HUGO GALVÃO CAIANO PEREIRA

# BIOTECHNOLOGICAL APPLICATIONS OF A PROMISING MARINE CHLOROPHYTE (*TETRASELMIS* SP. CTP4): A BIOREFINERY APPROACH


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Doutoramento em Ciências do Mar, da Terra e do Ambiente

Ramo Ciências do Mar

Especialidade em Biotecnologia Marinha

**Trabalho efetuado sob orientação de**

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FACULDADE DE CIÊNCIAS E TECNOLOGIA

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DECLARAÇÃO DE AUTORIA DE TRABALHO

Declaro ser o autor 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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(Hugo Galvão Caiano Pereira)

The author acknowledges the Portuguese Foundation for Science and Technology (FCT) for funding the PhD fellowship (Grant SFRH/BD/105541/2014).

## ACKNOWLEDGMENTS

At the end of this stage, there is not enough space and words to express my gratitude to everyone who contributed to the accomplishment of the work developed during the last four years. However, I would like to very briefly highlight some of those who helped me on this long quest.

This thesis could not have been brought to completion without the help of my supervisor, Professor João Varela, for all the incredible support and guidance during all these years but above all for being a very good friend.

I am also deeply grateful to my supervisor, Dr. Luisa Gouveia, for her constant positivity in supporting my ideas and work as well as to all the wisdom, friendship and companionship.

I would also like to acknowledge my supervisor, Professor Luísa Barreira, for her guidance, support and knowledge passed on over the years.

To all my colleagues of the MarBiotech group and CCMAR that shared my unavoidable laboratory troubles, Peter Schulze, Carolina Bruno de Sousa, Vera Gomes, Lisa Schüler, Catarina Pereira, Marta Oliveira, Alexandre Ribeiro and others… A special thanks to Tamára Santos for being a real good friend, for all the support in these long four years and for being a key researcher in most of the work carried out in this thesis. To my good friend Dr. Katkam Gangadhar, with whom I have gained most of my lipid chemistry skills, for all the knowledge, support and companionship. Also, to Mariana for the support, motivation and friendship in the last moments of this endeavor.

A sincere acknowledge to all the students and friends that I had the opportunity to train in MarBiotech, Algafarm and Necton, which were fundamental for my personal growth as well as for the work undertaken: Inês Maia, João Salazar, Henrique Carvalho, Ivo Monteiro, Eunice Santos, Ana Raposo, Pedro Leitão, Pedro Quelhas, Mafalda Trovão, Adriana Machado, Joana Silva, Tânia Magina, Bernardo Carvalho, among others.

A special thanks to the entire Allmicroalgae (Algafarm) team with whom I had the pleasure of working daily for more than a year, for having welcomed me as one of them, in a truly incredible experience. A special credit to Jaime, for his friendship and for being a real supervisor of most of the work I have done at Algafarm. Also, to Joana L. Silva for constantly

supporting me during my stay at Algafarm as well as nowadays, but most of all for the friendship and patience.

I want to express my gratitude to the entire Necton team for welcoming me so well. With special emphasis to João Navalho, who was responsible for the opportunity to collaborate with Necton and Algafarm, but also for all knowledge and friendship.

To the entire Sparos team for the help and support during the time I spent in the company. Particularly to Jorge Dias for allowing me to carry out the experiment, for all the wisdom and help with the writing of the scientific manuscript. I am also very grateful to Manuel Sardinha, for all the help throughout the course of this thesis, but above all for being a very good and close friend.

I am particularly grateful to professor Maria Teresa Dinis, who was my master's thesis supervisor, for having guided me on the early stages of my scientific path.

To Dr. Teresa Lamela for all the know how she gave me regarding the production of microalgae, knowledge, that still today I pass to all the students that I have the opportunity to train.

To Professors Sara Raposo and Raúl Barros, for helping me in important stages of the work carried out in this thesis.

To all my dear friends. Although I was probably far away from most of you during this last year, you were always present in my mind.

I am also deeply grateful to my incredible family, for the unconditional support and care since ever. It is my sincere conviction that I could have not been more luckily in having you in my life.

Finally, but not least, I would like to dedicate this dissertation to my mother, father and brother. For being my heroes, the meaning of my life, for all love, for everything!

*“In the attempt to make scientific discoveries,*
*every problem is an opportunity –*
*and the more difficult the problem,*
*the greater will be the importance of its solution.”*

*Edward O. Wilson*

# KNOWLEDGE DISSEMINATION

## In the scope of this dissertation

Papers in international scientific periodicals with referees

1. Pereira, H., Gangadhar, K. N., Schulze, P., Santos, T., Bruno de Sousa, C., Schüler, L., Custódio, L., Malcata, F. X., Gouveia, L., Varela, J., & Barreira L. (2016). Isolation of a euryhaline microalgal strain, *Tetraselmis* sp. CTP4, as a robust feedstock for biodiesel production. Scientific Reports, 6, 35663.
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3. Pereira, H., Paramo, J., Silva, J., Gouveia, L., Barreira, L., & Varela J. (2017). Laboratory, pilot-scale and industrial production of *Tetraselmis* sp. CTP4: Growth performance, culture medium recirculation and harvest of biomass. Algae Europe 2017, Berlin, Oral presentation.
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5. Pereira, H., Páramo, J., Silva, J., Santos, T., Gangadhar, K. N., Barreira, L., Varela, J., & Gouveia, L. (2018). Growth performance, biomass harvesting and life cycle analysis of *Tetraselmis* sp. CTP4 produced in industrial photobioreactors for different biorefinery-based biotechnological applications. 3rd EUALGAE Training School, Florence, Oral presentation.
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7. Pereira, H., Sardinha, M., Santos, T., Barreira, L., Varela, J., Dias, J., & Gouveia, L. (2019). Can residual microalgal biomass (*Tetraselmis* sp. CTP4) replace soybean meal in the aquafeeds of juvenile gilthead seabream (*Sparus aurata*)? Final EUALGAE COST conference, Madrid, Oral presentation.

## THESIS OVERVIEW

The present thesis is divided in eight chapters. The first chapter describes a general introduction and state of the art on the topic (chapter I), followed by six chapters that describe the original research work performed throughout the course of the thesis (chapters II-VII) and a final chapter with the general discussion and conclusions of the work undertaken (chapter VIII).

In chapter I, an extensive bibliographic revision was performed to give a general overview of the microalgal biotechnology field, focusing on the main topics that were addressed in the scope of the present dissertation.

The methodological approach used to isolate the microalgal strain investigated in this thesis, *Tetraselmis* sp. CTP4, was described in chapter II. In addition, the preliminary evaluation at lab-scale of the biotechnological potential of this strain was addressed.

Chapter III refers to the potential of *Tetraselmis* sp. CTP4 for industrial-scale biomass cultivation and for $CO_2$ sequestration applications. The work performed also describes the industrial scale-up of this strain and the operational optimization in tubular flow-through photobioreactors.

Thereafter, a low-cost harvesting procedure was developed for *Tetraselmis* sp. CTP4 focusing on the natural sedimentation properties of these cultures. The work performed describing the procedure and process metrics is described in chapter IV.

An extensive biochemical characterization and toxicological evaluation of *Tetraselmis* sp. CTP4 biomass grown in industrial-scale 100-$m^3$ tubular photobioreactors was performed in chapter V, upon which possible nutritional applications were discussed.

In chapter VI, a novel downstream processing considering a biorefinery approach was described in order to fractionate *Tetraselmis* sp. CTP4 biomass into different streams, followed by their chemical characterization and value upgrade into different bioproducts.

In chapter VII, the residual biomass of *Tetraselmis* sp. CTP4 obtained during the downstream process was tested for its potential as an ingredient in aquafeeds geared towards juvenile sea bream (*Sparus aurata*).

Finally, an integrated discussion of all the original work conducted in the scope of this dissertation as well as the main conclusions achieved, and future perspectives foreseen are given in chapter VIII.

## ABSTRACT

*Tetraselmis* sp. CTP4 was selected from a bioprospection screening as a promising candidate for industrial cultivation and exploitation of different biotechnological applications. At lab-scale, several experiments revealed that this strain is highly robust to environmental conditions, has the ability to accumulate significant amounts of lipids under nitrogen depletion, displays also high growth and sedimentation rates. In collaboration with Allmicroalgae (Algafarm, Secil, Leiria, Portugal), experiments at industrial-scale in 100-$m^3$ tubular photobioreactors showed that this strain is able to attain promising areal productivities, remaining a monoalgal culture throughout the whole trial. Thereafter, a low-cost pilot-scale harvesting system enabled the recovery of 97% of the total biomass by natural sedimentation, reducing the harvesting costs by 93%. Biochemical characterization of industrially produced biomass revealed a high content of proteins and dietary fibres as well as interesting levels of chlorophyll, carotenoids and vitamins, whereas microbial pathogens and contaminants analysed were absent from the biomass. Later, upon the development of a biorefinery platform, the wet biomass was extracted with ethanol and fractionated using a liquid-liquid triphasic system, leading to four different streams: non-polar (NP), colloidal (CP) and water (WP) phases as well as the residual biomass (RB) leftover of the ethanolic extraction. The CP was characterized as a source of high value molecules, while the NP, WP and RB were successfully upgraded into biodiesel, bioethanol and biogas, respectively. The RB was also tested as an ingredient for juvenile seabream, showing to be a promising substitute to replace soybean meal in aquafeeds. Overall, *Tetraselmis* sp. CTP4 was successfully produced at industrial scale, a low-cost harvesting system was established, and the produced biomass had a promising composition suitable for prospective nutritional applications. In addition, the biorefinery approach implemented led to the production of different streams that were effectively upgraded into different bioproducts, namely biofuels and aquaculture feed.



**Keywords:** Biofuels; Biorefinery; Biotechnological applications; Industrial biomass production; Microalgae; Nutrition; *Tetraselmis* sp. CTP4.

## RESUMO

No âmbito de um esforço de bioprospeção de novas microalgas para desenvolvimento biotecnológico realizado pelo grupo MarBiotech (Centro de Ciências do Mar, Universidade do Algarve), a estirpe *Tetraselmis* sp. CTP4 foi selecionada para o cultivo de biomassa à escala industrial e exploração de diferentes aplicações biotecnológicas. À escala laboratorial, ensaios preliminares revelaram que esta estirpe apresenta elevada taxa de crescimento e robustez para tolerar diversas condições ambientais. Adicionalmente, esta estirpe tem a capacidade de acumular quantidades significativas de lípidos, que podem chegar a 33% do peso seco da biomassa produzida, quando as culturas são submetidas a limitação de azoto no meio de crescimento. Estes ensaios revelaram também que a *Tetraselmis* sp. CTP4 apresenta uma composição lipídica muito interessante, e que após a conversão da fração de lípidos em biodiesel, as propriedades do biocombustível produzido estão dentro dos parâmetros regulados pelas normativas europeia e americana.

Dado o elevado potencial desta microalga, foi estabelecida uma colaboração com a Allmicroalgae (Algafarm, grupo Secil, Leiria, Portugal), a maior unidade de produção de microalgas em sistemas fechados da Europa, para avaliar o crescimento das culturas desta estirpe em condições exteriores, utilizando fotobiorreatores tubulares industriais com um volume de 100 $m^3$. Os ensaios realizados no exterior mostraram que foram necessárias oito semanas para realizar o aumento de escala de uma placa de agar até aos reatores de produção industrial. Durante o aumento de escala, realizou-se a otimização da produção em sistemas tubulares à escala piloto. Esta otimização mostrou melhores produtividades de biomassa quando a velocidade de cultura se encontra entre 0,65 a 1,35 m/s e com o valor de pH de 8,0 para injeção de $CO_2$ na cultura. À escala industrial, verificou-se uma adaptação imediata das culturas aos sistemas de produção tubulares com produtividades areais muito promissoras (10-20 $g/m^2/d$) e elevada eficiência fotossintética (3,5% da irradiância solar total), sendo possível manter uma cultura monoalgal durante todo o período de produção (60 dias). A sequestração de $CO_2$ foi seguida no fotobiorreator de 100 $m^3$, revelando uma eficiência média de mitigação de $CO_2$ de 65% e uma relação de biomassa/carbono de 1,8.

Posteriormente, foi desenvolvido um sistema de colheita piloto de baixo custo da biomassa de *Tetraselmis* sp. CTP4, nas instalações da Algafarm. Para este fim, adaptou-se um tanque cilindro-cónico para recolher a biomassa através da sedimentação natural

das células sem custos energéticos. Os ensaios mostraram que após se introduzir a cultura no tanque, as células conseguem sedimentar na parte inferior do tanque após 24 horas, sendo possível recolher o meio de cultura pelas entradas laterais do sistema. Através do processo desenvolvido é possível recuperar 97% da biomassa total por sedimentação natural, sendo que se perdem apenas 3% com a remoção do meio de cultura do sistema. Usando esta abordagem, 93% do volume total da cultura é recuperado do tanque, com um peso seco de 0,07 g/L, o que representa uma redução muito significativa dos custos associados à colheita da biomassa. A restante cultura (7%) é recuperada na forma de uma cultura concentrada e pasta húmida de microalgas com pesos secos aproximados de 20 e 273 g/L, respetivamente.

Na fase seguinte, pretendeu-se avaliar o potencial nutricional da biomassa de *Tetraselmis* sp. CTP4 produzida em fotobiorreatores industriais (100 $m^3$). Para este fim, realizou-se uma avaliação minuciosa da composição bioquímica, microbiológica e toxicológica da biomassa. As análises efetuadas mostraram que a biomassa contém elevadas quantidades de proteína (31,2 g/100 g), fibras alimentares (24,6 g/100 g), glícidos digestíveis (18,1 g/100 g) e cinzas (15,2 g/100 g), mas com baixo conteúdo lipídico (7,04 g/100 g). A biomassa apresentou ainda níveis interessantes de clorofila (3,5 g/100 g), carotenóides (0,61 g/100 g) e vitaminas (por exemplo, 79,2 mg de ácido ascórbico/100 g) e atividade antioxidante. Por outro lado, bactérias patogénicas, metais pesados, cianotoxinas, micotoxinas, hidrocarbonetos aromáticos policíclicos e pesticidas não foram detetados na biomassa produzida. De um modo geral, a biomassa produzida tem uma composição promissora para aplicações nutricionais em humanos e animais.

Subsequentemente, pretendeu-se desenvolver um novo método de processamento da biomassa tendo em conta o conceito de biorrefinaria, de modo a rentabilizar ao máximo todos os componentes bioquímicos presentes na biomassa de *Tetraselmis* sp. CTP4, para produzir diferentes bioprodutos. Neste contexto, realizou-se uma extração com etanol diretamente da biomassa húmida e o extrato resultante foi fracionado usando um sistema trifásico líquido-líquido (LTPS). No final deste processo, a partir da biomassa, obtiveram-se 4 frações distintas, nomeadamente as frações não polar (NP), coloidal (CP) e aquosa (WP), obtidas a partir do extrato etanólico, e a biomassa residual (RB) remanescente da extração etanólica. A fração CP foi caracterizada como fonte de moléculas de valor acrescentado, devido à presença de fosfolípidos e carotenóides, que apresentam uma elevada aplicabilidade para diferentes indústrias. As frações NP, WP e RB foram

convertidas com sucesso em diferentes biocombustíveis, nomeadamente, biodiesel, bioetanol e biogás, respetivamente.

No final desta dissertação, foi ainda realizado um ensaio em colaboração com a Sparos Lda. para testar a biomassa residual como um ingrediente para rações de aquacultura. Neste contexto, foi realizado um ensaio para avaliar o efeito de uma incorporação de 10% de biomassa residual de *Tetraselmis* sp. CTP4, em substituição da farinha de soja em juvenis de dourada (*Sparus aurata*). O ensaio foi realizado durante 61 dias e mostrou que os critérios gerais de desempenho (peso corporal final, índice de crescimento diário, taxa de conversão alimentar e taxa de eficiência proteica), composição corporal total e retenção de nutrientes não foram significativamente afetados pela introdução da biomassa residual. No entanto, a dieta com biomassa residual apresentou valores significativamente superiores nos coeficientes de digestibilidade aparente (ADC) de proteína, energia e fósforo, comparativamente à dieta com farinha de soja. No final, um teste de confinamento agudo mostrou uma resposta de cortisol significativamente menor nos peixes alimentados com a dieta com biomassa residual (120 ± 23 ng/mL) do que naqueles alimentados com a dieta com farinha de soja (160 ± 33 ng/mL). Os resultados gerais mostraram que a biomassa residual de *Tetraselmis* sp. CTP4 pode reduzir as elevadas necessidades de farinha de soja em alimentos para a aquacultura como atualmente se verifica.

Em conclusão, a estirpe *Tetraselmis* sp. CTP4, foi produzida com sucesso à escala industrial, o sistema de recolha de biomassa de baixo custo foi efetivamente estabelecido e a biomassa produzida revelou um alto potencial para aplicações nutricionais. Além disso, a metodologia desenvolvida para o processamento da biomassa tendo em conta o conceito de biorrefinaria, permitiu a produção de diferentes frações que foram posteriormente convertidas em diferentes bioprodutos, nomeadamente biocombustíveis e rações para aquacultura.

**Palavras-chave**: Biocombustíveis; Biorrefinaria; Aplicações biotecnológicas; Produção industrial de biomassa; Microalgas; Nutrição; *Tetraselmis* sp. CTP4.

# TABLE OF CONTENTS

# LIST OF ABBREVIATIONS

AA – Amino acid

AA – Arachidonic acid

ABTS – 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt

AD – Anaerobic digestion

ADC – Apparent digestibility coefficients

AFDW – Ash free dry weight

AIC – Akaike information criterion

ALA – $\alpha$-Linolenic acid

ANCOVA – Analysis of Covariance

ANOVA – Analysis of Variance

Ara – Arabinose

BHT – Butylated hydroxytoluene

BI – Bayesian inference

BOD – Biological oxygen demand

BODIPY – 4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene

CAPEX – Capital expenditure

CC – Cell concentration

CCA – Copper chelating activity

CFPP – Cold filter plugging point

CFU – Colony-forming unit

CMP – Cimentos Maceira e Pataias

CN – Cetane number

CP – Colloidal phase

CTRL – Control

DB – Double bonds

DBU – 1,8-diazabicyclo[5.4.0]undec-7-ene

DES – Deep eutectic solvents

DGI – Daily growth index

DHA – Docosahexaenoic acid

DIC – Differential interference contrast

DMCHA – N,N-dimethylcyclohexylamine

DP – Downstream processing

DPA – Dipropylamine

DPPH – 1,1-diphenyl-2-picrylhydrazyl

DW – Dry weight

EBA – N-ethylbutylamine

EDTA – Eethylenediamine tetra-acetic acid

EFSA – European Food Safety Agency

EPA – Eicosapentaenoic acid

ESI – Electrospray

EU – Europe Union

FA – Fatty acids

FAAE – Fatty acid alkyl esters

FACS – Fluorescence activated cell sorting

FAME – Fatty acid methyl esters

FBW – Final body weight

FC – Flow cytometry

FCR – Feed conversion ratio

FFA – Free fatty acids

FI – Feed intake

FITC – Fluorescein isothiocyanate

FL – Fluorescence

FM – Fishmeal

FP – Flat Panel

FRAP – Radicals, ferric reducing antioxidant power

FSC – Forward scatter

GC – Gas chromatography

GC-qMS – Chromatography-quadrupole mass spectrometry

HDPE – High-density polyethylene

HPH – High pressure homogenization

HPLC – High performance liquid chromatography

HPLC-FD – High performance liquid chromatography-Fluorescence detector

HPLC-RP – High performance liquid chromatography-Reversed phase

HRAP – High rate algal ponds

IAA – Indispensable AA

ICA – Iron chelating activity

ICP-OES – Atomic emission spectrometry with inductively coupled plasma

IL – Ionic liquids

IPA – 2-propanol

IV – Iodine value

LA – Linoleic acid

LCA – Life cycle assessment

LCSF – Long chain saturated factor

LEDs – Light emitting diodes

LNEG – Laboratório Nacional de Energia e Geologia

LTPS – Liquid-liquid triphasic system

MAM – Modified algal medium

MCA – Metal chelating activity

MCMC – Markov Chain Monte Carlo

ML – Maximum likelihood

MP-AES – Microwave plasma atomic emission spectrometry

MUFA – Monounsaturated fatty acids

N+ – Nutrient repletion

N− – Nutrient starvation

NAABB – National Alliance for Advanced Biofuels and Bioproducts

NP – Non-polar phase

NRD – Nutrient content in the reference diet

NTI – Nutrient content in the test ingredient

OD – Optical density

OPEX – Operational expenditure

PAD – Photodiode array detector

PAHs – Polycyclic aromatic hydrocarbons

PBRs – Photobioreactors

PBS – Phosphate buffered saline

PCH – 1,2-propanediol, choline chloride

PER – Protein efficiency ratio

PLE – Pressurized liquid extraction

PMAA – Partially methylated alditol acetates

PMMA – Polymethylmethacrylate

PUFA – Polyunsaturated fatty acids

RB – Residual biomass

ROS – Reactive oxygen species

RSA – Radical scavenging activity

RT – Room temperature

SBM – Soybean meal

SC-$CO_2$ – Supercritical $CO_2$

SFA – Saturated fatty acids

SFE – Supercritical fluid extraction

SSC – Side scatter

TAG – Triacylglycerols

TFA – Total fatty acids

TLC – Thin layer chromatography

TNF-α – Tumor necrosis factor-alpha

TSS – Total suspended solids

UPLC – Ultra-performance liquid chromatography

VLCPUFA – Very long chain polyunsaturated fatty acids

VSS – Volatile suspended solids

WHO – World Health Organization

WMB – Whole microalgal biomass

WP – Water soluble phase

WWTP – Wastewater treatment plant

Xyl – Xylose

YEPD – Yeast extract peptone dextrose

μ – Specific growth rate

# CHAPTER I

# INTRODUCTION

**Parts of this introduction were published as a review paper:**

Pereira, H., Schulze, P., Schuler, L., Santos, T., Barreira, L., & Varela, J. (2018) Fluorescence activated cell-sorting principles and applications in microalgal biotechnology. Algal Research, 30, 113–120.

## 1.1. MICROALGAE

### 1.1.1. GENERAL DESCRIPTION

Microalgae are a group of microscopic eukaryotic microorganisms and prokaryotic cyanobacteria (previously called “blue-green algae”) that are usually able to carry out oxygenic photosynthesis. They are often unicellular, but some of them are colonial and even pluricellular. Throughout evolution, these group of microorganisms colonized almost every known habitat, including aquatic, terrestrial and subaerial environments. They have a cosmopolitan universal distribution and can be found across both fresh and marine ecosystems as well as in extreme environments, such as hydrothermal vents, deserts and polar crusts (Pushkareva et al. 2016). Microalgae assume a crucial ecological role in the Earth’s carbon cycle, being responsible for more than half of the total $O_2$ on the planet and are also responsible for more than 40% of the global $CO_2$ fixation (Hannon et al. 2010; Scott et al. 2010).

Although some microalgal strains retained the capacity to grow heterotrophically, most microalgae grow photoautotrophically. Sunlight is by far the most sustainable source of energy present in our planet, representing about 3800 zettajoules of annual solar energy, which is still poorly exploited (Sayre, 2010). Photosynthetic organisms (including microalgae) only capture and incorporate 0.05% of this energy in the form of biomass (Zhu et al. 2008). Photoautotrophic microalgae are sunlight-driven cell factories able to convert inorganic carbon ($CO_2$) into organic carbon used for growth, through the process of photosynthesis. The organic carbon obtained from the photosynthetic process is afterwards converted through a series of biosynthetic pathways into proteins, lipids, carbohydrates, and nucleic acids that constitute the cells (Figure 1.1). The wide biodiversity of microalgae is responsible for a wide biochemical diversity among strains, which is one of the key reasons why microalgae are so promising for several biotechnological applications.

Because of their outstanding biotechnological potential, microalgae are currently considered as a green alternative to produce foods, feeds, fuels and bioactive molecules with high potential to close the loop of $CO_2$ or phosphorus when flue gases and/or wastewaters are used (Brahmaiah et al. 2017). Nevertheless, the production costs of the biomass still restrict the commercialization of microalgal products, such as pigments,

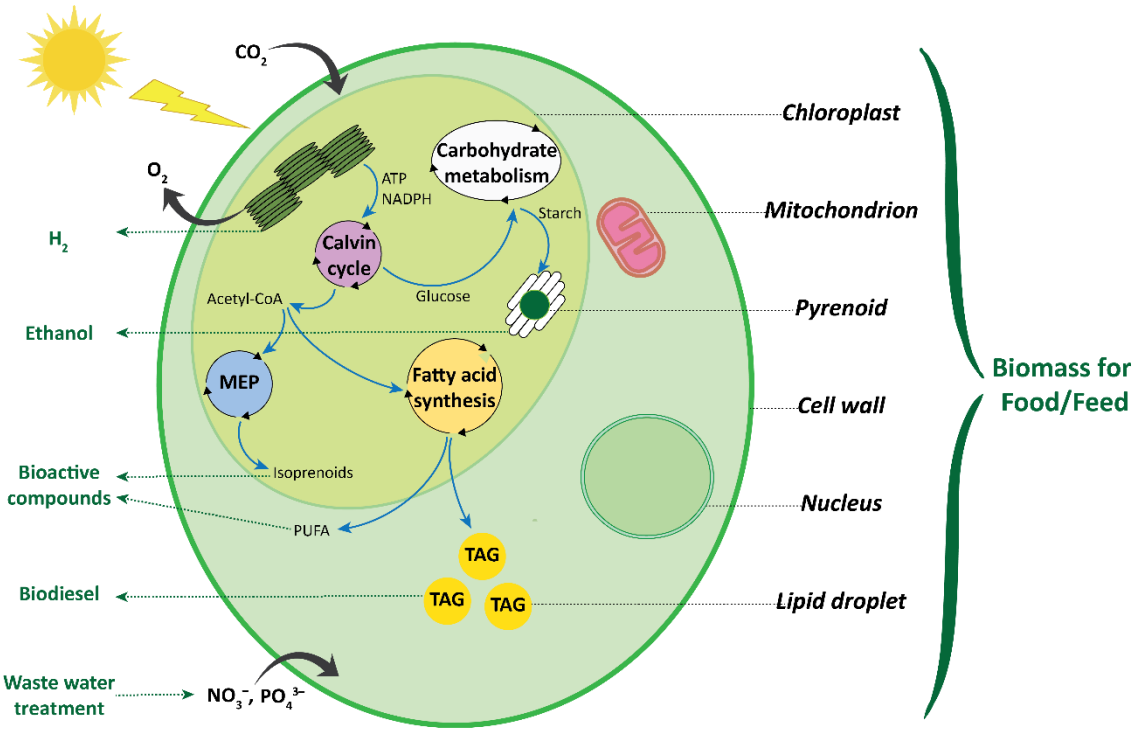


**Figure 1.1 -** Schematic representation of a microalga cell factory. Metabolic pathways for the synthesis of main macronutrients and secondary metabolites that occur inside the cells. The key biotechnological applications obtained from these cellular components are also presented (Raposo, 2017).

fatty acids or whole microalgal cells for niche markets applications, mainly in the food and feed sectors.

Although the high production costs of microalgal biomass have been widely discussed in the recent years, it is noteworthy that production of biomass in industrial scale facilities is still at an early stage. Compared to traditional agriculture, where the first records date from more than 15,000 years ago, the basis of cultivation techniques of microalgae in laboratory was developed in the late 1800's and early 1900's (Preisig and Andersen, 2005), whereas the first commercial production was performed in the 1960's (Spolaore et al. 2006). The industrial scale production was only vastly developed with the expansion of the aquaculture field and the need to mature technologies to grow massive volumes of phytoplankton. This was essential for the aquaculture sector for the rearing of different bivalve species and to enrich the zooplankton needed to produce fish larvae.

The wide biodiversity of microalgae and biotechnological applicability demands a high effort in screening programs for the bioprospection of novel species, in order to fully exploit their industrial potential.

### 1.1.2. BIOPROSPECTING FOR NOVEL STRAINS

The biodiversity of microalgae is largely unexploited concerning the isolation of suitable strains for sustainable biomass and production of different classes of biochemicals. However, the need for novel strains may be long overdue, considering that in 30 years of microalgal biotechnology only ~20 species have been commercially exploited, but the globally available pool of microalgae is no smaller than 72,500 species (Chu, 2012; Guiry, 2012; Barra et al. 2014). Among these species, microalgal strains have evolved and adapted to specific environments that led to unique metabolic pathways, which could be used for biotechnological purposes (Mata et al. 2010).

Several techniques and methodologies were developed and optimized for more than one century for the isolation of novel microalgal strains (e.g., single cell isolation, serial dilutions, agar streaking). Nevertheless, these classic isolation techniques are laborious procedures with low throughput, which consequently result in high recovery times to isolate microalgal strains, especially when dealing with a large number of environmental samples. In this context, fluorescence activated cell sorting (FACS) procedures were recently developed for more efficient high-throughput bioprospection of novel microalgae strains for different biotechnological applications.

Flow cytometry (FC) enables the detailed analysis of mono- or pluri-algal populations as well as complex environmental samples. Samples of cellular suspensions are directed through a draining system into the flow cell (Figure 1.2). Inside the flow cell, a narrow stream guides the sample into the sheath fluid (e.g., PBS or culture medium), where cells are coordinated to pass one at a time through a process called hydrodynamic focusing. At the interrogation point, each cell intercepts the lasers and the optic systems collect cell specific data. In microalgal research, the blue (488 nm) and red (633 nm) lasers are usually selected. The absorption and scattering of the light emitted by these lasers, due to the interaction of photons with the sample, is then measured by a series of detectors (see below). When light hits a cell, photons are scattered into multiple directions. Different detectors are then used to measure the light scattered by the cell, such as the forward scatter (FSC) and side scatter (SSC) sensors (Figure 1.3). It is often stated that FSC and SSC can be used to measure the relative cell size and inner cell complexity, respectively (Figure 1.3A). As eukaryotic cells are usually larger and more complex than unicellular prokaryotes, the former tend to generate higher FSC and SSC signals than the latter. For example, organelles, such as nuclei, mitochondria and

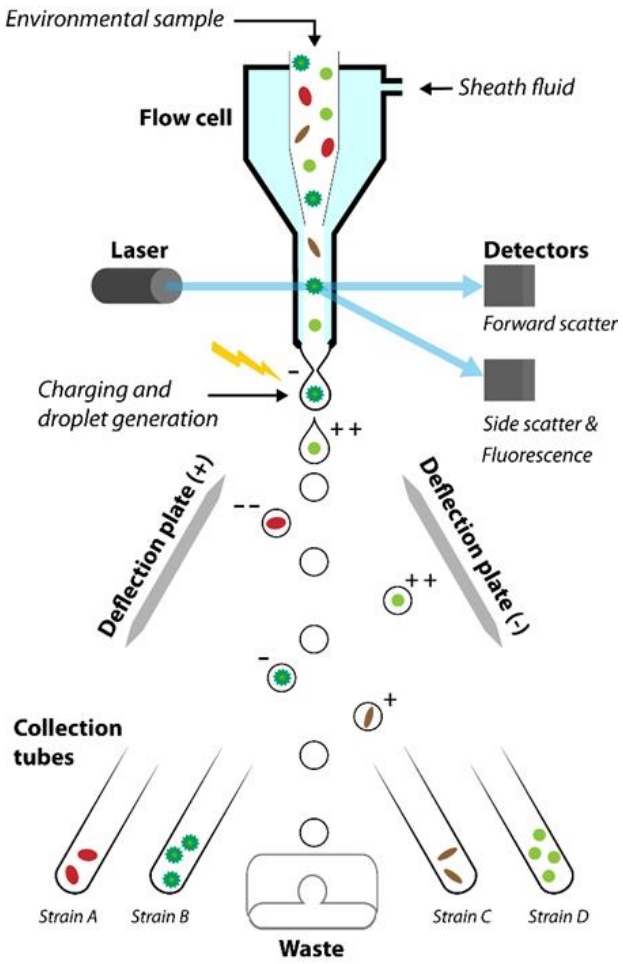


**Figure 1.2 -** Fluorescent activated cell sorting of environmental samples containing microalgae. Cells are restricted to a narrow band by a liquid stream (sheath liquid) in the flow cell. Through high-speed vibration of the nozzle of the flow cell, the liquid is divided into droplets usually containing no more than one cell, which is either positively or negatively charged. The cells are diverted into specific collection tubes by deflection plates according to the type and intensity of the electrical charge.

lysosomes present in eukaryotic cells are known to contribute significantly to higher SSC signals (Figure 1.3A) (Marina et al. 2012). Apart from the light scattering detectors, FC instruments are equipped with a series of detectors that are able to register the emission of fluorescence at distinct passbands (i.e. ranges of wavelengths that can pass through a filter). A key peculiarity of microalgae, which can be used in FC, is the presence of autofluorescent pigments (e.g., chlorophyll, phycoerythrin or allophycocyanin; Dubelaar and Jonker, 2000; Reckermann, 2000). In this way, photosynthetic cells can be distinguished from heterotrophic cells by the (auto)fluorescence of chlorophyll through a 695/40 nm bandpass filter after excitation with a blue laser (Figure 1.3B; Figure 1.4). Subsequently, detected light is converted to an electrical signal with a specific voltage. The generated data can be used to perform multiparametric analysis of all cells within the sample. At a later stage, signals representing events (e.g., a cell, salt grain or debris

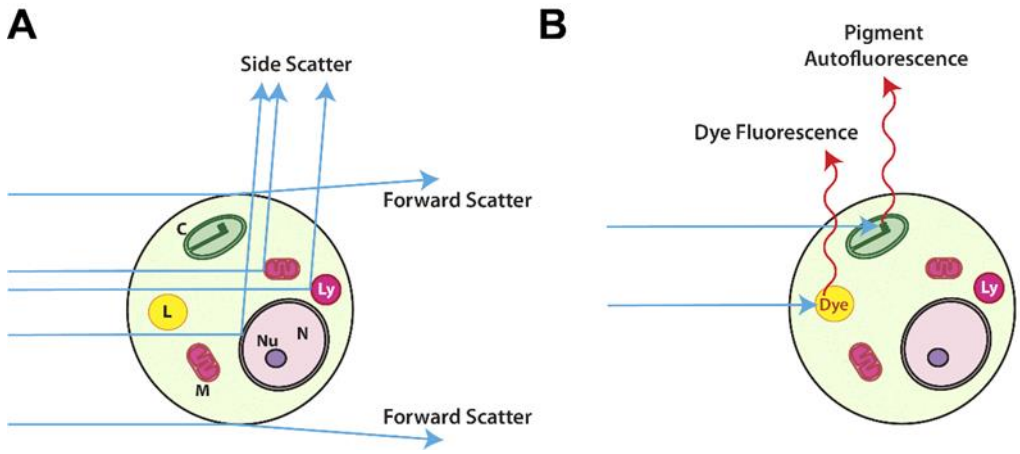


**Figure 1.3 -** Light scattering (A) and fluorescence (B) detected by flow cytometry. The cell wall was omitted for simplicity's sake and because there are microalgae that do not possess this cell covering. Pigment autofluorescence is N, nucleus usually detected by photomultipliers at high angles. C, chloroplast; L, lipid body; Ly, lysosome; M, mitochondrion; Nu, nucleolus.

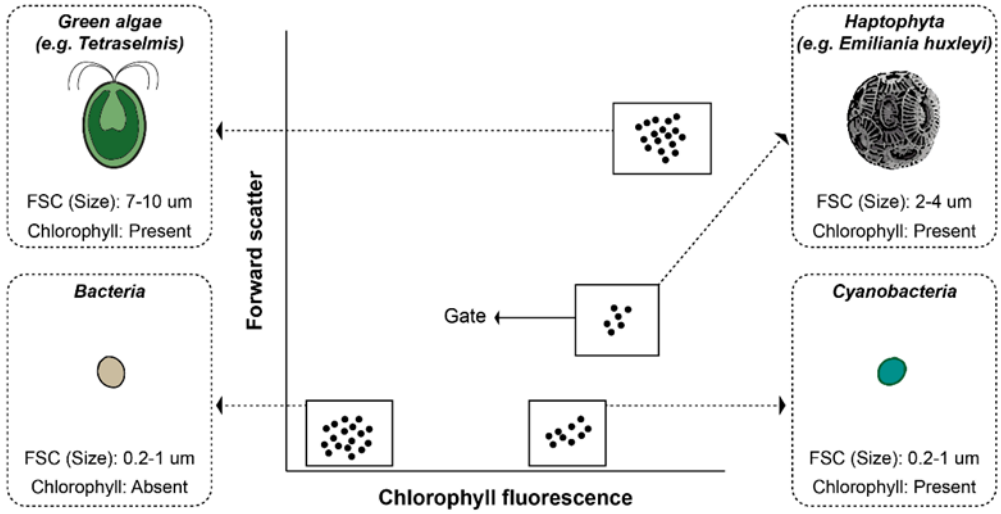


**Figure 1.4 -** Simplified schematic representation of a dot plot and gating procedure of an environmental sample. This example simulates the distribution of events of an environmental sample containing bacteria, cyanobacteria, *Emiliania huxleyi* and *Tetraselmis* sp., according to the signal obtained from the forward scatter (FSC), which often correlates with the relative cell size (y axis), and the chlorophyll autofluorescence (x axis). The squares represent the gates that could be used to discriminate the different populations acquired, allowing their isolation by fluorescence activated cell sorting (FACS).

particle) detected by the apparatus can be processed via gating on multiple two-dimensional dot plots (Cirulis et al. 2012), which allow the selection of subpopulations with shared properties in a multidimensional parameter space.

Through high-speed vibration of the nozzle of the flow cell, the liquid stream is split into droplets containing individual cells that have been selected by the gating procedure. As the apparatus recognizes a cell defined in the gate(s) at the interrogation

point, an electrical charge is later applied to that specific event when the droplet is generated in the nozzle. Afterwards, the stream of droplets passes through the deflection plates, and the droplets containing the cells are deviated to the desired collecting system based on the charge previously applied (Figure 1.2). Because of the multiparametric combination of individual cell properties gathered from the detectors (i.e., relative cell size, inner cell complexity and autofluorescence), distinct clusters/populations are obtained for different species present in an environmental sample that can be efficiently sorted to different sample collection tubes (Figure 1.4). Using the autofluorescence of chlorophyll, the non-photosynthetic cells naturally present in the environmental samples can be easily discriminated from photoautotrophs (Sensen et al. 1993). In addition, environmental samples can be stained with dyes in order to target a biochemical property of interest, as successfully achieved with Nile Red and BODIPY for the isolation of strains with promising features for lipid production (Doan et al. 2011; Pereira et al. 2011, 2013, 2016).

A successful approach to bioprospect for lipid-rich microalgae has relied on the use of a pre-enrichment step for promoting the isolation of fast-growing cells, which are able to withstand competition by other algae (Pereira et al. 2011; Larkum et al. 2012; Neofotis et al. 2015). Coupled with the use of BODIPY, the isolation effort is directed to cells or populations that in fact hold promise for biofuel production (Pereira et al. 2011; Larkum et al. 2012). FACS-based approaches allowed for shorter cell recovery times and higher culture growth rates, two developments that are crucial for the effective application of FACS to microalgal biotechnology in order to obtain fast-growing, lipid-rich microalgae (Pereira et al. 2011, 2016; Larkum et al. 2012).

The advantages of FACS for bioprospecting novel microalgae are obvious; cell sorting instruments enable screening procedures that lead to the isolation of multiple novel strains within few hours. In fact, over the last few years, several screening programs dedicated to microalgal bioprospection have been successfully implemented. For example, 57 unialgal dinoflagellate and raphidophyte cultures were isolated by Sinigalliano and co-workers (2009). On another screening program, 96 strains were isolated from Singapore coastal waters, from which 21 were further studied for their growth and lipid productivities (Doan et al. 2011). Elliott et al. (2012) used FACS methodologies to establish a culture collection of 360 microalgal strains from highly diverse ecosystems focused on bioenergy applications. Neofotis and colleagues (2015,

2016) isolated 2465 strains using FACS and traditional plating methods within the National Alliance for Advanced Biofuels and Bioproducts (NAABB), from which 30 potential strains are already being tested for production. Pereira et al. (2011, 2016), isolated 96 strains from coastal and lagoon waters in the South of Portugal, the major output being the isolation of a robust *Tetraselmis* sp. CTP4, which has been tested in an industrial production facility (Allmicroalgae, Secil group, Portugal). A recent screening effort in North Atlantic waters led to the isolation of 149 strains, from which 20 isolates were further assessed for growth rates and content of polyunsaturated fatty acids (PUFA; Steinrücken et al. 2017). Other screening projects using FACS such as the works performed by Surek and Melkonian (2004) bioprospected for several microalgal strains belonging to different phyla, while Wensel et al. (2014) isolated two promising haloalkaline-tolerant microalgae from soda lakes for two-stage cultivation.

In addition, it is noteworthy that the establishment of axenic cultures is a major advantage of FACS for bioprospecting novel microalgae, as it allows the isolation of uncontaminated unialgal cultures via cell sorting and direct plating onto 96-well plates (Sensen et al. 1993; Pereira et al. 2011). This can be key for bioprospecting novel strains when the establishment of axenic cultures is mandatory, in particular when microalgae need to grow under heterotrophic conditions.

## 1.2. BIOTECHNOLOGICAL APPLICATIONS

Microalgal biotechnology has gained increasing attention over the last few decades as a next-generation driver for obtaining food, feed and biofuels and to carry out bioremediation of effluents and $CO_2$ mitigation. As previously noted, the wide biodiversity and distribution of microalgae in nature is responsible for significant biochemical differences among the composition of species and even strains. This diversity turns microalgal biomass into a unique interesting feedstock for biotechnological applications, for the industrial exploitation of proteins, lipids, carbohydrates and secondary metabolites. A simplified scheme (Figure 1.5) denotes the most important general biochemical features of microalgae that are frequently investigated when their use has a biotechnological purpose.

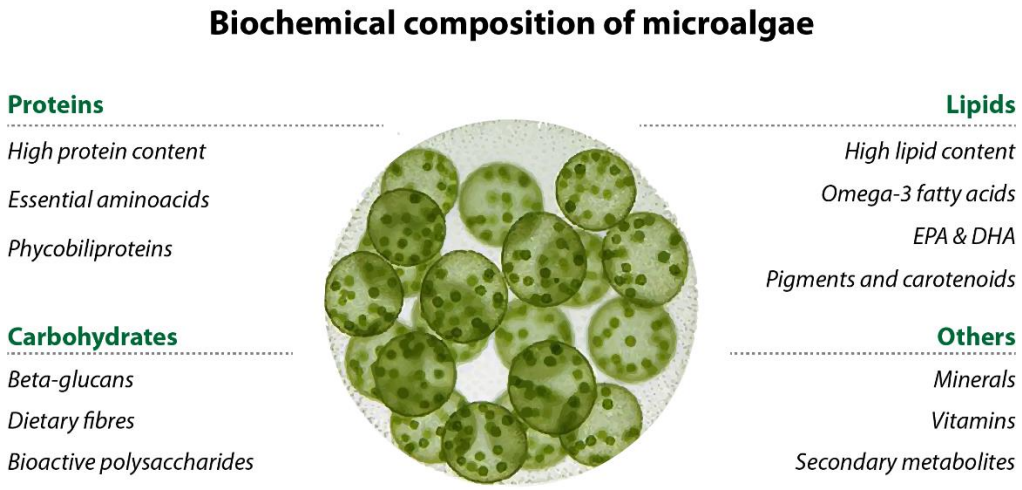


**Figure 1.5 -** General biochemical features that impact the biotechnological value of the microalgal biomass. EPA – Eicosapentaenoic acid; DHA – Docosahexaenoic acid.

With the growing funding of research on microalgae-based feedstocks, several novel innovative bioproducts obtained from microalgal biomass were developed for different applications, and some were already introduced in the market (e.g., Encapso® drilling oil and Thrive® cooking oil).

The expansion of the field in the last decade, supported by academia and industrial players, demonstrated that the biotechnological applications of microalgae biomass are almost limitless. An outstanding example on the development of microalgal biotechnology is the wide report of processes and technologies recently established to produce bioplastics from microalgal biomass (Rahman and Miller, 2017) as well as the potential for agricultural products, such as stimulants for germination and growth of plant crops, biofertilizers and pesticides (Bhalamurugan et al. 2018).

Actually, these are exciting times for microalgal biotechnology, as novel concepts and products are emerging at an ever-faster pace, almost on a daily basis. Because of the increasing applications of microalgal biomass, this section will focus on the biotechnological applications addressed in the following chapters of this dissertation, namely: human and animal nutrition, biofuels, high value applications, $CO_2$ sequestration and bioremediation.

### 1.2.1. NUTRITIONAL AND NUTRACEUTICAL APPLICATIONS

The use of microalgae biomass for human and animal nutritional applications as well as for nutraceutical ends started several decades ago and is currently a well-

established market. In this section, an overview of the history of the use of microalgae in nutraceuticals and in food and feed products and their novel emerging applications is discussed.

Although cyanobacteria have been exploited for thousands of years by indigenous populations for food consumption, efforts to industrially explore microalgae biomass for human nutrition only started in the early fifties (Jensen et al. 2001; Spolaore et al. 2006). In fact, effective commercial production of microalgae for food was only achieved in 1960's, 1970's and 1980's, by the Japanese company Nion producing *Chlorella* (Taipei, Taiwan), the Mexican company Sosa Texcoco S.A. producing *Arthrospira* (Mexico City, Mexico), and two Australian companies producing *Dunaliella salina* (Western Biotechnology and Betatene), respectively (Spolaore et al. 2006).

Microalgal biomass was mainly introduced in the food market as a natural food colorant or as a healthy supplement able to enhance conventional food products, and currently incorporated in several food products available in most countries (e.g., bars, pasta and cookies; Figure 1.6). They are also widely commercialized worldwide in the nutraceutical sector as food supplements, in the form of tablets and capsules (Becker,

**Figure 1.6 -** Microalgae powder and different food products manufactured with microalgae. All pictures were kindly provided by Allmicroalgae Natural Products S.A.

2004), with new commercial products appearing at an astounding rate (e.g., Spirulysat®). More recently, the application of microalgae in other food sectors bloomed, with novel products entering the beverage market (e.g., Springwave®) and other speciality markets. For instance, the first algae cooking oil (Thrive®) was successfully introduced in the USA market, and is already available in well-known retailers.

Regarding animal nutrition, the aquaculture sector alone is a fast-growing industry with an expected consistent yearly growth around of 4-5% (FAO, 2018). The first application of microalgae in the aquaculture sector was dated to the early 1970's (Bardach et al. 1972; Pulz and Scheibenbogen, 1998). The importance of microalgae for the aquaculture industry is inevitable, as microalgae are on the base of the natural food web of aquatic ecosystems. They are essential for the rearing of filter feeders (molluscs), shrimps and to enrich the zooplankton necessary to produce most fish larvae. The importance of microalgae in the rearing of most aquatic animals, is related with the supply of different nutrients that are essential for an effective development in the early growth stages. For such applications, the microalgae biomass is used directly (live or concentrates) in the production systems; however, the feed industry is nowadays looking at microalgae as possible macro-ingredient or as a feed additive that can be incorporated into the diets. The interest in microalgae for feed purposes was triggered by the increasing awareness of the dependence of the feed sector on non-sustainable feedstocks (e.g., fish/soybean meal and oils). Research efforts focused on finding innovative and sustainable sources of feed ingredients and several manufactures targeted microalgae as a promising venue to supply the high demand for proteins and oils in the near future. Nowadays, feed ingredients processed from microalgal biomass are a growing trend and some products are already commercialized (e.g., AlgaPrime™ DHA and Veramaris™) by key players of the industry.

The biochemical composition of microalgal biomass is the most important feature when developing food or feed ingredients. In addition, the biochemical stability of microalgal biomass produced at industrial scale is another important factor, which still poses a significant challenge that must be overcome in the near future. The chemical composition of microalgal biomass is known to vary from batch to batch, depending on the biotic (e.g., strain-specific genetics and associated microbiome) and abiotic (e.g., light, temperature, culture medium and reactor) factors (Becker, 2004). A brief overview

of the nutritional and nutraceutical applications of microalgal macronutrients and most important secondary metabolites is given below.

#### 1.2.1.1. Protein

The human population is expected to grow significantly in the forthcoming decades; some estimations project that the total population can reach 50 billion in 2050 (Austic et al. 2013). The growing world population has raised the awareness of insufficient protein supply, both for food and feed production, which led to a high research investment to find novel, alternative and unconventional protein sources (Becker, 2004).

In this context, protein sources used for human and animal nutrition must provide all the essential amino acids that humans and animals cannot synthesize. The amino acid profile reflects the nutritional quality of a protein source, in order to supply all essential amino acids in the diet. Similarly to terrestrial plants, microalgae synthesize all amino acids, coupling a high protein content with a balanced amino acid profile. Particularly freshwater strains can reach consistently 50-70% of biomass dry weight (DW), which is one of the main reasons to consider microalgae as very a promising single cell protein. *Chlorella vulgaris* and *Arthrospira platensis* (formerly known as *Spirulina platensis*) are two microalgal strains widely known for their high content of proteins and essential amino acids, which are marketed worldwide as a food supplement. Although the protein content of marine microalgae is lower than that of meat feedstocks, several strains contain values much higher than those observed in vegetable and emerging protein sources. For instance, *A. platensis* typically shows lower contents of some essential amino acids (methionine, cystine and lysine) than those observed for meat, eggs and milk but higher than most plant sources (Habib et al. 2008). Overall, microalgal biomass is currently considered as one of the most promising and sustainable feedstocks to support the future protein demand for the food and feed sectors.

#### 1.2.1.2. Lipids

The depletion of fish stocks raised the alarm on the future supply of PUFA for both human and animal nutrition, especially the very long chain PUFA (VLCPUFA), eicosapentaenoic (C20:5*n*-3; EPA) and docosahexaenoic (C22:6*n*-3; DHA) acids. The

replacement of fish oil, as a widely used commodity, is a global challenge, since these *n*- 3 PUFA are mainly found in marine species. In the last decades, in order to reduce the dependence on fish oil, research efforts focused on assessing different vegetable alternatives as lipid feedstocks, such as soybean and palm tree. However, as these feedstocks were being introduced in the market, high sustainability concerns were raised, as mass scale production of such feedstocks triggered the deforestation of tropical areas in third world countries. In this context, microalgae were proposed as a more sustainable lipid feedstock that can supply the demanding markets of human and animal nutrition.

From a nutritional point of view, the interest in microalgal biomass as a lipid feedstock is related with the high amounts of *n*-3 PUFA present in several marine species. These long chained carboxylic acids with two or more double bonds are present in all organisms and are important energy resources and indispensable nutrients for survival and growth (Kihara, 2012). $\alpha$-Linolenic (C18:3n-3; ALA) and linoleic (C18:2n-6; LA) acids are the pathway precursors of all *n*-3 and *n*-6 PUFA biosynthesis (Pereira et al. 2012). As vertebrates are unable to synthesize ALA and LA, these fatty acids (FA) are among the most important PUFA required in both food and feed, and thus they must be obtained through diet (Castro et al. 2012). Similarly, EPA and DHA acids are of the utmost importance for an adequate nutrition. In humans, the elongation of ALA into EPA and DHA is limited, 8% and 21% for EPA and 4% and 9% for DHA in men and women, respectively (Emken et al. 1994; Burdge et al. 2002a, 2002b, 2003). Therefore, it is important to supply an adequate amount of VLCPUFA through the diet. The PUFA composition in animal feed has a strong impact on the quality of food ingested by humans and may have consequences in the overall health of human populations (Bourre, 2005). Different microalgal strains possess high contents of EPA and DHA, and production of microalgae oils rich in PUFA is a high value market. Microalgae-based products rich in EPA and DHA are already commercialized worldwide for food (e.g., AlmegaPL®) and feed (e.g., AlgaPrime™ DHA) applications, and several more are expected to reach the market in the upcoming years.

#### 1.2.1.3. Carbohydrates

Carbohydrates are a complex group of molecules that includes sugars, starches and fibres. Many microalgae strains, as most vegetable feedstocks, present high levels of carbohydrates, as these are the main product of the photosynthetic process. Carbohydrates

are used by the cells as energy and carbon skeleton storage components as well as structural components in their cell coverings (e.g., scales, thecae, coccoliths, and cell walls). The most common carbohydrates found in microalgal biomass are found in the form of simple sugars (e.g., glucose, galactose, rhamnose, xylose, and mannose) and polysaccharides, such as cellulose, glycogen, and starch (Becker, 2004; Nakamura et al. 2005, Markou et al. 2012). The absence of hemicellulose and lignin in microalgal biomass promotes a high digestibility of the overall carbohydrates, suggesting that their introduction in both human and animal nutrition has no significant restrictions (Becker, 2004; Spolaore et al. 2006; Carrieri et al. 2010).

Although the wide trend in using microalgae for nutritional applications is not for their carbohydrate production, the introduction of bioactive carbohydrates, such as *β*-glucans, for nutritional ends has gained increasing interest. Several microalgal strains accumulate significant amounts of *β*-glucans as primary metabolites, with different chemical structures, as for example *Euglena gracilis* (paramylon), *Chlorella vulgaris* (zymosan) and *Phaeodactylum tricornutum* (chrysolaminarin). The effect of *β*-glucans on the immune status and disease control is well documented in mammals, fishes and invertebrates (Soltanian et al. 2009). The immuno-modulating effects of *β*-glucans are related with their capability to activate the immune system, namely macrophages and neutrophils (Gantner et al. 2003; Herre et al. 2004). The immunostimulant effect of *β*-glucans led to a significant investment of companies in developing commercial nutraceuticals from microalgae in the last years. For example, Kemin® recently launched in the market two products from *Euglena gracilis*, namely, BetaVia™ Complete (whole cell) and BetaVia™ Pure (paramylon).

#### 1.2.1.4. Secondary metabolites

Microalgae contain different secondary metabolites with high applicability in human and animal nutrition, such as pigments (e.g., carotenoids, phycobilins), protein (e.g., phycobiliproteins), phytosterols, vitamins, among others (Borowitzka, 2013; Barreira et al. 2015). Most of these compounds display biological activities that are in demand for nutritional and nutraceutical applications, namely as a source of natural antioxidants (e.g., astaxanthin and tocopherols).

The pigment composition (chlorophylls, carotenoids and phycobilins) of microalgae biomass has been widely studied for nutritional applications. Biomass rich in different pigments have a wide applicability and have been exploited and marketed for nutraceutical applications worldwide for a long time. Key examples are the different commercial products successfully established for *Dunaliella salina*, *Haematococcus pluvialis* and *Phaeodactylum tricornutum* rich in β-carotene, astaxanthin (AstaPure®) and fucoxanthin (Fucovital®), respectively. Biomass rich in these carotenoids have a high value and nutraceutical potential, mainly due to the potent antioxidant activity and different biological activities claimed for the different molecules, for example: i) *β*-carotene is known to act as a nontoxic vitamin A precursor (Linan-Cabello et al. 2002), ii) astaxanthin stimulates the immune system and has known anti-inflammatory and anti-cancer properties (Ambati et al. 2014), while, iii) fucoxanthin was consistently reported to display anti-diabetic and anti-obesity properties in *in vitro* and *in vivo* models (Maeda et al. 2009).

Regarding animal nutrition, astaxanthin is also of the outmost importance for the pigmentation of salmon and shrimp in the aquaculture sector. As fish cannot synthesize astaxanthin *de novo*, the pigment must be added to the commercial diets to allow an adequate pigmentation (Dominguez et al. 2005). Another striking example of the application of pigments for feed, is the use of *Phaeodactylum tricornutum* biomass rich in fucoxanthin in the diet of gilthead seabream (*Sparus aurata*). Results obtained in a previous study showed that a finishing diet containing *P. tricornutum* enhances the skin pigmentation of seabream leading to a higher commercial value of farmed fish (Ribeiro et al. 2017). Similar successful skin pigmentation results were observed using *Spirulina* and *Haematococcus* in the diets of *Pagrus pagrus* (Chatzifotis et al. 2011).

Another important group of secondary metabolites for nutritional and nutraceutical applications are phycobiliproteins. These protein-pigment complexes, encompasses different water-soluble molecules, namely, phycoerythrin, phycocyanin, and allophycocyanin. Phycobiliproteins are produced by several groups of microalgae, including, cyanobacteria, rhodophytes and some cryptophytes. In the last years, with awareness being raised on the toxicity of synthetic food colorants, phycobiliproteins gained increasing importance in the development of non-toxic and non-carcinogenic natural food colorants (Manirafasha et al. 2016). In addition, apart from the food colouring properties, the therapeutic potential of phycobiliproteins as nutraceutical agents

is also well established (Pandey et al. 2013). Nowadays, nutraceuticals based on microalgae phycobiliproteins are widely marketed worldwide, as for example, Spirulysat®, a 100% phycocyanin solution extracted from *Arthrospira*.

Vitamins are important secondary metabolites produced by microalgae with wide potential for human and animal nutrition (Fábregas and Herrero, 1990). The total vitamin content of microalgae is normally higher than those observed for higher plants, as for example for soybean and cereals (Buono et al. 2014). Microalgae are natural producers of all essential vitamins (A, $B_1$, $B_2$, $B_3$, $B_5$, $B_6$, $B_7$, $B_9$, $B_{12}$, C, E); however, most strains present higher amounts of niacin ($B_3$), ascorbic acid (C) and tocopherol (E). Recently, *Anabaena cylindrica* was proposed as a promising strain to produce vitamin $K_1$ (phylloquinone) for nutraceutical applications, able to provide the daily adult intake with only 1 g of dried biomass (Tarento et al. 2018). The introduction of microalgae biomass as a source of vitamins for human and animal nutrition is already in place, as this is one of the strongest arguments used by microalgae companies. In addition, the use of microalgae rich in tocopherol and other vitamins as a food preservative, is also expected to gain increasing relevance in the nutrition field, as the growing demand for natural antioxidants takes place (Weel et al. 1999).

Microalgae contain interesting contents of phytosterols (e.g., cholesterol, ergosterol and campesterol), with wide potential for nutritional applications. A wide variety of sterols are found among the different microalgae classes (Volkman, 2003), which surpasses the diversity found in land plants (Ponomarenko et al. 2004). The content of phytosterols in microalgae normally varies from 0.4-2.6% of DW, and *Pavlova lutheri* and *Tetraselmis* sp. M8 are apparently promising strains for their exploitation (Ahmed and Schenk, 2017). The potential of phytosterols for nutritional applications relies on their known effect in reducing blood cholesterol and preventing cardiovascular diseases (Luo et al. 2015). In addition, phytosterols are known to display different biological activities in mammals, such as, antioxidant, anti-inflammatory, anti-cancer activities as well as in the prevention of some nervous disorders (Luo et al. 2015; Ahmed and Schenk, 2017). Overall, due to the increasing interest in phytosterols for nutritional and nutraceutical ends, microalgae can be a key solution to meet the future market demands.

### 1.2.2. PHARMACEUTICAL AND COSMACEUTICAL APPLICATIONS

Microalgae are known sources of molecules able to enhance human welfare, and their use for therapeutic purposes has gained increasing attention in the last decade, through intensive screening programs performed worldwide. These drug discovery programs supported by high throughput screening methodologies, unravelled numerous novel bioactive molecules with promising biological activities that hold high potential for pharmaceutical applications. Several biological activities were already described in different microalgae species, such as antioxidant, antibiotic, antifungal, antiviral, antidiabetic, antitumoral, anti-inflammatory and neuroprotective properties (Guedes et al. 2011; Custódio et al. 2012; Borowitzka, 2013). Although it is not possible to cover all the biological activities and compounds previously reported in microalgae, a brief description of the most relevant activities for pharmaceutical applications and potential as feedstock for cosmeceutical applications is given below.

The antibiotic, antifungal and antiviral activities, are among the most studied biological activities, and are well documented for several microalgae strains. Research efforts in this field are of vital importance, and the increase of multidrug-resistant bacteria and drug-resistant viral variants urge researchers to find novel agents with distinct biochemical mechanisms of action (Amaro et al. 2011). In this context, several screening efforts carried out using microalgal extracts revealed a high amount of positive hits (Lauritano et al. 2016; de Vera et al. 2018). However, although strong inhibition of microbial growth is commonly observed *in vitro* upon the application of these extracts, only a very limited number is active in *in vivo* models (Borowitzka, 2011). The chemical structures of metabolites with antibiotic, antifungal and antiviral activities vary widely; and terpenoids, fatty acids, alkaloids, peptides and polysaccharides were frequently reported as the most active molecules (Borowitzka, 1995).

The search for microalgal metabolites with cytotoxic, antitumoral and antineoplastic activities has been long overdue, as cancer is one of leading death causes worldwide. There is a wide number of reports focusing on the anti-cancer activity of microalgae. *Chlorella ellipsoidea*, *Chlorella sorokiniana*, *Chaetoceros calcitrans*, *Amphidinium carterae*, *Heterocapsa psammophila* and *Skeletonema marinoi* are among the several strains reported to have metabolites effective against different cancer cell lines (Kwang et al. 2008; Nigjeh et al. 2013; Samarakoon et al. 2013; Goh et al. 2014; Shah et al. 2014; Lauritano et al. 2016; Martínez-Andrade et al. 2018). Despite the enormous

potential of microalgae in this challenging field, up to now there are still no compounds from microalgae in the cancer drug discovery pipeline. Nonetheless, astaxanthin and *β*-carotene are two widely known carotenoids reported to deliver high cytotoxicity against different cancer cell lines (Gloria et al. 2014; McCall et al. 2018).

The anti-diabetic activity of microalgae was also widely studied, and several strains have shown interesting activities, both in *in vitro* and in *in vivo* models (Nuño et al. 2013; Lauritano and Ianora, 2016). Screening efforts carried out by different authors identified *Chlorella* sp., *Chlorella zofingiensis*, *Chlorella protothecoides*, *Nitzschia laevis* and *Isochrysis galbana* as promising strains for the development of anti-diabetic pharmaceuticals (Sun et al. 2010, Ingebrigtsen et al. 2015; Lauritano and Ianora, 2016). The carotenoids and PUFA present in these microalgae strains are apparently the compounds responsible for the inhibition of important enzymes related with diabetes control (e.g., protein tyrosine phosphatase 1B, α-amylase and α-glucosidase) as well as in the prevention of the accumulation of advanced glycation end-products in the ARPE-19 cell-based model (Sun et al. 2010, 2011; Su and Chen 2012; Ingebrigtsen, 2015; Lauritano and Ianora, 2016).

Microalgae have also been reported as a promising source of anti-inflammatory compounds. Metabolites with anti-inflammatory are of extreme importance for pharmaceutical purposes, because inflammation is highly associated with different chronic illnesses as, for example, cardiovascular disease, diabetes, cancer and arthritis (Allen and Barres, 2009; Pangestuti and Kim, 2011). Different screening works assessed and identified several microalgae species that hold potential for the development of anti-inflammatory pharmaceuticals (Samarakoon et al. 2013; Lauritano et al. 2016). One key example is the potent anti-inflammatory activity of the polysaccharides of *Porphyridium* sp. inhibiting the tumour necrosis factor-alpha (TNF-α) inflammation in human coronary artery endothelial cells (Levy-Ontman et al. 2017). Soontornchaiboon and co-workers (2012) also reported that violaxanthin isolated from *Chlorella ellipsoidea* displayed interesting anti-inflammatory properties in lipopolysaccharide-stimulated RAW 264.7 mouse macrophage cells.

Conversely, the neuroprotective potential of microalgae was only investigated to a limited extent. Neurodegenerative disorders (e.g., Alzheimer and Parkinson diseases) are a group of diseases with very limited effective therapeutic agents, which mostly alleviate the neuropsychiatric symptoms associated with the disease. In this context, some

authors investigated microalgal extracts as a source of neuroprotective molecules, mainly related with the inhibition of cholinesterases and tyrosinase, which are enzymes known to be implicated in Alzheimer's and Parkinson diseases, respectively. In this context, several microalgal species, including different chlorophytes, rhodophytes and eustigmatophytes, were considered as valuable sources of neuroprotective compounds, able to effectively inhibit acetylcholinesterase, butyrylcholinesterase and tyrosinase (Custódio et al. 2012, 2014, 2015; Pereira et. al. 2015).

The increasing awareness of consumers to the potential harmful of chemical ingredients, natural based cosmetic ingredients, has gained increasing interest in this industry. Microalgae have recently attracted significant relevance in the development of different cosmetics, mainly in skin health and beauty. The interest in microalgae whole cells, extracts and ingredients as feedstocks for cosmetics, relies on the presence of natural molecules as for example, antioxidants and anti-inflammatory compounds. Different metabolites present in microalgal biomass are known to prevent oxidative stress and protect skin from sunlight-induced damage and, therefore, promote its natural aging and depigmentation. In addition, antioxidants naturally present in most microalgae are also important to prevent lipid oxidation, preserving the organoleptic properties of developed cosmetics (Wang et al. 2015).

There are several metabolites of microalgae with high relevance for cosmeceutical applications, namely, PUFA, polysaccharides, carotenoids and mycosporine-like amino acids (Ryu et al. 2015). However, conversely to macroalgae, where a well-established large-scale production of cosmetic ingredients (e.g., carrageenan and alginic acid) is in place (Bixler and Porse, 2011), microalgae-based cosmetic applications are still at a young stage of development. Nowadays, some innovative skin care products containing microalgae are already in the market (Jaspars, 2016), as for example, Dermochlorella D/DP®, Protulines®, Algenist®, Pepha®-Tight and Pepha®-Ctive.

### 1.2.3. BIOFUELS

Microalgae have been considered as one of the most promising feedstocks for large-scale production of biofuels, with potential to meet the high demand for transportation fuels in the medium term (Wijffels and Barbosa, 2010). In recent years, several start-up companies and pilot production facilities have been deployed worldwide

aiming the development of microalgae-based biofuels (Chisti, 2013). Nevertheless, commercialization of biofuels from microalgae is still effectively inexistent. Although different technologies to produce different biofuels from microalgae are available, the industrial development is rather challenging (Chisti, 2007) and not economically competitive due to the current high costs of production compared to fossil fuels, restricting the commercialization of biofuels (Georgianna and Mayfield, 2012). A simplified diagram of biofuels that can be obtained from microalgal biomass is presented in Figure 1.7. There are four main groups of biofuels that can be obtained from microalgae biomass: biogas, bioethanol, bio-oil and biodiesel.

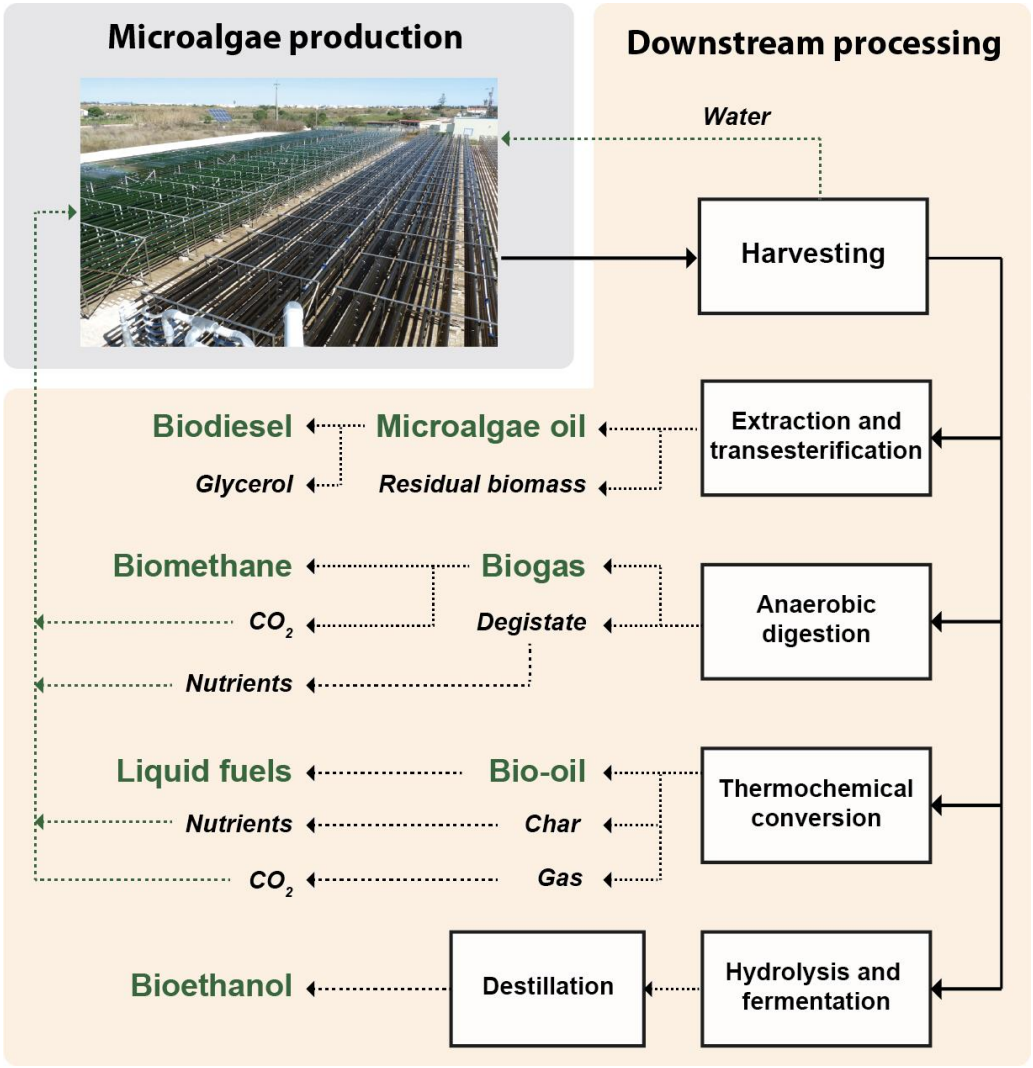


**Figure 1.7 -** Different processes that can be applied to produce biofuels from microalgal biomass, and the by-products obtained during their processing.

#### 1.2.3.1. BIODIESEL PRODUCTION

Biodiesel is a mixture of fatty acid alkyl (i.e., methyl, ethyl or propyl) esters (FAAE), which can be derived from a wide variety of renewable sources, including microalgae. Processing of biomass for biodiesel is commonly performed in two consecutive steps, namely (i) lipid (oil) extraction and (ii) lipid (oil) transesterification (biodiesel) or directly from the biomass *in situ* (Gouveia et al. 2016).

Microalgal lipids useful for biofuel production are mainly present in the form of triacylglycerols (TAG) within distinct lipid droplets inside the cells. However, because of the mechanically robust cell coverings (e.g., thecae and cell walls) of many microalgae a pre-treatment (cell disruption) step prior to lipid extraction might be required (e.g., bead milling, sonication and enzymatic treatment). Extraction of oil from microalgal biomass can be done using methodologies similar to those developed for the extraction of oil from oleaginous seeds of land plants (Ramesh, 2013), although these have been developed and optimized for feedstocks with low moisture content. A thorough explanation of the most common procedures and technologies for an effective extraction of lipids is given in section 1.4. After lipid extraction, TAG need to be converted to FAAE by esterification/transesterification with an alcohol and a suitable catalyst.

The preparation of biodiesel from microalgae oils can be particularly challenging due to the presence of residual water and high amount of free fatty acids (FFA) that can affect the transesterification process (Chen et al. 2012a). When base catalysts are used (e.g., NaOH or KOH) in the presence of water and FFA, soaps are formed and the catalyst performance is negatively affected (Endalew et al. 2011). Soap formation creates serious problems of product separation and ultimately lowers the FAAE yield substantially (Sharma et al. 2008). For this reason, base-catalysed transesterification is not suitable for moisture-containing oil, although the reaction is faster (≈ 4,000 ×) than acid-catalysed reactions (Freedman et al. 1984). Acid catalysts such as $H_2SO_4$, HCl and $H_3PO_4$ are more suitable for wet feedstocks than base catalysts, since they are not affected by the presence of FFA and water and can catalyse esterification and transesterification simultaneously (Zhang et al. 2010; Chen et al. 2012a). Several reports have demonstrated the potential for transesterification of acid-based catalysts in different wet feedstocks. Nonetheless, they have a few cost-related drawbacks, since acid reactions require more energy and acid catalysts are more difficult to separate from homogenous reaction mixtures.

Another possible option is enzymatic transesterification. Biodiesel production using enzymes (e.g., novozyme 435 and lipozyme TL1M) as biocatalysts has several advantages. The use of enzymes is environmentally friendly and requires mild reaction conditions. Moreover, if the enzymes are immobilized, they can easily be separated from the reaction mixture and then reused (Ranganathan et al. 2008; Tran et al. 2012). Similar to acid catalysts, enzymes can operate in the presence of FFA and water, being able to catalyse both esterification and transesterification in a single-step reaction without soap formation. However, enzymatic transesterification has not been adopted industrially yet, because enzymes deactivate easily, and biocatalysts are usually significantly more expensive than inorganic catalysts.

Carbon-based materials such as sugar-based (Toda et al. 2005), glycerol-based (Devi et al. 2009) and residual microalgal biomass-based carbon (Fu et al. 2013) catalysts have been considered as ideal catalysts, because of their water tolerance, reduced price, high surface area, thermal stability and simple preparation protocols. Although carbon-based catalysts are still at an infant stage, the continuous optimization of these catalysts can be key to new developments in biodiesel production. Carbon-based catalysts have the potential to substantially reduce the cost of biodiesel production, because (i) they can be produced from a by-product (glycerol or waste biomass); (ii) they can be used in a continuous process; and (iii) being heterogeneous catalysts, they are easily separated from the reaction mixture (Konwar et al. 2014).

Another approach to prepare biodiesel from wet biomass relies on the combination of lipid extraction and transesterification steps in a single step through *in situ* transesterification. The key advantage of using a single step is the lower amount of organic solvents and energy required, with environmental and cost-effective advantages, in particular when large-scale production is considered (Johnson and Wen, 2009; Ehimen et al. 2010; Xu and Mi, 2011; Sathish et al. 2014; Gouveia et al. 2016). However, established *in situ* transesterification procedures are known for their reduced efficacy in biomass containing high amounts of moisture. Biodiesel yield is known to decrease abruptly with increasing water content, due to lower solvent accessibility, competition for protons and hydrolysis of biodiesel into FFA (Cao et al. 2013; Sathish et al. 2014). However, recent optimization of *in situ* transesterification procedures have been established, enabling single-step processing of biomass containing high moisture content. For example, Cao et al. (2013) reported that increasing the temperature of the reaction

from 90 °C to 120 °C improved the biodiesel yield from 10.3% to 92.4% for biomass containing 90% water. Alternatively, the addition of excess $H_2SO_4$ and methanol to the reaction at standard temperature (90 °C) also improves the biodiesel yield (81%) with biomass containing 84% water (Sathish et al. 2014).

The advantages and disadvantages of *in situ* transesterification was previously extensively reviewed elsewhere (Salam et al. 2016). Although these procedures may decrease solvent use and energy, effective procedures for *in-situ* transesterification of large-scale microalgal cultures are still under development. As these requirements significantly impact the energy balance of produced biodiesel, further studies are needed to address their usefulness in specific production pipelines.

#### 1.2.3.2. OTHER MICROALGAE-BASED BIOFUELS

One popular biofuel that can be obtained from microalgal biomass is biogas which can be generated through anaerobic digestion (AD). AD enables the processing of large amounts of biomass, and the technology is already in place for other sources of biomass and waste materials. This process is applied directly to wet biomass and leads to biogas generation, which is composed of a mixture of methane and $CO_2$. AD of microalgal biomass typically produces biogas consisting of 60% methane and 40% $CO_2$, showing acceptable methane yields in comparison with some other biomass, such as municipal solid waste, fruit and vegetable wastes, grasses, woody biomass, weeds and aquatic biomass (Gunaseelan, 1997). Although the process was proved effective for wet microalgal biomass, the main constraint for usage of microalgal biomass for biogas production is the high production costs compared to other biogas feedstocks commonly used (Benzie and Hynes, 2013). Therefore, AD of algal biomass is currently regarded as a non-cost-effective process.

Bioethanol is the most produced biofuel worldwide, obtained from different raw materials and sugarcane in USA and Brazil, respectively (Hill et al. 2006). The production of bioethanol was previously considered as a promising venue for microalgal feedstocks rich in carbohydrates. As previously stated, microalgae carbohydrates are found in different forms, including fermentable sugars (monosaccharides) and polysaccharides, and their content can reach more than 60% of the biomass dry weight in some microalgae strains (Choi et al. 2010). However, due to the complexity of microalgae polysaccharides

a pre-treatment step is crucial to increase the content of fermentable sugars and allow a proper fermentation process. Thereafter, bioethanol production is performed via sugar fermentation to ethanol using yeast (e.g., *Saccharomyces cerevisiae*), and further distillation to purify and upgrade the produced bioethanol is required (Miranda et al. 2012). Although bioethanol production from traditional feedstocks is cost-effective, the high costs of microalgae biomass are the main hindrance for the commercialization of bioethanol from microalgae (Li et al. 2014).

Thermochemical conversion processes to produce bio-oil are an alternative route for the conversion of microalgal biomass into biofuels, which gained increasing attention in the last decade. Bio-oil is a mixture of long chain hydrocarbons that can be later refined to a variety of fuel products (e.g., gasoline, diesel, and jet-fuel) through hydrocracking, a process already used in the petroleum industry. Thermochemical processes can be applied to dry (e.g., pyrolysis and gasification) or wet biomass (e.g., hydrothermal liquefaction), producing bio-char, bio-liquids and gases. Fermoso et al. (2017) and Barreiro et al. (2013) reviewed the work performed by various researchers regarding pyrolysis and hydrothermal liquefaction of microalgal biomass, respectively. Overall, the energy-intensive nature of thermochemical processes has been one of the drawbacks commented by researchers regarding these technologies (Twaiq et al. 1999; Twaiq et al. 2003; Chisti, 2008). Therefore, further research is needed to demonstrate the robustness of the processes as well as its sustainability in terms of feedstock loading and technical economic viability.

### 1.2.4. $CO_2$ SEQUESTRATION AND BIOREMEDIATION

The increasing concentration of atmospheric $CO_2$ observed in the last 100 years has been a key contributor for global warming, ocean acidification and consequent loss of biodiversity (Lewis and Nocera, 2006; Battisti and Naylor, 2008; Sayre, 2010). Carbon emissions have significantly raised since the beginning of the industrial era as a result of the increasing demand for energy production by the industry development as well as for transportation. In addition, all projections reported by the International Energy Agency indicate that the emissions of $CO_2$ will continue to increase steadily if effective actions are not undertaken. Therefore, in order to address what is currently considered the main environmental threat to our planet, it is of vital importance to develop technologies that can effectively decrease the emissions and accumulation of $CO_2$ in the Earth´s

atmosphere. In this context, there are several established and emerging technologies in place for capturing and recycling of $CO_2$ that hold high potential for industrial application (Quadrelli et al. 2011). From all technologies, biological $CO_2$ fixation is currently considered as the most promising venue for industrial scale $CO_2$ mitigation, both from an economic and environmental point of view (Ho et al. 2011, Kumar et al. 2011, Bhola et al. 2014).

Photosynthetic organisms naturally capture and convert $CO_2$ into the proteins, lipids and carbohydrates that compose their cells. Although higher plants effectively sequester massive quantities of atmospheric $CO_2$ that can be further enhanced with agroforestry strategies (Nair et al. 2009), microalgae are apparently the way forward. One of the main advantages of using microalgae biomass compared to land plants is that they display significantly higher photosynthetic and $CO_2$ fixation rates, normally 10 times higher than those of land plants (Pires et al. 2012). In addition, microalgae do not require arable land for growth, and they can be grown in seawater and wastewater streams, another key advantage as the supply and management of freshwater is another important environmental concern worldwide. It is normally assumed that 50% of microalgal biomass is composed of carbon and that per Kg of microalgae produced 1.8 Kg of $CO_2$ are captured (Chisti, 2007), showing the high potential of microalgae for carbon fixation. Moreover, $CO_2$ capture using microalgae-based technologies can be carried out directly from the atmosphere as well as from industrial flue gases. In the last decades, massive research efforts have been performed in this direction by the academia and industrial sectors. For example, several pilot and industrial scale microalgae production units were constructed in the vicinity of power plants (e.g., Seambiotic, Israel) and cement manufacturing facilities (e.g., Allmicroalgae, Secil group, Portugal), in order to evaluate the performance of microalgae in growing and capturing $CO_2$ from different flue gases. Most studies confirmed the effective growth of microalgae in crude flue gases containing different concentration of $CO_2$ and other combustion products, such as $NO_x$ or $SO_x$, which are used as a source of nutrients for culture growth (Olaizola, 2003). However, the effective capture of $CO_2$ by microalgae from these large emitting industries will require massive areas of cultivation. Therefore, novel developments in microalgae-based technologies are expected in order to enhance the prospects of using microalgae as part of the future solution for effective $CO_2$ recycling as well as for the bioremediation of different effluents.

Combining wastewater treatment with microalgal production has already been researched since 1950's (Oswald et al. 1957) and has received increasing interest in science and industry. Currently, microalgal based wastewater treatment is considered to be an economically and environmentally sustainable procedure to remove dissolved nutrients from effluents and to produce valuable biomolecules to offset water treatment costs. Particularly, the usage of high rate algal ponds (HRAPs) fed by wastewater, and/or $CO_2$ exhausts, is considered as the most promising strategy to clean wastewater streams and produce feedstock for microalgal based by-products such as biofuels, biofertilizers, bioplastics and feed (Park et al. 2011; Craggs et al. 2014; Posadas et al. 2017). Although the main focus of using microalgae has been the removal of nutrients (Christenson and Sims, 2011), the reintroduction of nutrients into the market as transformed bio-products would follow the circular economy principle, a requirement to sustain the present life standards in industrial nations (European commission 2015, IP/15/620). In Europe, on average, 0.51 Kg P and 2.52 Kg N per inhabitant and year are discharged in wastewater (EU-EEA, 2015). These nutrients are valuable and/or finite resources that can substitute expensive fertilizers for production of crops and algae (Vaccari, 2009). Nitrogen and phosphorus are currently removed through treatments involving biological nitrification followed by denitrification or precipitation (US-EPA, 2013). The nitrification step receives ammonia-rich wastewater from a biological oxygen demand (BOD) removal step. Ammonia is transformed into nitrate by nitrifying bacteria under an oxygen-rich environment. Subsequently, the nitrate-rich effluent enters the denitrification step where denitrifying bacteria transform nitrate into molecular nitrogen under anoxic conditions, which is stripped out as gas by gentle aeration. However, denitrifying bacteria require external carbon sources that are often of fossil origin (e.g., methanol). Wastewater with high phosphorus concentrations must be treated through a phosphorus removal step before discharge into protected areas. Here, effluents coming from the denitrification step are supplemented with flocculants (e.g., aluminium salts and lime stone) allowing the precipitation of phosphorus as insoluble salts. However, these procedures present additional costs and can cause deterioration of the biomass quality as feedstock of nutrients in a functional fertilizer. One reason for this is the contamination with toxic, metal-containing flocculants that remain bound to phosphorus (Christenson and Sims, 2011). Moreover, contrary to standard biological treatments, algae were found to further improve the final effluent quality through natural disinfection and incorporation of other contaminants, such as heavy metals, pharmaceuticals and endocrine disrupters (Correa-

Reyes et al. 2007; Devi et al. 2012; Craggs et al. 2014). The separation of microalgae cells from the treated water, however, remains a major bottleneck for the large-scale implementation of microalgal-based bioremediation facilities, as current technologies (e.g., centrifugation and flocculation) for biomass recovery have high costs in terms of energy and/or chemicals (Christenson and Sims, 2011).

## 1.3. INDUSTRIAL MICROALGAL PRODUCTION PIPELINE

The production of autotrophic microalgae biomass requires different inputs, namely, light, water, $CO_2$ and nutrients. However, compared to land plant feedstocks, the production of microalgal biomass normally displays a higher degree of specialization and the production costs are significantly higher.

The whole microalgal production pipeline from strain selection to the final biomass products encompasses three main processes: i) biomass production, ii) biomass harvest, and iii) biomass processing. A simplified diagram of different technologies that can be used to accomplish these main steps as well as the inputs and biomass products that can be obtained is shown in Figure 1.8. In this section, an overview of the most important processes and technologies to effectively grow microalgae focusing on industrial scale facilities is discussed.

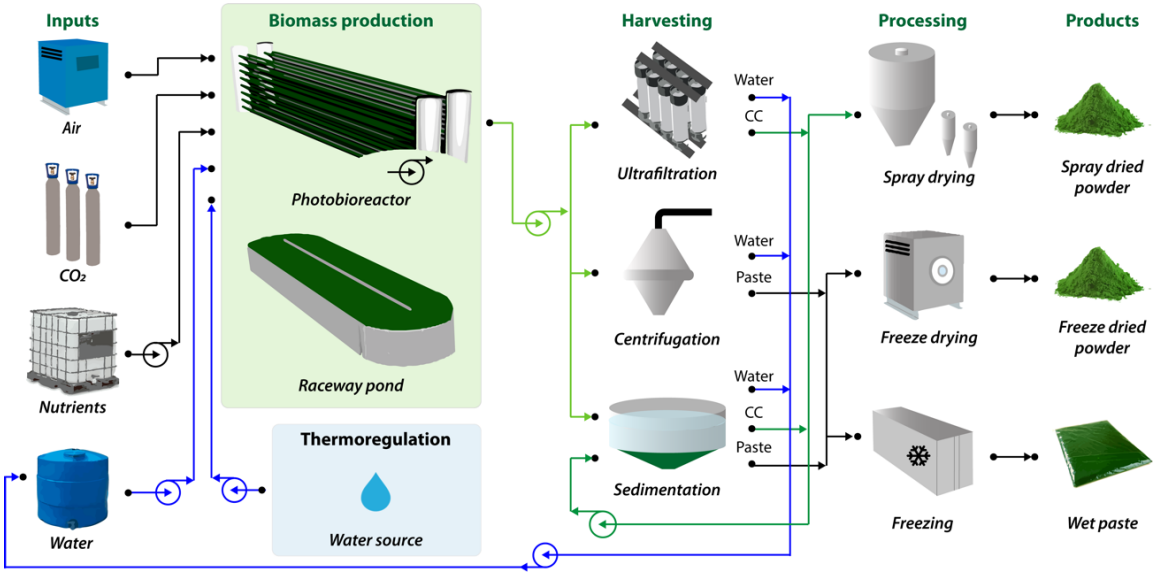


**Figure 1.8** – Schematic representation of a microalgae biomass production pipeline, including the production inputs and possible pathways for biomass production, harvesting, processing and products.

### 1.3.1. MICROALGAE BIOMASS PRODUCTION INPUTS

The light source used in industrial scale photoautotrophic production of microalgae is the sun, and therefore the production facilities should preferably be placed in a solar spot. However, a novel producing facility located in Iceland has successfully been using artificial lighting by means of light emitting diodes (LEDs) as light source. In this way, Algalif™ is able to produce high value products from microalgae all year round, namely, high-grade astaxanthin and *β*-glucans for different biotechnological applications in a weather-independent, stable, predictable manner.

The water source used for microalgae culture is of the outmost importance for successful production in industrial scale facilities and must be free of chemicals that can affect microalgae growth. The water source varies widely depending on the target strain and final biotechnological application. As previously mentioned, microalgae are effectively cultured in fresh-, sea-, brackish and wastewater media. Normally, freshwater microalgae are cultured with ground water, sterilized by mechanical (e.g., ultra-filtration) and chemical (e.g., hypochlorite) means. For marine cultures, the water is normally obtained from the sea (e.g., Necton, Portugal). However, if the company is located inland, synthetic sea water can be prepared and recirculated to efficiently grow most available strains (e.g., Allmicroalgae, Portugal). Industrial-scale production can also be achieved in urban and industrial wastewaters, as successfully demonstrated in the pilot-scale facility of Aqualia in Jerez de la Frontera (Cadiz, Spain).

$CO_2$ solubilization and availability in the growth media is a key parameter in industrial cultivation of microalgal biomass. At industrial scale, the $CO_2$ is commonly controlled by an automatic injection system that feeds the culture with $CO_2$ at a specific pH set-point, as the pH increases as a result of the photosynthetic process. As previously noted, atmospheric and commercial $CO_2$ as well as flue gases from different industries can be used as sources for industrial-scale production. However, most companies producing microalgae for human and animal nutrition as well as for high value applications use commercial food grade $CO_2$ to ensure a final high-quality product and meet certification standards. One commercial source of food grade $CO_2$ widely used for microalgae culturing is the $CO_2$ emitted as a co-product of the brewery industry.

Finally, as for lab-scale cultures, the industrial culture medium used in production facilities must provide all the necessary macronutrients, namely nitrogen, phosphorus and iron as well as silica in the culture of diatoms. In addition, several trace elements (e.g., K,

Mg, Mn and Se) are key to ensure effective growth of most microalgal strains and need to be supplied in the culture medium. Although the use of vitamins (thiamine, biotin and cobalamin) in industrial scale facilities is normally avoided, due to the high costs associated with massive culture volumes, for some high value strains the use of vitamins might be cost-effective. With the growing worldwide trend of producing organic feedstocks for both human and animal nutritional applications, the microalgae industry followed the market trends. In this context, different sources of organic nutrients have been widely tested, developed and successfully implemented in industrial facilities, in order to obtain the necessary certifications (e.g., Allmicroalgae).

### 1.3.2. MICROALGAE BIOMASS PRODUCTION SYSTEMS

In lab-scale systems, cultures are normally grown in glass Erlenmeyer flasks, round flat bottom flasks or tubes, air-lifts and bubble columns, and more recently in different high-tech benchtop photobioreactors (e.g., Algem®). However, the production of microalgae in industrial-scale facilities displays some key particularities that are often overlooked in lab-scale culture systems. With the increasing scale of production, problems arise, being an extraordinary challenge to maintain a culture monoalgal during a long production period. Contaminations are normally easily controlled in low culture volumes, but in industrial facilities many contaminants jeopardize the whole production process. Ensure the sealing of industrial-scale systems (in closed systems) as well as the sterility of the different inputs and water volumes used for culture renewal is highly demanding and non-optimized production protocols display a significant contamination threat. This is particularly important in large-scale facilities producing some of the most known commercial species. Examples are: i) *Chlorella vulgaris*, commonly contaminated with *Vampirovibrio chlorellavorus* and *Poterioochromonas malhamensis* (Ganuza et al. 2016; Ma et al. 2017), ii) *Scenedesums* sp. affected by the endotrophic parasite *Amoeboaphelidium protococcarum* (Letcher et al. 2013), and iii) *Haematococcus pluvialis* parasitized by *Paraphysoderma sedebokerensis* (Gutman et al. 2009). Although industrial-scale production of microalgal biomass is a challenging process, there are several production units, with matured production systems and protocols that successfully produce biomass and are well established in the market (e.g., Necton, Allmicroalgae, Fitoplancton Marino and AlgaTech). There are two main systems widely used to

effectively grow microalgae biomass in industrial-scale production facilities, namely, open and closed systems.

#### 1.3.2.1. Open systems

The production of microalgal biomass in open systems was thoroughly investigated in the last decades (Weissman and Goebel, 1987). Biomass production in open systems (Figure 1.9) was first achieved in open raceway ponds, and later in thin layer cascade systems (Borowitzka, 1999).


**Figure 1.9 -** Open production systems for large-scale production of microalgae at the University of Almeria (Spain). (A) Raceway pond; (B) Thin layer cascade.

Raceway ponds were developed several decades ago (since the 1950's) and consist of horizontal low-depth recirculating ponds that commonly operate with a culture water column of 10-30 cm (Chisti, 2012, 2016). Ponds are normally covered with a liner that can be composed of different materials, namely, clay, concrete, asphalt, fiberglass, and different polymers such as polyvinyl chloride, polyethylene, and polypropylene (Chisti, 2016). The culture is constantly mixed using a rotating paddlewheel, commonly at a velocity of 30 cm/s, which is the main energetic operational cost of raceway ponds. The main advantage of using raceway ponds for microalgal production, is the lower capital and operational costs over thin layer cascades and closed production systems. Therefore, open ponds are the production system chosen to produce microalgae for low-end applications, namely for bioremediation of effluents and $CO_2$ sequestration. However, significant problems in light penetration, mixing and $CO_2$ solubilization in the culture results in low biomass concentrations (0.5-1.0 g/L) and productivities. In addition, since cultures are constantly exposed to the atmosphere and possible contaminants, only a limited number of microalgal strains are able to grow in a challenging environment or

display high growth rates are effectively cultured. Typical examples of successful commercial-scale production in raceway ponds of strains that grow in extreme conditions, where common contaminants cannot proliferate, are *Dunaliella salina* (high salt concentration) and *Arthrospira* sp. (high pH).

Thin layer cascades were developed in Czech Republic in the 1960's by Šetlík et al. (1970). The production system consists of one or more slopped surface platforms, where a thin layer of microalgal suspension flows by gravity and cells perform the photosynthetic process. The name cascade comes from when the culture falls from the end of the slanted surface to a reservoir and the degassing process occurs; thereafter, a pump feeds the culture again to the upper section of the tilted surface. Generally speaking, the construction materials of the surface of thin layer cascades are similar to those used for open ponds, and similarly the main energetic cost associated is the pump used for culture mixing. Because of the optimal light path and high efficiency of the degasification process of thin layer cascade, the biomass concentrations obtained are much higher than those observed for open raceway ponds and most closed production systems, reaching values up to 35 g/L (Masojídek et al. 2011). Moreover, due to the high production rates, more microalgae strains can be cultured, since the production cycles in batch conditions are carried out in a short period of time. However, higher capital and operational costs are observed in thin layer cascades compared to raceway ponds.

Overall, the main advantage of both open production systems is the lower capital and operational costs compared to closed systems; however, since cultures in open systems are constantly exposed to the atmosphere, the culture of more sensitive microalgal strains and with lower growth rates can only be performed in closed systems.

#### 1.3.2.2. Closed systems

Production of microalgae in closed production systems is performed in specialized growth systems, commonly named photobioreactors (PBRs). The cultures in this case are contained from the external environment and there is no direct contact with the atmosphere. There are several designs of PBRs, but three main configurations exist, namely, flat panel, vertical columns and horizontal tubular PBRs (Figure 1.10).

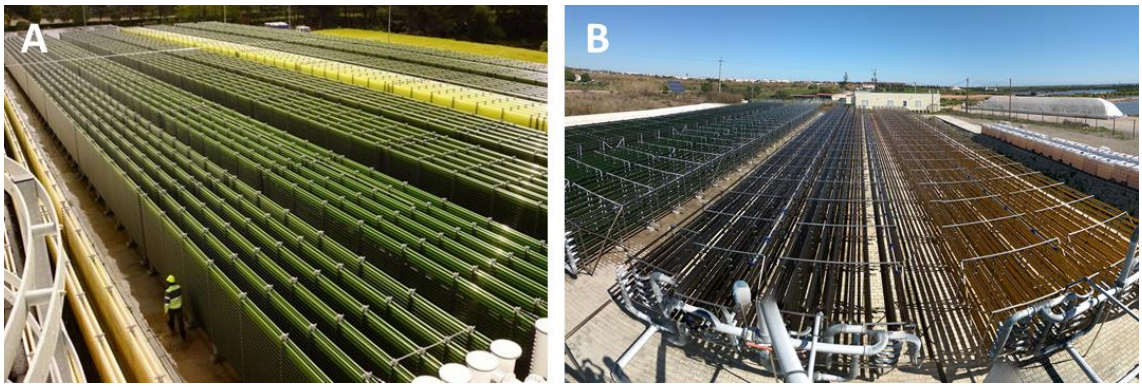


**Figure 1.10 -** Industrial microalgae production units using closed tubular flow through photobioreactors. (A) Algafarm (Allmicroalgae/Cimentos Maceira e Pataias, Secil group) plant located in Pataias (Leiria, Portugal); (B) Necton production plant located in Belamandil (Olhão, Portugal).

Vertical column PBRs (airlifts and bubble columns) are production systems widely used worldwide in aquaculture hatcheries to grow the phytoplankton needed for live prey enrichment. As the name suggests, the system is composed of a vertical column that can be composed of different materials, mainly glass, polymethylmethacrylate (PMMA) and high-density polyethylene (HDPE). The system is lit by artificial or natural light and culture mixing is achieved by injecting air through the bottom of the system (Tredici, 2004). These systems are regarded as cost-effective for small culture volumes and their productivity is dependent on the diameter of the column and consequent light path of the system.

Flat panel PBRs, were highly developed during the last decades and many different configurations are currently available. The reactors are composed of vertically displayed panels, characterized by a high illumination surface area and short light path. Alveolar flat panels were developed in the late 1980's and consisted of plates divided in narrow chambers, where a pump allowed the recirculation of cultures (Tredici, 2004). Thereafter, flat panels evolved to simple glass structures similar to a thin aquarium or HDPE bags supported by a net structure (e.g., Green Wall Panel®) mixed by continuous air bubbling. These flat panels are currently used by several companies for biomass production as well as for the scale-up of cultures from the laboratory to the industrial scale PBRs (e.g., Necton and Allmicroalgae).

Horizontal tubular PBRs are by far the most used closed culture systems for industrial-scale cultivation of microalgae biomass. Nowadays, they are the main system chosen to produce high quality biomass for different high-end applications (e.g., Necton, Allmicroalgae, Algatech and Algalif). Tubular PBRs are composed of two main sections,

the photosynthetic section composed of transparent tubes (glass or PMMA) and a dark section, normally a reservoir where culture degassing occurs. Different pumps are used for mixing, enabling a constant recirculation of the microalgae culture between the photosynthetic section and the degassing reservoir. The different rows of transparent tubes that compose the photosynthetic section can be connected by U-shaped connectors to form a serpentine or by using manifolds (Tredici, 2004). Another configuration of tubular PBRs widely developed in the past are the helical PBRs. Most characteristics of these systems are similar to all tubular PBRs systems, but the sunlit section is made from flexible tubes wrapped around a base metallic framework holding the tubes. Although high productivities were frequently reported in small scale helical PBRs, scaling is challenging and therefore they are not considered as suitable for industrial purposes.

The control of temperature in PBRs is a key process parameter in industrial scale facilities that needs to be effectively performed under hot weather conditions, mainly in the late spring and summer seasons. In order to maximise the biomass production yields, cultures must be kept under a controlled range of temperature. Standard production of most common microalgae strains is carried out between 20-30 °C; however, depending on the target species, different temperature set-points can be used. The thermoregulation of closed systems is normally achieved by using a water sprinkling system located on the higher section of the photosynthetic section of the PBR. Although water spraying is an effective thermoregulation system, it is a process that demands high quantities of freshwater and energy. Shading and heat exchangers can also be used for cooling, but their use at industrial scale can be challenging and is not cost-effective (Tredici, 2004).

The main advantage of closed systems is that contamination of cultures is often prevented and a higher control of most culture parameters, including thermal regulation, is effectively achieved, as previously explained. The effective control of process parameters enables high biomass concentrations and productivities of monoalgal cultures of most microalgal strains, including sensitive species for high-end applications (e.g., pharmaceutical grade). On the other hand, the main disadvantage of PBRs is the high capital and operational costs associated with these specialized culture systems, when compared to open production systems. Another constraining factor of PBRs is the high cell fragility of some microalgae that can undergo cell lysis due to the mechanical shear stress of standard pumps (e.g., *Pavlova* and *Isochrysis*) or air bubbling (e.g., *Emiliania huxleyi*).

### 1.3.3. BIOMASS HARVESTING

After biomass production, the following step in the production pipeline is harvesting, since microalgae biomass needs to be separated from the culture medium. Because of the small size and low density (similar to water) of microalgae cells as well as the low cell concentration commonly obtained in autotrophic cultures, biomass harvesting requires high energy inputs, which, at industrial scale, represents a major production cost in the whole pipeline (Barros et al. 2015). There is not an elected strategy that can be considered as universal, since different methodologies have their own advantages and disadvantages, and the final harvesting efficiency will highly depend on the target species and end product. Currently, there are several biomass concentration techniques available that can be divided into four main categories: mechanical, chemical, biological and electrical methods.

The most common methodologies used for microalgae harvesting are: centrifugation, filtration, chemical flocculation, flotation, bio-flocculation, gravity sedimentation and electricity-based processes (Barros et al. 2015). Nevertheless, at industrial scale, centrifugation and ultrafiltration, or a combination of both, are most used by different companies. Both centrifugation and filtration are highly efficient in the recovery of microalgal biomass from industrial-scale culture volumes, but they have high capital expenditure (CAPEX). Other pros and cons are also associated with these two processes; for instance, centrifugation rapidly processes high amounts of culture into a concentrated microalgae paste (20-30% DW), but requires high energy, which translates into high operational expenditure (OPEX), and shear stress-induced cell lysis may occur in fragile strains. On the other hand, filtration (e.g., microfiltration and ultrafiltration) is regarded as more cost effective (lower OPEX) and with less risk of cell damage, but lower biomass concentrations are achieved (2-7% DW) and membrane fouling may occur.

Previous reports identified chemical flocculation and flotation as more suitable for industrial-scale harvesting, due to lower costs and energy demand required for their implementation. However, both techniques require the use of chemical products to perform effectively, which can compromise the final quality of the biomass produced (Barros et al. 2015). Other emerging lab-scale technologies were previously suggested as promising for industrial-scale applications, such as electrocoagulation/flocculation (electrolytic process), ozonation-dispersed flotation, bio-flocculation, among others (Matos et al. 2013; Lananan et al. 2016; Singh and Patidar, 2018). Nonetheless, their

effective application and efficiency at large-scale, still needs to be demonstrated. Further improvement of emerging and established technologies as well as the combination of different harvesting techniques are expected, leading to higher recovery efficiencies and reduced costs thereof in the near future (Barros et al. 2015).

Upon biomass harvesting, the concentrated microalgal paste can be packed and stored at -20 °C for commercial ends. Wet microalgal paste containing different contents of water is widely marketed worldwide as, for example, to the aquaculture industry. However, for other biotechnological applications biomass drying is required and is the following step in the production pipeline.

### 1.3.4. BIOMASS DRYING

Different drying processes are currently available for microalgal biomass, mainly adapted from technologies that are normally used to dry seaweeds and other food products. Biomass drying is crucial to improve the stability of microalgal products, extending their shelf life (Shelef et al. 1984). However, drying is regarded as one of the most important techno-economical constraints, as it can be the most energy-consuming process in the whole production pipeline (Xu et al. 2011). Therefore, the improvement of the energy balances related to biomass drying is a key factor for the success of any entrepreneurial venture involving microalgae as a powder feedstock. Industrial-scale microalgal biomass drying can be achieved by using different methods, namely spray-, drum/rotary-, freeze, sun-drying, among others (Shelef et al. 1984; Sharma et al. 2013; Guldhe et al. 2014).

Spray-, drum - and freeze-drying are currently widely used in the food and pharmaceutical sectors, including different companies that dry microalgal biomass at industrial scale (e.g., spray-drying: Allmicroalgae; freeze-drying: Necton). The spray-drying operation involves the atomisation (spraying) of the concentrated microalgae-based feed with a hot air stream in a tower, which dries the cells almost instantly to a free-flowing powder. In drum-drying equipment a thin layer of microalgae is placed in a rotatory cylinder and the drum surface is heated with steam in order to remove the water from the biomass. Freeze drying is a gentler process consisting of a preliminary freezing step of the product, followed by the removal of the water crystals under vacuum by sublimation. One advantage of spray drying over drum- and freeze-drying is that the

previous harvesting process does not need to reach a high biomass DW; concentrated cultures still in a liquid form upon ultrafiltration (2-7% DW) are enough to feed the spray drying atomizer. On the other hand, in order to improve the process efficiency, both freeze- and drum-drying are normally operated with more concentrated inputs (10-30% DW). Although, spray-, freeze- and drum-drying are all mature technologies that effectively dry microalgal biomass, the high CAPEX and OPEX restrict their application to high-value applications (Prakash et al. 1997; Brennan and Owende, 2010).

Solar-drying of biomass has been used since ancient times to dry and preserve food products (Prakash et al. 1997) and has gained increasing interest over the last decades as a drying method for microalgal biomass. The process can be effectively accomplished by direct solar radiation or indirectly by means of hot circulated vents using solar collectors (Shelef et al. 1984). Obviously, the main advantage of solar drying is that the energy used for drying is natural and cost-free, and solar driers with low CAPEX and OPEX were already successfully demonstrated (Prakash et al. 1997). On the other hand, drying conditions are not constant and reproducible and maintaining the quality of the end product can be challenging, since biochemical composition, organoleptic properties and bacterial counts are significantly affected by overheating or if the drying process is too slow (Shelef et al. 1984; Prakash et al. 1997). Although some solar drier models overcome the overheating and process duration limitations (Prakash et al. 1997), the process is highly dependent on the weather conditions. This is a limitation for every-day use in large-scale production units, because it generates inconsistent, unpredictable variables in an industrial process. In addition, if large-scale drying is considered, large land areas are necessary to effectively dry the biomass (Sharma et al. 2013). However, from all technologies available, solar drying is the most cost-effective process and platforms for microalgal biomass are still at an infant stage of development (Sharma et al. 2013; Guldhe et al. 2014). Therefore, the continuous optimization of solar driers is crucial for low-end market products, such as biofuels, since all other drying processes are too costly (Mata et al. 2010) and solar drying seems the only viable option.

## 1.4. DOWNSTREAM PROCESSING: THE BIOREFINERY CONCEPT

Although the market of microalgal products has been growing rapidly, it is still far from its full potential. The high value compounds present in most microalgae can significantly improve the revenue of algae-based ventures, if the biomass produced is fractionated into different bulk and specialty products ('t Lam et al. 2018). The production of different bioproducts from microalgal biomass requires a multi-integrated downstream processing (DP) approach, often referred to as a "microalgae-based biorefinery" (Wijffels and Barbosa, 2010, Yen et al. 2013, Uggetti et al. 2014).

Analogous to a traditional petroleum refinery, the biorefinery concept relies on the conversion of biomass into marketable chemicals, fuels and products (Chew et al. 2017; Moreno-Garcia et al. 2017; Pérez et al. 2017). In a biorefinery approach, different biomass conversion processes are integrated in order to reduce the waste products and maximize the use of resources and overall profitability (Ferreira et al. 2013; Jung et al. 2013; Nobre et al. 2013; Brasil et al. 2017). Nowadays, established biomass biorefineries are already in place, for example, in the USA, Brazil and Germany to upgrade edible feedstocks (e.g., corn and soybean) into biofuels and other co-products (Jung et al. 2103; Brasil et al. 2017). The biorefinery concept has been identified as the most promising way to create a biomass-based industry (González-Delgado and Kafarov, 2011). Ideally, a microalgae-based biorefinery should integrate several biomass conversion processes to generate high value and bulk products as well as inputs of nearby industries required for microalgae production (e.g., $CO_2$ and nutrients), following the circular economy principle. The co-production of high added value products and environmental benefits is expected to offset the high production costs of mass cultivation of microalgae and support a microalgae-based bio-economy. However, biorefineries using microalgal feedstocks were mainly performed at lab-scale, as industrial scale processes are still under development and most companies focus on a single product (Ruiz et al. 2016).

Depending upon the target microalgae end products, the DP for an effective biorefinery may comprise a multitude of stages such as biomass cell disruption (pre-treatment), extraction of target biomolecules (e.g., TAG), purification of high value products and residual biomass upgrading. In this section, the most important stages to establish a microalgae-based biorefinery are discussed.

### 1.4.1. WET VS. DRY ROUTE

The DP of microalgal biomass can be accomplished using the wet or dry route, depending on the water content of the biomass chosen to obtain the final product (Lardon et al. 2009; Xu et al. 2011; Roux et al. 2017). The establishment of biorefineries using the dry route was extensively investigated by several authors, with promising set-ups and pipelines to exploit different high and low value products. A significant advantage is that normally the drying processes are known to disrupt the cell coverings of microalgae, cell walls in particular, reducing the costs associated with the biomass pre-treatment step. However, as previously noted, biomass drying is regarded as one of the most important techno-economical constraints. For this reason, drying can be avoided during the DP of microalgae, as the energy required for industrial drying comes at a high cost and accounts for most of the energy spent in the DP pipeline (Xu et al. 2011). Therefore, in order to prevent the high costs of biomass dewatering, thus improving the energy balances and biomass value, several authors proposed novel processes for successfully processing wet biomass during the DP stage.

For example, a life cycle assessment (LCA) of biodiesel production from microalgae indicated that although the energy required for extracting lipids from wet biomass is higher than from dry biomass, the energy needed for the drying process is clearly higher (Lardon et al. 2009). As a result, the total energy balance of the reported LCA was positive for the wet route (+105 MJ), whereas the dry route displayed a negative balance (-2.6 MJ). However, drying is not the only stage of the DP requiring high amounts of energy. To increase the efficiency of DP of microalgae biomass, previous reports demonstrated that a pre-treatment step to promote cell lysis might be essential for most microalgal strains.

### 1.4.2. BIOMASS PRE-TREATMENT

Several microalgal strains are known to present a thick, robust cell wall that reduces the efficiency of the DP and effective development of a biorefinery approach (Figure 1.11). Several reports revealed that a previous step of cell disruption substantially improved the extraction of oil and other biomolecules from microalgal biomass, resulting in higher recoveries and lower extraction time (Lee et al. 2010). The same trend was observed for the production of different biofuels. For example, anaerobic digestion of

microalgal biomass using a pre-treatment step to induce cell lysis led to improved methane conversion yields (Chen and Oswald, 1998), since the organic matter is more bioavailable for digestion by the microbiota (Sialve et al. 2009). In this context, different mechanical, biochemical and physical pre-treatments to disrupt the cell walls of different microalgal strains have been studied, such as bead/ball milling, high speed homogenization, high pressure homogenization, ultrasound, osmotic shock, temperature, autoclaving, chemical hydrolysis, pulse electric fields, microwave radiation, among others (Roux et al. 2017; 't Lam et al. 2018).

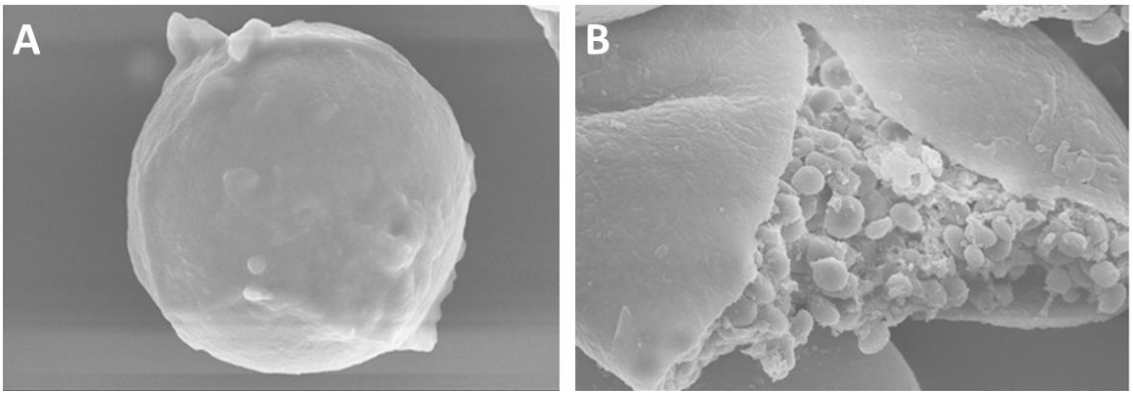


**Figure 1.11 -** Scanning electron microscopy of intact (A) and lysed (B) *Haematococcus pluvialis* cells (Huang et al. 2018).

From all available methods, bead/ball milling is probably the most suitable for industrial scale applications, since it is known to effectively lyse the cells of most microalgal strains and large-scale commercial units are already available (Günerken et al. 2015; Zinkoné et al. 2018). In addition, high pressure homogenization, high speed homogenization, ultrasounds and microwave treatments were also proposed as mature technologies for microalgal cell disruption already used at industrial scale or with high potential for scale-up (D'Hondt et al. 2017). The utilization of pulse electric fields was recently proposed as a promising pre-treatment, due to its lower OPEX compared to most technologies available, but the cell lysis efficiency is still being improved (D´Hondt et al. 2017; 't Lam et al. 2017, 2018). Is also noteworthy that the pre-treatment step of different methodologies must be performed at mild conditions, otherwise the functionality of high value metabolites might be lost, compromising their commercial value (Schwenzfeier et al. 2011; 't Lam et al. 2018; Zinkoné et al. 2018). In addition, the pre-treatment step has been considered as a major drawback for industrial application, due to the high demand of energy needed to process large amounts of biomass, leading to a significant increase of the DP costs (Khoo et al. 2011; Zinkoné et al. 2018).

### 1.4.3. BIOMASS EXTRACTION

The extraction of metabolites from biomass is a crucial step in the DP for effective establishment of a microalgae-based biorefinery. Therefore, different extraction procedures are used to recover different metabolites. Common methodologies include conventional solvent extraction, supercritical fluid extraction, pressurized liquid extraction as well as other emerging technologies.

#### 1.4.3.1 Extraction technologies

Conventional solvent extraction is the most used technique to extract different metabolites from food products as well as in industrial biorefineries (e.g., biodiesel production). In order to maximise the partition of crude microalgae biomass into high-value and bulk products in a biorefinery approach, the solvent extraction procedure followed must ensure the recovery of high value lipids, proteins and carbohydrates, and upgrade the residual biomass. Solvent-based extractions of different target compounds can be achieved by one- or multiple-step extraction, the latter being commonly used to enhance the selectivity of extracted fractions (López et al. 2015). Most biorefinery approaches focus on the extraction of hydrophobic molecules (e.g., carotenoids and PUFA), since they are normally the most valuable metabolites in microalgae, leaving the polar molecules and residual biomass as by-products ('t Lam et al. 2018).

Extraction of non-polar compounds from dry biomass is achieved by using low polarity solvents commonly used in the industrial oil seed processing. Different alkanes (e.g., heptane and cyclohexane) are widely used, but hexane is the most common solvent used in the plant oil industry as, for example, the extraction of rapeseed, soybean and other seed oils (Angles et al. 2017). Although hexane is easy to recover upon extraction, inexpensive and highly selective for neutral lipids, it is toxic, highly flammable and volatile, displaying significant solvent losses with long extraction times (Shin et al. 2014). Moreover, microalgae have a high amount of polar lipids (ca. 30% of total lipids) that will not be effectively extracted if hexane is used without a co-solvent (Wang and Wang, 2012). Therefore, different solvent systems using co-solvents (e.g., ethanol and methanol) were established that facilitated the extraction by non-polar solvents and proved to be very effective in extracting most, if not all, microalgal lipids (Wang and Wang, 2012).

However, to prevent the high costs of biomass dewatering, current research trends have been diverted to novel processes for successfully processing wet biomass.

Lipid extraction from wet biomass is more challenging, since cells are intact and common organic solvents are known to display reduced performance on wet biomass. The presence of water significantly lowers the lipid extraction efficiency, due to decreased contact of the microalga surface with the solvent system (Cooney et al. 2009). To overcome this limitation, novel solvent systems and extraction approaches have been established (Table 1.1). These methodologies, as described by the seminal works of Folch et al. (1957) and Bligh and Dyer (1959), increase mass transfer efficiency between the solvent system and the biomass. However, the use of solvents of increased polarity reduces the extraction selectivity, increasing the co-extraction of other biocompounds, such as proteins, carbohydrates and other organic compounds. For example, Chen et al. (2012b) used a mixture of hexane and ethanol (3:1) at 90 °C and 1.4 MPa, which allowed the extraction of 88% of total lipids (when compared to the Bligh and Dyer method). However, the sustainability and toxicity of organic solvents increased the relevance of green solvents for the extraction of different metabolites. Nowadays, several reports identified the advantage of food grade and green solvents for the effective extraction of microalgae biomass, as for example alcohols, switchable solvents, ionic liquids (IL) and deep eutectic solvents (DES).

**Table 1.1 -** Summary of selected procedures previously reported for extraction from wet biomass. n.d. = not described; n.a. = not applicable; r.t. = room temperature; ENZ = Enzymatic; SON = Sonication; HPH = High Pressure Homogenization

| Extraction system | Species | Water content | Extraction efficiency* | Temperature | Pre-treatment (cell disruption) | Reference |
|---|---|---|---|---|---|---|
| *Solvent-based* | | | | | | |
| EMIM# and methanol | *Chlorella vulgaris* | 70% | 75% | 65 °C | no | Young et al. (2010) |
| Hexane and ethanol | *Nannochloropsis* sp. | 65% | 88% | 90 °C | no | Chen et al. (2012b) |
| 2-propanol | *Nannochloropsis* sp. | 83-84% | 92% | 80 °C | no | Yao et al. (2012) |
| Chloroform and methanol | *Chlorella vulgaris* | 82% | 49% | 37-55 °C | ENZ and SON | Liang et al. (2012) |
| 2-ethoxyethanol | *Chlorella* sp. | n.d. | n.a. | 60 °C | no | Jones et al. (2012) |
| Ethanol | *Nannochloropsis* sp. | 88% | 68% | 80 °C | ENZ and SON | Wand and Wang (2012) |
| Ethanol | *Schizochytrium limacinum* | 90% | 95% | 80 °C | ENZ and SON | Wand and Wang (2012) |
| Hexane | *Nannochloropsis* sp. | 75-80% | 86% | 35 °C | HPH | Olmstead et al. (2013) |
| 1,2-Dimethoxyethane | *Botryococcus braunii* | 75-95% | 96% | r.t. | SON | Liu et al. (2013) |
| N,N-dimethylcyclohexylamine | *Tetraselmis suecica* | 80% | 126% | r.t. | no | Samorì et al. (2013) |
| Ethanol | *Picochlorum* sp. | 90% | 99% | r.t. | no | Yang et al. (2014) |
| N-ethylbutylamine | *Neochloris oleoabundans* | 95% | 100% | r.t. | no | Du et al. (2017) |
| Dimethylcyclohexylamine | *Haematococcus pluvialis* | 80% | 87% | r.t. | no | Huang et al. (2018) |
| *Others* | | | | | | |
| Acid/Base hydrolysis | Mixed culture | 84% | 60% | 90 °C | Acid treatment | Sathish and Sims (2012) |
| Aminoparticles | *Chlorella* sp. | n.a. | n.a. | r.t. | 1% $H_2O_2$ | Lee et al. (2013) |
| Supercritical $CO_2$ | *Scenedesmus* sp. | 93% | 53% | 50 °C | ENZ | Taher et al. (2014) |
| Functional membrane | *Aurantiochytrium* sp. | n.a. | n.a. | r.t. | no | Yoo et al. (2014) |

*Compared to a reference method

#1-ethyl-3-methyl imidazolium methyl sulfate

In this context, ethanol and 2-propanol (IPA) were reported as food grade solvents that effectively extract lipids from wet microalgae biomass (Wang and Wang, 2012; Yao et al. 2012; Yang et al. 2014). For example, IPA forms an azeotrope with water with a boiling point of approximately 80 °C, and the extraction of microalgal paste containing 70% of water at reflux temperature can reach an efficiency of 92%, without any cell disruption treatment (Yao et al. 2012). Similarly, ethanol was also reported as effective to extract lipids from *Picochlorum* sp. at room temperature and without cell disruption, with an extraction efficiency of 99.4% (Yang et al. 2014). The main disadvantage of ethanol when compared to IPA is that a larger volume of solvent is required since the azeotropic-mixture of IPA-water can accommodate more water than ethanol-containing aqueous solutions (Yao et al. 2012). More recently, Angles et al. (2017) screened and compared the extraction efficiency of wet microalgae biomass with several conventional solvents (cyclohexane, heptane, chloroform, toluene, methyl isobutyl ketone and ethyl acetate) and unconventional solvents from green chemistry (dimethyl carbonate, cyclopentylmethyl ether, methyl tert butyl ether, 2-methyl tetrahydofuran and R-limonene). The authors concluded that both methyl tert-butyl ether and cyclopentylmethyl ether, coupled high extraction efficiencies with low energy demand for solvent recovery, and were the most promising alternatives to conventional solvents.

The use of IL and DES is gaining increasing relevance in the development of microalgae biorefineries as greener and more sustainable alternatives to conventional solvents. IL are organic salts that are liquid below 100 °C with a strong ionic bond (Plechkova and Seddon, 2008), whereas DEP are liquid salts with a strong hydrogen bond obtained from a mixture of two solids that form a eutectic mixture (Dai et al. 2013). Examples of IL and DES applied to microalgal extraction are the use of 1-ethyl-3-methylimidazolium ethylsulphate [C2mim][$EtSO_4$] and PCH (1,2-propanediol, choline chloride, water; 1:1:1), respectively (Orr et al. 2016; Cicci et al. 2017). The main advantages of IL and DES is that both can be obtained from natural sources (e.g., organic acids, sugars and amino acids) and their physicochemical properties, namely their hydrophilic and hydrophobic nature can be tuned by changing the combination of salts (Dai et al. 2013). Moreover, other advantages of IL and DES include high chemical and thermal stability, high conductivity as well as their non-volatile and non-flammable properties (Seddon, 1997; Welton, 1999; Dai et al. 2013; Orr et al. 2016). They are also effective in the solubilization of different organic compounds, and one of the most

relevant features for microalgae extraction is that they are known to dissolve the polysaccharides present in the cell wall of several microalgae species, greatly improving the extraction efficiency and reducing pre-treatment costs (Kim et al. 2012; Teixeira, 2012; Orr et al. 2016). On the other hand, the non-volatile properties of IL and DES can be a significant limitation for the back extraction of target compounds ('t Lam et al. 2018), which can lead to an increase of the processing costs.

Switchable solvents have also gained increasing relevance for both the extraction of dry and wet microalgae. These liquid solvents made from amidines or secondary and tertiary amines have inducible polarity, and thus their polarity can be changed to non-polar by the introduction of $CO_2$ in the system and reverted to polar by using $N_2$ (Jessop et al. 2005). The non-polar form can be used to extract the lipids directly from wet biomass; afterwards the solvent can be recovered by removing the $CO_2$, which will reverse its polarity promoting its separation from the extracted non-polar compounds (Du et al. 2017). Thereafter, the solvent can be turn back into the non-polar form by sparging $N_2$ and reused for a novel extraction. This feature enables a major reduction in the costs related to back extraction, since the solvent is easily recovered ('t Lam et al. 2018). A recent report revealed that the use of switchable solvents is more cost effective than conventional solvent extraction and SFE (Du et al. 2015). Another cost-effective feature is that some switchable solvents were reported to promote cell disruption of microalgae (Huang et al. 2018). On the other hand, as the use of switchable solvents is relatively new, caution must be taken regarding the toxicity and volatility of the amines used (Kerton, 2016). The use of switchable solvents was widely applied for the extraction of different feedstocks, including for the extraction directly from wet microalgae biomass of hydrocarbons, lipids, carotenoids and other high value metabolites (Samorì et al. 2010; Du et al. 2015, 2017; Huang et al. 2018). The most common switchable solvents used in microalgae extraction to date are 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), *N*,*N*-dimethylcyclohexylamine (DMCHA), *N*-ethylbutylamine (EBA) and dipropylamine (DPA; Samorì et al. 2010, 2013; Du et al. 2015, 2017; Huang et al. 2018).

Supercritical fluid extraction (SFE) was widely investigated during the last years for the extraction of high value metabolites from different microalgal strains, such as pigments (e.g., fucoxanthin and astaxanthin), anti-microbials, PUFA and other lipid molecules (Mendiola et al. 2007; Macías-Sánchez et al. 2010; Nobre et al. 2013; López et al. 2015). Over the last few years, the technology has gained increasing relevance at

lab and commercial scale, because it is considered to be more sustainable, green and environmentally friendly than conventional solvent extraction processes (Lorenzen et al. 2017). SFE uses solvents above their critical pressure and temperature, resulting in supercritical fluids with a high extraction capacity. Under these conditions, the solvent combines physicochemical properties of liquids and gases, as the liquids solvating capacity and the gases fluidity (Herrero et al. 2006, 2015). Supercritical $CO_2$ (SC-$CO_2$) extraction is by far the most used SFE. As compared to other solvents, the use of $CO_2$ has several benefits, namely its negligible toxicity, non-flammability, widely availability and relatively low price (Zougagh et al. 2004; Poojary et al. 2016). In addition, solvent separation after extraction is not needed as $CO_2$ is a gas at ambient pressure. Therefore, the extractant can be easily recovered and recycled by depressurization (Taher et al. 2014; Yen et al. 2015). Key advantages of SC-$CO_2$ are the high extraction selectivity and fast extraction rate as well as the successful application in multi-stage microalgal biorefineries (López et al. 2015). However, SC-$CO_2$ is a hydrophobic solvent, and the presence of water is therefore a barrier for mass transfer (Catchpole et al. 2012). More recently, the extraction of lipids from wet microalgal biomass was also demonstrated, but the efficiency was lower than solvent extraction (Taher et al. 2014). The main drawbacks of supercritical $CO_2$ for industrial ends are its high CAPEX and OPEX, often due to, respectively, the investment needed for the required equipment and high energy costs of this technology (Yen et al. 2015).

Pressurized liquid extraction (PLE) is another green technique that has been reported to effectively extract high value metabolites from different microalgal strains, mainly carotenoids as well as polyphenols, antioxidants and antimicrobials to some extent (Herrero et al. 2006; Santoyo et al. 2009; Cha et al. 2010; Onofrejová et al. 2010; Plaza et al. 2010; Rodríguez-Meizoso et al. 2010; Herrero et al. 2015; Poojary et al. 2016). Extraction using PLE techniques is commonly performed with water; notwithstanding, organic (e.g., hexane and dichloromethane) and green solvents (e.g., ethanol and ionic liquids) are also widely used. PLE is operated at high temperature and elevated pressure (below critical point), and such conditions greatly improve the solubility and mass transfer between the solvents and target compounds (Herrero et al. 2015). Therefore, higher extraction rates and lower quantities of solvent(s) are observed in PLE techniques compared to those of conventional solvent extraction procedures (Herrero et al. 2015; López et al. 2015; Poojary et al. 2016). Although the use of PLE was widely proposed

and already used for multi-stage microalgae biorefineries (López et al. 2015), the high temperatures and pressures used for extraction result in high operational costs, and the degradation of heat sensitive bioactive molecules may occur (Suchan et al. 2004; Santos et al. 2012; Herrero et al. 2015). Moreover, PLE extractors are known to have a high CAPEX (Suchan et al. 2004), and aqueous PLE methodologies normally result in diluted extracts that must be further concentrated or purified (de la Guardia and Armenta, 2010).

Over the last years, novel procedures enabling a successful extraction of different molecules from microalgal biomass have been established. For example, Lee et al. (2013) developed an innovative procedure that couples the harvesting process with lipid extraction using amino particles. The use of these particles resulted in efficient flocculation of microalgal biomass in less than 5 minutes. Interestingly, upon treatment of the amino particles with 1% $H_2O_2$, the formation of free radicals led to cell damage and concomitant release of the internal lipids, which were later extracted with hexane. More recently, Yoo et al. (2014) applied a functional membrane composed of a tertiary-amine-containing polymer to microalgal cultures. This membrane was able to promote cell disruption as well as lipid release from wet microalgal biomass. This method allowed for a reduction of the costs associated with biomass drying and cell lysis. Although being at an early developmental stage, the authors strongly believe that, after optimization, this membrane-based procedure for processing wet biomass can be a forthcoming breakthrough.

#### 1.4.3.2. Extract fractionation

Upon biomass extraction, the extract can be used as a whole for different biotechnological applications or fractionated to further purify the different molecules that it contains. At lab scale, the fractionation of compounds from an extract for different applications (e.g., drug discovery and forensic technology) is commonly achieved using different methodologies, as for example, silica column, solid-phase extraction, thin layer chromatography, and preparative high-performance liquid chromatography, among others. However, these methodologies are not feasible to be employed in industrial separation of microalgae extracts for the production of commodities. Therefore, different approaches previously proposed to fractionate microalgae extracts with potential for industrial ends are succinctly described below.

A common approach to separate different molecules from a crude extract consists in changing the polarity of the solvent system of a given extract, in order to obtain different fractions of compounds. In this context, a simple approach reported by Yang et al. (2014) relies on the biomass extraction using ethanol, followed by the introduction of hexane and water in the solvent system that separates two layers: i) one layer containing the non-polar molecules dissolved in hexane, and; ii) another layer containing the polar compounds dissolved in ethanol and water. Thereafter, the layers can be easily separated using a conventional liquid-liquid extraction, generating two fractions from the crude ethanolic extract.

Another approach proposed by Veillette et al. (2015) for microalgae relies on the saponification of the crude lipid extract. The separation of the unsaponifiable matter is commonly used in the refining process of vegetable oils as well as to obtain added-value compounds (Ghosh, 2007). Through this approach, from a crude lipid extract, Peña et al. (2015) recovered different carotenoids contained in the unsaponifiable fraction, while the soap layer (i.e., saponifiable fraction) containing FA in the form of sodium/or potassium salts was further upgraded into biodiesel. More recently, Gangadhar et al. (2016) reported that the unsaponifiable matter obtained from a crude lipid extract of *Tetraselmis chui* was comprised of a mixture of carotenoids, phenolics and sterols, displaying antioxidant and metal chelating activities.

Membrane technology has gained increasing relevance in microalgal biotechnology in recent years, not only for microalgae biomass harvesting, but also for the fractionation of compounds from crude extracts (Gerardo et al. 2014; Safi et al. 2014, 2017). Membrane technology is already applied at industrial scale in different sectors as, for example, in the clarification of different beverages (e.g., beer and wine), wastewater treatment and desalination for fresh water supply (reverse osmosis). The growing interest in this technology relies on several key advantages, namely selective separation of compounds of different sizes, low OPEX, continuous operation and no chemicals are used (Gerardo et al. 2014). The membranes used for the separation of different molecules using this technology can be composed of different pore sizes (e.g., ultrafiltration and reverse osmosis), materials and configurations. Over the last few years, some publications have highlighted the potential of this technology for effective separation of microalgal compounds, since target metabolites have different sizes that can be effectively separated using membranes of different sizes. For example, Safi et al. (2014) used a two-stage

membrane filtration to fractionate a crude water extract, upon cell disruption, using high pressure homogenization, into three fractions enriched in different target molecules. The authors used, at a first stage, a 100 kDa membrane to separate a fraction rich in starch from a fraction rich in proteins and sugars. In a second stage, the later fraction was submitted to a 10 kDa membrane that effectively separated the proteins from the sugars. Overall, the continuous optimization of membrane technologies for highly selective fractionation of different compounds from microalgae extracts is expected to be a forthcoming breakthrough for the effective establishment of microalgae-based biorefineries.

### 1.4.4. UPGRADE OF RESIDUAL BIOMASS

Upon extraction of non-polar and/or polar compounds, the residual/spent biomass represents a high percentage of the feedstock, which depending on the extraction can range from 50-80% of dry weight of the biomass input. This co-product of a biorefinery is composed of compounds with interesting biotechnological applicability that need to be valorised in order to maximise the value and profitability of the whole pipeline. The residual biomass is normally rich in complex proteins that are not easily extracted as well as in the carbohydrates that compose the cell wall of microalgae and minerals (Maurya et al. 2016a). In this context, significant research has focused on the upgrade of the residual biomass for different end products, mainly for further application to the feed and biofuels sectors, but also as fertilizers, adsorbents, among other possible uses.

The application of this co-product to the feed industry was widely researched as a way of maximizing the final biomass profits, since feed feedstocks have a higher value than the feedstocks used for biofuels. The high amount of protein in the residual microalgal biomass after lipid extraction (defatted microalgae biomass) revealed a high potential for farming different livestock. In this context, several reports demonstrated the high potential of defatted biomass to feed different land animals, including rat models (Wistar) as well as swine and poultry (Austic et al. 2013; Ekmay et al. 2014; Gatrell et al. 2014; Leng et al. 2014; Vidyashankar et al. 2015). The same trend was observed for aquafeeds, with a significant number of reports showing the high applicability of defatted biomass in experimental feeds for white shrimp, Atlantic salmon and yellow perch (Ju et al. 2012, 2017; Patterson and Gatlin, 2013; Basri et al. 2015; Kiron et al. 2016; Sørensen

et al. 2017; Gong et al. 2018). Most reports using microalgal biomass for feed focus on the replacement of vegetable feedstocks and fish meal (aquafeeds). Depending on the target livestock and the source of defatted microalgal "meal" (i.e., the microalgal strain used), the inclusion of 10 to 25% is generally considered as safe (Jiang et al. 2018). Overall, the safe incorporation of residual algal biomass into feed formulations has the potential to improve animal and human food security and nutritional quality, while reducing the market demand for traditional unsustainable feedstocks.

Over the last few years, several research efforts have been carried out to upgrade the residual microalgal biomass into biofuels. The examples in the bibliography are many and, depending on the biochemical composition of the residual biomass, the production of biofuels as previously described in section 1.2.3 can be achieved. An example, often mentioned, is the use of anaerobic digestion to upgrade the residual biomass in the form of biogas after lipid extraction for biodiesel production in a biorefinery pipeline (Figure 1.12). Using this approach, the methane recovered from the biogas could be used for on-site energy, being burnt for heating or used in a combined heat and power unit, which could contribute to the energy balance of the whole biodiesel production process (Chisti, 2007). In alternative, produced biogas can be directly injected into the PBRs to supply the concentrated $CO_2$ required, which, in combination with pH control, has been shown to augment microalgal growth with concurrent $CO_2$ remediation (Olaizola, 2003; Doucha et al. 2005). Interestingly, the methane in the biogas appears not to adversely affect microalgae growth (Travieso et al. 1993; Mandeno et al. 2005; Heubeck et al. 2007). Several authors pointed out that coupling anaerobic digestion with biodiesel production

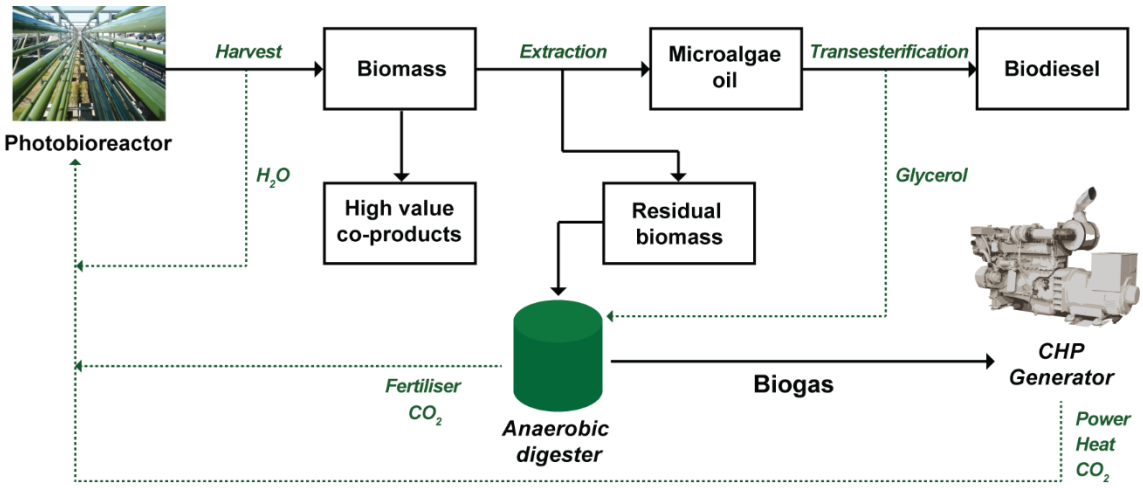


**Figure 1.12 -** Schematic representation of a microalgae-based biorefinery coupling the production of high-value co-products with biodiesel and biogas. The usage of the different by-products established in the different processes are also represented.

is one of the most promising ways to improve the net energy ratio of the whole process (Sialve et al. 2009, Uggetti et al. 2014).

Another relevant application of microalgal residual biomass, recently highlighted by several reports, is the high potential for the development of organic soil fertilizers. Although chemical fertilizers were the nutrient source predominantly used in traditional agriculture to date, an increasing market demand for organic fertilizers, stimulants and pesticides is in place, to ensure the future sustainability of agriculture. Fertilizers must supply all the nutrients essential for the growth of crops, namely, nitrogen, phosphorus and potassium as well as micronutrients (minerals). Accordingly, the residual microalgal biomass couples a high protein content (nitrogen), with the presence of phosphorus and minerals that seem ideal for the development of an organic substrate for soil fertilization. For example, the residual biomass (defatted) of *Chlorella variabilis* and *Lyngbya majuscula* was effectively used to reduce the demand for chemical fertilizers in the growth of maize (*Zea mays*; Maurya et al. 2016b). It is worth mentioning that although the application of residual biomass for soil fertilization is still under investigation, commercial fertilizers established from whole microalgae biomass were already introduced in the market (e.g., AgriAlgae® and Biorizon®).

More recently, Chandra et al. (2015) demonstrated that defatted microalgae biomass could be used as a low-cost adsorbent to remove dyes from aqueous solutions. The authors suggest that defatted microalgae biomass can be a promising alternative to non-renewable carbon-based adsorbents (e.g., coal-based activated carbon) in the removal of heavy metals and dyes from industrial wastewaters. Nevertheless, this is the lowest-end value application and other routes are preferable for residual biomass in order to improve the economics of a microalgae-based biorefinery (Maurya et al. 2016a).

## 1.5. *TETRASELMIS* SP. CTP4

The genus *Tetraselmis* (F. Stein, 1878) belongs to the phylum *Chlorophyta* and to the class *Chlorodendrophyceae* (Massjuk, 2006). The genus is composed of unicellular organisms with cordiform, elliptical or spherical cell shapes, with a single chloroplast, pyrenoid and eyespot (Guiry and Guiry, 2019). They are characterized by presenting four flagella of identical size, covered by hairs and scales (Melkonian, 1990; Arora et al. 2013). One particularity is the cell covering, which corresponds to a theca formed by a periplastic fusion of scales (Manton and Parke, 1965; Becker et al. 1998). Species of this genus are cosmopolitan and can be found in widespread marine and freshwater ecosystems (John et al. 2002). The most known species of this genus are *T. chui* and *T. suecica*, which have been widely used in the aquaculture industry for several decades.

*Tetraselmis* sp. CTP4 is a marine isolate selected from a screening effort carried out by the MarBiotech group (Centre of Marine Sciences) using FACS and BODIPY 505/515 staining. The strain was isolated from an environmental sample collected near a wastewater stream in the natural park of Ria Formosa, in the South of Portugal, and was selected because of several important features; laboratory experiments revealed that *Tetraselmis* sp. CTP4 is a fast-growing, robust microalgal strain that can withstand wide environmental conditions, namely temperatures ranging from 5 to 40 °C (Santos, 2014 and unpublished data), salinities between 1 and 100 ppm (Monteiro, 2014 and unpublished data) and light intensities from 50 to 400 µmol/$m^2$/s. This strain was effectively grown at lab-scale in unsterilized seawater, outcompeting for 30 days the natural bacteria and microalgae present in an urban wastewater effluent (Schulze et al. 2017). As this strain often lacks flagella and is large-sized (15 µm), harvest can be achieved by sedimentation alone, eliminating more than 90% of the water, and thus avoiding one of the major bottlenecks of microalgae processing. In addition, ethyl acetate extracts of CTP4 biomass have already demonstrated the ability to scavenge the ABTS radical (Santos, 2014), chelate iron and copper ions and inhibit butyrylcholinesterase (Monteiro, 2014). However, probably the most important factor is that this is an autochthonous strain from the Algarve, which is already adapted to the surrounding environmental conditions and can be exploited for industrial applications. Overall, these features turn *Tetraselmis* sp. CTP4 into a strong candidate for outdoor cultivation and for the exploitation of different biotechnological applications.

## 1.6. AIMS OF THE THESIS

The main aim of this dissertation was to identify the potential of a novel microalgal strain (*Tetraselmis* sp. CTP4), autochthonous from the Algarve coast, for industrial production and further use for different biotechnological applications using a biorefinery approach.

Although extensive research has been carried out in the characterization of the growth performance of different microalgal strains, most works were performed at lab-scale, which do not ensure that the target strain can be a viable feedstock for industrial application. In addition, reports focusing on the characterization of the biotechnological potential of a target strain are widely dispersed and are hardly comparable, and the majority do not consider a biorefinery platform for improved valorisation of microalgal biomass.

In this context, the following biological questions were addressed in the scope of the present work:

- Is *Tetraselmis* sp. CTP4 a viable feedstock for industrial production of microalgal biomass?
- Can the biomass be harvested by natural sedimentation at pilot-scale?
- What is the biochemical composition and high-value secondary metabolites of industrially produced biomass?
- What are the potential biotechnological applications of *Tetraselmis* sp. CTP4?
- Can we establish an effective biorefinery pipeline for the production of different bioproducts from *Tetraselmis* sp. CTP4?
- Can residual microalgal biomass of *Tetraselmis* sp. CTP4 replace soybean meal in the aquafeeds of juvenile gilthead seabream?

Overall, the present dissertation is expected to significantly contribute to the full biotechnological potential of *Tetraselmis* sp. CTP4, increasing the current knowledge on microalgae as emerging feedstocks to supply the next generation of foods, feeds, fuels, bioactive molecules and $CO_2$ capture.

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# CHAPTER II

# ISOLATION OF A EURYHALINE MICROALGAL STRAIN, *TETRASELMIS* SP. CTP4, AS A ROBUST FEEDSTOCK FOR BIODIESEL PRODUCTION

**Research article published in:**

Pereira, H., Gangadhar, K. N., Schulze, P., Santos, T., Bruno de Sousa, C., Schueler, L., Custódio, L., Xavier Malcata, F., Gouveia, L., Varela, J., & Barreira, L. (2016). Isolation of a euryhaline microalgal strain, *Tetraselmis* sp. CTP4, as a robust feedstock for biodiesel production. Scientific Reports, 6, 35663.

# ISOLATION OF A EURYHALINE MICROALGAL STRAIN, *TETRASELMIS* SP. CTP4, AS A ROBUST FEEDSTOCK FOR BIODIESEL PRODUCTION

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## ABSTRACT

Bioprospecting for novel microalgal strains is key to improving the feasibility of microalgae-derived biodiesel production. *Tetraselmis* sp. CTP4 (Chlorophyta, Chlorodendrophyceae) was isolated using fluorescence activated cell sorting (FACS) in order to screen novel lipid-rich microalgae. CTP4 is a robust, euryhaline strain able to grow in seawater growth medium as well as in non-sterile urban wastewater. Because of its large cell size (9–22 μm), CTP4 settles down after a six-hour sedimentation step. This leads to a medium removal efficiency of 80%, allowing a significant decrease of biomass dewatering costs. Using a two-stage system, a 3-fold increase in lipid content (up to 33% of DW) and a 2-fold enhancement in lipid productivity (up to 52.1 mg $L^{-1}$ $d^{-1}$) were observed upon exposure to nutrient depletion for 7 days. The biodiesel synthesized from the lipids of CTP4 contained high levels of oleic acid (25.67% of total fatty acids content) and minor amounts of polyunsaturated fatty acids with ≥4 double bonds (<1%). As a result, this biofuel complies with most of the European (EN14214) and American (ASTM D6751) specifications, which commonly used microalgal feedstocks are usually unable to meet. In conclusion, *Tetraselmis* sp. CTP4 displays promising features as feedstock with lower downstream processing costs for biomass dewatering and biodiesel refining.

## 2.1. INTRODUCTION

Microalgal biomass has drawn increasing attention for different biotechnological applications over the last few years, as shown by the significant developments in terms of funding allocated for microalgal research. The establishment of several start-up companies and commercial products from microalgae (e.g., DSM Life's® and Qualitas Health® nutraceuticals as well as Encapso® drilling oil) have confirmed that these microorganisms can be important feedstocks in different markets. Nevertheless, recent achievements observed in the field of microalgal biotechnology were mainly due to research and innovation efforts towards the development of microalgae-based biofuels (Borowitzka, 2013). Although the technology to produce biofuels (e.g., biodiesel) from microalgal biomass has been successfully demonstrated, several techno-economical reports concluded that the biofuels obtained from microalgal feedstocks are still unable to compete with fossil fuels (Chisti, 2007; Wijffels and Barbosa, 2010; Davis et al. 2011). To overcome the current constraints for the commercialization of microalgae-based biofuels, the optimization of the whole production pipeline is required. An important first step is strain selection, which has to take into account later steps, such as its robustness, easy and low cost downstream processing and the effective development of a biorefinery.

During the last decades, aquaculture has been the main market for microalgal biomass, particularly for rearing bivalves and enhancing the nutrition of live prey (Benemann, 1992; Borowitzka, 1997). Since unsaturated lipids are essential metabolites for the proper development of fish larvae and bivalve growth (Adarme-Vega et al. 2012), microalgal strains with high contents of polyunsaturated fatty acids (PUFA) have been selected and commercialized. However, microalgal feedstocks with high amounts of PUFA are not suitable for biodiesel production, because the large number of double bonds present in these fatty acids decrease the oxidation stability of the end product (Knothe, 2011, 2012; Gangadhar et al. 2016). In fact, both European (EN14214) and American (ASTM D6751) specifications impose strict limits concerning the presence of PUFA in biodiesel. Therefore, screening for strains containing high lipid content with low levels of unsaturated fatty acids is crucial to enhance the productivity and quality of the feedstock used for biodiesel production (Perrier et al. 2015; Piligaev et al. 2015; Gangadhar et al. 2016) and decreases the costs of biodiesel refining (Perrier et al. 2015).

Flow cytometry coupled to fluorescence activated cell sorting (FACS) is a powerful high-throughput technique for bioprospecting microalgae present in

environmental samples, because this technology enables the screening of thousands of cells in a short period of time for a specific purpose (e.g., high lipid contents). This can be accomplished by acquiring different signals, such as complexity and relative cell size coupled with the autofluorescence of photosynthetic pigments and the fluorescence of solvatochromic dyes (Acreman, 1994; Reckermann, 2000; Sinigalliano et al. 2009). Depending on the final product, bioprospection using FACS enables the isolation of strains with a desired biochemical profile through different selection approaches (e.g., dyes that only emit fluorescence in the presence of lipids), narrowing down the number of strains that have indeed a high potential for a given biotechnological application (Doan et al. 2011; Pereira et al. 2011). Concomitantly, selection procedures must also contemplate the growth performance and robustness of a given strain, as both features are crucial for the up-scaling and effective production in large-scale systems (Mutanda et al. 2011).

This work aimed to characterize and evaluate the potential of a novel euryhaline microalga isolated from a salt marsh near a wastewater stream in the south of Portugal as a feedstock for biodiesel production. *Tetraselmis* sp. CTP4 was selected from 96 isolates as a result of a screening effort using FACS to bioprospect for novel microalgal strains with biotechnological potential.

## 2.2. RESULTS

### 2.2.1. MICROALGAE ISOLATION AND IDENTIFICATION

*Tetraselmis* sp. CTP4 was isolated by FACS using the sorting procedure shown in Figure 2.1. Figure 2.1A presents the two-dimensional plot combining the SSC (side angle light scatter) and FL3 (fluorescence emission at 695 nm) signals, relating the relative inner cell complexity with chlorophyll autofluorescence, respectively. Through the combination of these signals, the first sorting trait was established in order to differentiate non-photosynthetic from photosynthetic cells; in this way, further analyses focusing only on photosynthetic cells were carried out (Pereira et al. 2011). Figure 2.1B shows the combination of the allophycocyanin autofluorescence signal (FL4) and the emission of BODIPY 505/515 (FL1), a lipid-staining solvatochromic dye. With this combination of signals, three clusters of cells representing three different microalgal species could be clearly distinguished. Two (P3 and P4) of the three clusters displayed

higher values of BODIPY fluorescence. The gates used in the sorting procedure were effective for the isolation of cells belonging to three different species as verified by microscopy upon sorting directly onto microscope slides (Figure 2.1C–E). The isolates were entitled CTP3, CTP4 and CTP5, according to the numbering of the clusters obtained in the cytometer. Interestingly, among all isolates, CTP4 presented the most dense cluster of events as compared with other microalgae found in the environmental sample (Figure 2.1B). This was an important first indicator that CTP4 was able to compete with other microalgae that were co-cultivated during the pre-enrichment step of the isolation process. Moreover, this microalga also showed the highest levels of BODIPY fluorescence (FL1), which strongly suggested that this microalga contained significant amounts of lipids. As a result, CTP4 was selected for further study.

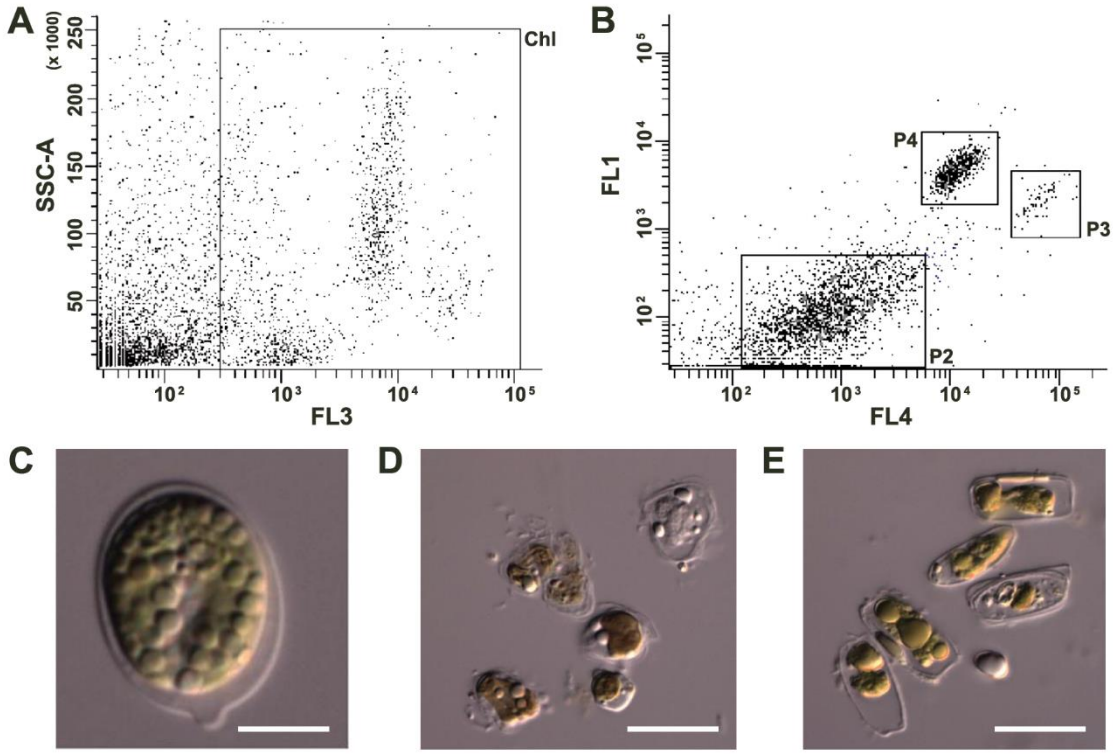


**Figure 2.1 -** Cell sorting procedure used to isolate CTP4 strain by means of fluorescence activated cell sorting (A,B), and different clusters sorted directly onto microscope slides observed with differential interference contrast (C–E). The first sorting trait (Chl gate) was applied through the combination of inner cell complexity, SSC-A, with chlorophyll autofluorescence, FL3 (A). The final gates used to isolate the CTP4 strain combined BODIPY fluorescence (FL1) with the signal of the allophycocyanin autofluorescence channel (FL4). Strains isolated from clusters P4 (C), P2 (D) and P3 (E). The first strain was named as CTP4 (C), whereas the second corresponded to an unidentified strain with apparent cell disruption (D) and the third strain was an unidentified diatom (E). Scale bar = 5 μm.

Chlorodendrophyceae 18S rDNA sequences were analysed by Bayesian (BI) and Maximum Likelihood (ML) inference and the consensus tree is shown in Figure 2.2 using equivalent sequences from Trebouxiophyceae algae as the outgroup. Topology of the BI and ML consensus trees indicates that *Tetraselmis* sp. CPT4, isolated in this study, belongs to the *T. striata/convolutae* clade with a posterior probability of 0.98 and a bootstrap value of 82%, respectively (Figure 2.2).

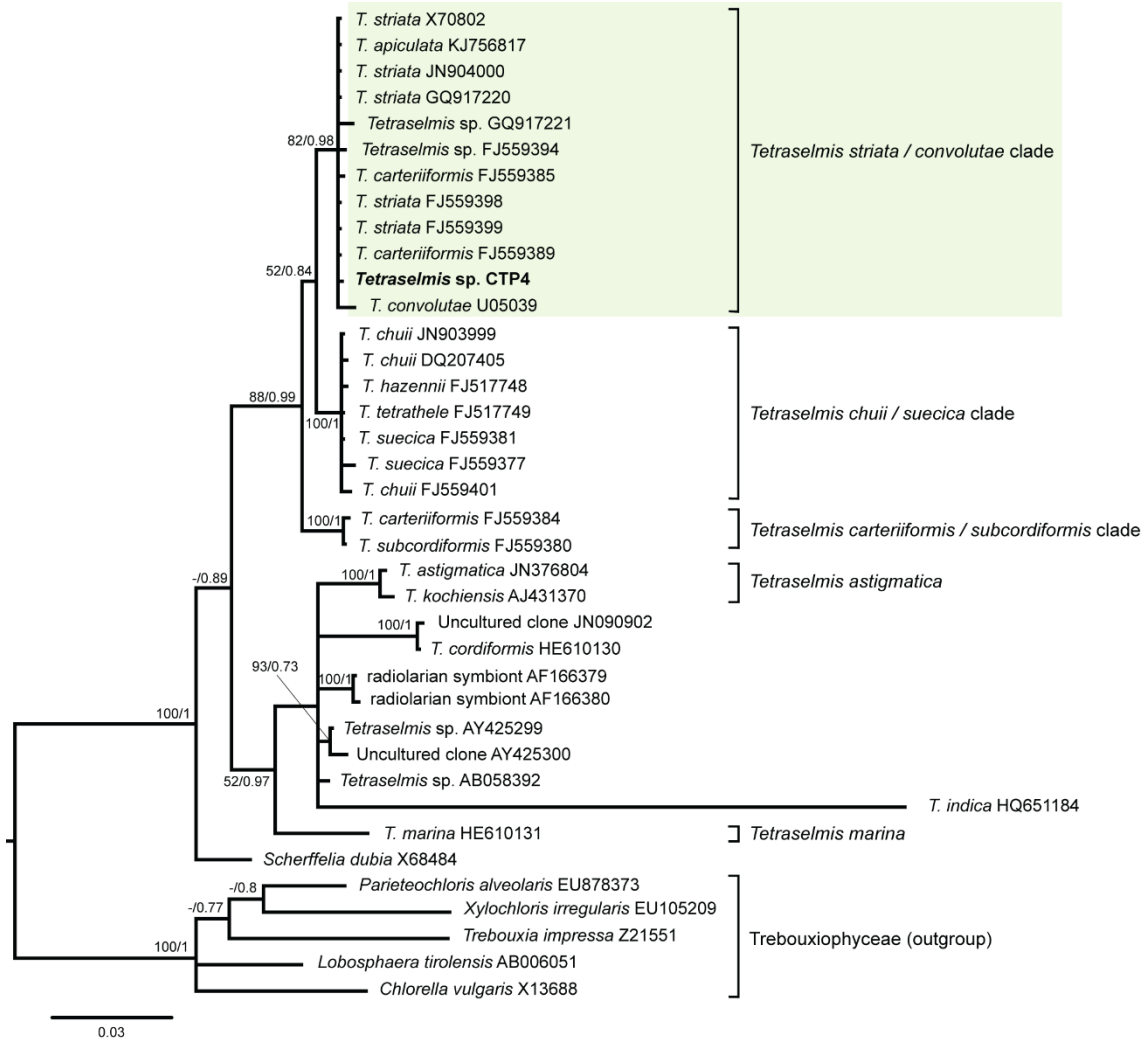


**Figure 2.2 -** Bayesian inference tree of species of the Chlorodendrophyceae class inferred using 18S rDNA sequences. Maximum-likelihood bootstrap values (> 50) and Bayesian inference posterior probabilities (> 0.70) are indicated at the branches, respectively. The CTP4 strain, isolated in this study, clustered with the *striata/convolutae* clade of the *Tetraselmis* genus.

### 2.2.2. CULTURE ROBUSTNESS AND DEWATERING

To further characterise the novel strain, *Tetraselmis* sp. CTP4 was cultivated in seawater-based Modified Algal Medium (MAM) and in a non-sterile, non-nitrified wastewater effluent (Figure 2.3). Cultures were grown until a cell concentration (CC) of

about 2.7 × $10^6$ cells $mL^{-1}$ was reached. Both batch cultures displayed similar growth curves, reaching stationary phase at day 8. Cultures were monitored daily by bright field microscopy and flow cytometry. Although bacterial and microalgal contaminants were frequently observed in the non-sterile wastewater culture, *Tetraselmis* sp. CTP4 remained the dominant specie throughout.

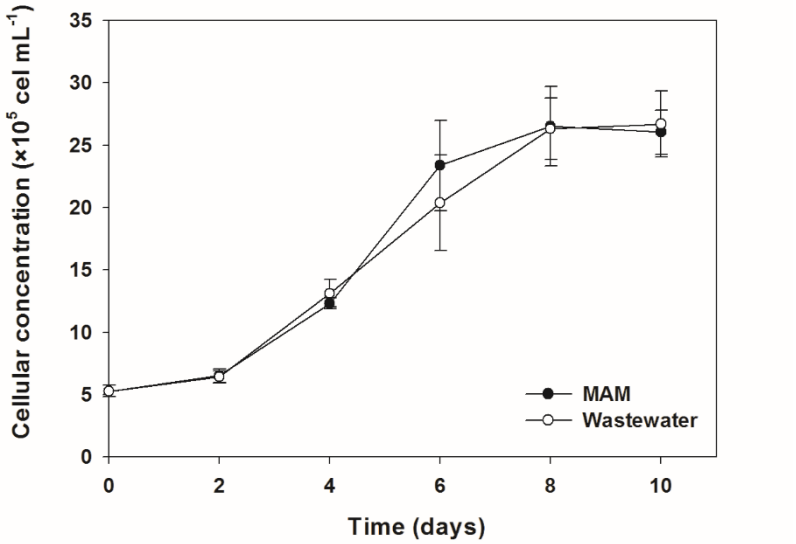


**Figure 2.3 -** Growth curves of *Tetraselmis* sp. CTP4 cultures grown in standard conditions and a wastewater effluent. Cultures were grown in Modified Algal Medium (MAM, salinity ≈ 3.6%) and non-sterile urban effluent (salinity ≈ 0.5%) for 10 days with a starting inoculum of 5 × $10^5$ cells $mL^{-1}$.

The natural sedimentation of cultures, presented as volume of settled culture per liter of culture, was investigated during the course of 8 hours using Imhoff cones (Figure 2.4). The settling of the cultures revealed a decreasing logarithmic curve, converging towards 18% of the initial volume over 6 hours.

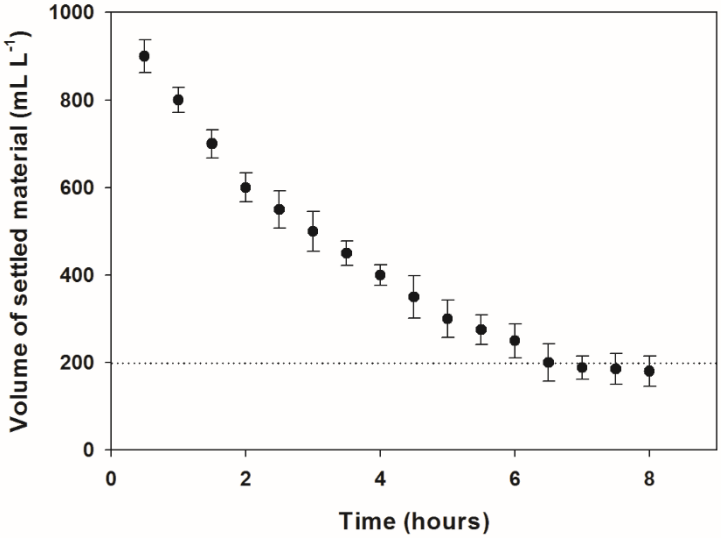


**Figure 2.4 -** Volume of settled material of *Tetraselmis* sp. CTP4 cultures in Imhoff cones (*n* = 3).

### 2.2.3. BIOMASS GROWTH AND LIPID INDUCTION

The growth curves of cultures grown under nutrient repletion (N+) and nutrient starvation (N−) in a two-stage growth system are shown in Figure 2.5A. Both cultures grew exponentially until the end of the 1st stage (day 10), reaching a CC of $2.9 \times 10^6$ cells $mL^{-1}$. The specific growth rate (μ) during this stage was similar in both culture conditions (0.29–0.31 $d^{-1}$). In the 2nd stage, N+ cultures continued to grow, although at a slower rate, reaching a final concentration of $3.3 \times 10^6$ cells $mL^{-1}$, whereas the N− cultures plateaued at approximately $3.0 \times 10^6$ cells $mL^{-1}$. At the beginning of the 1st stage the lipid content of both cultures was approximately 10% of DW (Figure 2.5B). During the exponential phase (between day 4 and 8), a decrease in the lipid content (5–8% of DW) was observed. During the 2nd stage (lipid accumulation stage), cultures supplemented with nutrients (N+) maintained the same lipid content, displaying a final lipid content of 10% of DW. However, the N− cultures reached a significantly higher lipid content: approximately 33% of DW. These results were confirmed by staining with BODIPY 505/515; microalgae grown under nutrient starvation contained a significantly higher amount of lipid bodies that stained positively for the solvatochromic dye (Figure 2.6). The biomass and lipid productivities obtained in the present study and previous reports with other *Tetraselmis* strains are presented in Table 2.1. The N+ cultures displayed higher biomass productivity compared to those in the N− treatment, yielding 0.29 and 0.25 g $L^{-1}$ $d^{-1}$, respectively. On the other hand, the final lipid productivity doubled under the N− conditions (52.1 mg $L^{-1}$ $d^{-1}$) when compared to N+ cultures (24.5 mg $L^{-1}$ $d^{-1}$).

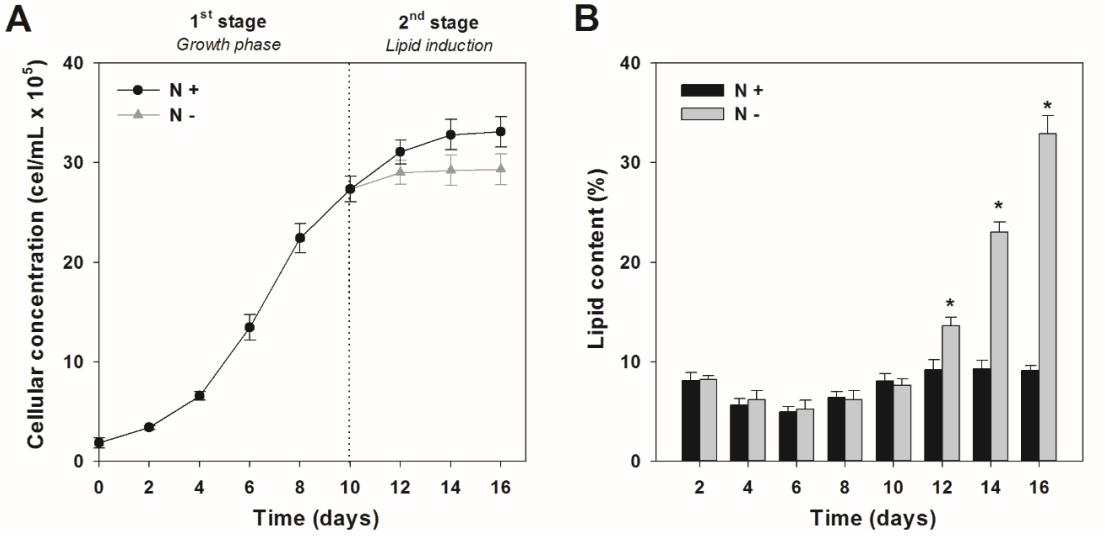


**Figure 2.5 -** Cultures grown in a two-stage system under nutrient repletion (N+) and nutrient depletion (N−) conditions. The growth curves (A) and the corresponding mean lipid content (B) of cultures exposed to both culture conditions is shown (n = 3). Dashed line shows the addition of nutrients to the N+ cultures (A).

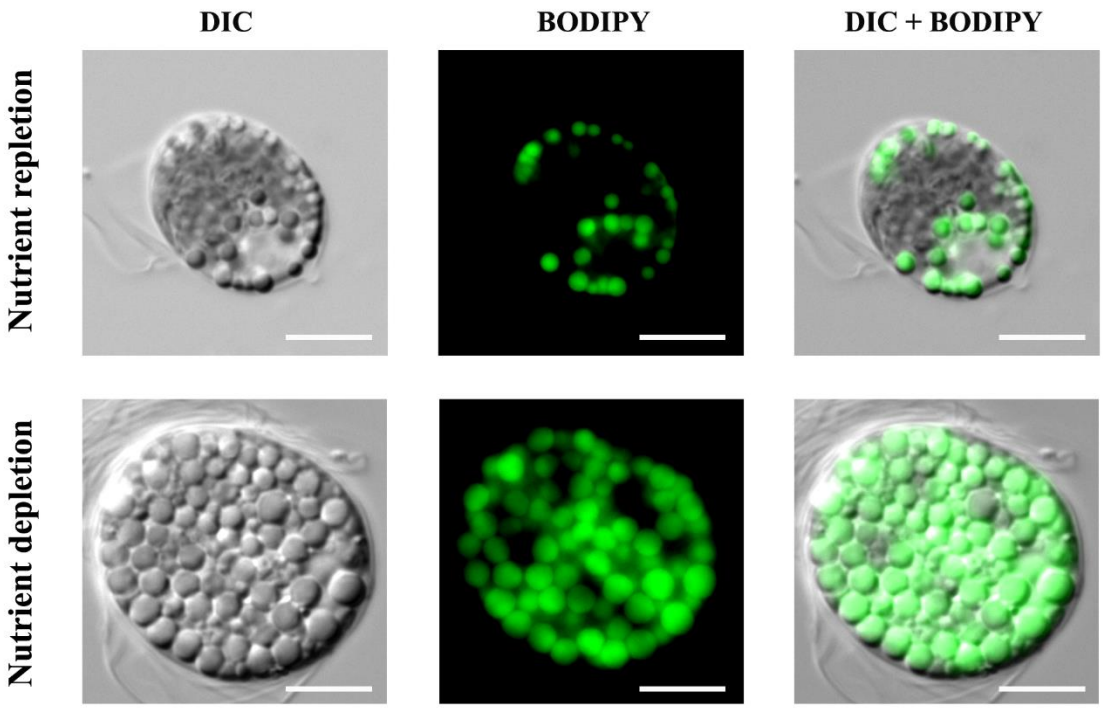


**Figure 2.6 -** BODIPY 505/515 staining of *Tetraselmis* sp. CTP4 cells. Images were acquired using differential interference contrast (DIC) and BODIPY fluorescence, showing the lipid bodies in cells grown under nutrient repletion (N+) and nutrient depletion (N−) media. Scale bar = 5 μm.

**Table 2.1 -** Biomass and lipid productivities previously reported for *Tetraselmis* species and in the present work. †Productivities were determined on an ash free dry weight basis.

| Species | Biomass productivity ($g\ L^{-1}\ d^{-1}$) | Lipid productivity ($mg\ L^{-1}\ d^{-1}$) | Reference |
|---|---|---|---|
| *Tetraselmis* sp. (F&M-M34) | 0.30 | 43.4 | Rodolfi et al. (2009) |
| *Tetraselmis suecica* (F&M-M33) | 0.32 | 27.0 | Rodolfi et al. (2009) |
| *Tetraselmis suecica* (F&M-M35) | 0.28 | 36.4 | Rodolfi et al. (2009) |
| *Tetraselmis* sp. | n.a. | 22.7 | Huerlimann et al. (2010) |
| *Tetraselmis* sp. | n.a. | 18.6 | Huerlimann et al. (2010) |
| *Tetraselmis* sp. | n.a. | 22.2 | Huerlimann et al. (2010) |
| *Tetraselmis* sp. (MUR 167) | 0.09† | 25.8† | Fon-Sing and Borowitzka, (2016) |
| *Tetraselmis* sp. (MUR 219) | 0.08† | 30.3† | Fon-Sing and Borowitzka, (2016) |
| *Tetraselmis* sp. (MUR 230) | 0.09† | 43.2† | Fon-Sing and Borowitzka, (2016) |
| *Tetraselmis* sp. (MUR 231) | 0.20† | 85.5† | Fon-Sing and Borowitzka, (2016) |
| *Tetraselmis* sp. (MUR 232) | 0.09† | 25.8† | Fon-Sing and Borowitzka, (2016) |
| *Tetraselmis* sp. (MUR 233) | 0.17† | 58.0† | Fon-Sing and Borowitzka, (2016) |
| *Tetraselmis* sp. (CTP4; N+) | 0.29 | 24.6 | Present work |
| *Tetraselmis* sp. (CTP4; N-) | 0.25 | 52.1 | Present work |

† *productivities were determined on an ash free dry weight basis.*

### 2.2.4. BIOCHEMICAL PROFILE AND PROPERTIES OF CTP4 BIODIESEL

Upon synthesis of biodiesel derived from the lipids extracted from CTP4 cells, the fatty acid methyl esters (FAME) profile of the biofuel was determined (Table 2.2). Palmitic (C16:0), oleic (C18:1) and linoleic (C18:2) acids were the major FAME detected, representing approximately 75% of the total fatty acids (TFA) in the biodiesel mixture. Other FAME also found at relatively high amounts were palmitoleic (C16:1) and hexadecatrienoic (C16:3) acids (10% of TFA), whereas only minor levels of hexadecadienoic (C16:2), linolenic (C18:3) and eicosapentaenoic (C20:5n-3, EPA) acids were detected.

**Table 2.2 -** Fatty acid profile of the biodiesel synthesised from *Tetraselmis* sp. CTP4.

| Fatty acid | Name | Biodiesel (%) |
|---|---|---|
| C16:0 | Palmitic acid | 24.13 |
| ∑SFA | | 24.13 |
| C16:1 | Palmitoleic acid | 14.70 |
| C18:1 | Oleic acid | 25.67 |
| ∑MUFA | | 40.37 |
| C16:2 | Hexadecadienoic acid | 0.44 |
| C18:2 | Linoleic acid | 23.17 |
| C16:3 | Hexadecatrienoic acid | 9.89 |
| C18:3 | Linolenic acid | 1.23 |
| C20:5 | Eicosapentaenoic acid | 0.77 |
| ∑PUFA | | 35.50 |

The properties of the synthesised biodiesel were then determined (Table 2.3) and compared to the limits established by the international biodiesel specifications (EN14214 and ASTM D6751). All FAME related properties, namely total FAME, cetane number (CN), iodine value (IV), cold filter plugging point (CFPP), linolenic acid and PUFA ≥ 4 double bonds (db) contents, were within or close to the values established in the EN14214 specification. The measured density, kinematic viscosity and oxidation stability were 0.85 Kg $L^{-1}$, 3.64 $mm^2$ $s^{-1}$ and 4.74 h, respectively. Produced biodiesel was devoid of glycerol (total and free) and acylglycerols (mono-, di- and triacylglycerols). The levels of group I (Na + K) and group II (Ca + Mg) metals were 0.45 and 0.05 mg $Kg^{-1}$, respectively. Hence,

both values were below the maximum limits specified by both standards. The phosphorous content was, however, higher than the specified limits.

**Table 2.3 -** List of biodiesel properties analysed in the biodiesel produced from lipids of *Tetraselmis* sp. CTP4 and the limits established by each standard (EN 14214 and ASTM D6751).

| Biodiesel properties | Unit | Biodiesel | EN 14214 | ASTM D6751 |
|---|---|---|---|---|
| FAME content | % (m $m^{-1}$) | 96.72 | ≥96.50 | - |
| Density (15 °C) | Kg $L^{-1}$ | 0.85 | 0.86-0.90 | - |
| Viscosity (40 °C) | $mm^2$ $s^1$ | 3.64 | 3.50-5.00 | 1.90-6.00 |
| Cetane number | - | 51.33 | ≥51 | ≥47 |
| Oxidation stability | hours | 4.74 | >6 | >3 |
| Iodine value | g I/100g | 110.63 | ≤120 | - |
| Linolenic acid | % (m $m^{-1}$) | 1.23 | ≤12.0 | - |
| PUFA ≥ 4 db | % (m $m^{-1}$) | 0.77 | ≤1.00 | - |
| Monoglyceride content | % (m $m^{-1}$) | <0.1 | ≤0.80 | - |
| Diglyceride content | % (m $m^{-1}$) | <0.05 | ≤0.20 | - |
| Triglyceride content | % (m $m^{-1}$) | <0.05 | ≤0.20 | - |
| Free glycerol | % (m $m^{-1}$) | <0.001 | ≤0.02 | ≤0.02 |
| Total glycerol | % (m $m^{-1}$) | <0.05 | ≤0.25 | ≤0.24 |
| Group I metals (Na+K) | mg $kg^1$ | 0.45 | ≤5.0 | ≤5.0 |
| Group II metals (Ca+Mg) | mg $kg^1$ | 0.05 | ≤5.0 | ≤5.0 |
| Phosphorus content | mg $kg^1$ | 24.02 | ≤4.0 | ≤10.0 |
| CFPP | °C | | | |
| Summer | | -8.89 | ≤ –5/+5* | |
| Winter | | | ≤ –5/–20* | |

**Country-dependent (values not included in EN 14214); limits describe the range of maximum values allowed by the legislation applied in Austria, France, Germany, Greece, Ireland, Italy, Netherlands, Portugal, Spain and United Kingdom.*

## 2.3. DISCUSSION

Microalgae intended for large-scale production need to be robust and present high growth rates in order to withstand wide environmental conditions and outgrow competitors and predators. In this sense, the enrichment step carried out before the FACS isolation step promoted the isolation of strains able to outcompete other cells also found in environmental samples. This step is thus crucial for the selection of robust microalgae

that are able to become dominant even under challenging conditions and in the presence of contaminants (Pereira et al. 2011, 2013). Strains of interest for biodiesel production should develop clusters with a higher number of events during FACS isolation, combined with a higher lipid-BODIPY signal. From an initial pool of 96 isolates obtained by authors' FACS-based methodology, *Tetraselmis* sp. CTP4 was selected as a promising biodiesel feedstock due to the combination of dominance over contaminants and lipid content. Strain identity was confirmed by phylogenetic analysis showing that *Tetraselmis* sp. CTP4 belongs to the *striata/convolutae* clade, in accordance with data reported by Arora et al. (2013).

The robustness of this euryhaline microalga was evaluated by growing cultures in non-sterile urban wastewater. *Tetraselmis* sp. CTP4 displayed similar growth curves in both the control MAM and the wastewater and, most importantly, it dominated over the microorganisms naturally present in the wastewater. Strains isolated in areas of wastewater discharges usually show high tolerance to oxidative stress and are often well suited for wastewater treatment (Osundeko et al. 2013). Moreover, recent trials have shown that *Tetraselmis* sp. CTP4 can grow at salinities ranging from ~1 to 100‰ (data not shown). Halotolerant strains display a key advantage for large-scale production, since the manipulation of the salinity (high vs. low salt shifts) in the culture medium can manage and contain possible contaminants, without affecting significantly the biomass productivity of the cultures. This feature is also a key feature to recycle the marine culture medium after dewatering, upon which wide variations of salinity can occur, as recently reported by Fon Sing et al. (2014) in pilot scale open raceways used to grow a euryhaline *Tetraselmis* strain.

Harvesting/dewatering of cultures is a main constraint in the whole microalgal production pipeline, due to the high-energy demands associated with biomass recovery from massive amounts of water (Uduman et al. 2010; Sharma et al. 2013). In this sense, the ability of *Tetraselmis* sp. CTP4 to settle down naturally is another crucial advantage, as it allows the removal of 80% of the culture medium after a 6-h sedimentation step without the addition of flocculants or the use of a pre-concentration procedure. Through this approach only 20% of the culture volume needs to be harvested using common methods (e.g., centrifugation and filtration), having thus the potential of decreasing significantly the costs of biomass dewatering.

To compare the lipid production of cultures, cells were cultured under nutrient repletion (N+) and depletion (N−) using a two-stage growth system. Results showed that cells grown under nutrient depletion yielded a 3-fold increase in the total lipid content and significantly higher cell size (15–22 μm) than the N+ treatment (9–12 μm). Lipid accumulation was only triggered upon nutrient starvation, which might affect amino acid levels needed for protein synthesis, providing a higher number of carbon skeletons that will be available for triacylglycerol (TAG) biosynthesis (Converti et al. 2009; Procházková et al. 2014). The two-stage approach used for inducing lipid accumulation in *Tetraselmis* sp. CTP4 effectively improved the lipid productivity of this microalgae, leading to a 2-fold increase in cultures exposed to nutrient depletion. The results of this two-stage system are in accordance with Gouveia et al. (2009) and Campenni' et al. (2013). However, they do not match the results recently published by Kim et al. (2016), where N+ cultures displayed higher lipid productivity. Although a strain-dependent response cannot be excluded, such difference may be explained by the short induction period of the 2$^{nd}$ stage (36 hours) used by these authors, which probably did not enable an effective lipid induction in the N− cultures. In the present work, the two-stage approach only promoted lipid accumulation in *Tetraselmis* sp. CTP4 after 48 hours of nutrient starvation.

The overall biomass and lipid productivities established in the present work for *Tetraselmis* sp. CTP4 matched those from previous reports on other *Tetraselmis* strains (Table 2.1). Moreover, chlorophytes and particularly those of the *Tetraselmis* genus have already been shown to be able to grow in outdoor systems, and can be promising feedstocks for the production of microalgae-based biofuels (Hu et al. 2008; Huerlimann et al. 2010; Zhou et al. 2011). The latter conclusion is confirmed by the fact that biodiesel synthesised from wet biomass of *Tetraselmis* sp. CTP4 displayed values within or close to the limits defined by the EN14214 and ASTM D6751 specifications (Table 2.3). From all properties investigated, the phosphorus content was the only parameter clearly outside the limits described in both specifications. However, this result was expected since, generally, microalgae oils present a significant amount of phospholipids (Lu et al. 2013; Iyer, 2016) that are co-extracted with the TAGs. Therefore, removal of phospholipids (e.g., degumming) from the microalgae oil is essential in order to reduce the content of phosphorus in microalgal biodiesel and fulfil the limits of both specifications (Dibenedetto et al. 2012; Iyer, 2016).

Most microalgae strains fail to address the properties related with the saturation of the lipid profile, namely, the content of linolenic acid, PUFA ≥ 4 db, IV, CN, and most importantly, the oxidation stability (Knothe, 2011; Stansell et al. 2012; Gangadhar et al. 2016). In fact, the oxidative stability of the produced biodiesel (4.74 h) is to the authors' knowledge the highest value reported for B100 microalgae-based biodiesel, except for that of *Scenedesmus* sp. (5.42 h; Chen et al. 2012a). Perrier et al. (2015) and Chen et al. (2012a, b) produced B100 biodiesel from *Chlorella protothecoides*, *Nannochloropsis* sp. and a dinoflagellate with induction periods of 4.52, 0.8–1.93 and 1.02 h, respectively. The oxidative stability of CTP4 biodiesel was also significantly higher than the values previously reported for the biodiesel produced from other vegetable sources, such as soybean (3.9 h), palm (3.52 h), rice bran (1.7 h) and sunflower (0.4 h) oils (Mittelbach and Schober, 2003; Knothe, 2007; El Boulifi et al. 2013; Botella et al. 2014). Such oxidative stability is probably related with the FAME profile of CTP4 and consequently of the produced biodiesel that revealed only trace values of long-chain PUFA, and high contents of palmitic, oleic and linoleic acids, accounting for nearly 75% of the TFA. The FAME profile of the biodiesel produced from CTP4 presents a lower degree of unsaturation than those of other *Tetraselmis* strains previously published in the literature (Grima et al. 1994; Montaini et al. 1995; Huerlimann et al. 2010; Gangadhar et al. 2016). The same is observed when the lipid profile of CTP4 is compared with that of most common commercial strains of microalgae, such as *Nannochloropsis oculata* and *Phaeodactylum tricornutum* (Gangadhar et al. 2016). The low unsaturation degree of *Tetraselmis* sp. CTP4 is a crucial advantage for biodiesel production, as recently highlighted in several reports (Knothe, 2011; Perrier et al. 2015; Piligaev et al. 2015; Gangadhar et al. 2016).

In conclusion, *Tetraselmis* sp. CTP4 displays several promising features as a biodiesel feedstock, including robustness, high biomass and lipid productivities, and potential for reduced downstream costs related with biodiesel refining and biomass dewatering.

## 2.4. MATERIALS AND METHODS

### 2.4.1. MICROALGAE ISOLATION AND CULTURE SCALE UP

*Tetraselmis* sp. CTP4 was isolated near a wastewater treatment plant in Ria Formosa, a coastal lagoon located in the south of Portugal (Algarve), by a microplate-based high throughput screening procedure described in Pereira et al. (2011). Briefly, water samples were supplemented with concentrated MAM17 and left exposed to indirect sunlight for approximately 1–2 weeks. Afterwards, aliquots were taken and stained with BODIPY 505/515 (4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene; Life Technologies Europe BV, Porto, Portugal) as described in Cooper et al. (2010) to prepare for flow cytometry. Stained samples were acquired in a Becton Dickinson FACS Aria II (BD Biosciences, Erembodegem, Belgium) equipped with a blue and red laser (488 and 633 nm, respectively) and FACSDiva (version 6.1.3) software. Four channels were used to record the fluorescence signal, namely FL1, FL2, FL3 and FL4 centred at 530/30, 585/42, 695/40 and 660/20 nm, respectively, after excitation with the blue (FL1-FL3) or red (FL4) laser. Cells emitting higher levels of fluorescence due to chlorophyll pigments and lipids stained with BODIPY were sorted directly onto 96-well microplates containing 250 μL of solid (agar) MAM and onto microscope slides. Colonies growing in the wells of the microplates were transferred to Petri dishes containing agar supplemented with MAM. The biomass growing on the Petri dishes was scrapped and transferred to 100-mL Erlenmeyer flasks with sterilized seawater and MAM and later transferred to 1-L photobioreactors with aeration.

### 2.4.2. MICROSCOPY

Microscopic images were acquired in a Zeiss AXIOMAGER Z2 microscope, with a coollSNApHQ2 camera and AxioVision software version 4.8 (Carl Zeiss MicroImaging GmbH, Göttingen, Germany), using the 100 × lens. Brightfield microscopy was carried out using differential interference contrast (DIC), while Zeiss 38 He filter set (Carl Zeiss MicroImaging GmbH, Gõttingen, Germany) for fluorescein isothiocyanate (FITC) was used to acquire the fluorescence images. Samples used for fluorescence microscopy were stained with BODIPY 505/515 as described for the flow cytometry analysis. Images were treated using Image J software (Research Service Branch, NIH, Bethesda, MD).

### 2.4.3. TAXONOMIC IDENTIFICATION

This strain was identified by means of 18S rDNA sequencing. DNA extraction was performed with the EZNA DNA plant extraction kit (Omega Bio-Tek, Norcross, GA) according to the manufacturer's guidelines. The obtained DNA was amplified by PCR with the primers 18SUnivFor (5′-ACCTGGTTGATCCTGCCAGT-3′) and 18SUnivRev (5′-TCAGCCTTGCGACCATAC-3′) as described in Pereira et al. (2011, 2013) and sequenced at an in-house DNA sequencing facility equipped with an Applied Biosystems 3130XL DNA sequencer (Life Technologies BV, Porto, Portugal). The obtained sequence was deposited in GenBank with the accession number KX278369 and compared with the GenBank database using BLASTn (https://blast.ncbi.nlm.nih.gov). The sequences were aligned and visually inspected using CLC Sequence Viewer (v. 7.6.1, Quiagen) and curated with Gblocks v. 0.91b software (Talavera and Castresana, 2007). Curation was performed allowing gap positions within the final blocks and a maximum of 8 contiguous nonconserved positions and a minimum block length of 5 nucleotides. Phylogenetic analysis was performed using Maximum-likelihood (ML) and Bayesian inference (BI). The substitution models that best fit the data set were selected using MrModeltest2 v.2.3 (Nylander, 2004) and PAUP* v.4.0b10 (Swofford, 2003) applying the Akaike information criterion (AIC; Akaike 1974). ML analysis was performed using RaxML v. 7.0.4 (Stamatakis, 2006), assuming a GTR + I + G substitution model with 400 bootstrap replicates. Posterior probabilities were determined by Markov Chain Monte Carlo (MCMC) sampling in MrBayes v. 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). MrBayes analyses were also conducted using the model GTR + I + G with 6 chains for 10,000,000 MCMC generations, sampling every 1,000$^{th}$ generation and using the default for all the other settings. The MCMC runs convergence and burn-in were determined through the analysis of the generations vs. log probability plot using the trace analysis tool TRACER v1.6 (Rambaut et al. 2014). The final tree was drawn with FigTree v.1.3.1 (Rambaut, 2006).

### 2.4.4. MICROALGAE GROWTH

All experiments were performed in a specialized growth chamber (Aralab Fitoclima S 600 PL clima plus 400), at 20 ± 0.5 °C, under continuous lighting (100 μmol $m^{-2}$ $s^{-1}$). Cultures were grown using 100 mL glass reactors, aerated continuously with

filtered (0.2 μm) compressed air. Standard culture medium was seawater (salinity = 36‰) supplemented with MAM. For the wastewater experiment, non-nitrified sewage effluent was supplied by Quinta do Lago wastewater treatment plant (WWTP, Quinta do Lago, Algarve, Portugal).

### 2.4.5. LIPID INDUCTION

Lipid induction assays were carried out using a two-stage growth system. Cultures were grown until day 10, under controlled conditions, as described in the previous section to allow the optimal growth of cultures reaching a high cell concentration (1$^{st}$ stage). At this stage, the nitrate content of the growth medium was completely depleted as determined by spectrophotometric methods described in APHA (1999). At day 10, the produced inoculum was either supplemented with concentrated MAM (N+, nutrient replete) or was left without nutrients (N−, nutrient depleted) to promote lipid induction (2$^{nd}$ stage). All the experiments were carried out in triplicate and average values are reported. Results were statistically analysed using SPSS (release 15.0, SPSS Inc., Chicago, IL) software, using analysis of variance (one-way ANOVA) and Tukey HSD post-hoc test with a confidence interval of 95%.

### 2.4.6. GROWTH EVALUATION AND CHEMICAL ANALYSIS OF CULTURES

#### 2.4.6.1. Determination of algal growth

Microalgal biomass concentration was determined by measuring the optical density of the cell culture in a 96-well plate spectrophotometer (Biotek Synergy 4) at 750 nm. CC was measured using a Neubauer counting chamber according to the manufacturer indications and through flow cytometry using CountBright™ absolute counting beads. For biomass concentration, expressed in a dry weight (DW) basis, 10 mL of algal suspension was filtered through a 0.45 μm cellulose acetate filter, washed with ammonium formate (37 g $L^{-1}$) and dried in an oven with forced air circulation at 60 °C until constant weight.

#### 2.4.6.2. Total lipid determination

Total lipid content was determined following the Bligh and Dyer (1959) with a few modifications as described in Pereira et al. (2011). Briefly, biomass was extracted with a mixture of chloroform, methanol and water (2:2:1), and homogenised with an IKA Ultra-Turrax disperser (IKA-Werke GmbH, Staufen, Germany) for 2 minutes. Phase separation was achieved by centrifugation, and the chloroform phase was transferred to new vessels with a Pasteur pipette. Afterwards, a known volume of chloroform (0.5–1 mL) was pipetted to pre-weighed tubes and evaporated overnight. The resulting dried residue was weighed and compared with the obtained DW to allow an accurate determination of the lipid fraction.

#### 2.4.6.3. Biodiesel synthesis

Lipids were extracted directly from wet biomass as described by Yang et al. (2015) with modifications. Briefly, 100 g of wet microalgae paste were dispersed in 250 mL of absolute ethanol at reflux temperature for 120 minutes (EtOH-1). Afterwards, the crude ethanol extract was separated from the remaining biomass by centrifugation (4000 g, 10 minutes). The biomass was further extracted using the same aforementioned conditions for 60 (EtOH-2, 200 mL) and 30 (EtOH-3, 150 mL) minutes. All extracts were pooled, and the ethanol was evaporated from the mixture using a rotatory evaporator.

Extracted lipids were converted to biodiesel by acid catalysed transesterification using the method described in Gangadhar et al. (2016) with modifications. Briefly, a solution of methanol and concentrated sulphuric acid (2% $H_2SO_4$ in methanol) was added to a round bottom flask containing the extracted lipids. The reaction mixture was stirred at reflux temperature for approximately 4 hours. The conversion of TAGs into FAME was followed by thin-layer chromatography, using hexane and ethyl acetate (95:5 v/v) as mobile phase. Upon reaction completion, the solvent was evaporated using a rotatory evaporator and the fatty acids were sequentially extracted three times with hexane. The resulting fractions were pooled and washed with distilled water to neutralize the acid.

#### 2.4.6.4. Fatty acid methyl esters profile

Produced biodiesel was analysed on a Bruker GC-MS (Bruker SCION 456/ GC, SCION TQ MS) equipped with a ZB-5MS (30 m × 0.25 mm internal diameter, 0.25 μm film thickness, Phenomenex) capillary column using helium as carrier gas. The temperature program was 60 °C (1 min), 30 °C $min^{-1}$ to 120 °C, 5 °C $min^{-1}$ to 250 °C, and 20 °C $min^{-1}$ to 300 °C (2 min). Injection temperature was 300 °C. For identification and quantification of the FAME total ion mode was used. Because of differences in the response factors, for each FAME separate calibration curves were determined in triplicate, using the Supelco® 37 Component FAME Mix (Sigma-Aldrich, Sintra, Portugal) commercial standard. In the case where no standard was available, the response factor of the most similar FAME, in terms of structure, was used. Results are expressed as a percentage of total FAME content.

#### 2.4.6.5. Biodiesel properties

The density of the produced biodiesel was determined at 15 °C using a certified Lenz pycnometer. Biodiesel kinematic viscosity was measured at 40 °C using a micro Ubbelohde viscometer in accordance with ISO 3105. Glycerol and acylglycerols contents were determined as per EN14105 method. Group I and II metals, as well as the phosphorous content, were determined by a microwave plasma atomic emission spectrometry (MP-AES 4200, Agilent technologies) according to Agilents' technical note (5990-9005EN).

The CN of the FAME mix ($CN_{mix}$) was estimated using the equation described in Knothe, (2014), relating the CN ($CN_c$) and relative amount ($A_c$) of each FAME in the biodiesel mixture:

$$CN_{mix} = \sum A_c \times CN_c \quad (1)$$

The oxidation stability was estimated using a Rancimat (model 743) according to the standard EN 14112:2003. IV was calculated using the factors estimated for different FAME according to the EN14214. The CFPP was calculated using the equations proposed by Ramos et al. (2009). This model relies on the estimation of the CFPP through

the determination of the long chain saturated factor (LCSF) in accordance with the following equations:

$$\mathrm{LCSF} = (0.1 \times \mathrm{C16}) + (0.5 \times \mathrm{C18}) + (1 \times \mathrm{C20}) + (1.5 \times \mathrm{C22}) + (2 \times \mathrm{C24}) \quad (2)$$

$$\mathrm{CFPP} = 3.1417 \times \mathrm{LCSF} - 16.477 \quad (3)$$

## ACKNOWLEDGEMENTS

The authors would like to thank Cristina Oliveira (PhD, LNEG, Portugal) for the kind determination of the oxidative stability of the biodiesel samples. The present work was funded by the Portuguese national budget and the CCMAR/Multi/04326/2013 grant of the Foundation for Science and Technology (FCT). H.P. (SFRH/ BD/105541/2014) and C.B.S. (SFRH/BD/78062/2011) are FCT doctoral research fellows. K.N.G. (SFRH/ BPD/81882/2011) and L.C. (IF/00049/2012) were supported by FCT as a post-doctoral research fellow and as FCT Investigator Programme recipient, respectively.

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# CHAPTER III

# SCALE-UP AND LARGE-SCALE PRODUCTION OF *TETRASELMIS* SP. CTP4 (CHLOROPHYTA) FOR $CO_2$ MITIGATION: FROM AN AGAR PLATE TO 100-$M^3$ INDUSTRIAL PHOTOBIOREACTORS

**Research article published in:**

Pereira, H., Páramo, J., Silva, J., Marques, A., Barros, A., Maurício, D., Santos, T., Schulze, P., Barros, R., Gouveia, L., Barreira, L., & Varela, J. (2018). Scale-up and large-scale production of *Tetraselmis* sp. CTP4 (Chlorophyta) for $CO_2$ mitigation: from an agar plate to 100-$m^3$ industrial photobioreactors. Scientific Reports, 8, 5112.

# SCALE-UP AND LARGE-SCALE PRODUCTION OF *TETRASELMIS* SP. CTP4 (CHLOROPHYTA) FOR $CO_2$ MITIGATION: FROM AN AGAR PLATE TO 100-$M^3$ INDUSTRIAL PHOTOBIOREACTORS

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## ABSTRACT

Industrial production of novel microalgal isolates is key to improving the current portfolio of available strains that are able to grow in large-scale production systems for different biotechnological applications, including carbon mitigation. In this context, *Tetraselmis* sp. CTP4 was successfully scaled up from an agar plate to 35- and 100-$m^3$ industrial scale tubular photobioreactors (PBR). Growth was performed semi-continuously for 60 days in the autumn-winter season ($17^{th}$ October – $14^{th}$ December). Optimisation of tubular PBR operations showed that improved productivities were obtained at a culture velocity of 0.65–1.35 m $s^{-1}$ and a pH set-point for $CO_2$ injection of 8.0. Highest volumetric (0.08 ± 0.01 g $L^{-1}$ $d^{-1}$) and areal (20.3 ± 3.2 g $m^{-2}$ $d^{-1}$) biomass productivities were attained in the 100-$m^3$ PBR compared to those of the 35-$m^3$ PBR (0.05 ± 0.02 g $L^{-1}$ $d^{-}$1 and 13.5 ± 4.3 g $m^{-2}$ $d^{-1}$, respectively). Lipid contents were similar in both PBRs (9–10% of ash free dry weight). $CO_2$ sequestration was followed in the 100-$m^3$ PBR, revealing a mean $CO_2$ mitigation efficiency of 65% and a biomass to carbon ratio of 1.80. *Tetraselmis* sp. CTP4 is thus a robust candidate for industrial-scale production with promising biomass productivities and photosynthetic efficiencies up to 3.5% of total solar irradiance.

## 3.1. INTRODUCTION

Most microalgae are unicellular photosynthetic organisms that through photosynthesis and several metabolic pathways convert inorganic carbon ($CO_2$) into organic carbon in the form of proteins, lipids, carbohydrates and nucleic acids. Therefore, the industrial production of microalgal biomass couples the mitigation of $CO_2$ with the production of biomolecules that can be purified or upgraded into bioproducts important for different biotechnological applications (e.g., food, feed, pharmaceuticals and biofuels). Although several microalgae ventures have been established in recent years (Chisti, 2013), the implementation of industrial biomass production is still at an infant stage (Enzing et al. 2014). Nevertheless, mass culture of microalgal biomass is currently considered as one of the most promising approaches to manufacturing next-generation foods, feeds, and biofuels with the concomitant capture of $CO_2$ from emitting industries and recycling nutrients from wastewaters (Jorquera et al. 2010; Quadrelli et al. 2011).

Mass culture of microalgae can be achieved in open (e.g., open ponds or raceways) or closed (e.g., photobioreactors; PBR) production systems (Figure 3.1). Open ponds are the system chosen by most companies producing microalgae at an industrial

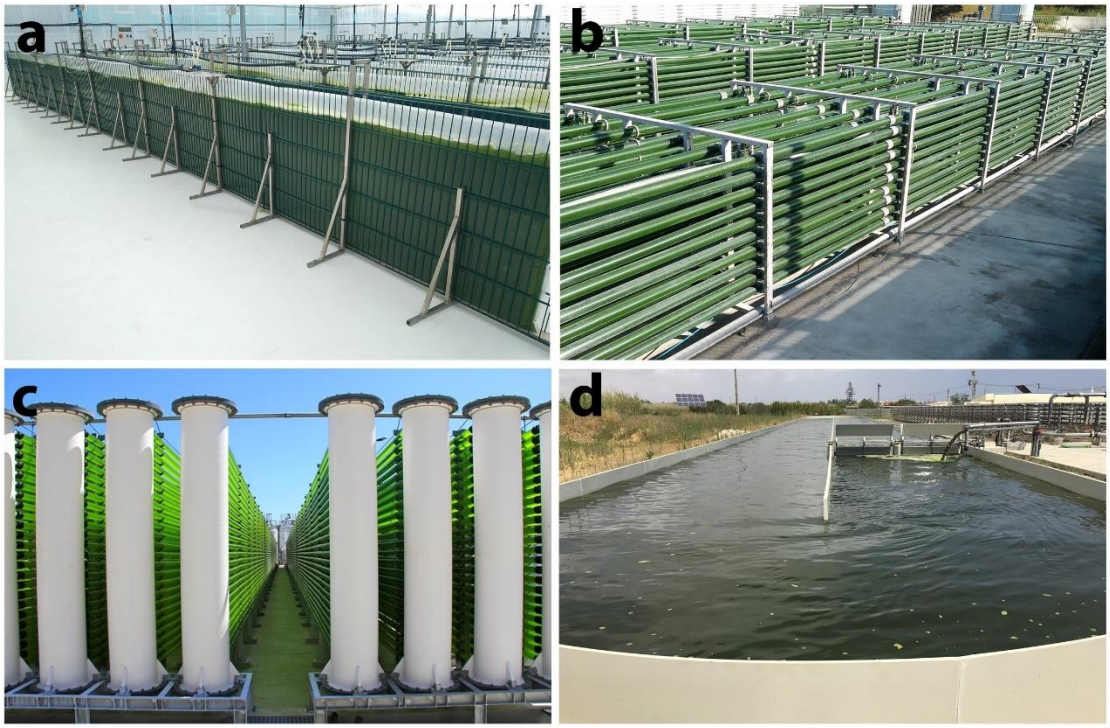


**Figure 3.1 -** Different large-scale systems currently used for the industrial production of microalgal biomass: (a) 1-$m^3$ Flat panel photobioreactor. (b) 2.5-$m^3$ pilot-scale tubular photobioreactor. (c) 100-$m^3$ industrial tubular photobioreactor. (d) 200-$m^3$ raceway. Pictures depicted were kindly provided by CMP, Secil group, Pataias, Portugal (a–c) and Necton S.A., Belamandil-Olhão, Portugal (d).

scale due to the low capital and operational costs (Acién Fernández et al. 1999; Borowitzka, 1999; Pulz, 2001; Chisti, 2007). However, as cultures are directly exposed to the atmosphere, the water and $CO_2$ losses and the probability of contamination are the main hindrances of open production systems (Pulz, 2001). In addition, the strict control of temperature and other culture parameters required to grow sensitive strains (e.g., diatoms) is rather challenging (Silva Benavides et al. 2013). On the other hand, closed systems display lower $CO_2$ and water losses, reduce the probability of contamination and allow a tighter control of growth conditions. This allows the cultivation of most microalgal strains (Richmond et al. 1993; Borowitzka, 1999; Ugwu et al. 2008) with higher areal and volumetric biomass productivities (Borowitzka, 1999; Pulz, 2001).

In order to meet the full potential of microalgal biomass, the selection of robust and fast-growing strains is crucial to develop feedstocks that can effectively grow in large-scale industrial facilities (Rodolfi et al. 2009; Rawat et al. 2013). Recently, *Tetraselmis* sp. CTP4 was isolated and characterised as a robust, euryhaline, lipid-rich microalga able to grow both in standard growth media, as well as in urban wastewater effluents (Pereira et al. 2016; Schulze et al. 2017). Apart from its high potential for bioremediation, *Tetraselmis* sp. CTP4 presents promising features as compared to common microalgal feedstocks. The biomass of this microalga can be recovered through natural cell sedimentation, decreasing the total culture volume down to 20% within 6 hours (Pereira et al. 2016). This property is essential to significantly decrease harvesting costs, one of the most costly steps of culturing and retrieving microalgae from an aqueous growth medium (Matos et al. 2013).

Because of the high potential of *Tetraselmis* sp. CTP4 for different biotechnological applications, the present work describes the scale-up procedure used to reach industrial production. To enhance the biomass production, the culture velocity and pH set point for $CO_2$ injection were tested and optimized in a pilot-scale tubular PBR. To the authors' knowledge, this is the first report addressing $CO_2$ mitigation as well as biomass and lipid productivities of microalgae cultures grown semi-continuously in an industrial-scale tubular PBR production system.

## 3.2. RESULTS

### 3.2.1. OPTIMIZATION OF CULTURE VELOCITY AND PH SET POINT

In a first experiment, the culture velocity was tested in 2.5 $m^3$ pilot-scale tubular PBR using three different culture velocities: 0.65, 1.01 and 1.35 m $s^{-1}$. The radiation during the trial was 10.3 ± 1.7 MJ $m^{-2}$ $d^{-1}$, while the temperature was 19.4 ± 2.9 °C (Figure 3.2a). Cultures under all conditions displayed similar growth patterns, without significant differences among them ($p > 0.05$), reaching the late exponential phase at day 13 and a final ash free dry weight (AFDW) of approximately 2.1 g $L^{-1}$. The same pattern was observed for the volumetric and areal biomass productivities (0.14–0.15 g $L^{-1}$ $d^{-1}$ and 12.9–13.6 g $m^{-2}$ $d^{-1}$, respectively), where no significant differences were observed ($p > 0.05$) under all velocities tested (Table 3.1). The same was found for the maximum biomass productivity under all conditions (0.36–0.43 g $L^{-1}$ $d^{-1}$ and 34.7–39.1 g $m^{-2}$ $d^{-1}$).

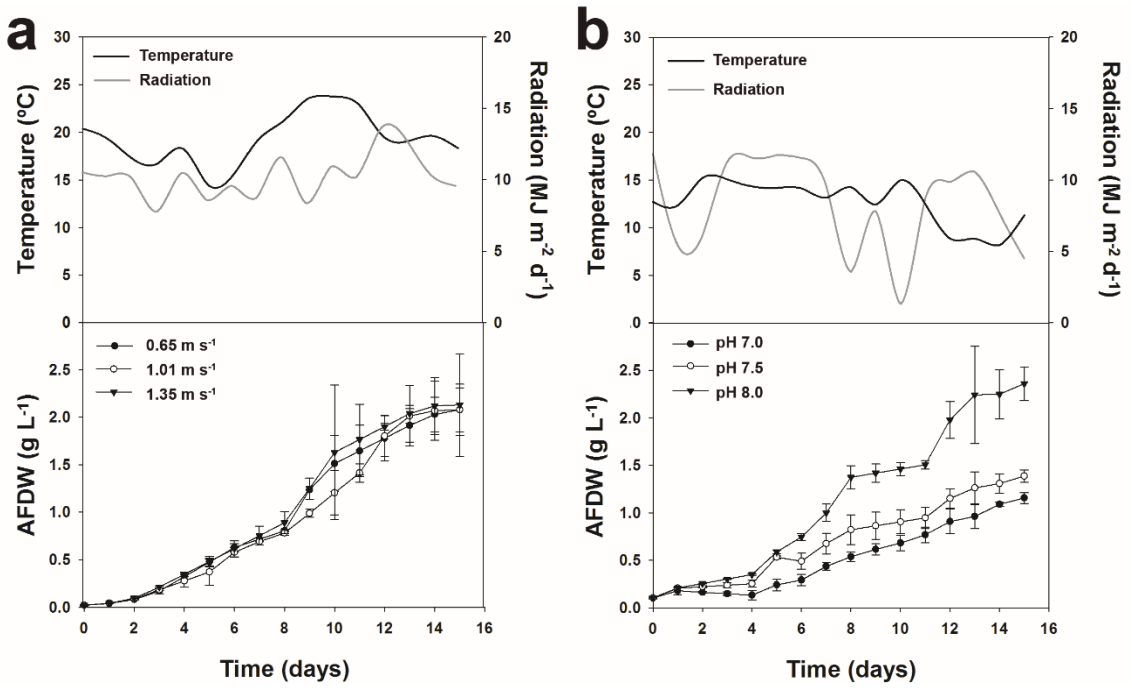


**Figure 3.2 -** Optimization of tubular photobioreactor operation in pilot-scale production systems. *Tetraselmis* sp. CTP4 growth in 2.5-$m^3$ tubular photobioreactors. (a) Culture velocity. (b) pH set point for $CO_2$ injection.

Afterwards, a trial was performed in the same PBRs (2.5 $m^3$) to assess the effect of different pH set points on $CO_2$ injection (Figure 3.2b). The temperature (12.7 ± 3.3 °C) and daily radiation (8.3 ± 3.3 MJ $m^{-2}$ $d^{-1}$) observed during this trial were lower than those of the previous experiment (Figure 3.2a). Interestingly, the different tested pH set points affected the growth of *Tetraselmis* sp. CTP4, displaying significant differences between cultures maintained at pH 8 compared to pH 7.5 and 7.0 ($p < 0.05$). Accordingly, best

growth was obtained at a pH set point of 8.0, with higher volumetric and areal biomass productivities (0.15 g $L^{-1}$ $d^{-1}$ and 15.9 g $m^{-2}$ $d^{-1}$, respectively). Cultures at pH 7.5 (0.08 g $L^{-1}$ $d^{-1}$ and 9.4 g $m^{-2}$ $d^{-1}$) and neutral pH (7.0) displayed the lowest growth performances (0.07 g $L^{-1}$ $d^{-1}$ and 7.8 g $m^{-2}$ $d^{-1}$). Consequently, faster growth (Figure 3.2b) and maximum biomass productivity (Table 3.1) were achieved at pH 8.0 (0.37 g $L^{-1}$ $d^{-1}$ and 34.1 g $m^{-2}$ $d^{-1}$), compared to that of pH 7.5 (0.20 g $L^{-1}$ $d^{-1}$ and 16.9 g $m^{-2}$ $d^{-1}$) and 7.0 (0.14 g $L^{-1}$ $d^{-1}$ and 13.1 g $m^{-2}$ $d^{-1}$).

**Table 3.1 -** Volumetric and areal biomass productivities presented in ash free dry weight of batch cultures grown in 2.5-$m^3$ outdoor tubular photobioreactors, using different culture velocities and pH set points for $CO_2$ injection. Different letters indicate significant differences within each parameter tested.

| PBR | Volumetric productivity | | Areal productivity | |
|---|---|---|---|---|
| | Total | Max | Total | Max |
| | g $L^{-1}$ $d^{-1}$ | g $L^{-1}$ $d^{-1}$ | g $m^{-2}$ $d^{-1}$ | g $m^{-2}$ $d^{-1}$ |
| **Culture velocity (m $s^{-1}$)** | | | | |
| 0.65 | 0.14 ± 0.02[a] | 0.43 ± 0.15[a] | 12.9 ± 1.44[a] | 39.1 ± 9.19[a] |
| 1.01 | 0.15 ± 0.01[a] | 0.39 ± 0.09[a] | 13.6 ± 0.52[a] | 35.4 ± 5.12[a] |
| 1.35 | 0.15 ± 0.02[a] | 0.36 ± 0.10[a] | 13.6 ± 2.01[a] | 34.7 ± 8.28[a] |
| **pH set point** | | | | |
| 7.0 | 0.07 ± 0.01[a] | 0.14 ± 0.02[a] | 7.8 ± 0.39[a] | 13.1 ± 1.85[a] |
| 7.5 | 0.08 ± 0.01[a] | 0.20 ± 0.05[a] | 9.4 ± 0.44[a] | 16.9 ± 3.28[a] |
| 8.0 | 0.15 ± 0.02[b] | 0.37 ± 0.04[b] | 15.9 ± 1.19[b] | 34.1 ± 8.90[b] |

### 3.2.2. GROWTH IN INDUSTRIAL SCALE PHOTOBIOREACTORS

After the optimization of the culture conditions, cells were grown semi-continuously in 35- and 100-$m^3$ industrial tubular PBR for approximately 60 days (Figure 3.3) and harvested four times, every 13–14 days. Experiments were carried out (17$^{th}$ October – 14$^{th}$ December) in a non-optimal season. In fact, the second half of this time range partially overlaps with the months when temperature and irradiance are lowest in the northern hemisphere. Ambient temperature decreased from 19.2 ± 2.9 °C during 17–30$^{th}$ October to 12.9 ± 2.5 °C between the 2$^{nd}$ November − 14$^{th}$ December. The same pattern was observed for the daily radiation, decreasing from 9.7 ± 1.9 MJ $m^{-2}$ $d^{-1}$ during the first 15 days to 7.9 ± 2.9 MJ $m^{-2}$ $d^{-1}$ due to higher cloud cover. Both PBRs were inoculated at a concentration of ~0.2 g $L^{-1}$. Notably, the 100 $m^3$-system displayed on average higher biomass concentrations than the 35 $m^3$ system ($p < 0.05$) with average concentrations of 1 g $L^{-1}$ and 0.8 g $L^{-1}$, respectively. As compared to the 35-$m^3$ system,

the 100-$m^3$ PBR registered higher volumetric (0.08 ± 0.01 vs. 0.05 ± 0.02 g $L^{-1}$ $d^{-1}$) and areal (20.3 ± 3.2 vs. 13.5 ± 4.3 g $m^{-2}$ $d^{-1}$) biomass productivities ($p < 0.05$; Table 3.2) as well as photosynthetic efficiencies (PEs; 3.35 ± 0.19 vs. 2.38 ± 0.27%; $p < 0.05$). In addition, the areal productivities were statistically higher during the first 30 days (35 $m^3$: 17.1 ± 1.9 g $m^{-2}$ $d^{-1}$; 100 $m^3$: 22.4 ± 3.5 g $m^{-2}$ $d^{-1}$) as compared to the last 30 days (35 $m^3$: 9.9 ± 0.6 g $m^{-2}$ $d^{-1}$; 100 $m^3$: 18.2 ± 0.4 g $m^{-2}$ $d^{-1}$) in both PBRs. This result can be explained by the lower temperatures and radiation observed on site.

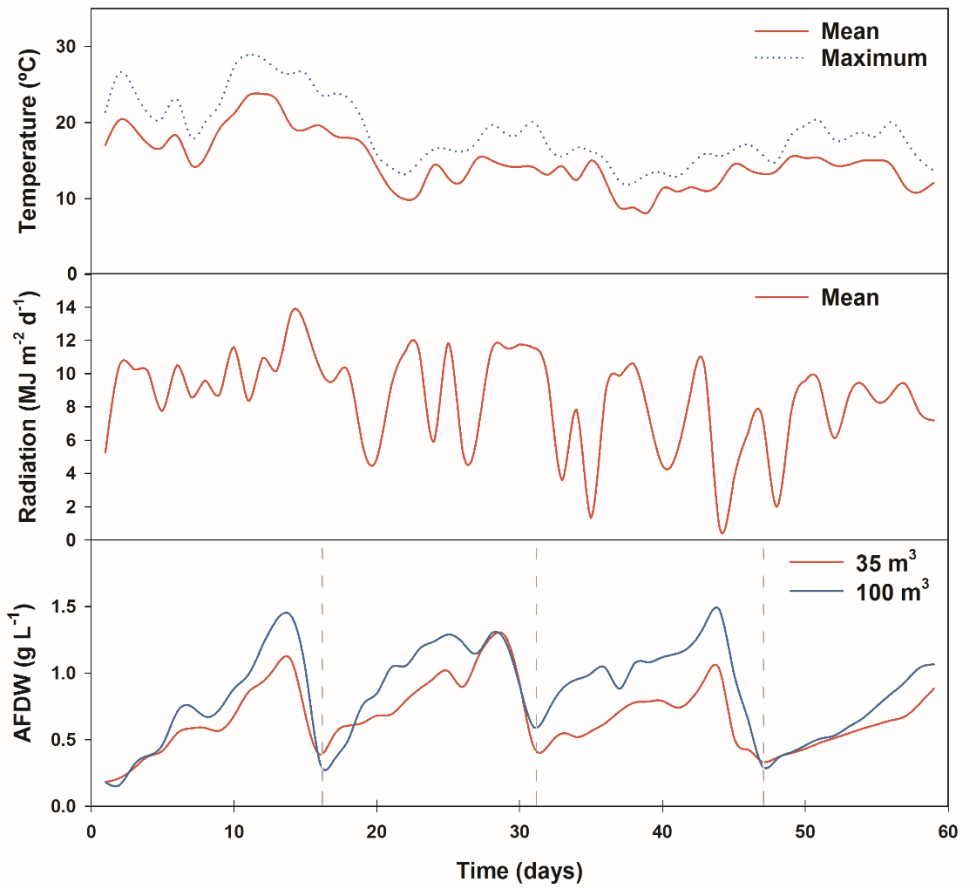


**Figure 3.3 -** Mean and maximum temperature and radiation registered during the growth of *Tetraselmis* sp. CTP4 in 35- and 100-$m^3$ industrial tubular photobioreactors grown semi-continuously. Cultures were harvested every 13–14 days for approximately 60 days, between 17th October and 15th December. Dashed grey line marks the start of the following growth period.

Taking into account the meteorological weather data and normalising the areal and volumetric productivities for both PBRs, a strong positive correlation between productivity and supplied irradiation (r = 0.97; $p < 0.05$) and temperature (r = 0.89; $p < 0.05$) was found. Temperature was also found to affect the PE, decreasing by 15% when this parameter dropped below 15 °C. This indicates that growth performance of this strain in both PBRs was strongly affected by light and temperature and that CTP4 tends to grow

better at temperatures above 15 °C. The maximum volumetric and areal productivities observed in the 35- (0.15 ± 0.03 g $L^{-1}$ $d^{-1}$ and 38.7 ± 8.4 g $m^{-2}$ $d^{-1}$) and 100-$m^3$ (0.17 ± 0.05 g $L^{-1}$ $d^{-1}$ and 37.2 ± 9.4 g $m^{-2}$ $d^{-1}$) PBRs were similar ($p > 0.05$), reaching the double of the average productivities in most growth periods.

**Table 3.2 -** Volumetric and areal biomass productivities of *Tetraselmis* sp. CTP4 grown semi-continuously in 35- and 100-$m^3$ tubular photobioreactors (PBRs) presented in ash free dry weight. The photosynthetic efficiency (PE) obtained in the different growth periods is also presented. Using a semi-continuous growth system, four different culture periods were established throughout the growth trial (17th Oct–15th Dec). Different letters indicate significant differences in productivity and PE between PBRs.

| PBR | Biomass productivity | | | | PE |
|---|---|---|---|---|---|
| | Total g $L^{-1}$ $d^{-1}$ | Max g $L^{-1}$ $d^{-1}$ | Total g $m^{-2}$ $d^{-1}$ | Max g $m^{-2}$ $d^{-1}$ | (%) |
| **35 $m^3$** | | | | | |
| 17th - 30th Oct | 0.07 | 0.18 | 18.4 | 46.8 | 2.62 |
| 2nd - 14th Nov | 0.06 | 0.15 | 15.8 | 39.1 | 2.59 |
| 17th - 29th Nov | 0.04 | 0.15 | 10.3 | 41.7 | 2.20 |
| 1st - 14th Dec | 0.04 | 0.10 | 9.5 | 27.1 | 2.09 |
| Mean | 0.05 ± 0.02[a] | 0.15 ± 0.03[a] | 13.5 ± 4.3[a] | 38.7 ± 8.4[a] | 2.38 ± 0.27[a] |
| **100 $m^3$** | | | | | |
| 17th - 30th Oct | 0.10 | 0.19 | 24.9 | 42.4 | 3.54 |
| 2nd - 14th Nov | 0.08 | 0.20 | 20.0 | 42.8 | 3.28 |
| 17th - 29th Nov | 0.07 | 0.18 | 18.5 | 40.4 | 3.46 |
| 1st - 14th Dec | 0.07 | 0.10 | 18.0 | 23.1 | 3.11 |
| Mean | 0.08 ± 0.01[b] | 0.17 ± 0.05[a] | 20.3 ± 3.2[b] | 37.2 ± 9.4[a] | 3.35 ± 0.19[b] |

The volumetric and areal lipid productivities were about 10% of the respective biomass productivities, since the lipid content in the biomass produced throughout the four growth periods and in both PBRs was quite stable, averaging 9.9 ± 0.3% of AFDW (Figure 3.4a). The results were confirmed by fluorescence microscopy of cells stained with BODIPY 505/515 (Figure 3.4b). Overall, obtained results revealed that the lipid content was not significantly affected by the volume of the PBR, temperature or light intensity ($p > 0.05$).

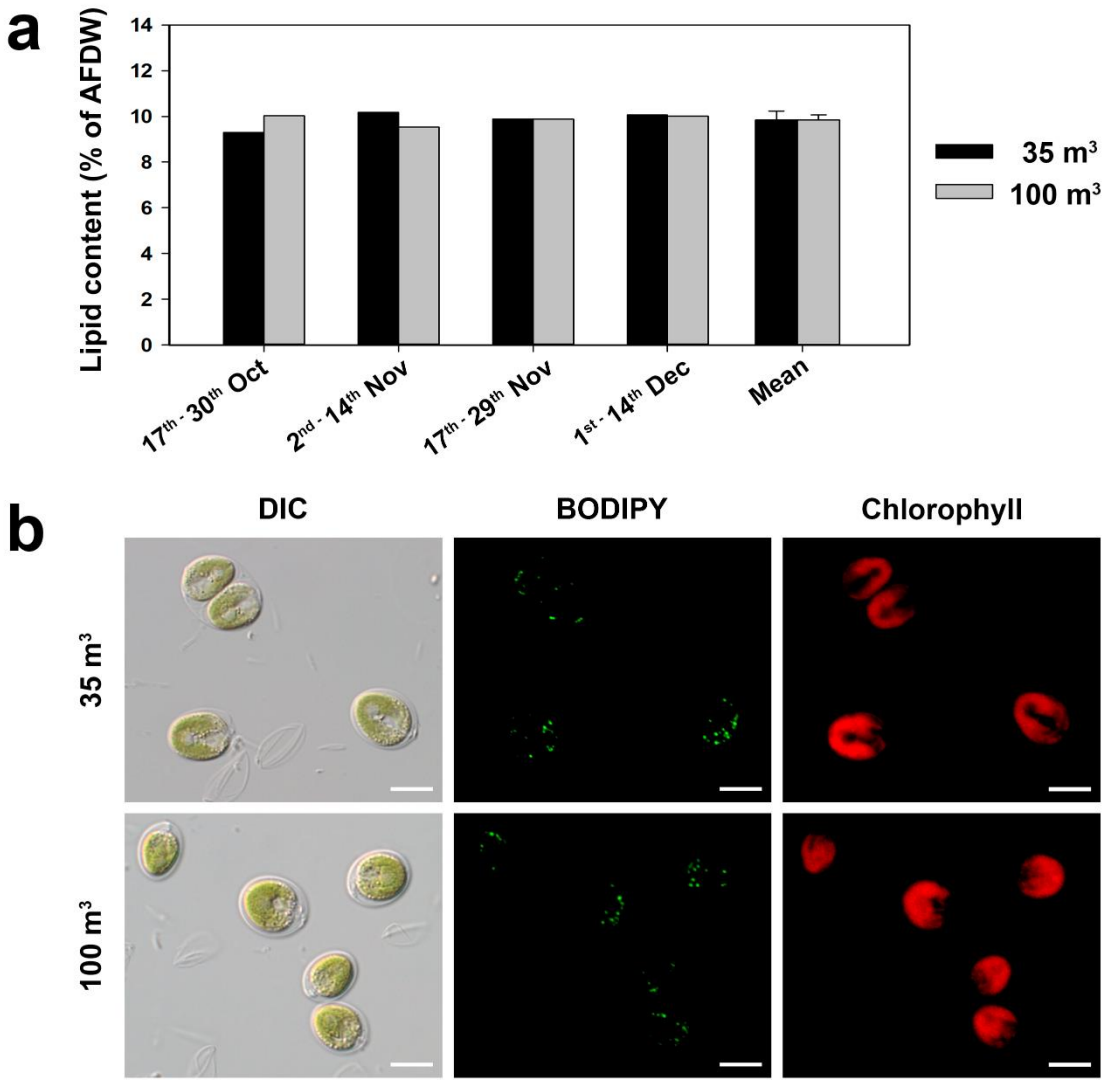


**Figure 3.4 -** Lipid content and fluorescence microscopy of *Tetraselmis* sp. CTP4 grown semi-continuously in tubular photobioreactors. (a) Lipid content of cultures grown industrially in four different growth periods and mean value obtained in the overall experiment. (b) Brightfield and fluorescence microscopy of cultures grown in the 35- and 100-$m^3$ tubular photobioreactors. Depicted pictures show the differential interference contrast (DIC), as well as BODIPY 505/515 and chlorophyll fluorescence of *Tetraselmis* sp. CTP4 cells. Scale bar = 10 µm.

### 3.2.3. $CO_2$ SEQUESTRATION

The capacity of *Tetraselmis* sp. CTP4 to mitigate $CO_2$ was investigated in the 100-$m^3$ PBR for 30 days ($17^{th}$ Oct – $17^{th}$ Nov). The mass balance of $CO_2$, considering the $CO_2$ that enters the system and the $CO_2$ exhausted from the PBR, was related with the C content of the biomass (determined by elemental analysis). In agreement with the elemental analysis of the biomass that showed mass contents of 49.1% C, 7.84% H and 5.80% N, the following approximate stoichiometry can be used to describe biomass formation from $CO_2$ and nitrate:

$$1.101CO_2 + 0.101NO_3^- + 1.008H_2O \rightarrow CH_{1.914}O_{0.568}N_{0.101} + 1.321O_2 + 0.101HCO_3^-$$

This equation shows that 1.80 g of $CO_2$ are consumed for the formation of 1.0 g of ash free algal biomass. Accordingly, the $CO_2$ mass balance in the 100-$m^3$ PBR was performed by quantifying the volume of injected $CO_2$ (99.99%), its content in the air used for degassing the culture (0.04%), and the $CO_2$ content of the exhaust gas (0.3–0.5%). Even though the volumetric flow of the later stream was not accurately measured due to operational impracticability of placing a rotameter in the exhaust section of the PBR, it can be assumed that its molar flow will be quite close to that of the compressed air, because it is two orders of magnitude higher than pure $CO_2$ injection. Our calculations (Supplementary data) show that 60–75% of the $CO_2$ introduced in the PBR is taken up by the culture, while 25–40% of the $CO_2$ is exhausted from the PBR to the atmosphere. In summary, a total of ~535 kg of $CO_2$ were consumed to produce ~296 Kg biomass in the 100-$m^3$ PBR during a 60-day operation.

### 3.2.4. SEASON COMPARISON USING AN ALGEM® PHOTOBIOREACTOR

A season comparison assay was performed using an Algem® PBR to simulate the Spring and Autumn seasons at the latitude and longitude of Algafarm using controlled artificial LED light. The main objective of the simulation was to estimate the growth potential of *Tetraselmis* sp. CTP4 under average abiotic conditions in order to expand the findings obtained outdoors. The Algem® built-in software defines a maximum light intensity of 700 and 1400 µmol $s^{-1}$ $m^{-2}$ and a mean temperature of 12 and 20 °C for Autumn and Spring, respectively (Figure 3.5). The growth conditions simulating Spring presented a higher growth rate, reaching the stationary phase in approximately 5 days with a final AFDW of 2.02 g $L^{-1}$, and a biomass productivity of 0.25 g $L^{-1}$ $d^{-1}$. On the other hand, cultures grown in conditions simulating Autumn displayed a lower growth performance, reaching a final AFDW of 1.76 g $L^{-1}$ and a biomass productivity of 0.12 g $L^{-1}$ $d^{-1}$ in the end of the assay (day 9). During day 1 and 9, the spring simulation yielded on average a significant higher biomass concentration (1.7 g $L^{-1}$) as compared to the winter conditions (1.3 g $L^{-1}$; $p < 0.05$). These results suggest that the expected growth rate of cultures and effective $CO_2$ mitigation has a marked seasonal dependence. This rate

is expected to be twice as high in spring, when compared to its value in autumn (0.45 g $CO_2$ $L^{-1}$ $d^{-1}$ vs 0.22 g $CO_2$ $L^{-1}$ $d^{-1}$).

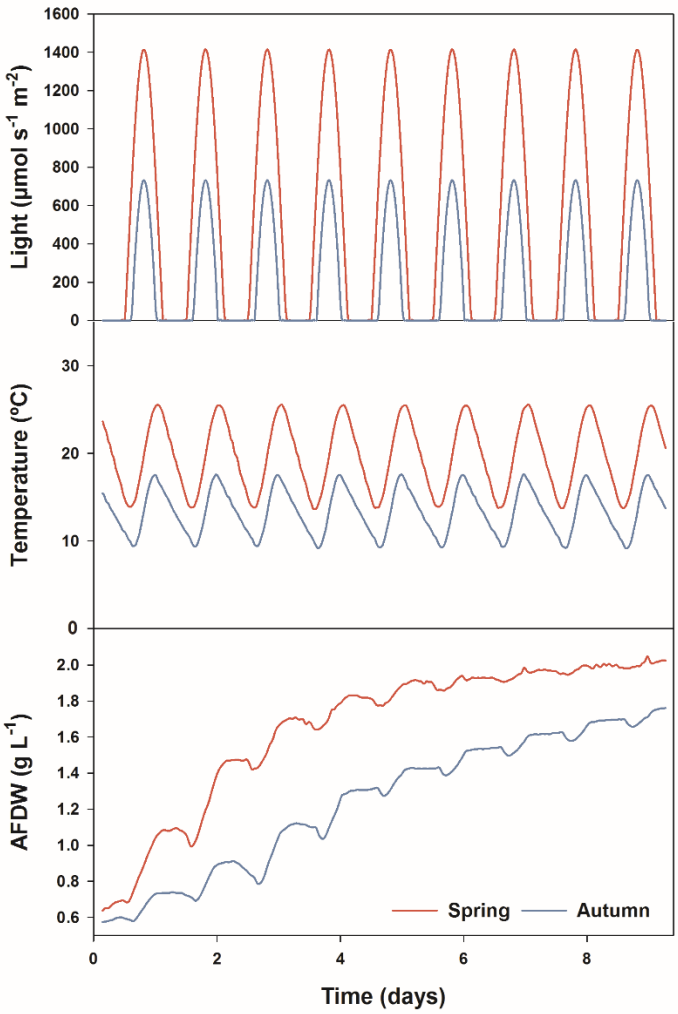


**Figure 3.5 -** Season comparison assay of *Tetraselmis* sp. CTP4 in Algem® photobioreactors. Growth curves of *Tetraselmis* sp. CTP4 using an Algem® photobioreactor simulating the conditions of growth in Spring and Autumn seasons on the West coast of Portugal.

## 3.3. DISCUSSION

The trend of microalgal biotechnology towards a medium sized market requires studies about the optimization of industrial scale cultivation systems and novel strains to widen the current portfolio for maximal production efficiency (Ruiz et al. 2016). The present study demonstrated the successful scale-up of the novel isolate *Tetraselmis* sp. CTP4 from an agar plate to a 100-$m^3$ industrial tubular PBR within eight weeks. *Tetraselmis* sp. CTP4 is thus a promising candidate for mass production of bulk products

due to high growth performance among various culturing systems and environmental conditions.

The pH of the culture medium is an essential parameter not only to obtain optimal growth, but it also determines the maximal amount of $CO_2$ dissolved in the medium (carbon balance). *Tetraselmis* sp. CTP4 performed best at a slight alkaline pH of 8.0, a result similar to that reported by Khatoon et al. (2014) for microalgae of the same genus. However, as the response to pH fluctuations is species-dependent, optimal growth of *Tetraselmis suecica* was achieved by Moheimani (2013) at pH 7.0 and 7.5. The culture velocities tested were all suitable for growing *Tetraselmis* sp. CTP4 in tubular PBRs. This might be explained by the low radiation observed on site during this time period, since it has been observed that under low light conditions the mixing rates are less important for the final productivity (Brindley et al. 2016). The opposite is expected in the spring-summer season during which the importance of velocity might increase due to its effect on the overall light availability to cells when grown under higher radiation. In addition, the optimization of velocity suitable to the microalgal culture inside the production tubes is important to avoid biomass deposition while cells travel through the photic section of the PBR and increase $CO_2$ availability (Zhu et al. 2013; Huang et al. 2017). Lower velocities can be used to reduce the energy costs in the production pipeline; however, this can lead to the formation of biofilm in the tubes, promoting light attenuation in the system (not observed in the present work). On the other hand, the use of higher culture velocities without lysing microalgal cells of interest can be important to manage and contain specific contaminations. This is particularly true for contaminants sensitive to the added turbulence and shear stress generated by faster velocities in the PBR (Brindley et al. 2016). An important factor for the successful implementation of a microalgal-based production pipeline is the proper management of predators and competing microalgae. Similarly, the euryhaline properties of *Tetraselmis* sp. CTP4 can be used to eliminate potential contaminants from large-scale production facilities by means of abrupt salinity shifts (Pereira et al. 2016), in particular if the contaminant does not have a cell wall or has reduced halotolerance. It is worth noting that during the scale-up procedure and all experimental trials, cultures of *Tetraselmis* sp. CTP4 remained monoalgal, i.e., no other microalgal species were detected. Although some common non-photosynthetic contaminants were observed they did not had a severe impact on productivity and did not take over the cultures (Figure 3.6). In fact, all reactors were grown without any culture

collapse in spite of the changing conditions of temperature and radiation. This is an important result, as some commonly used microalgal species (e.g., *Chlorella vulgaris* and *Haematococcus pluvialis*) are more susceptible to predators/parasites under industrial settings (e.g., *Chytridium* sp., *Amoeboaphelidium protococcarum* and *Vampirovibrio chlorellavorus*), which have a significant impact on culture viability and biomass productivity (Letcher et al. 2013; McBride et al. 2014; Ganuza et al. 2016).

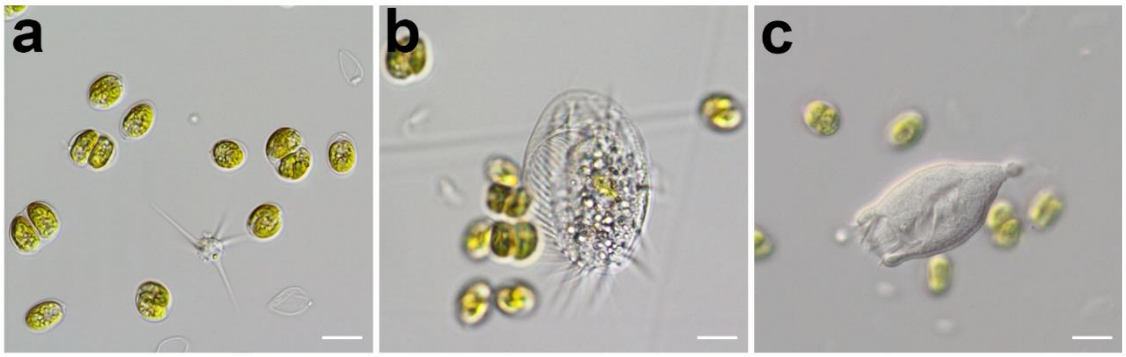


**Figure 3.6 -** Different environmental contaminants detected in the course of the present work throughout the growth in industrial scale production systems. (a) *Amoeba radiosa*. (b) Unidentified ciliate. (c) *Vorticella* sp. Scale bar = 10 µm.

Accordingly, semi-continuous growth of *Tetraselmis* sp. CTP4 at industrial scale was successfully achieved in both 35- and 100-$m^3$ PBR for a 60-day period. As expected, the growth of cultures was higher in the first two growth periods (late October – early November), which resulted in higher biomass productivities. These results are in accordance with the data obtained from the statistical correlations obtained for both industrial PBR as well as in the Algem® PBR, which strongly suggests that low temperatures (<15 °C) and radiation decreased the biomass productivities of this strain. However, *Tetraselmis* sp. CTP4 responded differently in the 35-$m^3$ and 100-$m^3$ PBRs, within the same time period. Interestingly, it was in the largest industrial PBR tested that higher productivities were consistently obtained. Volumetric biomass productivities in the industrial scale PBRs (35- and 100-$m^3$) were lower than when compared to the pilot-scale PBRs (2.5-$m^3$), while the reverse trend was apparent for areal productivities. The reason for such difference relies on the high stocking density of the horizontal tubes in the industrial reactors. Although the lower light penetration into the industrial PBRs tubes at lower layers reduces the volumetric production and the final biomass concentration in the system, the considerably higher culture volume in the same area results in higher areal productivities.

The areal productivity registered in the first growth period ($17^{th}$ – $30^{th}$ October) is similar to the productivity previously reported for other microalgal strains (e.g., *Phaeodactylum tricornutum* and *Nannochloropsis* sp.) (Griffiths and Harrison, 2009). In addition, the PEs of 3.35 ± 0.19% (100-$m^3$) were high despite the shifting temperature and light regimes during the time period tested.

The average mitigation efficiency of 65% of the $CO_2$ in the 100-$m^3$ PBR was notable, considering the industrial size of system. This efficiency is considerably higher than previous reports that addressed $CO_2$ mitigation using other microalgal strains and cultivation systems (Chiu et al. 2011; Li et al. 2011; Kurzbaum et al. 2017; Nithiya et al. 2017). However, the values here reported are similar to those reported by Keffer and Kleinheinz (2002) using *Chlorella vulgaris* (74% carbon mitigation efficiency) fed with an elevated $CO_2$ stream. Higher effective $CO_2$ removal (82.5–99%) has been reported when *C. vulgaris* is grown using a laboratory-scale sequential PBR array (Lam and Lee, 2013).

The biomass to carbon ratio of 1.80 obtained in this work is typical for non-stressed microalgae (Chisti, 2007; Rodolfi et al. 2009). This ratio can be increased by higher amounts of lipids in biomass that display higher carbon content per unit mass (76–77%) than proteins (53%) or carbohydrates (40–44%) (Klass, 2004). The values of the lipid content found in this work are in accordance with the data previously reported for *Tetraselmis* sp. CTP4 grown under nutrient repletion, about 10% of DW (Pereira et al. 2016). Under optimal growth conditions, cells shift the carbon flux towards the synthesis of carbohydrates rather than the accumulation of lipids. The latter are predominantly synthesized and accumulated under adverse environmental conditions, such as nutrient depletion. In this context, a two-stage growth system would be able to increase lipid productivities, and thus higher $CO_2$ fixation rates (Rodolfi et al. 2009; Pereira et al. 2016). In a first stage, cultures could be grown under optimal conditions to reach a high cell concentration, whereas at a later stage lipid induction is achieved via environmental stress (e.g., nutrient depletion, high light, salinity, temperature; Gouveia et al. 2009; Rodolfi et al. 2009; Campenni' et al. 2013).

However, the key strategy to enhance carbon mitigation is the optimization of culture growth. In subtropical or temperate climate zones, seasonal variations of solar irradiance and temperature often lead to impaired microalgal growth during winter (Jiménez et al. 2003; Grönlund et al. 2004; Hulatt and Thomas, 2011; Hindersin et al.

2014; Sutherland et al. 2014). Similarly to previous studies (Hulatt and Thomas, 2011; Hindersin et al. 2014), the season comparison assay under laboratory conditions (Algem® PBR) revealed that Spring conditions with higher temperatures and light intensities clearly enhance the growth rate and metabolism of *Tetraselmis* sp. CTP4 cultures. An additional enhancement of biomass and lipid productivities and consequently $CO_2$ sequestration requires optimization of growth media as well as effective light and $CO_2$ delivery into the cultures (the bottleneck of any PBR). In the present work, cultures were grown photoautotrophically, where growth depends on light and inorganic nutrients. However, a mixotrophic growth system that does not rely exclusively on $CO_2$ as a carbon source and use organic compounds such as acetic acid or glycerol could improve biomass production as reported for other species (Liang et al. 2009; Paranjape et al. 2016).

## 3.4. CONCLUSIONS

In conclusion, monoalgal cultures of *Tetraselmis* sp. CTP4 were successfully scaled up to industrial PBR and grown semi-continuously for 60 days without any culture collapse or contamination by a competing microalga. The growth data obtained in the autumn-winter season, demonstrate the robustness of this strain for large-scale production, as well as the interesting biomass productivities that can be obtained under non-optimal environmental conditions. However, as previously discussed, the productivities here presented do not represent the maximum that can be achieved with this microalgal strain. Large-scale production in spring-summer seasons will most probably lead to improved biomass productivity and carbon mitigation, due to the higher microalgal metabolism promoted by increased temperatures and solar radiation.

## 3.5. MATERIALS AND METHODS

### 3.5.1. MICROALGAE STRAIN AND CULTURE MEDIUM PREPARATION

All experiments described in the present work were performed at the facilities of CMP (Secil Group, Portugal), between 15$^{th}$ of August and 15$^{th}$ of December 2016. The microalgal strain selected for industrial growth, *Tetraselmis* sp. CTP4, was previously isolated, by the authors, near a wastewater stream in Ria Formosa, in the south of Portugal. The growth characterization under laboratory conditions was published

elsewhere (Pereira et al. 2016; Schulze et al. 2017). All experiments and scale-up were performed with artificial seawater (salinity of 20 g $L^{-1}$) prepared with commercial sodium chloride. Although *Tetraselmis* sp. CTP4 is a euryhaline strain that can withstand wide salt concentrations, the experiments carried out in the present work were performed in at 20 g $L^{-1}$ based on the higher growth performance of cultures previously demonstrated in the laboratory. Guillard's F2 culture medium adapted to the local water was used in all experiments; cultures were supplemented with the concentrated culture medium to reach a 5-mM concentration of nitrate (70 mg N $L^{-1}$).

### 3.5.2. SCALE-UP OF CTP4 CULTURES

The scale-up procedure (Figure 3.7) started with an agar plate (prepared according to Pereira et al. 2016) and reached after eight single steps the industrial scale (100 $m^3$ PBR). Each scale-up step lasted 7 days as follows: (i) cells were transferred to liquid medium by scrapping algal colonies from the agar plates directly to 100 mL Erlenmeyer flasks that were placed in an orbital shaker under low light intensity (50 µmol $s^{-1}$ $m^{-2}$); (ii) and (iii) the 100 mL cultures were inoculated in a vertical 1-L airlift with a 1 L capacity that was subsequently transferred to two 5 L airlifts; (iv) and (v) the cultures obtained in the two 5-L airlifts were used to inoculate a 125-L Flat Panel (FP), which was

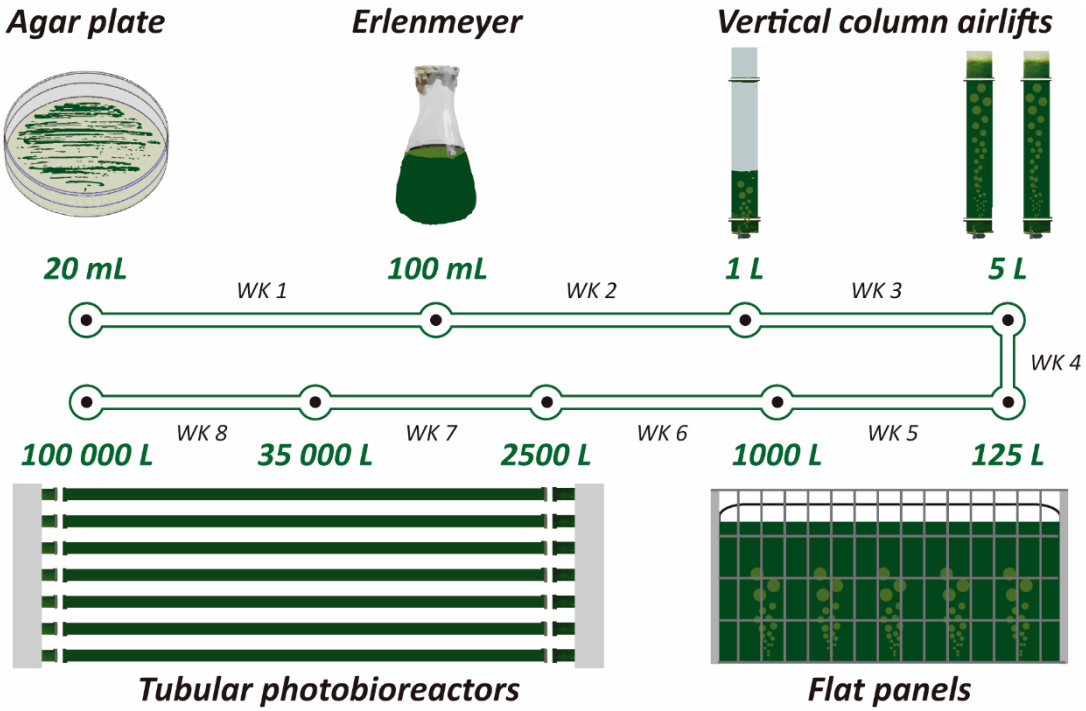


**Figure 3.7 -** Schematic representation of the scale-up procedure used in the present work. Cultures were transferred every week (WK) to a different production system; the corresponding culture volumes are represented for each system used.

then used to seed a 1-$m^3$ FP (Figure 3.1a); (vi) the culture grown in the 1-$m^3$ FP was used to inoculate two 2.5-$m^3$ pilot-scale tubular PBR (Figure 3.1b); (vii) the two pilot-scale PBRs were later used to inoculate an industrial-scale 35-$m^3$ tubular PBR; (viii) from this, approximately 30 $m^3$ were transferred from the PBR to inoculate the 100-$m^3$ tubular PBR (Figure 3.1c), while the remaining culture was regrown in the 35-$m^3$ PBR upon addition of culture medium.

### 3.5.3. OPTIMIZATION OF BIOMASS PRODUCTION

Experiments for the optimization of culture velocity and pH set point for $CO_2$ injection were performed in 2.5-$m^3$ tubular PBR in duplicates under batch conditions. Fixed culture parameters were chosen according to the results obtained by the previous trials (see Results section for details). Culture velocities of 0.65, 1.01 and 1.35 m $s^{-1}$ were tested at a fixed pH of 8.0, while three distinct pH set points (7.0, 7.5 and 8.0) were tested at a culture velocity of 1.01 m $s^{-1}$. The culture velocity was measured using a Dynasonics DXN (Portable Ultrasonic Measurement System). The pH was adjusted by an automatic $CO_2$ injection system (Yokogawa). The local temperature and radiation were registered using a RM Young meteorological station and an Apogee Logan UT SP-110 pyranometer, respectively.

### 3.5.4. INDUSTRIAL PRODUCTION OF BIOMASS

The industrial production of microalgae biomass was carried out in 35- and 100-$m^3$ horizontal tubular PBR, with an area of implementation of 133 and 405 $m^2$, respectively. The photic section of the production system was composed of polymethyl methacrylate (PMMA) tubes (Øi = 56 mm), having a total length and width of 48.2 × 2.5 m and 96.0 × 4.0 m for the 35- and 100-$m^3$ PBRs, respectively. The growth trial lasted for 60 days between 17$^{th}$ October and 15$^{th}$ December under a semi-continuous operation. Every 13–14 days, depending on available operational resources, approximately 70% of the total culture volume was harvested while the remaining culture was renewed with fresh growth medium. Both reactors were cultured at a salinity of 20 g $L^{-1}$, with a culture velocity of 1.01 m $s^{-1}$ and a pH set point for $CO_2$ injection of 8.0. An in-house system registered the turbidity, pH and temperature inside the PBR in real-time.

### 3.5.5. MICROSCOPY

The differential interference contrast (DIC) microscopic images were acquired with the 63 × lenses using a Nikon Eclipse Ni-U and a Zeiss Axioimager Scope A1. Fluorescence microscopy was performed with the Zeiss microscope with the 63 × lenses, using an Axiocam 503 color and Zeiss 64 and 65 HE filter sets. All images were treated with Zen v.2.3 (blue edition) software. Microalgae samples were stained with BODIPY 505/515 as described in Cooper et al. (2010) to evaluate the lipid content of the cells.

The presence of contaminants was evaluated by daily microscopic observations of three independent samples in ten microscopic fields. In addition, some samples were analysed by means of flow cytometry corroborating the microscopic results, as described in Schulze et al. (2017).

### 3.5.6. GROWTH ASSESSMENT

Microalgal biomass growth was assessed by means of optical density (OD) and dry weight (DW). The OD of cultures was determined using a Thermo Scientific Genesis 10 S UV-Vis spectrophotometer at a wavelength of 600 and 740 nm. DW was determined by filtering a known volume of culture through 0.45-µm fibreglass filters (VWR). The filter was sequentially washed with the same volume of ammonium formate (35 g $L^{-1}$) and of distilled water. The filters were dried and weighed in AnD MS-70 and Kern DBS 60-3 moisture analysers (120 °C). Ash content was determined by burning 1 g of biomass at 550 °C for 8 hours in a furnace (J. P. Selecta, Sel horn R9-L). A correlation between OD 600 and 740 and AFDW was used to establish the growth curves (previously determined).

### 3.5.7. LIPID DETERMINATION

The total content of lipids in the microalgal biomass was determined using a modified Bligh and Dyer (1959) method previously described in Pereira et al. (2011). Briefly, the microalgal pellet was extracted with a mixture of chloroform, methanol and water (2:2:1) using an Ultra-Turrax (IKA) disperser for 2 minutes. Phase separation was achieved by centrifugation for 10 minutes at 3500 *g*; the chloroform phase containing the lipids was removed using a Pasteur pipette and transferred to new vials. A known volume

of the lipid extract was then evaporated and the content of lipids was gravimetrically determined.

### 3.5.8. $CO_2$ SEQUESTRATION

In order to quantify the $CO_2$ mass balances, two rotameters were installed in the 100-$m^3$ industrial tubular PBR in the injection valve of the $CO_2$ supplying system and in the compressed air valve of the degassing system. To register the outputs of $CO_2$ from the PBR (every 5 minutes), a gas analyser (Madur, GA-21 plus) was coupled to the gas exhaust section of the PBR for 30 days ($17^{th}$ Oct – $17^{th}$ Nov). The $CO_2$ mitigation balance was calculated by the sum of $CO_2$ supplied by the automatic $CO_2$ injection system and the atmospheric $CO_2$ introduced from the degasser (compressed air), from which the $CO_2$ exhausted from the PBR, as quantified by the gas analyser, was subtracted.

### 3.5.9. ELEMENTAL ANALYSIS AND PHOTOSYNTHETIC EFFICIENCY

Elemental analysis of C, H and N in produced biomass was performed using a Vario el III (Vario EL, Elementar Analyser system, GmbH, Hanau, Germany) according to the procedure provided by the manufacturer. The higher heating value (HHV; KJ $g^{-1}$) of the biomass produced was calculated according to Callejón-Ferre et al. (2011) using the following equation:

$$HHV = -3.393 + 0.507[\%C] - 0.341[\%H] + 0.067[\%N]$$

where %C, %H and %N represent the carbon, hydrogen and nitrogen content in AFDW, respectively. PE was calculated by dividing the obtained HHV by the supplied irradiance during a given cultivation interval.

### 3.5.10. ALGEM® PHOTOBIOREACTORS SEASON COMPARISON

A season comparison assay was carried out using an Algem® PBR (Algenuity, Bedfordshire, UK), in order to assess whether the results obtained outdoors represent the maximum growth that can be obtained with this strain, since the microalga was cultivated in the autumn-winter season. Using the software provided with the equipment, the environmental conditions of Spring and Autumn seasons at the location of AlgaFarm

production plant (39.652936 N, −8.988986 W) were simulated. Cultures were mixed at 120 rpm, under constant aeration. $CO_2$ was injected automatically using a pH set point of 8.0. The PBR was set to register the optical density at 740 nm every hour.

### 3.5.11. STATISTICAL TREATMENT

One-way ANOVA followed by Tukey's post-hoc test and Analysis of Covariance (ANCOVA) were performed to detect statistical differences between continuous environmental variables (temperature and radiation) and the response variables (volumetric and areal biomass productivities, photosynthetic efficiency and lipid content) using Addinsoft XLSTAT (Version 2016.02.28451). Linear relationships were assessed via a two-tailored Pearson's test (r). Significance of correlations were tested for using Sigmaplot (Vers. 13, Systat Software Inc.). Significance level for all test was $\alpha = 0.05$.

## ACKNOWLEDGEMENTS

The authors would like to acknowledge all members of CMP for the kind support and help throughout this work. The present work was funded by the Portuguese national budget and the CCMAR/Multi/04326/2013 grant of the Foundation for Science and Technology (FCT), the 0055 ALGARED + 5 E - INTERREG V-A España-Portugal project, and the COST Action 1408 - European Network for Bio-products. H.P. (SFRH/BD/105541/2014) and P.S. were funded by PhD grants from FCT and the Nord University, respectively.

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## SUPPLEMENTARY INFORMATION

### $CO_2$ mitigation calculations

#### Inputs

**a) $CO_2$ injection** (99.99% at 1.5 bar) – Rotameter 20 L $min^{-1}$

Average value = 4870 L $day^{-1}$ (1.5 bar) = 13.20 Kg $day^{-1}$

**b) Degasser** (compressed air; 0.04% at 2.5 bar) – Rotameter = 180 L $min^{-1}$

Average value = 103.68 L $day^{-1}$ (2.5 bar) = 0.47 Kg $day^{-1}$

#### Sum of total $CO_2$ input (a + b)

$CO_2$ injection + Degasser = 13.67 Kg $day^{-1}$

#### Output

**Exhaust $CO_2$ -** $CO_2$ detected by gas analyser = 0.3-0.5% - Rotameter = 180 L $min^{-1}$

Exhaust $CO_2$ = 777.6-1296 L $day^{-1}$ = 3.51-5.85 Kg day-1

Average value = 1036.8 L $day^{-1}$ = 4.68 Kg day-1

#### $CO_2$ mitigation (average)

$$CO_2 \text{ mitigation } (\%) = \frac{(CO_2 \text{ input} - CO_2 \text{ exhaust})}{CO_2 \text{ input}} \times 100 = \frac{(13.67 - 4.68)}{13.67} \times 100 = 65.76\%$$

**$CO_2$ mitigation (max)** - Exhaust $CO_2$ = 0.3%

$$CO_2 \text{ mitigation } (\%) = \frac{(CO_2 \text{ input} - CO_2 \text{ exhaust})}{CO_2 \text{ input}} \times 100 = \frac{(13.67 - 3.51)}{13.67} \times 100 = 74.32\%$$

**$CO_2$ mitigation (min)** - Exhaust $CO_2$ = 0.5%

$$CO_2 \text{ mitigation } (\%) = \frac{(CO_2 \text{ input} - CO_2 \text{ exhaust})}{CO_2 \text{ input}} \times 100 = \frac{(13.67 - 5.85)}{13.67} \times 100 = 57.21\%$$

# CHAPTER IV

# GROWTH PERFORMANCE, BIOCHEMICAL COMPOSITION AND SEDIMENTATION VELOCITY OF *TETRASELMIS* SP. CTP4 UNDER DIFFERENT SALINITIES USING LOW-COST LAB- AND PILOT-SCALE SYSTEMS

**Research article published in:**

Trovão, M., Pereira, H., Silva, J., Páramo, J., Quelhas, P., Santos, T., Silva, J. T., Machado, A., Gouveia, L., Barreira, L., & Varela, J. (2019). Growth performance, biochemical composition and sedimentation velocity of *Tetraselmis* sp. CTP4 under different salinities using low-cost lab- and pilot-scale systems. Heliyon, 4, e01553.

# GROWTH PERFORMANCE, BIOCHEMICAL COMPOSITION AND SEDIMENTATION VELOCITY OF *TETRASELMIS* SP. CTP4 UNDER DIFFERENT SALINITIES USING LOW-COST LAB- AND PILOT-SCALE SYSTEMS

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## ABSTRACT

Biomass harvesting is one of the most expensive steps of the whole microalgal production pipeline. Therefore, the present work aimed to understand the effect of salinity on the growth performance, biochemical composition and sedimentation velocity of *Tetraselmis* sp. CTP4, in order to establish an effective low-cost pilot-scale harvesting system for this strain. At lab scale, similar growth performance was obtained in cultures grown at salinities of 5, 10 and 20 g $L^{-1}$ NaCl. In addition, identical settling velocities (2.4-3.6 cm $h^{-1}$) were observed under all salinities understudy regardless of the growth stage. However, higher salinities (20 g $L^{-1}$) promoted a significant increase in lipid contents in this strain compared to when this microalga was cultivated at 5 or 10 g $L^{-1}$ NaCl. At pilot-scale, cultures were cultivated semi-continuously in 2.5-$m^3$ tubular photobioreactors, refed every four days and stored in a 1-$m^3$ harvesting tank. Upon a 24-hour settling step, natural sedimentation of the microalgal cells resulted in the removal of 93% of the culture medium in the form of a clear liquid containing only vestigial amounts of biomass (0.07 ± 0.02 g $L^{-1}$ dry weight; DW). The remaining culture was recovered as a highly concentrated culture (19.53 ± 4.83 g $L^{-1}$ DW) and wet microalgal paste (272.7 ± 18.5 g $L^{-1}$ DW). Overall, this method provided an effective recovery of 97% of the total biomass, decreasing significantly the harvesting costs.


**Keywords:** Salt tolerance; Harvesting; Microalgae; *Tetraselmis* sp. CTP4; Pilot-scale

## 4.1. INTRODUCTION

Microalgae are microscopic photosynthetic microorganisms currently regarded as a promising feedstock for several biotechnological applications such as biofuels, bioremediation, human and animal nutrition, as well as a source of high value compounds (Huerlimann et al. 2010; Fon Sing et al. 2014; Pereira et al. 2016). Although recent reports suggested their wide biotechnological potential, the current production costs of microalgal biomass are still the main hindrance for large-scale commercialization. Therefore, to decrease production costs, it is necessary to address and optimize the whole microalgal production pipeline, from strain selection to the effective establishment of cost-effective harvesting and downstream processes.

Environmental factors (e.g., light, temperature, culture medium and salinity) strongly influence culture productivity and biomass composition (Lananan et al. 2013; BenMoussa-Dahmen et al. 2016). Relatively high salinities ("high salt") usually have a significant effect on microalgal cells, causing lower growth rates or even growth arrest (Ho et al. 2014; Zhu et al. 2016). In addition, salinity shifts may induce oxidative stress in the culture and alter its physiological and biochemical composition (Campenni' et al. 2013; Fon Sing et al. 2014). However, some microalgae, namely euryhaline and/or osmotolerant strains, are able to thrive on a wide range of salinities, which might be essential in industrial facilities when valuable metabolites (e.g., polyunsaturated fatty acids and carotenoids) need to be produced or when culture management techniques are needed to control contaminants (Skjånes et al. 2013; von Alvensleben et al. 2013; Zhu et al. 2016).

Apart from cultivation costs, harvesting and biomass dewatering processes are the most expensive steps in the whole production pipeline. In fact, the costs associated with harvesting and water removal can easily reach 30% of the total cost. Therefore, any cost savings in these steps can be a key factor in the economic profitability of the whole process (Chen et al. 2011; Acién et al. 2016; Show et al. 2017).

There are several biomass concentration techniques available. Novel lab-scale technologies are emerging with promising application to industrial purposes, such as electrocoagulation, bio-flocculation, electro-flocculation (electrolytic process), ozonation-dispersed flotation, among others (Lananan et al. 2016; Singh and Patidar, 2018). However, nowadays, industrial production units mainly use centrifugation,

ultrafiltration (membrane) or a combination of both methods in order to process large-scale culture volumes (e.g., Allmicroalgae and Necton S.A.). Although both techniques are highly efficient in microalgal biomass recovery, they have high CAPEX and significant advantages and disadvantages associated. Flocculation and flotation are described as more suitable for large scale due to lower costs and energy demands, but require the use of chemical products (Bilad et al. 2014; Yellapu et al. 2018; 't Lam et al. 2018). Natural sedimentation would be the perfect solution for the industry, however, most small size microalgae do not sediment or the sedimentation velocity restrains their recovery in a feasible period.

The *Tetraselmis* genus is considered as highly promising for different biotechnological applications, namely as a source of high value compounds (Pignolet et al. 2013; Sansone et al. 2017). In addition, *Tetraselmis* sp. CTP4 has previously been reported as a euryhaline, fast growing and robust microalgal strain, which holds high potential for scale-up in industrial production facilities (Pereira et al. 2018) as well as high sedimentation rates (Pereira et al. 2016).

Therefore, in this study we aim to expand our knowledge in order to understand the influence of salt concentration on growth and productivity rates, biomass composition and sedimentation velocity of *Tetraselmis* sp. CTP4. After a preliminary laboratory assay, the results of production and sedimentation were validated at pilot-scale, in a 2.5-$m^3$ photobioreactor and 1-$m^3$ sedimentation tank.

## 4.2. MATERIALS AND METHODS

### 4.2.1. MICROALGAE STRAIN

All experiments described in the present work were performed at the facilities of CMP (Secil Group, Portugal), between the 15$^{th}$ of September 2016 and 15$^{th}$ of August 2017. *Tetraselmis sp.* CTP4 was isolated from Ria Formosa (Portugal), as described in Pereira et al. (2016).

### 4.2.2. GROWTH UNDER DIFFERENT SALINITIES

Cultures were grown in laboratory conditions, in 5-L glass airlift reactors at ~25 °C under continuous lighting (100 μmol $m^{-2}$ $s^{-1}$), aerated with filtered compressed air (0.2

μm) supplemented with $CO_2$. Guillard´s F2 medium was used as culture medium in all experiments. Synthetic seawater was prepared using commercial sodium chloride at the following concentrations: 5, 10 and 20 g $L^{-1}$. All experiments were carried out in triplicate.

### 4.2.3. GROWTH ASSESSMENT

Microalgal growth was measured by optical density at 540 nm in a Thermo Scientific Genesis 10S UV-Vis spectrophotometer. Biomass dry weight (DW) was determined by filtration of samples through a 0.45-μm cellulose filter, washed with ammonium formate (25 g $L^{-1}$) and dried in an AnD MS-70 moisture analyzer at 120 °C. Cultures were monitored daily by means of microscopic observation (CX31RBSF, Olympus).

### 4.2.4. SEDIMENTATION RATE

Gravity-induced natural sedimentation was measured in cultures grown under different salinities and at different growth stages, following the guidelines used by Nollet (2000). The sedimentation rate was calculated in 100-mL measuring cylinders with a height of 16 cm. After the introduction of the culture in the sedimentation systems, they were kept in separate chamber without vibration. The height of settled culture was measured every 30 minutes, for 6 hours. Results are presented in cm $h^{-1}$.

After the sedimentation process, the supernatant was removed using a glass pipette and the remaining concentrated cultures were centrifuged for 5 min at 2000 *g* and later freeze-dried for biochemical analysis.

### 4.2.5. EVALUATION OF BIOCHEMICAL COMPOSITION

#### 4.2.5.1. Total lipids

Total lipids were extracted according to a modified Bligh and Dyer (1959) protocol described in Pereira et al. (2011). Briefly, lipid extraction was performed with a mixture of chloroform and methanol (1:2) and homogenized for 1 minute using an IKA Ultra-Turrax disperser. Afterwards, 1 mL of chloroform was added, and samples were

further homogenized for 30 s. At a later a stage, this step was repeated with 1 mL of water instead. Extracts were then centrifuged and the organic phase (chloroform) was transferred to pre-weighed tubes and dried overnight. Upon solvent evaporation, the extracted lipids were weighed, and the lipid fraction was estimated by gravimetry.

#### 4.2.5.2. Protein content

Total protein was estimated by Elemental analysis of C, H and N in the obtained biomass, using a Vario el III (Vario EL, Elementar Analyser system, GmbH, Hanau, Germany) according to the procedure provided by the manufacturer. Total protein was estimated by multiplying the nitrogen content by a factor of 6.25.

#### 4.2.5.3. Ash content

The determination of ash content was performed by burning 1 g of biomass for 8 hours at 550 °C in a muffle furnace (J. P. Selecta, Sel horn R9-L).

### 4.2.6. PILOT-SCALE PRODUCTION OF BIOMASS

Outdoor pilot-scale 2.5-$m^3$ tubular photobioreactors (PBRs) were used to grow *Tetraselmis* sp. CTP4 at a salt concentration of 10 g $L^{-1}$ ($n$ = 3). The pH was maintained at 8, by an automatic $CO_2$ injection system, while the temperature of cultures was kept between 25-30 °C, using a water sprinkling thermoregulation system. The flow rate of cultures (9 $m^3$ $h^{-1}$) was measured using a Dynasonics DXN (Portable Ultrasonic Measurement System). PBRs were inoculated at DW of approximately 0.2 g $L^{-1}$ and were allowed to grow until a DW of 2.3 g $L^{-1}$ (12 days; based on previous results, the beginning of stationary phase). At this stage, a semi-continuous approach was implemented and 25% of the culture volume (~600 L) was harvested (1$^{st}$ harvest) and fresh culture medium was added. The renewed culture was allowed to grow for 4 days and again 25% of the culture volume was replaced (2$^{nd}$ harvest). Finally, the cultures were allowed to grow for an additional 4-day period (3$^{rd}$ harvest, end of the trial) and the resulting culture was fully harvested. From the total volume of culture harvested at every step of the semi-continuous growth, only 250 L of each PBR was introduced in the pilot-scale sedimentation tank (see below for further details). Growth performance was daily assessed by means of optical

density and DW. A RM Young meteorological station and an Apogee Logan UT SP-110 pyranometer registered the local temperature and radiation, respectively.

### 4.2.7. PILOT-SCALE SEDIMENTATION OF CULTIVATED BIOMASS

The pilot-scale sedimentation experiment was performed in a 1-$m^3$ cylindrical-conical tank with a working volume of 0.75 $m^3$ at the end of each semi-continuous growth (Figure 4.1). As previously stated, a volume of approximately 0.25 $m^3$ of each 2.5-$m^3$ PBR was transferred to the sedimentation tank and the culture was allowed to naturally

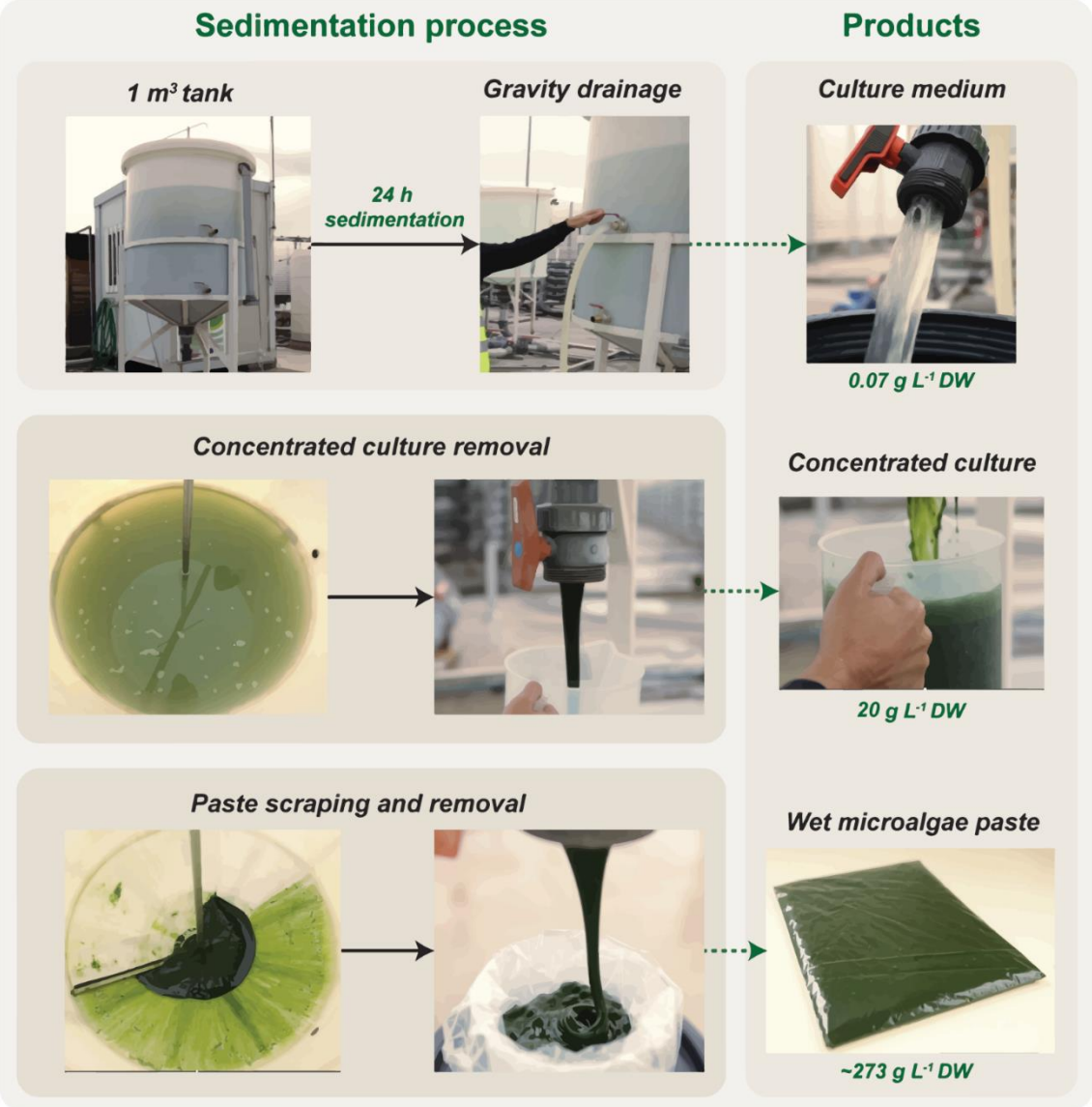


**Figure 4.1 -** Pilot-scale sedimentation process for low-cost harvesting of *Tetraselmis* sp. CTP4 biomass. Microalgal culture was settled by natural sedimentation (24 hours). Thereafter, the culture medium was recovered via taps connected to hoses located on the side section of the tank. Afterwards, the concentrated culture was removed using the bottom valve of the tank. The paste deposited in the bottom of the tank was retrieved by a homemade scraping system into the lower valve directly into plastic bags.

sediment for 24 hours. Afterwards, the supernatant was drained by gravity using the two taps located on the side of the tank, which were connected to hoses to better direct the liquid into storage vessels. The remaining concentrated culture was then removed through the valve located on the bottom of the tank. Since a thick microalgal paste remained attached to the lower part of the sedimentation tank, a squeegee was assembled at the bottom of the tank to scrap and recover the rest of the biomass via the bottom valve. All streams of the process (culture medium, concentrated culture and wet paste) recovered from the sedimentation tank were immediately analysed for their DW.

#### 4.2.8. STATISTICAL ANALYSIS

Experiments were performed at least in triplicate and results are expressed as mean ± standard deviation. Significance of differences was assessed by ANOVA using SPSS v24.0.

## 4.3. RESULTS AND DISCUSSION

### 4.3.1. EFFECT OF SALT CONCENTRATION ON GROWTH AND SEDIMENTATION PERFORMANCE

*Tetraselmis* sp. CTP4 was cultivated in laboratory conditions using three different salinities, namely, 5, 10 and 20 g $L^{-1}$ (Figure 4.2A) for 20 days. All cultures showed a growth curve similar to those previously obtained for this strain (Pereira et al. 2016). The lag phase took place for about 2 days and the stationary phase was reached at day 15. Cultures displayed similar growth without significant differences ($p$>0.05) among the three salinities tested, reaching a final biomass DW of 1.2-1.5 g $L^{-1}$. These results confirm the euryhaline properties of this strain and its capability to easily adapt to different salt conditions (Fon Sing et al. 2014; Das et al. 2016; Fon-Sing and Borowitzka, 2016; Pereira et al. 2016). This is a key feature for the successful growth of microalgae in large-scale industrial facilities. In open production systems (e.g., raceways), environmental factors, such as evaporation and local precipitation, can significantly increase and decrease, respectively, the salt concentration in the medium (Fon-Sing and Borowitzka, 2016). On the other hand, in closed systems (photobioreactors), salinity up- and downshifts can be crucial to manage natural occurring contaminants with lower halotolerance (Pereira et al.

2018). In addition, growing microalgal cultures in low-salt media might decrease production downstream costs related to the management of saltwater discharges.

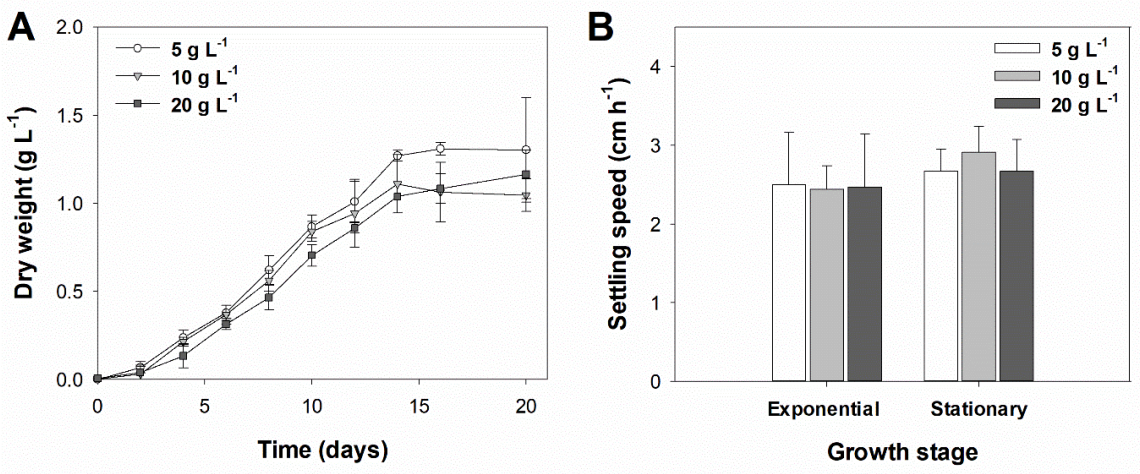


**Figure 4.2 –** (A) Batch growth of *Tetraselmis* sp. CTP4 in 5-L reactors under three different salinities (5, 10 and 20 g $L^{-1}$). (B) Sedimentation rate of cultures grown using different salinities at different growth stages (exponential and stationary), expressed in cm $h^{-1}$. Error bars represent the standard deviation calculated from three replicates.

The sedimentation rate of *Tetraselmis* sp. CTP4 cultures grown under three salinities in the exponential and stationary growth stages is shown in Figure 4.2B. The obtained data revealed no significant differences in the sedimentation rate of cultures grown within the range of salinities under study (5-20 g $L^{-1}$) at either exponential or stationary phase (2-3 cm $h^{-1}$; $p$>0.05). Although all cultures grown at different salinities displayed similar sedimentation rates, the concentration of salt is known to affect the growth and physiology of several microalgae species and should, therefore, be considered as a variable influencing cell buoyancy and hence algal sedimentation rates (Roik et al. 2016). On the one hand, it could be hypothesized that a medium of higher density, provided by increasing salt concentrations, would cause a lower sedimentation rate due to a higher buoyancy of the cell. On the other hand, higher NaCl concentrations might induce faster settling velocities in microalgae, because the ionic strength of saline solutions could affect the negative charges at the cell surface. This would decrease the zeta potential associated to the plasma membrane, cell coverings (e.g., cell wall) and other extracellular materials (Church et al. 2017; Wen et al. 2017). Overall, more experiments are needed to clarify the relation between salinity and sedimentation rate, perhaps with a wider range of concentrations, and also under different growth stages. These studies would also be of great importance to unravel the significance of this culture parameter on

culture sedimentation, which can be a key factor for decreasing the harvesting costs in microalgal production.

### 4.3.2. PROXIMATE COMPOSITION

The proximate composition of the biomass produced under the different salinities was further determined to assess the effect of salt on the biochemical composition (Table 4.1). Overall, the content of protein (ranging from 40.5 to 42.7% of DW) and ashes (7.5-8.2% of DW) in the biomass were quite similar across all salinities under study ($p$>0.05). On the other hand, lipid and carbohydrate contents differed significantly ($p$<0.05). Low and intermediate salt concentrations (5 and 10 g $L^{-1}$, respectively) showed lower total lipid contents (4.9 and 5.6% of DW) as compared to that (8.5% of DW) obtained at high salt (20 g $L^{-1}$; $p$<0.05). The increase in lipid contents occurred at the expense of carbohydrates ($p$<0.05), which decreased from 46.5 to 41.2% of DW as the salinity increased from 5 to 20 g $L^{-1}$.

**Table 4.1 -** Biomass composition of batch cultures grown in 5 L reactors under different salinities (5, 10 and 20 g $L^{-1}$). Values are the mean and corresponding standard deviation of three replicates. Different letters within each biochemical component indicate significant differences.

| Salt (g/L) | Proteins (%) | Lipids (%) | Carbohydrates (%) | Ashes (%) |
|---|---|---|---|---|
| 5 | $40.49 \pm 1.34^{a}$ | $4.86 \pm 1.00^{a}$ | $46.52 \pm 1.12^{a}$ | $8.04 \pm 0.15^{a}$ |
| 10 | $41.10 \pm 0.09^{a}$ | $5.58 \pm 0.06^{a}$ | $45.10 \pm 0.27^{a}$ | $8.22 \pm 0.30^{a}$ |
| 20 | $42.69 \pm 0.42^{a}$ | $8.54 \pm 0.09^{b}$ | $41.23 \pm 1.44^{b}$ | $7.53 \pm 0.52^{a}$ |

The metabolism of microalgal cells is highly affected by environmental factors, such as salinity, light, pH, temperature and nutrient availability. In turn, metabolic fluctuations influence growth and the biochemical composition of the biomass produced (Dammak et al. 2016). High salt combined with low salt growth conditions was previously reported to contribute to lipid and protein enrichment in the final biomass (Ho et al. 2014). Moreover, accumulation of lipids and proteins, induced by salinity shifts, might also promote higher $CO_2$ mitigation by microalgae due to the high carbon content of these biomolecules (BenMoussa-Dahmen et al. 2016).

Regarding the *Tetraselmis* genus, the effect of increasing salinities leading to higher lipid content has been previously reported (Khatoon et al. 2014; Dammak et al. 2016), as well as for other microalgal strains (Salama et al. 2013; Karpagam et al. 2015).

The rise of salinity in the medium might lead to an increment in osmotic pressure in the microalgal cells, which involves changes in cell metabolism and activation of several molecular physiological responses (Dammak et al. 2016). For example, upon a salinity upshift, cells usually accumulate osmoprotectant solutes, also known as osmolytes, such as glycerol (Salama et al. 2013; Talebi et al. 2013) and mannitol (Fon-Sing and Borowitzka, 2016). In addition, cells produce stress proteins to maintain stability and normal growth. However, it is noteworthy that the opposite effect has also been previously described by other authors (Renaud and Parry, 1994; Das et al. 2016). This difference might be explained by species/strains-specific effects, as well as through differences in their metabolism, biochemical composition and molecular responses. In accordance with the results of this study, a higher starch accumulation under low salinity has been previously reported for the *Tetraselmis* genus, which has been suggested to be associated or even enhanced by other factors, such as nitrogen deprivation (Yao et al. 2013). Lower salt concentrations force cells to increase their osmotic potential in order to reach an equilibrium with that of the surrounding medium. To this end, cells restrict the biosynthesis and accumulation of small osmolytes and channel the carbon flux to starch synthesis. Unlike other storage polysaccharides such as glycogen, and because of its crystalline structure and poor solubility in water, starch granules are osmotically inert (Ball et al. 2011). Therefore, the fact that this polysaccharide does not depress the osmotic potential might explain the observed trend for higher carbohydrate contents as salinity is decreased (Table 4.1).

### 4.3.3. PILOT-SCALE GROWTH

The results obtained in the laboratory were followed by a pilot-scale experiment using an outdoor 2.5-$m^3$ tubular PBR in semi-continuous mode at the intermediate salinity (10 g $L^{-1}$). The choice of this intermediate salinity for the scale-up step arose from two main factors, namely preventing the proliferation of possible contaminants, commonly found when the salinities are low, and limiting the use of salt when the growth medium is prepared. This balance is important in order to increase overall productivity and decrease production costs. Growth in the pilot-scale PBR was carried out at a stable mean temperature around 16 °C, with the exception of day 4, 5 and 6, where increasing temperatures were registered (~23 °C; Figure 4.3). Total radiation was stable during the first 14 days of the growth period (~17 MJ $m^{-2}$ $d^{-1}$), while in the last 6 days a decrease in

total radiation was observed. All PBRs were inoculated at a concentration of ~0.2 g $L^{-1}$ DW and reached the early stationary phase in 12 days, with a biomass concentration of 2.3 g $L^{-1}$ DW (1st growth stage). During this stage, cultures presented mean volumetric and areal productivities of 0.17 g $L^{-1}$ $d^{-1}$ and 16.09 g $m^{-2}$ $d^{-1}$, respectively. Upon refeeding the culture with fresh medium, the decrease in radiation led to lower growth rates of cultures. In this 2nd growth stage, cultures took 4 days to reach ~2.0 g $L^{-1}$ and displayed mean volumetric and areal productivities of 0.15 g $L^{-1}$ $d^{-1}$ and 13.91 g $m^{-2}$ $d^{-1}$, respectively. A similar pattern was seen in the 3rd growth stage, with a lower radiation observed on-site. Refed cultures grew slower, so that 5 days were necessary to reach ~2.0 g $L^{-1}$, whereas the mean volumetric and areal productivities were 0.12 g $L^{-1}$ $d^{-1}$ and 11.23 g $m^{-2}$ $d^{-1}$, respectively. These biomass productivities are quite similar to the results previously reported for this strain in the same production system (Pereira et al. 2018).

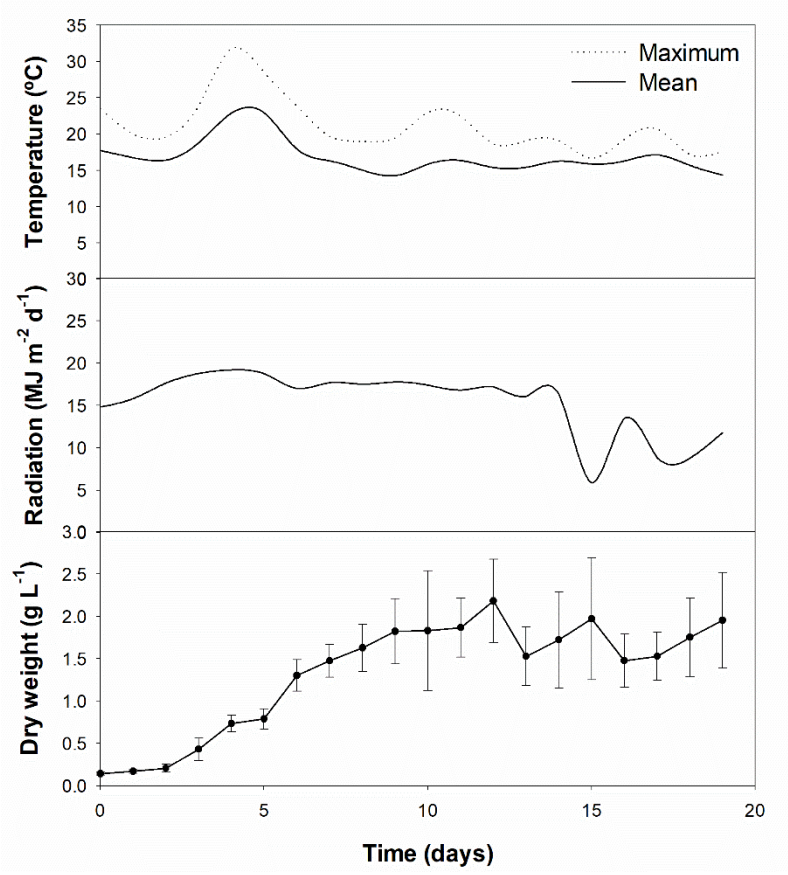


**Figure 4.3 -** Mean and maximum temperature and radiation registered on site during the growth of *Tetraselmis* sp. CTP4 in outdoor pilot scale photobioreactors (2.5 $m^3$) for 18 days. Error bars represent the standard deviation calculated from three replicates.

### 4.3.4. PILOT-SCALE SEDIMENTATION PROCESS

The biomass recovered using the semi-continuous cultivation approach was allowed to sediment in a pilot-scale tank; upon which the culture was refed three times, namely on the 6$^{th}$, 9$^{th}$ and 13$^{th}$ of October. The initial DW of cultures (process input) used to perform the sedimentation tests in the three distinct harvesting points was 2.18, 1.97 and 1.95 g $L^{-1}$ (Table 4.2). The performance of the sedimentation process (biomass removal efficiency) was quantified by measuring the DW of the culture medium removed via lateral taps connected to hoses, the concentrated culture recovered from the lower valve and the microalgal paste that settled at the bottom of the tank (Figure 4.1). Therefore, after the sedimentation period (24 hours), approximately 0.712 $m^3$ of culture medium were easily removed from the sedimentation tank by gravity drainage. This medium had the appearance of a clear liquid containing only vestigial biomass (0.07 ± 0.02 g $L^{-1}$). This volume corresponded to 93% of the total culture volume (Table 4.2). The culture volume that remained in the conical section of the sedimentation tank thus represented only 7% of the total culture volume. Afterwards, this highly concentrated culture was transferred to an appropriate container via the tap located at the bottom section of the tank (Figure 4.1), reaching a concentration of 19.53 ± 4.83 g $L^{-1}$. However, as part of the microalgal biomass settled at the bottom of the tank, this fraction was recovered from the lower section of the tank in the form of a microalgal paste using a scrapping device, with a mean biomass of 272.7 ± 18.5 g $L^{-1}$. Interestingly, this paste can thus be packed immediately, should this be the intended final product. Overall, these values represent a removal efficiency of 97% of the total biomass introduced in the sedimentation tank. In other words, this low-cost, gravity-dependent harvesting method only led to a biomass loss of 3% upon culture medium removal, without the use of any additional energy input.

**Table 4.2 -** Harvesting by sedimentation of *Tetraselmis* sp. CTP4, under a pilot-scale semi-continuous cultivation. Values are the mean and corresponding standard deviation of three replicates.

| Sedimentation process | Unit | Mean ± SD |
|---|---|---|
| ***Inputs*** | | |
| **Initial culture dry weight** | g $L^{-1}$ | 2.03 ± 0.13 |
| ***Outputs*** | | |
| **Culture medium** | g $L^{-1}$ | 0.07 ± 0.02 |
| **Concentrated culture** | g $L^{-1}$ | 19.53 ± 4.83 |
| **Microalgal paste** | g $L^{-1}$ | 272.7 ± 18.5 |
| ***Biomass*** | | |
| **Settling velocity** | cm $h^{-1}$ | 3.44 ± 0.10 |
| **Removal** | % | 96.64 ± 0.86 |

Biomass harvesting is considered one of the main costing steps of the whole microalgal production pipeline (Chen et al. 2011; Acién et al. 2016; Show et al. 2017). Microalgae harvesting requires high energy inputs, because of the small size of cells, low density (similar to that of water) and low cell concentration of autotrophic cultures (Bilad et al. 2014). Therefore, the method developed in the present work, based solely in the natural settling capacity of this strain, represents a major decrease in harvesting costs in the pipeline of biomass production. There are several authors that have corroborated this proof of concept although in different backgrounds. Yu et al. (2012) obtained 97.9% recovery of the microalga *Monoraphidium* sp. FXY-10 in 24 hours by natural sedimentation, but only in lab-scale experiments. Meanwhile, Hom-Diaz et al. (2017), in spite of working with different PBRs and sedimentation systems, reported a large-scale gravity sedimentation harvesting method with an 88% biomass recovery within 24 hours. Interestingly, there are other experiments reported for different species, namely *Tetraselmis suecica*, in which the microalgae *per se* were applied as bioflocculants in order to increase the sedimentation rate of non-flocculant cultures and decrease the energy requirements and costs of downstream processing (Salim et al. 2012).

## 4.4. CONCLUSIONS

Taking into account the robustness, stress tolerance and biochemical properties of *Tetraselmis* sp. CTP4 previously reported, here the authors report that the growth and sedimentation rate are not affected within the range of salinities studied. This work also shows that cultures can be easily harvested via a simple, cost-effective, gravity-dependent

process using cylindrical-conical reservoirs fitted with lateral taps, a scraper, and a bottom valve. This low-cost approach takes into account the specific properties of the strain in order to improve the profitability and sustainability of biomass harvesting. Nevertheless, further optimization of this method using a secondary sedimentation system and different tank geometries will most likely lead to improved reduction costs.

## ACKNOWLEDGMENTS

The authors would like to acknowledge all members of CMP for the kind support and help throughout this work. The present work was funded by the Portuguese national budget P2020 and the CCMAR/Multi/04326/2013 grant of the Foundation for Science and Technology (FCT), the ALGARED+ 1398 EP - INTERREG V-A España-Portugal project, the ALGACO2 project (nº 023310; Industrial cultivation of microalgae as a green technology for atmospheric $CO_2$ capture), and the COST Action 1408 - European Network for Bio-products. H.P. (SFRH/BD/105541/2014) was funded by a PhD grant from FCT.

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# CHAPTER V

# NUTRITIONAL POTENTIAL AND TOXICOLOGICAL EVALUATION OF *TETRASELMIS* SP. CTP4 MICROALGAL BIOMASS PRODUCED IN INDUSTRIAL TUBULAR PHOTOBIOREACTORS

**Research article submitted to *Journal of Applied Phycology*.**

# NUTRITIONAL POTENTIAL AND TOXICOLOGICAL EVALUATION OF *TETRASELMIS* SP. CTP4 MICROALGAL BIOMASS PRODUCED IN INDUSTRIAL TUBULAR PHOTOBIOREACTORS

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## ABSTRACT

Commercial production of microalgal biomass for food and feed has been a recent worldwide trend. The present work addresses the nutritional potential and a thorough microbiological and toxicological evaluation of *Tetraselmis* sp. CTP4 biomass produced in industrial photobioreactors (100 $m^3$). This microalga contained high amounts of protein (31.2 g/100 g), dietary fibres (24.6 g/100 g), digestible carbohydrates (18.1 g/100 g) and ashes (15.2 g/100 g), but low lipid content (7.04 g/100 g). The biomass displayed a balanced amount of essential amino acids, *n*-3 polyunsaturated fatty acids and starch-like polysaccharides. Significant levels of chlorophyll (3.5 g/100 g), carotenoids (0.61 g/100 g) and vitamins (e.g., 79.2 mg ascorbic acid /100 g) and radical scavenging activity were also found in the biomass. Conversely, pathogenic bacteria, heavy metals, cyanotoxins, mycotoxins, polycyclic aromatic hydrocarbons and pesticides were absent. In conclusion, the biomass produced has a promising nutritional composition for both human and animal applications.

**Keywords:** Microalgae; Biochemical composition; *Tetraselmis* sp. CTP4; Pigments; Vitamins; Antioxidants; Toxicological evaluation

## 5.1. INTRODUCTION

Microalgae are a polyphyletic group of highly biodiverse photosynthetic unicellular or colonial microorganisms that have been adapted to almost every known habitat during evolution. These microorganisms are currently considered to be one of the solutions to meet the high demand for food and feed caused by the expected growth of human population in the forthcoming decades (Austic et al. 2013). Moreover, microalgae usually couple a balanced nutritional profile with the presence of bioactive molecules. This combination can be used to implement new functional foods that are able to counteract risk factors for the development of different forms of non-communicative chronic conditions, such as cancer, cardiovascular and neurodegenerative disorders (Custódio et al. 2014).

Several microalgal strains are rich in *n*-3 fatty acids (e.g., eicosapentaenoic [EPA] and docosahexaenoic [DHA] acids) and/or carotenoids (e.g., lutein, astaxanthin and *β*-carotene), among others (Spolaore et al. 2006). Most importantly, these metabolites are known to have antioxidant and/or anti-inflammatory properties that could prevent medical conditions, such as cardiovascular and autoimmune diseases (Pereira et al. 2012). In addition, anticancer, metal chelating and neuroprotective activities have also been reported in microalgal extracts, which can be used in biomedical applications, including the manufacture of nutraceuticals and the development of promising leads for pharmaceutical drugs (Custódio et al. 2012, 2014).

Commercial production of microalgal biomass has been mainly accomplished for animal and human nutrition due to their high protein content and the presence of essential fatty acids and vitamins (Spolaore et al. 2006). In aquaculture, for example, they have an important role during the first stages of fish larvae rearing and bivalve cultivation. Regarding human nutrition, from the beginning of the 1960's, microalgae such as *Arthrospira* (formerly known as *Spirulina*) and *Chlorella* have been commercially produced for human nutrition either for direct consumption or as food supplements (Spolaore et al. 2006). In fact, the utilization of microalgae is a growing trend in Europe, with several authors claiming that microalgal biomass can be a "functional/super food" (Righi et al. 2016). Unfortunately, only a very limited number of microalgal species have been classified as food ingredients by the European Food Safety Agency (EFSA). For the introduction of a novel species in the Europe Union (EU) food market, a Novel Food

Dossier must be submitted, and the "novel food" status obtained. Dried *Tetraselmis chui* is one of those examples, having achieved the novel food status in 2014.

The *Tetraselmis* genus is known to hold promising nutritional properties, associated to different biological activities, including antioxidant, metal chelating, neuroprotective, cell repairing and cytotoxic activities (Custódio et al. 2012, 2014; Sansone et al. 2017). Although different reports detail the relevance of this genus as feedstock for carbohydrates (Ji et al. 2014), proteins (Schwenzfeier et al. 2011) and lipids (Pereira et al. 2016), it has become apparent that there are significant differences in the biochemical composition among strains grown in specific cultivation systems. *Tetraselmis* sp. CTP4 is a recently isolated strain displaying some interesting biotechnological properties as, for example, high growth rates under stressful conditions and robustness against potential predators and competitors (Pereira et al. 2016, 2018). Laboratory assays revealed that this strain has high potential for bioremediation (Schulze et al. 2017) and as a lipid feedstock (Pereira et al. 2016). Moreover, cultures of this strain are able to settle down by natural sedimentation (Pereira et al. 2016), a requisite to significantly decrease harvesting costs. Recently, industrial production of monoalgal *Tetraselmis* sp. CTP4 biomass was achieved in 35- and 100-$m^3$ tubular photobioreactors for 60 days (Pereira et al. 2018).

In this context, the present work aims to evaluate the nutritional potential of *Tetraselmis* sp. CTP4, addressing the proximate composition and biochemical characterization of the biomass produced in an industrial facility. In addition, to enhance biomass value, its antioxidant activity was assessed *in vitro* using different methodologies.

## 5.2. MATERIALS AND METHODS

### 5.2.1. MICROALGAE GROWTH

*Tetraselmis* sp. CTP4 was previously isolated as described in Pereira et al. (2016). The growth in urban wastewater as well as in laboratory and industrial systems was published elsewhere (Pereira et al. 2016, 2018; Schulze et al. 2017). Biomass was produced between 17 October and 14 November 2016, in 35- and 100-$m^3$ industrial tubular photobioreactors, as described in (Pereira et al. 2018). Produced biomass was concentrated in a Pall WUSP-6443 micro-filtration system and later dried in an MDR-

150 high-speed centrifugal spray drier. To present an accurate quantification of the biochemical profile of *Tetraselmis* sp. CTP4, all results were normalized taking into account the salt content of the biomass.

### 5.2.2. PROXIMATE COMPOSITION

Total protein content was determined with a Foss Kjeltec 2200 protein analyser system, while total lipids were determined by Soxhlet extraction, followed by solvent evaporation in a Buchi R-210 rotary evaporator. Dietary fibres were determined according to the AOAC 991.43 and AOAC 985.29 norms. Ash content was determined by burning the samples at 540 °C for 6 h in a muffle furnace (Nabertherm B180 MB2). Digestible carbohydrates were calculated by difference, whereas energy was calculated using standard equations (Reg. EU Nº 1169/2011).

### 5.2.3. AMINO ACID PROFILE

Amino acids were determined by ultra-performance liquid chromatography (UPLC) using the Waters Acquity UPLC equipped with an Accq-Tag Ultra C18 column (1.7 µm particle size (p.s.), 2.1 × 100 mm). The amino acids were released from protein by acid hydrolysis. The sulphur-containing amino acids methionine, cystine and cysteine were first subjected to performic acid oxidation into methionine sulphone and cysteic acid. A separate hydrolysis with lithium hydroxide was performed to release tryptophan from the matrix.

### 5.2.4. FATTY ACID PROFILE

The profile of fatty acid methyl esters (FAME) was analysed according to the procedure described in (Pereira et al. 2012). Briefly, samples were homogenized in a solution of methanol and acetyl chloride (20:1, v/v) with an IKA Ultra-Turrax T10B disperser for 2 min. Afterwards, samples were derivatised for 60 minutes at 90 °C and the FAME were sequentially extracted four times from the reaction mixture with *n*-hexane. The hexane extracts were dried with anhydrous sodium sulphate, filtered with 0.2-µm filter (Whatman® Puradisc, PTFE) and evaporated with a gentle stream of nitrogen. The

dried residue was resuspended in 500 µL of hexane and stored at -20 °C until the gas chromatography (GC) analysis.

FAME were analysed in a Bruker Scion 456/GC, Scion TQ MS coupled to a 30 m ZB-5MS capillary column with an internal diameter (i.d.) of 0.25 mm and film thickness of 0.25 µm (Phenomenex). Individual calibration curves were established for each FAME using Supelco® 37 Component FAME Mix (Sigma-Aldrich, Sintra, Portugal).

### 5.2.5. ANALYSIS OF CARBOHYDRATES

Neutral sugars were determined as alditol acetates by gas chromatography as described by Nunes et al. (2012). The monosaccharides were obtained after hydrolyses of the polysaccharides with sulphuric acid (1 M) at 100 °C for 2.5 hours. Monosaccharides were reduced with sodium borohydride and acetylated by acetic anhydride using methylimidazole as a catalyst. The alditol acetate derivatives formed were analysed by GC with a 30 m column DB-225 (i.d. of 0.25 mm and film thickness of 0.15 µm; J&W Scientific, Folsom, CA, USA) using a flame ionization detector (Perkin Elmer, Clarus 400). The monosaccharides were identified by retention time and quantified using 2-deoxyglucose as internal standard. The hydrolysis of all samples was done in duplicate and each one was injected at least twice.

Glycosidic-substitution analysis was determined by gas chromatography-quadrupole mass spectrometry (GC-qMS) of the partially methylated alditol acetates (PMAA) as described in (Oliveira et al. 2017). Samples were methylated with $CH_3I$ in alkaline medium. The methylated sample was hydrolysed with 2 M trifluoroacetic acid (1 mL) at 120 °C for 1h, and then reduced and acetylated as previously described for neutral sugar analysis (using $NaBD_4$ instead of $NaBH_4$). The PMAA were separated and analysed by GC–qMS (GC-2010 Plus, Shimadzu). The GC was equipped with a DB-1 (J&W Scientific, Folsom, CA, USA) capillary column (30 m of length, 0.25 mm of i.d., and 0.10 µm of film thickness). The GC was connected to GCMS-QP 2010 Ultra Shimadzu mass quadrupole selective detector operating with an electron impact mode at 70 eV and scanning the range m/z 50–700 in a 1-s cycle in a full scan mode acquisition.

### 5.2.6. DETERMINATION OF PIGMENTS

The pigments profile was determined according to Wright et al. (1991) using Waters Alliance 2695 high performance liquid chromatography (HPLC) and Waters 2996 photodiode array detector (PAD) coupled to a Waters Spherisorb column (5 µm, 4.6 × 250 mm). Briefly, samples were extracted with methanol, filtered through 0.2 µm syringe filters and injected in the HPLC. The standards of alloxanthin, diatoxanthin, lutein, neoxanthin, violaxanthin and zeaxanthin were obtained from DHI, while *β*-carotene was supplied by Sigma-Aldrich.

### 5.2.7. DETERMINATION OF VITAMINS

All vitamins were determined with an Agilent Technologies 1200 Series HPLC UV/VIS unless stated in contrary. Vitamins A and E were determined using a Kromasil 100-5-SIL column (5 µm, 4.6 × 250 mm), according to the EN 12823-1:2000 and EN 12822:14 standards, respectively. Samples for the determination of vitamin A were saponified with an ethanolic solution of sodium hydroxide and the extraction was carried out with *n*-hexane. Vitamin C was determined using a Waters Spherisorb column (5 µm, 4.6 × 250 mm) according to the EN 14130 standard. The extraction was performed with a solution of meta-phosphoric acid followed by a reduction of L(+)-dehydroascorbic and L(+)-ascorbic acids with a solution of L-cysteine. Vitamins B1 (thiamine), B2 (riboflavin) and B6 were determined using a fluorescence detector (HPLC-FD) coupled to an Atlantis dC18 column (5 µm, 4.6 × 150 mm) according to the Waters technical note (Vitamins B1 and B2) and the EN 14164:2008 standard (Vitamin B6). All samples were treated with hydrochloric acid followed by enzymatic digestion with clara-diastasa (Sigma Aldrich). A Licrospher 60 Rp-select B column (5 µm, 4.0 × 125 mm) was used to determine the content of vitamin B3 (niacin) in the samples, according to the EN 15652 standard.

Vitamin B5 (panthotenic acid) was determined by LC-MS-MS using Micromass Quattro Micro API y SCIEX Triple Quad 5500 coupled to a Zorbax Eclipse XDB-C8 column (3.5 µm, 3.0 × 100 mm). Vitamins B9 (folic acid) and B12 (cobalamin) were concentrated with immunoaffinity columns (Biopharm Rhône LTD) and an Atlantis dC18 column (5 µm, 4.6 × 150 mm) was used.

### 5.2.8. MINERAL ANALYSIS

The mineral composition was determined by the ALS Group, with a Varian 730-ES atomic emission spectrometry with inductively coupled plasma (ICP-OES) as per ISO 11885:2007. Iodine was determined by ICP-OES according to the EN 15111:2007 standard. Stoichiometric calculations of concentrations were established from measured values. All samples were prepared according to the CZ_SOP_D06_02_J02 (chap. 10.17.1, 10.17.2, 10.17.4, 10.17.7, 10.17.8). Prior to analysis, samples were homogenized and mineralized by acids and hydrogen peroxide.

### 5.2.9. EVALUATION OF *IN VITRO* BIOLOGICAL ACTIVITIES

For the evaluation of bioactivities, extracts were performed with selected solvents of different polarities, namely hexane, ethyl acetate, acetone, ethanol and distilled water. Homogenization was achieved by means of a disperser IKA Ultra-Turrax T10B, while the extraction occurred overnight at room temperature. All extracts were filtered through 0.7 μm pore glass fibre filters (VWR) and further concentrated in a rotatory evaporator (IKA, RV10 digital, Germany) at 40 °C under reduced pressure. Extracts were resuspended in DMSO and stored at -20 °C.

Extracts were evaluated for their antioxidant potential through complementary *in vitro* assays, namely radical scavenging activity on 1,1-diphenyl-2-picrylhydrazyl (DPPH), and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonicacid; ABTS) radicals, ferric reducing antioxidant power (FRAP) and metal chelating activities (MCA) on $Cu^{2+}$ and $Fe^{2+}$, using the methods described in Custódio et al. (2014) and Rodrigues et al. (2015). Butylated hydroxytoluene (BHT, E321) and ethylenediamine tetraacetic acid (EDTA) were used as positive controls for the radical scavenging activity (RSA) and FRAP, and MCA, respectively.

### 5.2.10. MICROBIOLOGICAL EVALUATION

All microbiological analyses were performed in laboratories certified by the ISO 17025. Total counts of aerobic microorganisms by EN ISO 4833-1:2013, enterobacteria by EN-ISO 8523:1991 and yeasts and moulds by NP 3277-1:1987. In addition, *Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella* spp.,

*Pseudomonas* spp. and *Vibrio* spp. were evaluated according to ISO 16649-2:2001, ISO 6888-2:1999, EN ISO 11290-1:1996, EN ISO 6579:2002, ISO 13720:2010, ISO/TS 21872-2:2007, respectively.

### 5.2.11. TOXICOLOGICAL EVALUATION

Determination of toxic metals was performed as described for the mineral analysis (section 2.7). Cyanotoxins were analysed by EPA Method 544 using a Liquid Phase Chromatograph Finnigan Surveyor (Thermo Scientific, San Jose, CA, USA), coupled with a spectrometry detector (MS Mass LCQ FleetTM ion trap), with electrospray (ESI) interface and a $C_{18}$ Hypersil Gold column (100 × 4.6 mm I.D., 5 μm, ThermoScientific, Waltham, MA, USA). The absence of microcystins-LR, -RR, -LA and cylindrospermopsin was confirmed by the non-existence of the precursor ion for each cyanotoxin, 995.5 $[M+H]^+$, 519.9 $[M+2H]^{2+}$, 910.5 $[M+H]^+$ and 416.5 $[M+H]^+$, respectively. Aflatoxins B1, B2, G1 and G2 were determined using an Agilent Technologies 1200 series HPLC coupled to a SPHERISORB column (4.6 x 250 mm, 5 μm ODS2, Waters) according to ISO16050:2003. The analysis of polycyclic aromatic hydrocarbons (PAHs) and pesticides was performed by Silliker Portugal S.A., using certified methods. PAHs were analysed using a 7890 Agilent GC-MS equipped with a J&W VF-17ms column (30 m × 0.25 mm, 0.25 μm, Agilent) according to F013550.0. Pesticides, both organochlorine (25 pesticides) and residues (about 250 pesticides) were evaluated using an Agilent 7890 gas chromatograph coupled to a 7000 Series MS according to the PS1052 e PS0001110 methods, respectively.

## 5.3. RESULTS AND DISCUSSION

### 5.3.1. PROXIMATE COMPOSITION

The macro composition of *Tetraselmis* sp. CTP4 is presented in Table 5.1. The biomass under study was compared with that of other *Tetraselmis* strains and to the well-known microalgae classified by EFSA as food ingredients, *Chlorella* and *Arthrospira*. The analysed biomass had low moisture content (~4 g/100 g). Although the protein content was identical to the values previously reported for industrially grown *T. chui* (~31 g/100 g; (Bernaerts et al. 2018), it was considerably lower than the values previously obtained at laboratory scale, which commonly reached 40 g/100 g (data not shown). Protein values ranging from 40-50 g/100 g have previously been reported for other strains of this genus (Molina et al. 1991; Tulli et al. 2012; Tibbetts et al. 2015). Nevertheless, marine microalgae commonly present lower protein contents than those obtained in freshwater species (e.g., *Arthrospira* sp. and *Chlorella* sp.; Table 5.1), which can easily reach 50-65 g/100 g (Tokuşoglu and ÜUnal 2003). Similarly to what has been previously reported for *Tetraselmis* sp. CTP4 (<10 g/100 g; Pereira et al. 2016), lipid contents of 7 g/100 g were achieved when cells were cultivated under nutrient repletion (Table 5.1). This lipid content is also observed in other strains of this genus (Molina et al. 1991) as well as in other microalgal strains (Table 5.1). Digestible carbohydrates represent about 18 g/100 g of *Tetraselmis* sp. CTP4 biomass. Microalgae belonging to the genus *Tetraselmis* are known to accumulate significant amounts of carbohydrates, and, according to the results obtained, the strain under study might be a promising feedstock for the exploitation of biotechnological applications for this purpose. The content of dietary fibres, 25 g/100 g of biomass (Table 5.1), was considerably higher as compared to that of other *Tetraselmis* strains (2-3 g/100 g; Tulli et al. (2012), and of *Chlorella* and *Arthrospira* (2-3 g/100 g; Table 5.1). The ash content of *Tetraselmis* sp. CTP4 was 15 g/100 g, being similar to the values obtained for other marine strains (Table 5.1; Bernaerts et al. 2018). On the other hand, freshwater strains usually display lower ash contents, as its content in the final biomass varies according to the concentration of salt used for growth. Finally, the energetic/calorific value of the biomass produced is similar to that of *Arthrospira* (1241 kJ/100 g, 297 kcal/100 g). From a nutritional point of view the energy value is low, mainly due to the low lipid content and the presence of significant amounts of fibres and ash.

**Table 5.1 -** Amino acid concentration (g/100 g DW) of *Tetraselmis* sp. CTP4 grown semi-continuously in industrial tubular photobioreactors. Values represent the mean % and corresponding standard deviation ($n$ = 3). Values in brackets represent the % of total amino acid.

| **Contents (g/100 g)** | ***Tetraselmis* sp. CTP4** | ***Tetraselmis chui*[1]** | ***Tetraselmis suecica*[2]** | ***Chlorella vulgaris*[3]** | ***Arthrospira* sp.[4]** |
|---|---|---|---|---|---|
| Moisture | 3.88 ± 0.35 | <7 | n.r. | 4.9 | 4.7 |
| Protein | 31.20 ± 0.48 | 35-40 | 48.7 | 56.9 | 57.5 |
| Lipids | 7.04 ± 0.42 | 5-8 | 8.0 | 7.5 | 7.7 |
| Digestible carbohydrates | 18.08 ± 4.18 | 30-32 | 22.4 | 19.2 | 20.3 |
| Dietary fibres | 24.60 ± 3.85 | 2-3 | 3.4 | 0.5 | 3.6 |
| Ash | 15.20 ± 0.80 | 14-16 | 17.5 | 10.9 | 6.2 |
| Energy (kJ/100 g) | 1241 ± 49 | n.r. | n.r. | n.r. | 1213 |
| Energy (kcal/100 g) | 297 ± 12 | n.r. | n.r. | n.r. | 290 |

[1] *Fitoplancton Marino S.L.*

[2] *Tulli et al. (2012)*

[3] *Allma product sheet*

[4] *United States Department of Agriculture (2018)*

*n.r. – not reported*

### 5.3.2. AMINO ACID PROFILE

The amino acid (AA) profile is of the utmost importance to assess the nutritional quality of a given food or feed. Regarding indispensable AA (IAA), according to the World Health Organization (WHO), the biomass of *Tetraselmis* sp. CTP4 shows high contents of leucine, valine, lysine and phenylalanine (Table 5.2). *Tetraselmis* sp. CTP4 presented a lower amount of IAA when compared to other marine microalgae, such as *T. chui* and *Phaeodactylum tricornutum* (Tibbetts et al. 2015), and some freshwater strains (Table 5.2). This difference might be explained by the lower amount of total amino acids found in CTP4 (10.7 g/100 g) when compared to *T. chui* (38.9 g / 100 g), *Chlorella* sp. (35.6 g/ 100 g) and *Arthrospira* sp. (24.3 g/100 g; (Tibbetts et al. 2015). Analysing the AA profile (% of total AA), CTP4 shows high IAA levels (41% of total AA; Table 5.2), similar to those reported for *T. chui* (36.9%), *Chlorella* sp. (45.4%), and *Arthrospira* sp. (41.7%). Although the relative percentages of IAA are similar, some differences could be observed. Overall, higher relative abundances of leucine and valine were reported for *Chlorella* sp., when compared to the strains shown in Table 5.2. Industrially produced *Tetraselmis* sp. CTP4 displayed higher percentages of leucine, isoleucine, valine, lysine, threonine and phenylalanine comparing to *T. chui*, whereas tryptophan, histidine and

cysteine were detected at lower percentages (Tibbetts et al. 2015). The IAA profile of *Tetraselmis* sp. CTP4 is quite similar to that of *Arthrospira* sp., displaying higher levels of lysine but lower isoleucine, valine and cysteine contents. Histidine was almost absent from the AA profile of *Tetraselmis* sp. CTP4, representing about 2% of the total AA of the remaining strains presented in Table 5.2. Regarding feed applications, the AA profile of CTP4 displays a strong similarity to that of a commercial non-algae-based feed. Hence, this microalga might provide all the required amino acids in a balanced way without the need for individual AA supplementation or compensation.

**Table 5.2 -** Amino acid concentration (g/100 g DW) of *Tetraselmis* sp. CTP4 grown semi-continuously in industrial tubular photobioreactors. Values represent the mean % and corresponding standard deviation ($n$ = 3). Values in brackets represent the % of total amino acid. n.r. – not reported.

| **Aminoacid** | ***Tetraselmis* sp. CTP4** | ***Tetraselmis chui*[1]** | ***Chlorella* sp.[1]** | ***Arthrospira sp.*[2]** |
|---|---|---|---|---|
| *Indispensable amino acids (IAA)* | | | | |
| Leucine | 2.28 ± 0.02 (8.83) | 7.3 (7.5) | 7.8 (9.9) | 4.9 (8.5) |
| Isoleucine | 1.12 ± 0.02 (4.34) | 3.4 (3.5) | 3.8 (4.8) | 3.2 (5.5) |
| Valine | 1.55 ± 0.02 (6.01) | 4.8 (4.9) | 5.3 (6.8) | 3.5 (6.0) |
| Lysine | 1.70 ± 0.09 (6.59) | 5.6 (5.7) | 5.2 (6.6) | 3.0 (5.2) |
| Threonine | 1.27 ± 0.05 (4.92) | 4.0 (4.1) | 4.0 (5.1) | 3.0 (5.1) |
| Tryptophan | 0.37 ± 0.03 (1.43) | 2.3 (2.4) | 0.8 (1.0) | 0.9 (1.6) |
| Methionine | 0.61 ± 0.03 (2.36) | 2.4 (2.5) | 2.2 (2.8) | 1.1 (2.0) |
| Phenylalanine | 1.44 ± 0.08 (5.58) | 4.7 (4.8) | 4.7 (6.0) | 2.8 (4.8) |
| Histidine | 0.04 ± 0.01 (0.15) | 1.6 (1.6) | 1.8 (2.3) | 1.1 (1.9) |
| Cystine + Cysteine | 0.28 ± 0.01 (1.08) | 2.8 (2.9) | n.r. | 0.7 (1.1) |
| **Total IAA** | **10.7 (41.3)** | **38.9 (39.8)** | **35.6 (45.4)** | **24.3 (41.7)** |
| *Non-indispensable amino acids (NIAA)* | | | | |
| Alanine | 2.04 ± 0.06 (7.90) | 6.0 (6.1) | 7.2 (9.2) | 4.1 (7.1) |
| Arginine | 1.70 ± 0.04 (6.59) | 9.4 (9.6) | 5.5 (7.0) | 5.8 (10.0) |
| Aspartic acid (Asx) | 2.89 ± 0.02 (11.2) | 14.1 (14.4) | 7.8 (9.9) | 8.4 (14.4) |
| Glutamic acid (Glx) | 3.64 ± 0.02 (14.1) | 12.0 (12.3) | 9.7 (12.4) | 3.1 (5.3) |
| Glycine | 1.58 ± 0.06 (6.12) | 6.5 (6.7) | 5.2 (6.6) | 2.4 (4.1) |
| Proline | 1.26 ± 0.03 (4.88) | 3.6 (3.7) | 4.2 (5.4) | 3.0 (5.2) |
| Serine | 1.19 ± 0.08 (4.61) | 4.2 (4.3) | 3.3 (4.2) | 2.6 (4.4) |
| Tyrosine | 0.85 ± 0.01 (3.29) | 3.0 (3.1) | n.r. | 4.9 (8.5) |

[1] *Calculated from Tibbetts et al. (2015)*

[2] *United States Department of Agriculture (2018)*

### 5.3.3. LIPID PROFILE

The fatty acid (FA) profile of *Tetraselmis* sp. CTP4 is mainly composed of palmitic (C16:0), palmitoleic (C16:1), oleic (C18:1), linoleic (C18:2), and α-linolenic (C18:3*n*-3) acids, which together are responsible for more than 80% of total FA (Table S5.1). Stearic (C18:0), hexadecatrienoic (C16:3*n*-3), and eicosapentaenoic (EPA; C20:5*n*-3) acids correspond to most of the remaining FA detected in *Tetraselmis* sp. CTP4. Polyunsaturated (PUFA) and monounsaturated (MUFA) fatty acids are the most abundant, while saturated fatty acids (SFA) are present in lower amounts. The sum of PUFA was equal to 36% of total FA, whereas *n*-3 PUFA corresponded to about 17% of the total FA, mainly represented by hexadecatrienoic (2.7% of total FA), EPA (2.8% of total FA) and α-linolenic acids (11.6% of total FA), which are important for different nutritional applications. Although *Chlorella* displays a higher amount of *n*-3 PUFA (Table S5.1), due to the high concentration of hexadecatrienoic and α-linolenic acids (12.7 and 32.9% of total FA, respectively), long-chain PUFA (>20 carbons; e.g., EPA) are generally absent from freshwater microalgal strains. In fact, EPA is a long-chain *n*-3 PUFA produced from marine biomass, being essential to several metabolic pathways in humans and animals and for an adequate nutrition of children, infants in particular. Overall, the FA profile here reported is similar to those reported for this strain in previous works (Pereira et al. 2016; Schulze et al. 2017), as well as to other strains belonging to the *Tetraselmis* genus (Table S5.1). Notable exceptions are the higher amounts of PUFA, including those of *n*-3 PUFA, and the absence of stearidonic acid (C18:4*n*-3), when *Tetraselmis* CTP4 is compared with other *Tetraselmis* strains (Table S5.1).

### 5.3.4. CARBOHYDRATES COMPOSITION

Sugar analysis showed that *Tetraselmis* sp. CTP4 biomass is composed mainly of glucose (Glc, 13.7 g/100 g), followed by galactose (Gal, 4.98 g/100 g) and mannose (Man, 1.33 g/100 g) in lower proportions (Table 5.3). Arabinose (Ara) and xylose (Xyl) were present in residual amount (< 0.2 g/100 g). This is in accordance with the literature, since glucose was the principal neutral sugar (75-85%, Table 5.3), while lower levels of galactose (11-16%), ribose (2-5%), mannose (2-3%), rhamnose, and arabinose (< 1%) were detected in *T. chui* and *T. suecica* (Brown, 1991; Kermanshahi-pour et al. 2014). The main intracellular polysaccharide described in this genus is starch (Kermanshahi-

pour et al. 2014). Starch is a storage polysaccharide common in green plants and algae. The 2-keto-sugar acids have been described as the main sugars of *Tetraselmis* species, such as *T. striata* and *T. tetrathele*, due to the presence of the theca, an extracellular cell wall organized in multilayered, fused scales (Becker et al. 1998). The acid sugars were not determined. Compared to the genus *Tetraselmis*, higher amounts of xylose, mannose and rhamnose were reported in *Arthrospira* sp. at the expenses of glucose and galactose (Shekharam et al. 1987).

Glycosidic-substitution analysis was performed to achieve more information about structural characteristics of *Tetraselmis* polysaccharides (Table S5.2). The main linkages observed were 1,4-linked Glc (57 mol%) and 1,4-linked Gal (22 mol%). 1,4-Glc is substituted at C6 (1,4,6–Glc) with a content of 4.4 mol%, which confirms the presence of starch-like polysaccharides containing a high percentage of branching residues. The *Tetraselmis* polysaccharides seem to be also constituted by a galactan with 1,4–Gal linkage in the backbone and substituted at C3, as inferred by the presence of 1,3,4–Gal (2 mol%). From a nutritional point of view, *Tetraselmis* sp. CTP4 is an interesting food as it could be a good source of energy provided by the starch-like polysaccharides.

**Table 5.3 -** Sugar composition of *Tetraselmis* sp. CTP4 grown semi-continuously in industrial tubular photobioreactors (g/100 g). Values from the literature for *Tetraselmis chui*, *Tetraselmis suecica* and *Arthrospira* sp. are also presented in % of total sugars. Values represent the mean % and corresponding standard deviation ($n$ = 3). Values in brackets represent the mol% of total sugars.

| **Contents** | ***Tetraselmis* sp. CTP4** | ***Tetraselmis chui*[1]** | ***Tetraselmis suecica*[1]** | ***Arthrospira sp.*[2]** |
|---|---|---|---|---|
| Arabinose | 0.18 ± 0.01 (1.0) | 0.41 | 0.90 | n.r. |
| Xylose | 0.10 ± 0.01 (0.6) | n.d. | n.d. | 7.0 |
| Mannose | 1.33 ± 0.02 (6.5) | 1.8 | 3.0 | 9.3 |
| Galactose | 4.98 ± 0.03 (24.5) | 11.3 | 15.7 | 2.6 |
| Glucose | 13.68 ± 0.07 (67.3) | 84.7 | 74.8 | 54.4 |
| Rhamnose | v. | 0.04 | 0.97 | 22.3 |
| Ribose | v. | 1.8 | 4.5 | n.r. |
| Others | - | n.r. | n.r. | 4.3 |

[1] *Brown (1991)*

[2] *Shekharam et al. (1987)*

*v. – vestigial (< 0.1 g/100g)*

*n.d. – not detected*

*n.r. – not reported*

### 5.3.5. PIGMENT PROFILE

Spray-dried *Tetraselmis* sp. CTP4 biomass (Table 5.4) contained high contents of chlorophyll (3531 mg/100 g), followed by neoxanthin (236 mg/100 g), lutein (226 mg/100 g), and violaxanthin (131 mg/100 g). Smaller quantities of zeaxanthin (11 mg /100 g) and *β*-carotene (8.4 mg/100 g) were also detected. Pigments can be added to foods as natural colouring agents and as antioxidants in healthy foods, to extend shelf life and prevent oxidation during food processing. All photosynthetic microalgae contain chlorophyll, which usually ranges between 500-1500 mg/100 g of dry weight (Baker and Günther, 2004). Interestingly, the chlorophyll contents of CTP4 dry biomass clearly exceeded this range (>3500 mg/100 g; Table 5.4). This high chlorophyll content may be beneficial to human health, since recent epidemiological studies provide evidence linking chlorophyll consumption to a decreased risk of colorectal cancer (Balder et al. 2006). Although the most common industrial source of lutein is usually the marigold flower, the microalgae *Muriellopsis* spp., *Scenedesmus* spp., *Chlorella* spp., and *Chlorella protothecoides* present significant contents. *Tetraselmis* CTP4 could also be a lutein source considering that its biomass contained about 0.2 g/100 g of this carotenoid (Table 5.4) and that improvement of the lutein contents of CTP4 might be achieved by the approach described in Cordero et al. (2011) for *Chlorella sorokiniana*.

**Table 5.4 -** Pigment profile of *Tetraselmis* sp. CTP4 grown semi-continuously in industrial tubular photobioreactors (mg/100 g). Values from the literature for *Tetraselmis chui*, *Tetraselmis suecica* and *Chlorella vulgaris* are also presented. Values represent the mean % and corresponding standard deviation ($n$ = 3).

| **Pigments** | ***Tetraselmis* sp. CTP4** | ***Tetraselmis chui*[1]** | ***Tetraselmis* sp. M8[1]** | ***Chlorella vulgaris*[2]** |
|---|---|---|---|---|
| Chlorophyll *a* and *b* | 3531.2 ± 152.1 | n.r. | n.r. | 2600 |
| Violaxanthin | 130.8 ± 5.7 | 54.6 | 22.9 | n.r. |
| Antheraxanthin | n.d. | 20.1 | 12.6 | n.r. |
| Neoxanthin | 236.4 ± 11.9 | n.d. | n.d. | n.r. |
| Zeaxanthin | 10.8 ± 1.3 | n.d. | n.d. | 626 |
| Lutein | 225.6 ± 8.5 | 62.4 | 66.5 | 1011 |
| *α*-carotene | n.d. | 17.4 | 3.0 | 6.92 |
| *β*-carotene | 8.4 ± 0.7 | 94.1 | 105.7 | 8.26 |

[1] *Ahmed et al. (2014)*

[2] *Allma product sheet*

*n.d. – not detected*

*n.r. – not reported*

Indeed, novel sources for this pigment might be important, because the lutein market size (USD 135 million in 2015) is estimated to generate significant gains in the near future (Chen et al. 2018). Strong application outlook in eye health supplements may favour product demand, since lutein from microalgae (E161g) has been approved both in EU and USA as a colour additive. The rising application of pigments in feed applications also accounted for over 30% of the carotenoid global demand in 2015, driven by growing consumer demand for meat, eggs and salmon with a healthy appearance and standardized colouring. Natural carotenoids market size may see over 4% gains by 2024. Germany, France, UK, and U.S. are key contributing countries, favouring the expansion of the bioingredient industry. In this sense, the microalga *Tetraselmis sp.* CTP4 could be part of this demand for natural pigments, especially due to its content in chlorophyll and lutein.

### 5.3.6. VITAMIN PROFILE

Ascorbic acid was the most abundant vitamin in *Tetraselmis* sp. CTP4 (79.2 mg/100 g), followed by tocopherol (20.28 mg/100 g) and niacin (7.98 mg/100 mg; Table 5.5). The vitamin C content of *Tetraselmis* sp. CTP4 biomass was higher than that reported for *Tetraselmis suecica* (19.1 mg/100 g; Fabregas and Herrero, 1990), but lower than that of *Tetraselmis* sp. CS-362 (300 mg/100 g; Brown et al. 1999). The biomass of *Tetraselmis* sp. CTP4 had also intermediate levels of vitamin E (20.3 mg/100 g). However, in this case, the highest values have been reported for *T. suecica* (20-50 mg/100 g; Carballo-Cardenas et al. 2003) as compared to those of *Tetraselmis* sp. CS-362 (7 mg/100 g; Brown et al. 1999). Although no results have been reported for the contents of niacin in *Tetraselmis*, *Tetraselmis* sp. CTP4 showed a concentration slightly lower than the usually found in microalgae (11-47 mg/100 g; Brown, 2002). Concerning the contents of the remaining vitamins, the values obtained here were lower than those described for *Tetraselmis* sp. and microalgae in general (Fabregas and Herrero, 1990; Brown et al. 1999; Brown, 2002; Carballo-Cardenas et al. 2003). These low values may be a consequence of the fact that *Tetraselmis* sp. CTP4 biomass was processed under industrial conditions by means of spray-drying rather than freeze-drying. Heat inactivation of vitamins is a known process that depends on the matrix, pH, oxygen, light and moisture (Lešková et al. 2006). As temperatures close to 50-80 °C can be attained in the process of spray-drying, it is possible that some thermal decay took place.

**Table 5.5 -** Vitamin contents of *Tetraselmis* sp. CTP4 biomass grown semi-continuously in industrial tubular photobioreactors. Values from the literature for *Tetraselmis* sp. as well as *Chlorella vulgaris* and *Arthrospira* sp. are also presented. Values represent the mean % and corresponding standard deviation ($n$ = 3).

| **Vitamins** | ***Tetraselmis* sp. CTP4** | ***Tetraselmis* sp. CS-362[1]** | ***Chlorella vulgaris*[2]** | ***Arthrospira sp.*[3]** |
|---|---|---|---|---|
| A – Retinol (µg/100 g) | <4 | 220 | <20 | 29 |
| B1 – Thiamin (mg/100 g) | 0.18 | 10.9 | 0.03 | 2.38 |
| B2 – Riboflavin (mg/100 g) | 0.53 | 2.6 | 0.05 | 3.67 |
| B3 – Niacin (mg/100 g) | 7.98 | n.r. | 0.10 | 12.8 |
| B5 - Pantothenic Acid (mg/100 g) | 0.65 | n.r. | 0.08 | 3.48 |
| B6 - Pyridoxal phosphate (mg/100 g) | 6.9 | 0.6 | 0.08 | 0.36 |
| B7 – Biotin (mg/100 g) | n.d. | 0.13 | n.r. | n.r. |
| B9 - Folic acid (µg/100 g) | 0.02 | 2000 | 30.6 | 94 |
| B12 – Cianocobalamin (µg/100 g) | 7.8 | 195 | 0.10 | - |
| C - Ascorbic acid (mg/100 g) | 79.2 | 300 | <100 | 10.1 |
| E – Tocopherol (mg/100 g) | 20.28 | 7 | 6.57 | 5 |

[1] *Brown et al. (1999)*

[2] *Allma product sheet*

[3] *United States Department of Agriculture (2018)*

*n.r. – not reported*

### 5.3.7. MINERAL COMPOSITION

Industrially produced biomass was mainly composed of the following minerals: potassium (4.2%), magnesium (2.08%), calcium (1.19%) sodium (1.18%), and phosphorus (0.71%, Table S5.3). *Tetraselmis* sp. CTP4 presented higher magnesium and potassium contents when compared to the values previously reported for *T. chui*, *C. vulgaris* and *Arthrospira* sp. Nevertheless, the phosphorus content observed in *Tetraselmis* sp. CTP4 was lower compared to those reported for *C. vulgaris* and *T. chui* (Tokuşoglu and ÜUnal, 2003; Tibbetts et al. 2015). Although there is a narrow threshold between recommended and toxic levels of trace elements, the values observed for *Tetraselmis* sp. CTP4 are within the values commonly reported for other microalgal strains. Iron, copper and zinc were detected at low concentrations (1.1-32.3 mg/100 g). Iron was the most abundant trace mineral in *Tetraselmis* sp. CTP4 (32.3 mg/100 g) with a concentration similar to that reported for *Arthrospira* sp. (28.5 mg/100 g). *T. chui* had a considerably higher concentration (173.4 mg/100 g), and *C. vulgaris* was reported to present considerably lower concentrations of this trace mineral (0.3 mg/100 g;

(Tokuşoglu and ÜUnal, 2003). Concentrations of zinc observed in CTP4 (2.9 mg/100 g) were similar to those of *C. vulgaris* (1.2 mg/100 g) and *Arthrospira* sp. (2.0 mg/100 g) but were lower than the values reported for *T. chui* (6.4 mg/100 g; Table S5.3).

Although low amounts of selenium and iodine were detected, it should be noted that both elements were not included in the industrial culture medium used for growth. Therefore, the addition of inorganic sources of both elements in the culture medium used for industrial production might allow the improvement of the concentrations obtained in the final biomass product, as previously described for other chlorophytes (Gojkovic et al. 2014). Bioaccumulation of selenium has also been observed in *Tetraselmis* sp. CTP4, mainly in the form of selenomethionine (data not shown).

### 5.3.8. ANTIOXIDANT ACTIVITY

Upon extraction with solvents of different polarities, several *in vitro* assays were used to determine the antioxidant activity of the biomass produced in industrial photobioreactors. Values of antioxidant activity are presented as the half maximal inhibitory concentration ($IC_{50}$) in mg/mL (Table 5.6). Extracts of intermediate polarity, namely, with ethyl acetate and acetone showed higher radical scavenging activity (RSA) than other solvents. In addition, these extracts were more efficient in scavenging the DPPH radical ($IC_{50}$=2.6 mg/mL) than the ABTS radical ($IC_{50}$=6.9 mg/mL). The other extracts were not able to scavenge more than 50% of the free radicals when tested at 10 mg/mL. The same trend was observed for the extracts capacity to reduce ferric iron (FRAP); both acetone and ethyl acetate extracts had the highest activities, with $IC_{50}$ values of 0.3 and 0.5 mg/mL, respectively. Nevertheless, it is noteworthy that both hexane and ethanol extracts showed an $IC_{50}$ of 1.1 mg/mL in this assay. The antioxidant nature of the samples tested might be related with the presence of phenolic compounds and/or carotenoid pigments. These compounds occur naturally in microalgae and many studies have demonstrated positive correlations between antioxidant activity and the concentration of these compounds (Sansone et al. 2017). Nevertheless, considering that microalgal extracts, particularly those using acetone and ethyl acetate, are generally more enriched in carotenoids than in phenolic compounds (Custódio et al. 2012, 2014; Sansone et al. 2017), the observed antioxidant activity is probably related with the carotenoids present in the extracts. Compounds with RSA have been in high demand, particularly

those from natural sources, as replacements of synthetic antioxidant food preservatives, such as BHT. This is mainly due to their protective role against oxidative stress and associated chronic disorders (Viña et al. 2004), and the safety concerns regarding the use of BHT in food and feed (Lanigan and Yamarik, 2002).

Chelation of redox metals, such as Fe and Cu, is also an effective way to prevent oxidative damage (Megías et al. 2009). Hence, the same extracts were tested for their copper (CCA) and iron (ICA) chelating activities and compared to the known chelating agent, EDTA. All extracts were ineffective in chelating both copper and iron. The only exception was the acetone extract, which was able to chelate 50% of the initial iron concentration at 6.1 mg/mL. Oxidative stress can have implications in the rise and development of neurological disorders such as Alzheimer's disease, therefore, the chelation of redox metals for this ailment was previously proposed (Megías et al. 2009). The results obtained with *Tetraselmis* sp. CTP4 were similar to those obtained by Custódio et al. (2014), also with acetone extracts of another *Tetraselmis* strain, which displayed a similar iron chelating activity. On the other hand, our extracts did not show CCA, which is consistent with data previously reported for microalgae of the same genus (Custódio et al. 2012, 2014). It is possible that compounds with CCA are not present in this strain or that the production system (including biomass processing) may hamper the preservation of this bioactivity in the biomass. Nonetheless, *Tetraselmis* sp. CTP4 can still be a potential source of bioactive compounds with antioxidant activity.

**Table 5.6 -** Radical scavenging activity on DPPH radical, ferric reducing antioxidant power (FRAP) and metal chelating activity on copper (CCA) and iron (ICA) of organic and water extracts of *Tetraselmis* sp. CTP4. Results are expressed as the mean $IC_{50}$ (mg/mL) and corresponding standard deviation ($n$ = 4).

| Sample | DPPH | ABTS | FRAP | CCA | ICA |
|---|---|---|---|---|---|
| Hexane | >10 | >10 | 1.1 ± 0.1 | >10 | >10 |
| Ethyl acetate | 2.6 ± 0.2 | 6.9 ± 0.4 | 0.5 ± 0.0 | >10 | >10 |
| Acetone | 4.9 ± 0.3 | 8.7 ± 0.3 | 0.3 ± 0.0 | >10 | 6.1 ± 0.2 |
| Ethanol | >10 | >10 | 1.1 ± 0.1 | >10 | >10 |
| Water | >10 | >10 | >10 | >10 | >10 |
| BHT | 0.14 ± 0.01 | 0.11 ± 0.01 | - | - | - |
| EDTA | - | - | - | 0.08 ± 0.01 | 0.03 ± 0.00 |

### 5.3.9. MICROBIOLOGICAL EVALUATION

A detailed microbiological profile of the biomass produced in closed photobioreactors was achieved according to the European Legislation for food (Table 5.7). Aerobic plate total counts and yeasts were $3.6 \times 10^2$ and $1.0 \times 10^2$ CFU/g, respectively. Enterobacteria and moulds were below the detection limits ($<1.0 \times 10^1$ CFU/g). The screened pathogenic bacteria were either below the detection limits or negative at 25 g. Overall, concerning microbiological specifications *Tetraselmis* sp. CTP4 biomass was considered *Premium* and free from pathogens although no microbiological criteria for microalgae is available in the EC NO 2073/2005.

**Table 5.7 -** Microbiological evaluation of *Tetraselmis* sp. CTP4 biomass grown semi-continuously in industrial tubular photobioreactors. CFU = Colony-Forming Unit

| | ***Tetraselmis* sp. CTP4** |
|---|---|
| Aerobic plate total counts (30 °C) | $3.6 \times 10^2$ CFU/g |
| Enterobacteria | $<1.0 \times 10^1$ CFU/g |
| *Staphylococcus aureus* | $<1.0 \times 10^1$ CFU/g |
| *Listeria monocytogenes* | $<1.0 \times 10^1$ CFU/g |
| *Escherichia coli* | $<1.0 \times 10^1$ CFU/g |
| *Salmonella* spp. | Negative (25 g) |
| *Pseudomonas* spp. | $<1.0 \times 10^1$ CFU/g |
| *Vibrio* spp. | Negative (25 g) |
| Yeasts (25 °C) | $1.0 \times 10^2$ CFU/g |
| Moulds (25 °C) | $<1.0 \times 10^1$ CFU/g |

### 5.3.10. TOXICOLOGICAL EVALUATION

In order to fully understand the potential for nutritional purposes, both human and animal, a thorough toxicological evaluation was carried out in accordance with the most important contaminants proposed by the World Health Organization (Table 5.8). Therefore, several toxic metals were analysed, and the results obtained revealed that all were below the quantification limit, except for cadmium, which was present in only trace amounts (0.2 μg/g) in the analysed biomass (Table 5.8). Nevertheless, cadmium content is below the limit regarded in the European legislation for foodstuffs (<3 mg/Kg; EU NO 488/2014). The cadmium detected in the biomass comes probably from the culture

medium used in the industrial production, as the elemental analysis of the concentrated culture medium also revealed the presence of low cadmium levels (data not shown).

**Table 5.8 -** Toxicological evaluation of *Tetraselmis* sp. CTP4 biomass grown semi-continuously in industrial tubular photobioreactors.

| **Toxic substances** | ***Tetraselmis* sp. CTP4** |
|---|---|
| **Toxic metals** | |
| Lead | <0.10 µg/g |
| Cadmium | 0.2 ± 0.0 µg/g |
| Mercury | <0.10 µg/g |
| Arsenic | <0.80 µg/g |
| Tin | <2.50 µg/g |
| **Cyanotoxins** | |
| Microcystin LR | n.d. |
| Microcystin RR | n.d. |
| Microcystin LA | n.d. |
| Cylindrospermopsin | n.d. |
| **Mycotoxins** | |
| Aflatoxins B1 and B2 | <0.5 ng/g |
| Aflatoxins G1 and G2 | <0.5 ng/g |
| **Dioxins** | |
| Benzo[a]pyrene | <0.5 ng/g |
| Benzo[a]anthracene | <0.5 ng/g |
| Benzo[b]fluoranthene | <0.5 ng/g |
| Chrysene | <0.5 ng/g |
| Other polycyclic aromatic hydrocarbons[α] | <0.5 ng/g |
| **Pesticides** | |
| Organochlorine pesticides[β] | <0.01 µg/g |
| Screening of >200 residues[ϕ] | <0.01 µg/g |

[α] Benzo(k)fluoranthene, Indeno[1,2,3-cd]pyrene, Dibenzo[a,h]anthracene, Benzo[ghi]perylene, Benzo[a]pyrene.

[β] 2,4'-DDD, 2,4'-DDE, 2,4'-DDT, 4,4'-DDD, 4,4'-DDE, 4,4'-DDT, Sum of DDD,DDE,DDT, Aldrin, Dieldrin, Sum of Aldrin and Dieldrin, alpha-Endosulfan, beta-Endosulfan, Endosulfan sulfate, Endosulfan (Sum of alpha- and beta-isomers and Endosulfan-sulphate), Hexachlorocyclohexane (HCH) alpha-isomer, Hexachlorocyclohexane (HCH) beta-isomer, delta-Hexachlorocyclohexane (delta-HCH), Lindane (Gamma-isomer (HCH)), Hexachlorocyclohexane (HCH), sum of isomers, except the gamma isomer, cis-Chlordane, Trans-Chlordane, Chlordane (sum of cis- and trans-Chlordane), cis-Heptachlor epoxide, trans-Heptachlor, epoxide, Heptachlor, Heptachlor (Sum of Heptachlor and Heptachlor epoxide), Endrin, Hexachlorobenzene (HCB), Isodrin, Metoxychlor.

[ϕ] Metalaxyl and metalaxyl-M (metalaxyl including other mixtures of constituent isomers including metalaxyl-M (sum of isomers)), Ethofumesate-2-keto, Ethofumesate, 3-Hydroxycarbofuran, Carbofuran, Sum of Carbofuran (including any carbofuran generated from carbosulfan, benfuracarb or furathiocarb) and 3-OH carbofuran), Abamectin, Acephate, Acetamiprid, Gibberellic acid, Aldicarb, Aldoxycarb, Aldicarb sulfoxide, Sum of Aldicarb, Haloxyfop-r-methyl, Aminocarb, Amitraz, n-(2,4-Dimethylphenyl)formamide, n-2,4-Dimethylphenyl-n´-methylformadine, n-2,4-Dimethylphenyl-n´-methylformanidine, Amitraz (amitraz including the metabolites containing the 2,4 -dimethylaniline moiety), Ancymidol, Asulam, Atrazine, Azadirachtin, Azinphos-ethyl, Azinphos-methyl, Azoxystrobin, Benalaxyl, Bendiocarb, Benfuracarb, Resmethrin, Boscalid, Bupirimate, Bupofrezin, Butocarboxim, Butralin, Cadusafos, Carbaryl, Carbendazim + Benomyl, Thiophanate-methyl, Sum of MBC, Carboxin, Carbosulfan, Cyanazine, Cyazofamid,

Cycloate, Cymiazole hydrochloride, Cymoxanil, Cinidon ethyl, Cyproconazole, Cyprodinil, Cyromazine, Clofentezine, Clomazone, Cloquintocet-1-methylhexyl ester, Chlorantranquiliprole, Chlorfluazuron, Chloridazon, Chlortoluron, Clothianidin, Thiamethoxan, Sum of thiamethoxan and clothianidin, Dementon-s-methyl, Dementon-s-methyl sulfone, Dementon-s-methyl sulfoxide, Sum of dementon-s-methyl + demeton-s-methyl sulfoxide, Desethylatrazine, Terbuthylazine-desethyl, Desmedipham, Desmethyl pirimicarb, Desmethylformamido pirimicarb, Pirimicarb, Sum of pirimicarb, Diafenthiuron urea, Diallate, Diazinon, Diclofuanide, Diclofop methyl,Dicrotophos, Diethofencarb, Diphenamid, Diflubenzuron, Diflufenican, Dimethenamid-p (dimethenamid-p including other mixtures of constituent isomers (sum of isomers)), Dimethenamide, Dimethoate, Omethoate, Sum of dimethoate and omethoate, Dimethomorph, Dinotefuran, Disulfoton, Dissolfoton sulfone, Disulfoton sulfoxide, Sum of Disulfoton, Diuron, Dodine, Emamectin benzoate B1a, Heptenophos, Hexaconazole, Ethiofencarb, Ethiofencarb sulfone, Ethion, Ethofenprox, Hexythiazox, Famoxadone, Fenamidone, Fenamiphos, Fenamiphos sulfone, Fenamiphos sulfoxide, Sum of fenamiphos, Fenazquin, Fenbuconazole, Fenhexamid, Phenmedipham, Fenoxaprop-p-ethyl, Fenoxycarb, Fenpyroximate, Fenpropathrin, Fenpropidin, Fenpropimorph, Fenthion, Fenthion sulfone, Fenthion sulfoxide, Fenthion oxon, Fenthion oxon sulfone, Fenthion oxon sulfoxide, Sum of fenthion, Fenuron, Fipronil, Flonicamid, Florasulam, Fluazifop-p-butyl, Fluazifop-p, Flufenacet, Flufenoxuron, Fluquinconazole, Flurprimidol, Flutriafol, Fonofos, Forchlorfenuron, Formetanate, Formothion, Phosphamidon, Phosmet, Fosthiazate, Furalaxyl, Furathiocarb, Imazalil, Imazamethabenz -methyl, Imazamox, Imazethapyr, Imidacloprid, Indoxacarb, Iprovalicarb, Isopropalin, Isoproturon, Kresoxim-methyl, Lenacil, Linuron, Malaoxon, Malathion, Sum of malathion and malaoxon, Mandipropamid, Mepanipyrim, Methabenzthiazuron, Methamidophos, Metamitron, Metazachlor, Methidathion, Methiocarb, Methiocarb sulfone, Methiocarb sulfoxide, Sum of methiocarb, Metobromuron, Methomyl, Thiodicarb, Sum of methomyl and thiodicarb, Methoxyfenozide, Metoxuron, Metribuzin, Mevinphos, Myclobutanil, Milbemectin A3, Milbemectin A4, Monocrotophos, Monolinuron, Monuron, Neburon, Oxadiazon, Oxamyl, Oxycarboxin, Paclobutrazol, Paraoxon, Pencycuron, Pendimethalin, Picolinafen, Pymetrozine, Piperonyl butoxide, Pyraclostrobin, Pyrethrins, Pyridaben, Pyridalyl, Pyridate, Pyrimethanil, Pirimiphos-ethyl, Pirimiphos-methyl, Pyriproxyfen, Prochloraz, Profenofos, Promecarb, Propachlor, Propamocarb, Propanil, Propaquizafop, Propiconazole, Propyzamide, Propoxur, Quinalphos, Quinoxyfen, Quizalofop-ethyl, Rotenone, Simazine, Spinosad, sum of spinosyn A and spinosyn D, Spirotetramat, Spiroxamine, tau-Fluvalinate, Tebuconazole, Tebufenozide, Tebufenpyrad, TEPP, Terbufos, Terbuthylazine, Tetraconazole, Thiabendazole, Thiacloprid, Thiobencarb, Tiocarbazil, Thiram, Tolyfluanid, Triadimefon, Triadimenol, Sum of tradimefon + triadimenol, Triazamate, Tricyclazole, Trichlorfon, Tridemorph, Trifloxystrobin, Triflumizole, Trioforine, Vamidothion, Zoxamide.

One important toxicological factor in industrially produced microalgal biomass is the presence of cyanotoxins. Cyanobacteria are common contaminants observed in large-scale production facilities, both in fresh and salt water systems. In accordance with the microscopic observations during the biomass production period where cyanobacteria were not detected (Pereira et al. 2018), a screening for microcystins-LR, -RR, -LA and cylindrospermopsin also revealed that they were absent from the produced biomass. We also evaluated the presence of mycotoxins that are common in some cereal grains. Therefore, aflatoxins B1, B2, G1 and G2 were analysed and the obtained results revealed that all were below the detection limit of the method (<0.5 ng/g). Finally, three distinct methods were used to analyse the presence of PAHs (9 compounds), organochlorine pesticides (24 compounds) and pesticides residues (about 250 residues). As in the industrial production of microalgal biomass no pesticides are used, the presence of PAHs and pesticides could only be due to their accumulation in the massive amounts of ground water used to produce the microalgal biomass. However, none of the analyses performed reveal any PAHs and pesticides in the industrially produced biomass.

Taken together, it can be concluded that industrially produced biomass is free from all common toxic factors tested, except for a residual amount of cadmium that can be eliminated from future production batches by using a different culture medium.

## 5.4. CONCLUSIONS

In conclusion, the biomass of *Tetraselmis* sp. CTP4 produced in an industrial facility displays a composition comparable to that of other strains belonging to the *Tetraselmis* genus and of other microalgae that are well established food products (*Chlorella* and *Arthrospira*). Overall, the microalga under study displays interesting concentrations of proteins, dietary fibres, carotenoids and vitamins coupled with moderate antioxidant capacity. In addition, the microbiological and toxicological evaluation revealed that most common pathogens and toxic factors were absent from the industrially produced biomass. Hence, the biomass of the CTP4 strain displays promising properties for both human and animal nutritional applications.

## ACKNOWLEDGMENTS

The authors would like to acknowledge CMP for the kind support and help throughout this work. The present work was funded by the Portuguese national budget (CI–ECO - POCI-01-0145-FEDER-007679; FCT UID /CTM /50011/2013 and Q–PNA - FCT UID/QUI/00062/2013), CCMAR/Multi/04326/2013 grant of the Foundation for Science and Technology (FCT) and the 0055 ALGARED+ 5E - INTERREG V-A España-Portugal project. H.P. (SFRH/BD/105541/2014), KNG (SFRH/BPD/81882/2011), and CN (SFRH/BPD/100627/2014) were funded by a PhD and post-doc grants from FCT, respectively.

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## SUPPLEMENTARY INFORMATION

**Table S5.1 -** Fatty acid profile of *Tetraselmis* sp. CTP4 grown in an industrial production facility. Values from the literature for *Tetraselmis chui*, *Tetraselmis suecica* and *Chlorella* sp. are also presented. Values represent the mean % of total fatty acids and corresponding standard deviation ($n$ = 3).

| FAME | *Tetraselmis* sp. CTP4 | *Tetraselmis chui*[1] | *Tetraselmis suecica*[2] | *Chlorella* sp.[1] |
|---|---|---|---|---|
| C14:0 | 0.91 ± 0.01 | 0.2 | 1.3 | 0.6 |
| C15:0 | n.d. | n.d. | n.d. | 0.5 |
| C16:0 | 20.72 ± 1.18 | 19.9 | 16.0 | 21.8 |
| C17:0 | 1.68 ± 0.03 | n.d. | n.d. | n.d. |
| C18:0 | 2.09 ± 0.02 | 0.3 | 3.1 | 0.8 |
| C21:0 | 1.74 ± 0.21 | n.d. | n.d. | n.d. |
| C24:0 | n.d. | 0.1 | n.d. | 0.3 |
| **∑ SFA** | **27.14** | **20.5** | **20.4** | **24.1** |
| C16:1 | 10.33 ± 0.92 | 3.6 | 3.3 | 7.9 |
| C18:1 | 25.37 ± 1.56 | 9.8 | 27.2 | 3.6 |
| C20:1 | 0.90 ± 0.01 | 2.5 | 2.3 | n.d. |
| **∑ MUFA** | **36.60** | **15.9** | **32.8** | **11.5** |
| C16:2 n-6 | 1.13 ± 0.02 | 0.1 | n.d. | 4.4 |
| C16:3 n-3 | 2.72 ± 0.10 | 1.5 | n.d. | 12.7 |
| C16:4 n-3 | n.d. | 15.9 | n.d. | n.d. |
| C18:2 n-6 | 16.51 ± 1.03 | 4.6 | 6.4 | 14.1 |
| C18:3 n-3 | 11.60 ± 0.95 | 25.2 | 16.3 | 32.9 |
| C18:3 n-6 | 0.50 ± 0.01 | 0.4 | n.d. | n.d. |
| C18:4 n-3 | n.d. | 6.1 | 10.4 | n.d. |
| C20:3 n-3 | n.d. | 0.1 | n.d. | n.d. |
| C20:4 n-6 | 0.98 ± 0.02 | 1.2 | 1.5 | n.d. |
| C20:5 n-3 | 2.82 ± 0.03 | 8.0 | 12.2 | n.d. |
| **∑ PUFA** | **36.26** | **63.1** | **46.8** | **64.1** |
| **∑ n-3** | **17.14** | **56.8** | **40.5** | **45.6** |

[1] *Dunstan et al. (2010)*

[2] *Patil et al. (2007)*

*n.d. – not detected*

**Table S5.2 -** Glycosidic-linkage analysis (mol%) of *Tetraselmis* sp. CTP4 grown semi-continuously in industrial tubular photobioreactors.

| **Sugar residues** | **Mol%** |
|---|---|
| t-Xyl | 1.1 |
| ***Total Xyl*** | ***1.1*** |
| 1,2,3,5-Araf | 3.5 |
| ***Total Ara*** | ***3.5*** |
| 1,3,6-Man | 2.8 |
| ***Total Man*** | ***2.8*** |
| 1,4-Gal | 21.5 |
| 1,3,4-Gal | 1.8 |
| ***Total Gal*** | ***26.0*** |
| t-Glc | 6.7 |
| 1,4-Glc | 57.4 |
| 1,6-Glc | 0.7 |
| 1,4,6-Glc | 4.4 |
| ***Total Glc*** | ***69.2*** |

**Table S5.3 -** Composition of minerals and heavy metals of *Tetraselmis* sp. CTP4 biomass grown semi-continuously in industrial tubular photobioreactors. Values from the literature for *Tetraselmis chui*, *Chlorella vulgaris* and *Arthrospira* sp. are also presented. Values represent the mean % and corresponding standard deviation ($n$ = 3).

| | ***Tetraselmis* CTP4** | ***Tetraselmis chui*[1]** | ***Chlorella vulgaris*[2]** | ***Arthrospira sp.*[3]** |
|---|---|---|---|---|
| **Minerals (g/100 g)** | | | | |
| Calcium | 1.19 ± 0.17 | 2.99 | 0.59 | 0.12 |
| Magnesium | 2.08 ± 0.30 | 0.43 | 0.34 | 0.20 |
| Phosphorus | 0.71 ± 0.10 | 1.46 | 1.76 | 0.12 |
| Potassium | 4.2 ± 0.61 | 1.86 | 0.05 | 1.36 |
| Sodium | 1.18 ± 1.15 | 0.89 | 1.35 | 1.05 |
| **Trace elements (mg/100 g)** | | | | |
| Iron | 32.3 ± 3.90 | 173.37 | 0.30 | 28.5 |
| Copper | 1.1 ± 0.10 | 10.22 | 0.06 | 6.1 |
| Selenium | <5 | 0.05 | 0.07 | 0.007 |
| Zinc | 2.9 ± 0.30 | 6.37 | 1.19 | 2.0 |
| Iodine | 0.14 ± 0.00 | n.r. | n.r. | n.r. |

[1] *Tibbetts et al. (2015)*

[2] *Tokusoglu and Ünal (2003)*

[3] *United States Department of Agriculture*

*n.r. – not reported*

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# CHAPTER VI

# AN INTEGRATED LIQUID-LIQUID TRIPHASIC SYSTEM IN A BIOREFINERY APPROACH TO PRODUCE DIFFERENT STREAMS FOR BIOFUELS AND HIGH-VALUE PRODUCTS FROM WET MICROALGAL BIOMASS (*TETRASELMIS* SP. CTP4)

**Research article in preparation.**

# AN INTEGRATED LIQUID-LIQUID TRIPHASIC SYSTEM IN A BIOREFINERY APPROACH TO PRODUCE DIFFERENT STREAMS FOR BIOFUELS AND HIGH-VALUE PRODUCTS FROM WET MICROALGAL BIOMASS (*TETRASELMIS* SP. CTP4)

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## ABSTRACT

The main goal of the present work was to upgrade the value of the biomass of a robust, thermotolerant, euryhaline, industrial microalgal strain (*Tetraselmis* sp. CTP4). For this purpose, a novel biorefinery approach was designed, which included biofuel and added-value streams. To avoid any drying costs, wet microalgal biomass was extracted with ethanol (yield: 25.6% of dry weight). Using a liquid-liquid triphasic system (LTPS), the crude ethanolic extract was fractionated into three streams: (i) non-polar (NP; 31.4%), (ii) colloidal (CP; 49.3%) and (iii) water soluble (WP; 19.3%) phases. Upon TLC, HPLC and GC-MS analyses, the presence of added-value molecules with antioxidant and metal chelating properties were detected in the CP fraction, namely phospholipids and carotenoids. The NP, WP fractions and the residual biomass (leftover of the ethanolic extraction) were upgraded into different biofuel streams, namely biodiesel, bioethanol and biogas, respectively. The biodiesel synthesized form the NP fraction was mainly composed of palmitic and oleic acid esters, with low amounts of polyunsaturated fatty acid esters. The WP was converted into bioethanol by yeast fermentation with a yield of 0.46 g ethanol/g fermentable sugar, due to the high amount of glucose present in this stream after enzymatic hydrolysis. Lastly, anaerobic digestion of the residual biomass and a treatment supplemented with glycerol resulted in a biomethane yield of 64 and 83%, respectively. Overall, this innovative lab-scale biorefinery approach enabled an effective separation of major compounds present in wet microalgal biomass, holding the potential of being scaled up to larger extractive systems.


**Keywords:** *Tetraselmis* CTP4, Biorefinery, LTPS, Biodiesel, Biogas, Bioethanol, High-value-added compounds.

## 6.1. INTRODUCTION

Microalgal biomass are currently recognized as a promising bio-based sustainable feedstock for the production of biofuels, food, feed and high value molecules as well as for the bioremediation of industrial wastewater and $CO_2$ sequestration (Amaro et al. 2011; Gangadhar et al. 2016a; Pereira et al. 2016). However, recent techno-economic reports highlighted that microalgal-based products cannot be economically competitive with traditional feedstocks, unless all compounds in the biomass are valorized for different market scenarios within a biorefinery platform (Chisti, 2013; Zhu, 2015; 't Lam et al. 2018). In this context, microalgae-based ventures and other industries are looking for appropriate biorefinery technologies to process the different biochemical components present in the microalgal biomass into different end-products (Eppink et al. 2017). Although complex biorefinery pipelines for the development of a wide array of bio-products were established, mainly at lab-scale, the fractionation/refining of microalgal crude extracts into different raw materials is still considered an underdeveloped area ('t Lam et al. 2018).

The extraction of metabolites from biomass is a crucial step in the effective establishment of a microalgae-based biorefinery, and different extraction procedures are used to recover different metabolites, including conventional solvent extraction, supercritical fluid extraction, pressurized liquid extraction as well as other emerging technologies (Plaza et al. 2010; Nobre et al. 2013; López et al. 2015). However, to date, conventional solvent extraction is still the most used technique to extract different metabolites from industrial food products as well as in industrial biorefineries (e.g., biodiesel production). Solvent-based extractions of different target compounds can be achieved by one- or multiple-step extraction (e.g., solid-liquid extraction, followed by liquid-liquid extraction), the latter being commonly used to enhance the selectivity of extracted fractions (López et al. 2015).

Microalgae are known to contain different lipid molecules, namely, triacylglycerols, phospholipids, glycolipids, wax esters, steryl esters and free fatty acids (Chen et al. 2012; Dong et al. 2013; Yang et al. 2014). In fact, most biorefinery approaches developed for microalgae focus on the extraction of high value lipid molecules (e.g., phospholipids, carotenoids and PUFA), since they are normally the most valuable metabolites, leaving the polar molecules and residual biomass as by-products (Dong et al. 2016). Hence, the efficient separation of valuable lipids with high potential

for cosmeceutical and nutraceutical industries (McClements and Gumus, 2016) is crucial to upgrade the total biomass value and thus offset the production costs of biofuels in a biorefinery platform (Zhu, 2015).

Therefore, in this work, we describe a novel downstream processing method able to fractionate the microalgal biomass of *Tetraselmis* sp. CTP4 into different end products, fulfilling the basis of a future biorefinery platform. This method couples an ethanolic extraction directly from wet paste with a Liquid-liquid Triphase System (LTPS) to fractionate the crude extract into three different streams. The obtained fractions were characterized for the development of added-value by-products and different biofuels: biodiesel, bioethanol and biogas. To the authors' knowledge, a similar biorefinery approach has never been purported.

## 6.2. MATERIALS AND METHODS

### 6.2.1. MICROALGAE GROWTH

Experiments were carried out in the laboratory of the MarBiotech group, Centre of Marine Sciences (CCMAR) of the University of Algarve, Faro, Portugal. The chemicals used were of analytical grade, namely ethanol (96%), hexane (95% purity), HCl (37%) and $H_2SO_4$ (96% purity) were purchased from VWR International (Leuven, Belgium).

### 6.2.2. PRODUCTION OF MICROALGAL BIOMASS

The microalga used in this work, *Tetraselmis* sp. CTP4, was isolated near a wastewater treatment plant in Ria Formosa (Algarve, Portugal) by means of fluorescence activated cell sorting (Pereira et al. 2011; Pereira et al. 2016). Biomass was grown for 15 days in 200-L plastic airlifts (air sterilised at 0.2 μm) at controlled temperature (22 ± 2 °C) under continuous light (100 μmol/$m^2$/s). Cultures were grown in modified ALGAL medium in triplicate (Pereira et al. 2011). Biomass was harvested by sedimentation (8 hours) followed by centrifugation (Avanti J-25, Beckman Coulter, CA, USA) at 4,000 *g* for 10 min, at room temperature (RT). The obtained microalgal paste was packed in plastic bags and stored at -20 °C until further analysis.

### 6.2.3. DETERMINATION OF MOISTURE CONTENT

Prior to lipid extraction, the moisture content was determined by drying 2 g of microalgal biomass in an oven at 60 °C for 72 h (*n*=3), until two identical weights were obtained. The percentage of moisture in the biomass was calculated as described below:

$$Moisture\ content\ (\%) = \left[(W_1 - W_2)\ \div\ \left(W_1 - W_p\right)\right] \times 100$$

Where, $W_1$ = weight of the wet biomass (with plate) before drying; $W_2$ = Weight of the dry biomass (with plate) after drying; Wp = Plate weight.

### 6.2.4. DOWNSTREAM PROCESSING OF WET BIOMASS

An effective biorefinery approach was established to process the wet biomass of *Tetraselmis* sp. CTP4 into different streams. A schematic representation that shows and summarizes the workflow of the downstream procedure carried out is shown in Figure 6.1.

#### 6.2.4.1. Ethanolic extraction of biomass

Wet microalgal biomass (80 g) was dispersed in ethanol (240 mL) at a ratio of 1:3 (w/v) and stirred at reflux temperature (78 °C) for 90 min using a procedure adapted from Yang et al. (2015). After reflux, the supernatant (i.e., the ethanolic portion) was immediately recovered by vacuum filtration using a Whatman (nº 4) filter paper and stored at 4 °C. Later, the residual biomass was further sequentially extracted using the same procedure with 160 and 80 mL of ethanol (1:2 and 1:1; w/v) for 45 and 30 min, respectively. All ethanol fractions were pooled together followed by filtration under vacuum using 1.2-μm glass microfiber filters (VWR) in order to remove the remaining debris; and were further dried under reduced pressure using a rotatory evaporator to obtain a crude lipid extract. The residual biomass (RB) was dried overnight in a fume hood at room temperature and stored in a desiccator.

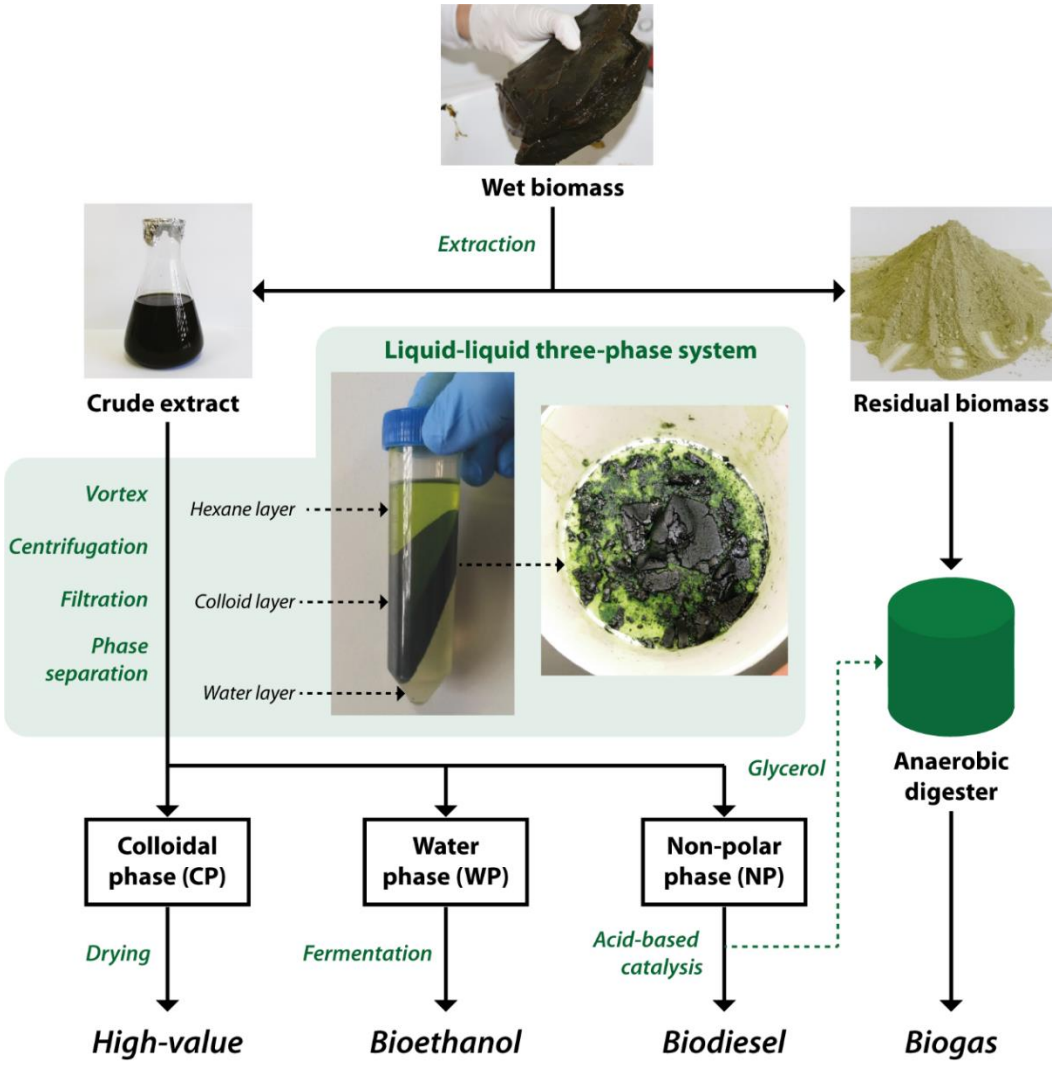


**Figure 6.1** - Schematic representation of the established downstream procedure. Wet microalgal biomass was directly extracted with ethanol at reflux temperature thrice. Upon extraction, the ethanol was evaporated and the extract was further partitioned with hexane and water into three distinct phases: non-polar (upper layer), colloidal (middle layer), and water/polar (bottom layer) phases.

### 6.2.4.2. Liquid-liquid Triphase System (LTPS)

The dried crude ethanolic extract (1.0 g) was dissolved in 80 mL of hexane and 80 mL of distilled water (1:1; v/v) and transferred to a separating funnel. Thereafter, approximately 20 mL of each layer mentioned above were transferred to Falcon tubes and vigorously shaken using a vortex to generate an effective emulsion. To improve layer separation, the mixture was centrifuged at room temperature at 11,000 *g* for 10 minutes. A thick layer of amphiphilic molecules (i.e., colloidal phase, CP) formed in the middle phase as depicted in Figure 6.1. This colloidal fraction was recovered by filtration. In order to maximize the yield of CP, the filtrate was vigorously shaken, centrifuged and filtered using the same conditions for two additional times. At the end of the procedure,

the three CP fractions were resuspended in a mixture of chloroform and methanol (2:1, v/v), pooled together and dried in rotatory evaporator at 40 °C. The hexane and water present in the filtrate were separated using a separating funnel, recovered to clean vials and dried in rotatory evaporator at 40 °C. This procedure led to generation of two different streams: a hexane-containing non-polar phase (NP) and a water phase (WP).

### 6.2.5. CHARACTERIZATION OF COLLOIDAL PHASE (CP)

#### 6.2.5.1. Quantification of carotenoid contents by HPLC-RP

Carotenoids were quantified by means of HPLC-Dionex 580 system equipped with a PDA 100 photodiode-array detector, P680 pump, ASI 100 automated injector and STH 585 column oven and analysed by Chromeleon software. Briefly, dried samples of crude ethanolic extract, NP and CP were dissolved in spectral grade methanol (2.5 mg/mL), filtered at 0.2 µm and 100 µL of sample were injected in a LiChroCART RP-C18 (5 µm, 250x4 mm, LiChrospher®) column. Carotenoids were eluted with a gradient mobile phase composed of solvent A (acetonitrile:water; 9:1, v/v) and solvent B (100% ethyl acetate). The program was initiated with 100% solvent A; 60% solvent A and 40% solvent B for about for 0-16 min, 40% solvent A and 60% solvent B for 16-30 min; and then solvent B (100%) from 30-32 min; and lastly solvent A (100%) up to 35 min at flow rate of 1 mL/min at 20 °C. Carotenoids were analysed at 450 nm; and identified as well as quantified using individual calibration curves for each analytical standard (1 mg/mL *β*-carotene in chloroform, 0.2 mg/mL lutein in chloroform, 0.2 mg/mL violaxanthin in ethanol, and 0.2 mg/mL neoxanthin in ethanol).

#### 6.2.5.2. Assessment of antioxidant activity

The antioxidant activity was evaluated using four *in vitro* assays. The radical scavenging activity (RSA) on 1,1-diphenyl-2-picrylhydrazyl (DPPH), and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonicacid) (ABTS) radicals, was performed using butylated hydroxytoluene (BHT, E321) as positive control (1 mg/mL), as described in Moreno et al. (2006) and Wang et al. (2007), respectively. The metal chelating activities on $Cu^{2+}$ and $Fe^{2+}$ were performed using ethylenediamine tetra-acetic acid (EDTA) as positive control (1 mg/mL), according to Megías et al. (2009).

### 6.2.6. UPGRADE OF STREAMS FOR THE PRODUCTION OF BIOFUELS

#### 6.2.6.1. Biodiesel synthesis from non-polar phase (NP)

Biodiesel was prepared according to the method described by Christie (1982) using 2% $H_2SO_4$ in methanol. Briefly, approximately 20-30 mg of sample (NP) were mixed in the methylating agent solution (2 mL) in screw-capped vials and the reaction mixture was heated in a water bath at reflux temperature (64–70 °C) for 3 h. After reaction completion, FAME were sequentially extracted three times with hexane. Hexane fraction was washed with distilled water until the acid was neutralized, followed by a brine solution treatment. The hexane fraction was dried over anhydrous sodium sulfate, filtered, and dried under nitrogen atmosphere to get the dried biodiesel. The resulting FAME were analysed with a Bruker GC-MS (Bruker SCION 456/ GC, SCION TQ MS) coupled to a ZB-5MS (30 m × 0.25 mm internal diameter, 0.25 μm film thickness, Phenomenex) capillary column. Helium was used as carrier gas, the injection temperature was 300 °C and the temperature program was 60 °C (1 min), 30 °C min to 120 °C, 5 °C min to 250 °C, and 20 °C min to 300 °C (2 min). Supelco® 37 FAME mix (Sigma-Aldrich, Sintra, Portugal) commercial standard allowed the preparation of individual calibration curves for each of the FAME identified.

#### 6.2.6.2. Bioethanol production from the water phase (WP)

##### 6.2.6.2.1. Enzymatic saccharification

The enzymatic saccharification of the WP was conducted using 100 μL of amyloglucosidase (300 amyloglucosidase units/mL, Sigma) and 50 μL α-amylase (from *Aspergillus oryzae*, 30 units/mg, Sigma). The batch saccharification reaction was performed in an Erlenmeyer flask, at 55 °C, pH 5.5 while shaking at 230 rpm for 8 h. Samples were collected periodically, and the amount of sugars was determined using the HPLC method described below.

##### 6.2.6.2.2. Batch experiments in shake flasks

For bioethanol production, *Saccharomyces cerevisiae* F13A (Lima-Costa et al. 2012; Rodrigues et al. 2015) was incubated on yeast extract peptone dextrose (YEPD)

broth (5 g/L peptone, 3 g/L yeast extract and 10 g/L glucose) in an orbital shaker (IKA KS4000i, Portugal) at 150 rpm and 30 °C, until the cultures reached the late exponential phase. Fermentations started with an initial cell concentration of about $1 \times 10^7$ cells/mL.

Batch fermentations were carried out in 250 mL shake flasks, containing 60 mL of medium (5 g/L peptone, 3 g/L yeast extract) supplemented with hydrolyzed WP, as carbon source, with an initial concentration of sugars of approximately 70 g/L. Two controls were performed: in control A, *S. cerevisiae* F13A was inoculated with the culture medium without the WP fraction, and control B with the culture medium supplemented with the non-hydrolyzed WP fraction. All cultures were incubated in an orbital shaker (NeifoPentlab, Portugal) at 150 rpm at 30 °C for approximately 36 h. Samples were taken immediately after inoculation and every 3 h the optical density, sugar consumption and ethanol production were measured. All tests were carried out in triplicate and the results presented are the mean of six values (three replicates of the process and two replicates of the analysis).

#### 6.2.6.2.3. Analytical Methods

Growth and monitoring of yeast cells were done by the measurement of absorbance using a spectrophotometer (GBC DBUV instrument Cintra 202, Australia) at 590 nm. Sugars and ethanol were determined using a HPLC (Hitachi LaChrom Elite HPLC, Japan) equipped with a refractive index detector (Hitachi L-2490, Japan). An Aminex HPX 87H column (Biorad, USA) with an isocratic elution of 10 mM $HNO_3$ at 65 °C and a flow rate of 0.5 mL/min were used for sugars and ethanol quantification.

### 6.2.6.3. Biogas production from the residual biomass (RB)

#### 6.2.6.3.1. Experimental set-up

An inoculum acquired from an anaerobic digestion reactor from Águas do Algarve, S.A. wastewater treatment plant (Lagos/ Silves) was used for the production of biogas. Experiments were carried out under controlled temperature (35 °C) using 100 mL clamp-top vials. Dried RB (1 g) was introduced in the vials and 40 mL of inoculum, 10 mL of distilled water and 0.5 g of sodium bicarbonate ($NaHCO_3$) were later added. A second treatment was performed using the same approach with the addition of 0.5 g of

analytical grade glycerol. Procedural controls were set using the same procedure but without the RB sample. To ensure anaerobic conditions, the head space was purged with nitrogen before the vial was sealed. All experiments were conducted in triplicate.

Biogas production was measured daily, and the head space pressure was registered using a manometer (Fisher Scientific FB57057). The pressure difference measured was converted into biogas volume using equation (1).

(1) $$V_{biogas} = \frac{\Delta P \times V_{head} \times C}{R \times T}$$

where $V_{biogas}$ is the biogas produced during the day (L), ΔP is the daily difference of absolute pressure (mbar), $V_{head}$ is the volume of head space of the digester (L), C is the molar volume (22.41 L/mol), R is the universal gas constant (83.14 L mbar/ mol K) and T is the absolute temperature (K).

The concentrations of carbon dioxide ($CO_2$, %), methane ($CH_4$, %), oxygen ($O_2$, %) and hydrogen sulphide ($H_2S$, ppm) in the biogas were determined using a Geotech Biogas 5000 Gas analyser.

#### 6.2.6.3.2. Analytical parameters

The content of C, H and N was determined by means of a Vario ELIII Elemental Analyser. Total suspended solids (TSS) and volatile suspended solids (VSS) were performed using the ESS Method 340.2.

## 6.3. RESULTS AND DISCUSSION

### 6.3.1. ETHANOLIC EXTRACTION AND LTPS FRACTIONATION

The *Tetraselmis* sp. CTP4 biorefinery established was initiated through the extraction of crude lipids directly from wet biomass paste (ca. 82.3% of moisture or 17.7% dry biomass). The wet biomass downstream processing route was selected, since drying is regarded as one of the most important techno-economic constraints in the whole production pipeline (Xu et al. 2011). Accordingly, wet microalgal biomass was directly extracted with ethanol at reflux temperature, yielding a crude ethanol extract of 25.6% of biomass dry weight (DW). As a result, 74.4% of RB was recovered after the extraction procedure (Table 6.1). It should be mentioned that the biomass used in this work was

grown under optimal conditions, leading to the observed low lipid contents. Therefore, higher extraction yields could have been achieved with cultures grown under nutrient starvation, a condition known to induce the accumulation of lipids in this strain (Pereira et al. 2016). Upon a 7-day nitrogen starvation trial, *Tetraselmis* sp. CTP4 is able to increase its lipid contents 3-fold (up to 33% of DW) and its lipid productivity 2-fold (up to 52.1 mg/L/d; Pereira et al. 2016).

**Table 6.1 -** Gravimetric yields of the lipid extract and residual biomass obtained after the ethanolic extraction from wet microalgal biomass (*Tetraselmis* sp. CTP4) and obtained streams after the fractionation using the liquid-liquid triphase system (LTPS) approach. Values are presented as means ± standard deviation ($n$ = 3)

| Fraction | Yield (%) |
|---|---|
| ***Ethanolic extraction*** | |
| Ethanolic extract (EE) | 25.6 ± 1.6 (of biomass DW) |
| Residual biomass (RB) | 74.4 ± 1.8 (of biomass DW) |
| ***LTPS fractionation*** | |
| Non-polar phase (NP) | 30.9 ± 2.9 (of EE DW) |
| Colloidal phase (CP) | 48.7 ± 1.1 (of EE DW) |
| Water phase (WP) | 20.4 ± 3.2 (of EE DW) |

Ethanol was chosen for microalgal biomass extraction, since it is already widely used at industrial scale for the extraction of lipids from different wet matrices. In addition, it is an eco-friendly and food-grade solvent that can inactivate cellular hydrolases, thereby decreasing the degradation of the extracted compounds, such as triacylglycerols (Yang et al. 2014). However, the extraction selectivity is reduced when ethanol is used, resulting in the co-extraction of other biocompounds, such as proteins, carbohydrates and other organic compounds (Yang et al. 2015).

In the second step of the established downstream process, a liquid-liquid triphase system (LTPS) was employed to separate the crude extract into three streams via centrifugation. Amphiphilic molecules (e.g., phospholipids and glycolipids) generated a stable colloidal phase in the form of a free-standing film, interfacing with two immiscible phases: hexane and water (Fan et al. 2015 and references are therein). As a result, the crude ethanolic extract was fractionated into three distinct streams, namely: i) a upper hexane layer containing mainly non-polar compounds (NP), ii) a thick colloidal phase (CP) in the middle; and iii) a lower layer, comprised of water-soluble/polar compounds

(WP). The fractionation yields were determined gravimetrically for the NP, CP and WP fractions, resulting respectively in 30.9, 48.7 and 20.4% of the crude ethanolic extract (Table 6.1).

Thereafter, the NP, CP and WP fractions were analyzed by thin layer chromatography (TLC) using a mixture of hexane and ethyl acetate (70:30, v/v) as developing solvent to assess the lipidic components (data not shown). Accordingly, the NP exhibited high amount of triacylglycerols (TAG) and free fatty acids (FFA), whereas carotenoids and sterols were less abundant. Conversely, WP did not show any lipids under iodine vapor. The CP showed the presence of phospholipids, carotenoids and sterols; without any TAG present in the fraction. The presence of this molecules in the CP was expected, since the LTPS approach relies on the use of a hexane-water solvent system. Such solvent combination is able to generate an effective emulsion, leading to the development of a thick colloid phase in the interface of the two solvents (Pichot et al. 2013). Different microalgal strains, for instance *Tetraselmis* sp., are known to display significant contents of amphiphilic molecules (e.g., phospholipids, glycolipids and betaine lipids; Cañavate et al. 2015). Interestingly, the presence of these molecules in the crude microalgal extracts of *Tetraselmis* sp. CTP4 greatly enhanced the stability of the emulsion obtained during the development of the LTPS approach. For example, phospholipids are often found in microalgal membranes, among other amphiphilic molecules (Yang et al. 2014). They are characterized by a hydrophilic phosphate group and a hydrophobic fatty acid chain, which promotes the formation of emulsions due to their self-assembly properties (Fan et al. 2015).

Microalgae are also a rich source of carotenoids, which are tetraterpenoids that can be classified as carotenes (e.g., $\beta$-carotene) and xanthophylls (e.g., neoxanthin, violaxanthin, lutein, zeaxanthin, canthaxanthin and astaxanthin), depending upon the absence or presence of oxygen, respectively. As carotenoids are high value compounds, learning the distribution of carotenoids among the ethanolic extract and the different LTPS streams is essential for the development of a biorefinery platform. HPLC-RP analysis of the crude ethanolic extract revealed that it contained two major carotenoids: 30.14% lutein and 41.71% of $\beta$-carotene in terms of total carotenoids (Table 6.2). The xanthophylls neoxanthin (8.68%), violaxanthin (8.13%) and zeaxanthin (2.34 %) were present at lower abundances. Concerning the LTPS streams, NP contained mostly $\beta$-carotene (70.94%), followed by lutein (22.95%), while the xanthophylls neoxanthin

(2.41%), violaxanthin (1.60%) and zeaxanthin (2.10%) were detected at lower quantities. Regarding the CP stream, only minor amounts of *β*-carotene (0.76%) were found, whereas this layer became particularly enriched in three xanthophylls, namely lutein (54.28%), neoxanthin (22.50%) and violaxanthin (20.86%). Zeaxanthin contents remained (1.60%) similar to those observed in the crude extract and NP. The higher content of xanthophylls in the CP might be related with the occurrence of polar hydroxyl groups in these carotenoids, imparting more amphiphilic characteristics to them. Overall, the CP couples the presence of different xanthophylls with other amphiphilic compounds, including other high value compounds (e.g., phospholipids) that could be used in different biotechnological applications.

**Table 6.2 -** HPLC analysis of carotenoid compounds (% of total carotenoids) of the crude ethanolic extract obtained from wet microalgal biomass (*Tetraselmis* sp. CTP4) as well as the non-polar phase (NP) and colloidal phase (CP) obtained after the fractionation using the liquid-liquid triphase system (LTPS) approach. Values are presented as means ± standard deviation ($n$ = 3).

| **Samples** | **Carotenoids (%)** | | | | |
|---|---|---|---|---|---|
| | **Neoxanthin** | **Violoxanthin** | **Lutein** | **Zeaxanthin** | **β-carotene** |
| Crude-extract | 8.68 | 8.13 | 30.14 | 2.34 | 41.71 |
| NP | 2.41 | 1.60 | 22.95 | 2.10 | 70.94 |
| CP | 22.50 | 20.86 | 54.28 | 1.60 | 0.76 |

### 6.3.2. ANTIOXIDANT ACTIVITY OF COLLOIDAL PHASE (CP)

The content of high value compounds present in the CP suggests that this fraction can hold a high antioxidant activity with potential for nutraceutical and cosmeceutical applications. Therefore, this fraction was analysed using four different *in vitro* assays to determine the antioxidant potential of this stream, namely the RSA against the DPPH and ABTS radicals as well as the metal chelating activities against copper (CCA) and iron (ICA) ions. The CP fraction revealed a dose-dependent effect in terms of RSA against DPPH and ABTS radicals as well as metal chelating activities (ICA and CCA). The lowest (1.52 ± 0.18 mg/mL) and highest (12.30 ± 1.97 mg/mL) $IC_{50}$ corresponded to the RSA against the ABTS and DPPH radicals, respectively (Table 6.3), whereas intermediate values, 7.80 ± 1.15 and 5.20 ± 0.58 mg/mL, were obtained for CCA and ICA, respectively.

**Table 6.3 -** Radical scavenging activity on DPPH and ABTS radicals as well as metal chelating activities on copper (CCA) and iron (ICA) of the colloidal phase (CP) obtained after the fractionation of the crude ethanolic extract of *Tetraselmis* sp. CTP4 using a liquid-liquid triphase system (LTPS). Results are expressed as the mean $IC_{50}$ (mg/mL) and corresponding standard deviation ($n$ = 4).

| **Antioxidant activity** | **$IC_{50}$ (mg/mL)** |
|---|---|
| DPPH | 12.28 ± 1.97 |
| ABTS | 1.52 ± 0.18 |
| CCA | 7.80 ± 1.15 |
| ICA | 5.20 ± 0.58 |

The antioxidant activities of the biomass of several microalgae have already been highlighted in previous works (Custódio et al. 2012, 2014; Safafar et al. 2015). A class of compounds known to display antioxidant properties are carotenoids, which are naturally present in microalgae to counteract the deleterious effects of reactive oxygen species (ROS) and triplet chlorophyll (Varela et al. 2015). Other compounds with known antioxidant properties are polyunsaturated fatty acids (PUFA) and phenolic compounds (Sansone et al. 2017), which are also abundant in microalgae. Thus, assessment of RSA in microalgae-based biorefinery is particularly important, because it enables us to determine which streams might have a protective role against oxidative stress and associated chronic disorders (Viña et al. 2004). Similarly, streams containing compounds able to chelate Fe and Cu ions might also contribute to prevent metal-catalysed oxidative damage (Megías et al. 2009; Gangadhar et al. 2016b). Over recent years, there has been an increased demand for antioxidants of natural origin in food and feed products. Rising safety concerns in the use of synthetic antioxidants (e.g., BHT, E321) has caused this trend to become apparent (Lanigan and Yamarik, 2002). The cosmeceutical industry follows the same pattern in the search of ingredients from natural sources. Previous works have concluded that antioxidants can be a promising option to avoid lipid peroxidation of cosmetics, improving the properties of skin protectors by preventing oxidative stress and sunlight-induced damage, and therefore decreasing natural aging and depigmentation (Wang et al. 2015).

### 6.3.3. BIODIESEL PRODUCTION FROM NON-POLAR PHASE (NP)

To further upgrade the value of the NP, a preliminary TLC analysis was carried out. This stream was composed of a large quantity of TAG and FFA, even though carotenoids were also present (data not shown). To determine whether the NP fraction could be converted into biodiesel, acid-catalysed transesterification into FAME was performed. The FAME profile was determined, as this is crucial to assess the quality and properties of the produced biodiesel.

The FAME profile revealed that produced biodiesel contained high levels of oleic (C18:1, 35.1% of total FAME) and palmitic (C16:0, 33.2% of total FAME) acids, which together accounted for more than 68.3% of total FAME (Table 6.4). Palmitoleic (C16:1) and linoleic (C18:2) acids were also detected in significant amounts, 8.0 and 9.9% of total FAME, respectively. All remaining fatty acids were detected at abundances lower than 4% of the total FAME, namely eicosenoic (C20:1), hexadecatrienoic (C16:3), hexadecadienoic (C16:2), arachidonic (C20:4) and eicosapentaenoic (C20:5) acids.

**Table 6.4 -** Fatty acid methyl esters (FAME) profile of *Tetraselmis* sp. CTP4 biodiesel synthesized from the non-polar phase (NP) obtained after the fractionation of the ethanolic extract using the liquid-liquid triphase system (LTPS). Values are presented as means ± standard deviation ($n$ = 3).

| FAME | % of total FAME |
|---|---|
| *C16:0* | 33.2 ± 2.1 |
| ∑*SFA* | 33.2 ± 2.1 |
| *C16:1* | 8.0 ± 0.2 |
| *C18:1* | 35.1 ± 2.4 |
| *C20:1* | 2.5 ± 0.1 |
| ∑*MUFA* | 45.6 ± 1.2 |
| *C16:2* | 2.8 ± 0.1 |
| *C18:2* | 9.9 ± 0.2 |
| *C16:3* | 2.5 ± 0.2 |
| *C20:4* | 3.2 ± 0.1 |
| *C20:5* | 2.9 ± 0.1 |
| ∑*PUFA* | 21.3 ± 0.1 |

Overall, the resultant FAME profile showed that the biodiesel synthesized from the LTPS NP stream has promising properties, because it is comprised of almost 80.0% of saturated (SFA) and monounsaturated (MUFA) fatty acids, containing only small

amounts of PUFA. As SFA and MUFA are less susceptible to oxidation than PUFA, high SFA and MUFA contents are essential for the production of biodiesel with high oxidation stability (Knothe, 2011; Piligaev et al. 2015; Gangadhar et al. 2016a).

In fact, biodiesel produced from *Tetraselmis* sp. CTP4 whole biomass was characterized in a previous report (Pereira et al. 2016), showing properties that comply with most of the European (EN14214) and American (ASTM D6751) specifications. Comparing this report with the results here obtained, the FAME profile was similar, with the exception of a higher content of PUFA with ≥ 4 double bonds. This difference is probably related with the growth conditions used to obtain the biomass of *Tetraselmis* sp. CTP4. As previously noted, in this work the biomass was grown under optimal conditions. This might have resulted in cultures containing high amounts of actively growing cells, which are known to produce membranes rich in PUFA (Molina Grima et al. 1999; Patil et al. 2005).

Although this work focused on the upgrade of the NP to biodiesel, it should be emphasized that the NP fraction can also be used for other biotechnological applications. The high content of TAG and carotenoids suggests that this fraction can also be a promising source for the production of edible oils for the food and feed industry (Xue et al. 2018). Nevertheless, further work must be conducted to evaluate whether the content of FFA and carotenoids in the NP is not detrimental for such applications.

### 6.3.4. BIOETHANOL FERMENTATION FROM WATER PHASE (WP)

In order to upgrade the LTPS water-soluble compounds, enzymatic saccharification was used to convert the WP carbohydrates into simple sugars for possible bioethanol production via fermentation. As microalgae are often rich in carbohydrates, these microorganims have usually high levels of reducing sugars that can be obtained via saccharification. This fact not only eases the saccharification process, but also turns microalgae into a sustainable feedstock for bioethanol production (Miranda et al. 2012a, 2012b). In this case, enzymatic treatment, catalyzed by amylase and glucoamylase, hydrolyzed the WP stream into simple sugars, which were fed to *Saccharomyces cerevisiae* F13A, an autochthonous and robust yeast adapted to high ethanol concentrations (Raposo et al. 2017).

The carbohydrates composition of microalgae is mainly polysaccharides which are entrapped within cell walls and intercellular matrices. Upon enzymatic hydrolysis, the main sugars identified in the WP stream were glucose (64.4%), galactose (23.2%), mannose (7.4%), and arabinose (4.9%). Interestingly, glucose was the monosaccharide preferably consumed by the yeast, followed by galactose (Figure 6.2). The initial concentration was 43.5 g/L, being practically depleted after 24 h. Taken together, these results are quite promising for a future biorefinery, since glucose was not only the most abundant sugar in the WP stream, but also was the monossacharide that was readily consumed by *S. cerevisiae* F13A.

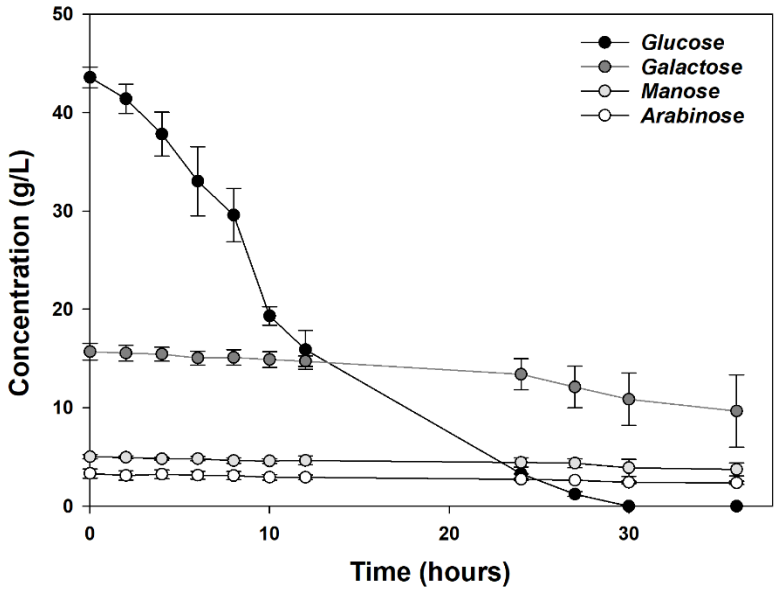


**Figure 6.2 -** Consumption of sugars during the fermentation period of the enzymatically hydrolysed water phase (WP) obtained after the application of the liquid-liquid triphasic system (LTPS). Values are presented as means ± standard deviation ($n$ = 3).

Starting at a concentration of 0.36 g/L, the yeast cells grew in the exponential phase with the maximum specific growth rate of 0.13 $h^{-1}$ (Table 6.5). The biomass concentration reached the stationary phase after approximately 13 h of fermentation, and the maximum cell biomass and ethanol concentration obtained during the fermentation were 6.3 g/L and 21.3 g/L, respectively. No growth was observed in Control A, a medium where the WP stream was omitted. In Control B, a medium where the polar fraction was not hydrolysed, only residual growth was observed with a specific growth rate of 0.07 $h^{-1}$ (Table 6.5). Interestingly, a low amount of glucose was detected in the medium (3 g/L), which could have been due to a thermal effect of the medium autoclavation. Although the

yield in ethanol was high, its concentration was substantially low, as compared to that obtained with the hydrolysed fraction (Table 6.5).

**Table 6.5 -** Growth parameters determined for *Saccharomyces cerevisiae* F13A, grown in enzymatically hydrolysed water phase (WP) obtained after the application of the liquid-liquid triphasic system (LTPS). Values are presented as means ± standard deviation (*n* = 3). Different letters within each treatment indicate significant differences.

| | $\mu_{máx}$ ($h^{-1}$) | $X_{máx}$ (g/L) | $Y_{x/s}$ | Et max (g/L) | $Y_{p/s}$ (g Et/g DW) | Inicial sugar (g/L) | Remaining sugar (g/L) |
|---|---|---|---|---|---|---|---|
| **CTRL A$^{\alpha}$** | - | - | - | - | - | 0 | 0 |
| **CTRL B$^{\beta}$** | $0.07 \pm 0.01^{b}$ | $1.72 \pm 0.10^{b}$ | $0.36 \pm 0.09^{a}$ | $1.98 \pm 0.00^{b}$ | $0.41 \pm 0.08^{a}$ | $9.04 \pm 0.01^{b}$ | $4.26 \pm 0.00^{b}$ |
| **Water Phase** | $0.13 \pm 0.03^{a}$ | $6.30 \pm 0.22^{a}$ | $0.09 \pm 0.01^{b}$ | $21.29 \pm 1.55^{a}$ | $0.46 \pm 0.04^{a}$ | $67.62 \pm 3.27^{a}$ | $15.76 \pm 9.01^{a}$ |

$^{\alpha}$ *culture medium without WP fraction*

$^{\beta}$ *culture medium supplemented with non-hydrolysed WP fraction*

No significant amount of ethanol was detected during the first 8 h. However, ethanol concentration increased continuously from 8 to 27 h, reaching a maximum value of 21.3 g/L (Figure 6.3). As fermentation progressed, the ethanol concentration was nearly constant. The fermentation duration was 36 h, during which around 20 g/L of ethanol was produced. Glucose concentration was consumed significantly, which was accompanied by a sharp increase in ethanol concentration, achieving an ethanol yield of 89.1%, relatively to the theoretical ethanol yield based on the amount of glucose released

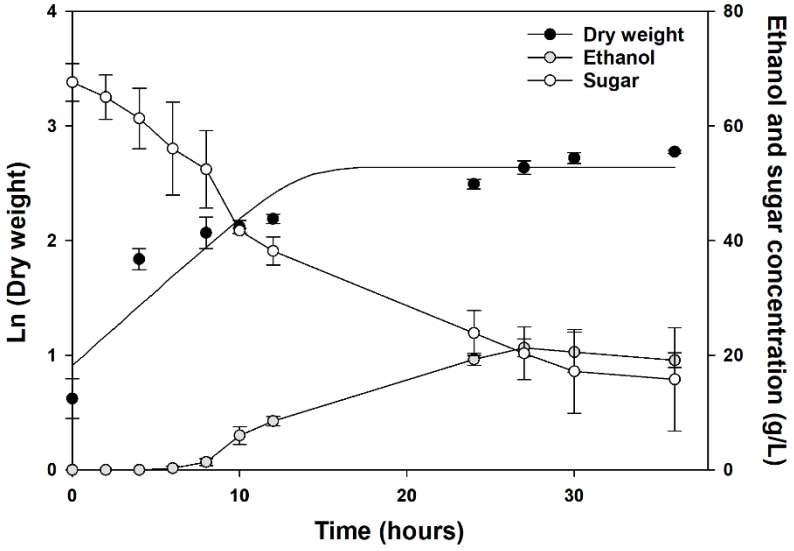


**Figure 6.3 -** Growth of *Saccharomyces cerevisiae* F13A, ethanol production and sugar consumption of the enzymatically hydrolysed water phase (WP) obtained after the application of the liquid-liquid triphase system (LTPS). Values are presented as means ± standard deviation (*n* = 3).

from microalgae after enzymatic hydrolysis (or 0.46 g ethanol/g fermentable sugar). According to the values presented in Table 6.5, the substrate was used preferentially for the production of bioethanol, being the biomass yield low. The fermentation route was favored, potentiating the production of bioethanol.

Similar results were obtained by Ho et al. (2013) for bioethanol production using biomass of *Chlorella vulgaris* upon enzymatic hydrolysis. Although the ethanol yields are similar, the concentration of ethanol (11.66 g/L) obtained was substantially lower, as compared to this work. The values of ethanol production and yield obtained are within those referenced in the literature, being in some cases higher than those obtained by other authors (Miranda et al. 2012b; Lee et al. 2015; Farias Silva and Bertucco, 2016; Phwan et al. 2018; Singh et al. 2018).

Overall, the hydrolysate from WP was converted into bioethanol by yeast fermentation with a yield of 0.46 g ethanol/g fermentable sugar (Table 6.5). In this study, it was shown that WP can indeed be successfully upgraded to bioethanol to improve the overall economic feasibility of the whole pipeline. However, it is also noteworthy that this fraction can also be used for other biotechnological ends. A previous characterization of the whole microalgal biomass of *Tetraselmis* sp. CTP4 revealed that the strain is rich in starch-like polysaccharides (data not shown), which could be used in nutritional applications. Therefore, future studies on how this fraction could be used for food or feed purposes might reveal novel ways of further upgrading the value of the WP stream in the form of nutraceuticals and feed additives. Eventually, these starch-like polysaccharides could also be transformed into bioplastics.

### 6.3.5. BIOGAS PRODUCTION FROM RESIDUAL BIOMASS (RB)

For biogas production, the RB collected after the ethanolic extraction was submitted to anaerobic digestion, with and without glycerol supplementation. The elemental analysis of the RB showed mass contents of 42.10% C, 6.52% H and 5.95% N, and a C:N ratio of 7.07. The inoculum used for the assay was gathered in a wastewater treatment plant digester and displayed a content of TSS and VSS of 27.0 and 23.0 g/L, respectively.

The daily biogas production is shown in Figure 6.4A. The plot clearly shows that higher biogas was produced in the treatment supplemented with glycerol during the whole digestion period, and that the first day was the most productive, probably due to the high availability of biodegradable components on the RB. Between days 3 and 5, a decrease in biogas production was observed, followed by an increase that peaked at the 9$^{th}$ day of the digestion, in both experimental treatments. Probably the consortium of bacteria of the

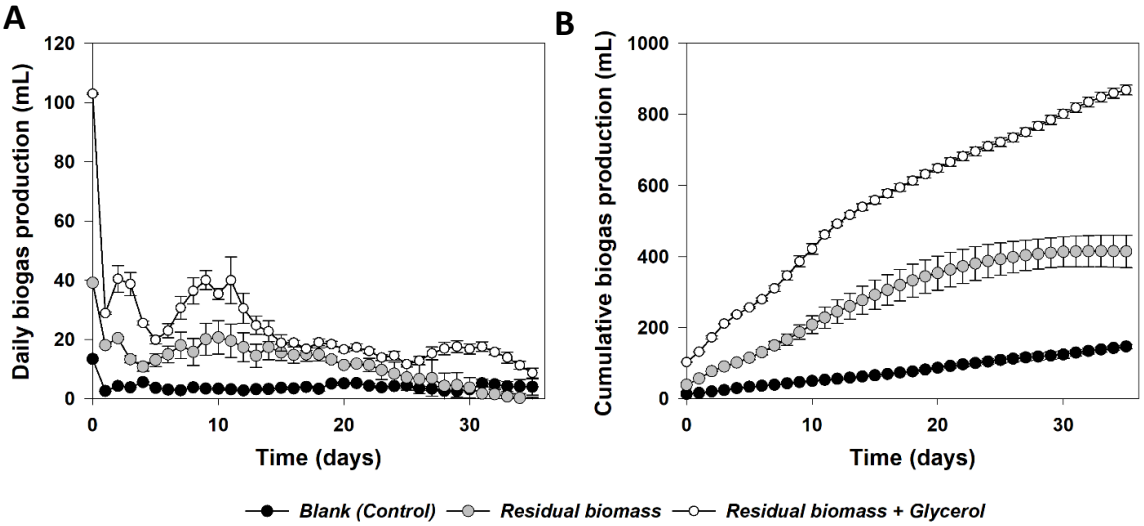


**Figure 6.4 -** Daily (A) and cumulative (B) production of biogas over a period of 36 days, using the residual biomass of *Tetraselmis* sp. CTP4 with and without the supplementation with glycerol. A blank control without residual biomass and glycerol was also carried out. Values are presented as means ± standard deviation ($n$ = 3).

inoculum has evolved over time to be more capable to digest the RB, which can justify this second increase. After the 9$^{th}$ day, the biogas production reduced gradually, until all the substrate was consumed.

The cumulative biogas production is shown in Figure 6.4B. After 36 days of incubation, the cumulative biogas production from RB was 507 mL/gVS, whereas the production of the treatment supplemented with glycerol was 1010 mL/gVS (Figure 6.4B). Therefore, the supplementation with glycerol led to a 2-fold increase in the total biogas production of the system, even though the increase in carbon available was less than 50%. The average concentration of methane present on the biogas was approximately 49.6% (v/v) in both experimental treatments (Table 6.6). Accordingly, the methane production and yields obtained for the RB treatment were 251 mL/gVS and 64%, while in the RB supplemented with glycerol were 501 mL/gVS and 83%.

**Table 6.6 -** Biogas, methane and theoretical methane production results obtained after anaerobic digestion of the residual biomass (*Tetraselmis* sp. CTP4), with and without supplementation with glycerol. Values are presented as means ± standard deviation ($n$ = 3). Different letters within each treatment indicate significant differences.

| Treatment | Theoretical methane production (mL/gVS) | Biogas production (mL/gVS) | Methane production (mL/gVS) | Methane yield (%) |
|---|---|---|---|---|
| **Residual biomass** | 392 | $507 \pm 16^{b}$ | $251 \pm 13^{b}$ | 64 |
| **Residual biomass + Glycerol** | 605 | $1010 \pm 14^{a}$ | $501 \pm 14^{a}$ | 83 |

The glycerol supplementation strategy was followed, since upgrading the NP to biodiesel would generate glycerol as co-product of the transesterification process. This otherwise waste product ($C_3H_8O_3$) was successfully used as a co-digestant to adjust the C:N ratio content of the RB, leading to a more acceptable ratio (C:N = 10.4). Nevertheless, both C:N ratios on the digesters were not ideal for the production of biogas, since C:N ratios of 20-30 are known to be optimal for biogas production (Zhong et al. 2012). Therefore, glycerol supplementation seems advantageous for microalgal biorefinery operations, since it is highly degradable by anaerobic digestion giving methane yields of 0.306 $m^3$ $CH_4$ $Kg^{-1}$ (Lopez et al. 2009).

As previously suggested by other authors (Chisti, 2007, 2013), anaerobic digestion can be a partial solution to overcome the restrictions of site placement and costly transport in large-scale microalgal production units, related with the addition of concentrated $CO_2$ and nutrient supplementation (N and P). Anaerobic digestion of residual algal biomass typically produces biogas consisting of 60% methane and 40% $CO_2$. Therefore, produced biogas can be directly injected into the photobioreactors to supply the concentrated $CO_2$ required, which, in combination with pH control, has been shown to augment microalgal growth with concurrent $CO_2$ remediation (Olaizola, 2003; Doucha et al. 2005). Interestingly, the methane in the biogas appears not to adversely affect growth (Travieso et al. 1993; Mandeno et al. 2005; Heubeck et al. 2007). Regarding nutrient supplementation, the residual digestate of anaerobic digestion can be a key source of nutrients for microalgal cultivation. Microalgae have a typically low C:N ratio (Elser et al. 2000), therefore, recycling the digestate after the anaerobic digestion of RB for

further algal culture could likely replace a substantial amount of the demand for macronutrients.

Although the RB was upgraded in the present work for the production of biogas, the biochemical composition of this fraction seems highly suitable for higher end applications. The high content of protein and minerals contained in the RB suggests a high potential for nutritional applications, both for human and animal consumption. Because of the high N content, this fraction is also of potential interest for the production of agricultural products, as a source of stimulants for germination and growth of plant crops as well as for the production of biofertilizers and biopesticides.

## 6.4. CONCLUSIONS

The LTPS approach produced three different streams from a crude ethanolic extract in an efficient manner, which could then be scaled up to a pilot-scale biorefinery. Most importantly, the CP fraction was characterized as a source of added-value molecules with antioxidant activity, containing lutein and phospholipids. In addition, biodiesel, bioethanol and biogas were successfully upgraded from the remaining fractions, offering the potential to increase the total revenue from the whole microalgae-based pipeline. Overall, this innovative microalgae-based biorefinery is expected to improve the sustainability of microalgal biomass downstream processing, through the production of multiple products in the form of high value products and biofuels.

## ACKNOWLEDGEMENTS

The present work was funded by the Portuguese national budget P2020 and the CCMAR/Multi/04326/2013 grant of the Foundation for Science and Technology (FCT), the ALGARED+ 1398 EP - INTERREG V-A España-Portugal project and the COST Action 1408 - European Network for Bio-products. KG (SFRH/BPD/81882/2011) and HP (SFRH/BD/105541/2014) would like to acknowledge FCT, for financial support as post-doctoral and doctoral research fellows under guidance of Prof. J. Varela and Prof. F. Xavier Malcata for their constant encouragement and support.

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# CHAPTER VII

# INCORPORATION OF RESIDUAL MICROALGAL BIOMASS (*TETRASELMIS* SP. CTP4) AT THE EXPENSES OF SOYBEAN MEAL AS A FEED INGREDIENT FOR JUVENILE GILTHEAD SEABREAM (*SPARUS AURATA*)

**Research article submitted to *Algal Research*.**

# INCORPORATION OF RESIDUAL MICROALGAL BIOMASS (*TETRASELMIS* SP. CTP4) AT THE EXPENSES OF SOYBEAN MEAL AS A FEED INGREDIENT FOR JUVENILE GILTHEAD SEABREAM (*SPARUS AURATA*)

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## ABSTRACT

The forecasted growth of the aquaculture sector requires the use of novel and sustainable ingredients in aquaculture feeds. A study was undertaken to evaluate the effect of a 10% incorporation of defatted microalgal biomass/residual biomass (RB) of *Tetraselmis* sp. CTP4, used at the expense of dehulled solvent-extracted soybean meal (SBM), on the growth performance, nutrient digestibility and physiological response to confinement stress in gilthead seabream juveniles. The trial comprised two dietary treatments: a control diet (CTRL) with relatively high levels of marine-derived proteins and 10% SBM; and a test diet (RB10) with the incorporation of 10% RB at the expenses of SBM, while maintaining a fairly constancy of all other ingredients. Triplicate groups of 30 fish, with a mean initial body weight of 6.0 ± 0.2 g were fed the experimental diets for 61 days. At the end of the trial, fish tripled their initial body weight, but the overall growth performance criteria (final body weight, daily growth index, feed conversion ratio and protein efficiency ratio), whole-body composition and nutrient retention were not significantly affected by the dietary treatments ($p>0.05$). The RB10 diet showed a significantly higher apparent digestibility coefficients (ADC) of protein, energy and phosphorus ($p<0.05$). When measured as an isolated feed ingredient, the RB had an ADC of protein, fat, energy and phosphorus of 87.9, 85.3, 75.5 and 41.4%, respectively. After an acute confinement stress test, fish fed with RB10 diet displayed a significantly lower plasma cortisol response (120 ± 23 ng/mL) than those fed with the control diet (160 ± 33 ng/mL) ($p<0.05$). Overall results showed that residual microalgal biomasses (RB), issued from biorefinery processes, could potentially spare the use of soybean meal in aquaculture feeds, contributing towards a reduction of the current protein deficit in the European market.


**Keywords:** Biorefinery; Residual microalgal biomass; Feed ingredient; Microalgae; Soybean; *Sparus aurata; Tetraselmis* sp. CTP4

## 7.1. INTRODUCTION

There is increasing interest in large-scale production of microalgal biomass as a sustainable lipid feedstock for different biotechnological applications, which include human and animal nutrition as well as biodiesel production (Wijffels and Barbosa, 2010). However, the downstream processing entailing the extraction of lipids from the biomass will generate massive amounts of defatted microalgal biomass (residual biomass, RB) as a co-product. Several reports have investigated the suitability of upgrading these RB into different biofuels to improve the net energy ratio of the whole production pipeline such as production of biogas, bioethanol and bio-oil (e.g., hydrothermal liquefaction or pyrolysis) in a biorefinery setting (Vardon et al. 2012; Rashid et al. 2013; Chandra et al. 2014; Ou et al. 2015). Although there is a high demand for renewable sources for global fuel supply from the market and policymakers, biofuels need to be relatively inexpensive in order to compete with fossil fuels. Therefore, to enable the commercial use of microalgae as feedstock for the generation of bioenergy, the production and processing costs have to be offset by higher-end commodities obtained from RB and other residues.

Whole microalgal biomass (WMB) and RB are feed ingredients not only as a solution to meet the high demand for feedstocks required by the feed industry, but also as a way to meet future demand caused by the expected growth of the human population in the forthcoming decades (Austic et al. 2013). Thus far, most studies have focused on the incorporation of WMB in feed without any processing, either as an additive or as a macro-ingredient (Fredriksson et al. 2006; Dallaire et al. 2007; Walker and Berlinsky, 2011; Ekmay et al. 2014; Sarker et al. 2016; Sørensen et al. 2016). Overall, most reports show that WMB is a promising feed ingredient with wide application in the farming of different livestock. Reports evaluating the applicability of RB as a feed ingredient for land animals have been published (Austic et al. 2013; Gatrell et al. 2014; Leng et al. 2014; Vidyashankar et al. 2015), as well as in aquaculture species (Ju et al. 2012; Patterson and Gatlin, 2013; Basri et al. 2015; Kiron et al. 2016; Ju et al. 2017; Sørensen et al. 2017; Gong et al. 2018). Indeed, over the last decades, alternative sources (e.g., vegetable protein sources, processed animal proteins, insect meals, krill meal) have been introduced in aquaculture feeds in order to reduce the dependency of fishmeal on aquafeeds. However, research is still needed for finding and fine-tuning innovative sources of feed ingredients for the aquaculture industry in order to decrease its dependence on non-

sustainable feedstocks and thus ensure the future sustainability of commercial fish supply (Naylor et al. 2000).

Among the alternatives proposed, soybean meal is one of the most used feedstocks for feed manufacturing. Dehulled solvent-extracted soybean meal (SBM) is a high-quality protein source with steady supply and competitive costs. This has triggered a significant rise in demand for soybean and derivatives (meal and oil) for livestock production and, more recently, for biodiesel production as well. However, sustainability concerns have been raised (Millazo et al. 2013a; 2013b), as extensive and ever-increasing soybean farming areas have become a major driver for worldwide deforestation and loss of biodiversity in developing countries, along with other environmental and social concerns (Steinfeld et al. 2006). According to recent European Union (EU) reports (European Commission, 2013; 2017), 60% of the world deforestation is related to the production of soybean and derivatives, which are mainly imported and consumed by EU countries. The EU animal feed market is highly dependent on protein feed imports and its self-sufficiency in soybean meal is extremely low (3%; de Visser et al. 2014). This situation makes the animal feed sector highly vulnerable to trade distortions, availability and price volatility of soybeans (Häusling, 2011; de Visser et al. 2014). A reduction of the EU protein deficit is a priority and requires the emergence of novel protein resources such as microalgae.

In this context, a nutritional study was undertaken to assess the effects of incorporating a residual microalgal biomass (from *Tetraselmis* sp. CTP4) at the expenses of soybean meal, on the growth performance, digestibility and nutrient retention of gilthead seabream (*Sparus aurata*) juveniles.

## 7.2. MATERIALS AND METHODS

### 7.2.1. RESIDUAL MICROALGAL BIOMASS

A residual microalgal biomass of *Tetraselmis* sp. CTP4 was generated upon the extraction of lipids directly from wet microalgal paste using an ethanolic extraction. The detail procedure used for biomass growth has been previously described by Pereira et al. (2016). The methodology used for lipid extraction was based on the protocol of Yang et al. (2015) with modifications. Briefly, wet microalgal paste was sequentially extracted (three times) with absolute ethanol at reflux temperature. After each extraction, the algae

cake (RB) was separated from the solvent by centrifugation (2,000 *g*, 10 min). Upon completion of the lipid extraction, RB was air dried at ambient temperature for 24 hours. Further drying of the biomass was achieved at 40 °C using a forced air-circulating oven until constant weight. The cake was later milled to powder and stored under vacuum in a desiccator until the manufacture of the experimental diets. The composition of the experimental residual microalgal biomass of *Tetraselmis* sp. CTP4 (RB) and its comparison to soybean meal and fishmeal (FM) is presented in Table 7.1.

**Table 7.1 -** Composition of residual microalgal biomass (RB), dehulled solvent-extracted soybean meal (SBM) and fishmeal (FM) (values expressed on a fresh matter basis).

| | **RB** | **SBM** | **FM** |
|---|---|---|---|
| Proximate composition | | | |
| Moisture, % | 0.90 | 9.20 | 6.60 |
| Crude protein, % | 40.63 | 42.04 | 71.85 |
| Crude fat, % | 1.29 | 2.00 | 6.90 |
| Ash, % | 14.57 | 5.54 | 18.07 |
| Total phosphorus, % | 0.93 | 0.60 | 1.92 |
| Gross energy, kJ/g | 17.10 | 17.45 | 19.78 |
| | | | |
| Amino acids (%) | | | |
| Arginine | 1.68 | 3.22 | 4.71 |
| Histidine | 0.04 | 1.14 | 1.75 |
| Isoleucine | 1.11 | 1.96 | 2.54 |
| Leucine | 2.26 | 3.32 | 5.10 |
| Lysine | 1.68 | 2.67 | 5.96 |
| Threonine | 1.26 | 1.71 | 3.50 |
| Tryptophan | 0.37 | 0.61 | 0.71 |
| Valine | 1.54 | 2.07 | 3.22 |
| Methionine | 0.60 | 0.58 | 2.61 |
| Cysteine | 0.28 | 0.61 | 0.33 |
| Phenylalanine | 1.43 | 2.18 | 3.38 |
| Tyrosine | 0.84 | 1.60 | 2.59 |
| Alanine | 2.02 | 1.89 | 4.38 |
| Aspartic acid | 2.86 | 4.89 | 6.92 |
| Glutamic acid | 3.61 | 7.74 | 8.92 |
| Glycine | 1.57 | 1.82 | 5.20 |
| Proline | 1.25 | 2.21 | 2.93 |
| Serine | 1.18 | 2.18 | 3.15 |

### 7.2.2. FORMULATION OF EXPERIMENTAL DIETS

The growth performance trial comprised two dietary treatments. A control diet (CTRL) containing relatively high levels of marine-derived proteins (fishmeal, fish hydrolysate and squid meal) and several plant proteins such as soy protein concentrate, wheat gluten, corn gluten meal and dehulled solvent-extracted soybean meal (SBM). Fish oil was used as the main lipid source. A second diet (RB10) was formulated with the incorporation of 10% RB at the expenses of soybean meal, while maintaining a fairly constancy of all other ingredients. Both diets were supplemented with monocalcium phosphate to avoid the risk of phosphorus imbalance: 0.5% and 0.3% for CTRL and RB10 diets, respectively. Overall, these two diets were isonitrogenous (crude protein: 58% DM), isolipidic (crude lipids: 16.6% DM) and isoenergetic (gross energy: 18.7 MJ/kg DM). A part of each experimental diet contained also 1% chromic oxide as an inert marker for digestibility measurements. One additional diet (RB ADC) containing 70% of the same basal mixture of the control diet with 1% chromic oxide and 30% of the test ingredient (residual microalgae biomass) was also manufactured to allow the measurement of the apparent digestibility of the individual test ingredient, according to the methodological approach recommended by NRC (2011).

Experimental diets were manufactured by SPAROS, Lda. (Olhão, Portugal). Ingredients were mixed according to target formulation and ground (< 250 μm) in a micropulverizer hammer mill (Hosokawa-Alpine, 1SH, Germany). Powdered ingredients and fish oil were mixed in a paddle mixer (MAINCA, RM90, Spain) and the blend moisturized with 25% water. Diets were manufactured by low-shear and low temperature extrusion (Italplast P55, Italy) at a pellet size of 1.0 mm. Upon extrusion, pellets were dried in a vibrating fluid bed dryer (TGC Extrusion, DR100, France). Throughout the trial, experimental feeds were stored at room temperature, but in a cool and aerated emplacement. Samples of each diet were taken for proximate composition (Table 7.2).

**Table 7.2 -** Formulation and composition of the three experimental diets: control diet (CTRL), a diet with 10% inclusion of residual microalgal biomass (RB10) and a diet for the ingredient apparent digestibility coefficient calculation (RB ADC).

| | CTRL | RB10 | RB ADC |
|---|---|---|---|
| Fishmeal[a] | 20.00 | 20.00 | 14.00 |
| Fish hydrolysate[b] | 5.00 | 5.00 | 3.50 |
| Fish gelatin[c] | 2.00 | 2.00 | 1.40 |
| Squid meal[d] | 12.50 | 12.50 | 8.75 |
| Soy protein concentrate[e] | 10.00 | 10.00 | 7.00 |
| Soybean meal[f] | 10.00 | - | 7.00 |
| Residual microalgal biomass[g] | - | 10.00 | 30.00 |
| Wheat gluten[h] | 8.00 | 8.00 | 5.60 |
| Corn gluten[i] | 8.00 | 8.70 | 5.60 |
| Wheat meal[j] | 9.50 | 8.90 | 6.65 |
| Fish oil[k] | 12.00 | 12.10 | 8.40 |
| Vitamin and mineral premix[l] | 1.50 | 1.50 | 1.05 |
| Soy lecithin[m] | 0.50 | 0.50 | 0.35 |
| Binder[n] | 0.20 | 0.20 | 0.14 |
| Antioxidant[o] | 0.20 | 0.20 | 0.14 |
| Sodium propionate[p] | 0.10 | 0.10 | 0.07 |
| Monocalcium phosphate[q] | 0.50 | 0.30 | 0.35 |
| Chromic oxide | 1.00 | 1.00 | 0.70 |
| | | | |
| Dry matter (DM), % | 96.5 | 97.4 | 97.2 |
| Crude protein, % DM | 58.3 | 57.8 | 54.1 |
| Crude fat, % DM | 16.5 | 16.7 | 16.0 |
| Ash, % DM | 6.07 | 6.41 | 7.89 |
| Total phosphorus, % DM | 1.20 | 1.20 | 1.29 |
| Gross energy, kJ/g DM | 18.7 | 18.8 | 19.2 |
| Chromic oxide, % DM | 1.28 | 1.18 | 1.22 |

[a] Fish meal NORVIK 70: 70.3% crude protein (CP) 5.8% crude fat (CF), Sopropêche, France; [b] CPSP 90: 83% CP, 9% CF, Sopropêche, France; [c] Fish gelatin: 96% CP, LAPI Gelatine SPA, Italy; [d] Squid meal: 83% CP, Sopropêche, France; [e] Soycomil P: 63% CP, 0.8% CF, ADM, The Netherlands; [f] Dehulled solvent extracted soybean meal: 46% CP, 2.3% CF, CARGILL, Spain; [g] residual microalgal biomass from *Tetraselmis* sp. CTP4: 41% CP, 1.3 CF; [h] VITAL: 80% CP, 1.7% CF, Roquette Frères, France; [I] Corn gluten meal: 61% CP, 6% CF, COPAM, Portugal; [j] Wheat meal: 10.2% CP; 1.2% CF, Casa Lanchinha, Portugal; [k] Fish oil, Savinor UTS, Portugal; [l] PREMIX Lda, Portugal (IU or mg kg-1 diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 1000 mg; inositol, 500 mg; biotin, 3 mg; calcium panthotenate, 100 mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg kg-1 diet): cobalt carbonate, 0.65 mg; copper sulphate, 9 mg; ferric sulphate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulphate,7.5 mg; sodium chloride, 400 mg; calcium carbonate, 1.86 g; excipient wheat middlings; [m] Lecico P700IPM, LECICO GmbH, Germany; [n] Guar gum: Seah International, France; [o] Paramega PX, KEMIN EUROPE NV, Belgium; [p] Sodium propionate: Disproquimica, Portugal; [q] MCP: 22% P, 18% Ca, Fosfitalia, Italy.

### 7.2.3. GROWTH TRIAL

All trials were performed at the experimental research facilities of SPAROS (Olhão, Portugal), and conducted by trained scientists (following category C FELASA recommendations) according to the European guidelines on protection of animals used for scientific purposes (Directive 2010/63/UE of European Parliament and of the European Union Council).

Gilthead seabream (*Sparus aurata*) juveniles, originated from a commercial hatchery (CUPIMAR, Cádiz, Spain), were adapted to the experimental conditions over a period of 15 days. Homogenous groups of 30 fish each, with a mean initial body weight of 6.0 ± 0.2 g, were stocked in 6 sub-square fiberglass tanks (volume: 60 L; water-flow rate: 3.5 L/min), supplied with thermo-regulated seawater, with 200% water renewal/hour (temperature: 20.3 ± 1.1 °C; dissolved oxygen: 6.0 ± 0.5 mg/L; salinity: 35‰). A 12L:12D photoperiod was maintained with daybreak set at 7.00 h. Each dietary treatment was tested in triplicate tanks over 61 days. Fish were fed to apparent satiety, by hand, three times a day (9.30 am, 2.00 pm and 5.00 pm) and utmost care was taken to avoid feed wastage and allow a precise quantification of feed intake. Light anesthetized fish (25 mg/L of MS-222, Germany) were group weighed at the start of the trial, at day 30 and day 61 for estimation of tank biomass. At the start of the trial, a pool of 15 whole fish from the initial stock and a pool of 5 whole fish per tank at the end of the trial were sampled and stored at -20 °C for subsequent analysis of whole-body proximate composition.

### 7.2.4. ACUTE CONFINEMENT STRESS TEST

At the end of the growth trial and two days after all associated samplings, the remaining fish (average body weight: 20.8 ± 0.4 g) were subjected to an acute confinement stress test. Eight fish from each replicate tank were transferred from the 60 L tanks with a rearing density of 10 kg/m$^3$, to a plastic container at a density of 60 kg/m$^3$. The water level was maintained at a minimum, forcing dorsal fin exposure, and the test was carried out for 15 minutes. Afterwards, a sample of blood (1 mL) was collected from all fish by puncture of the caudal vein with a heparinized syringe. Blood was placed in cooled 1.5 mL plastic tubes and centrifuged at 6,000 *g* for 5 min at 4 °C. Upon

centrifugation, supernatant plasma was transferred to Eppendorf tubes, snap-frozen in liquid nitrogen and stored at -80 °C until subsequent analysis of cortisol.

### 7.2.5. DIGESTIBILITY MEASUREMENTS

Following the growth trial and the acute confinement stress test, the apparent digestibility of nutrients and energy of the test ingredient and of the experimental diets was measured by the indirect method. The remaining fish (*n*=25 per tank) were maintained in the 60 L tanks equipped with a faeces settling column. Each group of fish was fed the same diet and reared under identical water conditions as those described for the growth trial. Fish were fed once a day (9.00 am) by hand in slight excess. Upon a thorough cleaning of the rearing tanks from any feed residues, faeces were collected daily for 10 consecutive days by means of a faeces decantation column (Guelph system). Faeces were collected approximately 18 hours after the meal. After removal of excess water, daily faeces were frozen at -20 °C. Pooled faeces from each group of fish were freeze-dried prior to subsequent analysis.

Apparent digestibility coefficients (ADC) of dietary nutrients and energy in the experimental diets were calculated according to NRC (2011):

$$\mathrm{ADC}(\%) = 100 - \left[\frac{\%\ \text{marker diet}}{\%\ \text{marker faeces}} \times \frac{\%\ \text{nutrient faeces}}{\%\ \text{nutrient diet}}\right]$$

Subsequently, the apparent digestibility coefficients of the test ingredient were calculated according to NRC (2011):

$$\text{ADC Test Ingredient (\%)} = ADC_{TD} + (ADC_{TD} - ADC_{RD}) \times (0.7 \times N_{RD}) / (0.3 \times N_{TI})$$

$ADC_{TD}$: ADC of test diet (%)

$ADC_{RD}$: ADC of reference diet (%)

$N_{RD}$: Nutrient content in the reference diet (% or kJ/g)

$N_{TI}$: Nutrient content in the test ingredient (% or kJ/g)

### 7.2.6. CHEMICAL ANALYSIS

The proximate composition analysis of the test ingredient, experimental diets, whole fish and feces was performed using the following analytical methods. Dry matter after drying at 105 °C for 24 h; total ash by combustion (550 °C during 6 h) in a muffle furnace (Nabertherm L9/11/B170, Germany); crude protein (N×6.25) by a flash combustion technique followed by a gas chromatographic separation and thermal conductivity detection (LECO FP428); total lipids were quantified by a modified Bligh and Dyer (1959) method, as described in Pereira et al. (2011); total phosphorus was determined according to the ISO/DIS 6491 method, using the vanado-molybdate reagent; gross energy in an adiabatic bomb calorimeter (Werke C2000, IKA, Germany); chromic oxide in feeds and feces was determined by spectrometry (SpectrAA 220 FS, Varian) according to Bolin et al. (1952) after perchloric acid digestion. The amino acid profile was determined by ultra-performance liquid chromatography (UPLC) as reported by Aragão et al. (2014). The concentration of cortisol in the plasma was evaluated by a radioimmunoassay as described in Rotllant et al. (2005).

### 7.2.7. STATISTICAL ANALYSIS

Growth performance data and ADC were expressed as means ± standard deviation of three replicates. Statistical analyses were performed with R computing software (Ihaka and Gentleman, 1996). Parameters expressed as percentage were subjected to arcsine square root transformation. Statistical significance was tested using analysis of variance (one-way ANOVA) and Tukey HSD post-hoc test at a 0.05 probability level.

## 7.3. RESULTS AND DISCUSSION

### 7.3.1. GROWTH PERFORMANCE

The overall growth performance of fish fed with the experimental diets over a period of 61 days is presented in Table 7.3. At the end of the growth trial, fish showed a 3-fold increase of their initial body weight. No significant differences were found among the two dietary treatments in terms of final body weight (FBW), daily growth index (DGI), feed conversion ratio (FCR) or protein efficiency ratio (PER) ($p$>0.05). However,

fish fed the RB10 diet showed a significantly reduction of the daily feed intake (FI, $p$=0.018).

**Table 7.3 -** Growth performance and whole-body composition of fish (IBWa=6.0 ± 0.2 g), fed both experimental diets: a control diet (CTRL) and a diet with 10% inclusion of residual microalgal biomass (RB10). Values are means ± standard deviation ($n$ = 3).

| | **CTRL** | **RB10** | ***p*-value** |
|---|---|---|---|
| FBW[b], g | 20.9 ± 0.27 | 20.7 ± 0.54 | 0.60 |
| FI[c], %ABW/d | 2.59 ± 0.03 | 2.50 ± 0.02 | **0.02** |
| DGI[d] | 1.56 ± 0.02 | 1.55 ± 0.04 | 0.54 |
| FCR[e] | 1.42 ± 0.02 | 1.40 ± 0.06 | 0.67 |
| PER[f] | 1.26 ± 0.02 | 1.29 ± 0.06 | 0.45 |
| | | | |
| Whole-body composition (%, wet weight) | | | |
| Moisture | 69.4 ± 0.33 | 68.9 ± 0.34 | 0.18 |
| Ash | 3.01 ± 0.34 | 2.99 ± 0.43 | 0.83 |
| Protein | 15.3 ± 0.57 | 15.2 ± 0.30 | 0.38 |
| Fat | 8.09 ± 2.71 | 8.01 ± 2.67 | 0.77 |
| Phosphorus | 0.64 ± 0.03 | 0.61 ± 0.04 | 0.21 |

[a] *Initial mean body weight.*

[b] *Final mean body weight.*

[c] *Feed intake per day: crude feed intake/Average body weigth/61 days.*

[d] *Daily growth index: ($FBW^{1/3} – IBW^{1/3}$)/61 days) × 100.*

[e] *Feed conversion ratio: wet weight gain/dry feed intake.*

[f] *Protein efficiency ratio: wet weight gain/crude protein intake.*

*Body composition of initial fish (% wet weight): 70.9% Moisture; 3.30% Ash; 15.4% Protein; 8.46% Fat; 0.33% Phosphorus;*

Patterson and Gatlin, (2013) reported the inclusion of RB from *Navicula* sp., *Chlorella* sp. and *Nannochloropsis salina* at the expense of fishmeal and soy protein concentrate in diets for juvenile red drum (*Sciaenops ocellatus*). The authors suggested a safe inclusion level of RB up to 10% of dietary protein without affecting significantly the fish performance, since higher incorporations of RB in the experimental diets led to decreased survival, weight gain and feed intake. Similarly, Ju et al. (2012) tested the replacement of fishmeal by *H. pluvialis* RB on the Pacific whiteleg shrimp *Litopenaeus vannamei*. In this study, it was shown that a dietary inclusion of RB up to 12.5% did not affect the growth parameters as compared to those of the CTRL feed. More recently, it was reported that an inclusion level up to 20% of *Desmodesmus* sp. RB and 10%

*Nannochloropsis oceania* RB in the feeds of Atlantic salmon did not affect the growth performance and health parameters (Kiron et al. 2016; Sørensen et al. 2017). In addition, inclusion levels up to 15% of different whole microalgal biomasses in striped bass did not impact the growth performance (Cruz et al. 2018).

The dietary inclusion of RB at the expense of SBM had no significant effect on the whole-body composition of fish in terms of moisture, protein, fat, ash, phosphorus and energy ($p$>0.05; Table 7.3). Values of whole-body composition are in accordance with those obtained in other experiments with seabream (Lupatsch, 2003; Gómez-Requini et al. 2004; Benedito-Palos et al. 2007; Kokou et al. 2012). This absence of effects of dietary RB inclusion on the whole-body composition of fish has been observed in several other studies (Ju et al. 2012; Patterson and Gatlin, 2013; Sørensen et al. 2017; Valente et al. 2019). Nonetheless, it is interesting to note that a reduction of whole-body fat associated with the dietary inclusion of microalgae, when used as whole biomasses, has been described in Japanese flounder (Kim et al. 2002), common carp (Nandeesha et al. 1998; Kiron et al. 2012), Atlantic salmon (Kiron et al. 2012) and gilthead seabream (Ribeiro et al. 2017). The mechanisms underlying this lipid-lowering effect are not completely understood. Commonly, these algal biomasses contain liposoluble carotenoids (e.g., fucoxanthin in *Phaeodactylum tricornutum*) which have been associated with lower accumulation of abdominal white adipose tissue in rodents, due to a depression of lipogenic enzymes activities and an increase on fatty acid oxidation (Ha and Kim, 2013; Maeda et al. 2015; Peng et al. 2011). Conversely, lipid extraction process applied to the current microalgal biomass of *Tetraselmis* sp. CTP4 may probably result in the removal of these carotenoids and therefore eliminate such effect.

Based on data from feed intake and whole-body composition of fish, nutrient and energy retention (expressed as percentage of intake) were calculated (Figure 7.1). Dietary treatments had no significant effect on the protein, fat, phosphorus and energy retention ($p$>0.05). Similar findings have been reported by Valente et al. (2019) in European seabass fed graded levels of a residual *Nannochloropsis* sp. biomass. Conversely, it was previously reported that the retention of protein and energy was significantly reduced in experimental diets fed to *Sciaenops ocellatus* containing 10% of RB (Patterson and Gatlin, 2013).

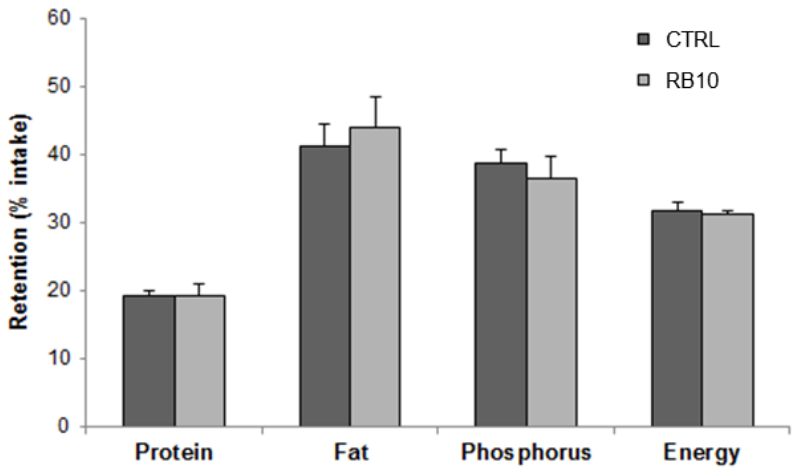


**Figure 7.1 -** Nutrient and energy retention in juvenile gilthead seabream, fed both experimental diets: A control diet (CTRL) and a 10% inclusion of residual microalgal biomass (RB10).

### 7.3.2. DIGESTIBILITY OF TEST INGREDIENT (RB) AND EXPERIMENTAL DIETS

Test ingredient, RB from *Tetraselmis* sp. CTP4, showed apparent digestibility coefficients (ADC) of protein, fat and energy of 87.0, 85.3 and 75.5%, respectively (Figure 7.2). A direct comparison with previously reported ADC values for SBM in *Sparus aurata* (Lupatsch et al. 1997) shows that RB had a similar protein digestibility and a slightly higher energy digestibility (72% in SBM). In gilthead seabream, the RB from *Tetraselmis* sp. CTP4 showed a moderate phosphorus digestibility (41.4%). Data on the phosphorus digestibility of microalgae biomasses is extremely scarce, but this value of

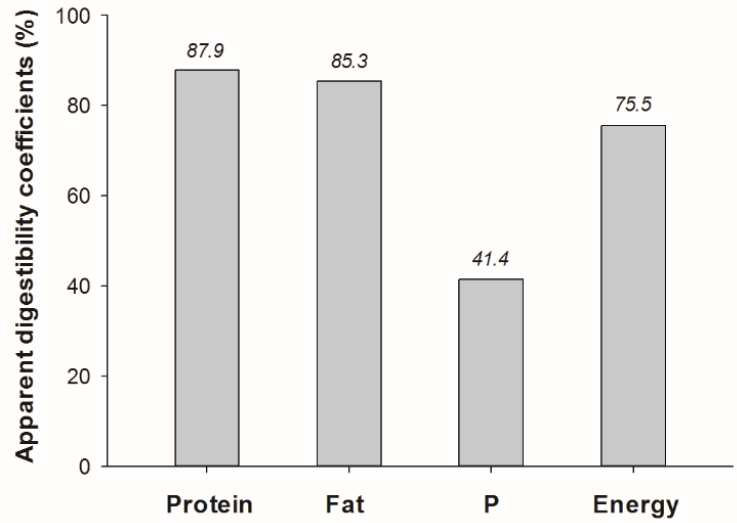


**Figure 7.2 -** Apparent digestibility coefficients of protein, fat, phosphorous and energy of the test ingredient: residual microalgal biomass (RB).

phosphorus ADC in *Tetraselmis* sp. is higher than values previously reported for SBM in European seabass (36.1%), Senegalese sole (27.6%) and rainbow trout (22%; Gomes da Silva and Oliva-Teles, 1998; Kaushik, 2005; Dias et al. 2010). Approximately 70% of the total phosphorus in plant feedstuffs is present in the form of phytic acid (myo-inositol 1,2,3,4,5,6-hexakisphosphate) and is largely indigestible by fish (NRC, 2011). Moreover, phytic acid has a strong binding affinity to other dietary minerals (e.g., calcium, iron, zinc) and proteins, inhibiting their absorption and therefore is generally considered as an antinutritional factor in fish (Kokou and Fountoulaki, 2018). Little information exists on the phosphorus forms present in microalgae (Mukherjee et al. 2015; Feng et al. 2016). Although requiring a thorough evaluation, there are indications that microalgae predominantly store inorganic phosphorus in vacuoles as polyphosphate granules, with variable positions of the phosphate groups on the inositol ring (Feng et al. 2016), and therefore may be more bioavailable for gastric liberation and intestinal absorption than phytic acid. Moreover, microalgae show the potential for tailoring their properties, and Erpel et al. (2016) recently reported the development of a *Chlamydomonas reinhardtii* mutant that expressed phytase activity that could contribute to enhance phosphorus digestibility in monogastric animals.

In the experimental diets (Table 7.4), the ADC of dry matter varied between 69 and 72%, with fish fed diet RB10 presenting a significantly higher digestibility, than those fed the CTRL diet ($p<0.05$). Similarly, the ADC of energy for RB10 (88%) was also significantly higher when compared to that of the CTRL diet (86.5%; $p<0.05$). A similar result was previously reported for *Navicula* sp. RB, where the ADC of energy of the diet containing 10% RB was higher than the reference diet (Patterson and Gatlin, 2013). However, other studies have also shown that the inclusion RB or whole microalgal biomasses tend to reduce energy digestibility in fish (Gong et al. 2016; Sørensen et al. 2017; Teuling et al. 2017; Sarker et al. 2018; Valente et al. 2019). This reduction of energy digestibility in microalgae-rich diets is often associated to an increase of dietary levels of complex carbohydrates, and particularly of non-starch polysaccharides (Teuling et al. 2017; Sarker et al. 2018; Valente et al. 2019). Most of these studies targeted a scenario of replacing fishmeal by microalgal meals that consequently results on an increase of dietary non-starch polysaccharides levels. However, in the present study, we used the *Tetraselmis* sp. RB to replace SBM, which is also a source of non-starch polysaccharides. Protein digestibility was similar in both CTRL and RB10 diets, 94.9 and

95.3%, respectively ($p$>0.05). The ADC of protein obtained in the present work was significantly higher compared to previous reports (80-85%) using RB from *Navicula* sp. in red drum (Patterson and Gatlin, 2013), *Nannochloropsis* sp. and *Desmodesmus* sp. in salmon (Gong et al. 2016) and a blend of *Tisochrysis lutea* and *Tetraselmis suecica* in *Dicentrarchus labrax* (Cardinaletti et al. 2018). Fat digestibility ranged 88.0% in both diets ($p$>0.05). Phosphorus digestibility was significantly enhanced in fish fed the RB10 diet ($p$<005). The exact mechanisms underlying this effect are unknown, but as mentioned before there are indications that, even though microalgae are photosynthetic organisms, they might show an arrangement of phosphate groups around the inositol ring with a higher bioavailability than phytic acid (Feng et al. 2016).

**Table 7.4 -** Apparent digestibility coefficients (ADC) of control (CTRL) and 10% diet inclusion of residual microalgal biomass (RB10). Values are means ± standard deviation ($n$ = 3).

| ADC (%) | CTRL | RB10 | *p*-value |
|---|---|---|---|
| Dry matter | 69.4 ± 0.4 | 72.0 ± 0.3 | **0.001** |
| Protein | 94.9 ± 0.2 | 95.3 ± 0.4 | 0.280 |
| Fat | 87.9 ± 0.5 | 88.0 ± 0.1 | 0.860 |
| Energy | 86.5 ± 0.8 | 88.0 ± 0.2 | **0.036** |
| Phosphorus | 73.8 ± 0.2 | 75.9 ± 0.8 | **0.017** |

The comparative carcass analysis combined with data on the ADC of both diets, allowed the calculation of the nitrogen and phosphorus mass balance (Figure 7.3). Regarding the nitrogen mass balance, values for daily nitrogen gain (428 to 446 mg N/kg ABW/day) were not affected by dietary treatment. On the other hand, a 10% diet inclusion of RB reduced fish total nitrogen losses (faecal and metabolic) when compared to fish fed with the CTRL diet, although only the faecal nitrogen losses were significantly lower ($p$<0.05). These results are linked to a slightly lower nitrogen intake allied with a slightly higher nitrogen digestibility, resulting in a lower faecal loss of fish fed RB10 experimental diet. Regarding phosphorus mass balance, no significant differences were observed on phosphorus gain (115 and 105 mg P/kg ABW/Day, respectively), as well as the metabolic and faecal losses in fish fed with CTRL and RB10 diets. The high faecal losses here obtained can be related with an excess of the total phosphorus supplied in both experimental diets (Kim et al. 1998). Although this mineral is of the outmost importance for fish development (e.g., synthesis of phospholipids and nucleic acids), excess addition

on the diets and consequent losses through faeces can lead to negative impacts in the environment (e.g., eutrophication).

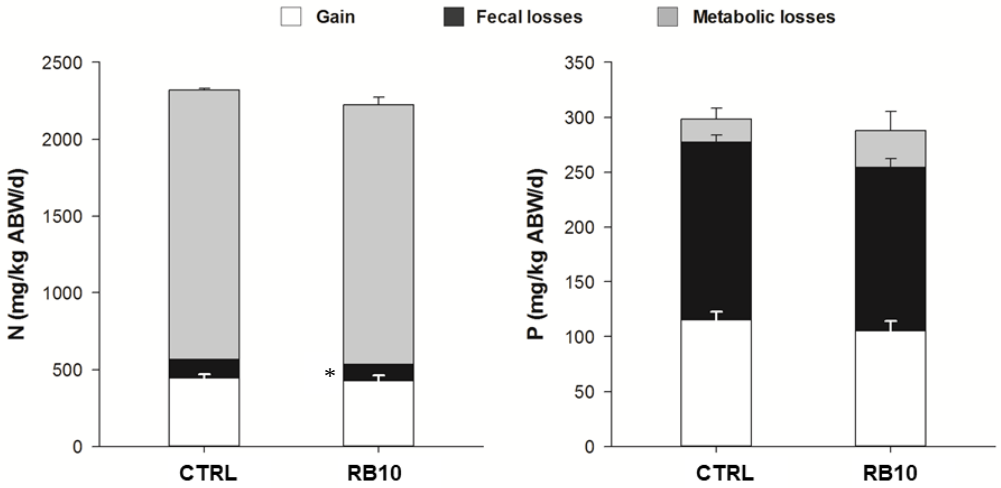


**Figure 7.3 -** Daily nitrogen and phosphorus balance in gilthead seabream fed experimental diets: control diet (CTRL) and a diet with 10% inclusion of residual microalgal biomass (RB10). Bars are means ± standard deviation ($n$ = 3). N/P Gain: (final carcass N/P content − initial carcass N/P content)/ABW/Days. Faecal N/P loss: crude N/P intake (mg/kg ABW/day) × (100−ADC Nitrogen/Phosphorus). Metabolic N/P losses: N/P gain – N/P faecal losses. * represent significant differences.

### 7.3.3. ACUTE CONFINEMENT STRESS

The basal cortisol values and response after an acute confinement stress of fish fed with both diets is presented in Figure 7.4. The basal cortisol values (black bars) of both treatments in the tanks that were not subjected to the acute confinement stress were similar (~10 ng/mL), and the values obtained were within those normally observed for seabream (Arends et al. 1999, Guerreiro et al. 2006). However, after the acute confinement stress, an effective cortisol response with significant differences ($p$<0.05) between both treatments was observed. Fish fed with RB10 displayed a lower cortisol response (120 ± 23 ng/mL) compared to those fed with the control diet (160 ± 33 ng/mL). Nath et al. (2011) also reported a slight decrease in cortisol values of guppy fry (*Poecilia reticulata*) fed with *Parietochloris incisa* compared to the CTRL diet after an acute confinement stress. Plasma cortisol values are normally used in fish physiology to study the effect of stress events, since cortisol is responsible for various physiological processes and is the main stress (corticosteroid) hormone in fish (Wendelaar Bonga and Pang, 1997); therefore, increased levels of plasma cortisol indicates higher physiological stress level. Even though significant differences were observed between both treatments, the analysis of a single hormone is not sufficient to claim a stress-protecting activity.

However, the results here obtained are a preliminary indication that the RB of *Tetraselmis* sp. CTP4 might reduce the stress in juvenile gilthead seabream. In fact, microalgae have previously been reported to contain anti-inflammatory and anti-stress bioactivities that seem to promote the health of aquatic animals (Ju et al. 2012 and references therein).

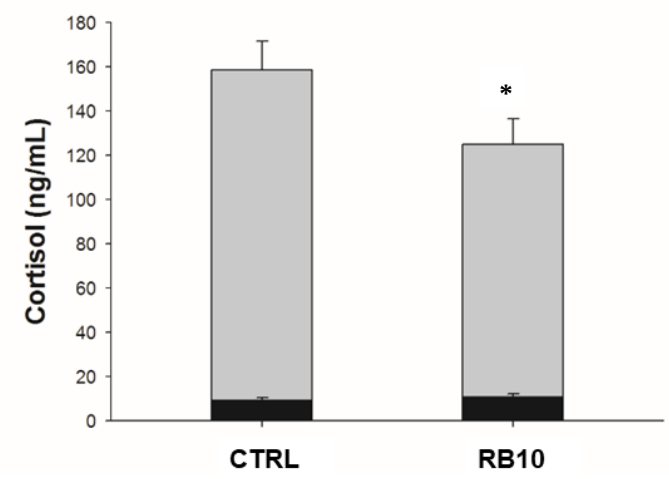


**Figure 7.4 -** Changes in plasma cortisol of seabream exposed to an acute confinement stress, fed with CTRL (control) and RB10 (10% inclusion of residual microalgal biomass) experimental diets. Black bars represent the basal values of cortisol in the plasma, while grey bars represent the cortisol response of stressed fishes.

## 7.4. CONCLUSIONS

In conclusion, the replacement of 10% SBM by RB from *Tetraselmis* sp. CTP4 did not affect overall growth performance, whole-body composition and nutrient retention in gilthead seabream juveniles. In addition, the ADC of protein, fat and energy in RB were also similar to those previously published for SBM. Therefore, if large-scale production of microalgae alongside with usage of edible oils or biofuels becomes a reality, RB can be considered as a promising alternative to complement soybean usage. This will certainly decrease the demand for soybean in the EU market, and also contributing to lower the deforestation rates caused by the need for ever larger land areas for growing such crops.

## ACKNOWLEDGMENTS

The authors would like to thank Prof. Adelino Canário and Elsa Couto (Centre of Marine Sciences) for the kind determination of cortisol. The present work was funded by Sparos, Lda., as well as by the Portuguese national budget and the

CCMAR/Multi/04326/2013 grant of the Foundation for Science and Technology (FCT). H.P. (SFRH/BD/105541/2014) was funded by a PhD grant form FCT.

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# CHAPTER VIII

# GENERAL DISCUSSION, CONCLUSIONS AND FUTURE PERSPECTIVES

## 8.1 GENERAL DISCUSSION

During the last decade, extensive work on microalgal biotechnology has been carried out worldwide, focusing on a wide array of microalgal strains and their exploitation for different biotechnological applications, including complex biorefinery pipelines for the development of different bio-products (Ferreira et al. 2013; Nobre et al. 2013; Ansari et al. 2017). However, a direct comparison between the present work and previously reported data is difficult, due to a wide gap between the culture systems, biotic and abiotic growth conditions, harvesting stage of biomass, and the analytical and processing procedures that have been used in each case (Moody et al. 2014). In addition, species- and strain-dependent responses to a particular condition further limit any sweeping conclusions to be drawn from a given experiment. In this context, the present thesis aimed to improve the knowledge of the potential of a novel, autochthonous microalgal strain, isolated from a sample obtained from Algarve coastal waters to be cultivated under industrial growth conditions. In addition, possible biotechnological applications under a biorefinery setting were explored in order to upgrade the final value of the produced biomass.

Accordingly, a complete process pipeline encompassing strain selection, biomass production in industrial photobioreactors, biomass harvesting using a low-cost settler, biorefining of the biomass and final upgrade to different bio-products was successfully established. Therefore, this chapter aims to allow a better integration of the research conducted throughout the present thesis in order to connect the different subjects addressed. A schematic representation that shows and summarizes the workflow of the research carried out in the scope of this dissertation is shown in Figure 8.1.

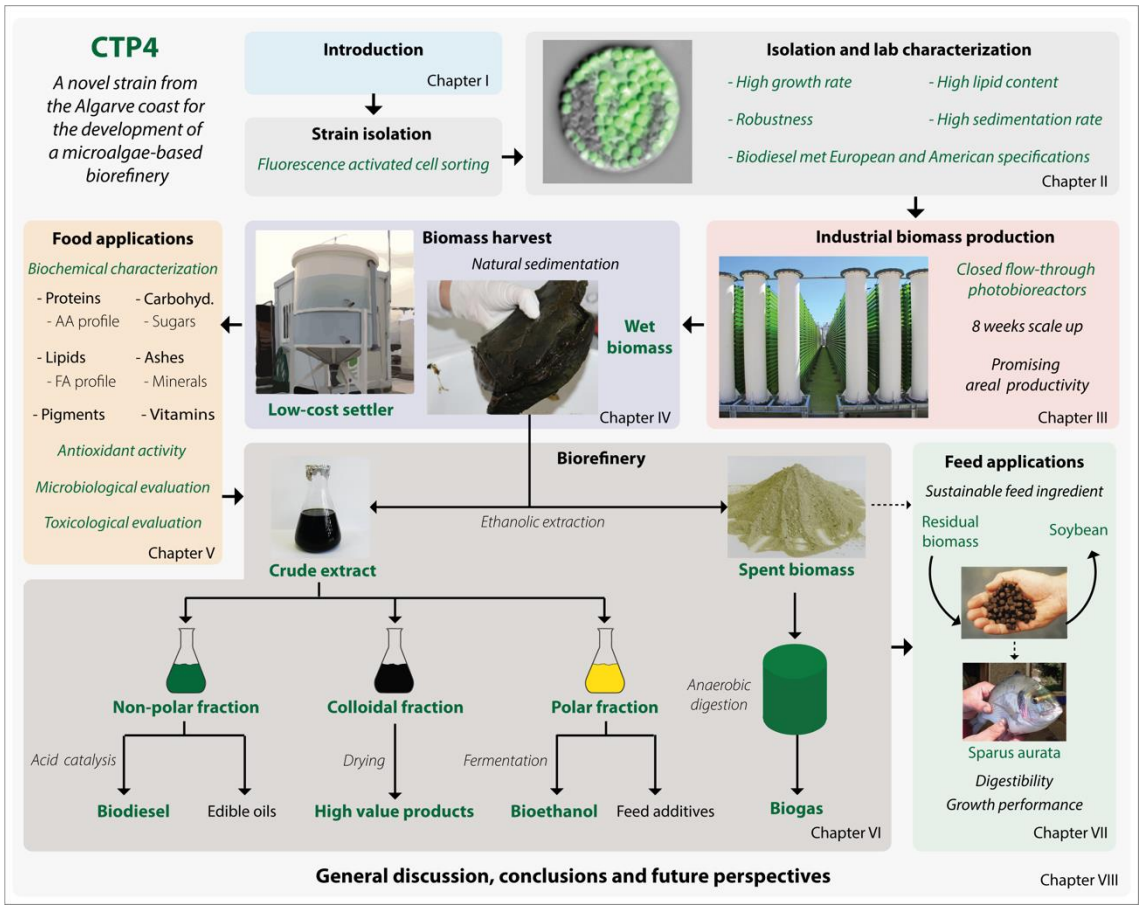


**Figure 8.1 –** Schematic representation of the workflow of the research carried out in the scope of this dissertation. The main activities performed in each of the chapters that compose the dissertation are briefly represented.

### 8.1.1 STRAIN SELECTION AND LAB-SCALE CHARACTERIZATION

Strain selection is one of the most important steps of any microalgae-based process pipeline. As discussed in the first chapter, several works targeted the bioprospection of novel microalgae strains for different biotechnological applications and research purposes (Sinigalliano et al. 2009; Mutanda et al. 2011; Elliott et al. 2012; Neofotis et al. 2015, 2016). Although thousands of strains have already been isolated and characterized under laboratory conditions, to date, only about 20 have consistently been cultivated in industrial facilities (Chu, 2012; Barra et al. 2014). Therefore, it is of the outmost importance to prove that a given strain can be effectively grown in massive culture volumes.

Accordingly, the work performed at the MarBiotech group of the Centre of Marine Sciences (CCMAR) has led to the isolation of 96 novel strains autochthonous from the Algarve coast (Pereira et al. 2011, 2016). This screening effort was achieved

using a high-throughput methodology by means of fluorescence activated cell sorting, by combining a pre-enrichment step to select for fast growing strains, with BODIPY staining to identify potential lipid hyper-producing strains (Pereira et al. 2011). However, establishing a monoalgal or axenic culture does not ensure that a given microalga can be used for industrial applications (Pereira et al. 2016). In this sense, a microalgal strain suitable for biotechnological development must have several features in order to maximize its full biotechnological potential and ensure the production of biomass and desired bioproducts in an effective manner (Wijffels and Barbosa, 2010; Mutanda et al. 2011). Among the strains previously isolated, *Tetraselmis* sp. CTP4 was selected to carry out the workplan of this PhD thesis. The reasons for this selection were identified during the preliminary laboratory characterization performed by the MarBiotech group (Monteiro, 2014; Santos, 2014) and the work described in Chapter II, and are described below.

A key feature for selecting a strain for further biotechnological development is its growth rate (Georgianna and Mayfield, 2012). The selection of fast-growing strains is advisable, since the scale-up to industrial photobioreactors is known to lead to a build-up of contaminants, a common event even in closed production systems (Wang et al. 2013). Therefore, the selection of fast-growing strains is essential to avoid competition with contaminants that can arise in production systems (Wijffells and Barbosa, 2010). In this context, the volumetric productivities obtained with *Tetraselmis* sp. CTP4 (0.25-0.29 g/L/day) at lab-scale seemed highly promising (Pereira et al. 2016), surpassing those observed in the literature for several species (Huerlimann et al. 2010; Fon-Sing and Borowitzka, 2016), except for the values published for *Tetraselmis* sp. F&M33 by Rodolfi et al. (2009).

Another important feature to consider in strain selection is the robustness of the cells to different environmental conditions (Wijffells and Barbosa, 2010; Georgianna and Mayfield, 2012), since outdoor culture conditions are only controllable to a limited extent, and major differences in abiotic and biotic parameters vary widely during the different production seasons (e.g., between winter and summer). In this context, preliminary work carried out at the MarBiotech group revealed that *Tetraselmis* sp. CTP4 can withstand wide environmental conditions (0.1-100 g/L NaCl; 5-40 °C; 100-400 µmol/$m^2$/s; Monteiro, 2014; Santos, 2014; unpublished data). This was a significant indication that this strain could be cultivated outdoors all year round. Moreover, as stated in Chapters III

and IV, the euryhaline behaviour of *Tetraselmis* sp. CTP4 (Pereira et al. 2016; Trovão et al. 2019) is also a crucial advantage for industrial ends. This particularity helps to manage potential contaminations that are sensitive to abrupt salinity shifts and to compensate the salinity changes promoted by natural evaporation, mainly when open systems are considered for industrial biomass production (Fon-Sing and Borowitzka, 2016; Pereira et al. 2018).

Another important evidence for the suitability of *Tetraselmis* sp. CTP4 to industrial applications is linked to the fact that this microalga was able to be cultivated in unsterilized wastewater effluents. Specifically, effluents supplied by a Portuguese company, Águas do Algarve, which is in charge of treating all the urban and industrial effluents of the Algarve region. This strain was able to maintain its dominance over contaminants naturally present in the urban wastewater effluent, showing a growth performance similar to that of the standard laboratory growth medium (Schulze et al. 2017). This led to the conclusion that urban effluents can be used as a viable source of nutrients to cultivate this strain, and that using wastewater can be a promising approach to reducing the costs associated with nutrient input in the production pipeline (Acién et al. 2012).

During laboratory characterization, *Tetraselmis* sp. CTP4 displayed another important feature, related with its ability to accumulate high amounts of lipids under specific culture conditions (Pereira et al. 2016), which is also an important feature in strain selection (Georgianna and Mayfield, 2012). Indeed, over the last few years, microalgae have been viewed as potential oleaginous feedstock for different biotechnological applications, particularly for the production of biodiesel and as a source of edible oils for human and animal nutrition (Wijffels and Barbosa, 2010). However, it is noteworthy that fast-growing strains commonly display low lipid contents, since cells actively dividing usually privilege the accumulation of carbohydrates over lipids as a way to store the energy obtained via photosynthesis (Montero et al. 2011). This storage can be essential for microalgae to survive when light is limiting and photosynthesis is unable to generate enough energy (Napan et al. 2015). Therefore, a two-stage growth system is commonly used to promote the growth of highly concentrated microalgal cultures (1$^{st}$ stage) followed by an induction step (2$^{nd}$ stage) upon which the accumulation of lipids in the cells is stimulated via, for example, nutrient starvation (Rodolfi et al. 2009; Campenni' et al. 2013; Sun et al. 2018). A similar approach was used in this dissertation

leading to a lipid accumulation of about 30% of the cell dry weight (DW), which is a promising value for industrial ends (Pereira et al. 2016).

Finally, as biomass harvesting is known to be one of the most important steps that significantly impact the costs of the whole microalgal production pipeline (Barros et al. 2015), the high sedimentation rate observed in Imhoff settling cones for *Tetraselmis* sp. CTP4 was also considered as a promising feature for selecting this strain for industrial exploitation (further discussed below in section 8.1.3).

### 8.1.2 INDUSTRIAL PRODUCTION OF BIOMASS

After the laboratory characterization, *Tetraselmis* sp. CTP4 was investigated for its potential as an industrial feedstock of microalgal biomass. The industrial biomass production trials were carried out in Algafarm, a large-scale production facility that belongs to the Portuguese company Cimentos Maceira e Pataias (CMP), which is part of the Secil group. The production facility of Algafarm is the largest unit operating in Europe, with photoautotrophic and heterotrophic production capacities of 1350 $m^3$ and 10 $m^3$, respectively (www.allmicroalgae.com).

Accordingly, the work carried out in the third chapter of this dissertation describes not only the biomass production performance, but also the $CO_2$ sequestration potential of *Tetraselmis* sp. CTP4 in an industrial setting using closed flow-through tubular photobioreactors. One important information gathered at this stage is that culture scale-up from an agar plate to the 100-$m^3$ photobioreactors takes eight weeks (Pereira et al. 2018).

While the scale-up process was undergoing, the operation in pilot-scale tubular flow-through tubular photobioreactors was successfully optimized, namely the optimum pH for $CO_2$ injection and culture flow rate. In this context, the optimum pH set point for $CO_2$ injection of 8.0 gave significantly better growth results compared to 7.0 and 7.5. Regarding the flow rate, reducing the pump speed to half (0.5 m/s) of the standard value (1 m/s) was an important finding, since it allowed us to reduce the energetic costs associated with mixing the cultures in these production systems (Pereira et al. 2018).

When the scale-up reached the production photobioreactors, the biomass production and processing pipeline of *Tetraselmis* sp. CTP4 encompassed three important steps, namely biomass production in tubular photobioreactors, harvesting by membrane ultrafiltration and drying using an industrial spray drier (Figure 8.2). During the 60 days of operation, the areal and volumetric biomass productivities obtained were considered as highly promising, because of the scale of the photobioreactors used as well as the history of biomass productivities previously registered for other microalgal strains in Algafarm. In addition, it should be pointed out that the trial was performed in the autumn-winter season, and lower productivities were expected. However, as previously stated, it is hard to compare the biomass productivities obtained in the 35- and 100-$m^3$ tubular photobioreactors with other works reported in the literature. To date, there is a significant gap of knowledge in the effective biomass productivities of most strains in industrial scale production systems. The main reason for this gap, relies in the different biomass productivities obtained at lab-scale and even outdoor at pilot scale that cannot be compared with those obtained at industrial-scale facilities, where culture volumes are several folds higher and the light path (and thus light availability) and mass transfer phenomena are markedly different.

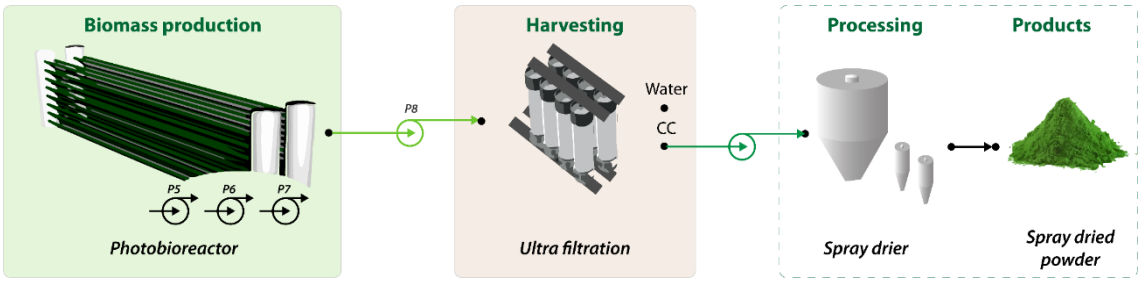


**Figure 8.2 -** Biomass production pipeline used at the facilities of Algafarm for the production of *Tetraselmis* sp. CTP4 biomass. The production was carried out in tubular photobioreactors and the resulting biomass was harvested using a membrane filtration system. Finally, the concentrated culture obtained after ultrafiltration was spray dried to obtain the final dried biomass product.

Regarding $CO_2$ sequestration potential, biomass production capacity is tightly related with $CO_2$ fixation and thus removal of this greenhouse gas from the growing system. In the course of this dissertation, it was possible to establish that around 1.82 Kg of $CO_2$ were sequestered per Kg of *Tetraselmis* sp. CTP4 biomass produced (Pereira et al. 2018), which is in accordance with the literature (Chisti, 2007). However, as the organic carbon present in the supernatant was not analysed, the actual $CO_2$ sequestration capacity estimated for *Tetraselmis* sp. CTP4 is probably underestimated. This fact was recently highlighted by Quelhas et al. (2019), who showed a higher $CO_2$ sequestration

using *Phaeodactylum tricornutum* when the total organic carbon of the supernatant was analysed and included in the stochiometric equation. Nevertheless, the high biomass production performance of *Tetraselmis* sp. CTP4 shows that this strain holds a high potential for industrial capture of $CO_2$.

### 8.1.3 BIOMASS HARVESTING

Upon the production of biomass at industrial scale, the following step of this dissertation focused on the establishment of a harvesting procedure. The reason for such effort was to reduce one of the most energy demanding and costliest steps of the whole processing pipeline (Chen et al. 2011; Barros et al. 2015; Acién et al. 2016; Show et al. 2017). Accordingly, a low-cost pilot scale system to harvest the cultures of *Tetraselmis* sp. CTP4 was implemented, based on its natural sedimentation capacity, as demonstrated in the second chapter.

At lab-scale, after the preliminary settling trials in Imhoff cones, we hypothesized that salinity would have a significant effect on the settling properties of the strain, since the medium osmolarity is known to affect the buoyancy of microalgal cells and the zeta potential of cell membranes (Church et al. 2017; Wen et al. 2017). Therefore, a preliminary trial was carried out using cultures grown at 5, 10 and 20 g/L of NaCl, which were later allowed to naturally sediment. The settling velocity was later calculated in measuring cylinders (Trovão et al. 2019). Interestingly, cultures cultivated at different salinities showed similar growth performance as well as settling velocities. These results led to the conclusion that the settling velocity of *Tetraselmis* sp. CTP4 was not affected under the range of salinities tested.

The pilot-scale harvesting system developed was achieved by adapting a cylinder-conical tank with two lateral openings in the side of the tank and a squeegee assembled in the conical section of the tank. The trials using *Tetraselmis* sp. CTP4 cultures at a concentration of 2 g/L DW revealed that a 24-hour sedimentation step is sufficient for recovering 96% of the culture medium by means of gravity drainage alone (Trovão et al. 2019). The remaining biomass was recovered in the form of a concentrated culture and wet microalgal paste with high biomass concentration. Overall, the harvesting system established in the course of this dissertation proved to be very effective, enabling a significant reduction of the harvesting costs, by recovering the biomass from the culture medium without the use of additional energy inputs.

### 8.1.4 BIOCHEMICAL PROFILE OF BIOMASS

Following the optimization of biomass production and an effective harvesting system, in order to explore the biotechnological potential of *Tetraselmis* sp. CTP4, the biomass produced at industrial scale was characterized and its potential nutritional applications were assessed (as described in chapter V). The composition of macronutrients, minerals and high value metabolites and a complete biochemical characterization is essential to unravel the nutritional value of a food or feed matrix (Brown, 2002). Accordingly, the industrially produced microalgal biomass of *Tetraselmis* sp. CTP4 can be considered to be a good source of proteins and dietary fibres, which accounted for more than 30% of the biomass DW. Conversely, lower contents of digestible carbohydrates and lipids were present. However, the former fractions contained starch-like polysaccharides and *n*-3 polyunsaturated fatty acids (PUFA), respectively, which are important molecules for nutritional purposes. Regarding the presence of high value metabolites, relevant levels of chlorophyll, carotenoids and vitamins were detected, as well as antioxidant activity in different *in vitro* models.

The nutritional data was further supported by a microbiological and toxicological evaluation performed in the industrially produced biomass by analysing several contaminants commonly found in food and feed products. This evaluation is of the outmost importance to ensure its safety for nutritional ends (Becker, 2004). The microbiological evaluation revealed that seven common pathogenic bacteria were absent from the biomass produced, whereas the total counts of bacteria, yeasts and moulds were within the values often observed in other food and feed meals. Regarding the toxicological evaluation, with the exception of a residual amount of cadmium (within lawful limits), no toxic metals were found in the biomass produced. Similarly, all the remaining contaminants analysed, including several cyanotoxins, mycotoxins, polycyclic aromatic hydrocarbons and pesticides were not detected in the biomass.

A comparison of the macronutritional composition of *Tetraselmis* sp. CTP4 with traditional, emerging and microalgal feedstocks is shown in Figure 8.3. As can be seen in this figure, *Tetraselmis* sp. CTP4 displays a composition similar to that of vegetables and emerging feedstocks (insects), with higher carbohydrate contents and lower amount of lipids (Ssepuuya et al. 2017; Caligiani et al. 2018). Dietary fibres were a major component of the carbohydrates detected in the biomass. Compared with *Chlorella* and “Spirulina” (*Arthrospira platensis*), two microalgae approved for human consumption, *Tetraselmis*

sp. CTP4 had a lower protein content, most probably explained by its higher carbohydrate content, which is a common pattern when freshwater and marine species are compared. Nevertheless, it is worth mentioning that currently *Tetraselmis* sp. CTP4 biomass cannot be used for food applications, since the species (*Tetraselmis striata/convolutae*) has not been approved yet by the European Food Safety Agency (EFSA) for human consumption. As previously stated in chapter V, in order to commercialize *Tetraselmis* sp. CTP4 for human nutrition, a novel food process must be envisaged, which is a costly procedure. Recently, Fitoplancton Marino (Spain) was able to get a novel food dossier approved for *Tetraselmis chui* (Fitoplancton Marino, 2014), which is a good indicator that the strains of *Tetraselmis* genus hold a high potential for human consumption. Overall, the industrially produced biomass of *Tetraselmis* sp. CTP4 displayed an interesting nutritional value and was free from contaminants and can be therefore considered to be a suitable for human and animal consumption.

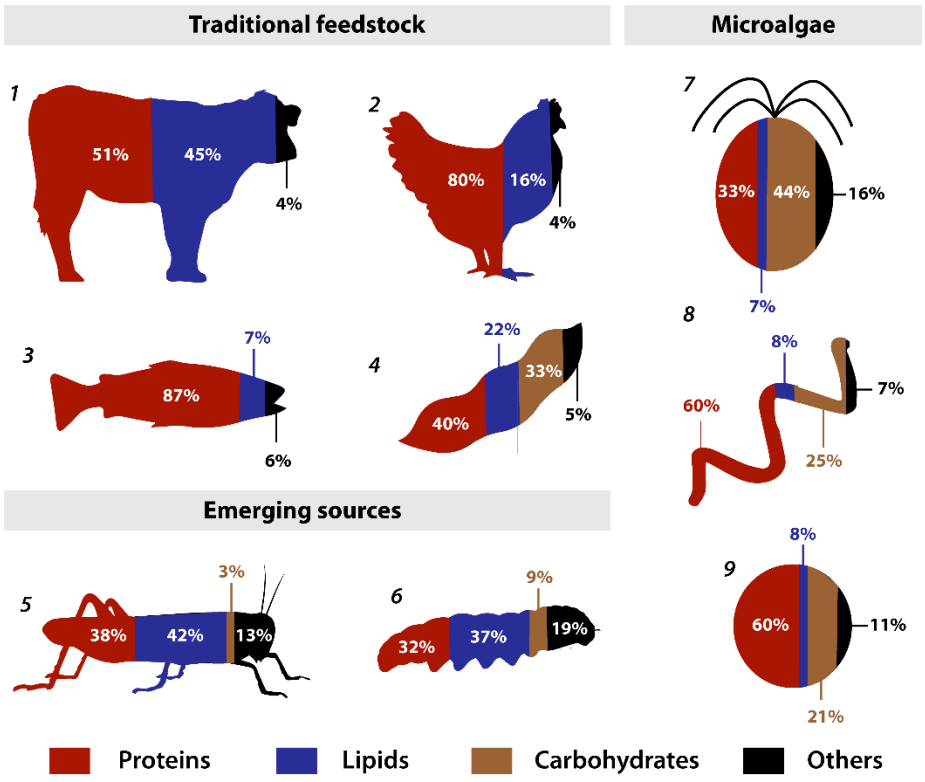


**Figure 8.3 -** Illustrative comparison of the macronutritional composition for human and animal nutrition of traditional (1-4), emerging (5,6) and microalgal feedstocks (7-9). 1: Beef (Food and Agriculture Organization); 2: Chicken (Food and Agriculture Organization); 3: Fish (Sea bream; Food and Agriculture Organization); 4: Soybean (Seeds raw; United States Department of Agriculture); 5: Grasshoper – *Ruspolia nitidula* (Ssepuuya et al. 2017); 6: Black soldier fly prepupae (Caligiani et al. 2018); 7: *Tetraselmis* sp. CTP4 (Present work); 8: *Spirulina* (Dried; United States Department of Agriculture); 9: *Chlorella vulgaris* (Allma product sheet)

### 8.1.5 BIOREFINERY INTO DIFFERENT BIOPRODUCTS

In the final part of the thesis, a biorefinery approach for *Tetraselmis* sp. CTP4 biomass was effectively implemented. The biorefinery strategy was conducted as a way to improve the revenue of the whole pipeline, by fractionating the wet biomass into different bulk and specialty bioproducts that can be later upgraded for varied biotechnological applications (Vanthoor-Koopmans et al. 2013: Ansari et al. 2017; Chew et al. 2017; ´t Lam et al. 2018). Therefore, a novel liquid-liquid triphasic system (LTPS) was developed and optimized in the course of this dissertation to fractionate different streams from wet microalgal biomass, as demonstrated in chapter VI.

Accordingly, at a first stage, an ethanolic extraction was carried out directly from the wet microalgal paste, leading to a crude ethanolic extract and the residual biomass leftover. The extraction procedure was performed with ethanol, because it is a highly available food grade solvent that effectively extracts the lipids from wet microalgal biomass (Yang et al. 2014). In addition, downstream processing using the wet route avoids the energetic costs associated with biomass drying (Xu et al. 2011). Thereafter, the LTPS process was applied, by using a solvent system based in hexane and water that after a vigorous mixing, generates a stable emulsion. The emulsion is generated by the presence of amphiphilic molecules (e.g., phospholipids) in the microalgal extract, which after centrifugation forms a solid colloidal fraction between the hexane and water layers. By applying the LPTS process, the crude ethanolic extract was fractionated into three different streams, namely: i) a non-polar hexane phase (NP), a colloidal phase (CP) and a water phase (WP).

The NP obtained from the LTPS process was characterized by thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC), revealing high contents of triacyclglycerols (TAG) as well as free fatty acids, and carotenoids at lower amounts. Subsequently, this fraction was upgraded to biodiesel by acid catalysed transesterification and the fatty acid methyl esters (FAME) profile was characterized by gas chromatography–mass spectrometry (GC-MS). Obtained results revealed that the biodiesel was mainly composed of saturated (SFA) and monounsaturated (MUFA) fatty acids, namely, palmitic and oleic acids. On the other hand, PUFA were detected at low amount, which is a good indicator of biodiesel quality, mainly of its oxidation stability (Knothe, 2011; Gangadhar et al. 2016). The FAME profile of CP was similar to that obtained in the preliminary characterization performed in chapter II, where the fuel

properties were assessed in collaboration with the National Laboratory of Energy and Geology (LNEG). Overall, produced biodiesel complied with most of the specifications legislated in the European (EN14214) and American (ASTM D6751) standards, without any blending or inclusion of additives (Pereira et al. 2016). Nevertheless, because of the low market value of biodiesel and the high production costs of the microalgal biomass, the NP should be used for higher value ends. Two important sectors where this fraction can have wide applicability are the food and feed markets, since the NP contains added value metabolites, such as carotenoids and *n*-3 PUFA that are in high demand for nutritional purposes.

The CP generated by the LTPS process, was also characterized as a source of added value compounds. Resorting to TLC, it was observed that the CP fraction was composed of phospholipids and carotenoids, and free fatty acids at a lesser extent. Subsequently, the profile of carotenoids was evaluated by HPLC, revealing an interesting content of xanthophylls, namely, neoxanthin, violoxanthin, lutein and zeaxanthin, while *β*-carotene was found in trace amounts. Interestingly, xanthophylls were more concentrated in the CP, probably due to the polar hydroxyl groups that confers a more amphiphilic nature to these molecules compared to carotenes. Because of the presence of xanthophylls, the antioxidant activity of the CP was evaluated using different *in vitro* models. Obtained results revealed that the CP displayed radical scavenging activity towards the DPPH (1,1-diphenyl-2-picrylhydrazyl) and ABTS (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) radicals as well as iron and copper metal chelating activities. Overall, the CP seems an interesting stream of added value molecules, containing phospholipids and xanthophylls as well as an antioxidant capacity that can be of interest for the nutraceutical and cosmeceutical industries.

The WP was mainly composed of starch like polysaccharides, since these are the main sugars present in *Tetraselmis* sp. CTP4 biomass, as previously identified in chapter V. Hence, this fraction was upgraded into bioethanol by means of yeast fermentation using *Saccharomyces cerevisiae* F13A. To this end, enzymatic saccharification (amylase and glucoamylase) was used to convert the polysaccharides contained in the WP into simple sugars. The enzymatic hydrolysis led to a high content of glucose in the WP broth (64% of total carbohydrates), and an initial concentration of 43.5 g/L was used to start the trial. Yeast fermentation was carried out for 36 hours. In the course of the experiment, glucose was fully consumed after 24 hours and an ethanol concentration of 21.3 g/L was

obtained at the end. In relation to the theoretical ethanol yield (based on the amount of glucose in the broth), a final ethanol yield of 89.1% was achieved in this experiment. Overall, a yield of 0.46 g ethanol/g fermentable sugar was obtained from the hydrolysed WP, showing that this fraction can be effectively converted to bioethanol. However, it is worth mentioning that the starch like polysaccharides are interesting also from a nutritional point of view and that the WP might hold potential for nutritional applications. Therefore, in order to improve the market value of this fraction, further studies must be conducted to evaluate the potential of this stream as a source of nutraceuticals and feed additives.

Regarding the residual biomass, two different biotechnological applications were evaluated in the course of this dissertation: upgrade for biogas production and the production of a sustainable ingredient for aquafeed. In a first trial, the residual biomass was upgraded into biogas, by means of anaerobic digestion, using an inoculum obtained from an anaerobic digester of Águas do Algarve (Lagos). Following the biorefinery concept, the biodiesel produced from the NP generates glycerol as a by-product; thus, an additional treatment with the residual biomass supplemented with glycerol was performed. This otherwise waste product is highly degradable by anaerobic digestion and was previously proposed as a way to improve methane yield (Ehimen et al. 2009). The digestion of the residual biomass was carried out for 36 days, and a 2-fold increase of biogas production was obtained in the treatment supplemented with glycerol. Interestingly, the average concentration of methane was similar in both treatments (~50%). Overall, a methane yield of 60 and 83% was obtained, for the residual biomass with and without glycerol supplementation, respectively. As previously mentioned in chapter VI, considering a biorefinery approach, the $CO_2$ from the biogas and the digestate obtained after the anaerobic digestion, can be a viable source of concentrated $CO_2$ (Zhao et al. 2013, 2015) and nutrients for microalgae cultivation (Zuliani et al. 2016), respectively. This strategy might overcome the limitations related with site placement and transport costs of two key inputs essential for industrial cultivation of biomass (Chisti, 2007, 2013).

A second trial was conducted in collaboration with Sparos Lda., to assess the potential of the residual biomass for aquafeeds. Compared to biofuels, feed products have higher market value and are a more interesting venue from a commercial point of view. In addition, there is an urgent need to find novel feedstocks that can replace the current

high demand for unsustainable ingredients in the feed sector, namely soybean and fish meals. At this stage, following the advice of the company, and since the biomass had been defatted the trial focused on using the residual biomass of *Tetraselmis* sp. CTP4 to replace soybean meal. The experimental trial was conducted in juvenile seabream (*Sparus aurata*), which is one of the most important species in Mediterranean aquaculture (Montero et al. 1999). Overall, the microalgal residual biomass-containing diet displayed similar results to those of the soybean-meal containing diet (control) concerning most of the zootechnical parameters analysed. The voluntary feed intake was, however, better (lower) in fish fed with the microalgal diet. In addition, fish fed with the diet containing the residual biomass of *Tetraselmis* sp. CTP4 showed higher apparent digestibility coefficients (ADC) of dry matter, phosphorus and energy as compared to those fed with the control diet. Regarding the ADC per ingredient, a high ADC for proteins and energy was observed, while a mild digestibility of phosphorus was observed. At the end of the trial, an acute confinement stress was performed, in which the fish fed with the residual biomass displayed lower cortisol production than those fed with soybean meal. This result suggested that some compounds present in the residual biomass of *Tetraselmis* sp. CTP4 might have a stress protecting effect.

## 8.2 CONCLUSIONS

In conclusion, a successful microalgal production pipeline, encompassing strain selection, industrial biomass production, pilot-scale biomass harvesting and biomass biorefining for different bioproducts was established in the course of this dissertation. In addition, the features that led to the selection of *Tetraselmis* sp. CTP4 in the beginning of the work here described proved to be highly suited throughout the remaining work that was carried out. The high growth rate and robustness detected in the early experiments performed at lab-scale were confirmed posteriorly at industrial scale. Furthermore, different biotechnological applications were assessed and effectively exploited revealing great potential of this strain in several industrial sectors.

It is my deep conviction that although several constrains need to be overcome in the near future, microalgae will definitely play a key role in several industrial biotechnological applications:

- The use of microalgae for $CO_2$ sequestration is far from being the solution to reduce the emission of this greenhouse gas; however, this will always be a positive feature when the mass production of microalgae is implemented for other biotechnological applications;
- The usage of microalgae to treat several wastewater effluents at industrial scale is already ongoing (e.g., Aqualia) and the benefits associated in closing the loop of nutrients, particularly phosphorus, are deemed to make microalgae as probably the best solution for water treatment processes;
- The production of microalgae for high value markets, including the nutraceutical, pharmaceutical and cosmeceutical sectors, is already in place, and the current research efforts will increase the number of microalgal products in these markets in the upcoming years;
- Finally, microalgae will definitely play a significant role in the supply of essential metabolites for both human and animal nutritional applications in the forthcoming years. One key example is the recent joint collaboration of DSM Life's® and Evonik, Veramaris™, which aims to ensure 15% of the eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids annual needs of the salmon aquaculture industry.

To summarize the conclusions section, the following biological questions posed in section 1.6 of this dissertation can now be accordingly answered:

- **Is *Tetraselmis* sp. CTP4 a viable feedstock for industrial production of microalgal biomass?**

Yes. The work conducted showed that *Tetraselmis* sp. CTP4 couples a high growth rate with a high robustness to a wide range of environmental conditions, ensuring that the production of biomass in industrial production facilities is not only possible, but also effective. During the course of this work, the strain was successfully cultivated in the winter season, where cultures are expected to perform worst, with promising volumetric and areal productivities.

- **Can the biomass be harvested by natural sedimentation at pilot-scale?**

Yes. The assays conducted at lab-scale were a good indicator that the high density of *Tetraselmis* ap. CTP4 cells enabled the development of a harvesting procedure that relied on natural sedimentation alone. At pilot-scale, the results were confirmed, and the cylinder conical tank adapted to harvest *Tetraselmis* sp. CTP4 cultures, performed well by efficiently separating the biomass from the culture medium via gravity drainage upon a 24-hour sedimentation step. The biomass was later successfully retrieved from the system, showing that the cultures of *Tetraselmis* sp. CTP4 can be easily harvested by natural sedimentation, significantly reducing the costs associated with this energy depending step of the whole microalgae production pipeline.

- **What is the biochemical composition and high-value secondary metabolites of industrially produced biomass?**

The biochemical composition of industrially produced biomass showed that *Tetraselmis* sp. CTP4 is rich in proteins and dietary fibres, while digestible carbohydrates and lipids are present at lower amounts. The amino acid profile displayed significant amounts of essential amino acids, namely leucine, isoleucine, valine, lysine, threonine and phenylalanine. The carbohydrate fraction was mostly composed of starch-like polysaccharides, while the fatty acid profile was dominated by palmitoleic, oleic, linoleic and $\alpha$-linolenic acids. The mineral composition of the biomass was rich in potassium, magnesium, calcium, sodium and phosphorus. The biomass was also rich in chlorophyll and other high value pigments, including violaxanthin, neoxanthin and lutein. Finally, several vitamins, namely, ascorbic acid, tocopherol and niacin were detected at relevant amounts. Taking these results together, *Tetraselmis* sp. CTP4 shows a promising nutritional value for food and feed applications.

- **What are the potential biotechnological applications of *Tetraselmis* sp. CTP4?**

The work carried out revealed that *Tetraselmis* sp. CTP4 can be used for several biotechnological applications. At lab-scale, the strain proved to be effective for the treatment of wastewater effluents, being able to thrive in unsterilized wastewater, outcompeting any contaminant naturally present in the effluent. Still at lab-scale, the fatty

acid profile of the strain proved to be highly suitable for the production of biodiesel, meeting most of the quality parameters regarded in the European and American legislation. At industrial scale, the high growth rate and robustness of this microalga led to the production of biomass in large-scale photobioreactors, which demonstrated the effectiveness of this strain for biomass production and $CO_2$ capture applications. The biochemical characterization of the biomass produced at industrial scale revealed that this strain is highly suitable for both human and animal nutrition as well as for nutraceutical applications. Finally, considering a biorefinery approach, using the LTPS process, the different streams obtained can be used for high value, nutritional and biofuel applications.

- **Can we establish an effective biorefinery pipeline for the production of different bioproducts from *Tetraselmis* sp. CTP4?**

Yes. In the course of the present thesis an effective biorefinery pipeline was established that led to the production of different bioproducts from the original biomass. Resorting to an ethanolic extraction, a crude ethanolic extract and the residual biomass that was left after the extraction procedure were obtained. The simple protocol used, relied in the extraction directly from the wet biomass, as a way to reduce the energetic costs associated with biomass drying. Thereafter, the LTPS approach enabled the fractionation of the crude ethanolic extract into three streams: NP, CP and WP. All fractions were later upgraded into different bioproducts with wide biotechnological applicability. The NP and WP were successfully converted into biodiesel and bioethanol, respectively. The CP was characterized as source of high value molecules for the development of added value products. Finally, the residual biomass was upgraded to biogas for the production of methane and successfully introduced in an experimental diet to rear juvenile seabream.

- **Can residual microalgal biomass of *Tetraselmis* sp. CTP4 replace soybean meal in the aquafeeds of juvenile gilthead seabream?**

Yes. The results obtained in chapter VII showed that the residual biomass of *Tetraselmis* sp. CTP4 after an ethanolic extraction can effectively replace soybean meal in the diets of juvenile seabream. Apart from the beneficial aspect of using a more sustainable feedstock, the residual biomass of *Tetraselmis* sp. CTP4 showed other advantages, such as, a lower voluntary feed intake and reduced losses in the assimilation

of phosphorus. In addition, after an acute confinement stress trial, fish fed with the experimental diet containing residual biomass revealed lower cortisol levels when compared to those fed with soybean meal.

## 8.3 FUTURE PERSPECTIVES

Looking ahead, several activities could be envisaged to further unravel and fully exploit the biotechnological potential of *Tetraselmis* sp. CTP4 biomass. Further research on assessing the life cycle analysis (LCA) of the whole biomass production and biorefining pipeline established in this work is of the outmost importance. An effective LCA will reveal if the energy and economical balances are positive and evaluate the feasibility of the whole process. In addition, the LCA can reveal the best pathways of the biorefinery process, allowing the identification of the steps that must be improved in regard to their energy consumption, and ultimately elect the most promising biorefinery approach and bioproducts that must be upgraded from an economic point of view.

Regarding the industrial production of biomass, the strain should be tested in open production systems, in order to improve the overall economics of the production pipeline, since higher production costs are obtained when flat panel and tubular photobioreactors are used. It is worth noting that, although this strain was never grown in raceways, the high sedimentation properties of *Tetraselmis* sp. CTP4 might be a problem that can hinder the production in flat open systems with large areas. Therefore, thin layer cascades might be more appropriate if an open production system is chosen for industrial production as a way to decrease the current production costs.

Although the pilot-scale harvesting procedure proved the concept of recovering both the culture medium and the produced biomass using a low-cost approach, increasing the scale of this procedure is a crucial step that should be undertaken. In order to reach this goal, the current technology used for wastewater seems a promising route to test the procedure at industrial scale.

Considering the biochemical characterization of industrially produced biomass, the characterization of the phospholipids classes and fatty acid distribution should be performed, since these compounds have wide applicability in the nutritional sector. In addition, the profile of phytosterols should also be evaluated, because of the known biological activities and emerging market for these high value molecules in the field of

nutraceuticals. The determination of biological activities was only performed to a limited extent in the scope of the present dissertation. Therefore, further work should also be achieved in assessing the biological activities of *Tetraselmis* sp. CTP4, as for example the anti-diabetic, anti-inflammatory and anti-parasitic activities. The chemical characterization of the compounds responsible for the different biological activities is also a field worth exploring, since the *Tetraselmis* genus is known to display different bioactivities that are relevant for different biotechnological applications.

Future work should also contemplate exploiting the effects of the residual biomass in other commercial species of fish commonly used in the aquaculture sector. The stress protecting effect obtained in the acute confinement assay should also be pursued, in order to identify the compound(s) responsible for this effect. Although cortisol is the main stress hormone in fish, other stress related hormones should also be evaluated following the same experimental approach, to fully confirm the stress protecting effect of the residual biomass of *Tetraselmis* sp. CTP4.

Finally, characterizing the potential of this strain for the production of biofertilizers, biostimulants and biopesticides should also be addressed, since this is an emerging field with high potential and market volume. Since most green microalgae are showing high potential for the development of different products for agriculture, the applicability of *Tetraselmis* sp. CTP4 biomass to these emerging opportunities should also be investigated.

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