



Nádia S. Guerra Correia

# Characterization and analysis of the biotechnological potential of a *Chlorococcum* strain isolated at the microalgae production unit — ALGAFARM

Master Dissertation in Biodiversity and Plant Biotechnology, supervised by Professor Doutor Leonel Pereira and Doutora Joana Laranjeira da Silva, presented at the Department of Life Sciences of the Faculty of Sciences and Technology of the University of Coimbra

June of 2018



UNIVERSIDADE DE COIMBRA

Front cover: *Chlorococcum* strain isolated at ALGAFARM facilities. Light microscope photograph by Nádia S. Guerra Correia.



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UNIDADE DE PRODUÇÃO DE MICROALGAS - PATAIAS



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Supervisor: Professor Doutor Leonel Pereira  
Co-Supervisor: Doutora Joana Laranjeira da Silva

Coimbra, June of 2018



*“Science is not only a disciple of reason but, also, one of romance and passion.”*

**Stephen Hawking**



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## Abstract

Currently, global warming, caused by the increase of greenhouse gases in the atmosphere, it is a serious concern among scientist. Beyond the direct atmospheric changes, the agricultural productions are also being affected and that are expected reductions in food production.

The increasing world's population and expanding economy leads to increasing demand on alternative sources of food as well as environmental friendly sources of biofuels.

Microalgae can be used to enhance the nutritional value of food and as supplement of conventional animal feed, positively affecting the health and growth of humans and animals.

Beyond, microalgae can efficiently use CO<sub>2</sub>, being responsible for more than 40 % of global carbon fixation. In this context, microalgal cultures can be considered one of the main biological CO<sub>2</sub> fixation process for the mitigation of CO<sub>2</sub> levels at the atmosphere and, consequently, the global warming.

As the work was developed at ALGAFARM (microalgae production company), the isolated strain was evaluated according to the needs for being suitable for the company. Medium with 10 mM of nitrate and 1 mM of nitrate were tested. For high biomass concentration and improved biochemical composition for food and feed, the 10 mM of nitrate medium is more suitable.

The strain of *Chlorococcum* was evaluated in scale-up reactors until industrial scale 10000 L PBR. The best volumetric productivities, maximum and global, were obtained in the pilot scale 2500 L PBR with 0.153 g.L<sup>-1</sup>.day<sup>-1</sup> and 0.098 g.L<sup>-1</sup>.day<sup>-1</sup>, respectively. However, the best areal productivities, maximum and global, were reached in industrial scale 10000 L PBR with 36.559 g.m<sup>-2</sup>.day<sup>-1</sup> and 17.419 g.m<sup>-2</sup>.day<sup>-1</sup>, respectively.

*Chlorococcum* better biochemical composition was obtained in the pilot scale 2500 L PBR with 55.72 % of protein content, 18.35 % of total lipids content (where 74.70 % are PUFAs), 17.71 % of carbohydrates and 7.61 % of ashes and reaching values of 4.02 % of total chlorophyll and 1.09 % of total carotenoids content (where 71.01 % is lutein and 16.57 % is β-carotene).

*Chlorococcum* strain isolated in the ALGAFARM facilities showed to have a great potential for feed and food applications since it reached high values of proteins content and it also contain relevant amount of polyunsaturated fatty acids.

This strain, without growth optimization, easily reached high values of total chlorophyll, which can be used as natural food colorant. It is also suitable for application in nutraceutical and pharmaceutical industries since showed a total carotenoids content of more than 1 % of biomass dry weight, mainly constituted by lutein (5.374 mg.g<sup>-1</sup>) and β-carotene (1.247 mg.g<sup>-1</sup>).

Despite of *Chlorococcum* sp. was reported in the literature to have the capacity to produce astaxanthin, this pigment was not found as component of total carotenoids content, since its production was not induced.

The isolated *Chlorococcum* was evaluated traditional microscopic observation and by molecular analysis to identify the specie. However, any homology was found. The results do not exclude the possibility of this strain could be a new specie, not yet identified. Although, it do now allows to be 100 % sure of it and more analysis is need.

**Keywords:** Microalgae, *Chlorococcum* sp., Biochemical composition, Molecular identification, Large-scale production.

## Resumo

Atualmente, o aquecimento global, causado pelo aumento dos gases do efeito de estufa na atmosfera, é uma preocupação séria entre os cientistas. Para além das alterações diretas na atmosfera, as produções agrícolas também estão a ser afetadas, sendo esperadas reduções na produção de alimento.

O aumento da população mundial e a expansão da economia levam ao aumento da procura por fontes alternativas de alimento, bem como por fontes de biocombustíveis amigas do ambiente.

As microalgas podem ser utilizadas para aumentar o valor nutricional dos alimentos e como suplemento das rações de animais convencionais, afetando positivamente a saúde e o crescimento de ambos, humanos.

Para além disso, as microalgas conseguem utilizar de forma eficiente o CO<sub>2</sub>, sendo responsáveis por mais de 40 % da fixação global de carbono. Neste contexto, as culturas de microalgas podem ser consideradas como um dos principais processos biológicos de fixação de CO<sub>2</sub> para a mitigação dos níveis de CO<sub>2</sub> na atmosfera e, conseqüentemente, atenuação do aquecimento global.

Como o trabalho foi desenvolvido na ALGAFARM (empresa de produção de microalgas), a estirpe isolada foi avaliada de acordo com a necessidade de se adequar à empresa. Foi testado meio com 10 mM de nitrato e 1 mM de nitrato. Para maiores concentrações de biomassa e composição bioquímica melhorada para alimentação humana e animal, o meio com 10 mM de nitrato é o mais adequado.

A estirpe de *Chlorococcum* foi avaliada em *scale-up* de reatores até ao PBR de escala industrial de 10000 L. As melhores produtividades volumétricas, máxima e global, foram obtidas no PBR de escala piloto de 2500 L com 0,153 g.L<sup>-1</sup>.dia<sup>-1</sup> e 0,098 g.L<sup>-1</sup>.dia<sup>-1</sup>, respetivamente. No entanto, as melhores produtividades areais, máxima e global, foram conseguidas pelo PBR de escala industrial de 10000 L com 36,559 g.m<sup>-2</sup>.dia<sup>-1</sup> e 17,419 g.m<sup>-2</sup>.dia<sup>-1</sup>, respetivamente.

A melhor composição bioquímica do *Chlorococcum* foi obtida no PBR de escala piloto de 2500 L com 55,72 % de conteúdo de proteína, 18,35 % de conteúdo de lípidos totais (onde 74,70 % são PUFAs), 17,71 % de hidratos de carbono e 7,61 % de cinzas e atingindo valores de 4,02 % de clorofila total e 1,09 % de conteúdo de carotenóides totais (onde 71,01 % é luteína e 16,57 % é β-caroteno).

O *Chlorococcum* isolado nas instalações da ALGAFARM mostrou ter grande potencial para alimentação animal e humana uma vez que atingiu valores elevados de proteína e, também, porque contém uma quantidade relevante de ácidos gordos polinsaturados.

Esta estirpe, sem otimização do crescimento, atingiu facilmente valores elevados de clorofila total, que pode ser utilizada como corante alimentar natural. Também é adequada para aplicação nas indústrias nutracêutica e farmacêutica uma vez que mostrou um conteúdo de carotenóides totais de mais de 1 % do peso seco da biomassa, maioritariamente constituído por luteína (5.374 mg.g<sup>-1</sup>) e β-caroteno (1.247 mg.g<sup>-1</sup>).

Apesar do *Chlorococcum* sp. ter sido reportado na literatura como tendo a capacidade de produzir astaxantina, este pigmento não foi encontrado como componente no conteúdo de carotenóides totais, uma vez que a sua produção não foi induzida.

O *Chlorococcum* isolado foi avaliado por microscopia eletrónica tradicional e por análise molecular com o objetivo de identificar a espécie. No entanto, não foi encontrada homologia. Estes resultados não excluem a possibilidade desta estirpe poder ser uma nova espécie, ainda não identificada. No entanto, não permite ter 100 % de certeza e são necessárias mais análises.

**Palavras-chave:** Microalgas, *Chlorococcum* sp., Composição bioquímica, Identificação molecular, Produção em larga-escala.

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## List of Abbreviation

AA	Arachidonic acid
AMD	Age-Related Macular Degeneration
ANOVA	Analysis of variance
APCER	Associação Portuguesa de Certificação
BIO	Biologic
BV	Biological value
CO <sub>2</sub>	Carbon dioxide
DC	Digestibility coefficient
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
DW	Dry weight
EPA	Eicosapentaenoic acid (C20:5 ω-3)
EtOH	Ethanol
FAME	Fatty acid methyl esters
FAO	Food and Agriculture Organization
GHG	Greenhouse gases
GLA	Octadecatrienoic / Gamma linolenic acid (C18:3 ω-6)
GS-MS	Gas Chromatography-Mass spectrometry
Gt	Gigatonne
GWP	Green wall panel
ha	Hectare
Kg	Kilogram
ISO	International Organization for Standardization
ITS	Internal transcribed spacer
LDPE	Low-density polyethylene
LA	Octadecadienoic / Linoleic acid (C18:2 ω-6)
m <sup>3</sup>	Cubic meter
MUFA	Monounsaturated fatty acids
N	Nitrogen

NaAc	Sodium acetate
NCBI	National Center for Biotechnology Information
NH <sub>4</sub> <sup>+</sup>	Ammonium
NO <sub>3</sub> <sup>-</sup>	Nitrate
NPU	Net protein utilization
OHSAS	Occupational Health and Safety Assessment Series
O <sub>2</sub>	Oxygen
P	Phosphorous
PBR	Photobioreactor
PER	Protein efficiency ratio
pH	potential of Hydrogen
PUFA	Polyunsaturated fatty acids
P-value	Significance value
rbcL	Ribulose-Bisphosphate Carboxylase gene
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
rpm	Rotations per minute
S	Sulphur
SD	Standard deviation
SDA	Saturated fatty acids
SEM	Scanning Electron Microscope
TAG	Triacylglycerol
TEM	Transmission Electron Microscope
ω	Omega

# 1. Introduction

## 1.1. Dissertation outline

The present work is organized into five distinct chapters. First, the current environmental problems are discussed and the microalgae cultivations are proposed as an alternative to solve those problems. The company producing microalgae, where all the work has been developed, is presented and the main intended objectives of this thesis are explained.

In the second chapter, the state of the art is reported, focusing on microalgae cultivation methods, added value products obtained from these microorganisms, market, and factors that influence their production. The specie of microalga adopted in this study, *Chlorococcum* sp., is introduced.

The third chapter illustrates the material specifications and methods used in microalgae production, control, and biomass analysis, as well as a statistical analysis for the data treatment.

The fourth chapter discusses the results obtained from the microalgae growth and the biomass analysis, focused on the biotechnological potential of *Chlorococcum* sp., in different growth conditions, in different scale bioreactors and their influence on the biochemical composition and protein, pigment and lipid yields.

The fifth and last chapter ends the dissertation with some general conclusions and future prospects.

## 1.2. Contextualization

Nowadays, the threat of climate change is debated with great concern by scientists from various fields. Global warming is mainly caused by the increase of the greenhouse gases (GHG) in the atmosphere, particularly the carbon dioxide (CO<sub>2</sub>) (Bhola *et al.*, 2013; Rasul *et al.*, 2017). This phenomenon will promote future changes in decisive factors that support life on the Earth as known today. They are expected: species extinction, changes in the amount and distribution of the precipitation, glacial melting and rise of the ocean level (Pires *et al.*, 2012).

Besides, the reduction in food production is another critical theme because the climate changes affects the agricultural production, and it cause areal reductions in geographically underprivileged fields (Altieri & Nicholls, 2013). Moreover, agricultural land suffers the soil deterioration due to the extensive manipulation, synthetic chemical

fertilizers and pesticides, which contributes to environmental degradation and harms human and animal health (Mostafa, 2012).

According to FAO (Food and Agriculture Organization), 33 % of global soil suffers from moderate to high degradation, by contrast it will be required to increase food production of about 60 % until 2050 to satisfy population necessities (FAO & ITPS, 2015).

It is important to implement new and more sustainable production strategies, which can satisfy mankind need and avoid or mitigate these environmental issues (Altieri & Nicholls, 2013).

The increasing world's population and the predictions of an insufficient protein supply led to the research of new alternative and unconventional protein sources. Algal biomass appeared as a good candidate for this purpose since its cells are able to synthesize all the essential aminoacids and their cultivation do not compete for arable land since they can grow in bioreactors (Spolaore *et al.*, 2006; Gouveia *et al.*, 2015).

Microalgae can be used to enhance the nutritional value of food and as supplement or substitute of conventional animal feed sources, from aquaculture to farm animals. Their chemical composition was already reported to positively affect the health and the growth of humans and animals (Spolaore *et al.*, 2006; Guedes & Malcata, 2012).

On another level, the increasing population and expanding economy leads to increasing demand of fossil fuel. Nevertheless, fuels supplies are finite and expected to decline over the next few decades (Borowitzka & Moheimani, 2013; Hannon *et al.*, 2014).

The replacement of conventional fuels requires: sufficient feedstock, competitive costs and to match standard specification of fuel quality (Harun *et al.*, 2010). Based on these requirements, microalgae have potential to be used as a raw material for biodiesel production, but it's fundamentally important the selection of the highly productive, oil-rich algal strains to achieve a cost-effective biofuel (Harun *et al.*, 2010; Hannon *et al.*, 2014).

Beyond all these benefits, microalgae can efficiently use CO<sub>2</sub>, being responsible for more than 40 % of global carbon fixation. The aquatic environment is the greatest active reservoir of carbon in the planet (38.000 Gt compared with 748 Gt in the atmosphere). In this context, microalgal cultures can be considered on the main the main biological CO<sub>2</sub> fixation process for the mitigation of the CO<sub>2</sub> levels released from industry, and, consequently, the global warming (Pires *et al.*, 2012).

### 1.3. Company SECIL | ALGAFARM

The activity of this dissertation was carried out at ALGAFARM, the unit of microalgae production of the company Cimentos Maceira e Pataias (CMP), SECIL Group, which is a leading cement producer in Portugal, located in Pataias, Alcobaça.

Founded in Portugal, SECIL also operates internationally in Angola, Tunisia, Lebanon, Cape Verde, The Netherlands and Brazil and produces an annual output of cement of 9 million tons (SECIL, 2018).

The main GHG resulting of the cement production is CO<sub>2</sub> from calcination of carbonates from raw material (60 % of emissions) and combustion of fuel in the ovens (40% of emissions). However, in the last years, SECIL has been improving the thermal and electrical efficiency and co-processing alternative fuels to reduce CO<sub>2</sub> emissions. In addition, the company is investing in innovative technologies for carbon sequestration with recourse to microalgal cultures in the facilities of ALGAFARM (SECIL, 2018).

ALGAFARM become operational in 2013 with the main goal of contributing to the carbon capture from the cement plant and it is the largest industrial unity of microalgae production in Europe. The plant has a total volume of 1300 m<sup>3</sup>, occupies more than 1 ha ground area and is able to produce 100 t of dried biomass per year (Fonseca *et al.*, 2016).

The production unit operates in closed photobioreactors (PBR) in autotrophic conditions, but the process for dark heterotrophic fermentation, in 200 L and 5000 L reactors, has been recently established for *Chlorella vulgaris* (Chlorophyta). The heterotrophic fermentation is aimed to reduce the scale-up time, which is a constringent factor in microalgae production. Then, ALGAFARM mixotrophic cultivation are established, when PBR are inoculated with heterotrophic inoculum of *C. vulgaris*. From this process results a premium quality biomass with biochemical composition higher than autotrophic cultivated *C. vulgaris* (Fonseca *et al.*, 2016; Silva *et al.*, 2017).

The next strategy, in the near future, goes through the construction of raceways to produce biomass with lower costs (Silva *et al.*, 2017).

The company's mission is to bring the best of microalgae biomass in the different market field such as human food, dietary supplements, animal nutrition, cosmetics and biofertilizers (SECIL, 2018). ALGAFARM is currently working with *Chlorella vulgaris*, *Chlorella vulgaris* BIO, *Nannochloropsis oceanica* (Ochrophyta), *Phaeodactylum tricorutum* (Bacillariophyta), *Tetraselmis chuii* and *Scenedesmus* sp. (Chlorophyta) and the quality of the final products are ensured by APCER: ISO 22000, ISO 9001, ISO 14001 and OHSAS 18001 (Allmicroalgae, 2018).



Figure 1 - Aerial view of ALGAFARM facilities and SECIL facilities (From Magazine *Pontos de Vista*, 2017).

#### **1.4. Research aims**

The main goal of this dissertation was the assessment of the biotechnological potential of a *Chlorococcum* sp. (Chlorophyta) isolated in ALGAFARM unit. The attention was focused on the content of protein, lipids, carbohydrates and pigments as well as the productivity as key parameter to define the microalgal strain applications and their reference market.

Biomolecular tests were also performed in order to identify the specie of the autochthon *Chlorococcum* adopted.

## **2. State of the Art**

### **2.1. Microalgae Biology and Growth Conditions**

Microalgae are an extremely heterogeneous group of photosynthetic organisms and the main producers of marine biomass. They are usually individual unicellular microorganisms (2-200  $\mu\text{m}$ ) but they can be organized in colony with little or no cell differentiation (Olaizola, 2003; Mutanda *et al.*, 2010). Some algae are derived from a

eukaryotic ancestor that acquired a photosynthetic cyanobacterium in a single endosymbiotic event, resulting in green algae, red algae and glaucophytes. In other algae, a secondary endosymbiotic event has occurred whereby a red or green alga was taken up by a eukaryotic ancestor (Sasso *et al.*, 2012).

Eukaryotic microalgae are used to be compared to primitive plants and can be either autotrophic, heterotrophic or mixotrophic. Autotrophic microalgae require only inorganic compounds such as CO<sub>2</sub>, nitrogen (N), sulphur (S), phosphorous (P) and light as an energy source for their growth and development. There are several forms of N which can be metabolized by algae such as ammonia, nitrate or urea. However, it is important to note that the form of N provided, can affect the cell composition, including the lipid content and fatty acid composition, as well as the growth rate and the culture stability (Borowitzka & Moheimani, 2013). Mixotrophic cultures are able to perform photosynthesis and to catabolize exogenous organic nutrients (Pignolet *et al.*, 2013).

The average elemental composition of freshwater algae is CH<sub>1.7</sub>O<sub>0.4</sub>N<sub>0.15</sub>P<sub>0.0094</sub>, but the N content can change according to the environmental conditions and nutrient status of the algae. In order to achieve maximum growth, these elements need to be supplied in sufficient quantity, otherwise they will be a limiting factor to growth (Borowitzka & Moheimani, 2013; Rasul *et al.*, 2017).

Microalgae can rapidly generate biomass from solar energy and use the CO<sub>2</sub> from atmosphere, and as they have minimal resource requirements, they can grow in harsh environments such as deserts, rocky areas, salt pans, brackish water or wastewaters and do not compete for land or important resources like drinking waters (Mostafa, 2012; Gouveia *et al.*, 2015). They tolerate a wide range of pH, temperature, turbidity, O<sub>2</sub>, and CO<sub>2</sub> concentration (Mutanda *et al.*, 2010; Barsanti & Gualtieri, 2014).

Generally, microalgae contain 40-70 % (w/w) of protein, 4-20 % (w/w) of lipids and 12-30 % (w/w) of carbohydrates. Although, when the cultures are in the stationary phase, the composition of microalgae may significantly change, e.g. nitrate limitation leads carbohydrate levels to double at the expense of protein (Guedes & Malcata, 2012; Ejike *et al.*, 2017).

## 2.2. Microalgae Identification

Microalgae are traditionally classified according to morphological and cytological characteristics of vegetative stages in their life cycle by means of microscope-based techniques. However, several studies have shown that the morphology could vary under different environmental conditions, which led to uncertain identification at the specie level (Darienko *et al.*, 2015). For more specific identification, conventional light microscopy has been extended to include fluorescence microscopy, phase-contrast microscopy, transmission electron microscopy (TEM) and scanning electron microscopy (SEM) (Bhola *et al.*, 2013). Anyway, as microscopy it is time-consuming and it requires significant experience in technical and taxonomic skills, molecular-based techniques have been adopted. Molecular identification enables rapid and precise monitoring, identification and quantification of microalgal species. The analysis of DNA regions for phylogenetic purposes include mitochondria genes, ribosomal RNA genes (rRNA), internal transcribed sequences (ITS), plastid genes (rbcL) and microsatellite DNA sequences. Then, a comparison with known 18S rRNA gene sequences collected in standard database (such as *NCBI*) is carried out. The specie is identified if a match of at least 90 % occurs (Bhola *et al.*, 2013; Bellinger & Sigeo, 2015).

## 2.3. *Chlorococcum* sp.

Cells of the genus *Chlorococcum* belong to the Chlorophyta phylum, Chlamydomonadales order and Chlorococcaceae family. They live as solitary vegetative cells or in temporary groups of indefinite form. The reproduction is mainly asexual, by zoospores (or aplanospores in case of water stress) or sexual, by isogametes, when the environmental conditions are not propitious such as light or nitrogen limitation (Watanabe & Lewis, 2017). *Chlorococcum* genus is distinguished from other spherical, zoospore-producing algae by three attributes (Bold & Parker, 1962):

- i. a hollow, parietal chromatophore (chloroplast) with or without an open surface;
- ii. one or more pyrenoids;
- iii. biflagellate zoospores which do not become spherical upon cessation of motility but retain an ovoid, ellipsoid shape for some days.

This genus is cosmopolitan. It has been isolated from hot springs in Central Asian and also in soils of Antarctica. This microalga can be collected from aquatic and aerial environments and also from soil and rocky areas (Watanabe & Lewis, 2017).



In the context of carbon sequestration, the genus *Chlorococcum*, *Chlorella*, *Scenedesmus* (Chlorophyta) and *Euglena* (Euglenozoa) (Bhola *et al.*, 2013) have been recognized as the most efficient. Indeed, their cultures can reach high density cultures, up to 84 g.L<sup>-1</sup> by using a flat-panel photobioreactor. Moreover, *Chlorococcum littorale* possess a high CO<sub>2</sub> tolerance (up to 65 % of CO<sub>2</sub>), which make it an interesting candidate for the CO<sub>2</sub> biofixation (Ota *et al.*, 2015).

*Chlorococcum* sp. has been reported as a potential source for biodiesel production since its lipid content reaches concentrations higher than 20 % (Prabakaran *et al.*, 2018). Usually algal oil containing saturated and polynsaturated fatty acids are used as feedstock for biodiesel production. *Chlorococcum humicola* oil contain around 95,4 % of C14-18 fatty acids (Santhoshkumar *et al.*, 2016; Shankar *et al.*, 2017).

Due to its relative fast growth rate, ease of cultivation in outdoor systems, and high tolerance to extreme pH and high temperature, *Chlorococcum* sp. is considered a promising comercial source of ketocarotenoids.

Lipid fraction of green vegetative cells, in some cases, consists mostly of lutein (75–80 %) (Lorenz & Cysewski, 2000) and the carotenoid composition changes as function of temperature and pH of the culture (Table 1 and 2) (Liu & Lee, 2000):

Table 1 - Influence of temperature on carotenoid content (% total carotenoids, w/w) of *Chlorococcum* sp. (Adapted from Liu & Lee, 2000).

<b>Carotenoid</b>	<b>20 °C</b>	<b>25 °C</b>	<b>30 °C</b>	<b>35 °C</b>
<b>β-Carotene</b>	25.3	14.5	6.79	5.21
<b>Astaxanthin</b>	6.8	7.9	9.51	11.6
<b>Total Carotenoids</b>	4.4	6.5	7.2	8.4

Table 2- Table 1 - Influence of pH on carotenoid content (% total carotenoids, w/w) of *Chlorococcum* sp. (Adapted from Liu & Lee, 2000).

<b>Carotenoid</b>	<b>pH 5</b>	<b>pH 6</b>	<b>pH 7</b>	<b>pH 8</b>	<b>pH 9</b>
<b>β-Carotene</b>	12.4	10.4	8.30	6.20	13.4
<b>Astaxanthin</b>	3.52	6.80	7.90	9.51	5.80
<b>Total Carotenoids</b>	3.82	2.05	5.50	2.42	1.77



classes of culture system are currently adopted for large-scale culture: closed photobioreactors (PBRs) and open ponds (Guedes & Malcata, 2012). Each of these system present some advantages and drawbacks and their selection should consider the selected microalgal strain and the main target product.

#### **2.4.1. Large-scale biomass production**

##### **Open ponds**

Open ponds can have different shapes and forms, and different agitation system. The location in which the pond is situated, the algal strain and the amount of light for photosynthesis are the main factors for the selection. The types that are currently used in the research and the industry are: raceways ponds, circular ponds tanks and shallow big ponds. This type of cultivation is limited by low light availability, temperature fluctuation, increase in the pH and dissolved oxygen concentration and exposure to contamination, thus, only some algal strains are able to grow in the ponds (Olaizola, 2003; Harun *et al.*, 2010; Rasul *et al.*, 2017). However, the costs of construction and operations of this cultivation are lower than the closed systems (Harun *et al.*, 2010; Guedes & Malcata, 2012).

##### **Photobioreactors**

Closed systems, mainly known as photobioreactors, gives a better control of the culture parameters, minimize of water evaporation, reduce of the contaminations by predators and competitive algal weeds and allows higher productivity (Rasul *et al.*, 2017).

A typical photobioreactor is essentially a four-phase system, consisting of: solid microalgal cells, a liquid growth medium, a gaseous phase and incident light radiance. There are several options of closed systems but the mainly used include tubular, flat plate and fermenter types (Guedes & Malcata, 2012).

Tubular photobioreactors are made with transparent materials and placed outdoor to use sunlight irradiation. Improved sunlight exploitation derive from their large surface area per volume unit (Guedes & Malcata, 2012).

The flat plate, also known as green wall panel, is a vertical plate photobioreactor mixed by air bubbling. It ensures high irradiance on the surface area and high biomass productivity. Moreover, the use of thin, transparent, cheap and flexible materials (like LDPE) reduce the construction costs with respect to the tubular photobioreactors (Rasul *et al.*, 2017).

Fermenters are controlled bioreactor, operating in indoor, generally used for bacterial and yeast growth. They have been proved to be competitive tools also for the heterotrophic microalgal growth. Indeed, the heterotrophic growth in fermenter allows the achievement of biomass concentration up to 200 g.L<sup>-1</sup>. This concentrated biomass can be used as inoculum of large scale PBRs reducing the time for the scale-up, which is a crucial factor for industry (Silva *et al.*, 2017).

## **2.5. Added-value compounds and market**

The first use of microalgae by humans, dates back 2000 years to the Chinese, who used *Nostoc* to survive during famine, but large-scale culture only started in the early 1960's in Japan with the culture of *Chlorella*. By 1980 there were 46 large-scale factories in Asia producing more than 1000 Kg of microalgae per month. In 1986 started the commercial production of *Dunaliella salina* (Chlorophyta) as a source of  $\beta$ -carotene and, more recently, began the production of *Haematococcus pluvialis* as a source of astaxanthin. Thus, the microalgal biotechnology industry has grown and diversified significantly. Nowadays, the microalgal biomass market produces about 5000 tons of dry matter per year (Spolaore *et al.*, 2006).

Between 1965 and 2006 almost 18.500 new compounds isolated from marine sources have been discovered, but their value is still limited if compared with almost 97 % of all existing marine compounds that have not yet been isolated (Guedes *et al.*, 2011b). Microalgae, that covers the majority of the marine environment and are responsible for over half the primary production at the base of the food chain, remain largely unexplored and may represent an opportunity to discover novel metabolites and produce them at lower costs (Guschima & Harwood, 2006; Guedes *et al.*, 2011b).

Currently, there is a great interest in microalgae as sources of renewable energy, biofuels and as a range of feedstocks such as for feed (Borowitzka & Moheimani, 2013; Zhu, 2015; Rastogi *et al.*, 2017) bioplastics, biofertilizers, biofuels and wastewater treatment (Gouveia *et al.*, 2015). Rather, the most profitable microalgae components find application in the cosmetic and pharmaceutical field and as high-value food and feed additives. These microalgae compounds include: minerals, vitamins, pigments, antioxidants, polyunsaturated fatty acids (PUFAs), proteins and polysaccharides (Harun *et al.*, 2010; Bholá, 2013).

The accumulation of high value metabolites is associated to secondary pathway of microalgal metabolism. The induction of secondary metabolism is influenced by

environmental conditions or developmental stages (Mostafa, 2012). The controlled induction of secondary metabolism is particularly interesting in biotechnology industry since it makes possible to increase the production of the desired valuable metabolite (Guedes *et al.*, 2011a).

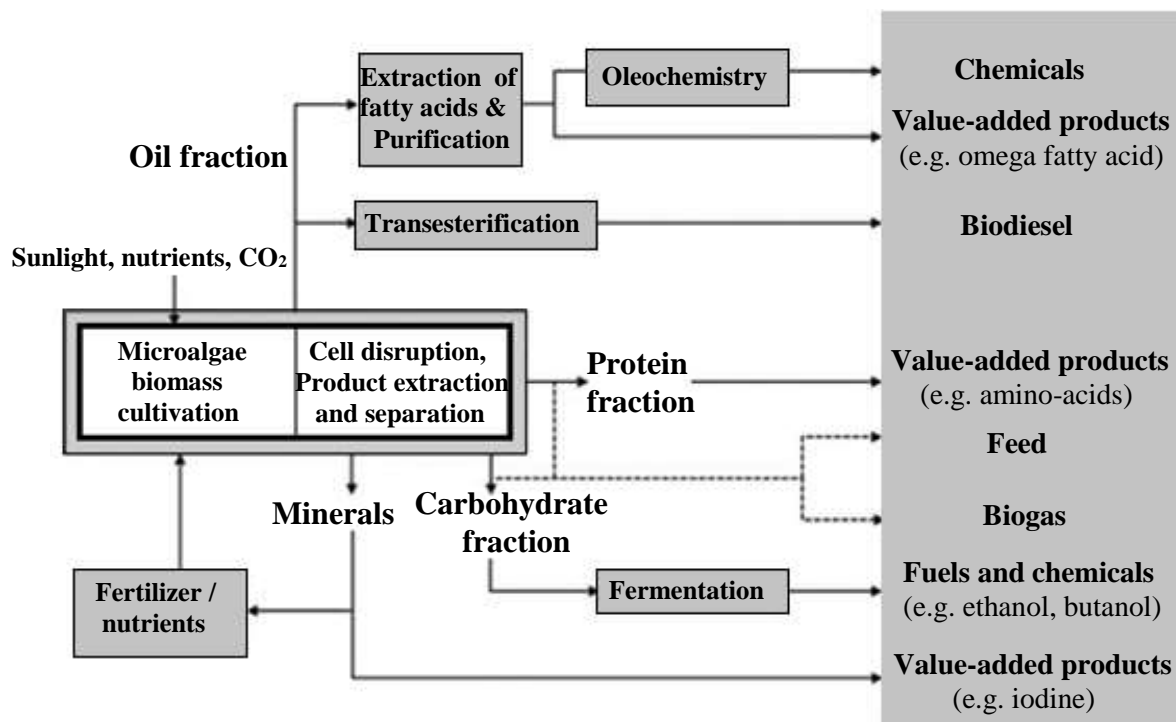


Figure 3 - Applications of microalgae (Adapted from Chew *et al.*, 2017).

### 2.5.1. Feed and Food application

#### Protein

Proteins are the main constituents of microalgae and one of the important products of microalgae biorefineries (Chew *et al.*, 2017). Peptides are very interesting for functional food application due to their demonstrated biological activities in human health conditions such as hypertension, oxidative stress, cancer, diabetes, inflammation, and immune disorders. These peptides exist as part of microalgae proteins and remain inactive until they are released by enzymatic hydrolysis via gastrointestinal protease action. The use of protein-derived bioactive peptides as functional ingredients in foods is a rapidly developing area of food innovation (Ejike *et al.*, 2017).

Microalgal proteins are also appreciated as additive to feed for a wide variety of animals ranging from fish (aquaculture) to pets and farm animals (Spolaore *et al.*, 2006).

Microalgae can synthesize all 22 amino acids and they can be considered unconventional sources of essential amino acids for human nutrition. The amino acid

composition of microalgal proteins is rather similar between species and it is affected by the growth phase and the light irradiance (Guedes & Malcata, 2012).

Moreover, chemical analyses and nutritional studies demonstrated that these algal proteins have high quality, comparable to conventional vegetable proteins, but lower biological values and digestibility if compared to casein and egg protein (Table 3) (Becker, 2007; Ejike *et al.*, 2017).

Table 3 - Comparative data on biological value (BV), digestibility coefficient (DC), net protein utilization (NPU) and protein efficiency ratio (PER) of different microalgae, casein and egg (based on Becker, 2007).

<b>Product</b>	<b>BV</b>	<b>DC</b>	<b>NPU</b>	<b>PER</b>
<b>Casein</b>	87.8	95.1	83.4	2.50
<b>Egg Protein</b>	94.7	94.2	89.1	-
<i>Scenedesmus obliquus</i>	71.9	77.1	55.5	1.20
<i>Chlorella sp.</i>	52.9	59.4	31.4	0.84

Even so, the consumption of alternative protein is growing rapidly and extracted protein from other sources such microalgae would cover up to 50 % market of the total alternative protein by 2054 (Khanra *et al.*, 2018).

### **Carbohydrates**

In microalgae, carbohydrates can be found mainly in the form of starch, glucose and other polysaccharides. Their high digestibility encourages the use of dried whole microalgae in foods or feeds without restriction (Spolaore *et al.*, 2006). Moreover, as the microalgae-based carbohydrates consist mainly of cellulose and starch without the lignin component characterizing vegetal sources, they also find application as readily available carbon sources for the fermentation industry aimed at biobutanol and bioethanol productions (Chew *et al.*, 2017).

Specific microalgal polysaccharides are able to modulate the immune system and inflammatory reactions with significant application as sources of biologically active and natural therapeutic agents (Chew *et al.*, 2017).

### **Lipids**

Microalgae produce significant amounts of lipids in natural conditions, but this lipid accumulation decreases when cultivation conditions are optimized to increase biomass

content. The lipids accumulation is generally associated to reduced growth rate then, the traditional approach for microalgae lipid production is based on two main steps: first, microalgae biomass production (growth phase) and second, lipid accumulation induced by nitrogen starvation (stress phase) (Shankar *et al.*, 2017).

Microalgae are mainly composed by polar lipids such as phospholipids and glycolipids, which are confined to cell organelle membranes, and neutral storage lipids, such as monoglycerides, diglycerides, triglycerides, free fatty acids, hydrocarbons, and pigments, which can be found mainly in vacuoles of the cell (Pignolet *et al.*, 2013).

In recent years, the fatty acids compositions of microalgae attracted considerable interest among researchers and new and high value application emerged beyond biodiesel production (Spolaore *et al.*, 2006; Mostafa, 2012). The microalgae adopted for fuel purpose have been diatoms, green algae, golden brown, prymnesiophytes and eustigmatophytes (Pires *et al.*, 2012).

Their fatty acids composition varies according to the species and their environmental conditions (Mutanda *et al.*, 2010). PUFAs are of the utmost importance for human metabolism. They are the major components of cell membrane phospholipids and may also be present in cellular storage oils (Pereira, *et al.*, 2012). Within them, some of the Omega-3 ( $\omega$ 3) and Omega-6 ( $\omega$ 6) families are of particular interest for human and animal nutrition. The functional sources of  $\omega$ 3 in microalgae are normally eicosapentanoic acid (EPA) and docosahexaenoic acid (DHA). These fatty acids are mainly obtained from fish oil, however, microalgae are self-producing  $\omega$ 3 and the production process is simple and economic (Harun *et al.*, 2010).

Most of the microalgal species exhibit considerable percentage of EPA (7 to 34 %), however, chlorophytes are, in general, deficient in both C20 and C22 PUFAs, only some species have small amounts of EPA (up to 3.2 %) (Guedes & Malcata, 2012).

These PUFAs have many health benefits. EPA has been used in clinical purposes, such as the treatment of coronary heart disease and inflammatory diseases. DHA helps the cancer therapy, maintaining lower the level of cholesterol so that it boosts the immune system and detoxifies the body (Harun *et al.*, 2010; Mostafa, 2012).

The applications of the polyunsaturated fatty acids include also the addition to infant formulas and nutritional supplements (Table 4). Moreover, they are very appreciated in aquaculture feed, where the ratios of DHA, EPA and arachidonic acid (AA) fulfil relevant importance (Spolaore *et al.*, 2006; Guedes & Malcata, 2012).

Table 4 - Particularly interesting microalgal PUFAs (Adapted from Spolaore *et al.*, 2006)

<b>PUFA</b>	<b>Structure</b>	<b>Potential application</b>
<b>Gamma Linolenic acid (GLA)</b>	18:3 ω6, 9, 12	Infant formulas Nutritional supplements
<b>Arachidonic acid (AA)</b>	20:4 ω6, 9, 12, 15	Infant formulas Nutritional supplements
<b>Eicosapentaenoic acid (EPA)</b>	20:5 ω3, 6, 9, 12, 15	Nutritional supplements Aquaculture
<b>Docosahexaenoic acid (DHA)</b>	22:6 ω3, 6, 9, 12, 15, 18	Infant formulas Nutritional supplements Aquaculture

### **Pigments**

Pigments from microalgae have their most important use as natural food colorant, as additive for animal feed and in cosmetic and nutraceutical industries (Campo *et al.*, 2007). Natural colorant has increasing demand in the last decades due to the raising attention of the consumers towards natural and healthy food additives. In particular, chlorophyll, due to its strong green colour, is used as natural in specific food formulation. Green algae cultured under optimum condition were reported to contain about 4% dry weight of chlorophyll (Harun *et al.*, 2010). Yellow, orange, and red carotenoids are also used in food products, but especially in cosmetics (Campo *et al.*, 2007).

The hydrocarbon carotenoids are named carotenes, whereas oxygenated derivatives are known as xanthophylls. All xanthophylls synthesized by higher plants (e.g., violaxanthin, zeaxanthin, neoxanthin and lutein), can also be synthesized by green microalgae. However, these have additional xanthophylls, (e.g., astaxanthin and canthaxanthin). In xanthophylls, oxygen can be present as OH groups (as in lutein), as oxi-groups (as in canthaxanthin), or in a combination of both (as in astaxanthin) (Guedes *et al.*, 2011a; Campo *et al.*, 2007).

Primary carotenoids, such as xanthophylls, are essential for the cells survival since they are structural and functional components of the photosynthetic apparatus. Secondary carotenoids, such as carotenes, are those produced at higher level by microalgae, after exposure to specific environmental stimuli, via carotenogenesis (Guedes *et al.*, 2011a).

A limited number of carotenoids, particularly β-carotene, astaxanthin, lutein, zeaxanthin, canthaxanthin and lycopene, are used commercially. However, the



competition with the less expensive synthetic form of the pigments, is still a critical issue (Spolaore *et al.*, 2006; Mostafa, 2012).

The effects of carotenoids on human health are, in general, associated with their antioxidant properties and, consequently, with the reduction of cancer risk (Mostafa, 2012). Other pigments, such as  $\beta$ -carotene, has a very important nutritional use due to the ability to act as provitamin A, so that increase the vitamin A production in the organism.  $\beta$ -carotene can also be used as a colouring agent with estimated market size of 10 tons to 100 tons per year and selling value of *ca.* 750 €<sup>1</sup> per Kg (Mostafa, 2012; Spolaore *et al.*, 2006).

Lutein is largely consumed as food colorant, reaching sales amount up to 129.000.000 €<sup>1</sup> at US (Fernández-Sevilla *et al.*, 2010), and as feed additives in aquaculture. Together with zeaxanthin, they are the essential component of the pigment present in the macula lutea in the eye retina and eye lens (Mostafa, 2012). In the eastern world there are a high percentage of people with ADM (Age-Related Macular Degeneration) disease and the recommended intake of lutein is 6 mg daily. Hereupon, the most feasible application of microalgae as lutein producers is for pharmaceutical or nutritional products for aquaculture (Fernández-Sevilla *et al.*, 2010).

Natural astaxanthin is the most expensive carotenoid, with an average price *ca.* 2,050 €<sup>1</sup> per Kg and its uses mostly concern aquaculture. Currently, this pigment is produced by *H. pluvialis* and its concentration can reach 1.5 % to 3 % of the biomass dry weight (Lorenz & Cysewski, 2000).

## **2.5.2. Environmental applications**

### **CO<sub>2</sub> biofixation**

Microalgae have higher photosynthetic efficiency, higher O<sub>2</sub> production rates and 10 times greater CO<sub>2</sub> fixation efficiency than terrestrial plants, whereby they are the most profitable option for CO<sub>2</sub> mitigation (Mostafa, 2012; Borowitzka & Moheimani, 2013). The increased CO<sub>2</sub> fixation can be attributed to the co-localisation of their photosynthetic apparatus and light capturing pigments within a single cell, allowing rapid energy use for biomass generation (Bhola, 2013).

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<sup>1</sup> Conversion factor at 18<sup>th</sup> June 2018: 1 USD = 0.863668 EUR

As shown in equation below, during photosynthesis, CO<sub>2</sub> is converted from its oxidized state to the reduced form such as carbohydrates (CH<sub>2</sub>O)<sub>n</sub>, by means of the light energy (Barsanti & Gualtieri, 2014):



CO<sub>2</sub> mitigation strategies globally implemented can be divided in physical and biological techniques. However, only the biological fixation appears to be an economical and environmentally sustainable technology. Microalgae are recognized as leading biological techniques for the capture of CO<sub>2</sub> from atmosphere, power plants, industrial flue gas and from soluble carbonate (Bhola *et al.*, 2013; Rasul *et al.*, 2017).

The levels of CO<sub>2</sub> in the atmosphere are generally low (approximately 0.036 %) when compared to industrial emissions, characterized by higher concentrations (within 3-30 %). Despite CO<sub>2</sub> is an effective supplement to stimulate microalgal growth, at high concentrations (above 5-10 %), microalgal growth can be prevented. This is generally attributed to the acidification of the culture due to the dissolved CO<sub>2</sub>. After all, effective CO<sub>2</sub> fixation in microalgae biomass can be reached (1 Kg of dry algal biomass can fix about 1.83 Kg of CO<sub>2</sub>), if hyper CO<sub>2</sub>-tolerant strains are isolated and selected (Brennan & Owende, 2010; Bhola, 2013).

## Biofuel

Biodiesel is a renewable fuel consisting of fatty acid methyl esters (FAME), generally produced by transesterification of vegetable oils or animal fats in order to achieve viscosity and flow properties close to that of fossil fuels (Figure 4) (Harun *et al.* 2010; Hoekman *et al.*, 2012).

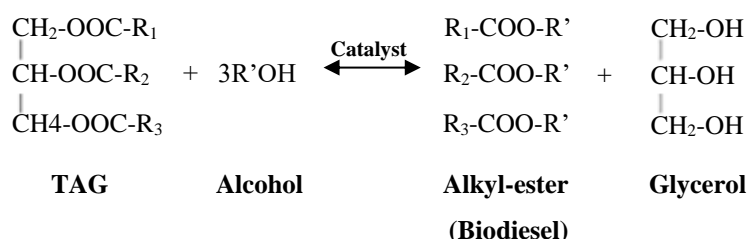


Figure 4 -Transesterification reaction for biodiesel production (Adapted from Hoekman *et al.*, 2012).

Conventional feedstocks for biodiesel production include rapeseed oil, sugarcane, soybean, sunflower, palm, and peanut oils, called the first generation of biofuels (Pinzi *et al.*, 2013). However, due to their impact on global food markets and on food security, mainly because of the use of arable lands, the production of biodiesel instead of food and

the associated increasing on food prices, questions were raised about their potential to replace fossil fuels (Brennan & Owende, 2010).

Thereby, has emerged the necessity to overcome this improper situation, and the second generation of biofuels has arisen. They were intended to produce fuels from agricultural residues and wood processing waste, although, the technology for conversion cellulosic materials was not well developed and expensive.

At the moment, have been exploited the third generation of biofuels, through microalgae, which can grow in wastewaters, do not competing for arable land and minimising associated environmental impacts and yet have higher yields (Brennan & Owende, 2010; Pinzi *et al.*, 2013; ENMC, 2018).

The fatty acid profile of biodiesel is dependent on the parent oil and the most common fatty esters are derived from palmitic (C16:0) acid, stearic (C18:0) acid, oleic (C18:1) acid, linoleic (C18:2) acid and linolenic (C18:3) acid (Pinzi *et al.*, 2013).

Microalgae rich in lipid and carbohydrate are considered as potential sources for renewable energy production, but it's fundamentally important to select the highly productive, oil-rich algal strains to achieve a cost-effective biofuel, otherwise its use it is not viable (Harun *et al.*, 2010; Mutanda *et al.*, 2010; Hannon *et al.*, 2014; Rasul *et al.*, 2017).

In conclusion, microalgae have been widely recognized as a cornerstone for the bioeconomy and biorefinery development. In Europe, macro and microalgae were recognized by SET-PLAN (Strategic Energy Technological Plan) as a key value chain for advanced biomaterials production. Portugal, as a coastal country, has excellent edaphoclimatic conditions and a great potential for microalgae culturing in a sustainable way. Hereupon, the partnership with fisheries and aquacultures could contribute to the development of the coastal communities as well as rural areas, for natural and sustainable feed and food production (Gouveia *et al.*, 2015).

### 3. Materials and Methods

#### 3.1. Microalgae strain and culture medium

All the culture experiments described in the present work were performed in the facilities of CMP/ALGAFARM (Secil Group, Portugal), between the 05th of January and the 25th of May 2018. The biochemical characterization of the produced biomass was performed at the MarBiotech group of the Centre of Marine Sciences (University of Algarve) between the 21st and 30th of May 2017.

The microalgal strain used in this work, *Chlorococcum* sp., was obtained from ALGAFARM culture collection and it was previously isolated as a contaminant of a *Chlorella vulgaris* culture.

The culture medium was Guillard's F/2 medium, adjusted to the local water composition and further supplemented with iron (25  $\mu$ M) (Watanabe, 2005).

#### 3.2. Scale-up of the cultures for industrial production

The culture system used in this study are reported in the Figure 5, in the order of size: A) 5 L air-lift; B) 125 L Green Wall Panel; C) 2500 L Photobioreactor; D, E) 10000 L Photobioreactor.



Figure 5 - Pictures from ALGAFARM unit: laboratory air-lifts 5 L (A); GWP 125 L (B); PBR 2500L (C); PBR 10000L (D and E).

Microalgae cultures were grown at 5 L laboratory air-lift reactors. In these reactors, compressed air was continuously injected by means of 0.2  $\mu\text{m}$  filters in order to sterilize it. The  $\text{CO}_2$  was manually injected to maintain pH values between 7.5 and 8. These reactors were placed under continuous light irradiance (24/24 h) of 6410 lux and at temperature of 24  $^\circ\text{C}$ .

Two 5 L reactors were used as the pre-inoculum for the outdoor 125 L Green Wall Panel (GWP). Then, the latter was used as inoculum for the 250 L GWP. The aeration system of the GWP was analogous to the 5 L air-lift and the sterilization of the air inlet was carried out with 0.2  $\mu\text{m}$  filters as well. The temperature of the GWP was maintained below 28  $^\circ\text{C}$  by a sprinkler-like irrigation system. The pH values were maintained between 7.5 and 8 by the manually controlling the pulses of  $\text{CO}_2$ .

Two 250 L GWP were used to inoculate a 2500 L tubular PBR and about 80 % of this PBR was used as an inoculum to start a 10000 L PBR. The tubular PBRs were kept under the same aforementioned conditions except for the pH assessment. The pH was controlled by automated system where the pH was measured in real time and activated the  $\text{CO}_2$  injection to keep the pH between 7.5 and 8 (for the 2500 L PBR) and between 6 and 6.5 (for the 10000 L PBR). The choice of lower pH in the larger scale PBRs was aimed to prevent virus contaminations.

The experiments in the laboratory air-lifts and the GWP were performed in triplicate at the same time. The PBR triplicates were performed one after the other.

### 3.3. Growth assessment

Microalgal growth was measured by the dry weight of biomass (DW). In brief, a known volume of culture was filtered by dried glass microfiber filters (0.7  $\mu\text{m}$ , VWR). Then dried and weighted using *Kern DBS* moisture analyzer. The DW of the biomass is the difference between the weight of the filter, after culture filtration, and the weight of the filter. The biomass concentration (X) was calculated as ratio between the weight of the biomass and the volume of sample filtered.

The volumetric biomass productivity (P) was calculated as the ratio between cell concentration at two different culture time ( $X_1$  and  $X_2$ ,  $\text{g.L}^{-1}$ ) and the corresponding time range ( $t_1$  and  $t_2$ , day) as shown in equation 1:

$$P (\text{g.L}^{-1}.\text{day}^{-1}) = \frac{X_2 - X_1}{t_2 - t_1} \quad (1)$$

The areal biomass productivity ( $P_a$ ) was calculated as the product of the volumetric biomass productivity ( $P$ ,  $\text{g.L}^{-1}\text{day}^{-1}$ ) and the volume of the PBR ( $V$ , L) divided by the irradiated area of PBR ( $A$ ,  $\text{m}^2$ ) as shown in equation 2:

$$P_a(\text{g} \cdot \text{m}^{-2} \cdot \text{day}^{-1}) = P \times V \cdot A \quad (2)$$

The specific growth rate of culture ( $\mu$ ,  $\text{day}^{-1}$ ) was determined according to the equation 3:

$$\mu(\text{day}^{-1}) = \frac{\ln(N_2/N_1)}{t_2 - t_1} \quad (3)$$

Where,  $X_2$  and  $X_1$  refers to biomass concentration ( $\text{g.L}^{-1}$ ) at the times  $t_2$  and  $t_1$  (days).

The photosynthetic efficiency was determined as the ratio between the higher heating value (HHV) and the sun irradiation that reached the reactor. The outside temperature and solar radiation were measured using a meteorological station (RM Young) and an Apogee Logan UT SP-110 pyranometer. The HHV was calculated according to a previous correlation reported by Callejón-Ferre *et al.* (2011), shown in equation 4:

$$HHV(\text{kJ} \cdot \text{g}^{-1}) = -3.393 + 0.507 \cdot C - 0.341 \cdot H + 0.067 \cdot N \quad (4)$$

Where, C is the percentage of carbon, H the percentage of hydrogen and N the percentage of nitrogen.

### 3.4. Biochemical composition

#### Elemental Analysis (CHN)

Elemental analysis of C, H and N was performed by Vario el III (*Vario EL, Elementar Analyser System, GmbH*, Hanau, Germany). Lyophilized biomass (5-10 mg) was weighed in specific aluminium caps, according to the procedure provided by the manufacturer. Total protein was estimated by multiplying the N content for the standard conversion factor 6.25 (Nunez & Quigg, 2015). The analysis was made in duplicates.

### Ash Content

Total ash was determined by the weight difference before and after combustion of the biomass. In brief, biomass was weighed and placed in small ceramic cups and treated for 8 h at 550 °C using a furnace (*J. P. Selecta, Sel horn R9-L*). Figure 6 A, B and C show the steps of the procedure explained. The experiment was performed in two replicates.



Figure 6 - A: Ceramic cup with biomass; B: Furnace with the samples inside; C: Ceramic cups with resulting ashes.

### Total Lipids determination

Total lipid content was determined following the Bligh & Dyer method (1959) with some modifications by Pereira *et al.* (2011). Lyophilized biomass was weighed in glass tubes, and 1 mL of chloroform, 2 mL of methanol and 0.8 mL of distilled water (1:2:0.8, v:v:v) were added. The sample was homogenized with an IKA Ultra-Turrax disperser (*IKA-Werke GmbH, Staufen, Germany*) on ice for 60 s.

Thereafter, 1 mL of chloroform was added and the mixture was again homogenized for 30 s. Finally, 1 mL of distilled water was added and homogenized for 30 s.

Afterwards, the mixture was centrifuged at 2000 g for 10 min to allow the phase separation. The organic phase (lower layer) was transferred into a clean tube with a *Pasteur* pipette (Figure 7 A). Then 0.7 mL of the organic phase were pipetted to a pre-weighed tube and dried at 60 °C to evaporate the chloroform (Figure 7 B). The final weight of the tube was measured by the precision balance (Figure 7 C).

The difference between the final weight of the tubes with dried samples and the initial one, represent the mass of lipids extracted. This assay was performed in duplicate.

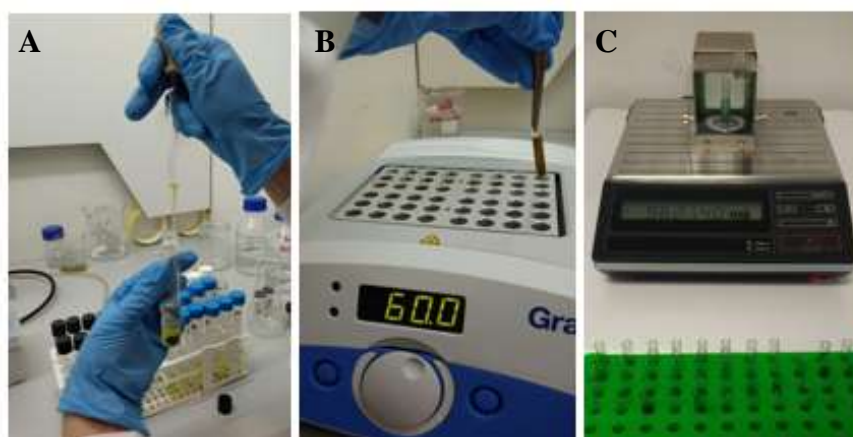


Figure 7- A: Separation of lipids with *Pasteur* pipette; B: Dry bath at 60 °C; C: Final weight of tubes in the precision balance.

### Carbohydrates content

Carbohydrates content was determined as the difference between the total weight of the biomass and the other macromolecules and ash measured.

### Fatty acids profile

Fatty acids were converted into the corresponding fatty acid methyl esters (FAME) according Lepage & Roy (1984) protocol, modified by Pereira *et al.* (2011). The FAME were then analysed by gas chromatography coupled with mass spectrometry analyzer.

The samples were weighted (20-40 mg DW) into the derivatization vessels and 1.5 mL of a solution containing methanol and acetyl chloride (20:1 v/v) were added. The mixture was homogenized on ice with an *Ultra Turrax* (12000 rpm) for 90 s. After, 1 mL of hexane was added and the mixture was heated for 1 hour at 70 °C. 1 mL of water was added and the samples were centrifuged at 3260 rpm (*Thermo Scientific Heraeus Megafuge 16R*) for 5 min. The organic phase was transferred to another vessel, filtered (0.22 µm), dried with anhydrous sodium sulphate, resuspended in 500 µL gas chromatography-grade hexane (Figure 8 A) and placed into the appropriate vials for GC.

FAME were analysed in a GC-MS analyzer (*Bruker SCION 456/GC, SCION TQ MS*) (Figure 8 B) equipped with a ZB-5MS column (length of 30 m, 0.25 mm of internal diameter, 0.25 µm of film thickness, by *Phenomenex*), using helium as carrier gas. The temperature program was 60 °C for 1 min, increase of 30 °C per min up to 120 °C, increase of 5 °C per min up to 250 °C, and final increase of 20 °C per min up to 300 °C. The temperature in the injector was 300 °C. For the identification and the quantification of FAME five different concentration of the standards *Supelco*<sup>®</sup> 37 component FAME Mix



(Sigma-Aldrich, Sintra, Portugal) were analysed in order to establish 37 different calibration curves for each of the standard. The analysis were performed in duplicates.

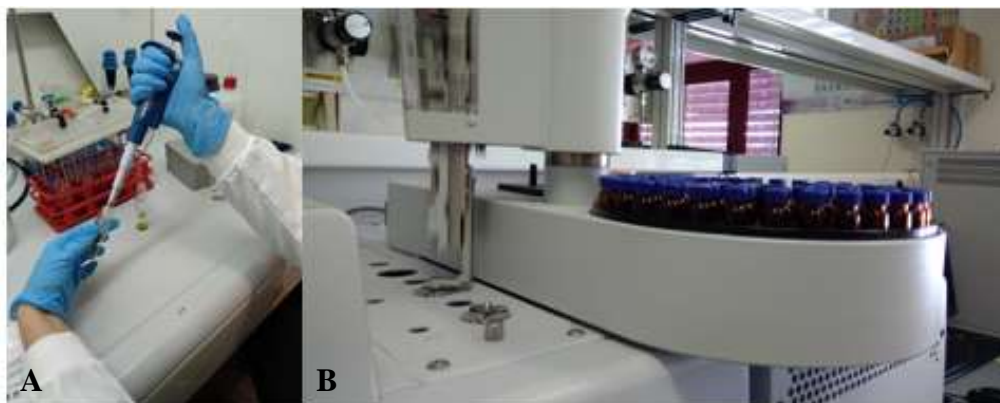


Figure 8 -A: Resuspension of dried sample with hexane; B: Injection of a vial sample at the GC-MS analyzer.

### **Pigments Content**

For the pigments extraction, 10 mg biomass were weighed in a glass tube and 1.5 mL of glass beads and added 6 mL of 100 % acetone were added and homogenized by vortex (*Velp Scientifica Classic Advanced*) for 10 min. Then, the sample was centrifuged (*HERMLE Z300*) at 3500 rpm for 10 min. These steps were repeated until the pellet lost all the colour.

The extracted pigment, recovered in the supernatant, was analyzed by *Genesys 10S UV-VIS* spectrophotometer in scanning spectrum (from 380 nm until 700 nm) and the data were analyzed by a specific intern data processing software from ALGAFARM unit.

For the characterization of total carotenoids by HPLC, the extract was completely dried under nitrogen flow, resuspended in HPLC grade methanol and filtered (0.22  $\mu\text{m}$ ) into brown HPCL vial. The separation and chromatographic analysis of pigments were performed by *Merck Hitachi LaCrom Elite HPLC* (Darmstadt, Germany) equipped with a diode-array detector, as described by Young *et al.* (1997), using a RP-18 column and a flow rate of 1 mL.min<sup>-1</sup>. The mobile phase consisted of ethyl acetate as solvent A and 9:1 (v/v) acetonitrile:water as solvent B. The gradient programme applied was: 0–16 min, 0–60 % A; 16–30 min, 60 % A; and 30–35 min 100 % A. The injection volume was 100 mL. Pigment detection was carried out at 450 nm.

All the analysis were performed in duplicates.

### 3.5. Molecular identification

For the DNA extraction, approximately 20-40 mg of wet biomass were placed into a 1.5 mL *Eppendorf* tube and the short protocol of the *E.Z.N.A.<sup>®</sup> Plant DNA kit* (*Omega Bio-Tek*, Norcross, GA) was performed 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').

For the PCR analysis, Mastermix was prepared with the composition in Table 5:

Table 5 - Mastermix composition.

<b>Ingredients</b>	<b>μL</b>
<b>MilliQ water</b>	13.3
<b>5x colorless GoTaq Flexi buffer</b>	5
<b>MgCl<sub>2</sub> solution (25 mM)</b>	2
<b>Primer 18SUnivF (10 μM)</b>	1
<b>Primer 18SUnivR (10 μM)</b>	1
<b>dNTPs (10 mM)</b>	0.5
<b>GoTaq G2 Flexi DNA polymerase</b>	0.2
<b>Total</b>	23

Thereon, 2 μL DNA were added to 23 μL of Mastermix and the PCR program was run following the cycle settings showed in Table 6:

Table 6 - PCR Program settings.

<b>Program step</b>	<b>Time</b>	<b>Temperature</b>	<b>Number of cycles</b>
<b>Initial denaturation</b>	5 min	94 °C	1
<b>Denaturation</b>	30 s	95 °C	35
<b>Annealing</b>	30 s	55 °C	
<b>Elongation</b>	1 min	72 °C	
<b>Final elongation</b>	10 min	72 °C	1
<b>Hold</b>	∞	10 °C	-

The PCR product was separated by agarose gel electrophoresis. The agarose gel (1 %) was prepared in 1x TAE buffer. 5 μL PCR product were added with 1 μL gel red (1:500 dilution) and 2 μL of green loading dye. The sample was loaded on the gel, as well

as 6  $\mu\text{L}$  of EZ Vision ladder (marker of molecular weight). The electrophoresis was run at 90 V for 30 min. Then, the gel was observed under UV to evaluate PCR product length and their purity.

For the purification step, 20  $\mu\text{L}$  of the PCR product were transferred to 1.5 mL *Eppendorf* tube. 10  $\mu\text{L}$  NaAc (3M) and 125  $\mu\text{L}$  EtOH (100 %) were added. The sample was centrifuged at maximum speed for 15 min and the supernatant was discarded. Then, 125  $\mu\text{L}$  EtOH (70 %) was added to the pellet and again centrifuged at maximum speed during 15 min.

The resulting pellet was dried at 50  $^{\circ}\text{C}$ , resuspended in 20  $\mu\text{L}$  MQ water and the concentration of DNA was measured on *NanoDrop*<sup>®</sup> ND-1000 (*Thermo Scientific*) (Figure 9 A).

For the sequencing analysis, 5  $\mu\text{L}$  of the purified PCR product were mixed with the primers and analyzed by *Applied Biosystems HITACHI 3130xl, Genetic Analyzers* (Figure 9 B). The obtained sequence was 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

The results are the average of at least two replicates. Phylogenetic analysis was performed using *Maximum-likelihood (ML)*.



Figure 9- A: *NanoDrop*<sup>®</sup>; B: *HITACHI 3130xl, Genetic Analyzers*.

### Statistical Analysis

Analyses of linear regression, *t student* and multivariable statistic tests were carried out using the software *SPSS 25.0*. Levene's test was used to test for homogeneity of variance with significance level of 0.05. When three or more conditions were analyzed *ANOVA* was performed with the multiple comparison of *Tukey-HSD*. For the comparison of groups of independent results, a *t-student* test was used. A confidence level  $\geq 95\%$  was set. For each test, triplicate mean and standard deviation were determined.

## 4. Results and Discussion

### 4.1. Effect of nitrate concentration on growth and biochemical composition

Nitrogen (N) is an important constituent of biomass and it is commonly provided in the form of nitrate ( $\text{NO}_3^-$ ) or ammonium ( $\text{NH}_4^+$ ) to microalgal cultures (Richmond, 2004). N availability affects the cell growth and biomass composition, including the lipid content and fatty acid profile (Borowitzka & Moheimani, 2013).

In this study, *Chlorococcum* sp. was cultivated in laboratory 5 L air-lifts at 1 mM  $\text{NO}_3^-$  and 10 mM  $\text{NO}_3^-$ . The growth lasted 21 days. The microalgal growth, pigments, proteins and lipids content are reported in the Figure 10 and 11, for the two conditions respectively.

In agreement with the literature, the availability of N influenced the cell growth and the biomass composition (Borowitzka & Moheimani, 2013). There is statistical significance ( $p < 0.05$ ) between the biomass dry weight between the two conditions. Cultures grown at 10 mM of  $\text{NO}_3^-$  (Figure 11) have shown higher biomass concentration ( $1 \text{ g.L}^{-1}$ ). In the first 8 days, the growth curves between the two conditions are comparable because nitrogen is available in both culture medium. However, the growth of the microalgae drastically decreases around 10th day for test at 1mM of  $\text{NO}_3^-$  (Figure 10). N was completely consumed and the N-depletion limited the growth.

In the cultures cultivated at 1 mM  $\text{NO}_3^-$ , chlorophyll content drastically decreased during the growth, especially after the N-depletion ( $p < 0.05$ ). The same trend was registered for total lipid content ( $p < 0.05$ ). Total carotenoids and protein only registered values, with statistical difference ( $p < 0.05$ ), between exponential and stationary growth phases. In particular, carotenoids content decreased from 4 to 2  $\text{mg.g}^{-1}$  and the proteins decreased from 20 % to 10 %, from exponential to stationary growth phase.

In contrast, in cultures cultivated with higher  $\text{NO}_3^-$  concentration (10 mM), the content of total chlorophyll increased with the increase of biomass growth between latency and exponential phases ( $p < 0.05$ ), while not statistical differences ( $p \geq 0.05$ ) were registered between the exponential and stationary phase. The same results were registered for protein content. These results are extremely important for industrial cultivation since it allows to conclude that for chlorophyll and protein production (most demanded products by ALGFARM clients), there is no need of growing a culture of *Chlorococcum* beyond the exponential phase.

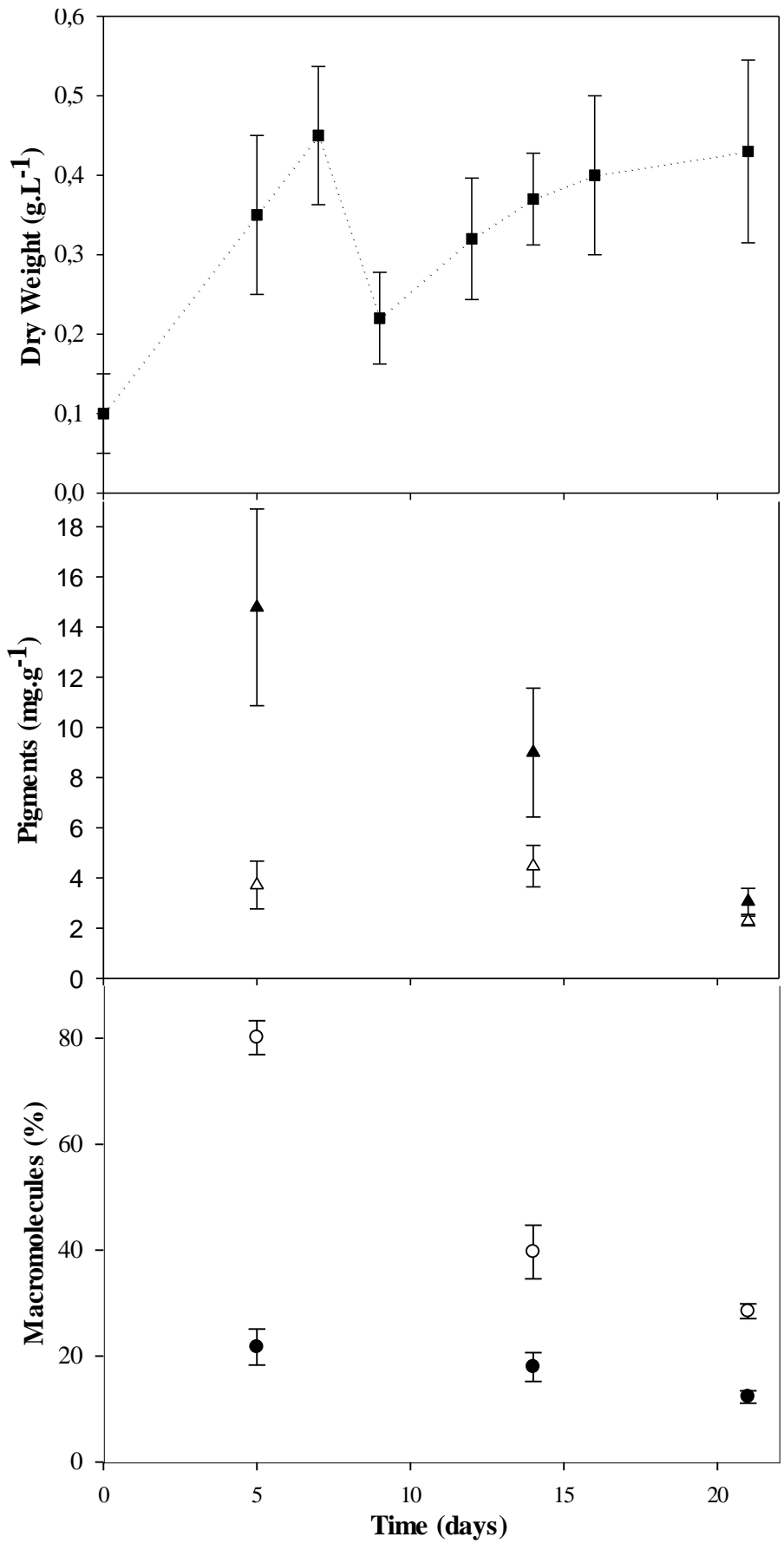


Figure 10 - Curve growth (■) of *Chlorococcum* cultivated at 1 mM of NO<sub>3</sub><sup>-</sup> and evolution of total chlorophyll (▲), total carotenoids (Δ), protein (●) and lipid (○) content during the growth.

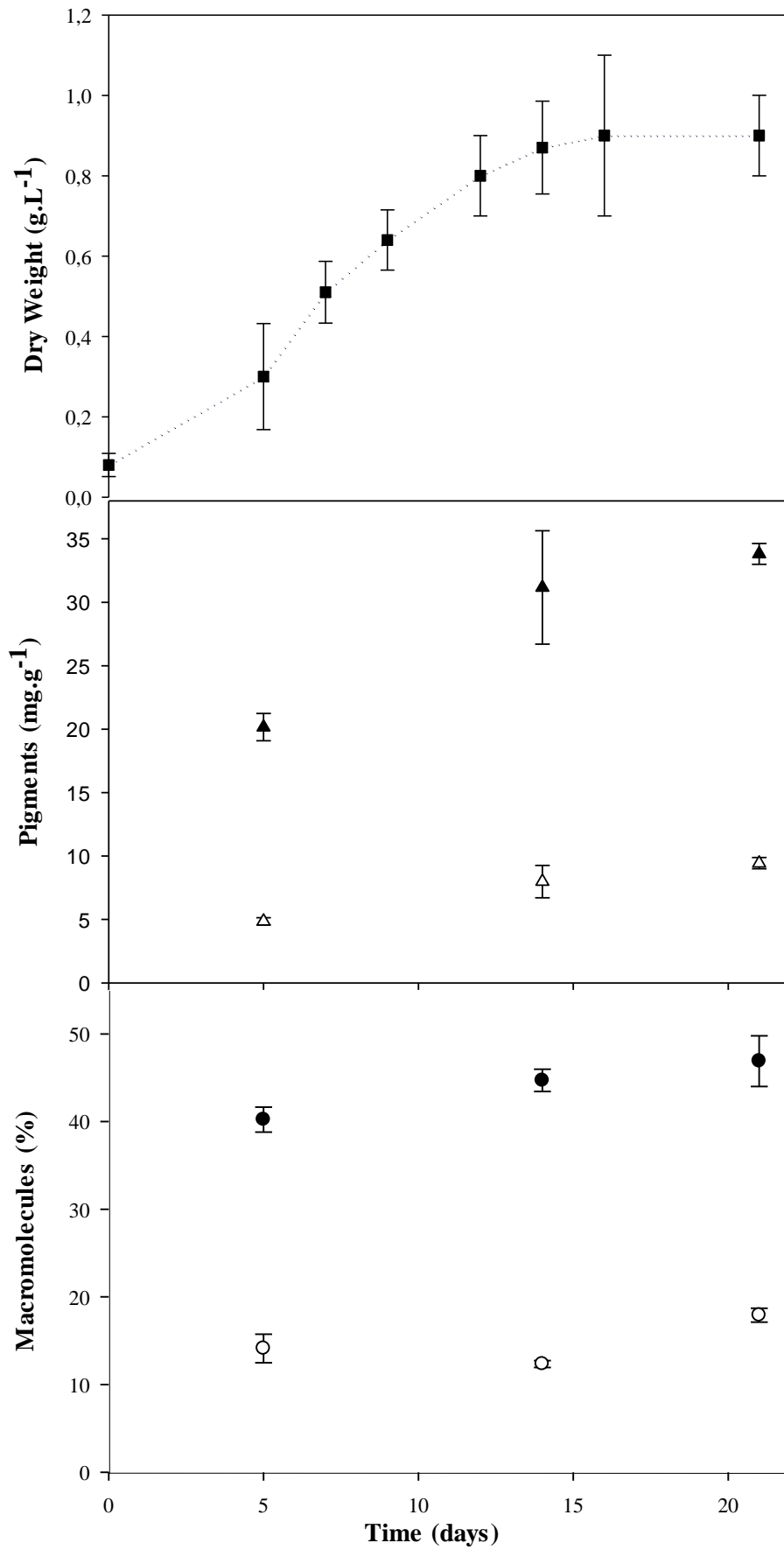


Figure 11 - Curve growth (■) of *Chlorococcum* cultivated at 10 mM of NO<sub>3</sub><sup>-</sup> and evolution of total chlorophyll (▲), total carotenoids (△), protein (●) and lipid (○) content during the growth.

Total carotenoid content increase along the growth phases with statistical differences ( $p < 0.05$ ), reaching a maximum in the stationary phase (Figure 11). The total lipids only shown an increasing value ( $p < 0.05$ ) between the exponential and stationary phase, due to the beginning of nitrogen depletion which stimulate the storage of energy in the form of lipids.

The two growth conditions were also compared in terms of the specific growth rates and the biomass productivity (maximum and global). The values are shown in Table 7:

Table 7- Values of productivities and specific growth rate of 5 L laboratorial air-lifts at 10 mM and 1 mM of  $\text{NO}_3^-$  cultivation.

Available N (mM)	Maximum volumetric productivity ( $\text{g.L}^{-1}.\text{day}^{-1}$ )	Global volumetric productivity ( $\text{g.L}^{-1}.\text{day}^{-1}$ )	Specific growth rate ( $\text{day}^{-1}$ )
<b>10</b>	$0.078 \pm 0.011$	$0.039 \pm 0.004$	$0.152 \pm 0.092$
<b>1</b>	$0.050 \pm 0.017$	$0.016 \pm 0.006$	$0.133 \pm 0.070$

Only for the global volumetric productivity were registered values with statistical significant differences ( $p < 0.05$ ) between the two conditions. However, the cultures cultivated with 10 mM  $\text{NO}_3^-$  showed best performance regarding the growth rate and the productivities, as expected (Borowitzka & Moheimani, 2013; Rasul *et al.*, 2017). As said before, the effect of the growth stages and the nitrogen depletion, on the growth and biomolecules accumulation, was already reported in the literature. Anyway, the present study is focused on a new isolated *Chlorococcum* specie and the physiological response to the environmental conditions needed to be confirmed before to start the real scale-up test.

#### **4.2. *Chlorococcum* sp. growth on large-scale photobioreactors**

The culture of *Chlorococcum* sp. was implemented from the laboratory scale to the large scale in outdoor conditions. The 5 L air-lifts of cultures grown at 10 mM of  $\text{NO}_3^-$  were used as inoculum for the 125 L GWP reactors, then scaled up to 250 L GWP reactors, after to the 2500L PBR. The final step reached the industrial scale 10000L PBR. The growth curves of *Chlorococcum* sp., in the different culture system mentioned, are shown in Figure 12:

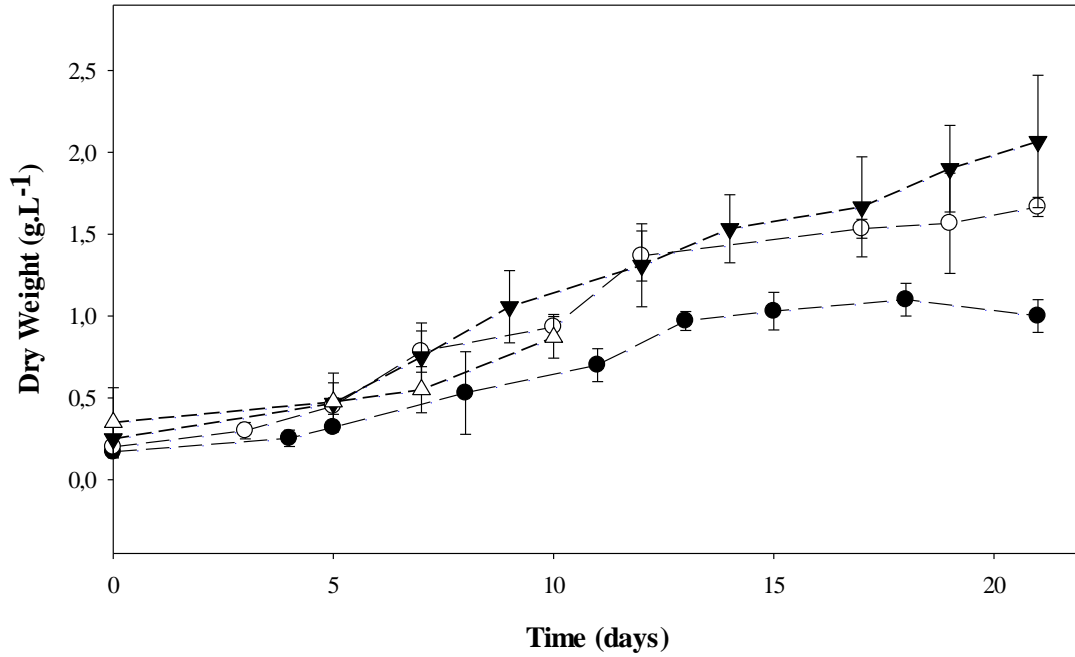


Figure 12 - Growth curves of scale-up: reactors 125 L GWP (●), 250 L GWP (○), pilot 2500 L PBR (▼) and industrial 10000 L PBR (△).

The experiments were carried out at different time periods, under different temperatures and light irradiance conditions. The 125 L GWP triplicates were cultivated during the winter, between January and February. The average of temperature was about 13.9 °C and of light irradiance was around 173 W.m<sup>-2</sup>. These were the worst growth conditions registered and might explain the lowest biomass concentration among the reactors. However, the 250 L GWP triplicates grown under similar conditions (13.6 °C and 190 W.m<sup>-2</sup>) and the biomass concentration among the growth was higher, although with no statistical significant difference ( $p \geq 0.05$ ).

The 2500 L PBR triplicates grown one after the other, from March to May. The average of temperature was 17.7 °C and of light irradiance was about 225 W.m<sup>-2</sup>. These results might explain the highest biomass concentration since the reactor grown under best climacteric conditions. Although, without statistical significant difference ( $p \geq 0.05$ ) from 250 L GWP.

The first triplicate of industrial 10000 L PBR grown during May and the other two, at the same time, from May to June. This reactor were cultivated under the highest light irradiance (about 260 W.m<sup>-2</sup>) and average of temperature 15.5 °C. Despite the good climacteric conditions, it registered biomass concentration values lower than the pilot reactor until day 10, without statistical significant difference ( $p \geq 0.05$ ). It was not possible to compare beyond 10 days of growth since this last experiment was carried out at the



production unit instead of the investigation unit, and the reactors can only be used at the times that are not being cultivated with cultures to commercialize. However, it was expected lower biomass concentration in comparison with the pilot 2500 L reactor since it is known from the company that the industrial reactors always use less light irradiation due to their height, which shade the lower tubes.

Additionally, the biomass productivities, specific growth rates and photosynthetic efficiency were calculated and the final values are shown in Table 8:

Table 8 - Values of volumetric and areal productivities of scale-up and industrial reactors.

Reactor	Volumetric Productivity (g.L <sup>-1</sup> .day <sup>-1</sup> )		Areal Productivity (g.m <sup>-2</sup> .day <sup>-1</sup> )	
	Maximum	Global	Maximum	Global
<b>GWP 125 L</b>	0.133 ± 0.029	0.040 ± 0.006	6.238 ± 0.675	3.175 ± 0.479
<b>GWP 250 L</b>	0.217 ± 0.018	0.070 ± 0.006	18.417 ± 1.503	5.937 ± 0.500
<b>PBR 2500 L</b>	0.153 ± 0.020	0.098 ± 0.007	16.250 ± 1.768	9.762 ± 0.673
<b>PBR 10000 L</b>	0.113 ± 0.005	0.054 ± 0.008	36.559 ± 1.521	17.419 ± 2.737

The higher overall volumetric productivity was obtained in the 2500 L PBR (p<0.05) despite the maximum volumetric productivity was reached by 250 L GWP (p<0.05).

However, when the areal productivity was analyzed, the most productive reactor was industrial 10000 L PBR (which registered the lowest volumetric productivity). The 10000 L PBR reached a maximum value of 36.559 g.m<sup>-2</sup>.day<sup>-1</sup> with statistically differences from the other reactors (p<0.05). These results are explained due to the geometry of reactors. Despite of industrial PBR uses less amount of the light irradiance that receives, as explained before, it affects negatively the volumetric productivity (which means that exist less biomass per litter), but because it is a greatly bigger reactor, the amount of biomass which is produced per area is also quite higher than within a pilot scale reactor. So, after all, it is better to grow microalgae at the industrial PBR due the highest amount of biomass collected at the end of the cultivation.

The specific growth rates and the photosynthetic efficiencies of *Chlorococcum* within the different reactors were calculated and the results are shown in Table 9:

Table 9 - Values of specific growth rates and photosynthetic efficiency on scale-up and industrial reactors.

<b>Reactor</b>	<b>Specific growth rate (day<sup>-1</sup>)</b>	<b>Photosynthetic efficiency (%)</b>
<b>GWP 125 L</b>	0.143 ± 0.007	0.406 ± 0.021
<b>GWP 250 L</b>	0.159 ± 0.003	0.735 ± 0.024
<b>PBR 2500 L</b>	0.126 ± 0.043	1.337 ± 0.127
<b>PBR 10000 L</b>	0.132 ± 0.048	1.399 ± 0.766

The growth of the cultures was faster in the 250 L GWP with specific growth rate of 0.159 day<sup>-1</sup>, without statistical significant differences ( $p \geq 0.05$ ). Despite registered the faster growth, the 250 L GWP shown low values of photosynthetic efficiency. This paradox has two main reasons: the first one, is the low light irradiance available during the cultivation period and the second one is the fact that the Green Wall Panel easily allows the fixation of biomass on the wall due to the worse aeration system, which gives less agitation, decreasing the absorbance of light.

The photosynthetic efficiency was higher in the 10000 L PBR, reaching values of 1.399 % with statistical significant differences from the 125 L and 250 L GWP reactors ( $p < 0.05$ ). This value was due to the good climacteric condition and sunlight exposure of the industrial PBR. There was no statistical significant difference in respect to the 2500 L PBR ( $p \geq 0.05$ ). It could be expected more photosynthetic efficiency from industrial scale 10000 L PBR since the photic area is substantially higher. However, the values are similar to the pilot scale 2500 L PBR because all the tubes in this last one are exposed to the sunlight irradiation and in the industrial PBR the light cannot reach the lowest tubes.

### 4.3. Biochemical composition of biomass grown in PBR

The biochemical composition of the biomass, harvested from the pilot scale 2500 L PBR and the industrial 10000 L PBR, were characterized and the results are shown in the Table 10:

Table 10 - Values of protein, total lipids, carbohydrates and ashes from 2500 L PBR and 10000 L PBR.

<b>Reactor</b>	<b>Protein %</b>	<b>Total Lipids %</b>	<b>Carbohydrates %</b>	<b>Ashes %</b>
<b>PBR 2500 L</b>	55.72 ± 2.846	18.35 ± 0.475	17.71 ± 3.730	7.61 ± 2.475
<b>PBR 10000 L</b>	46.71 ± 4.988	31.64 ± 4.640	5.76 ± 3.775	13.44 ± 0.346

Scientific studies reported that microalgae can reach 70 % of protein, 20% of lipid and 30 % of carbohydrates (Guedes & Malcata, 2012). In particular, for *Chlorococcum* sp. the content of lipids can reach values higher than 20 % (Prabakaran *et al.*, 2018).

The results of this study showed that proteins are the main component of *Chlorococcum* cells in both pilot and industrial scale PBR. The 2500 L PBR reached the highest protein content of 55,72 %. These results are comparable to that obtained in ALGAFARM for *Chlorella vulgaris* (55 % of protein) (Algae by Allma, 2018). The production of *Chlorella* and its protein content was optimized by the time, then it could be possible to increase the proteins content of *Chlorococcum* by medium adjustment or optimization of the growth conditions. The increase of the proteins content is a very important issue for companies involved in food and feed production, and *Chlorococcum* showed high potential in this context.

The lower value of protein measured for the 10000 L PBR was probably due to the stress conditions, characterizing that culture. Indeed, lower light irradiance were measured in that days with resulting lower photosynthetic efficiency (Table 9).

Although an increase on carbohydrates were expected, when proteins decrease (Ejike *et al.*, 2017), the content of carbohydrates was lower than in 2500 L PBR. On the contrary, the ashes content, during stress conditions, was reported increase for saline cultures. Then, more tests and in deep analysis of the metabolic pathways are needed to explain this behaviour in the industrial cultivation of this *Chlorococcum*.

The lipids content, in the largest culture, also increased up to 31.64 % with respect to the 2500 L PBR (18.35 %). The increased lipid content also suggests the existence of stress conditions.

The analysis of the fatty acid profile of this microalgal strain was carried for the biomass harvested in the two different PBRs. The fatty acid profile is a relevant factor influencing the food and feed applications of the whole microalgal biomass. Indeed, lipid fraction enriched in polyunsaturated fatty acids are characterized by higher market demand and high market value.

The fatty acids methyl esters (FAME) were analyzed by GC-MS and the results are shown in the Table 11:

Table 11 - FAME composition of *Chlorococcum* sp. on 2500 L PBR and 10000 L PBR.

FAME	% of Total FAME	
	2500 L PBR	10000 L PBR
<b>C14:0</b>	0.76 ± 0.094	2.88 ± 0.872
<b>C16:4 ω-3</b>	23.65 ± 2.201	21.94 ± 1.872
<b>C16:3 ω-3</b>	4.15 ± 0.399	2.75 ± 0.467
<b>C16:2 ω-6</b>	0.73 ± 0.079	0.90 ± 0.043
<b>C16:1</b>	2.59 ± 0.231	2.70 ± 0.587
<b>C16:0</b>	15.24 ± 1.603	18.82 ± 0.505
<b>C18:4 ω-3</b>	8.61 ± 1.150	19.29 ± 0.727
<b>C18:3 ω-3</b>	31.40 ± 2.800	27.58 ± 1.183
<b>C18:3 ω-6</b>	3.22 ± 1.258	3.21 ± 0.693
<b>C18:2 ω-6</b>	2.94 ± 0.781	3.73 ± 0.529
<b>C18:1</b>	5.34 ± 0.335	6.27 ± 0.759
<b>C18:0</b>	1.37 ± 0.559	2.71 ± 1.058
<b>SFA</b>	17.37 ± 2.415	23.92 ± 3.544
<b>MUFA</b>	7.93 ± 0.042	8.97 ± 1.356
<b>PUFA</b>	74.70 ± 2.456	64.30 ± 5.000

The main fatty acids produced in both reactors were C16:4ω3, C16:0, C18:4ω3 and C18:3ω3 (Table11). The functional sources of ω3 in microalgae are normally eicosapentanoic acid (EPA, C20) and docosahexaenoic acid (DHA, C22), however Chlorophytes are, in general, deficient in both C20 and C22 PUFAs. *Chlorococcum* sp. (belonging to Chlorophytes) has interesting ω3 fatty acids with lower carbon number, but with still significant applications in human and animal nutrition (Harun *et al.*, 2010; Guedes & Malcata, 2012).

The ratios of saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) are similar between the reactors. *Chlorococcum* sp. has demonstrated to be rich in PUFAs, with 74.70 % of total fatty acids in the pilot scale 2500 L PBR.

Fatty esters are derived from palmitic (C16:0) acid, stearic (C18:0) acid, oleic (C18:1) acid, linoleic (C18:2) acid and linolenic (C18:3) acid (Pinzi *et al.*, 2013). Despite of *Chlorococcum* fatty acids profile showed this components, the total lipids content it is not

high enough to consider this strain, since have been reported lipid contents as high as 85 % in certain microalgal strains (Rawat *et al.*, 2013).

The total chlorophyll and total carotenoids were analyzed by spectrophotometry for the pilot scale 2500 L PBR and for the industrial scale 10000 L PBR and the results are shown at Table 12:

Table 12 - Values of total chlorophyll and total carotenoids content by spectrophotometry on 2500 L PBR and 10000 L PBR.

<b>Reactor</b>	<b>Total Chlorophyll</b> mg.g <sup>-1</sup> DW	<b>Total Carotenoids</b> mg.g <sup>-1</sup> DW
<b>PBR 2 500L</b>	40.24 ± 7.941	10.87 ± 1.241
<b>PBR 10 000L</b>	25.81 ± 0.716	5.95 ± 1.365

For both chlorophyll and carotenoid content, the pilot scale 2500 L PBR reached the highest contents. Chlorophyll is one of the crucial factors indicative of healthy conditions of the culture.

Chlorophyll content of c.a. 4 % of dry weight is a great result for green algae (Harun *et al.*, 2010). In comparison, this *Chlorococcum* strain is able to easily achieve higher values of total chlorophyll than the optimized *Chlorella vulgaris* produced at ALGAFARM unit, which reach values of c.a. 2-3 % (Allmicroalgae, 2018).

With growth optimization, it could be possible reaching higher values of this pigment in *Chlorococcum* strain.

Also the total carotenoids value, of more than 1 % of DW, is higher than the current optimized produced *Chlorella vulgaris*, which cannot reach 1 % of DW (Allmicroalgae, 2018).

The reduced pigment content in industrial scale 10000 L PBR is probably due to the stress conditions or the change in the pH value (Liu & Lee, 2000) from the pilot reactor (7.5 - 8) to the industrial reactor (6 - 6.5).

To analyse the composition of total carotenoids, HPLC was performed and the results are shown in the Table 13:

Table 13 - Values of carotenoid composition by HPLC on 2500 L PBR and 10000 L PBR.

Carotenoids	PBR 2 500L		PBR 10 000L	
	mg.g <sup>-1</sup>	% Total carotenoids	mg.g <sup>-1</sup>	% Total carotenoids
Neoxanthin	0.63 ± 0.187	8.11 ± 1.303	0.14 ± 0.005	4.02 ± 0.004
Violaxanthin	0.33 ± 0.074	4.32 ± 0.338	0.13 ± 0.008	3.87 ± 0.104
Lutein	5.37 ± 0.780	71.01 ± 1.255	2.49 ± 0.086	73.27 ± 0.216
β-carotene	1.25 ± 0.138	16.57 ± 1.262	0.64 ± 0.028	18.84 ± 0.116

In both pilot and industrial scale reactors, lutein is the main component of total carotenoids, reaching values of more than 70 % of the total carotenoids. In the 2500L PBR, lutein reach 5.374 mg.g<sup>-1</sup>, which is similar to the values of several microalgae that have been reported as lutein producers such as *Scenedesmus almeriensis* and *Muriellopsis* sp. (Chlorophyta). These strains achieve lutein content of 4 to 6 mg.g<sup>-1</sup> in large-scale outdoor cultivation (Fernández-Sevilla *et al.*, 2010).

Since lutein is used as food dyes and as feed additives in aquaculture (Mostafa, 2012), *Chlorococcum* might be a good source for these applications. Also, it could be used for pharmaceutical and nutraceutical needs as treatment for people with AMD disease (Fernández-Sevilla *et al.*, 2010).

β-carotene is the second most abundant carotenoid in both tests and it is very important due to its ability to act as provitamin A (Mostafa, 2012). However, *Chlorococcum* has a low β-carotene content if compared to the main producer *Dunaliella salina* (11-21 mg.g<sup>-1</sup>) (Tinoco *et al.*, 2015).

Astaxanthin was not found as component of the carotenoids despite some species of *Chlorococcum* have the capacity to produce this pigment (Yuan *et al.*, 2002). However, in the present work, *Chlorococcum* was cultivated under proximate optimal conditions and the production of astaxanthin was not induced.

#### **4.4. Molecular identification of *Chlorococcum* sp.**

A molecular analysis was performed to identify the specie of *Chlorococcum* used in the present work. The strain was identified as *Chlorococcum* 0030CN (ALGAFARM collection identification).

In order to help on this specie identification, the knowledge and experience of Professor Fátima Santos (foundress of seaweed collection of University of Coimbra) in microalgae taxonomy were required. The results of her analysis suggested similarity with *Chlorococcum minutum*.

The results of the molecular analysis were organized in the diagram reported in the Figure 13. It is possible to confirm that there is an identity of 100% with *Chlorococcum* sp. but no homology is shown between the presented specie and the reported species. It is possible to observe that this strain has similarities with *C. aquaticum* and *C. minutum*, however, it is separated from them with branch value of 0.89.

This result does not exclude that the adopted strain could be a new specie, not yet identified. Another possibility is that the 18S rRNA gene is not sufficient to distinguish between *Chlorococcum* species. Further genes or ITS (internal transcribed spacer) must be tested in order to obtain more specific information about this strain (Bhola *et al.*, 2013; Bellinger & Sigee, 2015).

Morphology characters are also important to distinguish between *Chlorococcum* sp. and further analysis should be carried out, such as the shapes of vegetative cells, the thickness of the cell walls in old cultures, the size of cells in old cultures, and the stigma morphology of zoospores (Kawasaki *et al.*, 2015).

In addition, until now, *Chlorococcum* were classified mainly based on the morphology. Complete molecular analysis of the existing species could also notice that this strain belong to a specie which was misclassified (Kawasaki *et al.*, 2015).

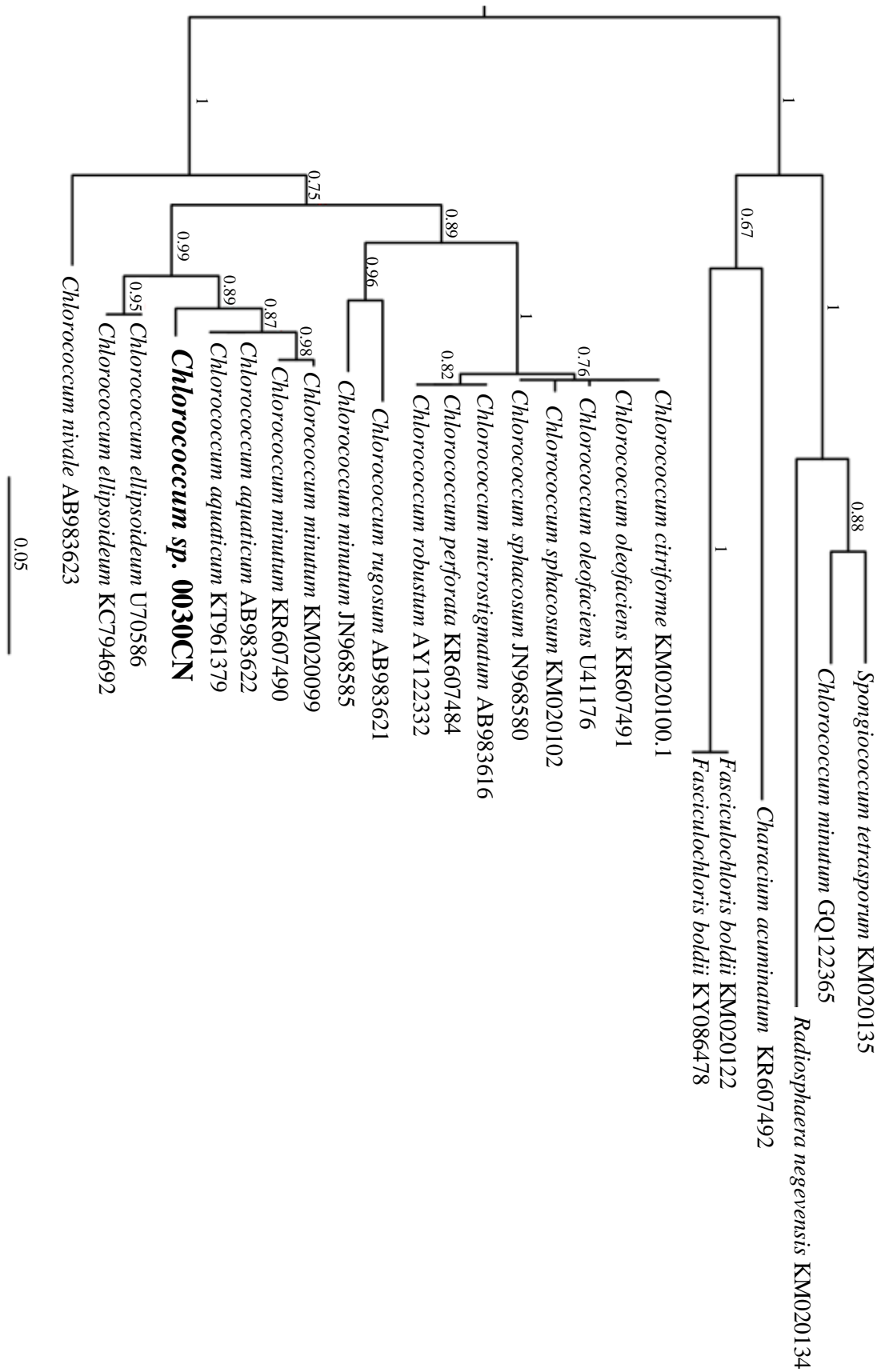


Figure 13 –Diagram obtained from molecular analysis of the isolated strain of *Chlorococcum* (0030CN) with *Maximum-likelihood* (ML).



## Conclusions

The *Chlorococcum* strain isolated in the ALGAFARM unit showed a great potential for feed and food applications, since it has plenty of protein and it also contain relevant amount of PUFAs. Chlorophyll and lutein content also find very interesting applications as natural food colorant, in nutraceutical and pharmaceutical industries. Then, *Chlorococcum* sp. biomass could be a promising environmental friendly alternative to the current consumer products.

The use of medium with 10 mM of nitrate was demonstrated to be more suitable for high biomass concentration and improved biochemical composition for food and feed applications (rich in protein, chlorophyll and total carotenoids). The industrial 10000 L PBR was less productive in terms of volumetric biomass productivity, although it reached highest areal productivity. The biochemical composition of *Chlorococcum* sp. was, in general, better in the pilot 2500 L PBR then in the larger PBR. Then, additional tests are needed for optimizing the growth and the biochemical composition of *Chlorococcum* in large scale culture system.

Interesting future perspectives about *Chlorococcum* sp. also regards astaxanthin production. Astaxanthin is synthesized from carotene by a pathway which differ from other astaxanthin-producing microorganisms. Then, further studies are needed to assess the potential of *Chlorococcum* sp. To accumulate astaxanthin and the optimization of the induction step should be carried out.

Moreover, the environmental application of *Chlorococcum* species as regard the CO<sub>2</sub> fixation is also a key issue for ALGAFARM company and there are literature data that refers some *Chlorococcum* species as good candidate for CO<sub>2</sub> fixation.

Finally, cost-effective cultivation of the autochthone *Chlorococcum* sp. could be reached by using open ponds culture system, which ALGAFARM is currently investing. This strain would be suitable for open culture because of its robustness and autochthone origins.

In addition, analyses of settling velocity suggest advantages in using *Chlorococcum* sp. since it has relatively big cells that would naturally precipitate when the agitation is stopped. In that way, harvesting costs could be reduced.

A polyphasic approach, including molecular phylogenetic, molecular structure and morphological comparison could allow an effective identification of *Chlorococcum* specie.

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