MARIA JOÃO SANTOS LOURENÇO

INDUCTION OF HIGH VALUE METABOLITES IN INDUSTRIAL MICROALGAL STRAINS USING ARTIFICIALLY FLASHING LIGHT

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2024

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Mestrado em Biotecnologia

Trabalho efetuado sob a orientação de:

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Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

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Maria Lourenço

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Agradecimentos

Antes de mais, gostaria de expressar os melhores agradecimentos aos meus supervisores, ao Dr. Peter Schulze (GreenCoLab) e ao Prof. João Varela (Universidade do Algarve, CCMar) por todo o apoio na orientação e realização na presente tese de mestrado, bem como na sua correção. Ao Dr. Hugo Pereira, pela oportunidade e confiança de fazer parte da equipa GCL. Um agradecimento especial, novamente, ao Peter, pelo constante incentivo e por não desistir mesmo quando as luzes eram tudo menos flashing light. Ah, e por atender sempre o telemóvel às 7h da manhã dum sábado quando eu precisava de ajuda. Peter, obrigado por tudo o que me ensinaste, incluindo a “lei do desenrasca”; não poderia ter pedido um melhor mentor.

Fazer uma tese sobre um tema que nada me era familiar, ademais em *outdoor*, não foi nada fácil. Afortunadamente, pude contar com o apoio de algumas pessoas que me ajudaram muitíssimo no período de adaptação: Ao Gustavo, Gabi e Sofia, que me ensinaram todo o essencial nos primeiros meses. Ao Pedro del Hierro, *master* dos tubulares, por ser o meu 3º “supervisor” e me ensinar a maior parte do que sei sobre culturas em *outdoor*, que me fez companhia a inocular às 6h da manhã quando estava com insónias, e ser o meu pronto-socorro quando alguma coisa corria mal. À Inês, por tudo o que também me ensinou sobre *outdoor*, por todas as dicas e os “para mim não faz sentido”, e por me ajudar sempre que tinha dúvidas. À Rita, pela cumplicidade, que me ouviu muitas das vezes quando tudo parecia não estar a resultar. Ao Bernardo, por se mostrar sempre disponível em ajudar, especialmente na fase crítica da escrita. Ao Daniel, por todos os excelentes conselhos (sim, porque ele é espetacular e só dá bons conselhos). À minha Joquinha, por toda a ajuda na estatística. Com cerca de 130 amostras e uma data de analíticas para fazer, não podia deixar de agradecer à Monya, pela ajuda, e em especial à Sara, por todo o conhecimento que partilhou comigo nos últimos meses, fazendo-me crescer muito, tanto a nível pessoal como profissional. A todos os membros do GreenCoLab, por me terem acolhido tão bem e por todos os bons momentos passados.

Por fim, quero agradecer à minha família, em especial ao meu avô, que fez com que realizar o mestrado fosse possível. Aos meus pais, em especial à minha mãe, por aturar os meus dramas durante todo este processo, pelo apoio e por terem sempre acreditado em mim. Ao meu irmão, que é um chato, e me fez aprender a ter mais paciência (ou talvez não). Ao meu namorado, pelo apoio incondicional, paciência e compressão das vezes que o deixei pendurado porque tinha de recolher amostras nos fins de semana. Não teria sido possível sem vocês.

Abstract

Algae are promising organisms with many applications as they can metabolise various (high-value) compounds, including pigments and fatty acids that present great potential as supplements in the human diet. As a result, algal biomass with an enhanced nutritional/biochemical profile regarding these target compounds has a high value on the market. A common strategy to improve microalgal biocompounds production is exposing the culture to stress conditions. However, these conditions often lead to biomass losses due to the long time needed (e.g., days or weeks). To overcome this problem, the time required for such stress conditions should be diminished. Thus, in this thesis, *Nannochloropsis oceanica* and *Phaeodactylum tricornutum* were exposed to stress conditions (flashing light - FL) for four days, under a two-stage outdoor cultivation approach, to accumulate higher protein, lipids, carbohydrates, fatty acids or pigments content. In the first stage, cultures were exposed to sunlight until they reached the desired concentration (~2 and 1 g DW L^{-1} for *N. oceanica* and *P. tricornutum*, respectively). The second stage started when cultures were exposed to sunlight combined with low-frequency FL (*f* = 0.5 (FL0.5), 5 (FL5) Hz; *DC*=0.05), continuous light (CL) or without additional light (SUN). Additionally, the effects of FL were benchmarked against different covariates (“trial”, “harvesting timepoint”, “induction time”, “biomass concentration” and “biomass productivity”). Generally, there were no significant differences between treatments (FL0.5, FL5, SUN and CL) in either species, with some exceptions. In *P. tricornutum*, protein and carbohydrate contents were significantly higher in cells exposed to FL5 compared to SUN and in cells exposed to CL and SUN compared to FL0.5, corresponding to 0.16 and 1.5-fold increases, respectively. In *N. oceanica*, pigment productivity had significant differences. Cells exposed to SUN conditions presented a 10.5-fold increase in violaxanthin productivity compared to those under FL0.5 and CL, whereas zeaxanthin productivity increased 2-fold under SUN conditions when compared to FL0.5. Regarding covariates, “trial” was the one with the highest effect on treatments. In conclusion, FL only affected protein and carbohydrate content, and violaxanthin and zeaxanthin productivities in *P. tricornutum* and *N. oceanica*¸ respectively, when considering the studied covariates.

Keywords: *Nannochloropsis oceanica*, *Phaeodactylum tricornutum*, flashing lights, biochemical composition, fatty acids, pigments

Resumo

As algas são organismos promissores com inúmeras aplicações, sendo utilizados como fontes para produtos farmacêuticos, alimentares, biocombustíveis e biomateriais. Estas conseguem metabolizar vários compostos com elevado valor, incluindo glícidos, proteínas, pigmentos, lípidos e ácidos gordos, que apresentam grande potencial como suplementos na dieta humana. Os pigmentos, incluindo os carotenoides, apresentam várias propriedades interessantes, tais como antioxidantes, antivirais, antimicrobianas, anti-inflamatórias e anticancerígenas. Uma vez que o ser humano e os animais não conseguem sintetizar carotenoides, estes devem ser absorvidos através da nossa alimentação, como por exemplo, através do consumo de microalgas. Os ácidos gordos, incluindo os ácidos gordos pol-insaturados (PUFA), nomeadamente o ácido eicosapentenóico (EPA) e o ácido docosahexenóico (DHA), conseguem prevenir, retardar e intervir em várias doenças, como a hipertensão, diabetes, doenças inflamatórias e auto-imunes, a depressão, alguns tipos de cancro, as doenças cardiovasculares, entre outras. Em consequência, a biomassa de algas com um perfil bioquímico melhorado em relação a estas biomoléculas alvo apresenta valores elevados no mercado. Por exemplo, a microalga *Phaeodactylum tricornutum*, uma das diatomáceas melhor caraterizadas até à data, apresenta uma composição bioquímica rica em ácidos gordos, nomeadamente EPA, e em pigmentos, nomeadamente a fucoxantina. Esta é uma espécie de interesse em aplicações biotecnológicas para uso em sumplementos alimentares e alimentos para peixes. Outra espécie com interesse biotecnológico, e também bastante conhecida, é a *Nannochloropsis oceanica*. Esta apresenta características bioquímicas igualmente interessantes, com um elevado teor em ácidos grodos, nomeadamente EPA e DHA, bem como pigmentos, incluindo as clorofilas, violaxantina e β-caroteno. Uma estratégia comum para melhorar a produção destes biocompostos é a exposição da cultura a condições de stress. No entanto, estas condições conduzem, geralmente, à perda de biomassa devido ao longo tempo necessário para induzir o stress. Para ultrapassar este problema, são implementadas estratégias de cultivo em duas fases. Numa primeira fase, são aplicadas condições ótimas de crescimento e, numa segunda fase, a produção de biocompostos é induzida através de condições de stress, tais como salinidade elevada, temperatura elevada e privação de nutrientes. Em alternativa, estudos recentes mostraram que o uso de luz intermitente (FL) de baixas frequências (f) e ciclos de trabalho (DC) curtos melhoravam a composição bioquímica das microalgas quando aplicada na segunda

fase duma abordagem em cultivo de duas fases. No entanto, os estudos anteriores centraram-se principalmente em experiências laboratoriais, ou seja, em pequena escala e em experiências em laboratório com parâmetros abióticos controlados. A aplicação de luz intermitente na segunda fase desmonstrou ser muito eficaz na indução de pigmentos e ácidos gordos nas microalgas. No entanto, falta uma prova de conceito deste processo à escala industrial. Nesta tese, duas microalgas marinhas, *N. oceanica* e *P. tricornutum*, foram cultivadas em estratégia de cultivo duas fases, em *outdoor*, em que as células, numa primeira fase, foram cultivadas sob condições ótimas de crescimento e, numa segunda fase, foram expostas a luz intermitente de baixa frequência (*f*=0,5 (FL0.5), 5 (Fl5) Hz; *DC*=0,05). Assim, o objetivo principal foi estudar o efeito da luz intermitente nestas duas espécies durante produção à escala piloto em exterior (*outdoor*) no crescimento e produtividade da biomassa, bem como no conteúdo e produtividade dos biocompostos (proteína total, lípidos totais, glícidos, ácidos gordos e pigmentos). Além disso, esta tese teve como sub-objetivos analisar o efeito de diferentes covariáveis relativamente ao efeito da luz intermitente e ao crecimento e composição bioquímica, incluindo "ensaios" ("trial"), "tempo de colheita" ("harvesting timepoint"), "dias de indução" ("induction time"), e "concentração de biomassa" ("biomass concentration"). De um modo geral, não se verificaram diferenças significativas entre os tratamentos (FL0.5, FL5, SUN e CL) em nehuma das espécies, com algumas exceções. Em *P. tricornutum*, os teores de proteínas e glícidos foram significativamente superiores em células expostas à condição de FL5 em relação a SUN e em células expostas à condição de CL e SUN comparado a FL0.5, o que corresponde a um aumento de 0,16 e 1,5 vezes. Em *N. oceanica*, a produtividade de pigmentos apresentou diferenças significativas entre os tratamentos. As células cultivadas sob condições de SUN apresentaram um aumento de 10,5 vezes na produtividade de violaxantina comparadas com aquelas sob FL0.5 e CL, enquanto que a produtividade de zeaxantina aumentou 2 vezes sob condições de SUN quando comparada a FL0.5. No entanto, as diferenças mencionadas só foram confirmadas quando controladas para todas as covariáveis analisadas. Relativamente às mesmas, "ensaio" ("trial") foi a covariável que teve o maior efeito nos tratamentos. Em conclusão, a FL apenas afectou o conteúdo de proteínas e glícidos, bem como a produtividade de violaxantina e zeaxantina em *P. tricornutum* e *N. oceanica*¸ respetivamente, quando consideradas todas as covariáveis. Uma vez que a experiência foi realizada ao ar livre (*outdoor*), as variáveis externas, como a temperatura, irradiância e fotoperíodo, podem ter tido um maior impacto no comportamento das microalgas do que o efeito da própria luz intermitente, o que pode ter contribuído para o efeito não significativo dos tratamentos. Assim, em estudos futuros, estas variáveis externas devem ser consideradas e medidas, bem como a realização de um maior número de ensaios.

Palavras-chave: *Nannochloropsis oceanica*, *Phaeodactylum tricornutum,* luz intermitente, composição bioquímica, ácidos gordos, pigmentos

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List of abbreviations

ALA	α-Linolenic acid
BHT	Dibutylhydroxytoluene
CCMAR	Centre of Marine Sciences
CF	Combustion furnace
CL	Continuous light
DC	Duty cycle
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
DW	Dry weight
EPA	Eicosapentaenoic acid
f	Frequency
FA	Fatty acids
FAME	Fatty acids methyl ester
FCP	Fucoxanthin-chlorophyll protein
filter_{fw}	Filter final weight
filter_{iw}	Filter initial weight
FL	Flashing light
FL0.5	Flashing light with a frequency of 0.5 Hz
FL5	Flashing light with a frequency of 5 Hz
HPLC	High pressure liquid chromatography
I_a	Average light intensity
I_l	Flash intensity
LED	Light emitting diode
MUFA	Monounsaturated fatty acids
NPQ	Non-photochemical quenching
OD	Optical density
ORPs	Open raceway ponds
PAR	Photosyntehthic active radiation

PBR	Photobioreactor
PPFD	Photosynthetic photon flux densitiy~
PSII	Photosystem II
PUFA	Polyunsaturated fatty acids
PWM	Pulse width modulator
RF	Reduction furnace
ROS	Reactive oxygen species
SFA	Saturated fatty acids
SUN	Sunlight
t_c	Flashing cycle
TCD	Thermal conductivity detector
t_d	Dark period
TFA	Total fatty acids
t_l	Flash period
WT1	Water trap 1
WT2	Water trap 2

1. Introduction

1.1. Microalgae

1.1.1. General overview

Microalgae can be defined as a diverse group of photosynthetic organisms, combining properties of higher plants, lower eukaryotes and prokaryotes [1–4]. These organisms are predominantly aquatic, growing in marine and freshwater habitats, which provides more opportunities and space for cultivation without disrupting the current water supply or competing for agricultural lands [5]. They can grow rapidly and their size ranges from a few micrometres to a few hundred micrometres [6]. Despite being typically free-living organisms, some microalgae can coexist in a symbiotic relationship with a variety of other organisms, such as bacteria and fungi [7,8].

As with other organisms, microalgae need carbon and energy sources to produce biomass. Depending on the sources used, they are capable of different kinds of trophy [1,2,9]. Regarding how they obtain their carbon source, microalgae can grow photoautotrophically and heterotrophically. Photoautotrophs use carbon dioxide (CO_2) as the carbon source, while heterotrophs use organic compounds (e.g., glucose) produced by other organisms [9]. The energy source classifies the organisms as phototrophs and chemotrophs. Phototrophs only use light as an energy source, which is converted into chemical energy through a process called photosynthesis. Conversely, chemotrophs use an exogenous organic or inorganic molecule as an energy source if they are chemoorganotrophs or chemolithotrophs organisms, respectively [10].

Phototrophic cultivation entails the use of production systems (PBRs, ponds, etc.) under optimal conditions to produce biomass or valuable compounds. Each system has its advantages and limitations in terms of scalability, productivity, management, controlled parameters and costs associated (See section 1.4.1. Cultivation systems for further details; **Table 1.3**).

Microalgae play an important role in the ecosystem balance as an essential component of food chains [4]. They also present the capacity to mitigate CO_2, helping to minimise environmental impacts, leading to climate change mitigation, one of the most critical challenges on our planet [4,11,12]. Additionally, phototrophic microalgae contribute to around 40% of global photosynthetic oxygen production [4,13]. Its market size has been growing due to its vast applications and its commercial and ecological interests (See section 1.1.3.2 High-value biocompounds for further details).

1.1.2. Biodiversity

Microalgae can be classified according to their photosynthetic pigments, cell wall composition, biochemical composition of storage products and cytological and morphological characteristics. Despite the approaches to identifying algal species, the taxonomy has changed over the years, and there is no consensus yet for which taxonomic system is the most correct. Still, the common classification system considers seven major phyla, belonging to two main domains – Prokaryota and Eukaryota – gathering four kingdoms – Protozoa, Chromista, Plantae and Eubacteria [3,4]. The Eukaryota domain includes seven main phyla, represented in **Figure 1.1**. Contrary to the latter, the Prokaryota domain comprises only one representative phylum of microalgae – Cyanobacteria. Regardless of the low representation in the Prokaryota domain, Cyanobacteria are one of the most abundant phylum of microalgae, as well as Chlorophyta and Heterokontophyta [4].

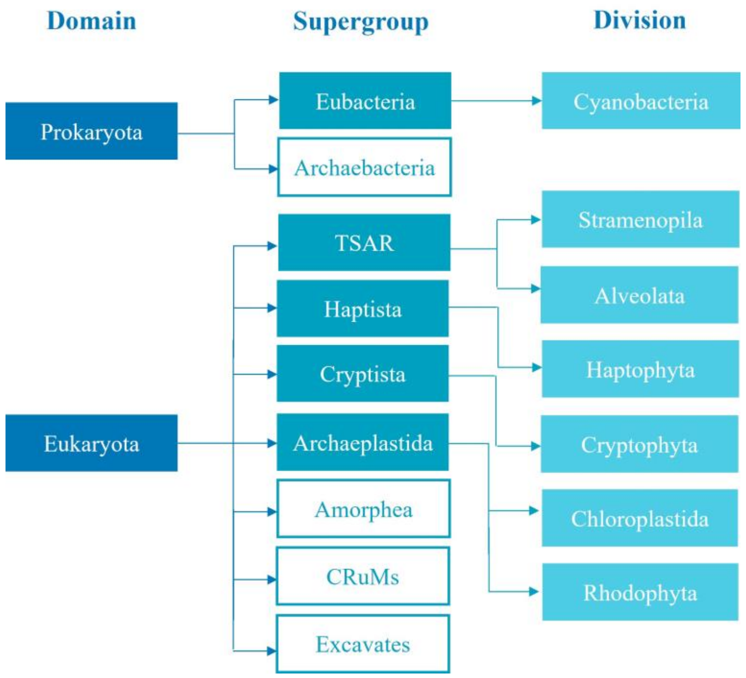

Figure 1.1 - Major microalgae division distribution classification scheme inspired from Levasseur et al., 2020. Microalgae supergroups are highlighted. Ref: [4,14].

1.1.3. Biochemical composition

1.1.3.1. Nutrients and biocompound biosynthesis

Algal growth depends mostly on nutrient requirements, temperature, CO_2 availability, pH, salinity, light intensity and quality and mixing rates. Nutrient limitation affects considerably the biochemical composition of microalgae (See section <u>1.5.1 Stress</u>

conditions for further details) [15]. Thus, the ideal culture medium must contain the micro- and macronutrients needed by microalgae. Macronutrients include nitrogen (N) and phosphorus (P). Micronutrients include sodium (Na), magnesium (Mg), sulfur (S), and trace elements, such as iron (Fe), copper (Cu), molybdenum (Mo), manganese (Mn), zinc (Zn) and cobalt (Co) [15–17].

The minimum nutritional requirements for microalgae growth depend on the species. N, P and C are the most important nutrients for autotrophic growth [15–17]. For instance, carbon contributes ~40-50% of the total biomass and is the main constitute of all organic substances synthesized by the cells, such as proteins, carbohydrates, nucleic acids and lipids. Inorganic carbon occurs in water in the form of CO_2, HCO_3^- and CO_3^{2-} [15,16]. Nitrogen is another important nutrient on microalgal growth, contributing with 1% to 14% of the total biomass [16–18], being used in the biosynthesis of proteins, nucleic acids, vitamins and photosynthetic pigments [15,16] and is mainly provided in its inorganic form (e.g., NH_4+, N_2 or NO_3^-) [15–17]. C and N quantification can give us information about nutritional quality, biomass yield and organic compounds. Additionally, we can analyse the C:N (carbon-to-nitrogen) ratio, a crucial parameter to understand macromolecular composition and interpret and predict the biochemistry of microalgae [19]. For instance, low C:N ratio, i.e., in nitrogen-rich conditions, microalgae focus on nitrogen assimilation and protein synthesis, prioritizing cell division. Contrary, high C:N ratio, i.e., in carbon-rich conditions, algae produces carbon storage compounds, such as carbohydrates and lipids [20,21]. Phosphorus is another essential nutrient for microalgal growth, contributing to around 1% of total biomass [15,16]. This element is mainly taken up in inorganic form (e.g., $H_2PO_4^-$ or HPO_4^{2-}) [15–17] and is required for various cellular metabolic activities, such as energy storage, biosynthesis of nucleic acids, cell signalling and energy transfer. The ratio of N:P strongly affects biomass productivity and lipids and carbohydrate contents [15,16].

1.1.3.2. High-value biocompounds

Humans' first use of microalgae dates back thousands of years by the Chinese to survive famine [18,22,23]. Species such as *Nostoc*, *Arthrospira* (*Spirulina*) and *Aphanizomenon* have been used as a source of food for thousands of years [22,23]. In fact, microalgae can produce biocompounds that can be used as feedstocks for biofuels, biomaterials (biodegradable bioplastic), bioactive compounds for nutraceutical, cosmeceutical and pharmaceutical applications and as supplements in human and animal diets (**Figure 1.2**) [2,5,24,25]. These biocompounds include proteins, lipids and carbohydrates.

Figure 1.2 - Potential applications of microalgae in various fields.

Proteins have important structural and metabolic functions in microalgal cells. They are an important component of the light-harvesting complexes and the membrane, serving a structural and metabolic role. Besides, they include numerous catalytic enzymes involved in cell metabolism and growth, i.e., photosynthesis [1,26]. The protein contents of microalgal cells depend on the species and its growth conditions, as well as the growth phase at the harvesting timepoint and the extraction efficiency (See section 1.4.2 Harvesting stages for further details) [23]. For instance, green microalgae (e.g., *Chlorella* sp. and *Tetraselmis* sp.) have rigid cell walls, which can lower the extraction efficiency of intracellular proteins, resulting in lower digestibility in humans. Moreover, qualitatively, microalgae contain all of the essential amino acids that mammals are unable to synthesize [1]. Several species can possess high protein contents, ranging from 40% to 60% of dry weight [1,23]. For instance, *Chlorella vulgaris* and *Arthrospira* sp. contain protein levels up to 58% and 70 % of dry weight (DW), respectively. *Nannochloropsis* sp. and *Phaeodactylum tricornutum* can reach up to 35% and 30% (DW) of protein content, respectively [23,27].

Carbohydrates, both monomers and polymers, present structural (cell wall) and metabolic functions (e.g., energy storage) [1,26,28,29]. They can be found attached to proteins (e.g., glycoproteins) or lipids (e.g., glycolipids) [1]. Microalgae produce glucose and starch-like energy storage products, which are the primary carbon-containing products from photosynthesis, serving as the starting point for the synthesis of other biochemicals [1,26]. Microalgae polysaccharides exhibit numerous properties, including antioxidant, anti-inflammatory, antitumor and antimicrobial effects, with great potential in the food, feed and agricultural sectors [29]. Besides, these are promising for food packaging development due to their biodegradability and sustainability [29]. Sources of polysaccharide production include plants, crustaceans, squid pens, fungi, or microalgae. The advantages of the latter source are significant as they present higher safety, stability, biocompatibility, and biodegradability, contributing to the promotion of human life quality [29]. Polysaccharide composition and content in microalgal cells depends on several factors, such as species and growth conditions (See section 1.4.2 Harvesting stages for further details). For instance, *Porphyridium* sp. can reach up to 57% (of DW) of carbohydrate content, whereas *P. tricornutum* and *Nannochloropsis* sp. carbohydrate content is only 16% and 10% (of DW), respectively [1].

Also lipids present structural (membranes) and energy storage functions [26]. The structural lipid fraction is composed of polyunsaturated fatty acids (PUFA), while the storage lipid fraction is composed of monounsaturated fatty acids (MUFA) and saturated fatty acids (SFA) [30]. Microalgae are mainly composed of polar lipids (phospho- and glycolipids), free fatty acids, and nonpolar lipids [1,26,30]. Nonpolar lipids usually store saturated fatty acids, while polar lipids are more associated with unsaturated fatty acids [1,26,30]. The primary interest and goal of microalgal lipids was the production of biodiesel. Nonetheless, omega fatty acids have gained significant commercial value due to their health benefits, including inflammatory and autoimmune effects [31–33]. Lipid production depends on the species and growth conditions, particularly on the growth phase, nutrient availability, temperature, pH, salinity, and light intensity (See section 1.4.2 Harvesting stages for further details). The lipid content of many microalgal species is well documented, representing often 20% to 50% of the DW [1]. For instance, lipid contents in *Chlorella* sp., *Nannochloropsis* sp. and *P. tricornutum* reached up to 57% of DW, respectively [27,30].

In addition to proteins, carbohydrates and lipids, microalgal biomass contains high-value compounds with a wide spectrum of biological activities, particularly pigments (as carotenoids) and PUFA [1,2]. They have great potential as supplements in the human diet for the prevention, management and treatment of health problems [1]. Therefore, microalgae interest in biotechnology has increased over the past years. Actually, the estimated microalgae market value worldwide is around 10.8 billion EUR with an estimated annual growth of 8% [34]. Compounds with a high market value include pigments (**Figure 1.3**), such as astaxanthin (2925-8190 €/Kg) and *β*-carotene (351-1755 €/Kg), and PUFA (94-187 €/Kg) [1,35].

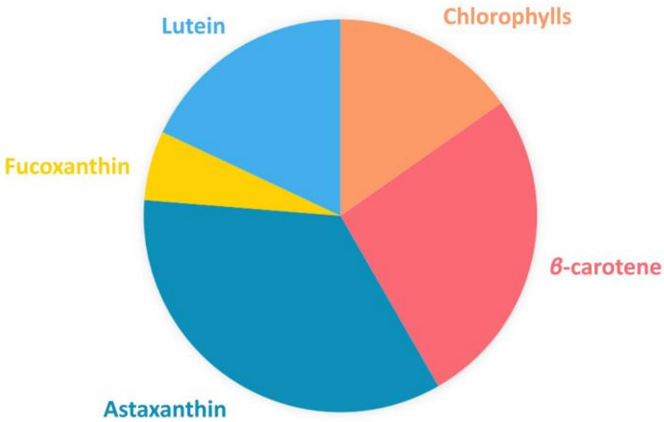

Figure 1.3 - Global market analysis of algae-based pigments expressed in percentage (%). Ref: [34,36–42].

Carotenoids are responsible for the yellow, orange and red colorations of flowers, fruits, vegetables and microalgae. These pigments present several interesting properties, including antioxidants, antivirals, antimicrobials, anti-inflammatory, and anticancer [12,43]. Thus, carotenes (*β*-carotene and lycopene) and xanthophylls (astaxanthin, fucoxanthin, lutein, zeaxanthin) can be applied as food and feed ingredients and be used in the formulation of nutraceuticals, cosmetics and pharmaceuticals [12,37,39]. Since humans and animals cannot synthesize carotenoids, these must be absorbed from our diet [44]. Besides plants and some species of bacteria, archaea and fungi, microalgae can also synthesize carotenoids [12,44]. In comparison to plants, microalgae grow up to 5-10 times faster and contain up to 10 times more carotenes [12,43,45,46]. For instance, marigold flowers of the genus *Tagetes*, well known as a commercial plant source of lutein, present a typical lutein concentration of 0.18-5.70 mg g^{-1} DW, depending on the species [45,47]. In microalgae, lutein contents are usually higher, reaching up to 7.60 mg g^{-1} DW, under normal conditions, whereas upon exposure to stress, it can reach up to 17.3 mg g^{-1} DW

[45]. More precisely, studies on *Chlorella sorokiniana* and *Dunaliella salina*, under normal conditions, showed a lutein content of 5.88 and 6.6 mg g^{-1} DW, respectively [48,49]. Under stress conditions, *Parachlorella* sp. and *Chlorella sorokiniana* presented lutein contents of 11.8 and 17.3 mg g^{-1} DW, respectively [49,50]. Despite the advantages of fungi, namely the wide range of nutrients and inorganic and organic sources that can be used and the fact that their carotenoid production is not affected by climate, as they are usually grown indoors or under controlled conditions (fermenters), microalgae present a faster growth rate and have multiple metabolic modes (auto-, hetero- and mixotrophic), being able to produce a wealth of biological products according to light conditions and carbon availability. Besides, the sources of carotenoids from fungi include lycopene, *β*-carotene, astaxanthin and canthaxanthin, while microalgae carotenoid sources include the latter plus *α*-carotene, lutein, zeaxanthin, violaxanthin and fucoxanthin [51]. Nonetheless, astaxanthin sources in fungi are produced by only one species – *Xanthophyllomyces dendrorhou*. For instance, astaxanthin content of *X. dendrorhous* and *Chlorella zofingiensis* can go up to 2.5 and 6.5 mg g^{-1} DW, respectively [46–48]. The most exploited algal carotenoids are *β*-carotene and astaxanthin, produced by *Dunaliella salina* and *Haematococcus pluvialis*, respectively [12,43,44]. Fucoxanthin is also gaining a lot of interest in dietary supplements and aquafeed. This pigment is produced by brown algae and diatoms, as *P. tricornutum* [16,43,52,53].

Polyunsaturated fatty acids (PUFA) are another class of interesting compounds known for their health benefits. These include preventing or delaying various diseases, such as hypertension, diabetes, inflammatory and autoimmune disorders, depression, some cancers, and cardiovascular diseases, among others. Depending on their chemical structure, PUFA can be divided into two main groups: ω-3 and ω-6. ω-3 PUFA include *α*-linolenic (ALA), eicosapentaenoic (EPA), docosapentaenoic (DPA) and docosahexaenoic (DHA) acids [32,33]. Among these, EPA and DHA have been extensively studied and demonstrated for their health benefits, exerting positive effects on neurological disorders [31]. The human body can produce many fatty acids, including PUFA (EPA and DHA), but not at sufficient levels to meet our body's nutritional and physiological requirements [32,33]. Hence, supplementation of natural EPA and DHA in the human diet is recommended [54]. Natural resources include fish, shellfish, or microalgae [32,33]. Although the first two are the main sources of PUFA, current concerns about animal consumption, overfishing, global warming and short supply for the global population have created the need to find alternative, vegan solutions. Therefore, PUFA-rich microalgae, such as *P. tricornutum* and *Nannochloropsis* sp. are an attractive solution [32,33].

1.2. *Phaeodactylum tricornutum*

Diatoms are unicellular photosynthetic small-size (5-200μm) microalgae, functionally single cells, but they can appear in different forms in nature: as filaments, cell chains, or colonies, living freely in the water column or attached to any single substratum [55–57]. Its siliceous cell wall is transparent, allowing the entrance of light to the cell chloroplasts, and porous, allowing the diffusion and transport of materials [55,56]. They are the main primary producers in the ocean and one of the most productive and environmentally adaptable microalgae in the world, responsible for 20% of global carbon fixation [58,59]. Diatoms are highly abundant and diversified, counting for about 20,000 species, representing 27.5% of total algal species [56,58–60]. The complex fucoxanthin-chlorophyll protein (FCP), constituted by the harvesting pigments chlorophyll *a*, chlorophyll *c* and fucoxanthin, is responsible for light harvesting [59,61]. Fucoxanthin is the most abundant pigment, which provides the typical golden-brown colour of these microalgae. This pigment has been shown to have health-conductive effects, including antioxidant, anti-inflammatory, antiobesity and anticancer activities. In addition, diatoms contain high amounts of lipids, including PUFA, as being a main building block for cell membranes, comprising usually between 10 and 25% of their biomass [60]. One of the best-characterized diatoms so far is *Phaeodactylum tricornutum* [62]. This species can exhibit three different morphotypes – oval, triradiate and fusiform – being the latter the most common (**Figure 1.4**), which is approximately 25μm long [57,62]. Biochemical composition can be altered and improved according to different culture conditions, such as nutrient depletion and high light intensities (See section 1.5.1 Stress conditions for further details) [55,58,59,63]. Its biochemical characteristics (**Table 1.1**), high biomass production rates and tolerance to high pH make this a species of interest for biotechnological applications, such as food supplements and aquafeed, making it suitable for large-scale cultivation [16,52,53,59,64]. For instance, this species is exploited by AlgaTechnologies Ltd. (Ketura, Israel), Simris group AB (Hammenhög, Sweden) and Allmicroalgae S.A. (Pataias, Portugal) [65–67].

Figure 1.4 - Micrographs obtained by light microscopy of live *Phaeodactylum tricornutum* cells.

Table 1.1 - Biochemical composition (lipids, fatty acids, protein, carbohydrates and fucoxanthin contents) of *Phaeodactylum tricornutum* expressed on % of DW. Refs: [27,55,56,59–61,64,68,69].

Lipids	Fatty acids	Protein	Carbohydrates	Fucoxanthin
15–57 %	15–25 %	30–36.4 %	8.4–26.1 %	1–6 %

1.3. *Nannochloropsis* sp.

Eustigmatophytes are a small group of unicellular photosynthetic small-size (2-8µm) green cells [70]. The cells are oval, spherical, elliptical or coccoid-shaped with polysaccharide cell walls [70,71]. This microalgal group is known for its high lipid content, constituted of a large amount of essential PUFA, in particular EPA, and pigment content, as *β*-carotene [72]. It contains the photosynthetic pigments chlorophyll *a*, violaxanthin and *β*-carotene [60]. Chlorophyll *a* is the most abundant pigment, which provides their typical green colour [30]. One of the most known and studied eustigmatophytes belongs to the genus *Nannochloropsis* (**Figure 1.5**). To date, seven species are described, comprising species now transferred to the *Microchloropsis* genus: *N. oculata*, *N. oceanica*, *N.* (*Microchloropsis*) *gaditana*, *N. granulata*, *N.* (*Microchloropsis*) *salina*, *N. limnetica* and *N. australis* [70,73]. The biochemical composition of *Nannochloropsis*, in particular its lipid content, can be influenced by different culture conditions, such as nutrient depletion and high temperatures and salinities (See section <u>1.5.1 Stress conditions</u> for further details) [70]. Its biochemical characteristics (**Table 1.2**) and high biomass production rates make this a species of interest for biotechnological applications – cosmetics and food and feed applications

[70,73]. For instance, this species is exploited for chlorophyll, proteins and EPA production by AlgaTechnologies Ltd. (Ketura, Israel) and Allmicroalgae S.A. (Pataias, Portugal) [66,74].

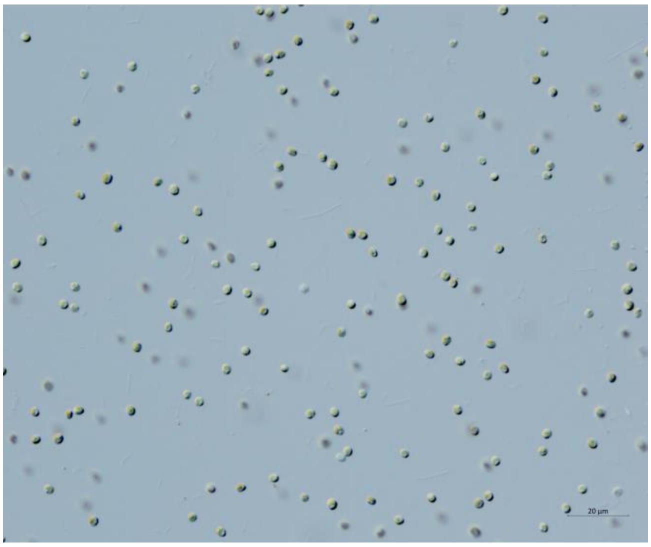

Figure 1.5 - Micrographs obtained by light microscopy of live *Nannochloropsis oceanica* cells.

Table 1.2 - Biochemical composition (lipids, fatty acids, protein, carbohydrates, *β*-carotene and violaxanthin contents) of *Nannochloropsis oceanica* expressed on % of DW. Refs[70,73,75–79].

Lipids	Fatty acids	Protein	Carbohydrates	Chlorophylls	*β*-carotene	Violaxanthin
14–56 %	2–47.5 %	14.5–35 %	4.7–39 %	1.8–3.9 %	0.1–1.7 %	0.5–5.8 %

1.4. Biomass production

1.4.1. Cultivation systems

There are two main types of mass-cultivation systems: photobioreactors (PBRs) and open raceway ponds (ORPs). A PBR is defined as a closed system in which photoautotrophs, *i.e.*, photosynthetic microorganisms, such as microalgae or cyanobacteria, are grown to achieve a photobiological reaction – photosynthesis [15,80]. Each cultivation system has its advantages and disadvantages (**Table 1.3**). PBRs are more expensive when compared to the capital, operational and energetic costs of ORPs. However, PBRs can lower the risk of contamination, avoid evaporation and weather exposure, improve light-use efficiency, and maximise biomass productivity [1,15]. These qualities are particularly important for food or pharmaceutical products that need to have a constant quality rich in desired high-value biocompounds and free of significant bacterial contamination. Biomass productivity can double when comparing PBRs with ORPs [81–83]. For instance, in the south of Spain, the productivity of ORPs was 27 tons/ha/year while closed systems (PBRs) presented a greater productivity of 34–61 tons/ha/year [84]. Depending on the dimensions and format of the closed culture system, PBRs offer good control of culture conditions and growth parameters (pH, CO_2, temperature) high solar radiation availability and thus high biomass productivity [15].

The design of the PBRs differs for the type of cultivation due to the high dependence of light (energy source) by photoautotrophs. As a result, a prior consideration in the PBR design is the availability, distribution, and use of light [15]. Other common stress factors, including contaminants and grazers, nutrients and CO_2 availability, photooxidation and cellular death due to excess accumulation of O_2 during the day, and significant changes in culture pH, can affect culture growth and physiological performance [1]. However, when the cultivation system is provided with optimal levels of nutrients and CO_2 and contamination loads are under control, the limiting factor will always be the availability of light [1,2]. Several PBR types, including flat panels and tubular, have been designed and tested to cultivate different species phototrophically, such as *P. tricornutum* and *Nannochloropsis* sp. (**Figure 1.6**) [60,81,85]. Among these, flat panels PBRs (**Figure 1.6 A, B**) were revealed to accomplish low shear stress and effective illumination, resulting in higher biomass and biochemical content productivity [60,81,86]. With decreasing light path, better light is distributed to cells within a culture [87,88]. Additionally, mixing rates (e.g., aeration) should also be considered to decrease the probability of photoinhibition that may occur. In tubular PBRs, mixing is achieved by circulating the microalgal cells with the help of a pump through the whole system, including the gas-exchange unit, i.e., the degassing unit, and the tubular unit. Over time, oxygen generated by photosynthesis is produced in the tubular unit of the PBR. Eventually, a saturating oxygen concentration will be achieved, causing oxidative damage to the cells [1]. To avoid this problem, one of the strategies commonly used, mainly in small tubular PBRs, is the equipment of a degassing tank, i.e., deoxygenation tank. This allows the removal of dissolved gases – oxygen – from the microalgal culture, preventing O_2 accumulation [89]. However, even at short light paths and optimal mixing rates, PBRs, including flat panels, will reach a critical biomass concentration, leading to low productivities in terms of target biocompounds [15].

Table 1.3 – Advantages and disadvantages of the three most used microalgae cultivation systems: open raceway ponds (ORPs), and tubular and flat panel photobioreactors (PBRs). Ref: [1,15,81].

Cultivation system	Advantages	Disadvantages
ORP	•Easy maintenance •Easy construction and operation •Low cost due to less energy consumption	•Poor light utilization •Poor biomass productivity •Water losses through evaporation •High risk of contamination •Large land area required
Tubular PBR	•Large illumination surface area •High biomass productivity •Low water losses •Low risk of contamination •Control of growth parameters	•Fouling •Large land area required, but less than ORP •High costs associated with energy consumption and materials •Hard to maintain and operate
Flat panel PBR	•Optimal light path •High biomass productivity •Low water losses •Medium risk of contamination •Control of growth parameters •Low power consumption •Easy maintenance and operation	•Scale-up requires many support materials such as piping and tubing, gaskets and seals, panel materials (e.g., polyethene bags) •Low land area required •Labour intensive

Figure 1.6 - Microalgae cultivation in different PBRs (photobioreactors). *Phaeodactylum tricornutum* cultivated in flat panels PBRs (A) and *Nannochloropsis oceanica* cultivated in flat-panels (B) and tubular (C) PBRs.

1.4.2. Harvesting stages

There are various sequential steps that are usually involved in the production of microalgal products. After microalgae cultivation, cells need to be harvested following three systematic processes: biomass recovery, dewatering, and drying [90–92]. Large volumes are required to process due to the small size of microalgae and low density, making the harvesting process very energy-intensive. Indeed, it can account for up to one-third of the biomass production costs, being consequently the bottleneck of the downstream processes [91,92]. Thus, it also represents a major challenge for the commercial production of low-cost products, such as biofuels [90]. This process would only be economically viable if a high microalgae productivity existed and if the biomass harvested were to be used in the production of high-value products, such as nutraceuticals

[90–92]. Therefore, improvements in biomass and biocompound productivity in microalgae are necessary.

The harvesting timepoint is also essential to maximise cost productivity. Microalgal biomass should be harvested at the end of the exponential phase, right before reaching the early stationary phase, where cells are still healthy, and the culture presents the highest biomass concentration. The growth phase of the culture is also important as it can change the culture's biochemical composition. For instance, towards late growth phases, protein and pigment contents (both chlorophyll and carotenoids) tend to decrease, while lipids or carbohydrate contents increase in a species-dependent manner [93,94]. Indeed, studies on a diatom, *Thalassiosira pseudonana*, showed that protein content was higher in the exponential phase, whereas carbohydrates and lipid contents increased within the stationary phase [94]. Further studies on *Tetraselmis suecica*, *Cyclotella cryptica* and *P. tricornutum* showed that protein and pigment contents decreased towards the stationary phase, while fatty acids and carbohydrate content increased [95]. It has been suggested that harvesting algae biomass in the afternoon might reduce respiration losses and increase the cultivation system's productivity. Studies in *N.* (*Microchloropsis*) *gaditana* indicated that lipid content in samples collected at sunset was significantly higher than in samples collected at sunrise [96]. Likewise, total fatty acid contents in *Rhodomonas* sp. were maximal in samples collected at sunset than in samples collected at sunrise [97]. Pigment content per cell in *N. gaditana* is higher when samples are taken by the end of the light period compared to the ones collected in the morning. This happens because, by the end of the light period, biomass concentrations are higher, contributing to the higher biochemical composition productivities, while during night respiration, microalgae utilise these energy-containing molecules for their metabolism. Previous results demonstrate that microalgae often start cell division during the first moments of the dark phase, while lipids are mainly consumed and carbohydrates and proteins decrease, though at a lower rate [98].

1.5. Biochemical productivity

As mentioned in Section 1.4 Biomass production, despite the vast application of microalgae, the commercialization of microalgal products is associated with low product yields and high costs related to biomass production and downstream processes [1,25]. To ensure the sustainable production of microalgal biomass with nutritional and health benefits, enhancing product yields and reducing overall costs, the improvement of biochemical profiles is a prerequisite, particularly pigments and fatty acids [1,15,25].

1.5.1. Stress conditions

Microalgae composition is intrinsically dependent on growth conditions, including temperature, light, pH, nutrient availability, and salinity. These factors affect microalgal metabolism and, subsequently, their composition [72]. Thus, to alter the biochemical composition of microalgae, these parameters are commonly manipulated [99].

Various approaches have been suggested to enhance biochemical productivity in different microalgal species, including *P. tricornutum* and *Nannochloropsis* sp. [72,73,100–105] (**Table 1.4**). Nutrient starvation and changes in light intensities, temperature, and salinity are some of the most commonly used stress-induced strategies (**Table 1.4**) [73,99,101,106]. For instance, nutrient stress, such as nitrogen depletion and phosphate starvation, have been well investigated to stimulate lipid production in microalgal species [99]. Changes in salinity, which cause osmotic and oxidative stress to algae, stimulate pigments, lipids or starch production [107,108]. In fact, under extreme environmental conditions, microalgae are known to upregulate metabolic pathways to protect the cells from oxidative damage [99–101,109]. This results in the accumulation of antioxidants or energy reserves [107,108]. Changes in light intensity can also increase lipids and pigment content [73,110,111]. In general, high light intensities stimulate storage lipids biosynthesis (e.g. MUFA), while lower light intensities enhance the levels of structural lipids (e.g., PUFA) [101]. These changes are related to alterations in chloroplast volume and thylakoid membranes, as well as energy storage mechanisms [112]. Changes in temperature can induce PUFA, which are needed by cells to maintain membrane structure and functionality [26,101]. Despite the increase in microalgal biochemical content, when such stresses are applied, microalgae shift to increased production of lipids, carbohydrates and hydrogen and decrease in growth rates [73,99,113]. To overcome this problem, a common strategy employed is a two-stage cultivation approach.

Table 1.4 - Impacts of different abiotic stress in microalgal biochemical composition of several algae.

Abiotic stress	Conditions	Microalga	Effects	Refs.
Light intensity	High intensity (150 µmol s^{-1} m^{-2})	*Chlorella zofingiensis*	Zeaxanhin, cantaxanthin, and astaxanthin increased ~430.3, 117.9 and 178%, respectively, after 72h of exposure compared to 0h.	[73]
	High intensity (500 µmol s^{-1} m^{-2})	*Nannochloropsis oceanica*	Total lipids content reached ~50%, being 1.6-fold higher than low light (50 µmol s^{-1} m^{-2}).	[114]
	Low intensity (30 µmol s^{-1} m^{-2})	*Nannochloropsis oculata*	Overall EPA content reached 15mg/L, being 2.2-fold higher compared to the control (75 µmol s^{-1} m^{-2}).	[73]
Nutrient	Nitrogen deprivation	*Chlorella* sp.	Lipid content reached 42.10%, being ~2-fold higher than the control (BG11 medium).	[115]
	Nutrient deprivation	*Dunaliella salina*	Lipid content reached 54.15±2.71%, being ~2-fold higher than the control (f/2 medium).	[116]
	Nutrient starvation ($NaNO_3^-$)	*Phaeodactylum tricornutum*	Lipid content reached 42.5±0.19%, being ~1.2-fold higher than the control (N+).	[104]
Salinity	Low salinity (15 ppt)	*Chlorella vulgaris*	Lipid content reached 63.50%, being 1.8-fold higher than the control (30 ppt).	[117]
	High salinity (16%)	*Desmodesmus* sp.	Starch content reached 19.55 mg g^{-1} DW, being 1.05-fold higher than the control (0% salinity).	[118]

	High salinity (400 mM)	*Scenedesmus* spp.	Lipid and carbohydrate contents reached 33.10% and 35.91%, being 1.8- and 1.7-fold higher than the control (0 mM), respectively.	[119]
Temperature	High temperature (35◦C [a]; 38◦C [b])	*Acutodesmus dimorphus*	[a] Chl *a* and *b* and carotenoids contents reached 10.68, 5.65 and 1.96μg/mL, being 1.4-, 1.5- and 1.3- fold higher than the control (25◦C). [b] Carbohydrate content reached 35% DW, being 1-fold higher than the control (25◦C).	[120]
	Low temperature (10◦C)	*Nannochloropsis oceanica*	Chl *a* and *b* and carotenoids contents reached 60.18, 2.41 and 33.25 mg g^{-1} DWextract, being 1.3-, 1.7- and 1.3- fold higher than the control (25◦C).	[72]
	Low temperature (10◦C)	*Phaeodactylum tricornutum*	EPA content reached 24.2%, being 1.8-fold higher than the control (25◦C).	[121]

1.5.2. Two-stage cultivation systems

Two-stage cultivation strategies are common approaches to induce target biocompounds. In a first stage, biomass is produced under growth promoting conditions and, in a second stage, biocompound production is induced by exposing the cells to different stress conditions [73,113]. Furthermore, it has been suggested that this approach provides conditions that can limit bacterial contamination [113]. Despite its wide application in industry, this approach is time-consuming. Most of the experiments for biocompound productivity enhancement focus on the extensive manipulation of abiotic parameters as stress conditions [73]. Such induction strategies often take several days or weeks, while preventing the cultures from growing, often leading to biomass loss due to respiration and, thus, decreased productivity [122–124]. Hence, novel alternative strategies to improve the biochemical composition of microalgae need to be implemented. Recent studies showed that flashing (or pulsed-) LEDs, i.e., the periodic emission of light flashes alternated by dark periods, stimulate the production of target metabolites. Furthermore, the time needed to induce high-value compounds, such as fatty acids and pigments, is significantly shorter (e.g., hours or a few days) under flashing light (FL) regimes compared to traditional two-stage cultivation approaches using stress conditions as a trigger for the accumulation of target metabolites [125,126].

1.6. Flashing light

While previous studies showed the advantages of using wavelength tailored LEDs to improve microalgal culture growth, the potential benefits of FL are yet to be confirmed. FL is generated when the power supply of LEDs is controlled via a pulse-width modulator (PWM), acting as a dimmer (**Figure 1.7**). This tool generates a rectangular pulse wave signal with an asymmetrical shape [122]. The ballast is a type of electrical resistor used to control and regulate the supplied current during different operating phases and voltages (i.e., during the light and dark periods) [127]. The wall plug efficiency is the product of all devices' efficiency, including PWM efficiency, ballast efficiency and LED efficiency (**Figure 1.7**). Flashing LEDs can emit high light intensity during a short period (known as “light period”, “flash period” or t_l), while also alternating with extended dark periods (or t_d) (**Figure 1.7**). One light period (t_l) followed by a dark period (t_d) is defined as a flashing cycle (t_c). The ratio between the light period and the whole flashing cycle is the duty cycle (DC). The number of flashing cycles (t_c), i.e., the number of light-dark intervals, that occur per second (s^{-1}), is defined as the frequency (f). The light intensity (in µmol photons s^{-1} m^{-2}) during the light period is defined as flash intensity (I_l); whereas, during the dark period, no light is emitted. The time-averaged light intensity is used to compare flashing light treatments with continuous light treatments (I_a) during one flashing cycle (t_c), and is expressed as per the following equation: $I_a = DC \times I_l$ [122].

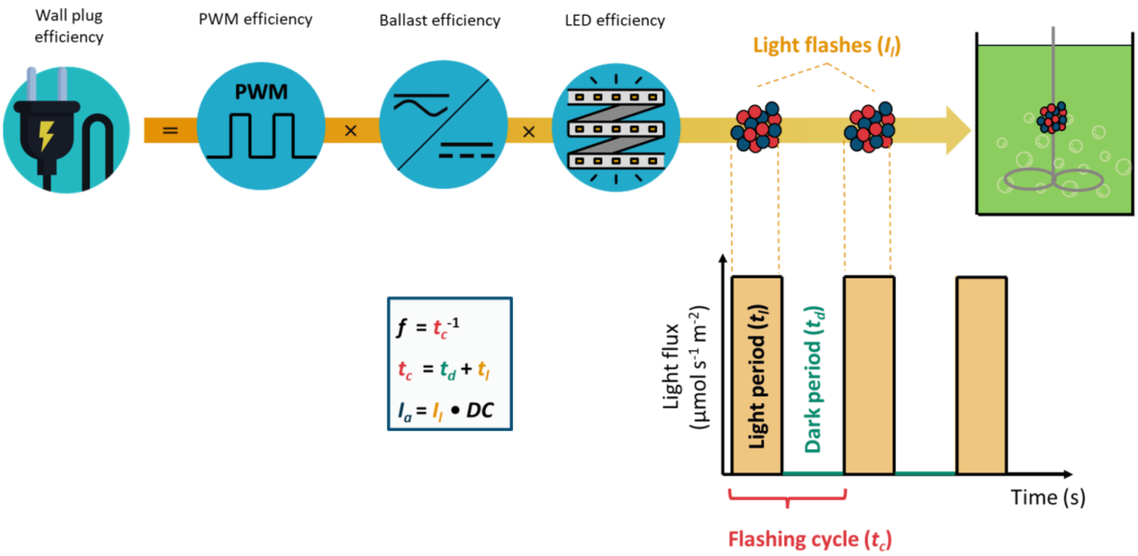

Figure 1.7 - Simplified diagram of a microalgal production unit using flashing light (FL) and how it works. Adapted from [122].

1.6.1. Effects of FL on microalgal growth

The potential advantages of FL discussed in previous studies differed significantly. Most studies focused on flashing light conditions of low frequencies (f <100 Hz) and relatively high duty cycles (DC>0.1), but the results were inconclusive (**Table 1.5**). Flashing light emitted with the same time-averaged light intensity (I_a) as continuous light was suggested to mitigate light attenuation in PBRs and perhaps improve photosynthetic performance of microalgal cells [122]. Flash intensities (I_l) increase light penetration depth, delivering photons deeper into the culture and, thus, maximising growth and stimulating the accumulation of target metabolites [122,125,126]. Since such intense light flashes may cause photodamage and inhibition of the phototroph, the repetition rate of light-dark intervals (f) and the proportion of light and dark period (DC) should be adjusted to the biological reaction kinetics of the photosynthetic apparatus [122].

Previous studies in *Tetraselmis striata*, *Nannochloropsis gaditana*, *Koliella antarctica* and *Tetraselmis chui* demonstrated that algae exposed to higher FL frequencies (e.g., f=500 Hz) displayed higher biomass productivity when compared to lower FL frequencies (f=0.5, 5 and 50 Hz), although in most cases productivities were still lower than continuous light (with the same averaged light intensity of the FL treatments) and different between species, indicating that the responses to FL treatment are species-specific (**Table 1.5**) [125,126]. However, on a two-stage cultivation approach, biomass productivities were higher compared to a one-stage approach. This suggests that applying the FL treatment to a higher biomass concentration may cause a better light distribution to a higher number of algal cells in the culture, hence limiting photoinhibition and improving growth [125,126,128].

Table 1.5 - Impacts of different flashing light treatments in microalgal growth or biomass productivity of several algae (CL: continuous light, *DC*: duty cycle, FL: flashing light, *f*: frequency, I_a: averaged light intensity, I_l: flash intensity, LED: light emitting diode, PBR: photobioreactor).

Microalgae	Conditions/Outcome	Refs.
Chlamydomonas reinhardtii	Frequencies of 100 Hz resulted, on average, in a 35% higher biomass yield when compared to CL. *DC* = 0.5; I_a = 220 µmol s^{-1} m^{-2}.	[129]
Chlorella vulgaris	Exposure to 1000 Hz presented highest biomass compared to CL. *DC* = 0.6; I_a = 200 µmol s^{-1} m^{-2}. 6 days of FL exposure.	[130]
Chlorella vulgaris	Cells exhibited highest growth rate at *f* = 0.1, 10 and 100 Hz under 7000 µmol s^{-1} m^{-2} compared to control treatments, being highest at 10 Hz. *DC* = 0.5.	[131]
Diacronema lutheri	All FL treatments (*f* = 0.5, 5 and 50 Hz) affected growth of *D. lutheri*, being CL the best.	[126]
Dunaliella salina	Biomass production under low FL frequencies (0.05 and 0.005 Hz) was lower than the control CL. Higher frequencies (0.5, 5 and 50 Hz) increased biomass remarkably compared to CL, with an increase of 17, 26 and 39%, respectively. I_a = 400 µmol s^{-1} m^{-2}.	[132]
Dunaliella salina	FL dry biomass is three times as much as higher than CL. PBR equipped with flashing panels of red (650–665 nm) and blue (450–470 nm) LEDs. *DC* = 0.5; *f* = 0.1 Hz; I_a = 18.2 µmol s^{-1} m^{-2}.	[133]
Isochrysis galbana	Cultivation under intermittent blue light showed the highest increase in cell density among the four light regimes (white CL and intermittent white, red and blue light). *DC*= 0.5; *f* = 10000Hz; I_a = 52 µmol s^{-1} m^{-2}; I_l = 104 µmol s^{-1} m^{-2}. 6 days of cultivation.	[134]
Isochrysis galbana	Cell densities were higher in all frequencies (1, 10, 100, 1k, 2.5k, 5k, 10k, 30k, 50k and 100k Hz) compared to CL, being *f* = 10 k the highest. *DC* = 0.5.	[135]

	Higher *DC* (40 - 80%) produced greater growth than lower *DC* (11 and 30%). Cell densities were highest under *DC* = 60% and *f* = 10k Hz.	
Isochrysis galbana	Cell concentration was highest under blue FL, followed by white FL, compared to other treatments (white CL and intermittent red and green light). White FL presented 1.3 times higher concentration than CL on the sixth day of experiment. *DC*= 0.5; *f* = 10 kHz; I_a = 52 µmol s^{-1} m^{-2}.	[136]
Koliella antarctica	Highest biomass productivity when exposed to FL with of 500 Hz compared to CL. Exposure to 5 and 50 Hz caused photoinhibition. *DC* = 0.05; I_a = 300 µmol s^{-1} m^{-2}.; I_l = 6000 µmol s^{-1} m^{-2}. Two-stage approach; FL exposure for 4 days.	[125]
Micractinium sp.	Biomass concentrations and specific growth rates increased 58 and 40% at 5 and 50 Hz, respectively, compared to the control (I_a = 1000 µmol s^{-1} m^{-2}). *DC* = 0.5; I_a = 2000 µmol s^{-1} m^{-2}; I_l = 2000 µmol s^{-1} m^{-2}.	[137]
Nannochloropsis gaditana	Lowest biomass productivities under FL regimes (*f* = 5, 50 and 500 Hz) compared to CL. Exposure to 5 Hz caused photoinhibition. *DC* = 0.05; I_a = 300 µmol s^{-1} m^{-2}; I_l = 6000 µmol s^{-1} m^{-2}. Two-stage approach; FL exposure for 4 days.	[125]
Nannochloropsis gaditana	Cells grown in CL and FL of 500 Hz presented higher growth than the other two conditions analysed (*f* = 5 and 50 Hz).	[138]
Scenedesmus dimorphus	FL at I_l = 375 µmol s^{-1} m^{-2} indicated 2.86 times higher biomass productivity compared to CL. *DC* = 0.2; *f* = 10 kHz; I_a = 75 µmol s^{-1} m^{-2}.	[139]
Tetraselmis chui	Highest biomass productivities when exposed to FL with of 50 and 500 Hz compared to CL. *DC* = 0.05; I_a = 300 µmol s^{-1} m^{-2}; I_l = 6000 µmol s^{-1} m^{-2}. Two-stage approach; FL exposure for 4 days.	[125]

Tetraselmis striata	FL of 5 and 50 Hz presented similar growth as CL. 0.5 Hz led to growth inhibition. DC = 0.05; I_a = 300 μmol s^{-1} m^{-2}; I_l = 6000 μmol s^{-1} m^{-2}. Two-stage approach; FL exposure for 4 days.	[126]

1.6.2. Effects of FL on biochemical composition

Flashing light has been demonstrated to improve the composition of target metabolites on microalgal cells (**Table 1.6**). Flashing light of low frequencies (e.g., f <100 Hz) and high flash intensities (e.g., I_l = 6000 µmol s^{-1} m^{-2}) were suggested to improve the contents of target compounds that are connected to photo-protection and energy dissipation mechanisms, also referred to as non-photochemical quenching (NPQ). Under low frequencies and short duty cycles, phototrophs are exposed to short high light flash (light) periods and extended dark periods. If phototrophs are not able to process photon excess under high light conditions, excitation dissipation mechanisms are activated to prevent the formation and evolution of reactive oxygen species (ROS). High levels of ROS suppress protein synthesis, which is essential for repairing plastoquinone oxidoreductase, commonly called photosystem II (PSII), upon photodamage of microalgal cells. To avoid excess ROS evolution during high light flash periods and maintain their metabolism during extensive dark periods, phototrophs employ different energy quenching and storage strategies, including accumulation of protective pigments and storage of carbohydrates and lipids [122].

Previous studies demonstrated that FL with a $f \leq 50$ Hz, a $DC \leq 0.75$ and $I_a \leq 1000$ µmol s^{-1} m^{-2} improved biochemical production (proteins, lipids including PUFA, and pigments) in *Microchloropsis gaditana*, *Koliella antarctica*, *Tetraselmis chui*, *Tetraselmis striata*, *Diacronema lutheri* and *Micractinium* sp. (**Table 1.6**) [125,126,137,140,141]. Nevertheless, most reports were conducted based on laboratory-scale investigations. Thus, large-scale trials and economic feasibility studies of these strategies are still needed to verify their application.

Table 1.6 - Impacts of different flashing light treatments on the biochemical composition of several algae (CL: continuous light, DHA: docosahexaenoic acid, *DC*: duty cycle, EPA: eicosapentaenoic acid, FL: flashing light, *f*: frequency, I_a: averaged light intensity, I_l: flash intensity, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, SFA: saturated fatty acids, TFA: total fatty acids).

Main product	**Microalgae**	**Conditions/Outcome**	**Refs.**
Lipids and Fatty acids			
Lipids	*Isochrysis galbana*	Lipid content was highest under blue FL, followed by white FL compared to other treatments (white CL and intermittent red and green light). *DC*= 0.5; *f* = 10 kHz; I_a = 52 µmol s^{-1} m^{-2}.	[136]
Lipids, MUFA	*Isochrysis galbana*	Cultivation under intermittent blue light showed the highest lipid content among the four light regimes (white CL and intermittent white, red and blue light). In glycolipids, MUFAs content was higher under blue intermittent light (24.3%). In phospholipids, the percentage of PUFAs cultivated under red intermittent light was the smallest (55.6%), and the percentage of PUFAs cultivated under blue intermittent light was the highest (62.0%.). *DC*= 0.5; *f* = 10 kHz; I_a = 52 µmol s^{-1} m^{-2}; I_l = 104 s^{-1} m^{-2}. 6 days of cultivation.	[134]
Lipids, MUFA	*Nannochloropsis gaditana*	Lipids gradually increased from the CL to the FL treatments (25 and 250 Hz), being highest at the latter. MUFAs content was highest at 2500 Hz. *DC* = 0.25. I_a = 70 µmol s^{-1} m^{-2}; I_l = 280 µmol s^{-1} m^{-2}. Culture medium with nutrient depletion.	[142]

PUFA	*Koliella antarctica*	Exposure to 5 and 50 Hz FL for 4 days accumulated higher PUFA contents compared to CL. *DC* = 0.05; I_a = 300 µmol s^{-1} m^{-2}.	[125]
PUFA	*Tetraselmis chui*	Exposure to FL of 5 and 50 Hz for 4 days accumulated higher PUFA contents compared to CL and FL 500. *DC* = 0.05; I_a = 300 µmol s^{-1} m^{-2}.	[125]
PUFA, EPA	*Nannochloropsis gaditana*	Exposure to FL of 5 and 50 Hz for 4 days accumulated higher PUFA and EPA contents compared to CL and FL 500. *DC* = 0.05; I_a = 300 µmol s^{-1} m^{-2}.	[125]
PUFA, DHA, EPA	*Diacronema lutheri*	FL exposure (5 Hz) increased PUFAs, EPA and DHA productivities 1.5-, 1.3- and 1.3-fold compared to CL, respectively. *DC* = 0.05; I_a = 300 µmol s^{-1} m^{-2}.	[126]
PUFA, MUFA, SFA, TFA	*Tetraselmis striata*	The highest TFA productivity was achieved with FL 5 Hz after 3 days of exposure, reaching 95.9±8.1 mg L^{-1} d^{-1}, which corresponds to a 1.2-fold increase compared to CL. FL 5 Hz led to an increase of SFA, MUFA and PUFA after 3 and 6 days of FL exposure. *DC* = 0.05; I_a = 300 µmol s^{-1} m^{-2}.	[126]
Pigments			
Astaxanthin	*Haematococcus pluvialis*	Increased yield of astaxanthin production per supplied photon (60% higher). *DC*<0.01; I_a = 16.4 µmol s^{-1} m^{-2}; I_l = 1700 µmol s^{-1} m^{-2}.	[141]
β-carotene, Lutein, Total chlorophyll, Total carotenoids, Violaxanthin	*Koliella antarctica*, *Nannochloropsis gaditana*, *Tetraselmis chui*	β-carotene and violaxanthin were on average 3-4 times higher compared to cultures under CL, while total chlorophylls and total carotenoids (as well as lutein) increased moderately by 1.7-2.3 times in FL (5 Hz) compared to CL. *DC* = 0.05; I_a = 300 µmol s^{-1} m^{-2}. 4 days of exposure to FL.	[125]

β-carotene, Lutein, Neoxanthin, Violaxanthin, Zeaxanthin	*Tetraselmis striata*	Six days of treatment with FL of 5 Hz led to a 1.3-fold increase in lutein productivity compared to CL and showed significantly higher zeaxanthin productivity of 0.014±0.001 mg L^{-1} d^{-1} than cultures under other treatments (0.5 and 5 Hz and CL). Three days of treatment with FL of 5 Hz presented the highest violaxanthin productivities, which decreased after 6 days of exposure. Three days of exposure and f = 50 Hz presented the best treatment for high productivity for neoxanthin and β-carotene, reaching 0.12±0.01 and 0.23±0.04 mg L^{-1} d^{-1}, respectively. DC = 0.05; I_a = 300 μmol s^{-1} m^{-2}.	[126]
Chlorophyll a, Diatoxanthin, Fucoxanthin	*Diacronema lutheri*	Six days of treatment under low-frequency FL (0.5 and 5 Hz) led to enhance fucoxanthin productivities, which was 1.2- and 1.3-fold higher compared to CL. Six days of treatment at frequency of 0.5 Hz increased diatoxanthin productivity over 4-fold compared to CL. Chlorophyll *a* productivities under FL of 5 Hz for three days led to a 4-fold increase compared to CL, reaching 1.60±0.19 mg L^{-1} d^{-1}. *DC* = 0.05; I_a = 300 μmol s^{-1} m^{-2}.	[126]
Total carotenoids	*Nannochloropsis gaditana*	Total carotenoid content was highest under 2500 Hz FL. *DC* = 0.25; I_a = 70 μmol s^{-1} m^{-2}; I_l = 280 μmol s^{-1} m^{-2}. Culture medium with nutrient depletion.	[142]
Proteins			
	Koliella antarctica	Proteins content differed among FL treatments, being highest in FL of 5 and 50 Hz-treated cultures. *DC* = 0.05; I_a = 300 μmol s^{-1} m^{-2}.	[125]

Tetraselmis chui	Protein contents were significantly higher under FL treatments of 5 and 50 Hz. *DC* = 0.05; I_a = 300 µmol s^{-1} m^{-2}.	[125]

1.6.3. Industrial application

The application of FL in microalgae cultivation has several industrial applications. They can be used to optimize growth or enhance the composition of target metabolites. It was first reported that under very short flashes, algal cells could present the same maximum carbon dioxide uptake and oxygen production rates as those under continuous light [143]. However, this FL regime can present other advantages over continuous light, including energy-saving consumption and costs [144]. This is particularly important and most needed when working in pilot and industrial scale. Additionally, FL regimes can be optimized to stimulate the production of high-value biocompounds, including pigments and lipids [125,126]. This is of particular interest in industries such as cosmetics, pharmaceuticals, and nutraceuticals. Moreover, flashing light can improve the efficiency of nutrient uptake in microalgae [130]. The application of optimal and efficient illumination increases biomass productivity and, thus, nutrient uptake efficiency [145]. This is important for bioremediation, including wastewater nutrient removal, where cells acclimated to high-frequency flashing light (e.g., f=1000 Hz) can result in a maximum nitrate and phosphate removal of 97% and 70%, respectively [130].

Although laboratory-scale microalgal cultivation allows for precise control of numerous parameters, including pH, photoperiod, and light availability, replicating these conditions on an outdoor industrial scale is challenging. This is due to several constantly changing environmental variables, such as daily light cycles, sunlight intensity or temperature shifts. Therefore, to fully benefit from the flashing light regime in outdoor cultivation, it is important to take these factors into account. For instance, during the early growth stages in outdoor photobioreactors, shades or cooling systems are needed to protect the microalgal cells from the high photosynthetic photon flux density (PPFD) of sunlight to prevent photoinhibition [144].

1.7. Justification of the dissertation

Algal biomass with an enhanced nutritional/biochemical profile is known to reach higher market values due to its vast applications and properties, including antioxidant and anti-inflammatory. Enhancing high-value target metabolites productivity in microalgal strains relevant for algae-based industries, such as *P. tricornutum* and *N. oceanica*., allows companies to attain better prices per unit of biomass produced. Common strategies to improve microalgal biocompound production are exposing the cells to stress conditions. Nevertheless, these induction strategies often take several days or weeks, during which cultures do not grow and sometimes lose biomass due to respiration. Alternatively, recent studies showed that flashing light with low frequencies and short duty cycles improved the composition of target metabolites in microalgae when applied in the second stage of a two-stage cultivation approach. Nonetheless, while previous studies mainly focused on laboratory experiments, it is unclear whether flashing light of low frequencies (e.g., $f \leq 100$Hz) and short duty cycles (e.g., $DC \leq 0.1$Hz) can indeed improve the composition of target metabolites in large-scale cultivations. Therefore, this master thesis investigates, in the scope of the Algaesolutions project (Portugal 2020, 17/SI/2019 ID: 71486), and in collaboration with the microalgae company Allmicroalgae S.A. (Pataias, Portugal) and GreenCoLab (Faro, Portugal) the potential of using flashing light at pilot scale under outdoor conditions.

1.7.1. Objectives

The main objective of this thesis was to study the effect of flashing LED light on the growth and biochemical composition, including total proteins, total lipids, total carbohydrates, fatty acids and pigments, of the two distinct commercially exploited microalgal species *Phaeodactylum tricornutum* and *Nannochloropsis oceanica* during outdoor cultivation. Furthermore, the effect of FL was benchmarked against other covariates for both growth and biochemical composition. For instance, the covariates considered for growth included “trial”, “harvesting timepoint” and “induction time”, whereas those for biochemical composition encompassed the aforementioned covariates plus “biomass concentration” or “biomass productivity”.

1.7.2. Hypothesis

When microalgae are exposed to low-frequency FL (f=0.5 and 5Hz), and different covariates are accounted for in statistical analysis, growth or biochemical composition, i.e., target metabolites, including proteins, lipids, carbohydrates, fatty acids or pigments will improve compared to microalgae exposed to sunlight and continuous light conditions, as FL may cause stress to microalgal cells, leading to the accumulation of photoprotective pigments and storage of lipids and carbohydrates, enhancing bioactive compounds production.

2. Methodology

2.1. Microalgae

Two marine microalgae species, *P. tricornutum* and *N. oceanica* provided by Allmicroalgae S.A., Pataias, Portugal, were cultivated and tested in association with GreenCoLab laboratories, University of Algarve, Portugal, and CCMAR (Centre of Marine Sciences), University of Algarve, Portugal. Experiments were conducted during the optimal growth seasons of each species, winter and summer, respectively.

2.2. Experimental setup

The experiment was conducted in tubular and flat panel PBRs under a two-stage cultivation approach. The tubular PBR had an operational volume of 1000L and a light path of 0.15m. The flat panel PBR had an operational volume of 80L, a length of ~1.30m, a height of ~1.10m and a light path of ~0.10m. The growth of *P. tricornutum* and *N. oceanica* was studied under two flashing light regimes, with a frequency (*f*) of 0.5 (FL0.5) and 5 Hz (FL5) and compared to continuous light (CL) and sunlight (SUN) in a two-stage setup. In the first stage, *P. tricornutum* and *N. oceanica* were cultivated under sunlight for nine and seven days, respectively, followed by an induction stage, where FL was applied for four days. The experiment for each microalgae was performed twice (trials 1 and 2). For instance, *P. tricornutum* trials 1 and 2 were conducted in February and May, respectively, and *N. oceanica* trials 1 and 2 were conducted in June and July, respectively.

2.2.1. One stage: growth conditions

N. oceanica growth was carried out in tubular PBRs (**Figure 2.1**) with an operational volume of 500L. For the microalgae *P. tricornutum*, growth was performed in the flat panel PBRs. The growth medium consisted of treated seawater from the Atlantic coastal waters of Faro, Portugal (salinity: 30 ppt) and supplied with: macronutrients (1020 mgL^{-1} $NaNO_3$; 81.6 mgL^{-1} KH_2PO_4), micronutrients (0.41 mgL^{-1} $ZnCl_2$, 0.54 mgL^{-1} $ZnSO_4•H_2O$, 0.59 mgL^{-1} $MnC1_2•4H_2O$, 0.72 mgL^{-1} $Na_2MoO_4•2H_2O$, 0.07 mgL^{-1} $COCl_2•6H_2O$, 0.07 mgL^{-1} $CuSO_4•5H_2O$, 7.20 mgL^{-1} EDTA-Na, 1.47 mgL^{-1} $MgSO_4•7H_2O$) and iron (16.20 mgL^{-1} $FeCl_3$, 22.20 mgL^{-1} EDTA-Na). For the diatom *P. tricornutum*, silica (90 mgL^{-1} $Na_2O_3Si•9H_2O$) was additionally added. To maintain optimal growth conditions, temperature and pH were measured through a probe

(HI2114P, Hanna instruments, Amorim, PT) and controlled. The temperature was set to a maximum of 30°C, in which higher temperatures activated a cooling mechanism in the cultivation system. This mechanism involved the activation of water sprinkles, which dispersed cool water over the surface of the cultivation system, effectively lowering the temperature of the culture to the desired. Similarly, the pH was set to 8. To prevent deviations, an automated CO_2 injection system was employed. When pH exceeded the set value, the system would release CO_2 into the culture, effectively lowering the pH to the desired.

Figure 2.1 - Tubular PBR inoculated with *Nannochloropsis oceanica.*

2.2.2. Two stage: induction treatment

The second stage was performed in flat panel PBRs. Each side of the flat panel PBR was equipped with six strips of warm white LEDs with a 0.15m distance from each other (**Figure 2.2**). The cultures were exposed to continuous light (CL) or flashing light of 0.5 (FL0.5) and 5 Hz (FL5) 24 hours per day. As a control, cultures were grown under sunlight without additional artificial light (SUN). The employed flashing light conditions consisted of a frequency (*f*) of 0.5 (FL0.5) and 5 (FL5) Hz at a duty cycle (*DC*) of 0.05 with an average light (I_a) and flash intensity (I_l) of 100 and 2000 µmol s^{-1} m^{-2}, respectively. FL regimes were adjusted by PWM-OCX (RMCybernetics Ltd, Alsager, UK) pulse width modulators (PWMs) powered by bench KPS6020D DC PS (NANKADF, CN) 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 the power supply units. The voltages and currents supplied to the LEDs were regulated to adjust the target light intensity and compensate for switching and working losses at the LEDs and PWMs. The supplied light intensity (i.e., photosynthetic active radiation; PAR) was measured inside the flat panel in the middle of two light arrays, for 1 min, (SPQA 5234 spherical quantum sensor connected to a data logger LI-1500, Li-Cor, Lincoln, USA) and integrated over time. Air was continuously pumped through a specific aeration tube which consists of an L-shaped metal tube with tiny holes at the bottom to spread air and thus mix the culture. Overheating of the culture due to high solar irradiances was prevented by water sprinklers that were turned on every 2 minutes for 30 seconds. A culture pH of 8.00 was maintained by a pH controlling unit, which consisted of a pH controller connected to an electro valve and a pH probe (**Figure 2.3**). From each culture, samples were taken before the second stage started and daily in the morning and evening for optical density (OD) readings and biochemical analyses. During the FL exposure, biomass samples for biochemical analysis were taken during the 4 days of FL treatment exposure, at 8-time points, each 12 h apart, to have samples at both times of the day (day and night). *P. tricornutum* cultures were harvested from each treatment at the exponential phase (1 g L^{-1}) and *N. oceanica* at the late exponential phase (2 g L^{-1}).

Figure 2.2 – Flat panel PBR inoculated with *Phaeodactylum tricornutum*. From left to right, SUN, CL, FL0.5 and FL5 treatments.

Figure 2.3 – A pH controlling unit, composed of pH controllers (a), electro valves for CO_2 injection (b) and 24 and 12V power sources (c).

2.3. Growth parameters

To assess the biomass concentration (g DW L^{-1}) in the cultures during the experiment, a batch cultivation was previously performed in 900-mL flasks and in the flat panels (**Figure A1**, **Figure A2**). Every two days samples were collected to determine optical density (OD_{750}), using the UH5300 UV/VIS spectrophotometer (Hitachi, Tokyo, JP) and total dry weight (g DW L^{-1}) to obtain a linear correlation.

Total dry weight determination was performed according to Zhu & Lee (1997) with slight changes [146]. Culture samples were filtered in glass fibre filters (pore size ø = 0.7 µm; Whatman, UK) and weighted as follows: filters were washed with a 0.5M ammonium formate solution (31.5 g to 1000 mL of distilled water) in a vacuum filter, dried overnight in the oven (100˚C) and weighed ($filter_{iw}$). Then, the previously weighted filter was wettened with the ammonium formate solution and subsequently a known volume of culture that results in a total dry weight on the filter of no more than 25 mg to avoid clogging (e.g., 5 mL of a culture with a concentration of 5 g DW L^{-1}) was filtered. Then, the filter including the biomass was washed with 20 mL ammonium formate solution. Afterwards, the filter was dried and weighed again ($filter_{fw}$). Finally, the total dry weight of the known culture volume is calculated as follows:

$$\text{Total DW} = filter_{fw} - filter_{iw} \quad (1)$$

where $filter_{fw}$ (mg) is the dry weight of the filter after filtrating the culture and $filter_{iw}$ (mg) is the dry weight of the filter before filtrating the culture.

Biomass concentration (g DW L^{-1}) was then used to determine biomass productivity (g DW L^{-1} d^{-1}), while biocompound concentrations (% or mg g^{-1} of DW, depending on the specific biocompound) were used to determine the respective biocompound productivity (% or mg g^{-1} of DW d^{-1}). Productivities were calculated as follows:

$$\text{Biomass or biocompound productivity} = (X_2 - X_1) / (t_2 - t_1), \quad (2)$$

where X_2 is the biomass or biocompound concentration at time t_2 (d) and X_1 is the biomass or biocompound concentration at time t_1 (d).

2.4. Biochemical analysis

P. tricornutum and *N. oceanica* samples collected during the second stage were centrifuged at 5000 rpm for 7 min (Centrifuge 5430 R, Eppendorf, Madrid, ES). The supernatant was discharged and the pellets were frozen at -80°C until freeze-drying and storage at -80°C for further analysis.

2.4.1. Total proteins

The protein content was determined by measuring total N, according to the Dumas method. This method relies on the combustion of algal biomass material and measures the resulting elemental nitrogen. A sample of 1-2 mg of freeze-dried biomass was put into tin capsules (Santis Analytical, 5x9mm) and folded carefully. The analysis was then performed in a CHN analyser/Elemental Analyzer Vario EL III (Elementar, Langenselbold, GE). A combustion furnace (CF) was maintained at 950-1100°C to burn the sample, obtaining elemental compounds. Then, water was removed by a first physical trap (WT1) and a second chemical one (WT2). Between these two, the elemental compounds passed through a reduction furnace (RF). Finally, the CO_2 absorbers allowed only the elemental nitrogen to pass, being then detected by the thermal conductivity detector (TCD). Summing up, this method converts the nitrogen present in the sample into gaseous Nox by complete combustion, where the final product (Nox) is then reduced to N_2 and measured using the TCD. Total protein was then calculated by multiplying the total N by the conversion factor of 6.25 as determined by Kjeldahl (1883) [147].

2.4.2. Total lipids

Total lipids were determined according to the Bligh and Dyer (1959) gravimetric method, with some modifications [148]. A sample of 20 mg of freeze-dried biomass, along with glass beads (425–600 μm), were transferred to 2mL centrifuge tubes (Eppendorf, Hamburg, GE), adding posteriorly 0.8 mL of distilled water to soften the samples. Upon a 15 min incubation period at room temperature, 0.5 mL of methanol and 0.2 mL of chloroform were added, and algal cells were disrupted using a MM 400 Mixer Mill (RETSCH, Düsseldorf, GE) at 30 Hz for 3 min. After, biomass was transferred to glass tubes and the 2mL centrifuge tubes were cleaned with 1.5 mL methanol and 0.75 mL of chloroform and added to the glass tubes. After mixing in vortex, 1 mL chloroform was added and mixed again, where 1 mL distilled water was added. Samples are mixed and homogenised again before phase separation. Phase separation was achieved by centrifugation at 2500 × *g* for 10 min (Centrifuge 5430 R, Eppendorf, Madrid, ES). Subsequently, a known volume of the bottom phase (extract), containing the chloroform and the lipids was transferred into new, pre-weighed tubes. The extracted was evaporated at 60°C using a dry block heater (Grant QBD4, Geraardsbergen, BE) for at least 3h followed by a 3-h cooling-down step in a desiccator at room temperature. Finally, lipids were determined gravimetrically as follows:

$$\%\ \text{total lipids} = \frac{\frac{(\text{final weight} - \text{initial weight}) \times \text{total chloroform}}{\text{evaporated chloroform}}}{\text{sample weight}} \times 100 \qquad (3)$$

where final and initial weight (mg) correspond to the final and initial weights of the glass tubes, respectively; total chloroform (mL) is the total volume of chloroform used in the extraction procedure; evaporated chloroform (mL) is the volume of the evaporated extract; sample weight (mg) corresponds to biomass weight.

2.4.3. Fatty acids

Fatty acids were determined based on a modified method of Lepage and Roy (1984) [20], where the fatty acids are directly transesterified (without extraction and previous purification) with a derivatization solution (methanol/acetyl chloride), extracted with an organic solvent (hexane) and finally analysed by GC-MS according to Pereira et al. (2012) [21].

A sample of approximately 20 mg of freeze-dried biomass was transesterified with 1.5 mL of the derivatization solution methanol/acetyl chloride (20:1, v/v). Samples were then homogenized, along with glass beads (425–600 μm) in 2 mL centrifuge tubes in the MM 400 Mixer Mill (RETSCH, Düsseldorf, GE). After, 1 mL of n-hexane was added to wash the 2 mL centrifuge tubes, which was then placed in the water bath (12L VWR® Unstirred Water Baths, Leuven, BE) at 70 °C for 60 minutes. After the samples cooled down, on ice, they were transferred to glass centrifuge tubes and 1 mL of distilled water and 4 mL of n-hexane (part by part) were added. Samples were then mixed in a vortex and centrifuged at 2000 ×g for 5 min at RT (Centrifuge 5430 R, Eppendorf, Madrid, ES). The hexane fraction was then transferred to new glass vials. The *n*-hexane addition and later steps were repeated twice. After extraction, anhydrous sodium sulphate (Na_2SO_4) was added to the glass vials to dry the organic phase of the extracts and the samples were filtered (0.22 μm PTFE) to new vials. The hexane was then evaporated under a gentle nitrogen gas flow until dryness. Samples were resuspended in 500 µl gas chromatography-grade hexane and transferred to vials, along with 2 μL of internal standard to each sample for GC-MS (Agilent 6890 Network GC System/5973 Inert Mass Selective Detector, Agilent, California, USA) analysis with a DB5-MS Plus capillary column (25m × 0.25mm internal diameter, 0.25μm film thickness, Agilent Tech, California, USA), with a runtime of 60min. Fatty acids quantification was performed through calibration curves of standard Supelco® 37 Component FAME Mix (Merck, Darmstadt, GE).

2.4.4. Pigments

Pigments were analysed according to Schüler et al. (2020) with modifications [149]. A sample of 5 mg freeze dried biomass was transferred into 2mL centrifuge tubes along with glass beads (425–600 μm). Biomass was then disrupted with 1 mL 100% methanol containing 0.03% butylhydroxytoluene (BHT). Cells were then disrupted in MM 400 Mixer Mill (RETSCH, Düsseldorf, GE) at 25 Hz for 3 min. Afterwards, cells were centrifuged at 15,000 × *g* for 6 min (Centrifuge 5430 R, Eppendorf, Madrid, ES). The supernatant was transferred to amber glass vials. The extraction was then repeated (disruption and supernatant transfer) until both the pellet and the supernatant became colourless. The extract (methanol) was then dried using gentle nitrogen flow. Afterwards, samples were resuspended in 0.5 mL of 100% methanol HPLC grade and filtered (0.22 μm PTFE) prior to HPLC injection. The pigment extracts were analysed with an HPLC system (Hitachi Chromaster, VWR, Carnaxide, PT) and recorded at 450 nm with a Diode array detector (Hitachi 5430 DAD, VWR, Carnaxide, PT) and with a Purosphere® STAR RP-18 endcapped column (250mm × 4mm internal diameter, 5µm pore size, Merck, Darmstadt, GE). A gradient program was applied, with 9:1 (v/v) acetonitrile:water as solvent A and ethyl acetate as solvent B with a runtime of 40min at 27°C. Finally, pigment quantification was performed through calibration curves of standard pigments.

2.4.5. Ash

The ash content was determined by heating 50-100mg of freeze-dried biomass samples at 550 °C in a muffle furnace (Narberthern, Lilienthal, GE) for 4 hours. This allows to remove all moisture and volatiles (as vapours) and organics (as CO_2 and NO_x).

2.4.6. Carbohydrates

Carbohydrates content was determined by difference, subtracting the measured protein, fat, ash, and water from the total weight.

2.5. Data analysis

Statistics were performed using Jasp software (version 0.18.3). ANCOVA analysis was performed to detect differences in biochemical contents among treatments while considering the effects of potential covariates. For microalgal growth parameters (Section 3.1 Effects of FL on microalgal growth), the covariates considered were “trial” (1 and 2), “harvesting timepoint” (i.e., morning vs. evening) and “induction time” (0 – 3 days). For biochemical composition (Section 3.2 Effects of FL on biochemical composition), the covariates considered were “trial”, “harvesting timepoint”, “induction time” and “biomass concentration” (g DW L^{-1}). Still in this section, the covariates considered in biocompounds productivity and content variation results, i.e., the increase or decrease of that compound in the biomass during the different days, are the same with slight modifications: the covariate “induction time” refers to total days of induction (1, 2, 3) and “biomass concentration” is replaced with “biomass productivity” (g DW L^{-1} d^{-1}). Data were tested for normality using the Shapiro-Wilk test. A significance level of 0.05 was used for all tests. The Type III sum of squares analysis was considered. The output *p*-values were used to describe the impact of treatment and the different covariates on the analysed data (growth, C and N, proteins, lipids, carbohydrates, fatty acids and pigments).

The adjusted means with 95% confidence interval from Tukey's post hoc tests were used to illustrate the differences among the treatments in the figures and tables. Notably, the adjusted means represent the differences among treatments while considering the effects of the mentioned covariates.

3. Results and Discussion

3.1. Effects of FL on microalgal growth

In the present study, we could not detect significant differences between treatments in *P. tricornutum* and *N. oceanica,* both in terms of microalgal growth (p>0.05) (**Figure 3.1**) and biomass productivity (p>0.05) (**Table 3.1**), when normalised to the effects of the studied covariates. Additionally, none of the covariates affected either species in microalgal growth (p>0.05). Nevertheless, biomass productivity (0.16±0.02 g DW L^{-1} d^{-1}) in *P. tricornutum* was, on average, two-times higher compared to that of *N. oceanica* (0.09±0.02 g DW L^{-1} d^{-1}). A reason for this discrepancy might be because while *P. tricornutum* was at the peak of exponential growth, *N. oceanica* was already at the end of exponential growth, reaching the linear growth phase.

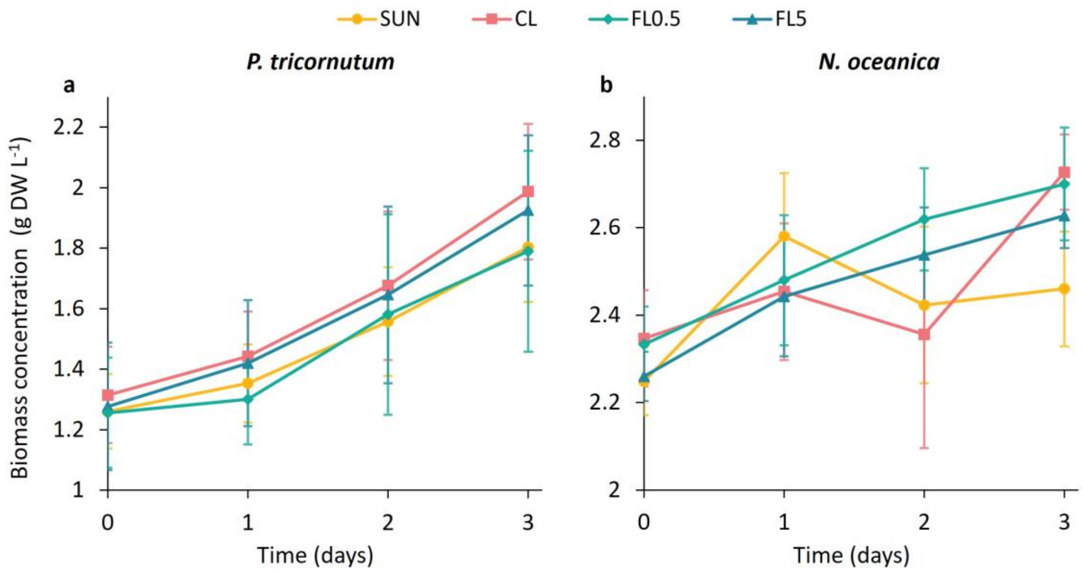

Figure 3.1 - Biomass (dry weight; DW) evolution of *Phaeodactylum tricornutum* (a) and *Nannochloropsis oceanica* (b). FL0.5 and FL5 were tested at a duty cycle of 0.05. Shown are the adjusted means ± 95 % confidence interval (ANCOVA) from data of the induction stage (n=4) adjusted for the effects of the covariates "trial", "induction time" and "harvesting timepoint". Statistical analysis revealed no significant differences among treatments (p>0.05).

Notably, biomass productivity (**Table 3.1**) was not affected by the "harvesting timepoint" (i.e., morning vs. evening) (p>0.05) in either species. Conversely, ANCOVA results showed that there was an effect of "trial" on biomass productivity in *P. tricornutum* (p<0.001). Biomass productivity significantly decreased from trial 1 (0.21±0.03 g DW L^{-1} d^{-1}) to trial 2 (0.10±0.03 g DW L^{-1} d^{-1}). This change might be explained by the period of cultivation. *P. tricornutum* is a wintertime species in southern Europe, with optimal cultivation temperatures between 15-20°C. The first trial was conducted in February, when 18°C was the maximum temperature. However, the second trial was conducted in May, where maximum temperatures could go up to 30°C. Besides, biomass concentration at the beginning of the FL exposure in trial 2 (1.01 g DW L^{-1}) was slightly lower than biomass concentration in trial 1 (1.32 g DW L^{-1}). As mentioned before, higher biomass concentration is associated with a high number of cells in the culture and better light distribution, thus limiting photoinhibition and improving growth compared to less concentrated cultures.

Table 3.1 - Biomass productivities (g DW L^{-1} d^{-1}) of *Phaeodactylum tricornutum* and *Nannochloropsis oceanica*. FL0.5 and FL5 were tested at a duty cycle of 0.05. Shown are the adjusted means ± 95 % confidence interval (ANCOVA) from data of the induction stage (n=4) adjusted for the effects of the covariates "trial", "induction time" and "harvesting timepoint". Statistical analysis revealed no significant differences among treatments (p>0.05).

	Biomass productivity (g DW L^{-1} d^{-1})			
	SUN	CL	FL0.5	FL5
P. tricornutum	0.14±0.02	0.17±0.05	0.14±0.05	0.17±0.05
N. oceanica	0.05±0.02	0.11±0.02	0.09±0.02	0.09±0.02

Our results differ from previous studies, where algae exposed to FL frequencies (e.g., f = 5 Hz) displayed higher biomass productivity when compared to CL [125,126,132]. For instance, previous studies on *N. gaditana* showed the lowest biomass productivities under FL regimes (f = 5, 50 and 500 Hz) compared to CL, and higher growth under higher FL frequencies compared to lower ones [125,138] However, in the present study, *N.* oceanica displayed similar growth under all treatments, with no significant differences among them. Additionally, previous studies in different microalgae created an assumption, in which cell size was inversely related to cells being prone to photoinhibition. This assumption can be related to the possibility that larger cells are less prone to photoinhibition and thus might adapt better to the long-lasting high-light intensities of low-frequency FL (e.g. $f = 0.5$ Hz) [125,150]. For instance, *Nannochloropsis* sp. presents a 2-4μm or 4.5-6μm diameter for round-shaped and cylindrical cells, respectively [151] and the most common morphotype – fusiform – of *P. tricornutum* is 18-25μm long and 2-3μm wide [62,152]. Hence, according to the previous assumption, *P. tricornutum* should absorb light better, using it for photosynthesis, and cope better with long-lasting high light intensities, i.e., low FL frequencies, as it is less susceptible to photoinhibition. Results of the present study show indeed that *P. tricornutum* presented higher biomass productivity when compared to *N. oceanica* (**Table 3.1**). However, since productivities are not significantly different among treatments, this verdict applies to all treatments and not only to low-frequency FL. Nevertheless, previous studies that were in accordance with this assumption, despite employing the same *DC* as the one in our study ($DC = 0.05$), the authors used a higher light intensity ($I_a = 300$ μmol s^{-1} m^{-2}), resulting in a higher flash intensity as well ($I_l = 6000$ μmol s^{-1} m^{-2}) [125,126,138]. Under FL regimes, cells receive light at higher intensities during the light period (i.e., flash intensity) [143]. Thus, it is important to choose an adequate light intensity to not exceed light saturation points [153]. At lower light intensities, microalgae are cultivated below their light saturation point, i.e., they are not yet limited by the availability of light for photosynthesis. Accordingly, they can efficiently use the available light pulses from the FL without experiencing light stress or saturation effects that may be detrimental to the photosynthetic apparatus and the cellular metabolism in general [144,154]. Therefore, cells exposed to a lower light intensity show better light efficiencies under FL regimes [155]. This postulation might explain the acceptable growth of both species under FL, assuming that the light intensity ($I_a = 100$ μmol s^{-1} m^{-2}) applied in this study did not cause photoinhibition under low frequencies, unlike when high intensities (e.g., $I_a = 300$ μmol s^{-1} m^{-2}) were applied in previous studies on *Nannochloropsis* sp. Despite the existing literature on the effects of FL on microalgal growth (**Table 1.5**), there is a scarcity of studies specifically investigating these effects on *P. tricornutum* or similar species.

3.2. Effects of FL on biochemical composition

To obtain an overall understanding of the biochemical composition of the studied microalgae, the results will first be discussed in terms of proximate composition (C and

N, protein, lipid and carbohydrate) followed by profile analysis (fatty acids and pigments).

3.2.1. Proximate composition

3.2.1.1.C and N

According to ANCOVA results, there were no significant differences between treatment in N and C contents or in C:N ratio in either species (**Figure 3.2**, **Figure 3.3**). However, the covariate “trial” significantly predicted N content in *N. oceanica* (p<0.001), with higher content in trial 2. C content was significantly affected by “trial” in both *P. tricornutum* and *N. oceanica* (p<0.05), with higher content in trials 1 and 2, respectively. Regarding the C:N ratio, “trial” (p=0.001; p<0.001) and “harvesting timepoint” (p<0.05; p<0.001) affected this ratio significantly in both *P. tricornutum* and *N. oceanica*, respectively. A higher C:N ratio was noted in trial 1 in *P. tricornutum* and trial 2 in *N. oceanica* and in samples collected in the evening in both species. During daylight, microalgae perform photosynthesis, i.e., they use CO_2 as a substrate for carbon fixation and biomass production. This leads to the assimilation of C into organic molecules, including carbohydrates, lipids and proteins, leading to a higher C:N ratio at the end of the day [156]. Additionally, the covariate “biomass concentration” (p<0.05) significantly predicted the C:N ratio in *P. tricornutum*, whereas “induction time” (p<0.001) significantly predicted the C:N ratio in *N. oceanica*, being higher on days 2 (12.24±0.12) and 3 (12.34±0.13) compared to day 0 (11.21±0.14).

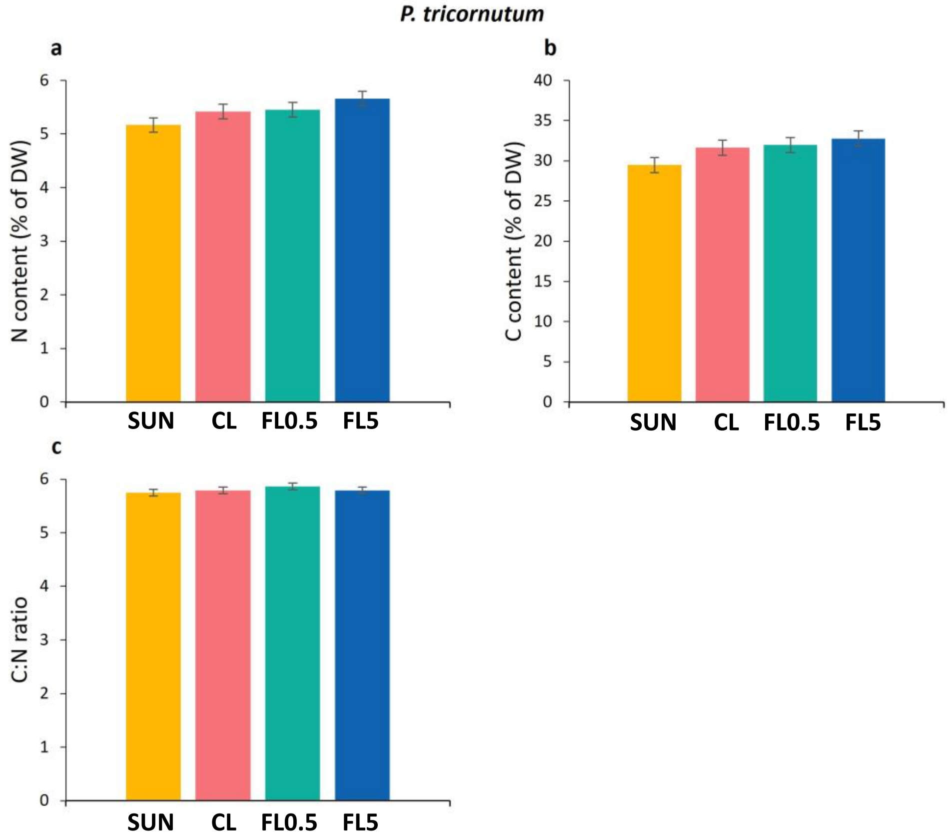

Figure 3.2 - Total N (a) and C (b) content (% of DW) and C:N ratio (c) of *Phaeodactylum tricornutum*. FL0.5 and FL5 were tested at a duty cycle of 0.05. Shown are the adjusted means ± 95 % confidence interval (ANCOVA) from data of the induction stage (n=16) adjusted for the effects of the covariates “trial”, “induction time”, “harvesting timepoint” and “biomass concentration”. Statistical analysis revealed no significant differences among treatments (p>0.05).

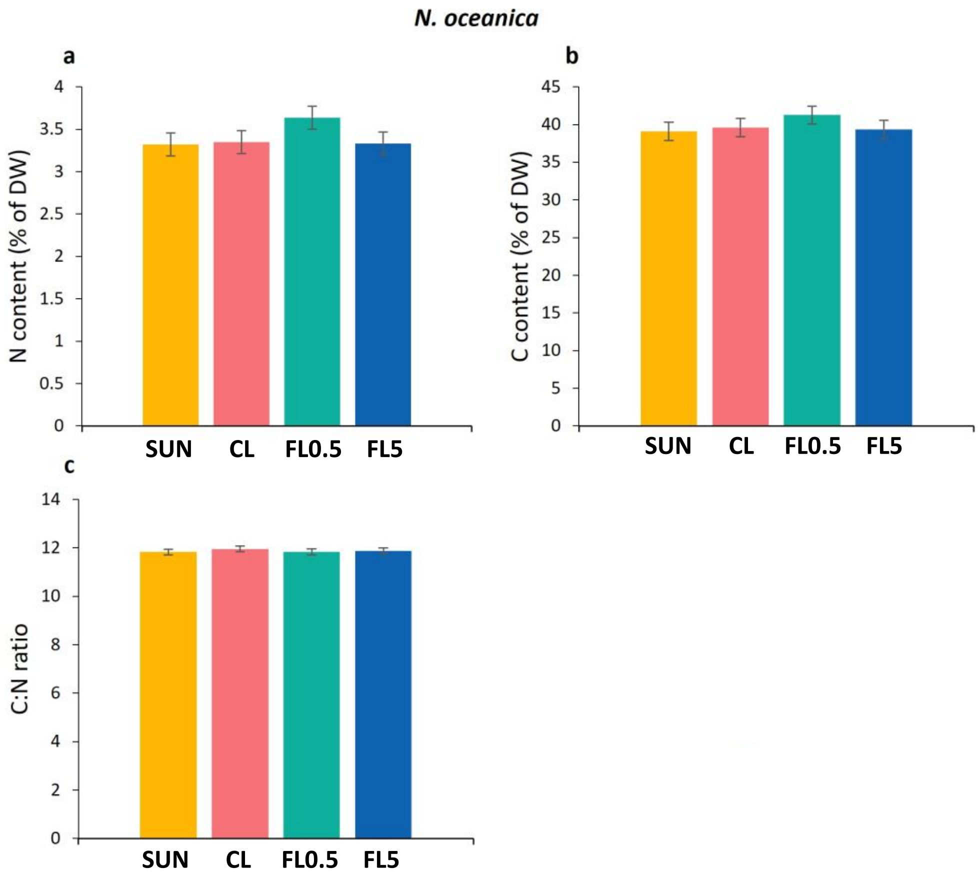

Figure 3.3 - Total N (a) and C (b) content (% of DW) and C:N ratio (c) of *Nannochloropsis oceanica*. FL0.5 and FL5 were tested at a duty cycle of 0.05. Shown are the adjusted means ± 95 % confidence interval (ANCOVA) from data of the induction stage (*n*=16) adjusted for the effects of the covariates "trial", "induction time", "harvesting timepoint" and "biomass concentration". Statistical analysis revealed no significant differences among treatments (*p*>0.05).

Overall, *N. oceanica* presents higher C content (39.11-41.25% of DW) (**Figure 3.3b**), whereas *P. tricornutum* presents higher N content (5.17-5.66% of DW) (**Figure 3.2a**). Thus, and in accordance with the obtained results, *N. oceanica* presents a higher C:N ratio (11.83-11.96) compared to *P. tricornutum* (5.75-5.86) (**Figure 3.2c, Figure 3.3c**). These results suggest that *N. oceanica* has higher carbohydrate and lipid content, whereas *P. tricornutum* shows higher protein content.

3.2.1.2. Total proteins

The effect of FL on protein content is species-dependent (**Figure 3.4**). Overall, cells under FL treatments presented a higher or similar protein content as those of SUN and CL. Nonetheless, these differences were only significant in *P. tricornutum* (**Figure 3.4a**). The ANCOVA model showed that the treatment significantly affected protein content (p<0.05). Post hoc testing using Tukey's correlation revealed that cells under FL5 (35.24±1.60% of DW) had significantly higher protein contents compared to cells under SUN conditions (32.30±1.61% of DW) after controlling for all covariates ("trial", "induction time", "harvesting timepoint" and "biomass concentration"). This result suggests that microalgae exhibited a stress response mechanism under FL5, leading to the upregulation of specific proteins involved in the response to stress conditions (e.g., HSPs – heat shock proteins), leading to higher total protein content [157]. All tested covariates did not affect protein content (p>0.05). Regarding *N. oceanica*, there were no significant differences between treatments regarding protein content (p>0.05), ranging from 20.77±0.85 to 22.72±0.85% of DW among all treatments (**Figure 3.4b**). Nevertheless, it was significantly affected by the covariate "trial" (p<0.001), where values on trial 2 (24.33±1.48% of DW) were significantly higher than trial 1 (18.29±1.48% of DW). Generally, the overall protein composition is in accordance with the typical levels of protein content found in both species (**Table 1.1**,**Table 1.*2***). Additionally, protein content (**Figure 3.4**) follows the same trend as N (**Figure 3.2a, Figure 3.3a**) and C (**Figure 3.2b**, **Figure 3.3b**) content in both species. This shows that both nitrogen and carbon availability correlate with protein synthesis. This phenomenon might be due to their interdependent nature since nitrogen and carbon are the main constituents of proteins (amino acids). This seems to be supported by the fact that the microalga with the highest protein content was *P. tricornutum*.

Previously published studies of FL effects regarding protein content in *Nannochloropsis* sp. did not report significant differences among the light regimes assayed (CL and FL of 5, 50 and 500 Hz), reaching values of 12-49% in *N. gaditana* (*DC* = 0.05; I_a = 300 μmol s^{-1} m^{-2}). Nonetheless, the same study, in marine green microalgae, such as *Koliella antarctica* and *Tetraselmis chui*, reported significantly higher protein content under FL treatments of 5 and 50 Hz [125]. Nevertheless, these studies were performed on a laboratory scale, under a controlled environment. To the best of the author's knowledge, there are no published studies yet reporting an effect of FL in protein content in *P. tricornutum*.

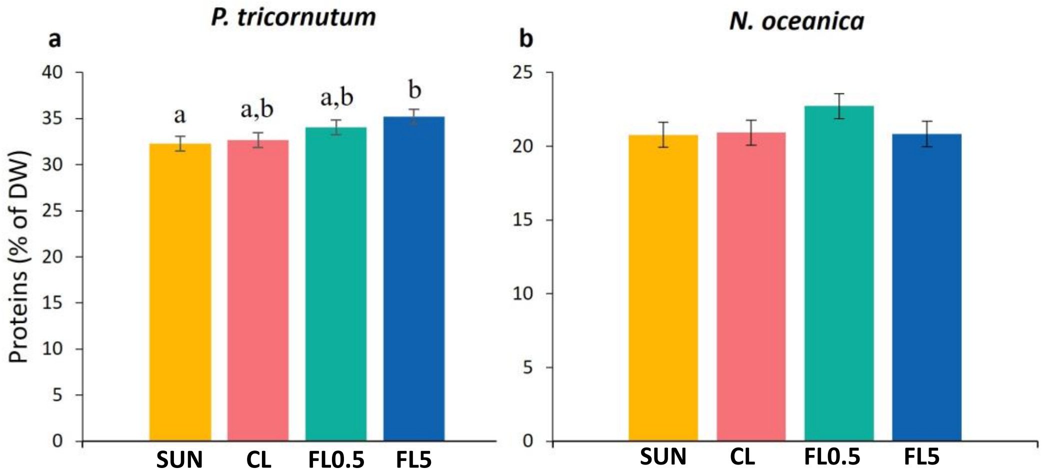

Figure 3.4 - Total protein content (% of DW) of *Phaeodactylum. tricornutum* (a) and *Nannochloropsis oceanica* (b). FL0.5 and FL5 were tested at a duty cycle of 0.05. Shown are the adjusted means ± 95 % confidence interval (ANCOVA) from data of the induction stage (*n*=16) adjusted for the effects of the covariates "trial", "induction time", "harvesting timepoint" and "biomass concentration". Treatments that do not share the same letter are significantly different from each other (a). Statistical analysis revealed no significant differences among treatments (p>0.05) (b).

Regarding protein productivity (% of DW d^{-1}) and cumulative protein content variation (% of DW), there were no significant differences between treatments (p>0.05) in either species (**Table 3.2**). Protein contents (% of DW) ranged from 1.01±2.31 to 6.24±4.16% of DW among treatments and induction time in *P. tricornutum* and from -10.38±8.41 to 2.73±8.48 % of DW among treatments and induction time in *N. oceanica* (**Table 3.2**). Nonetheless, the covariate "trial" significantly affected protein productivity (p<0.05) in both species, with higher contents on trial 1 (4.12±1.74% of DW d^{-1} in *P. tricornutum* and 3.860±3.48% d^{-1} of DW in *N. oceanica*) and negative values on trial 2. An explanation for this behaviour is that, on trial 2, protein degradation rates were possibly higher than protein synthesis rates.

Table 3.2 – Protein productivity (% of DW d^{-1}) and cumulative protein content variation (% of DW) during the 1, 2 and 3 days of induction of *Phaeodactylum tricornutum* and *Nannochloropsis oceanica.* FL0.5 and FL5 were tested at a duty cycle of 0.05. Shown are the adjusted means ± 95 % confidence interval (ANCOVA) from data of the induction stage (n=4) adjusted for the effects of the covariates "trial", "induction time", "harvesting timepoint" and "biomass productivity". Statistical analysis revealed no significant differences among treatments (p>0.05).

		Protein productivity (% of DW d^{-1})	Cumulative protein content variation (% of DW)		
			Induction time (d)		
			1	2	3
P. tricornutum	SUN	2.99±2.21	1.71±4.22	3.17±4.00	4.11±4.04
	CL	4.16±2.22	0.99±4.20	5.23±4.21	6.24±4.16
	FL0.5	-0.05±2.19	-1.01±2.31	-0.85±2.51	1.79±4.02
	FL5	2.26±2.19	2.64±4.17	2.56±3.99	1.59±3.15
N. oceanica	SUN	-3.815±4.69	-10.38±8.41	0.59±8.38	-1.64±5.12
	CL	-1.67±4.72	-1.39±5.72	-0.72±7.37	-2.78±3.16
	FL0.5	0.46±4.70	-0.27±8.08	-1.19±6.07	2.67±8.55
	FL5	1.79±4.70	2.73±8.48	1.18±8.39	1.41±8.52

3.2.1.3. Total lipids

According to ANCOVA results, treatments did not affect total lipid content after controlling for all covariates ("trial", "induction time", "harvesting timepoint" and "biomass concentration") (p>0.05) (**Figure 3.5**). However, the covariate "trial" significantly affected lipid content in both species (p<0.05 in *P. tricornutum* p<0.001 in *N. oceanica*). In *P. tricornutum*, lipid content in trial 1 (16.77±0.90% of DW) was significantly higher than in trial 2 (14.35±0.90% of DW) after controlling for all covariates (p>0.05). Contrarily, in *N. oceanica*, lipid content in trial 2 (20.51±0.85% of DW) was significantly higher than in trial 1 (15.560±0.85% of DW). Besides "trial", the covariate "induction time" also had a significant effect on *P. tricornutum* (p<0.05) between day 0 (13.67±1.02% of DW) and the remaining three days (day 1: 15.62±0.95% of DW; day 2: 16.72±0.92% of DW; day 3: 16.22±1.07% of DW). Generally, the overall total lipid content is in accordance with the typical levels of lipid content found in both species (**Table 1.1**, **Table 1.2**). Moreover, total lipid content (% of DW) (**Figure 3.5**) and C content (% of DW) (**Figure 3.2b**, **Figure 3.3b**) follow a similar trend, indicating that the high lipid contents correspond to high carbon contents. This occurs due to the intrinsic relationship between both since lipids are composed of carbon molecules. This similar behaviour indicates that both species are using efficiently the available C for lipid synthesis.

Previously published studies of FL effects regarding total lipid content in *N. gaditana* reported significant differences (p<0.05) among treatments (CL and FL of 5, 50 and 500 Hz), where cells under CL conditions presented the highest, whereas cells under FL0.5 conditions presented the lowest lipid contents [125]. However, the authors reported values of 23-43% in *N. gaditana*, while in our study we obtained a lipid content of 17-19% [125]. Nevertheless, conditions differed between studies. Although the same *DC* was applied, their light intensity was higher (I_a = 300 μmol s^{-1} m^{-2}), as well as frequencies (5 and 50 Hz) [125]. Another study, on the same species, also reported higher lipid content under FL treatments (f = 25 and 250 Hz), being highest at the latter [142]. Nonetheless, besides frequency, light intensity (I_a = 70 μmol s^{-1} m^{-2}) and duty cycle (*DC* = 0.25) were also different. These changes might have been enough to cause stress-induced responses. Under stress conditions, such as high light intensity, to prevent photoinhibition and oxidative stress, microalgae redirect its resources towards lipid production as a protective mechanism [158]. Regarding the effects of FL on lipid content in *P. tricornutum*, to the best of the author's knowledge, there are no published studies yet.

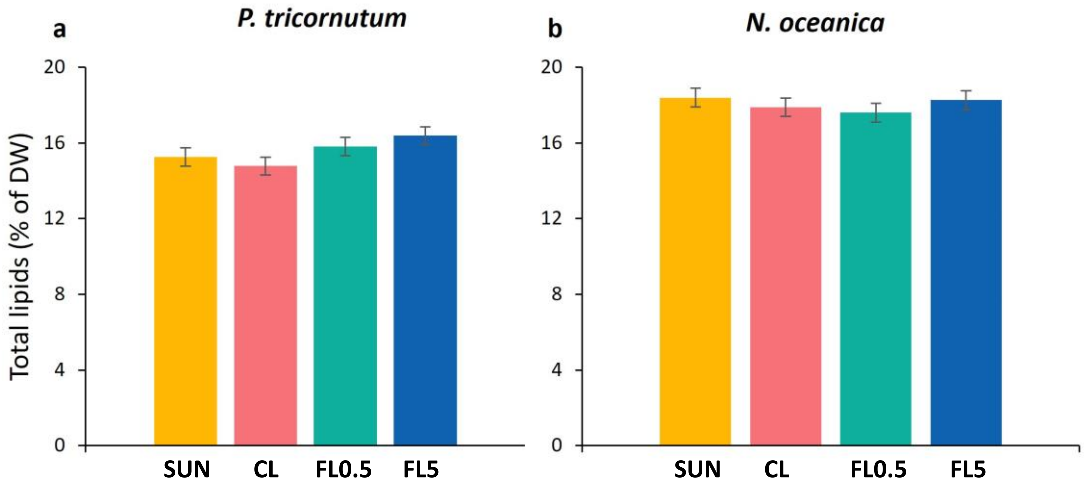

Figure 3.5 - Total lipid content (% of DW) of *Phaeodactylum tricornutum* (a) and *Nannochloropsis oceanica* (b). FL0.5 and FL5 were tested at a duty cycle of 0.05. Shown are the adjusted means ± 95 % confidence interval (ANCOVA) from data of the induction stage (n = 16) adjusted for the effects of the covariates “trial”, “induction time”, “harvesting timepoint” and “biomass concentration”. Statistical analysis revealed no significant differences among treatments (p>0.05).

Regarding total lipids productivity (% of DW d^{-1}) and cumulative lipid content variation (% of DW) , there were no significant differences between treatments (p>0.05) in either species (**Table 3.3**). Lipid contents (% of DW) ranged from 1.04±2.56 to 4.59±2.58% of DW among treatments and induction time in *P. tricornutum* and from -2.87±2.81to 2.04±2.84% of DW among treatments and induction time in *N. oceanica* (**Table 3.3**). Nonetheless, the covariate “trial” significantly affected total lipid productivity (p<0.05) in both species, reaching higher values on trial 1 (3.49±1.00% of DW d^{-1} in *P. tricornutum* and 2.33±1.14% of DW d^{-1} in *N. oceanica*). Additionally, the covariate “harvesting timepoint” also affected total lipid productivity (p<0.05) in *P. tricornutum*, with higher productivity in samples collected in the morning (3.96±1.26% of DW d^{-1}) than in samples collected in the evening (1.50±0.99% of DW d^{-1}). One of the reasons may be due to temperature. Since these experiments were conducted in an outdoor environment and temperature could not be perfectly regulated, this is a factor that may play a crucial role in microalgae behaviour. During the night and sunrise, i.e., early hours of the day, temperatures tend to be lower compared to temperatures during the day until sunset. Lower temperatures decrease membrane fluidity [159]. To maintain proper membrane function and integrity under cold stress, microalgae enhance lipid biosynthesis, stimulating lipid accumulation by producing unsaturated fatty acids [160–

162]. Moreover, besides FL, microalgal cultures were illuminated by sunlight. In this case, it is possible that the cells were under stressful conditions due to the combination of both FL and sunlight exposure. These high levels of stress might have led to lipid degradation, resulting in the rerouting of these carbon skeletons to produce protective molecules, such as antioxidants. The same might have happened under the CL treatment. Conversely, under less stressful conditions, i.e., during the night, where only FL or CL was applied to the culture, a better response and production of lipids might have taken place.

Table 3.3 – Total lipids productivity (% of DW d^{-1}) and cumulative lipid content variation (% of DW) during the 1, 2 and 3 days of induction of *Phaeodactylum tricornutum* and *Nannochloropsis oceanica.* FL0.5 and FL5 were tested at a duty cycle of 0.05. Shown are the adjusted means ± 95 % confidence interval (ANCOVA) from data of the induction stage (n=4) adjusted for the effects of the covariates "trial", "induction time", "harvesting timepoint" and "biomass productivity". Statistical analysis revealed no significant differences among treatments (p>0.05).

		Total lipids productivity (% of DW d^{-1})	**Cumulative lipid content variation (% of DW)**		
			Induction time (d)		
			1	**2**	**3**
P. tricornutum	**SUN**	1.87±1.39	1.26±2.59	1.30±2.46	2.82±2.48
	CL	3.42±1.39	2.97±2.58	4.59±2.58	2.94±2.56
	FL0.5	3.14±1.37	2.92±2.73	4.17±2.60	2.33±2.46
	FL5	1.94±1.37	1.04±2.56	2.35±2.45	2.44±2.54
N. oceanica	**SUN**	1.12±1.65	1.30±2.84	0.93±2.83	1.68±2.83
	CL	-1.45±1.67	-2.35±1.84	1.04±2.97	-2.87±2.81
	FL0.5	1.14±1.66	0.17±2.86	2.04±2.84	1.11±2.88
	FL5	-0.94±1.66	-0.63±1.60	-1.09±0.64	-1.14±0.60

3.2.1.4. Carbohydrates

The effect of FL on carbohydrate content is species-dependent (**Figure 3.6**). Treatment affected significantly carbohydrate content in *P. tricornutum* ($p<0.05$) (**Figure 3.6a**). Post hoc testing using Tukey's correlation revealed that cells under CL (18.56±3.21% of DW) and SUN (18.0±3.13% of DW) resulted in significantly higher carbohydrate content compared to those under FL0.5 (11.95±3.30% of DW). In *N. oceanica*, there were no significant differences between treatments ($p>0.05$) (**Figure 3.6b**). In *P. tricornutum* the covariate "trial" significantly affected carbohydrate content ($p<0.05$), with higher content on trial 1 (20.30±3.09% of DW). Regarding *N. oceanica*, carbohydrate content was significantly affected by the covariate "induction time" ($p<0.001$). Post hoc testing using Tukey's correlation revealed that days 2 (32.23±2.61% of DW) and 3 (33.30±2.85% of DW) resulted in significantly greater carbohydrate content compared to day 0 (25.02±2.99% of DW). Generally, the overall carbohydrate composition is in accordance with the typical levels of carbohydrate content found in both species (**Table 1.1**, **Table 1.2**).

Previously published studies of FL effects on carbohydrate content in *N. gaditana* did not report significant differences among light regimes (CL and FL of 5, 50 and 500 Hz; DC = 0.05; I_a = 300 µmol s^{-1} m^{-2}) either, reaching values of 7-14% in *N. gaditana* [125]. However, in our study, we could reach values in *N. oceanica* of up to 31%. Nonetheless, the same study in a marine green microalga, *Tetraselmis chui*, presented significant differences between treatments ($p<0.05$), with 40% higher content under CL than FL5 [125]. Regarding the effects of FL on carbohydrate content in *P. tricornutum*, to the best of the author's knowledge, there are no published studies as yet.

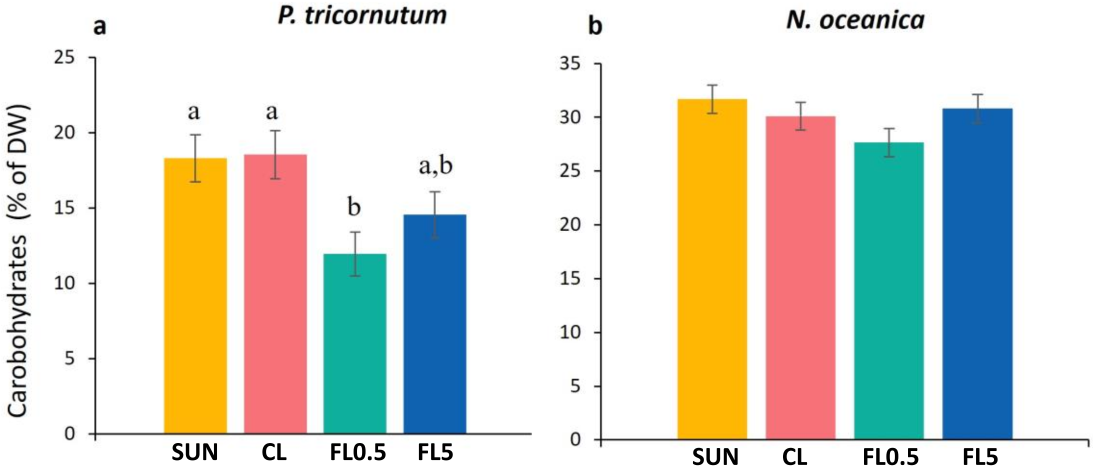

Figure 3.6 - Total carbohydrate content (% of DW) of *Phaeodactylum tricornutum* (a) and *Nannochloropsis oceanica* (b). FL0.5 and FL5 were tested at a duty cycle of 0.05. Shown are the adjusted means ± 95 % confidence interval (ANCOVA) from data of the induction stage (*n*=16) adjusted for the effects of the covariates "trial", "induction time" "harvesting timepoint" and "biomass concentration". Treatments that do not share the same letter are significantly different from each other (a). Statistical analysis revealed no significant differences among treatments (p>0.05) in *Nannochloropsis oceanica* (b).

Regarding carbohydrate productivity (% of DW d^{-1}) and cumulative carbohydrate content variation (% of DW), there were no significant differences between treatments in either species (p>0.05) (**Table 3.4**). Carbohydrate contents (% of DW) ranged from -8.56±5.20 to 12.62±10.02% of DW among treatments and induction time in *P. tricornutum* and from -1.72±7.16 to 11.32±7.06% of DW among treatments and induction time in *N. oceanica* (**Table 3.4**). None of the covariates affected carbohydrate productivity in *P. tricornutum.* Nonetheless, the covariate "harvesting timepoint" significantly affected carbohydrate productivity (p<0.001) in *N. oceanica*. Samples harvested in the morning presented a significantly higher carbohydrate productivity (8.18±2.78% of DW d^{-1}) than those harvested in the evening (1.21±2.78 % of DW d^{-1}). Similar to total lipid productivity (% of DW d^{-1}), one of the reasons may be the excessive light stress by the combination of both FL or CL treatment and sunlight during the day, causing carbohydrate degradation to produce protective molecules, such as antioxidants. Thus, there would be a better response and production of carbohydrates during less stressful conditions, i.e., during the night, when only FL or CL is applied to the culture. Furthermore, although the duration of light exposure until harvesting at sunrise is short, the light conditions, including sunlight intensity and angle, and atmospheric conditions during that time may be particularly optimal for carbohydrate production, leading to a fast increase in carbohydrate productivity. Nonetheless, to have a better understanding of this behaviour, other parameters or variables, such as antioxidants analysis, sunlight intensity and irradiance, and atmospheric variables (e.g., temperature) or specific carbohydrate quantification should be studied in the future.

Table 3.4 – Carbohydrate productivity (% of DW d^{-1}) and cumulative carbohydrate content variation (% of DW) during the 1, 2 and 3 days of induction of *Phaeodactylum tricornutum* and *Nannochloropsis oceanica.* FL0.5 and FL5 were tested at a duty cycle of 0.05. Shown are the adjusted means ± 95 % confidence interval (ANCOVA) from data of the induction stage (n=4) adjusted for the effects of the covariates “trial”, “induction time”, “harvesting timepoint” and “biomass productivity”. Statistical analysis revealed no significant differences among treatments (p>0.05).

		Carbohydrate productivity (% of DW d^{-1})	**Cumulative carbohydrate content variation (% of DW)**		
			Induction time (d)		
			1	**2**	**3**
P. tricornutum	**SUN**	-2.76±4.80	-4.28±8.96	-0.63±7.21	-2.35±8.53
	CL	1.34±4.99	5.30±8.91	-8.56±5.20	0.05±10.08
	FL0.5	7.27±4.95	6.38±9.47	3.61±8.94	12.62±10.02
	FL5	0.17±4.72	1.26±8.45	-1.57±5.30	0.89±8.77
N. oceanica	**SUN**	7.71±3.85	4.07±7.07	7.76±7.04	11.32±7.06
	CL	3.95±3.87	0.05±7.14	5.28± 7.40	6.59±7.32
	FL0.5	5.21±1.91	3.95±7.13	5.43±7.09	6.20±7.18
	FL5	1.893±1.90	4.27±37.13	3.10±7.05	-1.72±7.16

3.2.2. Profile analysis

3.2.2.1. Fatty acids

The contents of the analysed fatty acids were similar between treatments, with no significant differences in either species (p>0.05) (**Figure 3.7**, **Figure 3.8**). In *P. tricornutum*, SFA, MUFA and PUFA contents (% of TFA) ranged from 24.93±2.55 to 28.33±2.55%, 20.15±2.34 to 23.19±2.34% and 52.52±2.49 to 52.79±2.48%, respectively (**Figure 3.7a,b,c**). EPA (C20:5n3) contents, the main FA commonly synthesised in this species, ranged from 28.97±1.47% to 29.67±1.47% (**Figure 3.7d**). The fatty acids methyl ester (FAME) profile is represented in **Table A1**. In *N. oceanica*, SFA, MUFA and PUFA contents (% of TFA) ranged from 47.43±2.61 to 45.32±2.60%, 32.21±1.64 to 33.35±1.64% and 19.05±3.63 to 20.37±3.65%, respectively (**Figure 3.8a,b,c**). Palmitic acid (C16:0) and EPA (C20:5n3) contents, the main FAs commonly synthesised in this species, ranged from 38.15±2.67 to 39.15±2.67% and 15.47±3.05 to 16.70±.06, respectively (**Figure 3.8d,e**). Fatty acids methyl ester (FAME) profile is represented in **Table A2**. The fatty acids profile obtained are in accordance with those previously reported on the genera *Nannochloropsis* [162] and *Phaeodactylum* [163]. SFA, MUFA and PUFA contents (% of TFA) were not significantly affected by any of the covariates in either species (p>0.05). Additionally, C16:0 content in *N. oceanica* was also not significantly affected by any covariate (p>0.05). Nonetheless, EPA content (% of TFA) was significantly affected by the covariate "trial" in both species (p<0.05). In *P. tricornutum*, EPA contents were higher in trial 1 (31.06±1.44% of TFA) compared to trial 2 (28.42±0.68% of TFA). In *N. oceanica*, EPA contents were higher in trial 2 (18.97±2.67% of TFA) compared to trial 1 (13.97±2.67% of TFA). Additionally, the covariate "harvesting timepoint" also affected EPA content significantly in *P. tricornutum* (p<0.05) with higher contents in the morning (30.82±1.20% of TFA) compared to the evening (28.67±1.20% of TFA). These results are in accordance with the ones obtained in the previous analyses, including growth and C and lipid contents, where trials 1 and 2 presented the highest values in *P. tricornutum* and *N. oceanica*, respectively. These results suggest a positive correlation between EPA and lipid content. EPA is typically found in the lipid fraction of microalgae, particularly in triacylglycerols (TAGs), which serve as the main storage of lipids [26,30,158]. Therefore, an increase in lipid content may result in a proportional increase in EPA content. Moreover, the specific behaviour of EPA may vary depending on the metabolic priorities of microalgae. For instance, microalgae may prioritize EPA synthesis or limit EPA degradation during the night to maintain membrane integrity [164]. Additionally, during respiration, ROS may be generated. In cases like this, TAG, in particular those containing EPA may act as antioxidants to counterattack oxidative stress, resulting in a higher content of EPA during the night, i.e., in samples collected in the morning [164].

Previously published studies of FL effects regarding fatty acids content reported strain-specific behaviours [125]. Likewise, in our study, FA composition and content vary between species. While *P. tricornutum* presents higher contents (% of TFA) of PUFA, *N. oceanica* presents higher contents (% of TFA) of SFA and MUFA. Additionally, the same study reported a typical increase in the major PUFA under low-frequency FL compared to MUFA (DC = 0.05; I_a = 300 µmol s^{-1} m^{-2}) [125]. For instance, *N. gaditana* accumulated more EPA under FL5 and FL50, whereas C16:0 tended to decrease [125]. Nonetheless, under FL500, the FA profile was similar to that of cells under CL [125]. Other studies reported an increase in EPA contents under low light conditions (30 – 750 µmol s^{-1} m^{-2}) in *Nannochlorpsis* sp. and *P. tricornutum*, which has been related to increased thylakoid membrane synthesis under low light to improve photosynthetic efficiency [125,162,163]. However, as previously indicated, we did not find significant differences between treatments in our study in either species.

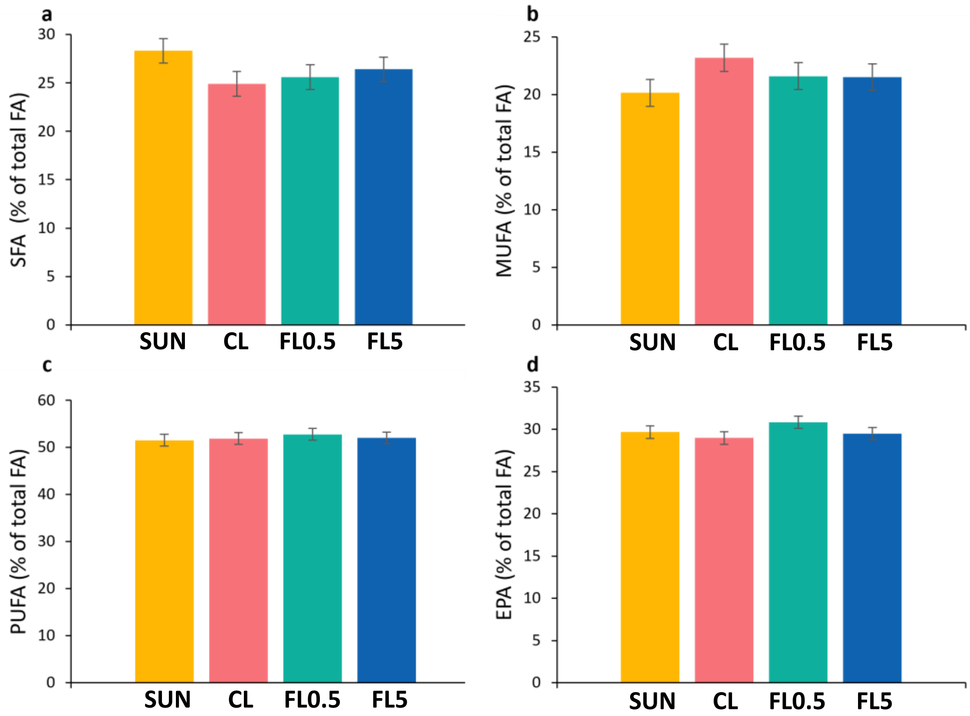

Figure 3.7 – Major fatty acids (% of total FA) of *Phaeodactylum tricornutum*, including SFA (a), MUFA (b), PUFA (c) and EPA (d). FL0.5 and FL5 were tested at a duty cycle of 0.05. Shown are the adjusted means ± 95 % confidence interval (ANCOVA) from data of the induction stage (n=16) adjusted for the effects of the covariates “trial”, “induction time”, “harvesting timepoint” and “biomass concentration”. Statistical analysis revealed no significant differences among treatments (p>0.05).

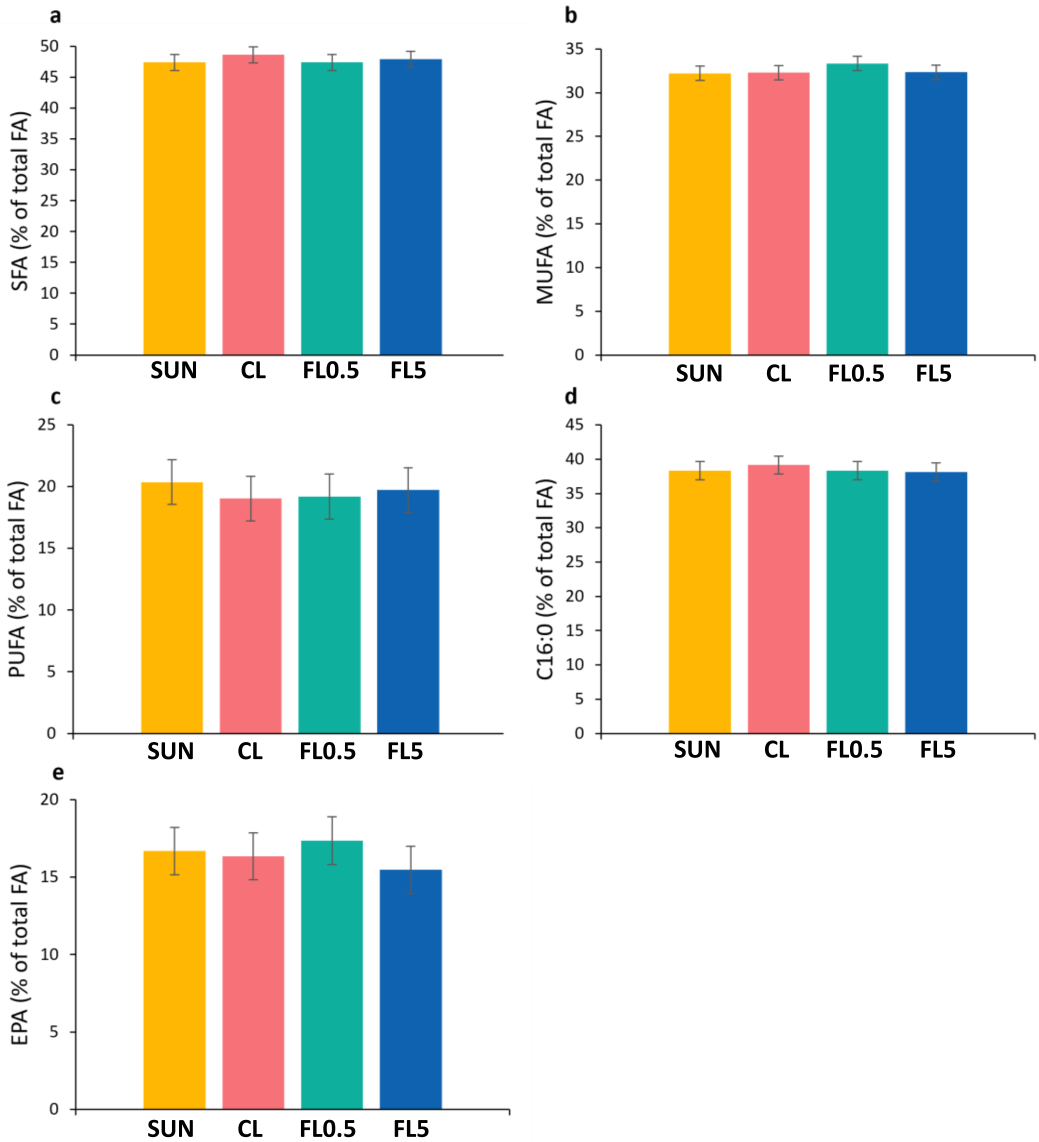

Figure 3.8 – Major fatty acids (% of total FA) of *Nannochloropsis oceanica*, including SFA (a), MUFA (b), PUFA (c), C16:0 (d) and EPA (e). FL0.5 and FL5 were tested at a duty cycle of 0.05. Shown are the adjusted means ± 95 % confidence interval (ANCOVA) from data of the induction stage (n=16) adjusted for the effects of the covariates "trial", "induction time", "harvesting timepoint" and "biomass concentration". Statistical analysis revealed no significant differences among treatments (p>0.05).

Regarding FA productivity (% of total FA d^{-1}) and cumulative FA content variation (% of total FA), there were no significant differences between treatments (p>0.05) in either species (**Table 3.5**, **Table 3.6**). SFA, MUFA and PUFA contents (% of total FA) ranged from -3.25±6.56 to 6.56±6.58%, -4.34±7.96 to 5.37±8.02% and -9.23±6.63 to 2.18±6.89% among treatments and induction time in *P. tricornutum* (**Table 3.5**) and from -4.07±5.56 to 3.61±6.58%, -0.32±3.50 to 8.93±3.54% and -11.96±8.61 to -0.14±8.55% among treatments and induction time in *N. oceanica*, respectively (**Table

3.6). EPA contents (% of total FA) ranged from -5.08±3.65 to 1.96±3.78% and -11.81±7.74 to 2.16±7.61% among treatments and induction time in *P. tricornutum* (**Table 3.5**) and *N. oceanica* (**Table 3.6**), respectively. C16:0 content variation (% of total FA) ranged from -6.04±6.86 to 1.26±6.99% among treatments and induction time in *N. oceanica*, respectively (**Table 3.6**). SFA and EPA productivities (% of total FA d^{-1}) were not affected by any of the covariates in *P. tricornutum* (p>0.05). Nonetheless, "harvesting timepoint" significantly affected MUFA and PUFA productivities (p<0.05). MUFA productivity (% of total FA d^{-1}) was higher in samples collected in the morning (4.74±2.99%) compared to the evening (-1.80±2.99%), presenting negative values. Conversely, PUFA productivity (% of total FA d^{-1}) was higher in samples collected in the evening (1.08±2.70%) compared to morning (-4.86±2.70%), presenting negative values. Although both (PUFAs and MUFAs) may be found in storage lipids (e.g., TAGs), serving as the primary energy reserves for microalgae, storage lipids predominantly contain MUFAs. During cellular respiration over night, the cellular energy demand is lower and microalgae may prioritize the synthesis and accumulation of storage lipids, i.e., MUFAs. Moreover, storage lipids can be produced by microalgae to provide energy for cellular processes during periods of stress [164]. Although we did not find significant differences between treatments, algae could have been stressed during nighttime. The increase in PUFA content in the evening may be related to the fact that, during photosynthesis, there is a higher availability and production of energy and C precursors, which may stimulate PUFA synthesis. Additionally, PUFAs are essential in membrane structure and fluidity, being required for membrane synthesis and other cellular processes during active growth and photosynthesis [23, 27, 170]. Thus, MUFA may have been prioritised for rich storage of lipids for energy metabolism and cell maintenance during the night, while PUFA may have been prioritised to support photosynthetic growth during day [26,30,158]. MUFA, PUFA, EPA (C20:5n-3) and C16:0 productivities (% of total FA d^{-1}) were not affected by any of the covariates in *N. oceanica* (p>0.05). Nevertheless, the covariate "trial" significantly affected SFA productivity (p<0.05). SFA productivity (% of total FA d^{-1}) was higher in trial 1 (2.36±2.52%) compared to trial 2 (-1.54±2.52%), presenting negative values. This is in line with total lipids productivity, both with higher productivities in trial 1, suggesting that this trial may have been more favourable for lipid accumulation and synthesis of SFA.

Previous published studies of FL effects regarding FA productivity reported an increase of EPA productivity of 1.4-fold under FL5 in *N. gaditana* and 1.9-fold in *K. antarctica* (DC = 0.05; I_a = 300 µmol s^{-1} m^{-2}) [125]. Other studies have reported that the highest productivity of C16:0 was achieved under CL compared to FL treatments in *D. lutheri*, decreasing significantly after six days of low-frequency FL exposure (e.g., FL0.5 and FL5) (DC = 0.05; I_a = 300 µmol s^{-1} m^{-2}) [126]. Interestingly, when cells were exposed to FL5 for only three days, EPA increased 1.3-fold compared to CL. Nonetheless, in our study, we did not find significant differences between treatments in all the analysed FA in either species.

Table 3.5 – FA productivity (% of total FA d^{-1}) and cumulative FA content variation (% of total FA) during the 1, 2 and 3 days of induction of *Phaeodactylum tricornutum*. FL0.5 and FL5 were tested at a duty cycle of 0.05. Shown are the adjusted means ± 95 % confidence interval (ANCOVA) from data of the induction stage (n=4) adjusted for the effects of the covariates "trial", "induction time", "harvesting timepoint" and "biomass productivity". Statistical analysis revealed no significant differences among treatments (p>0.05).

		FA productivity (% of total FA d^{-1})	**Cumulative FA content variation (% of total FA)**		
			Induction time (d)		
			1	**2**	**3**
SFA	**SUN**	0.50±3.73	-1.99±6.66	5.79±6.32	-2.53±6.37
	CL	-1.06±3.73	0.57±6.63	-0.24±6.64	-3.25±6.56
	FL0.5	-0.53±3.69	0.03±7.03	1.11±6.67	-2.76±6.34
	FL5	2.78±3.69	6.56±6.58	1.03±6.29	0.77±6.45
MUFA	**SUN**	4.43±4.22	4.76±8.057	3.44±7.65	5.21±7.70
	CL	2.67±4.22	5.37±8.02	1.44±8.03	1.08±7.93
	FL0.5	0.30±4.17	-0.82±8.50	-0.11±8.06	1.75±7.66
	FL5	-1.53±4.17	-4.34±7.96	0.37±7.61	-0.52±7.92
PUFA	**SUN**	-4.93±3.78	-2.77±6.99	-9.23±6.63	-2.68±6.68
	CL	-1.62±3.78	-5.94±6.96	-1.20±6.97	2.18±6.89
	FL0.5	0.23±3.74	0.79±7.73	-1.00±6.99	0.92±6.65

	FL5	-1.26±3.74	-2.13±6.91	-1.40±6.60	-0.25±6.86
EPA	**SUN**	-2.74±2.15	-1.45±3.85	-5.08±3.65	-1.56±3.68
	CL	-2.06±2.15	-2.79±3.83	-0.76±3.84	-2.76±3.79
	FL0.5	-1.07±2.13	-0.52±4.06	-2.99±3.85	0.31±3.66
	FL5	0.56±2.13	-0.56±3.81	0.27±3.64	1.96±3.78

Table 3.6 – FA productivity (% of total FA d^{-1}) and cumulative FA content variation (% of total FA) during the 1, 2 and 3 days of induction of *Nannochloropsis oceanica*. FL0.5 and FL5 were tested at a duty cycle of 0.05. Shown are the adjusted means ± 95 % confidence interval (ANCOVA) from data of the induction stage (n=4) adjusted for the effects of the covariates "trial", "induction time", "harvesting timepoint" and "biomass productivity". Statistical analysis revealed no significant differences among treatments (p>0.05).

		FA productivity (% of total FA d^{-1})	**Cumulative FA content variation (% of total FA)** Induction time (d)		
			1	**2**	**3**
SFA	**SUN**	1.07±3.64	3.61±6.58	-0.28±6.56	1.87±6.58
	CL	0.79±3.67	0.59±6.65	0.20±6.89	1.57±6.81
	FL0.5	1.39±3.66	3.00±6.64	-0.24±6.60	1.40±6.69
	FL5	-1.60±3.65	0.35±6.64	-4.07±5.56	-1.09±6.67
MUFA	**SUN**	2.45±2.11	1.57±3.51	1.37±3.50	4.41±3.51
	CL	2.82±2.12	2.68±3.54	2.76±3.67	3.08±3.63
	FL0.5	3.61±2.11	8.93±3.54	0.38±3.52	1.47±3.56
	FL5	1.57±2.11	2.68±3.54	-0.32±3.50	3.17±3.55
PUFA	**SUN**	-3.51±4.90	-3.17±8.53	-1.09±8.50	-6.29±8.52
	CL	-3.62±4.94	-3.29±8.61	-2.99±8.93	-4.62±8.23
	FL0.5	-5.07±4.92	-11.96±8.61	-0.14±8.55	-3.08±8.66

	FL5	-0.03±4.91	-2.22±8.60	-4.39±8.50	-2.25±8.64
EPA	**SUN**	-1.76±4.38	-2.43±7.64	0.88±7.61	-3.72±7.63
	CL	-2.15±4.41	-0.61±7.71	0.89±7.99	-6.03±7.90
	FL0.5	-5.01±4.40	-11.81±7.741	-0.02±7.66	-3.27±7.76
	FL5	1.43±4.38	1.84±7.69	2.16±7.61	0.27±7.74
C16:0	**SUN**	0.20±3.65	0.81±6.88	-0.49±6.86	0.29±6.88
	CL	0.10±3.67	0.71±6.95	-0.67±7.20	0.23±7.12
	FL0.5	0.41±3.66	0.76±6.95	-0.77±6.90	1.26±6.99
	FL5	-3.21±3.65	-0.94±6.94	-6.04±6.86	-2.64±6.97

3.2.2.2. Pigments

The contents of all individual pigments in *P. tricornutum* are similar between treatments, with no significant differences (p>0.05) (**Figure 3.9**). Fucoxanthin and neoxanthin were both significantly affected by the covariates “trial” and “induction time” (p<0.05). Both contents were higher in trial 2 (6.46±0.39% of DW in fucoxanthin and 3.14±0.39% of DW in neoxanthin) compared to trial 1 (5.51±0.39% of DW in fucoxanthin and 2.38±0.39% of DW in neoxanthin). Likewise, β-carotene content was also significantly affected by the covariate “trial”, with higher content in trial 1 (2.74±0.43% of DW) compared to trial 2 (0.17±0.43% of DW). These results are the opposite of what happened, in general, to biomass, protein, lipid and carbohydrate contents or productivities, as they all presented the highest values on trial 1. This may be explained because during this trial cells grew under optimal conditions for growth, as well as proximate composition. Thus, it is possible that, in trial 2, the physiological response to, for instance, higher temperature, might have led to different metabolic priorities, including fucoxanthin and neoxanthin synthesis [165,166]. On the other hand, β-carotene accumulation coincided with higher biomass, lipid and carbohydrate contents in trial 1. As an accessory pigment, β-carotene contributes to optimizing photosynthetic efficiency by not only absorbing excess light but also playing a role in the transfer of light energy to chlorophyll molecules, an event necessary for photochemical reactions to take place

[167]. Thus, higher *β*-carotene contents may correlate with enhanced photosynthetic activity and biomass accumulation under favourable growth conditions with optimal light availability. Regarding "induction time", post hoc testing using Tukey's correlation revealed that day 3 (6.26±0.21% of DW; 3.27±0.47% of DW) resulted in significantly greater content compared to day 0 (5.45±0.46% of DW; 2.23±0.45% of DW) ($p<0.05$) in both fucoxanthin and neoxanthin, respectively. Chlorophyll *a* content was not significantly affected by any of the covariates ($p>0.05$). Fucoxanthin content, the most abundant pigment in *P. tricornutum* is in accordance with the typical levels found in different studies (**Table 1.1**). FL treatments applied in this study did not stimulate the accumulation of targeted pigments.

Regarding pigment productivity (mg g^{-1} DW d^{-1}) and cumulative pigment content variation (mg g^{-1} DW) (**Table 3.7**), there were no significant differences between treatments ($p>0.05$) in fucoxanthin, neoxanthin and *β*-carotene contents. These ranged from -0.50±1.20 to 2.49±1.18, 0.06±1.01 to 1.86±0.10 and -1.20±1.25 to 0.59±1.24 mg g^{-1} DW among treatments and induction time, respectively (**Table 3.7**). However, treatment affected significantly ($p<0.05$) chlorophyll *a* productivity (**Table 3.7**). Post hoc testing using Tukey's correlation revealed that cells exposed to CL (0.01±0.08 mg g^{-1} DW d^{-1}) and SUN (0.07±0.08 mg g^{-1} DW d^{-1}) presented greater productivity compared to cells exposed to FL5 (-0.13±0.07 mg g^{-1} DW d^{-1}) (**Table 3.7**). Chlorophyll *a* is the main pigment related to photosynthesis, as it captures the energy and starts the photosynthetic electron transport chain. SUN and CL may represent more favourable growth environments for microalgae growth. Under these treatments, microalgae may optimize their photosynthetic apparatus to efficiently use available light energy for carbon fixation and biomass production, leading to the upregulation of chlorophyll *a* synthesis to maximise light capture and energy conversion [167,168]. Additionally, the covariates "trial" ($p<0.001$) and "harvesting timepoint" ($p<0.001$) affected chlorophyll *a* productivity (mg g^{-1} DW d^{-1}). Productivities on trial 1 were significantly higher (0.07±0.06 mg g^{-1} DW d^{-1}) when compared to trial 2 (-0.11±0.06 mg g^{-1} DW d^{-1}). Moreover, samples collected in the morning (0.08±0.06 mg g^{-1} DW d^{-1}) presented greater productivity than samples collected in the evening (-0.13±0.06 mg g^{-1} DW d^{-1}). On the first hours of the morning, as light levels increase with sunrise, microalgae start photosynthesis, synthesizing chlorophyll *a* to capture light energy for photosynthetic reactions [167–169]. On the contrary, at sunset, there is minimal light available for photosynthesis. In fucoxanthin, none of the studied covariates had a significant effect ($p>0.05$). However, the covariate "trial" played a significant effect on neoxanthin productivity ($p<0.05$), with higher productivity on trial 1 (1.36±0.40 mg g^{-1} DW d^{-1}) compared to trial 2 (0.42±0.37 mg g^{-1} DW d^{-1}). The covariate "harvesting timepoint" significantly affected *β*-carotene productivity ($p<0.05$), where samples collected in the evening (0.35±0.49 mg g^{-1} DW d^{-1}) presented greater productivity than samples collected in the morning (-0.65±0.49 mg g^{-1} DW d^{-1}). As mentioned before, *β*-carotene is a secondary photoprotective pigment that plays an important role during photosynthesis, preventing ROS production [167].

Previously published studies of FL effects regarding pigment productivity in *Diacronema lutheri*, also a diatom, reported significant differences ($p<0.05$) among treatments. Contrary to our study, fucoxanthin productivity was enhanced 1.2- and 1.3-fold under low-frequency FL of 0.5 and 5 Hz, respectively, compared to CL, under 6 days of induction. Chlorophyll *a* productivity was 4-fold higher under FL of 5 Hz compared to CL, during 4 days of induction. Nonetheless, although the same *DC* (0.05) and frequencies (0.5 and 5 Hz) were applied, light intensity (I_a = 300 μmol s^{-1} m^{-2}) was higher [126]. In our study, we did not find significant differences regarding pigment contents, and differences in pigment productivity are better under SUN and CL rather than FL treatments. Pigments function in light harvesting and photoprotection of the photosynthetic apparatus, being important for the cells to adapt to light intensity changes; particularly, in diatoms, the diadinoxanthin (Ddx) cycle [126,163,170]. Under high light stress, the biosynthesis shifts towards diatoxanthin production to protect the photosynthetic apparatus from damage [126,163,170]. Thus, the quantification of this pigment would be of great importance to understand if stress was indeed being induced in microalgal cells. In our study, *β*-carotene, fucoxanthin and neoxanthin contents and productivities did not present significant differences between treatments.

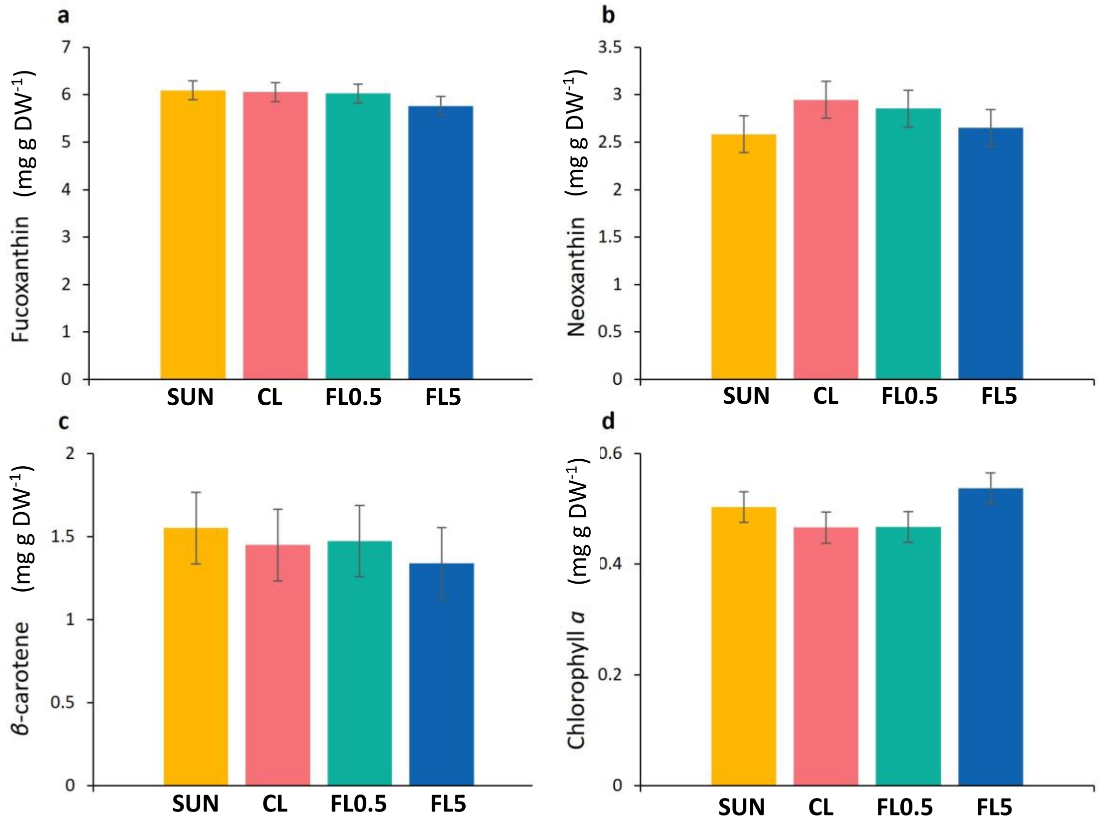

Figure 3.9 – Pigment profile (mg g^{-1} DW) of *Phaeodactylum tricornutum*, including Fucoxanthin (a), Neoxanthin (b), *β*-carotene (c) and Chlorophyll *a* (d). FL0.5 and FL5 were tested at a duty cycle of 0.05. Shown are the adjusted means ± 95 % confidence interval (ANCOVA) from data of the induction stage (n=32) adjusted for the effects of the covariates "trial", "induction time", "harvesting timepoint" and "biomass concentration". Statistical analysis revealed no significant differences among treatments (p>0.05).

Table 3.7 – Pigment productivity (mg g^{-1} DW d^{-1}) and cumulative pigments content variation (mg g^{-1} DW d^{-1}) during the 1, 2 and 3 days of induction of *Phaeodactylum tricornutum.* FL0.5 and FL5 were tested at a duty cycle of 0.05. Shown are the adjusted means ± 95 % confidence interval (ANCOVA) from data of the induction stage (n=4) adjusted for the effects of the covariates "trial", "induction time", "harvesting timepoint" and "biomass productivity". Statistical analysis revealed no significant differences among treatments (p>0.05), except for chlorophyll *a*. Treatments that do not share the same letter are significantly different from each other.

Pigment productivity	**Cumulative pigment content variation (mg g^{-1} DW)**

		($mg\ g^{-1}$ DW d^{-1})	Induction time (d)		
			1	2	3
Fucoxanthin	**SUN**	0.76±0.70	-0.50±1.20	0.87±1.14	1.66±1.15
	CL	0.86±0.70	0.31±1.19	1.37±1.19	1.16±1.18
	FL0.5	0.40±0.70	0.15±1.26	1.05±1.20	-0.01±1.14
	FL5	1.27±0.70	0.70±1.19	0.64±1.13	2.49±1.18
Neoxanthin	**SUN**	0.55±0.55	0.06±1.01	0.59±0.96	0.97±0.97
	CL	1.29±0.55	0.90±1.01	1.15±1.01	1.84±0.10
	FL0.5	0.89±0.54	0.70±1.07	1.17±1.01	0.80±0.96
	FL5	0.90±0.54	0.47±1.00	0.38±0.96	1.86±0.10
***β*-carotene**	**SUN**	-0.84±0.70	-0.92±1.31	-0.48±1.24	-1.20±1.25
	CL	0.07±0.70	0.34± 1.31	0.56±1.30	-0.63±1.29
	FL0.5	-0.22±0.70	0.24±1.38	-0.03±1.31	-0.86±1.24
	FL5	0.37±0.70	0.54±1.29	0.59±1.24	0.01±1.28
Chlorophyll *a*	**SUN**	0.07±0.08[a]	0.04± 0.15	0.05±0.14	0.11±0.14
	CL	0.01±0.08[a]	0.03±0.15	0.04±0.15	-0.03±0.15
	FL0.5	-0.05±0.08[a,b]	-0.07±0.16	-0.03±0.15	-0.04±0.14
	FL5	-0.13±0.07[b]	-0.18±0.15	-0.14±0.14	-0.07±0.15

The contents of all pigments in *N. oceanica* are similar between treatments, with no significant differences (p>0.05) (**Figure 3.10**). Regarding lutein, chlorophyll *a* and *α*-carotene contents, none of the covariates had a significant effect on them (p>0.05). Violaxanthin was significantly affected by the covariate "trial" (p<0.05), with higher content on trial 2 (1.01±0.07 mg g^{-1} DW) compared to trial 1 (0.84±0.07 mg g^{-1} DW). Likewise, zeaxanthin content was also affected by "trial" (p<0.05), with higher content on trial 2 (0.08±0.008 mg g^{-1} DW) compared to trial 1 (0.06±0.008 mg g^{-1} DW). Additionally, "induction time" (p<0.001) and "harvesting timepoint" (p<0.05) significantly affected zeaxanthin content. Post hoc testing using Tukey's correlation revealed that days 1 (0.07±0.01 mg g^{-1} DW), 2 (0.08±0.01 mg g^{-1} DW) and 3 (0.08±0.01 mg g^{-1} DW) presented greater content than day 0 (0.05±0.01 mg g^{-1} DW). Samples collected in the evening (0.07±0.01 mg g^{-1} DW) were greater than samples collected in the morning (0.06±0.01 mg g^{-1} DW).

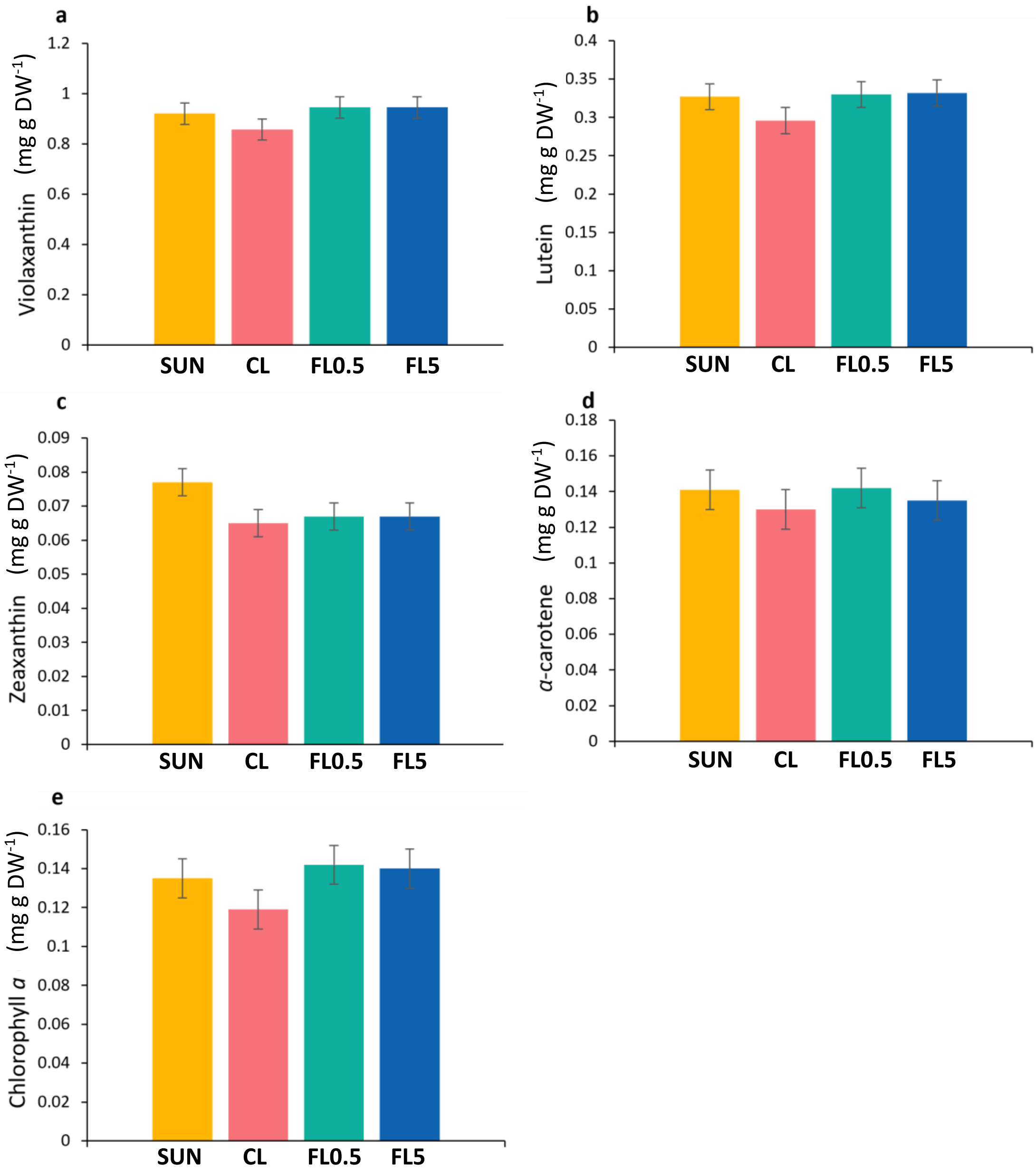

Figure 3.10 - Pigment profile (mg g^{-1} DW) of *Nannochloropsis oceanica*, including Violaxanthin (a), Lutein (b), Zeaxanthin (c), α-carotene (d) and Chlorophyll *a* (e). FL0.5 and FL5 were tested at a duty cycle of 0.05. Shown are the adjusted means ± 95 % confidence interval (ANCOVA) from data of the induction stage (*n*=32) adjusted for the effects of the covariates "trial", "induction time", "harvesting timepoint" and "biomass

concentration". Statistical analysis revealed no significant differences among treatments ($p>0.05$).

Regarding pigment productivity (mg g^{-1} DW d^{-1}) and cumulative pigment content variation (mg g^{-1} DW) (**Table 3.8**), there were no significant differences between treatments ($p>0.05$) in chlorophyll *a* and *α*-carotene. These ranged from -0.01±0.05 to 0.04±0.05 and -0.04±0.06 to 0.32±0.06 mg g^{-1} DW among treatments and induction time, respectively (**Table 3.8**). However, it affected significantly violaxanthin ($p<0.05$), lutein ($p<0.05$) and zeaxanthin ($p<0.05$) productivities (**Table 3.8**). Regarding violaxanthin, post hoc testing using Tukey's correlation revealed that cells exposed to SUN (0.21±0.10 mg g^{-1} DW d^{-1}) presented a greater productivity compared to cells exposed to FL0.5 (0.02±0.10 mg g^{-1} DW d^{-1}) and CL (-0.05±0.10 mg g^{-1} DW d^{-1}) (**Table 3.8**). Lutein productivity followed the same trend, with greater productivity in cells exposed to SUN (0.08±0.04 mg g^{-1} DW d^{-1}) compared to cells exposed to FL0.5 (-0.01±0.04 mg g^{-1} DW d^{-1}) and CL (-0.03±0.04 mg g^{-1} DW d^{-1}) (**Table 3.8**). Likewise, zeaxanthin productivitiy is greater in cells exposed to SUN (0.04±0.01 mg g^{-1} DW d^{-1}) compared to cells exposed to FL0.5 (0.01±0.01 mg g^{-1} DW d^{-1}) (**Table 3.8**). Sunlight provides a broader spectrum of wavelengths compared to artificial light sources (CL and FL), which may stimulate the synthesis of specific carotenoids, like violaxanthin, lutein and zeaxanthin [171]. Moreover, sunlight exposure may provide optimal conditions for light harvesting and pigment accumulation. The covariate "trial" also affected violaxanthin ($p<0.001$), lutein ($p<0.001$) and zeaxanthin ($p<0.001$) productivities (mg g^{-1} DWd^{-1}), with higher productivities on trial 1. This is in accordance with total lipid productivity (% of DW d^{-1}), which was also higher in trial 1. These results suggest that trial 1 may have provided more favourable light conditions, such as optimal light intensity and quality, and photoperiod, enhancing photosynthetic activity and C assimilation, leading to increased pigment and lipid productivities. Furthermore, optimal temperatures (trial 1) promote enzymatic activities involved in lipid biosynthesis and carotenoid metabolism, supporting higher productivities of these [171,172]. Additionally, also the covariate "harvesting timepoint" affected violaxanthin ($p<0.05$), lutein ($p<0.001$), zeaxanthin ($p<0.05$) and chlorophyll *a* ($p<0.05$) productivities (mg g^{-1} DW d^{-1}), with higher values on samples collected in the morning. In the first hours of the morning, as light levels increase with sunrise, microalgae start photosynthesis, synthesizing chlorophyll *a* to capture light energy for photosynthetic reactions [167,168]. On the contrary, at sunset, there is minimal light available for photosynthesis. Additionally, the transition from darkness to light may stimulate the synthesis of photoprotective pigments, such as violaxanthin, lutein and zeaxanthin [173,174]. The covariate "biomass productivity" affected violaxanthin (p<0.05) and lutein (p<0.05) productivities. Regarding α-carotene content variation, none of the covariates had a significant effect (p>0.05).

Table 3.8 - Pigment productivity (mg g^{-1} DW d^{-1}) and cumulative pigment content variation (mg g^{-1} DW) during the 1, 2 and 3 days of induction of *Nannochloropsis oceanica*. FL0.5 and FL5 were tested at a duty cycle of 0.05. Shown are the adjusted means ± 95 % confidence interval (ANCOVA) from data of the induction stage (n=4) adjusted for the effects of the covariates "trial", "induction time", "harvesting timepoint" and "biomass productivity". Statistical analysis revealed no significant differences among treatments (p>0.05), except for violaxanthin, lutein and zeaxanthin. Treatments that do not share the same letter are significantly different from each other.

		Pigment productivity (mg g^{-1} DW d^{-1})	**Cumulative pigment content variation (mg g^{-1} DW)**		
			Induction time (d)		
			1	**2**	**3**
Violaxanthin	**SUN**	0.21±0.10^{a}	0.20±0.17	0.22±0.17	0.21±0.17
	CL	-0.05±0.10^{b}	-0.11±0.17	-0.002±0.17	-0.04±0.17
	FL0.5	0.02±0.10^{b}	-0.04±0.17	0.04±0.17	0.07±0.17
	FL5	0.01±0.10^{a,b}	0.16±0.17	0.08±0.17	0.05±0.17
Lutein	**SUN**	0.08±0.04^{a}	0.08±0.07	0.08±0.07	0.07±0.07
	CL	-0.03±0.04^{b}	-0.04±0.07	-0.004±0.07	-0.03±0.07
	FL0.5	-0.01±0.04^{b}	-0.03±0.07	-0.01±0.07	0.008±0.07
	FL5	0.02±0.04^{a,b}	0.06±0.07	-0.02±0.07	0.003±0.07
Zeaxanthin	**SUN**	0.04±0.01^{a}	0.04±0.02	0.04±0.02	0.04±0.02
	CL	0.02±0.01^{a,b}	0.01±0.02	0.03±0.02	0.02±0.02
	FL0.5	0.01±0.01^{b}	0.007±0.02	0.01±0.02	0.02±0.02
	FL5	0.02±0.01^{a,b}	0.02±0.02	0.03±0.02	0.02±0.02

***α*-carotene**	**SUN**	0.01±0.06	-0.01±0.06	0.01±0.06	0.02±0.06
	CL	0.01±0.06	0.01±0.06	0.02±0.06	0.02±0.06
	FL0.5	0.02±0.06	0.01±0.06	0.02±0.06	0.05±0.06
	FL5	0.09±0.06	0.01±0.06	0.32±0.06	-0.04±0.06
Chlorophyll *a*	**SUN**	0.02±0.03	0.03±0.05	0.03±0.05	0.002±0.05
	CL	-0.01±0.03	-0.01±0.05	-0.04±0.05	0.01±0.05
	FL0.5	0.01±0.03	-0.01±0.05	0.04±0.05	-0.01±0.05
	FL5	0.01±0.03	0.03±0.05	-0.002±0.05	-0.01±0.05

Previously published studies of FL effects regarding pigment contents in *N. gaditana*, reported significant differences ($p<0.05$) among treatments [126]. *β*-carotene, violaxanthin, and lutein contents exposed to FL treatments (f = 5 and 50 Hz) were, on average, 3-4 times and 1.7-2.3 times higher compared to CL [125]. In our study, we did not find significant differences between treatments on all mentioned pigments. Likewise, neoxanthin, violaxanthin and *β*-carotene productivities were, on average, 2-3 times higher under FL (f = 5 and 50 Hz) compared to CL, whereas lutein productivity was enhanced 1.1-1.6 times [125]. In the present study violaxanthin, lutein and zeaxanthin productivities were greater under SUN compared to FL treatments (f = 0.5 Hz). Other studies on marine green microalgae, such as *Tetraselmis striata*, demonstrated that lutein productivity increased 1.3-fold under FL5 compared to CL, under 6 days of exposure [126]. Violaxanthin reached the highest productivity under FL5 and neoxanthin and *β*-carotene productivities were higher under FL50, all under 3 days of exposure [126]. Pigments function in light harvesting and photoprotection of the photosynthetic apparatus, being important for the cells to adapt to light intensity changes; particularly, in chlorophytes, the violaxanthin (Vx) cycle [126,163,170]. Under high light stress, the biosynthesis shifts towards zeaxanthin production to protect the photosynthetic apparatus from damage [126,163,170]. However, in our study, zeaxanthin contents were similar between treatments and productivity was greater under SUN compared to FL treatments (FL0.5). Conversely, violaxanthin accumulates under low-light stress, also to protect the microalgae cells from photodamage. Likewise, in our study, violaxanthin contents were similar between treatments and productivity was greater under SUN compared to FL treatments (FL0.5). Therefore, the observed results in this study suggest that there was not an activation of the xanthophyll cycle, suggesting that the cells were not light stressed.

4. Conclusions

Two marine microalgal species, *P. tricornutum* and *N. oceanica*, were studied under a two-stage cultivation strategy using flashing light as a stress condition to improve growth and biocompound accumulation. Regarding growth and biomass productivity, no significant differences were observed between the different treatments (SUN, CL, FL0.5, FL5) in either species, regardless of considering the studied covariates ("trial", "harvesting timepoints", "induction time"). Likewise, regarding biochemical composition, there were only minor significant differences among treatments in both contents and productivities. For instance, protein and carbohydrate contents (% of DW), in *P. tricornutum*, were significantly greater in cells exposed to FL5 compared to SUN (0.16-fold) and CL and SUN compared to FL0.5 (1.5-fold), respectively. In *N. oceanica*, regarding pigment productivity (mg g^{-1} DW d^{-1}), SUN-treated cells presented higher productivity compared to cultures under FL0.5 and CL in terms of violaxanthin productivity, leading to a 10.5-fold increase. When compared to cells under FL0.5, SUN-treated cells showed a 2-fold increase in zeaxanthin productivity. Nonetheless, the mentioned differences were only supported when the covariates "trial", "induction time", "harvesting timepoint" and "biomass concentration" or "biomass productivity" were statistically considered. Hence, we reject our hypothesis that FL as a finishing step can enhance meaningful biomass production or biochemical composition during outdoor production. Regarding the studied covariates, statistics indicated that they had a stronger effect on most measured parameters compared to the FL. The covariate "trial" had the strongest impact, whereas "harvesting timepoint" only showed minor effects on most of the tested parameters. The differences between trials were characterised by changing temperatures, light cycles and intensities, rainfall among other environmental parameters which have a stronger impact on microalgal behaviour than FL itself. Nonetheless, the present study, to the best of the author's knowledge, was the first study to analyse the effects of FL on the microalgae *P. tricornutum*, including total proteins, lipids and carbohydrates. Additionally, to the best of the author's knowledge, this was also the first study investigating the effects of FL on pilot-scale outdoor cultivation of microalgae.

5. Future perspectives

Future studies should quantify the different environmental factors (temperature, light cycles, irradiance, etc.) to identify which was the main contributor for the observed changes. For instance, trials should be performed during all year (e.g., 3 months apart, to have data from each season) or at specific times of the year (e.g., during optimal conditions: winter for *P. tricornutum* and summer for *N. oceanica*). Furthermore, the number of biological replicates should be increased to have a more robust statistical analysis, increasing the statistical power and reliability of the analysed data. Applying FL at large-scale production is not recommended due to the high resource requirements, including human resources, cleaning procedures, and equipment required. Nonetheless, implementing advanced monitoring and automation technologies in outdoor cultivation systems could provide real-time adjustments to environmental conditions. Additionally, with long-term study of the effects of environmental parameters on biochemical compounds, it would be possible to develop smart models that predict the best “harvesting timepoint” for high quality biomass.

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Anexx

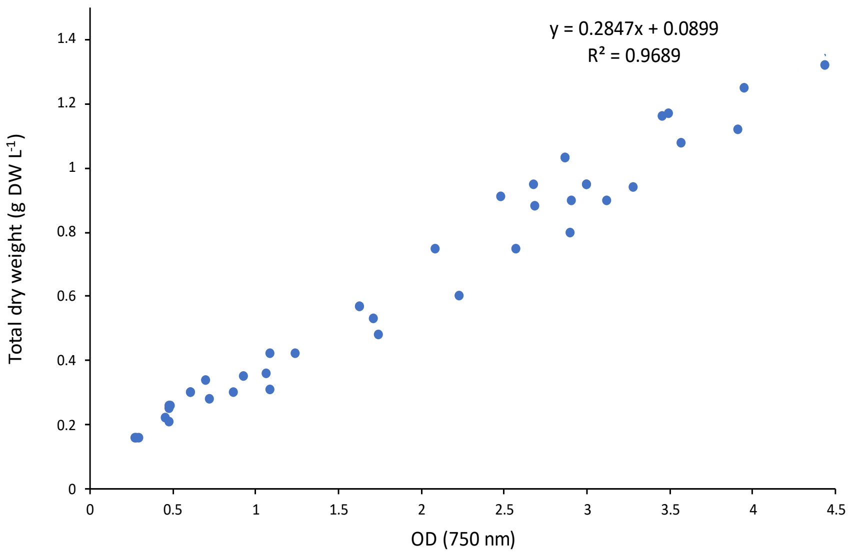

Figure A1 - Correlation curve of *P. tricornutum* dry weight. y = 0.28478x + 0.0899 and R^2 = 0.9689

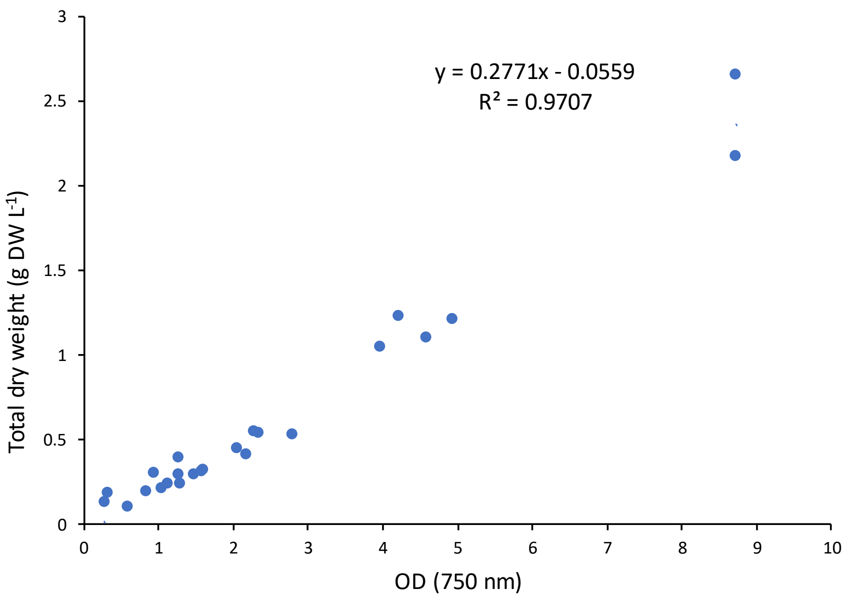

Figure A2 - Correlation curve of *N. oceanica* dry weight. y = 0.2771x + 0.0559 and R^2 = 0.9707

Table A1 - Fatty acids methyl ester (FAME) profile (% of total FA) of *Phaeodactylum tricornutum*. FL0.5 and FL5 were tested at a duty cycle of 0.05. Shown are the adjusted means ± 95 % confidence interval (ANCOVA) from data of the induction stage (n=16) adjusted for the effects of the covariates "trial", "induction time", "harvesting timepoint", and "biomass concentration". Statistical analysis revealed no significant differences among treatments (p>0.05), except for C18:2n-6. Treatments that do not share the same letter are significantly different from each other.

Fatty acids	% of total fatty acids			
	Sun	**CL**	**FL0.5**	**FL5**
C14:0	7.99±0.78	7.08±0.78	8.40±0.77	8.28±0.77
C15:0	0.29±0.15	0.42±0.15	0.21±0.15	0.27±0.15
C16:0	17.05±2.34	14.32±2.34	13.77±2.33	14.90±2.32
C18:0	0.71±0.27	0.72±0.27	0.94±0.26	0.59±0.26
C22:0	0.14±0.06	0.12±0.06	0.19±0.06	0.145±0.06
C24:0	2.05±0.24	0.24±0.24	2.10±0.24	2.25±0.24
C16:1	18.05±1.87	19.20±1.87	18.23±1.86	17.73±1.86
C18:1	0.81±1.24	2.39±1.24	1.23±1.23	1.84±1.23
C24:1	0.37±0.08	0.39±0.08	0.31±0.08	0.39±0.08
C16:2	11.45±0.078	11.61±0.78	11.98±0.78	12.83±0.78
C16:4	3.026±0.19	2.70±0.19	2.80±0.19	2.70.19
C18:2 n-6	2.60±0.19[a]	2.21±0.18[b]	2.28±0.18[a,b]	2.24±0.18[b]
C18:3 n-6	1.73±0.65	1.12±0.65	1.52±0.65	1.07±0.65
C20:3	0.91±0.14	1.03±0.14	1.08±0.14	1.03±0.14
C22:6 n-3	1.17±1.59	3.08±1.59	1.54±1.57	1.44±1.58
C22:5 n-3	29.67±1.47	28.97±1.47	30.82±1.46	29.51±1.46

Table A2 - Fatty acids methyl ester (FAME) profile (% of total FA) of *Nannochloropsis oceanica*. FL0.5 and FL5 were tested at a duty cycle of 0.05. Shown are the adjusted means ± 95 % confidence interval (ANCOVA) from data of the induction stage (n=16) adjusted for the effects of the covariates "trial", "induction time", "harvesting timepoint" and "biomass concentration". Statistical analysis revealed no significant differences among treatments (p>0.05).

Fatty acids	% of total fatty acids			
	Sun	**CL**	**FL0.5**	**FL5**
C14:0	7.46±0.36	7.69±0.36	7.79±0.18	8.01±0.36
C12:0	0.32±0.16	0.36±0.16	0.21±0.17	0.38±0.16
C16:0	38.34±2.67	39.15±2.66	38.35±2.68	38.16±2.66
C18:0	1.16±0.16	1.31±0.16	1.08±0.16	1.12±0.16
C16:1	24.00±1.07	24.13±1.07	25.32±1.07	24.42±1.07
C18:1	8.14±0.63	8.13±0.63	8.01±0.63	7.83±0.63
C18:2 n-6	1.69±0.25	1.71±0.25	1.71±0.25	0.189±0.25
C22:5 n-3	16.70±3.06	16.36±3.05	17.36±3.07	15.47±3.05