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Phototrophic microalgal cultivation in cold and light-limited environments

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Preface

This thesis is submitted in partial fulfilment of the requirements for the Degree of Philosophiae (PhD) at the Faculty of Biosciences and Aquaculture (FBA), Nord University, Bodø, Norway. The studies included in this thesis represent original research conducted at the Faculty of Aquaculture and Bioscience and the collaborating institutions University of Almeria, University of Algarve and Wageningen University from September 2016 to June 2019. The presented work is part of the project Bioteknologi– en framtidsrettet næring funded by Nordland County Government in Norway. Peter S.C. Schulze was only involved in fatty acid analysis in Paper II and did not draft the manuscript. Serena Lima and Peter S.C. Schulze share the first authorship in Paper V; Serena Lima will use portions of the study in her doctoral thesis at the University of Palermo. Supplementary research data from the thesis of Peter S.C. Schulze that could not be included in the thesis because of its large size, can be made available upon request by contacting the Faculty PhD administration.

The PhD project team consisted of the following members:

Peter S.C. Schulze, MSc, FBA, Nord University, PhD Student

Kiron Viswanath, Professor, FBA, Nord University, primary supervisor

René H. Wijffels, Professor, FBA, Nord University; Professor, Bioprocess Engineering, Wageningen University, co-supervisor

Peter S.C. Schulze

Bodø, August 2019

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List of abbreviations

DC	Duty cycle
EPA	Eicosapentaenoic acid
EPS	Extracellular polymeric substances
LED	Light emitting diode
MUFA	Monounsaturated fatty acids
NPQ	Non-photochemical quenching
PAR	Photosynthetic active radiation
PUFA	Polyunsaturated fatty acids
PWM	Pulse width modulator
RuBisCO	Ribulose-1,5-bisphosphate-carboxylase/oxygenase
SFA	Saturated fatty acids
TAG	Triacylglycerols
TFA	Total fatty acids

Abstract

Microalgal production at extreme latitudes (e.g., Bodø, Norway) require culture heating and artificial light for a year-round operation due to low ambient temperatures and solar irradiance. Cold-adapted microalgae can be used to save heating costs because they grow well at low temperatures and accumulate high amounts of biotechnologically-relevant compounds including fatty acids, proteins or pigments used in foods and feeds. To stimulate growth and biomolecule induction, previous studies suggested to adjust environmental triggers such as temperature and light intensities or using flashing light, which is the periodical supply of light pulses alternated by dark periods.

A first study, screening twenty cold-adapted strains, identified *Chlamydomonas* sp. (RCC 2488), *Tetraselmis chui* (SAG 1.96) and *Pseudopleurochloris antarctica* (SAG 39.98) as promising strains to produce proteins and (polyunsaturated-) fatty acids. The Arctic isolate *Chlamydomonas* sp. (RCC 2488) grew better at 8°C compared to 15°C and showed high productivities of protein and polyunsaturated fatty acids (PUFA) (70 and 54 mg L^{-1} d^{-1}, respectively). PUFA productions up to 85 mg L^{-1} d^{-1} were reached when the alga was cultivated continuously under nutrient sufficient conditions. Under nitrogen starvation, carbohydrates were induced, while light intensities (50-500 µmol s^{-1} m^{-2}) or salinities (0-70 ppt) only showed minor effects on biochemical composition. *T. chui* was a robust, fast growing strain reaching high biomass productivities among all treatments (up to 1 g L^{-1} d^{-1}), containing up to 50 mg PUFA g^{-1} and 15% proteins. *P. antarctica* (SAG 39.98) grew well at 15°C (0.4 g L^{-1} d^{-1}) and yielded highest eicosapentaenoic acid (EPA) productivity (7.6 mg L^{-1} d^{-1}). Lastly, a well-growing *Chlorella stigmatophora* strain (0.4 g L^{-1} d^{-1} at 15°C) was identified that excreted extracellular polymeric substances.

Exposing *T. chui* and *C. stigmatophora* to flashing light emitting diodes (LEDs) that emit light pulses more than 200-500 times per second (*i.e.*, frequency; $f \geq 500$ Hz), did not improve their growth compared to that under continuous light. Lower repetition rates affected growth negatively depending on culture concentration, light intensity and light acclimatisation stage of the cultures. Strikingly, low-frequency flashing light (5,

50 Hz) efficiently induced proteins, polyunsaturated fatty acid or pigments; in *Nannochloropsis* grown under mesophilic temperature conditions (20°C) as well as in the Antarctic strains *T. chui* and *Koliella antarctica* grown at psychrotrophic conditions (15°C). Under these conditions, pigment production (e.g., *β*-carotene) was improved up to three times compared to continuous light. Higher frequencies (*f*= 500 Hz) did not affect biochemical profiles or growth.

Notably, the biochemical profile of microalgae was mostly affected by the prevailing growth stage of the culture rather than the tested environmental parameter. In summary, artificial light-based microalgal production at extreme latitudes can employ cold-adapted microalgae and flashing lights to maximise PUFA and pigment production, taking advantage of the low ambient temperatures as cheap cooling sources for LEDs and cultures. In addition, minimising exposure time of triggers that inhibit growth but induce compounds (e.g., low frequency flashing light) is of utmost importance to maximise PUFA and pigment productivities.

1. Introduction

Biotechnological innovations employing microalgae can ensure sustainable supply of feedstock rich in proteins, lipids, pigments and other biomolecules to the food and feed industry (Ruiz et al. 2016). Microalgal production does not necessarily compete with agriculture as no arable land or fresh water is needed for cultivation (Gouveia and Oliveira 2009; Schenk et al. 2008). Furthermore, microalgal industry is poised to substitute lipids or proteins from unsustainable resources such as those from fisheries or soybean (Ryckebosch et al. 2014; Taelman et al. 2015). However, today´s microalgal production is in its infancy and high production costs (~5-30 € Kg^{-1}) allows to target mostly high-value products such as omega-3 fatty acids, pigments and functional compounds (Ruiz et al. 2016). To decrease production costs and broaden the market portfolio, industries require novel strains and better light management, the latter being a major bottleneck in any phototrophic microalgal production (Blanken et al. 2013; Ooms et al. 2016; Ruiz et al. 2016).

Presently, only ~20 microalgal species are commercially exploited, *i.e.*, a small subset of the globally existing species (Guiry 2012). Recent studies suggested that microalgal strains from cold regions are rich in lipids, fatty acids or pigments, and can grow at low temperatures (<15°C) with the same pace as meso- and thermophilic strains cultured under warm conditions (e.g., >20°C; Cvetkovska et al. 2014; Hulatt et al. 2017; Suzuki et al. 2018). These high growth rates under low temperatures make them ideal models for production during cold seasons or environments, because expensive heating required to culture meso- and thermophilic strains can be minimised (Pankratz et al. 2017). In addition to low temperature-adaption, microalgal cultivation at extreme latitudes (e.g., Bodø, Norway) must cope with low solar irradiances, indicating the need for artificial lighting for a year-round production. This approach increases production costs of the biomass to 25-30€ Kg^{-1} (Blanken et al. 2013). Nevertheless, microalgae that are produced indoors with artificial light score a high market value because cultures can be kept clean and a tight control of environmental parameters (temperature, light) stimulates growth and induction of target biomolecules (Blanken et al. 2013; Schüler et

al. 2017). The competitiveness of artificial light-based production strongly depends on the efficiency of the lamp to emit light that stimulates photosynthesis in algae cells and produce biomass rich in desired biocompounds. Flashing light, which is the periodical supply of high intense light flashes alternated by extended dark period, is considered promising to promote growth and induce biochemical compounds in microalgal cultures (Katsuda et al. 2008, 2006; Lunka and Bayless 2013; Schulze et al. 2017). The studies in this thesis identified the potential of cold-adapted marine microalgae from cold environments by investigating the efficacy of environmental factors (e.g., temperature, light intensities and growth stage) to trigger production of biomass and biocompounds. In addition, promising cold-strains were cultivated under various flashing light conditions, a plausible method to improve artificial light-based microalgal production at extreme latitudes.

1.1. Cold adapted microalgae

Through ~1.5 billion years of evolution, microalgae have adapted to different habitats such as hot fountains, permafrost regions, deep oceans or mountains by developing unique metabolic mechanisms (Falkowski et al. 2004). Cold environments are characterised by high water viscosity, ice formation, hypersaline (brine-) channels in pack ice or high irradiances due to light reflection by ice and snow (Varshney et al. 2015). For example, during polar winter, sun light and nutrients are scare and ice formation concentrates salts, forming ion-rich brine channels that inhabit microalgae (Horner 2017; Jones et al. 2001). Additionally, ice and snow can lead to strong light variations due to reflection of solar light by snow and ice. Towards polar spring, increasing solar irradiance permits higher photosynthetic rates by phototrophs and nutrients become readily available when the increasing temperature melts the ice, draining nutrients and minerals from soils into the rivers and oceans. Consequently, often large algae blooms occur in polar regions, a process detailed by Leu et al. (2015).

Due to extreme seasonal changes and low ambient temperatures, microalgae inhabiting polar oceans must develop high metabolic flexibility and endure intracellular ice and nucleation formation, tighter packing of molecules, protein misfolding and low metabolic reaction kinetics (Seckbach et al. 2013). As response, cold-adapted microalgae have effective protective mechanisms including the synthesis of cold-adapted enzymes, long chain-polyunsaturated flatty acids (PUFA), pigments and cryo-protective and chaotropic molecules such as modified α–amylase, extracellular polymeric substances (EPS), teichoic acid or fumarate and glycerol (de Jesús Paniagua-Michel 2014; Hulatt et al. 2017; Huston 2008; Katsuda et al. 2004; Seckbach et al. 2013; Suzuki et al. 2018; Varshney et al. 2015; Table 1). Cold-adapted microalgae can use carotenoids (e.g. xanthophylls) and PUFA to counterbalance variations in osmotic pressures or temperatures that otherwise would hinder cells to control molecule exchange with the environment through the lipid bilayer (*i.e.*, plasma membrane; Dieser et al. 2010). Therefore, low temperatures were found to trigger photoprotective pigment metabolism in cold-adapted *Fragilariopsis cylindrus* and *Haematococcus pluvialis* compared to high temperatures (Klochkova et al. 2013; Mock and Hoch 2005).

In praxis, many cold-adapted strains were tested on lab scale (Table 1) for the production of proteins, pigments or fatty acids, including *Haematococcus pluvialis* (Chekanov et al. 2014; Klochkova et al. 2013), *Chlamydomonas* sp. (Mou et al. 2012), *Koliella antarctica* (Fogliano et al. 2010) or *Monoraphidium* sp. (Řezanka et al. 2017). Additionally, outdoor production scenarios at cold climates were investigated (Table 2; Franco et al. 2012; Varshney et al. 2015). Notably, most of the tested strains were from freshwater habitats and included only few strains from marine or saline environments. However, it should be noted that the marine strains are rich in PUFA or pigments as they are at the base of polar marine food chains and are the source of omega-3 and pigments found in higher animals living in these environments. Therefore, the exploitation of these key compounds from cold-adapted microalgae holds promises, but suitable environmental triggers for production at cold environments remains to be investigated.

Table 1. Biotechnologically-relevant cold-adapted microalgae and cyanobacteria.

Main Product	Relevant Microalga/ Class	T (°C)	Conditions/Outcome	References
Pigments				
Astaxanthin	*Haematococcus pluvialis*, *Chlamydomonas nivalis*	+4 to +20	Cold-water adapted *Haematococcus pluvialis* produced 40-55 mg g^{-1} astaxanthin. *C. nivalis* produced 14 mg g^{-1} secondary carotenoids (mostly astaxanthin). Most *Chlamydomonas* isolated from snow and ice habitats are promising astaxanthin producers.	(Chekanov et al. 2014; Fujii et al. 2010; Klochkova et al. 2013; Remias et al. 2010; Remias et al. 2015; Remias et al. 2005)
Astaxanthin, Lutein	*Koliella antarctica* SAG 2030	+4 to +15	*K. antarctica* has lutein and astaxanthin as the major pigments. Both pigments are accumulated in biomass, with aging cultures containing up to 14 mg g^{-1} at the late exponential phase.	(Fogliano et al. 2010; La Rocca et al. 2015)
Xanthophyll, α-tocopherol, Lutein	Raphidonema, Chlorophyta	+10	Two *Raphidonema* species displayed a high pool of primary xanthophyll cycle pigments and are promising α-tocopherol producers. *R. nivale* and *Chlorococcum* sp. accumulate 37.4 and 30.1 mg lutein per100g fresh biomass, respectively.	(Leya et al. 2009)
Diatoxanthin, Fucoxanthin	*Fragilariopsis cylindrus*	+7, -1	*F. cylindrus* accumulated 361% more diatoxanthin and 84% more fucoxanthin when the alga was transferred from 7°C to -1°C.	(Mock and Hoch 2005)
Lutein, β-Carotene, Zeaxanthin	*Parietochloris incisa* (mesophilic snow algae)	+25	Moderate light intensities and N-sufficient conditions induced highest total carotenoid levels in *P. incisa*, while low-light increased the lutein fraction (max. 49.1% of total carotenoids). High-light and in nitrogen-depleted condition caused accumulation of zeaxanthin (max. 21.8% of total carotenoids).	(Solovchenko et al. 2009; Solovchenko et al. 2008; Solovchenko et al. 2010)

Lipids and Fatty acids

EPA	*Melosira arctica*, diatoms	n.a.	Environmental samples of *Melosira arctica* (Coscinodiscophyceae) contained up to 35% eicosapentaenoic acid (EPA) of total fatty acids.	(Falk-Petersen et al. 1998)
EPA, DHA	*Koliella antarctica* SAG 2030*	+10, +15	*K. antarctica* contained more EPA and DHA (6.8 and 16.2% of TFA, respectively) when cultivated under 10°C as compared to 15°C. EPA and DHA contents increased in aging cultures.	(Fogliano et al. 2010)
EPA, AA	*Pseudopleurochloris antarctica* SAG 39.98	+10	Cultures of *P. antarctica* obtained from SAG culture collection, maintained at 10°C, contained high amounts of EPA (20%) and AA (4.5%).	(Lang et al. 2011)
Fatty acids	*Chlamydomonas* sp. ArM0029A	+15	*Chlamydomonas* sp. ArM0029A contained high amounts of PUFA, which is composed of C16:3, C16:4, C18:2 and C18:3. Hexadecatrienoic acid (C16:3*n*-3) was induced at low salinities (7 ppt). Authors recommend the use of ArM0029A in cold climates for lipid and PUFA production via salinity adjustments (*T*= 15°C).	(Ahn et al. 2015)
TAG, AA	*Parietochloris incisa* (mesophilic snow algae)	+25	*P. incisa* contained arachidonic acid (AA), which is its major fatty acid (33-50% of TFA). The TAG fraction contained up to 77% AA. *P. incisa* is a promising candidate to produce TAG and AA simultaneously. Nitrogen starvation induces AA. High-light (270 µmol s^{-1} m^{-2}) increases total fatty acid content in biomass (up to ~35%). (*T*= 25°C)	(Bigogno et al. 2002; Khozin-Goldberg et al. 2002; Solovchenko et al. 2010)

Proteins, genes and secondary metabolites

Anti-freeze proteins	*Chaetoceros neogracile*	+4	*C. neogracile* has antifreeze protein gene (Cn-AFP), and the gene was successfully isolated, cloned and expressed in *Escherichia coli*. AFP can be used in crop plants against cold temperatures. (T=4°C)	(Gwak et al. 2010)
Anti-freeze proteins	*Coccomyxa subellipsoidea* C-169	+25	The genome of *C. subellipsoidea* C-169 was completely sequenced. Through in silico analysis information about the genes encoding antifreeze lipoprotein as well as those of exopolysaccharides, glycoproteins or lipid biosynthesis can be obtained (*T*= 25°C).	(Blanc et al. 2012)

Bioactive compounds	Diatoms	+4 to +9	Temperature and light affected the bioactivities of *Attheya longicornis*, *Chaetoceros socialis*, *Chaetoceros furcellatus*, *Porosira glacialis* and *Skeletonema marinoi* isolated from the North Atlantic. The extracted compounds had antioxidant and immunomodulatory properties and demonstrated bioactivities against diabetes II, cancer, and infection (T4-9°C).	(Ingebrigtsen et al. 2016)
Phycotoxins as bioactive compounds	Diatoms, dinoflagellates	-2 to +4	Cold water-adapted marine species are sources of natural bioactive compounds. The diatom *Nitzschia pungens f.* multiseries is a producer of domoic acid, the dinoflagellate *Alexandrium tamarense* produces saxitoxin, *Dinophsis acuta* accumulates pectenotoxins, *Alexandrium ostenfeldii* contains spirolides and *Protoceratium reticulatum* has yessotoxin (*T*= -2 to +4°C).	(Lebar et al. 2007)
MAA	Cyanobacteria, diatoms	-1 to +14	Diatoms and cyanobacteria from Environmental water samples from melt water and brine channels were dominated by diatoms and cyanobacteria that displayed considerable levels of mycosporine-like amino acids (MAAs) and carotenoids through their absorption spectra. (*T*=-1 to 14°C)	(Rajanahally et al. 2014; Vincent and Quesada 1994)
Extracellular polymeric substances (EPS)	Diatoms, cyanobacteria	n.a.	Cold-adapted algae and cyanobacteria use EPSs to attach to ice; form microaggregates or form a microenvironment to cope with low temperatures or hypersaline brine channels in ice. EPS from cold-adapted microalgae could be used as biosurfactants and bioemulsifiers for food/feed or pharmaceutics.	(de Jesús Paniagua-Michel 2014; Krembs et al. 2002; Mundy et al. 2011; Rontani et al. 2016)

Abbreviations: **n.a.**, not available; **AA**, Arachidonic acid; **DHA**, Docosahexaenoic acid; **EPA**, eicosapentaenoic acid; **EPS**, extracellular polymeric substances; **MAAs**, mycosporine-like aminoacids; **PUFA**, Polyunsaturated fatty acid; **T,** cultivation temperature; **TAG**, Triacylglycerol; **TFA**, Total fatty acid. Additional information about cold-adapted algae and cyanobacteria for biotechnology are provided by Varshney et al. (2015).

1.2. Cultivation at cold and light-limited environments

Today´s most commercially employed strains are warm water-adapted (*i.e.*, meso- or thermophilic) microalgae with an optimal growth temperature in the range of 20-35 °C (Borowitzka 2013; Leya et al. 2009). These mesophilic strains do not grow well when temperature were too high during summer (e.g., $T > 40$°C), a scenario that has been addressed by employing thermo-tolerant mutants (Ong et al. 2010; Pires et al. 2012). On the other hand, when temperatures drop below 15°C and solar irradiance decreases during winter or in cold climates, growth of microalgae is limited (Table 2) as reported for productions in Spain (Jiménez et al. 2003), New Zealand (Sutherland et al. 2014) or Portugal (Pereira et al. 2018). Even though the commonly used meso- or thermophilic strains require expensive and energy demanding heating systems to keep productivities high during winter (Ruiz et al. 2016; Williams and Laurens 2010), only few researchers focused on developing cold-tolerant mutants (Shukla et al. 2013)

An alternate approach to mutants is the use of microalgae isolated from cold environments that grow well under psychrophilic or psychrotrophic conditions (e.g., $T \leq 15$°C) while producing biomass rich in fatty acids and other valuable biocompounds (Leya et al. 2009). However, outdoor productions at extreme latitudes (e.g. >50°) are usually restricted to summer months (Table 2) due to insufficient solar irradiance for photosynthesis during the winter and ice formation that destructs the production systems (Grönlund et al. 2004; Hindersin et al. 2014; Hulatt and Thomas 2011; Pankratz et al. 2017; Williams and Laurens 2010). Therefore, a year-round microalgal biomass production at extreme latitudes requires an artificial light-based indoor cultivation (Baliga and Powers 2010). Such systems consume vast amounts of energy for the maintenance of optimum cultivation temperatures and to power lamps. Cold-adapted strains are apt for such indoor systems because energy use for culture heating can be minimised and low ambient temperatures serve as cheap cooling source. Notably, optimising cultivation parameters including temperature, light intensity, nutrient availability and photon emission tailoring of the light sources can effectively improve growth and biochemical composition (Ooms et al. 2016; Schüler et al. 2017).

Table 2. Summary of studies about outdoor cultivation of microalgae at extreme latitudes in cold climates or winter conditions.

Microalga	Growth	Location	T (°C)	Purpose or product	Conditions/Outcome	Reference
Porphyridium purpureum	47.04 mg L^{-1} d^{-1}	51°N 4°W	23.5 ± 2.62	Exopolysaccharides	Tubular PBR, *V*= 600 L, ø=4 cm, greenhouse cultivation, August to October, PFD= ~16 mol d^{-1} m^{-2}.	(Fuentes-Grünewald et al. 2015)
Scenedesmus obliquus	11.31 mg L^{-1} d^{-1}	53°N 4°W	10.8-24.1	Lipids, bioenergy	Tubular PBR, *V*= 500 L, ø=2.8 cm, March to September, PFD= 55.81 mol d^{-1} m^{-2}.	(Hulatt and Thomas 2011)
Scenedesmus obliquus	0.68-2 g L^{-1} d^{-1}	53°N 10°E	15.3-20.7	Flue gas cleaning, biomass	Solar tracked flat panel PBR, light path length 15-22 mm, heated, PFD: 10 mol m^{-2} d^{-1} during winter.	(Hindersin et al. 2014)
Chlorella mirabilis	$\mu \leq 0.89$ d^{-1}	49°N 14°E	n.a.	Fatty acids, biomass	Micro-pond, *V*= 2 L, ø= 2 mm, *I*=~250 µmol s^{-1} m^{-2}, heated culture, March-April.	(Shukla et al. 2013)
Phaeodactylum tricornutum *Oscillatoria* sp.	n.a.	56°N 3°E	4-23	Wastewater treatment	Cascade raceway, Area= 0.6 m^2, V= 2.1 L, July to November.	(Craggs et al. 1997; Craggs et al. 1995)
Algal consortia	μ~0.22d^{-1}	63°N 14°E	0-25	Wastewater treatment	HRAPs (Raceways), greenhouse, additional artificial light during autumn, averaged *I*= 270 µmol s^{-1} m^{-2}, April to November.	(Grönlund 2002; Grönlund et al. 2010; Grönlund et al. 2004)
Phonnidium sp.	n.a.	Lab-study	5-35	Wastewater treatment, pigments	Among 49 mat-forming isolates, the cyanobacteria *Phonnidium* sp. was found efficient for N and P removal at low temperatures. On warm summer days (25°C), green algae performed better.	(Tang et al. 1997; Tang et al. 1997)

Unidentified MA2H1; 100 isolates tested	93 mg L^{-1} d^{-1}	Lab study	10, 22	Wastewater treatment, lipids	Among 100 isolates from ~45-46°N, the strain MA2H1 displayed highest productivities of biomass (93.3 mg L^{-1} d^{-1}) and lipids (29.3 mg L^{-1} d^{-1}) compared to those grown at 22°C.	(Abdelaziz et al. 2014)
Cyanobacteria	n.a.	Lab study	5, 15, 25	Wastewater treatment	Four isolates from Arctic and Antarctic were tested under different temperatures (5-25°C) and irradiances (80-1470 µmol s^{-1} m^{-2}). At 15 and 25°C, growth was sufficient for most isolates.	(Chevalier et al. 2000)

Abbreviations: n.a., not available; ***ø***, Light path length; ***µ***, growth rate; **HARPS**, high rate algal ponds; ***I***, light intensity in µmol s^{-1} m^{-2}; **PBR**, photobioreactor; **PFD**, photosynthetic photon flux density in mol m^{-2} d^{-1}; **T,** cultivation temperature.

1.3. Triggers to produce biochemical compounds

Temperature, salinities, light intensity and nutrient supply are the environmental factors that are commonly altered to manipulate the biochemical composition and growth of microalgae (Schüler et al. 2017). For example, lowering the culture temperatures can effectively induce PUFA, which are needed by cells to maintain membrane fluidity, flexibility, and functionality (Hulatt et al. 2017; Lyon and Mock 2014; Morgan-Kiss et al. 2006). On the other hand, salinity changes cause osmotic and oxidative stress to algae. As a result, microalgae upregulate metabolic pathways connected to the expression of antioxidants or energy reserves such as pigments, lipids or starch (Leya et al. 2009; Wei et al. 2017; Zhu et al. 2016). Currently, microalgal industries use high salinities to enhance the production of lipids and high-value antioxidants including beta-carotene (e.g., *Dunaliella* spp.; Ben-Amotz et al. 1982; BenMoussa-Dahmen et al. 2016) but also to limit contaminations in the culture (Zhu et al. 2016). Another trigger is light intensity. For example, low-light intensities can induce PUFA in many microalgae, whereas high-light intensities trigger pigment and TAG induction by (Schüler et al. 2017). These changes in lipids and pigments are related to alterations in not only chloroplast volume and thylakoid membrane structure (Fisher et al. 1998) but also energy storage mechanisms. Lastly, nutrient limitation is a major stressor, which can induce reactive oxygen species or free radicals. The response of microalgae to nutrient limitation is similar to osmotic stresses: A strain-specific biosynthesis of antioxidants including pigments or the energy reserve molecules lipids and carbohydrates, being often accompanied by decreasing intracellular protein contents (Schüler et al. 2017). Microalgal producers often adopt nutrient deprivation as a strategy to induce pigments (e.g., *Haematococcus*), lipids (e.g., *Nannochloropsis*) or other strain-specific metabolites (Schüler et al. 2017). Notably, cultures in late growth stages (e.g., stationary phase) are often subject to nutrient limitation, which causes strain-specific biochemical changes such as degradations of PUFA, pigments or proteins and accumulations of carbohydrates, lipids or saturated fatty acids during late growth stages (Brown et al. 1996; Lv et al. 2010; Zhu et al. 1997).

1.4. Artificial light for indoor production

Phototrophic cultivation of microalgae requires CO_2, nutrients (e.g., nitrogen, phosphorus, trace minerals) and sun- or artificial light (Blanken et al. 2013). Sunlight is the most cost-effective and environmentally friendly photon source for microalgae production and should be used whenever possible (Blanken et al. 2013). However, the use of artificial lighting becomes a necessity when production takes place at locations or seasons where natural light is limited or at instances when a reliable and predictable production is required. Artificial light-based production increases the production costs by four to five times compared to sunlight-based production (Blanken et al. 2013). These costs cover those for lamp acquisition, energy consumption and engineering efforts. To cut costs and improve the environmental fingerprint of artificial light-based microalgal production, light emission by lamps should be optimised.

A viable artificial light-based microalgal production depends on efficient conversion of electrical energy into light (technical factor), which in turn must be efficiently converted into biomass by an alga (biological factor). In addition, the efficient delivery of photons to cells within a culture to overcome self-shading by cells (or light attenuation) is of utmost importance (physical factor; Schulze et al. 2017a).

LEDs are the today´s preferred light source for microalgal production because of their long life time (<25-50,000h) and high efficiency to transform more than 40-50% of electrical energy into light that can be utilised by phototrophs for photosynthesis (e.g., photosynthetic active radiation; PAR; Ooms et al. 2016). Innovative artificial light-based microalgal producers can use LEDs that emit light of appropriate wavelengths or flashing light to control growth and biochemical composition of the culture (Ooms et al. 2016; Schulze et al. 2017, 2014). Nevertheless, adjusting the LED´s photon emission to mitigate self-shading is the most challenging task in today´s artificial light-based microalgal production (Ooms et al. 2016).

1.4.1. Flashing light to counter light attenuation in microalgae cultures?

In any photobioreactor, cells located at the periphery absorb most of the incoming light and can become photoinhibited, whereas cells at the inner layers remain in the

dark and become photolimited (Lee 1999). Exposure of cells at the surface to high irradiance causes dissipation of the absorbed light energy as heat, instead of using the harvested photons to fix CO_2 into biomass (de Mooij et al. 2016). On the other hand, cells located in the dark use up intracellular energy reserves (e.g., starch) for maintaining cell functions (*i.e.*, respiration) because no light is available to fix CO_2 via photosynthesis. As a result, the rates of respiration exceed photosynthesis, causing a loss of biomass. The light penetration depth into a culture depends primarily on the biomass concentration, the absorptive capacity of the cultured cells, light intensity and wavelengths, cell morphology (e.g. cell size) and biochemistry (e.g. pigment content; Lee 1999). Previous studies recommended to use LEDs that are tailored to emit appropriate spectra (de Mooij et al. 2016; Schulze et al. 2014) or flashing light (Abu-Ghosh et al. 2015; Park et al. 2000; Schulze et al. 2017) to mitigate self-shading and stimulate growth. While wavelength-tailoring has been shown promising to improve the growth of microalgal cultures (de Mooij et al. 2016; Ooms et al. 2016; Schulze et al. 2016), potential benefits of flashing light are yet to be confirmed.

Flashing light (Fig. 1) emitted with the same time-averaged light intensity (I_a) as continuous light was suggested to mitigate light attenuation in photobioreactors and perhaps improve photosynthetic performance of cells (Abu-Ghosh et al. 2016; Iluz et al. 2012; Martín-Girela et al. 2017; Park et al. 2000; Park and Lee 2001). Flashing LEDs can provide light of high intensity within a short period (e.g., few nano- to microseconds, hereafter called light *flash period* or t_l), alternating with extended *dark periods* (t_d). One flash period followed by a dark period is defined as a flashing cycle (t_c, in which $t_c = t_l + t_d$). The use of high flash light intensities (I_l) enables a deeper photon penetration into the culture, serving photosynthesis of cells in deeper layers of the culture (Lee 1999; Park and Lee 2001, 2000). To prevent photo-damage and inhibition of the phototroph by too intense light flashes, the repetition rate of the light-dark transition (*i.e.*, flashing light frequency, f) and the relative proportion of the light and dark period (*i.e.*, the *duty cycle*, $DC = t_l \; t_c^{-1}$) should be adjusted to the biological reaction kinetics of the photosynthetic apparatus or energy dissipation mechanisms (also referred to as non-photochemical quenching; NPQ; Schulze et al. 2017a).

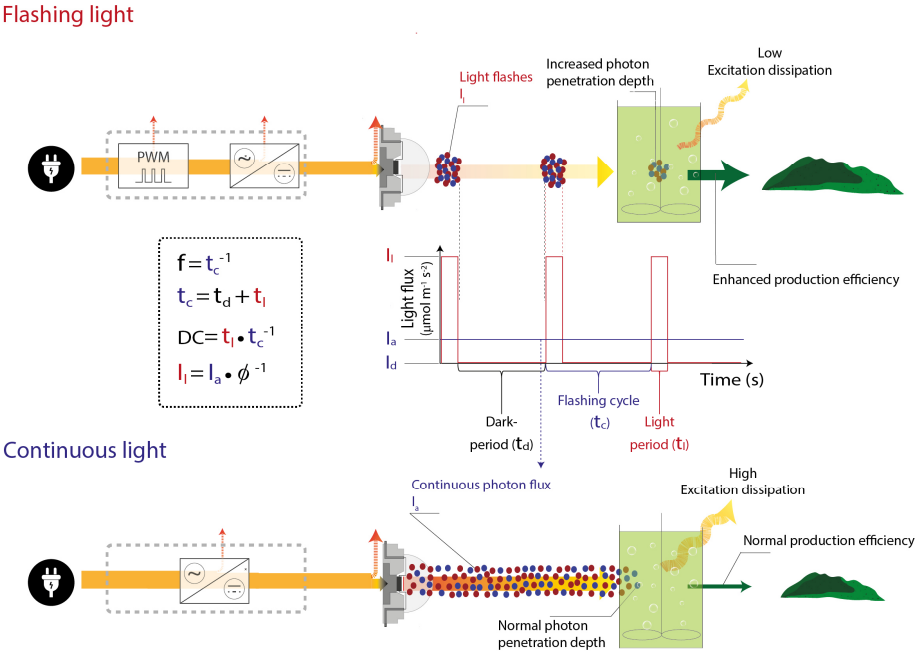

Figure 1. Theory of flashing light applied to microalgal cultivation.

Depicted is a microalgal production unit that uses flashing (upper panel) and continuous light (bottom panel) employing light emitting diodes (LEDs). Flashing light devices emit light (t_l) and dark periods (t_d) in an approximately rectangular waveform. The number of light-dark intervals that occur per second (s^{-1}) is the frequency (f). The duty cycle (DC) is the ratio between the flash period and the whole flashing cycle. The light intensity (in µmol photons s^{-1} m^{-2}) during t_l is defined as flash intensity (I_l), while during t_d no light is emitted (e.g., I_d = 0 µmol photons s^{-1} m^{-2}). Under this condition, the time-averaged light intensity (I_a) during one flashing cycle can be expressed as $I_a = DC \times I_l$ and is used to compare flashing with continuous light treatments. The flash intensity and the duty cycle are inversely proportional at a given averaged light intensity.

Furthermore, flashing light of low frequencies and high flash intensities were suggested to induce biochemical compounds that are connected to photo-protection and energy dissipation (Schulze et al. 2017). Flashing light with low frequencies activates NPQ mechanisms that help the phototrophs to cope with the excess light received during the flash period. As a result, cells often show a high-light-response (Combe et al. 2015; Sastre 2010) and alter pigment composition, intracellular ultrastructure,

expression of protective proteins (e.g. proton gradient protein PGR5, the ratio between photosystem I and II (PSI, PSII), light harvesting antenna size, ribulose-1,5-bisphosphate-carboxylase/oxygenase (RuBisCO) activity or starch content (Abu-Ghosh et al. 2015; Allahverdiyeva et al. 2014; Combe et al. 2015; Gris et al. 2014; MacKenzie and Campbell 2005; Mouget et al. 1995; Park and Lee 2000; Porcar-Castell and Palmroth 2012; Sastre 2010; Sforza et al. 2012). However, flashing light conditions that trigger NPQ mechanisms also impair biomass productivity because the light energy is dissipated as heat or used in alternative electron transfer pathways rather than directing the harvested photon energy towards carbon-fixing metabolism (e.g., Calvin-Benson cycle, Schulze et al. 2017a). Therefore, improvement in a target biomolecule productivity using low-frequency flashing light may require a two-stage cultivation approach: In the first stage, microalgae can be exposed to growth-stimulating light such as continuous- or flashing light (e.g. $f > 500$ Hz, $DC < 0.1$), while the subsequent induction phase could provide flashing light of low frequencies (e.g. $f < 10$Hz) and short duty cycles (e.g. $DC < 0.1$). In this second stage, the light phase should be characterised by a high flash intensity that lasts long enough to trigger the desired biomolecule pathway, whereas the dark phase should be short enough to prevent its downregulation (Schulze et al. 2017).

Until today, studies about the application of flashing light on microalgae focused mostly on flashing light conditions present in cultures, where cells shift through mixing between the light- to dark zones within a PBR and thus experience a gradual transition between light and dark phases (Abu-Ghosh et al. 2016). To mimic these conditions, researchers usually employed frequencies $f < 100$ Hz or relatively high duty cycles ($DC > 0.1$; Grobbelaar 2009; Iluz et al. 2012; Liao et al. 2014; Raven and Ralph 2014; Vejrazka et al. 2012). It is unclear if (1) flashing light of high frequencies and short duty cycles could indeed improve growth performance of microalgal cultures (e.g., $f > 100$ Hz, $DC < 0.1$; Schulze et al. 2017a) and (2) if low-frequency flashing light and short duty cycles could induce biochemical compounds. The present thesis studies these effect of flashing light for microalgal cultivation, which may allow more efficient indoor productions required at places with low solar irradiances.

2. Objectives

The objective of this thesis is the identification of promising cold-adapted microalgae for biotechnological applications and the examination of environmental factors that stimulate growth and biomolecule induction (e.g., PUFA, pigments) in artificial light-based indoor cultivation. These objectives were addressed by five papers (Fig. 2):

1. Identification of potential fast-growing cold-adapted microalgae from marine origins through literature survey, and selection of environmental triggers (e.g., temperature, salinity, nutrient supply, prevailing growth stage and light intensity) for biosynthesis of fatty acids, carbohydrates, lipids or proteins in the algae (Paper I, II).
2. Evaluation of the ability of flashing light to improve growth of microalgae (Paper III, IV).
3. Induction of biomolecules including fatty acids and pigments in microalgae, using flashing light (Paper III, V).

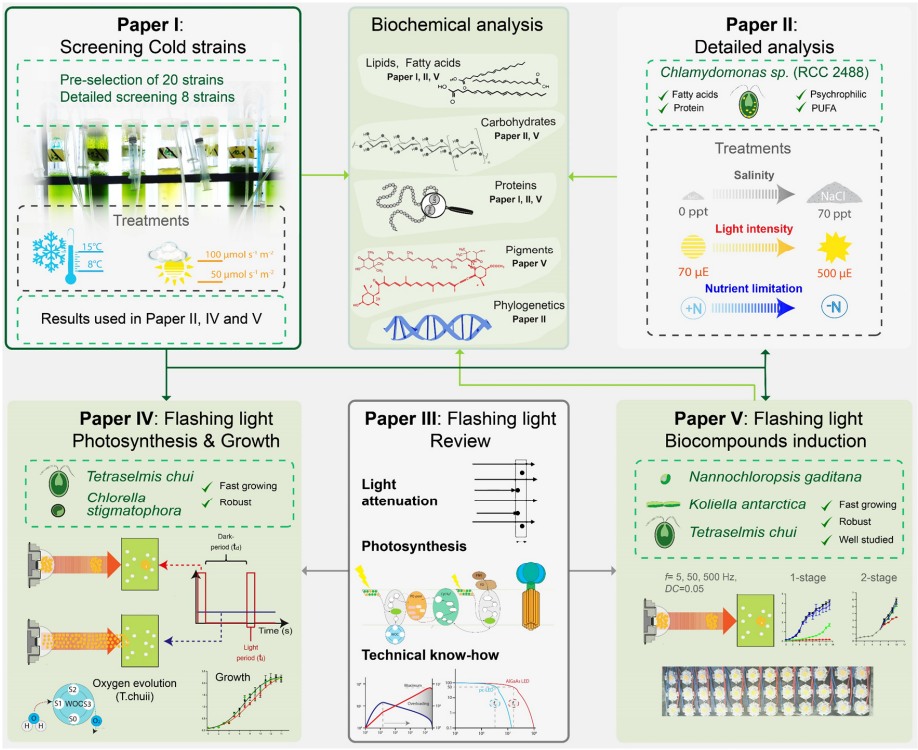

Figure 2. Schema of the thesis including five studies (Paper I-V).

Paper I: Screening of strains from cold environments; **Paper II**: detailed analysis of one potential cold adapted strain identified in Paper I; **Paper III**: Flashing light review to gather knowledge in order to perform experiments in Paper IV and V; **Paper IV**: Screening flashing light conditions to improve growth; **Paper V**: Using specific flashing light conditions to induce biochemical compounds. Biomass produced in Paper I, II and V was biochemically analysed (light green arrows). Promising strains from Paper I were used in Paper II, IV and V (dark green arrows). Knowledge acquired in Paper III was used in Paper IV and V (grey arrows).

3. Methods

To identify promising cold strains for microalgal cultivation (**Paper I**), a literature survey was conducted to select 20 promising strains (Fig. 2). They were then obtained from culture collections (Roscoff Culture Collections (RCC) at the Station Biologique de Roscoff, France, and the Culture Collection of Algae (SAG) at the Department of Experimental Phycology at Göttingen University, Germany. These strains were pre-cultivated and the eight best-growing strains were used for further studies; to understand their growth and biochemical composition under different temperatures (8 and 15°C) and light intensities (50 and 100 µmol s^{-1} m^{-2}). Biomass samples were taken after 10 and 14 days of cultivation and analysed for fatty acid contents and proteins. Analysis of covariance (ANCOVA) was employed to identify differences among strains, and effects of treatments and the prevailing growth stage (a function of biomass concentration in the medium) as one of the important covariates influencing the biochemical composition of the microalgae.

Phylogenetic relationship of the only true psychrophilic microalga *Chlamydomonas* sp. (RCC2488) with other members of the order Chlamydomonadales was conducted (**Paper II**). Different strategies –modification of light intensities (*I*= 70-500 µmol s^{-1} m^{-2}), salinities (0-80 ppt) and nitrogen levels– were adopted to understand the optimum environmental factors that yield maximum biomass and biomolecules. Knowledge about the effects of light, salinity and nitrogen starvation on total protein, lipid (TAG and neutral lipids and their fatty acid composition) and carbohydrate contents was also gathered. A connection between cold-adaption and phylogenetic relationship with other members of the Chlamydomonadales order was assessed.

For the flashing light experiment, first a comprehensive literature survey was conducted (**Paper III**); to gather knowledge about photosynthetic processes and dynamics in microalgal cultures. In addition, technical knowledge that was essential to conduct experiments, was acquired and a review was published (Schulze et al. 2017).

The first flashing light experiment (**Paper IV**) was carried out in collaboration with Jose M. Fernandez (University of Almeria, Spain). Employing the technical knowledge (**paper III**), a state-of-the-art flashing light system was built to understand the effect of flashing light on the growth of two strains. Growth responses of *Tetraselmis chui* and *Chlorella stigmatophora* were tested for frequencies from 0.01 Hz-1 MHz, duty cycles of 0.001-0.7 and average light intensities of I_a= 50, 500 and 1000 µmol s^{-1} m^{-2}. Using an oxygen probe, oxygen evolution rates (indicator of photosynthetic performance) of the diluted and concentrated *T. chui* cultures were measured. Because oxygen evolution performance of cells may change from day to day or hour to hour, a normalisation of the oxygen evolution rates (P_n) was performed ($P_n = P_f\ P_c^{-1}$) every 3-4 hours; oxygen evolution rate from flashing light experiments (P_f) and from continuous light treatments (P_c). In the general discussion (Section 4.2) these normalised oxygen evolution rates were used to compare the evolution rates from different cultures (concentrated or dilute) or light intensities. In addition, batch culture experiments were conducted for 14 days at f= 40 and 400 Hz (DC= 0.05) and I_a= 50 and 200 µmol s^{-1} m^{-2}.

Lastly, the effects of flashing light on biochemical composition (**Paper V**) of three microalgal species (*N. gaditana*, *T. chui* and *Koliella antarctica*) were evaluated at Nord University in collaboration with Serena Lima (University of Palermo, Italy) and CCMar (Algarve University, Faro, Portugal). The effects of flashing light (f= 5, 50 and 500 Hz, DC= 0.05) on biomass productivity and biochemical composition were tested by growing microalgae in batch cultures, in a one- and a two-stage production approaches. In the two stage cultivation, algae were fist grown for six days under continuous light, and then exposed to flashing light. Continuous light was used as a control treatment across all experiments employing the same average supplied light intensity as used under flashing light (I_a=300 µmol s^{-1} m^{-2}). Biomass harvested at the end of the experiment was analysed for proteins, carbohydrates, total lipids, fatty acids, pigments, and elemental (C, H, N) composition.

4. General Discussion

The general aim of this thesis was to understand the ideal conditions that stimulate algae growth; to suggest improvements in the current methods for the production of biomass and (valuable-) biomolecules in microalgae produced employing artificial light at extreme latitudes. Until now, only few strains have been commercially exploited for the production of high-value products such as PUFA, pigments or whole microalgal cells (Blanken et al. 2013; Ruiz et al. 2016). To broaden the portfolio of potential strains and provide more sustainable solutions for production during cold seasons or facilities in cold climates, this thesis addresses in five papers the identification of novel strains, induction of biochemical compounds and application of flashing light.

In **Paper I** the growth potential of microalgae isolated from cold marine habitats under different temperatures (8 and 15°C) and light intensities (50 and 100 µmol s^{-1} m^{-2}) is examined. Effects on fatty acid and protein contents were also tested. **Paper II** reports detailed optimisation of environmental parameters for the production of fatty acids, proteins, carbohydrates and total lipids by the psychrophilic alga *Chamydomonas* sp. (RCC 2488). A review about the potential of applying flashing light to microalgal production is given in **Paper III**, which includes the description of the technology and possible enhancements of culture growth or induction of biomolecules. In **Paper IV**, effect of flashing light–of various frequencies, duty cycles and light intensities–on the production of *T. chui* and *C. stigmatophora* was evaluated based on oxygen evolution rates or growth responses under batch cultivation. The effects of specific flashing light conditions on the production of fatty acids, proteins, carbohydrates, lipids and pigments by *N. gaditana*, *K. antarctica* and *T. chui* are investigated in **Paper V**.

4.1. Characteristics of cold-adapted microalgae

Out of the twenty microalgal strains from cold marine habitats, obtained from different culture collections, eight that demonstrated good growth were tested for their response to different temperatures and light regimes. The combined effects of

temperature and light intensities on growth, total protein and fatty acids were tested by ANCOVA. The environmental parameters, temperature and light, affected the growth of the tested strains. However, most of the assessed biomolecules changed as a result of prevailing culture growth stage but not due to the applied treatments.

Among the eight promising strains, only the Arctic isolate *Chlamydomonas* sp. (RCC 2488) grew better at 8°C compared to 15°C (up to 0.5 g L^{-1} d^{-1}), indicating a clear psychrophilic trait, which is the ability to grow actively at low temperatures (e.g., T< 15°C; Remias et al. 2015). *Chlamydomonas* sp. RCC 2488 surpassed all strains in their productivity of protein (69.6±13.7 mg L^{-1} d^{-1}), total fatty acids (TFA; 91.0±5.1 mg L^{-1} d^{-1}) and PUFA (54.1±3.1 mg L^{-1} d^{-1}) when cultivated at T= 8°C, I= 100 µmol s^{-1} m^{-2} **(Paper I)**. In **Paper II**, the phylogenetic relation of *Chlamydomonas* sp. RCC 2488 with an Antarctic *Chlamydomonas* isolate (UWO 241) was revealed. *Chlamydomonas* sp. RCC 2488 exposed to a combination of low salinity (20 ppt) and nutrient limitation induced large amounts of carbohydrates (~49% in biomass) but not lipids (~32% in biomass). The alga exhibited a maximum PUFA productivity of 85 mg L^{-1} d^{-1} under a light intensity of 250 µmol s^{-1} m^{-2} and nutrient sufficient conditions in a flat panel photobioreactor. Interestingly, this alga could grow in salinities ranging from 0-80 ppt, though an inhibition in growth at 80 ppt was observed. This high salt tolerance of the strain could be connected to the isolation habitat (Beaufort Sea), where extreme seasonal temperature variations drive freeze-thawing cycles of ice that causes salinity shifts.

Another promising strain identified in **Paper I** was *Pseudopleurochloris antarctica* (SAG 39.98). This alga grew well at 15°C (0.4 g L^{-1} d^{-1}) and produced biomass with 23% protein and up to 34% fatty acids containing up to 30% eicosapentaenoic acid (EPA). *P. antarctica* achieved high production rates of protein (58.8±3.4 mg L^{-1} d^{-1}), EPA (7.7±1.1 mg L^{-1} d^{-1}) and monounsaturated fatty acids (MUFA; 64.9±3.9 mg L^{-1} d^{-1}). The EPA productivity was comparable to that of known EPA producers such as *Nannochloropsis* and *Isochrysis* cultivated under higher temperatures (e.g., ≥20°C), but similar light conditions (Chen et al. 2015; Grima et al. 1992; Meng et al. 2015; Mitra et al. 2015; Sukenik 1991). Worth mentioning is the floc forming feature of *P. antarctica*;

the flocs settled rapidly as soon as culture aeration stopped, which could help saving dewatering costs in future production scale up scenarios.

The tolerance of *Chlamydomonas* sp. RCC 2488 and *P. antarctica* to psychrophilic and psychrotrophic growth conditions (*T*= 8-15°C) make them ideal candidates for production of proteins and PUFA in cold climates. These results **(Paper I, II)** support previous findings that cold-adapted microalgae indeed contain higher amounts of lipids and fatty acids as compared to mesophilic strains making them a valuable biotechnological resource (Cvetkovska et al. 2014; Remias et al. 2015). Higher yields of PUFA may be obtained in a continuous cultivation approach (Del Campo et al. 2007; Schüler et al. 2017). For example, Řezanka and colleagues (2017) reported a production of up to 97 mg PUFA L^{-1} d^{-1} by the cold-adapted *Monoraphidium* sp. CCALA (strain no 1094) at ~10°C in a continuous thin-layer photobioreactor.

Another promising strain identified in **Paper I** was *T. chui* (SAG 1.96). This strain achieved the highest biomass productivities (~0.7-1 g L^{-1} d^{-1}) among all strains and test conditions, and therefore can be considered as the most robust strain in this study **(Paper I)**. Due to this reason, *T. chui* was used for the experiments with flashing light **(Paper IV, V)**. However, the productivity of food and feed relevant biomolecules such as protein, TFA and PUFA was rather low (e.g., up to 44.5±10.2, 32.6±5.3 and 13.7±1.7 mg L^{-1} d^{-1}, respectively; **Paper I, V**), traits that are typical for *Tetraselmis* strains. Indeed, strains of the *Tetraselmis* genus are usually tolerant to various environmental parameters such as salinity, light, temperature, contaminants, which makes them suitable for removing pollutants from wastewaters (Pereira et al. 2016; Pereira et al. 2018; Schulze et al. 2017). Interestingly, previous studies demonstrated the successful application of cold-adapted microalgae and cyanobacteria to treat wastewater in cold climates, which might be also a promising application for the presently tested *T. chui* strain (Abdelaziz et al. 2014; Chevalier et al. 2000; Craggs et al. 1997; Grönlund et al. 2010; Tang et al. 1997)

Lastly, *C. stigmatophora* (RCC 661) achieved biomass productivities of 0.4 g L^{-1} d^{-1} at 15°C and produced extracellular polymeric substances (EPS; **Paper I**). Previously,

Chlorella, including *stigmatophora* strains were reported to secrete EPS, a promising bio-resource for valuable bioactive compounds (Kaplan et al. 1987; Mishra et al. 2011; Xiao and Zheng 2016; Zhao et al. 2015). Naturally, EPS serve to protect the excreting cells from competitive species, contaminants or environmental conditions (e.g., high or low temperatures). However, EPS released to the growth medium increase medium viscosity, which hinders separation of media and algae, a bottleneck researchers try to tackle by, e.g., using ultrafiltration (Xiao and Zheng 2016). Hence, the major application of *C. stigmatophora* may not be for the production of the tested intracellular fatty acids or proteins, but for EPS, a source of bioactive compounds.

4.2. Effects of flashing light on growth

The phototrophic production of microalgae at extreme latitudes requires artificial light. However, these lighting systems should be energy efficient to decrease operational expenditures and for sustainability. Researchers have suggested that for microalgal production, in addition to wavelength tailoring, the light should be supplied in a discontinuous way (*i.e.*, flashing- or pulsed light) rather than continuously. This approach was suggested to improve light delivery and photosynthetic performance of microalgal cultures (reviewed in **Paper III**) because short intense light flashes can penetrate deep into the culture to stimulate photosynthesis of cells in dark layers (Park and Lee 2001, 2000). Naturally, microalgae in any photobioreactor are subject to light attenuation and are exposed to intermittent light as a result of culture mixing, which move cells from light to dark layers (Abu-Ghosh et al. 2016). The mitigation of light attenuation by employing flashing light enhances the growth of microalgae (Abu-Ghosh et al. 2016; Brindley et al. 2011; Grobbelaar et al. 1996; Lunka and Bayless 2013; Park and Lee 2001, 2000; Sastre 2010). However, there are inconsistencies in published results and lack of clear evidences about the benefits of using flashing light. Light delivery and growth were suggested to be particular enhanced if single flashes are extreme intense (e.g., at short duty cycles) while repeating fast enough to prevent inhibition of photosynthesis (e.g., $f \geq 500$ Hz; **Paper III**). These high frequencies and short duty cycles were investigated in **Paper IV** by using a flashing light system with state-of-

the-art LEDs and solid-state components. This system allowed the emission of light flash periods as short as $t_f \geq$ 100 nanoseconds and flash intensities of $I_f \leq$ 100,000 µmol s^{-1} m^{-2}. Using this system, the growth response of the promising cold-adapted microalgae *T. chui* (SAG 19.52) and *C. stigmatophora* (RCC 661; identified in **Paper I**) to different frequencies (e.g., 0.01 Hz-1 MHz), duty cycles (e.g., 0.001-0.7), light intensities (I_a= 50-1000 µmol s^{-1} m^{-2}) and culture concentrations (~0.1-4.7 g L^{-1}) was tested (**Paper IV**).

Contrary to what was previously suggested (**Paper III**; Carvalho et al. 2011), the results from **Paper IV** indicated that flashing light has no beneficial effect on microalgal growth. The results demonstrated that frequencies <200 Hz were most discriminative for impairing photosynthetic oxygen evolution. Frequencies higher ~200-500 Hz were necessary to obtain the biological flashing light effect, the threshold where photosynthesis under flashing light is similar to continuous light. These findings, however, support the theory from **Paper III** that the photosynthetic apparatus needs an approximate threshold frequency of at least ~ 200-300 Hz, regardless of the adjusted duty cycle to match the turnover rates (3-5 ms) of the linear electron transfer chain (e.g., Q_0 acceptor in the Cytochrome b_6f complex; **Paper III**).

Paper IV tested the oxygen evolution response of low, medium and high-light adapted diluted *T. chui* cultures exposed to flashing light with the same average light intensity (I_a=50, 500 and 1000 µmol s^{-1} m^{-2}). The low culture concentration (0.1 g L^{-1}) was characterised by a minimal light attenuation, which allowed all single cells in the culture to receive flashing light close to the full adjusted average light intensity. Notably, such low density cultures are required to test biological responses including photosynthetic performance of single cells because self-shading as an interacting factor can be minimised (Brindley et al. 2010; Vejrazka 2012; 2011). Thus, the flashing light effect on the photosynthetic apparatus could be described for low, medium and high-light adapted cells (I_a=50, 500 and 1000 µmol s^{-1} m^{-2}) in low culture concentrations. The results showed that the oxygen evolution rates in 50 µmol s^{-1} m^{-2}-adapted cells were higher compared to 500 or 1000 µmol s^{-1} m^{-2}-adapted cells at low frequencies (f= 10-200 Hz). In 50 µmol s^{-1} m^{-2}-adapted cells, the average flashing light intensity was below the saturation intensity (α=250 µmol s^{-1} m^{-2}) and cells may have processed flashing light

better as if the average light intensity was saturating (e.g., I_a= 500 µmol s^{-1} m^{-2}, α= 456 µmol s^{-1} m^{-2}) or oversaturating (e.g., I_a=1000 µmol s^{-1} m^{-2}, α= 559 µmol s^{-1} m^{-2}). On the other hand, high-light adapted cultures (I_a=1000 µmol s^{-1} m^{-2}) tolerated better low-frequency flashing light than cultures adapted to moderate light intensities (I_a= 500 µmol s^{-1} m^{-2}). Here, high-light adapted cells may have accumulated more photo-protective metabolites (e.g., pigments such as carotenoids) that protected cells from long-lasting high-light flashes (e.g., low frequency and short duty cycle; Katsuda et al. 2006, Mouget et al. 1995, Sastre 2010, Schüler et al. 2017) as compared to cells adapted to moderate light intensities (I_a= 500 µmol s^{-1} m^{-2}).

Cultures at frequencies from 10-100 Hz always showed a higher normalised oxygen evolution rate as compared to diluted cultures. However, as frequencies decreased f< 8Hz, oxygen in the medium decreased, indicating a higher rate of respiration than photosynthesis in concentrated cultures. At same frequencies, diluted cultures showed neither evolution nor consumption of oxygen, indicating an equilibrium between photosynthesis and respiration. To explain these findings, a deeper look into the physiological state of the used concentrated cultures is required. It was suggested that cells in concentrated cultures experience a more complex light regime, as they move across (1) high-light zones at the periphery of the photosynthetic chamber gradually to, (2) low-light layers and (3) dark zones (Abu-Ghosh et al. 2016). The concentrated cultures used in **Paper IV** were characterised by a high biomass concentration of ~5 g L^{-1} and a light path of 2 cm which causes a high proportion of dark-to-light zones, leaving cells most of the time in the dark and only for limited periods in the (low-) light zones (Loomba et al. 2018). An average cell in such culture becomes low-light adapted, characterised by downregulation of photoprotective pigments (Jahns et al. 2009; Schüler et al. 2017). A lack of photoprotective metabolites in low-light adapted cells (e.g., pigments) hinder microalgae at the periphery (1) to dissipate energy from intense light flashes, leading eventually to photodamage and increasing respiration rates (Schulze et al. 2017). This assumption was validated by experiments where low-light (I= 50µmol s^{-1} m^{-2})-adapted diluted cultures were exposed to flashing light of a higher average light intensity (I_a=500 µmol s^{-1} m^{-2}; **Paper IV**).

On the other hand, cells in the low-light layers (2) of concentrated cultures are subject to attenuated flashing light due to mutual shading (Abu-Ghosh et al. 2016; Brindley et al. 2011). Experiments with dilute cultures or plants have shown that a decreasing average light intensity increases light utilisation efficiency by phototrophs under low frequency flashing light (**Paper IV**, Jishi et al. 2015; Xue et al. 2011). Therefore, it is suggested that concentrated cultures exposed to low-frequency flashing light benefit from light attenuation, achieving higher normalised photosynthetic oxygen evolution rates compared to dilute cultures exposed to the same initial light intensity (500 and 1000 µmol s^{-1} m^{-2}; f= 10-100 Hz; **Paper IV**).

Cells in the dark zones (3) receive insufficient light to drive photosynthesis. Oxygen evolution in this particular zone of the culture is often negative due to a high respiration rates (Abu-Ghosh et al. 2016; Brindley et al. 2016). However, measurements inside concentrated cultures (**Paper IV**) have shown that high intense light flashes indeed penetrated deep into dense cultures although the average light intensity was as low as under continuous light (I_a≈0 µmol s^{-1} m^{-2}). Cells in the dilute cultures achieved similar photosynthetic efficiency only under flashing light compared to continuous light at a given light intensity. Therefore, even though, light flashes penetrate deep into concentrated cultures, no enhancement of growth could be found because the average light intensity was damped by the culture as under continuous light.

T. chui and *C. stigmatophora* cultures were cultivated under flashing light to investigate possible long-term effects such as cell acclimatisation. Previously, Grobbelaar et al. (1996) reported that *Scenedesmus obliquus* cultures did not acclimatise to flashing light (tested range: 0.05-50 Hz, exposure time: 72 h). This trend was confirmed in the present batch experiments (**Paper IV**) for both algae and average light intensities of I_a=50 and 200 µmol s^{-1} m^{-2}. However, unlike short exposure in the photosynthetic chambers (10-20min) or experiments carried out by Grobbelaar and colleagues (1996), batch cultures change their biomass concentration significantly, increasing the light attenuation over time. The lag phase in batch cultures is characterised by low-light attenuation and biomass concentration (~0.15 g L^{-1}). During this period, cells were inhibited by low frequency flashing light as confirmed by

experiments with diluted cultures in the photosynthetic chamber. Over time, biomass concentration and self-shading increased and effects of low frequency flashing light (40 Hz) were less distinct to continuously supplied light as the total photon dose per cell decreased with increasing cells in the culture. At a lower average light intensity (50 µmol s^{-1} m^{-2}), growth was not significantly affected, neither during lag nor exponential growth phases. These results confirmed findings from the photosynthetic measurements (**Paper IV**) in diluted *T. chui* cultures where inhibiting effects of low-frequency flashing light decreased with decreasing average light intensities.

In summary, results from **Paper IV** suggest that the observations in concentrated cultures is a result of (1) inhibition of cells in high-light zones; (2) better light utilisation efficiency of cells in low- or moderate-lit zones and (3) respiration rates of cells in the dark zones. It was further concluded that the specific threshold–where a given duty cycle, frequency or average light intensity of flashing light becomes inhibiting–depended on acclimatisation stage of the algae and light attenuation potential of the culture that affects the total photon dose received by an average cell inside the culture.

4.3. Effects of flashing light on biochemical composition

As reviewed in **Paper III**, the induction of biomolecules with low-frequency flashing light in microalgae holds promise (Katsuda et al. 2008; Kim et al. 2014; Schulze et al. 2017).Therefore, **Paper V** examines growth and changes in total proteins, carbohydrates, lipids, fatty acids and pigments in three EPA-producing microalgae: (1) *Nannochloropsis gaditana* as a representative model strain as well as (2) *Tetraselmis chui* (SAG 1.96) and (3) *Koliella antarctica* (SAG 20.30) as promising fast-growing, Antarctic strains identified in previous studies (**Paper I**; Suzuki et al. 2018). The algae were exposed to flashing light (f= 5, 50 and 500 Hz, DC= 0.05) and cultivated in a one- and a two-stage batch approaches.

The experiments showed that a flashing light frequency of 500 Hz reached similar growth as compared to continuous light while lower frequencies (f= 5, 50 Hz) usually caused a strain-dependent growth inhibition. These results match findings from **Paper**

IV and previous studies on microalgae and plants (Combe et al. 2015; Jishi et al. 2015; Simionato et al. 2013), but contradicts those of studies that reported flashing light-induced growth enhancement (Abu-Ghosh et al. 2015; Lunka and Bayless 2013; Park and Lee 2001). However, *T. chui* cultures coped better with low frequency flashing light with, e.g., only ~30% less biomass productivity compared to continuous light under *f*= 50 Hz. On the other hand, *N. gaditana* and *K. antarctica* displayed ~70% less biomass productivity at *f*= 50 Hz. Indeed, a strain-specific growth response to flashing light was found previously for other phototrophs (Jishi et al. 2015; Nedbal et al. 1996), which may be linked to morphology or species-specific photoprotective strategies and the cell´s ability to store photosynthetic intermediates (**Paper V**; Jishi et al. 2015). In **Paper V**, all cultures grown in a two-stage system showed considerably higher biomass productivities even under the most growth inhibiting frequency (e.g., *f*= 5Hz, 0.34-0.43 g L^{-1} d^{-1}) as compared to the one-stage cultivation approach (e.g., *f*= 5 Hz, <0.05 g L^{-1} d^{-1}). In the two-stage cultivations, during the first stage a biomass concentration of ~2 g L^{-1} was achieved prior to applying the flashing light during the second stage. As discussed for **Paper IV**, a higher biomass concentration decreased the total photon dose per cell as compared to the lag phase of the one-stage cultivation where all cells received the full applied light intensity (e.g., I_o= 300µmol s^{-1} m^{-2}; **Paper III, IV**). Therefore, the two-stage cultivation approach yielded a higher biomass productivity under growth-inhibiting, low-frequency flashing light (*f*= 5, 50 Hz) as compared to the one-stage approach.

Although low frequencies of flashing light (e.g., *f*= 5 and 50 Hz, **Paper V**) inhibited cell growth, their intracellular contents or productivities of PUFA, chlorophyll and carotenoids (lutein, beta carotene, violaxanthin and neoxanthin) increased compared to continuous light (**Paper V**). For example, protein levels were usually higher in cultures exposed to flashing light frequencies of 5 and 50 Hz as compared to continuous light. On the other hand, total lipids in *N. gaditana* and carbohydrates in *K. antarctica* and *T. chui* tend to decrease under low-frequency flashing light (*f*= 5, 50 . productivities of proteins increased slightly by 20-30% in low frequency flashing light (*f*= 5 Hz, 50 Hz)-treated cultures, while carbohydrates and lipids productivity decreased by 20-50% compared to

continuous light. The finding in **Paper V** match previous studies that reported minor effect of flashing light (*f*= 1 to 30 Hz, *DC*= 0.05-0.5) on the protein, carbohydrate or lipid contents in *N. salina, Scenedesmus obliquus* and *Chlorella pyrenoidosa* (Gris et al. 2014; Matthijs et al. 1996; Mouget et al. 1995; Sforza et al. 2012; Yoshioka et al. 2012).

Analogous to the trend of increasing proteins, and decreasing carbohydrates or lipids under low frequency flashing light, a species-specific accumulation of PUFA and degradation of MUFA was observed in the study. On the other hand, continuous light or flashing light with *f*= 500 Hz showed a contrary pattern. For example, *N. gaditana* accumulated more C20:5*n*-3 under *f*= 5 Hz and 50 Hz-treatments (up to 17-20% EPA) while the MUFA C16:1*n*-4 and 18:1*n*-9 tend to decrease, contrary to what was observed in continuous- and 500 Hz flashing light exposed cultures. Similarly, major PUFA in *K. antarctica* (C18:3*n*-3 and C20:5*n*-3) increased under the expense of C18:1*n*-9 when exposed to flashing light (*f*= 5 and 50 Hz). Lastly, *T. chui* showed higher amounts of C16:4*n*-3, C18:3*n*-3 and C18:4*n*-3, accompanied by lower C18:1*n*-9. However, no effect on C20:5*n*-3 was observed in this alga. Likewise, previous studies have found no responses when microalgal cultures were exposed to flashing light of high frequencies (e.g., *f*> 100Hz; Choi et al. 2015; Yoshioka et al. 2012), while low frequencies (e.g., *f*< 50Hz) indeed caused rising PUFA levels (Choi et al. 2015).

As for the pigments, the contents of *β*-carotene, violaxanthin and neoxanthin (in *T. chui* and *K. antarctica*) were on average three to four times higher in cells exposed to low frequency flashing light (5, 50 Hz) as compared to continuous or 500 Hz-flashing light. Total chlorophyll, total carotenoids and lutein contents increased by up to two times under low-frequency flashing light. By employing a two-stage cultivation system, the productivity of *β*-carotene, violaxanthin and neoxanthin was enhanced on average three fold under low frequency flashing light treatments (*f*= 5, 50 Hz) as compared to continuous or flashing light of high frequencies (*f*= 500 Hz). T. The lowest tested frequency (5 Hz) tended to be more efficient in producing pigments. Under this treatment, *N. gaditana* produced maximum chlorophyll, total carotenoids, and violaxanthin (2.97, 1.34 and 0.45 mg L^{-1} d^{-1}, respectively), *T. chui* produced chlorophyll, neoxanthin, lutein and *β*-carotene (2.97, 0.04, 0.47 and 0.35 mg L^{-1} d^{-1}, respectively),

while *K. antarctica* did not exceed any of these productivities. Similarly, low frequency flashing light enhanced the astaxanthin production in *Haematococcus pluvialis* and increased light energy usage by up to four times (e.g., f= 3.5 Hz, DC= 0.05) as compared to continuous light (Katsuda et al. 2008, 2006; Kim et al. 2006).

Summarizing the observations in **Paper V**, the combined accumulations of proteins, PUFA or pigments under low-frequency flashing light (f= 5, 50 Hz) are often connected to an adaption to low-light conditions (He et al. 2015; Schüler et al. 2017). Under such conditions, the thylakoids increase and they contain more light harvesting pigments that facilitate the absorption of photons (Berner et al. 1989). The bigger thylakoid membranes and higher pigment contents require more membrane lipids including PUFA but also proteins that are bound to accessory light harvesting pigments such as carotenoids (Jahns et al. 2009; LaRoche et al. 1991; Schüler et al. 2017). Noteworthy, both, high-light typical pigments such as lutein or β-carotene (Mulders et al. 2014) as well as low-light typical pigments such as violaxanthin and chlorophyll (Couso et al. 2012; Jahns and Holzwarth 2012) were simultaneously induced under low frequency flashing light treatments. As reported in **Paper III**, under low frequency flashing light (f= 5, 50 Hz; **Paper V**), phototrophs respond to the instantaneous light intensities (I_l and I_d) within a flashing cycle. The treatments 5, 50 Hz characterized by long flash durations (e.g., t_l= 1-10 ms) with a high instantaneous flash intensity (I_l= 6000 µmols^{-1} m^{-2}) perhaps induced moderately high-light responses in cells, while a lack of light during the prolonged dark phase (t_d= 19-190 ms) caused photo-limitation. Most of these trends were distinct under 5Hz treatments compared to the 50 Hz-treated cultures, indicating that the magnitude of low- or high-light stresses increased with decreasing frequencies (**Paper III**).

Besides the effects by flashing light, growth-stage effects were often more relevant to explain biocomponent changes in the tested microalgae (**Paper V**). Indeed, the prevailing growth stage of the culture is well-known to induce intracellular changes in microalgae (Fuentes et al. 2000; Mansour et al. 2003). In **Paper V**, we observed species-specific drop of total proteins and accumulation of carbohydrates and lipids as the culture biomass concentration increased, a common trend in aging microalgal cultures (Brown et al. 1996; Lv et al. 2010; Zhu and Lee 1997). Similarly, with increasing culture

concentration, strain-specific PUFA decreased, MUFA increased and pigments decreased (**Paper V**). These findings are often connected to depletion of nutrients or limitation of light during late growth stages, as a result of which cells downregulate their photosynthetic activity and intracellular pigments (e.g., chlorophyll, violaxanthin; **Paper V**; Oukarroum 2016).

It is suggested (**Paper V**) that the application of low frequency flashing light in a two-stage cultivation system is a promising method to maximise the production of proteins, PUFA, pigments or compounds that are induced by low- or high-light stresses. However, consideration of the growth stage and the right exposure time to growth inhibiting but biomolecule-inducing flashing light will be of utmost importance in next generation artificial light-based microalgal production.

5. Conclusions

Indoor microalgal production at extreme latitudes necessitates the application of artificial lighting and culture heating. However, cold-adapted rather than mesophilic strains should be used in these regions where the low ambient temperature demands culture heating to maintain productivities (e.g., during winter or at extreme latitudes). At higher latitudes, cold-adapted strains and appropriate LEDs can be biotechnologically optimised for the production of biomass and biomolecules; low ambient temperatures provide optimal conditions for the cultures and cool LEDs will have more efficiency and longer life expectancy. The present study identified promising cold-adapted microalgae from marine habitats as efficient producers of fatty acids, proteins and pigments. By adjusting environmental triggers (temperatures, salinities, lighting conditions or nutrient levels) or using low-frequency flashing LED light, evidences were gathered for the enhanced production of proteins, PUFA or pigment. The applied low-frequency flashing light inhibited growth, and the biochemical responses indicated both —low- and high-light responses— of microalgal cells. On the other hand, contrary to previous expectations, high frequency flashing lights with short but intense light pulses did not improve biomass productivity of microalgal cultures compared to continuous light. The major effects on biochemical compounds were often caused by the prevailing growth stage of the culture rather by the treatment itself (e.g., temperature, flashing light, light intensity).

6. Limitation of the thesis

This work shed light into the potential of cold-adapted microalgae and the efficacy of flashing light in improving the biomass and biocomponent productivity. A more conclusive pattern could have been observed if the following aspects were also considered:

1. Cold strains were only grown under laboratory conditions. This approach does not reflect the real production performance on an industrial scale. Therefore, large scale production trials are necessary to identify their real performance i.e. to produce biomass, PUFA or pigments.
2. **Paper I** was conducted only on duplicates in order to screen as much species as possible. To draw a more conclusive answer about optimal cultivation conditions of the most promising strains (e.g., *P. antarctica*, *Chlamydomonas* sp. RCC 2488) future studies may focus on specific strains subjected to more selected environmental parameters, but with more replicates for statistical validity.
3. Only frequencies >10 Hz were systematically screened in **paper IV**, but results showed that lower frequencies were highly discriminative (when comparing different cell acclimatisation stages, culture concentrations and light intensities). Hence lower frequencies of flashing light should be also examined in future studies.
4. Induction experiments (**Paper V**) were only tested for frequencies ≥5Hz and minimum exposure time was five days. Lower frequencies and shorter exposure times might be more effective to produce pigments or fatty acids.

7. Future Perspectives

Cold-adapted microalgae may find their application in (i) outdoor cultivation in cold climates during summer, (ii) outdoor cultivation during winter in warm climates (e.g. Mediterranean zone or subtropics) or (iii) indoor, artificial light-based cultivation at cold climates. To commercialise these promising bioresources, additional studies are needed

to test toxicity, digestibility and content of bioactive compounds. Only after demonstrating the safety of microalgae, the European Food Safety Authority will approve it as novel foods. Market introduction will ultimately broaden the portfolio of commercially cultivated species approved as food and feed. These are key milestones for the development of microalgal biotechnology sector.

The production of microalgae at extreme latitudes will be sustainable only through the use of i) renewable energies and ii) applied to local processes that cannot be outsourced to countries with higher solar irradiance (e.g., wastewater treatment) or iii) by producing valuable metabolites that score a high market value (e.g., EPA, pigments). The producers at extreme latitudes seek ways to minimise energy usage for artificial light sources (e.g., personal communication with Keynatura efh., Hafnarfjörður, Iceland and FjordAlg AS, Svelgen, Norway). Although electrical efficiencies of LEDs are constantly being improved, the effective use of emitted light by microalgal cultures remains a bottleneck. The present study showed that flashing light does not improve biomass productivities of microalgal cultures. However, a promising approach is the adaption of wavelengths to a culture´s optical properties to stimulate photosynthetic performance. One approach is the usage of wavelengths that are not directly absorbed by algal cells at the periphery but serve photosynthesis of cells in deeper layers of the culture (e.g., green-yellow light for green algae; de Mooij et al. 2016). Additionally, single colour LEDs or laser diodes that emit only narrow wavebands can be considered as promising light sources for microalgal production. If the photons from these light sources are concentrated on a short waveband they may not be absorbed quickly compared to the same amount of photons distributed across the whole absorption spectra of an alga (e.g., typical for broad band spectrum light sources). Lastly, decreasing the light path between alga and light source by using free-swimming, submersed wireless light emitters seems promising to enhance the performance of photobioreactors (Heining and Buchholz 2015; Heining et al. 2015; Sutor et al. 2014). Taken together, it is suggested that biotechnological innovations with cold-adapted strains, flashing light, waveband tailoring and narrow light paths hold promise for an efficient artificial light-based microalgal production at extreme latitudes.

8. References

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Paper I

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Fatty acids and proteins from marine cold adapted microalgae for biotechnology

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ABSTRACT

Cold-adapted microalgae display unexpectedly high biomass production, pointing to their potential to produce high-value bioproducts under cold and light-limited conditions. From culture collections, we screened eight cold-adapted strains of different genera (*Chlamydomonas, Chlorella, Tetraselmis, Pseudopleurochloris, Nannochloropsis* and *Phaeodactylum)* for the production of fatty acids and proteins under low temperature and light regimes (T = 8, 15 °C; I = 50, 100 μmol s^{-1} m^{-2}). Among the strains, the Arctic isolate *Chlamydomonas* sp. (RCC 2488) had better growth at 8 °C compared to 15 °C (up to 0.5 gDW L^{-1} d^{-1}) and highest productivities of protein and polyunsaturated fatty acids (PUFA) (70 and 65 mg L^{-1} d^{-1}, respectively). Two tested *Tetraselmis* strains (SAG 1.96, RCC 2604) achieved highest biomass productivities (0.7–1 gDW L^{-1} d^{-1}), containing up to 50 mg PUFA gDW^{-1} and 15% proteins. *Pseudopleurochloris antarctica* (SAG 39.98) grew well at 15 °C (0.4 g L^{-1} d^{-1}), with 23% proteins in biomass and the highest eicosapentaenoic acid (EPA) productivity (7.6 mg L^{-1} d^{-1}). *Chlorella stigmatophora* (RCC 661) achieved productivities of 0.4 gDW L^{-1} d^{-1} at 15 °C and produced extracellular polymeric substances (EPS). The major cause for the observed shifts in biochemical profiles was biomass concentration, which is an indicator for the prevailing growth stage. Based on the current experimental design, *Chlamydomonas* sp. (RCC 2488), *T. chuii* and *P. antarctica* can be suggested as the most promising strains for the production of protein and (polyunsaturated-) fatty acids at low temperatures. However, additional strain-specific studies are necessary to statistically validate these findings.

1. Introduction

Microalgae are sustainable sources of proteins or fatty acids, and their areal biomass production potential is several times higher compared to traditional crops [1]. Microalgae-based products used in food or feed supplements are considered as health-benefitting substances that contribute to the well-being of humans and animals [2]. Demand for sustainable and healthy consumables can drive the future development of microalgal biotechnology [2]. Today's fledgling microalgal industry focuses on high-value products such as polyunsaturated fatty acids (PUFA), pigments or whole microalgal cells, but current high production costs of > 5–25€ kg^{-1} of dry algal biomass erode the profit margin [3,4]. To improve the financial viability and push microalgal biotechnology-based products towards larger markets, researchers and industries are searching for new strains that rapidly produce high amounts of valuable biocomponents [5]. Bioprospecting novel strains from cold environments such as polar oceans, upwelling areas, mountains or snowfields is promising as these microorganisms biosynthesise PUFA, pigments or antioxidants [5–8]. These cold strains have adapted to specific light conditions, rapid freeze-thawing cycles or salinity shifts and can achieve biomass productivities at low temperatures (≤ 15 °C) similar to those of meso- or thermophilic strains cultivated at ≥ 20 °C [9]. This ability could be exploited by microalgal outdoor production during cold seasons or cultivation facilities located in cooler regions (e.g. Northern Europe [5,6]), thereby minimising expensive heating and artificial lighting [11]. For the production of protein, lipid, PUFA and pigments under cold- and light-limited conditions, researchers have proposed cold-adapted strains such as *Haematococcus pluvialis* [12,13], *Chlamydomonas* sp. [14], *Koliella antarctica* [15,16] or *Monoraphidium* sp. [17]. Few marine strains have been examined, even though they are known to contain high amounts of PUFA, and form the base of the marine food chain in cold oceans.

In this work we aimed to understand the production potential of marine cold-adapted microalgal strains in cold and light-limited

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Table 1
List of tested strains obtained from Roscoff Culture Collection (RCC) and Culture Collection of Algae in Göttingen (SAG).

Strain name	Class	Strain ID	Isolation	T (°C)[1]	Lat.	Long.
Chlamydomonas sp. MALINA	Chlorophyceae	RCC 2488	Beaufort Sea	4	69°48 N	138°26 E
Chlamydomonas sp. CEFAS	Chlorophyceae	RCC 2607	North Sea	15	54°77 N	2°99 W
Chlorella stigmatophora	Trebouxiophyceae	RCC 661	North Sea	4	70°33 N	140°48 W
Tetraselmis chuii	Chlorodendrophyceae	SAG 1.96	Ekho Lake[2]	15	68°52 S	78°26 W
Tetraselmis sp.	Chlorodendrophyceae	RCC 2604	North Sea	15	55°17 N	0°0 W
Pseudopleurochloris antarctica	Xanthophyceae	SAG 39.98	Wood Bay, Pack-ice	10	74°36 S	165°40 W
Nannochloropsis granulata	Eustigmatophyceae	RCC 2478	English Channel	15	48°45 N	3°57 O
Phaeodactylum tricornutum	Bacillariophyceae	RCC 641	North Sea	13	54°11 N	7°54 W

[1]Approximate cultivation temperature at culture collection. [2]Hypersaline (72 ppt), heliothermal (15 °C), pH: 8.4.

conditions. From a pre-selected 20 potentially interesting cold-water strains obtained from culture collections, we cultivated eight strains under different low temperatures (T = 8, 15 °C) and light intensities (I = 50, 100 μmol s^{-1} m^{-2}), and assessed the growth, accumulation of proteins and fatty acids.

2. Materials and methods

Microalgae of different evolutionary lineages were obtained from Roscoff Culture Collections (RCC) at the Station Biologique de Roscoff, France, and the Culture Collection of Algae (SAG) at the Department of Experimental Phycology at Göttingen University, Germany (Table 1; Table A.1 in Supplementary material). Based on a literature review, a total number of 20 strains were selected and pre-cultivated as described in Table A.1. From these 20 strains, the eight best-performing species were chosen for further testing. Of the eight strains, five belong to the Plantae kingdom; *Chlamydomonas* sp. MALINA (RCC 2488), *Chlamydomonas* sp. CEFAS (RCC 2607), *Chlorella stigmatophora* (RCC 661), *Tetrasemis chuii* Butcher (SAG 1.96), *Tetraselmis* sp. (RCC 2604). The remaining three strains belong to the Chromista kingdom; *Nannochloropsis granulata* (RCC 2478), *Phaeodactylum tricornutum* (RCC 641) and *Pseudopleurochloris antarctica* (SAG 39.98). The *Chlamydomonas* strains RCC 2488 and RCC 2607 are referred to as *Chlamydomonas* sp. MALINA and *Chlamydomonas* sp. CEFAS, respectively; the suffixes indicate the name of the cruise during which the strains were isolated. All microalgae were saline species isolated from cold marine environments.

2.1. Experimental setup

Inocula for all experiments were grown in Erlenmeyer flasks placed on orbital shakers for 14 days, at 8 or 15 °C. The flasks were illuminated from above at an intensity of 30 μmol s^{-1} m^{-2} photosynthetically active radiation (PAR) supplied by cool-white fluorescent lamps (Philips TLD 840, Amsterdam, Netherlands). Seawater from the North Atlantic shoreline of Bodø,-Norway-(Salinity 35 ppt) was enriched with a modified F-medium [18] consisting of 5.3 mM $NaNO_3$, 0.22 mM $NaH_2PO_4H_2O$, 35 μM $FeCl_3$ $6H_2O$, 35 μM Na_2EDTA $2H_2O$, 0.12 μM $CuSO_4$ $5H_2O$, 0.078 μM Na_2MoO_4 $2H_2O$, 0.23 μM $ZnSO_4$ $7H_2O$, 0.126 μM $CoCl_2$ $6H_2O$, 2.73 μM $MnCl_2$ $4H_2O$, 8.88 μM thiamine HCl, 0.06 μM biotin and 0.012 μM cyanocobalamin was used for all experiments. The medium for *P. tricornutum* was additionally supplemented with 318 μM Na_2SiO_3 $9H_2O$.

All algae were cultivated for 14 days in duplicate using glass bubble-tube photobioreactors filled with 310 mL culture and placed in a climate chamber [9]. Four growth conditions that are relevant for microalgal production under cold and light-limited conditions were tested: (A) low temperature and low light (T = 8 °C, I = 50 μmol s^{-1} m^{-2}); (B) low temperature and high light (T = 8 °C, I = 100 μmol s^{-1} m^{-2}); (C) high temperature and low light (T = 15 °C, I = 50 μmol s^{-1} m^{-2}); and (D) high temperature and high light (T = 15 °C, I = 100 μmol s^{-1} m^{-2}). The cultures were mixed by aerating the tubes with humidified and 0.2 μm filtered air enriched with CO_2 (1%v/v; GMS-150, Photon Systems Instruments, Drasov, Czech Republic) at a flow rate of 110 mL/min. Biomass samples for biochemical analysis were collected at 10 and 14 days after inoculation.

2.2. Growth monitoring

Optical density at 540 nm (OD_{540}) was measured in quadruplicate for all algal cultures on a daily basis using 96-well plates (Tecan Sunrise A-5082, Männedorf, Switzerland). The biomass concentration in the cultures was determined at day 10 and 14 for each treatment. A known volume of algal suspension was filtered using 47 mm glass fibre filters (pore size ø = 0.7 μm; VWR), washed twice with 10 mL ammonium bicarbonate (0.5 M) and dried at 105 °C for 24 h. Dry weight (DW) was determined gravimetrically. Upon plotting OD_{540} against DW from different experiments, significant linear correlations were obtained for each alga ($r^2 \geq 0.9$, $p < 0.05$), allowing the estimation of DW via OD_{540} measurements on a daily basis.

Cultures harvested at day 10 and 14 were centrifuged (5000 *g*, 5 min), washed (ammonium bicarbonate, 0.5 M), freeze-dried and stored at −80 °C until further analysis. Ash weight was determined by incinerating 50 mg freeze-dried biomass at 560 °C for 12 h.

2.3. Fatty acid and protein analysis

Approximately 6 mg of freeze-dried microalgal biomass was suspended in 4 mL chloroform:methanol solution (2:2.5 v/v) containing an internal standard (Tripentadecanoin, C15:0 Triacylglycerol, Sigma-Aldrich, Oslo, Norway). Cells were disrupted using bead milling (0.1 mm glass beads; Precellys Evolution, Bertin Technologies, Montigny-le-Bretonneux, France). Next, 2.5 mL Tris-buffer (6 g L^{-1} Tris, 58 g L^{-1} NaCl) was added, mixed with a vortex mixer and centrifuged (2000 *g*) to separate the phases. The lower chloroform-phase containing the lipids was transferred into a new glass tube and evaporated under a gentle nitrogen flow to prevent fatty acid oxidation. Subsequently, 3 mL methanol with 5% H_2SO_4 was added and kept for 3 h at 70 °C to convert the fatty acids into their methyl esters. Thereafter, 3 mL hexane was added, mixed for 15 min in an orbital shaker and the fatty acid methyl esters (FAMEs) in the hexane phase were quantified using gas chromatography equipped with a Flame Ionisation detector (SCION 436 m Bruker, Massachusetts, US) and a CP-Wax 52 CB column (Agilent, Santa Clara, US) using split-less mode. To identify and quantify the most common FAMEs, an external 37-component standard (Supelco, Bellefonte, US) was used.

The protein content of the algal biomass was determined with a Bio-Rad DC™ Protein Assay (Bio-Rad Ltd., Hemel Hempstead, UK). Water-soluble proteins from freeze-dried biomass were extracted by re-suspending 6 mg freeze-dried biomass in a lysis buffer containing 60 mM tris (hydroxymethyl) aminomethane (Tris-) and 6.9 mM $NaC_{12}H_{25}SO_4$ (SDS) and subsequent bead milling (three cycles of 60 s, 6500 rpm, 120 s break between bead milling; Precellys Evolution). The samples along with glass beads and lysis buffer were first incubated at 100 °C for 30 min and then they were centrifuged (2000 *g*, 10 min). The water-

soluble proteins contained in the supernatant was measured according to Bio-Rad *DC* Protein Assay manual, at an optical density of 750 nm (Dr3900, Hach Lange, Salford Quays, UK).

2.4. Data treatment

Growth parameters were estimated according to Ruiz, et al. [19,20] and are detailed in Table A.2 (Supplementary material). Protein and fatty acid productivities were calculated by multiplying the total amount of a given biocomponent in the biomass (% of DW) by the biomass concentration in the culture (gDW L^{-1}) and dividing by the total cultivation time (time in days; d). Explanatory variables in the present study were strain, temperature, light intensity and biomass concentration. We defined the growth stage as a function of the biomass concentration in the culture at the time point of harvesting (X_t in gDW L^{-1}). A high biomass concentration indicates an advanced growth stage and a low biomass concentration an early growth stage. This consideration was necessary to separate the effects of the treatments (light and temperature) from culture maturation (or aging) effects, which is a well-known factor that impacts the biochemical composition of algae [21]. Biomass data were log10 transformed to meet the assumption of linearity. ANCOVA tests were conducted to check differences among strains while controlling the effects of temperature, light or biomass concentration (XLStat, Vers. 2016.02.27444, Addinsoft, New York, US). Pearson's correlations (r) were used within the ANCOVA models to quantify the effects of temperature and light intensity on biomass productivity. For the protein content and fatty acids, the co-variates temperature, light and biomass concentration were considered in the ANCOVA. The closer the r-value to 1 or − 1, the stronger the positive or negative effect of an explanatory variable, respectively. Type III sum of squares analysis was used to describe the impact on the response variables (biomass productivity, protein and major fatty acid classes). Normality of the response variables was checked using Shapiro-Wilk test. A significance level (α) of 0.05 was used for all tests. All biochemical data are detailed in Table A.3 (Supplementary material).

3. Results and discussion

3.1. Growth performance

There was a significant difference in biomass productivities of all strains, after controlling for light and temperature. Temperature ($p = 0.013$) and microalgae strain ($p < 0.001$) significantly affected the ANCOVA model, while light ($p = 0.057$) did not (Table A.4, Supplementary material).

The low temperature treatments ($T = 8$ °C; $I = 50$, 100 µmol $m^{-2} s^{-1}$) reduced the growth rate and prolonged the lag phase of *Chlamydomonas* sp. CEFAS, *C. stigmatophora*, *T. chuii*, *Tetraselmis* sp., *P. antarctica, N. granulata* and *P. tricornutum* as compared to 15 °C (Fig. 1). At 8 °C, these strains did not reach their stationary phase during the 14-day cultivation period. Only *Chlamydomonas* sp. MALINA reached the stationary phase under 8 °C ($I = 50$, 100 µmol $m^{-2} s^{-1}$). *N. granulata* and *P. tricornutum* did not grow well under any tested conditions.

Among all treatments, *T. chuii* displayed the highest biomass productivity (0.83 ± 0.19 g $L^{-1}d^{-1}$ Fig. 2A). *P. antarctica* achieved a biomass productivity of 0.35 ± 0.07 g $L^{-1}d^{-1}$ among all treatments, similar to *Tetraselmis* sp. (0.55 ± 21 g $L^{-1}d^{-1}$), *C. stigmatophora* (0.29 ± 0.18 g $L^{-1}d^{-1}$), *Chlamydomonas* sp. MALINA (0.26 ± 0.21 g $L^{-1}d^{-1}$) and *Chlamydomonas* sp. CEFAS (0.20 ± 0.12 g $L^{-1}d^{-1}$). *N. granulata* and *P. tricornutum* did not grow well and achieved the lowest biomass productivities among all strains (0.07 ± 0.09 and 0.11 ± 0.15 g L^{-1} d^{-1}, respectively).

The biomass productivity of *Chlamydomonas* sp. CEFAS, *C. stigmatophora*, *P. antarctica* and *N. granulata* correlated positively with temperature (Fig. 2B); these algae had highest biomass production at high temperature ($T = 15$ °C). On the contrary, the biomass productivity of *Chlamydomonas* sp. MALINA correlated negatively with temperature, i.e. productivity was higher at low temperatures (0.53 ± 0.03 g L^{-1} d^{-1} at $T = 8$ °C and $I = 100$ µmol $m^{-2} s^{-1}$). Light correlated positively only with the biomass production of *T. chuii*, showing maximal biomass productivities at high light treatments ($I = 100$ µmol $m^{-2} s^{-1}$).

Visual observation of the cultures revealed a strong floc formation of *P. antarctica* cells that settled quickly when aeration was stopped (50–100 cells per floc; Fig. A.1 in Supplementary materials). *Tetraselmis* cells also settled after stopping the aeration but at a slower pace, as previously reported for this genus [22]. On the contrary, *C. stigmatophora* remained suspended in the medium due to high amounts of extracellular polymeric substances (EPS) exuded by the alga. These EPS increased the viscosity of the medium and hindered the separation of cells by centrifugation.

Tetraselmis strains performed best in terms of biomass productivity and maximum biomass concentration, a trait that is common for algae of this genus [23]. Notably, many Chlorophytes from cold environments are psychrotrophic ($T_{opt} > 15$ °C; [10,15,24-28]) with few psychrophilic ($T_{opt} < 15$ °C; [29]) or mesophilic ($T_{opt} > 20$ °C) exceptions [30]. Also in the present study, most tested chlorophytes grew best at 15 °C, with the exception of *Chlamydomonas* sp. MALINA that had highest biomass productivities at 8 °C. Thus, we identify *Chlamydomonas* sp. MALINA as one of the few psychrophilic Chlorophytes. Despite being a psychrophilic strain, the maximum biomass productivity (0.53 g L^{-1} d^{-1}) was lower compared to *T. chuii* cultivated under a suboptimal temperature ($T = 8$ °C and $I = 100$ µmol $m^{-2} s^{-1}$; $P_{max} = 0.73$ g L^{-1} d^{-1}). *N. granulata* and *P. tricornutum* displayed atypical growth rates; lower than normally reported for this genus [31-33], suggesting that the tested conditions were not optimal. The highest tested temperature (15 °C) was similar to the temperature of the location from where these species were isolated or maintained at the culture collection. Such temperatures may be too low because the optimum growth temperatures usually lie 6–7 °C higher than that of the natural habitat [34,35]. Likewise, the Chlorophytes *Tetraselmis sp., T. chuii* and *Chlamydomonas* sp. CEFAS were previously maintained at ~15 °C by the culture collections, and so better growth performances may be achieved at higher temperatures (e.g. 20 °C). The present study aimed to identify strains that grow well under low temperatures and additional studies are needed to ascertain the true optimum temperatures for these strains.

In contrast to any other tested strain, the biomass productivity of the polar microalga *P. antarctica* was similar among all treatments with only up to 21% variation (Fig. 2A). Previously, Andreoli, et al. [36] described *P. antarctica* as a species abundant in pack ice drill cores, that prefers a salinity of 35‰ for growth (among the tested range 0 to 35‰) at a temperature of $T = 4$ °C [37]. Despite the occurrence of the alga at lower temperature, as in pack ice (t ≈ 0 °C), our results indicate that *P. antarctica* is a psychrotrophic species (growth was higher at 15 °C than at 8 °C and 50–100 µmol $m^{-2} s^{-1}$; Fig. 1, 2B). In addition, a psychrotrophic rather than a mesophilic trait was confirmed by SAG; their results indicated that *P. antarctica* cannot grow actively in mesophilic growth conditions ($t = 20$ °C, $I = 40$ µmol $m^{-2} s^{-1}$; data not shown; personal communication with Dr. Maike Lorenz, SAG).

3.2. Biochemical composition

Each strain and genus had different biochemical profiles and response patterns at different growth stages or treatments. The following sections present the contents of proteins, and fatty acids of the algal strains and results of the statistical analyses. The complete set of data is provided in the supplementary materials (Table A.3).

3.2.1. Proteins

Protein contents differed among the strains ($p < 0.001$) after controlling for temperature, light intensity and biomass concentration

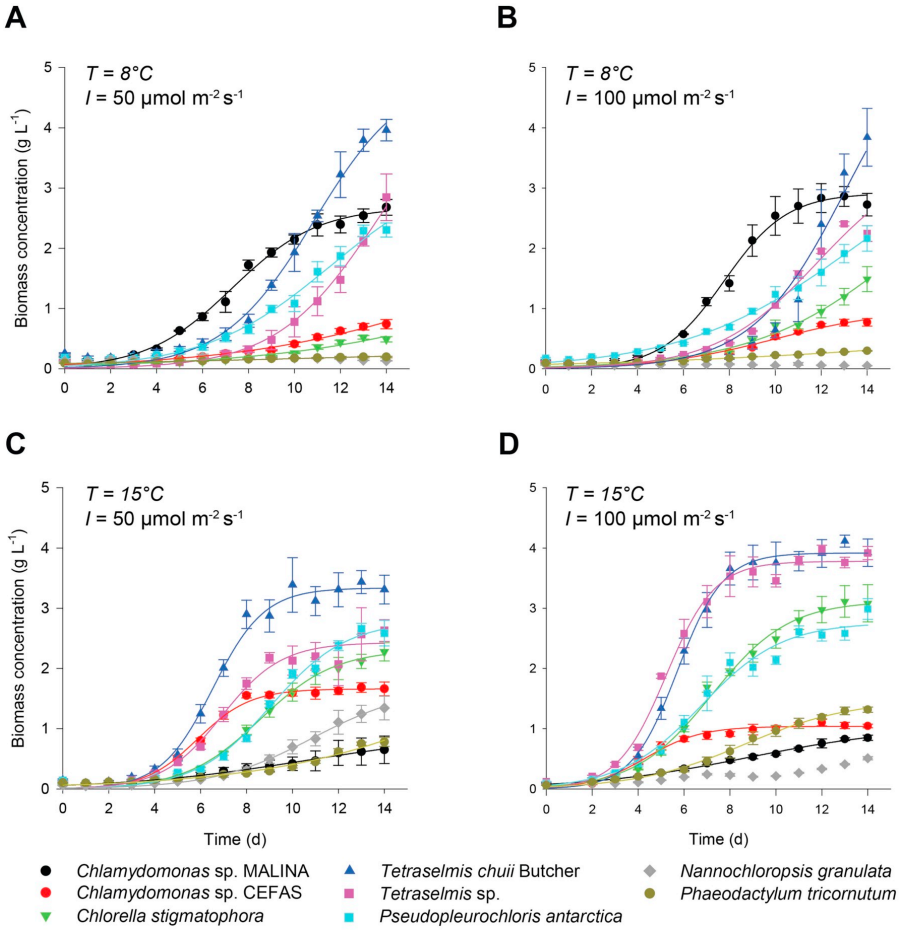

Fig. 1. Growth curves during 14 days of cultivation. The tested strains were exposed to temperatures of $T = 8\,^{\circ}C$ and $15\,^{\circ}C$ and photon flux densities of 50 and 100 µmol $m^{-2}\,s^{-1}$ (A-D). Solid lines are fitted using a sigmoid growth model, based on the growth data (symbols at each time point). No model fit was obtained for *Nannochloropsis granulata* cultivated under 100 µmol $m^{-2}\,s^{-1}$ (no line plotted). All model parameters are given in the Supplementary material (Table A.2). Data points at each day are shown as mean ± SD, $n = 2$.

(Table A.4). The ANCOVA model is significantly affected by biomass concentration and algal strain ($p < 0.001$) and to a lesser extent by temperature ($p = 0.031$). Light intensity did not affect proteins ($p = 0.968$).

Among all treatments, *Chlamydomonas* sp. MALINA, *N. granulata* and *P. tricornutum* contained 37.7 ± 15.0, 41.6 ± 10.4 and 37.7 ± 16.6% protein of DW, respectively (Fig. 3A). The remaining strains contained on average 24.3 ± 12.8% proteins of DW. The observed protein contents of all tested strains were generally in the range of other meso- and thermophilic strains belonging to the respective genus (e.g., *Chlamydomonas*: 12–48%; *Chlorella*: 10–58%; *Tetraselmis*: 9–50%; *Nannochloropsis*: 10–43%; *Phaeodactylum*: 40–65%; [21,38-47]).

The relationship between the protein content and biomass concentration in the cultures was modelled across all the tested algae, revealing an exponential decrease in protein content with increasing biomass concentration (Fig. 3B; $r^2 = 0.59$, $p < 0.01$). Notably, when algae enter the exponential growth stage, protein levels in their cells and nitrogen levels in the growth media drop rapidly, an effect described as growth-stage-dependent protein drop [41]. This growth-stage dependent protein drop was more discriminative ($F = 96.5$, $p < 0.001$) for the prediction of protein contents in biomass compared to the environmental test conditions temperature and light ($F < 5.0$).

3.2.2. Fatty acids

The ANCOVA models for TFA and MUFA were affected by the biomass concentration ($p < 0.001$), but not by temperature and light ($p > 0.05$; Table A.4, Supplementary material). Interestingly, the total PUFA content was affected by all co-variates and differed among strains ($p < 0.01$). More specifically, the effects of temperature ($F = 14.0$), light ($F = 10.8$), biomass concentration ($F = 37.9$) and strain ($F = 67.8$) (Table A.4), indicate that PUFA differed mostly among the strains (highest F-value). The ANCOVA model for SFAs was not affected by any of the tested co-variates, except for alga strains ($p < 0.001$). Strain-specific effects of the temperature, light intensity and biomass concentration on fatty acids are discussed below and given in Table A.5

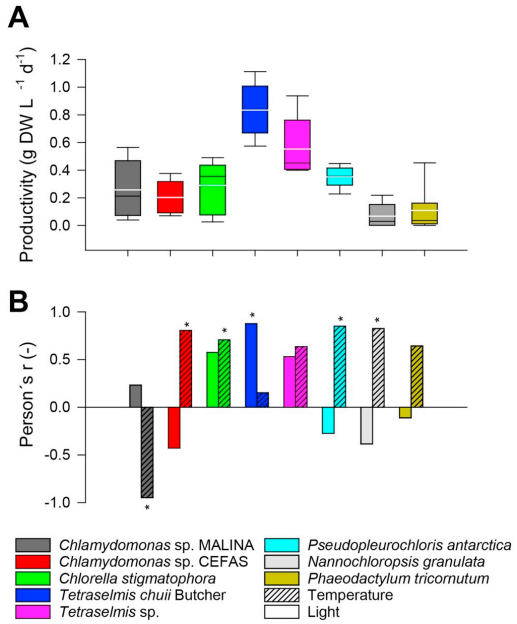

Fig. 2. Biomass productivities of microalgae and their correlation with temperature and light. The maximum biomass productivity ranges (g dry weight (DW) L^{-1} d^{-1}) of each strain shown in box plots (A) are from cultures grown in duplicates ($n = 2$) under different temperatures (8 and 15 °C) and light intensities (50 and 100 µmol m^{-2} s^{-1}). Black and white lines (in A) indicate the medians and means of all data points, respectively. The effect of light and temperature on the biomass productivity of each strain are given by Pearson's r correlation coefficients (B). Significant positive or negative correlations of temperature (dashed bars) and light intensity (empty bars) with biomass productivity are indicated by asterisks.

(Supplementary material).

The TFA contents in *Chlamydomonas* sp. MALINA, *P. antarctica* and *N. granulata* was among all treatments and harvesting time points 243.5 ± 87.8, 240.3 ± 68.6 mg TFA gDW^{-1} and 239.0 ± 66.3 mg TFA gDW^{-1}, respectively (Fig. 4). Both *Tetraselmis* strains displayed the lower TFA contents (average: 74.3 ± 11.6 mg TFA gDW^{-1}). Such low TFA content together with the low protein contents (~10% of DW; Fig. 3A), suggests a strong accumulation of carbohydrates during the late growth stage, a trend similar to mesophilic *Tetraselmis* strains [41].

Similar, high PUFA contents were found across all treatments in *Chlamydomonas* sp. MALINA (149.1 ± 52.5 mg PUFA gDW^{-1}) and *Chlamydomonas* sp. CEFAS (91.8 ± 28.9 mg PUFA gDW^{-1}), while *Tetraselmis strains* contained less (average: 35.1 ± 7.5 mg PUFA gDW^{-1}). *P. antarctica* and *N. granulata* accumulated high amounts of monounsaturated fatty acids (MUFAs) in their biomass (169.1 ± 58.8 and 116.6 ± 40.3 mg MUFA gDW^{-1}, respectively). In addition, *N. granulata* contained high SFA levels (79.8 ± 28.9 mg SFA gDW^{-1}).

Major fatty acids (given in % of TFAs) in the tested green algae were the PUFA C16:4*n*-3 (hexadecatetraenoic acid, 8–20%) and C18:3*n*-3 (α-linolenic acid; ALA, 8–32%; Fig. 5 A-E), as previously reported for this algal group [17,48,49]. *Chlamydomonas* sp. MALINA accumulated high levels of the omega-3 fatty acid C18:3*n*-3 (27–31%), C18:1*n*-9, oleic acid (20–32%), and C16:0, palmitic acid (6–12%), [50]). The fatty acid profile of *C. stigmatophora* matched those of other strains belonging to the same genus, with C18:2*n*-6 (linoleic acid; LA) and C18:3*n*-3 as major fatty acids [50]. However, the tested *C. stigmatophora* strain had higher C18:1*n*-9 (up to 61%) than previously reported for this species (~6.5%). Only *C. augustoellopsoidea* [50] had similar C18:1*n*-9 levels as *C. stigmatophora* in the present study. On the other hand, contents of C16:0, C18:1, C18:3*n*-3 and eicosapentaenoic acid (EPA, C20:5*n*-3) of the Antarctic isolate *T. chuii* and the North Sea isolate *Tetraselmis* sp. were similar to those reported in earlier studies [21,41,50] for other mesophilic *Tetraselmis* strains. Akin to previous findings, *P. antarctica* had only three major fatty acids (C16:1*n*-7, C18:1*n*-9 and C20:5*n*-3 [50]) contributing ~80% to the TFAs. Although Lang, et al. [50] reported ~30% EPA for this strain, in the present study such values could only be achieved when biomass concentrations were low, indicating an early growth stage (~1 gDW L^{-1} at $T = 8$ °C, $I = 50$ µmol m^{-2} s^{-1}, day 10; Table A.3, Supplementary material). At this sampling point, cells were growing exponentially at high nutrient conditions (excess N, P)

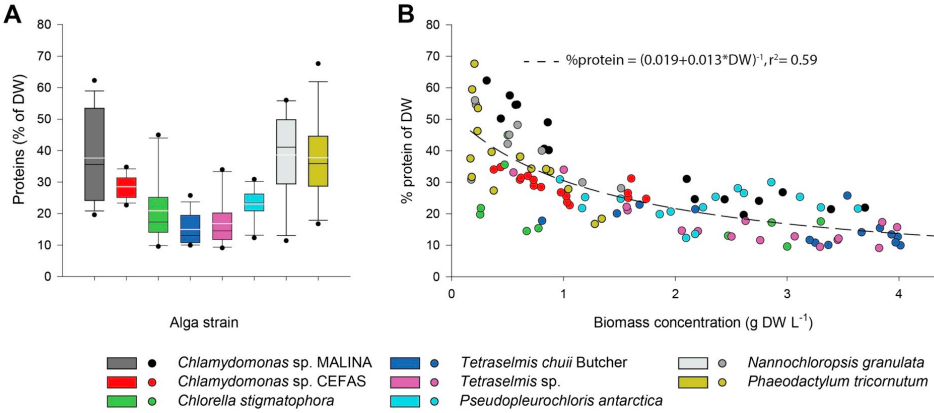

Fig. 3. Protein content of the tested microalgae and the relationship with the biomass concentration. The protein content ranges (in % of dry weight; DW) of each strain shown in box plots (A) were obtained from cultures grown in duplicates ($n = 2$) under different temperatures ($T = 8$, 15 °C) and light intensities ($I = 50$, 100 µmol s^{-1} m^{-2}) and harvested at two time points (day 10, 14). Black and white lines indicate the medians and means of all data, respectively. By performing regression analysis on protein data and biomass concentration across all time points, treatments and algal strains, an exponential relationship (B) was obtained ($p < 0.05$), explaining 59% of the variability in protein content by the biomass concentration.

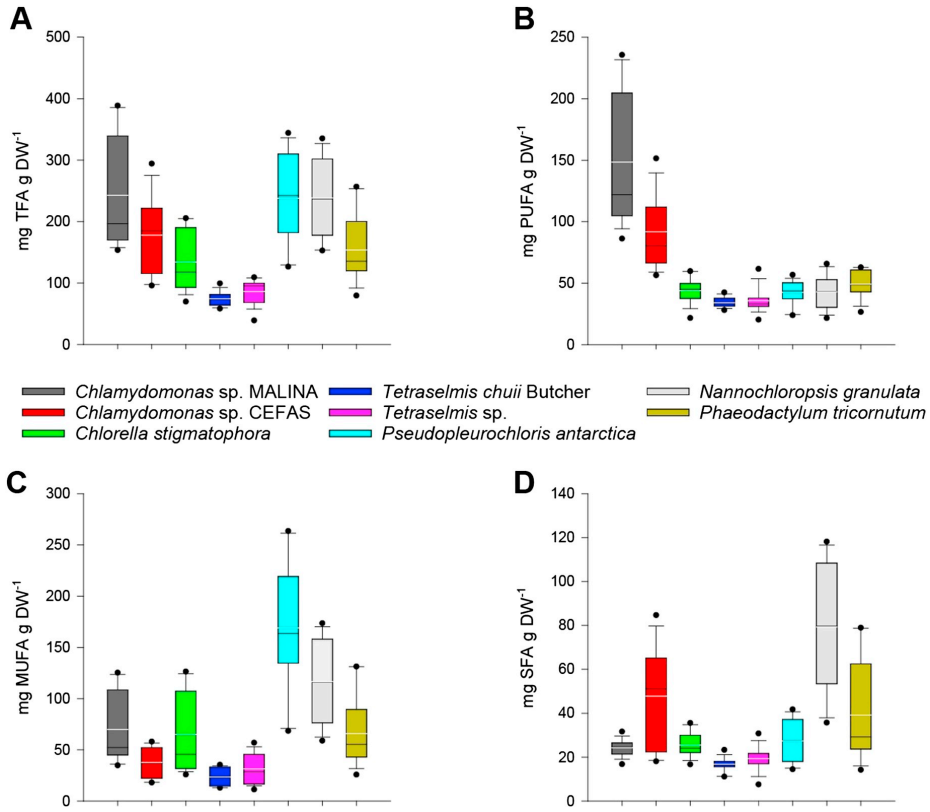

Fig. 4. Content of the total fatty acids, TFAs (A), polyunsaturated fatty acids, PUFA (B), mono-saturated fatty acids, MUFAs (C) and saturated fatty acids, SFAs (D) of the tested algae. Data (ranges in dry weight, mg gDW^{-1}) shown in these box plots were obtained from cultures grown in duplicates ($n = 2$) under different temperatures ($T = 8$, 15 °C) and light intensities ($I = 50$, 100 µmol s^{-1} m^{-2}) and harvested at two time points (day 10, 14). Black and white lines indicate the medians and means of all data, respectively. The effects of temperature, light and growth stages on fatty acids are provided in Table A.5 (Supplementary material).

that are commonly associated with maximum EPA levels [51]. As biomass concentrations in the medium increased, EPA levels dropped to ~10%. Due to the simple fatty acid profile and the prominence of EPA, *P. antarctica* can be a promising candidate for long-chain PUFA production, e.g., by means of selection, genetic modification or cultivation engineering.

3.2.3. Treatment effects on fatty acids

Temperature and biomass concentration correlated with the fatty acid composition (PUFA, MUFA and SFA) and single fatty acids in most of the tested strains (Table A.5, Supplementary material). TFAs usually correlated with conditions that resulted in best growth (e.g. low temperatures in *Chlamydomonas* sp. MALINA or high temperatures in *Chlamydomonas* sp. CEFAS) or biomass concentrations. Long chain PUFA such as EPA and DHA were generally higher in algae exposed to low temperatures or early growth stage (low biomass concentrations). For example, *P. antarctica* and *T. chuii* showed on average ~25% and 33% more EPA, respectively, at 8 °C compared to 15 °C. Low light treatments tend to induce PUFA (negative correlation of EPA and C18:2*n*-6 with light intensity; $r \leq -0.62$) and decreased the MUFA fraction (C18:1*n*-9, $r = 0.69$) in *P. antarctica*. In *Chlamydomonas* sp. MALINA cultures, the fraction of PUFA in TFAs was not affected by any treatment. Low temperatures and light intensities can induce PUFA in many microalgae, whereas nutrient limitation can cause a decrease in PUFA levels [51]. Our results of all tested algae, generally confirm these trends except of the psychrophilic microalgae *Chlamydomonas* sp. MALINA. At low temperatures, this alga achieved high biomass concentrations but also high PUFA levels, indicating that cold-adapted microalgae are potential fatty acid producers since PUFA contents in biomass remain high even at late growth stages.

3.3. Production performance and applications

Biocomponent productivities of each strain under different cultivation conditions are given in the Supplementary materials (Table A.3). *Chlamydomonas.* sp. MALINA surpassed all strains in their protein (69.6 ± 13.7 mg protein L^{-1} d^{-1}), TFA (91.0 ± 5.1 mg L^{-1} d^{-1}) and PUFA (54.1 ± 3.1 mg L^{-1} d^{-1}) productivity when cultivated at $T = 8$ °C, $I = 100$ µmol m^{-2} s^{-1}.

P. antarctica achieved second highest protein production rates (58.8 ± 3.4 mg protein L^{-1} d^{-1} at $T = 15$ °C, $I = 100$ µmol m^{-2} s^{-1}) and EPA and MUFA productivities of up to 7.7 ± 1.1 and 64.9 ± 3.9 mg L^{-1} d^{-1}, respectively ($T = 8$ °C, $I = 100$ µmol m^{-2} s^{-1}). Such EPA productivity is comparable to that of known EPA producers such as *Nannochloropsis* and *Isochrysis* cultivated under similar conditions [52-56]. The tolerance of *Chlamydomonas* sp. MALINA

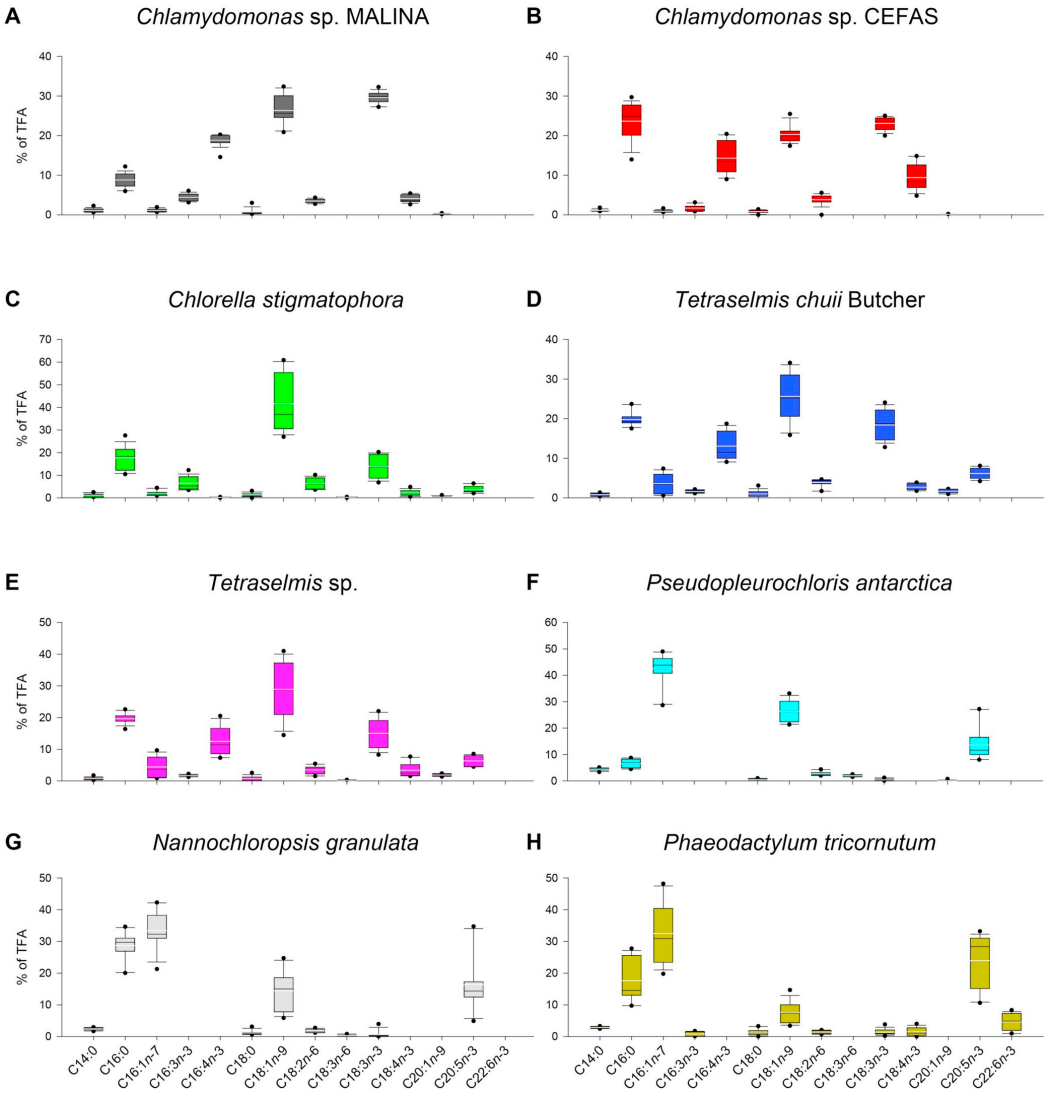

Fig. 5. Major fatty acids (in % of total fatty acids, TFAs) of *Chlamydomonas* sp. MALINA (A), *Chlamydomonas* sp. CEFAS (B), *Chlorella stigmatophora* (C), *Tetraselmis chuii* Butcher (D), *Tetraselmis* sp. (E), *Pseudopleurochloris antarctica* (F), *Nannochloropsis granulata* (G) and *Phaeodactylum tricornutum* (H). Data shown in box plots were obtained from cultures grown in duplicates ($n = 2$) under different temperatures ($T = 8, 15\,^{\circ}C$) and light intensities ($I = 50, 100\,\mu mol\,s^{-1}\,m^{-2}$) and harvested at two time points (day 10, 14). Black and white lines indicate the medians and means of all data, respectively. Effects of treatments and growth stages on each fatty acid were analysed by ANCOVA (Table A.5 in Supplementary material).

and *P. antarctica* to psychrophilic and psychrotrophic growth conditions ($T = 8$–$15\,^{\circ}C$) and low light levels make them ideal candidates for production of proteins and PUFA under cold and light-limited climates. However, continuous cultivation can greatly influence PUFA yields [51,57] and should be considered in future studies. For example, Řezanka, et al. [17] reported that the cold-adapted *Monoraphidium* sp. CCALA (strain no 1094) produced up to 97 mg PUFA $L^{-1}\,d^{-1}$ when cultivated at ~10 °C in a continuous thin-layer photobioreactor. On the other hand, *T. chuii* is a temperature tolerant, fast growing (up to 1 gDW $L^{-1}\,d^{-1}$) strain that only had low protein, TFA and PUFA productivities (e.g., max 44.5 ± 10.2, 32.6 ± 5.3 and 13.7 ± 1.7 mg $L^{-1}\,d^{-1}$, respectively). Since such biomass quality may only pose a low market value, this strain could be used for the removal of nutrients from cold marine wastewater streams (~15 °C) to produce biomass suitable for biofuel production [41]. A similar scenario was previously suggested for a mesophilic *Tetraselmis* strain to clean effluents ($T = 20\,^{\circ}C$) from wastewater treatment plants in warm climates [41]. Notably, also in cold climates, psychrophilic and psychrotrophic microalgae were tested for nutrient removal from wastewaters [35,58–64], which may be a promising application for the presently

tested *Tetraselmis* strains. The auto-settling abilities of *Tetraselmis* and *P. antarctica* can also save dewatering costs in future production scale up scenarios.

The major application of *C. stigmatophora* may not be for the production of intracellular fatty acids or proteins, but for EPS as sources of bioactive components. Indeed, *Chlorella* and *C. stigmatophora* strains were often reported for their EPS secretion, a promising bioresource for high-value bioactive components [65-67]. However, the separation of EPS containing media and algae remains a bottleneck that researchers try to tackle using e.g., ultrafiltration [68].

4. Conclusions

The present study tested eight strains isolated from cold marine habitats for the combined effects of temperature and light regimes on growth, proteins and fatty acids. Based on the collected data, *Chlamydomonas* sp. MALINA, *T. chuii* and *P. antarctica* are the most promising biomass, protein or fatty acids producers at low temperature and light regimes ($\leq$ 15 °C, $I \leq 100\,\mu mol\,m^{-2}\,s^{-1}$). The protein and total fatty acid contents among all tested strains were mainly affected by the growth stage rather than temperature and light treatments. Our results demonstrated that including biomass concentration of the culture as a quantitative indicator for the prevailing growth stage should be considered when evaluating effects of any treatment applied to microalgal cultures. Notably, only PUFA were affected by temperature and light intensity. Hence, these two factors could be adjusted together with the harvesting time point to maximise PUFA yields. Using cold water-adapted strains can benefit microalgal outdoor production during cold and light-limited seasons because expensive cooling or additional artificial light could be minimised. In addition, indoor productions located at extreme latitudes can use the usual low ambient temperatures to provide favourable conditions for growing cold-adapted strains and operating the commonly used LED lamps as artificial light sources.

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Declaration of authors' contributions

P.S. (peter.schulze@nord.no) designed the experiments, performed the laboratory work, data analysis, and drafted the manuscript. C.H. (christopher.j.hulatt@nord.no) contributed to laboratory work and revised the article for intellectual contents. D.S-M (daniela.morales-sanchez@nord.no) contributed to manuscript drafting, discussion and critical revision of the article for important intellectual content. R.W. (rene.wijffels@wur.nl) and V. K. (kiron.viswanath@nord.no) conceived the main project, contributed to the design, reviewed and edited the article and provided the administrative support for the research project. The authors agree with the authorship of this work and give final approval of the version to be submitted and any revised version.

Declaration of Competing Interest

The authors confirm that there is no known conflict of interest related with this publication and there has been no significant financial support for this study that could have influenced its outcome.

Statement of Informed Consent

No conflicts, informed consent, human or animal rights applicable.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2019.101604.

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Paper II

Manuscript style adapted to journal guidelines of Journal of Applied Phycology.

Production of carbohydrates, lipids and PUFA by the polar microalga *Chlamydomonas* sp. RCC2488

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Highlights:

- RCC 2488 is closely related to other psychrophilic algae: UWO 241 and SAG 75.94.
- RCC 2488 grows at low temperature (8 °C), in both freshwater and seawater.
- Maximum biomass productivity was achieved at a light intensity of 250 μmol m^{-2} s^{-1}.
- Nitrogen deprivation promotes carbohydrate synthesis and maintains lipid content.
- The lipid fraction is mainly composed of polyunsaturated fatty acids.

Abstract

The polar *Chlamydomonas* sp. RCC2488 grows at low temperatures and produces high amounts of lipids mainly composed of polyunsaturated fatty acids (PUFA). However, the phylogenetic relationship with other Chlamydomonadales members is not clear and the optimum growth conditions for maximum biomass productivity have not yet been identified. Here, a phylogenetic analysis was performed to determine the closest relatives of *Chlamydomonas* sp. RCC2488 within the Chlamydomonadales order. To select the best growth conditions for maximum biomass productivities in cultivations performed at 8°C, different salinities (0-80 ppt) and light intensities (70-500 μmol m^{-2} s^{-1}) were tested, using bubble column and flat-panel photobioreactors. The effect of nitrogen limitation was tested to determine if RCC2488 can accumulate carbohydrates and lipids. Phylogenetic analysis confirmed that RCC2488 is closely related to the psychrophilics *Chlamydomonas* sp. UWO 241 and *Chlamydomonas* sp. SAG 75.94, and to the mesophilic *C. parkeae* MBIC 10599. The highest biomass (527 mg L^{-1} day^{-1}), lipid (161.3 mg L^{-1} day^{-1}) and PUFA (85.4 mg L^{-1} day^{-1}) productivities were obtained at a salinity of 17.5 ppt, light intensity of 250 μmol m^{-2} s^{-1} and nitrogen replete conditions. Strikingly, the marine RCC2488 can also grow in fresh water. While the intracellular lipid content remained unchanged under nitrogen deprivation, the carbohydrate content increased (up to 49.5 % w/w), and the protein content decreased. The algal lipids were mainly comprised of neutral lipids, which were primarily composed of PUFA. *Chlamydomonas* sp. RCC2488 is a polar microalga suitable for high biomass, carbohydrate, lipid and PUFA productivities at low temperatures.

Keywords: Chlamydomonas, salinity, nitrogen deprivation, light intensity, PUFA, proteins

1. Introduction

Polyunsaturated fatty acids (PUFA) have gained interest in the pharmaceutical industry due to its beneficial properties for human and animal health (Ruxton et al. 2004; Khozin-Goldberg et al. 2011; Martins et al. 2013). PUFA used for human nutrition are mainly obtained from fish oil (Martins et al. 2013; Guihéneuf and Stengel 2013). However, obtaining PUFA from fish have several limitations, such as possible depletion of the resource, contamination with heavy metals, variability in the oil composition and quality, unpleasant odor, and environmental negative impacts like degradation of marine habitats (De Swaaf et al. 2003; Martins et al. 2013; Greene et al. 2013; Guihéneuf and Stengel 2013). On the other hand, through the food chain, fish obtain PUFA from microalgae via bioaccumulation (Guihéneuf and Stengel 2013; Morales-Sánchez et al. 2017). PUFA from microalgae can be a sustainable, environmentally friendly, and "vegetarian" alternative (Wijffels and Barbosa 2010; Khozin-Goldberg et al. 2011; Suzuki et al. 2018). Moreover, its production can be enhanced by using polar or cold adapted microalgae, which are the ideal candidate due to its naturally occurring high PUFA content (Morgan-Kiss et al. 2006). The synthesis of PUFA in polar microalgae is induced at low temperatures because these compounds help to maintain the fluidity, flexibility, and functionality of the cellular membranes, which is a crucial adaptive strategy to support the cellular metabolism at such temperatures (Morgan-Kiss et al. 2006; Suzuki et al. 2018). Also, polar microalgae have developed mechanisms to successfully adapt to low temperatures, oscillating light conditions, osmotic pressure, and oxidative or nutrient stresses (Řezanka et al. 2008; Leya et al. 2009; Lomsadze et al. 2012; Lyon and Mock 2014). Such adaptation to a wide range of environmental conditions has bestowed these microorganisms with a high degree of phenotypic plasticity (Pocock et al. 2011; Lyon and Mock 2014), which makes them interesting organisms for the production of PUFA, and other high-value metabolites. Polar or cold-adapted microalgal species that have shown evidence of high biomass and/or PUFA productivities at low temperature include *Chlamydomonas pulsatilla, C. klinobasis, Chloromonas platystigma*, *Raphidonema sempervirens, Koliella Antarctica,* and *Chlamydomonas* sp. RCC2488 (Hulatt et al. 2017a; Suzuki et al. 2018; Schulze et al. 2019). *Chlamydomonas* sp. RCC2488 (called RCC2488 hereafter) is a polar microalga isolated from the Beaufort Sea of the Arctic Ocean (Balzano et al. 2012). In our previous study, *Chlamydomonas* sp. RCC2488 had high levels of PUFA when cultivated at 8°C compared to 15°C (Schulze et al. 2019). However, the phylogenetic relationship of RCC2488 within the Chlamydomonadales order and its best growth conditions for obtaining desired biomass

and metabolites are yet to be investigated. In this study, we performed a phylogenetic analysis of the 18S rRNA gene sequence of RCC2488 and available *Chlamydomonas* strains. We report the performance of RCC2488 under different experimental conditions, such as salt concentrations, light intensities, and nitrogen stress, to identify the optimal growth conditions at which this microalga produces commercially important metabolites, such as lipids, carbohydrates, and PUFA.

2. Materials and methods

2.1. Strain

The marine microalgae *Chlamydomonas* sp. RCC2488 was obtained from the Roscoff Culture Collection, France (RCC). This strain was isolated from the Beaufort Sea, within the Arctic Ocean, at latitude 69°48N and longitude 138°26E (Balzano et al. 2012).

2.2. Phylogenetic analysis

The sequence of the 18S ribosomal RNA gene of RCC2488 strain was obtained from the Roscoff Culture Collection with accession number JN934686. 18S rRNA related genes were identified by BLASTN searches against GenBank and NCBI, using RCC2488 18S rRNA gene sequence as a query (JN934686). Sequences were aligned by Muscle (Edgar 2004); all gaps and missing data were eliminated. The evolutionary history was inferred by constructing a phylogenetic tree using the Maximum Likelihood method based on the Tamura-Nei Model (Tamura and Nei 1993). Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016).

2.3. Culture conditions

Stock cultures were maintained on agar plates containing modified F2 media. Inocula for all experiments were cultured in 250 mL Erlenmeyer shake-flasks (100 rpm) containing 100 mL of modified F2 at 8 °C with an irradiance of ~120 μmol m^{-2} s^{-1} (Phillips TLD 840 fluorescence lamps) and ambient levels of CO_2. For the media preparation, we used seawater from the North Atlantic shoreline of Bodø (Norway) containing a salinity approximately of 35 ppt. The modified F2 media comprised the following macronutrients (in mM): $NaNO_3$ 31.8, $NaH_2PO_4H_2O$ 1.32, $FeCl_3 \bullet 6H_2O$ 0.105, $Na_2EDTA \bullet 2H_2O$ 0.105, trace elements (in μM): $CuSO_4 \bullet 5H_2O$ 0.36, $Na_2MoO_4 \bullet 2H_2O$ 0.234, $ZnSO_4 \bullet 7H_2O$ 0.69, $CoCl_2 \bullet 6H_2O$ 0.378, $MnCl_2 \bullet 2H_2O$ 8.19, and vitamins (in μM): thiamine

HCl 26.6, biotin 0.18 and cyanocobalamin 0.036. The initial biomass concentration of all experiments was around 0.2 g of dry cell weight (DCW) L^{-1}. For salinity experiments, RCC2488 was cultured in modified F2 media for 10 days at 0, 17.5, 35, and 80 ppt, using combinations of fresh water (0 ppt) and seawater (35 ppt) or supplementing with NaCl for higher salinity (80 ppt). For testing of different light intensities and nitrogen stress, a salinity 17.5 ppt was used. For light intensity experiments, RCC2488 was grown in modified F2 media for 10 days at 70, 120, 250 and 500 μmol m^{-2} s^{-1}. For nitrogen availability experiments, RCC2488 was pre-cultured in modified F2 media and 120 μmol m^{-2} s^{-1} until the middle logarithmic phase was reached (day 5). At this growth stage, cells were collected by centrifugation, washed twice with water (salinity:17.5 ppt), and re-suspended in modified F2 media with nitrogen (F2+N) or without nitrogen(F2-N) at a biomass concentration of 1.5 g_{DCW} L^{-1}. Cultures were grown for five days at a light intensity of 250 μmol m^{-2} s^{-1}. After five days, cells were harvested by centrifugation (2000 *g*, *t*= 5 min), washed with 0.5M ammonium formate, centrifuged again (2000 *g*, *t*= 5 min), and the pellets were stored at -70 °C for further analyses.

2.4. Cultivation in photobioreactors

Salinity and nitrogen stress experiments were conducted in bubble column photobioreactors (Table 1) designed by Hulatt et al. (Hulatt et al. 2017a). Briefly, the arrangement consisted of glass tubes (Friedel, Oslo, Norway) measuring 35 mm internal diameter with 300 ml of working volume, fitted with sealed silicon stoppers and autoclaved as complete units at 121 °C for 20 min. The media was autoclaved separately in 1L flasks. Filtered air (0.2 μm, Acrodisc ® PTFE filters, Pall Corporation, USA) containing 1% CO_2 was supplied to each photobioreactor at a flow of 1 vvm (300 mL min^{-1}) using a rotameter (Omega, Manchester, UK). A mass flow control system (GMS-150, Photon Systems Instruments, Czech Republic) was used to control the CO_2 concentration by mixing it with compressed air. These photobioreactor systems were placed in a temperature-controlled environment chamber at 8 °C (Termaks AS, Bergen, Norway) fitted with nine fluorescent lamps (cool daylight, 36 W, Phillips) illuminated from one side at a light intensity of 120 μmol m^{-2} s^{-1}.

Light intensity experiments were carried out in autoclaved flat-panel photobioreactors (Algaemist-S, Ontwikkelwerkplaats, Wageningen UR, The Netherlands) (Table 1), fully described previously (Hulatt et al. 2017b). The working volume was 380 mL with a light path of 14 mm. The cultures were aerated at 1 vvm with 0.2 μm filtered air mixed with 1 % CO_2. Continuous light intensity of 70, 120, 250 and

500 μmol m^{-2} s^{-1} was provided by warm-white LEDs. The cultivation temperature of 8 °C ± 0.5 °C was controlled by the Algaemist software. Initial pH was 7 without control along the cultivation period. All experiments were carried out in triplicate for 10 days.

Table 1. Different conditions used in the treatments tested in the present work.

Treatment	Salinity (ppt)	Light intensity (μmol m^{-2} s^{-1})	Nitrogen availability	Photobioreactor
Salinity	0	120	+	Bubble column
	17.5	120	+	
	35	120	+	
	80	120	+	
Light intensity	17.5	70	+	Flat-panel
	17.5	120	+	
	17.5	250	+	
	17.5	500	+	
Nitrogen stress	17.5	250	+	Bubble column
	17.5	250	-	

+: Nitrogen replete conditions
-: Nitrogen deplete conditions

2.5. Growth measurement

Samples of the culture (0.5 – 1 mL) were collected daily to measure the absorbance at 750 nm in a 1 cm micro-cuvette using a spectrophotometer (Hach-Lange DR3900, Hach, International). The DCW was evaluated gravimetrically by filtrating 5-10 mL of culture through a pre-dried and pre-weighed 0.45 μm pore size fiber glass membrane filter (Milipore). For daily calculation of the biomass concentration (W), a calibration curve between the absorbance measured at 750 nm (A750) and DCW was established ($W = 0.884 \cdot A_{750} + 0.0117$, R^2=0.99).

2.6. Lipid, protein, and carbohydrate analyses

Total lipids from RCC2488 were extracted using organic solvents, and fatty acid methyl esters (FAMEs) from triacylglycerols (TAGs) and polar lipids were identified and quantified by gas chromatography (GC) as previously described by Breuer et al. (Breuer et al. 2013), with some modifications. Briefly, 10 mg of freeze-dried microalgal biomass was weighed using a precision balance (MX5, Mettler-Toledo, USA), then the lipids were extracted and gravimetrically quantified. For the extraction, a mix of chloroform:methanol (2:2.5 v/v) containing an internal standard (Tripentadecanoin, C15:0 Triacylglycerol, Sigma-Aldrich, USA) was added to the samples to extract the lipids, and then the cells were disrupted using a bead mill (Bertin technologies, Precellys

Evolution, France, 0.1 mm glass beads). Methanol from the solution containing the extracted lipids was separated by adding an aqueous solution of Tris buffer (6 g L^{-1} Tris, 58 g L^{-1} NaCl, pH 7.5). The chloroform phase containing the lipids was removed and dried under a stream of nitrogen. To determine the fatty acid composition, lipid samples were chemically derivatized to fatty-acid methyl-esters (FAMEs) using 5% H_2SO_4 in methanol and heated at 70 °C for 3 h. Methanolic H_2SO_4 from the solution containing the FAMEs was separated by adding a mix of hexane:H_2O (1:1). Finally, samples containing the FAMEs and hexane were placed into chromatographic vials. The obtained organic phase was analyzed in a GC fitted with a Flame Ionization Detector (Scion 436, Bruker, USA) and an Agilent CP-Wax 52 CB column (Agilent Technologies, USA) using splitless injector. To identify and quantify the most common FAMEs, external Supelco® 37-component standards (Sigma-Aldrich, USA) were used. Blanks were included in the extraction process to eliminate background trace peaks.

For carbohydrate determination, samples were hydrolyzed with HCl to yield simple sugars, and the resultant monosaccharides were quantified using the phenol-sulphuric acid method (Thompson 1950).

For protein determination, lysis buffer (60mM Tris pH 9, 2% sodium dodecyl sulfate) was added to 10 mg of freeze-dried biomass samples prior to cells disruption in bead milling system as described before, and then protein content was determined using the Lowry method (Lowry et al. 1951).

2.7. Calculations

The cellular growth kinetic and productivity were calculated accordingly with a 4-parameter logistic function (Hulatt et al. 2017b) (Eq 1:

$$C_x = \emptyset 1 + \frac{\emptyset 2 - \emptyset 1}{1 + \exp\left(\frac{\emptyset 3 - t}{\emptyset 4}\right)} \qquad (1)$$

Where C_x is the dry weight (g L^{-1}) at time t (days), $\phi 1$ is the lowest asymptote (minimum C_x), $\phi 2$ is the upper asymptote (maximum C_x), $\phi 3$ is t at 0.5$\phi 2$ (the inflection point), and $\phi 4$ is the scale parameter (Hulatt et al. 2017b). From the previous equation, the volumetric productivity was calculated between two time points, accordingly with equation 2:

$$P_i = \frac{C_{x,i} - C_{x,i-1}}{t_i - t_{i-1}} \qquad (2)$$

Where P is the productivity (g L^{-1} day^{-1}), $C_{x.i}$ and $C_{x.i-1}$ are the concentrations of the biomass (g L^{-1}) at two time points, and t_i and t_{i-1} are the time of cultivation (days).

2.8. Statistical analysis

For all treatments, the normal distribution of data was confirmed using Saphiro-Wilk test, and the homogeneity of the variance between treatments was validated using Brown-Forsythe test. For salinity and light intensity treatments, one-way analysis of variance (ANOVA) and *post-hoc* Tukey's multiple comparison test were used. For nitrogen stress treatments, a *t*-test was applied. *P* values smaller than 0.05 were considered statistically significant.

3. Results and discussion

3.1. Phylogenetic analysis

Previous results indicated that RCC2488 belongs to the order Chlamydomonales (Balzano et al. 2012), we confirm this result (data not shown). We constructed a phylogenetic tree by the comparison of the sequences of 18S rRNA gene of isolate RCC2488 and only the Chlamydomonales order (Fig. 1)**.** In this tree, the RCC2488 strain is placed in a lineage closely related to *C. parkeae,* within the Moewusinia clade. RCC2488 is closely related to *Chlamydomonas* sp. UWO 241 (UWO 241 hereafter, score = 2983 bit, identity = 99%), *Chlamydomonas* sp. SAG 75.94 (score = 2942 bit, identity = 99%) with a strong bootstrap (BS = 100). All the sequences form clusters with bootstrap support of no less than 50%.

Previous phylogenetic studies indicated that this microalga cluster together with *C. raudensis* CCAP 11/131 and *C. parkeae* within the Moewusii clade (Balzano et al. 2012). It is clear that RCC2488 belongs to the Chlamydomonadales order, mainly composed by freshwater flagellates within the Chlorophyceae (Balzano et al. 2012). However, RCC2488 in the present phylogenetic analysis appeared more closely related to UWO 241 and *C. parkeae* MBIC10599 than to *C. raudensis* that is being grouped in a sister clade (Fig. 1). The reason for this discrepancy is that UWO 241 was earlier misidentified as *C. raudensis* CCAP 11/131 (Pocock et al. 2004). But recent studies on phylogenetic analysis of nuclear and plastid DNA sequences revealed that UWO 241 is in fact closely affiliated to the marine strain *C. parkeae* SAG 24.89 and strongly differs from *C. raudensis* SAG 49.72 (Possmayer et al. 2016).

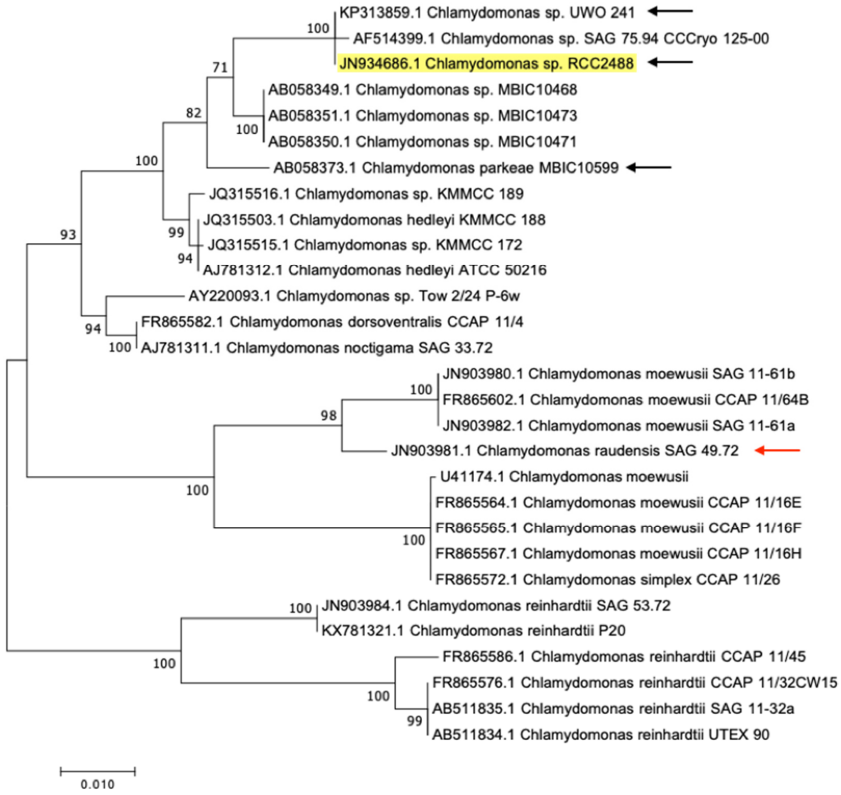

Fig. 1 Phylogenetic tree. Maximum-likelihood phylogenetic tree based on the alignment of the 18S rRNA gene sequences from *Chlamydomonas* sp. RCC 2488 (yellow highlighted) and several *Chlamydomonas* strains. Number of branches indicates the percentage of 1000 bootstrap replication supporting a particular node. Black arrows identify the position of *Chlamydomonas* sp. RCC 2488, UWO 241 and *C. parkeae* MBIC10599 as closest relatives in the same cluster. A red arrow denotes the position of *C. raudensis* SAG 49.72 in a sister clade.

3.2. Effect of salinity concentration

Highest biomass concentration (5.02 g_{DCW} L^{-1}) and productivity (480 mg_{DCW} L^{-1} d^{-1}) were attained at salinities of 17.5 and 35 ppt (p>0.05, Figs. 2a and b). The growth and maximal biomass concentration decreased 5-fold at the highest salinity tested (80 ppt) (Figs. 2a and b). *Chlamydomonas* sp. RCC2488 is a marine microalga, originated from the Beaufort Sea located in the Arctic Ocean (Balzano et al. 2012). There, during late summer when most phytoplankton blooms are occurring, salinities shift between 28 and 32 ppt due to freshwater inflow from rivers and melting ice (Sherr et al. 2003; Wang et al. 2005; Balzano et al. 2012). RCC2488 may have adapted to these salinity shifts, explaining the high growth performance at salinities ≤35 ppt. As demonstrated in RCC2488 closest relative, UWO 241, one possible explanation of the salinity tolerance of RCC2488 is the modulation of the redox signal (redox state of plastoquinone pool), affecting later the expression of genes responsible of high salinity acclimation (Pocock et al. 2011; Dolhi et al. 2013).

Protein content in treatments from 0 to 35 ppt had similar values (26.1 – 27.6 % of DCW, p>0.05, Fig. 2c). A lower protein content was found in cells cultivated at 80 ppt as compared to 17.5 ppt (p<0.05), probably as a result of a lower metabolic activity. The carbohydrate content in cells cultivated at ≤35 ppt was not statistically different (24.5 – 26.1 % of DCW; p>0.05). Carbohydrate accumulation up to 33.2 % of DCW was stimulated by high salinities, resulting in a linear trend from salinities 17.5 to 80 ppt (data not shown). In the case of total lipid content, the statistical analysis (one-way ANOVA and *post-hoc* Tukey HSD) demonstrated that there were no significant differences between the salinity treatments.

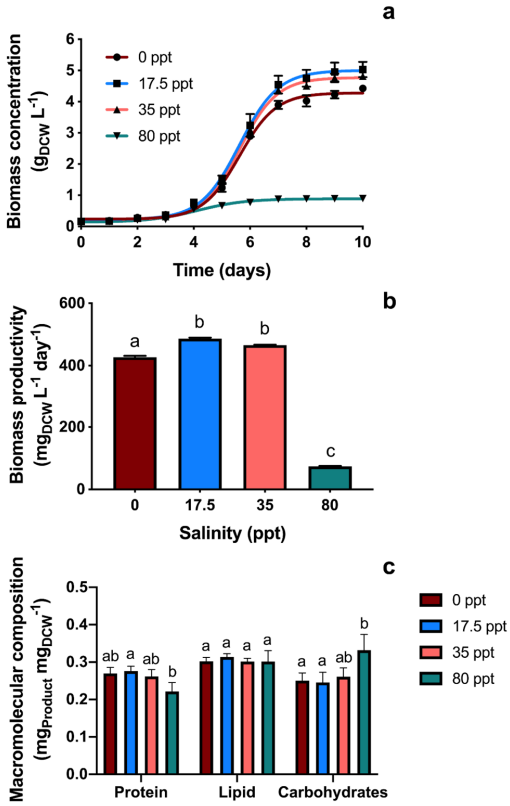

Fig. 2 Effect of salinity. Growth kinetics (a) and biomass productivity (b) of RCC2488 after 10 days of batch cultivation in tubular photobioreactors in response to salinity concentrations. The macromolecular composition (c) was analyzed in the middle of exponential growth phase (day 5). Values on the Y-axis indicate the mean and standard deviation of three independent experiments. Different lowercase letters indicate a significant difference among means of different groups (one-way ANOVA with *post-hoc* Tukey HSD test, $p<0.05$).

The fatty acid content and class were similar in the treatments at salinities ≤35 ppt ($p>0.05$, Fig. 3). Cells cultivated at salinities ≤35 ppt synthesized the double of polar lipids (60-68.5 $mg_{POLAR\ LIP}$ g_{DCW}^{-1}) than cells grown at 80 ppt (30.2 $mg_{POLAR\ LIP}$ g_{DCW}^{-1}, $p<0.01$, Fig. 3a). In general, the TAG pool comprised the major lipid fraction in all treatments, ranging from 230.1 to 267.4 mg_{TAG} g_{DCW}^{-1}. Specifically, into this TAG pool, cells cultivated at ≤35 ppt mainly synthesized PUFA (hexadecatetraenoic acid, C16:4*n*-3 and α-linolenic acid, C18:3*n*-3) (Figs. 3b and 8a).

Maximal PUFA productivity of 65.1 mg_{PUFA} L^{-1} day^{-1} was obtained in cells cultivated at 17.5 and 35 ppt (p<0.05, Fig. 3b). Interestingly, the high biomass productivity at ≤35 ppt did not compromise the cellular total lipid content as being usually found for microalgae (Oh et al. 2013; Vaidyanathan et al. 2016). For the ongoing experiments, a salinity of 17.5 ppt was chosen.

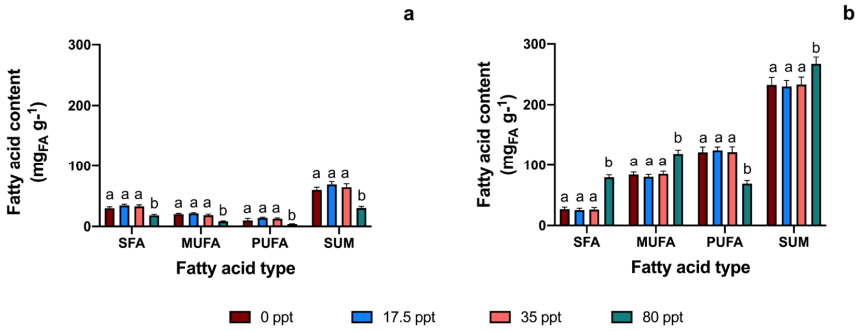

Fig. 3 Effect of salinity on the fatty acid content. Fatty acid content in the polar lipid pool (a) and the TAG pool (b) of RCC2488 at day 5 of batch in response to salinity concentrations. SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, TAG: triacylglycerols. Values on the Y-axis indicate the mean and standard deviation of three independent experiments. Statistical comparison was performed individually for each macromolecular component and each class of fatty acid (polar, TAGs, SFA, MUFA, PUFA and sum) among the treatments. Different lowercase letters indicate a significant difference among means of different groups (one-way ANOVA with *post-hoc* Tukey HSD test, p<0.05).

3.3. Effect of light intensity

The highest biomass concentration of 5.3 g L^{-1} and overall productivity of 527.4 mg_{DCW} L^{-1} day^{-1} was found at 250 µmol m^{-2} s^{-1} (p<0.05, Figs. 4a and b). Strikingly, RCC2488 could grow at low but also at high light intensities, suggesting that this microalga may be high light tolerant. The ability of RCC2488 to grow at high light intensities was not found in UWO 241, which did not grow above light intensities of 250 µmol m^{-2} s^{-1} (Pocock et al. 2007, 2011). Probably, one reason could be that UWO 241 lacks PsbS, which is a protein that plays a key role in photoprotection (Dolhi et al. 2013), however the presence of this protein in RCC2488 is unknown. Therefore, for further studies, it would be an interesting phenomenon to investigate the effect of even higher light intensities on the physiology, metabolism, and genome of RCC2488. Nevertheless, the high performance of RCC2488

in a broad range of light intensities may be a consequence of living in the Arctic, where RCC2488 had to adapt to low light levels occurring during winter and to high light irradiances during summer (Morgan-Kiss et al. 2006). Adaptations to light variabilities include evolution to a structurally and functionally distinct photosynthetic apparatus, and augmented light-harvesting apparatus (Pocock et al. 2004; Morgan-Kiss et al. 2006).

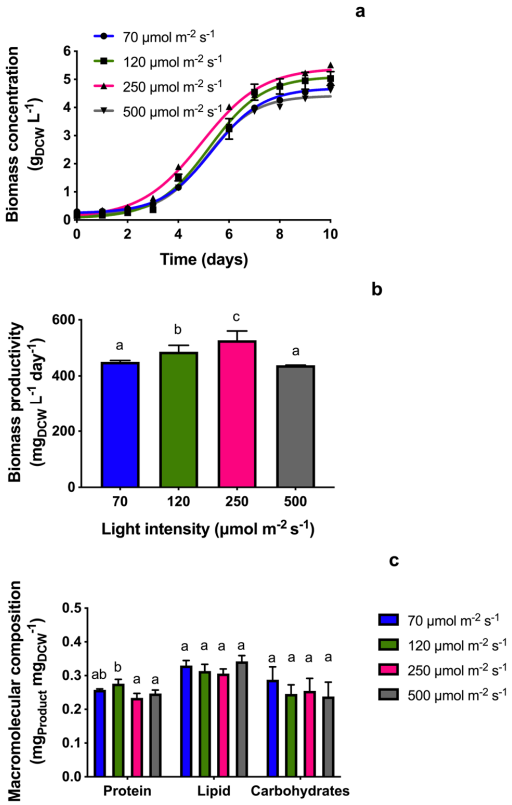

Fig. 4 Effect of light intensity. Growth kinetics (a) and biomass productivity (b) of RCC2488 after 10 days of batch cultivation in flat-panel photobioreactors in response to light intensities. The macromolecular composition (c) was analyzed in the middle of exponential growth phase (day 5). Values on the Y-axis indicate the mean and standard deviation of three independent experiments. Different lowercase letters indicate a significant difference among means (one-way ANOVA with *post-hoc* Tukey HSD test, $p<0.05$).

The highest protein content was found in the treatments at 70 and 120 μmol m^{-2} s^{-1} (27.6 % of DCW, p=0.2325, Fig. 4c). The content of carbohydrate and lipid had no significant differences (p>0.05) among all light intensities, suggesting that light intensities, ranging from 70 to 500 μmol m^{-2} s^{-1}, did not have an effect on the synthesis of energy reserve metabolites in RCC2488. This response is contradictory to what has been observed with other microalgal strains. For example, diminution of the protein and carbohydrate content but an increase in the lipid content are typical responses to high light intensity in several strains (Liu et al. 2012; Draaisma et al. 2013; Gwak et al. 2014; Ho et al. 2014; He et al. 2015).

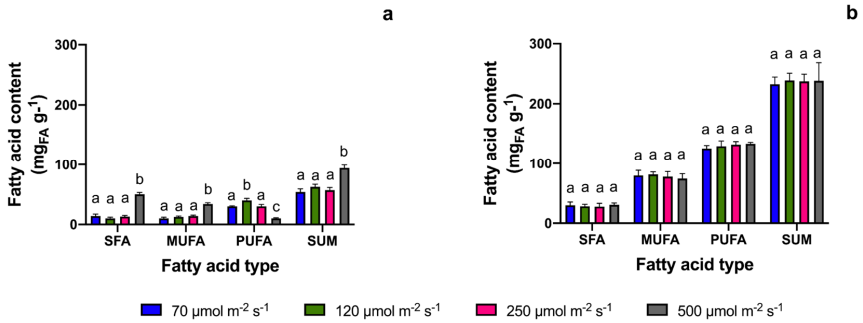

Fig. 5 Effect of light intensity on the fatty acid content. Fatty acid content in the polar lipid pool (a) and the TAG pool (b) of RCC2488 at day 5 of batch cultivation in flat-panel photobioreactors in response to light intensities. SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, TAG: triacylglycerols. Values on the Y-axis indicate the mean and standard deviation of three independent experiments. Statistical comparison was performed individually for each macromolecular component and each class of fatty acid (polar, TAGs, SFA, MUFA, PUFA and sum) among the treatments. Different lowercase letters indicate a significant difference among means of different groups (one-way ANOVA with *post-hoc* Tukey HSD test, p<0.05).

Nevertheless, in all treatments the lipid and carbohydrate content were relatively high for cells taken in the mid exponential phase of growth. As discussed below, probably another stressor, such as temperature, induced high lipid and carbohydrate synthesis.

The highest light intensity (500 μmol m^{-2} s^{-1}) induced higher content of polar lipids (p<0.01; Fig. 5a), specifically SFA (C14:0, C16:0 and C18:0; Fig. 8b) and MUFA (C16:1n-7, C18:1n-9, C18:1n-7; Fig. 8b). This increase in the polar lipids pool was probably due to remodeling or relocation of membrane lipids in response to the high light intensity (Gwak et al. 2014; Hu et al. 2017; Seiwert et al. 2017). For example, the glycolipid

digalactosyl diacylglycerol (DGDG) is known to stabilize, structurally and functionally, the chloroplast which led to cell survival (Gwak et al. 2014; Hu et al. 2017; Seiwert et al. 2017). Photoprotection is another function of polar lipids like monogalactosyl diacylglycerol (MGDG) at high light intensities (Gwak et al. 2014; Hu et al. 2017; Seiwert et al. 2017). Indeed, high levels of membrane lipids were also found in UWO 241 (RCC2488 closest relative), where polar lipids were mainly composed of MGDG, DGDG, and sulfoquinovosyldiacylglycerol (Dolhi et al. 2013).

In all treatments, lipids were mainly TAG with similar composition (Fig. 5b; $p>0.05$). This TAG fraction comprises primarily of PUFA such as C16:4*n*-3 and C18:3*n-3* (Fig. 8b). The maximal total PUFA productivity of 83.8 mg_{PUFA} L^{-1} day^{-1} was found in the treatments at light intensities of 120 and 250 μmol m^{-2} s^{-1} ($p>0.05$).

3.4. Nitrogen stress

Nitrogen stress condition was applied to RCC2488 cells to test if energy reserve metabolites can be accumulated. Due to a halt in cell division, the final biomass concentration and productivity of cells maintained under nitrogen deprivation (F2-N) were ~2 times lower than cultures at replete nitrogen conditions (F2+N, Figs. 6a and b, $p<0.0001$, *t-test*).

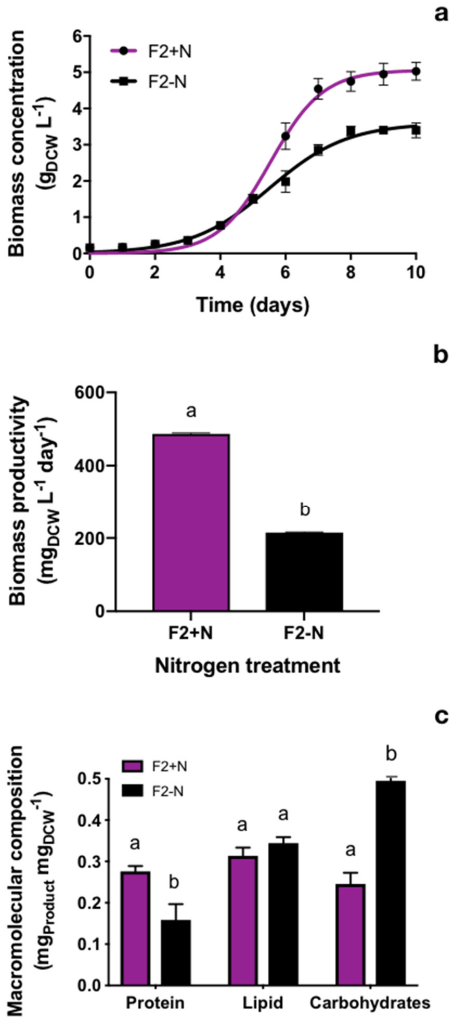

Fig. 6 Effect of the nitrogen availability. Growth kinetics (a) and biomass productivity (b) of RCC 2488 after 10 days of batch cultivation in tubular photobioreactors in response to nitrogen availability. The macromolecular composition (c) analysis was performed after 5 days of nitrogen availability treatments. Values on the Y-axis indicate the mean and standard deviation of three independent experiments. Different lowercase letters indicate a significant difference among means (Student´s *t*- test, $p<0.05$).

Nitrogen deprivation stimulated the accumulation of carbohydrates (up to 49.5 % DCW, $p<0.01$, *t*-test, Fig. 6c), at protein synthesis expenses (12 % DCW decrease, $p<0.01$, Fig. 6c). One of the most preferred strategies to increase reserve metabolites production in several microalgal species is a nutrient stress, like nitrogen deprivation (Chen et al. 2017). When nitrogen availability is limited, cells synthesize energy storage compounds such as carbohydrates, while reducing nitrogen-containing compounds such as proteins (Procházková et al. 2014). Hence, this strategy was effective for carbohydrate but not for lipid accumulation. The lipid content had no significant differences among the treatments ($p>0.05$; Fig. 6c). Nevertheless, the lipid content of RCC2488 is relatively high in both treatments (average 32.5 % of DCW), compared with other *Chlamydomonas* strains, such as the mesophilic *C. reinhardtii*, that can accumulate up to 19% at nutrient replete conditions (Li-Beisson et al. 2015).

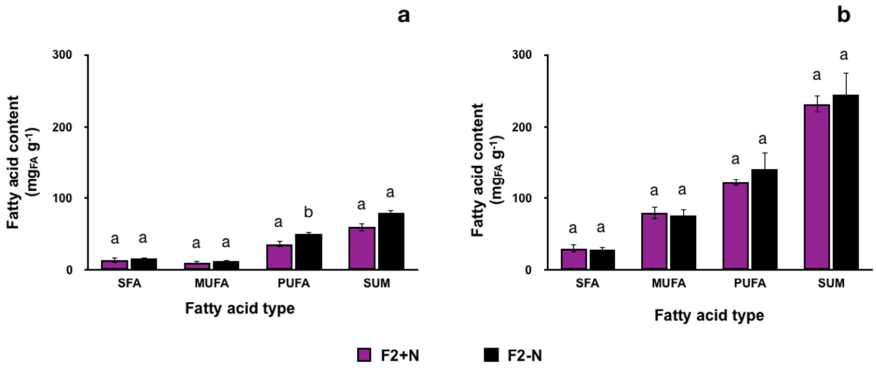

Fig. 7 Effect of nitrogen availability on the fatty acid content. Fatty acid content in the polar lipid pool (a) and the TAG pool (b) of RCC2488 after 10 days of batch cultivation in tubular photobioreactors in response to nitrogen availability. SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, TAG: triacylglycerols. Values on the Y-axis indicate the mean and standard deviation of three independent experiments. Statistical comparison was performed individually for each macromolecular component and each class of fatty acid (polar, TAGs, SFA, MUFA, PUFA and sum) among the treatments. Different lowercase letters indicate a significant difference among means (Student´s *t*- test, $p<0.05$).

This high lipid content has been reported in other polar and mesophilic algal species as an essential player for temperature acclimation and adaptation (Renaud et al. 1995, 2002; Cao et al. 2016). Consequently, a possible explanation is that this polar algal strain was already under a temperature stress at 8 °C, which induced lipid accumulation in all treatments tested in this study. Nevertheless, we advise additional studies at lower temperatures to support this observation.

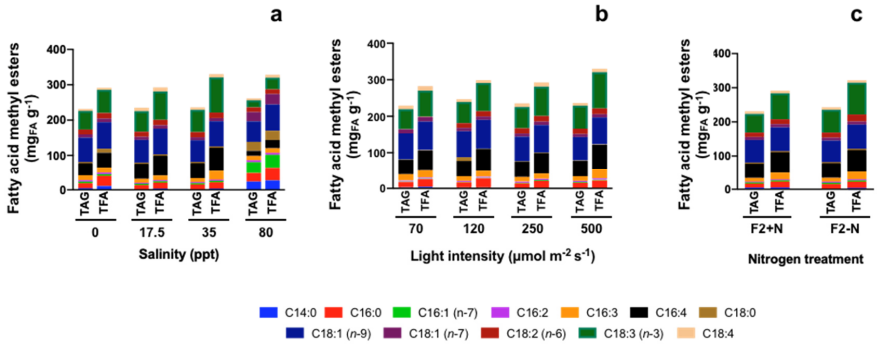

Fig. 8 Fatty acid profiles in the different treatments. Effect of salinity (a), light intensity (b) and nitrogen stress (c) treatments on the fatty acid profile of total fatty acids (TFA) and fatty acids in triacylglycerols (TAG) contained in RCC 2488 cells. Values on the Y-axis indicate the mean and standard deviation of three independent experiments.

The total PUFA productivity of cells cultivated in F2+N was 76.9 mg_{PUFA} L^{-1} day^{-1}, while the PUFA productivity of cells maintained in F2-N was substantially lower (40.9 mg_{PUFA} L^{-1} day^{-1}). In both cases, PUFA was mainly comprised of C16:4*n*-3 and C18:3*n*-3 (Fig. 8c). The proportion of total polar lipids and total TAG was similar in both treatments (p>0.05, Figs. 7 and 8c). The PUFA content in the TAG pool (Fig. 7b) was similar in F2+N and F2-N (p>0.05). However, the PUFA content in the polar lipid pool was significantly higher in F2-N (p<0.05) (Figs. 7a and 8c). The effect of low temperature on the membrane lipid composition is well-known (D'Amico et al. 2006; Morgan-Kiss et al. 2008), like the contribution of C16:4*n*-3 in the transition from liquid-crystalline to gel phase (Dolhi et al. 2013). This phenomenon has been also observed in the psychrophilic UWO 241, which contains high levels of C16:4*n*-3 (Morgan-Kiss et al. 2002). Nevertheless, the combined effect of low temperature and nitrogen deprivation on the membrane lipid composition is not yet understood and requires further studies.

4. Conclusions

The arctic green alga *Chlamydomonas* sp. RCC2488 is a novel polar microalga that belongs to the Chlamydomonadales order, closely related to the well-studied Antarctic strain *Chlamydomonas* sp. UWO 241. Both strains share similar physiological features such as tolerance to a wide range of salinities, high lipid content composed mainly by C16:4*n*-3 and C18:3*n*-3. However, RCC2488 tolerated high light intensities, a trait that was not found for UWO 241. RCC2488 achieved maximum productivities of biomass (527 mg L^{-1} day^{-1}), lipids (161.3 mg L^{-1} day^{-1}) and PUFA (85.4 mg L^{-1} day^{-1}) under nitrogen replete conditions using brackish water (salinity: 17.5 ppt) and a light intensity of 250 μmol m^{-2} s^{-1}. Nitrogen deprivation triggered the accumulation of carbohydrates in cells (up to 49.5 % w/w) at the expense of proteins but without compromising lipid biosynthesis. RCC2488 is a polar microalga suitable for biomass, lipid, PUFAs and carbohydrate production at low temperatures (8°C).

5. Acknowledgments

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6. Contributions

DMS designated the study and collected the data. DMS and PS performed the lipid and fatty acid analysis. DMS, PS, RHW, and KV contributed to manuscript drafting, discussion and critical revision of the article for important intellectual content.

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Paper III

Trends in Biotechnology

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Review

Flashing LEDs for Microalgal Production

Peter S.C. Schulze,[1,2] Rui Guerra,[3] Hugo Pereira,[2] Lisa M. Schüler,[2] and João C.S. Varela[2,*]

Flashing lights are next-generation tools to mitigate light attenuation and increase the photosynthetic efficiency of microalgal cultivation systems illuminated by light-emitting diodes (LEDs). Optimal flashing light conditions depend on the reaction kinetics and properties of the linear electron transfer chain, energy dissipation, and storage mechanisms of a phototroph. In particular, extremely short and intense light flashes potentially mitigate light attenuation in photobioreactors without impairing photosynthesis. Intelligently controlling flashing light units and selecting electronic components can maximize light emission and energy efficiency. We discuss the biological, physical, and technical properties of flashing lights for algal production. We combine recent findings about photosynthetic pathways, self-shading in photobioreactors, and developments in solid-state technology towards the biotechnological application of LEDs to microalgal production.

Trends

Light sources require technical fine-tuning for efficient emission of intense light flashes that match the kinetics of the photosynthetic apparatus and are able to penetrate deeper into microalgal cultures.

Flashing light may decrease the energy required to achieve a given productivity compared to continuously supplied light.

Flashing light systems require higher light output during the light period than standard light sources, and this can be achieved by increasing the current supplied to the LED.

Efficient flashing light emitters are single-color LEDs and laser diodes rather than organic LEDs (OLEDs) or phosphor-converted white (pc-)LEDs.

Laser diodes will be promising for future flashing-light sources and may induce beneficial quantum effects on microalgae.

Artificial Light in Microalgal Production

Microalgae are a promising biological resource for the mass production of lipids, sugars, polymers, or proteins for the food, feed, and chemical industries [1]. The coproduction of high-value biomolecules such as polyunsaturated fatty acids, carotenoids, β-glucans, and phycobiliproteins for nutraceutical, pharmaceutical, and biomedical applications increases the value of microalgal biomass and the economic feasibility of microalgae-based biorefineries [2]. Presently, high production costs of €5–25 kg^{-1} hinder the economic feasibility of microalgal-based commodities [3,4]. The European Commission is supporting research and development of microalgal biotechnology by allocating ~€40 million annually between 2007 and 2017 to microalgae-based ventures. For **photo- and mixotrophic** (see Glossary) microalgal cultivation, light is one of the most important growth parameters; it can come from natural (sun) or artificial (lamps) sources [4,5]. Although artificial light costs more than sunlight, it allows tight control of microalgal biochemistry and growth, increasing the reliability of industrial processes for the production of high-value biomolecules [4,5].

However, the competitiveness of any artificial light-driven microalgal production hinges on energy consumption. Decreased energy costs require improvements in photon harvesting by microalgae and the photon conversion efficiency of light sources. Better light energy usage by phototrophs can be achieved by tailoring species-specific emission spectra of artificial light sources [5,6]. Another strategy concerns not the light quality but light delivery. Instead of using continuous illumination, recent studies propose using flashing lights (Figure 1, Key Figure). Flashing light is **intermittent light** that can provide highly intense light flashes with a short duration (called the light 'flash period' or t_l) alternating with extended dark periods (t_d). One flash period followed by a dark period can be defined as a flashing cycle (t_c, in which $t_c = t_l + t_d$). The use of high light flash intensities (I_l) enables light to penetrate deeper into the culture and mitigate **light attenuation** [7–9] in photobioreactors, maintaining high **photosynthetic efficiency** in

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Key Figure

Simplified Diagram of a Microalgal Production Unit Using Flashing (Upper Panel) and Continuous Light (Bottom Panel) Emitted by Light-Emitting Diodes (LEDs)

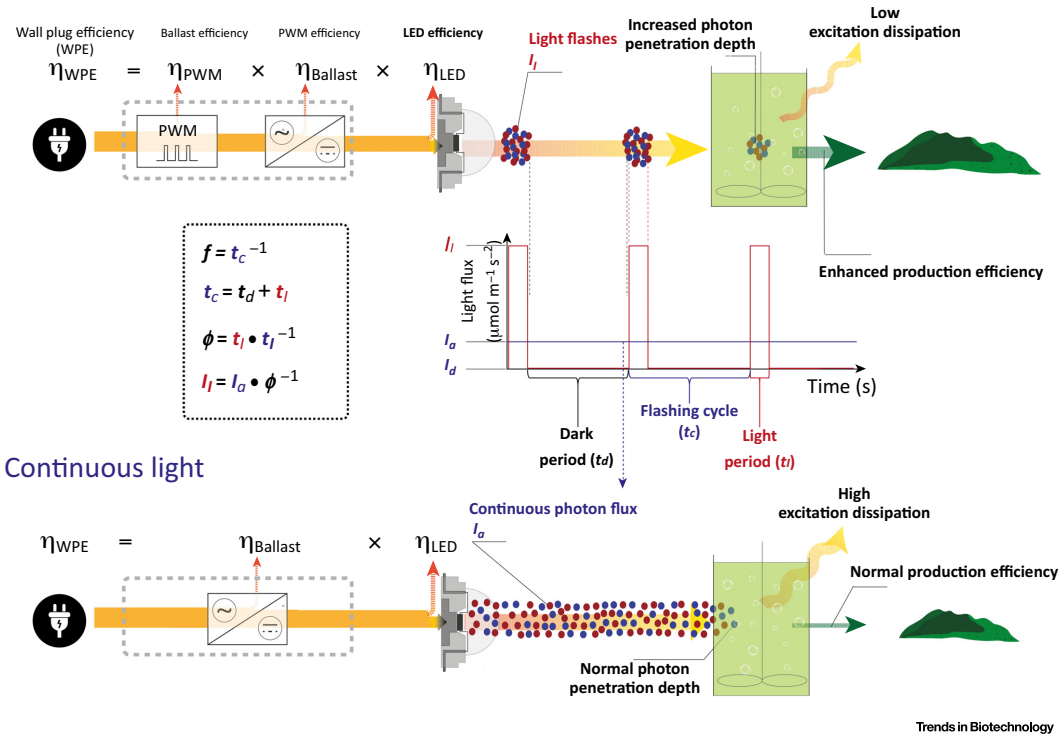

Figure 1. Flashing or pulsed light can increase light penetration depth and decrease excitation dissipation mechanisms, improving biomass productivity. A flashing light emitting system can transmit tailored wavebands that increase further photon penetration depth (e.g., green light for chlorophytes and Stramenopiles–Alveolata–Rhizaria species) or stimulate metabolic pathways and biochemical composition (e.g., blue and red light). The wall-plug efficiency (η_{WPE}) is the product of the efficiency factors of all devices between power source and light output, including the efficiencies of the ballast ($\eta_{Ballast}$), pulse-width modulator (η_{PWM}), and the LED (η_{LED}). Flashing light devices emit light flashes (t_l) and dark periods (t_d) in an approximately rectangular waveform. This waveform is commonly described by the flashing light frequency (f) and the duty cycle (ϕ). The frequency (in Hertz, Hz) is the number of light–dark intervals (flashing cycles, t_c) that occur per second (s^{-1}). The duty cycle is the ratio between the flash period and the whole flashing cycle. The light intensity (in μmol photons m^{-2} s^{-1}) during t_l is defined as flash intensity (I_l), while during t_d no light is emitted (e.g., I_d = 0 μmol photons m^{-2} s^{-1}). Under these conditions the time-averaged light intensity (I_a) during one flashing cycle can be expressed as $I_a = \phi \times I_l$ and is used to compare flashing with continuous light treatments. Moreover, the flash intensity and the duty cycle are inversely proportional at a given mean light intensity.

concentrated cultures [10,11]. To prevent photo-damage and inhibition of the phototroph under cultivation by over-intense light flashes, the repetition rate of the light–dark transition (i.e., flashing light frequency, f) and the relative proportion of the light flash period (i.e., the 'duty cycle', ϕ) within the flashing cycle should be adjusted to the biological reaction kinetics of photosynthetic processes and energy dissipation mechanisms (also often referred to as non-

photochemical quenching, NPQ). Nonetheless, well-engineered light sources are essential to emit efficiently flashing light regimes that are advantageous for phototrophic cultivation [4,12]. Balancing these factors, flashing light can result into higher growth performance per input energy than the same light energy supplied in a continuous way [4,12]. We discuss the techno-biological threshold for an efficient flashing light system in terms of (i) biological, (ii) physical, and (iii) technical factors that are crucial for applying this promising tool to microalgal cultivation.

Biological Boundaries

Microalgal cultures can display similar or higher photosynthetic rates under flashing light than under continuous light at the same mean light intensity. This is referred to as the 'flashing light effect' [13,14] or the 'light integration effect' [15] and occurs if the photosynthetic apparatus is working close to its full capacity (a biological factor). However, in cultures with light attenuation, the flashing light effect can additionally be achieved by enhanced light delivery into the culture (a physical factor), even though the photosynthetic apparatus is operating at rates that are far lower than its full capacity. In this section we define the flashing light conditions (e.g., frequency or duty cycle) at which the photosynthetic apparatus perceives a flashing light effect as a biological boundary. Generally, the flashing light effect refers to the response of a phototroph to mean light intensity (I_a) during the flashing cycle, and not to the instantaneous light intensity of the light (I_l) or dark (I_d) periods (Figure 1). When exposed to frequencies that are too low (e.g., f = 1–10 Hz) with duty cycles that are also too low (e.g., $\phi > 0.5$), no flashing light effect takes place and phototrophs quench excess energy during the light period, and might experience enhanced respiration during the dark period (e.g., post-illumination respiration) [13,15,16]. This results in less growth and biomass losses. Moreover, molecular responses to stress in the phototroph under cultivation can also be activated.

The biological boundary depends on the reaction kinetics of the energy dissipation mechanisms, energy storage, and the linear electron transfer chain (Box 1). Flashing light studies on single leaves of land plants or microalgal cultures with low light attenuation potential can identify the biological boundary, and may be described as a function of frequency (f) and duty cycle (ϕ), in other words $f_{(\phi,f)}$ [17,18]. For example, Jishi and colleagues [17] identified this flashing light effect for lettuce (*Lactuca sativa*). Interestingly, their model also fits the photosynthetic performance of microalgal cultures with low biomass concentrations (<0.1 g L^{-1}) or short light path lengths (<1–2 cm), and of land plants (e.g., tomato) under various flashing light conditions [19–27].

Excitation Dissipation and Energy Storage Efficiencies

Absorbed light energy is able to bring chlorophyll from its ground state (Chl) to a singlet excited state (^{1}Chl*). ^{1}Chl* can pass its excitation energy via resonance or excitation energy transfer to adjacent chlorophyll molecules in the light-harvesting complexes or the reaction centers of photosystem I or II (PSI or PSII). In the reaction centers, charge separation takes place and excitons can be photochemically quenched by provoking the transfer of electrons to the photosynthetic linear or cyclic electron transfer chains [28]. These photosynthetic pathways are essential for the production of ATP and reducing equivalents such as plastoquinol and NADPH [28]. If the reaction centers are 'closed', in other words if they are not able to process photon excess under high light conditions, ^{1}Chl* can dissipate absorbed energy as heat through excitation dissipation mechanisms or re-emit a photon (fluorescence) when falling back to its ground state (Chl). Both processes prevent the formation of triplet Chl (^{3}Chl*) which causes the production of reactive oxygen species (ROS) [28]. If, for example, the storage capacity for reducing equivalents cannot cope with the excess electrons under high light intensity, the likelihood of ROS accumulation increases. Such high ROS levels suppress protein synthesis, which is essential for repairing PSII upon photodamage [29]. To avoid excess ROS evolution during high light (flash) periods and maintain their metabolism during prolonged dark

Glossary

Intermittent light: includes flashing or pulsed light and fluctuating, flickering, or oscillating light. Light and dark periods of flashing or pulsed light conditions shift in an all-or-nothing rectangular waveform. Fluctuating or oscillating light is a fluent transition between high and low light periods, whereas instantaneous light intensities alter continuously over time, usually following a sinusoidal waveform. Sunflecks or cells moving from light to dark zones within a photobioreactor through mixing usually follow fluctuating light patterns.

Light attenuation: self-shading by microalgal cells is the most challenging bottleneck limiting the productivity and maximal achievable cell concentration in photobioreactors. Cells located at the periphery of a culture absorb most of the incoming light and may become photoinhibited, whereas cells at the inner layers remain in the dark and become photolimited. This results in high respiration and energy dissipation rates, causing inefficient photobioreactor use. The depth of the light penetration depends primarily on absorption by cells under cultivation, the incoming light intensity and wavelength, and cell morphology (e.g., cell size) and biochemistry (e.g., pigment contents).

Mehler and Mehler-like reactions: these control light-dependent O_2 consumption. Unlike the Mehler reaction, the Mehler-like reaction involves flavodiiron proteins to reduce O_2 without evolution of reactive oxygen species (ROS). Mehler-like reactions enable cyanobacteria, microalgae, and plants to cope efficiently with intermittent light regimes.

Minimal response times (t_{r_min}): the minimal response time of LEDs and transistors can be calculated by $t_{r\_min} = \phi \times f_c^{-1}$, where ϕ is the duty cycle and f_c is the flashing frequency at 'cut-off' obtained from a frequency response graph (i.e., Bode plot).

Photosynthetic efficiency: this refers to how much light (e.g., amount in photons or energy) is required by a phototroph to fixate CO_2 or produce O_2 through photosynthesis (e.g., μmol CO_2 or O_2 per μmol of photons). The effects

periods, phototrophs employ different energy quenching and storage strategies under flashing light with a low duty cycle (e.g., ϕ <0.1; Figure 2) [14,16,30,31]. Usually, alternation between light and dark periods longer than seconds, minutes, or hours (implying frequencies <1 Hz) are referred to light that is supplied intermittently, discontinuously, or through light/dark cycles or photoperiods. For the sake of convenience, however, the term 'flashing light' and associated parameters will be used in all timescales.

In this context, mechanisms of short-term energy storage (fs–ps timescale) follow the laws of quantum dynamics, and energy transport takes place via quantum coherence. The excitation energy delivered by light flashes with a duration of fs–ps can be stored in pigment cofactors (e.g., chlorophylls, carotenoids, or phylloquinones) as excitons or through inter-protein hopping within the light-harvesting complexes [32]. If reaction time permits, energy may be stored in reaction center II. In this timescale, excess energy may be dissipated through ultrafast reacting quenchers (e.g., chlorophyll *a*) [32,33], resulting in pigment internal thermal decay or fluorescence.

Medium-term energy storage (ns–μs timescale) may take place via components and products of the non-cyclic photosynthetic electron transfer chain. Examples are the plastoquinone bound to PSII (Q_a^-), plastoquinol (PQH_2) in the plastoquinone (PQ) pool [34], protons in the stroma coming from the water-splitting reaction catalyzed by the water oxidizing complex, and ATP produced by the ATP synthase in the thylakoid [35]. However, if the previous storage mechanisms are unable to handle excess energy, other non-photochemical quenching reactions and biomolecules seem to play a protective role: for example **Mehler-like reactions**, the proton gradient regulator PGR5, the ferredoxin-plastoquinone reductase PGRL1, the serine/threonine-protein kinase STN7, and several flavodiiron proteins [8,36–38].

For long-term energy storage (ms–s timescale), phototrophs produce reduced equivalents (e.g., NADPH) or 'high-energy' chemical bonds via ATP-dependent nitrogen and sulfur assimilation as well as carbon fixation. The last process yields Calvin–Benson cycle intermediates containing ATP-dependent high-energy bonds, such as bisphosphoglycerate or triose phosphate [30]. At this timescale, excess energy can be quenched via reoxidation of the PQ pool through the quinol terminal oxidase, phosphorescence, or through the initiation of diadinoxanthin–diatoxanthin, violaxanthin–astaxanthin–zeaxanthin, or lutein epoxide cycles [39–41].

Energy storage for an even longer term is possible. Biochemical processes at timescales of minutes and hours, such as the accumulation of non-structural low molecular weight sugars, starch, or amino acids (e.g., glutamine as the first amino acid resulting from nitrogen assimilation) can be used to store energy [42]. Under these conditions, excess energy can be quenched through the same photoprotective pigment cycles as under ms–s conditions, but also through high-energy state (qE) quenching and photoinactivation of PSII (here referred to as qI) [33,41,43]. To decrease excess energy that phototrophs receive under longlasting light periods, reversible phosphorylation of the light-harvesting complex II and a decrease in the light-harvesting antenna size might occur [37,39].

Generally, the ratio between non-photochemically and photochemically quenched energy, and the probability of damaging the photosystems by ROS evolution, as a result of a failure of the excitation dissipation mechanisms in place, increases with light flash period duration, causing a drop in photosynthetic efficiency. Under frequencies and duty cycles that are too low and too short, respectively, for obtaining a biological flashing light effect, phototrophs use more-complex and energy-demanding excitation dissipation mechanisms during the light period

of flashing light on the photosynthetic efficiency of single cells or chloroplasts and whole cultures should be distinct. Dilute cultures with narrow light paths and negligible light attenuation are usually used to identify the effects of flashing light on single cells or chloroplasts (biological boundary). However, flashing light was mostly found to improve photosynthetic efficiency of whole microalgal cultures with high light attenuation (e.g., highly concentrated cultures).

Photo- and mixotrophy: phototrophic organisms use light as an energy source to fix inorganic carbon dioxide in organic compounds. Heterotrophic organisms obtain energy and carbon from organic sources (glucose or acetate). A few mixotrophic microalgae are able to obtain energy and carbon skeletons by means of photosynthesis, active predation, endocytosis, and membrane-bound transport systems. Some others are even able to steal chloroplasts from other microalgae using a mechanism known as myzocytosis.

Pulse-width modulation: a tool used to control the power supply (e.g., dimming) of electrical devices such as LEDs. It generates a pulse wave signal (i.e., rectangular pulse wave) with an asymmetrical shape (i.e., the duration of the on–off cycle) described by the duty cycle.

Box 1. Kinetics of the Linear Electron Transfer Chain (LET)

Photons emitted from a light source are absorbed through light-harvesting pigment complexes within femtoseconds. About 300–500 ps are necessary to transfer shared excited energy states (excitons) through inter-protein hopping and magnetic resonance to the reaction centers of PSII (reaction center II or P680), causing the excitation of an electron. The reaction center requires two electrons for reduction and 'closure' (i.e., P680*; Figure I) [32]. Once the reaction center is in the 'closed' state, further excess photon energy cannot be transferred to the reaction center II and is released through energy dissipation mechanisms. The low redox state of the P680* reduces the primary electron acceptor pheophytin within 3–8 ps, becoming oxidized ($P680^+$). The electrons from pheophytin are transferred to the primary (Q_a) and secondary (Q_b) acceptor sites within ~200–500 ps and 700–1200 μs, respectively. Upon Q_b reduction, this site acquires protons from the stroma, forming plastoquinol. In turn, plastoquinol diffuses towards the PQ pool in the thylakoid membrane upon exchange with one PQ molecule, which binds to the Q_b site. The PQ pool serves as an energy store. The energy is retrieved upon the oxidation of plastoquinol by the cytochrome b_6f complex via the q-cycle [77].

The high redox potential of $P680^+$ initiates an electron transfer from the water-oxidizing complex through the intermediate electron carrier tyrosine, which reduces $P680^+$ in a succession of steps (S0–S3). Full oxidation of two water molecules and the release of four electrons takes place in about 1–2 ms [35]. As P680 is formed, the reaction center II reopens and the subsequent exciton capture takes place.

The slowest (~3–5 ms) and thus limiting step in the linear electron transfer chain is the oxidation of plastoquinol by cytochrome b_6f. Two protons are released into the thylakoid lumen and electrons are transferred towards plastocyanin. Plastocyanin transfers electrons towards PSI within 150–550 μs. In PSI, electrons are passed to the electron donor P700 (reaction center I), forming P700* through photon energy delivered by the light-harvesting complex I within femtoseconds. Electrons are passed to the electron acceptors A_o, A_1, and the 4Fe–4S iron–sulfur centers Fx, Fa, and Fb within picoseconds, reducing the final electron acceptor, ferredoxin. Because of these short turnover times, the reactions in P700 are considered to be a spontaneous reaction [32,34]. Ferredoxin can donate electrons to ferredoxin-$NADP^+$ reductase to form NADPH, completing the LET. The cyclic electron transfer chain is activated to produce additional ATP and NAPDH required for carbon assimilation via the Calvin–Benson cycle.

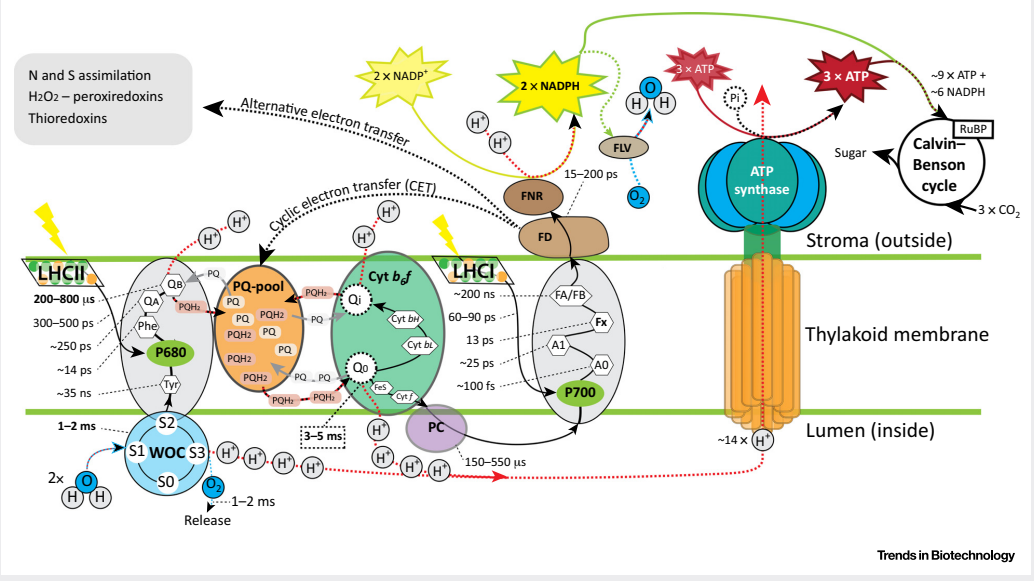

Figure I. Simplified Diagram of the Major Kinetics of Electron Transfers in Phototrophs, Showing the Linear Electron Transfer Chain (LET) and Other Alternative Pathways. Reaction times and pathways are summarized from [35,39]. Note that the stoichiometric values for H^+, ATP, and NADPH are variable. To balance electron flow under fluctuating light regimes, phototrophs use, in a species-dependent manner, different Flv proteins to reduce oxygen to water at the expense of NADPH or electrons from the photosystems [74]. Abbreviations: A_1, phylloquinone-based electron acceptor; A_o, chlorophyll based electron acceptor; Cytb_6f, cytochrome b_6f complex; Cyt $b_{L/H}$, *f*, b-type hemes cytochrome $b_{L/H}$, *f*; FA/FB and Fx, electron acceptor 4Fe–4S iron–sulfur centers; FeS, Rieske iron–sulfur protein; FD, ferredoxin; Flv, flavodiiron protein; FNR, ferredoxin-$NADP^+$ reductase; LHC, light-harvesting complex; P680, photosystem II; P700, photosystem I; PC, plastocyanin; Phe, pheophytin; Pi, inorganic phosphorus; PQ, plastoquinone; PQH_2, plastoquinol; Q_A, primary acceptor site; Q_B, secondary acceptor site; Q_i, quinone reductase; Q_o, quinol oxidase; RuBP, ribulose-1,5-bisphosphate; Tyr, tyrosine; WOC, water-oxidizing complex.

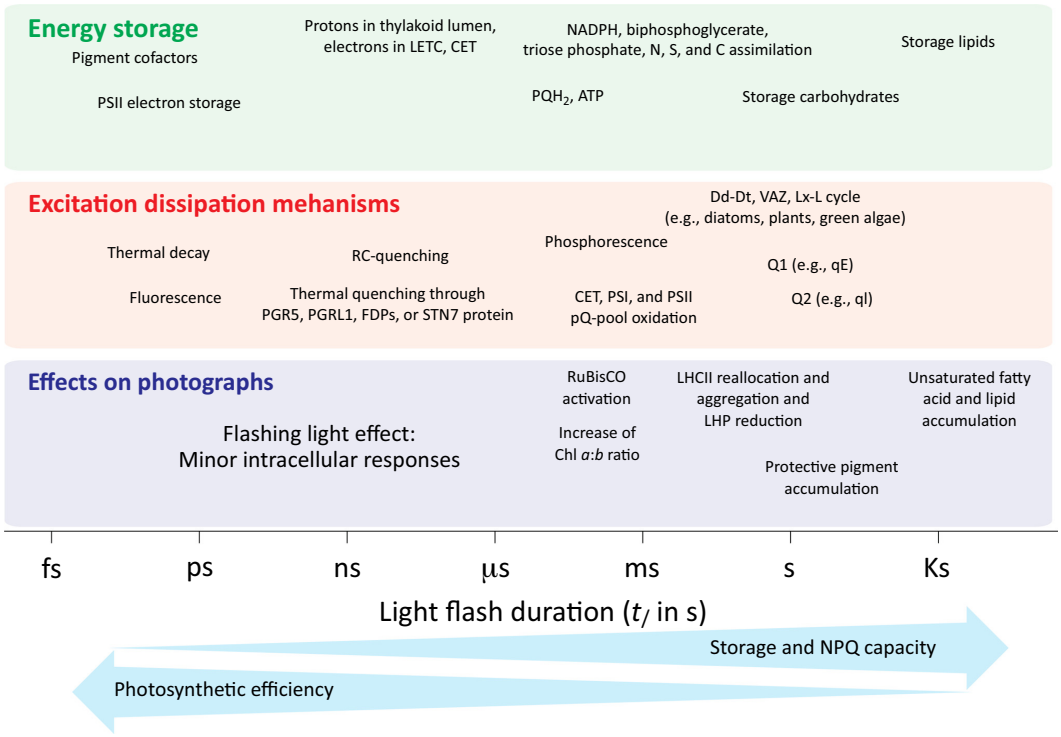

Figure 2. Response Timescales of Phototrophs Exposed to Different Flash Period Durations (t_l) with High Flash Intensity (I_l) That Arise if the Flashing Light Is Composed of a Short Constant Duty Cycle (e.g., $\phi < 0.1$) and a Saturating Mean Light Intensity (I_a). A given flash period duration is inversely proportional to the flashing light frequency (f). Timescales of events were obtained from results summarized in Tables 1,S1 and elsewhere [33,34,37]. Bar length represents the approximate timescale of the initiation of a given event. Abbreviations: CET, cyclic electron transfer; Chl, chlorophyll; Dd-Dt, diadinoxanthin–diatoxanthin; FDPs, flavodiiron proteins; LETC, linear electron transfer chain; LHC, light-harvesting complex; LHP, light-harvesting pigment; Lx-L, lutein epoxide; NPQ, non-photochemical quenching; PGR5, proton gradient regulator 5; PGRL1, a ferredoxin-plastoquinone reductase that is apparently involved in CET in chlorophytes; PQ, plastoquinone; PQH_2, plastoquinol; PS, photosystem; Q1/2, quenching sites 1 and 2; qE, energy state quenching; qI, photoinactivation of photosystem II; RC, reaction center; RuBisCO, ribulose-1,5-bisphosphate-carboxylase/oxygenase; STN7, a serine/threonine-protein kinase involved in the adaptation to changing light conditions; VAZ, violaxanthin–astaxanthin–zeaxanthin.

(e.g., photoprotective pigment synthesis or high-energy state quenching [33]), and respiration rates exceed photosynthetic rates during the extended dark period [15,44,45]. Both situations will ultimately decrease or restrict net photosynthetic efficiency [27] and alter the biochemical profile and appearance of microalgal and cyanobacterial cells. Changes include cell size, pigment composition, intracellular ultrastructure, the expression of protective proteins (e.g., PGR5 or STN7), the ratio between PSI and PSII [8], light-harvesting antenna size, ribulose-1,5-bisphosphate-carboxylase/oxygenase (RuBisCO) activity, and sugar and starch contents [8,11,30,46–51]. These changes are typical for responses of microalgae to intense light [49,50], and thus can be used as indicators if the frequency and duty cycle are inadequate for a phototroph to experience the biological flashing light effect. Conversely, if phototrophs are exposed to increasing frequencies (e.g., $f > 10$ Hz; $\phi = \sim 0.1–0.5$), these changes become less obvious [46–48,52,53] because a phototroph buffers and quenches photoenergy delivered

during the light period with a similar efficiency to that under continuous light. Nevertheless, lower intracellular chlorophyll *a* and carotenoid contents are probably not good indicators for the flashing light effect because lower amounts of these pigments occur in several species under a wide range of flashing light conditions (e.g., $f = 0.1–100$ Hz; Table 1) [44,46–49].

Table 1. Impact of Flashing Light with Different Duty Cycles and Frequencies on Microalgal Composition[a,b]

Microalga	Frequencies (f)	Duty cycles (ϕ)	Outcome	Refs
Chlamydomonas reinhardtii	1–100 Hz	0.5	Absorption spectra unaffected by flashing light, no obvious shift in carotenoid:chlorophyll ratio in absorption spectra.	[20,21]
Chlamydomonas reinhardtii	0.5–5 Hz	0.5	Fatty acid profile and total lipids were mostly unaffected by flashing light.	[75]
Chlamydomonas reinhardtii	0.00138–1 Hz	0.5	Decreasing chlorophyll *a* content with increasing frequency (I_a = 220 μmol photons m^{-2} s^{-1}). The lowest amount of chlorophyll *a,b* and carotenoids was under f = 1 Hz.	[44]
Chlorella kessleri	5Hz–37 kHz	0.5	Higher intracellular chlorophyll concentrations under flashing light as compared to continuous light.	[11]
Chlorella pyrenoidosa	2.5–25 kHz	0.0125–0.125	Immediate sugar accumulation when exposed to saturating light flashes for 18 h. Dark periods lasting only 6 h led in turn to an accumulation of nucleic acids and complete consumption of accumulated sugars. Protein and chlorophyll levels unaffected.	[24]
Dunaliella salina	0.017–5 Hz	0.4–0.66	Chlorophyll *a* content was usually lower under flashing light (I_a = 400 μmol photons m^{-2} s^{-1}) conditions as compared to continuous light. A f = 5 Hz gave similar results to continuous light. *D. salina* exposed to flashing light conditions always displayed a lower total lipid content.	[50]
Haematococcus pluvialis	25–200 Hz	0.17, 0.33, 0.67	Final astaxanthin and biomass concentrations in the medium were higher under flashing light as compared to continuous light. With increasing duty cycle but the same frequency, the final volumetric astaxanthin concentration rose. The use of flashing light lowered energy consumption for astaxanthin production by up to 70%.	[76]
Isochrysis galbana	10 kHz	0.5	No effects on total lipid content in *I. galbana* or cell weight. Fatty acid profile was similar under flashing and continuous light.	[52]
Nannochloropsis oceanica CY2	7,8,9 Hz	0.5	No significant differences in EPA content between cells under flashing and continuous light.	[53]
Nannochloropsis salina	1–30 Hz	0.1, 0.33	Flashing light had no effect on total lipid content and usually caused reduced accumulation of chlorophyll *a* and carotenoid:chlorophyll ratios (except f = 10 Hz, ϕ = 0.33).	[48]
Porphyridium purpureum	0.17–100 Hz	0.17, 0.5	A frequency of 0.17 Hz increased the intracellular phycoerythrin and chlorophyll *a* content as compared to either continuous light or 25 Hz and ϕ = 0.33. Bound and free polysaccharides were affected marginally. Production rates of phycoerythrin and free polysaccharides were highest under f = 100 Hz, ϕ = 0.5 (I_l = 540 μmol photons m^{-2} s^{-1}).	[49]
Scenedesmus bicellularis	~100 Hz	0.5	Long-term exposure to flashing light did not affect total lipids, proteins, carbohydrates, fatty acids, or amino acids. However, flashing light slightly lowered chlorophyll *a* and *b* levels, increased chlorophyll *a/b* ratios, decreased carotenoid content, and increased the carotenoid/chlorophyll *a* ratio. RuBisCO initial activity (not activated) and RuBisCO total activity (activated) were significant higher only under flashing light at a moderate irradiance of I_a = 175 μmol photons m^{-2} s^{-1}, whereas low (I_a = 87.5 μmol photons m^{-2} s^{-1}) and high (I_a = 350 μmol photons m^{-2} s^{-1}) mean light intensities had no effect.	[47]
Scenedesmus obliquus	5, 10, 15 Hz	0.5	Carotenoid:chlorophyll ratio and chlorophyll *a* content in cells were lower under flashing light. Carbohydrate, lipid, and protein contents were unaffected.	[46]

[a]Refer to Table S1 for a detailed overview of relevant flashing light studies on microalgae, cyanobacteria, and plants.
[b]Abbreviations: EPA, eicosapentaenoic acid; I_a, mean light intensity during a flashing cycle; I_l, light flash intensity; RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase.

Limits of Flashing Light on the Electron Transfer Chain

Emerson and Arnold [54] demonstrated that a short light period with an adequate flash intensity can excite all 'open' reaction centers, whereas a sufficiently long dark period allows all reaction centers to 'reopen' and harvest most of the incoming photons of the next light flash. A later study by Radmer and Kok [55] quantified that a light-harvesting complex containing 400 chlorophyll molecules harvests ~2000 electrons per second under full sunlight, whereas the subsequent carbon fixation reactions are able to process only 100–200 electrons per second. They showed that the photosynthetic apparatus could only use a small portion of incident light under continuous light, while, most of the time, reaction centers are closed and light is non-photochemically quenched.

Recent findings about excitation dissipation mechanisms and the quenching role of the PQ pool may allow other approaches to determine the optimal flashing light settings required for photosynthesis. It has been suggested that the species-dependent storage capacity of the PQ pool for plastoquinol may define the threshold frequency, duty cycle, and the required mean light intensity beyond which the flashing light effect occurs [26,56]. Vejrazka and colleagues [21] and Hüner and colleagues [34] pointed out that, if excess plastoquinol is generated, the plastoquinol:plastoquinone (PQH_2:PQ) ratio becomes too high, leading to over-reduction of the PQ pool, which will prevent the reaction center II from reopening [34]. Such over-reduction occurs if transfer rates of photonic energy exceed the kinetics of its use by metabolic pathways that promote growth, including those involved in nitrogen, sulfur, and carbon utilization. Detrimentally, excess energy can lead to photoinhibition and photo-oxidative damage due to ROS evolution. Phototrophs can dissipate this energy by, for example, (i) activating the cyclic electron flow, (ii) phosphorylating and migrating the light-harvesting complex II towards PSI to reinforce the cyclic electron transfer used to oxidize the PQ pool, or (iii) activating the xanthophyll cycle [39]. However, to avoid photoprotective mechanisms and thus inefficient photonic energy usage, light supply should take place in balance with the reaction kinetics of the linear electron transfer chain. Interestingly, a light flash lasting picoseconds is already sufficiently long to excite and close the PSII reaction centers [26]. To restore the ground state of PSII, and thus to reopen the reaction center II, requires the transfer of this charge from the PSII to the PQ pool in the form of plastoquinol [57] and the reduction of PSII through the water-oxidizing complex. To avoid excess of plastoquinol reducing equivalents in the PQ pool, the reduction rate of PQ to plastoquinol at the Q_b site of PSII should be similar to the plastoquinol oxidation rate at the Q_o site of the cytochrome b_6f complex. However, an imbalance easily arises because the oxidation of one plastoquinol takes longer (~3–5 ms) than does reducing PSII by the water-oxidizing complex (~1–3 ms). To mitigate such imbalances in the linear electron transfer chain, flashing light may be tailored to a flash period duration of a few hundred picoseconds to reduce efficiently reaction center II without triggering excitation dissipation mechanisms, and a dark period of 3–5 ms to allow the timely oxidation of plastoquinol, avoiding over-reduction of the PQ pool.

However, these kinetics would correspond to a duty cycle of only $\phi = \sim 10^{-8}$ and a frequency of f = 300–500 Hz. Such settings require low switching times, which are problematic to implement with currently available technologies (Box 2). Indeed, frequencies higher than 300 Hz usually resulted in a flashing light effect in most phototrophs if the mean light intensity was near saturation (e.g., $I_a \geq 100$ μmol photos s^{-1} m^{-2}; Table S1 in the supplemental information online). However, decreasing the mean light intensity towards sub-saturating levels appears to require higher frequencies to obtain the flashing light effect [26,58]. More specifically, Martín-Girela and coworkers [58] found a CO_2 fixation efficiency of 6.2 photons per fixed CO_2 molecule, which was beyond theoretical limits (~8 photons CO_2^{-1}) at a frequency of 10 000 Hz ($\phi = 0.05$) with a mean light intensity that was only 5% of the photosaturating intensity.

Box 2. Technical Limits of Flashing Light Sources

The efficiency of a flashing light system depends on working and switching losses at transistors, built-in pulse-width modulators, controlling units, and LEDs that interact differently with applied current, frequency, and duty cycle. For example, work losses in transistor switches increase if duty cycle and currents are high, but they are frequency-independent. Switching losses increase with frequency, although they are current-independent [73]. Photon extraction potential from LEDs is higher if forward current is increased (i.e., LED overloading), and if light and dark periods are sufficiently short or long, respectively, to allow sufficient heat dissipation from the LED-chip (Figure I). In this case, frequency and duty cycle are interdependent.

Overloading is a valuable option for flashing light applications, permitting the operation of an LED under higher currents that exceed nominal levels (e.g., by increasing the supplied voltage), resulting in maximal photon flux during the duty cycle and heat dissipation during the dark period [11,24]. Through overloading, the LED operates at current densities beyond which the 'droop effect' occurs. As result, the photon conversion efficiency drops with increasing forward currents, and maximal photon extraction cannot exceed a given threshold [78]. During overloading and a shortened duty cycle, the maximal extractable mean light intensity (I_a) per LED decreases, but the maximal applicable instantaneous forward current and extractable flash intensity increases.

Another parameter is the LED **minimal response time** (t_{r_min}), which is the major cause for electrical losses when emitting flashing light. The depletion region as the central internal element of any LED chip creates capacitances that limit the response of single-color LEDs to a few nanoseconds (t_{r_min} = ~1–50 ns). Note that organic LEDs display high capacitances while phosphor-converted LEDs have long (electro)luminescence decay times, and this restricts t_{r_min} to approximately ≥ 1 μs [79–81]. Standard laser diodes display very low capacitance, allowing t_{r_min} < 500 ps [82]. Overloading and chip size increases these capacitances and thus response times, limiting maximal adjustable frequencies or duty cycles. Lowering the response times of (organic) LEDs is an active field of research aimed at facilitating efficient visible light communication or screens [80,82,83].

Broadband flashing light research at nanosecond scales can use widely available signal generators connected to high power single-color LEDs or laser diodes as a cheap solution (e.g., <€2000). For larger-scale systems with high light output, a more robust and cheaper system may be used, based on a slower-responding open source system (≥1 μs) consisting of an Arduino microcontroller coupled with standard LED luminaries (for examples see [50,84]).

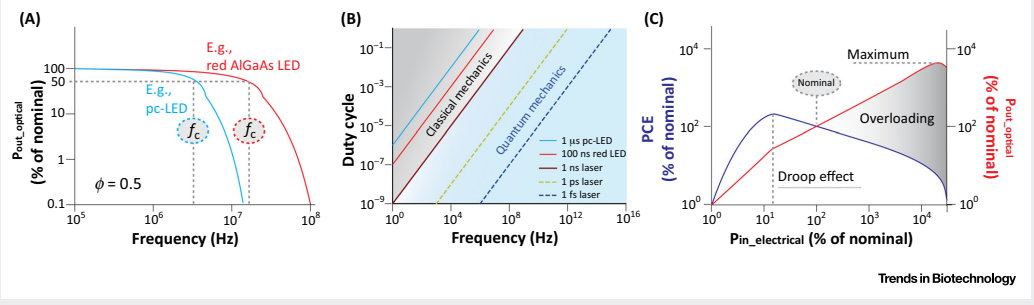

Figure I. The Efficiency of LEDs or Laser Diodes under Pulsed Power Supply Can Be Calculated from Frequency Response Graphs (Bode Plots) (A). The cut-off frequency (f_c) refers to the maximal adjustable frequency with maximal 50% power losses (commonly referred to as the 3 dB point). From f_c, response times and possible adjustable flashing light regimes can be calculated for different light sources (B). If switching regimes are below 1 ns, a shift from the laws of classical to quantum mechanics occurs. Overloading (C) is achieved if the input power ($P_{in\_electrical}$) increases due to higher forward currents that exceed nominal levels. Nominal conditions are obtained when LEDs operate under continuous power supply and rated (or nominal) currents at a given temperature. Under a nominal power input ($P_{in\_electrical}$), an LED achieves its nominal light output ($P_{out\_optical}$) and nominal efficiency (e.g., photon conversion efficiency; PCE = $P_{out\_optical}/P_{in\_electrical}$) as defined by the manufacturer. LED light output comes at the cost of efficiency after passing a critical current density beyond which the droop effect occurs (dashed line), and usually reaches a peak (maximum) with subsequent decline. All values are examples and may differ among diodes. Abbreviations: pc-LED, phosphor-converted LED; AlGaAs LED, gallium–aluminium–arsenide LED.

On the other hand, it remains to be seen how phototrophs respond if they are exposed to extremely low duty cycles (e.g., $\phi < 10^{-8}$), with a flash intensity ~10^8-fold higher than the mean light intensity, which corresponds also to a photon penetration depth eightfold higher than that of continuous lighting [7]. Under such a timescale, dissipation of excess energy may only take place via fluorescence or thermal decay, and other more complex and energy-demanding quenching mechanisms (e.g., mediated by PGR5, PGRL1, flavodiiron proteins, or STN7) cannot be activated in time [37,59]. This may lead to a more efficient light utilization and higher photosynthetic efficiencies. On the other hand, these conditions could also increase flash intensities above a threshold that would instantly cause photodamage. If so, an under-saturating mean light intensity could be sufficient to achieve the same or even enhanced

photosynthetic rates compared to continuous lighting with a saturating light intensity, leading to lower power consumption of artificial lighting. Notably, flash intensities that are inhibitory if emitted continuously do not inhibit the phototroph if the frequency is high and the duty cycle is sufficiently short (e.g., $f > 1$ kHz, $\phi < 0.1$) for a given mean light intensity [24–26,60–62]. For example, Tennessen *et al.* [26] exposed tomato leaves to photoinhibitory flash intensities ($I_f = 5000$ μmol s^{-1} m^{-2}) at a short duty cycle ($\phi = 0.01$) and a high frequency ($f = 5000$ Hz) without impairing photosynthesis. Nevertheless, Ley and Mauzerall [63] found that flash intensities higher than 22 000–37 000 μmol photons m^{-2} s^{-1} (e.g., 10^{16} photons cm^{-2} supplied during light periods of 450–750 ns) can indeed cause a decline of oxygen evolution rates in *Chlorella vulgaris* cultures ($t_d = 2$ s). These findings indicate that mean light intensity, flash intensity, frequency, and duty cycle are interdependent and must be well balanced to reap the benefits of artificial flashing light-based phototrophic cultivation.

Physical Boundaries

The most important physical factor of flashing light is the potential to mitigate light attenuation and increase light delivery in concentrated microalgal cultures [13,49,61,64,65]. Current efforts to enhance light delivery include intensive mixing, light path minimization, antenna size reduction, waveband tailoring, or the inclusion of fibers and nanoparticles as waveguides into the photobioreactor [9,66–69]. In addition to these approaches, high light intensities can increase photon penetration depth in suspensions as defined by the Beer–Lambert law [7]. This law describes a linear increase of light penetration depth into microalgal cultures with exponentially rising light intensity, although the effects of fluorescence or light scattering by different algae are not considered.

Light intensities that are too high cause photoinhibition of microalgae at the periphery of the photobioreactor, an effect that is mitigated by increasing mixing rates. Higher mixing rates minimize the retention time of cells in the high light zone near the walls and in the dark zones in the middle of the reactor. These high mixing rates improve illumination and can provide light–dark cycles that are sufficiently fast to obtain the flashing light effect [13,14]. Particularly high productive cultivation systems benefit from high culture concentrations and light intensities [70]. However, these conditions require extremely high mixing velocities, resulting in high energy consumption [44,62,71], shearing, cavitation, and pressure changes that impair the physiology and viability of microalgal cells [70]. Alternatively, a light source can directly emit flashing light, and this allows the generation of intense light flashes at frequencies and low duty cycles that do not occur in nature or in any culture vessel merely by adjusting the mixing velocity of the growth medium.

Because mitigating light attenuation is one of the main arguments for flashing light-induced growth enhancement [13,49,61,64,65], production systems that operate at high cell concentrations or culturing vessels with long light path lengths are promising targets for flashing light-related power savings. Although this trend has only been observed in a few studies [11,61,70], the true potential of mitigating light attenuation in dense microalgal cultures remains uncertain, particularly at extremely high light flash intensities (e.g., $I_f > 10\,000$ μmol photons m^{-2} s^{-1}), delivered at low duty cycles (e.g., $\phi < 0.01$) and high frequencies (e.g., $f > 1$ kHz).

Technical Boundaries

In artificial light-based microalgal production, light with low and high light periods (e.g., flickering or fluctuating light) is naturally emitted by common gas discharge lamps, whereas flashing light is generated when the light intensity of LEDs is controlled via **pulse-width modulation** (e.g., Figure 1). The intensity of light emitted by fluorescent lamps and mercury or sodium vapor lamps changes between maximum and minimum values (often referred as 'flickering light') at a ballast- and grid-dependent frequency [47]. For example, fluorescent lamps driven by a

conventional magnetic or electronic ballast emit flickering light at frequencies of 100–120 Hz and 40–120 kHz, respectively. On the other hand, induction lamps operate at frequencies ranging from hundreds of kHz to tens of MHz [72]. Therefore, it becomes clear that neither induction nor gas discharge lamps should be used as 'non-flashing' controls in any flashing light study. In addition, gas discharge lamps are inefficient in terms of photon conversion efficiency if operated at low duty cycles, and may be unsuitable for customized flashing light modulation. Alternatively, amplitude- or pulse width-modulated LEDs can efficiently emit continuous and flashing light, respectively. However, commonly available pulse width-modulated dimmers generate flashing light only between frequencies of 150 and 300 Hz, which may be not sufficient to obtain a flashing light effect if dimmed, although higher frequencies and lower switching times are possible (Box 2).

For microalgal production, a promising flashing LED device may operate at a (sub)saturating mean light intensity, which requires a light flash intensity that increases in inverse proportion to the duty cycle. The emission of high light flash intensities is possible if the stock densities of LEDs in a luminary array are increased, which has additional costs. Alternatively, the number of photons emitted per light emitter can be enhanced under flashing light if the forward current to an LED is increased far beyond the nominal currents used under continuous operating conditions. This so-called 'overloading' demands precise switching regimes to extract the highest number of photons with the highest efficiency possible. Considering all losses between power source and light emission of a flashing light system (referred to as wall plug efficiency), most discriminative parameters include (i) response time and photon conversion efficiency of the LED, (ii) operating frequency and duty cycle, and (iii) the efficiency factor of the electronic ballast and pulse-width modulation unit. Generally, the wall plug efficiency of a flashing lighting system decreases with increasing frequency, decreasing duty cycle, and increasing forward current because of working and switching losses at transistors and LEDs [73]. The efficiency drop can be damped if transistors and LEDs display low response times as well as low working and switching losses under the flashing light condition and currents employed. A joint effort between physicists and biologists will thus be necessary to develop efficient flashing light systems that enhance energy use in artificial light-based microalgal production.

Concluding Remarks and Future Perspectives

Using flashing lights is a promising strategy to supply photonic energy to phototrophic organisms, thereby increasing biomass productivities and reducing power consumption in artificial light-based production systems. Flashing light can also be applied to established methods employed to improve the photosynthetic performance of microalgal cultures, such as mixing, light guides, or waveband tailoring. The optimal settings of a flashing light regime should correspond to a frequency that is sufficiently high to obtain the same or higher photosynthetic efficiencies than those under continuous light (e.g., $f > 300$–500 Hz; biological factor) at the shortest possible duty cycle to obtain the highest possible photon penetration depth (physical factor), but both within the range of adequate power consumption (technical factor). So far, most studies have tested flashing light conditions with low frequencies ($f < 100$ Hz) and relatively high duty cycles ($\phi > 0.1$) to mimic conditions that are present in mixed microalgal cultures. However, data beyond these conditions will be of particular interest for artificial light-based microalgal production. Mainstream flashing light research and industrial application will benefit specifically from inexpensive and technically mature LED modules as light sources.

Nevertheless, current LED technology cannot modulate flashing light in response timescales close to the boundaries imposed by the biological responses, such as light-harvesting events within the range of femto- to picoseconds. A possible solution to this limitation is the use of faster-responding laser diodes (see Outstanding Questions) which could replace common LEDs in cutting-edge research, as well as in future industrial production facilities. Cultures

Outstanding Questions

How do concentrated microalgae cultures respond to frequencies >100 Hz and duty cycles <0.1?

Flashing light can induce pigments, fatty acids, and possibly other biomolecules in microalgae. What are the best flashing light settings to induce a given target biomolecule in microalgae?

Do phototrophs downregulate energy dissipation mechanisms with decreasing duty cycles if the frequency is ≥300 Hz?

How does mixing quantitatively affect the actual supplied flashing light conditions to which an algal cell is exposed?

Does flashing light with light flashes lasting only femto- to picoseconds and extended dark periods of 3–5 ms improve photosynthetic efficiencies in light harvesting?

Flashing light supplies light in an approximately square waveform to a microalgal culture. Light and dark periods with the kinetics of photosynthesis (e.g., quantum coherence states, electron states at the LETC) or travel velocities of cells in a mixed culture should be coordinated. What is the effect and potential of coordinating flashing light with photosynthetic energy transfer rates and the physiology of microalgal cultures?

exposed to sub-nanosecond light flashes might obey the laws of quantum mechanics, which can result in unforeseeable effects on photosynthesis and growth of phototrophs. Research on charge transfer on quantum level, as for example implemented by the EU project H2020-MSCA-QuantumPhotosynth, may shed new light on the limits of photosynthesis and more efficient photon utilization by microalgae.

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Supplemental Information

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Paper IV

Manuscript style adapted to journal guidelines of Bioresource Technology.

Flashing light does not improve microalgal growth

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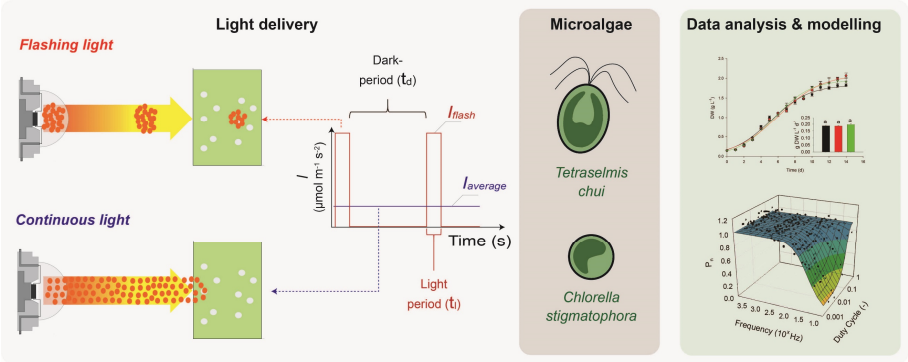

Highlights:

- Flashing light did not improve microalgal growth compared to continuous light.
- Flashing light effect obtained at maximal 500 Hz (tested duty cycles: 0.001-0.7).
- Frequencies <200-500 Hz inhibited microalgal growth.
- Respiration exceeded photosynthetic rates in dense cultures exposed to <8 Hz.
- Flashing light at low light intensities inhibited less than at high intensities.

Abstract

Self-shading of cells inside a photobioreactor is the major bottleneck in microalgal production. Flashing light was previously proposed as a promising strategy to mitigate this light attenuation, as high intensity light flashes penetrate deep into a culture, promoting photosynthesis. Here, *Tetraselmis chui* and *Chlorella stigmatophora* were exposed to flashing light using different frequencies (0.01 Hz-1 MHz), duty cycles (0.001-0.7) and light intensities (50-1000 µmol s^{-1} m^{-2}). Compared to continuous light, *T. chui* cultures revealed no improvement of growth or photosynthetic oxygen evolution under flashing light at frequencies >500 Hz regardless to the adjusted duty cycle, light intensity or culture concentrations. Also, the combination of flashing- and continuous light did not benefit photosynthetic performance of concentrated *T. chui* cultures. Lastly, batch cultures of *C. stigmatophora* and *T. chui* showed no productivity improvement under flashing light (*f*= 40, 400 Contrary to previous studies, we conclude no benefit of flashing light for microalgal growth.

Key words: Pulsed light, *Tetraselmis*, *Chlorella*, Respiration, Photosynthesis

1. Introduction

Research and development on microalgal biotechnology dates back to the 19th century, and the first commercial cultivation was reported in the 1960s (Milledge 2011). Since then, technologies have evolved and innovative production systems such as tubular or flat panel photobioreactors (PBRs) have been employed to improve the biomass throughput per area. Nevertheless, the limitation of any PBR is the inefficiency in delivering photons at optimum wavelengths and quantities to drive photosynthesis in all microalgae cells within a culture (de Mooij et al. 2016). The cells at the periphery of the PBR prevent profound penetration of light into the culture, limiting the photosynthetic efficiency of the whole culture and, eventually, its productivity (Abu-Ghosh et al. 2016).

To improve the delivery of photons to cells in a culture, light intensities as well as culture mixing velocities should be increased. While high-intense light penetrates deeper into a PBR, appropriate culture mixing rates allow the algal cells to move faster from the light-limited (or dark) to light-saturated zones (Abu-Ghosh et al. 2016; Brindley et al. 2016). In the latter case, the fast transition from light- to dark zones helps to avoid photoinhibition of cells at the periphery but ensures the sufficient absorption of light energy by all cells to carry out photosynthesis and convert this energy into energy-bound compounds (e.g., sugars in the Calvin cycle, NADPH, ATP).

In most production systems, as soon as a high cell concentration is reached, the proportion of light zones to dark zones becomes too low and the retention time of cells in the dark area becomes too long, which increases respiration, leading to substantial biomass losses. To maximise growth at these high cell concentrations and to avoid photoinhibition, the supplied light as well as the mixing velocity should be increased. However, this approach has its drawbacks; the high energy consumption of mixing pumps and light-emitting lamps increase the production costs, and high mixing velocities can impair growth (Brindley et al. 2004). Alternatively, maintaining growth at high cell concentrations can be achieved by narrowing the light path between the light source and the culture (e.g., by light guides or decreasing thickness of the PBR) or tailoring wavelengths (de Mooij et al. 2016; Schulze et al. 2014). Yet, another approach is the use of flashing (or pulsed-) light emitting diodes (LEDs) to generate high-light flashes artificially, which penetrate deep into the culture (Abu-Ghosh et al. 2015; Schulze et al. 2017). Flashing LEDs emit periodically flashing cycles, which are composed of short light flash periods (or pulses; t_l) and extended dark periods (t_d). Choosing an ideal repetition rate (*i.e.*, frequency) for the flashing cycle is important to accomplish the so-called

"flashing light effect", where maximal photosynthetic rates are reached. Previous studies obtained contradictory results about the benefits of flashing light; they focused on flashing conditions such as low frequencies (f < 100 Hz) and relatively high duty cycles (DC > 0.1) that are present in mixed cultures (Grobbelaar 2009, 2006; Iluz et al. 2012; Raven and Ralph 2014; Sager and Giger 1980). Moreover, it is not certain if flashing light of high frequencies and short duty cycles (e.g., f > 100 Hz, DC < 0.1) can significantly improve growth performance of microalgae. In Schulze et al. (2017), we reviewed the potential of flashing light to improve microalgal growth, *i.e.* proportional to the duty cycle, and suggested a minimum theoretical frequency threshold of f ~ 300-500 Hz necessary to obtain the biological flashing light effect in microalgae. In addition, previous studies have indicated a threshold frequency at which microalgae experience a short enhancement of growth (Nedbal et al. 1996; Vejrazka et al. 2012). We used the latest LED and solid-state technologies to examine the growth response of the two green microalgae *Tetraselmis chui* Butcher and *Chlorella stigmatophora* by exposing them to different frequencies (e.g., 0.01 Hz - 1 MHz), duty cycles (e.g., 0.001 - 0.7), light intensities (50-1000 µmol s^{-1} m^{-2}) and culture concentrations. In addition, we have compared previously reported results with ours to understand if flashing light has a significant benefit on microalgal production.

2. Methods

2.1. Trials in photosynthetic chamber

In a first trial, we tested the oxygen evolution response of *T. chui* (SAG 19.52) cultures to flashing light inside a photosynthetic chamber; here the factors are the culture´s biomass concentration, supplied averaged light intensity or adjusted duty cycles and frequencies. Diluted *T. chui* cultures had a biomass concentration (dry weight per litre; g DW L^{-1}) of 0.13 g DW L^{-1} (±10%) and were exposed to three different average light intensities: I_a= 50, 500 and 1000 µmol s^{-1} m^{-2}. Concentrated *T. chui* cultures with a biomass concentration of 4.7 g DW L^{-1} (±10%) were exposed to two average light intensities: I_a= 500 and 1000 µmol s^{-1} m^{-2}. The lowest light intensity (I_a= 50 µmol s^{-1} m^{-2}) did not yield a positive oxygen evolution rate, and hence excluded from the concentrated culture trials. The range of tested frequencies and duty cycles ranked from 10-10,000Hz and 0.01-0.7, respectively.

In addition to the above-mentioned broad-scale screening, supplementary trials were conducted to understand the effect of extreme flashing light conditions on diluted and concentrated cultures. Test conditions were f= 0.01 Hz-2 MHz, DC < 0.01 with maximal

instantaneous flash intensities of up to I_l=100,000 µmol s^{-1} m^{-2} whenever technically feasible. Also, the simultaneous supply of continuous background light (150 µmol s^{-1} m^{-2}) emitted with flashing light (350 µmol s^{-1} m^{-2}) was tested on concentrated *T. chui* cultures (f = 1-10,000 Hz, DC = 0.05 and 0.1, I_a =500 µmol s^{-1} m^{-2}), as a promising approach for microalgal production (Abu-Ghosh et al. 2015).

2.2. Trials under batch conditions

In a second trial, we cultivated *C. stigmatophora* (RCC 661) and *T. chui* under batch conditions for 14 days using flashing light to understand long-term effects of flashing lights on different microalgal species. Both strains were grown for 14 days under flashing light at frequencies of 40 and 400 Hz and a duty cycle of 0.05 using an average light intensity of I_a= 200 µmol s^{-1} m^{-2}. Here we considered the technical feasibility of these conditions for industrial artificial light-based cultivation systems. To check the effects of flashing light of under-saturating average light intensities, *T. chui* was additionally grown at 40 and 400 Hz (DC= 0.05) with an average light intensity of I_a= 50 µmol s^{-1} m^{-2}.

All average light intensities (I_a) under flashing light are the time integrated obtained during one flashing cycle, which is composed of the light flash period (t_l) with a given instantaneous light intensity I_l and the dark phase t_d, where no light is emitted (e.g., I_a= 0 µmol s^{-1} m^{-2}; see Schulze et al. (2017) for further details). The instantaneous light intensity and the duty cycle are inversely proportional at a given average light intensity. The applied average light intensities (I_a=50-1000 µmol s^{-1} m^{-2}) were measured (QSL-100; Biospherical Instruments, San Diego, CA) inside the distilled water-filled photosynthetic chamber or cultivation flask.

2.3. Stock cultures

Tetraselmis chui was cultivated continuously (dilution rate: 0.7 d^{-1}; target biomass concentration: 1.5 g DW L^{-1}) in a 2 L-bubble column PBR under a light intensity of 400 µmol s^{-1} m^{-2}. This culture was kept inside a climate chamber (T= 15°C) and was used as inoculum for all experiments, referred as *stock (1)*.

For the experiments with the diluted cultures, around 150 mL of *stock (1)* was transferred into a second PBR containing 2 L fresh medium to obtain a final biomass concentration of ~0.13 g DW L^{-1}, referred as *stock (2)*. This dilute culture was maintained at average light intensities of I_a= 50, 500 or 1000 µmol s^{-1} m^{-2}; referred as *stock 2.1*, *2.2* and *2.3*, respectively. After one day of acclimation to a given average light intensity, the

culture was used for the flashing light experiments in the photosynthetic chamber as described further below.

For the experiments with the concentrated cultures, the overflow of *stock (1)* was connected to a second PBR with an adjusted light intensity of 2000 µmol s^{-1} m^{-2} to obtain a higher biomass concentration of ~4.7 g DW L^{-1}, referred as *stock (3)*.

The growth medium for *stock (1)* and *stock (2)* was a modified F-medium consisting of 5.3 mM $NaNO_3$, 0.22 mM $NaH_2PO_4H_2O$, 35 µM FeCL3*6H2O, 35 µM $Na_2EDTA*2H_2O$, 0.12 µM $CuSO_4*5H_2O$, 0.078 µM $Na_2MoO_4*2H_2O$, 0.23 µM $ZnSO_4*7H_2O$, 0.126 µM $CoCl_2*6H_2O$, 2.73 µM $MnCl_2*4H_2O$, adjusted to a salinity of 35 ppt using artificial sea salt (PRODAC International S.r.l., Cittadella, Italy). For *stock (3)*, twice the aforementioned nutrient concentration was used to ensure nutrient-sufficient growth conditions. Non-flashing light (current controlled warm white LEDs, 2700K) was supplied 24 h/day to all stock cultures. The adjusted light intensities were measured inside the PBR when filled with distilled water (QSL-100; Biospherical Instruments, San Diego, CA). All cultures were aerated and supplemented with CO_2 on demand at a pH of 7.5 (Hanna BL 931700 pH controller instruments, Bedfordshire, UK). The cultures were microscopically checked daily for contaminations and cell viability. The cultures were maintained at 15°C using a heat exchanger (F250, Julabo GmbH, Seelbach, Germany), which was connected to the water jacket of each PBR.

2.4. Experimental setup

Oxygen evolution measurements were conducted according to Brindley et al. (2010), using a flat panel reactor with a working volume of 200 mL as the photosynthetic chamber. This chamber had side lengths of 10x10 cm, providing a front surface area of 100 cm^2 and a light path length of 2 cm. The chamber was lit from the front (100 cm^2), and the water jacket around the chamber was connected to a heat exchanger (F250, Julabo GmbH) to maintain the temperature of the culture inside the chamber at 15°C. A rubber cover on the top of the chamber served as an insulation, minimising gas exchange with the environment. The rubber cover had three openings to fit different accessories; (1) a mixer, (2) a funnel to fill the chamber with fresh culture and (3) an oxygen probe (Crison 6050 oxygen, Crison Instruments S.A., Barcelona, Spain) to measure the oxygen evolution. The mixer kept the microalgal cells in suspension and facilitated the gas exchange between the culture and the oxygen probe. The oxygen probe was connected to a Mettler Toledo O24100 transmitter (Mettler-Toledo S.A.E., Barcelona, Spain), which

transferred the oxygen evolution data to a data logger (Jabjack U12, LabJack ltd. Lakewood, US).

The following procedures were adopted for each trial: (1) filling of the photosynthetic chamber with the fresh stock cultures (*stock 2.1, 2.2, 2.3* or *3*); (2) monitoring the oxygen evolution rate under a given light condition for 10-20 min; (3) draining the culture through an opening at the bottom of the chamber into an Erlenmeyer flask; (4) washing and filling of the chamber with fresh stock culture for the next experiment; and (5) transferring the already tested culture back to the stock culture. Every day, after the experiment, the chamber was cleaned and disinfected with ethanol (70% v/v).

Every 3-4 h, we measured the oxygen evolution of the cultures under continuous light at the same average intensity as used for the flashing light trials (e.g., I_a=50, 500 or 1000 s^{-1} m^{-2}); to detect possible shifts in oxygen evolution due to growth-stage changes or cell attachment to the corners or walls of the chamber. These control measurements were used to normalise the data obtained under flashing light, allowing comparison of results from different days, culture concentrations or light intensities.

A photosynthesis-irradiance (P-I) curve was generated to estimate the photo-acclimation stage of the stock cultures. Here, we measured the oxygen evolution rates of dilute cultures adapted to 50 (*Stock 2.1*), 500 (*Stock 2.2*) and 1000 (*Stock 2.3*) µmol s^{-1} m^{-2} and concentrated cultures (*stock 3*) under incrementally increasing actinic light intensities (0-10,000 µmol s^{-1} m^{-2}). For these trials, control measurements at a fixed continuous light intensity of 500 µmol s^{-1} m^{-2} were taken every 3-4 h to detect possible shifts in oxygen evolution rates due to cell attachment to the chamber or changing growth stage of the stock culture.

All batch cultures were carried out in 1-L borosilicate glass flasks (diameter; *d*=8 cm) filled with 900 mL algal culture (triplicates) in a climate chamber maintained at 15°C, using the inocula obtained from *stock (1)*. Air enriched with 1% CO_2 was used to mix the cultures. The growth medium was the same as used for *stock (3)*. The biomass concentrations in all cultures used was determined spectrophotometrically at 750 nm (OD_{750}) (CM-3500D, Minolta Co., Ltd., Osaka, Japan). In addition, at different time points during batch cultivation, culture samples were filtered through pre-dried glass fibre filters (pore size ø = 0.7 µm; VWR), washed twice with 0.5 M ammonium bicarbonate, dried (*T*= 70°C) and weighed to determine the dry biomass weight per litre (DW L^{-1}). Upon plotting OD_{750} data vs. dry weight, a linear correlation was obtained ($p< 0.05$) and used to determine biomass concentrations of all cultures on a daily base.

2.5. Light supply

Sixteen warm-white high-power LEDs (2700 K, MHDGWT-0000-000N0HK427G-SB01, Opulent Americas, Raleigh, US)–mounted on an actively cooled aluminium heat sink (10 × 10 cm)–lit the photosynthetic chamber. All LEDs were operated in parallel and were connected to the output of a pulse width modulator (PWM-OCX, RMCybernetics Ltd, Alsager, UK). The pulse signal was provided by a function generator (TG4001, TTi, Huntingdon, UK) and controlled via Waveform Manager Plus software (V. 4.01). Bench power supplies were used (EA-PS 2042-10B and EA-PS 2084-05B; EA Elektro-Automatik, Viersen, Germany) to power the pulse width modulator and LEDs. The voltages and currents supplied to the LEDs were regulated by the power sources to adjust the average light intensities and to compensate for switching and working losses by the LEDs and the pulse width modulator. The light flashes generated by this system were as short as 100 nanoseconds and the peak flash intensities were up to I_f = 100,000 µmol s^{-1} m^{-2} inside the photosynthetic chamber. We were able to attain the highest flash intensities and maximal overloading potential only at a frequency threshold of $f \approx$ 400-1000 Hz. At frequencies beyond this threshold, maximum flash intensities decreased to 20,000 µmol s^{-1} m^{-2} (e.g., at f = 1 Hz; duty cycle depended). This reduced the possible range of test frequencies and duty cycles under a given average light intensity.

For the batch culture experiments, 36 warm-white LEDs (2700 K, MHDGWT-0000-000N0HK427G-SB01) were mounted on an actively cooled aluminium heat sink (L=300cm, H=75cm, W=40cm), and the test flashing light conditions (f = 40, 400 Hz, DC=0.05) were adjusted by the same pulse width modulators and bench power sources that are mentioned above. For the continuous light control treatment, the LEDs were directly connected to a current-controlled power source (EA-PS 2084-05B, EA Elektro-Automatik).

The flashing light output of the LEDs was measured with a high-speed photodiode (OSI FCI-125G-006HRL, kindly provided by OSI Optoelectronics, Inc, Hawthorne, US) connected to an electrical resistor. To measure the intensity of flashing light that reached the microalgal cultures, the photodiode was mounted waterproof inside a glass tube and submerged into the cultures. The voltage drop at the resistor, which corresponded to the light output of the LEDs, was displayed on an oscilloscope (Picoscope 3000, Pico Technology Ltd., Cambridgeshire, UK) and the values were analysed using PicoScope 6 software (V.6.12.9.2917, Pico Technology Ltd.). These measurements confirmed that the adjusted conditions in the pulse width modulators were emitted by the LEDs.

2.6. Data treatment

The effects of flashing light on oxygen evolution and biomass productivities were examined using different analysis of variance (ANOVA). Because not all flashing light conditions could be tested for all cultures due to technical and biological limitation, we conducted step-wise analysis: First (1), we evaluate effects of *frequency* (10-10,000 Hz) and *duty cycle* (0.001-0.3) on diluted cultures adapted to 50 µmol s^{-1} m^{-2} (*Stock 2.1*; Fig. 2 B,C), based on both the F-statistic and Tukey's tests. Next, a general ANOVA (2) tested the effects of the factors *culture concentrations* (dilute and concentrated), *averaged light intensities* (I_a= 500 and 1000 µmol s^{-1} m^{-2}), *frequencies* (10-10,000 Hz) and *duty cycles* on dilute (*Stock 2.2, 2.3*) and concentrated (*Stock 3*) cultures. This analysis was done to identify the major factor (based on F-statistics) influencing the oxygen evolution rates. Based on this analysis (2), we examined the effect of the main factor by a third (3) and fourth (4) ANOVA that tested the effects of *duty cycle* (0.03-0.7), *frequency* (10-10,000 Hz) and *light intensity* (500 and 1000 µmol s^{-1} m^{-2}) on dilute (*Stock 2.2, 2.3*) and concentrated (*Stock 3*) cultures, respectively (Fig. 3 E, F). Lastly, a fifth ANOVA (5) was conducted on all diluted cultures (*Stock 2.1-2.3*) employing the factors *light intensity* (50, 500 and 1000 µmol s^{-1} m^{-2}), *frequency* (10-10,000 Hz) and *duty cycle* (0.03-0.3).

The F- and p-values resulting from the Type III sum of squares analysis were used to describe the impact of a given factor on the response variable photosynthetic oxygen evolution in the main text. The adjusted means with standard error from Tukey´s post hoc tests from ANOVA (1), (3) and (4) were used to illustrate the impact of frequency and duty cycle on a given culture (Fig. 2 B, C; 3 E, F). Post hoc results from ANOVA (5) are used to describe effects of different light intensities in the main text. The reader may refer to Table A.1 for test-statistics values.

Growth parameters for the cultivation experiments were estimated according to (2013); Ruiz et al. (2013). A significance level of $p< 0.05$ was considered for all tests.

To quantify the photo-acclimation stage for each culture (*Stock 2, 3*), oxygen evolution rates from the P-I curve were modelled, as described by Rubio et al. (2003). Their model provides the photosynthetic parameters (i) α, the saturation constant which is the light intensity (in µmol s^{-1} m^{-2}) where neither the metabolic rates nor light are limiting the photosynthetic rates, (ii) κ, the half saturation constant of the Calvin cycle (no unit) and (iii) P_m, the maximum rate of photosynthesis (mgO_2 g^{-1} s^{-1}; eq. 1):

$$P = P_m * \frac{I}{2\propto} * \left[\left(1 + \kappa + \frac{\alpha}{I}\right) - \sqrt{\left(1 - \kappa - \frac{\alpha}{I}\right)^2 + 4\kappa}\right] \quad [1]$$

The parameters α, P_m and κ were estimated via curve fitting (Sigma Plot software 13.0.0.83, Systat Software Inc, San Jose, US) to obtain the oxygen evolution rate (P) under a given continuous light intensity (I). Maintenance respiration (m) was subtracted from the P data prior to regression.

Because oxygen evolution performance of cells may change from day to day or hour to hour, we normalised the oxygen evolution rates (P_n) from flashing light experiments (P_f) in relation to the continuous light measurements (P_c) (eq. 2):

$$P_n = \frac{P_f}{P_c} \quad [2]$$

When P_n approaches a value of one, oxygen evolution rates under flashing- and continuous light become similar, while a P_n lower than one indicates that oxygen evolution under flashing light was lower than under continuous light.

A Sigmoidal model (eq.3) was applied to describe oxygen evolution response (P) to frequency or duty cycle (x).

$$P = y_0 + \frac{a}{1+\exp\left(-\frac{x-x_0}{b}\right)} \quad [3]$$

Where y_0, a, x_0 and b are parameters determined via iteration using Sigma Plot software.

In order to visualise and interpret data from the flashing light experiment, a mathematical model was designed to predict the oxygen evolution rates obtained in the experiment (P) by the variables frequency (f) and duty cycle (DC) and four parameters a, b, c and d (eq. 4).

$$P = \frac{1+e^{-a-b*(DC-c+d)}}{1+e^{-(a-b*\log 10(f))*(DC-c+d*\log 10(f))}} \quad [4]$$

Data from the low-light adapted cultures (I_a= 50 µmol s^{-1} m^{-2}) were applied to a model proposed by Fernández et al. (2018):

$$\frac{P}{P_m} = \frac{f}{f_\beta} \cdot \left(1 - e^{-\frac{f_\beta \cdot I_a}{f \cdot \alpha}}\right) \quad [5]$$

Where f is the frequency of the flashing cycle and f_β is the frequency at which the maximum photosynthetic performance (P_m) is reached. This equation was developed as a special case of the Camacho-Rubio model (2003) by using the following restrictions: very short flashes (low duty cycles) and non-saturating averaged light intensities ($I_a < \alpha$). The data sets of flashing light at moderate average light intensity (I_a=500 µmol s^{-1} m^{-2}) and high average light intensity (I_a=1000 µmol s^{-1} m^{-2}) were not suitable for fitting to equation [5] because in both cases $I_a > \alpha$, violating the non-saturating irradiance restriction.

3. Results and discussion

3.1. P-I Curve

A photosynthetic oxygen evolution rate *vs* irradiance curve (P-I curve) of *T. chui* cultures obtained under continuous irradiance shows the photo-acclimation stages of cultures exposed to the average light intensity used in the flashing light experiments (I_a= 50, 500 and 1000 µmol s^{-1} m^{-2}; *Stock 2, 3*; Fig. 1). The maintenance respiration (oxygen evolution rate measured in the darkness) of the 50, 500 and 1000 µmol s^{-1} m^{-2} adapted cultures (*Stock 2.1, 2.2, 2.3*) was m= −0.088, −0.16 and −0.26 µmol O_2 g^{-1} s^{-1}, respectively, and in concentrated cultures (Stock 3) −0.2 µmol O_2 g^{-1} s^{-1}. The P-I curves of all diluted cultures followed a typical pattern, with the following characteristics: (1) An increase of oxygen evolution with increasing light intensities, (2) a maximum and (3) a subsequent decrease of photosynthetic performance with further rising light levels. The ratio between enzymatic and photochemical rate constants (α in µmol s^{-1} m^{-2}) increased with acclimation light intensity (α= 250, 456 and 559 µmol s^{-1} m^{-2} for the 50, 500 and 1000 µmol s^{-1} m^{-2} acclimatised cultures, respectively). The α-value of cultures acclimatised to 50 µmol s^{-1} m^{-2} was higher than the average light intensity supplied (α=250 µmol s^{-1} m^{-2} > I_a=50 µmol s^{-1} m^{-2}), indicating that photosynthetic rates are limited by the supplied light but not by metabolic turnover rates. The 500 µmol s^{-1} m^{-2}-adapted cultures had an α-value that was similar to the light intensity supplied (α= 456 µmol s^{-1} m^{-2}), indicating that neither the metabolic rates nor light intensity were limiting the photosynthetic performance of these cultures. Lastly, the 1000 µmol s^{-1} m^{-2}-adapted cultures showed a significantly lower α-value than the supplied light intensity (α= 559<1000 µmol s^{-1} m^{-2}), indicating that photosynthetic rates were maximal for *T. chui* cells and only metabolic turnover rates were limiting the photosynthetic performance.

The half saturation constant of the Calvin cycle (κ, dimensionless) was lower in 500 and 1000 µmol s^{-1} m^{-2}-acclimatised cultures (κ=0.236 and 0.497, respectively) as compared to 50 µmol s^{-1} m^{-2}-adapted cells (κ=0.675). The kappa values can be related to the photo-acclimation strategies of cells including adjustment of the amount of PSUs, chlorophyll molecules per PSU or others (Dubinsky and Stambler 2009; Fernández et al. 2018; Fisher et al. 1996; Quigg et al. 2006; Rubio et al. 2003).

The maximum rate of photosynthetic oxygen evolution (P_m) increased with light adaption levels ($P_{m\_50\ \mu mol}$=2.094, $P_{m\_500\ \mu mol}$=2.545 and $P_{m\_1000\ \mu mol}$=3.260 µmolO_2 g^{-1} s^{-1}). Here, the maximum photosynthetic rates were reached at light intensities ranking from

I= 1000-2000 µmol s^{-1} m^{-2} for the 50 µmol s^{-1} m^{-2}-light-adapted cultures, and *I*=1000-4000 µmol s^{-1} m^{-2} for the 500 and 1000-µmol s^{-1} m^{-2}-adapted cultures (Fig. 1). Similar trends were previously reported for dilute microalgal cultures acclimatised to low, moderate and high light intensities (García-Camacho et al. 2012).

In addition, we tested the oxygen evolution of concentrated cultures in response to increasing light (Fig. 1). Generally, photosynthetic rates per cell were ~10 times lower compared to dilute cultures and a minimum light intensity of 200 µmol s^{-1} m^{-2} inside the chamber was necessary to achieve photosynthetic oxygen evolution. Therefore, subsequent flashing light experiments in concentrated cultures (Fig. 3, 4) were only tested with average light intensities of I_a= 500 and 1000 µmol s^{-1} m^{-2}, but not with I_a= 50 µmol s^{-1} m^{-2}. The model could not fit the data for concentrated cultures, as no maximum was reached at light intensities of 1-10,000 µmol s^{-1} m^{-2}.

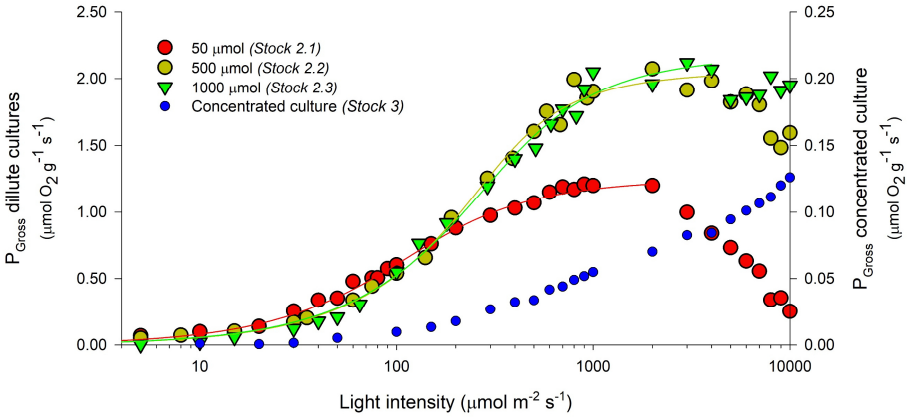

Figure 1. Photosynthesis-irradiance (P-I) curves of stock cultures used in the flashing light experiments. The dilute (0.13 g L^{-1}) stock cultures were adapted to continuous light with a light intensity of 50 (*Stock 2.1*), 500 (*Stock 2.2*) and 1000 (*Stock 2.3*) µmol s^{-1} m^{-2} while the concentrated culture (4.7 g L^{-1}; *Stock 3*) was previously cultivated under 2000 µmol s^{-1} m^{-2}. Oxygen evolution rates are plotted over incrementally increasing actinic light intensities (*I*= 0-10,000 µmol s^{-1} m^{-2}). Solid lines are fitted to the gross photosynthetic rates using the P-I model described Rubio et al. (2003) until maximal photosynthetic oxygen evolution was achieved. We did not obtain any model for concentrated cultures or for light intensities under which photoinhibition occurred (no line plotted). All model parameters and raw data are given in the supplementary material (Table S1).

The low α value of 50 µmol s^{-1} m^{-2}-adapted dilute cultures indicates that this culture was low-light-adapted, while the high α value of 1000 µmol s^{-1} m^{-2}-adapted dilute

cultures shows that they were high-light-adapted. The 500 µmol s^{-1} m^{-2}-adapted cultures were moderately adapted to high light. The concentrated cultures did not show a plateau or any signs of decreasing oxygen evolution rates with increasing light intensities tested. A possible explanation is that the concentration of the cultures did not allow sufficient mixing of cells using the present methodology in order to respond to the average light intensity supplied. Such insufficient mixing conditions let cells respond to the instantaneous light intensity, which is considered a flashing light regime caused by mixing as described by Terry (1986). Notably, concentrated cultures in *stock (3)* and those in the photosynthetic chamber were characterised by high biomass concentration which brings a high proportion of dark layers. Consequently, a high residence time of cells in the dark layers (Abu-Ghosh et al. 2016) promotes high respiration rates and the adaption of cells to low light intensities, resulting in low photosynthetic efficiencies observed in concentrated cultures.

3.2. Effect of flashing light

The effects of frequencies, duty cycles and light intensities on dilute cultures and concentrated cultures were evaluated and modelled (Fig. 2, 3). Among all tested conditions, cultures exposed to flashing light did not exceed the photosynthetic rates obtained under continuous light. However, depending on culture concentration and average light intensity, inhibitory effects of flashing light on photosynthesis differed significantly.

3.2.1. Dilute low-light adapted cultures

Flashing light was tested at a low average light intensity (I_a=50 µmol s^{-1} m^{-2}) on dilute microalgal cultures adapted to the same light intensity *(Stock 2.1*) among different frequencies (10-10,000 Hz) and duty cycles (0.001-0.3). The obtained model shows that the duty cycle and frequency significantly affected the oxygen evolution rate (p<0.01). Considering the F-values, we conclude that duty cycle (F= 5.7) was affecting the oxygen evolution rate less than frequency (F= 20.9). Oxygen evolution rates rose sigmoidally with increasing duty cycles (Fig. 2B). An analysis of the adjusted means from the Tukey´s post hoc test indicated that duty cycles between 0.07-0.3 achieved the higher oxygen evolution rate, than compared to 0.003, 0.007 and 0.01 (p< 0.05; Fig. 2B, Table A.1). Duty cycles of 0.03, 0.05, 0.0005 and 0.0001 did not differ significantly from the aforementioned groups (p> 0.05). A sigmoidal model (r^2_{adj}= 0.98) includes the initial exponential rise of oxygen evolution rate over frequencies (Fig. 2C). Frequencies between 10-50 Hz produced significantly lower oxygen evolution rates while higher

frequencies (≥60 Hz) did not differ to continuous light treatment (P_n≈1, Table A.1). Additional modelling according to Fernández et al. (2018) identified the threshold frequency under which the flashing light effect occurs in *stock (2.1)* between ~130 and 180 Hz at duty cycles ≤0.01 (Table A.1).

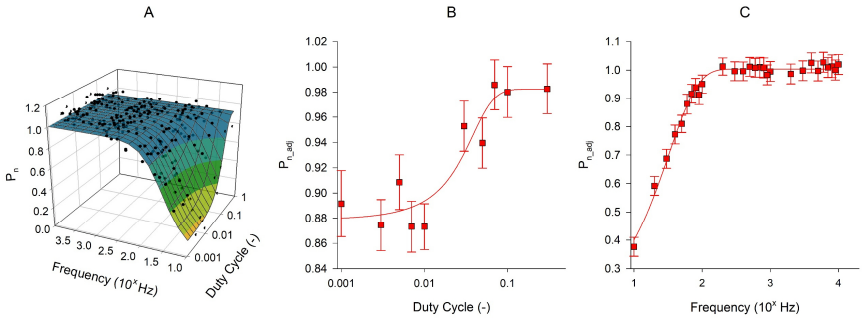

Figure 2. Effects of flashing light with I_a=50 µmol s^{-1} m^{-2} on dilute *T. chui* cultures adapted to the same average light intenstiy (*Stock 2.1*) among frequencies of 10-10,000 Hz and duty cycles of 0.001-0.3. Data (black points in A) of normalised oxygen evolution rates (P_n) are described by a mathematical model (surface area) as a function of duty cycles and frequencies (A). An ANOVA (1) of the data was used to quantify the effects of duty cycle (B) and frequency (C, x-axis values are $10^{\text{the labels}}$), followed by Tukey´s post hoc tests. Error bars indicate the standard error from the ANOVA model (r^2=0.73). Detailed statistical data are given in supplemental materials (Table A1).

3.2.1. Concentrated vs. dilute cultures

Concentrated and dilute *T. chui* cultures (*Stock 2.2, 2.3* and *3*) were exposed to average light intensities of I_a= 500 and 1000 µmol s^{-1} m^{-2} and frequencies of 10-10,000 Hz and duty cycles of 0.01-0.7 whenever technically feasible. An overall ANOVA (2) model identified all parameters (duty cycle, frequency, culture concentration and averaged light intensity) as significantly affecting the oxygen evolution rates (p< 0.01, Table A.1). The culture concentration had the strongest effect on oxygen evolution rates, indicated by the highest F-value (F= 62.2), compared to duty cycle (F= 23.8), frequency (F= 31.6) and light intensity (F= 11.1; Table A.1). Based on the above result, to better understand the effects of culture concentration, we conducted separate ANOVA (3 and 4) only on oxygen evolution data from either (i) concentrated or (ii) dilute cultures among both average light intensities tested (I_a= 500 and 1000 µmol s^{-1} m^{-2}). In

both models, oxygen evolution rates were mostly affected by duty cycle (F_{dil_DC}= 24.7, F_{conc_DC}= 10.6) and frequency (F_{dil_f}= 34.6, F_{conc_f}= 10.6) and to a lesser extent by average light intensity (F_{dil_I}= 13.7, F_{conc_I}= 5.4; Table A.1). A plot of the adjusted means of oxygen evolution in response to duty cycle and frequency (Tukey´s test; Fig. 3E, F) revealed that concentrated cultures scored usually higher oxygen evolution rates at frequencies <200 Hz compared to dilute cultures. Generally, oxygen evolution rates were higher at duty cycles from 0.3-0.7, while shorter duty cycles caused significantly lower rates (Table A.1). Similar to experiments with *Stock (2.1)* exposed to 50 µmol s^{-1} m^{-2} (Fig. 2), adjusted means of oxygen evolution exponentially with frequency, approaching its maximum at around ~200 Hz.

A final ANOVA (5) was conducted on diluted cultures (*Stock 2.1-2.3*) exposed to 50, 500 and 1000 µmol s^{-1} m^{-2} to quantify effects of different light intensities (Table A.1). Cultures exposed to average light intensity of 50 µmol s^{-1} m^{-2} showed higher oxygen evolution rates as compared to those exposed to 1000 µmol s^{-1} m^{-2} (P_{adj}=0.996 vs. 0.850). Lowest oxygen evolution rates were found in 500 µmol s^{-1} m^{-2}-adapted cultures (P_{adj}=0.777; p<0.01).

Frequencies between 10 and 200 Hz were most discriminative for changes of the oxygen evolution, while frequencies above 200-400 Hz usually result in similar photosynthetic rates than obtained under continuous light. These findings confirm that the photosynthetic apparatus needs a frequency of at least 200 Hz, regardless of the adjusted duty cycle; to match the turnover rates (3-5 milliseconds) of the linear electron transfer chain [(e.g., Q_0 acceptor in the cytochrome b_6f complex; (Schulze et al. 2017)].

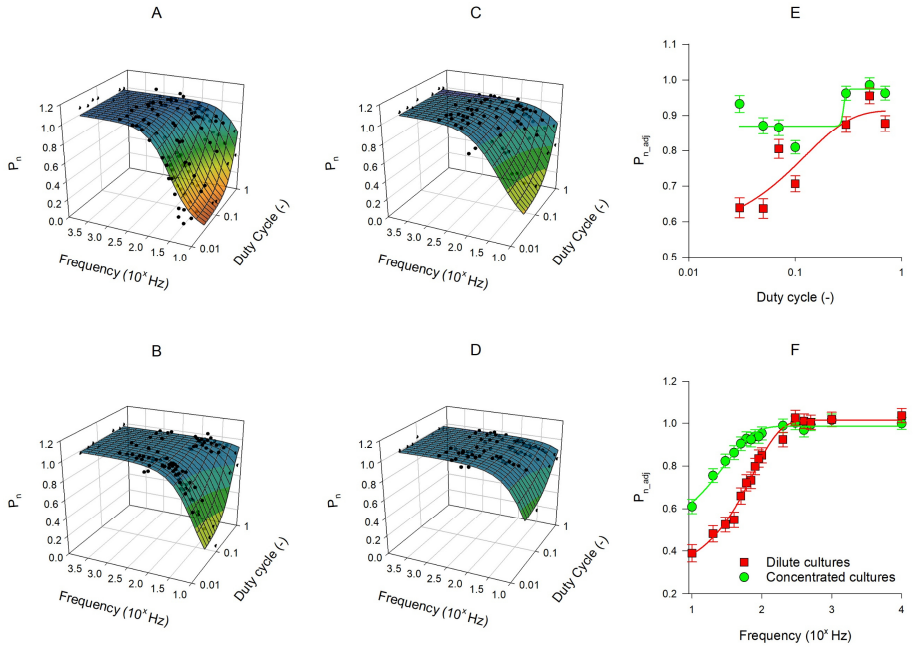

Figure 3. Surface response of oxygen evolution for concentrated and dilute *Tetraselmis chui* cultures exposed to flashing light at frequencies from 10-10,000 Hz and duty cycles of 0.01-0.7. Dilute cultures adapted to 500 µmol s^{-1} m^{-2} (*Stock 2.2,* A) and concentrated cultures (*Stock 3*; B) were exposed to I_a= 500 µmol s^{-1} m^{-2}. Dilute cultures adapted to 1000 µmol s^{-1} m^{-2} (*Stock 2.3*; C) and concentrated cultures (*Stock 3*; D) were exposed to I_a=1000 µmol s^{-1} m^{-2}. Black points are the normalised oxygen evolution rates (P_n) and surface plot is the fitted model. The adjusted means from Tukey´s test for duty cycle (E) and frequency (F) are given for concentrated and dilute cultures. Error bars indicate the standard error from the ANOVA (3, 4) model (r^2=0.60-0.80). Table A1 in supplemental material provides model parameters, raw data and statistical differences among the treatments.

All cells in dilute cultures received the flashing light with the adjusted average light intensity as light attenuation by self-shading was minimal (Brindley et al. 2010; Vejrazka et al. 2011), and the effect on the photosynthetic apparatus (*i.e.*, biological flashing light effect; Schulze et al 2017) could be tested for low, medium and high light-adapted cells (I_a=50, 500 and 1000 µmol s^{-1} m^{-2}; *Stock 2.1-2.3*). Our results showed that the normalised oxygen evolution rates in 50 µmol s^{-1} m^{-2}-adapted cells was higher compared to 500 or 1000 µmol s^{-1} m^{-2}-adapted cells ($P_{adj\_50\mu mol} > P_{adj\_500\text{-}1000\mu mol}$) at low frequencies (e.g., *f*= 10-50 Hz; Table A.1). Because the average flashing light intensity in 50 µmol s^{-1} m^{-2}-adapted cultures was below the saturation constant ($I_a<\alpha_{50\mu mol}$), we suggest that cells may have processed flashing light better than if I_a was similar or higher than the saturation constant (e.g., $I_a \geq \alpha$ in 500 and 1000 µmol s^{-1} m^{-2}-adapted cultures). Similar to these results, Jishi et al. (2015) and Xue et al. (2011) reported a decrease of light use efficiency under low-frequency flashing light by *Spirulina platensis* and land plants as the supplied average light intensity increased. Jishi et al. (2015) indicated that pool size of photosynthetic intermediates [e.g., plastoquinone; (Schulze et al. 2017)] is responsible for efficient flashing light utilization for photosynthesis. It has been reported that the lower the supplied average light intensity, the better the light use efficiency of phototrophs under low-frequency flashing light (Jishi et al. 2012; Xue et al. 2011). Contrary to these findings, in the present study, 1000 µmol s^{-1} m^{-2}-adapted cultures (*Stock 2.3*) tolerated better low-frequency flashing light than 500 µmol s^{-1} m^{-2}-adapted cultures ($P_{adj\_500}<P_{adj\_1000}$; ANOVA 5, Table A.1). The 1000 µmol s^{-1} m^{-2}-adapted cultures that were high light-adapted ($I_a>\alpha$; Fig. 1) may have accumulated more photo-protective metabolites (e.g., *β*-carotene) that protect the cells from long-lasting high light-flashes [e.g., low frequency and short duty cycle; (Katsuda et al. 2006; Mouget et al. 1995; Sastre 2010; Schüler et al. 2017)] compared to 500 µmol s^{-1} m^{-2}-acclimatised cells ($I_a \approx \alpha$). However, to our knowledge no such high average light intensities (I_a=1000 µmol s^{-1} m^{-2}) were tested previously on high light-adapted cultures and further studies are needed to confirm the present findings.

The higher oxygen evolution rates of concentrated over diluted cultures at flashing light frequencies from 10-200 Hz and duty cycles <0.1 (Fig. 3E, F) may come from a higher light attenuation in concentrated cultures (Abu-Ghosh et al. 2016). Through culture mixing, cells could move from potentially light-inhibiting zones at the periphery towards low-light layers. Cells travelling through the low-light layers receive a lower average light intensity than emitted by the LEDs. As shown in our experiments with dilute cultures; lower light intensities were less inhibiting than flashing light of high

averaged light intensities (e.g., $P_{adj\_50\mu mol} > P_{adj\_500\text{-}1000\mu mol}$, $p<0.01$). These dynamics in concentrated cultures may have allowed the cells to cope better with flashing light at high intensities (I_a=500-1000 µmol s^{-1} m^{-2}) than diluted cultures where single cells were fully exposed to the applied light regime.

3.2.2. Extension of flashing light frequency and duty cycle

Previous studies found higher photosynthetic rates of dense and dilute microalgal cultures under flashing light compared to continuous light when the duty cycles are short or frequencies are high (Liao et al. 2014; Park and Lee 2001; Vejrazka et al. 2012; Yago et al. 2012). We were not able to confirm this finding at frequencies between 10 and 10,000 Hz and duty cycles ≥0.01 (Fig. 2, 3), adopted in our study. In addition, previous studies by Abu Gosh and colleagues (2015) showed an enhancement of the photosynthetic performance when flashing light was combined with continuous light. To get more conclusive results, we conducted additional experiments, extending the frequency range to 0.01 Hz-1 MHz and duty cycles <0.01 (whenever technically feasible) at a given average light intensity (Fig. 4). We also tested flashing light in combination with continuous light ($I_{a\_flashing}$=350 µmol s^{-1} m^{-2} + $I_{a\_continuous}$=150 µmol s^{-1} m^{-2}).

Short duty cycles of 0.005 (500-800 Hz) and 0.007 (200-1000 Hz) were tested for cultures exposed to I_a=500 µmol s^{-1} m^{-2} (Fig. 3A). Dilute cultures showed slightly reduced oxygen evolution rates (P_n= 0.7-0.9) when exposed to a duty cycle of 0.007, f=200-300 Hz and a duty cycle of 0.005, f=400-500 Hz, while higher frequencies resulted in similar rates compared to continuous light. Oxygen evolution rates of concentrated cultures were similar to continuous light among the same test conditions.

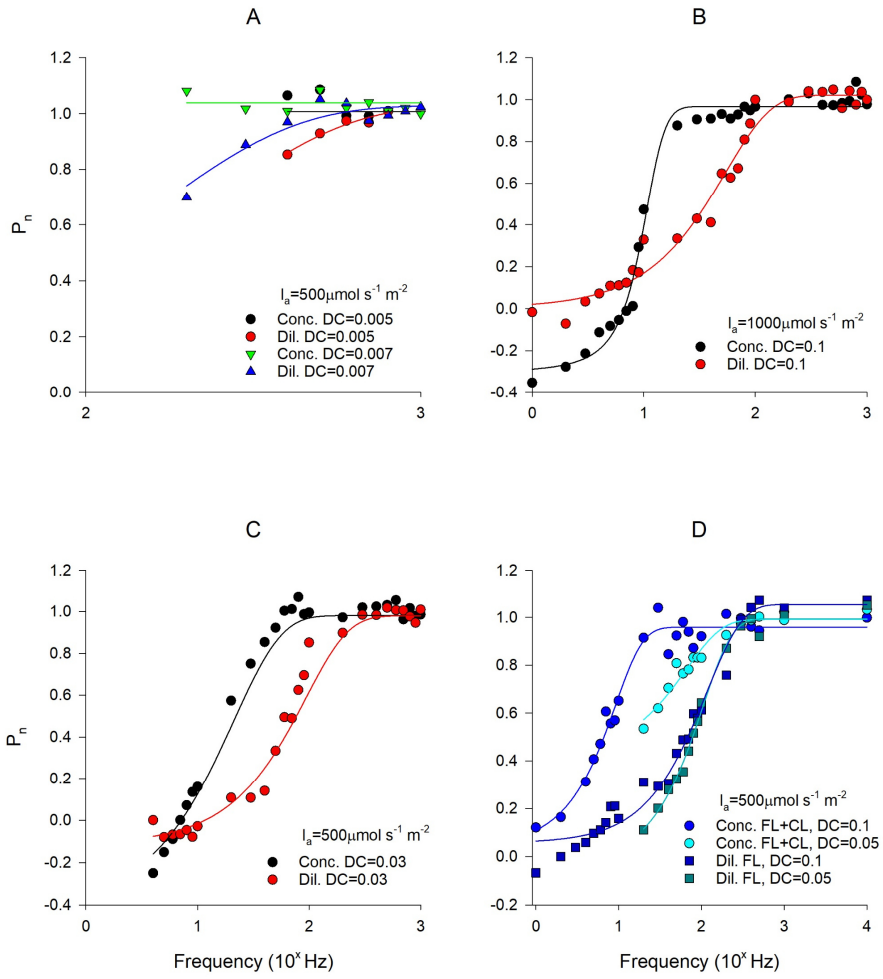

Figure 4. Effects of extreme flashing light conditions with average light intensities of I_a= 500 and 1000 µmol s^{-1} m^{-2}. Duty cycles < 0.01 were tested on cultures exposed to I_a= 500 µmol s^{-1} m^{-2} (A; *f*= 200-800 Hz; *Stock 2.2, 3*). Frequencies < 10 Hz were tested on dilute and concentrated cultures with an average light intensity of I_a= 1000 µmol s^{-1} m^{-2} (B; *Stock 2.3, 3*) and I_a= 500 µmol s^{-1} m^{-2} (C; *Stock 2.2, 3*). Flashing light with I_a= 350 µmol s^{-1} m^{-2} combined with 150 µmol s^{-1} m^{-2} continuous light (D; *Stock 3*) was tested for a duty cycle of 0.1 and 0.05 and compared with flashing light in dilute cultures (*Stock 2.2*) exposed to only flashing light at the same average light intensity. Sigmoidal models (solid lines) were fitted to the normalised oxygen evolution rates (P_n). All data and model parameter are given in Table A1 (supplemental material).

Low frequencies until 0.01 Hz were tested for cultures exposed to I_a= 1000 µmol s^{-1} m^{-2} at a duty cycle of 0.1 (Fig. 3B) and f≥ 4 Hz for cultures exposed to I_a= 500 µmol s^{-1} m^{-2} and a duty cycle of 0.03 (Fig. 3C). In dilute cultures, oxygen evolution rates at frequencies ≤10 Hz approached to P_n≈ 0, indicating that rates of photosynthesis and respiration were similar. In concentrated cultures, frequencies ≤7 Hz caused a consumption of oxygen (negative oxygen evolution rates), indicating that rates of respiration were higher than the photosynthetic rates. Oxygen evolution rates reached a minimum of P_n= −0.32 when exposed to f= 1 Hz, I_a= 1000 µmol s^{-1} m^{-2} and DC= 0.01. Additional testing of frequencies of 0.1 and 0.01 Hz showed that oxygen evolution rates did not drop below those obtained under dark (no light) conditions ($P_{n(Dark)}$=-0.6; Table A.1 in supplemental material).

At frequencies (f> 7-10 Hz), oxygen evolution rates increased sigmoidally in dilute and concentrated cultures with rising frequencies until oxygen evolution rates became similar to those obtained under continuous light (P_n≈ 1). In concentrated cultures, the maximum photosynthetic rate was reached at lower frequencies (f≈ 30-50 Hz) as compared to dilute cultures (f= 100-400 Hz).

At a duty cycle of 0.1, frequencies up to 1 MHz were tested for 50-µmol s^{-1} m^{-2}-adapted diluted cultures and up to 200,000 Hz for concentrated cultures exposed to I_a=500 µmol s^{-1} m^{-2} (Data provided in Table A.1 in supplemental material). Similar to our previous findings for frequencies up to 10,000 Hz (Fig. 2, 3), photosynthetic rates remained similar compared to continuous light-lit cultures.

Lastly, concentrated cultures were exposed to continuously emitting LEDs with I_a=150 µmol s^{-1} m^{-2} in combination with flashing LEDs (I_a= 350 µmol s^{-1} m^{-2}; DC= 0.05, 0.1). Again, this treatment was not found to improve oxygen evolution rates of concentrated cultures that were exposed to continuous light alone. However, oxygen evolution rates were usually higher when compared to the application of only flashing light at frequencies from of 10-200 Hz (Fig. 4D).

Similar to our previous results (Fig. 2, 3), shortening the duty cycles to <0.01 or increasing the frequency to 0.2-1 MHz did not improve photosynthesis of dense or dilute cultures compared to continuous light. Dilute cultures adapted to I_a= 500 and 1000 µmol s^{-1} m^{-2} did neither show oxygen evolution nor consumption at low frequencies and short duty cycles (Fig. 4 B, C), as previously reported for other strains under similar conditions (Brindley et al. 2010; Nedbal et al. 1996; Takache et al. 2015; Vejrazka et al. 2015). In mass cultures, insufficient mixing can also lead to short duty

cycle and low frequency-light regimes, which comes with high residence time of cells in the dark layers. Under these conditions, the low light availability could have been compensated by depleting intracellular photochemical energy, thereby resulting in limited growth (Brindley et al. 2016; Terry 1986). To maintain a high productivity, Grima et al. (1999) proposed to design PBRs and scale-up cultures by defining an acceptable duration of the dark and light periods.

In the present study, concentrated cultures exposed to low frequencies ($f \leq 7$ Hz) showed negative oxygen evolution rates, indicating higher respiration than photosynthetic rates. Such negative growth for high average light intensities was also reported by Xue et al. (2011). Furthermore, Brindley et al. (2011) predicted the oxygen evolution rates for similar conditions. Towards higher frequencies, oxygen evolution rates reached those obtained under continuous light, as previously reported for other strains (Brindley et al. 2010; Matthijs et al. 1996; Nedbal et al. 1996; Vejrazka et al. 2013; Vejrazka et al. 2012, 2011; Vejrazka et al. 2015).

The light regimes in concentrated cultures are complex and were studied extensively (Brindley et al. 2016; Loomba et al. 2018; Melis 2009; Melis et al. 1998). For the sake of simplicity, we considered three light zones in concentrated cultures: (1) A high-light zone at the surface of the PBR where cells are exposed to most of the emitted light; (2) a low- to moderate-light zone i.e., a few millimetres after the high light zone, where cells are exposed to low average light intensity; and (3), a dark zone where cells do not receive any light and respiration exceeds photosynthetic rates.

Our cultures with biomass concentrations of ~4.7 g DW L^{-1} and a light path of 2 cm were characterised by a high proportion of dark to light zones; leaving cells most of the time in the dark and only for short durations in the (low-) light zones (Loomba et al. 2018). The cells will have high retention time in the dark regions and hence will experience only short exposure to light, causing a low-light adaption of most cells in the culture (Brindley et al. 2010). Such a low light adaption comes with a downregulation of photoprotective metabolisms (Schüler et al. 2017), and the microalgal cells passing through the high light zone will not be able to cope with high light flashes, eventually leading to both photodamage (Schulze et al. 2017) and increase in respiration rates. If light adaption can account for the negative oxygen evolution rates found at frequencies <8 Hz, then a low light-adapted diluted culture (e.g., I= 50 µmol s^{-1} m^{-2}, *Stock 2.1*) exposed to low-frequency flashing light with short duty cycles at a high average light intensity (e.g., I_a=500 µmol s^{-1} m^{-2}, DC=0.1, f=10 Hz) should also trigger a negative oxygen evolution rate. In an additional experimental set up, we tested these conditions

(Table A.1, supplementary material) and indeed observed that 50 µmol s^{-1} m^{-2}-adapted dilute cultures (*Stock 2.1*) exposed to 500 µmol s^{-1} m^{-2}-flashing light showed a negative oxygen evolution rate at low frequencies (f= 10-80 Hz, DC= 0.05, 0.07).

When the cells were stimulated with frequencies from 10 to 100 Hz, the growth of cells in the periphery of dense cultures were less inhibited, and cells in the low- to medium-lit zones could already use this flashing light efficiently for photosynthesis, as shown in our results with dilute cultures that achieved a higher normalised oxygen evolution rate at low average light intensities as compared to higher intensities (ANOVA 5). Such effect could explain why concentrated cultures exposed to frequencies of f=10-100 Hz achieved higher oxygen evolution rates than dilute cultures in the same flashing light range.

Regardless of the flashing light conditions, cells in dark zones were likely to show higher respiration rates than photosynthetic rates, as these cells received no light, adding up the total respiration of the culture.

In summary, cells in each zone of concentrated cultures responded differently to flashing light, depending on the prevailing average light intensity. We suggest that the observations in concentrated cultures are a result of three factors: (1) inhibition of cells in high light intensity zones; (2) better light utilisation efficiency of cells in low- or moderate-lit zones and (3) high respiration rates of cells in dark zones.

3.3. Batch experiments

Previous studies about microalgae cultivation using flashing light indicated practically better growth as compared to continuous light (Katsuda et al. 2006; Liao et al. 2014; Lunka and Bayless 2013; Park and Lee 2000; Sastre 2010; Vejrazka et al. 2012; Xue et al. 2011; Yago et al. 2014; Yoshioka et al. 2012). Even though photoproduction processes occur within milliseconds (Bernardi et al. 2017), the short exposure time of cultures to flashing light during the oxygen evolution trials (10-20 min; Fig. 1-4) could not detect beneficial effects that may come from cell acclimation to flashing light or from changing biomass concentration (or optical properties) of cultures. Therefore, we cultivated *C. stigmatophora* and *T. chui* under flashing light with frequencies of 40 and 400 Hz at a duty cycle of 0.05 during 14 days in cultivation flasks with a maximum light path of d= 8 cm (Fig. 5). At a light intensity of I_a= 200 µmol s^{-1} m^{-2}, 40 Hz-treated *C. stigmatophora* and *T. chui* cultures had lower biomass productivities (0.18±0.01 and 0.49±0.01 g DW L^{-1} d^{-1}, respectively) compared to continuous and 400 Hz-flashing light

treated ones (p<0.01). Continuous light did not differ significantly to 400 Hz-flashing light treatments (average: 0.21±0.01 g DW L^{-1} d^{-1} for *C. stigmatophora* and 0.59±0.02 g DW L^{-1} d^{-1} for *T. chui*, p> 0.05).

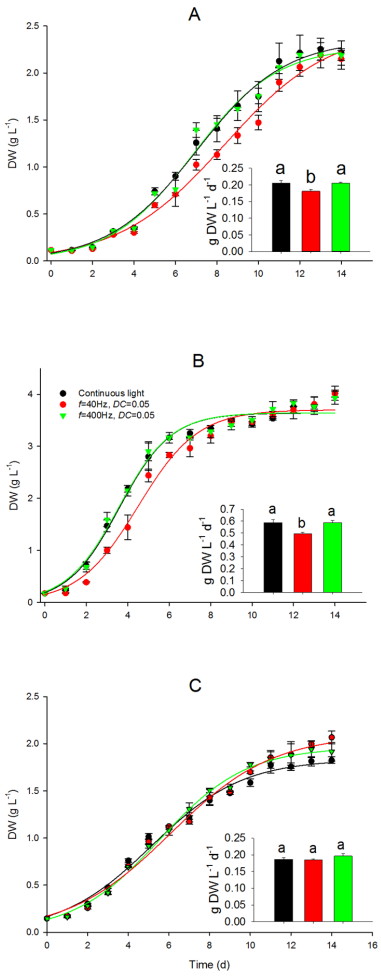

Figure 5. Batch cultivation under flashing light with frequencies of 40 and 400 Hz at a duty cycle of 0.05. *Chlorella stigmatophora* (A) and *Tetraselmis chui* (B) were cultivated under an averaged light intensity of I_a=200 µmol s^{-1} m^{-2}. *T. chui* was additionally cultivated at I_a= 50 µmol s^{-1} m^{-2} (C). Solid lines are fitted using a sigmoidal growth model, based on the growth data (symbols at each time point). All model parameters are given in the supplementary material (Table A.1). Data are shown as mean ±SD, n=3.

Because the effects of flashing light on algal growth depends also on supplied average light intensity (Fig. 2-4) and previous reports indicated that flashing light at under-saturating intensities is particular promising to improve photosynthesis of microalgae (Martín-Girela et al. 2017), we tested a lower average light intensity of I_a= 50 µmol s^{-1} m^{-2} on *T. chui* cultures. As a result, biomass productivities did not differ among all treatments (average: 0.19±0.01 g DW L^{-1} d^{-1}; Fig. 5C; p> 0.05; Table A.1). These results confirm our findings that flashing light of lower average light intensities inhibit cells less than high average light intensities. In conclusion, our data indicate that cells do not acclimatise to flashing light in accordance with the report of Grobbelaar et al. (1996), and flashing light has no enhancing effects in batch cultures where culture concentrations change over time.

3.1. Comparison with other publications

We derived P_n from several flashing light studies conducted on various microalgal species (Fig. 6, Table A.1). The studies employed different culture densities, light intensities and light adaption levels. For most studies that reported improved growth or photosynthetic performance, the P_n value was ~1.2 (20% better growth compared to continuous light) [e.g., Fig. 6, (Liao et al. 2014; Nedbal et al. 1996; Park and Lee 2000; Vejrazka et al. 2012; Vejrazka et al. 2015; Yago et al. 2014; Yago et al. 2012)]. Only few studies have reported higher values (Lunka and Bayless 2013; Luzi et al. 2019). It was often suggested that photosynthesis does not require continuous light and that short intense light flashes can penetrate deeper into the water column so that the cells in deeper layers can carry out photosynthesis (Park and Lee 2000; Vejrazka et al. 2011; Vejrazka et al. 2015). At present, self-shading is one of the most discussed factors for explaining flashing-light-induced growth enhancement in microalgal cultures (Abu-Ghosh et al. 2016; Brindley et al. 2011; Grobbelaar et al. 1996; Lunka and Bayless 2013; Sastre 2010). We previously suggested flashing light conditions that favour light penetration (e.g., short duty cycle) and do not inhibit photosynthesis (e.g., frequencies ~500 Hz) as promising approaches to overcome self-shading and increase culture performance (Schulze et al. 2017). Such conditions were tested in our experiments with concentrated cultures and batch cultures. To our surprise, we did not find any improvements of flashing- over continuous light, even at shortest technically possible duty cycles (e.g., 0.005) with highest possible flash intensities (e.g., I_f= 100,000 µmol s^{-1} m^{-2}) or frequencies (≤1 MHz). By measuring the light pattern of flashing light with a photodiode inside concentrated cultures, we could confirm that the light flashes indeed penetrated deep into a dense culture (data not shown). However, the average light

intensity of flashing light decreased to the same extent as continuous light with increasing light path. Since the (biological-) flashing light effect only causes similar photosynthetic rates as under continuous light (Jishi et al. 2015; Schulze et al. 2017) and cells respond to the average light intensity when frequencies are above 200-500 Hz, flashing light could not improve photosynthetic performance under any conditions.

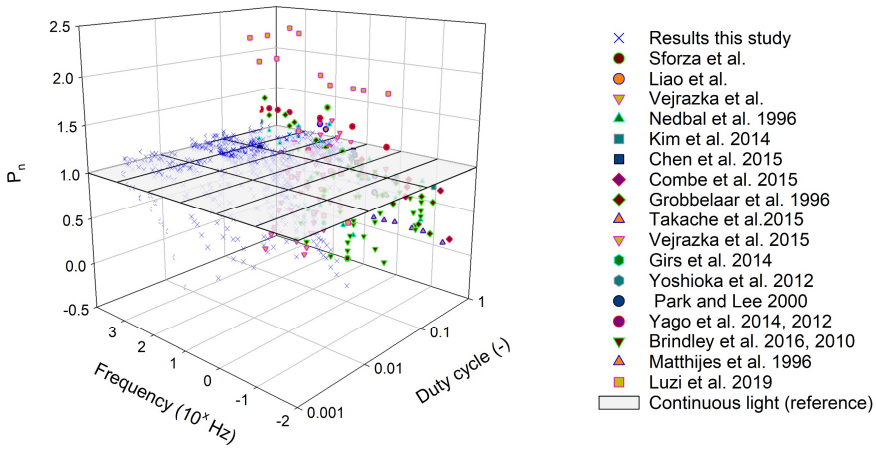

Figure 6. Summary of studies with flashing light on microalgae and cyanobacteria. Data from different studies were normalised to a continuous light reference and is given as relative growth parameter (P_n). The horizontal plane (frequency-duty cycle plane) that passes through P_n = 1 indicates the maximum value of the oxygen evolution rate obtained under continuous light. Data for each study are provided in the supplemental material (Table A.1).

If flashing light becomes inhibiting for a culture, a given duty cycle, frequency or average light intensity seems to depend on the light acclimation stage of cells and the light attenuation potential of their culture. These factors influenced the threshold frequency where growth was inhibited (e.g., f= 200-500 Hz). Among all experiments, oxygen evolution rates of cultures exposed to flashing light with frequencies >500 Hz were only similar (but not higher) to cultures exposed to continuous light with the same average light intensity. Nevertheless, the penetration of light flashes deep inside the culture may be still used to deliver signals to cells that trigger a desired metabolic pathway [e.g., pigment or fatty acid biosynthesis; (Sastre 2010)]. Until now, only few studies are found in this field and the conditions to induce a desired biocompound without impairing growth remain to be identified.

4. Conclusion

The microalgae *T. chui* and *C. stigmatophora* exposed to flashing light of various frequencies (0.01 Hz-1MHz), duty cycles (0.001-0.7) and light intensities (50-1000 µmol s^{-1} m^{-2}) always showed similar or less growth compared to continuous light. Frequencies <100 Hz and duty cycles <0.1 were inhibitory for most cultures while frequencies ≤7 Hz caused higher respiration than photosynthesis in concentrated cultures. We conclude that flashing light cannot improve growth performance of photosynthetic microalgal cultures, but may remain a promising tool to induce photoprotective metabolites such as pigments or fatty acids.

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6. Declaration of authors' contributions

P.S. (peter.schulze@nord.no) designed the experiments, performed the laboratory work, data analysis, and drafted the manuscript. C.B. (cbrindle@ual.es), J.M.-F. (jfernand@ual.es) and R.R. (ralf.rautenberger@nibio.no) contributed to discussion and critical revision of the article for important intellectual content. H.P. (hgpereira@ualg.pt) contributed to graphical design of the graphs and revised the manuscript. R.W. (rene.wijffels@wur.nl) and K.V. (kiron.viswanath@nord.no) conceived the main project, contributed to the design, reviewed and edited the article and provided the administrative support for the research project. The authors agree with the authorship this work and give final approval of the version to be submitted and any revised version.

7. Conflict of interest

The authors confirm that there is no known conflict of interest related with this publication and there has been no significant financial support for this study that could have influenced its outcome.

8. Supplementary material

Supplementary research data from the thesis can be made available upon request by contacting the Faculty PhD administration.

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Paper V

Manuscript style adapted to journal guidelines of Algal Research.

Induction of proteins, polyunsaturated fatty acids and pigments in microalgal cultures using flashing light

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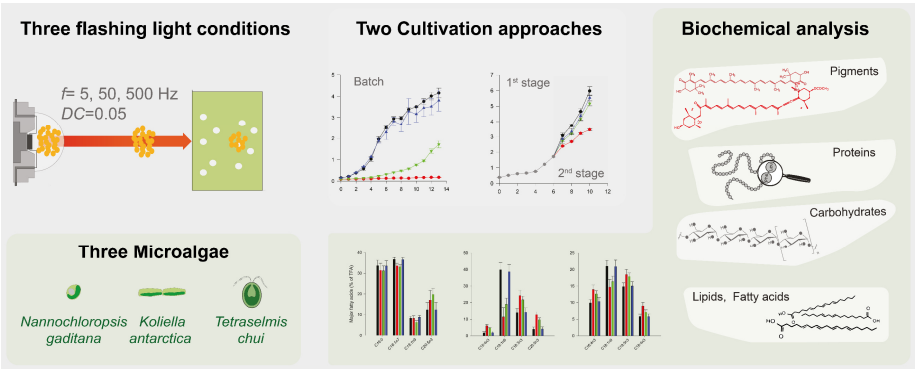

Highlights:

- Low frequency flashing light inhibits species-specific growth.
- Microalgae respond similarly to high frequency flashing light and continuous light.
- Flashing light used in a two-stage cultivation system improves pigment productivity.
- Microalgae under low frequency flashing light show low- and high-light responses.
- Protein, PUFA or pigment contents increased under low-frequency flashing light.

Abstract

Pigments, fatty acids or proteins from microalgae can be tapped by nutrition and nutraceutical industries. Industrial phototrophic microalgal cultivation is commonly achieved outdoors using sunlight as a photon source. However, countries in high latitudes face low solar irradiances, making the use of artificial light indispensable for the commercial production of microalgae. Light emitting diodes (LEDs) are often used to overcome this hindrance, and these photon sources can be tailored to emit flashing light that could improve the production of biomass and target biomolecules. Therefore, we assessed the effect of continuous and flashing light (f = 5, 50 and 500 Hz; duty cycle = 0.05) on the biomass productivity and biochemical composition of *Nannochloropsis gaditana*, *Koliella antarctica* and *Tetraselmis chui* grown in batches. In addition, a two-stage cultivation approach was implemented, where the flashing light regimes were applied only after six days of cultivation under continuous light. The biomass productivity and biochemical composition of all strains cultivated under 500 Hz were not different compared to continuous light. At lower flashing light frequencies (e.g., f= 5 and 50 Hz), a strain-dependent growth inhibition and induction of proteins, polyunsaturated fatty acids (PUFA), chlorophyll and carotenoids (lutein, *β*-carotene, violaxanthin and neoxanthin) was observed. By applying low frequency flashing light in a two-stage cultivation, productivities of eicosapentaenoic acid (EPA), violaxanthin *β*-carotene and neoxanthin was two to three times higher compared to continuous light-treated cultures. Overall, the effect of biomass concentration on the culture as indicator for the prevailing growth stage was identified as a major factor affecting the observed biochemical changes in all the tested strains. In conclusion, protein, PUFA or pigment productivities can be maximised by applying low-frequency flashing light at exponential or late growth stages and harvesting the culture at a well-defined time point.

Key words: Pulsed light, Duty cycle, Fatty acids, Pigments, Polyunsaturated fatty acids.

1. Introduction

Microalgae are being commercialized, mainly for the nutraceutical and aquafeed sectors. They are also currently considered as emerging feedstocks in the fields of bioenergy, biomaterials and bioremediation (Richmond and Hu, 2013). Beside their great potential for different biotechnological applications, current industrial scaled phototrophic microalgal cultivations faces low biomass productivities, deriving from inefficient light delivery to cells inside the culture (Ruiz et al., 2016).

The light sources, both natural and artificial, can be used for the photoautotrophic production of microalgal biomass (Blanken et al., 2013). Large scale production facilities use sunlight as the cheapest light source, and the systems are therefore commonly located in places with high annual solar irradiances (e.g., countries near the equator). On the other hand, microalgae production facilities in high latitudes rely on expensive artificial light due to insufficient year-round natural irradiance (Kim and Choi, 2014; Powers and Baliga, 2010). High costs of these production systems are connected to artificial lighting, as a result of electric energy consumption and acquisition of lamps (Blanken et al., 2013).

To cut costs and improve biomass and biocompound productivities of microalgal cultures, light emitting diodes (LEDs) can be used to emit wavelengths tailored to the strain under cultivation (Schulze et al., 2014; De Mooij et al., 2016). Alternatively, LEDs can emit flashing or pulsed light, which is the periodic supply of high intense light flashes alternated by extended dark periods. Similar to the optimal supply of light with specific wavelengths, flashing light can be tailored to improve production of microalgal biomass and accumulation of target biocompounds (De Mooij et al., 2016; Glemser et al., 2016; Phillips and Myers, 1954; Schulze et al., 2017b). Although flashing light has not always caused an improvement in microalgal growth over continuous light (Vejrazka et al., 2012; Yoshioka et al., 2012), the use of flashing light has the potential to promote the induction of target biomolecules (Katsuda et al., 2008; Kim et al., 2014, 2006). However, it has become clear that microalgae respond differently to flashing light, and hence change their protein, carbohydrate, lipid or pigment contents (Park and Lee, 2000; Schulze et al., 2017b; Sforza et al., 2012). Such differences can be due to strain-specific genetics, the use of distinct culture systems, the occurrence of unique biotic and abiotic growth conditions, and the analytical and processing procedures that are employed in each case (Moody et al., 2014). In addition, the prevailing culture growth stage at the time of harvesting is a major cause for intracellular changes of the biochemical profile in any microalga (Rebolloso Fuentes et al., 2000; Su et al., 2013).

In this context, the present study examines the growth and changes in the protein, carbohydrate, lipid, fatty acid and pigment contents of the Eustigmatophyte *Nannochloropsis gaditana*, and of the chlorophytes *Tetraselmis chui* and *Koliella antarctica* exposed to flashing light. Flashing light conditions that effectively induce biocompounds often inhibit growth (Schulze et al. 2017b), necessitating a production system that employs a two stage cultivation approach. In the first stage, enough algal biomass is produced under growth-stimulating light conditions, while the second stage that employs flashing light helps in induction of target biocompounds. Hence, we have employed one- and two-stage cultivation systems to assess the efficiency of flashing LEDs in artificial light-based facilities located in countries with low solar irradiances (e.g., in Norway).

2. Materials and methods

2.1. Experimental setup

Nannochloropsis gaditana (CCAP 849/5) (Ochrophyta, Eustigmatophyceae) was obtained from the Scottish Association for Marine Science (Oban, Scotland); *Tetraselmis chui* (Chlorophyta, Chlorodendrophyceae), Butcher (SAG 1.96) and *Koliella antarctica* (SAG 2030) (Chlorophyta, Trebouxiophyceae) were obtained from the Culture Collection of Algae at Göttingen University (SAG, Germany). *N. gaditana* was used as a representative mesophilic model strain, whereas *T. chui* and *K. antarctica* were tested because of their promising growth performance at extreme latitudes (Schulze et al., unpublished data, Suzuki et al., 2018). The cultivation temperature for *N. gaditana* was 20 °C, while *T. chui* and *K. antarctica* cultures were grown at 15°C.

The inocula used for all experiments were grown in Erlenmeyer flasks placed on orbital shakers for 14 days. The flasks were illuminated from above at an intensity of 100 µmol s^{-1} m^{-2} supplied by cool-white fluorescent lamps (Philips TLD 840, Amsterdam, Netherlands). Seawater from the North Atlantic shoreline of Bodø (Norway, 35 ppt) was enriched with a modified F-medium consisting of 5.3 mM $NaNO_3$, 0.22 mM $NaH_2PO_4H_2O$, 35 µM $FeCl_3$ $6H_2O$, 35 µM Na_2EDTA $2H_2O$, 0.12 µM $CuSO_4$ $5H_2O$, 0.078 µM Na_2MoO_4 $2H_2O$, 0.23 µM $ZnSO_4$ $7H_2O$, 0.126 µM $CoCl_2$ $6H_2O$ and 2.73 µM $MnCl_2$ $4H_2O$.

Tissue culture flasks (Falcon Scientific, Seaton Delaval, UK) with a total volume of 250 mL and 30 mL were filled with 200- and 25-mL cultures for one-stage or two-stage cultivation systems, respectively. The light paths were 3.7 and 2.0 cm in the 250- and 30-mL flasks, respectively. The cultures were placed on different shelves inside a climate

chamber and illuminated from the front by 36 LEDs (2700 K, MHDGWT-0000-000N0HK427G-SB01, Opulent Americas, Raleigh, US, emission spectrum provided in Table A.1) with the following light regimes: continuous light and flashing light with a duty cycle of 0.05 with the frequencies of 5, 50 and 500 Hz (see Table A.1 for definition of flashing light conditions). The time-averaged light intensity was I_a= 300 µmol s^{-1} m^{-2} in all conditions, which corresponded to an instantaneous flash intensity of I_f= 6000 µmol s^{-1} m^{-2}. Each shelf was covered in aluminium foil to prevent light interference among treatments. The cultures were mixed by aeration, with humidified and 0.2 µm-filtered air enriched with CO_2 (1% v/v; GMS-150, Photon Systems Instruments, Drasov, Czech Republic) at a flow rate of 160 mL min^{-1}. All algae were cultivated during the one-stage batch cultivation for 13 days (n = 3). The two-stage cultivation approach consisted of a first stage (1) during which cultures were grown for 6 days at I_a= 300 µmol s^{-1} m^{-2} under continuous light in the same flasks used in the first experiment, and a second stage (2) where the cultures (n = 3) were split into 30-mL flasks and cultivated for five days under the same (flashing-) light treatments used for the one-stage approach. Continuous light was used as a control treatment across all experiments employing the same average supplied light intensity as used under flashing light (I_a=300 µmol s^{-1} m^{-2}).

Optical density at 540 nm (OD_{540}) was measured daily (n = 4) for each algal culture using a 96-well plate spectrophotometer (Tecan Sunrise A-5082, Männedorf, Switzerland). To determine the biomass concentration in the culture in grams of dry weight (DW) per litre, a known volume of algal suspension was filtered using glass fibre filters (pore size ø = 0.7 µm; VWR), washed twice with 10 mL ammonium bicarbonate (0.5 M) and dried at 105°C for 24 h. The dry weight was determined gravimetrically. Significant linear correlations between OD_{540} and dry weight were obtained for each microalga (r^2≥ 0.9, p< 0.05).

Culture samples for biochemical analysis were taken at the end of all experiments. The harvested cultures were centrifuged (5000 *g*, 5 min), washed (0.5 M ammonium bicarbonate), freeze-dried and stored at -80 °C until further analysis. Furthermore, at the end of the cultivation an aliquot of sample was diluted to reach the concentration of approximately 1g DW L^{-1} and centrifuged at 5000 *g* for 5 min at 10 °C. The pellet was stored at -20 °C for spectroscopic quantification of total carotenoids and chlorophylls.

2.2. Light source

An array of 36 warm-white LEDs (2700 K, MHDGWT-0000-000N0HK427G-SB01), mounted on an actively cooled aluminium heat sink, with a total length, height and width of 300, 75 and 40 cm, respectively, was used as an artificial light source. The test flashing light conditions (f= 5, 50, 500 Hz, DC= 0.05) were adjusted by PWM-OCX (RMCybernetics Ltd, Alsager, UK) pulse width modulators (PWMs) powered by bench EA-PS 2084-05B (EA Elektro-Automatik) power supply units. The pulse signal was provided by a TG4001 (TTi, Huntingdon, UK) function generator or directly through the PWMs. For the continuous light control, the LEDs were directly connected to a current-controlled EA-PS 2084-05B power supply units. The voltages and currents supplied to the LEDs were regulated by the power supply units to adjust I_a to 300 µmol s^{-1} m^{-2} and compensate switching and working losses at the LEDs and PWMs. The supplied light intensity (i.e., photosynthetically active radiation) was measured for 1 min at the same position as the flasks (SPQA 5234 connected to a data logger LI-1500, Li-Cor, Lincoln, USA) and averaged over time.

2.3. Total lipids, proteins and carbohydrates

Total lipids were determined gravimetrically using a modified Bligh and Dyer (1959) method as described in Pereira et al. (2011). Briefly, 10 to 30 mg of freeze-dried microalga samples were weighed and transferred to glass tubes. Afterwards, 0.8 mL of distilled water was added to soften the samples and kept for 20 min. Subsequently, 2 mL methanol and 1 mL chloroform were added and homogenized with an IKA T18 Ultra-Turrax disperser (IKA-Werke GmbH & Co. KG, Staufen, Germany) in an ice bath for 60 seconds. A 1-mL aliquot of chloroform was added and samples were homogenized for 30 seconds, followed by the addition of 1 mL of distilled water and further homogenized for 30 seconds. Phase separation was performed by centrifugation (2000 *g* for 10 min). Subsequently, 1 mL of the organic phase was transferred into a new pre-weighed tube, and the chloroform was evaporated at 60 °C in a dry bath overnight and cooled down to room temperature in a desiccator. Finally, the remaining lipids in the tube were determined gravimetrically.

Protein contents of the algal biomass was determined with a Bio-Rad DC™ Protein Assay (Bio-Rad Ltd., Hemel Hempstead, UK). Water-soluble proteins from freeze-dried biomass were extracted by re-suspending ~7 mg freeze-dried biomass in 4 mL NaOH (1 M) and bead milled using three cycles of 60 s (6500 rpm, 120 s break between bead milling; Precellys Evolution, Bertin technologies, Montigny-le-Bretonneux, France). The

samples along with glass beads were centrifuged (2000 *g*, 10 min, 20°C) and the supernatant was transferred into clean vials. The water-soluble proteins contained in the supernatant was measured according to Bio-Rad *DC* Protein Assay manual, at a wavelength of 750 nm (Dr3900, Hach Lange, Salford Quays, UK).

An elemental analysis of the harvested biomass for carbon (C), hydrogen (H) and nitrogen (N) was conducted (Vario EL iii, Elementar Analysensysteme GmbH, Langenselbold, Germany). Thereafter, the protein contents obtained by the Bio-Rad DCTM Protein Assay were confirmed by multiplying the nitrogen content in the biomass (%N) by 4.78 (Lourenço et al., 2004).

Total carbohydrate content was determined according to Trevelyan et al. (1952). Briefly, 10 mg of freeze-dried biomass were suspended in 3 mL HCl 37% (v/v) and hydrolysed in a water bath for 1 h at 100°C. Subsequently, 4 mL of a fresh anthrone solution (Sigma-Aldrich, Oslo, Norway, 2 mg mL^{-1} in 99% H_2SO_4) were added to 1 mL of sample extract. The absorbance of each sample was read at 620 nm (Dr3900, Hach Lange, Salford Quays, UK). Aliquots of different glucose concentrations (0.02-0.1 mg L^{-1}) were prepared and processed in the same way as microalgal extracts, to obtain a calibration curve.

The contents of protein, carbohydrates and total lipids in microalgal biomass were expressed as % of DW.

2.4. Fatty Acid Analysis

For fatty acid analysis, 4 mL of a chloroform:methanol solution (2:2.5 v/v) containing an internal standard (Tripentadecanoin, C15:0 Triacylglycerol, Sigma-Aldrich, Oslo, Norway) were added to ~6 mg of freeze-dried microalgal biomass. Cells were disrupted by bead milling using 0.1 mm glass beads (Precellys Evolution, Bertin technologies, Montigny-le-Bretonneux, France). Thereafter, 2.5 mL tris-buffer (6 g L^{-1} Tris, 58 g L^{-1} NaCl) were added, mixed with a vortex mixer and centrifuged (2000 *g*) to separate the phases. The lower chloroform-phase containing the lipids was transferred into a new glass tube and evaporated under a gentle nitrogen flow to prevent fatty acid oxidation. Subsequently, 3 mL of a methanol solution containing 5% H_2SO_4 were added and kept for 3 h at 70 °C, to convert the fatty acids into the corresponding methyl esters. After the reaction, 3 mL of hexane were added and mixture was shacked for 15 min in an orbital shaker. Finally, the fatty acid methyl esters (FAMEs) in the hexane phase were quantified using gas chromatography equipped with a Flame Ionisation Detector (SCION 436m Bruker, Massachusetts, US) and a CP-Wax 52 CB column (Agilent, Santa Clara, US)

using split-less mode. To identify and quantify the most common FAMEs, an external 37-compound standard (Supelco, Bellefonte, US) was used.

2.5. Spectrometric Pigment Analysis

For the chlorophyll and total carotenoid extraction, beat beads were added to the tube with the algae pellet (containing ~1 mg DW) together with 1.5 mL of methanol (100%). Tubes were beat milled for three cycles of 20 s at 6000 rpm (Bertin technologies, Precellys Evolution, Montigny-le-Bretonneux, France). Subsequently, tubes were stored in ice in darkness for 2 h. Afterwards, the extract along with glass beads were mixed (Vortex mixer, Stuart, UK) and centrifuged (7000 *g*, 10 min, 10°C). Finally, a 1-mL aliquot of the supernatant was transferred into a micro-cuvette (1 mL, Polystyrene cuvettes VWR, Oslo, Norway) and the spectrum from 400 to 700 nm was recorded by means of a Uviline 9400 (Schott, Mainz, Germany) spectrophotometer. All analyses were done under dimmed light. Chlorophyll *a* (C_a) and *b* (C_b) and total carotenoids (C_{carot}) were determined according to Lichtenthaler and Wellburn (1983) by applying the OD measurements at 666, 653 and 470 nm (A_{666}, A_{653}, A_{470}) from the methanol extracts to equations **1**, **2** and **3**:

$$C_a = 15.65\ A_{666} - 7.34\ A_{653} \quad \textbf{1}$$

$$C_b = 27.05\ A_{653} - 11.21\ A_{666} \quad \textbf{2}$$

$$C_{carot} = (1000\ A_{470} - 2.86\ C_a - 129.2\ C_b)/221 \quad \textbf{3}$$

Because *Nannochloropsis* microalgae do not contain chlorophyll *b*, a modified formula according to Henriques et al. (2007) was used (equations **4** and **5**):

$$C_a = 15.65\ A_{666} \quad \textbf{4}$$

$$C_{carot} = (1000\ A_{470} - 44.76\ A_{666})/221 \quad \textbf{5}$$

Results obtained from equations **1**-**5** in $\mu g_{pigments}\ mL^{-1}$ were divided by the sample concentration (1 g DW L^{-1}) to obtain the pigment concentration in terms of algal biomass ($\mu g_{pigment}\ g\ DW^{-1}$).

2.6. HPLC Pigment Analysis

For extraction of single carotenoids (lutein, *β*-carotene, violaxanthin and neoxanthin), ~3 mg of freeze-dried biomass was transferred into a tube together with 50 µL deionized water. Afterwards, 3 mL acetone and 0.7 g of glass beads were added; the samples were then vortexed for 2 min (Vortex Mixer, Stuart, UK) and centrifuged (5000 *g*, 5 min). Subsequently, the supernatant was transferred into a clean and light-proof tube and the extraction was repeated sequentially thrice. Acetone was evaporated under a gentle nitrogen stream and resuspended in methanol prior to HPLC injection. Carotenoid extracts were analysed with a Dionex 580 HPLC System (DIONEX Corporation, Sunnyvale, United States) equipped with a PDA 100 Photodiode-array detector and STH 585 column oven set to 20°C. Separation of the compounds was achieved using a LiChroCART RP-18 (5 µm, 250x4 mm, LiChrospher, Merck KGaA, Germany) column with a mobile phase consisting of acetonitrile:water (9:1; v/v) as solvent A and ethyl acetate as solvent B and a constant flow of 1 mL min^{-1}. The gradient program applied was: (i) 0–16 min, 0–60% B; (ii) 16–30 min, (iii) 60% B; (iv) 30–32 min 100% B and (v) 32-35 min 100% A (adapted from Couso et al. 2012). The injection volume of the samples was 100 µL, chromatograms were recorded at 450 nm and analysed by Chromeleon Chromatography Data System software (Version 6.3, ThermoFisher Scientific, Massachusetts, US). For the quantification of individual carotenoids, calibration curves for lutein, *β*-carotene, violaxanthin and neoxanthin were used. All pigment standards were supplied by Sigma-Aldrich (Sintra, Portugal). All HPLC grade solvents were purchased from Fisher Scientific (Porto Salvo, Portugal).

2.7. Data analysis

Growth parameters were estimated according to Ruiz et al. (2013) and are detailed in Table A.1 (supplementary material). Productivities of target biocompounds were calculated for the two-stage cultivation system by multiplying the total amount of a given biocompound in the biomass (e.g., mg gDW^{-1}) by the biomass concentration in the culture (g DW L^{-1}) and dividing by the total cultivation time (10 days).

ANCOVA analysis were performed to detect differences in intracellular biochemical contents among *treatments*, *strains* and *cultivation approaches* (one- or two stage) while considering the co-variate *biomass concentration* at the time point of harvesting (X_t in g DW L^{-1}) as indicator for the prevailing growth stage. The inclusion of biomass concentration in the statistical analysis was necessary to separate the effects of the treatments (e.g., flashing light) and strains from culture maturation (or aging) effects. Two-way ANOVA was used to detect differences in productivity data using the explanatory variables *strains* and *treatment*.

To compare biomass productivities or biochemical contents among different treatments and strains, all data were normalised with the control treatment (continuous light). In this case, productivities approaching a value of 1 are similar to continuous light, while values lower or higher than 1 indicate a lower or higher productivity under a given treatment, respectively.

Biomass concentration data were log10-transformed to meet the assumption of linearity and homogeneity of variance. The Type III sum of squares analysis was considered and the output *F*-values together with *p*-values were used to describe the impact of treatment, biomass concentration, strain or cultivation approach on the response variables (biomass productivity, protein, carbohydrates, lipids, fatty acids or pigments). The adjusted means with 95% confidence interval from Tukey´s post hoc tests were used to illustrate the results of the ANCOVA and ANOVA analysis in the figures.

Pearson´s correlations (*r*) were used within the ANCOVA analyses to quantify the effects of biomass concentration on the response variables. The closer the *r*-value to 1 or -1, the stronger the positive or negative effect of culture maturation, respectively. A significance level (α) of 0.05 was used for all tests. Normality of the response variables was tested using the Shapiro-Wilk test.

3. Results and discussion

3.1. Growth performance

The growth curves of *N. gaditana*, *K. antarctica* and *T. chui* were obtained for three flashing light conditions (5, 50 and 500 Hz) and a control grown under continuous light (CL), for one-stage (Fig.1 A, C, E) and two-stage (Fig. 1 B, D, F) cultivation-systems. The results obtained revealed that the microalgal cultures growing under the highest flashing light (FL) frequency tested (*f* = 500 Hz; FL 500) reached similar growth as those under CL. Conversely, microalgae under FL at low frequencies (*f* = 5 and 50 Hz) often showed slower growth (Fig. 1; Table 1). Using the one-stage cultivation approach, all tested strains under FL at 5 (FL 5) and 50 (FL 50) Hz displayed lower biomass productivity and a prolonged lag phase as compared to those under FL 500 and CL. Notably, *T. chui* cultures performed better at FL 50 when compared to those of other species (Fig. 1, Table 1). For example, biomass productivity of *T. chui* was only about 30% lower than cells under CL, whereas *N. gaditana* and *K. antarctica* cultures displayed a 70% decrease in biomass productivity (Table A.1).

In the two-stage cultivation approach, all strains treated with the lowest frequency (FL 5) consistently presented lower biomass productivities when compared to control cultures under CL. On the other hand, only *K. antarctica* under the FL 50 treatment showed decreased biomass productivity, while *N. gaditana* and *T. chui* showed similar values as obtained under CL (Table 1). However, all strains cultured in the two stage approach and exposed to FL 5 reached considerably higher biomass productivities (0.34-0.43 g DW L^{-1} d^{-1}) as compared to those cultivated in the one-stage approach (<0.05 g DW L^{-1} d^{-1}).

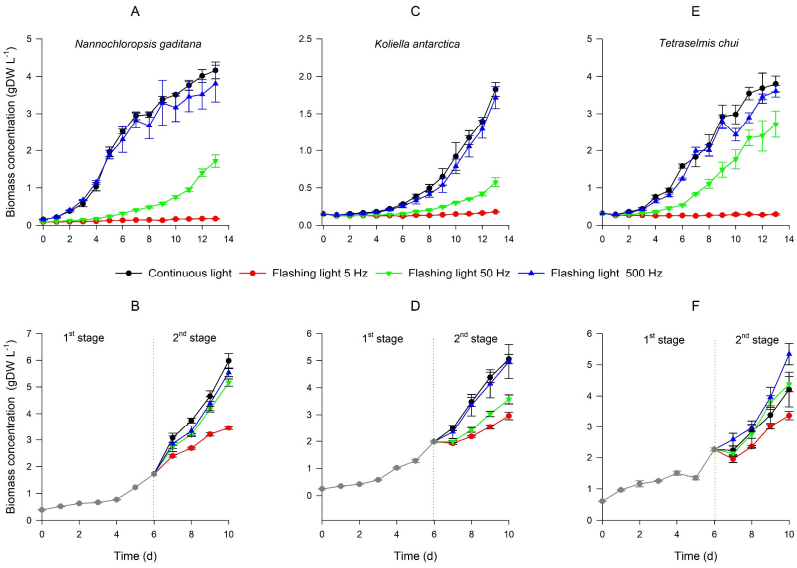

Figure 1. Growth curves of algae grown in a one- and two stage batch culture under different flashing light conditions. *Nannochloropsis gaditana* (A, B), *Koliella antarctica* (C, D) and *Tetraselmis chui* (E, F) were cultivated under continuous light or flashing light with a duty cycle of 0.05 and frequencies of 5, 50, 500 Hz at an average light intensity of I_a= 300 µmol s^{-1} m^{-2}. Data points at each day are shown as mean ±SD, *n*=3.

Previous studies conducted in various algae and plants (e.g., *Dunaliella salina, Nannochloropsis salina*, *Lactuca sativa* (Combe et al., 2015; Jishi et al., 2015; Simionato et al., 2013a) indicated that if the FL frequency is high enough, the photosynthetic apparatus cannot distinguish between single FL pulses and CL (Grobbelaar et al., 1996), a condition referred to as the "flashing light effect" (Schulze et al., 2017b) . Other studies (Abu-Ghosh et al., 2015; Lunka and Bayless, 2013; Park and Lee, 2000; Grobbelaar et al., 1996) have suggested that microalgal cultures could benefit from intense light flashes, which improve photon penetration into the culture. In the present study, the authors could not identify a significant growth enhancement of the strains tested. However, biomass productivities under FL became similar to CL at higher frequencies (e.g., FL 500). The thresholds of specific flashing light parameters (e.g., frequency and duty cycle) beyond which growth performance becomes similar to those for CL depends on various factors, including the applied average light intensity of flashing light and species. For example, in both higher plants (Jishi et al. 2015) and cyanobacteria (Xue et al. 2011), the inhibitory effects of low-frequency FL increase when the applied average light intensity

is higher. In the present study, microalgae-specific responses were observed; *T. chui* responded better to low-frequency FL (e.g., FL 50) as compared to *K. antarctica* or *N. gaditana.* Similarly, Nedbal et al. (1996) tested different strains under various flashing light conditions and identified strain-specific threshold frequencies. Jishi et al. (2015) showed that such thresholds depend on various factors, including morphological or photoprotective strategies of phototrophs. In microalgae, cell architecture and size determines the amount of light that gets absorbed and that is used for photosynthesis inside the cell (Dubinsky et al., 1986). As regards the cell size, *T. chui* cells that have an oval-shape (13 × 5 × 4 µm; Bottino et al., 1978) and an estimated volume of about 260 μm^3 are larger than those of other tested strains; *N. gaditana*, round-shaped, with a diameter of 2-4 µm and a volume of 40 μm^3 (Rocha et al., 2003), or *K. antarctica*, cylindrical shaped, 7.5 × 2.5 µm (La Rocca et al., 2009) with a volume of 60 μm^3. Since light absorbing characteristics of the cell will determine the light available for photosynthesis (Dubinsky et al. 1986), larger cells may withstand better low-frequency FL conditions than smaller cells, because they are less susceptible to photoinhibition, as confirmed by Key et al. (2010), and cope better with long-lasting high-light intensities applied at low flashing frequencies (e.g., FL 5).

Table 1. Biomass productivities (in g DW L^{-1} d^{-1}) of *Nannochloropsis gaditana*, *Koliella antarctica* and *Tetraselmis chui* under continuous light (CL) and flashing light at frequencies of 5 (FL 5), 50 (FL 50) and 500 (FL 500) Hz and a duty cycle of 0.05 using a one or two-stage cultivation approach. Productivities that do not share the same letter for a given alga in a particular cultivation approach are significantly different from each other.

	One-stage cultivation								Two-stage cultivation							
	CL		FL 5		FL 50		FL 500		CL		FL 5		FL 50		FL 500	
Nannochloropsis gaditana	0.361 (0.003)	a	0.008 (0.000)	c	0.085 (0.009)	b	0.329 (0.046)	a	0.686 (0.056)	a	0.431 (0.012)	b	0.602 (0.016)	a	0.653 (0.035)	a
Koliella antarctica	0.117 (0.021)	a	0.042 (0.025)	b	0.032 (0.004)	b	0.106 (0.013)	a	0.588 (0.024)	a	0.340 (0.012)	b	0.407 (0.023)	b	0.636 (0.069)	a
Tetraselmis chui	0.255 (0.022)	a	0.000 (0.000)	c	0.183 (0.031)	b	0.246 (0.024)	a	0.510 (0.029)	a	0.393 (0.002)	b	0.531 (0.029)	a	0.523 (0.032)	a

In the adopted two-stage cultivation approach, low-frequency FL led to a less pronounced decrease in growth compared to those observed with the one-stage approach. This might be due to differences in biomass concentration prior to the application of the FL regime. For example, in the two-stage approach, a higher biomass concentration (≈2 g DW L^{-1}) during the second stage allowed the distribution of the total dose of supplied photons to more cells. On the other hand, the one-stage cultures started at a low initial biomass concentration (0.1 g DW L^{-1}) and all cells received the full

applied photon dose (e.g., I_a= 300 µmol s^{-1} m^{-2}, I_l=6000 µmol s^{-1} m^{-2}). Therefore, the perceived average light intensity by each microalgal cell was lower in the two-stage compared to the one-stage cultivation approach. As mentioned by Xue et al., (2011), a low average light intensity is less disruptive than the higher intensities under low-frequency FL conditions. In addition, the higher biomass concentrations and productivities in the two-stage cultures stem from shorter light paths (L= 2.0 cm) compared to those in the flasks used for the one-stage approach (L= 3.7 cm).

3.2. Biochemical composition

3.2.1. Proteins, carbohydrates and lipids

The proximate composition of macronutrients in the three strains, grown under different light regimes, was evaluated with an ANCOVA analysis and Tukey´s post hoc test (Fig. 2, Table A.1). The results obtained revealed that the total protein content registered for *N. gaditana* (20.5±1.8%) was similar to that of *K. antarctica* (23.8±1.9%). However, *T. chui* showed the lowest protein contents (14.6±1.9%). The ANCOVA model for protein content was mostly affected by the growth stage (F= 55.8, p< 0.01) and to a lesser extent by the species (F= 16.7) and light treatments (F= 5.7). The content of total carbohydrates was lowest in *N. gaditana* (9.6±2.7%, p< 0.01) compared to the values obtained for *K. antarctica* (28.3±2.9%) and *T. chui* (26.7±2.8%), which displayed similar values. Regarding total lipids, *N. gaditana* (36.8±2.3%) displayed the highest content, followed by *K. antarctica* (15.7±2.4%), whereas *T. chui* (11.6±2.4%) presented the lowest values. The overall proximate composition results match those previously reported for *Nannochloropsis*, *Koliella* and *Tetraselmis* strains (Camacho-Rodríguez et al., 2015; Dinesh Kumar et al., 2018; Fogliano et al., 2010; Hulatt et al., 2017; Khatoon et al., 2018; Schulze et al., 2017a; Suzuki et al., 2018).

When the light regimes are compared, the protein levels (Fig. 2A) tended higher for all strains under FL 5 and FL 50 as compared to CL. On the contrary, a decrease of carbohydrates (Fig. 2B) and lipids (Fig. 2C) was only observed in *T. chui* and *N. gaditana* cultures, respectively. No noteworthy effects on proteins, carbohydrates and lipids were found for FL 500 treated cultures compared to CL.

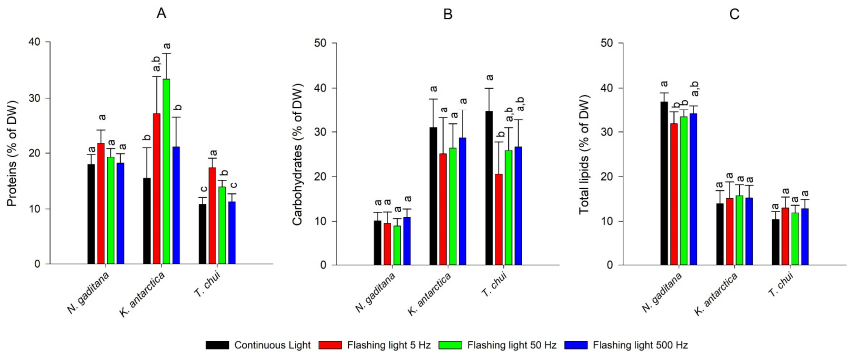

Figure 2. Biochemical composition of *Nannochloropsis gaditana*, *Koliella antarctica* and *Tetraselmis chui* exposed to flashing light. Total proteins (A), carbohydrates (B) and lipids (C) expressed as % in dry weight (DW) are shown by the adjusted means ±95% confidence interval obtained from Tukey´s post hoc test (ANCOVA) for one- and two stage cultivation (*n*= 3). Treatments that do not share the same letter are significantly different from each other.

Previous studies hardly reported effects of low frequency flashing light on the protein, carbohydrate and lipid content of *Scenedesmus bicellularis* (*f*= 100-120 Hz, *DC*=0.1-0.5), or *Scenedesmus obliquus* (*f*= 5,10,15 Hz, *DC*= 0.5) (Gris et al., 2014; Mouget et al., 1995). In the present study, effects of flashing light on proteins, carbohydrates and lipids seemed to be species-dependent. For example, *K. antarctica* exposed to FL 50 doubled its protein contents (from 15 to > 30%) when compared to cultures under CL (Fig. 2A). Low solar irradiance at extreme latitudes make polar strains more prone to low-light adaption, which is also connected to dense packaging of pigments and binding proteins. Furthermore, polar microalgae seem to have a higher number of ribosomal proteins to counteract cold stress (Lyon and Mock, 2014; Toseland et al., 2013). The high protein levels found in *K. antarctica* cells exposed to low-frequency FL may be thus explained by its natural cold-adaptation and a response to low-light. Unlike *K. antarctica*, the tested *T. chui* strain seemed not to be a true psychrotroph (Schulze et al., unpublished data) and naturally only contained low amounts of proteins, which could explain the minor effects of FL 5 and 50 treatments on protein contents. However, future work should be carried out to investigate if similar trends could be also observed in other polar strains.

On the other hand, *Nannochloropsis* is known to upregulate lipid biosynthesis under high-light conditions (Packer et al., 2011; Solovchenko et al., 2008). In the present study, *N. gaditana* showed lower lipid and high protein levels when exposed to FL 5 and FL 50

(Fig. 2C), which also indicates a low-light adaption. On the other hand, *T. chui* showed lower carbohydrate contents in cultures exposed to FL 5 and FL 50, which was previously described as low-light response in *Tetraselmis* (Michels et al., 2014). Indeed, low-light responses of microalgae exposed to low-frequency flashing light was reported by Grobbelaar et al., (1996). At high frequencies, we did not find any differences in protein, carbohydrate and lipid levels of the strains compared to CL, indicating no inhibition by high frequency flashing light, as previously reported (e.g., f> 100Hz; Yoshioka et al., 2012). In terms of productivity in the two-stage cultivation system, the most promising species for protein production were *N. gaditana* and *K. antarctica* (average: 75.4±4.3 and 77.8±17.1 mg L^{-1} d^{-1}, respectively). *T. chui* and *K. antarctica* were most promising for carbohydrate production (average: 119.8±49.1 and 137.7±14.2 mg L^{-1} d^{-1}, respectively), while lipids (average: 136.7±46.2 mg L^{-1} d^{-1}) were most efficiently produced by *N. gaditana*. Notably, protein productivities were on average 1.1-1.3 times higher for all microalgae when cultivated under flashing light (FL 5 and FL 50) compared to CL, while carbohydrate and lipid productivity decreased by 20-50% (Table A.1).

In general, protein levels correlated negatively with the biomass concentration of all species (r= -0.8, p< 0.01). This trend can also be described as growth stage-dependent protein drop and a strain-specific accumulation of intracellular carbohydrates or lipids at late growth stages (Brown et al., 1996; Lv et al., 2010; Zhu et al., 1997). *N. gaditana* is a lipid-accumulating microalga, which becomes evident from the positive correlation of the biomass concentrations with the lipid contents (r= 0.8, p< 0.01; Table A.1) and the negative correlation with carbohydrate contents (r=-0.6, p<0.05; Table A.1). On the other hand, the biomass concentration of *K. antarctica* and *T. chui* cultures correlated positively with the total carbohydrate levels (r= 0.8, 0.4; p< 0.05, p= 0.88, respectively) but not with lipids (r= -0.2 and -0.4, p> 0.05). Indeed, these results match data from previous studies describing carbohydrate or lipid accumulation in *Nannochloropsis* and *Tetraselmis* strains during the late growth stages (Dunstan et al., 1993; Kim et al., 2016). We did not find any previous reports connected to the macronutrient accumulation in the genus *Koliella*.

Taken together, these results suggest that processes regulating the biosynthesis of proteins, carbohydrates and lipids depend on the growth stage and the flashing light conditions. The biochemical response to low frequency flashing light suggests a low-light adaption in all tested species, indicated by lower carbon-bound compounds (carbohydrate or lipids) and higher proteins compared to CL. Lastly, the magnitude of

low-frequency flashing light effects on a given compound is species-specific (e.g., lipids in *Nannochloropsis*, proteins in *Koliella* and carbohydrates in *Tetraselmis*).

3.2.2. Fatty acids

The fatty acid profile (given in % of total fatty acids; TFA) differed among species and treatments (Table A.1 and Fig. 3). The major fatty acids in *N. gaditana* were C16:0 (palmitic acid, 9-40%), C16:1*n*-7 (23-40%) and C20:5*n*-3 (eicosapentaenoic acid; EPA, 6-41%). In *K. antarctica,* C16:0 (8-13%), C18:1*n*-9 (oleic acid; 9-43%), C18:2*n*-6 (linoleic acid; 3-13%), C18:3*n*-3 (α-linolenic acid; ALA, 10-34%) and C20:5*n*-3 (4-14%) were the most abundant fatty acids. Regarding the fatty acid profile of *T. chui,* mostly C16:0 (6-16%), C16:4*n*-3 (hexadecatetraenoic acid; 9-16%), C18:1*n*-9 (10-23%), C18:3*n*-3 (11-20%) and C20:5*n*-3 (9-13%) were found. The fatty acid profiles obtained are in accordance with those previously reported for the genera *Nannochloropsis* (Hulatt et al., 2017), *Koliella* (Fogliano et al., 2010; Suzuki et al., 2018) and *Tetraselmis* (Lang et al., 2011; Mohammadi et al., 2015; Rasoul-Amini et al., 2014). *N. gaditana* showed the highest productivities of fatty acids (104.3 mg TFA, 16.5 mg SFA, 66.1mg MUFA and 19.7 mg PUFA L^{-1} d^{-1}), confirming that this oleaginous species presented the highest lipid contents among the microalgae tested (Fig 2C).

The TFA (Fig. 3A) content did not differ under any flashing light treatment compared to CL in all tested strains. Concerning SFA (Fig. 3B), *N. gaditana* and *K. antarctica* did not show any difference between treatments, whereas *T. chui* contained less SFA under the FL 50 treatment. Interestingly, across all algae, the MUFA (Fig. 3C) fraction tended to be lower in microalgae under FL 5 and FL 50 compared to those under FL 500 and CL. On the other hand, higher PUFA contents (Fig. 3D) were obtained in all species tested under FL 5 and FL 50 compared to those grown under FL 500 and CL.

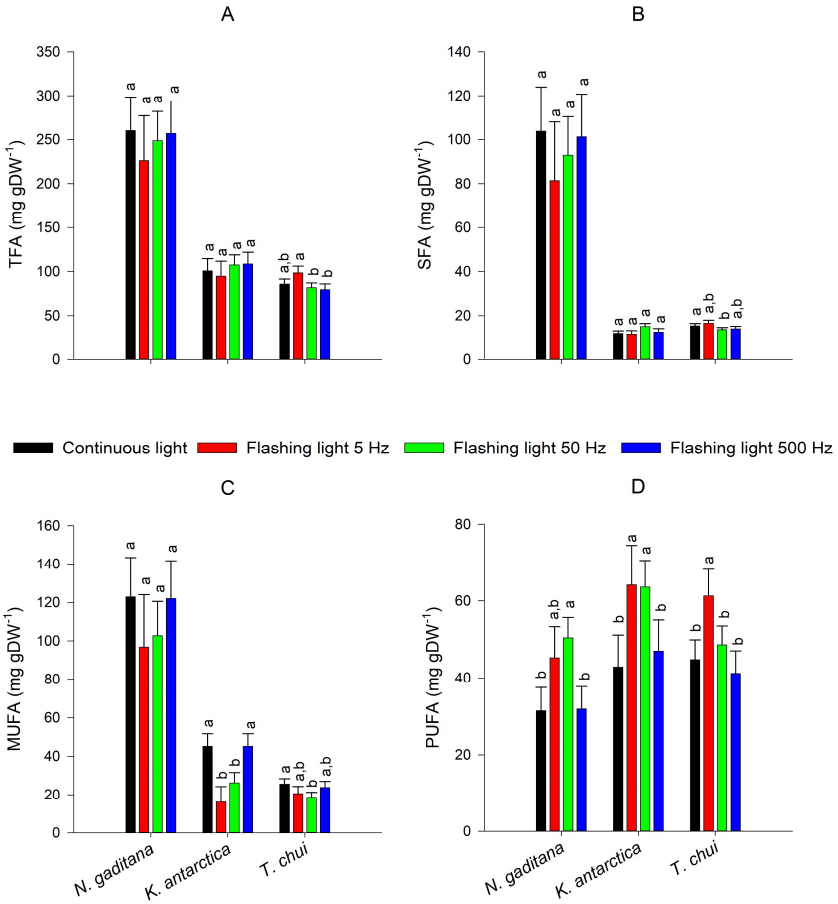

Figure 3. Major fatty acid classes for *Nannochloropsis gaditana*, *Koliella antarctica* and *Tetraselmis chui* exposed to flashing light. Contents of total fatty acids, TFA (A), saturated fatty acids, SFA (B), monounsaturated fatty acids, MUFA (C) and polyunsaturated fatty acids, PUFA (D) in dry weight (mg g DW^{-1}) are given as adjusted means ± 95% confidence interval obtained from Tukey´s post hoc test (ANCOVA) for one- and two stage cultivation (n= 3). Treatments that do not share the same letter are significantly different to each other.

Low frequency flashing light (FL 5 and 50) caused often an accumulation of species-specific PUFA at the expenses of MUFA (Fig. 4). For example, *N. gaditana* accumulated more C20:5*n*-3 under FL 5 and FL 50, whereas the MUFA, C16:1*n*-4 and 18:1*n*-9, tended to decrease (Fig. 4A). Conversely, when exposed to FL 500, this microalga showed a fatty acid profile comparable to cells under CL. Similarly, *K. antarctica* increased the major PUFA, C18:3*n*-3 and C20:5*n*-3, at the expense of the MUFA C18:1*n*-9 under FL 5 and 50,

compared to CL and FL 500. Lastly, *T. chui* showed higher amounts of C16:4*n*-3, C18:3*n*-3 and C18:4*n*-3 and lower C18:1*n*-9 contents upon exposure to FL 5 and FL 50, whereas no effect on C20:5*n*-3 was observed. Similarly, productivities of C20:5*n*-3 increased in FL 5 and FL 50 treated *N. gaditana* and *K. antarctica* cultures by 1.4-1.9 times, while *T. chuii* showed a 1.4 times higher C18:4*n*-3 productivity compared to CL (Table A.1).

Apart from effects caused by flashing light, significant correlations between fatty acids and biomass concentration were observed. For example, biomass concentration correlated positively with TFAs, SFA and MUFA in *N. gaditana* (r= 0.8 to 0.9, p< 0.05) and negatively in *T. chui* (r= -0.8 to -0.9, p< 0.01). Conversely, PUFA contents correlated negatively with biomass concentration in all microalgae (r= -0.5 to -0.8; Table A.1). However, a significant effect was noted only for the ANCOVA model for *N. gaditana* (p< 0.05; Table A.1). Major PUFA in *N. gaditana,* C20:5*n*-3, decreased with increasing biomass concentration (r= -0.9, p< 0.01). This effect was stronger (F= 48.9) compared to that of flashing light treatments (F= 5.7, p< 0.01). In *K. antarctica*, C18:3*n*-3 and C20:5*n*-3 tended to decrease with increasing biomass concentration (r= -0.8 to -0.6, p<0.5), an effect that was not as strong (F=0.6, 8.9) as that of the flashing light treatments (F=9.2, 21.5). In *T. chui*, a correlation between biomass concentration and C18:3*n*-3 (r= 0.7, p< 0.01) and C18:4*n*-3 (r= -0.9, p< 0.01) levels was stronger (F=26-78) as compared to flashing light treatments (F= 9). Notably, strong effects of the prevailing growth stage on major fatty acids have previously been reported for these genera (Fernández-Reiriz et al., 1989; Hodgson et al., 1991; Suzuki et al., 2018).

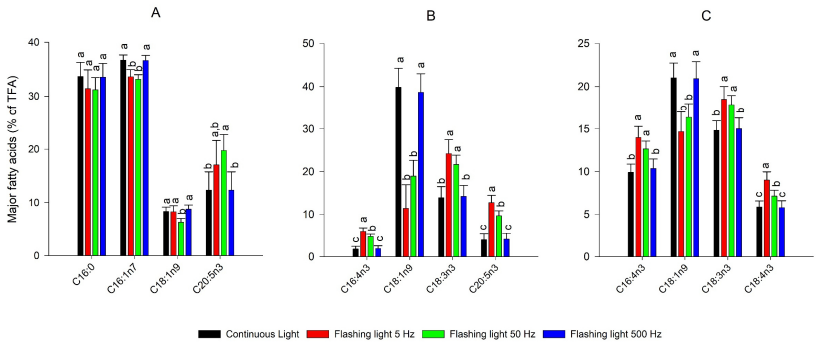

Figure 4. Major fatty acids of *Nannochloropsis gaditana* (A), *Koliella antarctica* (B) and *Tetraselmis chui* (C) exposed to flashing light and continuous light. Data given are adjusted means ±95% confidence interval obtained from Tukey´s post hoc test (ANCOVA) for one- and two stage cultivation. Treatments that do not share the same letter are significantly different to each other. A detailed fatty acid profile is available in the supplementary material (Table A.1).

The fact that the fatty acid profiles are usually more affected by the biomass concentration than the flashing light treatment in *N. gaditana* and *T. chui* (Table A.1) may be linked to a higher biomass productivity and faster transition from one growth stage to another (e.g., lag, exponential and stationary phases), as compared to the slower growing *K. antarctica* (Fig. 1, Table 1). Similar species-specific fatty acid shifts under flashing light have been reported previously. For example, *Chlamydomonas reinhardtii* was not affected by flashing light (*f*= 0.05-5 Hz, *DC*= 0.5, Kim et al., 2014), while *Isochrysis galbana* accumulated more phospholipids and docosahexaenoic acid (DHA) when exposed to blue flashing LEDs (e.g., *f*= 10 KHz, *DC*= 0.5, Yoshioka et al., 2012).

From our results, the prevailing growth stage had the strongest effect on the fatty acid profiles of a given microalga (Statistical analysis in Table A.1). However, higher PUFA contents consistently obtained under low frequency flashing light (FL 5) indicate that these cells are under stress linked to the production of reactive oxygen species caused by highly intense light flashes (Schulze et al., 2017b; Wiktorowska-Owczarek et al., 2015). However, unlike previous suggestions that flashing light might induce a response similar to that obtained in cells under high light (Schulze et al., 2017b), high PUFA contents usually arise from exposure to low light (Schüler et al., 2017).

3.2.3. Pigments

The contents of most pigments were significantly higher in all microalgae exposed to FL 5 and FL 50 as compared to those under CL and FL 500 ($p < 0.05$; Fig. 5A). The carotenoids *β*-carotene, violaxanthin, and neoxanthin (in *T. chui* and *K. antarctica*) were on average three to four times higher in all microalgae as compared to cultures under CL. Total chlorophyll and total carotenoids as well as lutein increased moderately by 1.7-2.3 times in FL 5 compared to CL treatments (Fig. 5A).

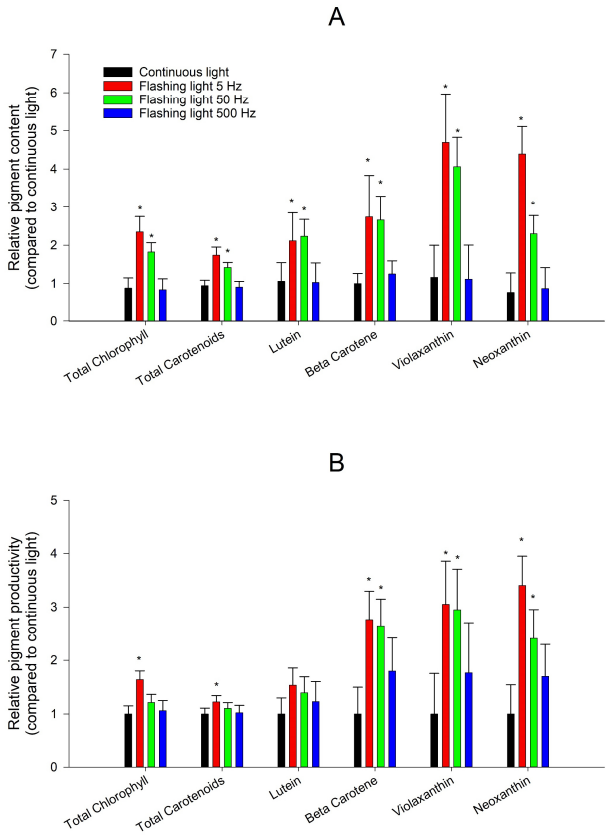

Figure 5. Pigment contents (A) and productivities (B) for *Nannochloropsis gaditana*, *Koliella antarctica* and *Tetraselmis chui* exposed to flashing light (*f*= 5, 50, 500 Hz ; *DC*= 0.05). Data for each microalgal species were normalised to results obtained under continuous light (= 1) and are given as adjusted means ±95% confidence interval obtained from a Tukey´s test. Asterisks indicate significant differences compared to continuous light. Neoxanthin was only detected in *T. chui* and *K. antarctica*. Original pigment data are available in the supplementary material (Table A.1).

Interestingly, the productivity of the accessory light harvesting pigments neoxanthin and violaxanthin and the photoprotective pigments *β*-carotene and lutein (Mulders et al., 2014) were on average two to three times higher in all microalgae under low frequency flashing light (FL 5 and FL 50) compared to cultures under CL or FL 500 (Fig. 5B). However, productivities of total carotenoids, chlorophyll and lutein were only slightly enhanced (1.1-1.6 times higher) under FL 5 and FL 50 compared to cells exposed to CL.

Intracellular contents of total chlorophyll and carotenoids ranked among all strains from 1.8-13.8 mg g DW^{-1} and 0.7 to 7.4 mg g DW^{-1}, respectively. The major carotenoid was violaxanthin (0.24-2.07 mg g DW^{-1}) in *N. gaditana*, whereas lutein (0.27-2.05 mg g DW^{-1}) was predominant in *K. antarctica*. *T. chui* contained mostly lutein (0.29-2.2 mg g DW^{-1}) and *β*-carotene (0.40-1.25 mg g DW^{-1}, Table A.1). These findings are similar to previous reports for these algae (Ahmed et al., 2014; Fogliano et al., 2010; Simionato et al., 2013b). Under FL 5, *N. gaditana* was the most productive in terms of chlorophyll, total carotenoids and violaxanthin (2.97, 1.34 and 0.45 mg L^{-1} d^{-1}, respectively), whereas *T. chui* was efficient in producing chlorophyll, neoxanthin, lutein and *β*-carotene (2.97, 0.04, 0.47 and 0.35 mg L^{-1} d^{-1}, respectively; Table A.1). Conversely, *K. antarctica* was unable to reach any of these productivities. Flashing light has previously been reported to induce pigments and improve their production. Production of astaxanthin in *Haematococcus pluvialis* under CL was the same as that in cells under flashing light (*f*= 100 Hz, *DC*= 0.67, Katsuda et al., 2006), whereas low frequency flashing light (e.g., *f*<30 Hz) often increased pigment production (Katsuda et al., 2008). Furthermore, Takache et al. (2015) found that *Chlamydomonas reinhardtii* accumulated more carotenoids when exposed to lower frequencies (*f*=0.00138-1 Hz, *DC*=0.5). Likewise, the results presented here showed a trend towards higher pigment contents in cells under FL 5 compared to those exposed to FL 50.

The violaxanthin-antheraxanthin-zeaxanthin (VAZ) cycle is an important regulator for the adaptation to different light intensities. Shifts towards the biosynthesis of zeaxanthin occurs in plants and algae under high-light stress to avoid photodamage of the photosynthetic apparatus (Jahns et al., 2009). Conversely, violaxanthin accumulates under low-light stress to allow efficient light harvesting (Jahns et al., 2009). In the present study, cultures exposed to FL 5 tend to contain more violaxanthin compared to FL 50 treatments, while high frequency FL conditions (FL 500) were not different to CL. Both in Chlorophytes such as *Koliella* and *Tetraselmis* and in Eustigmatophytes (i.e., *Nannochloropsis*) pigments are bond to proteins in order to constitute light harvesting complexes (LHC; Basso et al., 2014; Jahns et al., 2009; Sukenik et al., 1992; Thornber, 2013). In addition, thylakoids number can increase resulting in more membranes containing PUFA and LHC and, thus, leading to a more efficient light harvesting (Berner et al., 1989). Therefore, the concomitant increase of proteins (Fig. 2), PUFA (Fig. 3, 4) and pigments (Fig. 5) could be attributed to an increase in photosynthetic units in cells treated with low-frequency flashing light which indicates an acclimation of cells to low-light conditions (He et al., 2015; Schüler et al., 2017), as suggested earlier for microalgae exposed to low-frequency FL (Grobbelaar et al., 1996; Yarnold et al., 2015).

Furthermore, biomass concentration correlated negatively with all the pigments (r= -0.4 to -0.9, p< 0.01-0.7; significance strain-dependent; Table A.1). Generally, cells in aging cultures are subjected to nutrient depletion or light limitation, leading to downregulation of their photosynthetic activity and decrease in photosynthetic pigments (e.g., chlorophyll, violaxanthin, Oukarroum, 2016). Our statistics revealed that flashing light seemed to counteract this effect significantly (Fig. 5) because higher pigment contents were found under low frequency FL when considering the co-variate *biomass concentration*. Therefore, our results suggest that the long flash duration (e.g., 1-10 ms) and high instantaneous intensity (I_f= 6000 µmol s^{-1} m^{-2}) of FL 5 and FL 50 treatments may still stimulate protein, PUFA and pigment biosynthesis even at advanced growth stages where otherwise proteins and pigments decrease or the fatty acids become saturated. Because increasing levels of *β*-carotene and lutein found in cultures exposed to low-frequency FL are connected to photoprotection (Mulders et al., 2014), cells may experience also a moderate high-light stress. This indicates that future studies are needed to better understand the high-light responses of microalgal cells exposed to low frequency FL. Such studies should focus on high-light typical pigments such as zeaxanthin (e.g., in *Nannochloropsis*) or diatoxanthin (e.g., in diatoms) as well as metabolic pathways using transcriptomic approaches.

4. Conclusions

The effects of flashing light were most discriminative at low frequencies (5 and 50 Hz, *DC*=0.05), whereas cultures exposed to 500 Hz showed similar growth and biomass composition compared to cells cultivated under continuous light. The effects on growth was strain- and culture concentration-depended. Low-frequency flashing light conditions (f= 5 and 50 Hz) induced intracellular biocompound that typically accumulate under low-light conditions, including protein, PUFA, chlorophyll, lutein and *β*-carotene. Strikingly, the productivity of these compounds was highly improved when using a two-stage cultivation system. Our statistical analysis revealed that most biomolecules were strongly affected by the biomass concentration in the medium–which is an indicator for the prevailing growth stage of a culture–rather than the flashing light treatments applied. We conclude that microalgal cultivation at high latitudes can benefit from employing artificially emitted low frequency flashing light (e.g., f≤ 50 Hz) to produce high-value microalgal biomass rich in high value metabolites, including PUFA or pigments.

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6. Declaration of authors' contributions

S.L. (serena.lima@unipa.it) and P.S. (peter.schulze@nord.no) contributed equally to this study. They designed the experiments, performed the laboratory work, analysed protein and carbohydrate contents and drafted the manuscript. P.S. carried out the statistical data as well as the fatty acid analyses, and revised the article for intellectual contents. S.L. and L.S. (lmschueler@ualg.pt) analysed the pigment profiles (HPLC) and revised the article for intellectual contents. T.S. (tamarafilipasantos@gmail.com) analysed total lipids and elemental composition (CHN) of biomass. H.P. (hgpereira@ualg.pt) contributed to the artwork and revised the intellectual content of the manuscript. J.V. (jvarela@ualg.pt) and F.S. (francesca.scargiali@unipa.it) contributed to the critical review of the manuscript and gave administrative support for the project. R.W. (rene.wijffels@wur.nl) and K.V. (kiron.viswanath@nord.no) conceived the main project, contributed to the design, reviewed and edited the article and provided the administrative support for the research project. All authors agreed with the authorship of this work and gave the final approval of the version to be submitted.

7. Conflict of interest

The authors confirm that there is no known conflict of interest related with this publication and there has been no significant financial support for this study that could have influenced its outcome.

8. Supplementary material

Supplementary research data from the thesis can be made available upon request by contacting the Faculty PhD administration.

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Marine cold-adapted microalgae are a promising bioresource to replace unsustainable fish oil or soybean-based proteins and their production does not compete with traditional agriculture for arable land. Even though low ambient temperatures at high latitudes (e.g., Northern Norway) are optimal for their cultivation, low solar irradiance limits photosynthesis and therefore expensive artificial light is needed. The present thesis identified cold-adapted microalgae that grow fast at temperatures 15°C or below and contain valuable polyunsaturated fatty acids, (PUFA), proteins or pigments that could be used either as food or as feed components. By optimising cultivation parameters such as temperature, light intensity and nutrient or salt supply, PUFA production could be greatly enhanced. Furthermore, a technological advance in microalgal cultivation was made through the application of light emitting diodes (LEDs) that periodically emit short but intense light pulses (i.e., flashing light). This alternative way of artificial light supply substantially improved PUFA or pigment production, compared to continuous light. Both the optimisation of cultivation conditions and the supply of flashing light will be key for high latitude production of cold-adapted microalgae rich in valuable biomolecules.

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