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INDUSTRIAL PRODUCTION OF DIATOMS *SKELETONEMA COSTATUM* AND *CHAETOCEROS CALCITRANS*

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Mestrado em Biologia marinha

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Declaração de autoria de trabalho

INDUSTRIAL PRODUCTION OF DIATOMS *SKELETONEMA COSTATUM* AND *CHAETOCEROS CALCITRANS*

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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Carolina Rebelo de Vela Bastos

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Resumo

Os oceanos são um bioma com um enorme potencial devido aos diversos recursos que provêm dos mesmos, cuja relevância económica é evidenciada por as várias indústrias que dependem e lucram dos oceanos. Uma indústria que tem emergido com contínua e crescente inovação é a biotecnologia marinha. Através desta indústria foi possível expandir a aplicabilidade dos recursos marinhos para diversas áreas, desde farmacêuticos, comidas saudáveis, energia renovável, cosméticos, biomateriais, entre outras. Fundamentalmente, a biotecnologia marinha permite a transformação de matérias-primas em productos de alto valor acrescentado, maioritariamente através do uso de microrganismos, entre eles as microalgas. As microalgas são organismos fotossintéticos unicelulares e/ou coloniais, capazes de converter luz em energia química através da fotossíntese. Este processo resulta também na produção de diversos compostos de interesse (ex: ácidos gordos polinsaturados, carotenoides, polissacáridos sulfatados), cujos principais mercados são a aquacultura, cosmética e a indústria alimentar. Para cada aplicação são escolhidas espécies específicas de microalgas, tendo em conta os seus perfis bioquímicos e características de interesse. Ao nível industrial, os sistemas de produção podem ser divididos em sistemas abertos (ex: *raceways*, tanques), ou em sistemas fechados (ex: fotobiorreactores tubulares, *Flat panels*), dependendo se, respetivamente, as culturas de microalgas estão diretamente expostas ao ambiente que as rodeia ou se existe uma barreira física que as separa. Tipicamente, espécies de microalgas mais robustas e resistentes a condições extremas são cultivadas em sistemas abertos. Os sistemas fechados permitem, então, uma maior variedade de microalgas que podem ser cultivadas, embora menos robustas e com um maior custo de produção associado. As diatomáceas são um grupo de microalgas castanhas cuja produção em larga escala é reportada em sistemas fechados e abertos, geralmente dependendo do produto final que se pretende obter. As diatomáceas têm um elevado interesse ao nível de produção industrial devido ao seu enorme potencial económico e biotecnológico. Este potencial deriva principalmente da sua composição em lípidos, pigmentos e sílica, assim como das suas capacidades de bioabsorção e biorremediação. Além disso, as elevadas taxas de duplicação, a plasticidade na alocação de carbono no interior de componentes celulares, a resiliência a alterações ambientais, assim como as elevadas taxas de sedimentação das diatomáceas incrementam o interesse da produção industrial deste grupo de microalgas. Com o objetivo de reduzir os custos de produção em simultâneo, estas indústrias investem

na otimização do cultivo das suas culturas. O cultivo de diatomáceas requer o fornecimento de H_2O, CO_2, energia e nutrientes. Considerando o papel fulcral dos nutrientes, a adição e manipulação do meio de cultura fornecido às diatomáceas é uma das principais estratégias de otimização da sua produção. Geralmente, o meio de cultura é composto por macro-, micronutrientes e vitaminas, sendo que estas últimas são principalmente fornecidas apenas à escala laboratorial. Considerando a relevância de cada nutriente no metabolismo das diatomáceas, nitratos, fósforo e sílica são considerados macronutrientes, enquanto outros elementos como ferro, magnésio e zinco são definidos como micronutrientes. As vitaminas são tendencialmente compostas por biotina, tiamina e vitamina B12. *Skeletonema costatum* e *Chaetoceros calcitrans* são duas espécies de diatomáceas cêntricas de alto valor, cosmopolitas e formadoras de colónias, cujos principais mercados são as indústrias de aquacultura e cosméticos. Com base nas publicações existentes, a produção industrial destas diatomáceas é realizada maioritariamente em sistemas abertos; contudo, é sugerido que o seu cultivo seja realizado em fotobiorreactores. Com o contínuo interesse na obtenção de bioprodutos provenientes de diatomáceas em paralelo com o crescente potencial do mercado da biotecnologia marinha, o futuro da industrialização destas espécies aparenta ser promissor. Como tal, os objetivos deste trabalho são otimizar a produção de biomassa e avaliar a consequente composição bioquímica de duas espécies de diatomáceas de alto valor: *S. costatum* e *C. calcitrans*. De forma a cumprir estes objetivos: (1) foi realizado um processo gradual de otimização no qual as concentrações ótimas dos principais nutrientes do meio de cultura Nutribloom plus® foram determinadas ao nível laboratorial para cada espécie; (2) a validação deste processo foi realizada à escala piloto em *Flat Panels* no exterior, para a espécie *S. costatum*; (3) os efeitos do meio de cultura otimizado na composição bioquímica foram determinados para ambas as diatomáceas. O sucesso deste trabalho irá fornecer as ferramentas base para a empresa Necton S.A. aperfeiçoar vários produtos de *aquafeed* que já se encontram no mercado. No Capítulo 2, o processo de otimização do meio de cultura Nutribloom plus foi realizado num sistema de 12 colunas de bolhas de 1 L, separadamente para cada espécie. Neste sistema, as culturas foram sujeitas a um regime constante de luz durante um período de 7 dias. O processo gradual de otimização teve início com sílica, seguido de nitratos, fósforo, ferro e, por fim, micronutrientes. Com o uso de triplicados, foram avaliadas 4 concentrações de cada nutriente, sendo que a ótima foi selecionada previamente ao início do ensaio seguinte. Para a espécie *S. costatum*, o meio de cultura otimizado foi validado à escala piloto pela

inoculação de 6 *Flat Panels* de 100 L no exterior, 3 com o meio de cultura Nutribloom plus® otimizado e 3 com o meio de cultura Nutribloom plus® controlo. Diariamente, para os ensaios realizados ao nível laboratorial e no exterior, foram avaliados o peso seco (g L^{-1}), o pH e a temperatura das culturas, sendo que a cada 2 dias foram medidos os nitratos, sílica e fosfatos e foi readicionado o meio de cultura. Para cada ensaio, foi guardada biomassa no último dia, através da qual a análise bioquímica foi realizada, onde se obteve a composição de proteínas, lípidos, glícidos, cinzas e ácidos gordos. Para ambas as diatomáceas, os resultados obtidos salientam a relevância da otimização do meio de cultura para o aumento da biomassa produzida. Para *S. costatum* e *C. calcitrans*, as condições controlo resultaram em menor crescimento, onde os pesos secos obtidos foram $2,00 \pm 0,03$ gL^{-1} e $0,76 \pm 0,03$ gL^{-1}, respetivamente. Frequentes indícios de stress ao nível morfológico foram observados em ambas as espécies cultivadas sob condições controlo, principalmente ao nível de aumento do comprimento celular e distrofia celular. A otimização da concentração de sílica resultou numa elevada melhoria de ambas as culturas, com um aumento significativo de crescimento e melhoria da performance celular, onde as concentrações deste nutriente consideradas como ótimas foram 2,4 e 1,2 mM para *S. costatum* e *C. calcitrans*, respetivamente. Este aumento do fornecimento de sílica em 6 e 3 vezes resultou em pesos secos de $3,51 \pm 0,17$ gL^{-1} e $2,03 \pm 0,06$ gL^{-1} nas culturas de *S. costatum* e *C. calcitrans*, respetivamente. A otimização da concentração de nitratos demonstrou que as condições controlo (4 mM) eram ótimas para ambas as espécies, e destacou a elevada necessidade deste nutriente, visto que o fornecimento de nitratos a concentrações inferiores resultou na diminuição do crescimento das culturas com uma elevada deterioração celular associada. Para ambas as diatomáceas, a otimização das concentrações de fósforo, ferro e micronutrientes resultaram em menores diferenças ao nível de crescimento, entre as distintas concentrações testadas. Para *S. costatum* e *C. calcitrans*, a concentração ótima de fósforo foi de 100 µM, enquanto as concentrações de ferro foram 20 e 80 µM, respetivamente, e a de micronutrientes foi de 0,5 mL L^{-1}. Através da validação da otimização do meio de cultura para *S. costatum* em *Flat Panels*, foi registado um menor crescimento para as condições controlo e otimizadas, $0,97 \pm 0,03$ gL^{-1} e $0,86 \pm 0,05$ gL^{-1}, respetivamente, quando comparado com os resultados obtidos a nível laboratorial. Este resultado pode ser atribuído à ausência de iluminação constante das culturas crescidas no exterior. Não obstante, as culturas fornecidas com o meio de cultura otimizado cresceram significativamente mais do que as culturas com condições controlo, sendo que a disparidade entre ambas teve início no quinto dia de

ensaio. Desta forma, à escala piloto, o fornecimento do meio de cultura otimizado deve ser feito para níveis de peso seco superiores a 0,6 gL^{-1}. Para as culturas de *S. costatum* ao nível laboratorial, as análises bioquímicas da composição proximal revelaram uma descida da % de peso seco de proteína e hidratos de carbono, sendo que a % de peso seco de cinzas aumentou e a % de peso seco manteve-se, ao longo do processo de otimização. As análises bioquímicas da composição proximal das culturas de *C. calcitrans* demonstram a ausência de diferenças significativas ao longo do processo de otimização. De forma semelhante, as análises bioquímicas da composição proximal das culturas de *S. costatum* cultivadas em *Flat Panels* revelam uma ausência de diferenças significativas. Relativamente à composição de ácidos gordos das culturas de *S. costatum* e *C. calcitrans* cultivadas ao nível laboratorial, os perfis de ácidos gordos de cada espécie foram caracterizados e foi possível detetar um aumento significativo no conteúdo de EPA ao longo da otimização dos nutrientes, sendo que para *S. costatum* este aumento também foi registado ao nível do conteúdo de DHA. Para ambas as espécies, o processo de otimização resultou no aumento significativo do conteúdo de PUFAs em detrimento dos conteúdos de SFAs e MUFAs, devido à redução das condições de stress. Contudo, esta disparidade não foi observada nas culturas de *S. costatum* cultivadas no exterior devido ao estado de crescimento ativo das culturas quando a biomassa foi recolhida. Adicionalmente, foram registados elevados valores de PUFAs. Em geral, através do presente trabalho foi possível aumentar a produtividade da biomassa obtida em cerca de 1,8 e 3,2 vezes em culturas de *S. costatum* e *C. calcitrans*, respetivamente. Para ambas as diatomáceas, os principais nutrientes cruciais para a otimização da biomassa em termos de crescimento foram sílica e nitratos. O processo de otimização resultou em poucas alterações ao nível da composição proximal de ambas as espécies. Contudo, ao nível da composição de ácidos gordos, foi possível registar aumentos significativos no conteúdo de PUFAs, especialmente ao nível dos ácidos gordos de valor acrescentado como EPA e DHA. Estes resultados denotam a aplicação promissora e relevante de produtos de *S. costatum* e *C. calcitrans* nas indústrias de aquacultura e nutracêuticos. O processo de otimização aplicado no presente trabalho é indispensável para o incremento da produção industrial destas espécies de diatomáceas de alto valor acrescentado, onde o aumento de produtividade em termos de biomassa e composição bioquímica é associado à redução de custos de produção.

Palavras-chave: Aumento biomassa, sílica, nitratos, fósforo, ferro, micronutrientes

Abstract

The marine biotechnology industry is a fast-growing industry that primarily uses microorganisms, such as microalgae, to obtain high-value products. Microalgal species like diatoms are currently produced industrially, given their economic and biotechnological potential. One of the main strategies to optimise their cultivation is the addition and manipulation of the culture medium. *Skeletonema costatum* and *Chaetoceros calcitrans* are two cosmopolitan high-value centric diatoms, with a rich nutritional profile. These diatoms are commercialized mainly as products for aquaculture, such as feed for molluscs and crustaceans. The purpose of the following work was to optimise the cultivation protocol of *S. costatum* and *C. calcitrans* in terms of the culture medium composition. Optimisation of this medium was performed in a stepwise fashion, where the supply of different nutrients was optimised in the following order: silicate, nitrate, phosphate, iron, and micronutrients. Furthermore, this optimisation was validated for *S. costatum* at pilot-scale in exterior flat panels. The biochemical profile of all cultures was characterized regarding proximal composition of protein, lipid, ash, and carbohydrates contents as well as fatty acid profile. The results obtained revealed an increase in biomass productivity for both diatoms when the supply of nutrients was optimised, with a 1.8 and 3.2-fold increase in biomass produced by *S. costatum* and *C. calcitrans*, respectively. Silicate and nitrates were the key nutrients impacting the growth of both diatoms, whilst phosphate, iron and micronutrients had smaller effects. The validation of the optimised culture medium in *S. costatum* cultures grown outdoors demonstrated smaller differences in biomass productivity. Furthermore, lower growth was registered for both cultures under control and optimised conditions when compared with that of cultures grown under controlled laboratory conditions, which can be attributed to the absence of constant illumination in outdoors conditions. The biochemical profile showed relevant results regarding fatty acid composition, where an increase in high-value PUFAs such as EPA was observed for both diatoms, whilst an increase in DHA was only detected for *S. costatum* cultures, all regarding laboratory and outdoor conditions. The present study supports the indispensable application of the described optimisation process in the industrial production of diatoms *S. costatum* and *C. calcitrans* for the increment of biomass and nutritional composition productivity of these species, which are highly relevant regarding aquaculture and nutraceuticals applications.

Keywords: biomass growth, silicate, nitrate, phosphate, iron, micronutrients

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Chapter 1: Introduction

Chapter 2: Production Optimization and Nutritional Value of Two Diatoms, *Skeletonema costatum* and *Chaetoceros calcitrans*, Grown in a Bubble Column Photobioreactor System

List of abbreviations and acronyms

ALA – α – Linolenic Acid

ATP – Adenosine Triphosphate

DAM – Diatom Artificial Medium

DHA – Docosahexaenoic Acid

DIC – Differential Interference Contrast

DM – Diatom Medium

DNA – Deoxyribonucleic Acid

DW – Dry Weight

EFSA – European Food Safety Authority

EPA – Eicosapentaenoic Acid

ESAW – Enriched Seawater Artificial Seawater

FA – Fatty Acid

FAME – Fatty Acid Methyl Esther

FAO – Food and Agriculture Organisation

FDA – Food and Drugs Administration

LA – Linoleic Acid

LDM – Locally Developed Medium

LEDs – Light Emitting Diodes

MUFA – Monounsaturated Fatty Acid

NB^+ – Nutribloom Plus

NEPCC – Northeast Culture Collection Protocol

OD – Optical Density

PBR – Photobioreactor

PTFE – Polytetrafluoroethylene

PUFA – Polyunsaturated Fatty Acid

SFA – Saturated Fatty Acid

UV – Ultraviolet

1. Chapter 1: Introduction

1.1. Ocean innovation

The oceans are the largest biome on earth, covering roughly 70% of the earth's surface (Daniotti & Re, 2021). Indisputably, they are a crucial source of food, energy, and minerals, being also a regulator of weather, climate, and a source of oxygen (Rayner *et al*., 2019). Though many biotic and abiotic resources already come from the oceans, unknown or unexplored new resources further boost the potential of this biome (Dobretsov, 2019). The economic relevance of marine resources is highlighted by the many industries already established, while new ones are continuously emerging (OECD, 2016; Rayner *et al*., 2019). Consequently, the interest in ocean resources has risen side-by-side with terms such as "Blue Growth" and "Blue economy", where win-win scenarios can occur for humans and the environment itself (Vieira *et al*., 2020).

One industry emerging with expanding innovation and applicability is marine biotechnology (Rotter *et al*., 2020). This industry has allowed the expansion of marine resources from feed, biofuel, agriculture, and food to the development of novel products in the areas of pharmaceuticals, health foods, renewable energy, cosmetics, biomaterials, among others (Blasiak *et al*., 2018; Lauritano & Ianora, 2018). Ultimately, marine biotechnology has permitted the transformation of raw materials into high-value products, mainly by means of microorganisms (Felício *et al*., 2012). As a result, the marine biotechnology market value is continuously growing, and it is estimated that, by the year 2025, it will reach 6.4 billion dollars (Hurst *et al*., 2016; Blasiak *et al*., 2018). More specifically, one group of organisms gaining increasing relevance in the biotechnology industry over the last few decades is microalgae, whose high biodiversity holds tremendous potential for different biotechnological applications. In fact, a vast array of microalgae-based bioproducts have already been established in several commercial areas, along with a growing potential for novel applications in various fields (Fig. 1.1, Olaizola, 2003; Gujar *et al.*, 2019).

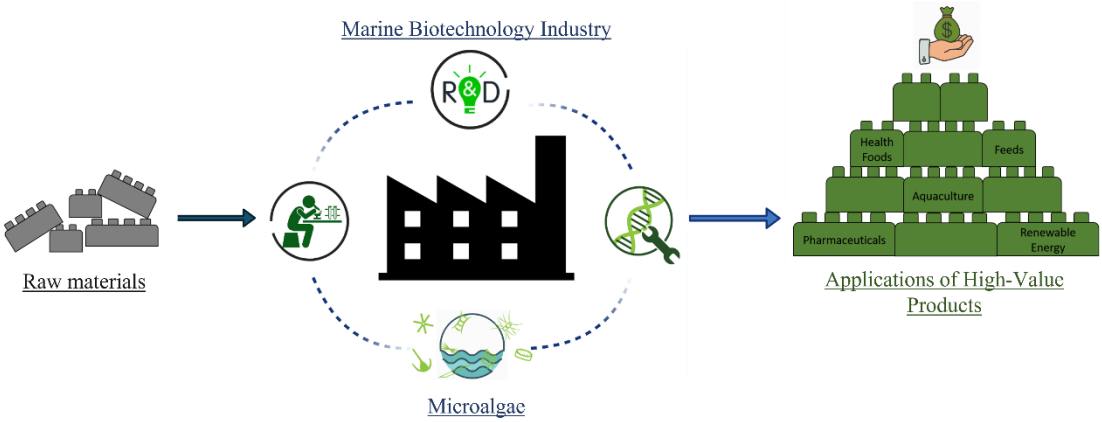

Figure 3.1: Schematic representation of the marine biotechnology industry, transforming raw materials into high-value products with applications in fields such as aquaculture, pharmaceuticals, renewable energy, and health foods, through the use of microorganisms like microalgae.

1.2. Microalgae

Microalgae are unicellular and/or colonial photosynthetic organisms that represent the basis of the food chain in different ecosystems. They can be divided into a polyphyletic assemblage of eukaryotes, with the ability to convert light into chemical energy through photosynthesis. Consequently, other organisms are eminently dependent on microalgae to provide them with basic essentials such as food and oxygen (Hamed, 2016). The unique physiology, metabolism, and biodiversity of microalgae highlight their potential to provide innovative biomasses and high-value compounds (Wan *et al*., 2020).

The contemporary biotechnological relevance of these organisms can be understood through the study of phylogenetics. Eukaryotes can currently be grouped into twelve major groups, with microalgae belonging to four of these, namely: SAR (Stramenopiles, Alveolates, and Rhizarians), Haptista, Cryptista, and Archaeplastida (Keeling & Burki, 2019). Microalgae are most probably some of the oldest eukaryotic organisms on earth, and hence, their remarkable capacity of quick adaptation to extreme conditions has been reported as an evolutionary mechanism of survival (Bradshaw & Hardwick, 1989; López-Rodas *et al*., 2009; Lyon & Mock, 2014). The marine biotechnology industry has exploited these organisms for commercial purposes, given the variety of high value bioproducts currently originated from microalgae feedstocks. Several compounds (e.g., polyunsaturated fatty acids, carotenoids, and sulphated polysaccharides) produced by different microalgal strains are in high demand for different areas, like human and animal nutrition, aquaculture, cosmetics, and medicine (Alam *et al*., 2020). In addition, the

potential of microalgae as a more ecological solution for food and feed production, because of their CO_2 consumption and ability to grow on systems placed in non-arable land, has increased the interest in applying these organisms in biorefineries. In turn, biorefineries can be used to upgrade the value of biomass as different streams, which may include bioenergy by-products, with a concomitant reduction of greenhouse gases emissions and substitution of traditional energy sources. As a result, microalgae-based industries can become more sustainable, being part of a more circular bioeconomy (Cheah *et al*., 2015; Wan *et al*., 2020).

1.2.1. Microalgal Biotechnology

Despite the recent increasing biotechnological interest in microalgae, microalgal biomass or components thereof have been used in diverse areas for years. In fact, their history in biotechnology goes back thousands of years to when microalgae were used as food and, recently, to when Arnold Nobel created dynamite with the help of fossil diatoms (Pulz & Gross 2004; Milledge, 2011). However, the actual exploitation of microalgae biomass for commercial purposes began in the 1970's, following the rise of fish farming (Couteau & Coiffard, 2018). At present, the primary markets for microalgae-based products are aquaculture, cosmetics, pharmaceuticals, and nutritional supplements (Fig. 1.2, Hemaiswarya *et al*., 2011; Acién *et al*., 2019). With the rising biotechnological innovation, the potential of these microorganisms expands their applications to other

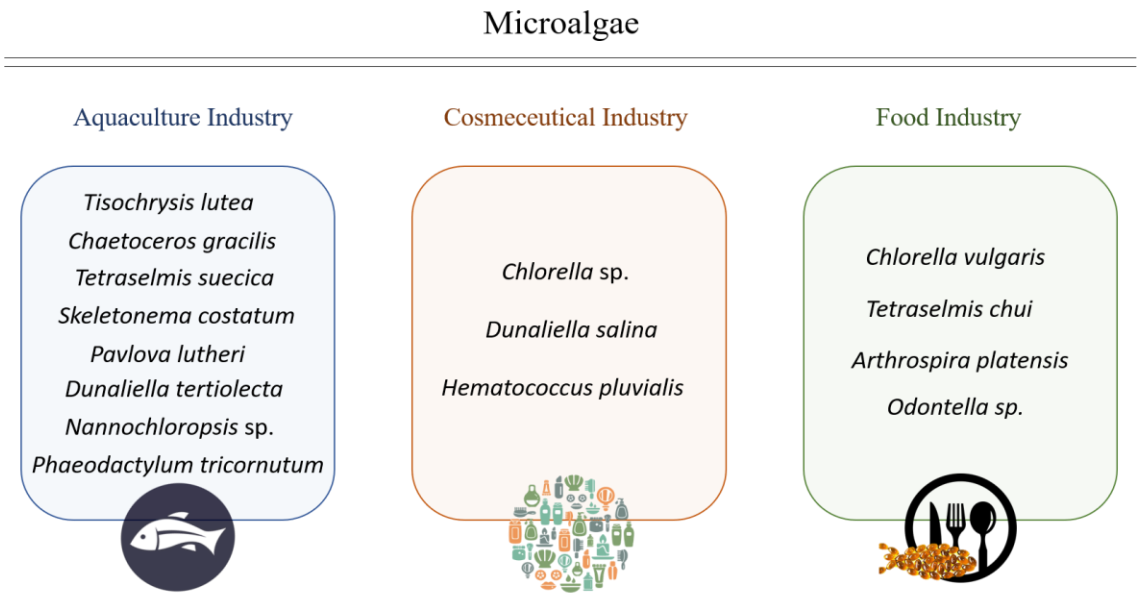

Figure 1.2: Schematic representation of the biotechnological applications of different microalgae species in 3 main commercial markets currently investing in microalgal biomass and components thereof.

markets, with research focusing on biofuels, wastewater treatment, high-value biomolecules and CO_2 sequestration (Levasseur *et al*., 2020).

1.2.1.1. Aquaculture Industry

The continuous growth of the human population leads to the unsustainable use of a limited resource: fisheries (Pauly *et al*., 2002; Merino *et al*., 2012). A promising solution to invert the ratio of demand being higher than supply is the aquaculture market, which is currently responsible for nearly 50% of human fish consumption (FAO 2018; Gasco *et al*., 2018). One of the influencing factors affecting the nutritional value of these aquaculture products is the quality of the feeds supplied. However, the formulation of most aquafeeds comes from the production of fishmeal and fish oils whose present supply is unsustainable, due to high inputs of lower-trophic species that derive from nearly a third of processed global fisheries (Pauly & Zeller, 2017; Yarnold *et al*., 2019). Therefore, as alternatives began to emerge, the inclusion of these unsustainable products in aquaculture has been reduced to a minimum (Gasco *et al*., 2018). In addition, the co-production of nutrient-rich wastewater increases the levels of pollution caused by aquaculture, creating yet another problem for this fast-growing industry (Cao *et al*., 2007).

Over the last decade, researchers have proposed microalgae as potential problem-solvers to improve the sustainability of feed ingredients in the aquaculture sector (Han *et al*., 2019; Chen *et al*., 2020; Yang *et al*., 2021). Several reports highlight the presence of different classes of compounds in microalgae biomass with growth-promoting and immunostimulant components (e.g., polyunsaturated fatty acids, *β*-carotene, and astaxanthin) that hold a high potential to substitute and/or decrease the use of unsustainable ingredients traditionally used in aquafeeds (Roy & Pal, 2015; Chen *et al*., 2020). To obtain balanced diets and further improve the positive benefits of microalgae, mixtures of different strains are usually supplied, either directly, as is the case for bivalve molluscs, or indirectly through the enrichment of zooplankton (e.g., rotifers) as live feed for fish larvae (Knuckey *et al*., 2006; Rico-Villa *et al*., 2006; Priyadarshani & Rath, 2012). The most common microalgae species used in aquaculture are *Tisochrysis lutea*, *Chaetoceros gracilis, Tetraselmis suecica*, *Skeletonema costatum*, *Pavlova lutheri*, *Dunaliella tertiolecta, Nannochloropsis* sp., *Phaeodactylum tricornutum,* and *Chlorella* sp. (Hemaiswarya *et al.*, 2011; Shah *et al.*, 2018). Additionally, recent studies have given a strong indication that applying microalgae to the treatment of wastewater from

aquaculture can lead to the implementation of biorefineries. In such scenarios, microalgae consume the excess nutrients from wastewater, decrease O_2 depletion and mitigate CO_2 evolution, while producing valuable compounds that can be used for aquaculture or for other purposes, linking bioremediation with the production of bioresources (Lu *et al*., 2019; Andreotti *et al*., 2020; Hawrot-Paw *et al*., 2020). Thus, the integration of algal production systems with fish farming might be the basis for a more eco-friendly and circular aquaculture industry (Yarnold *et al*., 2019).

1.2.1.2. Cosmeceutical Industry

One business that currently benefits from microalgae compounds is the highly profitable cosmetics industry (Vieira *et al*., 2020). In 2016, this industry had a global market value of $425 billion with a $72 billion valuation in Europe (Couteau & Coiffard 2018; 2016). Following a continuous search for innovative products, microalgae are, once again, microorganisms of interest whose application is either an excipient (thickener, stabilizer, or emulsifier) or an active ingredient (Ariede *et al*., 2017; Nethravathy *et al*., 2019). For example, several compounds can be used to maintain skin health and prevent dermatological issues, owing to their strong antioxidant activity, as is the case of *β*-carotene, astaxanthin, and polyunsaturated fatty acids (PUFAs) (Wang *et al.*, 2015; Guillerme *et al*., 2017; Couteau & Coiffard, 2018).

Microalgae-based compounds are of high appeal, given the growing demand for natural and more sustainable sources and eco-friendly products to substitute synthetic compounds (Ariede *et al*., 2017; Tang *et al*., 2020). Some of the few strains that are currently produced at an industrial level and are of interest to the cosmetics industry are *Chlorella* sp., *Dunaliella salina,* and *Haematococcus pluvialis* (Couteau & Coiffard, 2018).

According to Ryu *et al*. (2015), natural products coming from microalgae can also be used as pharmaceutical agents if the desired goal is a cosmetic with health benefits – cosmeceutical[1]. Even though the pharmacological properties of microalgae components have already been researched extensively in different fields (e.g., cardiovascular diseases, metabolic syndromes, neurodegenerative diseases, vaccines, among others), several of these studies have only been performed *in vitro*, demonstrating that there is still a great

[1] Cosmeceuticals are generally described as products for topical application, with specific ingredients that may provide a prolonged effect associated with medicinal properties. Since the term is not recognized by most governmental offices worldwide, the regulation of these products is mainly performed by the cosmetic industries (Espinosa-Leal & Garcia-Lara 2019).

deal to be done to validate these findings *in vivo* (Rumin *et al*., 2020). Ultimately, in cosmetics and cosmeceuticals, microalgae-derived ingredients are mainly used as thickening, moisturizer, anti-aging, natural pigments, and skin-care agents (Guillerme *et al*., 2017; Levasseur *et al*., 2020).

1.2.1.3. Food Industry

Microalgae consumption as a food product is already a traditional norm in Asiatic countries, and a significant growing trend in European countries (European Commission, 2016). Several different studies have focused on integrating microalgae and microalgae components as nutraceuticals in functional foods, whose ultimate purpose is to strengthen everyday foods with additional health benefits (Shahidi, 2009; Matos *et al*., 2017). This growing trend comes from the presence of highly relevant compounds for human nutrition, such as polysaccharides, lipids (e.g., PUFA), essential amino acids (e.g., leucine), pigments (e.g., *β*-carotene), vitamins (e.g., vitamin B12), and micronutrients (e.g., iron) (Villarruel-López *et al*., 2017; Galasso *et al.*, 2019). Consequently, extensive research regarding the benefits of microalgae products in health-related issues are available, which are related with conditions such as cardiovascular diseases, oxidation-associated diseases and inflammation, cancer, neurodegenerative diseases, and diabetes. In addition, microalgae may be used to fight infectious disease due to their anti-microbial and anti-viral properties (Pulz & Gross, 2004; Plaza *et al.*, 2010; Ejike *et al.*, 2017; García *et al*., 2017) as well as decrease malnutrition and infant death in sub-Saharan Africa due their nutritional value (Alam *et al*., 2020).

Currently, the main species produced and approved for human consumption in the EU are *Chlorella vulgaris*, *Tetraselmis chui*, and *Arthrospira platensis* (commonly known as "Spirulina"), although other microalgae such as *Odontella* sp. have also been accepted for human nutrition and have recently gained more popularity in the food industry (Kaur, 2020; Remize *et al*., 2021). Nowadays, microalgae biomass and derived compounds have already been incorporated into products available in the food market, mainly in the form of cookies/biscuits, tablets or liquids, pasta and seafood-flavouring agents, where microalgal components are used as colouring agents and/or as ingredients in "functional foods"[2] by strengthening their nutritional content and biomedical value. For example,

[2] Functional foods are industrially processed or natural foods that have potentially additional positive health effects (e.g, reduce the risk of disease), when consumed in appropriate levels with a diverse diet. Furthermore, proper clinical trials along with significant experimental evidence of safety and functionality are required to establish a food product as a functional food (Granato *et al.,* 2020).

specific bioactive compounds such as eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids present in the microalgal biomass have been incorporated into dietary supplements as “nutraceuticals” in the form of pills and capsules (Caporgno & Mathys, 2018; Batista *et al*., 2019; Alam *et al*., 2020; Tang *et al*., 2020).

Despite the high potential of microalgae biomass in food products, their use as food ingredients is still in its infancy in non-Asiatic countries (Priyadarshani & Rath, 2012; European Commission, 2016). This is mainly due to strict food safety regulations imposed by entities like the European Food Safety Authority (EFSA) and the Food and Drugs Administration (FDA), which have resulted in the approval of a limited number of microalgal species for human consumption. In addition, high production costs of the microalgal biomass together with a relative shortage of studies regarding the actual bioaccessibility and bioavailability of microalgae-derived bioactive compounds introduced in novel food matrixes have delayed the confirmation of the nutritional value and benefits of these products, thus further hindering their successful commercialization (Pulz & Gross, 2004; Batista *et al*., 2012; Alam *et al*., 2020; Nova *et al*., 2020).

For each application, specific microalgae species can be chosen due to their extensively diverse biochemical traits and characteristics of interest, which can furthermore be shaped by different culturing methods.

1.3. Industrial production: room for improvement

Throughout the years, the lack of commercially appealing products along with the challenges associated with mass production at an industrial scale, led to the commercialization of a low number of different microalgal strains (Olaizola, 2003). Nowadays, the improvement of biotechnology has resulted in the successful production of different microalgal strains at an industrial level (Couteau & Coiffard, 2018).

This type of production is based on a scale-up process where cultures are firstly grown in small volumes, usually under controlled laboratory conditions, and later transferred to higher volumes, passing through pilot-scale units until they finally reach industrial-scale photobioreactors (Fig. 1.3). Considering the significant discrepancy between small-scale laboratory conditions and large-scale outdoor conditions, the challenges of microalgae mass-scale cultivation often present new issues, imperceptible at production steps at smaller scales (Olaizola, 2003). For this reason, several parameters must be considered for the production of photosynthetic cultures in the design of the final production system to achieve maximum production yields, namely: pH, temperature, light availability,

culture mixing, nutrients, amongst other influencing factors (Fernández *et al*., 2016; Acién *et al*., 2017; Pawlowski *et al*., 2017).

Currently, large scale industrial production systems can be divided into:

(i) open systems, such as natural or artificial circular ponds, raceway ponds, tanks, and thin-layer cascade systems, where microalgae are directly exposed to uncontrolled environmental conditions; and

(ii) closed systems, like tubular, flat panel, and column photobioreactors (PBR), where there is a physical barrier between the cultures and the surrounding environment (Dragone *et al.*, 2010; Acién *et al.*, 2017).

Open systems have the advantage of being easier and cheaper to build. However, they often have several technical and biological constraints related to high evaporation, easy contamination, difficult control of the effective photosynthetic efficiency, and uncontrolled environmental conditions (Dormido *et al*., 2014). This results in a selective number of microalgal strains, often resistant to extreme conditions (e.g., high salinity or pH levels) and/or able to grow fast, which can successfully be cultured in these systems. Examples of these strains are the highly resistant microalgae *Chlorella* spp., *Nannochloropsis* spp., *Scenedesmus* spp. and *Dunaliella* spp., as well as the cyanobacterium *A. platensis* (Carlsson *et al*., 2007; Acién *et al*., 2017). On the other hand, closed systems are more expensive and require more maintenance. Still, they provide higher control over the production parameters of microalgae cultures, including pH and temperature control as well as decreased risk of contamination and lower evaporation, ultimately leading to higher production rates (Dormido *et al*., 2014). Flat panels and tubular PBRs are the most popular closed systems used and, with the correct design and sensors (O_2, temperature and pH), optimum culture growth can be achieved (Dormido *et al*., 2014; Hamed, 2016). In addition, these systems allow for the cultivation of a wide range of strains, generally less robust and thus not optimal for open systems, such as *H. pluvialis*, *T. lutea,* and *Porphyridium cruentum* (Acién *et al*., 2017).

One highly relevant microalgae group that is presently industrially produced for aquaculture-related purposes is diatoms, whose mass-scale production is reported mainly in open systems, while only pilot-scale photobioreactors have been used in closed systems (Wang & Seibert, 2017; Valenzuela *et al*., 2018; S. Wang *et al*., 2018).

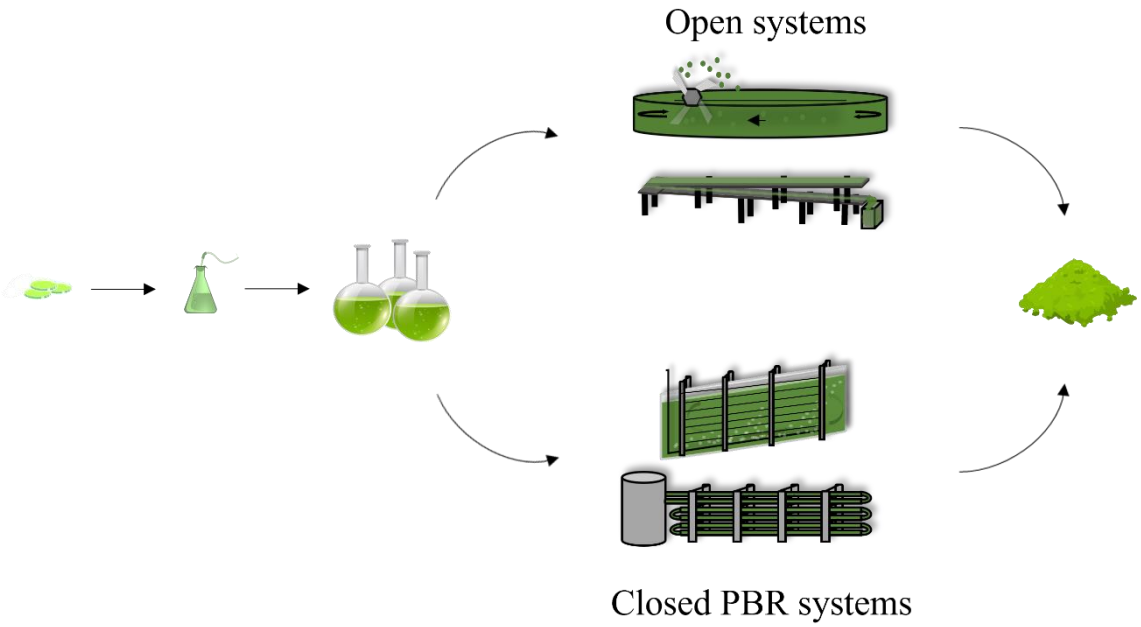

Figure 1.3: Schematic representation of the scale-up process of microalgae cultivation, from smaller volumes (agar-plate) to larger production volumes (open systems or closed photobioreactor systems), until the desired.

1.4. Diatoms

Prevalent in marine, freshwater and terrestrial ecosystems, diatoms represent a major group of eukaryotic microalgae (H. Wang *et al*., 2017). As a major constituent of benthic and planktonic algal communities, diatoms are responsible for approximately 40% of the ocean's primary productivity and 20% of the earth's photosynthesis (Amin *et al*., 2012; Gautam *et al*., 2019). Moreover, they are critical players in the biogeochemical cycles of carbon, nitrogen, phosphorous, silicon, and iron (Buesseler, 1998; Allen *et al*., 2006). These unicellular photosynthetic eukaryotic organisms belong to the class *Bacillariophyceae* in the "megagroup" Stramenopiles. They can occur in colonies or as solitary cells, with most species ranging between 10-50 µm (Merz & Main, 2015).

One unique characteristic of diatoms is the intricately patterned exoskeleton structure known as frustule, made of the biomineralization of proteins, carbohydrates, and mainly silica, which divides them into two major groups: pennate (Bacillariales) or centric (Biddulphiales) (Fig. 1.4, Bozarth *et al*., 2009; Kuppusamy *et al*., 2017). In addition, due to their secondary endosymbiotic origin, their chloroplasts are surrounded by two extra

membranes: the endoplasmic reticulum and the periplastidic membrane (Gibbs, 1979; Lang *et al*., 1998). Diatoms display a diplont life cycle, with a meiosis at the end of the gametogenesis, with the zygote developing into an auxospore. Their vegetative reproduction occurs by binary fission, where two new individuals are formed within the parent cell frustule, resulting in a size reduction of the daughter cells, since each receives one parent cell theca as their epitheca (Gvethe & Syve 1990; Hildebrand *et al*., 2009).

Considering all their unique biological, physiological, and metabolic characteristics, diatom dominance over other phytoplankton communities quickly occurs in upwelling regions where the right conditions such as light climate, inorganic nitrogen, phosphorous, silicon, and other trace elements are present (Morel & Price, 2003; Sarthou *et al*., 2005). Consequently, in coastal waters, diatoms provide the basic needs for fisheries, while in the open ocean, a significant portion of their organic matter sinks and either serves as food for the deep-water organisms or settles in the ocean floor, eventually contributing to petroleum reserves (Sarthou *et al*., 2005; Armbrust, 2009).

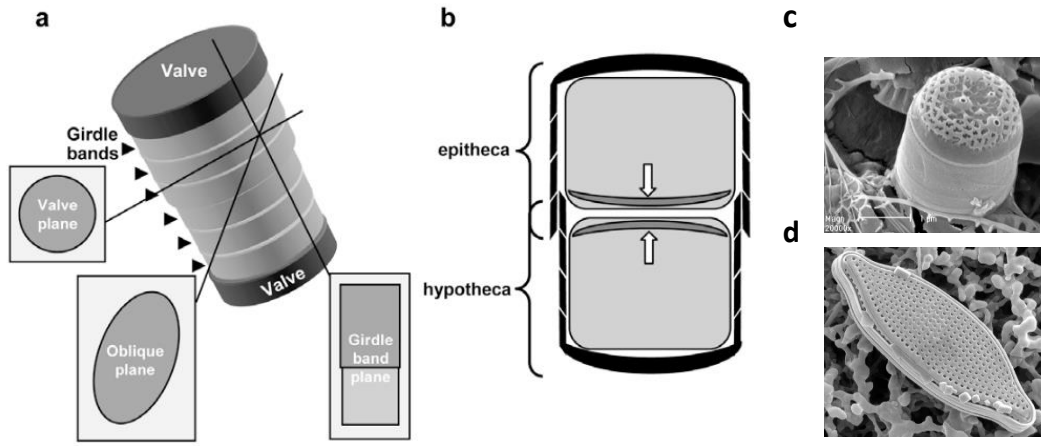

Figure 1.4: (a) Schematic diagram of the exterior of a *Thalassiosira pseudonana* cell. (b) Cross-sectional diagram of a *Thalassiosira pseudonana* cell containing two daughter cell protoplasts. (c) Scanning electron microscopy (20000× lens) of a centric diatom. (d) Scanning electron microscopy (8000× lens) of a pennate diatom. Adapted from Hildebrand *et al*., (2009) and Young *et al*., (2020).

1.4.1. Applications of Diatoms

Besides having a crucial role in their natural habitats, diatoms' applicability in human commercial activities can be vast. In recent years, their enormous economic potential has resulted in the generalized recognition of the biotechnological applications of diatoms (Lebeau & Robert 2003a). In addition to their extensive use in aquaculture, novel emerging commercial applications in other areas have been proposed, such as: nitrogen-

fixing biofertilizers, cosmetics, pharmaceuticals, renewable energy, health foods, fluid fuel production, raw materials production, wastewater detoxification, nanotechnology, and even in the field of forensic sciences (Fig. 1.5, Piette & De Letter, 2006; Bozarth *et al*., 2009; Wang & Seibert, 2017; Liu *et al*., 2020). These applications are mainly related to their contents of silicate and lipids and their capacity to bioremediate pollutants through, for example, biosorption.

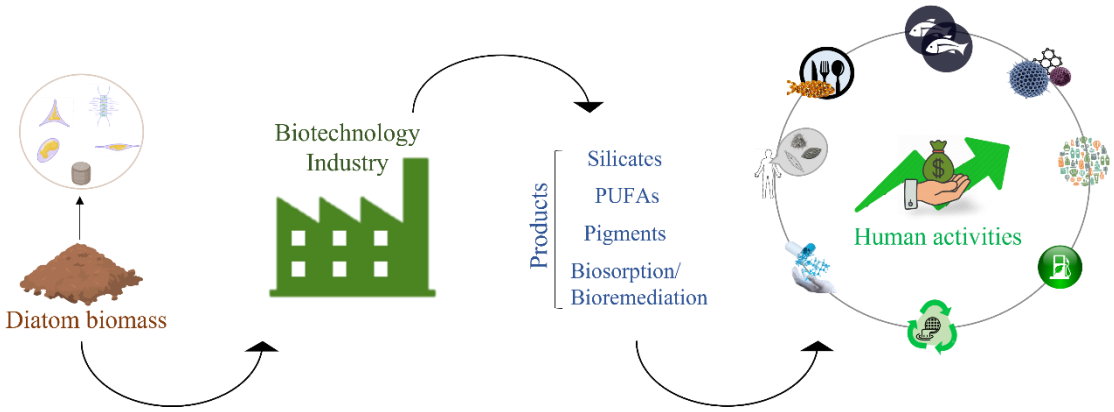

Figure 1.7: Schematic representation of the biotechnological applications of diatoms and components thereof. This process begins with the harvesting of biomass, and its application to the biotechnology industry, where the main components/characteristics of the diatoms are refined to be further exploited in several human activities.

1.4.1.1. Silicates

Frustules differentiate diatoms from any other microalgae, surpassing modern engineering capabilities with their minute, complex morphology, which allows them to play an important role in the field of nanotechnology (Townley *et al*., 2008; Bozarth *et al*., 2009). Diatom-derived silica, along with non-silica based structures, can be incorporated into micro/nanodevices and can have several applications in biochemical analysis, computing, microsensors, micro-robotics and micro-fuel cells (Hildebrand, 2005; Gordon *et al*., 2009). Furthermore, given that their frustules are covered with nano-sized gaps, they are commonly used as filters applied in the filtration of liquids, DNA purification, and absorption of heavy metals (Gilmore *et al*., 1993; Al-Degs *et al*., 2001). They can also be used in potential applications regarding antibody arrays and techniques like immunoprecipitation (Townley *et al*., 2008).

1.4.1.2. Lipids

Lipids are the primary carbon storage mechanism of diatoms, typically containing high lipid contents, with a significative portion of *n*-3 PUFA, which have led researchers to propose diatoms as attractive sources for the development of supplements and feeds (Yi *et al*., 2017; Bhattacharjya *et al*., 2020).

i. *Polyunsaturated fatty acids*

Throughout the past two decades the relevance of PUFAs has been recognised by health organisations worldwide (Sahena *et al*., 2009). These crucial fatty acids are known to affect a variety of health-related conditions such as cardiovascular disease, immunological and inflammatory responses and psychiatric illnesses (Glaser *et al*., 2011; Bellino *et al*., 2021; Farley *et al*., 2021; Fu *et al*., 2021). All vertebrates synthesize *n-3* and *n*-6 PUFA from the essential fatty acids linoleic (LA) and α-linolenic (ALA) acids that act as precursors. However, the biosynthesis in vertebrates of *n*-3 PUFAs EPA and DHA is insufficient (Pereira *et al*., 2012; Sartaj & Prasad, 2020). Although there is not a precise quantitative estimate for the recommendation of *n*-6 intake (e.g., LA), due to the scarcity of data available, the already proven detrimental effects of *n*-3 deficiency (e.g., EPA and DHA) have facilitated the establishment of precise guidelines for *n*-3 intake recommendations by organisations like the Food and Agriculture Organisation, FAO (FAO, 2010; Hsu *et al*., 2018; Tobin *et al*., 2018). Thus, the consumption of *n*-3 PUFAs EPA and DHA through dietary methods and/or supplementation is already a usual practice for many people, besides being recommended by FAO (FAO, 2010; Mozaffarian & Wu, 2012; Dyall, 2015; Hamed *et al*., 2015). Diatoms are a known source of EPA and DHA, which display several health-related benefits, not only in humans but also in animals (Li *et al*., 2014; Remize *et al*., 2021).

In the aquaculture sector, due to their rich lipidic content in *n*-3 PUFAs, diatoms have been shown to be crucial for shrimp, bivalve larvae and post-larvae hatcheries as well as a food source for zooplankton (Benemann, 1992; Borowitzka, 1997; Lebeau & Robert 2003b; Merz & Main, 2015). Additionally, their growth-promoting, immunostimulant, and antibacterial activities are reported to decrease mortality rates and improve the quality of the farmed aquatic organisms. In a study evaluating seabream diets, Ribeiro *et al.*, (2017) concluded that the addition of the diatom *Phaeodactylum tricornutum* to the feeds reduced the whole-body fat and improved the coloration of the fish. In another study, Gordon *et al*., (2006) reported higher survival growth rates of post-larval abalone when a mixture of diatoms composed of *Amphora luciae* and *Navicula cf. lenzii* was used as feed, instead of single-species diets.

Currently, the market-ready source of these PUFA is mainly fish oils, but considering the negative economic and ecologic impact of these products, microalgae-based PUFAs come across as an exciting and possible alternative to fish oils (Zhang *et al*., 2019; Remize *et al*., 2021). Nowadays, regarding human consumption, many products containing EPA,

DHA or the metabolic precursor ALA are already in the market, with a few of them deriving from diatoms, as is the case of *Odontella aurita* from Inovalg (France). Other products contain biomass of highly marketed species such as *Chlorella*, which can be used in food and food supplements, as commercialised by Allmicroalgae (Portugal) under the brand name Allma (Gouveia *et al*., 2009; Raja *et al*., 2018). Nonetheless, the potential use of diatoms as a source of *n*-3 PUFAs is substantial (Li *et al*., 2014).

1.4.1.3. Pigments

Diatoms contain two types of pigments: chlorophylls and carotenoids. Within the chlorophylls, diatoms have chlorophyll *a* with a central role in the conversion of energy during photosynthesis, and chlorophyll *c*, which functions as an accessory pigment, broadening the range of light absorption (Kuczynska *et al*., 2015). Carotenoids can be accessory light-harvesting pigments as well as crucial photoprotective components of the photosynthetic machinery. These pigments can be further divided into carotenes, such as *β*-carotene, and xanthophylls (oxygenated derivatives of carotenes), such as fucoxanthin, diadinoxanthin, diatoxanthin, zeaxanthin, violaxanthin and antheraxanthin (Kuczynska *et al*., 2015; Pagels *et al*., 2020).

These components display valuable health-related features with antioxidant, anticancer, anti-inflammatory and neuroprotective activities, as well as natural colorant properties, which enables diatoms to be applied to the cosmetic, pharmaceutical, and food industries (Elias *et al*., 2008; Pangestuti & Kim, 2011; Ambati *et al.*, 2019). Recently, fucoxanthin has gained more attention due to its medical-related properties like antioxidant, anti-inflammatory, anticancer, anti-obesity activities, among others (Abidov *et al.*, 2010; Zarekarizi *et al*., 2019). This pigment binds to a chlorophyll *a* and *c* protein complex, playing a role in light-harvesting (Owens, 1986; Gelzinis *et al*., 2015). Examples of diatoms with high fucoxanthin content are *Phaeodactylum tricornutum* (15.71–16.28 mg g^{-1} dry-weight (DW)) and *Odontella aurita* (14–15 mg g^{-1} DW), which have the potential to outperform current fucoxanthin sources such as macroalgae (Xia *et al*., 2013; Bhattacharjya *et al*., 2020).

1.4.1.4. Biosorption and bioremediation

The biosorption and bioremediation properties of diatoms have been reported throughout the years (Schmitt *et al*., 2001; Lebeau & Robert, 2003b; Sbihi *et al*., 2014). Recently, this capacity has been examined in wastewater treatment, where the authors Marella *et al.* (2019) successfully tested the treatment of urban wastewater in India by

using a consortium of microalgae mainly composed of diatoms (>90%). The fact that diatoms can tolerate extreme conditions of diverse temperatures and high nutrient concentrations while maintaining their competitiveness and high growth rates explains their success in these treatment systems (Marella *et al*., 2020). Moreover, in addition to diatom cultures having the potential to be applied to the treatment of several wastewaters, their biomass can later be exploited for high-value products (Olguín, 2012).

1.4.2. Mass-scale Production of Diatoms: Nutritional requirements

Adding to their potential in several biotechnological applications, the industrial cultivation of diatoms has gained momentum due to their high duplication rates, plasticity in carbon allocation within cellular components, resilience to environmental changes, and high sedimentation rates, which allows for reduced harvesting costs (Vella *et al*., 2019).

The main requirements for the mass-scale production of diatoms are H_2O, CO_2, energy and nutrients. Considering the vital role of nutrients in the cell metabolism and biosynthesis of biological macromolecules (proteins, chlorophyll, nucleic acids), their adequate provision through a controlled culture medium is vital for successful mass production (Hao *et al*., 2020). These nutrients can be classified as either macro- or micronutrients, depending if their requirement in the culture medium for microalgae is in large (g L^{-1}) or trace (mg L^{-1}) amounts, respectively (Procházková *et al*., 2014). Ultimately, nutrients will be converted into biomass (15-35% of the cellular DW) by the microalga, through which carbon, oxygen and hydrogen are fixed by photosynthesis (Pandey *et al*., 2019). At an industrial level, the culture medium chosen needs to be optimised for each species to maximize culture growth rates, thus decreasing the associated production costs (Malek *et al*., 2016; Novoveská *et al.*, 2016). Furthermore, understanding what drives higher productivity and quality of the final product is imperative to optimize said production for specific applications of each microalga. Hence, as agricultural crops require the addition of fertilizers to the soil, one of the most important factors that can be used to enhance biomass or the accumulation of a given compound in microalgae is the addition and manipulation of the culture medium, preferably with a species-specific composition (Dinis *et al*., 2009; Pandey *et al.*, 2019). Some culture media frequently reported for diatoms cultivation are Erd-Schreiber's, Provasoli, modified Walne's, and Guillard's F2 media (Table 1.1, Lebeau and Robert, 2003a; Bindhu KB, 2013; Endar *et al*., 2013).

At the laboratory scale, the culture medium is mainly composed of 3 main solutions (macronutrients, micronutrients, and vitamins), which are subjected to sterilisation methods such as autoclaving. However, considering industrial production, it is highly important that the culture media is formulated as a unique optimised solution to maximise biomass production, where the presence of vitamins is rare due to associated high costs and the sterilisation process is executed through filtration (Andersen, 2005; Dinis *et al*., 2009). Usually, macronutrients in the culture medium are composed of primary nutrients present in higher concentrations, namely, nitrates and phosphates. The micronutrients comprise all trace elements (e.g., zinc, copper, iron, magnesium, potassium) (Dinis *et al*., 2009; Pandey *et al*., 2019). Therefore, the cultivation of diatoms can be performed with artificial culture media, where the exact composition of each element is known, or with enriched culture media, where nutrients are added to seawater if marine species are used (Andersen, 2005).

Table 1.1: Compilation of different culture media used for the growth of diatoms. *Additions to the culture media are not exhibited. DAM: diatom artificial medium, DM: diatom medium, ESAW: enriched seawater, artificial seawater, LDM: locally developed medium, NEPCC: northeast culture collection protocol.

Diatom species	Culture medium*	Source
Amphora hyaline	DAM	(Gagneux-Moreaux *et al*., 2007)
Bacillaria paradoxa	DAM	(Gagneux-Moreaux *et al*., 2007)
Biddulphia mobiliensis	Foyn's "Erd-Schreiber's"	(Gross *et al*., 1937)
Biddulphia sinensis	Erd-Schreiber's	(Volkman *et al*., 1980)
Chaetoceros calcitrans	f/2	(Krichnavaruk *et al*., 2005)
	Walne's	(Réveillon *et al*., 2016)
Chaetoceros didymus	Foyn's "Erd-Schreiber's"	(Gross *et al*., 1937)
Chaetoceros gracilis	f/2	(Mortensen *et al*., 1988)
	Walne's	(Mortensen *et al*., 1988)
	Daigo's IMK	(Kajikawa *et al*., 2016)
Chaetoceros pseudocritinus	Foyn's "Erd-Schreiber's"	(Gross *et al*., 1937)
Chaetoceros simplex	Erd-Schreiber's	(Chuecas & Riley, 1969)
***Chaetoceros* sp.**	DAM	(Gagneux-Moreaux *et al*., 2007)
***Cocconeis* sp.**	Walne's (modified)	(Uriarte *et al*., 2006)
Coscinodiscus excentricus	Foyn's "Erd-Schreiber's"	(Gross *et al*., 1937)
Coscinodiscus granii	Foyn's "Erd-Schreiber's"	(Gross *et al*., 1937)
	DAM	(Gagneux-Moreaux *et al*., 2007)
	L1	(Su *et al*., 2017)
Coscinodiscus radiatus	Foyn's "Erd-Schreiber's"	(Gross *et al*., 1937)
***Coscinodiscus* sp.**	Erd-Schreiber's	(Chuecas & Riley, 1969)

Coscinodiscus sub-bulliens	Foyn's "Erd-Schreiber's"	(Gross *et al*., 1937)
Cyclotella cryptica	f/2	(Botte *et al*., 2018)
Cylindrotheca closterium	Walne's (modified)	(Uriarte *et al*., 2006)
Ditylum brightwellii	Foyn's "Erd-Schreiber's"	(Gross *et al*., 1937)
***Entomoneis* sp.**	f/2	(He *et al*., 2016)
Haslea crucigera	DAM	(Gagneux-Moreaux *et al*., 2007)
Haslea ostrearia	Walne's (modified)	(Arsad *et al*., 2019)
	DAM	(Gagneux-Moreaux *et al*., 2007)
	Provasoli	(Nghiem Xuan *et al*., 2020)
	Provasoli	(Arsad *et al*., 2019)
Melosira borreri	Foyn's "Erd-Schreiber's"	(Gross *et al*., 1937)
Navicula ramosissima	DAM	(Gagneux-Moreaux *et al*., 2007)
***Navicula* sp.**	f/2	(J. Kim *et al*., 2019)
	Walne's	(Nurachman *et al*., 2012)
Nitzschia closterium	Erd-Schreiber's	(Chuecas & Riley, 1969)
	Walne's	(Gopinathan, 1986)
	Miquel's	(Gopinathan, 1986)
	Miquel's (modified)	(Dutton & Manning, 1941)
Nitzschia compressa	DAM	(Gagneux-Moreaux *et al*., 2007)
Nitzschia laevis	LDM	(X. Lu *et al*., 2018)
Nitzschia ovalis	Walne's (modified)	(Uriarte *et al*., 2006)
***Nitzschia* sp.**	f/2	(Chagoya *et al*., 2014)
	f/2	(Xing *et al*., 2018)
Odontella aurita	DAM	(Gagneux-Moreaux *et al*., 2007)
Phaeodactylum tricornutum	f/2	(Pudney *et al*., 2019)
	f/2	(Serif *et al*., 2018)
	Erd-Schreiber's	(Chuecas & Riley, 1969)
	Walne's	(Réveillon *et al*., 2016)
	Walne's	(Haro *et al*., 2017)
	Walne's (modified)	(Huang *et al*., 2019)
	DAM	(Gagneux-Moreaux *et al*., 2007)
	ESAW	(Fabris *et al*., 2020)
	ESAW	(Hamilton *et al*., 2016)
Pleurosigma intermedium	DAM	(Gagneux-Moreaux *et al*., 2007)
Porosira glacialis	f/2 (50 ×)	(Svenning *et al*., 2019)
Rhiziosolenia alata	Foyn's "Erd-Schreiber's"	(Gross *et al*., 1937)
Skeletonema costatum	f/2	(X. Chen & Gao, 2004)
	f/2	(Uddin & Zafar, 2007)
	Erd-Schreiber's	(Chuecas & Riley, 1969)

	Foyn's “Erd-Schreiber’s”	(Gross *et al*., 1937)
	DAM	(Gagneux-Moreaux *et al*., 2007)
***Skeletonema* sp.**	f/2	(Endar *et al*., 2013)
	Walne's (modified)	(Endar *et al*., 2013)
Staurosirella pinnata	DM	(Savio *et al*., 2020)
	DM	(De Angelis *et al*., 2016)
Streptotheca thamensis	Foyn's “Erd-Schreiber’s”	(Gross *et al*., 1937)
***Thalassionema* sp.**	DAM	(Gagneux-Moreaux *et al*., 2007)
Thalassiosira grauida	Miquel’s (modified)	(E. J. Allen, 1914)
Thalassiosira pseudonana	Walne’s	(Réveillon *et al*., 2016)
	NEPCC	(Kirkham *et al*., 2017)
***Thalassiosira* sp.**	Foyn's “Erd-Schreiber’s”	(Gross *et al*., 1937)
	Walne’s	(Nurachman *et al*., 2012)
Thalassiosira weissflogii	f/2	(Botte *et al*., 2018)

1.4.2.1. Silica

Silica is required for DNA replication and is a major component in diatom cell wall (Huysman *et al*., 2014). In contrast with other microalgae groups, silica is a crucial macronutrient for diatom growth and is one of the critical factors for the successful competition of diatoms over other phytoplankton communities in nature (Kiran *et al*., 2015), and a crucial nutrient for the successful mass cultivation of diatoms in commercial production units. Specifically, silicified cell walls provide diatoms protection against grazers and environmental elements, while its porosity facilitates the uptake of nutrients (Hamm *et al*., 2003; Losic *et al*., 2006). In addition, a study by Milligan & Morel (2002) suggested that their frustules act as a pH buffer that consequently allows for the quick conversion of bicarbonate into carbon dioxide, which can be quickly metabolized, thus resulting in higher growth. Furthermore, for plants with silicified cell walls, it has been demonstrated that the energy required for their biosynthesis is lower than that for organic cell walls that, if applied to diatoms, could also explain their success over other microalgae in natural populations (Raven, 1983).

1.4.2.2. Nitrates

The relevance of nitrates can be attributed to the fact that they are a major constituents of nucleic acids and are indispensable for the biosynthesis of chlorophyll and proteins, being linked to cellular biomass productivity and photosynthesis (Z. K. Yang *et al*., 2013; Hao *et al*., 2020). Nitrogen is a highly studied macronutrient, given its influence in the

productivity of algae, where its availability is known to be one of the key factors that might limit phytoplankton bloom formation in the marine environment (Rosenwasser *et al.*, 2014). For these reasons, nitrate deficit has been extensively evaluated in diatom cultures, where increased saturated lipid productivities are generally observed, though with associated low photosynthetic efficiency, low growth rates and low PUFA concentrations (Liefer *et al.*, 2018; Cointet *et al.*, 2019).

1.4.2.3. Phosphorus

Phosphorus is a non-renewable (extracted from phosphate rock) but crucial macronutrient for the cultivation of diatoms, being essential in photosynthetic processes and highly relevant for the synthesis of biological components such as phospholipids, nucleic acids, ATP and for the development of other metabolites (Chu *et al.*, 2013; Singh *et al.*, 2018). The availability of phosphorus is another factor known to affect microalgae growth and is recognized as a major driver of marine ecosystems (Dyhrman *et al.*, 2012; Singh *et al.*, 2018). Additionally, diatoms are recognized as important exporters of phosphorus (Dyhrman *et al.*, 2012). As a result, phosphorus deficit is reported to significantly alter key metabolic pathways in diatoms, such as shifting the carbon flow towards the production of triacylglycerols (Z. K. Yang *et al.*, 2014; Brembu *et al.*, 2017).

1.4.2.4. Iron

Iron is an important micronutrient associated with many biological processes related to enzymatic reactions, nitrogen metabolism, and chlorophyll synthesis (Raven, 1988; Sajjadi *et al.*, 2018). Furthermore, due to its low availability in the marine environment, iron is considered a limiting nutrient for primary productivity, especially when there is a high concentration of nutrients as nitrates and silica (Moore & Abbott, 2000; Moore *et al.*, 2001). Consequently, iron-limiting conditions have been shown to result in the downregulation of biological processes such as photosynthesis, mitochondrial electron transport and nitrate assimilation (Allen *et al.*, 2008).

1.4.2.5. Micronutrients

Micronutrients have crucial roles in microalgae metabolism. However, how microalgae interact with micronutrients that are indeed bioavailable in different combinations is not yet fully understood. It is nonetheless reported that the interactions of these micronutrients affect their functional roles in microalgal cells (Quigg, 2016). Some of the essential micronutrients required for microalgal growth and present in several

culture media are zinc, magnesium, molybdenum, manganese, copper, and cobalt. Regarding some of their main functional roles: zinc is an essential enzyme co-factor and may be involved in silica uptake (Quigg, 2016; Baeyens *et al*., 2018); magnesium is a central constituent of chlorophyll and has a role in the ribosome structure (Encarnação *et al*., 2012; Quigg, 2016); manganese is associated with the removal of toxic superoxide radicals and is essential in O_2 evolution by the photosynthetic process (Fox & Zimba, 2018; Polat *et al*., 2020); molybdenum is associated with nitrogen fixation and redox reactions (Quigg, 2016; Fox & Zimba, 2018); copper functions as a cofactor in enzymes that regulate redox reactions associated with photosynthesis (Kagalou *et al*., 2002; Quigg, 2016); cobalt is required in vitamins such as vitamin B_{12} (Quigg, 2016; Fox & Zimba, 2018). Consequently, nutrients such as zinc, copper and cobalt have been reported to limit diatom culture growth when present at low concentrations (Anderson *et al*., 1987; Baeyens *et al*., 2018).

The correct supply of a nutritive culture medium can thus affect not only the biochemical composition of diatoms, but also their photosynthetic efficiency. Considering the relevance of the mentioned nutrients, their optimization in diatom culture media could help the industry surpass the difficulties associated with the commercial production of different diatom species, such as *Skeletonema costatum* and *Chaetoceros calcitrans*, two highly relevant diatom species whose current production is mainly associated with the aquaculture and cosmetics markets, and whose additional detailed description is further provided in the following chapter.

1.5. Objectives

The main objective of this thesis is to significantly optimize the culture medium of *Skeletonema costatum* and *Chaetoceros calcitrans*, to improve the current biomass productivities of both strains from laboratory to industrial scale. The achievement of this objective will provide the baseline tools for Necton S.A. to improve several aquafeed products that are already in the market. In order to reach this main objective, several secondary goals will be attained, namely:

1) identify at laboratory scale the optimal concentrations of most important nutrients in the standard culture medium used by Necton S.A., Nutribloom® Plus, for the two diatom species mentioned above, using a stepwise optimization process;

2) validate the optimized culture medium obtained for the diatom *Skeletonema costatum*, in outdoor pilot-scale culture systems; and

3) assess the effect of the different nutrient concentrations and optimized culture media in the biochemical profile of both diatom strains.

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2. Chapter 2: Production Optimization and Nutritional Value of Two Diatoms, *Skeletonema costatum* and *Chaetoceros calcitrans*, Grown in Bubble Column Photobioreactors

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2.1. Abstract

Skeletonema costatum and *Chaetoceros calcitrans* are two cosmopolitan high-value centric diatoms, with a rich nutritional profile. These diatoms are presently commercialized mainly as products for aquaculture, such as feed for farming molluscs and crustaceans. The purpose of the present work was to optimise the culture medium of *S. costatum* and *C. calcitrans*. The optimisation of this medium was performed in a stepwise process, where the supply of different nutrients was optimised in the following order: silicate, nitrate, phosphate, iron, and micronutrients. Furthermore, this optimisation of the culture medium was validated for diatom *S. costatum* at pilot-scale in exterior flat panels. The biochemical profile of all cultures was characterized regarding proximal composition of protein, lipid, ash, and carbohydrates contents as well as fatty acid profile. The results obtained revealed an increase in biomass productivity for both diatoms when the supply of nutrients was optimised, with a 1.8 and 3.2-fold increase in biomass produced by *S. costatum* and *C. calcitrans*, respectively. For *S. costatum* and *C. calcitrans,* the optimal silicate and iron concentrations were 2.4 mM and 1.2 mM and 20 and 80 µM, respectively. Optimal nitrate, phosphate and micronutrients were, respectively, 4 mM, 100 µM and 0.5 mL L^{-1} for both diatoms. The validation of the optimised culture medium in *S. costatum* cultures grown outdoors demonstrated smaller differences and lower biomass productivity between treatments, which was associated with the absence of constant illumination under outdoors conditions. The biochemical profile showed an increase in high-value PUFAs such as EPA in both diatoms, whilst an increase in DHA was only detected in *S. costatum* cultures, all of them regarding

laboratory and outdoor conditions. The present study supports the indispensable application of the described optimisation process in the industrial production of diatoms *S. costatum* and *C. calcitrans* to enhance productivity as well as biomass quality, two factors which are highly relevant to aquaculture and nutraceutical applications.

[1] This chapter will be submitted for publication to Algae journal

2.2. Introduction

The optimization of culture conditions is critical to improve the current biomass productivities and nutritional value of microalgal cultures, since they are known to significantly affect growth and biochemical composition (Costa *et al*., 2018; Ye *et al*., 2018). One method often employed to induce the abovementioned variations in microalgae is the adjustment of nutrient supply (Goiris *et al*., 2015; Ran *et al*., 2019). For most microalgal species, the proper supply of nitrates, phosphates, and iron are key factors for promoting optimal growth that can readily change. However, in the particular case of diatoms, silica supplementation is of the utmost importance to ensure optimal growth (Procházková *et al.*, 2014; Kiran *et al.*, 2015). The ability of diatoms to endure highly polluted environments, aside from the discovery of high-value metabolites in their biomass, has emphasized their biotechnological potential (Lebeau & Robert, 2003; Mishra *et al*., 2017; Bhattacharjya *et al*., 2020). The optimization of production protocols for high-value diatom species, like *Skeletonema costatum* (Fig. 2.1A) and *Chaetoceros calcitrans* (Fig. 2.1B), is essential, given their outstanding potential for different biotechnological applications, for example, for the aquaculture sector.

S. costatum is a diatom that dominates coastal waters and has the capacity to withstand variable temperatures and salinity levels (Gao *et al*., 2019). In addition, it has also been reported to tolerate elevated nutrient levels such as nitrates, phosphates, and iron, ultimately causing severe harmful algal blooms (Li *et al*., 2009; Gao *et al*., 2018; Ogura *et al*., 2018). This strain forms straight chains linked by long, marginal, siliceous, tubular processes that can exceed the cell length (Cupp, 1962). Besides having a central nucleus, two chloroplasts can be observed, depending on the cell's position (girdle view) (Kumar & Prabu, 2014). Because of its nutritional profile, *S. costatum* is presently commercialized as an aquaculture feedstock, more specifically as live feed in farming larviculture units for different molluscs and crustacean species (Lestari *et al*., 2014; FAO, 2016). *S. costatum* extracts have also demonstrated antibacterial activity against several marine pathogenic bacteria (Naviner *et al*., 1999; Lauritano *et al*., 2018). In addition to being used for feeds, this diatom species is also very interesting for the exploitation of cosmetic products (Couteau & Coiffard, 2018).

C. calcitrans is a diatom whose cells have been described as having four long setae on their corners whose poles join cells together and form chains (Şirin *et al*., 2015). This strain is also widely used as feed for larval, early juvenile, broodstock stages of

bivalve molluscs, and as direct feed for crustaceans during early larval stages due to its balanced contents of polyunsaturated fatty acids (PUFAs) and vitamins (Brown, 2002; Khoi, 2006). In addition to aquaculture, this diatom has proven its applicability in other fields, such as the development of novel drugs, given its reported fucoxanthin-rich fraction with effective anticancer properties (Foo *et al*., 2019).

According to Guedes and Malcata (2012), the cultivation of these two diatoms should occur in closed photobioreactor (PBR) systems. However, similar reports are scarce, as most of the published literature proposes their cultivation in open systems, like large tanks or open ponds. Nonetheless, more research regarding the optimal nutritional requirement for growth and mass production has emerged (Krichnavaruk *et al.*, 2005; Kaspar *et al.*, 2014; Pérez *et al.*, 2017; Azmi *et al*., 2020). Following the rising interest in obtaining diatom-based bioproducts alongside the ever-increasing potential of the marine biotechnology market, the future of the industrialization of these cultures seems bright. The purpose of the present study was to optimise biomass production and assess the biochemical composition of two high-value diatoms: *S. costatum* and *C. calcitrans*. In order to determine the parameters for optimal growth performance, different nutrient concentrations were assessed. This process was first performed under controlled conditions in a laboratory, after which the trial for *S. costatum* was scaled up to outdoor pilot-scale flat panel photobioreactors under uncontrolled environmental conditions.

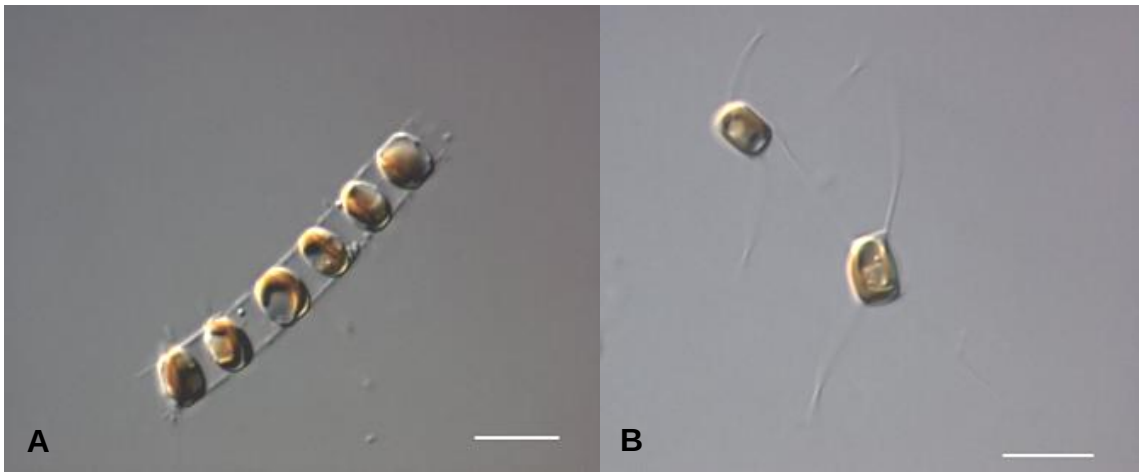

Figure 2.1: Microscopic observations of diatoms *Skeletonema costatum* (A) and *Chaetoceros calcitrans* (B) using differential interference contrast (DIC) and a 100× lens with additional 1.6× amplification via Optovar module. Scale bar = 20 µm. Source: Necton S.A.

2.3. Material and methods

2.3.1. Cultures of diatoms

Inocula of *S. costatum* and *C. calcitrans* were obtained from Necton´s culture collection. Both strains were continuously grown in 6-L round balloons, bubbled with a mixture of air and CO_2 (filtered through 0.22 µm PTFE filters), under controlled temperature (17 ± 2 ºC) and a 16:8 light:dark photoperiod. All inocula cultures were supplemented with Nutribloom® Plus (NB^+) culture medium at a final nitrate concentration of 4 mM. In addition, a concentrated silicate solution was also supplied to a final concentration of 0.4 mM.

2.3.2. Culture medium optimization

The culture medium optimization trials were conducted in 1-L bubble columns PBRs. Through a stepwise procedure, several parameters were tested in triplicates, in the following order: 1) silicate concentration: 0.4, 0.8, 1.2 and 2.4 mM; 2) nitrate concentration: 1, 2, 4 and 8 mM; 3) phosphate concentration: 50, 100, 200 and 400 µM; 4) iron concentration: 10, 20, 40 and 80 µmol and 5) micronutrients concentration: 0.5, 1-, 2- and 4-mL L^{-1}. As control conditions, NB^+ was supplied at a final nitrate, phosphate, and iron concentrations of 4 mM, 200 and 40 µM, respectively. Micronutrients and vitamins were provided upon a 1:500 dilution of the respective stock solutions. Silicate was provided at a final concentration of 0.4 mM.

Every two days, silicate, nitrate, and phosphate were measured and added to the medium to reach the tested concentrations. Iron and micronutrients were supplied by assuming that their levels were negligible before their addition to the cultures. The bubble column system was inoculated with an initial dry weight (DW) of 0.18 ± 0.01 g L^{-1} for *S. costatum* and 0.12 ± 0.02 g L^{-1} for *C. calcitrans*. Each trial had a duration of 7 days.

2.3.3. Experimental setup: laboratory-scale

Twelve 1-L bubble columns PBRs, made of transparent borosilicate glass (Normax LDA), were used to test the abovementioned conditions. These bubble column PBRs offer settings such as: light supplied by light-emitting diodes (LEDs; PrimeLux® lamps) at a constant photosynthetic flux density of 125 µmol photons m^{-2} s^{-1}, measured using a quantum sensor (Model US-MQS-B, Walz, Effeltrich, Germany); air flux at 800 mL min^{-1} filtered through 0.22 µm Whatman PTFE filters and controlled temperature at

19 ºC ± 1 ºC. CO_2 was supplied when cultures reached a pH higher than 8.5. All glass materials were rinsed with HCl (10%) and autoclaved (121 ºC, 20min) to decrease possible contaminations, and all inoculations were performed in a laminar flow chamber.

2.3.4. Experimental setup: pilot-scale

After the lab-scale optimization, a validation trial using *S. costatum* was conducted in outdoor flat panels PBRs. This decision was made as this particular diatom was deemed more valuable to be developed as an industrial strain than *C. calcitrans*. For this purpose, *S. costatum* was grown in six 100-L flat panels PBRs during a period of 9 days using the control and the final optimised culture media: 4 mM of nitrates, 100 µM of phosphate, 20 µM of iron, 2 mL L^{-1} of micronutrients, 2 mL L^{-1} of vitamins and 2.4 mM of silicate. The flat panels were inoculated with initial DW's of approximately 0.4 g L^{-1}. Filtered air flux was regulated and CO_2 was added when pH was higher than 8.5 using a pulse injection system. For both conditions, the culture media were re-supplied at a final nitrate concentration of 4 mM every two days. Silicate was re-supplied at final concentrations of 0.4 and 2.4 mM, for the control and optimised flat panels, respectively.

2.3.5. Culture monitoring

2.3.5.1. Growth assessment

Culture growth was evaluated through optical density (OD) measurements and DW determination. OD was measured using a UV-mini-1240 UV/VIS spectrophotometer (Shimadzu, Kyoto, Japan) at a wavelength of 450 nm. DW of the cultures was determined by filtration of a 10-mL sample through a pre-weighed 1.5-µm Whatman fiberglass filters that were then washed with 10 mL of ammonium formate (0.5 M) and dried at 60ºC, in an oven.

2.3.5.2. Nutrient concentration analysis

The consumption of nutrients in the cultures was evaluated by the determination of nitrate, silicate, and phosphate concentrations, calculated by calibration curves determined previously. Before each analysis, 20 mL of sample were centrifuged at 2700 *g* for 10 minutes.

Nitrate concentration was measured using a UV spectrophotometric method developed by Armstrong (1963) with modifications. Briefly, 0.5 mL of supernatant was added to a solution of 0.2 mL of HCl (83 mL L^{-1}) and 9.3 mL of a NaCl solution (35.5 g L^{-1}). The

solutions were measured in a spectrophotometer at 220 nm and 275 nm and the nitrate concentration was calculated using equation 1.

$$C\ (mM) = [(Abs220 - 2 \times Abs275) \times 0.3147] - 0.0052 \qquad (1)$$

The concentration of silicate was measured through a spectrophotometric method adapted from Smith & Milne (1981). Briefly, a mixed reagent made of sulphuric acid and ammonium heptamolybdate tetrahydrate, and solutions of oxalic acid (100 g L^{-1}) and ascorbic acid (17.5 g L^{-1}) were added to 3 mL of supernatant. The samples were measured in a spectrophotometer at a wavelength of 810 nm and the silicate concentration was calculated using equation 2.

$$C(mM) = \frac{(Abs - 0.0327) \div 0.0199}{1000} \qquad (2)$$

The concentration of phosphate was determined using a phosphate test Kit Spectroquant®, adapted to a spectrophotometric method. The phosphate determination was performed according to the manufacturer's procedure. The samples were then measured at a wavelength of 880 nm and the phosphate concentration was calculated using equation 3.

$$C\ (\mu M) = \frac{[(Abs - 0.0076) \div 0.24] \times 100}{9.5} \qquad (3)$$

2.3.6. Biochemical analysis

For biochemical analysis, samples were collected at the end of each trial and centrifuged for 10 min at 2700 *g*. The biomass was then subjected to lyophilization (LyoQuest Telstar, Terrassa, Spain) and stored at -20 ºC until further analysis.

2.3.6.1.Proximal composition

The protein content was evaluated by elemental analysis of C, H and N through Vario EL III (Elementar Analysensysteme GmbH, Germany), according to the manufacturer's procedure. Total protein content was obtained by the multiplication of the percentage of N by the conversion factor of 4.78 and 4.63 for *C. calcitrans* and *S. costatum*, respectively (Lourenço *et al*., 2004).

Total lipids were determined according to Bligh & Dyer (1959) method, modified by Pereira *et al.*, (2011). Here, the lipid content was obtained through an extraction method applying a mixture of chloroform, methanol, and water (2:2:1), and using an

Ultra-Turrax (IKA) disperser (2 min at 25000 rpm). Phase separation was obtained after centrifugation (10 min at 685 *g*), and the organic phase (chloroform fraction) was transferred to new glass tubes with a Pasteur pipette. Afterward, a known volume of this phase was transferred into previously weighed vessels and evaporated overnight in a dry bath of 60 ºC. The lipid content was determined gravimetrically.

Ash content was determined through gravimetry by burning 50 mg of the samples at 550 ºC for 8 h, in a furnace.

Carbohydrates were determined by the difference of the sum of total proteins, lipids, and ash content from the total percentage of biomass.

2.3.6.2. Fatty acids

The fatty acid profile was determined by using a modification of the Lepage & Roy (1984) protocol, described by Pereira *et al*., (2012). Briefly, 1.5 mL of an acetyl chloride/methanol (20:1, v/v) solution was added to 20 mg of freeze-dried biomass. Afterwards, an IKA Ultra-Turrax disperser was used to disrupt and homogenize the microalgal cells during two periods of 60- and 30-s. This was followed by the addition of 1 mL of hexane, where the direct extraction of the lipidic phase occurred. The derivatization step was achieved by heating the samples for 1 h in a 70 ºC bath. Afterwards, 1 mL of distilled water and 4 mL of hexane were added, followed by 60 s of vortex and 5 min of centrifugation (438 *g*). The hexane fraction was extracted, filtered, and evaporated (nitrogen gas flow). The samples were resuspended in 500 µL of HPLC-grade hexane for GC-MS analysis.

The analysis of the fatty acids was performed in a Bruker GC-MS (Bruker SCION 456-GC, SCION TQ MS) equipped with a ZB-5MS capillary column (30 × 0.25 mm of internal diameter with 0.25 µm film thickness; Phenomenex). The carrier gas was helium at 1 mL min^{-1} and the program of the temperature was set for 60 ºC (1min), then 30 ºC per min until 120 ºC, 4ºC per min until 250 ºC and 20 ºC per min until 300 ºC, (4 min hold), with 300 ºC of injection temperature in splitless mode. The identification of the fatty acids was done by using as standard a mix Supelco®37 component FAME Mix (Sigma-Aldrich, Sintra, Portugal) with different calibration curves. The results were expressed as percentages of total fatty acid content.

2.3.7. Statistical analysis

Statistical analyses were performed using IBM SPSS, version 26 (V Armonk, NY: IBM Corp.) using one-way ANOVA followed by Tukey tests ($p < 0.05$).

2.4. Results and Discussion

2.4.1. Culture optimization

2.4.1.1. *Skeletonema costatum*

The first trial with *S. costatum* established the relevance of a significant increase in the silicate concentration for cultures with a continuous light source under laboratory conditions. Control conditions (Standard NB^+ supplemented with 0.4 mM silicates) resulted in cultures with the lowest biomass recorded (2.00 ± 0.03 g L^{-1} DW). Higher growth performances were obtained with higher silicate concentrations, specifically 2.4 and 1.2 mM (Fig. 2.2A). A maximum biomass concentration of 3.51 ± 0.17 g L^{-1} DW was obtained with the highest silicate levels (2.4 mM) supplied to the culture, which grew significantly higher than at 1.2 mM ($p < 0.05$). No record was found in the literature regarding a similar silicate supply for diatoms. In fact, 0.11 mM is the standard concentration applied to culture media, such as F/2 culture medium (Gao *et al*., 2019; Abate *et al*., 2020; Gleich *et al*., 2020), and the maximum biomass concentration reported in *S. costatum* cultures, 1.52 g L^{-1} DW (Ebrahimi & Salarzadeh, 2016). Moreover, under control conditions, cell elongation and cell death were detected by microscopic observation. Indeed, particularly during the last days of the trial, culture deterioration was visible by microscopic observation, most probably due to lack of silicates, even though apparent growth was still recorded. As highlighted by Huysman *et al*., (2014), diatom abundance and success follow silicate availability. These results stress the relevance of silicate in the culture performance and are in agreement with the findings reported by Davis (1976), where *S. costatum* cultures grown under continuous illumination resulted in a growth halt due to silicate limitation. Additionally, the described morphological changes in *S. costatum* cells have been observed previously in silicate-deficient cultures (Harrison *et al*., 1977; Yamamoto & Tsuchiya, 1995). Both Vaulot *et al.*, (1987) and Brzezinski *et al*., (1998) demonstrated that the limitation of silicate induced cell cycle arrest at the G1/S boundary and the transition of G2/M in diatom cultures. These findings could explain the mentioned observations of longer and unhealthy-looking cells. Furthermore, significant depletion of silicate was detected in all treatments, which could

be explained by the fact that diatoms contain specialized silicate deposition vesicles (SDV), where the absorbed silicate is transported to and stored for later use (Heintze *et al.*, 2020).

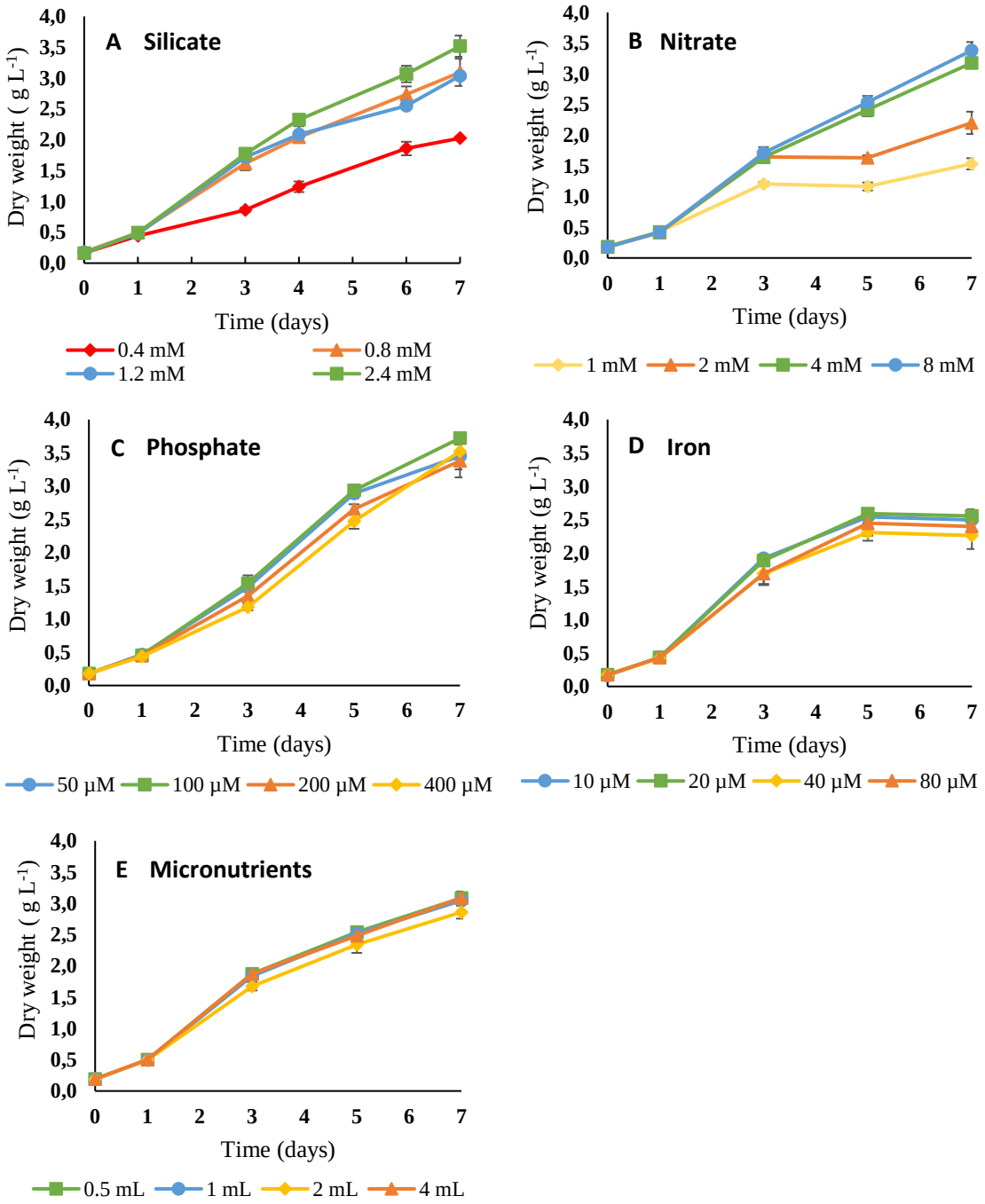

Figure 2.2: *S. costatum* growth performance in terms of biomass dry weight (g L^{-1}) obtained in 1-L bubble column PBRs in order to optimize the supply of a specific nutrient (A, silicate; B, nitrate; C, phosphate; D, iron) or micronutrient (E) concentrations (n=3). Values are represented as mean ± standard deviation.

The nitrate concentration optimization trial followed a similar trend to that obtained for silica optimization, where the cultures supplied with the lowest concentrations (1 and 2 mM) reached the stationary phase on the 3rd day of the experiment, with increased cell death from that point onwards (Fig. 2.2B). These results were expected considering that a low nitrate supply does significantly affect and stress microalgal cultures, thus being commonly used to increase the production of specific bioproducts, such as lipids (Yodsuwan *et al*., 2017; Gao *et al.*, 2019). Higher growth rates were obtained for the concentrations of 8 and 4 mM of nitrates. However, the growth obtained with a culture medium containing 8 mM of nitrates was not significantly higher than the one obtained with half that concentration ($p > 0.05$). For this reason, 4 mM was considered the optimal nitrate concentration at which the culture reached a DW of 3.18 ± 0.07 g L^{-1}, the highest value obtained in this experiment. Similar to what was observed for silicate, 0.8 mM of nitrates are usually reported for *S. costatum* cultures (Chen & Gao, 2004; Takabayashi *et al*., 2006; Gao *et al*., 2019), a value that is 5× lower than what was considered optimal in this study. Other studies supply even lower nitrate concentrations, as reported by Gao *et al.*, (2018), who performed a nitrate enrichment trial with a maximum concentration of 0.2 mM.

Regarding phosphate optimization, no significant differences were detected between treatments, despite the low levels of phosphate detected in all cultures of this trial. Nonetheless, the integration of microscopic observations allowed to conclude that 100 µM of phosphate was the optimal concentration for the supply of this nutrient, with a maximum DW of 3.72 ± 0.07 g L^{-1} (Fig. 2.2C). This conclusion was made considering cell fitness and culture health evaluated through microscopic observation of cell morphology. In a study performed in a similar bubble column system with *S. costatum*, Monkonsit *et al*., (2011) obtained similar results, where different concentrations of phosphate, even though lower than the ones applied in the present study, did not result in differences in growth, despite a high depletion rate. In this study, the authors proposed that the low levels of phosphate present in the medium might be explained by storing phosphate as energy in the form of ATP by the cells or by precipitation. More specifically, a mechanism known as "phosphorus luxury uptake" might justify the drastic depletion of phosphate from the culture medium. Phytoplankton are able to uptake phosphate in excess with this mechanism without immediate use by the cell, by storing the nutrient mainly in the form of polyphosphate (long-term storage), in addition to being stored as ATP (short-term storage) (Powell *et al*., 2009; Blank, 2012; Solovchenko *et al*., 2019). The

accumulation of polyphosphate as dense calcium-associated inclusions (e.g., acidocalcisomes) has previously been observed in *S. costatum*, thus supporting this hypothesis (Diaz *et al*., 2008; Sanz-Luque *et al*., 2020). Furthermore, alkaline and oxidising conditions can also result in the formation of precipitates whose surfaces are suitable for phosphate adsorption, mediating further precipitation of PO_4 (Slomp & van Raaphorst, 1993; Gunnars *et al*., 2002). Therefore, another explanation for the low levels of phosphate observed in the bubble column system is that phosphorus precipitated, possibly with cations such as Ca^{2+}, which are naturally present in the seawater supplied to the cultures (Larsdotter *et al*., 2007).

The determination of the optimal iron concentration provided unexpected results since, for all treatments, the exponential phase came to a halt on the 5th day of trial, resulting in a maximum DW of 2.60 ± 0.10 g L^{-1}, which was achieved at an iron concentration of 20 µM (Fig. 2.2D). Although not yielding a significantly higher biomass production than those of the remaining treatments ($p > 0.05$), the iron concentration of 20 µM was deemed optimal from microscopic analysis, resulting in a culture with the most suitable morphology. Furthermore, the iron optimum (20 µM) as determined in this study is supported by Sasireka & Muthuvelayudham (2015), where the highest biomass concentration was achieved at the same iron levels, with a maximum DW concentration of 1.62 g L^{-1}.

Finally, the optimal dilution of micronutrients was evaluated. However, no significant differences ($p > 0.05$) could be determined between treatments for this parameter (Fig. 2.2E). Consequently, the highest dilution (0.5 mL L^{-1}) of micronutrients was considered optimal, leading to a maximum biomass concentration of 3.09 ± 0.07 g L^{-1} DW. From this trial, it was possible to conclude that the micronutrient solution, mainly composed of magnesium, zinc, molybdenum and manganese, is only required in trace amounts, with the lowest supply being sufficient for optimal growth. From previous studies, it was possible to conclude that there is a lack of research regarding the effects of micronutrients on the growth of *S. costatum*. However, the evaluation of the effects of zinc on microalgal growth is recurrent in different studies, where usually its toxicity is tested (Wong *et al*., 2010; Zhang *et al*., 2016).

2.4.1.2. *Chaetoceros calcitrans*

Considering the optimisation of silicate supply, and similarly to *S. costatum*, higher growth (Fig. 2.3A) was obtained at higher silicate concentrations (2.4 and 1.2

mM). At a silicate concentration of 2.4 mM, the highest DW was obtained for *C. calcitrans* (2.10 ± 0.08 g L^{-1}), which is in agreement with the biomass concentrations (2.20 g L^{-1} DW) reported previously by Banerjee *et al.*, (2011). However, as this growth was not significantly higher than that at 1.2 mM (2.03 ± 0.06 g L^{-1} DW, $p > 0.05$), the lowest silicate concentration was accepted as the optimal condition for supplying silica to the cultures of this species. On the other hand, the control conditions resulted in the lowest biomass concentration (0.76 ± 0.03 g L^{-1} DW) obtained, which was accompanied by high cell death as observed by microscopy. Once again, the value considered optimal in this study highly differs from those found in the literature, where silica-replete conditions were attributed to a supply of circa 0.2 – 0.4 mM L^{-1} (Corzo *et al*., 2000; Tantanasarit *et al*., 2013). As seen for *S. costatum*, cell elongation was also observed in response to lower silica availability, although this morphology was less noticeable in *C. calcitrans*. Nonetheless, for silicate concentrations of 0.4 and 0.8 mM, a higher number of cells with abnormal morphology was detected.

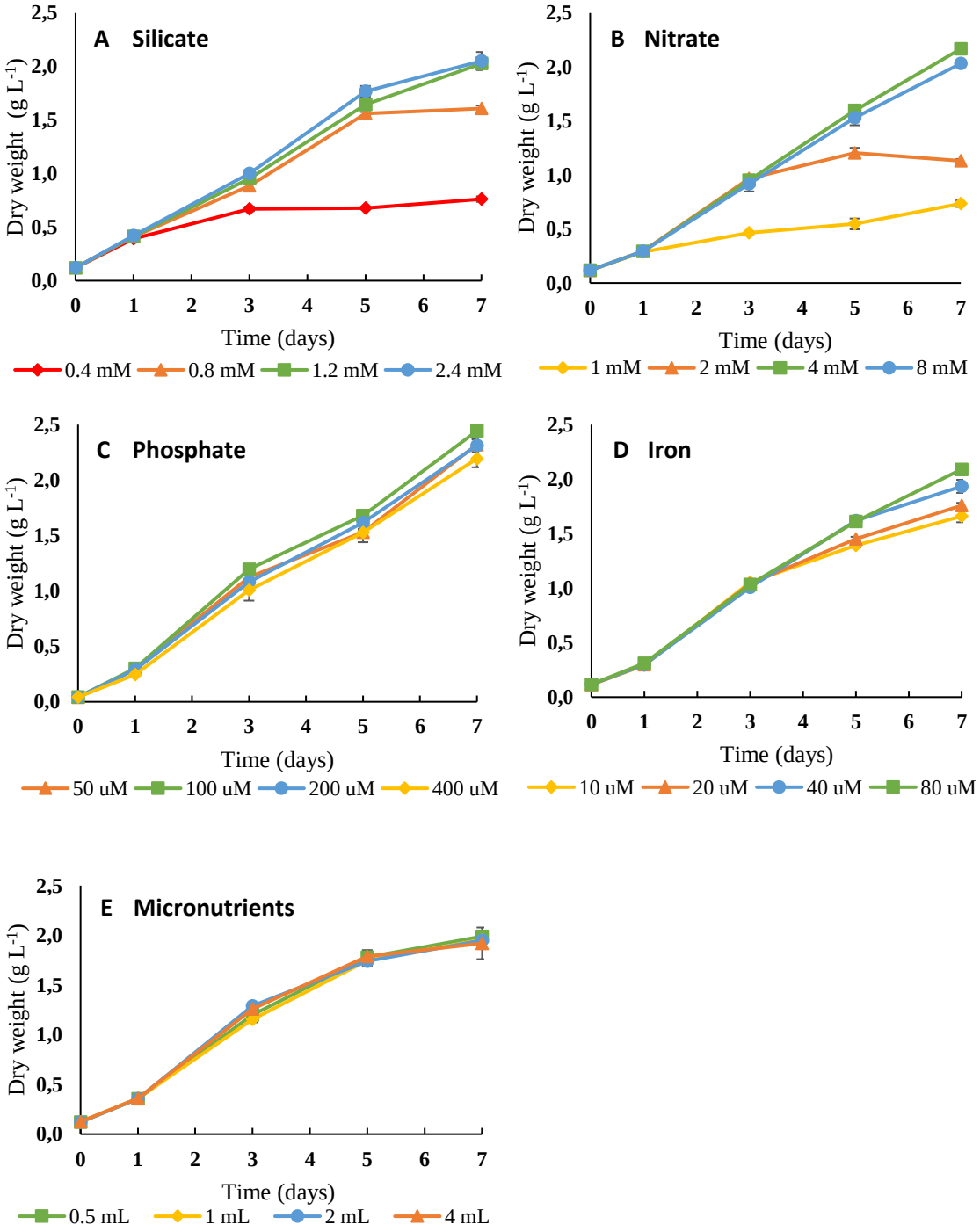

Figure 2.3: *C. calcitrans* growth performance in terms of biomass dry weight (g L^{-1}) obtained in in 1-L bubble column PBRs in order to optimize the supply of a specific nutrient (A, silicate; B, nitrate; C, phosphate; D, iron) or micronutrient (E) concentrations (n=3). Values are represented as mean ± standard deviation.

In the nitrate optimization trial, comparably to what was observed with *S. costatum*, higher growth was observed when the nitrate supply was the highest: 4 and 8 mM (Fig. 2.3B). However, a significantly higher growth was obtained for 4 mM of nitrate ($p < 0.05$), with a maximum DW of 2.17 ± 0.02 g L^{-1}, when compared to 8 mM (2.03 ±

0.02 g L^{-1} DW). As a result, 4 mM was considered the optimal nitrate concentration. Additionally, for cultures grown at nitrate concentrations of 1 and 2 mM, a significant increase in cell death alongside a 'mucilage' attached to the cells and in the medium was detected, especially for the treatment with 1 mM of nitrate. This could result from an increase in total carbohydrates accompanied by the release of free extracellular polysaccharides and sugar-containing compounds that became bound to the cell surface. Such a result has been ascribed to nitrate-deficient conditions, as previously reported for *Chaetoceros* sp. cultures (Corzo *et al.*, 2000). From what could be found in the literature, the concentration of nitrate supplied in cultures of *C. calcitrans* ranges between 0.4 and 2 mM (Corzo *et al.*, 2000; Tantanasarit *et al*., 2013). As found in the present study, these values are far below the concentration considered as optimal for this diatom.

Smaller differences were observed between the different treatments concerning the remaining trials, as observed for *S. costatum*. At the phosphate trial, higher growth was obtained at the concentration of 100 µM, where a maximum DW of 2.44 ± 0.02 g L^{-1} was obtained (Fig. 2.3C). However, this growth was not significantly different from the ones obtained under the remaining conditions ($p > 0.05$), except when the highest phosphate concentration, 400 µM, was used (2.19 ± 0.08 g L^{-1} DW, $p < 0.05$). As what happened with the trials of *S. costatum*, high depletion of phosphate was observed under all conditions. Nonetheless, and once again, the integration of microscopic observations with growth data allowed to conclude that the supply of 100 µM of phosphate was the optimal concentration, even though the standard phosphate concentration reported in the literature is usually circa 200 µM (Rivero-Rodríguez *et al*., 2007; Tantanasarit *et al*., 2013). A previous study reported increased mucilaginous polysaccharides in *C. calcitrans* cultures under phosphate limitation (Guerrini *et al.*, 1998). However, this trend was not observed for the concentrations used in the present study.

Regarding the iron optimization trial, higher growth was obtained at the highest iron concentrations, 40 and 80 µM (Fig. 2.3D). A maximum biomass of 2.10 ± 0.02 g L^{-1} DW was obtained at 80 µM, resulting in significantly more growth as compared to cultures grown at 40 µM (1.93 ± 0.06 g L^{-1} DW, $p < 0.05$). Thus, 80 µM was considered the optimal iron concentration. Depending on the species, iron uptake and further luxury uptake in diatoms have been associated with the protein ferritin and/or storage vacuoles. Besides being crucial to prevent cell damage, mainly due to reactive oxygen species and oxidative stress from free intracellular iron, ferritin and storage vacuoles can also facilitate iron uptake up to high levels (Marchetti *et al*., 2009; Marchetti & Maldonado,

2016). This superior storage ability of iron and other nutrients coincides with the bloom-forming capacity that diatoms are known to possess in natural-forming phytoplankton communities. Therefore, these results shed some light on the potential use of *C. calcitrans* in metal bioremediation.

At the final trial where micronutrients were tested, a maximum DW of 1.99 ± 0.05 g L^{-1} was achieved using the lowest supply: 0.5 mL L^{-1} (Fig. 2.3E). However, similar results were obtained under the remaining conditions ($p > 0.05$). As observed for *S. costatum*, there is a dearth of studies regarding the supply of micronutrients, and the few that exist are on the subject of the toxicity effects of micronutrients such as zinc and cobalt (Anu *et al*., 2016, 2018). Overall, the amount of research regarding growth optimization in cultures of *C. calcitrans* is highly scarce (Krichnavaruk *et al*., 2005). The final optimised results obtained for each diatom species are summarized in Table 2.1.

Table 2.1: Summary of non-optimized (control) and optimised biomass concentrations (g L^{-1} DW) obtained with *S. costatum* and *C. calcitrans* cultures grown in laboratory bubble column PBRs (n=3). DW values are represented as mean ± standard deviation.

	Tested conditions	Control	Silicate mM	Nitrate mM	Phosphate µM	Iron µM	Micronutrients mL L^{-1}
S. costatum	**Optimised conditions**		2.4	4	100	20	0.5
	DW (g L^{-1})	2.00 ± 0.03	3.51 ± 0.17	3.18 ± 0.07	3.72 ± 0.07	2.60 ± 0.10	3.09 ± 0.07
C. calcitrans	**Optimised conditions**		1.2	4	100	80	0.5
	DW (g L^{-1})	0.76 ± 0.03	2.03 ± 0.06	2.17 ± 0.02	2.44 ± 0.02	2.10 ± 0.02	1.99 ± 0.05

2.4.1.3. *Skeletonema costatum*: pilot-scale cultures

The trial with *S. costatum* cultures under outdoor conditions and pilot-scale volumes yielded lower maximum biomasses, respectively 0.97 ± 0.03 g L^{-1} and 0.86 ± 0.05 g L^{-1} for optimised and control conditions, when compared with the laboratory bubble column PBR system (Fig. 2.4). Nonetheless, the culture with the optimised culture medium grew significantly more than the culture under control conditions ($p < 0.05$). One hypothesis that explains the lower yields obtained is the absence of continuous illumination of the outdoor cultures, since continuous illumination with LEDs is able to increase biomass yields (Schulze *et al*., 2014). From this trial, it was only possible to

observe differences between cultures from the 5th day of trial onwards. As a result, we suggest a two-phase production system for *S. costatum* cultures grown under outdoor pilot-scale conditions, where the optimised culture medium is only supplied when the cultures reach a biomass of, approximately, 0.6 g L^{-1} DW, since for lower concentrations the cultures do not seem to require optimal nutrient concentrations.

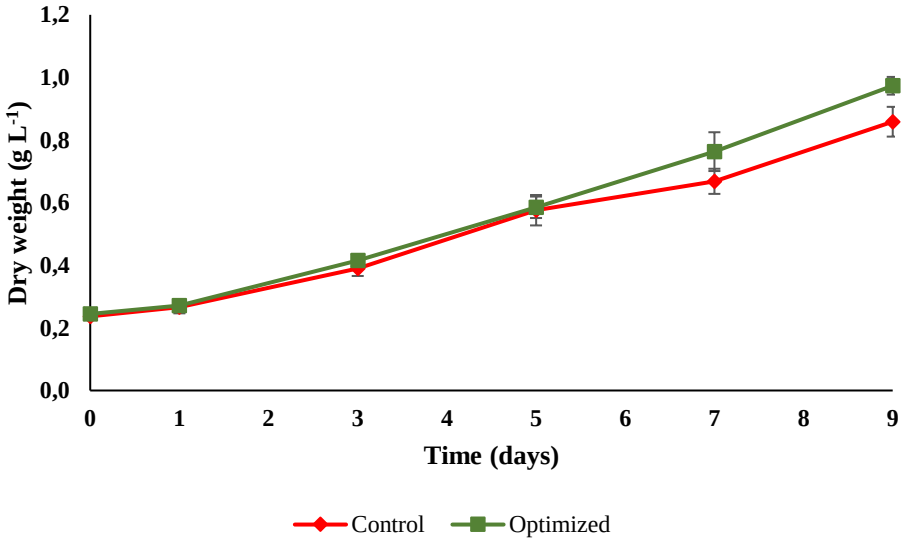

Figure 2.4: Growth performance in biomass dry weight (g L-1) obtained in control culture medium conditions and optimised culture medium conditions for *S. costatum* in 100-L flat panel PBRs (n=3, mean ± standard deviation).

2.4.2. Biochemical analysis: Proximal composition

2.4.2.1. *Skeletonema costatum*

The maximum protein content was obtained during the silicate and nitrate trials (19.6 ± 0.5% DW and 19.7 ± 0.1% DW, respectively), which did not significantly vary from that of the control conditions (18.8 ± 1.2% DW, $p > 0.05$; Table 2.2). However, in the following trials, the protein content was significantly reduced ($p < 0.05$), when compared to the control conditions, where 15.8 ± 0.3% DW, 14.3 ± 0.9% DW and 15.6 ± 0.3% DW were detected in the phosphate, iron and micronutrient trials, respectively (Table 2.2). These results suggest that a phosphate concentration shift from 200 to 100 µM (optimal conditions for growth) negatively affected the protein content in *S. costatum* cultures. Taking into consideration that phosphorus is an integrating nutrient in the biosynthesis of photosynthesis-related proteins and ATP (adenosine triphosphate), the decrease in protein content might be explained by a reduction in ATP synthesis, consequently resulting in the deficiency of available energy for the assimilation of

nitrogen, which is essential for protein synthesis (Liu *et al.*, 2013; Singh *et al.*, 2018). In addition, the maximum values of total protein content obtained, although slightly lower, were similar to the ones found in the literature, where protein content ranged between 23.3–31% DW (Brown, 1991; Monkonsit *et al.*, 2011; Lestari *et al.*, 2014; Van Houcke *et al.*, 2017).

Table 2.2: Proximal composition of optimised biomasses of diatoms *S. costatum* and *C. calcitrans* grown in bubble column PBR system, regarding total protein, lipid, ash, and carbohydrate contents as % DW (n=3, mean ± standard deviation). a, b: different letters denote significant differences between values from control cultures and cultures grown in the remaining optimization trials ($p < 0.05$).

Proximal composition (% DW)	Optimization Trial	*Skeletonema costatum*	*Chaetoceros calcitrans*
Protein	**Control**	18.8 ± 1.2[a]	15.3 ± 0.9
	Silicate	19.6 ± 0.5[a]	16.5 ± 1.4
	Nitrate	19.7 ± 0.1[a]	16.9 ± 1.6
	Phosphate	15.8 ± 0.3[b]	15.4 ± 0.2
	Iron	14.3 ± 0.9[b]	16.0 ± 1.6
	Micronutrients	15.6 ± 0.3[b]	16.2 ± 1.2
Lipids	**Control**	14.5 ± 5.2	8.5 ± 2.6
	Silicate	10.7 ± 2.3	9.0 ± 2.2
	Nitrate	18.5 ± 2.6	10.8 ± 3.6
	Phosphate	16.2 ± 2.9	12.2 ± 1.4
	Iron	13.3 ± 2.0	12.6 ± 0.9
	Micronutrients	10.2 ± 3.1	11.0 ± 1.1
Ash	**Control**	28.7 ± 0.6[b]	50.5 ± 0.6
	Silicate	38.2 ± 2.4[a]	48.8 ± 1.9
	Nitrate	46.0 ± 0.3[a]	47.1 ± 0.2
	Phosphate	49.7 ± 0.4[a]	50.5 ± 9.6
	Iron	52.5 ± 1.5[a]	46.4 ± 2.7
	Micronutrients	48.0 ± 0.2[a]	49.7 ± 0.8
Carbohydrates	**Control**	38.4 ± 6.7[a]	25.7 ± 2.9
	Silicate	31.5 ± 4.8[a]	25.4 ± 2.4
	Nitrate	15.8 ± 2.7[b]	24.9 ± 4.1
	Phosphate	18.3 ± 2.6[b]	20.6 ± 11.2
	Iron	19.8 ± 2.6[b]	26.1 ± 1.6

	Micronutrients	26.2 ± 3.1[b]	23.4 ± 1.5

The maximum lipid content was obtained in the nitrate optimization trial (18.5 ± 2.6% DW), although this value was not significantly different from that of the non-optimized (control) culture and remaining trials ($p > 0.05$), where values ranged between 10.2–16.2% DW (Table 2.2). These results were in agreement with what has been reported in the literature where values ranged between 1.3–16.2% DW (Brown, 1991; Monkonsit *et al*., 2011; Lestari *et al*., 2014; Van Houcke *et al.*, 2017). The variability in the lipid content obtained between treatments can be explained by the evolutionary advantages and protective mechanisms against unfavourable environment conditions, which diatoms have been reported to possess (Bradshaw & Hardwick, 1989; López-Rodas *et al*., 2009; Lyon & Mock, 2014). Since the differences mentioned above between treatments correlated with differences in nutrient consumption and/or differences in growth, we suggest that the fast shift in lipidic content might be a protective response mechanism of *S. costatum* to changes in the surrounding environment.

The highest percentage of ash content was detected in the iron trial (52.5 ± 1.5% DW), which was significantly different from the control conditions and remaining trials ($p < 0.05$), at the exception of the phosphate trial (Table 2.2). The control conditions presented the lowest percentage of ashes (28.7 ± 0.6% DW), significantly lower than those of the remaining trials, which ranged between 38.2–52.5% DW ($p < 0.05$) (Table 2.2). This result was expected and may be explained by the increased silicification of the cell walls, due to the increase in silicate concentration present in the medium (Reynolds, 2006). Furthermore, although there is a general shortage of information available regarding ash content in *S. costatum* cultures, a study by Lestari *et al*., (2014) reported ash content of 55.6% DW. This study, along with the presented work, demonstrate the high nutrient storage capacity of diatoms as well as an elevated rate of cell wall silicification, which could explain the elevated ash contents described in *S. costatum* cultures (Raven, 1987; Hildebrand *et al*., 2012).

The maximum carbohydrate content was obtained under control conditions (38.4 ± 6.7% DW), which was significantly higher from all trials ($p < 0.05$), with the exception of the silicate trial (31.5 ± 4.8% DW, $p > 0.05$) (Table 2.2). Additionally, the carbohydrate content of the remaining trials ranged between 15.8–26.2% DW (table 2.2). The carbohydrate content detected in this work was similar to what is found in the literature,

where values ranged between 4.6–26.4% DW (Brown, 1991; Monkonsit *et al*., 2011; Van Houcke *et al.*, 2017). The high carbohydrate content determined for cells under control conditions can be associated with the low silicate concentration, which is consequently associated with lower ash contents. Furthermore, the decrease in carbohydrate content throughout the optimisation process may also indicate that the cellular carbohydrates were converted into other biochemical constituents (Granum *et al*., 2002). In addition, we suggest that the higher content of carbohydrates observed in the control conditions could be associated with the lower growth registered and the harvesting of cultures in the stationary phase. For these conditions, an increase in storage and cell wall polysaccharides occurs due to a lower rate of cell division, since structural polysaccharides are crucial constituents in the organic casing of the siliceous components of the diatom cell (Schmid *et al*., 1981; Gautam *et al*., 2019).

2.4.2.2. *Chaetoceros calcitrans*

Regarding *C. calcitrans* cultures, the maximum protein content was detected in the nitrate optimization trial 16.9 ± 1.6% DW, although this value was not significantly different from the ones obtained in the control conditions and remaining trials ($p > 0.05$), where values ranged between 15.3–16.9% DW (Table 2.2). These results differed from what has been reported in the literature for *C. calcitrans* cultures, where total protein content ranged between 31.3–41.6% DW (Brown, 1991; Banerjee *et al.*, 2011; Monkonsit *et al*., 2011; Velasco *et al*., 2016). Furthermore, the reduction in protein content from the control conditions to the optimised trials observed in the analysis of *S. costatum* was not detected for *C. calcitrans.*

The maximum lipid content was detected in the iron optimization trial, 12.6 ± 0.9% DW, although this value was not significantly different from that of the control conditions and remaining trials ($p > 0.05$), where values ranged between 8.5–12.2% DW (table 2.2). These results were similar to what has been reported in the literature for *C. calcitrans* cultures where total lipid content ranges between 8.7–23% DW (Brown, 1991; Banerjee *et al.*, 2011; Monkonsit *et al*., 2011; Velasco *et al*., 2016).

From the ash analysis, the maximum content was detected in the control conditions and the phosphate trial (50.5 ± 0.6% DW and 50.5 ± 9.6% DW, respectively). However, these values were not significantly different from the remaining trials ($p > 0.05$), where values ranged between 47.4–49.7% DW (Table 2.2). The information regarding ash content in *C. calcitrans* is scarce with Kudaibergenov & Khajiyeva (1987)

reporting an ash content of 19.5% DW in *C. calcitrans* cultures. For *S. costatum* cultures, the ash content was significantly influenced by the presence of silicates, however this trend was not observed in *C. calcitrans* cultures. In fact, the increase of silicates concentration in the medium did not seem to influence the ash content of *C. calcitrans* cultures.

Regarding carbohydrates, the maximum carbohydrate content was detected in the iron optimization trial, 26.1 ± 1.6% DW (Table 2.2), although this value was not significantly different from the remaining trials and control conditions ($p > 0.05$), where values ranged between 23.4–25.7% DW (Table 2.2). These results were consistent with what has been described in the literature where values range between 6–37% DW (Brown, 1991; Banerjee *et al.*, 2011; Monkonsit *et al*., 2011; Velasco *et al*., 2016).

2.4.2.3. *Skeletonema costatum: pilot-scale flat panels*

The protein analysis of the cultures that were scaled up and grown outdoors was similar to the ones obtained indoors, with the control conditions having higher protein content (22.3 ± 5.2% DW) although not significantly different ($p > 0.05$) when compared to the optimised conditions (18.8 ± 1.0% DW) (table 2.3).

The lipid analysis results were similar to the ones obtained under laboratorial conditions, with no significant differences detected between treatments ($p > 0.05$), where the control conditions showed higher protein content (14.1 ± 3.9% DW) when compared to the optimised conditions (13.8 ± 1.0% DW) (Table 2.3).

Although the ash content was lower in the control conditions (32.8 ± 9.5% DW), contrary to what was observed in laboratory conditions, this value was not significantly different from the optimised culture medium supply (42.7 ± 1.0% DW, $p > 0.05$) (Table 2.3). These results can be explained by the high cellular death rate in the laboratory control cultures, at the time of harvest, whilst the control cultures grown in the Flat panels were harvested during an active growth phase in which no significant cell death was observed; hence, the absence of differences regarding ash content between the control and optimised conditions. Additionally, diatoms are reported to thrive and dominate over other phytoplankton communities when nutrient conditions are replete, by taking up higher nutrient quantities in comparison to other phytoplankton organisms (Allen *et al*., 2006; Hildebrand *et al.*, 2012). From these results we suggest that this phenomenon of luxury uptake occurs in *S. costatum* cultures as a storage-response mechanism to nutrient replete conditions, thus resulting in increased final ash content.

At last, regarding carbohydrate content, the values obtained were similar to the laboratory results, with no significant differences between the control conditions and the optimised conditions (27.6 ± 5.3% DW and 24.7 ±1.0% DW, $p > 0.05$, respectively) (Table 2.3).

Table 2.3: Proximal composition of diatom *S. costatum* cultures grown in exterior pilot-scale 100-L flat panels, as % DW. Optimised culture medium was tested against control culture medium (n= 3, mean ± standard deviation). No significant differences were found between cultures under non-optimized and optimized conditions ($p < 0.05$).

Proximal composition (% DW)	Optimised	Control
Protein	18.8 ± 1.0	22.3 ± 5.2
Lipids	13.8 ± 1.0	14.1 ± 3.9
Ash	42.7 ± 1.0	32.8 ± 9.5
Carbohydrate	24.7 ± 1.0	27.6 ± 5.3

2.4.3. Biochemical composition: Fatty acid profile

Concerning the fatty acid (FA) composition of *S. costatum* and *C. calcitrans* cultures, a wide range of values can be found in the literature, naturally due to several influencing factors such as different culture conditions and different extraction methods. Regardless, microalgae fatty acid composition can be divided regarding their saturation in polyunsaturated (PUFA) monounsaturated (MUFA) and saturated (SFA) fatty acids (Paliwal *et al*., 2017).

2.4.3.1. *Skeletonema costatum*

For the control conditions, lower contents of eicosapentaenoic (EPA) and docosahexaenoic (DHA) were registered (8.9 ± 5.6% of total FA and 2.0 ± 1.2% of total FA, respectively), whilst for the remaining optimization trials higher values were obtained with ranges between 12.5–22.7% of total FA and 3.0–5.7% of total FA, respectively (Table 2.4). Regarding other FA, profiles were mainly composed of palmitoleic (C16:1), myristic (C14:0), palmitic (C16:0), hexadecatrienoic (C16:3), and stearidonic (C18:4) acids (Table 2.4) in cultures grown under all conditions tested. In general, EPA (C20:5) is normally found in *S. costatum* with significant values ranging between 6–23.5% of total FA, while DHA (C22:6) is reportedly found at lower values, ranging between 1.41–4% of total FA (Volkman *et al*., 1989; Pennarun *et al*., 2003; Guihéneuf *et al*., 2008; Van Houcke *et al*., 2017; Gao *et al*., 2019). The production of

both these FA is highly relevant given their essential role in human and animal nutrition (Pereira *et al*., 2012; Remize *et al*., 2021). The stepwise optimization process resulted in the increase of EPA and DHA contents where a marked increase was obtained with the optimisation of the concentrations of silicate and nitrate, until the final trial, where the composition of the culture medium NB^+ was optimal for *S. costatum* growth. These results reveal the successful manipulation of the culture medium where increased biomass associated with higher PUFA content was obtained, in detriment to lower growth and higher content of SFA and MUFA in the control treatments due to the stressful conditions present. Hence, *S. costatum* cultures can be an interesting source of *n*-3 PUFA, especially of EPA and DHA. Additionally, similarly to what was obtained in the present work, other FA have been reported with interesting values, such as myristic (14.1–31.79% of total FA), palmitic (10.7–45.36% of total FA), palmitoleic (14.78–31.15% of total FA), hexadecatrienoic (3.7–16.3% of total FA), and stearidonic (3.4–4.4% of total FA) acids (Volkman *et al.*, 1989; Pennarun *et al.*, 2003; Prartono *et al*., 2013; Van Houcke *et al.*, 2017; Rohit and Mohan, 2018; Gao *et al.*, 2019). Moreover, although Van Houcke *et al.*, (2017) reported 11.2 ± 0.6% of total FA of palmitidonic acid (C16:4) for *S. costatum* cultures, we detected trace amounts not only in the control conditions (0.6 ± 0.3% of total FA) but also for the remaining trials where values ranged between 0.6–2.2% of total FA (Table 2.4).

Table 2.4: Fatty acid profiles of *S. costatum* from biomass of non- (control) and optimised cultures from silicate, nitrate, phosphate, iron and micronutrient optimization trials (*n*=3, values are expressed as mean of total FAME percentages ± standard deviation). ∑SFA: total content of saturated fatty acids, ∑MUFA: total content of monounsaturated fatty acids, ∑PUFA: total content of polyunsaturated fatty acids. a, b: different letters denote significant differences between values from control cultures and cultures grown in the remaining optimization trials ($p < 0.05$).

Fatty acid (%)	Control	Silica	Nitrates	Phosphate	Iron	Micro
C14:0	31.4 ± 3.8	26.1 ± 1.7	16.8 ± 1.3	23.5 ± 1.7	16.9 ± 0.4	17.3 ± 0.3
C16:0	7.3 ± 1.4	6.0 ± 1.2	4.9 ± 0.2	4.5 ± 0.7	11.7 ± 0.0	4.9 ± 0.0
∑SFA	**36.3 ± 7.4 [a]**	**32.1 ± 2.7[a]**	**21.7 ± 1.3[b]**	**28.0 ± 2.2[a]**	**22.7 ± 7.9[a]**	**18.9 ± 2.8[b]**
C16:1	40.9 ± 2.9	29.2 ± 8.6	26.7 ± 3.1	27.6 ± 2.4	41.5 ± 4.3	23.6 ± 1.4
C18:1	2.6 ± 0.5	2.4 ± 0.4	1.3 ± 0.2	1.8 ± 0.2	3.5 ± 0.0	1.3 ± 0.0
∑MUFA	**42.2 ± 4.3[a]**	**31.7 ± 8.2[a]**	**28.0 ± 3.1[b]**	**29.3 ± 1.3[b]**	**43.2 ± 1.9[a]**	**24.9 ± 1.4[b]**
C16:4	0.6 ± 0.3	0.7 ± 0.1	1.2 ± 0.1	1.3 ± 0.2	0.6 ± 0.1	2.2 ± 0.1
C16:3	6.6 ± 2.4	15.5 ± 2.8	15.8 ± 1.1	15.0 ± 6.4	5.4 ± 0.7	20.1 ± 0.5

C18:4	3.2 ± 1.3	4.6 ± 0.7	5.4 ± 0.2	5.9 ± 0.7	4.1 ± 0.3	6.5 ± 0.1
C20:5	8.9 ± 5.6	12.5 ± 1.6	22.7 ± 0.8	16.4 ± 2	18.3 ± 1.7	22.2 ± 1.3
C22:6	2.0 ± 1.2	3.0 ± 0.5	5.1 ± 0.2	4.2 ± 0.5	3.7 ± 0.5	5.2 ± 0.3
∑PUFA	**21.6 ± 11.1[b]**	**36.3 ± 5.6[a]**	**50.3 ± 1.9[a]**	**42.7 ± 3.3[a]**	**32.0 ± 3.1[b]**	**56.2 ± 1.4[a]**

In general, the cultures of *S. costatum* under control conditions contained higher levels of SFA and MUFA, 36.3 ± 7.4% of total FA and 42.2 ± 4.3% of total FA respectively, whilst for the remaining conditions the values ranged between 21.7–32.1% of total FA and 24.9–43.2% of total FA, respectively (Table 2.4). In contrast, PUFA content was lower for *S. costatum* cultures under control conditions, 21.6 ± 11.1% of total FA, whereas for the remaining trials, PUFA content increased with values ranging between 32.0–56.2% of total FA (Table 2.4). From the available literature, for *S. costatum*, total SFA ranged between 17.3–73.6% of total FA, total MUFA ranged between 14.8–36.6% of total FA, and total PUFA ranged between 9.9–61.7% of total FA (Volkman *et al.*, 1989; Pennarun *et al.*, 2003; Guihéneuf *et al.*, 2008; Prartono *et al*., 2013; Van Houcke *et al.*, 2017; Gao *et al.*, 2019). Under stressful environmental conditions (i.e., nutrient limitation), cells favour the biosynthesis of SFA or MUFA in detriment to the biosynthesis of PUFA, since SFA and MUFA can store more energy and thus allow the cells to maintain homeostasis (Srinuanpan *et al*., 2018; Gao *et al*., 2019;). In addition, the same FA adjustment has been described for cultures collected in the stationary growth phase (Deshmukh *et al*., 2019). Despite the absence of changes in lipid content, these results suggest an adaptive strategy where, in the control cultures, the FA profile was shifted to a higher content of storage lipids (SFA and MUFA) as a response mechanism to the silica-deficit conditions, indicating a clear stressful state of the cultures. Furthermore, the higher content of MUFA (43.2 ± 1.9% of total FA) detected in the cultures collected from the iron optimization trial (Table 2.4) can be attributed to the described growth results where the stationary growth phase was reached on day 5 (Fig. 2.2D). These results support the ones described by Gao *et al.*, (2019), where silica limitation in *S. costatum* cultures resulted in the increase in SFA and MUFA contents in parallel to the decrease in PUFA content.

2.4.3.2. *Chaetoceros calcitrans*

For the control conditions lower contents of EPA and DHA were registered (10.5 ± 0.6% of total FA and 1.3 ± 1.2% of total FA, respectively), whilst in the remaining trials

the values were higher for EPA (18.8–23.7% of total FA) and did not vary for DHA (1.9–2.3% of total FA) when compared with the control conditions (Table 2.5). From the results obtained, the optimisation of silicate supply was the key-step to increment the content of EPA in *C. calcitrans* cultures. Regarding other FA, all trials were mainly composed of palmitoleic (C16:1), palmitic (C16:0), myristic (C14:0) and hexadecatrienoic (C16:3) FA (Table 2.5). The higher EPA contents compared to DHA levels is in accordance with values obtained previously, which range between 5–26.3% of total FA and 0.7–2.3% of total FA, respectively (Fernández-Reiriz *et al.*, 1989; Volkman *et al.*, 1989; Delaunay *et al.*, 1993; Rivero-Rodríguez *et al*., 2007; Kaspar *et al.*, 2014; Méndez-Martínez *et al.*, 2018). In addition, other FA have been reported in interesting amounts, such as myristic (9.2–23.2% of total FA), palmitic (10.7–29.7% of total FA), palmitoleic (17.4–32.5% of total FA), hexadecatrienoic (6.4–14.5% of total FA) acids, although there are reports of stearic (5.4–16.7% of total FA), and elaidic (9.8–12.1% of total FA) FA. In this work, however, these FA were only detected in trace amounts with values ranging between 0.9–1.6% of total FA and 1.7–4.6% of total FA, respectively (Fernández-Reiriz *et al.*, 1989; Volkman *et al.*, 1989; Delaunay *et al.*, 1993; Rivero-Rodríguez *et al*., 2007; Kaspar *et al.*, 2014; Méndez-Martínez *et al.*, 2018). As with *S. costatum*, these results suggest that *C. calcitrans* can be an interesting source of EPA. These results demonstrate that *C. calcitrans* is a good candidate for the aquaculture and nutraceutical industries where there is a high appeal for *n*-3 PUFAs, such as EPA and DHA (Nilsson *et al*., 2020; Pratiwy & Pratiwi, 2020), thus establishing the high-value potential of this strain.

Table 2.5: Fatty acid profiles of *C. calcitrans* from the optimised biomasses of the control, silicate, nitrate, phosphate, iron and micronutrient trials (n=3, values are expressed as mean of total FAME percentages ± standard deviation). ∑SFA: total content of saturated fatty acid, ∑MUFA: total content of monounsaturated fatty acid, ∑PUFA: total content of polyunsaturated fatty acid. a, b: different letters denote significant differences between values from control cultures and cultures grown in the remaining optimization trials ($p < 0.05$).

Fatty acid (%)	**Control**	**Silica**	**Nitrates**	**Phosphate**	**Iron**	**Micro**
C14:0	15.8 ± 1.2	15.2 ± 0.9	16.3 ± 1.2	14.9 ± 0.5	17.2 ± 0.6	13.1 ± 1.1
C16:0	32.9 ± 1.1	15.2 ± 0.9	12.6 ± 0.4	16.0 ± 0.4	15.0 ± 0.2	20.6 ± 1.6
C18:0	1.6 ± 0.4	1.2 ± 0.0	0.9 ± 0.0	1.1 ± 0.2	0.9 ± 0.1	1.4 ± 0.1
∑SFA	**50.3 ± 0.5^{a}**	**31.6 ± 0.2^{b}**	**29.8 ± 1.6^{b}**	**32.1 ± 0.2^{b}**	**33.4 ± 0.8^{b}**	**34.4 ± 0.8^{b}**
C16:1	27.2 ± 1.0	30.7 ± 0.5	32.4 ± 1.0	31.4 ± 0.7	31.9 ± 0.9	32.9 ± 0.7

C18:1	4.6 ± 0.6	2.6 ± 0.3	2.0 ± 0.0	2.4 ± 0.2	1.7 ± 0.4	2.8 ± 0.5
∑MUFA	**31.8 ± 0.6^{b}**	**33.3 ± 0.8^{b}**	**34.5 ± 1.0^{a}**	**33.9 ± 0.6^{b}**	**33.6 ± 1.2^{b}**	**35.7 ± 0.6^{a}**
C16:3	6.1 ± 0.5	10.8 ± 0.4	10.0 ± 0.2	8.9 ± 0.3	9.2 ± 0.1	9.2± 2.6
C20:5	10.5 ± 0.6	22.3 ± 0.2	23.7 ± 2.5	22.8 ± 0.4	21.7 ± 1.7	18.8 ± 2.3
C22:6	1.3 ± 0.2	2.0 ± 0.0	2.0 ± 0.3	2.3 ± 0.1	2.2 ± 0.1	1.9 ± 0.2
∑PUFA	**17.7 ± 0.3^{b}**	**35.0 ± 0.6^{a}**	**35.8 ± 2.6^{a}**	**34.1 ± 0.7^{a}**	**33.0 ± 1.8^{a}**	**29.9 ± 0.5^{a}**

In comparison to the different culture's conditions of *S. costatum*, higher values of SFA and MUFA were also detected in *C. calcitrans* cultures under control conditions. However, unlike *S. costatum*, the SFA (50.2% of total FA) contents were higher than those of MUFA (31.8% of total FA; Table 2.5). For the remaining conditions, SFA decreased significantly in relation to the control conditions, with values ranging between 29.8–34.4% of total FA ($p < 0.05$), whilst MUFA (32.9–35.4% of total FA) did not vary significantly, when compared with the control conditions (Table 2.5). On the other hand, PUFA were lower for the control conditions (17.9% of total FA) and increased in the remaining optimization trials with values ranging between 29.7–35.0% of total FA (Table 2.5). From the available literature, for *C. calcitrans*, total saturated (∑SFA), monounsaturated (∑MUFA) and polyunsaturated (∑PUFA) FA ranged between 11.7–50.2% of total FA, 19.6–39.1% of total FA, and 5.0–50.9% of total FA, respectively (Fernández-Reiriz *et al.*, 1989; Volkman *et al.*, 1989; Delaunay *et al.*, 1993; Rivero-Rodríguez *et al*., 2007; Kaspar *et al.*, 2014; Méndez-Martínez *et al.*, 2018). According Akbarnejad *et al.*, (2020), *C. calcitrans* cultures under unfavourable conditions increase the formation and accumulation of neutral lipids such as SFA. Therefore, these results suggest that under control conditions the cultures of *C. calcitrans* were under a stressful environment under which silica was limiting.

2.4.3.3. *Skeletonema costatum*: pilot-scale flat panels

The fatty acid analysis performed on the final biomasses of the optimised and control cultures grown in 100-L flat panels revealed no significant differences between them ($p > 0.05$) (Table 2.6). In comparison to the laboratory conditions, significantly higher contents of PUFA were detected ($p < 0.05$), for both the optimised and control conditions (63.0 ± 3.2% of total FA and 64.2 ± 2.2% of total FA, respectively), which agrees with the harvesting of the cultures during an active growth phase. In contrast,

significantly lower contents of SFA (17.5 ± 2.4% of total FA and 17.0 ± 1.6% of total FA, respectively) and MUFA (19.6 ± 0.8% of total FA and 18.9 ± 1.1% of total FA, respectively), were registered for both optimised and control conditions ($p < 0.05$) (Table 2.6). The correlation of these results with the ones from the laboratory conditions suggests that the changes in nutrient supply do not affect the biochemical composition of *S. costatum* cultures when a continuous light source is not provided. Additionally, a continuous light source in laboratory conditions results in a higher content of SFA and MUFA, possibly indicating a minor stress response of the cultures. Moreover, these results further support the laboratory findings of *S. costatum* cultures being an interesting source of PUFA, especially of EPA.

Table 2.6: Fatty acid profiles of biomasses of *S. costatum* cultures grown with optimised and control culture media (n=3, values are expressed as mean of total FAME percentages ± standard deviation). ∑SFA: total content of saturated fatty acid, ∑MUFA: total content of monounsaturated fatty acid, ∑PUFA: total content of polyunsaturated fatty acid. No significant differences were detected between the control and optimised conditions ($p > 0.05$).

Fatty acid (%)	Control	Optimised
C14:0	14.6 ± 1.3	14.6 ± 2.4
C16:0	2.4 ± 0.3	2.9 ± 0.1
∑SFA	**17.0 ± 1.6**	**17.5 ± 2.4**
C16:1	18.4 ± 0.6	19.0 ± 0.8
C18:1	0.4 ± 0.1	0.5 ± 0.2
∑MUFA	**18.9 ± 1.1**	**19.6 ± 0.8**
C16:4	7.2 ± 0.4	6.2 ± 0.3
C16:3	17.1 ± 2.2	19.6 ± 0.6
C18:4	6.3 ± 0.6	6.1 ± 1.3
C20:5	25.9 ± 1.4	23.7 ± 3.9
C22:6	7.7 ± 1.5	7.4 ± 0.8
∑PUFA	**64.2 ± 2.2**	**63.0 ± 3.2**

2.5. Conclusions

The present work established the relevance of applying a stepwise process when optimizing the biomass production of microalgae cultures. Under laboratory conditions, the key step to enhance the biomass production of *S. costatum* and *C. calcitrans* was, respectively, a 6- and 3-fold increase in silicate supply. Besides silicate, only nitrate-

deficient conditions resulted in growth arrest, with the already established supply proving to be optimal. Regarding the optimisation of the supply of phosphate, iron and micronutrients, it is possible to assume a smaller impact on growth enhancement. Hence the manipulation of the supply of silicate and nitrate are key factors at the nutrient level to increase biomass production for both diatoms. Under outdoor conditions, for pilot-scale production of *S. costatum*, smaller differences in growth were detected between the non-optimised and optimised cultures, where the quantity of silicate supplied was the most remarkable disparity. Taken together these results suggest that higher quantities of silicate should be provided in continuously illuminated cultures, under laboratory conditions, whereas in cultures being grown in pilot-scale production systems under outdoor conditions, only upon reaching a given threshold concentration. Overall, the culture medium optimisation led to a maximum 1.8-fold and 3.2-fold increase in the biomass produced by *S. costatum* and *C. calcitrans* cultures, respectively. The growth optimization process did not seem to significantly affect the proximal composition of both diatoms except for a higher ash content that appeared to correlate with higher nutrient supply, primarily silicate, in the case of *S. costatum*. Furthermore, a shift in the fatty acid composition was observed under laboratory conditions under which the control cultures, being in stress, contained higher amounts of MUFA and SFA to the detriment of PUFA. The increment in PUFAs EPA and DHA was detected and associated with a higher culture growth, establishing the immense potential of these strains regarding the production of high-value compounds. Through these results, the production in terms of growth, of both diatom species, was successfully optimized whilst the resulting biochemical profile was characterized and optimised specifically at the level of high-value EPA and DHA, denoting the promising and relevant application of *S. costatum* and *C. calcitrans* products in the aquaculture and nutraceutical industries. To conclude, the nutrient optimisation process described in the present work is of the utmost importance for the industrial production of these high-value diatom species, following the paradigm of increased productivity with reduced associated costs, two essential factors for the success of any industry.

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