et al. [29] calculated an annual biomass productivity for the green algae *Tetraselmis suecica* of 36 t $ha^{-1}\ yr^{-1}$ in Tuscany, Italy (240 operation days) and 66 t $ha^{-1}\ yr^{-1}$ when placing the reactors in North Africa (330 operation days). In regions of lower latitudes, productivities are increased due to higher irradiance and more operational days during a year. The longer day length during spring and summer that was assumed to promote productivity at higher latitudes could not compensate for this. However, the higher temperatures in southern regions demand

Table 2
Percent major fatty acids (> 2% TFA) identified for the three *Phaeodactylum tricornutum* strains during six-months repeated-batch cultivation in flat panel outdoor bioreactors in Bergen, western Norway. Values show average and standard deviation of all samples of the respective seasons (n = 30, 72 and 60 for spring, summer and autumn, respectively).

Strain	Season	14:0 Myristic	16:0 Palmitic	16:1 n-7 Palmitoleic	16:2 n-4 Hexadeca-dienoic	16:3 n-4 Hexadeca-trienoic	16:4 n-1 Hexadeca-tetraenoic	18:2 n-6 Linoleic	20:4 n-6 Arachidonic	20:5 n-3 EPA	24:0 Lignoceric	Rest
Fito	Spring	8.0 ± 0.6	10.9 ± 0.9	18.7 ± 1.9	4.3 ± 0.8	6.7 ± 1.5	4.5 ± 1.2	2.0 ± 0.6	1.5 ± 0.6	30.6 ± 1.8	2.0 ± 0.3	10.8
	Summer	7.9 ± 0.5	11.7 ± 1.6	18.9 ± 2.8	5.7 ± 0.8	5.6 ± 2.4	4.2 ± 1.2	2.0 ± 0.6	0.8 ± 0.5	30.8 ± 2.2	2.0 ± 0.3	10.4
	Autumn	8.0 ± 0.4	12.4 ± 2.1	17.4 ± 2.6	6.1 ± 1.3	3.4 ± 1.5	3.9 ± 1.4	3.0 ± 0.7	0.8 ± 0.4	31.4 ± 3.3	2.3 ± 0.4	11.3
M28	Spring	8.9 ± 0.4	10.2 ± 0.8	24.7 ± 2.0	1.8 ± 0.6	2.4 ± 0.7	4.8 ± 0.6	3.0 ± 0.8	2.1 ± 0.8	27.1 ± 1.9	2.4 ± 0.3	12.6
	Summer	7.5 ± 1.0	10.5 ± 1.2	24.5 ± 2.4	3.2 ± 1.1	3.1 ± 1.3	4.4 ± 1.0	2.7 ± 0.9	0.7 ± 0.5	27.5 ± 1.9	2.7 ± 0.5	13.2
	Autumn	5.0 ± 0.5	11.5 ± 1.5	25.1 ± 2.9	4.5 ± 1.4	2.9 ± 0.9	4.9 ± 1.2	1.9 ± 0.6	0.2 ± 0.1	25.7 ± 2.8	3.1 ± 0.5	15.2
B58	Spring	6.7 ± 0.2	11.7 ± 0.7	24.3 ± 1.7	3.7 ± 0.6	6.9 ± 1.2	2.6 ± 0.7	2.8 ± 0.7	6.5 ± 1.1	22.4 ± 1.7	1.9 ± 0.2	10.5
	Summer	6.0 ± 0.3	12.1 ± 1.1	23.2 ± 2.0	6.3 ± 1.8	6.2 ± 2.5	2.2 ± 0.7	2.9 ± 0.8	4.0 ± 1.6	24.2 ± 1.8	2.1 ± 0.3	10.8
	Autumn	6.2 ± 0.3	12.6 ± 1.7	22.1 ± 2.8	5.8 ± 0.9	5.8 ± 1.8	2.0 ± 1.2	3.4 ± 0.7	3.3 ± 1.4	25.2 ± 2.0	2.3 ± 0.3	11.3

intensive cooling of cultures. In Italy, cooling is necessary for 5–6 months of the year and corresponds to 11% of the energy input for plant operation [29]. Those inputs are likely to be reduced at higher latitudes, as cooling at our location was necessary only during days with high irradiance, and cost-efficient tap water could be used.

Although the lower productivities in our study were most likely connected to the limited irradiance at our cultivation site, alterations in cultivation conditions and operation modes could potentially increase productivities at the local site, suggesting the operation of the reactors in continuous mode by using either chemostat or turbidostat operation [30]. The light that is available for the microalgae in the culture strongly depends on the prevailing irradiance, but also on reactor design and biomass concentration of the culture. When applying repeated batch cultures, the constantly changing biomass concentrations, together with the fluctuating solar irradiances, make it difficult to ensure optimal irradiance available for cultures. Too high or too low irradiance can result in suboptimal operation, due to incomplete light absorption and photo-inhibition, or dark zones that reduce the productivity, respectively [31,32]. Keeping the DW between approximately 0.4 and 2.5 g L^{-1} turned out to be a suitable range for maintaining the strains at linear growth during spring and summer. However, during autumn, all strains appeared to become light-limited progressively earlier at DW below 2.5 g L^{-1}, probably as a result of the gradually decreasing day length and solar azimuth, as average daily irradiances were similar as in summer.

Maintaining nutrient replete conditions is important to measure biomass and EPA production potential accurately. Periodically-measured nitrate and phosphate concentrations in the media revealed that these nutrients were at no time point limiting to culture growth (data not shown). The silica concentration was not monitored as *P. tricornutum* cells usually have little or no silica requirements [2], and Zhao et al. found that silicon influenced the growth rate only under conditions of low temperature and green light [33]. Nutrient replete growth was also confirmed by the TFA content of the strains during the cultivation period. An increase in TFA due to accumulation of storage lipids is a typical response to nutrient starvation and an increase of TFA of up to 35% DW has been reported for nutrient-depleted *P. tricornutum* cultures [34]. Even though TFA content varied (10.0–17.4, 6.3–18.1 and 8.5–16.0% DW for Fito, M28 and B58, respectively) throughout the cultivation period, such an accumulation was not observed in our study. Similar TFA values (between 8.4 and 10% DW) were found for outdoor cultures of *P. tricornutum* by Sánchez Míron et al. [27]. The moderate variation of the TFA content observed in our study might reflect changing physiological processes in the cells during variations in the cultivation conditions. Moderate variations during the cultivation period were also observed for the EPA content in all three strains. Several studies have revealed that environmental factors such as nutrient availability, temperature, light, salinity, pH and cell density influence the microalgal lipid content and composition and thus EPA content [7,35–43]. Generally, the TFA and EPA contents were considerably lower after re-inoculation, and increased to higher levels with increasing cultivation time in all three strains; this was especially noticeable for the EPA content in strain Fito.

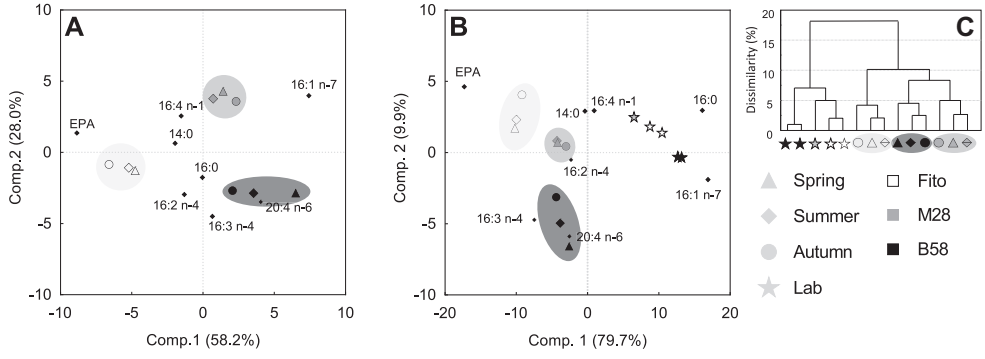

Fig. 6. Principal component analysis (A and B) and Euclidian Dendrogram (C) of the relative fatty acids composition (% TFA) for three *Phaeodactylum tricornutum* strains (Fito, M28 and B58). A: Nine objects representing the three strains at the three seasons (spring, summer, autumn) during six-months repeated-batch cultivation in flat panel outdoor bioreactors in Bergen, western Norway, and eight variables, representing the fatty acids with highest impact on the distributions. Values are average of all samples of the respective seasons (n = 30, 72 and 60 for spring, summer and autumn, respectively). B: Same variables and objects as in (A) including additional data from laboratory experiments (average of two measurement replicates) for Fito, B58 (two biological replicates) and M28 (one biological replicate). C: Dendrogram showing dissimilarities between the nine outdoor and five laboratory objects. Laboratory data are from exponential growth phase during batch experiments with experimental setup described for M28 in Steinrücken et al. [14].

4.2. Strain specific characteristics

Our investigations revealed a number of dissimilarities between the three *P. tricornutum* strains including morphology, phylogenetic relationship, productivity, EPA content, and relative FA composition. However, no correlation between biogeography and the other factors was observed. Even though the two local strains M28 and B58 were isolated from the same location (but at different times), strain B58 was phylogenetically closer to strain Fito than to strain M28. Strain M28 diverged from strains Fito and B58 at several positions in the ITS1-5.8S-ITS2 region of the genome, whereas strains Fito and B58 had identical sequences, with the exception of a single bp (position 553, 5′-3′), where strain B58 displayed thymine, whereas the sequencing chromatograms indicated that strain Fito displayed equal amounts of cytosine and thymine. There are several possible explanations for the divergence observed in strain Fito: (1) Strain Fito is composed of two strains, and has been since the beginning of the experiment. (2) Within strain Fito there has been a point mutation, which has been inherited and is now present in approximately half of the culture. (3) Strain Fito has two copies of the ITS1-5.8S-ITS2 gene complex with one bp difference between the two copies, resulting in the observed differences. The consistency of the genomic differences observed throughout the experimental period confirmed that strains were not mixed or substituted during the experiment. However, in terms of morphology, the two local strains M28 and B58 were more similar to each other than to strain Fito. Strain Fito exhibited only fusiform cells that were noticeably smaller than cells of the others two strains, whereas all three morphotypes were eventually detected for strains M28 and B58, both dominated by cells with fusiform morphology. This seems to indicate that polymorphism within the species *P. tricornutum* is strongly strain specific, and different studies have shown that strains vary in their tendency for pleiomorphy [44]. However, the conditions that promote growth and maintenance of a specific morphotype are still poorly understood. Often a specific morphotype predominates within a strain, whereby the fusiform morphotype is the most prevalent form [21].

Biomass productivity was similar for all three strains in spring and in summer with B58 having slightly higher productivities in spring. However, in autumn, after the third inoculation, strain M28 revealed a reduced biomass (and therewith EPA) productivity compared to the other strains, but at the same time the QY remained on the same level as during the previous cultivation (aside from a short drop after inoculation). This indicates that the physiological condition of the cells was not diminished, and that the reduced productivity might have been a strain-specific response to the progressively shorter day length. In that case, M28 would be an inferior candidate for outdoor cultivation at the given locations. In accordance with the biomass productivity, the EPA productivity was maximal in spring and lower in summer and autumn for all three strains. However, a significant difference between the strains was observed, with strain Fito revealing highest EPA productivities during the entire cultivation period. This was due to the high EPA content of the biomass in strain Fito (average of 4.4% DW) compared to M28 and B58 (average of 3.2 and 3.1%). Such a high EPA content has, to our knowledge, not been reported before for *P. tricornutum*, and compensates somewhat for the low biomass productivities. Thus, strain Fito was the most promising strain regarding EPA productivity at our location.

The same FA were detected for all thee strains with EPA, palmitoleic acid, palmitic acid, and myristic acid representing the major fraction of TFA. Similar relative FA composition for *P. tricornutum* were found by others [27,45]. However, although the FA composition and contribution to TFA were similar among the three strains, a principal component analysis revealed strain-specific differences. The average FA composition of the three seasons for each strain were very similar and grouped closely together, whereas the three strains were clearly separated. Thus, differences between the strains were greater than differences between the seasons for a strain, and the three *P. tricornutum* strains could easily be distinguished by their relative FA composition. The FA that were primarily responsible for the clustering, were EPA, palmitoleic acid, arachidonic acid and hexadecatrienoic acid. EPA correlated positively with strain Fito, thus, reflecting the predominantly higher amount of EPA in this strain compared to M28 and B58, whereas palmitoleic acid and arachidonic acid were negatively correlated to strain Fito, indicating lower relative amounts of these FA. However, comparing the FA composition with data from laboratory cultures from the same three strains, revealed a significantly different relative FA composition between indoor and outdoor strains. The respective indoor and outdoor strains did not cluster together, but distinct from each other, and the indoor strains were more similar to each other than to the corresponding outdoor strains. These data indicate that all strains changed their relative FA composition from indoor to outdoor conditions, but responses were different between the strains. Thus, a particular FA composition only accounted for the conditions of this outdoor experiment, and did not allow for general strain identification outside this experimental setup. Interestingly, indoor/outdoor cultivations had a particular influence on the EPA content, that was noticeably lower for all strains under laboratory conditions. This correlates well with the decreased EPA contents that were observed in the outdoor cultures just after inoculation. Significant differences in the lipid fractions between laboratory- and outdoor-grown *P. tricornutum* cultures were previously shown by López Alonso et al. [46], who found EPA content increasing within four different lipid classes from indoor to outdoor conditions, with an increase from 31 to 40% in monogalactosyldiacylglycerols.

The key regulatory factors accounting for these different responses are difficult to identify, but are presumably related to the different cultivation conditions used in laboratory and outdoor cultures, including culture densities, pH, culture media, and daily irradiance and temperature conditions. Laboratory cultures had much lower DW concentration (0.1–0.2 g L^{-1}), and constant temperature, irradiance and pH conditions (15 °C, 120 μmol m^{-2} s^{-1} for 24 h d^{-1} and pH 7.4). In contrast to this, outdoor grown strains had greater variation in DW concentrations (0.05–3 g L^{-1}), and were exposed to diurnal and seasonal changes in irradiance (intensity and duration) and temperatures, and a less stable and higher pH (average pH 7.8). Furthermore, the media for outdoor cultures had a considerably higher macronutrient concentration than media used for indoor cultures. Further laboratory experiments with alteration of settings of the different environmental factors, together with additional outdoor experiments might help identify which factor or combination of factors exert the primary influence on the dramatic change in FA profile. However, our results are significant in terms of strain selection for outdoor cultivation. A strain that might appear promising under laboratory conditions does not necessarily perform best under outdoor conditions. The same is true conversely, as strain Fito had the lowest EPA content under laboratory conditions and the highest content under outdoor conditions, and thus the highest EPA productivities of all strains.

Growing *P. tricornutum* on a large scale is realizable under the local climate conditions. However, the EPA productivity strongly depends on the strain chosen, and it can be further optimized by improving photobioreactor operation. To what extent large-scale cultivation is manageable in a sustainable and economic way, needs to be evaluated further.

5. Conclusion

Outdoor production of three different *P. tricornutum* strains was feasible during at least six months of the year at the climate conditions in Bergen, western Norway and productivities were maximal in spring (Biomass: 0.25, 0.26 and 0.30 g L^{-1} d^{-1}, EPA: 11.8, 8.0 and 9.2 mg L^{-1} d^{-1}, for Fito, M28 and B58, respectively) when irradiance was highest (mean of 36.3 mol m^{-2} d^{-1}). In summer and autumn, average irradiances were reduced by 45% and productivities decreased by approximately 30–45% in all strains, and by 65% in strain M28 in

autumn. Strain Fito revealed highest EPA productivities during the entire cultivation period due to an exceptionally high EPA content of the biomass (average of 4.4% DW compared to 3.2 and 3.1 for M28 and B58), that has to our knowledge, not been reported before for *P. tricornutum*. When related to studies from lower latitudes, biomass productivities of the three strains were lower, most probably due to the reduced irradiances at the given location. However, the comparatively high EPA content of our strains under outdoor conditions could partially compensate for the lower biomass productivities, and similar EPA productivities were obtained as found from studies in Italy. Microalgae cultivation at higher latitudes might be further enhanced by improving cultivation conditions, like maintaining more constant biomass concentrations, and a more accurate temperature and pH control.

The same FA were identified for the three strains, but a PCA revealed different relative abundances, allowing for discrimination between the three strains by their FA profiles, whereas the changing seasons had only little influence on the FA content. The FA that predominantly contributed to the distribution of the three strains were EPA, palmitoleic acid, arachidonic acid (20:4 n-6), hexadecatetraenoic acid (16:4 n-1), hexadecatrienoic acid (16:3 n-4) and hexadecadienoic acid (16:2 n-4); EPA correlated positively with strain Fito. Comparing the relative FA composition from the outdoor cultures with their respective FA profiles from laboratory experiments revealed stronger differences between outdoor- and laboratory-grown cultures, than between strains. Hence, the increased EPA content in strain Fito that resulted in a significantly higher EPA productivity was only observed during outdoor conditions, but not in laboratory experiments. Our results demonstrate the importance for empirical comparison of different strains at a given location to achieve maximal EPA productivities.

Declarations of interest

None.
No conflicts, informed consent, human or animal rights applicable.

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Appendix A

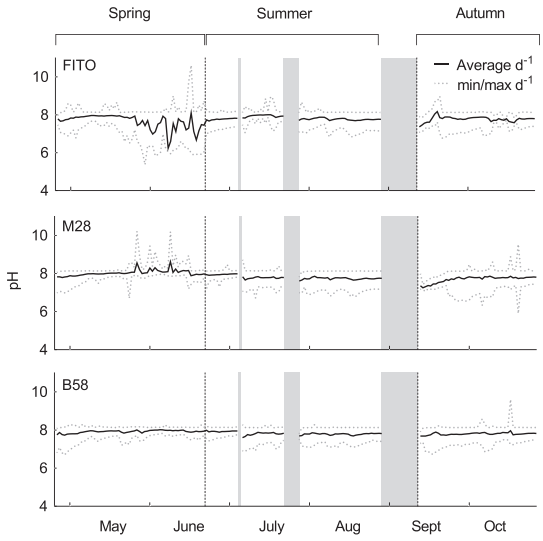

Fig. 1. Daily average, minimum and maximum pH during six-months repeated-batch cultivation of three *Phaeodactylum tricornutum* strains (Fito, M28, B58) in flat panel outdoor bioreactors in Bergen, western Norway. The three grey bars indicate interruptions of the cultivation process. After interruption (1) and (3), the position of the strains was rotated, and after (2) and (3), the panels were re-inoculated with backup cultures. Black dotted lines mark the division of the cultivation period into three seasons; spring, summer and autumn.

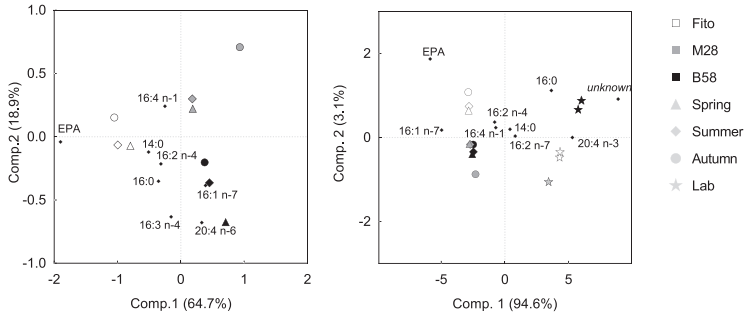

Fig. 2. Principal component analysis of the fatty acids composition (% DW) for three *Phaeodactylum tricornutum* strains (Fito, M28 and B58). A: Nine objects representing the three strains at the three seasons (spring, summer, autumn) during six-months repeated-batch cultivation in flat panel outdoor bioreactors in Bergen, western Norway, and eight variables, representing the fatty acids (FA) with highest impact on the distributions. Values are average of all samples of the respective seasons (n = 30, 72 and 60 for spring, summer and autumn, respectively). B: Nine variables and same objects as in (A) including additional data from laboratory experiments (average of three measurement replicates) for Fito, B58 (two biological replicates) and M28 (one biological replicate). Laboratory data are from exponential growth phase during batch experiments with experimental setup and analyses described for M28 in Steinrücken et al. [14].

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