

Integrated Master in Bioengineering

**Environmental benefits of *Chlorella vulgaris* and
*Pseudokirchneriella subcapitata***

CO₂ capture and bioenergy production

Dissertation for Master Degree in Biological Engineering

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Abstract

The increase of anthropogenic CO₂ emissions has been causing several environmental problems, due to the accumulation of this pollutant in atmosphere, the main responsible for the greenhouse effect and global warming. The search for effective systems to capture CO₂, has revealed the potential of microalgae to effectively perform this task. Apart from their application in carbon dioxide removal, these photosynthetic organisms are able to produce a wide variety of compounds that can have different utilities in diversified areas. Although many studies have focused on the use of microalgae as an effective biological method for CO₂ sequestration, further research is required to increase biomass productivities and to reduce production costs.

In this study, different applications of microalgae were exploited using the species *Chlorella vulgaris* and *Pseudokirchneriella subcapitata*: i) to verify the effect of light intensity, photoperiods, and medium composition on microalgal growth, allowing to infer the best culture conditions for CO₂ uptake; ii) to evaluate lipid productivities from microalgae grown under different light intensities and photoperiods using a colorimetric method; and iii) to study the ecological interaction between the microalga *P. subcapitata* and the cyanobacterium *Synechocystis* sp.

Results have shown that increasing light intensities leads to an increase in biomass production. However, continuous light supply and higher light intensities (between the range studied) leads to cell damage due to the photooxidation phenomenon. In general, higher nutrient concentration results in higher biomass productivities. It was observed that lipid productivities also increased with light intensity. However, higher light intensities studied were apparently responsible for the degradation of fatty acids, a consequence of photooxidation too. Mixed cultures of the organisms *P. subcapitata* and *Synechocystis* sp. revealed that *P. subcapitata* has higher ability to uptake inorganic phosphorus even at low concentrations, being suitable for wastewater treatment. Furthermore, the study of surface properties of these organisms has shown that further research in this field is required, as it seems that the interactions between these organisms may be very useful in biotechnological processes, such as the production of biomolecules, biofilm formation or harvesting techniques (bio-flocculation).

Key-words: *Chlorella vulgaris*, *Pseudokirchneriella subcapitata*, CO₂ capture, Lipid production, Ecological interactions, *Synechocystis* sp.

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Nomenclature

Abbreviations

TFA	Total fatty acids
ACCase	Acetyl-CoA carboxylase
BSA	Bovine serum albumin
CDW	Cell dry weight
DNS	Dinitrosalicylic acid
FA	Fatty acids
FFA	Free fatty acids
GHG	Greenhouse gas
IL	Ionic liquid
MAE	Microwave assisted extraction
OD	Optical density
P _i	Inorganic phosphorus
PBR	Photobioreactor
PEF	Pulsed electric field
s.d.	Standard deviation
SCCO ₂	Supercritical fluid extraction using CO ₂
TEA	Triethanolamine
TFF	Tangent flow filtration
UAE	Ultrasonic assisted extraction
DLVO	Derjaguin-Landau-Verwey-Overbeek
XDLVO	Extended Derjaguin-Landau-Verwey-Overbeek

Indexes

max	Maximum
sws	Between two entities of a given surface, when immersed in water
l	Liquid
LW	Lifshitz-van der Waals component
AB	Lewis acid-base component
TOT	Total

+	Electron acceptor parameter of the Lewis acid-base component (γ^{AB})
-	Electron donor parameter of the Lewis acid-base component (γ^{AB})
F	Formamide
W	Water
B	α -bromonaphthalene

Measure units

μ	Specific growth rate	d^{-1}
X	Biomass concentration	$g L^{-1}$
t	Time	d
P	Biomass productivity	$g L^{-1} d^{-1}$
K_I	Half saturation constant	$\mu E m^{-2} s^{-1}$
I	Light intensity	$\mu E m^{-2} s^{-1}$
K_P	Half saturation constant	$\mu g L^{-1}$
[P]	Phosphate concentration	$\mu g L^{-1}$
A	Affinity	$L \mu g^{-1} d^{-1}$
ΔG	Free energy of interaction	$mJ m^{-2}$
γ	Surface free energy	$mJ m^{-2}$
θ	Contact angle	$^{\circ}$

CHAPTER 1

Work outline

1.1. Background and project presentation

The continuous use of fossil fuels as the major source of energy has led the world to an energy and environmental crisis, due to the depletion of this source of energy and the increase of exhaust gases emissions that accumulate in the atmosphere and cause global warming. The search for sustainable sources of energy has showed that producing biodiesel from vegetable oil crops, such as palm, rapeseed, and soybean, and animal fats could be a promising alternative (Amaro et al., 2011; Demirbas, 2011; Lee et al., 2010; Ranjan et al., 2010). However, oil production yields from vegetable crops and animal fats do not achieve the current demand for transport fuels (Chisti, 2007; Demirbas, 2011). Furthermore, producing biodiesel from vegetable crops is time consuming and requires great areas of arable land that would compete with the one used for food crops, leading to starvation in developing countries (Costa & de Moraes, 2011; Demirbas, 2011; Demirbas & Demirbas, 2011).

Due to these limitations, attentions are now focused on using microalgae. When growing autotrophically, microalgae assimilate CO₂ and produce biomass and other compounds, such as lipids, that can be used to produce biodiesel. Through photosynthesis, microalgae also assimilate other nutrients, such as nitrogen and phosphorus, which can be obtained, for example, from wastewater (Carvalho Júnior et al., 2011). Apart from their uses in the production of biodiesel, microalgal biomass can have a lot of different applications. The combination of CO₂ uptake with effluent treatment, as well as the synthesis of various products of commercial value provides a promising alternative to actual biodiesel production techniques (Carvalho Júnior et al., 2011; Chisti, 2007). Additionally, microalgae present several advantages over oil crops, including (Chisti, 2007; Clarens et al., 2010; Lee et al., 2010; Mercer & Armenta, 2011): i) higher lipid contents – oil levels of 20-50% by weight of dry biomass are common in several microalgae; ii) higher growth and biomass production rates – normally microalgae double their biomass within 24 hours; iii) shorter maturity rates; iv) require far less land, thus not compromising food production and supply.

1.2. Main objectives

The main aim of this study is to evaluate the potential of the microalgae *Chlorella vulgaris* and *Pseudokirchneriella subcapitata* in environmental applications, such as CO₂ capture and bioenergy production.

To evaluate the ability of these microalgae to efficiently capture CO₂ from the atmosphere, different experiments should be performed to determine the more appropriate conditions that lead to higher biomass productivities. In this sense, both microalgae were grown in flasks with the same volume and the following assays were carried out: i) batch experiments were performed for both microalgae using the same experimental conditions (working volume, temperature, pH, light intensity, photoperiod and culture medium); ii) different light intensities, photoperiods and concentrations of the culture medium were applied, to determine the best growth conditions; iii) cell growth and kinetic parameters were evaluated by daily monitoring optical density (OD) at 683 nm.

As microalgae are a great source of a wide variety of compounds, it is also an objective to evaluate if these organisms have the ability to produce compounds of interest, such as proteins and polysaccharides in the tested conditions. The presence of extracellular proteins and polysaccharides in microalgal cultures was studied by applying the Bradford (1976) and Miller (1959) methods, respectively.

Another aim of this study is the determination of oil contents of these microalgae and the effect of different light intensities and photoperiods on lipid yields. Quantification of intracellular lipids was performed using a colorimetric method, to determine the growth conditions that increase lipid yields.

It will also be studied the interaction between the microalga *P. subcapitata* and the cyanobacterium *Synechocystis* sp. These interactions were determined by growing these organisms together and analyzing physicochemical properties of microalga and cyanobacterium surface (zeta potential and hydrophobicity). These experiments give information about the behavior of these organisms when growing together and under stress conditions and also about their ability to adhere to different surfaces, which can be very useful for application of these organisms in wastewater treatment or in a technological process called bio-flocculation. In this area, the following assays were realized: i) batch experiments were performed for both organisms in separate flasks and also mixed cultures using the same experimental conditions; ii) a stress condition (in this case, limited phosphorus concentration) was induced to verify the response of the organisms; iii) cell growth and kinetic parameters were evaluated by cell

counting; iv) zeta potential and hydrophobicity of each organism's surface were determined as major characteristics of physicochemical cell surface properties.

1.3. Thesis organization

This work is divided in six chapters. Chapter 1 presents the main goals, context, and motivations for the development of this study. It consists in a guideline for the overall work presented in the further chapters.

Chapter 2 provides a brief review of the literature. Characteristics of microalgae, as well as the major applications are described. The main technologies applied in microalgal cultivation and downstream processing are also referred in this chapter.

Chapter 3 focuses the effect of light intensity, photoperiod, and growth medium concentrations on the ability of *C. vulgaris* and *P. subcapitata* to capture CO₂ from the atmosphere. Batch experiments were carried out with synthetic wastewater at room temperature with artificial illumination and all the cultures were aerated with CO₂ at atmospheric concentration.

Chapter 4 shows how different light supplies can affect lipid productivities of the microalgae *C. vulgaris* and *P. subcapitata*. Lipid contents were determined using a colorimetric method, to easily determine in which conditions these microalgae have higher lipid productivities.

Chapter 5 provides the study of the interaction of the microalga *P. subcapitata* with the cyanobacterium *Synechocystis* sp. when grown under limiting phosphorus concentrations with physicochemical characterization of their surface.

Chapter 6 presents an overview of all the developed work, with emphasis on the main conclusions and on the perspectives for further research.

CHAPTER 2

Literature review

This chapter focuses on the major applications of microalgae, with emphasis on the technological processes associated with CO₂ removal and bioenergy production.

2.1. Microalgae

Microalgae are prokaryotic or eukaryotic photosynthetic microorganisms that can be found in aquatic or terrestrial ecosystems (Gouveia, 2011; John et al., 2011; Mata et al., 2010). The number of microalgal species is not known, but in some references the numbers estimated round the 200 000 to some millions of species (Norton et al., 1996).

Due to their unicellular or multicellular simple structure, microalgae have a photosynthetic mechanism similar to land-based plants; they can grow rapidly and in a wide variety of environmental conditions. Furthermore, being submerged in an aqueous environment allow microalgae to easily access to water, CO₂ and other nutrients, thus converting more efficiently solar energy into biomass (Gouveia, 2011; John et al., 2011).

These organisms are classified in different groups, according to some parameters like morphologic characteristics, cell wall and photosynthetic pigments composition, and chemical nature of their by-products (Brennan & Owende, 2010; Tomaseli, 2004). Prokaryotic cells, known as cyanobacteria (*Cyanophyceae*), resemble more to bacteria rather than algae. They do not have membrane-bound organelles, such as plastids, mitochondria, nuclei, Golgi bodies and flagella. Examples of eukaryotic microalgae include green algae (*Chlorophyta*), red algae (*Rhodophyta*) and diatoms (*Bacillariophyta*). These organisms have the above mentioned organelles, which are responsible for cell control, reproduction and survival (Brennan & Owende, 2010; Mata et al., 2010).

Microalgae are autotrophic organisms: they reproduce themselves using photosynthesis, requiring only inorganic compounds such as CO₂ to convert solar light energy into chemical energy (Brennan & Owende, 2010; Mata et al., 2010). However, some microalgal species are also able to use exogenous organic nutrients as energy source. These algae are known as mixotrophic, as they can grow both autotrophic or heterotrophically (Brennan & Owende, 2010).

2.2. Applications of microalgae

Due to its high biomass productivity and to the ability to perform photosynthesis, biological conversion of light energy into chemical energy in the form of organic carbon compounds (Falkouski & Raven, 2007), microalgae have numerous applications in diversified areas (Brennan & Owende, 2010; Mata et al., 2010; Spolaore et al., 2006).

Through photosynthesis, microalgae are able to assimilate CO₂ from the atmosphere, as well as from flue gas emissions, thus reducing the risk of exhaust gases accumulations in atmospheric air (Ho et al., 2011; Tang et al., 2011). Additionally, these photosynthetic organisms assimilate other compounds, such as nitrogen and phosphorus. These nutrients are frequently found in wastewaters, meaning that microalgae can be a promising alternative in wastewater treatment (Rawat et al., 2011; Silva-Benavides & Torzillo, 2012). Furthermore, microalgal biomass can have a lot of applications, in such different areas (Mata et al., 2010; Parmar et al., 2011; Spolaore et al., 2006). Algal biomass is suitable for human food and animal feed, as they are an important source of natural vitamins, minerals and fatty acids. They can be used to feed different animals, such as cats, dogs, aquarium fish, birds, horses and cows (Hu, 2004; Spolaore et al., 2006). A lot of high-valued compounds from microalgae, like pigments, antioxidants, β -carotenes, proteins, polysaccharides, triglycerides, fatty acids and vitamins, can be used as raw materials for the production of cosmetics, drugs and functional food (Hu, 2004; Mata et al., 2010; Singh et al., 2005). The fatty acids produced by microalgae can be extracted and used for biodiesel production and products such as proteins and residual biomass can be fermented to produce ethanol or methane (Brennan & Owende, 2010; John et al., 2011; Parmar et al., 2011).

2.3. Microalgal cultivation

Autotrophic cultivation of microalgae requires light supply, CO₂, water, and inorganic salts. Several variables are known for their influence in the cultivation process: light distribution and saturation, temperature, pH, salinity, quality and amount of nutrients, concentration of dissolved oxygen, and toxicity of the medium, which is mainly due to the presence of heavy metals (Chisti, 2007; Eriksen, 2008; Pires et al., 2012). Microalgal growth medium requires the presence of the inorganic elements that constitute algal cells: nitrogen, phosphorus, and iron. Marine microalgae are commonly grown in sea water supplemented with nitrate and phosphate fertilizers, whereas other microalgae can be cultivated in wastewaters, thus reducing cultivation costs and providing wastewater treatment (Molina Grima et al., 1999). CO₂ from the atmosphere or from flue gas emissions should be supplied continuously during light periods, thus enabling carbon dioxide mitigation. Alternatively, light must be supplied using solar light, artificial light,

or both. Microalgae cultivation can be carried out in closed systems, where culture conditions are strictly controlled, or in open systems.

2.3.1. Closed photobioreactors

Using closed photobioreactors (PBRs) for microalgal growth can be more advantageous, as culture conditions and growth parameters, such as pH, temperature, mixing, CO₂ and O₂ concentrations are strictly controlled; evaporation and contaminations can be easily avoided, and higher cell concentrations can be achieved. Despite these advantages, PBRs have some limitations in terms of overheating, difficulties in scale-up, and higher costs of construction (Posten & Schaub, 2009). The most commonly used PBRs include flat plate reactors, bubble-column reactors, and tubular reactors (Posten, 2009; Ugwu et al., 2008; Xu et al., 2009). A schematic representation of these geometries is shown in Figure 2.1.

Flat plate PBRs are known for their large illumination surface area (Ugwu et al., 2008; Xu et al., 2009). Higher biomass productivities are achieved with these PBRs, which make them suitable for outdoor cultures. Additionally, the use of flat plate bioreactors is very good for immobilization of microalgae (Ugwu et al., 2008). Accumulation of dissolved oxygen is relatively low, when compared with horizontal tubular reactors. In terms of costs and operating/maintenance conditions, these reactors are relatively cheap, easy to clean-up and readily tempered (Lee, 2001; Ugwu et al., 2008; Xu et al., 2009). The main disadvantages of flat plate bioreactors include difficulties in scale-up, due to the requirement of many compartments and support materials, and difficulties in temperature control (Lee, 2001; Ugwu et al., 2008).

Bubble-column PBRs have been extensively used in bioprocessing, wastewater treatment, and in chemical process industry (Xu et al., 2009). Generally, bubble-column reactors are vertical and compact – requiring less land, relatively cheap, and easy to operate. The high mass transfer coefficient and extremely low physical stress obtained in these reactors make them suitable for microalgal culture, especially for aquaculture (Sánchez Mirón et al., 2000; Xu et al., 2009). The main problems posed by these reactors are high energy costs associated to mass circulation, the possibility of cell sedimentation, and difficulties in scale-up, as an increase in column diameter causes an exponential decrease in light penetration (Xu et al., 2009).

The majority of tubular PBRs is made of transparent plastic or glass and consists in the arrangement of tubes to form a straight, coiled, or looped pattern. Microalgae are recirculated

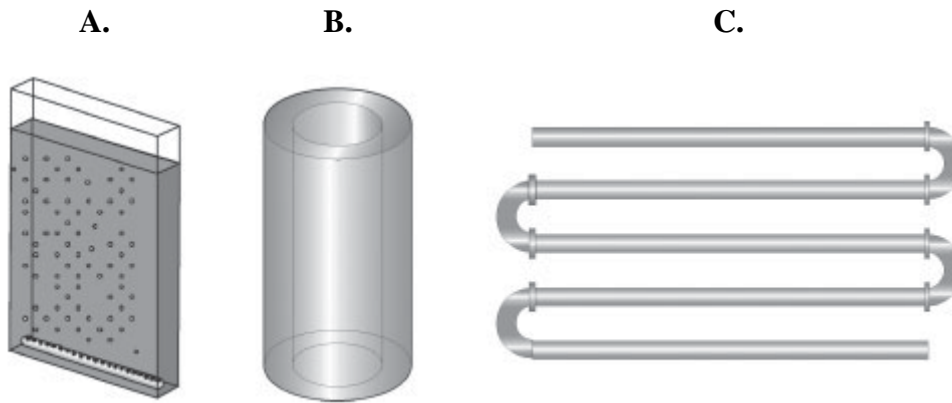


Figure 2.1. Most common PBR geometries: A. flat plate reactor; B. bubble-column reactor; C. tubular reactor (Posten, 2009).

along the tubes using a pump or airlift technology (Ugwu et al., 2008; Xu et al., 2009). Tubular bioreactors can be horizontal, near horizontally, conical, and inclined (Ugwu et al., 2008). The large illumination surface area of these systems is ideal for outdoor culture of microalgae. Additionally, these reactors are low-cost and high biomass productivities can be achieved (Ugwu et al., 2008; Xu et al., 2009). The main problem associated with the use of tubular reactors is the poor mass transfer, which poses several difficulties, especially in terms of scale-up (Molina Grima et al., 2001). Some operational conditions are difficult to control due to temperature oscillations, formation of pH gradients along the tubes, and accumulation of high O₂ concentrations. Using these reactors also requires larger areas of land (Molina Grima et al., 2001; Ugwu et al., 2008; Ugwu et al., 2003).

2.3.2. Open ponds

In the past few years, cultivation of microalgae using open systems has been extensively studied. These systems can be divided into two categories: natural ponds, which include lakes, lagoons, and ponds, and artificial ponds or containers (Lee, 2001; Ugwu et al., 2008). The most commonly used systems include shallow big ponds, tanks, circular ponds and raceway ponds (Lee, 2001; Parmar et al., 2011; Ugwu et al., 2008).

Microalgae production in open systems is less expensive in terms of construction and operation and has larger production capacity (Borowitzka, 1999; Posten & Schaub, 2009; Pulz, 2001). Biomass productivities achieved with these systems are lower than those achieved with closed PBRs, which is mainly due to insufficient mixing, oscillations in the culture conditions, and higher susceptibility to the co-existence of other organisms that act as competitors and predators. Additionally, it is common in these systems the diffusion of CO₂

to the atmosphere, evaporative losses of water, and poor light utilization by cells (Lee, 2001; Posten & Schaub, 2009; Pulz, 2001; Ugwu et al., 2008).

2.4. Downstream processing of microalgae

Producing biofuels or other compounds from microalgal metabolites and biomass requires a set of processes that include harvesting of biomass, dewatering and drying, and also extraction and purification of the metabolites of interest. As one of the aims of this work is bioenergy production using microalgae, the following sections will emphasize the downstream processes involved in the recovery of microalgal metabolites, with focus on biodiesel production from microalgal lipids.

2.4.1. Harvesting

Cell harvesting consists in the separation of algal cells from the culture broth using one or more solid-liquid separation procedures. Since microalgae have small sizes, ranging from 3 to 30 μm , and after the cultivation step cell densities are very low ($0.3\text{-}5.0\text{ g L}^{-1}$), recovery of algal biomass can be very difficult and cost-effective (Molina Grima et al., 2003; Uduman et al., 2010). To reduce costs, cell harvesting often occurs in a two-step process: i) bulk harvesting, where biomass is firstly separated from the bulk suspension by the processes of flocculation, flotation, or gravity sedimentation, reaching a final concentration of about 2-7% total solid matter; ii) thickening, where algal cells are further concentrated through centrifugation or filtration, resulting in a paste of 15-25% total solid matter (Olaizola, 2003). Selection of the adequate harvesting technique depends on some parameters, such as cell size and density, value of target product, biomass concentration, and culture conditions (Brennan & Owende, 2010; Olaizola, 2003; Uduman et al., 2010). The major techniques used for cell harvesting include centrifugation, filtration, and flocculation (Molina Grima et al., 2003; Oh et al., 2001; Rossignol et al., 1999). Table 2.1 shows the main advantages and disadvantages of the referred harvesting methods.

In centrifugation, solid particles suspended in the liquid phase are allowed to deposit by the action of centrifugal forces. Among the different harvesting methods, centrifugation is the preferred one, as it has the ability to process large volumes relatively faster than other methods and to produce concentrates that remain fully contained during recovery (Molina Grima et al., 2003; Pires et al., 2012; Sim et al., 1988). However, this method presents several disadvantages, including: i) the possibility to damage cells due to the high shear stress forces

Table 2.1. Principal advantages and disadvantages of the most commonly used harvesting methods

Harvesting method	Advantages	Disadvantages
Centrifugation	<ul style="list-style-type: none"> • High biomass recovery • High capacity • Fast 	<ul style="list-style-type: none"> • High costs • Cell damage
Filtration	<ul style="list-style-type: none"> • Low cost 	<ul style="list-style-type: none"> • Slow process • Possibility of fouling • Possibility of cell damage
Flocculation	<ul style="list-style-type: none"> • Simple and fast • Low cost • Recycling of the clarified culture medium 	<ul style="list-style-type: none"> • Toxicity of flocculants • Some flocculants are very expensive

involved; ii) energy demanding; and iii) relatively expensive (Knuckey et al., 2006; Lee et al., 2009; Sim et al., 1988). For these reasons, it is thought that centrifugation is ideal for the recovery of high-valued products (Lee et al., 2009; Molina Grima et al., 2003). Furthermore, centrifugation is not suitable to harvest microalgae from a saline medium because salt concentration is responsible for an increase in corrosion (Pires et al., 2012).

In the filtration process, the suspension is allowed to pass through a membrane, where the solid particles are retained. This is a low-cost process, but it is not appropriate for processing large volumes, as it is very slow. Additionally, the use of filter presses strongly depends on cell size. It is suitable for the recovery of large cells, such as *Coelastrum prosboscideum* and *Spirulina platensis*, but it is not able to retain cells whose diameter is similar to that of bacteria like *Scenedesmus*, *Dunaliella*, and *Chlorella* (Molina Grima et al., 2003). Another bottleneck of this method is the accumulation of matter in the filtering membrane (fouling), which is responsible for a reduce in the permeability of these filters, diminishing process velocity and efficiency (Rossi et al., 2004). As well as in the centrifugation process, the shear forces reached in filtration can cause damage to some cells (Jaouen et al., 1999). For this reason, it is common to apply tangent flow filtration (TFF), such as microfiltration or ultra-filtration, to harvest microalgal biomass. In TFF processes, the bulk flow is parallel to the membrane and perpendicular to the permeation flux, reducing shear stress forces involved (Pires et al., 2012; Rossignol et al., 1999).

Flocculation occurs when suspended particles interact with flocculants and aggregate in large flocks that settle to the bottom of the tank owing to gravitational and fluid drag forces (Harith et al., 2009; Knuckey et al., 2006). This method has been successfully applied in the clarification of wastewater and drinking water and color removal in paper industry and is

appropriate for the harvesting of large particles, such as microalgal cells (Harith et al., 2009). It is a simple and fast process and has reduced costs, when compared with other solid-liquid separation methods (Harith et al., 2009; Knuckey et al., 2006; Molina Grima et al., 2003). There are three different types of flocculation: chemical flocculation, bio-flocculation, and electro-flocculation. The most commonly used flocculants are aluminum sulfate, aluminum chloride, and ferric chloride. To select the most appropriate flocculant, it is important to clearly define the aim of the separation process and to ensure that the flocculant will not pose problems in the downstream processing of microalgal biomass. Furthermore, flocculants applied should be cheap, effective even for low flocculant concentrations, and non-toxic (Lee et al., 2009; Oh et al., 2001; Rossignol et al., 1999).

2.4.2. Drying

After harvesting of biomass, water content in microalgal paste is still very high, which difficults the choice for a cost-effective dewatering process. The main drying processes used for microalgal biomass include sun-drying, spray-drying, and freeze-drying (Molina Grima et al., 2004).

Sun-drying is the dewatering process that is applied to the majority of crops. This is a low-cost process, but the high water content present in the biomass is responsible for a huge increase in drying times. In addition, this method requires large drying surfaces (Desmorieux & Decaen, 2005; Molina Grima et al., 2004; Prakash et al., 1997). Due to the highest demand for food and fuels verified in the last decades, this method is not suitable when the final aim of biomass is the production of food and bioenergy (Molina Grima et al., 2004).

In spray-drying, atomizers or gas-liquid jets are used to form a fine spray of solution droplets, which are brought into continuous contact with hot air in a large chamber. These systems have several advantages: i) continuous operation; ii) the final product (powder) does not require further size reduction; and iii) rapid drying. However, this is a high costly method, implying that it is only suitable for the recovery of high-valued products (Leach et al., 1998).

In freeze-drying or lyophilization, algal biomass is frozen and the ice crystals sublimed by slight warming without thawing. Lyophilization comprises three steps: i) freezing to solidify the material; ii) sublimation drying to reduce moisture to below 20% w/w; and iii) desorption to reduce bound moisture to the required final value ($\approx 1\%$ w/w) (Snowman, 1996). This drying method has high equipment and energetic costs, being suitable for fine applications, where it is important to keep biochemical properties of the biomass constant.

However, it is a fast and efficient process for water removing (Brennan & Owende, 2010; Molina Grima et al., 2004).

2.4.3. Cell disruption and extraction of microalgal metabolites

There are several methods described for the extraction of microalgal metabolites, those being enzymatic extraction, pulsed electric field extraction (PEF), ultrasound and microwave assisted extraction (UAE and MAE), expeller press extraction, organic solvent extraction, supercritical fluid extraction (Amaro et al., 2011; Mercer & Armenta, 2011; Taher et al., 2011), and ionic liquid mediated extraction (Kim et al., 2011). The referred extraction procedures can be divided into three groups: i) those that can be applied directly after the harvesting process; ii) those that are strongly affected by the presence of water, requiring previous drying; and iii) those that can be applied to both wet and dried biomass. Extraction procedures, such as enzymatic and pulsed electric field, can be applied directly to the harvested microalgal paste (containing about 15-25% total solid matter), without further dewatering. UAE, MAE, and expeller press extraction are examples of extraction procedures that can be applied to both wet and dried biomass. The extraction procedures that require previous drying include organic solvent extraction, supercritical fluid extraction, and ionic liquid mediated extraction.

Enzymes can be applied in the extraction metabolites from microalgae, as they can mediate the hydrolysis of cell walls, facilitating the release of their contents into an appropriate solvent. Enzymes can be applied directly to wet biomass and their application with little volumes of organic solvents can improve recovery yields, as well as extraction times (Mercer & Armenta, 2011). In general, enzymes used to cell wall degradation include lipases and cellulases. Despite being expensive, enzymes offer several advantages over other cell wall disruption methods. Enzymes present higher degradation selectivity than other mechanical disruption methods. Furthermore, algal cell walls are more recalcitrant than cell walls from other microorganisms, being very resistant to degradation. Thus, the use of mechanical disruption methods will require higher energy amounts (Sander & Murthy, 2009; Shah et al., 2004).

The use of PEF technology seems to be a potential alternative for the recovery of algal metabolites (Taher et al., 2011). This technique applies brief pulses of a strong electric field to cells, allowing the permeabilization of cell matrices, as well as the mass transport across the cell membranes (Guderjan et al., 2005; Taher et al., 2011). Although high recovery efficiencies can be increased employing organic solvents, this method requires far less

organic solvents than the conventional organic solvent extraction methods (Guderjan et al., 2007; Guderjan et al., 2005).

UAE and MAE improve extractions of co-products from microalgae significantly, with high efficiencies, reduced extraction times and increased yields, as well as moderate costs and negligible added toxicity (Mercer & Armenta, 2011). In UAE metabolites from microalgal cells can be recovered by cavitation. The cavitation phenomenon occurs when vapor bubbles of the liquid are formed in an area where pressure of the liquid is lower than its vapor pressure. As these bubbles grow when pressure is negative and compress under positive pressure, a violent collapse of the bubbles is promoted. When bubbles collapse near cell walls, damage can occur, leading to the release of cell contents (Cravotto et al., 2008; Mercer & Armenta, 2011; Taher et al., 2011). Application of this ultrasound-assisted method to both dry and wet biomass can improve extraction efficiencies by reducing extraction times and increasing recovery yields. In turn, MAE is supported by the principle that microwaves directly affect polar solvents and materials. Even when they are applied to dried cells, trace amounts of moisture are affected: temperature increases due to microwaves, moisture is evaporated, and pressure in the cells increases, leading to a damage or rupture of the cell wall, with the release of its contents (Balasubramanian et al., 2011; Mandal et al., 2007). Microwave theory and the extraction principle are described in detail in Mandal et al. (2007). The use of microwaves followed by organic solvent extraction using small amounts of solvent contributes to an efficient and inexpensive extraction procedure, which does not require previous dehydration of biomass.

Pressing techniques lie on the principle that when microalgal cells are submitted to high pressures, they start to crush, releasing their contents to an adequate solvent. As the methods described before, pressing can be advantageous when used as a pretreatment for organic solvent extraction, for example (Shen et al., 2009a; Taher et al., 2011). Although this method is very simple and inexpensive, it presents some disadvantages, such as high power consumption and maintenance costs (Taher et al., 2011).

The use of organic solvents to extract microalgal contents is the most applied method, especially for lipid recovery. The most used organic solvents are hexane, cyclohexane, benzene, ethanol, acetone, and chloroform (Brennan & Owende, 2010; Mercer & Armenta, 2011; Molina Grima et al., 2003). These solvents have shown to be quite effective in the extraction of microalgal metabolites, especially from dried biomass. A good solvent may present the following characteristics: i) to be insoluble in water; ii) to have high affinity for the metabolites of interest, iii) to have a low boiling point to facilitate its removal after

extraction; iv) to have a considerably different density from that of water. Furthermore, the applied organic solvent should be inexpensive, non-toxic, and reusable (Mercer & Armenta, 2011; Ranjan et al., 2010). The main disadvantages of this method are associated with the high volumes of toxic and harmful organic solvents required.

One alternative to avoid the use of volatile and toxic organic solvents for metabolite extraction (especially lipid extraction) from microalgae is to use supercritical fluids as solvents (Amaro et al., 2011; Halim et al., 2011; Mercer & Armenta, 2011). Supercritical fluids are compounds that behave both as a liquid or a gas when exposed to temperatures and pressures above their critical temperature and pressure points. The most used supercritical fluid is CO₂ (SCCO₂) because it presents low critical temperature (31.1 °C) and pressure (72.9 atm) (Mercer & Armenta, 2011). SCCO₂ extraction presents several advantages over the traditional organic solvent extraction procedures, such as: tunable solvating power, low toxicity and flammability, favorable mass transfer rates, and production of solvent free extracts because at room temperature CO₂ behaves as a gas (Amaro et al., 2011; Crampon et al., 2011; Halim et al., 2011; Macías-Sánchez et al., 2007). The main disadvantage associated with this method is its expensiveness, which is mainly due to the high costs of the infrastructure and the required operational conditions (Halim et al., 2011).

Ionic liquids (ILs) have been reported as an attractive alternative for volatile and toxic organic solvents because of their non-volatile character, thermal stability, and high solvation capacity (Kim et al., 2011; Lateef et al., 2009). ILs are salts of relatively large asymmetric organic cations coupled with smaller organic or inorganic anions. These organic salts can be liquid at room temperatures or low melting point solids (<100 °C) (Khodadoust et al., 2006; Lateef et al., 2009; Young et al., 2010). By altering the nature of both cation and anion of the ionic liquid, either hydrophilic or hydrophobic ionic liquids can be prepared, to make them suitable for different applications (Lateef et al., 2009).

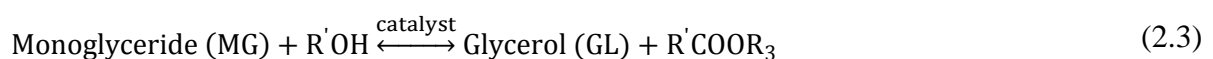
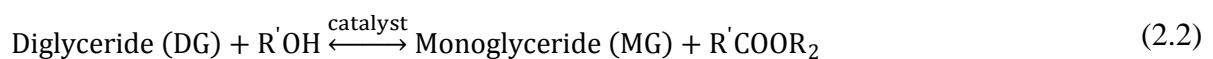
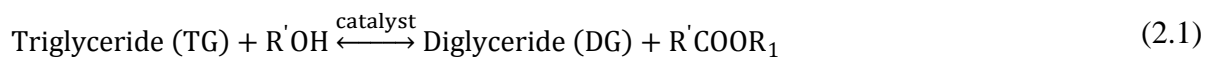
After the extraction procedures, the metabolites recovered should be refined and purified according to their purpose and applications. For example, lipids can be used for biodiesel production, thus requiring further processing, as referred below.

2.4.4. Biodiesel production

Biodiesel constitutes the best candidate to substitute diesel fuel, as it can be used directly as fuel, requiring some engine modifications, or blended with petroleum diesel and used in diesel engines with few or no modifications (Leung et al., 2010). Chemically, biodiesel is a mixture of esters with long-chain fatty acids, such as lauric, palmitic, stearic,

oleic, etc. (Demirbas & Demirbas, 2010). Recently, this biofuel has become more attractive due to its environmental benefits: it is biodegradable and has lower sulfur and aromatic content than diesel fuel, meaning that it will emit less toxic gases. Furthermore, it presents several advantages over conventional petroleum diesel, such as higher combustion efficiency and cetane number. The main disadvantages of biodiesel include the high costs of production, its higher viscosity, lower energy content, and higher nitrogen oxide emissions (Demirbas & Demirbas, 2010; Leung et al., 2010). Biodiesel can be produced from extracted lipids through four different methods: direct use or blending of oils, microemulsification of oils, thermal cracking or pyrolysis, and transesterification, also known as alcoholysis (Balat & Balat, 2010; Leung et al., 2010; Ma & Hanna, 1999). Additional information of the referred methods can be obtained in Balat & Balat (2010) and Ma & Hanna (1999). Transesterification constitutes the most applied method for biodiesel production, as it presents several advantages over the others. For example, blending and microemulsification cause some problems, such as carbon deposition and oil contamination, whereas pyrolysis is responsible for the production of low valuable products, as well as the production of gasoline instead of diesel (Sharma & Singh, 2009). Therefore, transesterification, the chemical conversion of triglycerides in glycerol and esters in the presence of an alcohol, seems to be the most appropriate method for biodiesel production.

As shown in Equations 2.1 to 2.3, the transesterification is a multi-step reaction where triglycerides are converted into diglycerides, monoglycerides, and finally into glycerol during three reaction steps. These reactions are reversible and each one results in the formation of 1 mol of fatty acid ester (Leung et al., 2010; Ma & Hanna, 1999).



R represents a small hydrocarbon chain, whereas R₁, R₂, and R₃ represent long-chain hydrocarbons, also known as fatty acid chains.

Variables affecting biodiesel yields during transesterification are: i) the employed alcohol and molar ratio; ii) type of used catalyst; iii) amount of free fatty acids (FFA); iv) water content; and v) reaction temperature and time (Ehimen et al., 2010; Ma & Hanna, 1999; Miao et al., 2009; Sharma & Singh, 2009; Wahlen et al., 2011).

There are two types of transesterification: transesterification applied to the extracted oil and transesterification directly applied to the oil source, without previous lipid extraction, also known as transesterification *in situ*. Although both methods are very similar, it is thought that transesterification *in situ* could lower the production costs of biodiesel fuel, as this process can produce biodiesel directly without the extraction step (Ma & Hanna, 1999).

This literature review shows the wide variety of applications of microalgae: from CO₂ uptake and wastewater treatment to the production of metabolites with interest in diversified areas, such as cosmetics, pharmaceuticals and biofuels. The following chapters provide three different studies that have been performed to have an overview of some of the referred applications of microalgae.

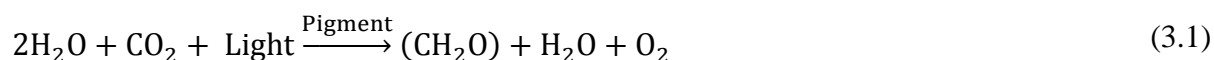
CHAPTER 3

The effect of light supply and medium composition on CO₂ capture

3.1. Introduction

Anthropogenic activities are responsible for an increase in the atmospheric greenhouse gas (GHG) concentrations, which, in turn, are responsible for global warming (O'Neill & Oppenheimer, 2002). In the last decades, emissions of CO₂, one of the main contributors of greenhouse effect and global warming, have drastically increased, raising its concentration to approximately 30% higher than levels in the pre-industrial period (Mikkelsen et al., 2010). A solution for this environmental problem is CO₂ capture, both from flue gases (4-14% CO₂) and from the atmosphere (\approx 0.04% CO₂). Recently, a lot of different studies have focused the CO₂ capture from flue gases and only a few have mentioned the capture from atmospheric air, due to the high CO₂ levels present in the flue gases (Abu-Zahra et al., 2007; Pires et al., 2011; Rao & Rubin, 2002; White et al., 2003). However, this process may be not enough to stabilize the CO₂ level in atmosphere, which leads to the necessity of CO₂ capture from the atmosphere (Pielke Jr, 2009).

Actually, there are three ways of CO₂ removal from a gaseous stream or mixture: CO₂ sequestration using physical methods, CO₂ capture by chemical reactions, such as amine-based absorption systems (Rao & Rubin, 2002), and CO₂ capture by biological processes (Workman et al., 2011). In biological processes, CO₂ is captured by photosynthetic organisms, according to the following expression (Falkouski & Raven, 2007):



Besides CO₂ removal, the biomass produced during photosynthesis can be used to produce electricity. However, this process will release the acquired CO₂ to the atmosphere, eliminating its effect. Thus, carbon capture should be coupled with the production of other compounds of interest, such as proteins and polysaccharides, that can be further used in the production of cosmetics, drugs and functional food (Hu, 2004; Singh et al., 2005).

Biological processes have an important role in the equilibrium of the atmospheric CO₂ concentration. For instance, the photosynthesis that naturally occurs in the ocean is responsible

for approximately 40% of the overall amount of carbon annually fixed on the planet (Jacob-Lopes et al., 2008). However, there are concerns that the ocean contribution in CO₂ removal will not be the same in the future (Gent, 2012; Swart & Fyfe, 2012). A promising capture process is the culture of microalgae, that use the solar energy with efficiency ten times greater than terrestrial plants (Pires et al., 2012). However, this process is still not economically viable. Research studies are required, mainly regarding the culture conditions (light intensity, photoperiod, temperature, pH and nutrient qualitative and quantitative profiles) and the photobioreactor design in order improve the knowledge on microalgae technology and its application.

This chapter aims to analyse the influence of light intensity, photoperiod and nutrient quantitative profile in the growth and consequent CO₂ uptake of two microalgae (*Chlorella vulgaris* and *Pseudokirchneriella subcapitata*), which cultures were aerated with atmospheric CO₂ at room temperature. Excretion of proteins and polysaccharides was also analysed, to observe if these microalgae were able to produce other compounds of interest at the same time.

3.2. Materials and methods

3.2.1. Microorganisms and culture medium

Stock solutions of the freshwater green algae *Chlorella vulgaris* and *Pseudokirchneriella subcapitata* obtained from American Type Culture Collection – ATCC (Figure 3.1 A and B, respectively) were prepared in OECD test medium (OECD, 2011), with the following composition (per liter): 12 mg MgCl₂·6H₂O, 18 mg CaCl₂·2H₂O, 15 mg

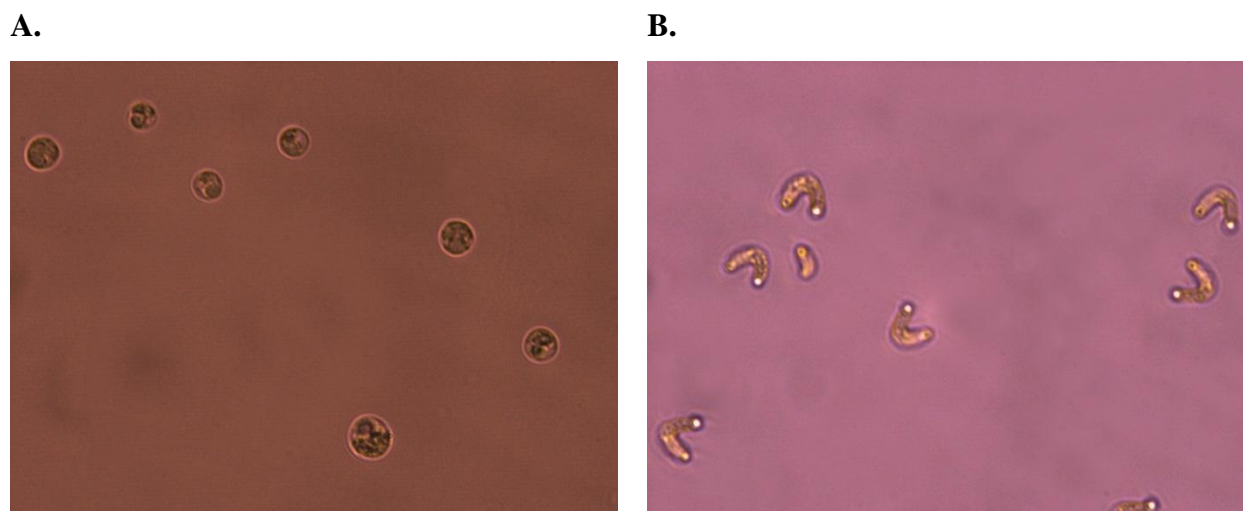


Figure 3.1. Microscopic photographs of the microalgae *C. vulgaris* (A) and *P. subcapitata* (B) taken from a Leica DM LB2 microscope (Leica Microsystems, Germany) incorporated with a DFC300 FX camera and the acquisition software IM50. Photographs were obtained using a 100× oil immersion objective.

MgSO₄·7H₂O, 1.6 mg KH₂PO₄, 0.08 mg FeCl₃·6H₂O, 0.1 mg Na₂EDTA·2H₂O, 0.185 mg H₃BO₃, 0.415 mg MnCl₂·4H₂O, 3 µg ZnCl₂, 1.5 µg CoCl₂·6H₂O, 0.01 µg CuCl₂·2H₂O, 7 µg Na₂MoO₄·2H₂O, and 50 mg NaHCO₃. Nitrogen was supplied in the form of NaNO₃ for *C. vulgaris*, and in the form of NH₄Cl for *P. subcapitata* (15 mg L⁻¹). The cells were incubated in 500 mL flasks at room temperature, under continuous fluorescent light with an intensity of 72 µE m⁻² s⁻¹ at the surface of the flasks. Agitation was obtained by bubbling atmospheric (filtrated through a 0.22 µm cellulose acetate membranes, Orange Scientific, Belgium) air in the bottom of the flasks.

3.2.2. Experimental setup

Experiments were performed in 500 mL flasks operating in batch with a working volume of 450 mL. Cells were cultivated for 12 days in the following experimental conditions: initial cell concentration of 0.05-0.08 g L⁻¹, room temperature (22±1 °C), and continuous aeration with the injection of atmospheric air in the bottom of the flasks. The assays were carried out under different light intensities: 36, 72, 96, and 126 µE m⁻² s⁻¹. For each light intensity, different light cycles were evaluated: 10:14, 14:10, and 24:0 (light:dark). Additionally, for all light intensities and photoperiods tested, two different nutrient concentrations were used: the one described above, M₁, and other where concentrations of all nutrients were doubled, M₂. All the experiments were conducted in duplicates. The experimental units are represented in Figure 3.2.



Figure 3.2. Experimental unit for microalgal growth.

3.2.3. Analytical methods

Light intensity was monitored using an IsoTech Lux-1335 light meter. Duplicate samples were collected at 24 h intervals and biomass concentration was determined by measuring optical density at 683 nm, OD₆₈₃ (Kwon et al., 2005), using a V-1200 spectrophotometer (VWR, Germany). Each sample was diluted to give an absorbance in the range of 0.1–1.0. The relationship between OD₆₈₃, y , and the dry cell weight, x , of *C. vulgaris* and *P. subcapitata* was established by linear regression (see Figure A.1): $y = (1.841 \pm 0.013)x$ ($R^2 = 0.997$) and $y = (2.732 \pm 0.027)x$ ($R^2 = 0.993$), respectively. The pH of the cultures was also determined everyday using a HI 8424 pH meter (HANNA Instruments, USA).

3.2.4. Quantification of extracellular proteins and polysaccharides

One milliliter samples of each culture were collected daily and centrifuged at $16\,500 \times g$ for 10 min in an Eppendorf 5424 centrifuge (Eppendorf, Germany). The supernatants were stored at $-20\text{ }^\circ\text{C}$ in eppendorfs until being analyzed. Proteins and polysaccharides contained in thawed supernatants were then analyzed using the Bradford (1976) and Miller (1959) methods, respectively. Both methods were optimized to be applied in a 96-well polystyrene microtiter plates.

For protein quantification, standards with concentrations ranging from 0 to 0.01 g L^{-1} were prepared from a stock-solution (10 g L^{-1}) of BSA (bovine serum albumin), purchased from Sigma-Aldrich, USA. Colorimetric detection of extracellular proteins was determined by adding $160\text{ }\mu\text{L}$ of both standards and samples to different wells, followed by the addition of $40\text{ }\mu\text{L}$ of Bradford reagent (see composition and preparation protocol in the Annex A.2). After an incubation period of 5 minutes at room temperature, absorbance at 595 nm was measured using a SpectraMax M2 (Molecular Devices, USA). Water ($160\text{ }\mu\text{L}$) with $40\text{ }\mu\text{L}$ of Bradford reagent was used as blank. Protein concentration was then determined by linear regression of absorbance measured at 595 nm *versus* BSA concentration in g L^{-1} (see Figure A.2), according to the expression: $y = (28.043 \pm 0.755)x$ ($R^2 = 0.990$).

Standards of glucose were prepared with concentrations ranging from 0 to 1.5 g L^{-1} from a stock-solution of 5.0 g L^{-1} . Quantification of reducing sugars started by placing $25\text{ }\mu\text{L}$ of each sample and each standard in different eppendorfs, followed by the addition of $25\text{ }\mu\text{L}$ of DNS (dinitrosalicylic acid) reagent (see composition in Annex A.4). Each eppendorf was incubated at $80\text{ }^\circ\text{C}$ for 5 min in a thermostatic bath. After this incubation period, $250\text{ }\mu\text{L}$ of water were added to stop the reaction. Finally, $200\text{ }\mu\text{L}$ of the solution present in the

ependorfs was transferred to different wells and absorbance was measured at 540 nm using the SpectraMax M2 (Molecular Devices, USA). The used blank was water submitted to the same procedure as samples. Polysaccharide concentration was then determined by linear regression of absorbance measured at 540 nm *versus* glucose concentration in g L⁻¹ (see Figure A.3), according to the expression: $y = (0.534 \pm 0.019)x$ ($R^2 = 0.989$).

3.2.5. Kinetic parameters

Cell concentration values obtained everyday were used to determine specific growth rates (μ , d⁻¹), maximum biomass concentration (X_{max} , g L⁻¹), and biomass productivities (P , g L⁻¹ d⁻¹) of each microorganism, as described by Tang et al. (2011). Specific growth rates were calculated from Equation 3.2:

$$\mu = \frac{\ln(X_1/X_0)}{t_1 - t_0} \quad (3.2)$$

where X_1 and X_0 were the biomass concentration (g L⁻¹) on days t_1 and t_0 , respectively.

Biomass productivities were calculated from the variation in biomass concentration (g L⁻¹) within a cultivation time (d), according to Equation 3.3:

$$P = \frac{X_1 - X_0}{t_1 - t_0} \quad (3.3)$$

Specific growth rate values for different light intensities (I , $\mu\text{E m}^{-2} \text{s}^{-1}$) were then used to determine the mathematical Monod model (Fergola et al., 2007), expressed by Equation 3.4:

$$\mu = \frac{\mu_{max} \cdot I}{K_I + I} \quad (3.4)$$

where μ_{max} is the maximum specific growth rate and K_I is the half saturation constant. This model was fitted to the experimental data using a non-linear minimization function (NonLinearRegress) of the software package Mathematica (Wolfram Mathematica 8). These parameters were chosen to minimize the χ^2 function given by the sum of squared residuals $\sum_i e_i^2$.

3.2.6. Statistical analysis

The kinetic parameters were determined from two independent experiments and data was analyzed using Paired-Samples *t*-test from the statistical software SPSS 17.0 (Statistical Package for the Social Sciences). Significance level for the separation was set at $p < 0.05$.

3.3. Results and discussion

As with all plants, microalgae also require light to perform photosynthesis, converting inorganic carbon into biomass. The light energy is converted into chemical energy (stored in chemical bonds of produced compounds). The microalgal growth depends on light intensity, spectral quality and photoperiod. The sunlight is available in a discontinuous mode and its intensity varies during the day. Taking into account the application of microalgae cultures for CO₂ capture, the light effect on microalgal growth should be studied to evaluate the requirements of artificial light for their cultures. Additionally, nutrient profile and concentration also influence microalgal growth, thus becoming necessary studying the effect of different nutrient concentrations in CO₂ capture from microalgae.

Figure 3.3 and Figure 3.4 show the growth curves of *C. vulgaris* and *P. subcapitata*, respectively, for different light and culture medium conditions at room temperature and aerated with CO₂ at atmospheric concentration. For almost all cultures, it was observed the lack of an adaptation phase or a very short one. For example, for almost all cultures the exponential phase started before completing the first day of culture. However, for photoperiod of 10:14 and low light intensities, both microalgal species showed longer adaptation phases. Generally, almost all cultures presented an exponential growth phase with the same duration, reaching the stationary phase at the seventh day of culture. Similar behavior was observed by Jacob-Lopes et al. (2009), when the effect of light cycles on the cultures of the cyanobacterium *Aphanothece microscópica Nägeli* was analyzed. However, for cultures where nutrient concentration was doubled and light supply was higher (longer light periods and higher light intensities), stationary phase was not reached at all before the end of the experiment. This result indicates that nutrients can be a growth limiting factor in cultures where its concentration was lower. Two possible limiting nutrients were nitrogen and phosphorus. According to the study performed by Bhola et al. (2011), *C. vulgaris* reached its maximum concentration for nitrogen and phosphorus concentrations of 5 g L⁻¹ and 0.04 g L⁻¹, respectively. In this study, nitrogen and phosphorus were supplied at 0.03 g L⁻¹ and 0.0032 g L⁻¹, respectively. As these values are lower than those used in the study performed by Bhola et al. (2011), the possibility of nutrient limitation is very high, especially for the assays conducted under lower nutrient concentrations.

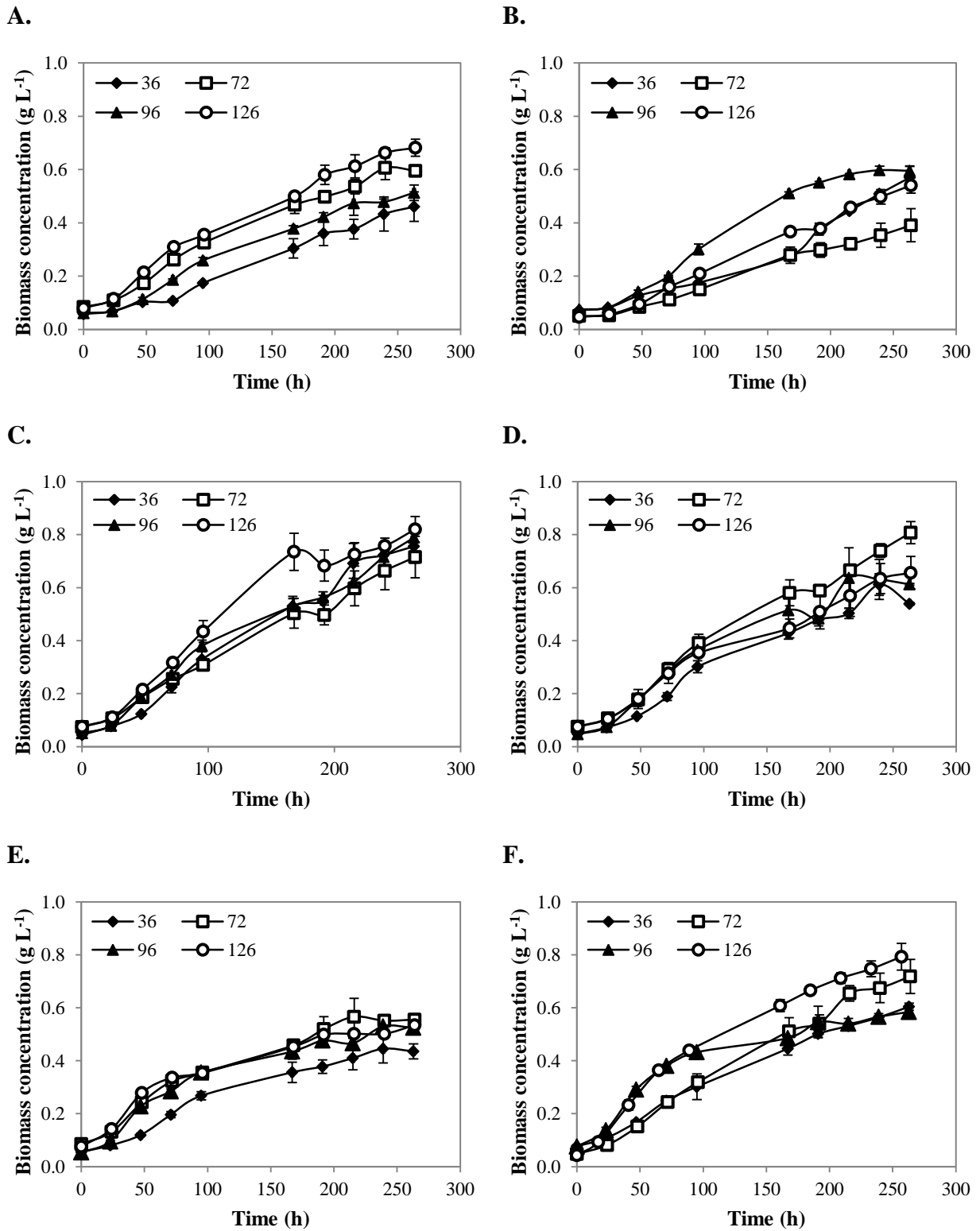


Figure 3.3. Growth curves of *C. vulgaris* grown in M₁ (A, C and E) and in M₂ (B, D and F) for different light conditions: light intensity (36, 72, 96 and 126 $\mu\text{E m}^{-2} \text{s}^{-1}$) and photoperiod (10:14 – A and B; 14:10 – C and D; 24:0 – E and F). Values are presented as the mean \pm s.d. of two independent experiments.

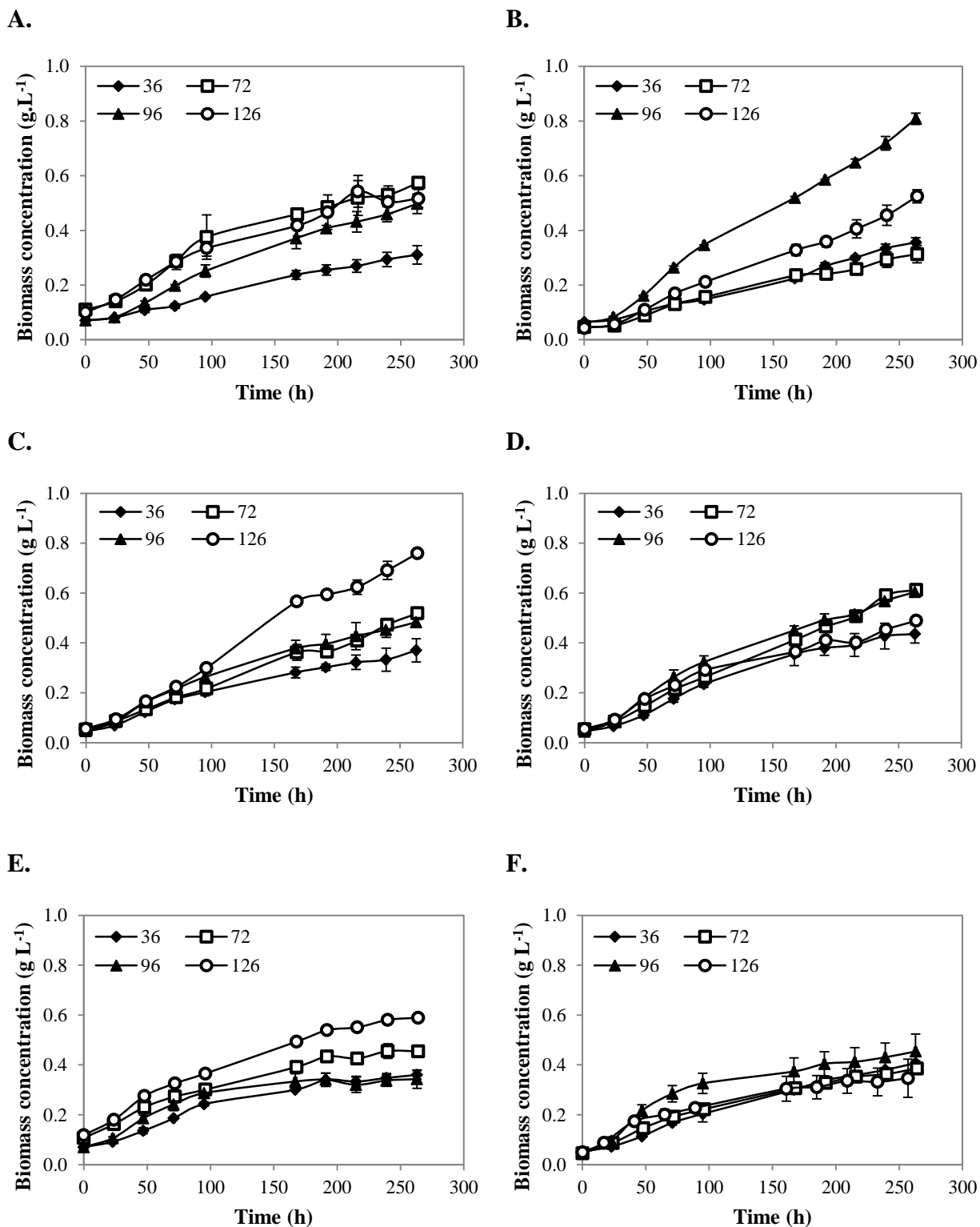


Figure 3.4. Growth curves of *P. subcapitata* grown in M₁ (A, C and E) and in M₂ (B, D and F) for different light conditions: light intensity (36, 72, 96 and 126 μE m⁻² s⁻¹) and photoperiod (10:14 – A and B; 14:10 – C and D; 24:0 – E and F). Values are presented as the mean ± s.d. of two independent experiments.

Table 3.1 and Table 3.2 show the main kinetic parameters (μ , X_{\max} , and P_{\max}) for cultures of *C. vulgaris* and *P. subcapitata*, respectively. Specific growth rates obtained with the different culture conditions range from 0.225 ± 0.044 to 0.977 ± 0.043 d⁻¹. Different studies have reported similar values. For example, Chiu et al. (2008) obtained a specific growth rate of 0.248 d⁻¹ for a *Chlorella* strain grown with CO₂ at 0.04% (v/v) and de Moraes & Costa (2007) reported a value of 0.25 d⁻¹, for *C. vulgaris* grown with the same amount of CO₂. Higher specific growth rates of 0.993 d⁻¹ were achieved by *C. pyrenoidosa* when grown with 5% (v/v) CO₂ (Tang et al., 2011). In general, specific growth rates increase with an increase in light intensity and in the light period. This value is also higher for cultures where nutrient concentration was doubled. From *t*-test ($p < 0.05$), the maximum value of specific growth rate achieved by *C. vulgaris* was 0.977 ± 0.043 d⁻¹. This value was obtained with constant supply of light energy (photoperiod of 24:0) with light intensity of $126 \mu\text{E m}^{-2} \text{s}^{-1}$ and doubled nutrient concentration, M₂. Maximum specific growth rate achieved by *P. subcapitata* was 0.754 ± 0.040 d⁻¹, for continuously illuminated cultures with light intensity of $96 \mu\text{E m}^{-2} \text{s}^{-1}$ and doubled nutrient concentration, M₂. This value was not statistically different ($p > 0.05$) from the one obtained with the same photoperiod (24:0) and light intensity $126 \mu\text{E m}^{-2} \text{s}^{-1}$. These results support the assumption that in cultures with lower nutrient concentrations there is a lack of nutrients that limits microalgal growth.

Regarding the maximum biomass concentration achieved in each culture, data present in Table 3.1 and Table 3.2 show that, in general, this value was higher for the medium composition M₂, for both microalgae. This result confirms the possibility of nutrient limiting concentrations in M₁. Maximum biomass concentrations achieved by both microalgae were obtained in a for photoperiod 14:10. These values were statistically higher ($p > 0.05$) than those obtained for photoperiod 24:0. The achievement of the highest value in a discontinuous light supply (already observed in Figure 3.3 and Figure 3.4 for both medium compositions) may be related with possible photooxidation (Chisti, 2008; Molina Grima et al., 2001). The oxygen generated by photosynthesis may accumulate in culture medium, reaching values that in combination with intense light can form free radicals that are responsible for the conversion of fatty acids into lipid peroxides, which can damage the cell membrane and can even lead to cell death. During the dark period, microalgae do not perform photosynthesis and the oxygen may be released from the culture by the constant aeration. On the other hand, the cells get energy by oxidizing the compounds produced during the light period. Consequently, the O₂ concentration in the culture decreases and the microalgae could repair the photo-induced damage (Merchuk et al., 1998).

Table 3.1. Kinetic parameters for cultures of *C. vulgaris* using different nutrient concentrations and light conditions

	M ₁				M ₂			
	Light intensity ($\mu\text{E m}^{-2} \text{s}^{-1}$)	10:14	14:10	24:0	10:14	14:10	24:0	24:0
μ (d^{-1})	36	0.291±0.072 ^{a,1}	0.526±0.029 ^{a,2}	0.393±0.002 ^{a,3}	0.287±0.091 ^{a,1}	0.439±0.011 ^{a,2}	0.399±0.029 ^{a,2}	
	72	0.359±0.057 ^{b,1}	0.464±0.054 ^{b,1}	0.523±0.052 ^{b,2}	0.259±0.019 ^{a,1}	0.429±0.049 ^{a,2}	0.577±0.025 ^{b,3}	
	96	0.313±0.017 ^{b,1}	0.659±0.112 ^{a,2}	0.738±0.077 ^{c,2}	0.325±0.126 ^{b,1}	0.669±0.107 ^{b,2}	0.686±0.036 ^{c,2}	
	126	0.494±0.077 ^{c,1}	0.525±0.081 ^{b,1}	0.650±0.014 ^{c,2}	0.352±0.088 ^{c,1}	0.437±0.059 ^{a,1}	0.977±0.043 ^{d,2}	
X_{max} (g L^{-1})	36	0.460±0.055 ^{a,1}	0.756±0.063 ^{a,2}	0.445±0.053 ^{a,1}	0.570±0.042 ^{a,1}	0.611±0.041 ^{a,1}	0.604±0.013 ^{a,1}	
	72	0.606±0.045 ^{b,1}	0.716±0.078 ^{a,1}	0.566±0.070 ^{b,2}	0.391±0.062 ^{b,1}	0.808±0.042 ^{b,2}	0.718±0.065 ^{b,2}	
	96	0.513±0.029 ^{a,1}	0.789±0.029 ^{a,2}	0.530±0.023 ^{b,1}	0.595±0.018 ^{a,1}	0.613±0.003 ^{a,1}	0.584±0.015 ^{a,1}	
	126	0.682±0.032 ^{c,1}	0.821±0.048 ^{a,2}	0.534±0.000 ^{b,3}	0.541±0.029 ^{a,1}	0.656±0.061 ^{a,2}	0.793±0.050 ^{c,3}	
P_{max} ($\text{g L}^{-1} \text{d}^{-1}$)	36	0.066±0.003 ^{a,1}	0.162±0.081 ^{a,1}	0.077±0.002 ^{a,1}	0.103±0.023 ^{a,1}	0.112±0.027 ^{a,1}	0.081±0.012 ^{a,1}	
	72	0.090±0.007 ^{a,1}	0.080±0.002 ^{a,1}	0.111±0.005 ^{a,2}	0.043±0.009 ^{b,1}	0.113±0.024 ^{a,2}	0.114±0.005 ^{a,2}	
	96	0.073±0.009 ^{a,1}	0.110±0.006 ^{a,1}	0.132±0.001 ^{b,1}	0.109±0.011 ^{a,1}	0.134±0.038 ^{a,1}	0.180±0.038 ^{a,1}	
	126	0.100±0.009 ^{a,1}	0.117±0.023 ^{a,1}	0.146±0.013 ^{b,1}	0.080±0.007 ^{a,1}	0.097±0.002 ^{a,1}	0.140±0.016 ^{a,1}	

Values are mean ± s.d. of two independent experiments; within the same column (and the same kinetic parameter), means having different superscript letters are significantly different ($p < 0.05$) by *t*-test; within the same row (and the same microalgal specie), means having different superscript numbers are significantly different ($p < 0.05$) by *t*-test.

Table 3.2. Kinetic parameters for cultures of *P. subcapitata* using different nutrient concentrations and light conditions

	M ₁				M ₂				
	10:14	14:10	24:0	10:14	14:10	24:0	10:14	14:10	24:0
Light Intensity ($\mu\text{E m}^{-2} \text{s}^{-1}$)									
36	0.225±0.044 ^{a,1}	0.516±0.036 ^{a,2}	0.335±0.031 ^{a,3}	0.240±0.063 ^{a,1}	0.456±0.033 ^{a,2}	0.399±0.029 ^{a,2}			
72	0.300±0.031 ^{b,1}	0.465±0.019 ^{a,2}	0.384±0.020 ^{b,3}	0.322±0.022 ^{b,1}	0.502±0.029 ^{a,2}	0.580±0.034 ^{b,3}			
96	0.325±0.096 ^{b,1}	0.635±0.016 ^{b,2}	0.496±0.037 ^{c,3}	0.487±0.032 ^{c,1}	0.708±0.019 ^{b,2}	0.754±0.040 ^{c,2}			
126	0.392±0.001 ^{b,1}	0.543±0.016 ^{a,2}	0.421±0.002 ^{b,3}	0.462±0.018 ^{c,1}	0.586±0.036 ^{b,2}	0.736±0.037 ^{c,3}			
36	0.311±0.034 ^{a,1}	0.370±0.047 ^{a,1}	0.360±0.017 ^{a,1}	0.356±0.017 ^{a,1}	0.436±0.036 ^{a,2}	0.407±0.009 ^{a,2}			
72	0.574±0.007 ^{b,1}	0.519±0.023 ^{b,2}	0.455±0.003 ^{b,3}	0.313±0.032 ^{b,1}	0.613±0.009 ^{b,2}	0.386±0.024 ^{b,3}			
96	0.497±0.036 ^{c,1}	0.483±0.017 ^{b,1}	0.343±0.037 ^{a,2}	0.808±0.021 ^{c,1}	0.604±0.017 ^{b,2}	0.454±0.070 ^{a,3}			
126	0.517±0.033 ^{c,1}	0.760±0.013 ^{c,2}	0.589±0.005 ^{c,3}	0.525±0.024 ^{c,1}	0.489±0.002 ^{a,1}	0.347±0.076 ^{c,2}			
36	0.034±0.001 ^{a,1}	0.054±0.005 ^{a,1}	0.057±0.005 ^{a,1}	0.045±0.008 ^{a,1}	0.066±0.004 ^{a,1}	0.055±0.008 ^{a,1}			
72	0.087±0.007 ^{a,1}	0.069±0.023 ^{a,1}	0.089±0.029 ^{a,1}	0.042±0.001 ^{a,1}	0.085±0.009 ^{a,1}	0.059±0.003 ^{a,1}			
96	0.063±0.007 ^{a,1}	0.078±0.007 ^{a,1}	0.079±0.015 ^{a,1}	0.142±0.054 ^{b,1}	0.093±0.009 ^{a,1}	0.121±0.025 ^{b,1}			
126	0.071±0.023 ^{a,1}	0.089±0.007 ^{a,1}	0.115±0.028 ^{a,1}	0.069±0.016 ^{b,1}	0.089±0.008 ^{a,1}	0.086±0.016 ^{b,1}			

Values are mean±s.d.. of two independent experiments; within the same column (and the same kinetic parameter), means having different superscript letters are significantly different ($p<0.05$) by *t*-test; within the same row (and the same microalgal specie), means having different superscript numbers are significantly different ($p<0.05$) by *t*-test.

Taking into account the maximum biomass productivity (that is proportional to CO₂ fixation rate) (Jacob-Lopes et al., 2009), the highest value obtained for *C. vulgaris* was $0.180 \pm 0.038 \text{ g L}^{-1} \text{ d}^{-1}$, for the photoperiod of 24:0, light intensity of $96 \mu\text{E m}^{-2} \text{ s}^{-1}$, and for the highest nutrient concentration, M₂. This value did not statistically differ from the values obtained with other photoperiods (maintaining the light intensity) and other light intensities (maintaining the photoperiod). On the other hand, maximum biomass productivity achieved by the microalga *P. subcapitata* was $0.142 \pm 0.054 \text{ g L}^{-1} \text{ d}^{-1}$, for the photoperiod of 10:14, light intensity of $96 \mu\text{E m}^{-2} \text{ s}^{-1}$, and for the highest nutrient concentration, M₂. This value did not statistically differ from the values obtained with other photoperiods (maintaining the light intensity). In conclusion, higher biomass productivities were obtained for cultures where nutrient supply was higher, which is in accordance with the higher specific growth rates achieved in these cultures and with the assumption that in M₁ nutrients were a growth limiting factor.

The influence of light intensity in microalgal growth was modeled by Monod function (Equation 3.4). Table 3.3 shows the model parameters for each microalga and for each experimental condition obtained by the non-linear minimization function (Wolfram Mathematica 8). For both microalgae and both nutrient concentrations, there is, in general, an increase in maximum specific growth rates with longer light periods. Values of half saturation constant are not always feasible because, in some cases, experimental data did not follow correctly the Monod kinetic model. These results can be explained by the use of only four

Table 3.3. Monod model parameters of microalgal growth: maximum specific growth rates, (μ_{max} , d⁻¹) and half saturation constants, (K_I , $\mu\text{g L}^{-1}$). Values are presented as the mean \pm s.d. of two independent experiments

	Photoperiod	M ₁		M ₂	
		μ_{max} (d ⁻¹)	K_I ($\mu\text{E m}^{-2} \text{ s}^{-1}$)	μ_{max} (d ⁻¹)	K_I ($\mu\text{E m}^{-2} \text{ s}^{-1}$)
<i>C. vulgaris</i>	10:14	0.568 \pm 0.224	40.9 \pm 25.9	0.358 \pm 0.062	11.9 \pm 13.7
	14:10	0.528 \pm 0.062	3.1 \pm 3.7	0.569 \pm 0.199	10.6 \pm 12.7
	24:0	1.011 \pm 0.318	55.8 \pm 34.9	3.443 \pm 2.177	340.9 \pm 306.1
<i>P. subcapitata</i>	10:14	0.535 \pm 0.070	53.6 \pm 17.9	0.857 \pm 0.367	96.7 \pm 79.7
	14:10	0.590 \pm 0.106	6.4 \pm 12.7	0.758 \pm 0.191	24.7 \pm 24.9
	24:0	0.529 \pm 0.099	20.8 \pm 17.4	1.202 \pm 0.252	70.9 \pm 33.0

experimental points. To obtain a closer fit to the Monod model, more light intensities, especially lower values, should be studied. The closeness of the fit can be observed in Figure A.4 and Figure A.5 by examining the theoretical curve super-imposed over the experimental points. For example, the model was not closely fitted for all the experiments performed with a 14:10 photoperiod. However, for the other experiments, it is possible to state that with respect to other models quoted in the literature (Edwards, 1970; Hansen & Hubbell, 1980), the model here considered give the lowest residual standard error (between 3.1×10^{-3} and 6.2×10^{-2}). The majority of half saturation constant values range between 3 and $70 \mu\text{E m}^{-2} \text{s}^{-1}$, indicating that the cultures could achieve half of their maximum specific growth rates with low light intensities.

Taking into account the achieved results, cultures of the studied microalgae present better growth rates with discontinuous light supply. Thus, artificial light may not be necessary for CO₂ capture by microalgae, which will reduce biomass production costs. However, higher nutrient concentrations should be applied, so that nutrient limitation could be avoided.

Results obtained with extracellular proteins and polysaccharides quantification have shown that both microalgae were not able to produce these compounds in the conditions studied and quantified by the selected colorimetric methods (data not shown).

3.4. Conclusions

The CO₂ sequestration by microalgae is a promising technology for application in countries with elevated solar light availability. The study of effect of photoperiod and light intensity is relevant to evaluate the need to use artificial light during night period. Both studied microalgae showed that the continuous light supply did not bring any significant improvement on their growth. In addition, taking into account the achieved biomass concentration at the end of 12 days of culture, the continuous light had a negative effect on microalgae (photooxidation). The maximum kinetic values were achieved by *Chlorella vulgaris*: i) specific growth rate of $0.977 \pm 0.043 \text{ d}^{-1}$; ii) maximum biomass concentration of $0.821 \pm 0.048 \text{ g L}^{-1}$; and iii) biomass productivity of $0.180 \pm 0.038 \text{ g L}^{-1} \text{ d}^{-1}$. The study of the effect of nutrient concentration also showed interesting results. Lower nutrient concentrations led to growth limitation.

CHAPTER 4

Effect of light supply on lipid production

4.1. Introduction

Apart from their applications in CO₂ mitigation, microalgae are also known for their high lipid contents (20-50% of dry weight) and high growth rates, being a promising energetic source (Chisti, 2007; Clarens et al., 2010; Lee et al., 2010; Mercer & Armenta, 2011). However, producing biodiesel from microalgal lipids is a costly process and still requires several optimizations to reduce production costs and to increase productivities, in order to achieve current demand for biofuels (Brennan & Owende, 2010). Different studies have reported that lipid contents differ according to the microalgal strain and their cultivation conditions (Demirbas, 2011; Liu et al., 2008; Shen et al., 2009b; Weldy & Huesemann, 2007). To analyze the lipid content of microalgae, the most applied method is organic solvent extraction followed by chemical characterization of lipid profiles using gravimetric analysis (Bligh & Dyer, 1959), thin-layer chromatography, high-performance liquid chromatography, or gas chromatography (Goutx et al., 1990; Volkman et al., 1989). However, this procedure is costly, time-consuming, and requires large amounts of toxic and harmful solvents and of biomass (Amaro et al., 2011; Brennan & Owende, 2010; Halim et al., 2011; Mercer & Armenta, 2011; Molina Grima et al., 2003). Additionally, extraction efficiencies of this method strongly depend on water content present in microalgal biomass, requiring previous drying (Amaro et al., 2011; Mercer & Armenta, 2011).

Colorimetric methods showed to be simple and rapid procedures for screening lipid productivities from microalgae. These methods require little amounts of biomass and reagents and allow the rapid determination of the lipid content on microalgae (Chen et al., 2009; Lee et al., 1998). Nile-red (a lipid-soluble fluorescent dye) has been frequently used in the determination of neutral lipid contents in animal cells (Genicot et al., 2005), bacteria (Izard & Limberger, 2003), and microalgae (Chen et al., 2009; Elsey et al., 2007; Lee et al., 1998). This method has received much attention due to the rapid and extraction-free determination of neutral lipids and also due to its sensitivity (Bertozzini et al., 2011; Chen et al., 2009). Although it is very sensitive, this method is not universal, as the applied concentrations of Nile-red should be defined for each organism. The establishment of a specific amount of Nile-red for each organism is required because this dye acts by penetrating the cells and binding to cytosolic neutral lipids,

which means that different affinities of cell membranes to uptake Nile-red will result in false determination of lipid contents (Chen et al., 2009; Chen & Vaidyanathan, 2012). Another methodology used to easily determine microalgal lipids is to quantify free fatty acids (FFA). Lipids can be accumulated in free or bound forms; in microalgae, they are mainly stored as bound fatty acids (FA). Thus, previous saponification (hydrolysis of FA) will allow the quantification of FFA, as they can react with copper or cobalt salts, forming colored complexes that can be detected by spectrophotometric methods (Ayers, 1956; Duncombe, 1963; Iwayama, 1959). The method applied in this study is based on the fact that FFA can react with copper or cobalt salts with the formation of colored complexes that can be extracted by chloroform in the presence of triethanolamine (TEA). The amount of copper in the chloroform phase is then colorimetrically determined by adding diethyldithiocarbamate to develop a yellow colored product that can be detected by measuring absorbance at 440 nm. This method was derived from the methods proposed by Wawrik & Harriman (2010) and Chen & Vaidyanathan (2012).

In this study, the effect of light on lipid productivity was assessed by applying the described method to cultures of *Chlorella vulgaris* and *Pseudokirchneriella subcapitata* grown under different light intensities (36, 72, 96, and 126 $\mu\text{E m}^{-2} \text{s}^{-1}$) and photoperiods (10:14, 14:10, and 24:0). Performing the experiments under several growth conditions and with different microalgal species can be important to infer about the sensitivity of colorimetric lipid quantification.

4.2. Materials and Methods

4.2.1. Microorganisms cultivation and harvesting

C. vulgaris and *P. subcapitata* (obtained from American Type Culture Collection – ATCC) were cultured in modified OECD test medium (OECD, 2011), with the following composition (per liter): 24 mg $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 36 mg $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 30 mg $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 3.2 mg KH_2PO_4 , 0.16 mg $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.2 mg $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$, 0.37 mg H_3BO_3 , 0.83 mg $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 6 μg ZnCl_2 , 3 μg $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.02 μg $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 14 μg $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, and 100 mg NaHCO_3 . Nitrogen was supplied in the form of NaNO_3 for *C. vulgaris*, and in the form of NH_4Cl for *P. subcapitata* (30 mg L^{-1}). Experiments were performed in 500 mL flasks operating in batch with a working volume of 450 mL. Cells were cultivated for 12 days using the growth medium described above. The experimental conditions were the following: initial cell concentration of 0.05-0.08 g L^{-1} , room temperature (22 ± 1 °C), and continuous aeration with the injection of atmospheric air (filtrated through a 0.22 μm cellulose acetate membranes, Orange Scientific, Belgium) in the bottom of the flasks. The assays were carried

out under different light intensities: 36, 72, 96, and 126 $\mu\text{E m}^{-2} \text{s}^{-1}$. For each light intensity, different photoperiods were evaluated: 10:14, 14:10, and 24:0 (light:dark). All the experiments were conducted in duplicates.

4.2.2. Standard curves

Sodium salts of each fatty acid (decanoic acid, lauric acid, myristic acid, sarkosyl acid, and palmitic acid) were purchased from Sigma-Aldrich, USA ($\geq 98\%$ purity). Stock solutions (5 mM) of each salt were prepared in a solvent mixture of chloroform and methanol (2:1), according to the method proposed by Chen & Vaidyanathan (2012). Serial dilutions were prepared and 300 μL of each dilution was used for lipid quantification, as described below.

4.2.3. Saponification of cellular lipids

For each performed experiment, two samples of 1.5 mL were collected in the first and last day of culturing. These algal suspensions were centrifuged at $16\,500 \times g$ for 10 min in an Eppendorf 5424 centrifuge (Eppendorf, Germany) and cell pellets were stored at $-20\text{ }^\circ\text{C}$ until lipid extraction. Cell pellets were thawed and resuspended in 20 μL of Tris HCl (1 M, pH 8.0). Then, 480 μL of saponification reagent (25% methanol in 1 M NaOH) and approximately 50 mg of 0.1 mm glass beads (Sigma-Aldrich, USA) were added. Cell disruption was promoted by vigorous vortexing for 3 min. Finally, another 500 μL of saponification reagent were added and the mixture was saponified at $90\text{ }^\circ\text{C}$ for 30 min and vortexed every 5 min to hydrolyze ester bonds of membrane lipids and triglycerides.

4.2.4. Lipid extraction and colorimetric detection

After saponification, samples were cooled down to room temperature and 600 μL of the saponified mixture was added to an eppendorf containing 900 μL of a solvent mixture of chloroform and methanol (2:1). After vortexing for 2 min, samples were centrifuged at $7\,000 \times g$ for 2 min in an Eppendorf 5424 centrifuge (Eppendorf, Germany). 500 μL of the organic phase was then transferred to a new eppendorf containing copper reagent (9 vol. aq. 1 M TEA, 1 vol. 1 M acetic acid, 10 vol. 6.45% w/v $\text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$). The procedure applied to standards was similar: to 300 μL of standard solutions, 300 μL of saponification reagent and 300 μL of a chloroform and methanol mixture (2:1) were added; after vortexing and centrifugation in the same conditions, 500 μL of the organic phase was added to a new eppendorf, containing the same volume of copper reagent. Samples and standards were vortexed and centrifuged again. After this step, 200 μL of the organic phase was carefully

transferred to a quartz cuvette, where 200 µL of color developing reagent (1% w/v sodium diethyldithiocarbamate in 2-butanol) was added. In this step, it is important that none of the aqueous phase is transferred, since it contains copper ions that will produce false signal during colorimetric detection. Absorbance at 440 nm was then measured using a V-1200 spectrophotometer (VWR, Germany). A solution containing 200 µL of a chloroform and methanol (2:1) mixture supplied with 200 µL of color developing reagent was used as blank.

4.2.5. Statistical analysis

Lipid quantification was performed in duplicate and data was analyzed using Paired-Samples *t*-test from the statistical software SPSS 17.0 (Statistical Package for the Social Sciences). Significance level for the separation was set at $p < 0.05$.

4.3. Results and discussion

The method described in this study enables the simple and rapid quantification of lipids from small amounts of microalgal biomass during cell growth experiments. Long-chain fatty acids used in this study showed linear responses for concentrations between 0 and 190.5 mg L⁻¹, as shown in Figure A.6. One-Sample *t*-test showed that the extinction coefficients, in L mg⁻¹ cm⁻¹, for all fatty acids was not significantly different ($p > 0.05$), indicating a similar behavior between fatty acids of different chain lengths. For this reason, lipid quantification was assessed by using an average of extinction coefficients for all fatty acids (Figure A.7): $y = (1.352 \times 10^{-2} \pm 1.496 \times 10^{-4})x$ ($R^2 = 0.998$), where y is absorbance measured at 440 nm and x is fatty acids concentration in mg L⁻¹.

Figure 4.1 shows the lipid yields for *C. vulgaris* (A) and *P. subcapitata* (B) determined for different light supplies. Lipid yields achieved range from 4.611±0.939 to 28.234±0.581 mg g⁻¹ CDW (cell dry weight) for *C. vulgaris* and from 8.562±1.204 to 39.287±1.879 mg g⁻¹ CDW for *P. subcapitata* (see Table 4.1). Similar values were reported by different authors (Lee et al., 2010; Ranjan et al., 2010). Ranjan et al. (2010) achieved lipid yields between 6.0 and 60 mg g⁻¹ CDW when applying organic solvent extraction to recover lipids from the microalga *Scenedesmus* sp. Using the same extraction procedure, Lee et al. (2010) achieved lipid yields of 49.0 mg g⁻¹ CDW for *C. vulgaris*. The effect of light intensity in lipid yields was similar for both microalgal strains. Lipid yields were significantly lower ($p < 0.05$) for the lowest light intensity (36 µE m⁻² s⁻¹). Concerning the other light intensities, lipid yields were not statistically different ($p > 0.05$). Similar results were obtained by Solovchenko et al. (2008), who showed that the

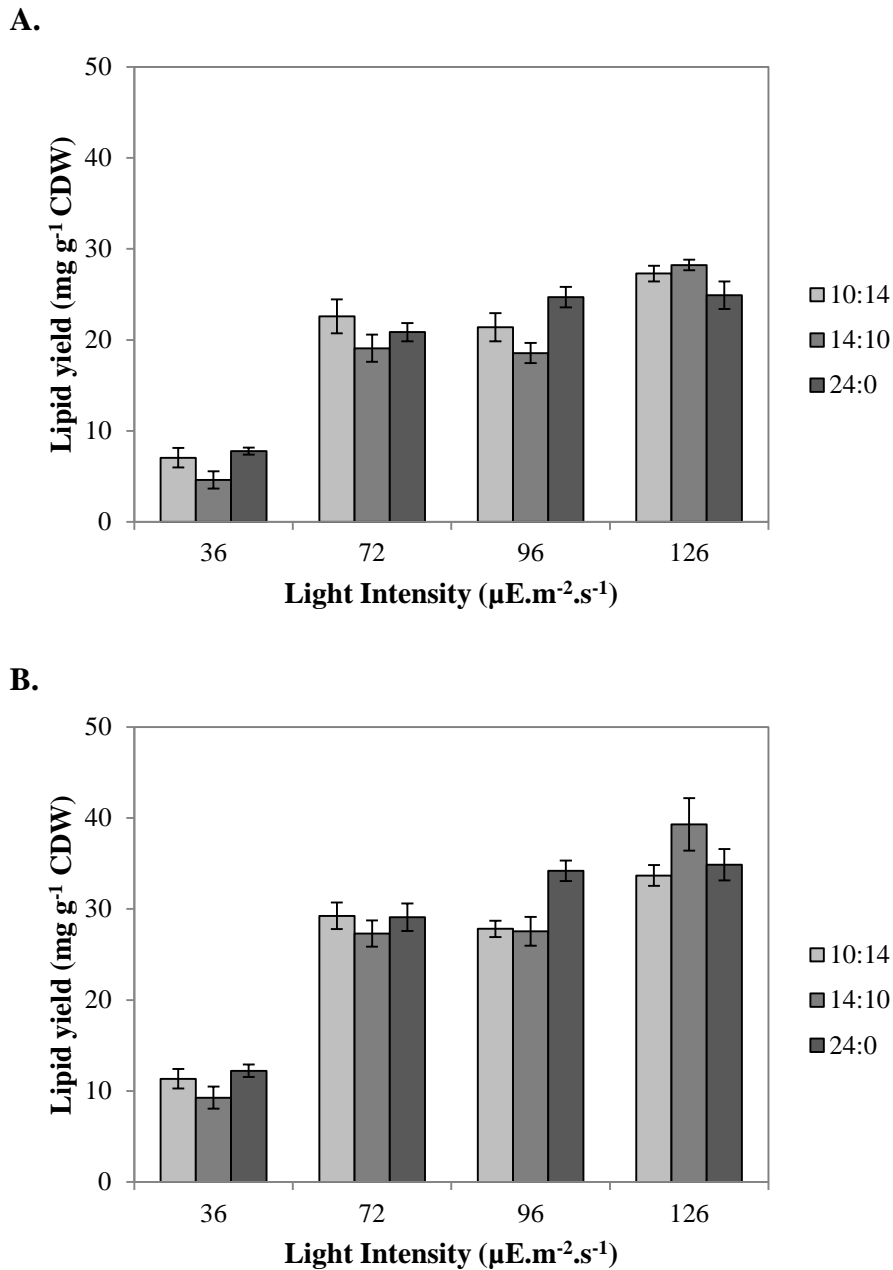


Figure 4.1. Lipid yields in mg g^{-1} CDW determined for *C. vulgaris* (A) and *P. subcapitata* (B) under different light intensities and different photoperiods. Values are presented as the mean \pm s.d. of two independent experiments.

accumulation of total fatty acids by the microalga *Parietochloris incisa* increased with light intensity (35, 200, and $400 \mu\text{E m}^{-2} \text{s}^{-1}$). Renaud et al. (1991) also demonstrated that lipid yields from *Isochrysis* sp. grown under light intensities ranging from 107 to $140 \mu\text{E m}^{-2} \text{s}^{-1}$ increased from 7.9 to $10.5 \text{ pg cell}^{-1}$. The same study has revealed that for higher light intensities, lipid yields did not significantly increase (Renaud et al., 1991). The study performed by Weldy & Huesemann (2007) also showed that lipid concentration in *Dunaliella salina* cells increased with higher light intensity. Different photoperiods did not influence lipid yields achieved for both

microalgae; however, for 96 $\mu\text{E m}^{-2} \text{s}^{-1}$, continuous light supply led to a significant increase in lipid yields. To our knowledge, this is the first study demonstrating the effects of different photoperiods in lipid yields from microalgae. Higher lipid yields observed for higher light intensities may be due to the storage of excessive light energy into chemical energy in the form of FA, thus avoiding photooxidative cell damage (Asada, 1994). Non-increasing lipid yields under higher light intensities, 96 and 126 $\mu\text{E m}^{-2} \text{s}^{-1}$, and for longer light periods is a consequence of the photooxidative mechanism: when exposed to high light intensities, chlorophyll molecules are excited forming a very unstable form, which, in turn, react with dissolved oxygen and transfers energy to it. The excited oxygen then reacts with fatty acids to form lipid peroxides, resulting in the reduction of FA concentrations (Carvalho et al., 2011). Another possible reason is that cells can store light energy into chemical energy in the form of other compounds, such as polysaccharides (Falkowski & Raven, 2007). Figure 4.1 also shows higher lipid yields for the microalga *P. subcapitata*. These results can be explained by the lower maximum biomass concentrations achieved by this microalga (see Table 4.1). Nutrient limitation, mainly nitrogen limitation, has resulted in decreased specific growth rates with higher

Table 4.1. Lipid yields in mg g^{-1} CDW and maximum biomass concentrations in g L^{-1} obtained for *C. vulgaris* and *P. subcapitata* obtained for different light intensities and different photoperiods. Values are presented as the mean \pm s.d. of two independent experiments

Photoperiod	Light Intensity ($\mu\text{E m}^{-2} \text{s}^{-1}$)	<i>C. vulgaris</i>		<i>P. subcapitata</i>	
		X_{max} (g L^{-1})	Lipid yield (mg g^{-1} CDW)	X_{max} (g L^{-1})	Lipid yield (mg g^{-1} CDW)
10:14	36	0.570 \pm 0.042	7.059 \pm 1.072	0.356 \pm 0.017	11.351 \pm 1.060
	72	0.391 \pm 0.062	22.579 \pm 1.869	0.313 \pm 0.032	29.252 \pm 1.458
	96	0.595 \pm 0.018	21.398 \pm 1.537	0.808 \pm 0.021	27.814 \pm 0.908
	126	0.541 \pm 0.029	27.301 \pm 0.862	0.525 \pm 0.024	33.683 \pm 1.130
14:10	36	0.611 \pm 0.041	4.611 \pm 0.939	0.436 \pm 0.06	9.272 \pm 1.204
	72	0.808 \pm 0.042	19.088 \pm 1.497	0.613 \pm 0.009	27.294 \pm 1.433
	96	0.613 \pm 0.003	18.552 \pm 1.105	0.604 \pm 0.017	27.553 \pm 1.580
	126	0.656 \pm 0.061	28.234 \pm 0.581	0.489 \pm 0.002	39.287 \pm 2.879
24:0	36	0.604 \pm 0.013	7.794 \pm 0.386	0.407 \pm 0.009	12.227 \pm 0.677
	72	0.718 \pm 0.065	20.859 \pm 1.007	0.386 \pm 0.024	29.092 \pm 1.526
	96	0.584 \pm 0.015	24.691 \pm 1.124	0.454 \pm 0.070	34.180 \pm 1.127
	126	0.793 \pm 0.050	24.922 \pm 1.516	0.347 \pm 0.076	34.870 \pm 1.734

FA accumulation. According to Lv et al. (2010), lipid accumulation during photosynthesis consists in the conversion of acetyl-CoA into malonyl-CoA and then into FA after repeated cycles. This reaction is catalyzed by acetyl-CoA carboxylase (ACCase) in the chloroplasts. FA biosynthesis is then regulated by this enzyme. Activity and concentration of ACCase strongly influence FA levels in microalgal cells. In nitrogen limiting conditions, a reduction in the cellular concentration of ACCase is observed, leading to a decrease in the enzyme activity and in lipid biosynthesis. However, in these conditions, there is a trend to lipid accumulation because cell division is almost ceased (Sukenik & Livne, 1991).

4.4. Conclusions

The use of colorimetric/spectrophotometric methods for rapid screening of microalgal lipids constitutes a promising and accurate technique to determine lipid productivities from microalgae. The effect of light intensity and photoperiod has shown that higher light supplies are responsible for higher lipid accumulation. However, results obtained for higher light intensities and longer light periods have suggested that photooxidation is responsible for the formation of lipid peroxides, decreasing FA contents. Another possible reason is the storage of excessive light energy into chemical energy in the form of other compounds, such as polysaccharides.

CHAPTER 5

Interactions between microalgae and cyanobacteria: kinetic modeling and surface interactions

5.1. Introduction

Along this work, two main applications of microalgae were studied: CO₂ capture and lipid synthesis with possible application in biofuel production. In this chapter, a study was performed to ascertain the growth kinetic benefits of microalgae-cyanobacteria interaction and to understand the physicochemical aspects involved in the inter-kingdom association. The association of different photosynthetic microorganisms is an emerging area because synergistic or competitive effects between these organisms can be exploited to: i) improve biomass production rates in adverse conditions; and ii) study the potential of these interactions to other biotechnological processes, such as bio-flocculation and biofilm formation for wastewater treatment. Furthermore, the study of interactions between different microalgal species or between microalgae and cyanobacteria is of great importance to understand their behavior in aquatic environments. Aquatic photoautotrophs often face severe competition for resources, either space, light or nutrients (Gross, 2003; Hulot et al., 2001). In these competitive environments, microorganisms tend to produce secondary metabolites, known as allelochemicals. The story of the biosynthesis and mode of action of these compounds, also identified as the chemical ecology of microalgae, has received much attention in the last few years, due to their importance in natural products chemistry and in several biotechnological processes, such as bio-remediation and wastewater treatment (Cembella, 2003; Fergola et al., 2007).

Allelopathy, the direct or indirect harmful effect of one species on another through the production of chemicals released to the environment, occurs essentially under stress situations, such as nutrient limitation. Target organisms might be more susceptible to allelochemicals under stress, and/or donor organisms might induce or increase the production of allelopathically active compounds in such conditions (Fergola et al., 2007; Gross, 2003). Phosphorus limitation, for example, has shown to affect growth of some microalgae and cyanobacteria. Polyphenolic compounds produced by some organisms interfere with alkaline phosphatase, an exoenzyme

used by several algae and cyanobacteria to overcome inorganic phosphorus (P_i) limitation (Gross, 2003).

The study of the interactions between different organisms also includes the study of how these organisms interact with each other and with different surfaces in terms of adhesion. The adhesion process is commonly studied for bacteria, especially to determine their ability to form biofilms. This is a very complex process, which depends on several factors, such as i) the physicochemical characteristics of microorganisms (hydrophobicity, surface charge); ii) the properties of surfaces' material (chemical composition, surface charge, hydrophobicity, roughness and texture); and iii) the environmental factors (temperature, pH, time of exposure, microorganisms concentration, chemical treatment or the presence of antimicrobials and fluid flow conditions) (An & Friedman, 1998). Therefore, determining the surface charge of microorganisms and physicochemical properties, such as surface tension, charge and hydrophobicity, allow a better understanding about the interactions between different microorganisms and between the microorganism and the environment.

In this study, the cyanobacterium *Synechocystis* sp. and the microalga *Pseudokirchneriella subcapitata* were grown in mixed cultures under P_i limiting concentrations, to observe: i) the predominant microorganism, the one with higher ability to uptake phosphorus at low concentrations; and ii) some evidence about the production of allelochemicals. To allow a better understanding about the interactions between both microorganisms, a common procedure applied for the determination of surface properties in bacteria was used. The classical Derjaguin-Landau-Verwey-Overbeek (DLVO) theory was applied, where the electrostatic and van der Waals forces have been combined. DLVO theory has been extended by the inclusion of acid-base interactions (XDLVO) which accounts for the hydrophobicity of the surfaces involved (Simões et al., 2010).

5.2. Materials and methods

5.2.1. Microorganisms and culture medium

The cyanobacterium *Synechocystis* sp. was obtained from CIIMAR (Centre of Marine and Environmental Research of the University of Porto) and the microalga *P. subcapitata* was obtained from American Type Culture Collection (ATCC). Stock solutions of the cyanobacterium and the microalga (Figure 5.1 A and B, respectively) were prepared in OECD test medium (OECD, 2011), with the following composition (per liter): 30 mg NaNO₃, 24 mg MgCl₂·6H₂O, 36 mg CaCl₂·2H₂O, 30 mg MgSO₄·7H₂O, 3.2 mg KH₂PO₄, 0.16 mg FeCl₃·6H₂O, 0.2 mg Na₂EDTA·2H₂O, 0.37 mg H₃BO₃, 0.83 mg MnCl₂·4H₂O, 6 µg ZnCl₂, 3 µg CoCl₂·6H₂O, 0.02 µg CuCl₂·2H₂O, 14 µg Na₂MoO₄·2H₂O, and 100 mg NaHCO₃.

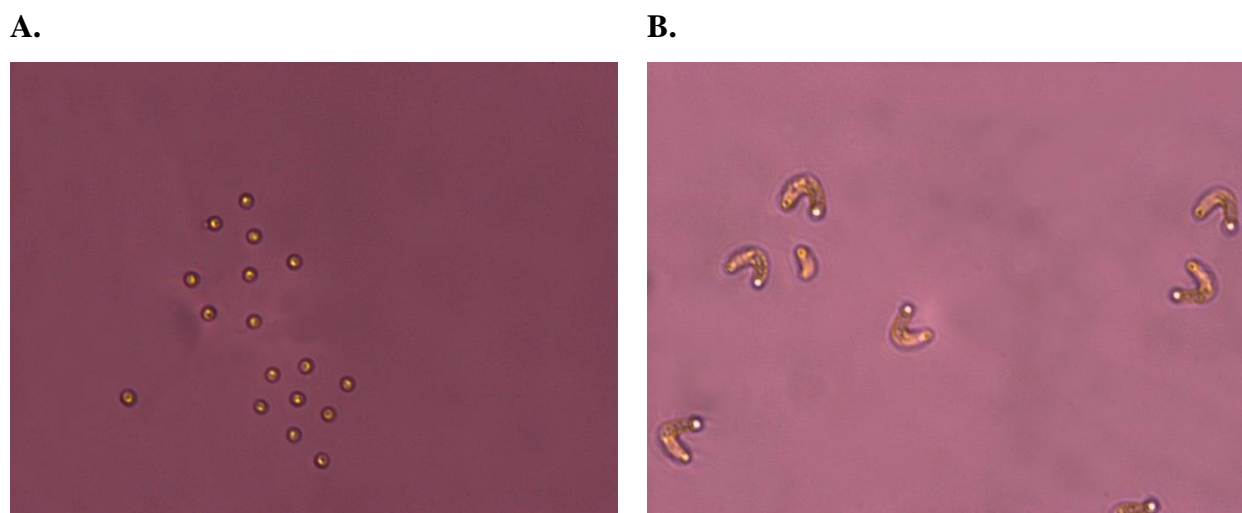


Figure 5.1. Microscopic photographs of the cyanobacterium *Synechocystis* sp. (A) and the microalga *P. subcapitata* (B) taken from a Leica DM LB2 microscope (Leica Microsystems, Germany) incorporated with a DFC300 FX camera and the acquisition software IM50. Photographs were obtained using a 100× oil immersion objective.

The cells were incubated in 500 mL flasks at room temperature, under continuous fluorescent light with an intensity of $72 \mu\text{E m}^{-2} \text{s}^{-1}$ at the surface of the flasks. Agitation was obtained by bubbling atmospheric air (filtrated through a $0.22 \mu\text{m}$ cellulose acetate membranes, Orange Scientific, Belgium) in the bottom of the flasks.

5.2.2. Growth under different P_i concentrations

Batch experiments, either with each single species or with mixed cultures of both species, were performed to study the effect of different P_i concentrations. Initially, both species were collected by centrifugation at $2\,900 \times g$ for 30 min, in an Eppendorf 5810 R centrifuge (Eppendorf, Germany), washed twice, and suspended in P_i -free growth medium. Culture medium described above with P_i concentrations ranging from 1.5 to $24 \mu\text{g L}^{-1}$ was then inoculated in order to obtain an initial cell concentration of approximately 1.0 to 2.0×10^6 cells mL^{-1} . Cells were cultivated for 12 days in 500 mL flasks (working volume of 450 mL), at room temperature (22 ± 1 °C), with continuous light intensity of $72 \mu\text{E m}^{-2} \text{s}^{-1}$, and continuous aeration with the injection of atmospheric air (filtrated through a $0.22 \mu\text{m}$ cellulose acetate membranes, Orange Scientific, Belgium) in the bottom of the flasks. All the experiments were performed in duplicates under aseptic conditions.

5.2.3. Analytical methods and kinetic parameters

Duplicate samples were collected at 24 h intervals and cellular concentration was determined using a Neubauer counting chamber (Marienfeld, Germany), under a Leica DM LB (Leica Microsystems, Germany) microscope.

Specific growth rates were determined using cell concentration values, according to Equation 3.2:

Specific growth rate values (μ , d⁻¹) for different phosphorus concentrations ([P], $\mu\text{g L}^{-1}$) were then used to determine the kinetic parameters μ_{max} (maximum specific growth rate) and K_p (half saturation constant), according to the mathematical Monod model (Fergola et al., 2007), expressed by Equation 5.1.

$$\mu = \frac{\mu_{max} \cdot [P]}{K_p + [P]} \quad (5.1)$$

This model was fitted to the experimental data using a non-linear minimization function (NonLinearRegress) of the software package Mathematica (Wolfram Mathematica 8). These parameters were chosen to minimize the χ^2 function given by the sum of squared residuals $\sum_i e_i^2$.

5.2.4. Quantification of extracellular proteins and polysaccharides

Extracellular proteins and polysaccharides were quantified in the first and last day of culturing using the methods described in Chapter 3, Section 3.2.4.

5.2.5. Zeta potential measurements

Samples of *Synechocystis* sp. and *P. subcapitata* grown with P_i concentration of 24 $\mu\text{g L}^{-1}$ were diluted in MilliQ water (Millipore) to obtain a final concentration of about 1.0×10^6 cells mL⁻¹. The zeta potential was measured using a ZetaSizer Nano ZS (Malvern Instruments, Worcestershire, UK). All the determinations were performed in a clear disposable zeta cell at approximately 25 °C and a light scattered at an angle of 17°. Mean values for each preparation were obtained by at least triplicate measurements of three samples of each microorganism.

5.2.6. Surface contact angles

The measurement of contact angles was performed using the sessile drop contact angle method. Lawns of each studied microorganism, grown with P_i concentration of $24 \mu\text{g L}^{-1}$, were prepared according to the method described by Busscher et al. (1984). The measurements were carried out at room temperature ($23 \pm 2 \text{ }^\circ\text{C}$) using three different liquids: water, formamide and α -bromonaphtalene (Sigma-Aldrich, Portugal). Determination of contact angles was performed automatically using a model OCA 15 Plus (DATAPHYSICS, Germany) video based optical contact angle measure instrument, allowing image acquisition and data analysis. Contact angle measurements (at least 25 determinations for each liquid and for each microorganism) were performed at three independent experiments for each microorganism tested.

5.2.7. Surface hydrophobicity

After contact angle measurements, hydrophobicity of cell surfaces was determined using the approach described by Van Oss et al. (1987; 1989; 1988). In this approach, the degree of hydrophobicity of a given surface, s , is expressed as the free energy of interaction between two entities of that surface when immersed in water, w : $\Delta G_{sws}^{\text{TOT}}$, in mJ m^{-2} . When $\Delta G_{sws}^{\text{TOT}} < 0$, the interaction between the two entities is stronger than the interaction of each entity with water and the material is considered hydrophobic. Alternatively, if $\Delta G_{sws}^{\text{TOT}} > 0$, the material is hydrophilic. $\Delta G_{sws}^{\text{TOT}}$ can be calculated through the surface tension components of the interacting entities, according to Equation 5.2:

$$\Delta G_{sws}^{\text{TOT}} = -2 \left(\sqrt{\gamma_s^{\text{LW}}} - \sqrt{\gamma_w^{\text{LW}}} \right)^2 + 4 \left(\sqrt{\gamma_s^+ \gamma_w^-} + \sqrt{\gamma_s^- \gamma_w^+} - \sqrt{\gamma_s^+ \gamma_s^-} - \sqrt{\gamma_w^+ \gamma_w^-} \right) \quad (5.2)$$

where γ^{LW} accounts for the Lifshitz-van der Waals component of the surface free energy and γ^+ and γ^- are the electron acceptor and electron donor parameters, respectively, of the Lewis acid-base component (γ^{AB}), being $\gamma^{\text{AB}} = 2\sqrt{\gamma^+ \gamma^-}$.

The surface tension components of a surface, s , were obtained by measuring the contact angles of three pure liquids, l : α -bromonaphtalene (apolar) and water and formamide (both polar), followed by the simultaneous resolution of three equations of the form of Equation 5.3. Surface tension of liquid components were obtained from literature (Janczuk et al., 1993).

$$(1 + \cos \theta) \gamma_1^{\text{TOT}} = 2 \left(\sqrt{\gamma_s^{\text{LW}} \gamma_1^{\text{LW}}} + \sqrt{\gamma_s^+ \gamma_1^-} + \sqrt{\gamma_s^- \gamma_1^+} \right) \quad (5.3)$$

where θ is the contact angle and $\gamma^{\text{TOT}} = \gamma^{\text{LW}} + \gamma^{\text{AB}}$.

5.2.8. Statistical analysis

The kinetic parameters (μ , m , and a) were determined from two independent experiments and data was analyzed using Paired-Samples t -test from the statistical software SPSS 17.0 (Statistical Package for the Social Sciences). Significance level for the separation was set at $p < 0.05$.

5.3. Results and discussion

The effect of P_i concentrations on specific growth rates for *Synechocystis* sp. and *P. subcapitata* in single and mixed cultures is represented in Table 5.1. Specific growth rates in all cultures showed an increase with increasing P_i concentrations. The decrease of specific growth rates with decreasing P_i concentrations has already been reported. For example, in the study performed by Litchman et al. (2003), the effect of two different concentrations of P_i (P-sufficient versus 1 μM) on the microalgae *Nitzschia* sp. and *Sphaerocystis schroeteri* and on the cyanobacterium *Phormidium luridum* has shown that specific growth rates for cultures grown in P-sufficient conditions were significantly higher than those obtained for P_i limiting conditions. The results presented in Table 5.1 show that specific growth rates of *Synechocystis* sp. were significantly lower ($p < 0.05$) than those obtained for *P. subcapitata* in both single and mixed cultures. In single cultures, specific growth rate values for the cyanobacterium range from

Table 5.1. Specific growth rates (d^{-1}) for different P_i concentrations ($\mu\text{g L}^{-1}$) for single and mixed cultures of *Synechocystis* sp. and *P. subcapitata*. Values are presented as the mean \pm s.d. of two independent experiments

[P] ($\mu\text{g L}^{-1}$)	Single cultures		Mixed cultures	
	<i>Synechocystis</i> sp.	<i>P. subcapitata</i>	<i>Synechocystis</i> sp.	<i>P. subcapitata</i>
24.0	0.381 \pm 0.047	0.676 \pm 0.096	0.191 \pm 0.034	0.645 \pm 0.086
12.0	0.214 \pm 0.052	0.689 \pm 0.031	0.114 \pm 0.007	0.582 \pm 0.106
6.0	0.182 \pm 0.059	0.477 \pm 0.007	0.096 \pm 0.014	0.542 \pm 0.092
3.0	0.153 \pm 0.028	0.197 \pm 0.028	0.080 \pm 0.004	0.263 \pm 0.037
1.5	0.071 \pm 0.017	0.244 \pm 0.046	0.056 \pm 0.026	0.103 \pm 0.020

0.071±0.017 to 0.381±0.047 d⁻¹, while the same values for the microalga range from 0.244±0.046 to 0.676±0.096 d⁻¹. Similar orders of magnitude were obtained for two different strains of the cyanobacterium *Trichodesmium* (0.05-0.26 d⁻¹) when growing under P_i concentrations of 0 to 20 µM (Fu et al., 2005). These results indicate that limiting P_i concentrations favor the growth of *P. subcapitata*. Furthermore, specific growth rate values of *Synechocystis* sp. grown in single cultures are statistically lower ($p < 0.05$) than specific growth rates obtained for the same microorganism grown in mixed cultures (0.056±0.026 to 0.191±0.034 d⁻¹). On the other hand, looking at specific growth rate values for *P. subcapitata*, no significant difference ($p > 0.05$) is observed between single and mixed cultures (0.103±0.020 to 0.645±0.086 d⁻¹).

The values presented in Table 5.1 were then used to perform mathematical modeling to evaluate the kinetic parameters associated with each culture studied. Different mathematical models are suggested in the literature (Edwards, 1970; Hansen & Hubbell, 1980). However, in this study, the Monod uptake function expressed by Equation 5.1 was applied. The kinetic models were determined based on experimental data and the results are shown in Figure 5.2. By examining the theoretical curves superimposed over the experimental data, it is possible to evaluate the closeness of the fit. Actually, comparing to other models cited in the literature (Edwards, 1970; Hansen & Hubbell, 1980), this model seems to correctly define the behavior of specific growth rates with different P_i concentrations for the studied cultures, presenting residual standard errors ranging from 4.0×10⁻⁴ to 7.0×10⁻³. Figure 5.2 shows that specific growth rates tend to achieve saturation, as P_i concentration increases. In fact, when the microalga *P. subcapitata* was grown with the same light supply, but with P_i concentration of approximately 0.32 mg L⁻¹, specific growth rate was not higher than the specific growth rate values achieved in this study for the maximum P_i concentration: 0.551±0.019 d⁻¹ (see Chapter 3, Table 3.2). Similar results were obtained for *P. subcapitata* in the study performed by Fergola et al. (2007) and for the cyanobacterium *Trichodesmium* in the study performed by Fu et al. (2005). Analysis of Figure 5.2 confirms that specific growth rates are lower for the cyanobacterium (A and C), especially in mixed cultures (C).

Kinetic parameters established through mathematical modeling are presented in Table 5.2. Values of maximum specific growth rates (μ_{max} , d⁻¹) and half saturation constants (K_P , µg L⁻¹) for both *Synechocystis* sp. and *P. subcapitata* in single and mixed cultures were determined by this model, while the affinity, A , of both microorganisms to grow under P_i limiting concentrations was calculated as the ratio between μ_{max} and K_P (Healey, 1973). Maximum

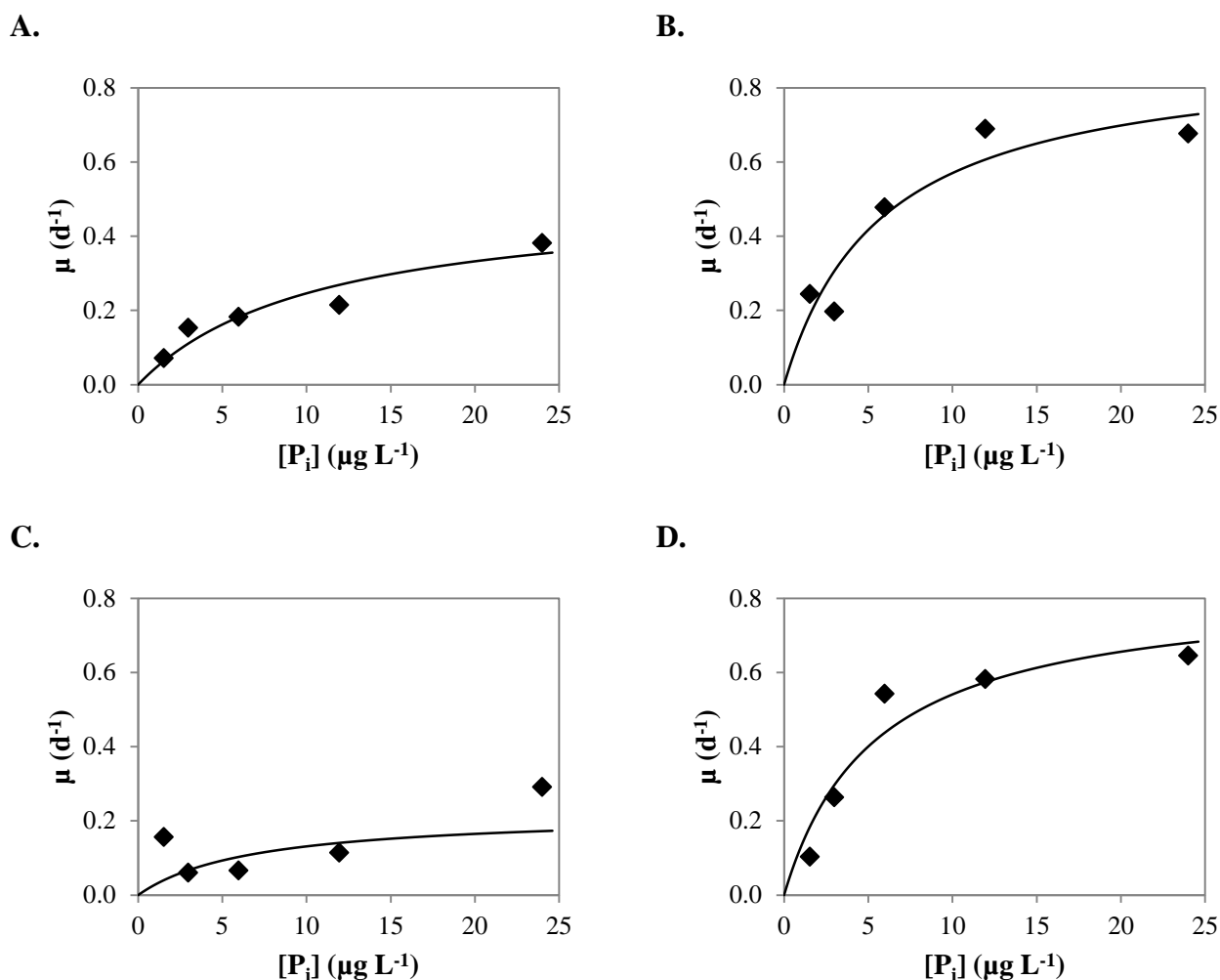


Figure 5.2. Model fit of Monod equation to the experimental data for *Synechocystis* sp. and *P. subcapitata* in single cultures (A and B) and in mixed cultures (C and D).

specific growth rates for *P. subcapitata* were very similar ($p > 0.05$) for both single and mixed cultures. The same parameter was significantly lower ($p < 0.05$) for *Synechocystis* sp. These results suggest that limiting P_i concentration can be a growth limiting factor to this microorganism. Additionally, μ_{max} value was lower ($p < 0.05$) for the cyanobacterium when growing in mixed cultures. Lower K_p values obtained for the microalga indicate that this organism is better adapted to take up phosphate even at low concentrations (Fu et al., 2005). This assumption can be confirmed by the higher affinities, A , to limited P_i concentrations showed by the microalga, approximately 5-fold higher (Fu et al., 2005; Healey, 1973). Owing to these results, the use of *P. subcapitata* in single cultures seems to be a promising alternative for wastewater treatment, as it has the ability to grow under such different P_i concentrations: these experiments have shown that this microalga has high affinity to this nutrient, which is a limiting

Table 5.2. Kinetic parameters determined by Monod model for both *Synechocystis* sp. and *P. subcapitata* in single and mixed cultures: maximum specific growth rates (μ_{\max} , d^{-1}), half saturation constants, (K_p , $\mu\text{g L}^{-1}$), and affinities (A , $\text{L } \mu\text{g}^{-1} \text{d}^{-1}$). Values are presented as the mean \pm s.d. of two independent experiments

	Single cultures		Mixed cultures	
	<i>Synechocystis</i> sp.	<i>P. subcapitata</i>	<i>Synechocystis</i> sp.	<i>P. subcapitata</i>
μ_{\max} (d^{-1})	0.512 \pm 0.135	0.902 \pm 0.169	0.223 \pm 0.048	0.833 \pm 0.144
K_p ($\mu\text{g L}^{-1}$)	10.8 \pm 2.2	3.8 \pm 1.9	6.9 \pm 2.7	3.4 \pm 1.6
A ($\text{L } \mu\text{g}^{-1} \text{d}^{-1}$)	0.047	0.236	0.032	0.246

component in several wastewaters, being apparently suitable to grow under these environments. Using wastewaters as growth medium for the production *P. subcapitata* in large quantities has different advantages: i) wastewater treatment; and ii) reduced costs with nutrient supply. Apparently, mixed cultures did not enhance microalgal growth; however, the existence of contaminations with this cyanobacterium will not be a problem, as there is no apparent indication of a negative impact, other than reduced growth ability, such as production of allelochemicals, of *Synechocystis* sp. over *P. subcapitata*: *P. subcapitata* growth was not inhibited by the presence of the cyanobacterium. In fact, analysis of extracellular proteins and polysaccharides has shown that the production of these extracellular compounds was negligible for both cyanobacterium and microalga in the conditions studied and with the colorimetric methods applied (data not shown). These results suggest that apparently there is no production of allelochemicals from each of the studied organisms.

Aspects involved in the interactions established between the two studied microorganisms were evaluated by the characterization of their physicochemical surface properties. Table 5.3 shows the surface properties of both *Synechocystis* sp. and *P. subcapitata* when growing with P_i concentration of $24 \mu\text{g L}^{-1}$. Zeta potential measurements give information about the charge of cell surfaces. *Synechosystis* sp. and *P. subcapitata* are negatively charged with similar values of zeta potential ($p > 0.05$). In fact, most microorganisms present a negatively charged surface, mainly due to the presence of anionic groups, such as carboxyl and phosphate, in their membranes/cell walls (Ferreira et al., 2011). Similar zeta potential values indicate that repulsive forces are higher than attractive ones and that both cyanobacterium and microalga are not expected to aggregate.

The hydrophobicity of microorganisms' surface is defined as the energy of attraction between apolar or slightly polar cells immersed in an aqueous phase. Although there are several

Table 5.3. Zeta potential (mV), contact angles (in degrees) with water (θ_w), formamide (θ_F), and α -bromonaphtalene (θ_B), surface tension parameters, and hydrophobicity (ΔG_{sws}^{TOT}) between two entities of the surface (s) when immersed in water (w). Values are presented as the mean \pm s.d. of three independent experiments

		<i>Synechocystis</i> sp.	<i>P. subcapitata</i>
Zeta potential (mV)		-23.9 \pm 1.8	-23.0 \pm 1.4
Contact angle ($^\circ$)	θ_w	93.36 \pm 5.58	35.70 \pm 3.73
	θ_F	36.69 \pm 2.56	33.72 \pm 2.35
	θ_B	67.78 \pm 12.21	44.69 \pm 2.43
Surface tension parameters (mJ m ⁻²)	γ^{LW}	36.04	37.24
	γ^+	0.01620	0.003843
	γ^-	1.234	54.18
Hydrophobicity (mJ m ⁻²)	ΔG_{sws}^{TOT}	-81.12	41.99

methods for the determination of surface tension parameters, contact angle measurement is the most common method applied to correctly determine the hydrophobicity of microorganisms' surface (Cerca et al., 2005). The surface hydrophobicity of *Synechocystis* sp. and *P. subcapitata* was determined using the approach of van Oss et al. (1995), which allows the assessment of the absolute degree of hydrophobicity of any surface in comparison with their interaction with water.

Table 5.3 shows that the cyanobacterium *Synechocystis* sp. has a hydrophobic surface, while the microalga *P. subcapitata* presents a hydrophilic surface. The hydrophobic and hydrophilic character of surfaces can be estimated from surface tension parameters: the higher the value of LW component, more apolar is the surface and, therefore, lower would be its affinity for polar liquids (Simões et al., 2007). The hydrophobic and hydrophilic character of the studied microorganisms cannot be determined by this parameter, since the Lifshitz van der Waals (γ^{LW}) value for both was not very different. Higher γ^+ value for *Synechocystis* sp. indicates that this cyanobacterium is an electron acceptor, while higher γ^- value for *P. subcapitata* indicates that this microalga is an electron donor (Simões et al., 2007). Surface hydrophobicity can also be determined by calculating the energy of hydrophobic attraction, ΔG_{sws}^{TOT} , through the expressions proposed by van Oss (1987; 1989; 1988). As referred before, negative values of ΔG_{sws}^{TOT} indicate

that surface molecules have less affinity for water than among themselves, being considered hydrophobic. On the other hand, positive $\Delta G_{\text{sws}}^{\text{TOT}}$ values indicate that interaction between the molecules of a surface in water is strongly repulsive and that the surface is hydrophilic (Simões et al., 2010). Therefore, surfaces of *Synechocystis* sp. and *P. subcapitata* are considered hydrophobic and hydrophilic, respectively. To this moment, there are no reports in the literature with the determination of surface properties of both microalgae and cyanobacteria. The hydrophobic character of the cyanobacterium indicates that this organism can have an application in bio-flocculation, as it tends to form aggregates and settle down to the bottom (Lee et al., 2009; Oh et al., 2001). Furthermore, the ecological interaction of this cyanobacterium with the microalga *P. subcapitata* has shown that the cyanobacterium did not cause any damage to the microalga and did not compete for the limiting nutrient, phosphorus.

5.4. Conclusions

The ecological effect of the cyanobacterium *Synechocystis* sp. and the microalga *P. subcapitata* was studied by culturing under a stress condition: P_i limitation. Growing both microorganisms in different P_i concentrations has showed that the microalga *P. subcapitata* has higher affinity to uptake phosphorus even at low concentrations. At the same time, there is no evidence of the production of secondary metabolites with high biological activity from these organisms. These results suggest that the microalga *P. subcapitata* constitutes a promising microorganism for application in wastewater treatment due to its ability to grow under diverse P_i concentrations.

The study of surface characteristics of both cyanobacterium and microalga revealed that the two organisms have similar surface charges. However, cyanobacterium surface had a hydrophobic character, while the microalga surface was considered hydrophilic. In terms of biotechnological applications, it seems unlikely that the association of *P. subcapitata* and *Synechocystis* sp. is not relevant, either in the production of biomolecules, biofilm formation or harvesting techniques (bio-flocculation).

CHAPTER 6

Concluding remarks and research needs

6.1. General conclusions

In this study different technological aspects of microalgae were assessed: i) CO₂ capture (Chapter 3); ii) lipid production with possible applications in biofuel production (Chapter 4); and iii) the kinetic benefits of a microalga-cyanobacterium association and the role of inter-kingdom interactions in biotechnological applications (Chapter 5).

The effect of different light intensities and photoperiods on microalgal CO₂ uptake is very important to evaluate if it is necessary to use artificial light during night period. For both microalgae studied, continuous light supply did not bring any significant improvement on their growth. Additionally, it was observed that in the last days of culturing, cultures under continuous light were photoinhibited. The study of the effect of nutrient concentration on microalgal growth has shown that lower nutrient concentrations led to growth limitation.

Lipid production from microalgae is also influenced by light intensity. Higher light supplies are responsible for higher lipid accumulation. However, results obtained for higher light intensities and longer light periods have suggested that photooxidation is responsible for a decrease in FA contents. Another possible reason is the storage of excessive light energy into chemical energy in the form of other compounds, such as polysaccharides.

Growing the cyanobacterium *Synechocystis* sp. and the microalga *P. subcapitata* in mixed cultures has showed that the microalga has higher affinity to uptake phosphorus even at low concentrations. These results suggest that the microalga *P. subcapitata* constitutes a promising microorganism for application in wastewater treatment. Apparently, the production of allelochemicals from these organisms in the studied conditions is negligible. The study of surface characteristics of both cyanobacterium and microalga revealed that cyanobacterium surface had a hydrophobic character, while the microalga surface was considered hydrophilic. These results were not very conclusive and further research in this area is required because it seems that ecological interactions between these organisms can play an important role either in the production of biomolecules, biofilm formation or harvesting techniques (bio-flocculation).

6.2. Research needs

Although different growth conditions were assessed in this study, more experiments using different operational parameters are required in order to obtain a cost effective method of CO₂ uptake and lipid production.

Additional photoperiods should be studied, as this parameter showed to be fundamental for microalgal growth and consequently an important criterion for the design of photobioreactors for CO₂ sequestration. Furthermore, different studies should be performed using other nutrient concentrations, to determine the one that maximizes microalgal growth and consequent CO₂ capture. Air streams with different CO₂ concentrations should be also studied, to verify if microalgae are able to uptake CO₂ at higher levels and to evaluate their potential in removing CO₂ from flue gas emissions.

For higher lipid productivities, nitrogen should be removed after the achievement of higher biomass concentration, as it is reported in the literature that nitrogen limitation induces the biosynthesis of lipids. Monitoring of other components, such as polysaccharides, should be also performed because microalgae store the excessive light energy into chemical energy in the form of lipids and polysaccharides. Studying the production of these compounds allow the determination of the fraction of light energy that is used from microalgae to produce lipids.

The last study included in this work lacks a lot of other experiments that were not possible to perform due to time constraints and material resources. This is an emerging area and only a few studies have reported the use of a mixed culture or a consortium for CO₂ capture, wastewater treatment, and other applications associated with microalgae. Therefore, the interaction of different microorganisms, other than *Synechocystis* sp. and *P. subcapitata*, should be studied, to determine a synergic system capable of producing higher amounts of biomass, capture CO₂ and produce high-valued compounds at higher rates. The studies involving the interaction between different microorganisms should be complemented with the monitoring of allelochemicals synthesis by these organisms. Finally, surface properties of different microalgae and cyanobacteria are also an important issue because information in this field allows the comprehension of interactions between different species and between organisms and surfaces, which can be very useful in biotechnological processes, such as bio-flocculation and biofilm formation (with potential application in wastewater treatment).

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Annexes

A.1. Calibration curves optical density *versus* biomass concentration

Calibration curves of OD₆₈₃ *versus* biomass concentration for *C. vulgaris* and *P. subcapitata* are represented in Figure A.1.

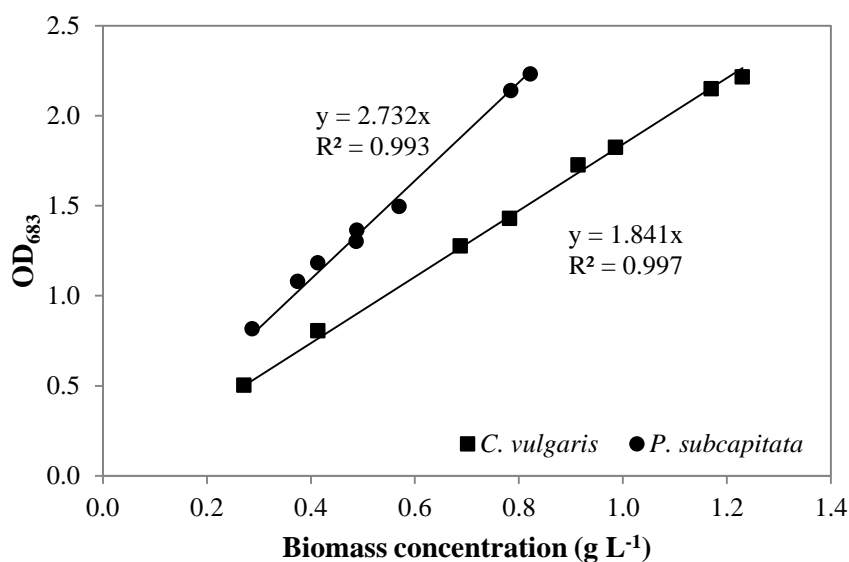


Figure A.1. Calibration curve of OD₆₈₃ as a function of biomass concentration, in g L⁻¹, for *C. vulgaris* and *P. subcapitata*.

A.2. Bradford reagent

A.2.1. Composition

Composition of the Bradford reagent used for protein quantification can be consulted in Table A.1.

Table A.1. Composition of the Bradford reagent (Bradford, 1976)

Reagents	Concentration (g L ⁻¹)
Coomassie Brilliant Blue G-250	0.1
Ethanol	47.5
Phosphoric acid	85.0

A.2.2. Preparation

Bradford reagent was prepared according to the method described by Bradford (1976). Coomassie Brilliant Blue G-250 (100 mg) purchased from Sigma-Aldrich, UK, was dissolved in 50 mL 95% ethanol. To this solution, 100 mL of 85% (w/v) phosphoric acid from were added. The resulting solution was diluted in distilled water to a final volume of 1 L.

A.3. Calibration curve for protein quantification

Calibration curve of absorbance at 595 nm *versus* BSA concentration in g L⁻¹ is represented in Figure A.2.

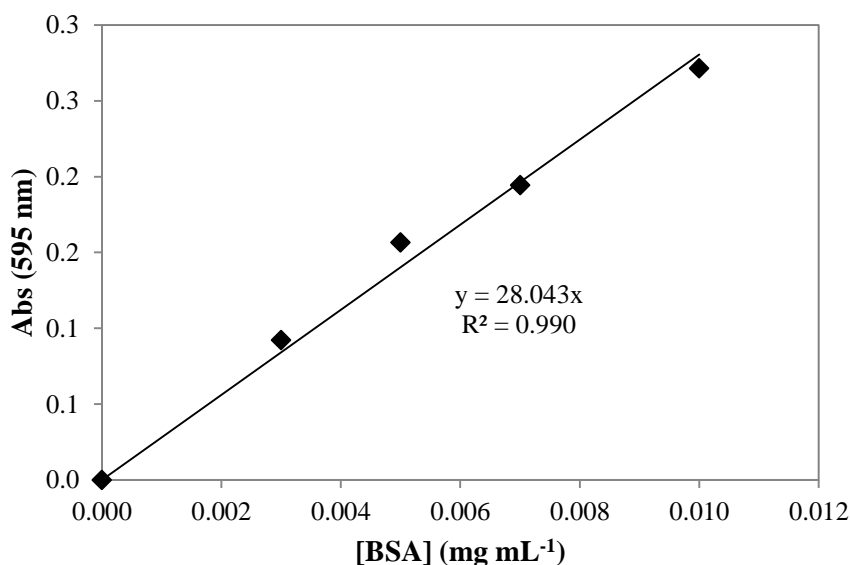


Figure A.2. Calibration curve of absorbance at 595 nm as a function of BSA concentration in g L⁻¹.

A.4. DNS reagent

A.4.1. Composition

Composition of the Bradford reagent used for protein quantification can be consulted in Table A.2.

Table A.2. Composition of the DNS reagent (Miller, 1959)

Reagents	Concentration (g L ⁻¹)
Dinitrosalicylic acid	10.0
Phenol	2.0
Sodium sulfite	0.5
Sodium hydroxide	10.0

A.5. Calibration curve for polysaccharides quantification

Calibration curve of absorbance at 540 nm *versus* glucose concentration in g L⁻¹ is represented in Figure A.3.

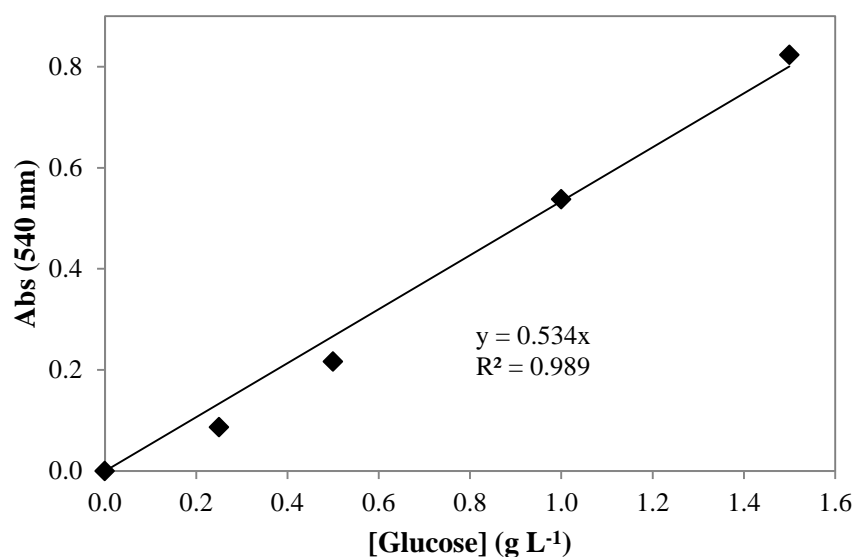


Figure A.3. Calibration curve of absorbance at 540 nm as a function of glucose concentration in g L⁻¹.

A.6. The effect of light intensity on microalgal growth through Monod model

Figure A.4 and Figure A.5 show how the Monod equation fits the experimental data (specific growth rates, μ , obtained for different light intensities studied) for both microalgae under different nutrient concentrations and with different photoperiods.

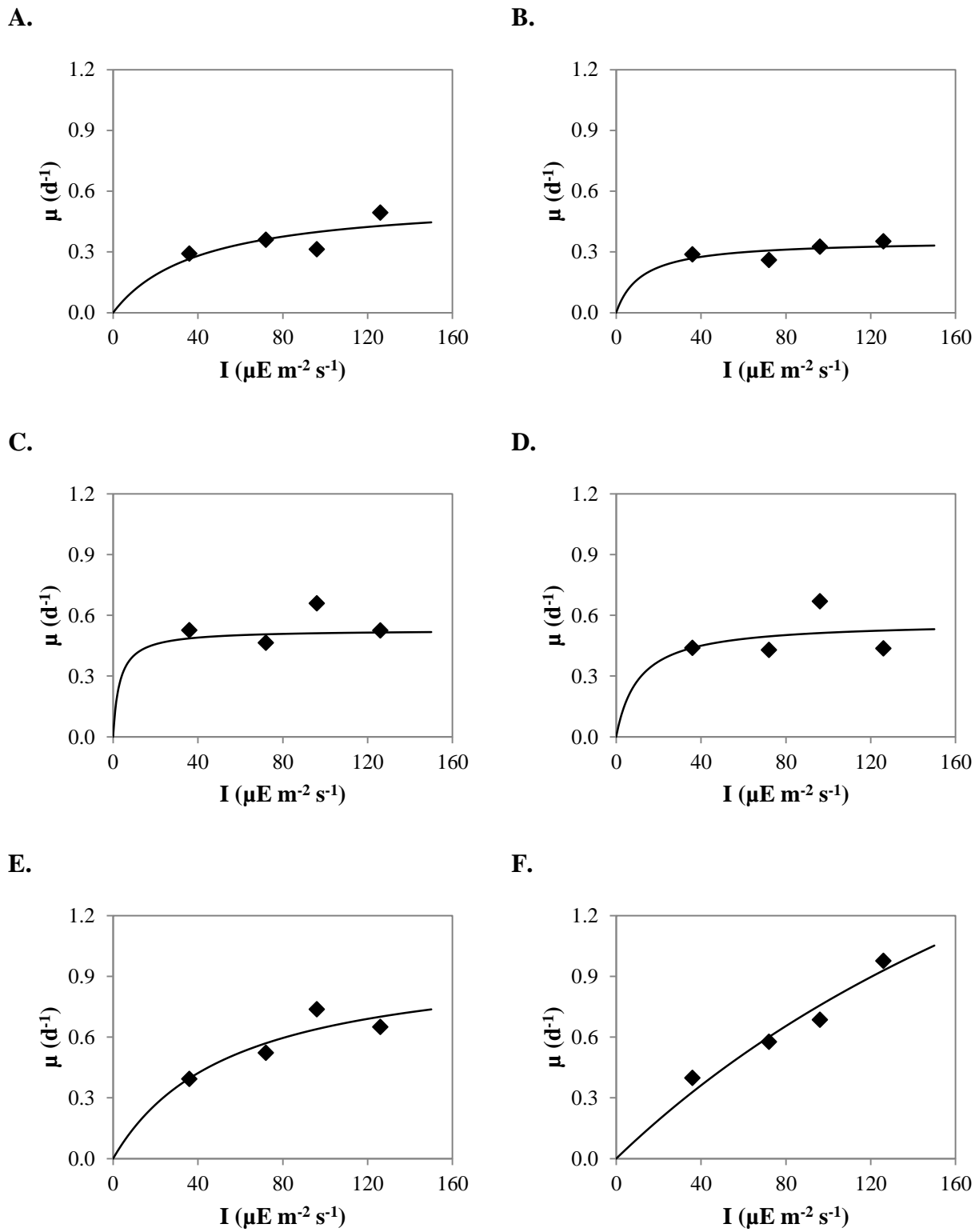


Figure A.4. Model fit of Monod equation to the experimental data for *C. vulgaris* grown in M₁ (A, C and E) and in M₂ (B, D and F) for different photoperiod (10:14 – A and B; 14:10 – C and D; 24:0 – E and F).

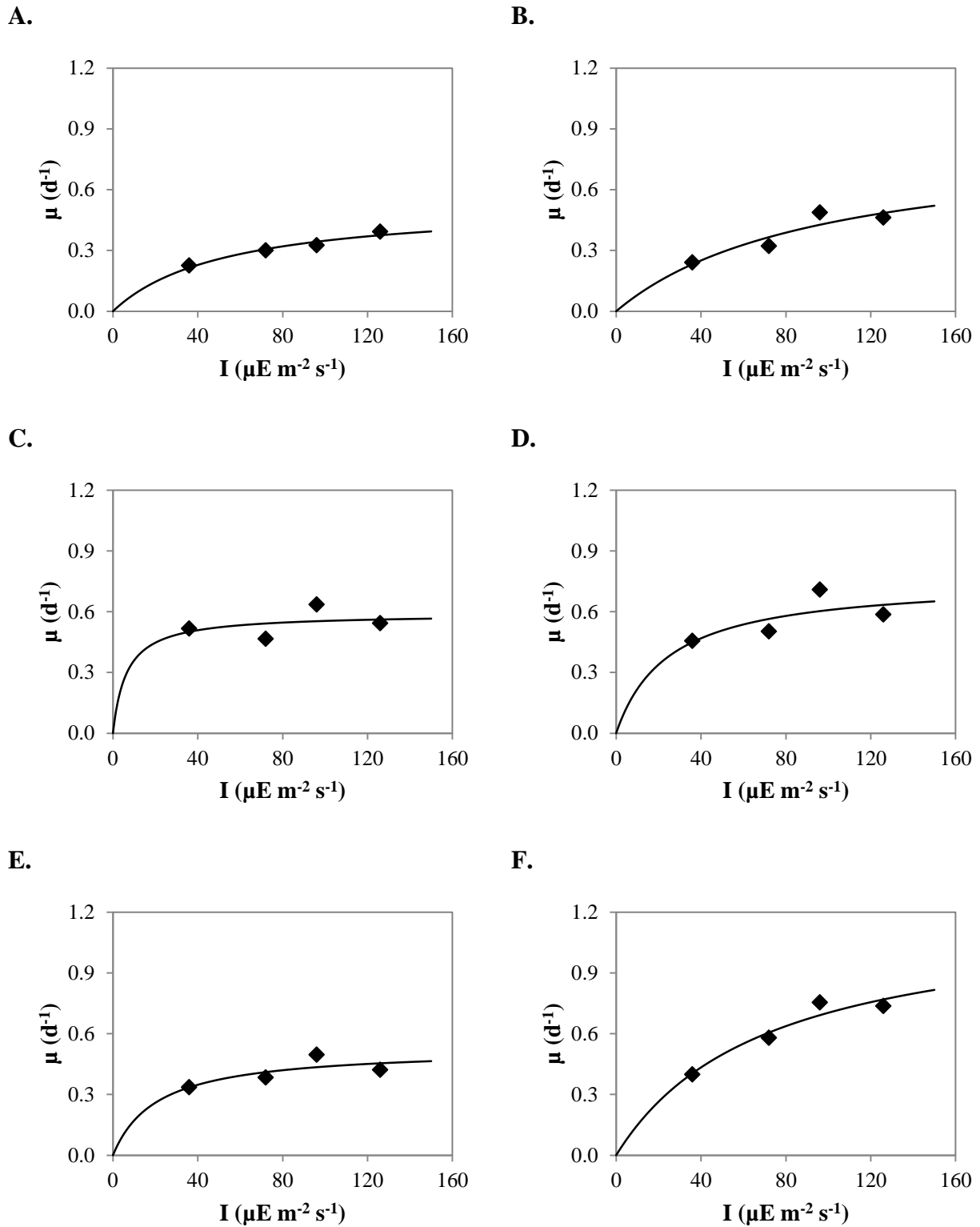


Figure A.5. Model fit of Monod equation to the experimental data for *P. subcapitata* grown in M₁ (A, C and E) and in M₂ (B, D and F) for different photoperiod (10:14 – A and B; 14:10 – C and D; 24:0 – E and F).

A.7. Calibration curves for lipid quantification

Figure A.6 shows the calibration curves of absorbance at 440 nm *versus* fatty acid concentration in mg L⁻¹ for each long-chain fatty acid used in this study.

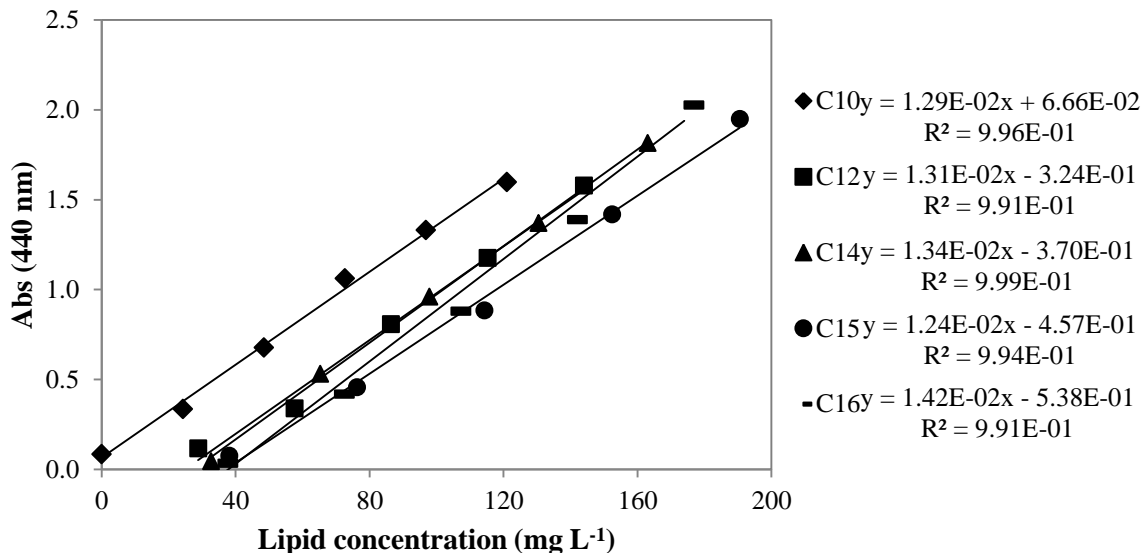


Figure A.6. Calibration curve of absorbance at 440 nm as a function of fatty acids concentration in mg L⁻¹ for different long-chain fatty acids.

An average of different extinction coefficients obtained for each long-chain fatty acid resulted in the calibration curve presented in Figure A.7.

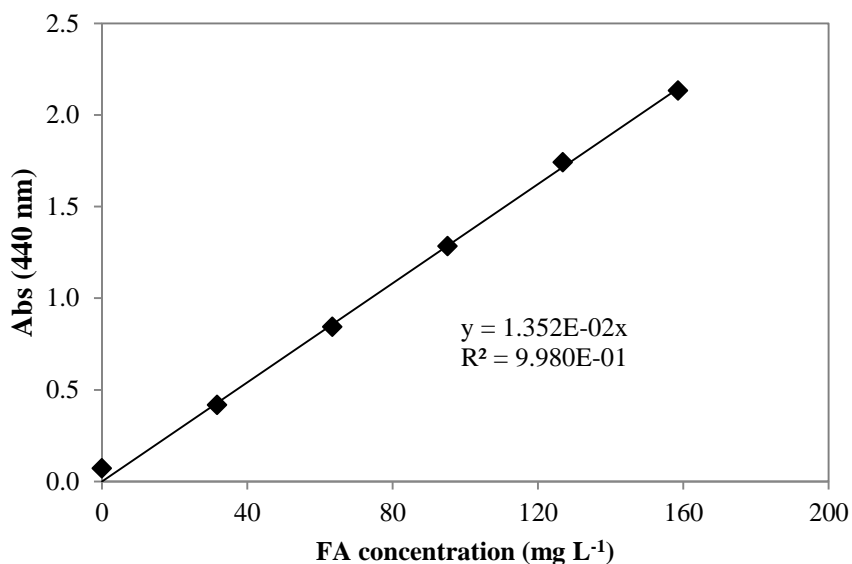


Figure A.7. Calibration curve of absorbance at 440 nm as a function of fatty acids concentration in mg L⁻¹ using an average of extinction coefficients obtained for each long-chain fatty acid.