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Screening of *Pavlova* sp. and scale-up to outdoor photobioreactors

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STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

ABSTRACT

SCREENING OF *PAVLOVA* SP. AND SCALE-UP TO OUTDOOR PHOTOBIOREACTORS

Microalgae are a promising source of several bioactive compounds such as proteins, lipids, carbohydrates, carotenoids and vitamins, which are valuable for commercial use and can be explored and used for diverse applications. Furthermore, microalgae are known producers of polyunsaturated (PUFA) omega-3 fatty acids, including eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids and, consequently, cultivation of microalgae as a supplement to marine animals is increasing. The marine microalgae *Pavlova* sp. is also widely used in aquaculture to feed crustacean, bivalves, and fish.

In the present study, the growth of four *Pavlova* species (*P. lutheri, P. gyrans, P. pinguis* and *P. granifera*) was evaluated at lab-scale in order to select the most suitable species for outdoor and large-scale production. A preliminary trial comparing all strains showed that *P. gyrans* and *P. pinguis* had a higher final volumetric productivity, 0.087±0.005 g L^{-1} d^{-1} and 0.077±0.002 g L^{-1} d^{-1}, respectively. In accordance, higher specific growth rates and maximum volumetric productivity were achieved by these species. The biochemical composition of all strains understudy was analyzed and the amount of PUFA (DHA and EPA) demonstrated to be the same for all *Pavlova* species. In addition, 30% of inoculum revealed to be the optimum inoculation rate to obtain higher global productivity when compared to 5, 10 and 20%.

Furthermore, a screening outdoor assay was carried in 55 L flat panels using *P. gyrans* and *P. pinguis*. Both demonstrated a similar growth and global volumetric productivity, 0.058±0.007 and 0.079±0.019 g L^{-1} d^{-1}, respectively. The FAME profile showed a higher PUFA content of 48% of total fatty acids (TFA) in *P. gyrans*, followed by 37% of TFA in *P. pinguis*. Outdoor cultures proved to have an increased lipids content and a higher level of EPA (30-40% of TFA) and DHA (9% of TFA), when compared with lab-scale production.

Different types of outdoor photobioreactors (PBR) were used to investigate and compare their impact on *P. pinguis* scale-up: flat panel, tubular PBR and raceway pond. The results showed that flat panel was the system with the highest global productivity of 0.077 g L^{-1} d^{-1} and, similarly, the highest specific growth rate of 0.316 d^{-1}.

Keywords: DHA, EPA, Growth optimization, Industrial-scale production, *Pavlova*

RESUMO

RASTREIO DA *PAVLOVA* SP. E AUMENTO DE ESCALA PARA FOTOBIORREATORES EXTERIORES

As microalgas são uma fonte promissora de vários compostos bioativos como proteínas, lípidos, carbohidratos, carotenoides e vitaminas, valiosos para uso comercial e podem ser explorados e usados em muitos aplicações. Para além disso, as microalgas são as únicas produtoras dos ácidos gordos polinsaturados ómega-3, nomeadamente os ácidos eicosapentanóico (EPA) e docosahexanóico (DHA), e, consequentemente, o cultivo de microalgas como suplemento para os animais marinhos tem aumentado. A microalga marinha *Pavlova* sp. é amplamente usado em aquicultura para alimentar crustáceos, bivalves e peixes graças à sua acumulação de elevadas quantidades de ácidos gordos polinsaturados, especialmente EPA e DHA.

O crescimento das quatro espécies *Pavlova* (*P. lutheri, P. gyrans, P. pinguis* e *P. granifera*) foi avaliado em escala laboratorial de forma a selecionar a melhor espécie para escala industrial. *P. gyrans* e a *P. pinguis* demonstraram valores maiores de produtividade global final, 0.087±0.005 g L^{-1} d^{-1} e 0.077±0.002 g L^{-1} d^{-1}, respetivamente. Similarmente, obtiveram uma maior taxa especifica de crescimento e produtividade máxima. A composição bioquímica da microalga foi analisada e a produção de ácidos gordos polinsaturados (EPA e DHA) demonstraram ser iguais em todas as espécies *Pavlova*. Decorreu outro ensaio com a *P. gyrans* para testar o efeito da percentagem de inóculo no crescimento. Resultados mostraram que 30% é a concentração que potencia uma maior produtividade global, comparado com 5, 10 e 20 % de inóculo.

Para além disso, um ensaio exterior decorreu em painéis de 55 L com *P. gyrans* e *P. pinguis,* mas ambas demonstraram um crescimento e produtividade global similar, 0.058±0.007 e 0.079±0.019 g L^{-1} d^{-1}, respetivamente. O perfil de ácidos gordos mostrou um maior conteúdo de 48% do total de ácidos gordos na *P. gyrans*, seguido de 37% na *P. pinguis*. Culturas no exterior mostraram um aumento no conteúdo lipídico e um maior nível de EPA (30-40% do total de ácidos gordos) e DHA (9% do total de ácidos gordos), quando comparadas com o laboratório.

Diferentes tipo de fotobiorreatores foram usados no aumento de escala da *P. pinguis*: painéis planos, fotobiorreator tubular e tanques abertos, para investigar e comparar o impacto dos sistemas no crescimento da *Pavlova* sp.. Resultados demonstraram uma maior produtividade nos painéis, 0.077 g L^{-1} d^{-1} e uma maior taxa especifica de crescimento 0.316 d^{-1}.

Palavras-chave: DHA, EPA, Otimização do crescimento, *Pavlova*, Produção industrial

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LIST OF GENERAL NOMENCLATURE

ADP	*Adenosine Diphosphate*
ANOVA	*Analysis of variance*
ATP	*Adenosine Triphosphate*
a.u.	*Arbitrary unit*
CH	*Carbohydrates*
DNA	*Deoxyribonucleic Acid*
DW	*Dry Weight*
EPA	*Eicosapentaenoic Acid*
FA	*Fatty Acids*
FAME	*Fatty Acid Methyl Esters*
HHV	*Higher Heating Value*
MUFA	*Monounsaturated Fatty Acids*
NADPH	*Nicotinamide Adenine Dinucleotide Phosphate*
PBR	*Photobioreactor*
p	*p value*
P	*Global volumetric productivity*
P_a	*Productivity per unit of area per unit of time*
PUFA	*Polyunsaturated Fatty Acids*
μ	*Specific Growth Rate*
R^2	*Correlation Coefficient*
RNA	*Ribonucleic Acid*
SFA	*Saturated Fatty Acids*
t	*Time*
TAG	*Triacylglycerols*
TFA	*Total Fatty Acids*

1. CONTEXTUALIZATION

Microalgae are unicellular microorganisms that represent an important focus of interest for different industrial applications (Wojciechowski et al.,2013). Currently, several studies are exclusively dedicated to the study of microalgae and their biotechnological potentialities (Raja et al., 2008). Many of these studies have highlighted the role of microalgae on biofuel production, capture of carbon dioxide (CO_2) and its integration on effluent treatments (Brennan & Owende, 2010).

The excessive use of natural resources, mainly in the industrial processes, is a current and growing concern allied to higher industrial pollution potential (Barcellos et al., 2009). Thus, more and more waste has been generated and the use of natural resources is increasing, which is becoming scarce and often polluted and degradated (Schmitz et al., 2012). Therefore, there is an urgent need to promote the use of renewable/alternative energy sources for a sustainable future (Ndimba et al., 2013).

Toward this scenario of scarce resources and high pollution levels, it is urgent to seek new and effective ways of avoiding pollution and environmental impact, through the development of new products, the seek of alternative processes and efficient techniques (Schmitz et al., 2012). This is dependent on a huge investment by the government, in order to contribute to research improvement and to obtain reliable scientific information.

One of the most promising approaches is the application of microbial processes in order to degrade and minimize the pollutants and transform raw materials, generating innocuous or less polluting products and releasing them to the environment (Gadd, 2009; Schmitz et al., 2012). In this context, the application of microorganisms such as microalgae in effluent treatments, CO_2 fixation, biofuel production, biosorption of heavy metals, among others, become of reinforced importance (Brennan & Owende, 2010).

Known by possessing photosynthesis mechanisms, microalgae play an important role in building and preserving the Earth's atmosphere since they are responsible for producing oxygen while consuming CO_2 (Muller-Feuga et al., 2007). The high photosynthetic potential related to microalgae is widely recognized due to their efficient utilization of solar energy, face to superior plants (Priyadarshani & Rath, 2012; Wojciechowski et al., 2013). When compared with the

production of other macro and microorganisms, microalgae have the advantage of minimizing the anthropogenic effect related to combustion processes, using CO_2 as a carbon source, as well as nitrogen and phosphorus present in residual waters (Khan et al., 2018). Furthermore, microalgae have the advantage of not requiring arable land to their growth (Cai et al., 2013).

Microalgae are the source of several metabolites such as proteins, lipids, carbohydrates (CH), carotenoids or vitamins and food additives (Brennan & Owende, 2010; Khan et al., 2018). These bioactive compounds are valuable for commercial use and can be explored and used to many applications (Borowitzka, 1995; Priyadarshani & Rath, 2012).

However, in spite of microalgae being an interesting source of biofuel production and other products with high commercial value, there are many limitations to its production, which cause difficulties in the scaling-up process. The biggest challenge has been the increase of cultures growth rate and synthesis of added-value compounds as well as biomass harvesting. (Khan et al., 2018).

1.1. Historical aspects of SECIL/ALGAFARM

The present Master's thesis was conducted in AlgaFarm production facility, belonging to SECIL group, whose activity is based on cement production, ready-mixed concrete, aggregates, mortars, prefabricated concrete, and hydraulic lime.

SECIL group, beyond being the largest concrete producer in Portugal, integrates companies which operate in different but complementary areas, for which the primordial goal is to develop solutions related to environmental preservation and use wastes as an energy source (Secil Group, 2019). The group was founded in 1930 as a result of the fusion of two companies. Consolidated in Portugal, where it is originated from, SECIL group is expanded to Angola, Tunisia, Lebanon, Cape Verde, Holland, and Brazil. Currently, SECIL operates three cement factories in Portugal located in Outão, Maceira, and Cibra. Therefore, operating in eight factories, seven countries and four continents, it is responsible for an annual output of more than 9 million tons of cement (Secil Group, 2019).

The release of greenhouse gases to the atmosphere has been a permanent concern of the group. Due to the high costs of advanced technology to reduce the emission of polluting gases, such as CO_2, SECIL has been looking for alternatives to mitigate this kind of pollution. The

implementation of a microalgae production unit, known by AlgaFarm appears in this context. AlgaFarm has a mission to reduce CO_2 emissions. The industrial-scale production unit resulted from the expansion of the initial pilot-scale unit and it is, currently, in commercial operation and directed to *Chlorella vulgaris* production for the food industry (Rabaçal et al., 2017).

AlgaFarm unit is divided into four main fields: resources, production, processing, and control. Each sector includes various systems that, together, possess the largest microalgae closed production unit around the world, with a production volume of 1300 m^3 (Rabaçal et al., 2017).

Allmicroalgae is the supplier of Allma *Chlorella*, with continuous production in Algafarm, located in Pataias. Allmicroalgae's products are focused on microalgae production in order to use them as supplements, food, animal feed, and cosmetic applications. In Algafarm unit, *Chlorella vulgaris*, *Nannochloropsis oceanica*, *Phaeodactylum* sp. and *Tetraselmis* sp. are grown in closed tubular systems and/or fermenters, minimizing contamination and ensuring maximum purity (Allmicroalgae, 2019). The Algafarm company, represented in Figure 1, is certificated by ISO 220000 in order to ensure product safety and quality.

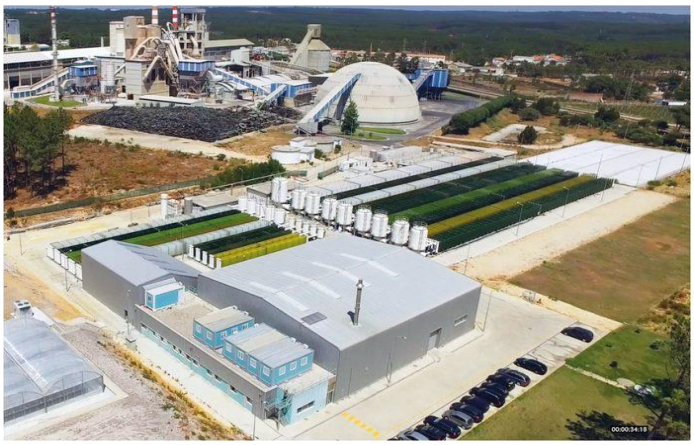

Figure 1 - Aerial view of Algafarm and SECIL facilities.

1.2. Thesis outline

The present thesis dissertation is organized in 5 chapters, as follows:

In Chapter 1, there is a contextualization of the thesis and a brief description of the history, vision, and work developed by SECIL and Algafarm.

Chapter 2, the introduction, reviews the concept of microalgae and their importance for the industry, as well as the cultivation of *Pavlova* sp. and their possible applications. The main differences in open and closed systems and the challenges found in this area are also mentioned. Besides, a brief description of the most important parameters for microalgae cultivation is referred, together with a review of existing literature. To conclude this chapter, the main objectives of the present work are exposed.

Chapter 3, material and methods, describes the methodology used in the laboratory followed by the scale-up experiments, the materials, and instruments required for the techniques described and the experimental procedure. The statistical tools used for data analysis are also presented, along with the tests used to obtains growth curves.

Chapter 4, results and discussion, presents the results obtained from the standard protocols referred in the previous chapter, supported by a discussion of those results based on published literature.

The final conclusions of the work are presented in Chapter 5, as well as the future perspectives.

2. INTRODUCTION

2.1. Microalgae

Microalgae, defined as a polyphyletic group, are unicellular and eukaryote organisms which are capable of converting solar into chemical energy, through CO_2 fixation. Known as thallophytes (lower plants), they possess similarities with superior plants since both have photosynthesis mechanisms (Priyadarshani & Rath, 2012; Wojciechowski et al., 2013). However, microalgae have been proved to be more efficient concerning to solar energy conversion due to their cellular simplicity, which consequently leads to tenfold increase in efficiency when compared with superior plants (Priyadarshani & Rath, 2012; Raja et al., 2008; Sathasivam et al., 2019).

Microalgal metabolism involves numerous bioactive compounds of interest with application in nutrition, cosmetics and in the pharmaceutical industry (Das et al., 2011; Khan et al., 2018; Muller-Feuga et al., 2007; Richmond, 2004). Additionally to its cellular simplicity, microalgae present a wide geographic location resulting in metabolic plasticity that allows species to adapt to a range of environmental conditions (Khan et al., 2018; Muller-Feuga et al., 2007; Wojciechowski et al., 2013). They can be virtually found in all terrestrial conditions, even under extreme environments, with some species showing great resistance to dryness, high salinity, low light, among others (Evangelista et al., 2008; Wojciechowski et al., 2013). However, microalgae are mainly found in the aquatic environment playing an important role in the CO_2 fixation, organic matter consumption and in the release of oxygen to the atmosphere (Wojciechowski et al., 2013).

Microalgae classification is based on their photosynthetic pigments, macromolecules composition, and constituents of the cell wall. Their similarities to superior plants are due to the presence of CH, proteins, and photosynthetic pigments. Microalgae have chlorophyll *a* as a primary pigment and others such as carotenoids, phycocyanin, and phycoerythrin, whose distribution is limited. Furthermore, microalgae are also classified regarding cytologic and morphologic aspects such as flagellum structure, cellular division, nucleus formation, among others (Wojciechowski et al., 2013). Some microalgae species are represented in Figure 2.

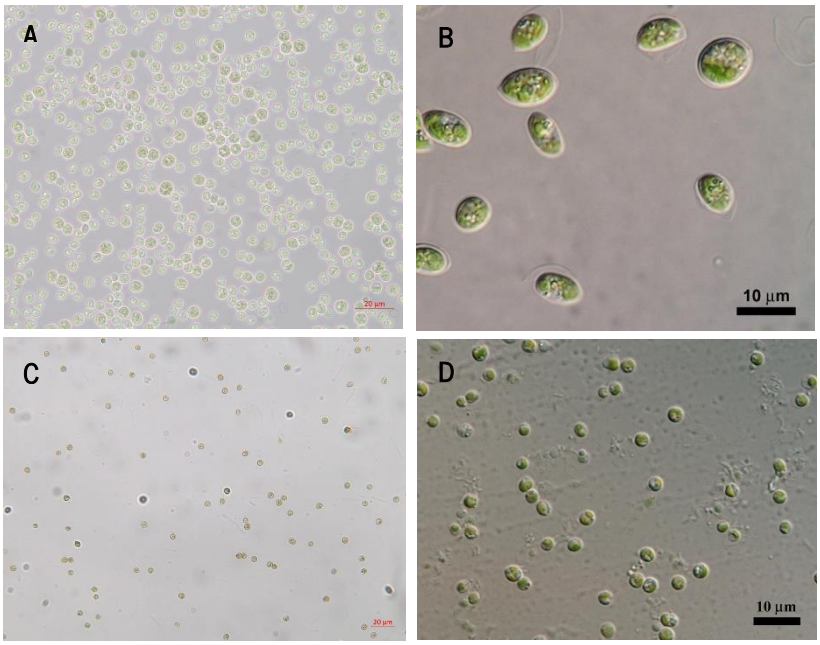

Figure 2 - Microscopic image: (A) *Chlorella vulgaris*, (B) *Scenedesmus obliquus*, (C) *Pavlova lutheri,* and (D) *Nannochloropsis oceanica*.

Chinese were the first population resorting to microalgae (*Nostoc* sp.) as food, in time of famine, about 2000 years ago. However, the biotechnology and commercialization related to microalgae just started to develop in the middle of the last century (*Chlorella* sp. and *Spirulina* sp.) as healthy foods in countries such as Japan, Taiwan, and Mexico (Sathasivam et al., 2019).

Nowadays, there are numerous microalgae applications, not restricted to the food supply. They are used to increase the nutritional value of food owing to their chemical composition; have a huge contribution to aquaculture and can be incorporated in cosmetics (Priyadarshani & Rath, 2012). These microorganisms are a source of polyunsaturated fatty acids (PUFA) that are added to infant formulas and nutritional supplements and pigments which are sold as natural dyes (Priyadarshani & Rath, 2012).

Three relevant aspects give microalgae a huge potential to be converted into new products and investigated in different fields from commercial and biotechnological point of view: i) microalgae are a group of microorganisms with a wide range of physiological and biochemical characteristics; ii) they absorb ^{13}C, ^{15}N and ^{2}H isotopes, used to facilitate the structure determination of proteins, CH and nucleic acids, and incorporate them into their biomass and into the various compounds that produce; iii) microalgae are part of a huge group of microorganisms under-explored and investigated and, consequently, could form a potential source of undeveloped products (Bux, 2013; Priyadarshani & Rath, 2012).

For those reasons, cited before, it is important and urgent to proceed with microalgae investigation.

2.2. Microalgae cultivation

Microalgae growing on an industrial scale aims to contribute, in a sustainable way, to industrial development through biomass production and products with high commercial value and a good price-benefit ratio. Nevertheless, information about some microalgae strains with high industrial interest is still needed in order to improve these processes.

These microorganisms can be cultivated using different methods and conditions attending to their general and specific needs. Microalgae can be grown in the presence of light in photoautotrophic culture, or in the presence of both light and fixed organic carbon in mixotrophic culture, or, in certain cases, in the presence of organic carbon in darkness, in heterotrophic culture (Das et al., 2011).

In the particular case of photoautotrophic cultivation, they require a light energy source so that they can convert absorbed water and CO_2 into biomass, through photosynthesis. To grow, they need nitrogen and phosphorus as main nutrients and others such as sodium, magnesium, calcium, potassium (Cai et al., 2013; Walker, 1954). Those nutrients, as well as micronutrients, can be provided from residual waters that are considered a good source to the same and will contribute to microalgae feeding (Cai et al., 2013).

Nitrogen and phosphorus are the nutrients with a higher importance in the culture medium, the N:P rate is one of the most important factors when designing culture media (Cai et al., 2013).

2.2.1. Light intensity

Light intensity is one of the most important confining factors in autotrophic microalgae growing (Khan et al., 2018; Muller-Feuga et al., 2007). The photoperiod and light intensity directly affect microalgae photosynthesis and impact its biochemical composition and biomass growth efficiency (Boussiba et al., 1987; Brown et al., 1996; Liang et al., 2006; Roessler, 1990). The ideal light intensity should be determined experimentally in order to maximize CO_2 absorption. The optimal level of light intensity, for the most microalgae, is usually found between 200-400 $\mu mol\ m^{-2}\ s^{-1}$ (Schuurmans et al., 2015).

A balance between periods of light and absence of light must be maintained for microalgae photosynthesis. Periods of light results in ATP (adenosine triphosphate) and NADPH (nicotinamide adenine dinucleotide phosphate) synthesis while in absence of light are produced carbon skeletons (Khan et al., 2018; Sun et al., 2018).

2.2.2. Temperature

Temperature is one of the most important abiotic factors in microalgae growth and it influences directly its metabolism. It is important to highlight that each species possess its own optimal temperature that improves its cellular growth (Khan et al., 2018). However, increasing or decreasing this optimal temperature leads to a reverse performance, delaying or even stopping exponential growth. Above optimal value of the temperature of each species, the rate of viability loss increases. This increase can be explained by the fact that algal death is most likely due to degradation of key enzymes and membrane denaturation (van Boekel, 2002).

Moreover, a lower temperature will affect photosynthesis through decreasing in carbon assimilation, whereas if it is too high it will reduce photosynthesis, affecting negatively cell energy balance and inactivating photosynthetic proteins. It also contributes to cell size reduction and affects their respiration as well (Khan et al., 2018).

Generally, the optimal temperature of most microalgae species is between 20 and 30 °C (Singh & Singh, 2015).

2.2.3. pH

Another parameter affecting microalgae growth is pH, whereas its optimal value varies with the microalgae strain being cultured (Khan et al., 2018). The majority of microalgae has an optimal pH between 6 and 9 and are sensitive to pH variations, in contrast to *C. vulgaris*, which is able to withstand a wide range of pH values (Khan et al., 2018; Lam & Lee, 2012). Unusual variations in pH result in chemical modifications of some substances such as CO_2, phosphate, iron, among others, and it will directly interfere in microalgae metabolism, affecting membrane permeability, ionic transport, and the enzymatic reactions (Khan et al., 2018)(Wojcechowski et al., 2013).

pH and CO_2 concentration are directly related. When dissolved in the water, CO_2 forms carbonic acid (H_2CO_3), helping to acidify the culture media. If there is not a supply of CO_2, this compound is consumed by microalgae from the culture medium and the pH increases

(Wojciechowski et al., 2013). Alkaline media may result in toxic ammoniacal production through dissolved salts, which may inhibit microalgae production (Chisti, 2013). The use of nitrate (NO_3^-) supply as a source of nitrogen helps controlling the pH however, microalgae appear to have a preference for ammonia (Scherholz & Curtis, 2013). A CO_2 injection system is often designed and installed in the reactor in order to control the pH during the various stages of microalgae production (Chisti, 2013).

2.2.4. Nutrients supply: macro and micronutrients

Different microalgae species vary in their nutrient needs, however, nitrogen, phosphorus, and carbon are the macronutrients required for the growth of any microalgae and a deficiency in these inorganic nutrients affects directly microalgae growth, resulting in lower biomass productivity (Cai et al., 2013; Khan et al., 2018; Walker, 1954).

Nitrogen is an important element in the microalgae metabolism, being required in high quantities, immediately after carbon. It integrates the molecular composition of proteins, enzymes, and nucleic acids (Wojciechowski et al., 2013). Usually, nitrogen obtained through NO_3^- and urea constitutes a good and economical source when compared with other inorganic sources (Khan et al., 2018).

Phosphorus also plays important roles in microalgae growth and energy (ATP/ADP) production, DNA (deoxyribonucleic acid) and RNA (ribonucleic acid) structure and it is part of the plasmatic membrane (phospholipids) (Wojciechowski et al., 2013). The main phosphorus forms are dihydrogen phosphate ($H_2PO_4^-$) and hydrogen phosphate (HPO_4^-), and they can be incorporated into organic components through phosphorylation with the release of energy (Cai et al., 2013). Microalgae are able to store an excess amount of phosphorus, in the form of polyphosphate (Larsdotter, 2006).

Carbon can be added to microalgae cultures in organic form, glycerol, and acetate, or inorganic form, CO_2. However, at the industrial scale, it is recommended to use environmental CO_2 as a carbon source, which is more economical and allows CO_2 fixation (Khan et al., 2018). Although atmospheric CO_2 concentration is not enough and extra injection of CO_2 is often necessary (Chisti, 2016). Microalgae are composed of 50% carbon, which makes it an important element to microalgae growth and the main when considering autotrophic metabolism (Chisti, 2016).

Regarding micronutrients, such as manganese, potassium, sodium, cobalt, iron, magnesium, boron, zinc, among others, they are required in vestigial quantities (Walker, 1954; Wojciechowski et al., 2013). At high concentrations, micronutrients are toxic to the majority of microalgae species (Khan et al., 2018; Monteiro et al., 2011). Usually, they are added to the culture media together with a chelating agent, which avoids them to achieve toxic concentrations (Larsdotter, 2006). Micronutrients play roles related to enzymatic activity and microalgae structure. However, their addition is not always mandatory and shows to be more relevant in synthetic media (Khan et al., 2018; Wojciechowski et al., 2013).

2.2.5. Mixture and aeration

The standardization between nutrients, air and CO_2 in microalgae culture is achieved through mixture and aeration of the culture. This procedure allows nutrients dissolution and uniform distribution of light inside microalgae culture and, at the same time, avoid biomass settlement and its consequent aggregation. In case of absence of mixture and air supply, biomass productivity is significantly minor and, therefore, microalgae culture must be continuously mixed (Khan et al., 2018).

2.3. Large scale biomass production

Microalgae cultivation is increasingly being researched and explored and has shown remarkable signs of progress. There are several configurations that contribute to the success of the mass cultivation of microalgae, according to the species (Khan et al., 2018; Thukral & Sharma, 2015). The nutritional value of microalgae and their application should be an important parameter to consider when proceeding to large scale biomass production. The selection of the accurate culture system should take into account some parameters such as light efficiency, pH and temperature control, productivity, hydrodynamic stress, the need for an axenic culture, harvesting, and feasibility of scale-up (Guedes & Malcata, 2012; Tamiya, 1956).

Nowadays, microalgae cultivation at large scale can be carried out in open and closed cultivation systems (Carvalho et al., 2006). The first one allows contact with the atmosphere and the other does not allow this contact (Fonseca et al., 2016). The choice of the most suitable system

is a decision dependent on the species to be produced and the final purpose intended, these two parameters play a major role in this decision (Carvalho et al., 2006).

There are five main types of open systems: water tanks, raceway ponds, circular ponds, thin layer cascade, and shallow big ponds (Fonseca et al., 2016; Narala et al., 2016).

The first step in the direction to microalgae production was achieved through a raceway pond (Figure 3) and, since then, there was extensive research in microalgae cultivation in open systems (Borowitzka, 1999; Carvalho et al., 2006; Johnson et al., 1988). These systems have some economic advantages when compared with closed systems, not requiring high capital and operating costs, are easier to clean and do not need much energy for culture mixture (Narala et al., 2016). However, open systems present also space issues, a higher probability of occurring contaminations, are dependent on favorable climate, have evaporation losses, CO_2 diffuses easily into the atmosphere, present an inefficient use of light and lower productivity rates (Fonseca et al., 2016; Narala et al., 2016).

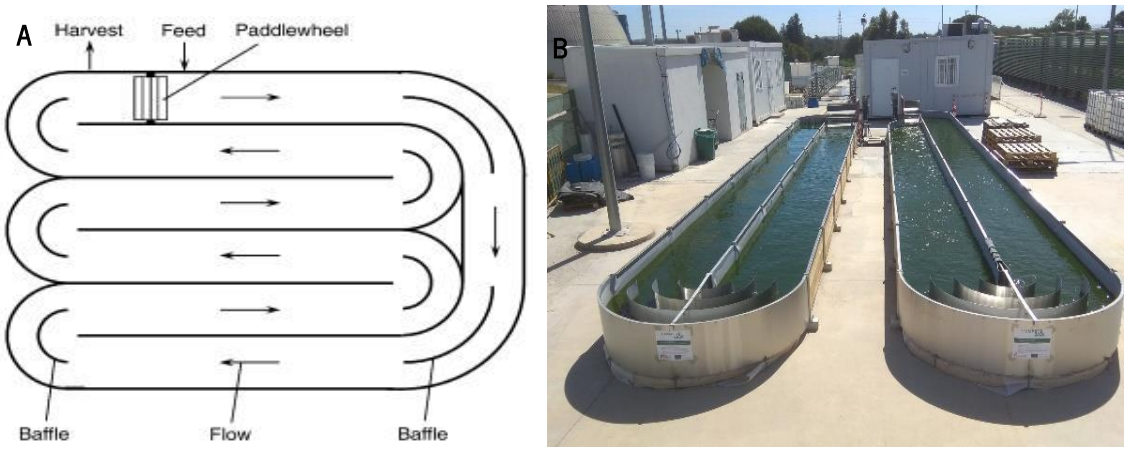

Figure 3 - Raceway pond cultivation systems. (A) Schematic design (Chisti, 2007) (B) Photography of a raceway pond in AlgaFarm facilities.

Raceway ponds are known by their channels with an oval and closed-form, designed to flow the culture in a loop, with a paddlewheel paddling the microalgae culture in one direction. The paddlewheel should be in continuous operation to prevent sedimentation and to ensure proper mixing of the nutrients (Brennan & Owende, 2010; Show et al., 2017). Therefore, the mixture and

circulation are needed in order to stabilize and maximize microalgae growth and productivity. The depth of the system is usually between 0.2 and 0.5 m in order to enable the entrance of light (Brennan & Owende, 2010). In this type of reactors, CO_2 injection is usually done from the surface air, but submerged aerators may be installed to enhance CO_2 absorption (Terry & Raymond, 1985).

On the other hand, closed systems proved to be more efficient than the previous and allow better control of the culture parameters. Inside of the closed systems, mainly known as photobioreactors (PBR), they can appear with different configurations: tubular reactors, and flat panel reactors (Carvalho et al., 2006). The difference between these PBR lies in aspects such as manufacturing material, layout, the passage of light and the aeration. Wherefore, the operation of each system is influenced by the species concerned, climate conditions and associated costs (Carvalho et al., 2006; Narala et al., 2016).

Closed systems are used to overcome the open systems' disadvantages, allowing better efficiency and achieving higher biomass concentrations, as far as the operating conditions can be easily controlled and contaminations can be prevented. Nevertheless, the huge problem associated with this type of reactors is the higher cost of using them (Narala et al., 2016). Configurations of tubular reactors are mostly vertical and horizontal tubular reactors.

Vertical PBR can be categorized into airlift and bubble column reactors (Figure 4) based on their liquid flow patterns inside the PBR (Mirón et al., 2000). Bubble columns and airlift PBR can be useful for culturing phototrophic organisms. Light availability in bubble columns and airlift reactors is influenced by aeration rate, gas holdup, and culture mixing (Mirón et al., 2000). They are normally built-in polyethylene or glass tubes that have sufficient transparency to allow the penetration of the light in the reactor (Carvalho et al., 2006). And as they are manufactured with common and non-expensive materials, are considered financially accessible. In these bioreactors, the air is bubbled from the bottom of the reactor and, resorting to a gas sparger system, the inlet air is converted into tiny bubbles. This way, reactors with this configuration can provide good mixture, enough O_2 removal, and CO_2 supply (Carvalho et al., 2006; Thukral & Sharma, 2015). The difference between a bubble column and an airlift reactor is the draft tube that allows culture circulation (Figure 4B) (Behin, 2012).

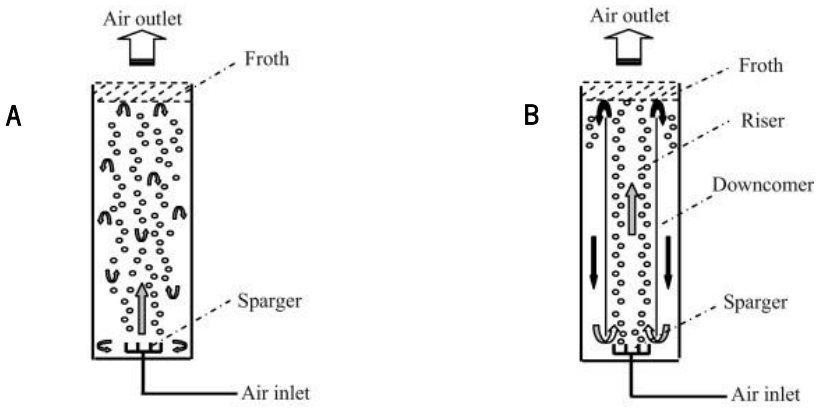

Figure 4 - Schematic design of reactors (A) bubble column reactor and (B) internal loop airlift reactor (Behin, 2012).

Tubular PBR with horizontal configurations, represented in Figure 5 are widely used for algae production and are made of transparent polypropylene acrylic or polyvinyl chloride pipes with a small intern diameter in order to facilitate the penetration of the light (Borowitzka, 1994; Thukral & Sharma, 2015). In this type of reactor, the gas transfers occur at the pipe connection or in a gas exchange unit. An air pump or an airlift system to provide the necessary agitation and gas exchange can also be added to the system (Thukral & Sharma, 2015). Such systems have the advantage of managing large culture volumes once the risk of contamination is lower than in the open systems (Borowitzka, 1994; Carvalho et al., 2006). However, a considerable amount of heat is often an operational concern (Richmond, 1987). Therefore, temperature control is necessary, increasing the installation and operational cost (Carvalho et al., 2006). Furthermore, due to the large illumination area that they are submitted, they present some limitations during scale-up such as oxygen buildup and photo-inhibition (Borowitzka, 1994; Carvalho et al., 2006; Mirón et al., 1999).

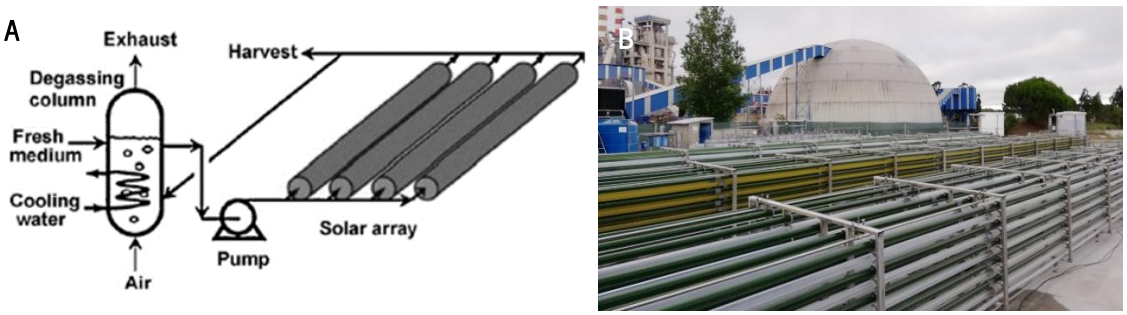

Figure 5 - Tubular photobioreactor (PBR) with horizontal configuration. (A) Schematic design (Singh & Sharma, 2012) (B) photography of a tubular PBR in AlgaFarm facilities.

Flat panel PBR (Figure 6A) consists of two rectangular panels made of glass or acrylic and may be inclined in order to capture the optimal quantity of radiation into the material of the reactor. The culture mixing is provided by an aeration system and facilitates the removal of oxygen resulted from photosynthesis. CO_2 should also be added by this aeration system whereas the temperature is controlled by spraying water over the surface (Thukral & Sharma, 2015). However, the disadvantages associated involve space and high solar energy requirements, difficult maintenance and low efficiency in terms of mass production (Thukral & Sharma, 2015). Nevertheless, as an alternative to replace the operational costs and to overcome the problems of scale-up, a cheaper design was proposed by Rodolfi et al. (2009), where the previously referred materials are replaced by plastic bags (Figure 6B). These systems use large sterile plastic bags and are fitted with a system for aeration. Normally, these systems are operated in batch mode (Borowitzka, 1999).

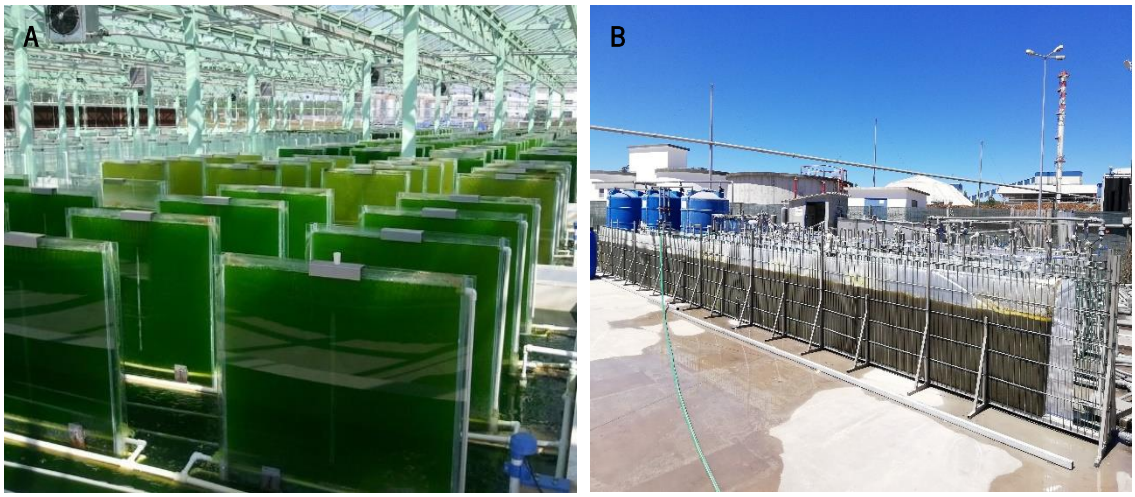

Figure 6 - (A) Conventional flat panel photobioreactor and (B) flat panel reactor constituted by plastic bags in AlgaFarm facilities.

All reactors above involve photoautotrophic microalgae production, using natural or artificial lighting. (Thukral & Sharma, 2015). On the other hand, fermenters, represented in Figure 7, are the conventional reactors used to heterotrophic microalgae cultivation, where the source of carbon is organic. The major advantage of this type of reactors is the precise control of some process parameters. Another advantage is the fact of heterotrophic fermenters allowing the achievement of higher biomass values (Carvalho et al., 2006). Therefore, it is common to use this concentrated

biomass as inoculum to a large scale tubular PBR in order to gain pigments and to reduce the time of scale-up (Barros et al., 2019).

Figure 7 - Industrial fermenter with an operational volume of 5000 L located in AlgaFarm facilities.

Generally, closed systems allow an increase in microalgae cultivation efficiency, when compared with open systems (Carvalho et al., 2006; Narala et al., 2016; Thukral & Sharma, 2015). However, although closed systems have a configuration that overcomes the disadvantages of open systems, they also present some disadvantages (Narala et al., 2016). This type of systems is associated with fouling, overheating, cleaning issues and oxygen accumulation, which can lead to a decrease in culture growth (Brennan & Owende, 2010; Carvalho et al., 2006; Narala et al., 2016). In addition, the facilities require huge capital investment and operational costs (Brennan & Owende, 2010; Narala et al., 2016).

2.4. Microalgae biotechnological applications

The commercial culture of microalgae targeted at their metabolites has been taking place for many years (Richmond, 2004). The main microalgal species grown at industrial scale and commercialized are *Chlorella* sp. and *Spirulina* sp. for healthy foods, *Dunaliella salina* for *β*-carotene, *Haematococcus pluvialis* for astaxanthin and several other minor species, such as *Pavlova* sp., for aquaculture (Borowitzka, 1999).

Due to their chemical composition, microalgae are able to enhance the nutritional content of conventional food. Microalgae represent also a non-conventional alternative to incorporate food products due to the high protein content of various microalgae species. In fact, microalgae are able to synthesize almost all amino acids and, for this reason, they can provide them to humans and animals (Table 1) (Spolaore et al., 2006).

The CH present in microalgae composition assume forms of starch, sugars and other polysaccharides (Spolaore et al., 2006). As their specific digestibility is high, CH do not represent a limitation to their incorporation in food and feed products (Richmond, 2004).

Also, the lipid content in microalgae cells varies between 1% and 70% of biomass dry weight (*DW*) (Metting, 1996). There are two major lipid classes in microalgae: polar lipids, which are usually present in membranes and are constituted by phospholipids and glycolipids, and neutral lipids (mainly triacylglycerols), which are storage lipids. Polar lipids are usually constituted by PUFA, while triacylglycerols (TAG) contain more saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) (Dunstan, Volkman et al., 1993; Guihéneuf & Stengel, 2013; Roessler, 1990). Of all fatty acids (FA) present in microalgae cells, omega-3 and omega-6 FA have a particular interest in industrial applications (Spolaore et al., 2006).

Table 1 - General composition of different human food sources in comparison with different microalgae species (% of dry weight (*DW*)) (Amos Richmond, 2004)

Product	Protein	Carbohydrate	Lipid
Bakers' yeast	39	38	1
Meat	43	1	34
Milk	26	38	28
Rice	8	77	2
Soybean	37	30	20
Anabaena cylindrica	43-56	25-30	4-7
Chlamydomonas rheinhardii	48	17	21
Chlorella vulgaris	51–58	12-17	14-22
Dunaliella salina	57	32	6
Porphyridium cruentum	28–39	40-57	9-14
Scenedesmus obliquus	50–56	10-17	12-14
Spirulina maxima	60–71	13-16	6-7
Synechococcus sp.	63	15	11

The aquatic environment has been recognized not only as a rich source of food but also an important source of minerals and natural products (Richmond, 2004). Microalgae, particularly, have demonstrated huge potential in various fields as nutrition, medical, pharmaceutical, fertilizers, research and environmental.

Besides all microalgal applications, their applicability goes beyond that and microalgae have been targeting interesting studies in the most diversified areas, such as effluent treatment, biological detoxification, and heavy metals removal, bioindicator of the presence of nutrients or substances with toxicity (Brennan & Owende, 2010). In the farming field, microalgae are a good alternative to proceed to soil's parameters control, wherefore its biomass can be used as fertilizer (Wojciechowski et al., 2013). Furthermore, microalgae contribute to the synthesis of a wide range of bioactive molecules with specific properties and a high potential to be applied in the medical field (Wojciechowski et al., 2013). Another area that has been investigated is the area of renewable energies, contributing to biofuel production (Brennan & Owende, 2010). Finally, microalgae play a key role in the mitigation of greenhouse gases, leading to CO_2 fixation resulted from the burning of fossil fuels (Brennan & Owende, 2010). Table 2 represents some products obtainable by microalgae.

Table 2 - Products obtained from microalgae (Derner et al., 2006; Spolaore et al., 2006)

	Products	Applications
Biomass	Biomass	Healthy food; Functional food; Food additives; Aquaculture; Soil conditioner
Dyes and antioxidants	Xanthophyll; Lutein; β-carotene; Vitamin C and E	Food additives; Cosmetics
Fatty acids	Eicosapentaenoic acid (EPA); Docosahexaenoic acid (DHA); Gamma-linolenic acid (GLA); Linoleic acid	Food additives; Nutritional supplements; Aquaculture
Enzymes	Superoxide dismutase; Phosphoglycerate kinase; Restriction Enzymes	Healthy food; Research; Medicine
Polymers	Polysaccharides; Starch; Poly-β-hydroxybutyrate	Food additives; Cosmetics; Medicine
Special products	Peptides; Toxins; Isotopes; Amino acids; Steroids	Research; Medicine

2.5. *Pavlova* species: general overview and applications

Pavlova species belong to the phylum Haptophyta because they present a haptonema, a third appendage used mainly for attachment and food handling, and fit in the class of Pavlovophyceae family, known for possessing cells with knob scales. In the order Pavlovales, they possess motile cells with two unequal flagella – the longer with an investment of small knob scales and fine hairs and the shorter sometimes vestigial (Eikrem et al., 2016).

Table 3 - Taxonomic classification of *Pavlova* sp.

Domain	Eukaryota
Kingdom	Chromista
Phylum	Haptophyta
Class	Pavlovophyceae
Order	Pavlovales
Family	Pavlovaceae
Genus	Pavlova
Species	*P granifera* *P. gyrans* *P. lutheri* *P. pinguis*

Regarding *Pavlova* sp. propagation, they have a motile and a palmelloid (non-motile) phase. It is during the palmelloid stage that occurs longitudinal division (Butcher, 1952). The movements of *Pavlova* cells are very diversified. Sometimes they can be found twisting and turning their own axis, moving rapidly in one direction or even going forward with a vibration motion (Butcher, 1952).

Pavlova sp. exhibits different and varied forms and sometimes it appears as a spherical or amoeboid form while at other times it arises a cylindrical compressed morphology (Figure 8). The yellow-brown coloration at high densities is due to accessory carotenoid pigments. This unicellular microorganism has a 7-8 μm length and is only adapted to grow in a saline environment. Cells are flagellated and cultures are commonly grown in autotrophic conditions (Eikrem et al., 2016).

Many microalgae species are able to grow in both mixotrophic and heterotrophic conditions, which could result in a growth several times higher than under photoautotrophic conditions (Heredia-Arroyo et al., 2010; Wen & Chen, 2000). Bashir et al. (2019) studied the effects of

different growth conditions in *P. lutheri*. However, although the growth of *P. lutheri* was inhibited under heterotrophic conditions, it was enhanced in mixotrophy, when compared to microalgae growth under light intensity alone.

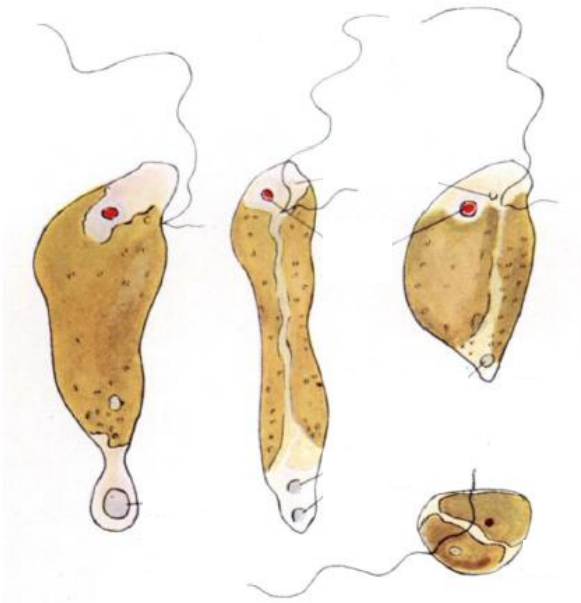

Figure 8 - The most frequently observed shapes of *P. gyrans* (Butcher, 1952).

Organisms belonging to Haptophyta phylum are very rich in FA and a large amount of these FA may correspond to the valuable omega-3 PUFA, namely eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids (Eikrem et al., 2016).

It is well known the importance of PUFA in human health and, although the major source of these FA is incorporated in a diet composed by fish, it is known that fish, by themselves, synthesize EPA and DHA only to a limited extent (Dyerberg, 1986; Grima et al., 1993; Pereira et al., 2012). Therefore, these compounds must be supplemented in their feed, including PUFA-rich microalgae (Borowitzka, 1997; Guedes & Malcata, 2012; Grima et al., 1993).

Therefore, *Pavlova* sp. can become one of the most important feed sources in aquaculture and is currently used in mariculture hatcheries to feed crustacean, bivalves, and fishes (Meireles et al., 2003). Microalgae are one of the most promising sources of EPA and DHA and, consequently, cultivation of microalgae, especially Haptophytes, as a supplement of fish and other marine animals is increasing day by day (Eikrem et al., 2016). The first species of microalgae used

in aquaculture were selected because they presented a natural growth in the marine environment and, therefore, exhibited to be the easiest microalgae to grow for this purpose. Subsequently, other species were investigated and showed to be most nutritionally efficient than the previously used ones (Richmond, 2004).

2.6. Research aims

The main objective of this Master's thesis was to study the growth of four species of *Pavlova* at lab-scale and determine the one which demonstrates better performance under the same light-regime, salinity, and temperature conditions, and at a given inoculum concentration. Furthermore, to discover if the biochemical composition of microalgae is easily modified through different system configurations, light intensity and photoperiod, an assay using *P. gyrans* and *P. pinguis* cultures was carried out in outdoor scale. Finally, to study and evaluate the influence of the cultivation system in *Pavlova* sp. cultures growth, an assay was carried out on a large scale: flat panel, tubular PBR, and raceway pond.

3. MATERIALS AND METHODS

The growth assessment experiments were performed at Algafarm facilities, located in Pataias, Portugal, between February 06th and August 23th 2019. The biochemical profile of produced biomass was performed at MarBiotech, a group belonging to the Centre of Marine Sciences (University of Algarve) between the July 24th and August 08th 2019.

3.1. Microalgae strain and inoculum preparation

Pavlova granifera, Pavlova gyrans and *Pavlova pinguis* were obtained from Roscoff Culture Collection, while *Pavlova lutheri* was obtained from AlgaFarm Culture Collection.

All autotrophic experiments were carried out using non-axenic cultures as inoculum. *Pavlova* sp. cultures have grown in t-flasks expose to natural light and at room temperature. These cultures were later scaled-up to 1.5 L and 5 L reactors which were grown at room temperature, under continuous light irradiance of ~100 µmol m^{-2} s^{-1}, with aeration and injection of CO_2.

Guillard's F/2 (10 mmol L^{-1} of NO_3^-), supplemented with iron (Fe^{2+}) and Conway's vitamin solutions, was used as culture medium, with a salinity adjusted to 30 g L^{-1} (Tompkins et al., 1995). The pH of all cultures was daily adjusted to 8 (Carvalho & Malcata, 2005; Carvalho & Malcata, 2000; Shah et al., 2014).

All *Pavlova* sp. cultures were operated in batch mode and were used to inoculate all the autotrophic reactors used in the present work. These conditions were used in all the assays performed, except when mentioned.

3.2. Lab-scale assays of *Pavlova* sp.

The autotrophic assays were conducted in 1 L bubble column reactors set with constant aeration of 600 L min^{-1}, containing 1% CO_2 used to manually maintain pH at 8. Cultures were kept at 24 °C under continuous LED radiation exposure of 700 µmol m^{-2} s^{-1}. Samples were taken every two days together with the addition of water to compensate for evaporation losses. The water used in the inoculation and in the evaporation losses was local water provided by SECIL group and was

chemically treated with sodium hypochlorite and neutralized with thiosulfate. All experiments were conducted in triplicates.

3.2.1. *Pavlova* sp. selection

The first laboratory assay consisted of testing the growth of the different species of *Pavlova*. Accordingly, *P. lutheri*, *P. granifera*, *P. gyrans* and *P. pinguis* were grown in 1 L bubble column reactors.

The assay was intended to start at, nearly, 0.20 g L^{-1} of biomass. The culture conditions used in this assay were the same previously mentioned. In the first 12 days, cultures were maintained at continuous radiation of ~100 µmol m^{-2} s^{-1} and then the light exposure was increased to ~700 µmol m^{-2} s^{-1}. All experiments were done in triplicate.

3.2.2. Effect of inoculum concentration in *P. gyrans* growth

The assay was performed in 1 L bubble column reactors. *P. gyrans* was inoculated at 0.15, 0.23, 0.35 and 0.49 g L^{-1}, corresponding to 5, 10, 20 and 30% inoculum. Samples were taken every two days during the trial, where the growth performance (optical density at 750 nm), pH, salinity and NO_3^- concentration were monitored.

3.3. Outdoor scale-up of *Pavlova* sp.

Culture systems used in this study are represented in Figure 9, with increasing culture volumes: A) 1.5 and 5 L balloon reactors; B) 600 L flat panels; C) 2500 L tubular PBR; D) 3500 L raceway.

Microalgae cultures were grown in 1.5 and 5 L laboratory balloon reactors. The reactors were continuously aerated with 0.2 µm filtered air and CO_2. pH was maintained around 8. The reactors were under continuous light irradiance of ~100 µmol m^{-2} s^{-1} and at room temperature.

Figure 9 - *Pavlova* sp. growing in different PBR at AlgaFarm facilities: (A) 1.5 and 5 L laboratory balloon reactors, (B) 600 L flat panels, (C) 2500 L tubular PBR and (D) 3500 L raceway pond.

3.3.1. Scale-up of *Pavlova* sp. to outdoor flat panel reactors

Four 5 L reactors were used as pre-inoculum for the outdoor 60 L flat panel and successive scale-ups were made until the achievement of a 600 L flat panel reactor. Optical density, salinity, and NO_3^- consumption were daily monitored.

The sterilization of the air inlet was carried out with 0.2 µm polypropylene filters (Whatman™, United Kingdom). pH was registered using a pH meter (Combo pH/Conductivity/TDS Tester, Hanna instruments, Portugal) three times a day and maintained around 8 by the manual control of CO_2 pulses, injected by the same system used in the air inlet. The temperature was maintained below 25 °C by a system of spraying water over the flat panel surface.

3.3.2. Scale-up of *Pavlova* sp. to the outdoor tubular PBR

Two 600 L flat panels were used to inoculate a 2500 L horizontal tubular PBR with *P. gyrans*, which started at 0.4 g L^{-1}. Another 2500 L tubular PBR was inoculated with *P. pinguis* using a 600 L flat panel at 0.4 g L^{-1}. pH was controlled by an automated system and CO_2 injected in order to keep the pH at the optimal value of 8. Besides, culture mixing was achieved using a centrifugal pump at, maximum, 18 Hz. One sample was taken per day during the trial, where the growth performance, salinity, and NO_3^- consumption were monitored.

3.3.3. Scale-up of *Pavlova* sp. to the outdoor raceway

A preliminary growth assay was performed in a 28.8 m² raceway pond. In this trial, a 600 L flat panel reactor of *P. pinguis* was used to inoculate a 3500 L raceway at 0.25 g/L. The water line was kept at 10.5 cm and the paddlewheel operating at 6 Hz. One sample was taken per day during the trial. Every two days, water was added to cover evaporation losses.

3.4. Growth assessment

The culture growth was determined by cell density and *DW*. As an indirect measurement of the growth of the culture, optical density (*OD*) was determined in a Zuzi spectrophotometer (model Zuzi 4251/50, Spain) at 750 nm.

DW was determined by filtering a known volume of culture and then washed twice with ammonium formate 35 g L^{-1} in 0.7 µL glass microfiber filters (VWR, Portugal)(Guihéneuf & Stengel, 2017). After that, the filters were dried on a moisture analyzer (MA 50.R, RADWAG, Poland). Then, the final value of *DW* was obtained in a precision balance. This procedure was to establish the calibration curve of *OD* at 750 nm versus *DW*, present in Equation 1.

$$OD_{750nm} = 2.7067\ DW\ (g\ L^{-1}) - 0.1464 \qquad R^2 = 0.9377 \qquad (1)$$

The calibration curve generated is present in Annex A.

The specific growth rate of culture (μ) was estimated by the quotient between the growth rate (r_X) and the concentrations of biomass (X) at that moment. The development of the integral results in the simplified equation that is the fraction between the Neo-logarithmic of biomass concentration (X_Y) on time later (t_Y) divided by biomass concentration (X_X) on time previous (t_X) and the time interval (t_Y less t_X). The specific growth rate was measured using Equation 2.

$$\mu(day^{-1}) = \frac{r_X}{X} = \frac{1}{X}\frac{dX}{dt} = \frac{\ln(X_y/X_x)}{t_y - t_x} \qquad (2)$$

Global volumetric productivity (*P*) during the logarithmic phase was calculated through Equation 3 and was determined by the division of *DW* in g L^{-1} differences (X_2 and X_1) and the time interval (t_1 and t_2 represent the total time in d^{-1} of the trial).

$$P\left(g\ L^{-1}day^{-1}\right)=\frac{X_2/X_1}{t_2-t_1} \quad (3)$$

Productivity per unit of occupied-land area per unit of time (P_a) was determined by multiplying the volumetric biomass productivity by the volume of the PBR in L (*V*) and divided by the area of the PBR, in m^2 (*A*). Pa was calculated through Equation 4.

$$P_a\left(g\ m^{-2}day^{-1}\right)=\frac{P\ x\ V}{A} \quad (4)$$

The photosynthetic efficiency was determined as the ratio between the higher heating value (*HHV*) and the solar irradiation that reached the reactor. The solar radiation was measured using a meteorological station (RM Young) and an Apogee Logan UT SP-110 pyranometer. *HHV* was calculated according to a previous correlation reported by Callejón-Ferre et al. (2011), shown in Equation 5.

$$HHV\ (kJ\ g^{-1}) = -3.393 + 0.507\ C - 0.341\ H + 0.067\ N \quad (5)$$

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

3.5. Nitrate determination

Samples collected were centrifuged for 20 min at 3500 rpm (MiniStar silverline, VWR, Portugal). NO_3^- were determined according to Armstrong (1963). Briefly, the collected supernatant was diluted (ratio of 1:80) and an hydrochloric acid solution (1 mol L^{-1}) was added. Samples were

measured at 220 and 275 nm. NO_3^- concentration was calculated from a previously established calibration curve.

3.6. Analytical determinations

After cultivation, the microalgal biomass obtained during the growth phase was harvested by centrifugation at 3500 rpm for 15 min. The obtained biomass pellet was frozen at -18 °C and was then freeze-dried in a Telstar LyoAlfa 15 until a constant weight was achieved.

3.6.1. Total lipid determination

Total lipid content (Figure 10) was determined following the Blingh and Dyer (1959) method, with a few modifications by Pereira et al. (2012). Lyophilized biomass was weighed (w_i) in glass tubes and 0.8 mL of distilled water, 2 mL of methanol and 1 mL of chloroform was added, followed by homogenization with an IKA Ultra-Turrax disperser (IKA-Werke GmbH, Staufen, Germany) on ice during 60 s. Thereafter, 1 mL of chloroform and 1 mL of distilled water were added one at a time to the mixture and homogenized for two intervals of 30 s. Afterwards, the mixture was vortexed and centrifuged at 2500 *g* for 10 min and, with a Pasteur pipette, the chloroform phase (lower layer) was extracted to a new glass tube. A well-known volume of chloroform (0.5-1 mL) was transferred to previously weighed tubes (w_1), which were placed in a dry bath at 60 °C to evaporate the chloroform. After that, the tubes were put in the desiccator and then weighed in a precision balance.

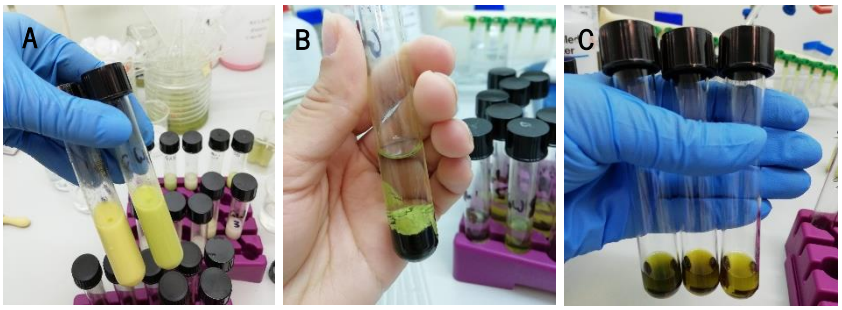

Figure 10 - Total lipid quantification procedure: (A) Lipids extraction on Ultra-Turrax, (B) Lipids extraction with phase separation after centrigugation (chloroform is present in the lower layer) and (C) Chloroform extraction.

The resulting dried residue was weighed (w_2) in a precision balance and the percentage of lipids in each sample was calculated according to Equation 6.

$$\% \text{ Total lipids} = \frac{\frac{(w_2 - w_1) * V \text{ total of chloroform}}{V \text{ evaporated chloroform}}}{(w_i)} \quad (6)$$

3.6.2. Fatty acid methyl esters determination

FA (Figure 11) were converted into the correspondent fatty acid methyl esters (FAME) following the Lepage and Roy method (1984) with a few modifications by Pereira et al. (2012). Lyophilized biomass was weighed (5-10 mg) to derivatization vessels and 1.5 mL of a methanol/acetyl chloride solution (20:1, v/v) was added. The mixture was homogenized on ice for 90 s with an Ultra-Turrax disperser. Afterwards, 1 mL of hexane was added and the vessels were placed in a water bath at 70 °C, for 60 min, and then in ice, for 15 min. Next, 1 mL of water and 4 mL of hexane were added to the mixture and then centrifuged at 2000 *g* for 5 min. With a Pasteur pipette, the hexane phase (higher layer) was extracted to another vessel. The previous two steps were repeated (just the hexane fraction). The extracted hexane was dried with the addition of anhydrous sodium sulfate (Na_2SO_4) and was filtered (0.22 µm) to new tubes. After that, the hexane was evaporated under nitrogen gas flow until dryness. The FAME were resuspended in 500 µL gas chromatography-grade hexane and transferred to vials for GC-MS analysis.

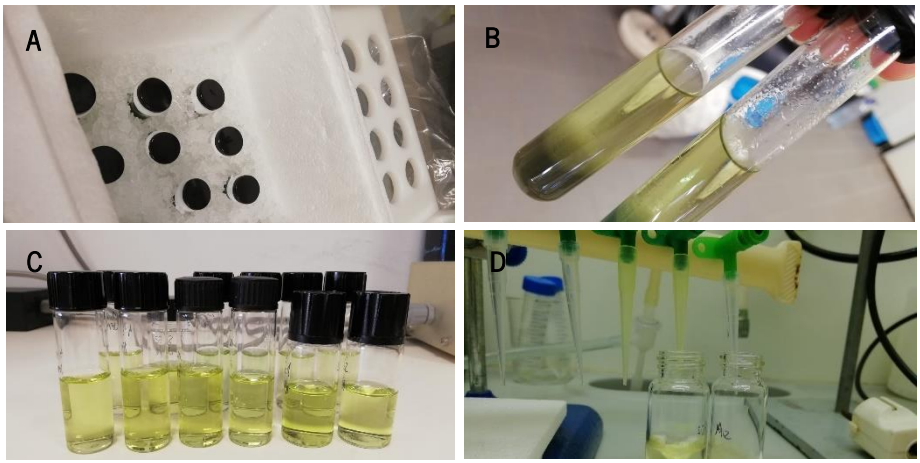

Figure 11 - Some steps of fatty acid methyl esters procedure: (A) Samples in ice for 15 min; (B) PUFA extraction with phase separation after centrifugation; (C) Samples with anhydrous sodium sulfate (D) Evaporation of the hexane fraction under nitrogen gas flow.

3.6.3. Elemental analysis (CHN)

Elemental analysis of carbon, hydrogen, and nitrogen was achieved using a vario EL III (Vario EL, Elementar Analyser system, GmbH, Hanau, Germany). The liophylized biomass (1 mg) was weighed in specific aluminium caps (Figure 12). Total protein was estimated by multiplying the N content by 4.59 (Lourenço et al., 2004).

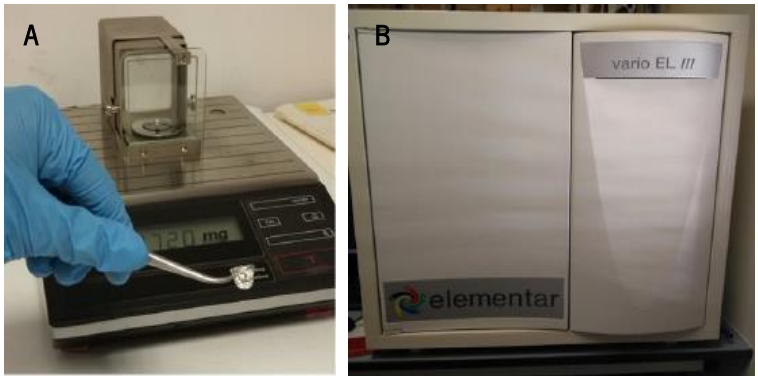

Figure 12 - (A) Precision balance to weigh the aluminum capsules with 1 mg of biomass and (B) Vario EL, Elementar Analyser system, GmbH, Hanau, Germany).

3.6.4. Ash content

Biomass was weighed and placed in small ceramic cups and burned for 5 h at 550 °C using a furnace. The ceramic cups were stored in a desiccator. Total ash was determined by the weight difference before and after burning the produced biomass (Figure 13).

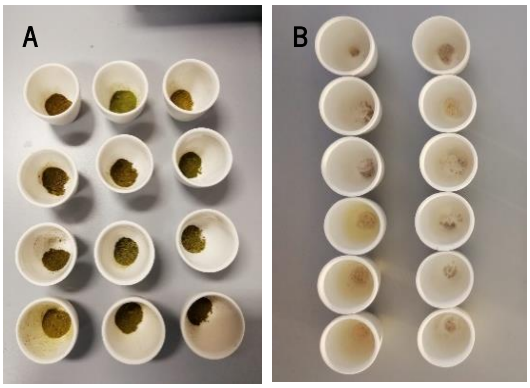

Figure 13 - (A) Initial biomass wheiged in ceramic cups and and (B) Ashes obtained after biomass burned at 550 °C.

3.6.5. Carbohydrates content

CH were determined by the difference between the other principal macromolecules referred previously.

3.7. Statistical analysis

All statistical analysis were done using R software (version 3.6.1), through the RStudio IDE (version 1.2.1335). ANOVA was performed for tests using three or more conditions with a multiple comparison of *Tukey-HSD*. Experimental results are presented with 95% confidence level ($p<0.05$). For each test, the mean and standard deviation from three replicates were determined.

4. RESULTS AND DISCUSSION

4.1. Screening of *Pavlova* sp. and biochemical composition

The marine microalgae *Pavlova* sp. is widely used in mariculture and is currently one of the most promising producers of high levels of EPA and DHA (Guihéneuf et al., 2015; Hu et al., 2008a). The aim of this thesis was to evaluate the growth and compare four different *Pavlova* species: *P. granifera*, *P. gyrans*, *P. pinguis* and *P. lutheri* (Figure 14). The screening assay lasted 18 days and occurred under the effect of two different light intensities.

Pavlova species have different growth curves, differing in exponential and lag phases. *P. gyrans* and *P. pinguis* have grown exponentially at the beginning of the experiment, overtaking the lag phase. In contrast, *P. granifera* and *P. lutheri* have grown exponentially later on and had an initial lag phase of 6 and 4 days, respectively. The stationary phase was not possible to achieve under 700 µmol m^{-2} s^{-1}. *P. gyrans* and *P. pinguis* had consumed the available NO_3^- in culture medium faster, remaining 0.4 and 1.8 mmol L^{-1} after 18 days, respectively. *P. granifera* and *P. lutheri* ended with a concentration of 11.6 and 10 mmol L^{-1}, respectively (Annex B).

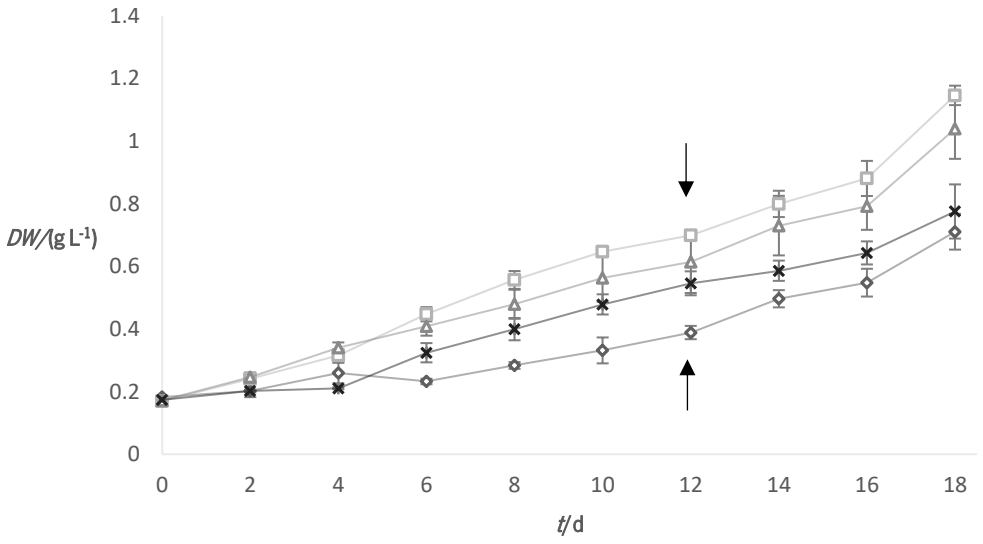

Figure 14 - Batch growth of *P. granifera* (◇), *P.gyrans* (□), *P. pinguis* (△) and *P. lutheri* (×) in 1 L bubble column reactors. Increasing of light intensity at day 12 (↓). Each point represent a mean of three replicates and respective standard deviation (n=3; mean±sd)

P. gyrans and *P. pinguis* always showed a higher total biomass production, during the 18 days, independently of the light intensity used. On the other hand, *P. granifera* and *P. lutheri* demonstrated a slower production at the same conditions ($p<0.05$). Under a light intensity of 100 µmol m^{-2} s^{-1}, just *P. gyrans* and *P. pinguis* have shown similar growth ($p\geq0.05$) while differences between the other species were noticed ($p<0.05$). After light increase, there were not observed significant differences also in the growth of *P. lutheri* and *P. granifera* ($p\geq0.05$).

In this study, the maximum value of biomass obtained was 1.15 g L^{-1} in *P. gyrans* and 1.04 g L^{-1} in *P. pinguis*, followed by 0.77 g L^{-1} and 0.71 g L^{-1} in *P. lutheri* and *P. granifera*, respectively. Other authors report having achieved a biomass of 0.45 g L^{-1} in 250 mL and 0.43 g L^{-1} in 1 L *P. lutheri* cultures under 130 µmol s m^{-2} s^{-1} and a light-regime of 24:0 (L:D) (Shah et al., 2014). Guihéneuf et al., (2009), in turn, obtained in his study a maximum density of 0.9-1 a.u. (arbitrary unit) at 750 nm in *P. lutheri* cultures under 100 µmol m^{-2} s^{-1}, while in the present study was obtained a higher culture density of between 1.3-1.8 a.u. in *P. lutheri, P. gyrans* and *P. pinguis*.

The growth rates and biomass productivities for each *Pavlova* species were calculated and are represented in Table 4.

Table 4 - Volumetric productivity on biomass during the whole assay, maximum value, and specific growth rate on the exponential phase for each *Pavlova* sp. in the study. All data represent the mean (±sd) of three individual replicates

Species	Light Intensity (µmol m^{-2} s^{-1})	Maximum volumetric productivity (g L^{-1} d^{-1})	Global volumetric productivity (g L^{-1} d^{-1})	Specific growth rate (d^{-1})
P. granifera	100	0.042±0.006	0.017±0.002	0.086±0.007
	700	0.081±0.011	0.053±0.008	0.130±0.014
P. gyrans	100	0.069±0.019	0.044±0.001	0.161±0.008
	700	0.133±0.019	0.087±0.005	0.132±0.024
P. pinguis	100	0.059±0.008	0.037±0.008	0.171±0.013
	700	0.124±0.011	0.077±0.002	0.136±0.006
P. lutheri	100	0.061±0.012	0.031±0.003	0.213±0.052
	700	0.066±0.027	0.047±0.014	0.091±0.033

Under a lower light intensity there was no significant difference in global productivities between *P. pinguis* and *P. gyrans* and between *P. pinguis* and *P. lutheri* ($p\geq0.05$). In contrast, statistical differences were found for the specific growth rate of *P. granifera* compared to the other

three species (p<0.05). No statistical differences were found regarding the maximum volumetric productivity (p≥0.05).

Under a light intensity of 100 µmol m^{-2} s^{-1} the maximum value of specific growth rate was between 0.161 and 0.213 d^{-1}, obtained by *P. lutheri, P gyrans* and *P. pinguis* without statistical differences (p≥0.05). Previous studies have seen a similar specific growth rate, 0.173-0.181 d^{-1} in *P. lutheri* cultures grown under 100 µmol m^{-2} s^{-1} (Guihéneuf et al., 2009).

Besides, in Shah et al. (2014) experiment, they achieved a lower volumetric productivity (0.026 g L^{-1} d^{-1}), while in this study were obtained higher values (0.031 and 0.044 g L^{-1} d^{-1}) at similar light intensity and under the same photoperiod. In relation to the specific growth rate, a lower value was observed in the previously reported study (0.12 d^{-1}).

In the case of microalgae under a higher light intensity, no statistical differences were found between growth rates and volumetric productivities between *P. gyrans* and *P. pinguis* as well as in *P. granifera* and *P. lutheri* (p≥0.05). Guihéneuf et al. (2009) observed values of specific growth rate at higher light intensity (340 µmol m^{-2} s^{-1}) of 0.136-0.145 d^{-1} in *P. lutheri*, while in this work were obtained similar values in *Pavlova* sp.. Analyzing this data, it should not be necessary to use a too high light intensity since the same results are obtained at lower light intensity. It can be concluded that there is a limiting light intensity to these species since, comparing the two different light intensities, there was a higher performance of *Pavlova* sp. under a higher light intensity. Moreover, this result is not according to Guihéneuf et al. (2009 and 2017) studies in *P. lutheri*, which detected a decrease in the growth cultures when increasing the light intensity, especially under high temperatures.

Results obtained in the laboratory conditions tested indicate to proceed to *P. gyrans* or *P. pinguis* cultivation on the pilot-scale, moving cultures to outdoor conditions once they showed higher growth and volumetric productivity when compared to the other *Pavlova* species.

Furthermore, the values of global and maximum productivity increased with the increase of radiation (p<0.05), except the maximum volumetric productivity of *P. lutheri* cultures, which remained the same. The opposite occurred with the specific growth rate in each species, where only *P. granifera* has shown an increase in this value. This should be related with the high concentration of the other *Pavlova* species, limiting the light penetration (Boussiba et al., 1987).

Similarly, in a previous study, lower values of specific growth rates were obtained when comparing two different light intensities, namely 100 and 340 μmol m^{-2} s^{-1} (Guihéneuf et al., 2009).

The high concentration of proteins, CH and FA contained in microalgae make them essential food for zooplankton, larvae and juvenile stages of mollusks, crustaceans and certain herbivorous fish (Dorner et al., 2014). The biochemical composition of the biomass under study was characterized and is present in Table 5.

Table 5 - Proximate composition of batch cultures grown in 1 L bubble column reactors comparing different species growth. All data represent the mean (±sd) of three individual replicates

Species	Proteins (%)	Lipids (%)	Ashes (%)	Carbohydrates (%)
P. granifera	26.67±1.52	26.92±3.51	14.49±2.93	31.92±3.66
P. gyrans	39.97±0.99	14.17±0.48	8.35±0.91	37.52±1.74
P. pinguis	38.36±1.60	12.60±1.80	9.75±2.32	39.29±1.07
P. lutheri	26.63±2.70	29.30±1.97	13.84±0.73	30.23±3.40

The highest lipids content was observed in *P. granifera* and *P. lutheri* ($p \geq 0.05$), while *P. gyrans* and *P. pinguis* cultures had the lowest percentage of lipids and showed no differences between them ($p \geq 0.05$). Lipids quantification of *P. pinguis* in this study is not in agreement with previous studies, in which the content of lipids found was around 19-24% of *DW*, although a 12:12 light:dark cycle and lower light intensities (70-220 μmol m^{-2} s^{-1}) were used in the previous studies (Brown et al., 1998; Ponis et al., 2006b). However, a study conducted by McCausland et al. (1999) obtained a lipid content of 13% of *DW* in *P. pinguis* cultures under continuous radiation, which is similar to the value obtained in the present work. Differences are most likely related to different light intensity used and photoperiod. Furthermore, *Pavlova* sp. was shown to have a lipid content of 27% of biomass *DW* (Martínez-Fernández & Southgate, 2007), which are in agreement with the results obtained in *P. granifera* and *P. lutheri* cultures.

The higher amount of proteins occurred in *P. gyrans* and *P. pinguis* cultures, without any statistical differences between each other ($p \geq 0.05$). While the lowest content was verified in *P. lutheri* and *P. granifera* ($p \geq 0.05$). Under stress conditions, there is often observed a decrease in protein content per cell (Dunstan et al., 1993). Consequently, microalgae cells have the tendency to store energy in the form of lipids, mostly triacylglycerol, and CH (Dunstan et al., 1993; Roessler,

1990). Also, the addition of CO_2 on *Pavlova* sp. cultures could trigger the increase of protein content. Brown et al. (1997) noticed increases in protein content of several groups of microalgae species when cultures were enriched with CO_2. In the present study, CO_2 was added firstly in *P. gyrans* and *P. pinguis* cultures to lower pH, which could justify the higher protein content. Other authors, detected a protein content of 33-63% in *Pavlova* sp., which are in agreement with the results obtained in this study (Brown et al., 1998; Martínez-Fernández & Southgate, 2007). A study focused in *P. pinguis* reported of 38.1% of protein while, in accordance with the values obtained during the present work (Ponis et al., 2006b).

The percentage of ashes in each culture was also analyzed and there are no statistic differences in the ashes content between the *Pavlova* strains ($p \geq 0.05$) except when comparing *P. gyrans* with *P. granifera* and *P. lutheri* ($p < 0.05$), which have a higher ash content. According to the obtained values, previous studies observed ash content of 5-12% in *Pavlova* sp. (Brown et al., 1998; Ponis et al., 2006b).

Statistically, there were differences in the CH content of *P. pinguis* when compared to *P. granifera* and *P. lutheri*, and *P. gyrans* and *P. lutheri* ($p < 0.05$). Lower values of CH (3%) were detected in a previous study of Martínez-Fernández & Southgate (2007). The higher amount of CH can be explained with the fact of not existing a dark period in the experiment (Terry, 1983; Terry et al., 1985). According to Terry (1983), polysaccharide molecules are rapidly produced during the day and consumed at night. In the absence of a dark phase, polysaccharides will not be consumed as CH reserves. The results of CH are in agreement with previous studies with a photoperiod of 24:0 (L:D), in which a higher amount of CH was detected, around 29-38% (McCausland et al., 1999; Ponis et al., 2006b).

FA composition is classified into SFA, MUFA and PUFA classes whose percentages are shown in Table 6, as well as the identification and quantification of individual compounds of FAME.

The main FA produced in all *Pavlova* species were tetradecanoic (C14:0), hexadecanoic (C16:0), hexadecenoic (C16:1), EPA (C20:5n-3) and DHA (C22:6n-3) acids, which are in agreement with those found in the bibliography (Patil et al., 2007; Ponis et al., 2006b; Tatsuzawa & Takizawa, 1995; Thompson, Harrison, & Whyte, 1990; Volkman et al., 1989).

Table 6 - Fatty acid methyl esters (FAME) composition of *Pavlova* sp. in 1 L bubble column reactor. All data represent the mean (±sd) of three individual replicates

FAME	% of total FAME			
	P. granifera	*P. gyrans*	*P. pinguis*	*P. lutheri*
Saturated (SFA)				
C14:0	8.75±0.52	24.83±1.37	30.71±1.04	10.97±1.05
C16:0	30.61±0.40	22.88±2.54	14.76±2.06	33.06±3.26
Sum	39.36±0.67	47.71±1.35	45.47±1.02	44.04±2.25
Monounsaturated (MUFA)				
C16:1	33.97±1.37	28.39±0.93	34.44±2.64	26.95±4.7
C18:1	0.16±0.22	-	-	1.46±0.70
Sum	34.13±1.53	28.38±0.93	34.44±2.64	28.41±4.73
Polyunsaturated (PUFA)				
C18:3n-3	0	0	0	0.09±0.13
C18:2n-6c	0	0	0	0.64±0.61
C20:5n-3 (EPA)	17.74±2.11	17.85±0.94	14.72±1.18	21.70±5.39
C22:6n-3 (DHA)	8.76±0.86	6.05±0.69	5.38±1.10	5.13±1.81
Sum	26.51±1.67	23.9±0.51	20.1±2.21	27.55±6.93
∑ (n-3)	26.51±1.67	23.9±0.51	20.1±2.21	26.92±7.17
∑ (n-6)	0	0	0	0.64±0.61
∑ (n-3) / ∑ (n-6)	-	-	-	9.68±7.36
PUFA/SFA	0.67±0.05	0.5±0.02	0.44±0.05	0.64±0.19

Previous analyses of FAME showed some differences between the constituents detected. FA such as octadecatrienoic (C18:3n-3), octadecadienoic (C18:2n-6c), octadecenoic (C18:1), octadecanoic (C18:0) and eicosatetraenoic (C20:4n-6), reported in the literature as minor constituents (Guihéneuf et al., 2009; Guihéneuf & Stengel, 2017; Patil et al., 2007; Ponis et al., 2006b) were not possible to quantify in the present study.

The biochemical composition of biomass produced by different species of *Pavlova* showed some differences between SFA, MUFA and PUFA fractions compared to previous studies (Patil et al., 2007; Ponis et al., 2006b; Volkman et al., 1989). Relatively to SFA, *P. gyrans* and *P. pinguis* ($p \geq 0.05$) demonstrated higher C14:0 accumulation, while *P. granifera* and *P. lutheri* ($p \geq 0.05$) showed to have tendency to C16:0 accumulation. Guihéneuf et al. (2009) obtained a C14:0 content

of 8-10% of total fatty acids (TFA) in *P. lutheri*, which is similar to those obtained in the present study for *P. granifera* and *P. lutheri*, but a lower content of C16:0 (16% of TFA) compared with this study (14-33% of TFA). Another study carried by Guihéneuf et al. (2011) observed in *P. lutheri* cultures higher values of C14:0 and C16:0, 22% and 28% of TFA, respectively.

Hexadecanoic acid (C16:1) was about 30% of TFA in all tested strains ($p \geq 0.05$) and it is the MUFA present at the highest percentage in *Pavlova* sp.. Although the results in this study are higher than the 17-23% of TFA previously reported, C16:1 is always reported as the major MUFA (Patil et al., 2007; Ponis et al., 2006b; Volkman et al., 1989). Differences should be explained by differences in the light intensity, since MUFA levels are increased with the increase of light intensity (Guihéneuf et al., 2009).

Several studies investigating the biochemical composition of microalgae with the aim of improving the nutritional value of algal foods (aquaculture) and/or to boost PUFA production (pharmaceutical industry) are exclusively dedicated to *Pavlova* sp., specially to *P. lutheri* (Brown et al., 1997; Guihéneuf et al., 2009). Many of these studies focus on the use of algae as feed in mariculture (Ponis et al., 2006a; Tatsuzawa & Takizawa, 1995; Volkman et al., 1989).

Pavlova sp. presents both EPA and DHA in its composition (Carvalho & Malcata, 2005). In this study, the production of DHA and EPA obtained was between 15-21% and 6-9% of TFA, respectively. No statistical differences in EPA and DHA content were detected in *Pavlova* species ($p \geq 0.05$). Previous studies measured a DHA content of 10-13% of TFA in *P. lutheri*, while 3% and 4% of TFA were verified in *P. gyrans* and *P. pinguis*, respectively (Patil et al., 2007; Ponis et al., 2006b; Volkman et al., 1989; Yang & Hur, 2012). However, other studies quantified DHA around 6.6% of TFA under the maximum light and temperature tested (18 °C and 200 μmol m^{-2} s^{-1}) (Guihéneuf & Stengel, 2017). Those differences between the current study and the previous ones should be related to combined effects of temperature and light intensity affecting the lipid content and the FA profile of *Pavlova* sp. (Guihéneuf et al., 2009; Tatsuzawa & Takizawa, 1995). However, it has been shown that the PUFA content, especially EPA, was significantly higher under low light intensity, whereas SFA content and DHA levels were significantly higher under high light intensity (Guihéneuf et al., 2009; Thompson et al., 1990). In contrast, EPA content found in this study was very similar to previous studies, on which the percentage of EPA was about 20% of TFA in *P. lutheri* and 15% of TFA in *P. pinguis,* although the light intensity used was lower (Ponis et al., 2006b; Volkman et al., 1989). However, EPA content found in *P. gyrans* and *P. lutheri* was higher in this

study, 18% and 21% of TFA, respectively, that what obtained by other authors, 8% EPA of TFA, under simultaneous high temperature and light intensity (Guihéneuf & Stengel, 2017; Yang & Hur, 2012).

Marine algae are major producers of omega 3-PUFA (EPA and DHA), while algae from fresh water are predominantly producers of SFA and MUFA (Patil et al., 2007). In this study, there was a higher quantity of SFA and MUFA and a lower content of PUFA ($p<0.05$), which is not in agreement with previous studies that observed a higher content of PUFA in *Pavlova* sp. (Dunstan et al., 1993; McCausland et al., 1999; Patil et al., 2007; Ponis et al., 2006b; Volkman et al., 1989). However, similar results to the present work were obtained by Guihéneuf & Stengel (2017), which combination of the highest temperature and continuous light intensity resulted in a higher SFA (40%) and MUFA (35%) content and decreased PUFA levels (22%). Another study carried by Guihéneuf & Stengel (2013) obtained a FA profile composed by 45% SFA, 32% MUFA and 21% of PUFA, in cultures grown under nutrient depletion.

According to Roessler (1990), higher levels of MUFA and SFA appear to be synthesized as an early response to growth under conditions when energy input – light intensity – exceeds the cellular capacity for energy utilization such as cell growth and division. Besides, many microalgae species have tendency to produce those FA under stress conditions, such as pH and salinity non-optimal and/or NO_3^- depletion (Breuer et al., 2012; Guihéneuf et al., 2015; Guihéneuf & Stengel, 2013; Hu et al., 2008b)(Breuer et al., 2012; Guihéneuf & Stengel, 2013; Q. Hu et al., 2008). In this study, *P. gyrans* and *P. pinguis* MUFA and SFA amounts could be related to nutrient depletion since NO_3^- were almost fully consumed (Annex B) (Breuer et al., 2012; Guihéneuf & Stengel, 2013). In the particular case of *P. granifera* and *P. lutheri* the high amount of those FA should be associated to higher quantities of light received, since *P. lutheri* has been proven to grow faster under lower light intensities (Guihéneuf & Stengel, 2017; Roessler, 1990) .

4.2. Optimization of inoculum quantity of *Pavlova gyrans* in culture growth

The present assay was performed with only one species of *Pavlova*. *P. gyrans* was the species chosen to continue the rest of the trials because of AlgaFarm's interests. Four different concentrations of *P. gyrans* were submitted to a batch assay to compare the microalgal growth performance among them. In Figure 15 the growth curves of *P. gyrans* cultures are presented. In all cultures, microalgae started their exponential growth in day 0, being not possible to see a lag

phase. Cultures reached a stationary phase at day 6, with the exception of the culture which started with 20% of *P. gyrans*, which extended the growth two more days. All cultures showed a decline phase at day 10 of the experimental assay.

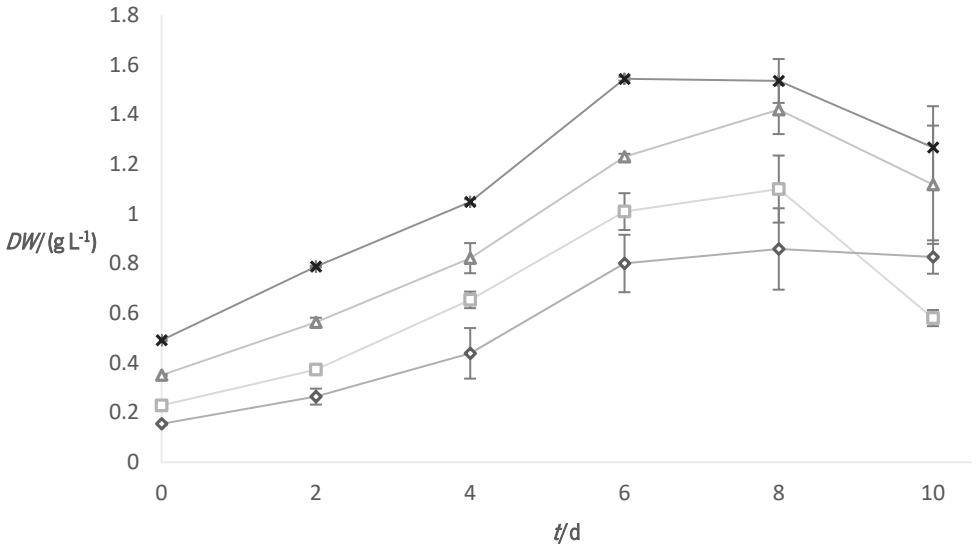

Figure 15 - Screening of the growth of *P. gyrans* in 1 L bubble column reactors using 5% (◇), 10% (□), 20% (△) and 30% (✕) of inoculum. Each point represent a mean of three replicates and respective standard deviation (n=3; mean±sd).

The different inoculum percentages showed different total biomass productions (p<0.05). The highest biomass concentration was achieved by the 30% inoculum volume of *P. gyrans* (1.544±0.007 g L^{-1}). Similar results were obtained in the study of the effects of initial population density on growth of *Nannochloropsis* sp., in which the microalgae cultured with higher concentration resulted in a higher biomass concentration (Chen et al., 2012).

The specific growth rates and the biomass productivities values for each culture of *P. gyrans* are presented in Table 7.

For the range of inoculum size tested, there were no significant differences comparing the specific growth rate of cultures starting 5 and 10% of *P. gyrans* and 20 and 30% (p≥0.05). The specific growth rate was lower when starting with 30 (0.191±0.002 d^{-1}) and 20% of *P. gyrans* inoculum (0.209±0.003 d^{-1}), while it was higher using 5% (0.273± 0.022 d^{-1}) and 10% of inoculum (0.247±0.013 d^{-1}). Accordingly, Wang et al., (2015) studied the influence of inoculum size in growth culture of *Nannochloropsis* sp. and found cultures with the lowest initial biomass demonstrated the highest specific growth rate. These results should be because cell concentration

in microalgae cultures affects the amount of light that reaches the individual cells of culture. Therefore, the higher cell density, the lower the specific growth rate to be expected in these culture systems (Boussiba et al., 1987).

Table 7 - Volumetric productivity on biomass during the whole assay, maximum value, and specific growth rate on the exponential phase for each percentage of inoculum in the study. All data represent the mean (±sd) of three individual replicates

% inoculum	Maximum volumetric productivity (g L^{-1} d^{-1})	Global volumetric productivity (g L^{-1} d^{-1})	Specific growth rate (d^{-1})
5% *P. gyrans*	0.187± 0,055	0.108±0.020	0.273± 0,022
10% *P. gyrans*	0.178±0.031	0.130±0.012	0.247±0.013
20% *P. gyrans*	0.208±0.027	0.134±0.013	0.209±0.003
30% *P. gyrans*	0.248±0.008	0.175±0.002	0.191±0.002

There were no statistical differences in the value of maximum productivity, according to different inoculum concentrations ($p \geq 0.05$).

Experimental results obtained by Li et al., (2017) in *C. vulgaris* indicate that high inoculum size was more favorable to increased daily biomass productivity. Results showed by Bohutskyi et al., (2016) also demonstrated a positive effect on volumetric productivity using higher concentrations of inoculum.

On the other hand, a study conducted by Dogaris et al., (2015) in *Picochlorum oculatum* cultures did not observe significant differences in the maximum biomass production and productivity value when inoculum sized varied from 10% to 15% and 20%. According to global volumetric productivity, in the present work, significant differences were found when starting with 30% inoculum or the other tested concentrations ($p < 0.05$). Based on these data, it will be of greater interest to start the cultures with 30% of inoculum, leading to a higher biomass in less time (0.175±0.002 g L^{-1} d^{-1}). On the other hand, there were no differences in the other inoculum sizes ($p \geq 0.05$), which suggest that the use of a culture with a less percent of inoculum, such as 5 %, could be a feasible option to reduce the costs associated with larger inoculum size preparation, being particularly important when considering industrial scales (Dogaris et al., 2015).

4.3. Outdoor screening of *Pavlova* sp. and biochemical composition

After the growth of *Pavlova* sp. at the lab-scale, a scale-up to 55 L flat panel reactors with initial biomass of 0.4 g L^{-1} was carried out. The species used to compare their growth in outdoor PBR were the ones that demonstrated higher growth in lab-scale: *P. gyrans* and *P. pinguis*.

Figure 16 resumes *Pavlova* sp. in outdoor reactors. Cultures had grown exponential for 11 days, having a 1 day lag phase. The growth ended after 12 days of assay and it was not possible to see a stationary phase. *P. gyrans* and *P. pinguis* had consumed the available NO_3^- in culture, remaining 3.7 and 0.1 mmol L^{-1} after 12 days, respectively (Annex C).

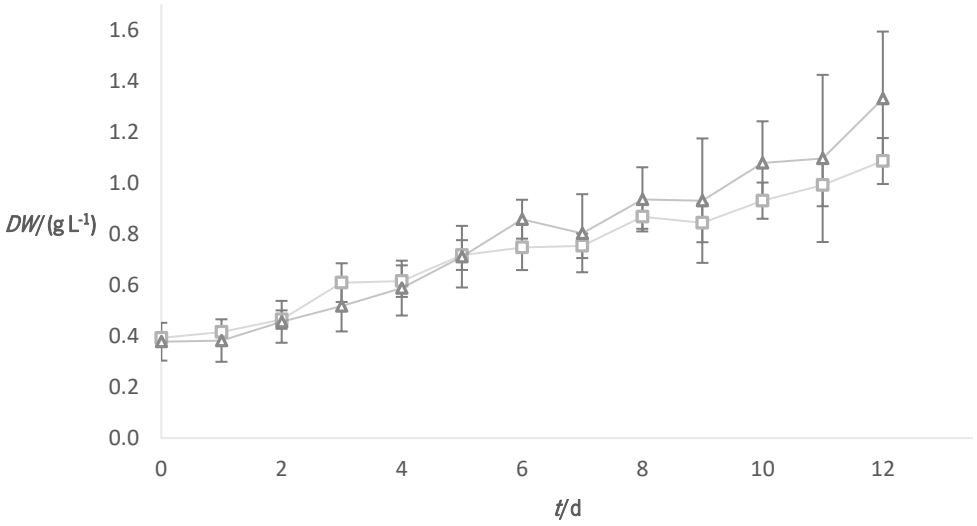

Figure 16 - Growth of *P. gyrans* (□) and *P. pinguis* (△) in 55 L flat panel reactors. Each point represent a mean of three replicates and respective standard deviation (n=3; mean±sd).

Triplicates were performed during the first half of June, with a maximum daily temperature of 23 °C and light exposure of 1592 µmol m^{-2} s^{-1}.

P. gyrans and *P. pinguis* have shown a similar total biomass during the outdoor experiment ($p \geq 0.05$) and the maximum biomass obtained during this assay was 1.33 g L^{-1} by *P. pinguis* and 1.09 g L^{-1} by *P. gyrans*. This result is lower than the values reported in the literature. A study in 4 L flat panel operating in batch and then in semi-continuous regime carried out by Ponis et al., (2008) during 35 days, achieving a final biomass of 3.5 g L^{-1} in *P. lutheri* cultures. Another study involving *P. lutheri* cultivation in 4 L flat panel PBR, under continuous light of 125 µmol m^{-2} s^{-1}, reached in 7 days a density of 1.9 g L^{-1} (Ponis, et al., 2006a). Differences related to the size of

the reactor and the continuous lightening can explain the low biomass obtained during the present study.

Furthermore, the culture in outdoor flat panels reached a higher biomass concentration than that obtained in bubble column reactors under 100 µmol m^{-2} s^{-1}. Flat panels allowed to obtain 1.33 g L^{-1} *P. pinguis* and 1.09 g L^{-1} *P. gyrans*, whereas lab-scale achieved 0.70 g L^{-1} and 0.61 g L^{-1} by *P. pinguis* and *P. gyrans*, respectively. Similar results were obtained in previous studies that demonstrated higher growth in cultures growing in flat panel reactors (Ponis et al., 2006a).

Volumetric productivity and specific growth rate were calculated and are present in Table 8.

Table 8 - Volumetric productivity on biomass during the whole assay, maximum value, specific growth rate on the exponential phase, and photosynthetic efficiency for each *Pavlova* sp. in outdoor flat panel reactors. All data represent the mean (±sd) of three individual replicates

Species	Maximum volumetric productivity (g L^{-1} d^{-1})	Global volumetric productivity (g L^{-1} d^{-1})	Specific growth rate (d^{-1})	Photosynthetic efficiency (%)
P. gyrans	0.083±0.001	0.058±0.007	0.152±0.011	0.453±0.053
P. pinguis	0.120±0.055	0.079±0.019	0.166±0.052	0.606±0.156

In accordance to the reported laboratory assays, *P. gyrans* and *P. pinguis* showed no differences in total biomass ($p \geq 0.05$), such as in global and maximum productivities ($p \geq 0.05$). Similarly, no significant differences were found in the growth rate of each species ($p \geq 0.05$). *P. gyrans* showed a lower global and maximum productivity in outdoor conditions compared to laboratory conditions at 700 µmol m^{-2} s^{-1} ($p < 0.05$), however, it is important to consider the differences in system geometry and culture conditions, such as temperature, pH, light intensity and light-regime, have a strong influence on biomass productivity (Ryu et al., 2012).

Comparing the global productivity obtained in flat panel and lab-scale at 100 µmol m^{-2} s^{-1}, the cultures growing in PBR attained to a higher productivity value. Ponis et al., (2006a) obtained the same results, under continuous light of 150 µmol m^{-2} s^{-1}, in which cultures grown in flat panel PBR attained a higher global productivity of 0.25 g L^{-1} with respect to that in carboys cultures, that achieved 0.10 g L^{-1}.

Differences in photosynthetic efficiency were not noticed in *Pavlova* sp. ($p \geq 0.05$).

Once the growth of these microalgae species was statistically similar in both lab- and outdoor scale, it was important to evaluate if biochemical profile suffered differences. Results are resumed in Table 9.

Table 9 - Proximate composition of batch cultures grown in flat panel reactors comparing different species growth. All data represent the mean (±sd) of three individual replicates

Species	Proteins (%)	Lipids (%)	Ashes (%)	Carbohydrates (%)
P. gyrans	40.13±0.58	19.54±1.75	12.22±2.47	28.11±2.05
P. pinguis	42.10±0.88	22.04±4.29	10.13±1.25	25.74±2.80

For the different *Pavlova* species tested in outdoor flat panels, there were no significant differences in the biochemical content ($p \geq 0.05$), showing similar contents of proteins, lipids, ashes and CH. However, statistical differences in biochemical content of lipids and CH were detected between growth in lab-scale and growth in outdoor scale for both *Pavlova* species ($p < 0.05$), such as the protein content of *P. pinguis* ($p < 0.05$). No differences in ashes content of both *Pavlova* sp. were found comparing the growth of lab-scale with the growth in outdoor flat panels ($p \geq 0.05$).

The results of this study indicated that the biochemical composition was influenced by the culture system and the conditions to which they were subjected, such as temperature, light intensity and light-regime, as suggested by other studies (Carvalho et al., 2009; Richardson et al., 1983; Uslu et al., 2009). Nevertheless, a previous study about the effect of the culture system and culture technique on biochemical characteristics of *P. lutheri* did not show to induce differences in the microalgae biochemical profile cultivated in flat panel reactor, comparing to lab-scale (Ponis et al., 2006a). However, a continuous illumination was used in both lab-scale and flat panel, whereas in the present work a light/dark cycle was used in outdoor reactors. A study realized by Wahidin et al. (2013) observed significant differences in the lipid composition of *Nannochloropsis* sp. under different light/dark cycles and the maximum total lipid content was obtained when the cultures were exposure to 18:6 light/dark, being the lowest content at 24:0 light/dark.

Therefore, and as described in the literature, light stands out as a key factor in microalgae growth and biochemical profile (Guihéneuf et al., 2015). Richardson et al. (1983) reported in their study that different light intensities and light-regimes exhibited various responses in the growth of microalgae and in their chemical composition. Moreover, it is proven that different light intensities

and light/dark cycles affect especially lipid profile of various microalgae species (Harwood, 1998). All of these should explain the differences obtained in lipid content of outdoor and indoor growth conditions. In the present work, the maximum total lipid content in *P. gyrans* and *P. pinguis* (19-22% of *DW*) was obtained while the cultures were exposed to a light regime of 18:6 (L:D) and a daily maximum light intensity of 1592 μmol m^{-2} s^{-1}, under outdoor conditions. Total lipid content decreased under 24:0 (L:D) regime, presenting a lipid content of 12-14% of *DW* and under a lower light intensity of 700 μmol m^{-2} s^{-1}. The results obtained are in accordance with the study realized by Wahidin et al. (2013), which studied the influence of photoperiod and light intensity on lipid content of *Nannochloropsis* sp. and proved to be an important factor.

Furthermore, during outdoor cultivation, there was a decreased in CH content. This should be associated to the existence of a light/dark regime, once it is described that polysaccharides are synthesized during the light period and consumed in the dark phase (Terry et al., 1985).

The FA profile of microalgae growing in outdoor flat panels was also investigated and are listed in Table 10. The FA showing the higher difference between lab-scale and flat panel was C16:0 (p<0.05). Outdoor cultivation led to a drastic decline in the value of this SFA, 23 and 15% of TFA in *P. gyrans* and *P. Pinguis* at lab-scale, respectively, and 2 and 5% of TFA at large scale. According to Wahidin et al. (2013), light is essential for triacylglycerol production and the light and regime appropriated vary with the species and for this reason *Pavlova* sp. growing at a lab-scale, in the absence of a dark period, produce higher quantities of C16:0. Furthermore, there was an increase in DHA levels, which could represent the lower accumulation of C16:0. According to Sahin et al., (2018), a decrease in SFA may be an indicator of a metabolic pathway that results in DHA production.

A study of *P. lutheri* growing in flat panels found C16:0, C16:1, EPA and DHA as the major FA presents. The amount of the major SFA and MUFA present in those cultures made up a total of 37% of TFA while in this study a total of 17% was detected (Ponis, Parisi, et al., 2006). However, the assays were carried under continuous light of 150 μmol m^{-2} s^{-1}, while in the present study a different photoperiod and light intensity were used.

Table 10 - FAME composition of *P. gyrans* and *P. pinguis* on 55 L flat panel reactors. All data represent the mean (±sd) of three individual replicates

FAME	% of total FAME	
	P. gyrans	*P. pinguis*
SFA		
C14:0	34.29±0.91	31.98±0.68
C16:0	1.62±0.51	5.33±1.13
Sum	35.91±1.03	37.31±1.75
MUFA		
C16:1	16.06±0.98	25.50±1.63
Sum	16.06±0.98	25.50±1.63
PUFA		
C20:5n-3 (EPA)	39.97±0.08	28.40±0.83
C22:6n-3 (DHA)	8.06±0.51	8.79±0.52
Sum	48.03±0.45	37.19±0.71
∑ (n-3)	48.03±0.45	37.19±0.71
∑ (n-6)	0	0
∑ (n-3) / ∑ (n-6)	-	-
PUFA/SFA	1.34±0.05	1.00±0.06

Furthermore, there was a decrease in SFA amounts of outdoor *Pavlova* sp. cultures compared to indoor conditions, which is not supported by bibliography. An increase in SFA content was observed in the study about the effect of different light intensities in the biochemical characteristics of *P. lutheri*, in which SFA increased from 24 to 30% of TFA with increasing light from 20 to 340 µmol m^{-2} s^{-1} (Guihéneuf et al., 2009). According to Roessler (1990), increasing light intensity commonly results in an increase in triacylglycerol levels. This differences should be justified with the absence of a light/dark cycle in the lab-scale, that was always under a 24 h photoperiod (Guihéneuf et al., 2009; Guihéneuf & Stengel, 2017). Studies conducted by Guihéneuf et al. (2009 and 2017) observed differences in SFA content using cultures under a continuous light intensity of 200 µmol m^{-2} s^{-1} (40%) and cultures under 340 µmol m^{-2} s^{-1} with a 14:10 (L:D) photoperiod (30%).

The C16:1 was detected at higher amounts in *P. pinguis* cultures ($p<0.05$) while it was not possible to conclude when the assays were performed in bubble column reactors ($p\geq0.05$).

Comparing lab with outdoor scale, there was a decrease in MUFA levels of both *Pavlova* species. Studies by Guihéneuf et al. (2009 and 2017) supported these results once 20% MUFA was obtained in *P. lutheri* cultures growing under a photoperiod of 14:10 (L:D), while cultures under continuous light showed 35% MUFA of TFA.

EPA content was significantly increased in the biomass produced in outdoor flat panel reactors in both species when compared with laboratory assays ($p<0.05$). However, in outdoor conditions, EPA content has higher values in *P. gyrans* than in *P. pinguis*, achieving 40% EPA. Both species had a higher percentage of DHA when cultured outdoor instead of lab-scale ($p<0.05$), achieving a DHA content around 9% of TFA. *P. lutheri* was reported to achieve high levels of EPA and DHA (25 and 10% of TFA, respectively) when cultivated in flat panel (Ponis et al., 2006a). Similar results were obtained with *P. pinguis* while a higher content of EPA was observed in *P. gyrans* (40% of TFA).

The proportion of PUFA, especially EPA, are usually higher under low light intensity and DHA levels are significantly higher under high light intensity (Guihéneuf et al., 2009). However, this was not observed for EPA levels in this study, which increased in cultures under high light intensity. Guihéneuf et al. (2009 and 2017), obtained 22% EPA of TFA under photoperiod of 14:10 (L:D) compared to 9% EPA of TFA under continuous light. EPA content in a culture seems to be strongly influenced by the light regime.

Another parameter that plays an essential role in lipid production, affecting the type of lipids accumulated by microalgae, is temperature (Roessler, 1990). The general trend toward increasing PUFA levels with decreasing temperature was previously observed in microalgae (Raison, 1985; Roessler, 1990). A study by Guihéneuf & Stengel, (2017) tested how different temperatures affected the biochemical profile of *P. lutheri* cultures. Results showed that PUFA levels were higher under lower temperature, independent of the light intensity used. Although outdoor assays have a maximum daily temperature close to lab-scale assays, outdoor assays were under a light/dark cycle, which means temperatures during the dark phase were lower.

Although in a previous study carried by Ponis et al. (2006a) they have not detected significant differences in EPA content between *P. lutheri* cultures growing in lab-scale and flat panel, the same results were not confirmed in the present work. Comparing FAME content at lab-scale with the outdoor production, it was possible to achieve a higher content of both EPA and DHA in outdoor cultures ($p<0.05$). The present study may increase further commercial mass cultures of the marine

microalgae *Pavlova* sp. for production of EPA and DHA, which could be recognized as one of the most promising sources of PUFA with an enormous potential for aquaculture (Hu et al., 2008).

Furthermore, a previous study by Volkman et al., (1989) observed similar results to this study in the biochemical profile of *P. pinguis*, showing contents of SFA, MUFA and PUFA of 36%, 20.4% and 42% of TFA, respectively, using a 12:12 light:dark cycle.

4.4. Growth of *Pavlova* sp. on large scale PBR

P. gyrans and *P. pinguis* were further cultivated in large-scale tubular PBR. The cultures were maintained for 12 days and the growth curves are represented in Figure 17. *P. gyrans* assay was carried in the mid July, with a maximum daily temperature of 23 °C and a light intensity of 1420 µmol m^{-2} s^{-1}, while *P. pinguis* in the beginning of August, under 24 °C and 1540 µmol m^{-2} s^{-1}.

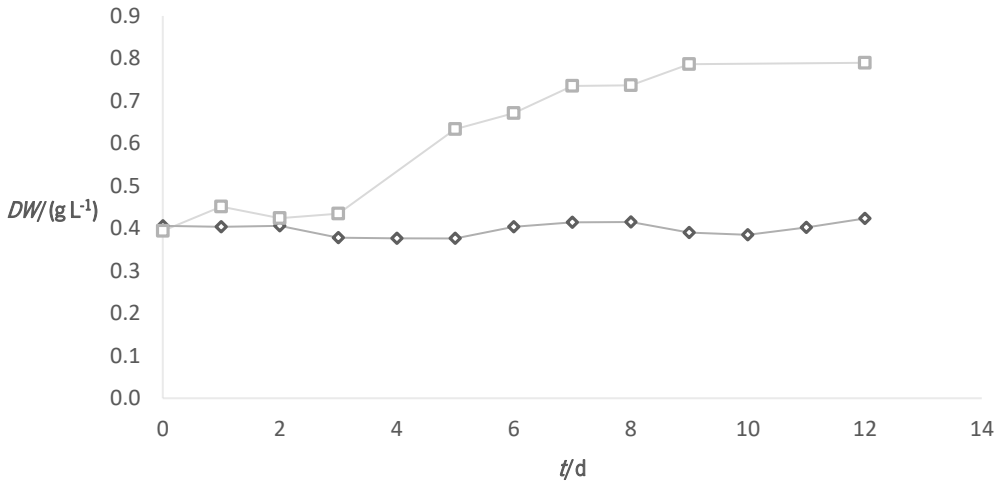

Figure 17 - Growth curves of *P. gyrans* (◇) and *P. pinguis* (□) in 2500 L tubular PBR.

These data evidence differences in the total biomass of two different tubular PBR with two distinct strains. NO_3^- consumption of both cultures is present in Figure 18.

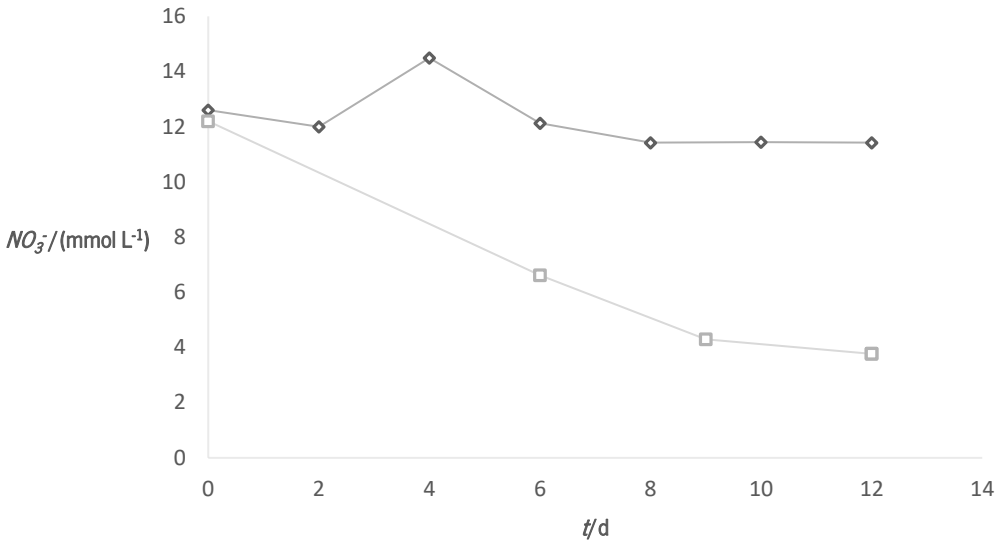

Figure 18 - Nitrate (NO_3^-) consumption of *P. gyrans* (◇) and *P. pinguis* (□) in 2500 L tubular PBR.

By comparing the initial and final concentrations of NO_3^- in these cultures, it can be observed that the fractional uptake of NO_3^- was higher in *P. pinguis* (71.1% was consumed) than in *P. gyrans* culture (10% was consumed). These results are in agreement with the growth of cultures. While the highest value of biomass achieved for *P. pinguis* was 0.79 g L^{-1}, *P. gyrans* was always around 0.4 g L^{-1}, during 12 days. The specific growth rates and biomass productivities are presented in Table 11.

Table 11 - Volumetric productivity on biomass during the whole assay, maximum value, and specific growth rate on the exponential phase for *Pavlova* sp. in outdoor tubular PBR

Species	Maximum volumetric productivity (g L^{-1} d^{-1})	Global volumetric productivity (g L^{-1} d^{-1})	Specific growth rate (d^{-1})
P. gyrans	0.028	0.001	-
P. pinguis	0.199	0.044	0.189

P. gyrans and *P. pinguis* cultured in tubular PBR showed significant differences at global and maximum productivity values and specific growth rate. *P. pinguis* achieved a higher maximum productivity of 0.199 g L^{-1} d^{-1} while *P. gyrans* only 0.028 g L^{-1} d^{-1}. Moreover, a global productivity of 0.044 g L^{-1} d^{-1} was obtained in *P. pinguis* while in *P. gyrans* was observed a global productivity

of 0.001 g L^{-1} d^{-1}. Studies related that centrifugal pumps could influence negatively the productivity of microalgae with a weak cell wall, due to cell damage (Carvalho et al., 2006). Specific growth rate was calculated in *P. pinguis* (0.189 d^{-1}) while in *P. gyrans* it was not taken into account because it was not observed an exponential phase. These differences could also be behind the culture mixing used in both cultures. *P. gyrans* experiment started with a 10 Hz pump frequency and in the next day, it was increased to 14 Hz due to a huge flocculation in PBR tubes and a lean mixture. After several days of assay, centrifugal pump was operating at 18 Hz. *P. pinguis* culture, in turn, was mixed from the beginning at 18 Hz.

The pump is the main source of shear in tubular PBR system and it can lead to cell damage and, consequently, cellular death (Annex D). A wide range of different types of pumps have been tested in tubular reactors (Borowitzka, 1994; Gudin & Chaumont, 1991). For microalgae production at the Production Unit, pump frequency is usually kept at 30 Hz, but in this specific case, it was reduced to 18 Hz. However, high levels of mixing are necessary to reach a turbulent flow of the culture in order to overcome light limitation (Borowitzka, 1994; Gudin & Chaumont, 1991). Furthermore, a good mixing avoids the possibility of sedimentation at the bottom of PBR tubes and consequently biomass losses. In *P. gyrans*, a cellular aggregation and consequently sedimentation of biomass was noticed, avoiding its growth and hindering nutrients assimilation.

A study based on the cultivation of shear stress-sensitive microalgal species in a tubular PBR operating a centrifugal pump showed that different species (e.g., *Isochrysis galbana, Skeletonema costatum* and *Chaetoceros muelleri*) were not able to grow in the tubular PBR operated with a centrifugal pump, not even at the lowest pumping speed (Michels et al., 2016). This same study also observed that the collapse of a shear stress sensitive culture is affected by the number of times it passes the pump. Carvalho et al. (2006) suggest that a tubular PBR operating with an airlift pump, instead of a centrifugal pump, may decrease the shear stress caused in cells and consequential cell damage. *I. galbana*, a species belonging to Haptophyta phylum with a morphology similar to *Pavlova* sp., have been reported to be successfully cultivated in 50 L tubular PBR by Grima et al., (1994) study, on which culture was recirculated using an airlift pump.

Based on the results previously obtained during the scale-up of *P. pinguis* in tubular PBR, the effects of different cultivation techniques on the grown of this species was investigated. Tests were performed using a single replicate. The experiments were carried out at the same time, under

the same temperature and light intensity, 24 °C and 1540 µmol m^{-2} s^{-1}, respectively. The growth of *P. pinguis* in the different culture systems is present in Figure 19.

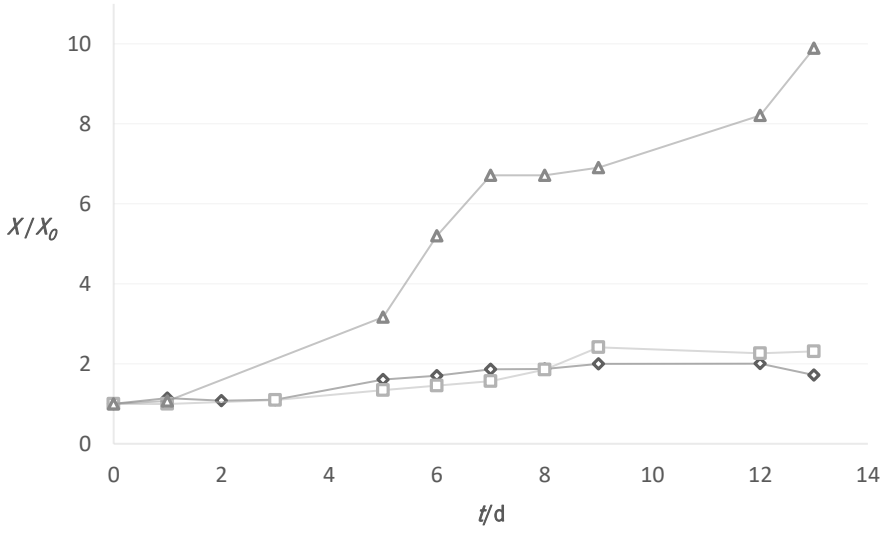

Figure 19 - Scale-up of *P. pinguis* in outdoor PBR: tubular PBR (◇), raceway (□) and flat panel (△). X_0/(g L^{-1}) refers to initial biomass and X/(g L^{-1}) to current biomass obtained in each system.

The highest biomass concentration was 1.12 g L^{-1}, reached in a flat panel reactor with an initial biomass of 0.11 g L^{-1}. In contrast, 0.79 g L^{-1} was obtained in tubular PBR and 0.57 g L^{-1} in raceway pond, which started at 0.39 and 0.25 g L^{-1}, respectively. A previous study, in which *P. lutheri* was cultured in 30 and 300 L tanks, achieved a maximum biomass of 0.39 and 0.35 g L^{-1}, respectively (Shah et al., 2014). The growth between the cultures in tubular PBR and raceway demonstrated to be similar. However, tubular PBR and raceway displayed significant differences in the growth when compared to the flat panel reactor.

The specific growth rates of *P. pinguis* growing in each one of the bioreactors and the biomass productivities were calculated and are shown in Table 12.

The system with the highest global volumetric productivity was the 600 L flat panel, achieving a value of 0.077 g L^{-1} d^{-1}. Besides, a higher maximum volumetric productivity was achieved in flat panels (0.230 g L^{-1} d^{-1}), followed by raceway pond (0.138 g L^{-1} d^{-1}) and tubular PBR (0.099 g L^{-1} d^{-1}).

Table 12 - Maximum volumetric productivity, global volumetric productivity and maximum specific growth rate for the different geometries of outdoor PBR where growth was analyzed

Microalgae System	Maximum volumetric productivity ($g\ L^{-1}\ d^{-1}$)	Global volumetric productivity ($g\ L^{-1}\ d^{-1}$)	Productivity per unit of area and time ($g\ m^{-2}\ d^{-1}$)	Specific growth rate (d^{-1})
Tubular PBR	0.099	0.044	4.128	0.188
Raceway pond	0.138	0.039	4.702	0.131
Flat panel	0.230	0.077	5.563	0.306

The productivity per unit of area per unit of time was higher in flat panel reactor (5.563 $g\ m^{-2}\ d^{-1}$), followed by raceway pond (4.702 $g\ m^{-2}\ d^{-1}$) and by tubular PBR (4.128 $g\ m^{-2}\ d^{-1}$). Interestingly, a higher productivity per unit of area per unit of time was obtained to raceway system but higher global productivity was shown in tubular PBR (0.044 $g\ L^{-1}\ d^{-1}$) compared with raceway pond (0.039 $g\ L^{-1}\ d^{-1}$), which could be explained by the differences in reactors geometry and the light amount they received. Productivity per unit of area per unit of time, in the case of tubular PBR, was calculated taking into account both pipes (photosynthetic area) and culture tank while in the raceway pond the productivity includes the whole photosynthetic area. Accordingly, a study comparing the three outdoor pilot-scale PBR used in this work was carried by De Vree et al., (2015) using *Nannochloropsis* sp. cultures and concluded that flat panel was the system with the highest global and maximum volumetric and productivity per unit of area per unit of time, followed by tubular PBR and raceway pond.

The highest specific growth rate was obtained in cultures growing in flat panel reactors (0.306 d^{-1}). The value of specific growth rate obtained in raceway (0.131 d^{-1}) cultivation in this project was similar to that found in the literature. Shah et al. (2014) experienced the cultivation of *P. lutheri* in 300 L open tanks at 0.12 d^{-1}. However, the same authors reported a global volumetric productivity , of 0.024 and 0.022 $g\ L^{-1}\ d^{-1}$ in 30 and 300 L open tanks, respectively, while a higher volumetric productivity of 0.039 $g\ L^{-1}\ d^{-1}$ was reported in this work (Shah et al., 2014).

The results obtained during this experiment demonstrated a higher specific growth rate in tubular PBR, compared to raceway pond, due to the efficient light penetration ensured by the tube diameter of tubular PBR. The light penetration in raceway pond is not as efficient once the water depth hampers the penetration of the light and affects the amount of light that reaches the individual cells of a culture (Boussiba et al., 1987; Carvalho et al., 2006). A higher global volumetric

productivity was also obtained in *Tetraselmis* sp. cultures cultivated in tubular PBR but according to literature, higher values of productivity are frequently associated with these reactors (Narala et al., 2016; Show et al., 2017). Similar results were obtained in *Nannochloropsis* sp. cultures, by De Vree et al., (2015), in which the raceway pond productivities were the lowest due to the long culture depth in the system.

Horizontal tubular PBR are the most commonly used for commercial production of microalgae because they are associated with higher productivities and their tubes diameter ensures an efficient light penetration (Show et al., 2017). Furthermore, they are capable of operating huge volumes with a lower risk of contamination under controlled parameters (Lee & Pirt, 1981; Pirt et al., 1983). However, these reactors consume more energy and require a large amount of capital investment (Carvalho et al., 2006). In contrast, open systems, such as raceway pond, have lower energy requirement but generally produce a lower amount of biomass for the same area and are more susceptible to contamination (Carvalho et al., 2006; Chisti, 2013; Mazlan & Hashim, 2016). However, no contamination was found in the present study.

The divergence between theoretical potential associated with tubular PBR and the biomass productivity obtained in *P. pinguis* is due to hydrodynamic stress caused on cells by pumping (Gudin & Chaumont, 1991).

Hu et al. (2008a) tried scaling-up *Pavlova viridis* using a 60 L tubular PBR, which demonstrated primarily a similar growth as laboratory assays, under high temperatures and light intensity. However, a second outdoor assay was carried out but with a lower temperature and light intensity, which caused slower growth in *P. viridis* culture.

Therefore, *Pavlova* sp. cultivation can be cultured either in open (raceway pond) or closed systems (tubular PBR and flat panel). However, the comparison between *Pavlova* sp. cultivated in different systems demonstrated the best performance of flat panel reactor operating in batch mode in terms of volumetric and productivity per unit of area per unit of time, as well as in specific growth rate. This type of reactors, which operate under controlled conditions, showed to be effective in the production of highly concentrated microalgal biomass and proved to be feasible for *P. pinguis* culture, maintaining high daily productivity and specific growth rate.

5. CONCLUSIONS AND FUTURE PERSPECTIVES

Pavlova sp. is rich in EPA and DHA and for that reason it is important to access different species growth and industrial potential. A comparison in lab-scale of four different *Pavlova* species – *P. granifera, P. gyrans, P. pinguis* and *P. lutheri* – was performed in this work. Results demonstrated a higher total biomass production in *P. gyrans* and *P. pinguis*, independent of the light intensity used. The biochemical composition of microalgae was analyzed and the production of PUFA confirmed to be similar for all *Pavlova* species. Besides, an optimization of the inoculum size of *P. gyrans* was performed and 30% of inoculum was the concentration with higher global productivity but no differences were observed between the other inoculum volumes tested – 5, 10 and 20%.

A scale-up to pilot flat panels was performed using *P. gyrans* and *P. pinguis*. cultures. Growth in outdoor PBR did not show differences between species, however, it evidenced a higher total biomass production than in lab-scale, during the same period of time, achieving the highest volumetric productivity and specific growth rate. Moreover, the biochemical composition and FAME profile of the produced biomasses exhibited some differences in lipid and CH contents. A higher level of EPA and DHA was obtained in cultures growing in outdoor PBR, showing that flat panels were effective for the production of *Pavlova* sp.. Therefore, it can be concluded that the biochemical composition of microalgae is easily modified through different systems configurations, light intensity, and photoperiod.

The marine microalgae *Pavlova* sp. has been successfully cultivated in large-scale flat panel, tubular PBR and raceway pond, achieving a higher volumetric and productivity per unit of area per unit of time, as well as a higher specific growth rate, in the flat panel PBR. Tubular PBR could be promising in the cultivation of microalgae although microalgae with a weak cellular wall, such as *Pavlova* sp., may require more studies to culture optimization. Consequently, there is a need to study the relation between shear stress-sensitive microalgae with the shear stress levels caused by tubular PBRs operating with a centrifugal pump.

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ANNEX A – CALIBRATION CURVES

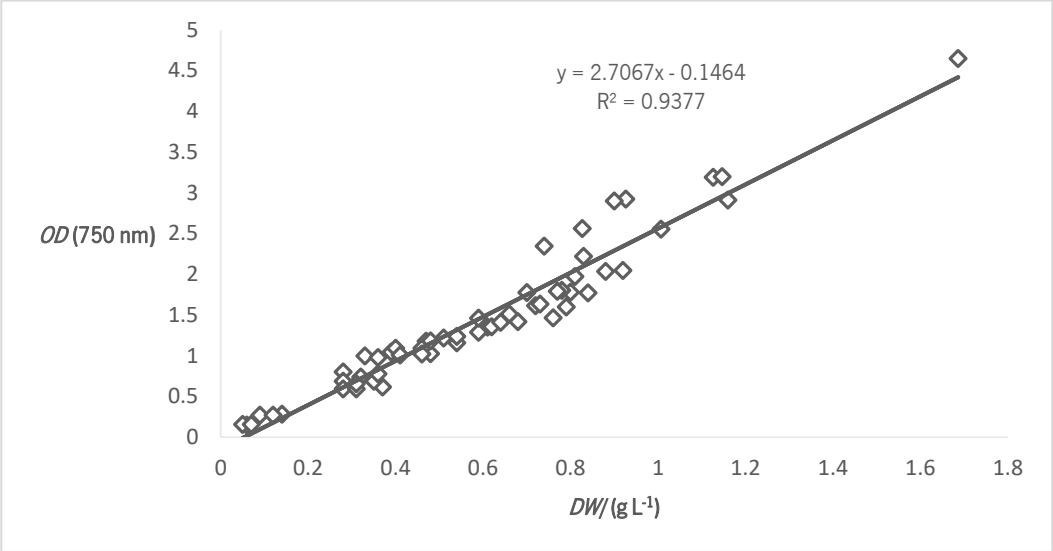

Figure A1 - Calibration curve of optical density (OD) at 750 nm versus DW of *Pavlova* sp. biomass.

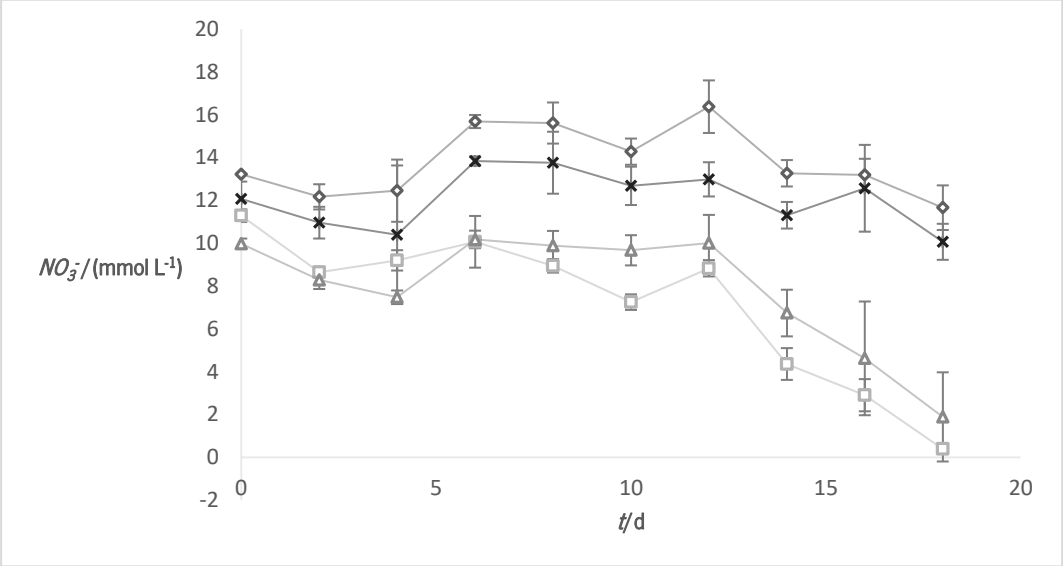

Figure B1 - NO_3^- consumption of *P. granifera* (◇), *P. gyrans* (✕), *P. pinguis* (△) and *P. lutheri* (□) cultivation in 1 L bubble column reactors.

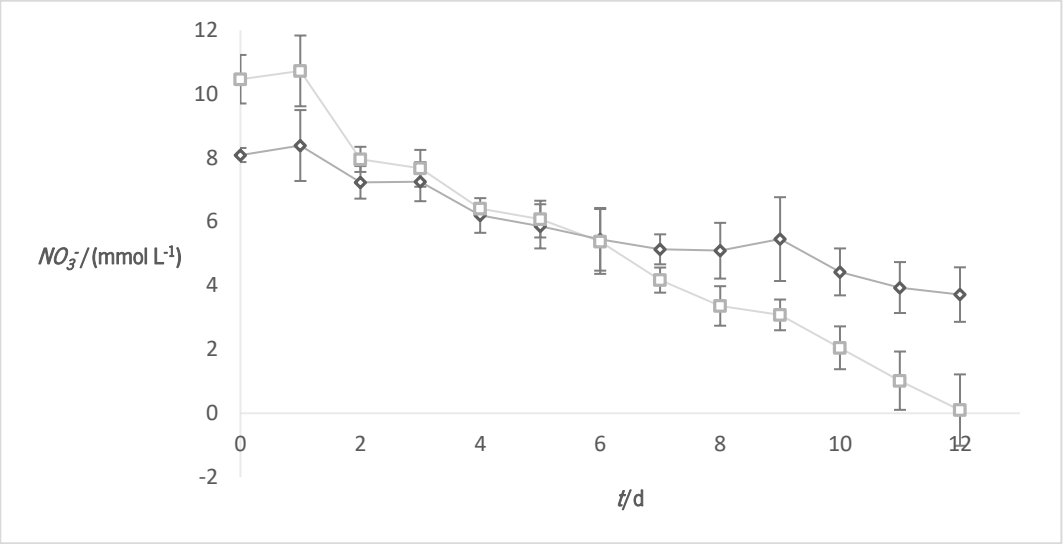

Figure C1 - NO_3^- consumption of *P. gyrans* (◇) and *P. pinguis* (□) over the time in flat panels cultivation.

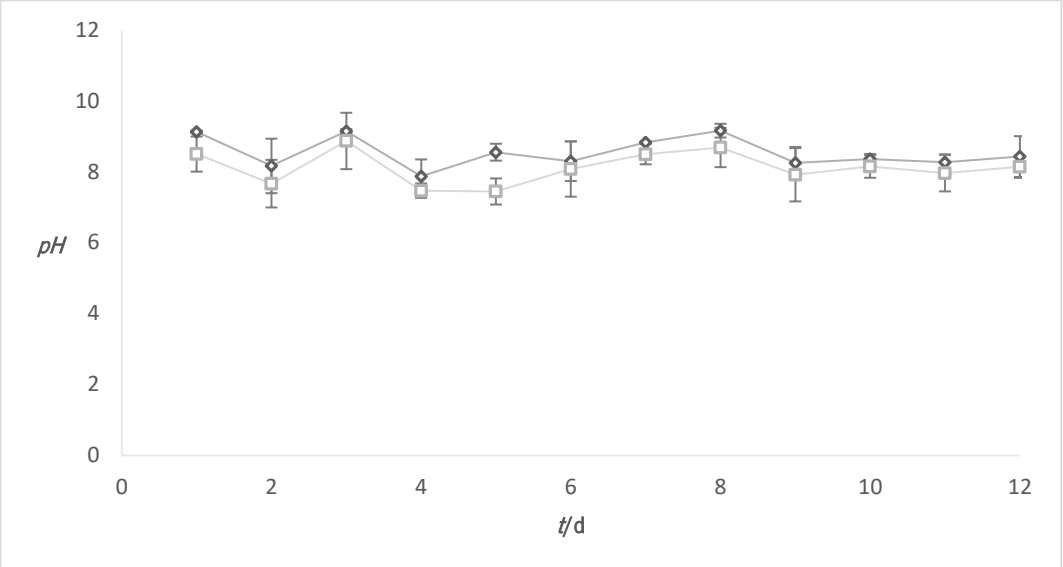

Figure C2 - *pH* measures over the time of cultivation of *P. gyrans* (◇) and *P. pinguis* (□) in 55 L flat panels.

ANNEX D – CELLS MORPHOLOGY IN THE SCALE UP OF *P. PINGUIS* IN TUBULAR PBR

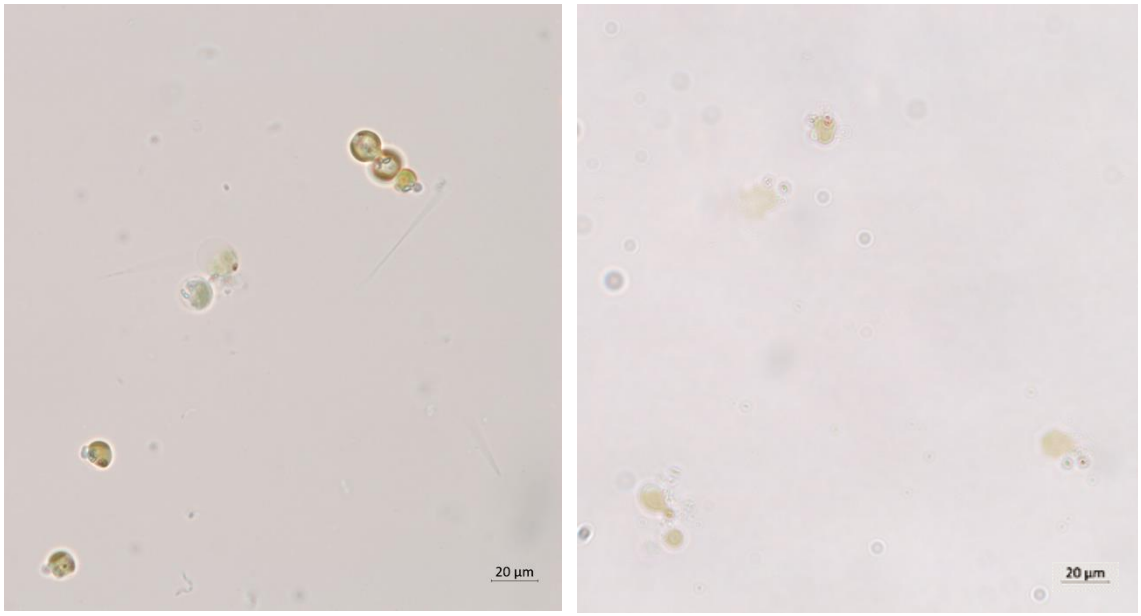

Figure D1 - Morphologies of *P. pinguis* observed on optical microscopic from daily samples.