TÉCNICO LISBOA

Comparison of microalgae grown at pilot-scale under different operation regimes

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Thesis to obtain the Master of Science Degree in

Biological Engineering

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November 2019

Statement

I declare that this document is an original work of my own authorship and that it fulfills all the requirements of the Code of Conduct and Good Practices of the Universidade de Lisboa.

Inês Funico Guerra

Acknowledgments

Em primeiro lugar, tenho de agradecer à Joana Laranjeira pela oportunidade incrível, pela sua permanente preocupação e pela confiança e à Professora Marília Mateus pela disponibilidade e, principalmente, por me ter encorajado a aceitar esta oportunidade.

Ao Hugo tenho também de dizer um obrigado gigante por me ajudar a construir a minha tese, por me ajudar a ultrapassar todos os obstáculos que com ela também vieram e por me incentivar sempre a continuar, mesmo quando parecia que já nada tinha solução.

Tenho de deixar também um enorme obrigado à Joana Teles por me ter ensinado tudo o que eu sei sobre PBRs e outdoor e por continuar mostrar uma confiança enorme no meu trabalho, mesmo depois de todas as asneiras que eu ia fazendo. À Margarida agradeço por mostrar-se sempre pronta a ajudar-me, e por toda a amizade.

Não posso também deixar de agradecer a todas as outras pessoas da Allmicroalgae. Desde o Nuno que tantas vezes me safava sondas de pH, à Ana e ao Dinis que arranjavam sempre maneira de me fornecer cultura para que eu pudesse fazer os meus ensaios, à Maria que descodificava as minhas imagens microscópicas, ao Bernardo que me ensinou a lei do desenrasca, o Quelhas que tirava todas as dúvidas de processamento, às meninas do laboratório, à Soraia e aos operacionais de turnos que, de uma maneira ou de outra, me ajudaram neste percurso.

Agradeço também ao Professor João Varela e à sua equipa do CCMAR que colocou o seu trabalho em segundo plano para que os sete invasores do seu laboratório pudessem fazer todas as análises necessárias. Um obrigado particular à Tamára, que teve a maior paciência do mundo para tirar as nossas dúvidas e resolver os nossos problemas.

Um agradecimento muito especial tem de ir para “Os estagiários”, o Chico, o Pedro, a Joana, a Susana, a Rosângela e a Sara. Obrigada por todo a ajuda que me deram, fosse a reparar fugas ou a carregar sacos de sal algum de vocês estava sempre disponível para me ajudar! Obrigada também por me desencaminharem a ir à praia, pelas gargalhadas, pelas memórias fantástica, desde os jantares no sushi aos almoços no cantinho. Foram um apoio fantástico e uma família fora de casa. Obrigada também à Mafalda que tinha sempre uma palavra amiga a dizer e que conseguiu partilhar um T2 com estes sete macacos.

Não posso também deixar de agradecer aos meus amigos de faculdade que me acompanharam durante estes 5 anos de curso. Obrigada especialmente ao Marco, ao Pedro, ao Bruno, à Joana e à Bia pelas longas tardes de estudo, e pelas horas de almoço que se prolongavam com jogos de cartas. Sem vocês não sei se teria sobrevivido ao técnico.

Obrigada também às melhores colegas de quarto do mundo, Inês e Beta, obrigada por todo o apoio, por me acolherem no vosso espaço e por transformarem, desde o primeiro dia, o LAFL num lar. Obrigada também às pequenas do LAFL, que contagiavam qualquer uma de nós com a sua permanete boa disposição!

A toda a minha família, desde os meus avós, à minha irma e, especialmente, aos meus pais, um obrigada enorme por me apoiarem incondicionalmente, incentivarem e suportarem financeiramente,

não só durante estes 5 anos, mas durante a minha vida toda.

Por fim, obrigada ao meu namorado, Gonçalo, por apoiar todas as minhas decisões, por todo o carinho e por conseguir sempre reconfortar-me, mesmo nos piores dias.

Resumo

As microalgas podem ser cultivadas em diferentes regimes de operação, nomeadamente *batch*, contínuo e semi-contínuo, apresentando vantagens e desvantagens entre eles, relacionadas especialmente com a esterilidade, produtividade e consistência da qualidade do produto. Neste contexto, os três regimes de operação foram comparados, a fim de selecionar o mais sustentável para a produção de *Nannochloropsis oceanica* e *Chlorella vulgaris*, nas instalações da Allmicroalgae, durante as estações primavera/verão em fotobiorreatores tubulares de 2,6 m^3. Os resultados obtidos revelaram que o cultivo de *N. oceanica* em semi-contínuo e contínuo levou um aumento de 1,5 vezes na produtividade volumétrica (0,165 $\pm$ 0,013 e 0,154 $\pm$ 0,021 g $L^{-1}dia^{-1}$, respectivamente) em relação ao *batch* (0,108 $\pm$ 0,011 g $L^{-1}day^{-1}$). No entanto, os regimes semicontínuo e contínuo gastaram quase o dobro da quantidade de água que o *batch* (0,04 $\pm$ 0,03 e 1,25 $\pm$ 0,14 comparado a 0,62 $\pm$ 0,07 m^3 por kg de biomassa produzida). Relativamente ao cultivo de *C. vulgaris*, o regime contínuo apresentou a maior produtividade volumétrica 0,436 g $L^{-1}dia^{-1}$, seguindo-se 0,313 e 0,261 g $L^{-1}dia^{-1}$, obtidos nos regimes semicontínuo e *batch*, respectivamente. A quantidade de água gasta foi significativamente menor para *C.vulgaris*, em relação à *N. oceanica*, com 0,30, 0,67 e 0,81 m^3 de água gasta por kg de biomassa produzida para os sitemas de operação *batch*, contínuo e semicontínuo, respectivamente. Finalmente, o perfil bioquímico da biomassa produzida usando os diferentes regimes de operação não mostrou diferenças significativas em termos de proteínas, lipídos e perfil de ácidos gordos, em ambas as espécies de microalgas. Estes resultados mostram que mudar o regime de operação não afetou a qualidade final da biomassa.

Palavras-chave: *Nannochloropsis oceanica*, *Chlorella vulgaris*, *Batch*, Contínuo, Semi-contínuo, Escala Piloto

Abstract

Microalgae can be grown using different operation regimes, including batch, continuous and semi-continuous. The referred production strategies present different advantages and disadvantages among them, especially related with sterility, productivity and product quality. In this context, the three operation regimes were compared in order to select the most suitable one for the production of *Nannochloropsis oceanica* and *Chlorella vulgaris*, in the facilities of Allmicroalgae. Therefore, several trials were conducted during Spring/Summer seasons in 2.6 m^3 tubular photobioreactors. Obtained results revealed that *N. oceanica* grown using the semi-continuous and continuous operation regimes enabled a 1.5-fold increase in volumetric productivity (0.165 $\pm$ 0.013 and 0.154 $\pm$ 0.021 g L^{-1} day^{-1}, respectively) compared to batch regime (0.108 $\pm$ 0.011 g L^{-1} day^{-1}). However, the semi-continuous and continuous regimes spent nearly the double amount of water than the batch regime (1.04 $\pm$ 0.03 and 1.25 $\pm$ 0.14 compared to 0.62 $\pm$ 0.07 m^3 per kg of produced biomass). Regarding *C. vulgaris*, the continuous regime displayed the highest volumetric productivity 0.436 g L^{-1} day^{-1}, followed by 0.313 and 0.261 g L^{-1} day^{-1} obtained in the semi-continuous and batch regimes, respectively. Interestingly, the amount of water required was lower for this microalga, with 0.30, 0.67 and 0.81 m^3 of culturing water per kg of produced biomass for batch, continuous and semi-continuous regimes, respectively. Finally, the biochemical profile of produced biomass using the different operation regimes did not show significant differences in terms of protein, lipids and fatty acid profile, in both microalgae strains. These results show that changing the operation regime did not affect the final biomass quality.

Keywords: *Nannochloropsis oceanica*, *Chlorella vulgaris*, Batch, Continuous, Semi-continuous, Pilot-scale

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Nomenclature

Abbreviations

CIP	Clean in Place
DHA	Docosahexaenoic acid
DW	Dry weight
EPA	Eicopentanoic acid
FAME	Fatty Acid Methyl Ester
GHG	Greenhouse gas
GWP	Green Wall Panel
IBC	Intermediate Bulk Container
MUFA	Mono Unsaturated Fatty Acids
OD	Optical density
PBR	Photobioreactor
PUFA	Poly unsaturated Fatty Acids
PUFA	Polyunsaturated fats
SFA	Saturated Fatty Acids
SIP	Sterilization in Place
TAGs	Triglycerides

Chemical species

C	Carbon
CH_4	Methane
CO_2	Carbon dioxide
CO_3^{-2}	Carbonate

H	Hydrogen
H_2CO_3	Carbonic acid
HCO_3^-	Bicarbonate
N	Nitrogen
NH_4^+	Ammonium
NO_2	Nitorgen dioxide
NO_3^-	Nitrate
O_2	Oxygen
P	Phosphors
PO_4^{-2}	Orthophosphate

Chapter 1

Introduction

1.1 Contextualization

Major climate changes have been observed since 1950, and human activities are one of the main cause of those changes. The emissions of greenhouse gases (GHG), including CO_2, CH_4 and NO_2, have been increasing since the pre-industrial era and still remain in the atmosphere, soil and oceans (IPCC, 2015).

In this context, it is the utmost importance to find ways of living that do not affect or, if possible, help the environment to bounce back. The reduction in the emission of GHG and the consumption of the already emitted GHG can limit their impact on global warming (IPCC, 2015).

CO_2 is the most abundant GHG emitted by human activities, constituting almost 55% of those (Roy, 2018). One of the currently used strategies for CO_2 mitigation is the biological approach, that allows the biological conversion of CO_2 to organic matter, mainly performed by terrestrial plants or other photosynthetic organisms. Microalgae are currently considered a promising venue for industrial-scale mitigation of CO_2, since they grow much faster than terrestrial plants and can fix CO_2 using solar energy with an efficiency up to 10 times higher (Pires et al., 2012). The stoichiometrical demand is about 1.7 kg of CO_2 per kg of produced dry biomass (Posten and Schaub, 2009). In addition, microalgae also have the advantage of being used as waste water treatment, removing pollutants like ammonia, nitrate, phosphate and even heavy metals, with the concomitant capture of CO_2 (Abdel-Raouf et al., 2012).

Adding to the climate change issue, another significant problem of the century is the fast growth of the population, which is expected to reach 11.2 billion by 2100 (United Nations, 2019). This overpopulation will trigger an increase in food requirements and energy demand, which can cause resource-exhausting agriculture and fossil fuel exhaust, leading to irreversible environmental damages (Singh et al., 2017). In order to improve food security, microalgae were widely proposed as a promising feedstock to overcome the dependence on traditional food feedstocks. Microalgae biomass presents a high nutritional value, containing good amount of protein with all the essential amino acids (Gouveia et al., 2008) and fatty acids (Khan et al., 2018). Some microalgae have even the capacity to produce and accumulate high quantities of energy-rich oils that can be used to make biofuels (Hannon et al., 2010). Moreover, these microorganisms can be cultivated in saline/brackish water/coastal seawater on non-arable land, that do

not compete for resources with conventional agriculture (Khan et al., 2009).

Even though microalgae are associated to several benefits, industrial-scale production still displays high production costs limiting the commercialization of microalgae biomass to high-value markets. It is important to optimise biomass production in order to spend the least amount of resources possible, namely water, medium, energy and even occupied space. In this context, it is essential to study the growth of microalgae in different reactors, using different conditions and operation regimes, in order to grow microalgae at the maximum efficiency, and lowest environmental impact.

1.2 Secil/Allmicroalgae

SECIL is a business group composed of eight cement plants across seven countries, namely Portugal, Angola, Tunisia, Lebanon, Cape Verde, the Netherlands and Brazil. In Portugal, SECIL is present in Outão, Maceira and Pataias. The company main work relies on production and sale of cement, ready-mixed concrete, aggregates, mortars, prefabricated concrete structures and hydraulic lime (SECIL,2019).

Following the climate changes observed over the past century there has been a major concern in reducing its carbon impact (UNFCCC. Conference of the Parties (COP),2015). In this context, SECIL is promoting the use of secondary raw material and alternative fuels, with the purpose of capturing a part of the CO_2 generated by the plant in the cement manufacturing process. Since CO_2 is the main emitted GHG, SECIL has made significant investments in the microalgae investigation field (SECIL,2019).

In 2010 a project was started to capture and mitigate the cement plant emitted CO_2 founding a microalgae production plant, near the cement plant of Pataias, Allmicroalgae (Figure 1.1) (SECIL,2019).

Figure 1.1: Allmicroalgae facilities, located in Pataias, Alcobaça (Pontos de Vista, 2019)

Allmicroalgae presents a current production volume of 1300 m^3 of photobioreactores (PBR) (autotrophic production systems) and up to 5 m^3 fermenter vessels (heterotrophic production systems). After biomass cultivation, microalgae are harvested by ultrafiltration and pasteurized. The final biomass

products can be supplied in terms of paste or powder and, depending on the microalgae, it can be sold for food, feed and beauty care being found in the market by the brand Allma. Allmicroalgae focuses mainly in the production of *Chlorella vulgaris*, *C. vulgaris* BIO and *Nannochloropsis oceanica* due to their high market demand. Additionally, *Phaeodactylum tricornutum*, *Tetraselmis chui* and *Scenedesmus* sp., are also produced and commercialized (Allmicroalgae, 2019).

The production facility possesses several APCER certification given (ISO 9001, OHSAS 18001, ISO 14001 and ISO 22000) that demonstrate the company's quality, environmental, occupation health and safety and food safety management systems (Allmicroalgae, 2019).

1.3 Microalgae general characteristics

Microalgae are unicellular photosynthetic organisms that can efficiently fix CO_2 and, through photosynthesis, converting it into organic matter (Wang et al., 2008). In applied phycology, the science that studies algae, cyanobacteria are included in the microalgae group, together with microscopic algae, because they share a significant number of characteristics (Pulz et al.,2001), however, in a taxonomic approach, these organisms can not be considered part of the algae (Rastogi et al.,2017).

Autotrophic eukaryotic microalgae cells have organelles and cell walls with a microfibrillar layer of cellulose that can be delimited by an amorphous layer. However, some species lack the cell wall. The chloroplasts contain a series of thylakoids, containing chlorophylls and associated pigments (Rastogi et al., 2017).

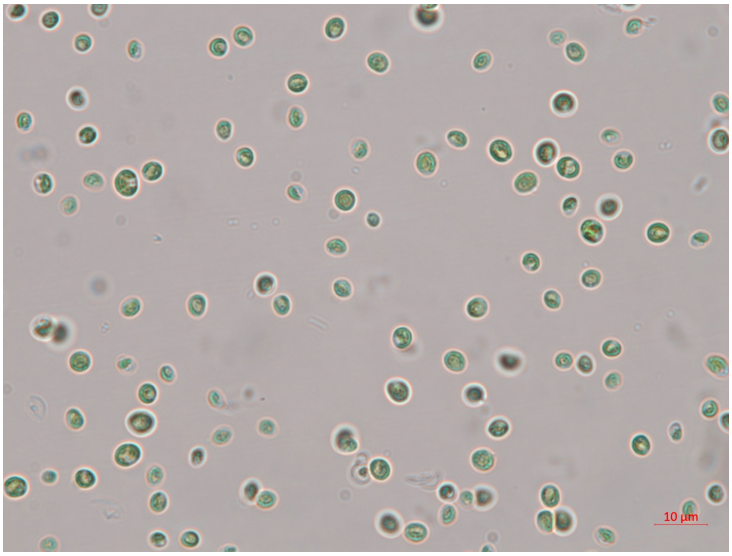


Figure 1.2: Microscopic view of *Chlorella vulgaris*, the first isolated microalgae. Image obtained with a 1000x magnification.

The current systems of classification of microalgae are based on the following main criteria: kinds of pigments, chemical nature of storage products and cell wall constituents. Additional criteria take into consideration the following cytological and morphological characters: occurrence of flagellate cells, structure of the flagella, scheme and path of nuclear and cell division, presence of an envelope of

endoplasmic reticulum around the chloroplast, and possible connection between the endoplasmic reticulum and the nuclear membrane (Richmond and Hu, 2004). In terms of abundance, these organisms can be divided in four big groups: diatoms represented by Bacillariophyceae, green algae represented by Chlorophyceae, blue–green algae represented by Cyanophyceae and golden algae represented by Chrysophyceae (Khan et al., 2009).

Microalgae are mainly found in the aquatic environments (freshwater and saline), even though they can also be found on the surface of soils and stone (Rastogi et al., 2017). They developed different physiological systems which allow them to support several, sometimes extreme, conditions. This way, they can even be found from deserts to polar seas (Rastogi et al., 2017).

In terms of energy and carbon source microalgae can be autotrophic, heterotrophic or sometimes even both at the same time and are capable to shift metabolism as a response to changes in the environment (Mata et al., 2010). Autotrophic microalgae require an inorganic source of carbon, salts and appropriate light source in order to grow, while heterotrophic use an external source of organic carbon and nutrients as their energy source (Bhola et al., 2014). Mixotrophic cultivation is a combination between the autotrophic and heterotrophic conditions where microorganisms have both a supply of inorganic carbon, to be fixed via photosynthesis, and some organic carbon source (Khan et al., 2018).

1.4 Nutritional needs

A basic microalgae cultivation medium must have nitrogen (N) and phosphorus (P) source as well as a set of needed micronutrients. It is also mandatory to have a carbon source, whether organic or inorganic (Carvalho et al., 2019).

Autotrophic microalgae use CO_2, and its derived aqueous chemical species, H_2CO_3, HCO_3^- and CO_3^{-2} as a carbon source, and light as energy source (Carvalho et al., 2019). The inorganic carbon conversion to organic mater, trough the use of light, corresponds to photosynthesis and occurs by the oxidation of water, releasing O_2 (Richmond and Hu, 2004). Heterotrophic algae use organic compounds, like glucose, acetic acid and fructose, as their carbon and energy source (Carvalho et al., 2019).

Nitrogen is, after carbon, the most important macronutrient since it's fundamental to the synthesis of amino acids and, consequently, protein and of any other N-containing molecules such as nucleic acids, poly-amines, quaternary ammonium compounds, and some hormones (Chen et al., 2017). This macronutrient can be supplied in the form of urea, nitrate (NO_3^-) and ammonia (NH_4^+)(Chen et al., 2017). NH_4^+ is the microalgae preferred nitrogen form since it can be directly incorporated into organic compounds (Richmond, 1986), however, is reported to be toxic at high concentration, therefore, the most widely used N-source for microalgae culture is the NO_3^- (Procházková et al., 2014).

Phosphorus is essential to the cell growth because it is a part of nucleic acid and phospholipid biosynthesis, and participates in modification of protein function and energy transfer (Moseley and Grossman, 2009). This way, it is involved in several crucial cellular functions, such as storing and exchange of energy and information in cells (Solovchenko et al., 2019). In microalgae cultivation is usually supplied as orthophosphate (PO_4^{-2}) and its uptake is energy-dependent (Procházková et al., 2014). The major

form in which cells acquire PO_4^{-2} is the inorganic one, since the organic compounds need to be first hydrolyzed by extracellular phosphatases (Procházková et al., 2014; Becker, 1994).

The most important micronutrients for microalgae are S, K, Na, Fe, Mg, Ca and trace elements such as B, Cu, Mn, Zn, Mo, Co, V and Se (Richmond and Hu, 2004). These micronutrients are crucial since they serve as cofactors in several essential metabolic pathways, and their requirements can differ, depending on the amount and form on which each of the micronutrients is available (Procházková et al., 2014).

Iron is the most important of the micronutrients since it fulfills fundamental catalytic roles and participates in photosynthetic electron transport chains (Raven et al., 1999), DNA synthesis and assimilative nitrate-reducing systems (Procházková et al., 2014).

Other parameters like light, temperature and salinity also severely affect microalgae growth and biochemical composition, thus has to be adequate (Richmond and Hu, 2004).

The light quality and quantity must be adequate in order to have a proper growth. Red, yellow and white lights, like the sun light, showed the best performed in microalgae growth rates (Wahidin et al., 2013). The intensity of light is also very important, as low-intensity light may not be able to deeply penetrate the culture, leading to lower productivities (Wahidin et al., 2013). The photoperiod also affects microalgae either in the culture growth, as in their biochemical composition, depending on the species and on the used light intensity (Wahidin et al., 2013; Rai et al., 2015).

After the light, temperature is the most important external factor for culturing microalgae, specially in outdoor. Normally microalgae can tolerate temperatures up to 15 °C bellow their optimal temperature, but beyond that temperature it's observable a drastic decrease in the growth rate, reaching lethal temperatures even after 2 °C in some species (Mata et al., 2010; Ras et al., 2013) .

Finally, salinity it is an important parameter as it affects microalgae growth and biochemistry through osmotic stress, ion stress and changes of the cellular ionic ratios due to the membrane selective ion permeability (Mata et al., 2010).

1.5 Microalgae cultivation

1.5.1 Industrial autotrophic scale production

When choosing a system for microalgae growth there are several factors to be considered, including the biology of the alga, the cost of land, the amount of man-work, energy and water consumption, nutrients requirements, climate to which are submited (if the culture is outdoors) and the type of final product (Borowitzka, 1992). For the autotrophic production of microalgal biomass there are two main used systems, namely the open and closed systems.

Open systems

Most very large scale commercial systems used today are open-air systems, because they are a more economically viable option (Borowitzka, 1999; Gupta et al., 2015). However, these systems are very

susceptive to contaminations, since they are constantly exposed to the atmosphere, thus only a small number of species resistant to extreme conditions can be grown in open pounds. For instance, the *Dunaliella* and *Spirulina* are currently produced in open systems using high salinity and alkalinity conditions, respectively (Pires et al., 2012).

The major types of open-air systems currently in use are natural water bodies, circular ponds, raceway ponds, and thin layer cascade systems. The raceway pound (Figure 1.3) is the most used design for industrial microalgae production since it allows achieving high productivities at a low cost (Bux, 2013).

Figure 1.3: Pilot Raceway pound. (Allmicroalgae, Pataias, Portugal)

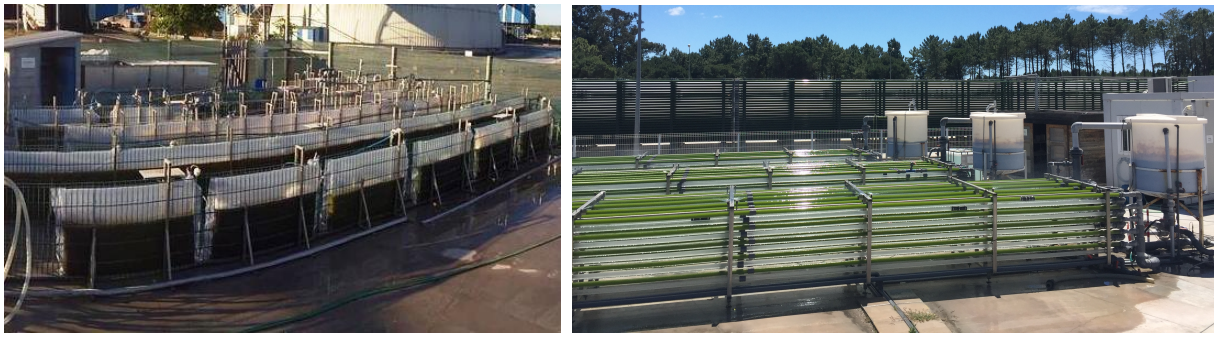
Although the fact that the low cost of open systems is a huge advantage, there are products for which the cultures must be maintained free of contaminants, as well as species of interest that do not grow in the referred highly selective environments.

Closed systems

Closed photobioreactors (PBRs) open a new world of possibilities since they allow to more easily control important physicochemical variables like pH, temperature, dissolved CO_2 and even salinity, as they have reduced evaporation rates, allowing to culture more sensitive species and obtain higher yields, whether in terms of growth or biochemical compounds (Suh and Lee, 2003; Mata et al., 2010). As they are closed, they strongly limit the direct gas exchange, reducing the contaminants in the culture making it easier to maintain it as a monoculture (Suh and Lee, 2003; Richmond and Hu, 2004). Some microalgae are used to obtain high-value products that are employed in pharmaceutical and cosmetics industries, being mandatory to reach a culture free of pollution, requiring growth in closed PBRs (Rastogi et al., 2017).

Industrial PBRs can be classified into three big groups, vertical column, flat panel and tubular PBRs (Wang et al., 2012).

Vertical column PBRs are usually cylinders with a small radius in order to increase the surface-volume ratio (Wang et al., 2012). The cultures are usually circulated by air pumped or an airlift system, having the advantage of low shear stress and a good mass transfer (Wang et al., 2012; Gupta et al.,



(a) Flat panel bioreactores, or Green Wall Panels.

(b) Pilot-scale serpentine tubular photobioreactor.

Figure 1.4: Closed autotrophic systems. (Allmicroagae, Pataias, Portugal)

2015). Vertical column reactors can be grouped into bubble columns or airlift reactors, in which the big difference is the existence of a physical separation of the riser and downcomer zones in the airlift reactor (Gupta et al., 2015).

Flat panel PBRs are also known as Green Wall Panel (GWP) and consist of vertical plate bioreactors mixed by air bubbling inserted directly from the bottom (Li et al., 2014). These PBRs are characterized by high surface to volume ratio that leads to high photosynthetic efficiencies. Although this characteristic allows flat panel systems to reach high cell concentrations, they are not very suitable for very large scale production due to difficulties in controlling the temperature, fouling and sterilization issues and high-stress damage caused by aeration (Gupta et al., 2015).

Tubular system reactors are one of the most promising and popular closed PBRs configurations (Molina et al., 2001; Wang et al., 2012; Gupta et al., 2015). These tubular PBRs are made of transparent materials and placed outdoor to use sunlight irradiation (Guedes et al., 2011). They can have several configurations such as i) serpentine, in which several straight transparent tubes are connected in series by U-bends to form a flat loop (the photo stage) that can be arranged either vertically or horizontally; ii) manifold, where a series of parallel tubes are connected at the ends by two manifolds, one for distribution and one for collection of the culture suspension and iii) helical where flexible tubes, generally of a small-diameter wound around (Richmond and Hu, 2004).

Tubular systems end up having a higher surface to volume ratio when compared to vertical columns because they can increase the length of the tubes while maintaining a small diameter without compromising the structural integrity (Wang et al., 2012). On the other hand, they hand up having problems with temperature control, as they overheat very easily (Wang et al., 2012). In comparison with flat panel systems, tubular PBRs have much higher areal productivities, but have higher accumulation of dissolved O_2 (Gupta et al., 2015).

A comparison between open and closed systems, more oriented towards raceway pounds and tubular PBRs, respectively, is presented in Table 1.1.

Table 1.1: Comparision between industrial microalgae cultivation in open and closed bioreactors. Adapted from Borowitzka,1999 and Pires et al., 2012. [a] Borowitzka,1999; Chisti,2007; De Vree et al., 2015. [b] Chini Zittelli et al., 1999; De Vree et al., 2015.

Factor	Open system (Raceway pound)	Closed system (Tubular PBR)
Ground space required	High	Low
Evaporation	High	No evaporation
Gas mass transference	Low	High
Mixing	Fair-good	Uniform
Light utilization	Fair-good	Excellent
Sterility	None	Achievable
Algal species	Restrictive	Flexible
Productivity	0.03-0.50[a]	0.10-0.90[b]
(g L^{-1} day^{-1}, in dry weight)		
Scale-up	Difficult	Easy
Harvest efficiency	Low	High
Harvest cost	High	Low
Capital investment	Low	High

1.5.2 Industrial heterotrophic production

Heterotrophic microalgae mass production is usually achieved by industrial fermenters (Figure 1.5).These systems have several advantages, they allow the production of algae in light independent process, being possible to reach higher cell densities (Chen, 1996) and higher productivities (Chen et al., 2015). However, the cell's biochemical characteristics often change during the absence of light, leading to microalgae with, for example, a more reduced percentage of pigments or protein, reducing the algae value (Barros et al.. 2019; Borowitzka, 1999). Another big problem of heterotrophic growth is that not all microalgae can be cultivated in this regime (Borowitzka, 1999).

Figure 1.5: Industrial 5000 L fermenter (Allmicroalgae, Pataias, Portugal).

1.6 Operation regimes

Biomass production in photobioreactors can be achieved using different operation regimes, namely batch, continuous and semi-continuous, which is a mixture of the first two.

The batch operation regime consists in the introduction of all the needed nutrients, with exception of carbon source for autotrophic cultivation, and a single culture, in the bioreactor, at the beginning of the batch, being this culture completely harvested after the production period (Zohri et al., 2017; Rastogi et al., 2017). In batch regime the growth ceases due to substrate depletion or growth-inhibiting product accumulation (Rastogi et al., 2017). In outdoor cultures, if not limited by substract, the culture can grow following an exponential phase, until their own cellular concentration doesn't allow the light to penetrate the culture, limiting, this way, its growth (stationary growth phase). The entire culture is usually completely harvested at mid to late logarithmic or stationary growth phase (Brown et al., 1993). The main advantage of batch production is its simplicity and flexibility, which allows to changes from species to species and fast fixing of system defects. Despite this, batch operation regime is not the most efficient one, since the cells go through various phases during the cultivation, which leads to productivity fluctuations (Rastogi et al., 2017). In addition, it presents a long non-productive time (for cleaning and sterilization), when comparing to other operation regimes (Lavens et al., 1996; Zohri et al., 2017).

In the continuous regime, the medium supplemented with all the needed nutrients (the feed) are continuously added to the cultivation system, while, simultaneously, the culture is continuously removed from the vessel, with the same flow of the added feed (Zohri et al., 2017). The continuous process can be divided into two categories: i) the turbidostat culture, in which the cell density is monitored and an automatic system maintains this parameter at a pre-set level by changing the dilution rate, or ii) a chemostat culture, in which a fresh flow of feed is introduced into the culture at a steady, pre-determined rate (Bharguram, 1999). This operation regime presents, most of the times, the highest producivities, since it is able to maintain the culture very close to the maximum growth rate, which also mean that they produce cells of more predictable quality (Lavens et al., 1996). However, these systems are often complex and costly. Currently, most microalgae continuous growth systems need constant illumination and temperature, being most of the time restricted to a small scale and indoors (Lavens et al., 1996; Zohri et al., 2017).

Semi-continuous operation regime may be regarded as a combination between the batch and continuous operations (Zohri et al., 2017). In this operation a percentage of the cultures (usually 10-50%) is removed when it reaches mid to late logarithmic phase and the volume is replaced with fresh medium (Brown et al., 1993). This process is repeated at constant intervals or when the culture reaches a specific cell density (Brown et al., 1993, Zohri et al., 2017). Semi-continuous production regime presents the advantages and disadvantages of both batch and continuous culture. It usually presents higher productivity than the batch regime, but is very susceptible to contaminants and metabolites eventual build up, that can render the culture unsuitable for further use (Lavens et al., 1996).

A comparison between the three operation regimes is resumed in Table 1.2.

In this context, the adequate production regime for effective microalgae production must be chosen

Table 1.2: Comparision between batch, semi-continuous and continuous operation systems. Zohri et al., 2017; Lavens et al.,1996; Bharguram, 1999

Factor	Batch	Semi-continuous	Continuous
Investment	Low	Higher	Higher
Operation control	Low	Medium-low	High
Sterility	High	Low	Low
CIP and SIP time	High	Low	Low
Possibility of species mutation	Low	High	High
Long term productivity	Low	High	Higher
Quality consistency	Low	High	Higher

considering the type of product and the already existing production systems in the facility. Although there is no overall better production method, the tendency in bioprocessing has been to adopt increasingly more continuous processes (Figure 1.6).

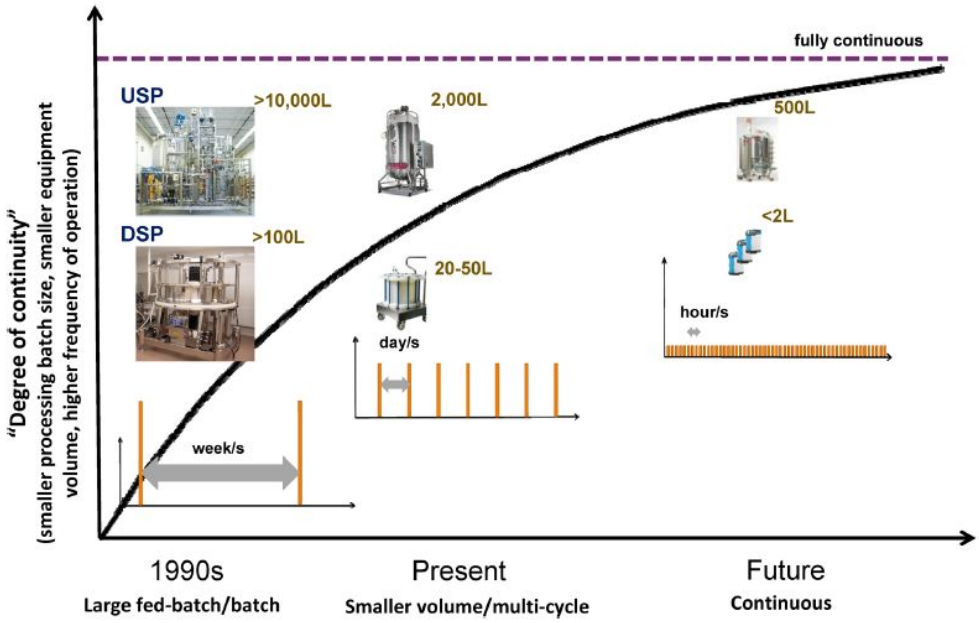


Figure 1.6: Bioprocessing trend over the last 20 years. (Croughan et al., 2015)

1.7 Biotechnological applications

The commercial culture of microalgae is now 50 years old, and had started with the cultivation of *Chlorella* and *Spirulina* for health food, *Dunaliella salina* for β-carotene, and *Hematococcus pluviavialis* for astaxanthin (Borowitzka, 1999).

Most these microorganisms have a complex chemical composition which makes them important resources for valuable and novel products in food, feed, pharmaceutical, ecological applications and research industry (Pulz and Gross, 2004).

Microalgae have great potential to be used as human food or supplements, since they have a protein

content as high as other food sources, namely eggs, meat and milk, presenting, in addition, all the essential amino acids, making it an adequate and sustainable food source (Gouveia et al., 2008). These microorganisms also present high content of polyunsaturated fatty acids (PUFA), principally omega-3 and omega-6 that are not produced by humans or animals (Khan et al., 2018). Examples of such omega-3, highly produced by some microalgae, are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) that are crucial to fetal development and cardiovascular function (Swanson et al., 2012). Regarding all this, microalgae are a completely vegan food alternative that is nutritiously rich and very sustainable (Bux and Chisti, 2016). Currently, there are only three microalgae approved for human consume, *Chlorella*, *Spirulina* (Vigani et al., 2015) and, most recently, *Tetraselmis chuii* (Segura et al., 2013). These products can be found in form of powder, tablets, capsules and liquids and can also be added to foods and drinks as a nutritional supplement or food colorant (Priyadarshani and Rath, 2012).

In the last years, has been an increase in the production of bioactive compounds from natural sources. Microalgae are a source caratenoids, like phycobiliproteins, phycocyanin, phycoerythrin, β-carotene, lutein and astaxanthin (Rao and Rao, 2007), that have anti-oxidant and anti-inflammatory effects (Zhang et al., 2016). In this context, these microorganisms are being used ever more to extract compounds that can be used either in cosmetics and in pharmaceuticals (Bhalamurugan et al., 2018).

Microalgae that present easy cultivation, lack toxicity, high nutritional value and a digestible cell wall can be used in feed (Hemaiswarya et al., 2011). In aquaculture microalgae are used to feed cultures of larvae and juvenile shellfish and fish (Chen et al., 2015), being these fed directly, or indirectly, through the use rotifers and daphnias previously fed by microalgae (Priyadarshani and Rath, 2012). Some of the microalgae generally used as feed are *Chlorella*, *Isochrysis*, *Phaeodactylum*, *Chaetoceros*, *Nannochloropsis*, *Tetraselmis*, *Dunaliella* and *Scenedesmus* (Bhalamurugan et al., 2018).

Some microalgae species, which have the capacity to accumulate high lipid percentages and have a rapid biomass production rate, are currently being studied to be used as biofuel feedstock with high areal productivities (Priyadarshani and Rath, 2012). Currently, several studies have been made for biofuel production through microalgae, in which *Chlorella*, *Scenedesmus*, *Dunaliella* and *Nannochloropisis* had shown great potential (Bhalamurugan et al., 2018). Microalgae have also been studied to produce biohydrogen and, resorting to microalgae with high starch accumulation capacities, bioethanol (Bhalamurugan et al., 2018).

In terms of CO_2 mitigation microalgae are promising candidates, since several species are capable of growing in, and consuming, high CO_2 concentrations from flue gas streams (Bhola et al., 2014). Wastewater treatment using microalgae is particularly attractive, because of their photosynthetic capabilities, converting solar energy into useful biomass and incorporating nutrients, such as nitrogen and phosphorus, otherwise causing eutrophication (Abdel-Raouf et al., 2012). Some microalgae used to wastewater treatment are *Chlorella*, *Spirulina*, *Scenedesmus* and *Chlamydomonas* (Mata et al., 2010; Salama et al., 2017).

Due to the high natural nitrogen and phosphorus content of microalgae they can be used as an alternative to the chemical biofertilizers, of which price are rising (Bhalamurugan et al., 2018). *Chlorella* (Jochum et al., 2018), *Spirulina* (Dineshkumar et al., 2019) and *Nannochloropisis* (Coppens et al., 2016)

are some of the microalgae that had already been studied as fertilizers and showed great potential.

1.7.1 *Nannochloropsis oceanica*

Nannochloropsis oceanica (Figure 1.7) is a unicellular small (2-4 μm in diameter) marine microalgae belonging to the eustigmatophyceae group and its taxonomic classification is given in Table 1.3 (Richmond and Hu, 2004; Khan et al., 2009).

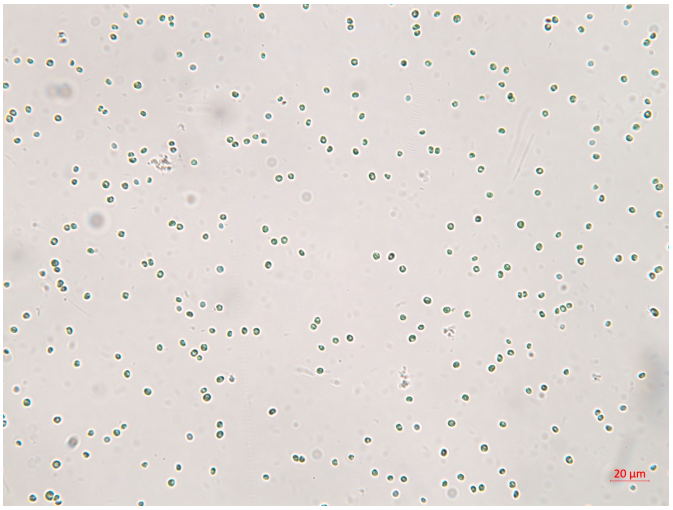


Figure 1.7: Microscopic view of *Nannochloropsis oceanica*. Image obtained with a 400x magnification.

Table 1.3: Taxonomic classification of *Nannochloropsis oceanica* (Worms - World Register of Marine Species, 2019)

Kingdom	Chromista
Division	Ochrophyta
Class	Eustigmaatophycea
Order	Eustigmatales
Family	Monodopsidaceae
Genus	Nannochloropsis
Specie	*Nannochloropsis oceanica*

These cells present a spherical to slightly ovoid shape, are non-flagellated and surrounded by a polysaccharidic cell wall (Richmond and Hu, 2004). Cells present a simple structure consisting primarily in a chloroplast, a mitochondrion with tubular cristae, and a nucleus (Cao et al., 2013).

The main pigments of *N. oceanica* are violaxanthin, vaucheriaxanthin and chlorophyll a. The lipid content of this algae is around 35 % (Ashour et al., 2019), with the most abundant fatty acids being palmitic (C16:0), palmitoleic (C16:1) and eicosapentaenoic (C20:5n-3, EPA) acids (Cao et al., 2013).

N. oceanica is commonly cultivated in fish hatcheries, to enrich zooplankton for fish larvae rearing, due to its high content of EPA (Nichols et al., 1996; Bhalamurugan et al., 2018). Due to the high rates

of lipid and biomass production *Nannochloropsis* sp. is a prominent candidate to the biofuel production (Liu et al., 2017). Between several *Nannochloropsis* species, *N. oceanica* is the one that presents one of the highest lipid contents and productivity, maintaining a considerable growth rate (Liu et al., 2017).

The most used cultivation mode to grow *N. oceanica* is autotrophy, although it can also be grown in mixotrophy (Ra et al., 2016; Mitra and Mishra, 2018). Regarding heterotrophy, there were not found studies reporting the successful grown of this species in literature (Přibyl and Cepák, 2019).

1.7.2 *Chlorella vulgaris*

Chlorella vulgaris (Figure 1.8) was the first microorganism to be isolated back in 1890 (Beijerinck, 1890, in Richmond and Hu, 2004). This microorganism is a unicellular fresh water cell included in the green algae group (Table 1.4). It has a diameter of 2-5 μm (Yamamoto et al., 2004), presents an ovoidal shape, it's nonmotile and can be cultivated in autotrophy, mixotrophy and heterotrophy (Richmond and Hu, 2004).

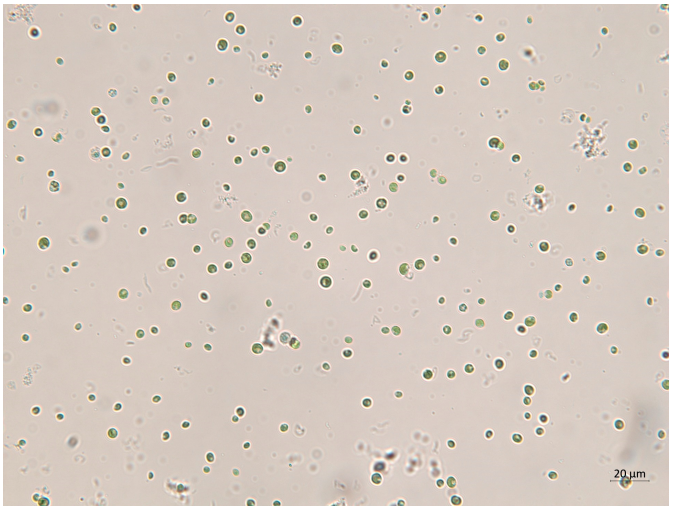


Figure 1.8: Microscopic view of *Chlorella vulgaris*. Image obtained with a 400x magnification.

Table 1.4: Taxonomic classification of *Chlorella vulgaris*. (Worms - World Register of Marine Species, 2019)

Kingdom	Plantae
Division	Chlorophyta
Class	Trebouxiophyceae
Order	Chlorellales
Family	Chlorellaceae
Genus	Chlorella
Specie	*Chlorella vulgaris*

C. vulgaris presents a high protein content, around 41-58 % (Priyadarshani and Rath, 2012), with a very good amino acid profile, as this species synthesizes essential and non-essential amino acids (Safi

et al., 2014). On the other hand, in order to maintain a good productivity *C. vulgaris* usually presents a low lipidic level, only around 20% of biomass dry weight (Liang et al., 2009).

Besides that, this algae presents interesting pigments being it's main chlorophyll a, that could reach 1-2 % of the cells dry weight, producing also several β-carotenes (González et al., 2015). These pigments are known by their antioxidant activities, regulating blood cholesterol, preventing chronic diseases and fortifying the immune system (Zhang et al., 2016).

Considering this, *C. vulgaris* revealed a huge potential to be used as human food, and even more as a food supplement (Safi et al., 2014).

1.8 Research aims

The main purpose of this dissertation is to assess how a biological system like microalgae will behave when grown under three different operation regimes, namely batch, continuous and semi-continuous. The main goals were to perceive which of the operation regimes would allow obtaining the highest volumetric and areal biomass productivities, using the least amount of resources possible, and still maintaining a good biochemical profile.

Chapter 2

Materials and methods

All the experiments described in this work were performed at the facilities of CMP/Allmicroalgae in Pataias (Secil Group, Portugal) between the 15^{th} of February and 15^{th} of September 2019. The biochemical profile of the cultured biomass was performed at MarBiotech group of the Centre of Marine Sciences (University of Algarve, Portugal) between 29^{th} of July and 9^{th} of August 2019.

2.1 Microalgae strain and culture media

The microalgae strains used for this work were *N. oceanica* and *C. vulgaris* from the culture collection of Allmicroalgae.

2.2 Scale-up of the cultures for industrial production

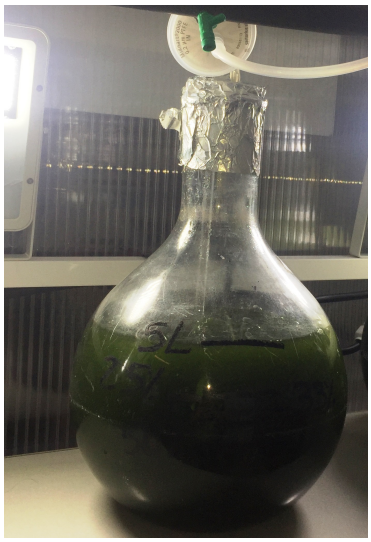
2.2.1 *Nannochloropsis oceanica*

N. oceanica was scaled-up from 5 L airlift reactors (Figure 2.1). The aeration of this reactors was made by compressed air pre-mixed with CO_2, in order to maintain the pH bellow 8.2, sterilized by 0.2 μm filters at the entrance. These reactors were maintained under a constant irradiance (24:0h photoperiod) of approximately 100 μmol of photons m^{-2} s^{-1} at ambient temperature ($\approx$ 25 $^{\circ}$C). The culture medium used to grown *N. oceanica* was modified Guillard's F/2 medium supplemented with iron, at a nitrate concentration of 5 mM, adjusted to a synthetic sea water made with local water, sea salt and magnesium water (salinity of 30 g L^{-1}).

Three of these 5 L reactors were used to inoculate a 125 L Flat Panel (FP) PBR which served as inoculum for an 800 L FP. The aeration conditions were similar to the 5 L reactors, although the CO_2 was added manually in order to maintain the pH bellow 8.2, while the temperature was maintained below 30 $^{\circ}$C with an irrigation system.

The 800 L FP served as inoculum to a 2.6 m^3 tubular PBR which latter was used as inoculum to three tubular PBRs. In these systems the agitation of the culture was performed by pumping the culture

through the PBR, using centrifugal pumps. The pH was measured in real-time and controlled at pH under 8.2 by an automated system that injected CO_2. The temperature was maintained bellow 30 °C through an irrigation system.



(a) 5 L bioreactor

(b) GW 125 L

(c) Three PBR 2.6 m^3

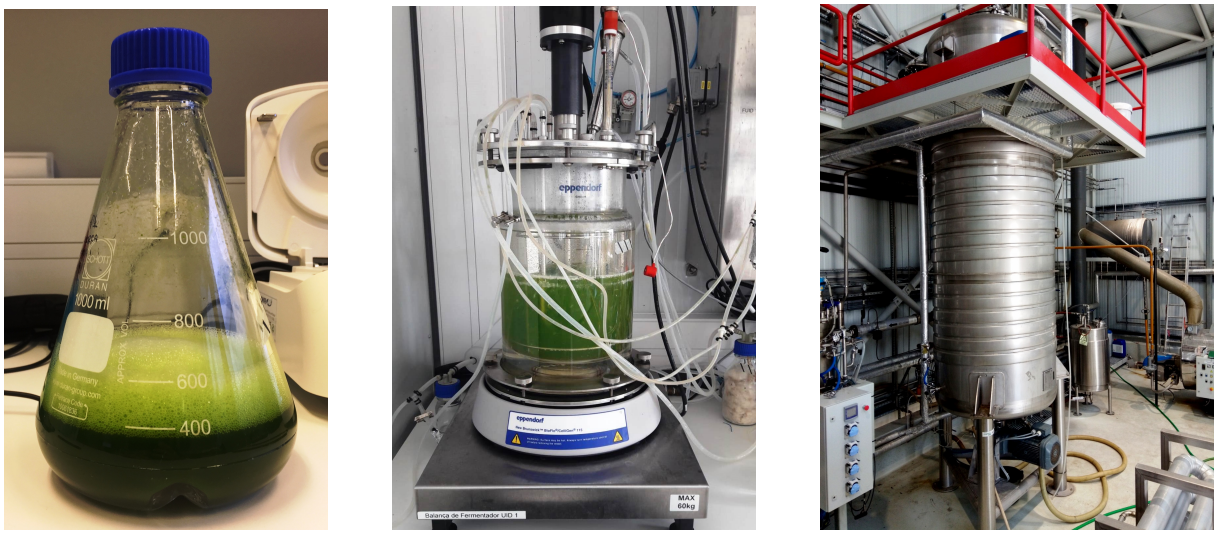
Figure 2.1: Some steps of the scale-up of the culture for industrial assays (Allmicroalgae, Pataias, Portugal).

After a first trial, the three 2.6 m^3 PBRs were inoculated with a percentage of a culture from a 35 m^3 tubular PBR in order to begin all the remaining rehearsals with the needed initial cell density.

2.2.2 *Chlorella vulgaris*

C. vulgaris was cultivated using a confidential Allmicroalgae biological medium, with ammonia as nitrogen source, adjusted to the local water composition. The three 2.6 m^3 PBRs were inoculated with a heterotrophic culture from Allmicroalgae production unit (Figure 2.2). This culture was initially scaled-up from a 50 mL Erlenmeyer flask, which functioned as inoculum to a 1 L Erlenmeyer. The Erlenmeyers

were supplemented with an in-house heterotrophic medium and kept in a climatized incubator at 28 °C and 200 rpm. This Erlenmeyer function as inoculum for a 5 L bench fermenter (New Brunswick BioFlo/CelliGen 115; Eppendorf AG, Hamburg, Germany) which latter inoculated a 5 m^3 industrial fermenter. The fermenters were mantained at 28 °C, using the above refered medium, with the pH maintained above 6.5 by the addition of a 24 % (m/m) ammonia solution. A percentage of the culture of the industrial fermenter was used to as inoculum for three 2.6 m^3 tubular PBRs for the following trials.



(a) 1 L Erlenmeyer (b) Bench fermenter (c) Industrial fermenter 5 m^3

Figure 2.2: Some steps of the scale-up of the culture for industrial assays (Allmicroalgae, Patais, Portugal).

2.3 Operation regime trials

For these assays, both species were grown in three tubular PBRs presented in Figure 2.3, each one of them being operated in batch, semi-continuous or continuous operation regimes. The tubular PBRs characteristics are presented in Table 2.1.

The culture was diluted to meet an initial dry weight of 0.3 g L^{-1} for the batch PBR and 0.9 g L^{-1} for the other two operation regimes, in a total volume of 2.6 m^3. The temperature was maintained below 30 °C by an irrigation system.

Table 2.1: Specifications of the pilot-scale photobioreactor used in this study. The presented ground area occupied is respective to two 2.6 m^3 PBR, as they share the same ground space.

Type of PBR	Configuration	Working volume (m^3)	Illuminated volume (m^3)	Illuminated surface(m^2)	Ground area occupied (m^2)
Horizontal tubular	Serpentine	2.60	1.60	144	52.8

The culture was left to grow for a day batchwise, in order to adapt to the new reactor conditions, and then each of the reactors was treated differently, depending on the correspondent operation regime.

Figure 2.3: Indutrial pilot PBRs used in the trials with 2.6 m^3 of working volume (Allmicroalgae, Pataias, Portugal).

In the semi-continuous mode, the culture was left to grow for further two days and, in the second day, was diluted with fresh ou saltwater, in order to reach 1 g L^{-1}. The medium was added manually when the nitrogen concentration fell under the desired one. This dilution process was repeated every two days until the end of the trial.

In the continuous regime, the culture was continuously being diluted by the introduction of fresh or saltwater by means of peristaltic pumps. At the same time, culture was removed from the reactor, by peristaltic pumps, at the same amount as the water was being added (scheme in Figure 2.4). The flow rate of the pumps was adjusted when needed in order to try to maintain the same cellular concentration in the reactor throughout the trial. The medium was added manually when needed, when the nitrogen concentration fell under the desired one.

The growth of each culture was evaluated daily, approximately at the same hour of the day for all the reactors, until the culture in the batch PBR reached the stationary phase. The nitrogen source concentration was measured, at least, once a day and this nutrient supplement added, manually, when needed.

Three consecutive replicates of each trial were attempted, always rotating the PBR used for the three used operation systems, in order to eliminate the error associated with the positioning of the reactor or even the reactor itself.

2.3.1 *Nannochloropsis oceanica*

During these trials the pH was measured in real-time and kept under 8.2 by an automated CO_2 injection system.

These cultures were grown at 30 g L^{-1} salinity. The salt-water used was synthetically prepared in independent 5 m^3 deposits by the addition of NaCl and magnesium water.

The nitrate concentration was maintained between 2 and 5 mM by the manual addition of medium. The iron was also manually added on the same proportion as the medium.

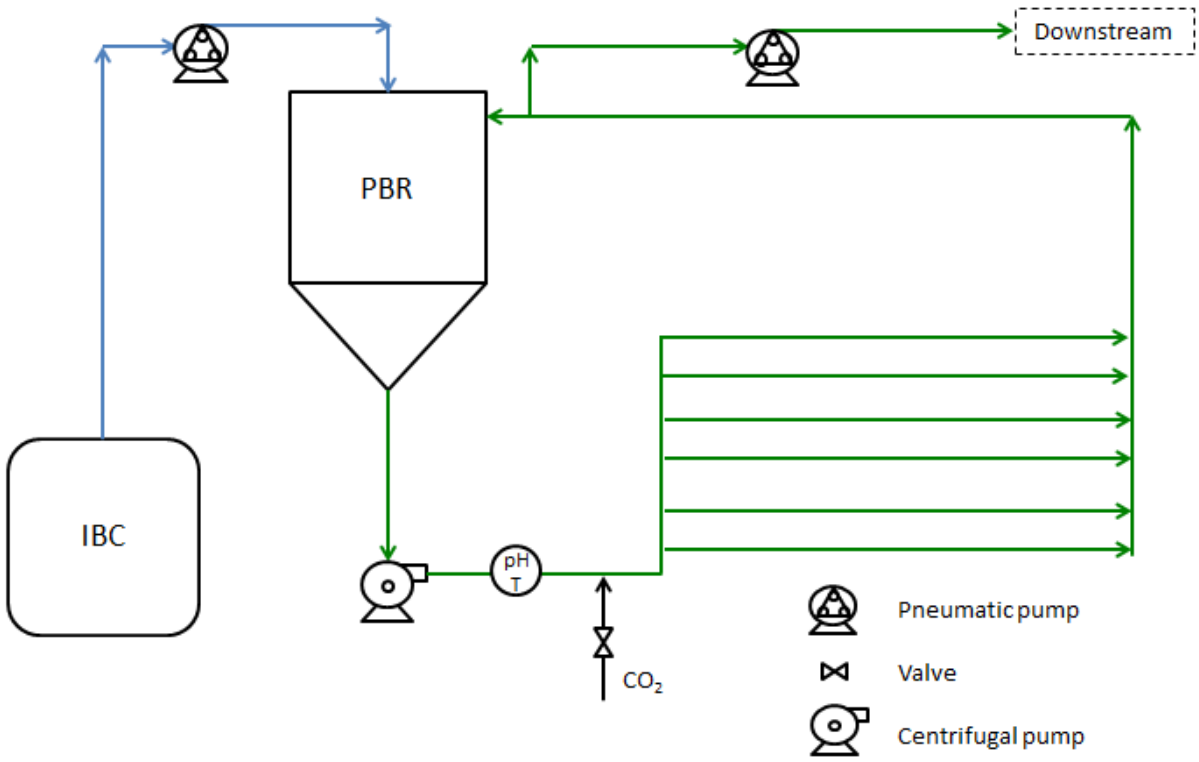


Figure 2.4: Schematic view of the tubular PBR functioning in the continuous operation regime. Blue arrows represent the water flow and green arrows represent the culture flow. pH and T represent sensors that measures simultaneously pH and temperature, respectively. The fresh or saltwater (depending on the species) was stored in the 1 m^3 intermittent bulk container(IBC) that was refiled when needed.

2.3.2 *Chlorella vugaris*

During the *C. vulgaris* trials, the pH was measured in real-time and kept under 6.5 by an automated CO_2 injection system.

The ammonium concentration was maintained between 0.5 and 2.0 mM, by the manual addition of medium. The iron and micronutrients were also manually added on the same proportion as the medium.

2.4 Growth assessment

The growth of microalgae was followed by optical density (OD) measured at a wavelength of 540 nm for *N. oceanica* and at 600 nm for *C. vulgaris* in an UV/Vis spectrophotometer (Zuzi, Spain and Thermo Fisher Scientific, USA).

The dry weight was obtained trough a correlation established by the calibration curve shown in Figure A.1 of the Apendix A and Figure A.2 of the Apendix A (equation 2.1 and equation 2.2, for *N. oceanica* and *C. vulgaris*, respectively).

$$DW = 0.244\, OD_{540} + 0.214 \tag{2.1}$$

$$DW = 0.427\ OD_{600} + 0.003 \tag{2.2}$$

Dry weight concentration (X, g L^{-1}) (equation 2.3) was obtained by filtering a known amount of culture in a 0.7 μm glass microfiber filter, which was later washed with an equal volume of ammonium formate (35 g L^{-1}) or distillate water, depending if the culture was cultivated in saline or fresh-water, respectively. This filter was previously and further dried and weighted using a *Kern DBS* moisture analyzer.

$$X(g\ L^{-1}) = \frac{filter\ weight_{after filtration} - filter\ weight_{before filtration}}{filtered\ volume} \tag{2.3}$$

The specific growth rate was calculated for the batch growth cultures through equation 2.4, where X_2 and X_1 represent, respectively, the cellular concentration in the end and beginning of the exponential phase and t_2 and t_1 are the times, in days, corresponding to those concentrations.

$$\mu\ (day^{-1}) = \frac{ln\frac{X_2}{X_1}}{t_2 - t_1} \tag{2.4}$$

The volumetric productivity (P) was calculated as the ratio of the sum of the produced biomass in each day ($m_{produced}$, g) by the total reactor volume (V_t, L) and correspondent time (t, day), as shown in equation 2.5. The produced biomass in each day (equation 2.6) was calculated by the difference in biomass amount existing in the reactor (given by the difference in the cell concentration, in two consecutive days, (X_1 and X_2, g L^{-1}) multiplied by the total reactor volume) summed to the amount of removed biomass in that day (given by the biomass concentration (X_2) multiplied by the corresponding removed volume on that day (V_r, L)).

$$P\ (gL^{-1}day^{-1}) = \frac{\sum m_{produced}}{V_t\ t} \tag{2.5}$$

$$m_{produced}\ (g) = (X_2 - X_1)V_t + X_2\ V_r \tag{2.6}$$

In the continuous operation regime the removed volume presents a floating concentration and it wasn't possible to measure that variance. Hence, for calculation purposes, it was considered that the concentration correspondent to the amount of volume removed in 24 hours was the concentration presented at the end of that time.

Areal biomass productivity (P_a) (equation 2.7) was determined by multiplying the volumetric biomass productivity by the volume of the reactor (V_t) divided by the ground area occupied by the reactor (A, m^2). The used reactors have their tubes meshed with another equal reactor, sharing the ground occupied area. This way, for the areal productive calculation, the considered volume was double the amount of the real culture volume.

$$Pa\ (gm^{-2}day^{-1}) = \frac{P\ V_t}{A} \tag{2.7}$$

The photosynthetic efficiency (PE) was determined by the ratio between the increase of the higher

heating value (HHV) and the total sun irradiation that reached the reactor (equation 2.8). The outside solar radiation and temperature were measured using a WatchDog 2000 weather station (Spectrum Technologies. Inc). The specific HHV (HHV_sp, kJ kg^{-1}) was calculated according to a previous correlation reported by Callejón-Ferre et al. (2011), present in equation 2.9, where C represents the percentage of carbon, H the percentage of hydrogen and N the percentage of nitrogen obtained by the CHN analysis to the final biomass.

$$PE\ (\%) = \frac{HHV * (biomass_{final} - biomass_{initial})}{Total\ incident\ radiation} \times 100 \tag{2.8}$$

$$HHV\ (kJ\ g^{-1}) = -3.393 + 0.507\ C - 0.341\ H + 0.067\ N \tag{2.9}$$

2.5 Nitrates and ammonium determination

For both analytical methods 1 mL of the samples was centrifuged for 15 min at 3500 rpm and only the supernatant was used.

Nitrates were determined according to Armstrong(1963). Briefly, 125μL of the collected supernatant were dilluted and 300 μL of hydrochloric acid (1 M) were added in a total volume of 10 mL. The absorbance of the samples was measured in as spectophotometer at 220 and 275 nm. The interference of the organic matter was corrected by subtracting two times the absorbance reading at 275 nm from the reading at 220 nm. The final absorbance was compared to the calibration curve, in order to obtain the nitrate concentration.

Ammonia concentration was determined through proper dilution of the sample and using an Ammonium-Ammonia Sera test (Sera, Heinsberg, Germany), according to the manufacture recomendations. The absorbance was measured at 697 nm and further compared to a calibration curve.

2.6 Biochemical composition

The biomass was collected and centrifuged at 3500 rpm for 15 minutes at the end of each culture trial. The resulting pellet was frozen and stored at -18 °C. Before the analysis the biomass was freeze-dried, being the powder used for all the needed analysis.

2.6.1 Elemental analysis (CHN)

Approximately 1 mg of lyophilized biomass was weighted in aluminium capsules. The capsules were inserted in Vario el III (Vario EL, Element Analyser System, GmbH, Hanau, Germany) in which the CHN composition was determined.

The total protein was determined by multipling the percentage of nitrogen by a factor of 6.25 for *Nannochloropsis* (Rasdi and Qin, 2015) and *Chlorella* (Molazadeh et al., 2019).

2.6.2 Ash content

The total ash content was determined using a gravimetric method. Samples were weighted before and after being burn in a muffle (J. P. Selecta, Sel horn R9-L) for 8 hours at 550 °C (Figure 2.5).

(a) (b)

Figure 2.5: Biomass before (a) and after (b) going into a muffle for 8 hours.

2.6.3 Total lipid determination

Total lipid content was determined following the Bligh Dyer method (1959) with few modifications by Pereira et al., 2011 (Figure 2.6). Approximately 10 mg of lyophilized biomass were weighed (w_i) in glass tubes and 0.8 mL of distilled water were added, followed by 1 mL of chloroform and 2 mL of methanol. Samples were homogenised in an IKA Ultra-Turrax disperser (IKA-Werke GmbH, Staufen, Germany) during 60 seconds on ice. Thereafter, a second extraction was performed by adding 1 mL chloroform and 1 mL of distilled water and homogenising again.

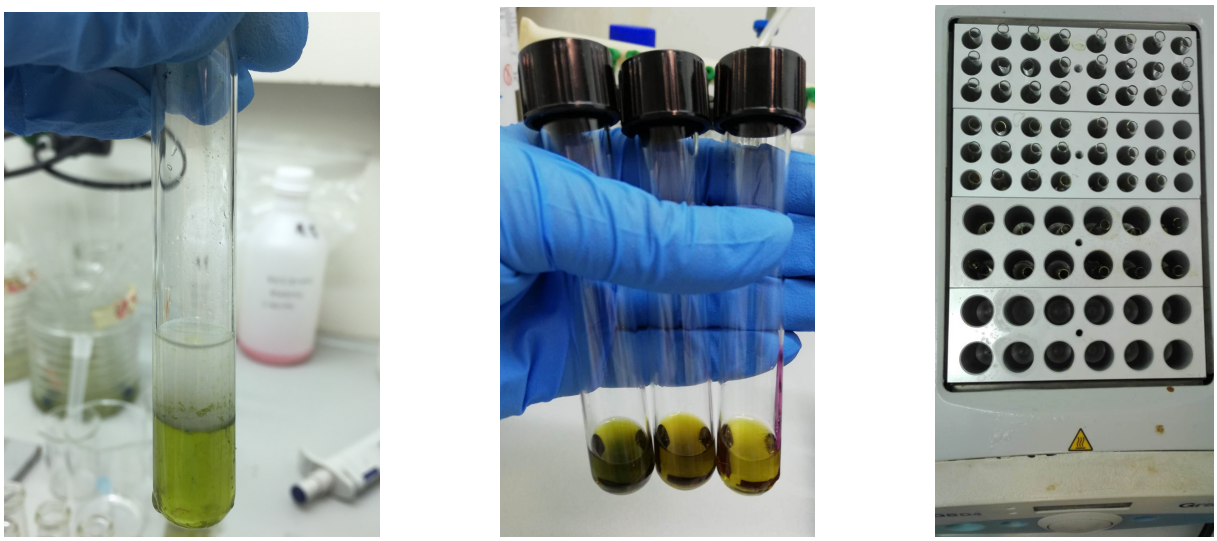
Afterwards, the mixture was centrifuged (Thermo Scientific Heraeus Megafuge 16Rat) at 2000 *g* for 10 minutes for phase separation. The organic phase (lower layer), containing mainly chloroform and lipids, was transferred to a clean tube with a Pasteur pipette. Later, a known volume of this organic phase (V_{evap}) was pipetted to another pre-weighed empty tube (w_1). This final tube was then placed in a dry bath at 60 °C to evaporate the chloroform.

The final tube with the dried residue resulting from the chloroform evaporation was weighed (w_2) in a precision balance. The mass percentage of lipids, in biomass dry weight, was calculated according to the formula present in equation 2.10:

$$Total\ lipid(\%) = \frac{\frac{(w_2 - w_1)\ Total\ vol.\ of\ chloroform\ (2\text{mL})}{V_{evap}}}{wi} \times 100 \tag{2.10}$$

2.7 Carbohydrates content

The carbohydrates content was determined by subtracting the weight of proteins, lipids and ashes to the total weight of biomass.



(a) (b) (c)

Figure 2.6: Some of the total lipid determination steps.(a) Separation of the organic and inorganic phases after centrifugation. (b) Chloroform with dissolved lipids ready to go into the final tubes wich will be placed in the dry bath. (c) Thermal dry bath.

2.8 Fatty acids profile

Fatty acids were converted into the corresponding fatty acid methyl esters (FAME) according to Lepage and Roy (1984), applying modifications described by Pereira et al. (2012) (Figure 2.7).

The samples were weighed into derivatization vessels and 1.5 mL of a solution containing methanol and acetyl chloride (20:1 v/v) were added. Then, the mixture was homogenized on ice with an Ultra Turrax for 90 seconds. Subsequently, 1 mL of hexane was added and the mixture was heated for 1 hour at 70 ^{o}C in a water bath.

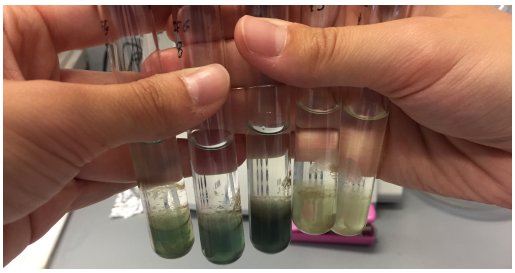
After this step, 1 mL of water was further added to the samples which were centrifuged at 3260 rpm (Thermo Scientific Heraeus Megafuge 16R) for 5 minutes.

The organic phase (top layer) was transferred to another vessel, dried with anhydrous sodium sulphate and filtered (0.22 μm). The filtrate was left to evaporate and further resuspended in 500 μL gas chromatography-grade hexane and placed into the appropriate vials for Gas Chromatography (CG).

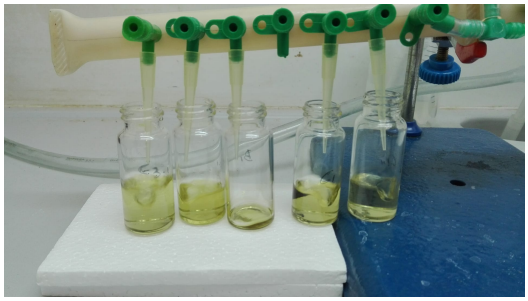
FAME were analyzed in a GC-MS analyzer (Bruker SCION 456/GC, SCION TQ MS) equipped with a ZB-5MS column (length of 30 m, 0.25 mm of internal diameter, 0.25 μm of film thickness, *Phenomenex*), using helium as carrier gas. The temperature program was 60 ^{o}C for 1 minute, increase of 30 ^{o}C per min up to 120 ^{o}C, increase of 5 ^{o}C per minute up to 250 ^{o}C, and final increase of 20 ^{o}C per min up to 300^{o}C. The temperature in the injector was 300 ^{o}C.

For the identification and the quantification of FAME five different concentration of Supelco 37 component FAME Mix standard (Sigma-Aldrich, Sintra, Portugal) were analyzed in order to establish 37 different calibration curves. Then the peak area of each component in each sample was compared to the corresponding calibration curve in order to have a quantitative analysis of that specific FAME.

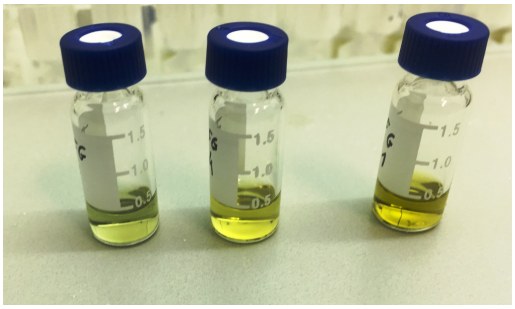
(a) Samples in the derivatization vessel, before homogenization.



(b) Separation of the phases after centrifugation.



(c) Evaporation phase.



(d) Sample in the gas chromatography vials.

Figure 2.7: Some steps of fatty acid sample preparation.

2.9 Statistical analyses

Statistical analyses were performed using R software (version 3.6.1) through RStudio IDE (version 1.2.1335). Experimental results are presented with 95 $\%$ confidence level, with a $p< 0.05$. Data were compared using one-way ANOVA, followed by a Tukey's multiple comparison tests. Whenever there were more than two replicates, the average and standard deviation were calculated.

Chapter 3

Results and discussion

3.1 *Nannochloropsis oceanica*

3.1.1 Preliminary assay

In order to access the optimal culture conditions to grow *Nannochloropsis oceanica* using the different operation regimes, a preliminary trial was conducted in three 2.6 m^3 PBRs. The cultures started all at the initial dry weight of 0.5 g L^{-1} (Figure 3.1).

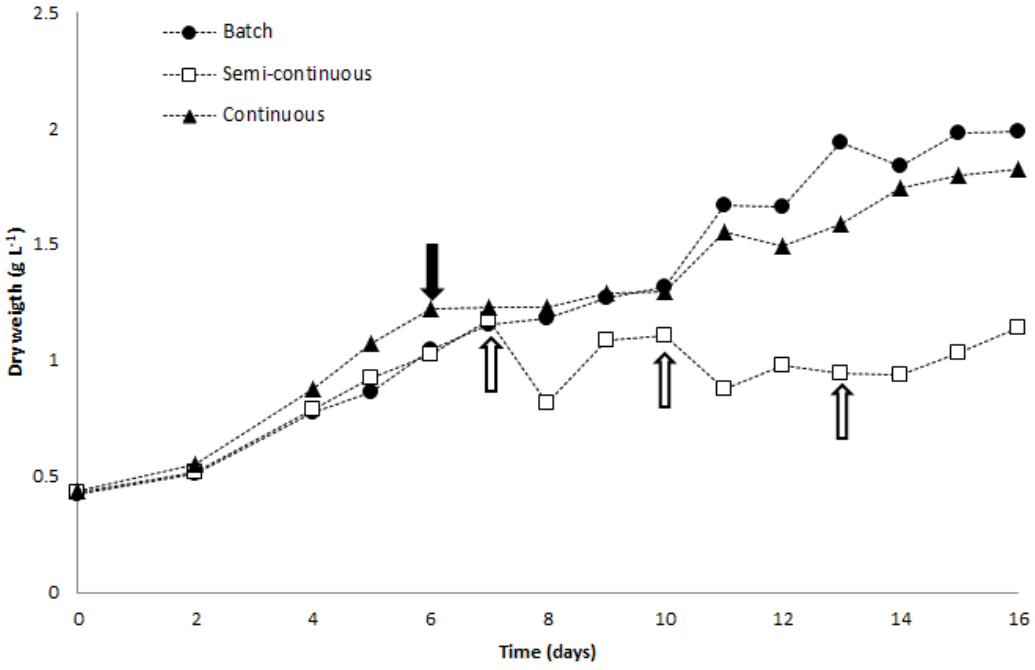


Figure 3.1: Growth of *Nannochloropsis oceanica* in 2.6 m^3 tubular PBRs using three different operation regimes: batch, semi-continuous and continuous. The black arrow represents the day in which the continuous regime PBR started to be diluted at an establish dilution rate of 0.10 day^{-1}, and the white arrows represent the moments of the semi-continuous regime PBR renovations.

The three cultures were grown during 16 days and presented a lag phase of 2 days. After the sencond day, the batch culture followed an exponential phase until the 7^{th} day, where the culture reached 1.7 g

L^{-1}. Hereinafter, the culture followed a slower growth phase, entering the stationary phase at the 13^{th} day, remaining like this until the end of the trial. The culture did not reach a decline phase and achieved a maximum dry weight of 2 g L^{-1}. The semi-continuous presented an exponential growth, from the 2^{nd} to the 7^{th} day, when it was renovated for the first time. The culture was again renovated on the 10^{th} and the 13^{th} day. After the 6^{th} day and until the end of the trial, the semi-continuous regime presented an dry weight between 0.8 and 1.2 g L^{-1}. Regarding the continuous operation regime, the culture presented an exponential growth from the 2^{nd} to the 6^{th}, when it started to be diluted, at a dilution rate of 0.10 day^{-1}. After this day, the culture maintained a dry weight of about 1.2 g L^{-1}, until the 10^{th} day. After this day the culture kept increasing in dry weight, reaching a maximum of 1.8 g L^{-1}. The continuous operation regime was the one which allowed to obtain the best results, reaching a productivity of 0.181 g L^{-1} day^{-1}, almost 2-fold than 0.097 and 0.096 g L^{-1} day^{-1}, obtained by the batch and semi-continuous operation regimes, respectively.

In the first 6 days the three PBRs were all at the same conditions, however, presented slightly different growth rates. This showed that, despite being side by side, some of the PBRs might get slightly different incident radiation rates or slightly different temperatures that affect the species growth. The discrepancy in the growth of the culture led to the start of the semi-continuous and continuous operation regimes in two different days, as they had been planned to start when they reached 1.0 g L^{-1}. After these results, the approach was changed, the batch being then started at 0.4 g L^{-1} and the continuous and semi-continuous at 1.0 g L^{-1}, in order to be able to start the two last regimes at the same time, with the same initial biomass concentrations, aiming to obtain more reliable results. With this in mind, the distribution of the operation regimes between the reactors was also more careful, having been all the operation regimes grown in all the reactors throughout the replicates.

In this first trial, the continuous regime was run as a chemostat, for which a previous defined continuous flow of 260 L day^{-1} of fresh media was added, which corresponds to a dilution rate of 0.10 day^{-1}. This proved not being very efficient, at least with the chosen flow, because the culture tended to a steady state at a high concentration, while the objective was to maintain it at a concentration in the exponential phase (between 0.5 and 1.15 g L^{-1}, as observed in the batch regime). Therefore, it was decided to grow the culture using a turbidostat approach,in which the biomass concentration was measured daily, and the flow was adjusted accordingly, in order to maintain the cultures around 1.0 g L^{-1}. This biomass concentration is in the exponential phase, that is the point with the highest growth rate.

The semi-continuous operation regime was, in this first assay, left to grow until 1.0 g L^{-1} and then taken the needed amount of volume to reach a 0.8 g L^{-1} concentration. As the continuous approach was being changed, it was thought that a better methodology would be to let the system grow for two days, and then collect the necessary amount of volume for it to reach a concentration of 1.0 g L^{-1}, in order to start at the mid-exponential phase concentration as well.

3.1.2 Growth in three different operation regimes

After the preliminary growth trial, *N. oceanica* was grown under batch, semi-continuous and continuous operation regimes in three 2.6 m^3 horizontal tubular photobioreactors, in triplicate.

The study occurred from, May 14^{th} until July 8^{th} and the main environmental abiotic parameters measured were the ambiance temperature and the incident solar radiation (Figure 3.2). The average ambiance temperature throughout the whole trial was 17.5 $\pm$ 0.8 °C, with a maximum and minimum of 21.1 $\pm$ 2.8 °C and 15.6 $\pm$ 2.3 °C, respectively. The temperature of the culture inside the reactor was usually higher than the ambiance temperature, and it was possible to maintain it bellow 30 °C through the thermoregulation system. The average daily solar radiation throughout the trial was 20.1 $\pm$ 3.5 MJ m^{-2}, with a maximum registered average daily radiation of 23.8 $\pm$ 2.4 MJ m^{-2} and a minimum of 15.4 $\pm$ 1.6 MJ m^{-2}.

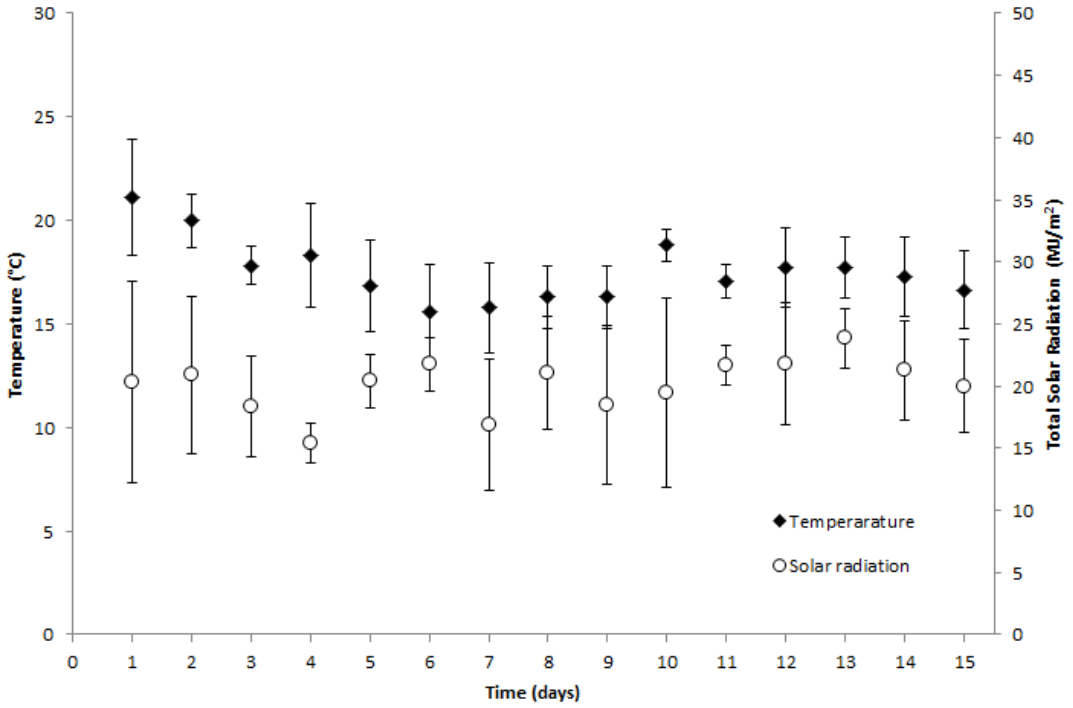


Figure 3.2: Medium daily ambiance temperature and total solar radiation incident in the 2.6 m^3 PBRs. The presented values are the average of the values obtained in three biologically independent replicates and the error bars represent the respective standard deviations.

Based on the preliminary assay, the cultures were grown during 15 days (growth curves in Figure 3.3) and started at 0.4 g L^{-1} for the batch regime and at 1.0 g L^{-1} for the semi-continuous and continuous operation regimes. In Figure 3.4, it's observable the growth curve of the culture in the semi-continuous and continuous operation regimes and also the daily percentage of renovated volume, in each one of those regimes.

The batch culture presented 1 day of lag phase, followed by an exponential phase of 8 days. It began entering the stationary phase around the 9^{th} day, ceasing the growth in the last day, reaching a maximum dry weight of 2.0 g L^{-1}. The maximum specific growth rate of the batch culture was 0.129 day^{-1}, calculated from the 4^{th} to 9^{th} culture day.

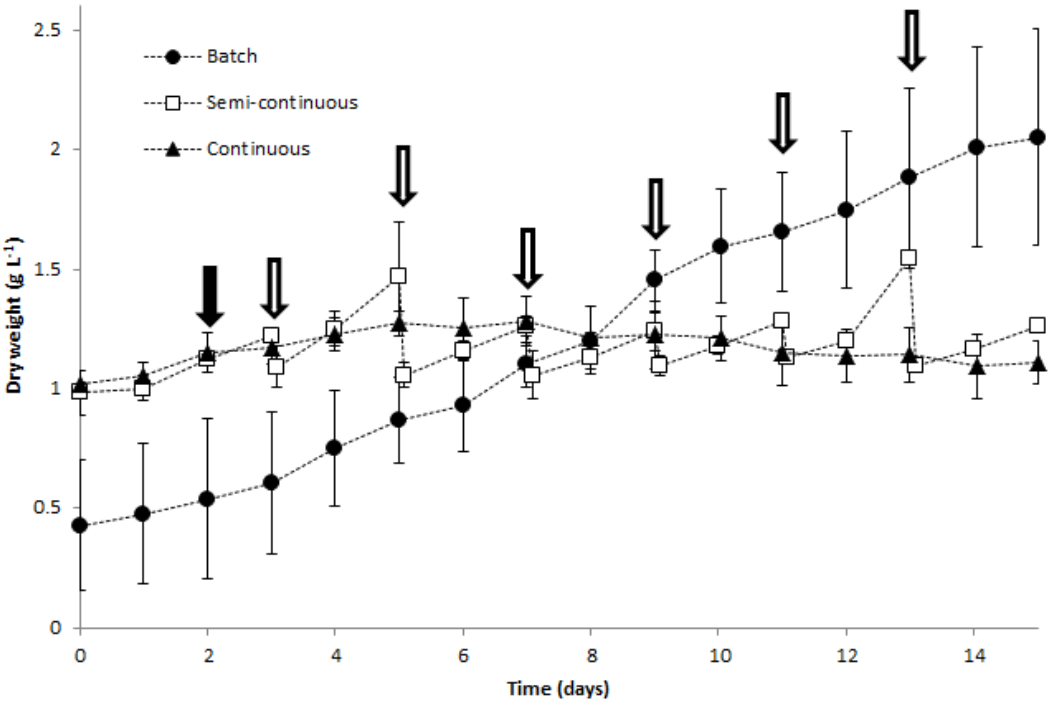
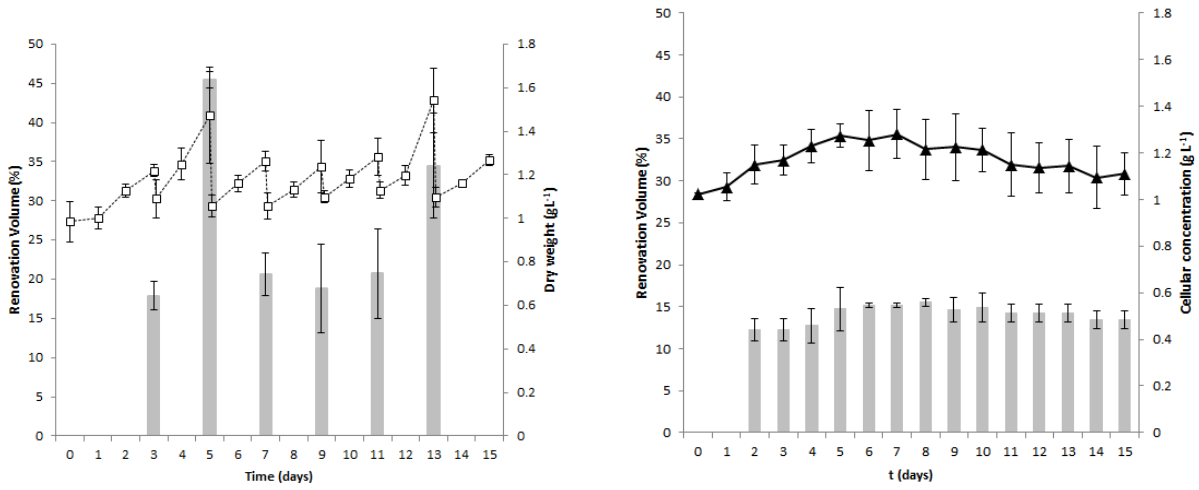


Figure 3.3: Growth, in triplicate, of *Nannochloropsis oceanica* in 2.6 m^3 tubular PBRs using three different operation regimes: batch, semi-continuous and continuous. The values presented are the average values of the three independent biological replicates and the error bars are the respective standard deviations. The black arrow represents the day in which the continuous regimes started to be diluted, and the white arrows represent the moments of the semi-continuous regime renovations.



(a) Semi-continuous

(b) Continuous

Figure 3.4: *Nannochloropsis oceanica* growth in a 2.6 m^3 PBRs in semi-continuous and continuous operation regimes. The bars represent the percentage of renovated volume. All the values are an average, with the respective standard deviation, of three biologically independent replicates.

The semi-continuous culture was renovated every second day, beginning at the 3^{rd} day and being renovated five more times during each trial. The renovation volume ranged between 17.8 $\pm$ 1.8 $\%$ and 45.4 $\pm$ 1.0 $\%$ of total volume, having an average dilution rate of 0.144 $\pm$ 0.013 day^{-1}. The culture dry weight ranged from 1.0 to 1.5 g L^{-1}.

The continuous culture started to be diluted on the 2^{nd} day, presenting a maximum and minimum renovation volume of 15.5 $\pm$ 0.5 $\%$ and 12.2 $\pm$ 1.4 $\%$ of total volume, respectively. The average dilution rate throughout the whole trial was 0.140 $\pm$ 0.010 day^{-1} . After the second day it was possible to simulate a steady-state, with the dry weight ranging from 1.1 to 1.3 g L^{-1}.

The amount of produced biomass per 2.6 m^3 PBR and per operation cycle was calculated through the growth curves, present in Table 3.1. In that table, and throughout this work, different letters in the same column represent significant different values ($p < 0.05$), while equal letters represent non significantly different values ($p \geq 0.05$).

The batch regime produced 4.23 $\pm$ 0.43 kg of biomass while the semi-continuous and the continuous systems produced significantly higher amounts of biomass of 6.44 $\pm$ 0.50 kg and 6.02$\pm$ 0.81 kg of biomass, per 2.6 m^3 PBR and per operation cycle, respectively. The latter operation regimes did not display significant differences among them.

Table 3.1: Produced biomass, per 2.6 m^3 PBR and per operation cycle, specific growth rate and average dilution rate of the three operation regimes. The values represent the average and standard deviation of three biologically independent replicates. Different letters within the same column represent significant different values.

Production regime	Produced biomass (kg)	Specific growth rate (day$^-1$)	Average dilution rate (day$^-1$)
Batch	4.23 $\pm$ 0.43 ^{a}	0.129 $\pm$ 0.020	-
Semi-continuous	6.44 $\pm$ 0.50 ^{b}	-	0.144 $\pm$ 0.013 ^{a}
Continuous	6.02 $\pm$ 0.81 ^{b}	-	0.140 $\pm$ 0.010 ^{a}

The total and maximum volumetric and areal productivities were calculated and the obtained values are present in Table 3.2.

Table 3.2: Volumetric and areal productivity of biomass during the whole test, and the maximum in a short interval, in the cultivation of *N. oceanica*, for each of the operation regimes in 2.6 m^3 PBRs. The values represent the average and standard deviation of three biologically independent replicates. Different letters within the same column represent significant different values.

Production regime	Volumetric productivity ($gL^{-1}day^{-1}$)	Maximum volumetric productivity ($gL^{-1}day^{-1}$)	Areal productivity ($gm^{-2}day^{-1}$)	Maximum areal productivity ($gm^{-2}day^{-1}$)
Batch	0.108 $\pm$ 0.011 ^{a}	0.333 $\pm$ 0.036 ^{a}	10.7 $\pm$ 1.1 ^{a}	31.6 $\pm$ 3.4 ^{a}
Semi-continuous	0.165 $\pm$ 0.013 ^{b}	0.427 $\pm$ 0.020 ^{b}	16.3 $\pm$ 1.3 ^{b}	40.4 $\pm$ 1.9 ^{b}
Continuous	0.154 $\pm$ 0.021 ^{b}	0.266 $\pm$ 0.028 ^{a}	15.2 $\pm$ 2.0 ^{b}	25.2 $\pm$ 2.6 ^{a}

The semi-continuous and continuous operation regimes reached productivity values non significantly different from each other, with a volumetric productivity of 0.165 $\pm$ 0.013 and 0.154 $\pm$ 0.021 g L^{-1}day^{-1}, respectively, and areal productivity of 16.3 $\pm$ 1.3 and 15.2 $\pm$ 2.0 g m^{-2}day^{-1}, respectively. On the other hand, these values were around 1.5-fold higher the volumetric (0.108 $\pm$ 0.01 g L^{-1}day^{-1}) and areal productivity (10.7 $\pm$ 1.1 g m^{-2}day^{-1}) obtained by the batch regime.

The batch productivity value was similar to the 0.15 g L^{-1}day^{-1} reported by Quinn et al. (2012) for flat panel outdoor batch growth of *Nannochloropsis* in the same period of the year (May to June) in the

north hemisphere (Colorado, USA).

For the semi-continuous operation regime higher volumetric productivity values were found by Ledda et al. (2015) and Chini Zittelli et al. (1999), of 0.48 g $L^{-1}day^{-1}$ in a 340 L vertical tubular reactor and 0.56 g $L^{-1}day^{-1}$ for a 36.6 L horizontal tubular reactor, in the same period of the year in the north hemisphere (Italy), respectively. These differences can be justified by the different temperature and irradiance felt, particularly in the first study. Additionally, a much smaller and different configuration reactors, with much higher ratio of incident radiation area to volume was used in the second study. In contrast, Ledda et al., 2015 reported a lower maximum areal productivity of 27 g $m^{-2}day^{-1}$, against 40.4 $\pm$ 1.9 g $m^{-2}day^{-1}$ in this study, meaning that the reactor configuration used by Ledda et al. in terms of used land is less efficient than the one used in this study.

The continuous operation regime presented a volumetric productivity of 0.154 $\pm$ 0.021 g $L^{-1}day^{-1}$, for a dilution rate of 0.14 day^{-1}. This values of productivity are much higher than the ones reported by James and Khars (1990), which presented a volumetric productivity of 0.058 $\pm$ 0.014 g $L^{-1}day^{-1}$ and 0.082 $\pm$ 0.017 g $L^{-1}day^{-1}$, using artificial light with an irradiance of 300 μE m^{-2} s^{-1} over 12:12 hours or 24:0 hours photo period, respectively. Despite this, San Pedro et al. (2014) reached a maximum volumetric productivity of 0.25 g $L^{-1}day^{-1}$ in a 340 L vertical tubular PBR with 0.1 day^{-1} dilution rate. This higher volumetric productivity might be consequence of the different configurations or smaller volumes used by the author.

Regarding the maximum volumetric and areal productivity, the batch and continuous operation systems presented similar values, respectively, 0.333 $\pm$ 0.036 g $L^{-1}day^{-1}$ and 0.266 $\pm$ 0.028 g $L^{-1}day^{-1}$, while the semi-continuous presented a significantly higher value of 0.427 $\pm$ 0.020 g$L^{-1}day^{-1}$. These values show that the semi-continuous production of *N. oceanica*, even though it did not present a significantly different volumetric productivity, exhibited a bigger potential than the continuous production regime, if optimized to work in that maximum productivity.

Other parameters, like the photosynthetic efficiency and the amount of culturing water and medium spent in each of the production regimes, must also be taken into account (Table 3.3).

Table 3.3: Photosynthetic efficiency and amount of culturing water and medium spent, either per cycle and PBR or per amount of produced biomass, in the growth of *N. oceanica* in 2.6 m^3 tubular PBRs, in three different operation regimes. The values represent the average and standard deviation of three biologically independent replicates. Different letters within the same column represent significant different values.

Operation regime	Photosynthetic efficiency (%)	Water/ cycle (m^3)	Medium/ cycle (L)	Water/ Produced biomass (m^3/kg)	Medium / Produced biomass (L kg^{-1})
Batch	0.358 $\pm$ 0.016^a	2.6 $\pm$ 0.0 ^{a}	33.0 $\pm$ 6.7^a	0.62 $\pm$ 0.07^a	7.75 $\pm$ 0.98^a
Semi-continuous	0.436 $\pm$ 0.043^a	6.7 $\pm$ 0.4 ^{b}	61.7 $\pm$ 1.0^b	1.04 $\pm$ 0.03^b	9.64 $\pm$ 1.20^a
Continuous	0.481 $\pm$ 0.073^a	7.4 $\pm$ 0.1 ^{c}	52.5 $\pm$ 7.3^b	1.25 $\pm$ 0.14^b	8.74 $\pm$ 0.84^a

Looking at the photosynthetic efficiency, there were no significant differences between the 0.358 $\pm$ 0.016, 0.436 $\pm$ 0.043 and 0.481$\pm$ 0.073 % obtained by the batch, semi-continuous and continuous operation regimes, respectively. These values are lower than the ones reported in the literature of 1.5-

1.8 % for the cultivation of *Nannochloropsis* sp. in an outdoor 0.56 m^3 horizontal tubular reactor (De Vree et al., 2015).

In terms of culturing water, the batch regime was, by far, the one that needed the least amount of culturing water, only 2.6 $\pm$ 0.0 m^3, followed by the semi-continuous 6.7 $\pm$ 0.4 m^3 and the continuous 7.4 $\pm$ 0.1 m^3. In terms of the medium the batch regime spent 33.0 $\pm$ 6.7 L, almost half when compared to the other two operation regimes (6.7 $\pm$ 0.4 L in the semi-continuous and 7.4 $\pm$ 0.1 L in the continuous regime).

Most important than looking at the spent amount of water, is to consider this value in the function of produced biomass. Table 3.3 shows that there were no significant differences in the culturing water used to produce one kg of biomass between the semi-continuous and continuous operation systems, but these values (1.04 $\pm$ 0.03 m^3 kg^{-1} and 1.25 $\pm$ 0.14 m^3 kg^{-1} of water for semi-continuous and continuous operation regimes, respectively) were almost double those needed in the batch regime (0.62 $\pm$ 0.07 m^3 kg^{-1}). However, these values do not have into account the water spent in cleaning the reactor. This would be much higher in the batch regime, since the reactor would have to be cleaned in the end of each cycle ($\pm$ every 15 days), whereas in the other two operation regimes the culture would only stop for cleaning if contamination occurred, or the algae were damaged in any other way. In terms of medium spent per kg of produced biomass there were no significant differences among batch, semi-continuous and continuous operation regimes.

These results indicate that the batch regime would be a better option in places where the water is a scarce and/or expensive resource, but if the process water would be directly collected from the sea, or reused it would be possible to obtain a higher productivity at a low cost and low environmental impact using semi-continuous or continuous operation regimes. On the other hand, the highest harvesting and downstream costs in the regimes that spent highers amounts of water, leading to lower biomass concentrations, must also be taken into account, in order to take a more informed decision.

3.1.3 Biochemical profile

Since the microalgal biomass was harvested in different stages of the growth curve, depending on the operation regime, the biochemistry of the cells was assessed for the different operation regimes, at the end of the trial (Table 3.4). The biochemistry results correspond only to the first two replicates, as the third one was contaminated by *Phaeodactylum tricornutum*, a salt-water diatom (Prestegard et al., 2016). This contamination wasn't probably strong enough to severely affect the culture growth but would affect the biochemistry results.

The macronutrient composition of *N. oceanica* biomass regarding carbohydrates, proteins, lipids and ashes, was constituted by 32.5-39.1 %, 28.6 $\pm$ 2.8 - 29.6 $\pm$ 3.6 %, 19.1 $\pm$ 2.5 to 24.0 $\pm$ 5.3 $\%$ and 10.7-14.9 % of biomass dry weight, respectively. Regarding the composition of macronutrients between the biomass produced among the different operation regimes, no significant differences were observed. This was not expected since the literature registered a higher amount of proteins, and lower amount of lipids in the semi-continuous cultures, than in the stationary phase of the batch culture in 85L bags, in

Table 3.4: Proximate composition, in percentage of total dry weight, of *N. oceanica* grown in 2.6 m^3 tubular PBRs, using different operation regimes. For proteins and lipids, the values represent the average and standard deviation of two biologically independent replicates and two analytical replicates. Different letters within the same column represent significant different values. Regarding ashes and, consequently, carbohydrates, the presented values are the average of two biological replicates and the minimum and maximum values.

Production regime	Protein (%)	Lipids (%)	Ash (%)			Carbohydrates (%)		
			Average	Min	Max	Average	Min	Max
Batch	29.6 $\pm$ 3.6 ^{a}	22.0 $\pm$ 3.1 ^{a}	10.7	9.4	11.9	37.8	34.7	40.8
Semi-continuous	28.6 $\pm$ 2.8 ^{a}	24.0 $\pm$ 5.3 ^{a}	14.9	13.0	16.8	32.5	26.4	38.6
Continuous	28.9 $\pm$ 2.5 ^{a}	19.1 $\pm$ 2.5 ^{a}	12.9	12.0	13.8	39.1	36.4	41.8

artificial light, over a 12:12 hours photo period (Brown et al., 1993).

The protein content obtained in this study for *N. oceanica* growth in outdoor tubular reactors is within the 21.1 - 39.2 % of biomass dry weight range reached by San Pedro et al. (2014) with a culture of *Nannochloropsis gaditana*, growth in continuous operation regime, outdoor, in 340 L tubular reactors. Regarding the lipid content, the same San Pedro et al. (2014) study reported a lipid content of 17.7 - 26.7 $\%$ of biomass dry weight, a value very similar to the one obtained in this work.

The FAME profile was also determined as it represents an important factor for food and feed applications of microalgal biomass. The obtained FAME profile is presented in Table 3.5 and shows only the fatty acids above 0.50 % by FAME weight.

Table 3.5: Fatty acid content and profile, in the percentage of the total FAME, of *N. oceanica* after growth of 2.6 m^3 of culture, in an outdoor tubular PBR. The values represent the average and standard deviation of two biologically independent replicates and two analytical replicates. Different letters within the same row represent significant different values.

FAME	Batch	Semi-continuous	Continuous
		($\%$) of total FAME	
C14:0	6.88 $\pm$ 0.44^a	5.99 $\pm$ 0.66 ^{a}	6.02 $\pm$ 0.39^a
C16:1	32.79 $\pm$ 0.55^a	32.28 $\pm$ 0.65 ^{a}	32.28 $\pm$ 1.79^a
C16:0	33.58 $\pm$ 1.76 ^{a}	38.24 $\pm$ 2.10 ^{b}	36.20 $\pm$ 0.94^{ab}
C18:2 ω6	0.75 $\pm$ 0.09^a	0.59 $\pm$ 0.05 ^{b}	0.76 $\pm$ 0.08^a
C18:1	11.96 $\pm$ 2.98^a	11.07 $\pm$ 0.76 ^{a}	12.40 $\pm$ 2.46^a
C18:0	1.05 $\pm$ 0.26^a	1.95 $\pm$ 0.26 ^{b}	1.58 $\pm$ 0.19^{ab}
C20:4 ω6	1.85 $\pm$ 0.48^a	1.43 $\pm$ 0.30 ^{a}	1.60$\pm$ 0.11^a
C20:5 ω3	11.12 $\pm$ 3.43^a	8.44 $\pm$ 2.00 ^{a}	9.16 $\pm$ 0.83^a

The major fatty acids observed in *N. oceanica* growth in an outdoor 2.6 m^3 tubular reactor were C16:1 and C16:0, together being more than 60% of total FAME, followed by C14:0, C18:1 and C20:5. This profile is in accordance with the one reported for growth of *Nannochloropsis* in outdoor tubular PBRs of 340 L, by San Pedro et al. (2014) and 97.9 L by Chini Zittelli et al. (1999).

Comparing the three operation regimes there were only significant differences in the C16:0 and in the C18:0, that were significantly higher in the semi-continuous than in the batch regime, not being

significantly different from the continuous.

In terms of saturation of the fatty acids (Table 3.6) in this study the obtained biomass presented slightly higher concentration of SFA, around 45 % of total FAME, against around 35 % of total FAME obtained by San Pedro et al. (2014). Additionally, in this study the percentage PUFA was slightly lower than the one reported in literature, 10-14 % of total FAME, against the 20 % of total FAME, reported by San Pedro et al. (2014).

Comparing the operation systems, the only significant difference was a higher percentage of SFA in the semi-continuous in relation to the batch one. This difference didn't spread to the PUFA/SFA ratio, that was not significantly different between the operation regimes and was lower that one, which is not optimal for food and feed applications.

Table 3.6: Saturation distribution of the fatty acid profile of *N. oceanica*, grown in 2.6 m^3 tubular PBR, in three different operation regimes. The values represent the average and standard deviation of two biologically independent replicates and two analytical replicas. Different letters within the same column represent significant different values.

Operation regime	SFA (%)	MUFA (%)	PUFA (%)	$\frac{\text{PUFA}}{\text{SFA}}$
Batch	41.52 $\pm$ 1.60^a	44.76$\pm$ 2.43 ^{a}	13.72 $\pm$ 4.00^a	0.33 $\pm$ 0.11^a
Semi-continuous	46.19 $\pm$ 1.64^b	43.36 $\pm$ 0.92 ^{a}	10.45 $\pm$ 2.31^a	0.23 $\pm$ 0.06^a
Continuous	43.80 $\pm$ 1.40^{ab}	44.68 $\pm$ 0.67 ^{a}	11.52 $\pm$ 0.88^a	0.26 $\pm$ 0.03^a

In terms of the ω3/ω6 ratio present in Table 3.7 there was no significant differences between the operation regimes, being all around 4. This was due to the high percentage of EPA (C20:5 $\omega 3$) of 8 - 11 % of total FAME, being this value slightly lower than the ones reported in literature of 13 - 25 % of total FAME (San Pedro et al., 2014).

Table 3.7: Essential fatty acids content of *N. oceanica*, growth in 2.6 m^3 tubular PBRs, in three different operation regimes. The values represent the average and standard deviation of two biologically independent replicates and two analytical replicas. Different letters within the same column represent significant different values.

Operation regime	$\omega 3(\%)$	$\omega 6(\%)$	$\frac{\omega 3}{\omega 6}$
Batch	11.12 $\pm$ 3.43^a	2.60$\pm$ 0.57 ^{a}	4.20 $\pm$ 0.40^a
Semi-continuous	8.44 $\pm$ 2.00^a	2.02 $\pm$ 0.34 ^{a}	4.13 $\pm$ 0.52^a
Continuous	9.16 $\pm$ 0.83^a	2.36 $\pm$ 0.07 ^{a}	3.89 $\pm$ 0.30^a

3.2 *Chlorella vulgaris*

C. vulgaris was grown outdoor under batch, semi-continuous and continuous operation regimes, in three horizontal tubular PBRs (Figure 3.6).

The culture was grown from July 12th to July 25th and the measured environmental parameters are in Figure 3.5. The average daily temperature was 19.5 $^\circ$C with a maximum and minimum daily registered

temperature of 22.0 °C and 17.8 °C, respectively. The culture temperature was usually higher than the ambiance temperature and, in this replica, unlike the others, it was possible to maintain this temperature below 30 °C through thermoregulation. In terms of solar radiation it was quite discrepant throughout this assay, having a maximum and minimum registered of total incident daily radiation of 21.3 MJ m^{-2} and 5.2 MJ m^{-2}, with an average of 14.0 MJ m^{-2}.

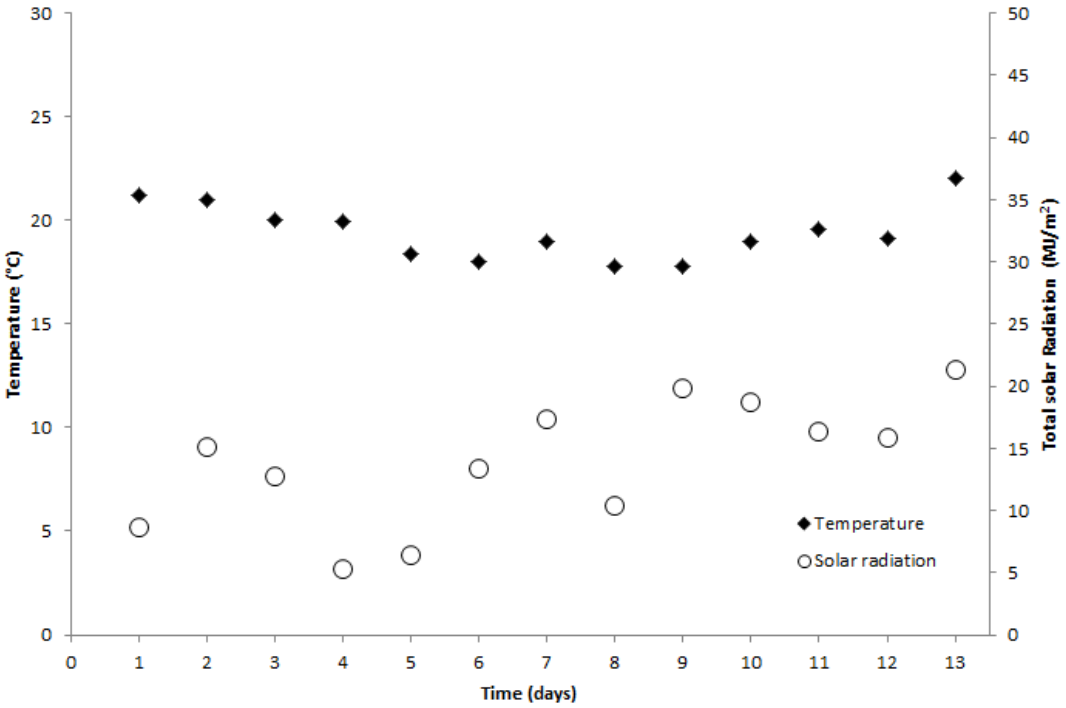


Figure 3.5: Medium daily ambiance temperature and total solar radiation incident in the tubular PBRs.

Two more attempts to replicate the assay were performed from August 13^{th} to September 9^{th} but, in the first one, the culture began to die at the 6^{th} day due to fungal contamination, and, in the second one, the culture stopped growing at the 7^{th} day due to a wave of extreme hot (average daily temperature from the 7^{th} to the 13^{th} of 22.4 °C) that did not allow to maintain the culture temperature below 30 °C trough thermoregulation and that affected the grown of all the existing algae in the facility (growth graphics in appendix B).

The initial dry weight of the cultures was 0.3 g L^{-1} for the batch regime and 0.8 g L^{-1} for the semi-continuous and continuous operation regimes. The cultures were left to grown for 15 days, presenting one day of lag phase.

After the first day, the batch regime followed an exponential phase until the 6^{th} day, with a dry weigth ranging from 0.4 to 2 g $L{-}1$. The culture entered the stationary phase after the 10^{th} day, reaching a maximum dry weight of 3.6 g L^{-1} on the 13 th day. In the last two days biomass lost was observable, representing a death phase. Thus, in order to follow the same scheme used for *N. oceanica*, henceforth will only be considered the first 13 days of growth for any calculation.

The semi-continuous operation regime also showed exponentially growth until the 3^{rd} day, when the culture was first renovated. The culture was renovated five more times throughout the trial, however, the

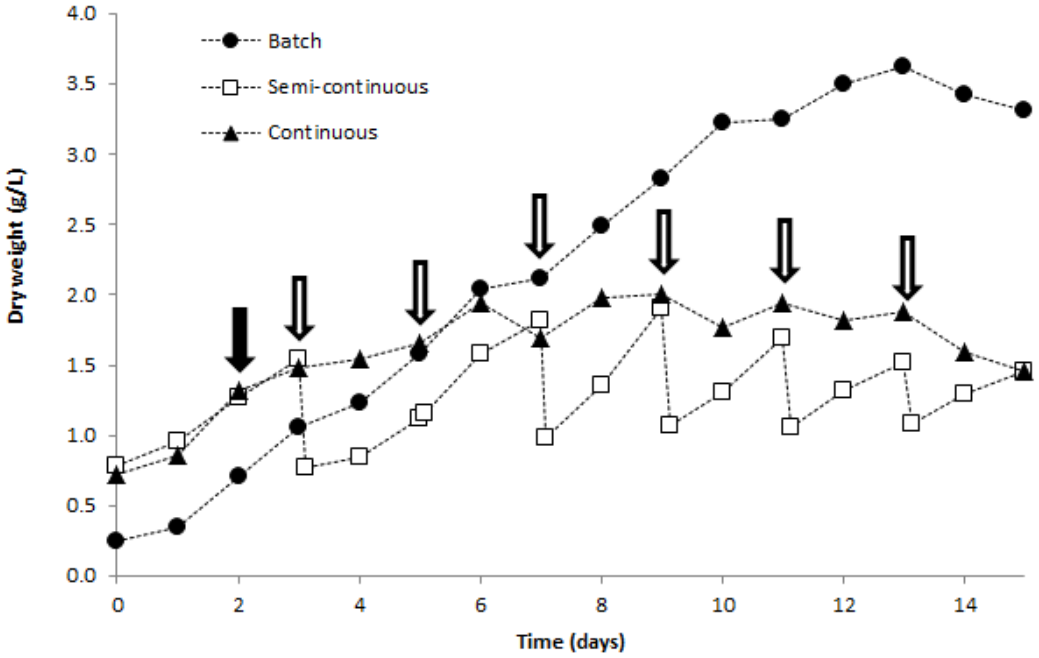


Figure 3.6: Growth of *C. vulgaris* in 2.6 m^3 tubular PBRs using three different operation regimes. The black arrow represents the day in which the continuous regimes started to be diluted, and the white arrows represent the moments of the semi-continuous regime renovations.

last one was not taken into account to the calculations. The culture dry weight ranged between 0.8 and 1.9 g L^{-1}.

The continuous regime started in the 2^{nd} day, when the culture presented a dry weight of 1.3 g L^{-1}. After this, the dry weight kept increasing until the 6^{th}, day reaching 1.9 g L^{-1}. From the 6^{th} to the 13^{th} day, a more appropriate dilution rate was reached, keeping the culture more stable, with the dry weight ranging between 1.7 and 2.0 g L^{-1}, values within the range of the exponential phase obtained in the batch regime.

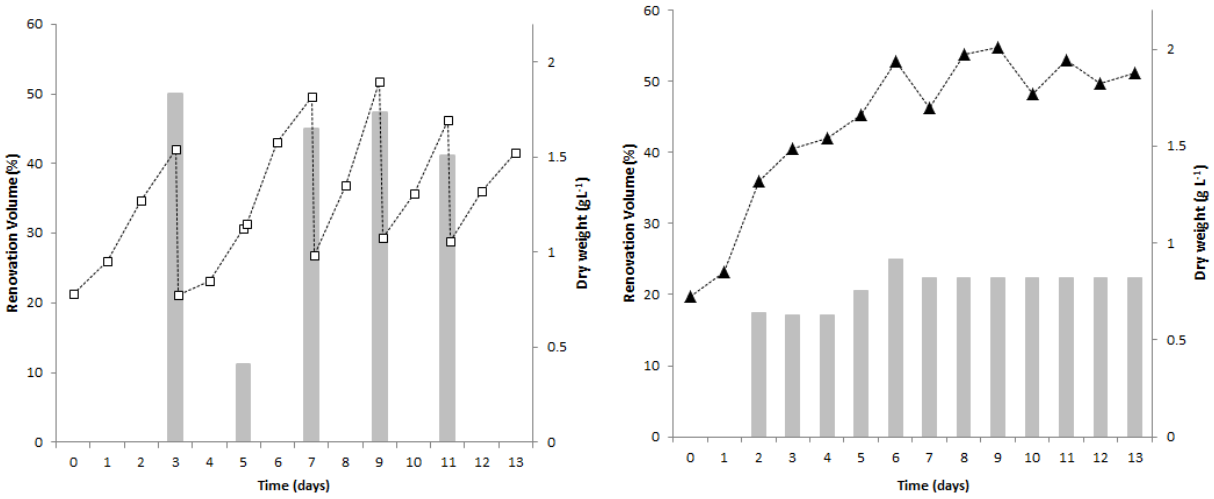
Taking into account the first 13 days, the batch regime was the one which produced the lowest amount of biomass, 8.8 kg, with a maximum specific growth rate of 0.356 day^{-1}, calculated from the 1^{st} to 6^{th} day. The semi-continuous operation regime produced 10.6 kg and the continuous one produced 14.7 kg of biomass, having the highest produced amount.

In Figure 3.7 is represented the daily percentage of the renovated volume that ranged between 11.1 % and 50 % of total volume in the semi-continuous regime, and between 17.2 % and 24.9 % of total volume in the continuous. The dilution rates (Table 3.8) were 0.181 day^{-1} for the semi-continuous and a slightly higher value of 0.212 day^{-1} for the continuous regime.

The volumetric and areal productivities were calculated for the three operation regimes and are present in Table 3.9, as well as the respective maximum productivities. The batch regime was the one that had the lowest volumetric and areal productivity of 0.261 g $L^{-1}day^{-1}$ and 25.7 g $m^{-2}day^{-1}$, respectively. This value of volumetric productivity is similar to the 0.212-0.247 g $L^{-1}day^{-1}$ obtained by Ortiz Montoya et al. (2014), in a 5 L indoor tubular reactor. The semi-continuous presented values of

Table 3.8: Produced biomass, per 2.6 m^3 PBR and per cycle, specific growth rate and average dilution rate of *C. vulgaris* growth in 2.6 m^3 PBRs in the three operation regimes.

Production regime	Produced biomass (kg)	Specific growth rate (day^-1)	Average dilution rate (day^-1)
Batch	8.8	0.356	-
Semi-continuous	10.6	-	0.181
Continuous	14.7	-	0.212



(a) Semi-continuous (b) Continuous

Figure 3.7: *Chlorella vulgaris* growth in 2.6 m^3 tubular PBRs in two different operation regimes. The bars represent the percentage of renovated volume.

0.313 g $L^{-1}day^{-1}$ in terms of volumetric productivity and 30.7 g $m^{-2}day^{-1}$ in terms of areal productivity.

Finally, the continuous operation regime presented the highest volumetric and areal productivity of 0.436 g $L^{-1}day^{-1}$ and 42.9 g $m^{-2}day^{-1}$, respectively. These values are lower, comparing to the 0.67-1.22 g $L^{-1}day^{-1}$ presented by Ugwu et al. (2005). This probably derived from the much lower volume used in that study (6 to 58 L), as diminished productivities are reported with an increase of the culture volume (Ugwu et al., 2005). Besides that, the maximum volumetric productivity in the present study, 0.660 g $L^{-1}day^{-1}$, is inside the range reported by Ugwu et al., 2005), showing the possibility of reaching much higher productivities through optimization.

Table 3.9: Volumetric and areal productivity during the whole test and the maximum in a short interval, for the cultivation of *C. vulgaris* grown in 2.6 m^3 tubular PBR, in each of the operation regimes.

Production regime	Volumetric productivity (g $L^{-1}day^{-1}$)	Maximum volumetric productivity (g $L^{-1}day^{-1}$)	Areal productivity (g $m^{-2}day^{-1}$)	Maximum areal productivity (g $m^{-2}day^{-1}$)
Batch	0.261	0.463	25.7	45.6
Semi-continuous	0.313	0.548	30.7	54.0
Continuous	0.436	0.660	42.9	65.0

Analyzing the photosynthetic efficiency (Table 3.10) the values were not very apart between the operation regimes, being the batch regime the one with the lowest efficiency (1.23%) and continuous

the one that had the highest efficiency (2.02%). These values are not very similar to the 5.1-6.4% reported by Scragg et al. (2002) in a 250 L tubular PBR with a 24:0 hour photoperiod. The different photoperiod or the slightly different configuration can probably explain these differences.

Table 3.10: Photosynthetic efficiency and amount of culturing water and medium, either per cycle and per PBR, or per amount of produced biomass, spent in the growth of *C. vulgaris* in 2.6 m^3 tubular PBRs for each of the operation regimes.

Operation regime	Photosynthetic efficiency (%)	Water/ cycle (m^3)	Medium/ cycle (L)	Water/ Produced biomass (m^3/kg)	Media / Produced biomass (L/kg)
Batch	1.23	2.6	13.8	0.30	1.57
Semi-continuous	1.43	8.6	19.9	0.81	1.89
Continuous	2.02	9.8	19.6	0.67	1.33

It's also important to analyze the results obtained in terms of spent resources, namely culturing water and medium. The amount of medium spent per produced biomass does not vary that much between the operation regimes, being continuous regime the one with the lowest amount of medium spent, 1.33 L kg^{-1} and the semi-continuous the one with the highest spent amount, 1.89 L kg^{-1}. Observing the culturing water, the batch regime was the one which allowed to save more water, needing only 0.30 m^3 per kg of produced biomass, less than half than the vales for the continuous and semi-continuous which needed, respectively, 0.67m^3 and 0.81 m^3 of culturing water per kg of produced biomass.

In an overall look, the batch regime would be the best option if the objective is to produce the highest amount of biomass, saving the most water possible. On the other hand, if the priority is to produce high amounts of biomass in less time, using the lowest amount of volume and area possible the continuous operation regime is the most suitable one, since it presents a much higher volumetric and areal productivities, having even the advantage of using less medium per amount of produced biomass.

3.2.1 Biochemical profile

At the end of the assay the composition of macronutrients of the produced biomass was evaluated, being the results presented in Table 3.11.

Table 3.11: Proximate composition, in percentage of total dry weight, of the *C. vulgaris* culture grown in 2.6 m^3 tubular PBRs using different operation regimes. The lipids values represent the average, minimum and maximum values of one biological replicate, with two analytical replicates. The values of the remaining parameters were obtained from one biological replicate, with one analytical replicate.

Production regime	Protein (%)	Lipids (%)			Ash (%)	Carbohydrates (%)
		Average	Min	Max		
Batch	54.0	20.0	17.8	22.2	9.4	16.6
Semi-continuous	56.9	20.5	17.5	23.4	10.6	12.1
Continuous	54.0	20.5	20.5	20.5	10.0	15.5

C. vulgaris presented as main macronutrient protein, reaching 54.0 % of the cells dry weight in the

batch and continuous regimes, and 56.9 % in the semi-continuous regime. The biomass also presented a good amount of lipids, between 20 to 20.5 % of the biomass dry weight. The ashes content ranged between 9.4 % for the batch regime, and 10.6 % for the semi-continuous operation regime, while the carbohydrates content ranged between 12.1 and 16.6 %, for the semi-continuous and batch regime, respectively.

The macronutrient values were similar between all the operation regimes, with the exception of a slightly higher amount of protein and a consequent lower amount of carbohydrates, in the semi-continuous regime. However more replicates accompanied by statistical analysis would be needed, to perceive if this different is actually significant.

The values of protein, are similar to the 52.18 % of biomass dry weight reported by Barros et al. (2019) in an outdoor tubular reactor of 100 m^3 and higher than the 32.86 - 42.55 % reported by Yusof et al. (2011) in an 1m^3 vertical outdoor reactor, with a 12:12h photoperiod. The lipid percentage of 20.0 - 20.5 % of dry weight are much higher than the 1.0 - 1.59 % of lipids reported by Yusof et al. (2011). The high protein and lipid reached values are an advantage for microalgae like *Chlorella* that are used as human food and supplementation.

The fatty acid profile was also determined and is present inTable 3.12.

Table 3.12: Fatty acid content and profile, in the percentage of the total FAME of *C.vulgaris* growth in 2.6 m^3 tubular PBRs in three operation regimes. The presented values are the average, minimum and maximum of two analytical replicates, coming from of one biological replicate.

	(%) of total FAME								
FAME	Batch			Semi-continuous			Continuous		
	Average	Min	Max	Average	Min	Max	Average	Min	Max
C14:0	1.31	1.31	1.31	2.08	2.00	2.16	1.68	1.64	1.71
C16:1	7.52	7.06	7.97	14.54	14.07	15.00	11.99	11.38	12.61
C16:0	59.91	59.85	59.97	55.95	55.44	56.46	60.99	60.41	61.56
C17:1	2.19	2.18	2.19	3.27	2.25	4.28	3.06	3.03	3.08
C18:3	0.53	0.52	0.54	ND	ND	ND	ND	ND	ND
C18:2	24.30	24.03	24.58	15.29	14.79	15.78	16.16	16.14	16. 17
C18:1	2.67	2.18	3.16	4.79	4.23	5.36	3.43	3.34	3.52
C18:0	1.57	1.26	1.88	4.09	1.88	6.29	2.70	2.62	2.78

The major fatty acids were C16:0, representing 55.91 - 60.99 % of the total FAME, followed by C18:2 and C16:1, representing 15.29-24.30 % and 7.52 - 14.54 % of the total FAME , respectively. Comparing the operation regimes, the biggest observed differences were in the C16:1 and C18:1, that were almost half the amount in the semi-continuous, when comparing it to the other two regimes. Additionally, the C18:2 content in the batch regime was more than 1.5-fold the values reached by the semi-continuous and continuous regimes. Another difference relies on C18:3, which appeared only in the batch regime, even though in small amount.

The obtained values were quite different from the literature, for example, cells cultivated in tanks with a 12:12 hour photoperiod (Yusof et al., 2011) reported a percentage of C18:3 of 21.1 - 27.5 % of total

FAME, which was almost inexistent in this work. Yusof et al. (2011) also reported considerable amounts of C20:0 and C17:0 which were not found in the present study. In a different work, with cells grown in a 5 L tubular reactor with a 24:24 hour photoperiod (Ortiz Montoya et al., 2014) there were more similar results reporting the C16:0 as the main fatty acid with a 45.1 %, not to distant of the 55.91-60.99 % of total FAME reached in this study and the C18:2 as the second most abundant fatty acid with a 19.4 %, also comparable to the 15.29-24.30 % of total FAME presented in this study. However, all the other FAME have no similar percentages especially the C18:3, that Ortiz Montoya et al. (2014) reports as being 13.6 % of the total fatty acids and was absent, or near it, in the biomass from this study.

When analyzing the FAME in terms of saturation (Table 3.13) it's noticeable that the final biomass of all the operation systems presented similar amount of SFA. The main difference relies on higher PUFA content of the batch regime 24.83 %, comparing to the 15.29 and 16.16 % of total FAME, in the semi-continuous and continuous operation regimes. The higher PUFA content results in a higher PUFA/SFA ratio (0.40, for the batch regime, in comparison to 0.25, for both continuous and semi-continuous operation regimes). However the value of the ratio was lower than 1, which is not ideal for the biomass to be used as food and feed, for which the higher this ratio, the better.

Table 3.13: Saturation distribution of the fatty acid profile of *C. vulgaris*, grown in 2.6 m^3 tubular PBR, in three different operation regimes.

Operation regime	SFA (%)	MUFA (%)	PUFA (%)	$\frac{\text{PUFA}}{\text{SFA}}$
Batch	62.79	12.37	24.83	0.40
Semi-continuous	62.12	22.59	15.29	0.25
Continuous	65.37	18.48	16.16	0.25

Chapter 4

Conclusions and future perspectives

Nannochloropsis oceanica and *Chlorella vulgaris* were successfully grown in pilot-scale tubular photobioreactors in batch, continuous and semi-continuous operation regimes.

The semi-continuous and continuous operation regimes showed to be much more productive, in comparison with the batch regime, in both *N. oceanica* and *C. vulgaris* trials. In terms of biochemical composition, both microalgae strains showed no significant differences in terms of protein, lipids, ashes, and neither in the fatty acid profile, when comparing the operation regimes.

From the different trials conducted with *N. oceanica*, it can be concluded that, even though the volumetric productivities of the semi-continuous and continuous operation regimes were not significantly different, the semi-continuous regime showed the highest potential, as it presented a maximum volumetric productivity 1.3-fold than the one in the batch and continuous operation regimes. On the other hand, the semi-continuous and continuous operation regimes needed non significantly different amounts of culturing water per kg of produced biomass but needed almost double of the amount, when comparing to the batch needs. The photosynthetic efficiency was not different between the operation regimes. In terms of the biochemistry of the cells, *N. oceanica* grown in pilot-scale reactors presented good amounts of proteins and lipids, being each within the range of 20-30% of biomass dry weight. The final biomass presented a good fatty acid profile, with an $\omega 3/\omega 6$ ratio around 4, and with 10% of total FAME being EPA.

Regarding *C. vulgaris*, the continuous operation regime was the one with the highest volumetric productivity, followed by the semi-continuous and, finally, the batch. The culturing water consumption per amount of produced biomass in the continuous operation regime, even though higher than the batch regime, was low, when compared to the semi-continuous. In addition, the amount of culturing water per produced biomass was much lower for this microalga, in comparison with *N. oceanica*. The continuous operation regime showed better results in terms of photosynthetic efficiency, compared with the other two regimes. The growth of *C. vulgaris* in tubular 2.6 m^3 PBRs allowed to obtain biomass with a protein percentage of 55-60% of the total dry weight, being proper for food usage.

In the future, it would be of most interest to replicate the study with *C. vulgaris*, in order to perceive the significance of the promising results. In addition, the strategy followed in this work should also be pursued with other strains of interest for industrial cultivation, as well as in other microalgae cultivation

systems, for example, open ponds.

The replicability of this work in industrial-scale PBRs (10 to 100 m^3), would also be of the most interest for Allmicroalgae, since those are the main reactors used for the production of both of these species.

An interesting approach would be to take the most productive operation regimes and try to optimize it. This could be done in terms of achieving the best steady-state cellular concentration, as well as the best dilution rate, for the continuous regime. In the case of semi-continuous, the study should be focused on discovering the best concentration to which the system should be renovated too, and in the waiting time between renovations.

Finally, it would be of most importance to perform an economic analysis. A life cycle assessment or a techno-economic assessment would allow perceiving the impact of the high amounts of spent water, in the most productive systems, either in terms of raw material costs, or in terms of downstream costs.

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Appendix A

Calibration curves

The calibration curves for *Nannochloropsis oceanica* (Figure A.1) and *Chlorella vulgaris* (Figure A.2) were obtained using experimental points from which was measured the optical density and the dry weight of the same sample.

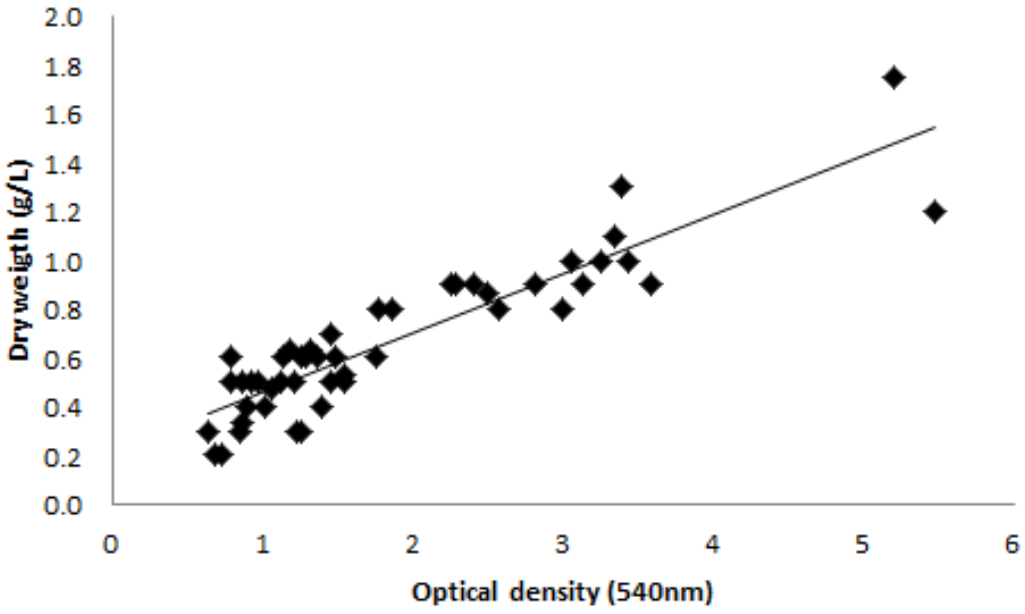

Figure A.1: Correlation between the optical density at 540 nm and the dry weight of an authotrophic culture of *Nannochloropsis oceanica*. The linear regression is given by the equation 2.1 with R^2=0.822 and n=48

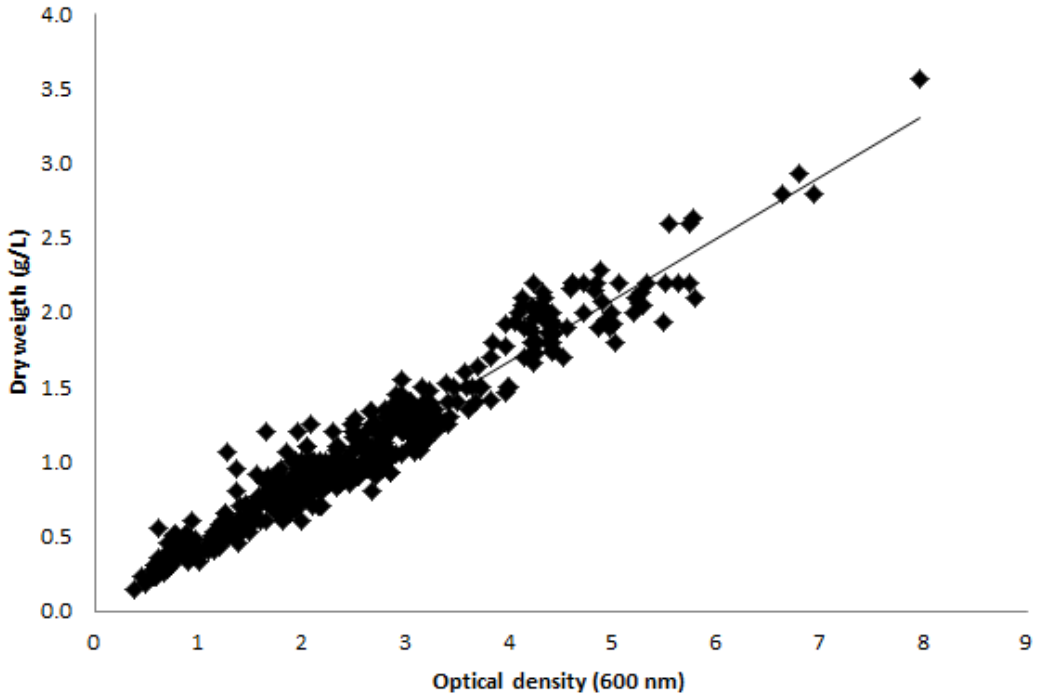

Figure A.2: Correlation between the optical density at 600 nm and the dry weight of an authotrophic culture of *Chlorella vulgaris*. The linear regression is given by the equation 2.2 with R^2=0.937 and n=506.

Appendix B

Chlorella vulgaris replicates

As mentioned in Chapter 2, two attempts to replicate the *C. vulgaris* assay were performed. The growth curves of those experiments are presented in Figure B.1 and Figure B.2, respectively.

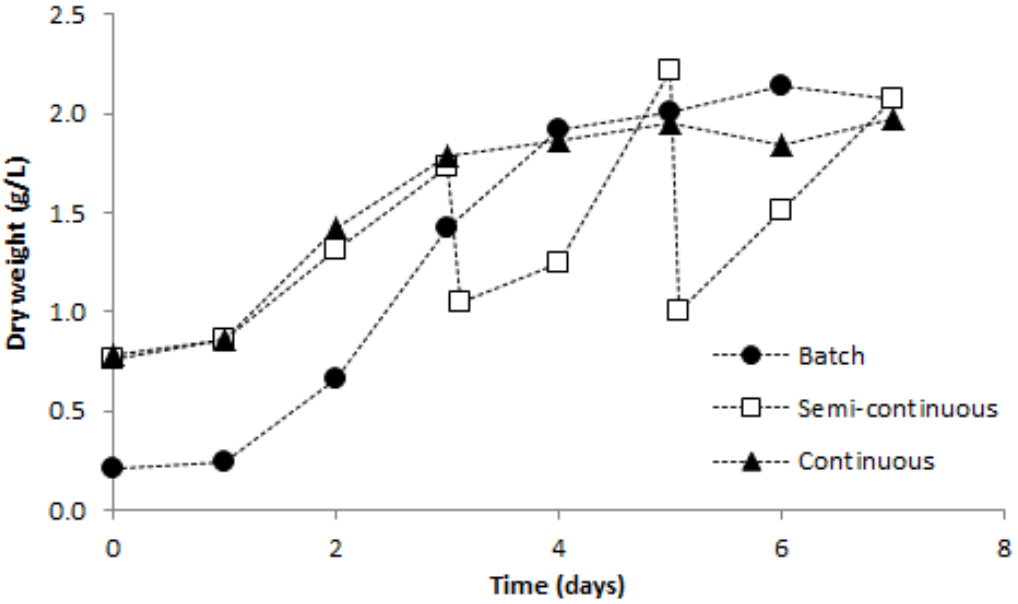

Figure B.1: Growth of *C. vulgaris* in three tubular 2.6 m^3 PBRs in three different operation regimes.

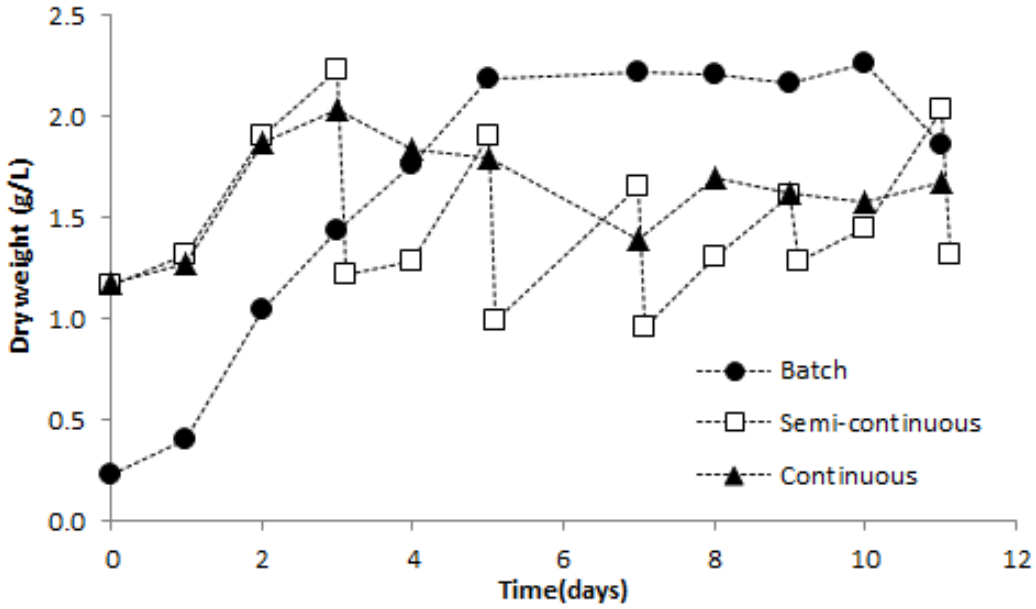

Figure B.2: Growth of *C. vulgaris* in three tubular 2.6 m^3 PBRs in three different operation regimes.