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CULTIVATION OF MICROALGAE IN OSCILLATORY-FLOW REACTORS: CO_2 MASS TRANSFER OPTIMIZATION

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Abstract

The increase in anthropogenic carbon dioxide (CO_2) emissions to the atmosphere is provoking an environmental crisis at global level, being this compound the main responsible for the greenhouse effect and consequent global warming. There is a constant search for effective systems to capture CO_2 and microalgal culturing appeared as an effective way to perform this task. Apart from the application of microalgae to capture CO_2, these microorganisms are also able to produce diverse compounds, making them a valuable resource for a wide range of applications.

Although microalgae have great potential in CO_2 capture, some improvements are still required to optimise microalgal biomass production, being the optimization of CO_2 mass transfer one important factor to consider, to increase the residence time of CO_2 in the culture medium and thus improve the efficiency of CO_2 utilisation by microalgae.

In this study, the growth of *Chlorella vulgaris* in an oscillatory flow reactor with smooth periodic constrictions was evaluated. In addition, the effect of several parameters (CO_2 concentration, oscillation amplitude and oscillation frequency) in CO_2 mass transfer was tested, to optimise CO_2 mass transfer and thus obtain higher biomass productivities in this reactor.

Results from mass transfer experiments showed that increasing the oscillation amplitude and frequency led to a decrease in bubble size in the reactor and hence an increase of CO_2 mass transfer coefficient values. Additionally, increasing CO_2 concentration also led to an increase in CO_2 mass transfer.

Regarding microalgal biomass production experiments, increasing the amplitude resulted in an increase in biomass growth, until it reached an optimum value, from which biomass growth started to decrease, because intense agitation (promoted by higher amplitudes) provoked flocculation of microalgal cells. An increase in CO_2 concentration resulted in flocculation at lower amplitudes, but it also means that, in these conditions (higher CO_2 concentrations), it is possible to achieve higher biomass concentrations at lower amplitudes (just before the amplitudes that provoke flocculation). The highest biomass productivities were obtained in the experiments performed with 1% (v/v) of CO_2, 19 mm of amplitude and 1.5 Hz of frequency. In these conditions, final biomass concentration was four times higher than the initial concentration, specific growth rate achieved was 0.369 d^{-1} and average biomass productivity was 145 mg $DW \cdot L^{-1} \cdot d^{-1}$, having a growth slightly higher than the one with atmospheric CO_2.

Keywords: *Chlorella vulgaris*; CO_2 capture; Mass transfer; Microalgae; Oscillatory flow reactor

Resumo

O aumento das emissões de dióxido de carbono (CO_2) para a atmosfera por meios humanos está a provocar uma crise ambiental a nível global, visto que este gás é o maior responsável pelo efeito de estufa e, consequentemente, pelo aquecimento global. Existe uma constante procura por meios eficazes em capturar CO_2 da atmosfera, e o uso de microalgas para este efeito surgiu como uma alternativa eficaz para reduzir a sua concentração. Para além do uso de microalgas para captura de CO_2, estes organismos também são capazes de produzir diversos compostos de interesse, que podem ser usados em várias áreas, fazendo com que estes organismos possam ser utilizados como matéria-prima para uma grande variedade de aplicações.

Embora as microalgas possuam um grande potencial para captura de CO_2, é necessário fazer melhorias para otimizar a produção de biomassa, sendo a otimização da transferência de massa de CO_2 um importante fator a considerar, para aumentar o tempo de residência de CO_2 na cultura e assim melhorar a eficiência de utilização de CO_2 pelas microalgas.

Neste estudo, foi avaliado o crescimento da microalga *Chlorella vulgaris* num reator de fluxo oscilatório com constrições periódicas suaves. Para além disso, foi testado o efeito de vários parâmetros (concentração de CO_2, amplitude e frequência de oscilação) na transferência de massa de CO_2, de forma a otimizar a transferência de massa e, consequentemente, obter maiores produtividades de biomassa neste reator.

Os resultados relativos ao estudo da transferência de massa mostraram que um aumento da amplitude ou frequência leva a um decréscimo no tamanho das bolhas no reator e um aumento na transferência de massa de CO_2. Adicionalmente, um aumento na concentração de CO_2 também leva a um aumento na transferência de massa de CO_2.

Relativamente aos ensaios de produção de biomassa, verificou-se que o aumento da amplitude provoca um aumento do crescimento de biomassa até atingir um valor ótimo, a partir do qual o crescimento da biomassa começa a decrescer, porque a agitação intensa leva à formação de agregados de células. Um aumento da concentração de CO_2 leva à formação de flocos, tornando possível o crescimento de biomassa a menores amplitudes. Os melhores resultados foram conseguidos nos ensaios realizados com 1% (v/v) de CO_2, 19 mm de amplitude e 1,5 Hz de frequência, onde houve um aumento de concentração da biomassa de quatro vezes o valor inicial, tendo-se obtido uma taxa específica de crescimento de 0,369 d^{-1} e uma produtividade de 145 $mg \cdot L^{-1} \cdot d^{-1}$ (peso seco), valor ligeiramente mais alto do que o obtido com CO_2 atmosférico.

Palavras-Chave: Captura de CO_2; *Chlorella vulgaris*; Microalgas; Reator de fluxo oscilatório; Transferência de massa.

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Nomenclature

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphate synthase
CCAP	Culture Collection of Algae and Protozoa
DW	Dry weight
HPLC	High performance liquid chromatography
$NADPH_2$	Nicotinamide adenine dinucleotide phosphate
OD	Optical density
OD_{680}	Optical density at 680 nm
OECD	Organisation for Economic Cooperation and Development
OFR	Oscillatory flow reactor
PBR	Photobioreactor
Pi	Inorganic phosphate
PS	Photosystem
RNA	Ribonucleic acid
rpm	Rotations per minute
$SC\text{-}CO_2$	Super critical carbon dioxide
SPC	Smooth periodic constrictions
vvm	Volume of gas per working volume per minute

D	Inner diameter of the straight section	mm
d_0	Inner diameter of the constrictions	mm
L_1	Constrictions length	mm
L_2	Straight section length	mm
f	Oscillation frequency	Hz
x_o	Oscillation amplitude	mm
ν	Fluid viscosity	$kg \cdot m^{-1} \cdot s^{-1}$
ρ	Fluid density	$kg \cdot m^3$
Re_o	Oscillatory Reynolds number	
St	Strouhal number	
k_L	Liquid-side mass transfer coefficient	$m \cdot s^{-1}$
a	Specific interfacial area	m^{-1}
k_La	Mass transfer coefficient	s^{-1}

C	Concentration in the liquid	$mg \cdot L^{-1}$
C*	Solubility	$mg \cdot L^{-1}$
t	Time	s
X	Biomass concentration	$mg\ DW \cdot L^{-1}$
V	Volume	L
μ	Specific growth rate	h^{-1}
P	Biomass productivity	$mg\ DW \cdot L^{-1} \cdot h^{-1}$
Q	Air flow	$mL \cdot min^{-1}$
C_i	Initial concentration of nutrients	$mg \cdot L^{-1}$
C_f	Final concentration of nutrients	$mg \cdot L^{-1}$

1. Introduction

1.1 Relevance and motivation

Anthropogenic activities are responsible for an intensive emission of greenhouse gases to the atmosphere, contributing to an increase of 35% of carbon dioxide (CO_2, one of the most important greenhouse gases) emissions between 1990 and 2010 (Cheah et al. 2015). A large fraction of these emissions results from the combustion of fossil fuels for energy production. In addition, there is an increasing demand for energy, particularly in the developing countries, which provokes an even higher increase in CO_2 emissions. In order to meet this demand for energy without increasing CO_2 emissions, it is necessary to do even more than just increase the efficiency of the energy production (Olaizola et al. 2003). For this reason, a high number of studies were made to find effective solutions to remove CO_2, from the atmosphere or directly from CO_2 emission sources, and to find effective green alternatives to fossil fuels, in order to produce energy without increasing the CO_2 concentration in the atmosphere.

Some of the most used techniques to capture CO_2 from the atmosphere are the use of absorption or adsorption to separate the CO_2 from the other gases. Absorption is commonly used to treat industrial air streams that contain various pollutants, including CO_2, (Majchrowicz et al. 2009) and usually uses amino solvents to separate these gases (Damen et al. 2006). However, this process has several drawbacks, like the limited CO_2 loading capacity and the high-energy consumption needed to regenerate the solvents (Knudsen et al. 2009). Adsorption is a process where CO_2 molecules adhere to a solid phase, being one of the most feasible processes for CO_2 capture. The biggest drawback of the adsorption technique is the need to cool and dry the flue gas and to remove sulphur oxide (SO_X), which is poison to several adsorbents (Li et al. 2008).

In last decades, the use of photosynthetic microorganisms, such as microalgae, appeared as an alternative to the most common techniques of CO_2 capture. These microorganisms use CO_2 during autotrophic growth, decreasing in this way the concentration of this compound in the atmosphere. In addition, during autotrophic growth, microalgae use CO_2 to produce biomass and other compounds, like lipids, which can be used in biodiesel production. Moreover, during photosynthesis, microalgae also assimilate other nutrients, like nitrogen and phosphorus, providing the possibility of growing microalgae while cleaning water with high concentrations of said nutrients, like wastewaters (Carvalho Júnior et al. 2011). This means that microalgae production can be an important solution for CO_2 capture, because the growing process can be a combination of CO_2 uptake from the atmosphere with effluent treatment and because several products with commercial value can be produced during autotrophic growth, enabling its use in other of the various additional microalgal applications (Carvalho Júnior et al. 2011).

However, the use of microalgae for CO_2 capture still has some limitations, namely related to the mass transfer of CO_2 because this gas presents a limited solubility in the culture medium, resulting in poor CO_2 utilisation efficiencies by microalgae. For this reason, it is necessary to improve de cultivation conditions to increase the CO_2 mass transfer and, consequently, the CO_2 capture by these photosynthetic microorganisms.

1.2 Objectives

The main objective of this study was to evaluate the possibility of using an oscillatory flow reactor (OFR) with smooth periodic constrictions (SPC) for microalgal growth and to optimise the efficiency of CO_2 usage during autotrophic growth using this system.

In order to optimise the efficiency of the process, it is necessary to improve the gas-liquid mass transfer of the system used, by varying several parameters. In this work, several batch experiments using the OFR-SPC were made, varying the oscillation amplitude and the fraction of CO_2 in the air mixture, to evaluate the influence of these parameters in biomass production of the microalga *Chlorella vulgaris*. These experiments were performed using the same temperature, pH, light intensity, photoperiod, oscillation frequency and culture medium.

In addition, the influence of the oscillation frequency and amplitude in gas-liquid mass transfer and in bubbles' size was evaluated in this study, maintaining the other conditions constant. These experiments were performed to determine the conditions that led to higher CO_2 dissolution in the culture medium.

1.3 Thesis structure

This work is divided in six different chapters. Chapter 1 acts as a preface to the rest of the work, explaining the relevance and motivations behind it, as well as the main objectives. Chapter 2 provides a review of the literature, describing the main characteristics of microalgae, as well as their applications. Cultivation methods and systems for microalgal growth, as well as the major factors influencing said growth, are also focused in this chapter. Chapter 3 presents the methodology behind this work. It describes how the different tests for mass transfer coefficient and microalgal growth were performed and how mass transfer and growth parameters were determined. Chapter 4 presents the results obtained from the experiences and their discussion. It contains a detailed analysis on the effect of different parameters (oscillation amplitude and frequency and CO_2 concentration) in the mass transfer coefficient and on microalgal growth. Chapter 5 presents an overview of the work, focusing on the main conclusions of the work and on the possibilities of future research in this area.

2. State of art

2.1 Microalgae

Microalgae, as the name suggests, are microscopic organisms found both in seawater and freshwater. They are a diverse group of unicellular organisms and can be classified as eukaryotic protozoa or prokaryotic cyanobacteria, also known as blue-green algae, being estimated that there are more than 25000 species. These microorganisms are capable of doing photosynthesis, which is an important mechanism to reduce the concentration of CO_2, a greenhouse gas, in the atmosphere. Microalgae are characterised by a short generation time, multiplying exponentially when the environmental conditions are favourable (Martinez-Porchas et al. 2014).

Regarding the energy and carbon sources used for their metabolism, microalgae can grow in three different ways: autotrophy, heterotrophy and mixotrophy. In autotrophic growth, microalgae produce the needed organic matter and energy by using CO_2 as carbon source and sunlight as energy source. Autotrophic cultivation is mostly performed in open pond systems and closed photobioreactors (PBRs). This is a low-cost method that promotes high pigments' and other valuable products' accumulation. The biggest drawback of autotrophic cultivation is the high dependence on external conditions, such as light and temperature, which can be difficult to control in outdoor open systems. Alternatively, highly specialised and cost-intensive reactors can be used to prevent these factors (Zhan et al. 2017). In heterotrophic growth, sunlight is not used as energy source, being organic carbon compounds used as both energy and carbon sources. The most commonly used carbon source in heterotrophic growth is glucose; however, some microalgae can grow with glycerol as carbon source (Zhan et al. 2017). Heterotrophic conditions can accelerate cell growth rate and provoke higher biomass production and lipid accumulation (Urrutia et al. 1995). The PBRs used in heterotrophic growth need less constraints than the ones used for autotrophic growth, making the design of said reactors easier (Zhan et al. 2017). In opposition, this method results in lower pigmentation, due to the absence of light, and CO_2 is produced instead of being consumed. The mixotrophic metabolism consists in a two-stage growth regime, with a heterotrophic first stage, that occurs when the organic carbon content is high, and an autotrophic second step, which begins with the induction of photosynthesis, usually when the organic carbon content is low. This method can be advantageous in natural light and dark cycles (also referred as photoperiod), where microalgae can grow through autotrophy and heterotrophy in optimum conditions. Using light and organic carbon as energy source, and CO_2 and organic carbon as carbon source, microalgae can take advantage of the best parts of both methods: (i) the biggest disadvantage of autotrophic growth, i.e, the need of a light source, can be overcome; (ii) CO_2 production that occurs in heterotrophic growth can be significantly reduced; and (iii) higher biomass productivities, lipid contents and pigments accumulation can be obtained. As main

drawbacks of this growth mode, it is possible to refer the higher production costs, higher susceptibility to contaminations and lower efficiencies in energy conversion (Zhan et al. 2017).

2.1.1 *Chlorella vulgaris*

C. vulgaris is a unicellular green microalga that grows in freshwater and has been present on Earth since the pre-Cambrian period, with a constant genetic integrity. It is consumed as medical treatment, having immune-modulating and anti-cancer properties. It is also capable of accumulating lipids, especially after nitrogen starvation, having a lipidic profile suitable for biodiesel production (Zheng et al. 2011).

It is a spherical microscopic cell with a 2-10 µm diameter (Yamamoto et al. 2005; Illman et al. 2000) and with cellular structural elements similar to those from plant cells, namely the chloroplasts, mitochondria, cell wall and cytoplasm. A schematic representation of a *C. vulgaris* cell is represented in Figure 2.1.

The cell wall of *C. vulgaris* is composed by a chitosan-like layer, cellulose, hemicellulose, proteins, lipids and minerals (Abo-Shady et al. 1993), which confers it a robust character. In terms of sugar contents, the cell wall is composed by a mixture of rhamnose, galactose, glucose, xylose, arabinose and mannose (Takeda 1993).

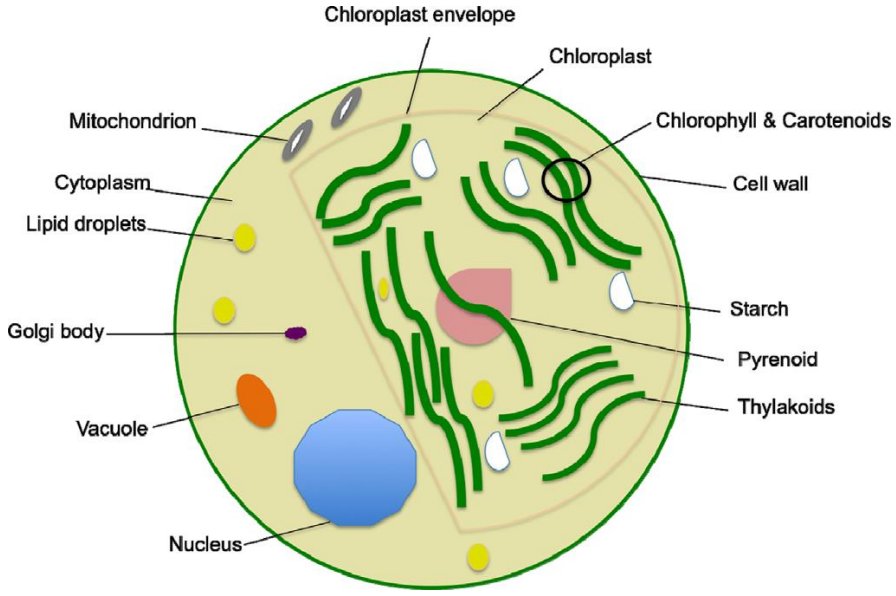

Figure 2. 1 - Schematic structure of a *C. vulgaris* cell representing different organelles (Safi et al. 2014).

C. vulgaris reproduces asexually and rapidly. Under optimal conditions, in 24 h one cell of this microalga multiplies by auto sporulation, forming four daughter cells with their own cell walls inside the mother cell (Yamamoto et al. 2005). After the maturation period, cell wall rupture of the mother cell occurs, being the cell wall remains consumed as feed by the newly liberated daughter cells. These daughter cells are almost perfect replicas of their mother cell and lack the

capacity to develop in zoospospores (Barsanti and Gualtieri 2013). This reproduction process is represented in Figure 2.2.

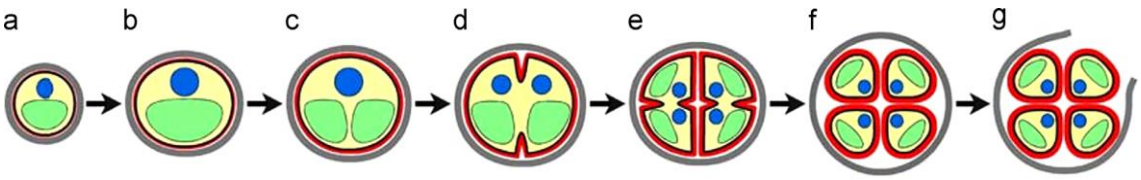

Figure 2. 2 - Phases of daughter cell-wall formation in *C. vulgaris*: (a) early cell-growth phase; (b) late cell-growth phase; (c) chloroplast dividing phase; (d) early protoplast dividing phase; (e) late protoplast dividing phase; (f) daughter cells (Yamamoto et al., 2005).

2.2 Microalgal photosynthesis

Photosynthesis is composed by two major types of reactions. The first reactions, commonly referred as light-dependent reactions, involve the capture of light energy and its conversion into nicotinamide adenine dinucleotide phosphate ($NADPH_2$) and adenosine triphosphate (ATP). The other reactions correspond to a sequence of reactions where inorganic carbon is fixed and reduced to organic carbon, being these reactions light-independent (Barsanti and Gualtieri 2013).

2.2.1 Light-dependent reactions

The main role of the light-dependent reactions is to provide the biochemical reductant, $NADPH_2$, and the chemical energy, ATP, necessary for the assimilation of inorganic carbon. These reactions take place in thylakoid membranes, which contain five major complexes: light-harvesting antennae, photosystems I and II (PS I and PS II, respectively), ATP synthase (ATPase) and cytochrome b_6f, which sustain photosynthetic electron transport and photophosphorylation.

The main role of the antennae system is light-harvesting and consequent energy transfer to the photosynthetic reaction centre (in the thylakoid membranes), releasing some heat during the transfer. PS I is an intermembrane complex composed of proteins and chlorophylls and performs the necessary reactions to reduce ferredoxin and produce $NADPH_2$. PS II, in turn, is a complex composed by a reaction centre, the oxygen-evolving complex and the inner light-harvesting antennae. The transport between PS II and PS I occurs via the cytochrome b_6f, assisted by plastiquinones, which are the electron carriers between PS II and the cytochrome, and via plastocyanin, which is responsible for electron transfer between the cytochrome and PS I. ATPase is a membrane-bound enzyme, which catalyses the synthesis of ATP from adenosine diphosphate (ADP) and inorganic phosphate (P_i).

The light energy is trapped in photoreactions carried out by PS I and II, which operate in series, connected by a chain of electron carriers, from a low to high (more positive) redox potential.

Briefly, in light-dependent reactions, when microalgae receive light energy, two electrons are extracted from water molecules with the release of oxygen (O_2), being transferred through an electron transport chain that ends with the production of $NADPH_2$. At the same time, protons are transported from the stroma into the intrathylakoid space, resulting in the formation of a pH gradient. This gradient drives ATP synthesis, which is catalysed by ATPase, in a reaction called photophosphorylation. These reactions can be expressed by Eq. 2.1 (Masojídek et al. 2004):

$$2\,\mathrm{NADP} + 2\mathrm{H_2O} + 2\mathrm{ADP} + 2\mathrm{P_i} \xrightarrow{\text{light energy/chlorophyll}} 2\mathrm{NADPH_2} + 2\mathrm{ATP} + \mathrm{O_2} \qquad \text{Eq. 2.1}$$

2.2.2 Light-independent reactions

The light-independent, or dark, reactions are, as the name suggests, a group of photosynthetic reactions that do not require the presence of light. These reactions include carbon assimilation and photorespiration.

The first step of carbon assimilation corresponds to CO_2 fixation using ATP and $NADPH_2$ produced in the light-dependent reactions, which can be represented by Eq. 2.2:

$$\mathrm{CO_2} + 4\mathrm{H^+} + 4\mathrm{e^-} \xrightarrow{2\mathrm{NADPH_2};\,3\mathrm{ATP}} \mathrm{CH_2O} + \mathrm{H_2O} \qquad \text{Eq. 2.2}$$

Although Eq. 2.2 represents a simplified version of the reaction of CO_2 fixation, this reaction (found out by Calvin and Benson) occurs in four distinct phases: carboxylation, reduction, regeneration and production. In the carboxylation phase, CO_2 is added to the 5-carbon sugar ribulose bisphosphate, forming two molecules of phosphoglycerate. During the reduction phase, phosphoglycerate is converted into 3-carbon products, in a two-step reaction that uses energy and reduction potential from ATP and $NADPH_2$, respectively. In the first step, phosphoglycerate is phosphorylated (with the help from ATP), forming diphosphoglycerate, which is reduced in the second step into phosphoglyceraldehyde (by $NADPH_2$). In the regeneration phase, ribulose phosphate is regenerated by a complex series of reactions combining sugar phosphates with 3-7 carbons. Finally, during the production phase, the end-products of photosynthesis are formed. In this step, carbohydrates are the main end-products obtained, but fatty acids, amino acids and organic acids are also formed in the CO_2 fixation process.

The photorespiration is a process where organic carbon is converted into CO_2 without any metabolic gain. This process depends on the relative concentration of O_2 and CO_2, being stimulated by a high O_2:CO_2 ratio (Masojídek et al. 2004).

2.3 Typical composition of microalgal biomass

Although microalgae have a great diversity, there are several compounds that are common to most of them. Many microalgal species are characterised by having high concentration of carbohydrates, proteins and lipids, as well as pigments (mainly carotenoids and chlorophyll). Moreover, microalgae have a high concentration of minerals and are a source of several kinds of vitamins.

2.3.1 Carbohydrates

Carbohydrates are a group of sugars and polysaccharides, being starch and cellulose the most frequently found in microalgal biomass. Starch is usually stored in the chloroplasts, being commonly used as an energy storage in the cells. On the other hand, cellulose is a highly resistant polysaccharide, being one of the main constituents of cell walls, where it is used as a protection barrier. Carbohydrates can reach up to 55% of biomass dry weight (DW), especially when grown under nitrogen-limiting conditions (Choix et al. 2012).

Total carbohydrates present in microalgal cells can be determined by the phenol-sulphuric method (Shi et al. 2007), where acidic hydrolysis of carbohydrates at 110 ºC is promoted and the resulting simple sugars can be quantified through high performance liquid chromatography (HPLC). To determine starch content in microalgal cells, an enzymatic method can also be applied (Dragone et al. 2011; B. Fernandes et al. 2012).

2.3.2 Proteins

Proteins have a central importance on microalgal composition, because they are involved in the growth, repair and maintenance of the cell, can serve as cellular motors, chemical messengers and regulators of the cellular activity and play an important role in defence against foreign invaders (Safi et al. 2014).

The main method applied for proteins extraction from microalgae is solubilisation of said proteins in an alkaline solution (Bajguz 2000; Rausch 1951; Safi et al. 2013). After the solubilisation, both trichloroacetic acid and hydrochloric acid can be used to precipitate the solubilised proteins and achieve a greater purification degree (Oliveira et al. 1999; Barbarino and Louren 2005). Some studies showed that microalgal proteins can also be separated by a two-stage ultrafiltration (Safi et al. 2014), where in a first phase starch is completely retained and in the second stage the proteins are retained.

2.3.3 Lipids

Lipids are a group of compounds that are defined by being soluble in non-polar solvents and insoluble in water (Bajguz 2000). The total lipids content of microalgae is mainly composed by three fractions: phospholipids, glycolipids and neutral lipids.

The lipids are synthesised in the chloroplasts and most of them have a structural role in both the cell wall and the membranes of different organelles, like the chloroplasts and mitochondria.

Lipids extraction from microalgae can be performed using hexane, petroleum ether or a mixture of chloroform and methanol (also known as the Bligh and Dyer method) (Phukan et al. 2011; Pavel et al. 2012; Liu and Wang 2008; Y. Lee 2001; J. Lee et al. 2010). A greener alternative to this extraction method is the supercritical carbon dioxide (SC-CO_2) extraction, which gives pure extracts without contamination. Extraction yields using this method can be increased by using a co-solvent, like ethanol, or using a preliminary cell disruption technique (Safi et al. 2014). Quantification of total lipids content can be done gravimetrically, after the evaporation of extracting solvent. Finally, total lipids profile can be determined through HPLC (Safi et al. 2014).

2.3.4 Photosynthetic pigments

Photosynthetic pigments are molecules that absorb light from the visible spectrum, playing an important role in light harvesting during light-dependent reactions. These molecules can be used as food additives and colorants and as pharmaceutical products (Begum et al. 2016). Microalgae are the best source of natural pigments, due to their ability to synthesise them in higher concentration than other photosynthetic organisms. The type of pigments produced depends on microalgal species (Mulders 2014): the most abundant pigments produced by microalgae include carotenoids, which are mainly synthesised by yellow, orange and red microalgae, and chlorophyll, present in green algae. Carotenoids are typically found in heterotrophic microalgae and have a therapeutic effect on humans, due to their strong antioxidant properties (Koyande et al. 2019). They are usually present in microalgal biomass with a concentration of about 0.1-0.2% DW (Koyande et al. 2019). On the other hand, chlorophyll is synthesised by photoautotrophic microalgae and is present in microalgal cells at concentrations up to 1.5% DW. Chlorophyll possesses antioxidant, anticarcinogenic, antigenotoxic and antimutagenic properties (García et al. 2017) and is a well-known detoxifying agent, having the potential to stimulate liver recovery. Chlorophyll improves the metabolism of lipids, proteins and carbohydrates in humans (Koyande et al. 2019).

Pigments extraction process involves the use of solvents, such as acetone, hexane and ethanol, ultrasound-assisted extraction (Cha et al. 2010; Li et al. 2002) or pressurised liquid extraction. The latter is useful for the simultaneous extraction of chlorophyll and carotenoids (Cha et al. 2010). Pigments extraction can also be performed through SC-CO_2 extraction, which provides higher selectivity (Safi et al. 2014). After extraction, quantification of the pigments can be conducted by spectrophotometry or HPLC (Ritchie 2006).

2.3.5 Minerals

Many minerals are important compounds used by microalgae as nutrients, playing an important role in photoautotrophic growth. These include potassium, sodium, iron, magnesium and calcium.

Quantification of minerals present in microalgal biomass is performed by atomic absorption spectrometry, after biomass incineration.

2.3.6 Vitamins

There are two types of vitamins, the water-soluble (vitamins B and C) and fat-soluble ones (vitamins A, D, E, and K). Microalgae have a good vitamin profile, being excellent sources of vitamin A, B1, B2, B6, B12, C and E. This means that they can be a good supplement for humans to (Safi et al. 2014; Brown et al. 1997): (i) promote cell growth (vitamin A); (ii) improve muscle function control and blood circulation (vitamins C and E); and (iii) help in enzymatic activities (various vitamins from the complex B). Despite being an important source of these vitamins, it is important to note that the vitamin content is higher for cells grown under heterotrophic conditions (Safi et al. 2014).

2.4 Microalgal applications

Microalgae can be used in various different areas, having several applications, from bioenergy production to pharmaceutics, being some types of microalgae used for human and animal nutrition. Autotrophic microalgae can also be used for CO_2 capture. There is several more areas in which microalgae can be used, as well as more potential applications for these microorganisms in addition to the ones used in the present.

2.4.1 CO_2 capture

Microalgae have emerged as a promising solution for CO_2 capture, because they can utilise CO_2 (from both the atmosphere or flue gas emissions) as a carbon source during the autotrophic growth. In addition, carbon is the most abundant element in microalgal biomass (Lam and Lee 2011), since the production of 1 kg of microalgal biomass requires the fixation of approximately 1.83 kg of CO_2 (Jiang et al. 2013).

Microalgae have high capacity of fixing CO_2, growing at a much faster rate than terrestrial plants, and being between 10 to 50 times more efficient than them at capturing CO_2, either from the atmosphere or from industrial sources (Ho et al. 2010). In addition, the efficiency of this process can be further enhanced by some engineering approaches, to make the process of CO_2 fixation by microalgae commercially feasible.

The biomass produced as a result of this process is rich in components that can be converted in biofuels or used in other of the various applications of microalgae, which means that the use of

microalgae for CO_2 capture, storage and sequestration can be considered one of the most effective and environmentally friendly approaches to reduce the atmospheric CO_2 concentration (Ho et al. 2013).

2.4.2 Wastewater treatment

Wastewaters have a complex mixture of natural and artificial compounds, on which are included important compounds for microalgal growth (ammonia, nitrates, phosphates and carbohydrates), meaning that it is possible to grow microalgae in wastewater (Goméz-Serrano et al. 2015). In addition, microalgae can assimilate dangerous compounds, such as heavy metals, and reduce the chemical oxygen demand in the wastewater (Han et al. 2018). Moreover, the use of wastewater as culture medium for microalgal growth is also a viable option in terms of process economics and environmental impact, as costs associated to nutrients supply can be avoided, thus reducing the overall production costs, and freshwater requirements can be significantly reduced.

2.4.3 Bioenergy production

Biodiesel produced from microalgae is considered one of the best alternatives to current biodiesel sources (with origin in crops like soybean, corn, rapeseed, among others) because microalgal biomass production does not compete with the area intended for food production and does not require arable land to grow (Singh et al. 2011). Microalgae can accumulate high amounts of lipids and analysis to their fatty acids profile has shown that they can be used for biodiesel production with properties that comply with the standards from most developed countries (Wang et al. 2013).

After fatty acids extraction, the remaining residue is rich in proteins and carbohydrates. These residues can be used in the production of bio-oil (by fast pyrolysis), which has poorer quality due to the high amount of nitrogen present in biomass residues (Wang et al. 2013). In addition, the starch present in the residues can be used for bioethanol production, achieving high ethanol conversion rates after saccharification and fermentation with yeasts (Hirano et al. 1997).

Microalgal biomass can also be used in the production of bio-crude by a reaction of the biomass in water at high temperature, also known as hydrothermal liquefaction (Ross et al. 2010). This reaction can occur in the presence (or not) of a catalyst and this method is more energetically efficient, as it uses the lipids, carbohydrates and proteins present in microalgal biomass instead of using the lipids exclusively (Biller and Ross 2011). In addition, it is possible to produce methane and electricity from microalgae, through anaerobic digestion of biomass (Tsapekos et al. 2018).

The biggest drawback of microalgal biofuels is the associated production cost, which is much higher than the one from fossil fuels. The high costs associated with microalgal biofuels

production make it difficult to compete with fossil fuels, hindering its commercialisation at an industrial scale. In addition, the economic sustainability of biofuels production in industrial scale is questioned by some, because it requires a high energy input when compared with other fuels production.

2.4.4 Human nutrition

Microalgae have been used as human food for thousands of years and their use for food and supplements can reduce the intense resource demand provoked by terrestrial food crops (Milledge 2011). Microalgae cultivation does not require large arable lands and needs less freshwater when compared with terrestrial corps, making it a more efficient cultivation method (Koyande et al. 2019).

Various microalgal species have similar amounts of proteins as those from traditional protein sources. In addition, due to severe environmental conditions and photoautotrophic growth, microalgae have developed the ability to produce antioxidants and pigments, both components being beneficial for human nutrition supplementation (Sampath-Wiley et al. 2008). Finally, microalgae are a good source of both vitamins and essential minerals, also very important for human nutrition (Koyande et al. 2019).

Although microalgae have several health benefits, it cannot be considered as a food product, due to the lack of legislation in terms of quality and requirements for microalgae (Gulati and Berry 2006). Accordingly, microalgae are considered as a nutraceutical.

2.4.5 Animal feed

Due to an increasing demand for food with natural composition, one third of all microalgae produced are used for animal feeding (Fernandes et al. 2012). Besides the very rich composition in terms of proteins and carbohydrates, in general, microalgae have high carotenoids contents and, when fed to animals, an improvement of their health and life expectancy can be observed (Becker 2007; Gouveia et al. 2002; Gouveia et al. 2007; Gouveia et al. 1996; Yamaguchi 1996). In addition, microalgal biomass has a protective effect against heavy metals and other harmful compounds (Vijayavel et al. 2007; Safi et al. 2014), as it reduces the oxidative stress induced by these compounds.

2.4.6 Cosmetic/pharmaceutical products

Microalgal extracts are widely used in cosmetics, mainly in face and skin-care products, like sun creams, anti-irritants or regenerative care products. As example, Dermochlorella, which stimulates collagen synthesis to skin tissue regeneration is extracted from *C. vulgaris* (Barsanti and Gualtieri 2013). Microalgae have also a wide application in pharmaceuticals, being used as food supplements and as a source of essential amino acids, antioxidants, vitamins, between others,

that can be used in various pharmaceuticals. Microalgae are also widely used as a therapeutic product, mainly in East Asia.

2.4.7 Agrochemical applications

Microalgae excrete substances that can influence plant growth and development. These substances include growth promoting factors, vitamins, amino acids, polypeptides, polymers and antibacterial and antifungal substances. Accordingly, microalgae can play an important role in biofertilisation, promoting the growth of many plants (Safi et al. 2014), due to the production of essential compounds, such as nitrogenase, nitrate reductase and minerals.

Studies showed that adding a liquid extract of *C. vulgaris* as bio fertiliser for plant growth, either directly to the culture medium or to the soil, provoked a significant increase in the fresh and dry weight of the seedlings from the crop, as well as an increase in pigments' content (Safi et al. 2014).

2.5 Microalgal cultivation methods

Microalgae can be produced by various methods, from batch cultures, which is a simple method where there are no inputs or outputs of materials, to continuous systems, where new fresh medium is constantly added and removed from the culture, to promote a continous biomass growth. It is also possible to combine both methods, in a semi-continuous culture.

2.5.1 Batch cultures

This operation mode is simple and cheap, which makes it the most common method used in microalgal cultures. In this type of system, the volume is limited and there is no input or output of materials, which means that this system's resources are finite and that microalgal growth occurs until the exhaustion of some limiting factor. Once the resources have been used by microalgae, the cultures may die by lack of nutrients, unless there is an additional supply of fresh medium.

The growth may not start right after inoculation, because most cells may not be in condition to start the division. The time interval needed by cells to adapt and start growing is called the lag or adaptation phase. During this phase, the growth rate is zero. Then, the growth starts to occur, and growth rate increases continually. The exponential phase begins when the growth rate is maximum, being the cell density growth rate constant. This phase is usually fast, since when the cell concentration increases, microalgal cells start to shade each other; and, consequently, the growth rate decreases, and the culture enters the retardation phase. In this phase, the concentration continues to increase until the achievement of a constant value (maximum) and the growth rate decreases until it reaches zero, indicating that the culture entered the stationary phase. After the stationary phase, the water quality deteriorates and nutrients concentration reach a level incapable of sustaining microalgal growth, leading to the achievement of negative growth rate values and,

therefore, the death phase. In this phase, cell concentration decreases and eventually the culture collapses. During the typical phases of batch cultures presented above, cell properties, such as size, nutrient composition and metabolic function, vary constantly.

Batch cultures are widely applied because of their simplicity and flexibility, which allows to solve defects rapidly. Batch cultures are harvested just prior to the beginning of the stationary phase, and thus can be maintained for a substantial period. However, the quality of the harvest can be less predictable than the one in the continuous systems and vary with the timing of the harvest. Other disadvantage of this method is the need to prevent possible contaminations during the first growth phases (Barsanti and Gualtieri 2013).

2.5.2 Continuous cultures

In these systems, it is possible to maintain a constant growth rate inside the reactor, by adding fresh medium at a rate proportional to microalgal growth rate and, in opposition, removing an equal volume of culture. Because fresh medium is constantly fed to the system, the resources are potentially infinite. This method permits to maintain the cultures very close to the maximum growth rates.

Continuous culture systems are widely used for industrial and research purposes. In these conditions, nutrients are supplied to the culture at the same flow rate as biomass produced is removed, and the volume is maintained constant, which allows the cell population to be at steady state (the total number of cells per millilitre of culture and the growth rate of the population remains constant).

There are two types of continuous cultures: the turbidostat and the chemostat. In the turbidostat, fresh medium is added when the cell concentration reaches a predetermined value and the same volume of culture is removed from the reactor. In chemostat, fresh medium is introduced into the culture at a steady rate, usually by a peristaltic pump or solenoid gate system, keeping constant the growth rate. At the same time, air is pumped into the culture through a sterile filter, to supply CO_2 and O_2 to the culture and help in the agitation and circulation of said culture. The flow rate of fresh medium in a continuous culture system is known as dilution rate and is equal to the culture division rate when the number of cells remains constant over time.

The advantage of continuous culture is the control of microbial growth by the dilution rate. When the dilution rate is lower than the maximum growth rate, the cell density increases to a point at which the cell division rate is balanced to the cell washout rate. On the other hand, when the dilution rate exceeds the maximum cell division rate, the cells are removed faster than they are produced, which eventually leads to a total washout of the cell population in the system. Additionally, continuous cultures normally produce biomass with a more predictable quality and requires reduced labour. On the other hand, this system is complex and expensive, needs constant

illumination and temperature and it is only feasible for small production scales (Barsanti and Gualtieri 2013).

2.5.3 Semi-continuous cultures

In these systems, the fresh medium flows into the culture all at once, the valve of the culture vessel is closed and microalgal cells are allowed to grow. After this period, the culture is transferred to a collecting vessel and new fresh medium is introduced in the system, repeating the procedure. This system can be used indoors or outdoors, but has an unpredictable duration time, due to contamination and existence of competitors and predators. The periodic partial harvesting followed by an immediate supplement of nutrients and topping up to the original volume allows the use of large tank cultures (Barsanti and Gualtieri 2013).

2.6 Microalgal cultivation systems

Algae can be produced either in laboratory, or in outdoor tanks, with closely controlled conditions or not. The latter are cheaper, but they are more easily contaminated by external factors, which difficult the growth of algal cultures for an extended period, and the former, although more expensive, allow the control of illumination, temperature, nutrient level, competition, between others.

For commercial scale production, there are several parameters that need to be considered, to choose which culture system to use. These parameters include microalgal biology, labour, energy, water and nutrients required and, if it is an outdoor system, climate. In addition, basic properties, such as the control of temperature and light utilisation efficiency, should also be analysed. With too many parameters to consider, the final choice is almost always a compromise between these considerations, so that an adequate outcome can be achieved. Microalgal culture systems used commercially range in volume from 10^2 to 10^9 L. Most microalgal species that are commercially produced (in which *C. vulgaris* is included) need a highly selective environment to grow, which means that they can be grown in both indoor and outdoor cultures, because this harsh conditions allow the system to remain relatively free from contamination, even in open-air cultures (Barsanti and Gualtieri 2013). Another parameter to consider in commercial-scale cultures is nutrients supply. Figures 2.3 to 2.6 show some examples of industrial scale systems for microalgae biomass production. The nutrient medium used in commercial scale cultures is similar to the one used for laboratory ones, but agricultural-grade fertilisers are used in opposition to the laboratory-grade reagents used in the latter.

There are various different systems to cultivate microalgae, from open ponds with different levels of control, to PBRs, which can take various different forms, from vertical column to flat-plate to horizontal tubular, between others.

Figure 2. 3 - Open pond (Adapted from: Roshdy 2019).

Figure 2. 4 - Vertical column PBR (Adapted from: Huo et al. 2018).

Figure 2. 5 - Flat-plate PBR (Adapted from: Ojamae 2019).

Figure 2. 6 - Horizontal tubular PBR (Adapted from: Climate-Kic 2019).

2.6.1 Open ponds

Various types of ponds for microalgal cultivation have been designed, varying in size, shape, mixing devices and construction materials. Large outdoor ponds can be unlined, using a natural bottom, or lined, using lining materials, such as clay, brick, cement or expensive plastics. The former suffers from heavy contaminations, percolation and silt suspension and can only be used in a few specific conditions and with few microalgal species, which means that lined outdoor ponds are the most commonly used. If the climatic conditions are suitable and there are enough nutrients in the system, natural systems, such as eutrophic lakes, can be used to produce microalgae. Some natural ponds do not even need mixing and only need little environmental control. An example of an open pond can be seen in Figure 2.3.

Algal production in outdoor ponds is not expensive but cannot be maintained for a long period of time, due to problems with contamination by predators, parasites and opportunistic algae that tend to dominate regardless of the species used as inoculum. In addition, outdoor cultures are only suitable for a few species and are characterised by unpredictable crashes, caused by variation in parameters that cannot be controlled and by a poor batch-to-batch consistency (Barsanti and Gualtieri 2013).

2.6.2 Photobioreactors

PBRs are closed systems that do not allow direct exchange of gases between the algal culture and the atmosphere. The tubes in PBRs are usually made of glass or acrylic, needing to be transparent to allow light penetration, and consequently, microalgal photosynthesis. In PBRs, a great proportion of the light does not go directly to the cell surface, passing first through the reactors' wall. These systems provide a more protected and controlled environment to the cultures, protecting them from contamination by other microorganisms and controlling parameters, such as pH, temperature and O_2 and CO_2 concentrations. These devices also attain higher productivity, by preventing evaporation and CO_2 losses by outgassing. However, this stricter control of the process makes these systems more expensive to build and operate in comparison with open ponds.

Since the construction of the first vertical tubular reactor, several experiments were done using different PBR designs (in small-scale systems), but only a few have been later implemented at commercial level (Barsanti and Gualtieri 2013).

2.6.2.1 Vertical column photobioreactors

A vertical column PBR (Figure 2.4) consists in vertical tubes, made of glass or acrylic. An air sparger is placed at the bottom of the reactor to convert the gas in tiny bubbles that give the driving force for mixing and to allow the mass transfer of CO_2. Based on the liquid flow patterns inside the reactor, vertical column PBRs can be divided into bubble column and airlift reactors. The former are cylindrical vessels with height greater than twice the diameter, and the latter consist in a vessel with two interconnected zones. In the first, named gas riser, the gas mixture flows upward, and in the other, named downcomer, the medium flows down towards the bottom allowing gas bubbles to circulate within the riser and downcomer (Yen et al. 2014).

2.6.2.2 Flat-plate photobioreactors

This PBRs consist in translucent flat plates, made of glass or optical light film, illuminated on one or both sides and stirred by aeration at the bottom of the plate, as in vertical column PBRs (Figure 2.5). Flat-plate PBRs can have high light energy requirements and low efficiency in terms of mass production per unit of space, because the productivity in said PBR is highly dependent on space between the panels and aerial productivity constraints (in outdoors) and the various effects of the light provided to the PBR and temperature (indoors). In addition, there is an increase in hydrostatic pressure with the increase in volume, which makes the scale-up of flat-plate PBRs difficult (Slegers et al. 2011).

2.6.2.3 Horizontal tubular photobioreactors

These systems are usually made of transparent polypropylene, acrylic or polyvinylchloride pipes, characterised by a small internal diameter to increase light penetration into the culture. The

horizontal pipes can be arranged vertically or horizontally. These systems contain an air pump to provide air circulation and thus, mixing and agitation of the culture. In comparison with the vertical column PBRs, these PBRs provide increased gas residence times (Briassoulis et al. 2010). Figure 2.6 shows an example of a horizontal tubular PBR.

2.7 Factors influencing microalgal growth

A culture can be divided in three different components: the culture medium, which is contained in a suitable vessel, the microalgal cells growing in said medium and the surrounding air, necessary for the exchange of CO_2 between the medium and the atmosphere.

The most important parameters to regulate microalgal growth are light, temperature, pH, nutrient quantity and quality and turbulence. Optimal parameters, as well as tolerated ranges, are species-specific.

2.7.1 Light

Light, namely light intensity and photoperiod (light:dark cycle), greatly affects microalgal photosynthesis, cell composition and metabolic pathways and consequently, the efficiency of the cultivation process in autotrophic conditions (Blanken et al. 2013; He et al. 2015). In addition, light:dark cycles play a fundamental role on the synthesis of organic compounds, like glucose, and on nutrient metabolism, which also affect the growth rate and productivity of microalgae (Patel et al. 2019).

Light intensity is a fundamental parameter in microalgal growth, but their requirements vary greatly with the characteristics of the culture, such as cell density and depth of the culture. For example, higher light intensities are required in cultures with higher depths or higher cell concentrations. However, the increase in light intensity needs to be done with precaution, because too much light may result in photo-inhibition of the culture (Barsanti and Gualtieri 2013).

2.7.2 Temperature

The temperature affects microalgal growth rate at different levels. Enzymatic activity is the parameter that is the most influenced by temperature. To temperatures below the one which provides optimal growth, an increase in temperature has a positive effect in the growth rates, following the Arrhenius Law (Serra-Maia et al. 2016). In opposite, to temperatures above the optimal one, an increase in temperature results in a decrease in growth rates, because high temperature provokes heat stress that affects the enzymatic activity (Ras et al. 2013). Culture temperature also affects the mass transfer rate and solubility of both CO_2 and O_2 in the culture medium, and the kinetics of the reactions related to the solubility (Renaud et al. 2002). Finally, the temperature can also influence the cell mortality rate: high temperatures (above the optimum) can be dangerous because they induce protein degradation and cause oxidative damage (due to

the stress induced in reactive oxygen species dynamics), leading to cell mortality (Serra-Maia et al. 2016).

With the model of Hinshelwood (1945), which states that the growth rate is the difference between the growth and mortality, and between others, depends on temperature, it is possible to determine the optimal temperature for microalgal growth and maximum net growth temperature. This parameter is also important to consider because with temperatures above this maximum, the mortality rate is higher than the growth rate (net growth rate is negative) (Serra-Maia et al. 2016).

2.7.3 pH

The pH of microalgal cultures is very important because it determines the concentrations of carbonaceous species in water (Qiu et al. 2017), i.e., the pH determines the solubility of CO_2 and nutrients and consequently, their availability to microalgae, which greatly affects microalgal metabolism (Chen and Durbin 1994). The reaction between CO_2 dissolved in water and water can be expressed by Eq. 2.3:

$$CO_2(g) + H_2O\ (l) \leftrightarrow H_2CO_3(aq) \qquad \text{Eq. 2.3}$$

The element formed is the carbonic acid and, when dissolved in water, can form two different salts, bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}), being the equilibrium between the three represented by Eq. 2.4:

$$H_2CO_3(aq) \leftrightarrow HCO_3^-(aq) + H^+(aq) \leftrightarrow CO_3^{2-}(aq) + 2H^+(aq) \qquad \text{Eq. 2.4}$$

According to Eq. 2.4, one can see that the pH greatly influences the presence of the various carbonated species in water. Figure 2.7 shows the fraction of the three different carbonated species present in water for different pH values. For low pH values (<5), there is already a high concentration of cations H^+ dissolved in water and the reaction expressed in Eq. 2.4 tends to the formation of the carbonic acid, as one can see in Figure 2.7, being this species predominant at these pH values. On the other side, at high pH values (>11), the concentration of the same cation in water is low, and the reaction tends to the formation of carbonate to release the cations H^+ to the water, reason why carbonate is the most common species in solution in these conditions (Figure 2.7). At pH close to the neutrality, the predominant species is bicarbonate.

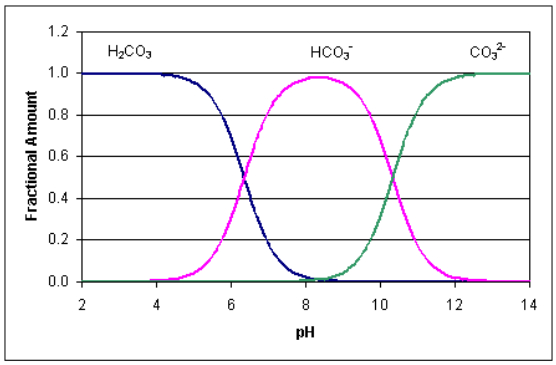

Figure 2. 7 - Fraction of the different carbonated species in water at different pH (Adapted from: Department of Chemistry and Biochemistry, USU 2004).

The optimal pH for biomass production varies for each species and usually is narrow and strain-specific (Moheimani 2013). Due to uptake of inorganic carbon by microalgae present in the culture, the pH of microalgal cultures raise during the light period. High pH values (>11) limit the availability of carbonaceous species that can be assimilated by microalgae and, consequently, inhibit cell growth and biomass production (Chen and Durbin 1994), but, on the other hand, high pH values can suppress some undesired contaminants (Bartley et al. 2014).

2.7.4 Nutrients

As important macronutrients for microalgal growth, the concentration of nitrogen and phosphorus in the growth medium has a direct influence on microalgal growth kinetics and consequently, on nutrients removal and lipid accumulation (Xin et al. 2010). Nitrogen or phosphorus limitation has a great negative effect in the productivity of microalgae, because these are two important constituents of microalgal biomass. The nitrogen limitation influences the amino acids supply, reducing the synthesis of proteins. Nitrogen limitation also reduces the photosynthetic, growth and respiratory rates. In the case of phosphorus, limiting concentrations of this nutrient result in the inhibition of nucleic acid synthesis, either at genome replication level or at ribonucleic acid (RNA) synthesis level, and thus an inhibition of protein synthesis, which affects cell metabolism. This limitation can also affect energy conversion process during photosynthesis, thus inhibiting microalgal growth (Barsanti and Gualtieri 2013).

2.7.5 Operational conditions

In order to achieve an optimum microalgal growth and, in cases of microalgal cultivation for wastewater treatment, optimum nutrients removal rates, it is necessary to evaluate some operational and physical factors, which directly influence both microalgal growth and nutrients uptake. In open ponds, where lower control of the culturing conditions is observed, operational conditions play an even greater role in microalgal growth (Eltanahy et al. 2018).

Mixing of the culture is critical to assure an efficient use of light and nutrients by microalgal cells, as well as to promote the diffusion of gases in the liquid medium, to maintain a constant temperature throughout the culture and to prevent the sedimentation of the culture (Grobbelaar 1994). The mixing of the culture is usually a turbulent one, however, some types of microalgae (mainly marine ones) are not accustomed to turbulent flows, and the mixing must be slower for these microorganisms (Barsanti and Gualtieri 2013).

Turbulent mixing can be obtained by aeration or by mechanical agitation (with paddle wheels, turbines, jet pumps or propellers, for example) (Barsanti and Gualtieri 2013). Figures 2.8 and 2.9 show diagrams representing the mixing of bioreactors by aeration and mechanical agitation, respectively.

Turbulence provoked by different media can result in different sensitivities for different microalgal species, or even a complete inhibition of some of them (Thomas and Gibson 1990). The effect of mixing is more visible in high density cultures, where this agitation is fundamental to provide light and nutrients to microalgae (Eltanahy et al. 2018).

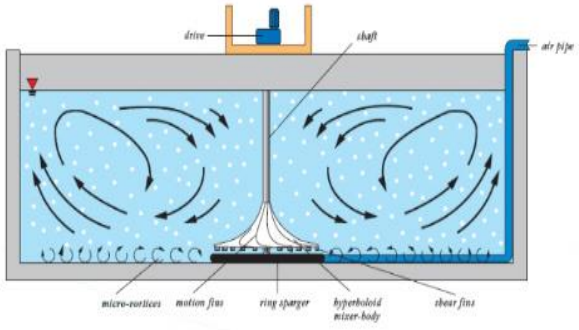

Figure 2. 8 – Schematic representation of mixing by aeration (Adapted from: Jonassen Industrial Projects Ltd 2019).

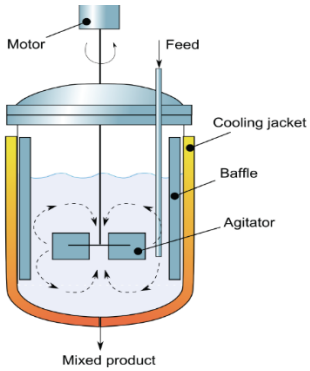

Figure 2. 9 – Schematic representation of mixing by mechanical agitation (Adapted from: AIChE 2018).

3. Materials and methods

3.1 Microorganisms and culture medium

The microalga used in this study, *C. vulgaris* CCAP 211/11B, was obtained from Culture Collection of Algae and Protozoa (CCAP, United Kingdom). This microalga was selected based on the following factors (Safi et al. 2013; Safi et al. 2014; Gonzalez and Bashan 2000): (i) it is a widely used microalga for both laboratory and commercial scales; (ii) has a very rigid cell wall, which grants its protection against invaders and harsh environmental conditions, thus enabling its growth in various environments; and (iii) it possesses a very rich nutritional composition, having really favourable lipidic and carbohydrate profiles, and great proteins, vitamins and pigments profiles, which allows its use in such a wide variety of biotechnological applications.

Stock solutions of *C. vulgaris* were prepared in 100-mL Erlenmeyer flasks, with working volume of 50 mL, using the modified OECD (Organisation for Economic Cooperation and Development) test medium (OECD, 2011), with the following composition (per litre): 250 mg $NaNO_3$, 12 mg $MgCl_2 \cdot 6H_2O$, 18 mg $CaCl_2 \cdot 2H_2O$, 15 mg $MgSO_4 \cdot 7H_2O$, 45 mg KH_2PO_4, 0.08 mg $FeCl_3 \cdot 6H_2O$, 0.1 mg $Na_2EDTA \cdot 2H_2O$, 0.185mg H_3BO_3, 0.415 mg $MnCl_2 \cdot 4H_2O$, 3 µg $ZnCl_2$, 1.5 µg $CoCl2 \cdot 6H_2O$, 0.01 µg $CuCl_2 \cdot 2H_2O$, 7 µg $Na_2MoO_4 \cdot 2H_2O$ and 50 mg $NaHCO_3$.

Erlenmeyer flasks were maintained in a thermostatic cabinet (Lovibond®, United Kingdom) at constant temperature (25 °C) and continuous light supply of approximately 6.50 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Agitation was promoted through an Unimax 1010 orbital shaker (Heidolph, Germany), set at 100 rpm (rotations per minute). At the beginning of each mass transfer and microalgal growth experiment, dilutions of the stock solutions were made to obtain an inoculum with optical density at 680 nm (OD_{680}) between 0.25-0.50 (corresponding to a biomass concentration of approximately 164-274 mg $DW \cdot L^{-1}$).

3.2 Experimental apparatus

Mass transfer and microalgal growth experiments were performed in an OFR-SPC, as schematically represented in Figure 3.1. The straight section of the OFR-SPC has an inner diameter (D) of 16 mm and length (L_2) of 22 mm and the constrictions have an inner diameter (d_0) of 7 mm and length (L_1) of 32 mm. The reactor is equipped with a water jacket that can be coupled with a thermostatic bath for temperature control. Oscillation was promoted by a CAT R100C motor (Cat Scientific, United States), which allows the operation at different oscillation amplitudes and frequencies. The experiments were performed using a mixture of air and CO_2 at the gas inlet. To control both CO_2 concentration and gas flow rate, two precision gas flow controllers (Alicat Scientific, United States) were used, one for air-K, composed by 79% of nitrogen (N_2) and 21% of O_2, and other for CO_2.

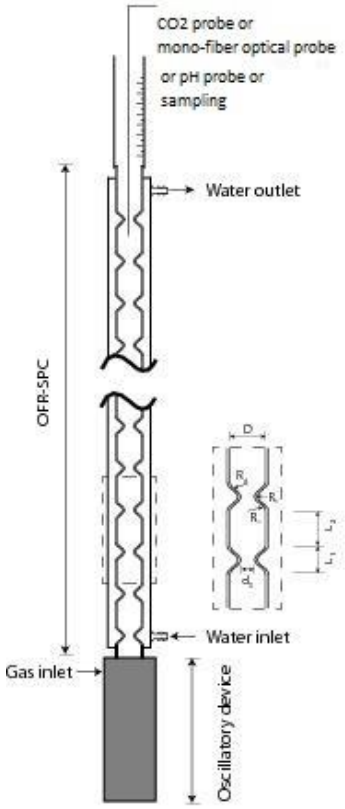

Figure 3. 1 - Schematic diagram of the OFR-SPC used in the experiments. OFR-SPC - oscillatory flow reactor with smooth periodic constrictions; D - inner diameter of the straight section; d_0 - internal tube diameter in the constrictions; L_1 - constriction length; L_2 - straight tube length; R_c - radius of curvature of the sidewall of the convergent subsection; R_d - radius of the sidewall of the divergent subsection; R_t - radius of curvature of constriction centre (Adapted From: Ferreira et al. 2015)

3.3 Mass transfer experiments

To have a complete understanding about CO_2 mass transfer in the OFR-SPC using microalgal cultures, CO_2 mass transfer experiments were performed for different operational conditions, regarding CO_2 concentration in the gas inlet, oscillation amplitude and oscillation frequency. The different conditions tested are summarised in Table 3.1. These experiments were conducted in the two-phase system (gas-liquid) at constant temperature (25 ºC) and with superficial gas velocity controlled by the gas flow controllers. The liquid phase was the microalgal inoculum with an OD_{680} of 0.25-0.50 and the gas phase was a mixture of N_2 and CO_2 (with the flow rates adjusted to obtain a CO_2 concentration of 1, 2 or 5% v/v, according to the condition tested).

Table 3. 1 - Conditions tested in mass transfer experiments

CO_2 concentration (% v/v)	Oscillation Amplitude, x_o (mm)	Oscillation frequency, f (Hz)
1	0	0.0
1	9	1.0
1	9	1.5
1	9	2.0
1	9	3.0
1	4	1.5
1	6	1.5
1	9	1.5
1	12	1.5
1	19	1.5
2	4	1.5
2	6	1.5
2	9	1.5
2	12	1.5
2	19	1.5
5	4	1.5
5	6	1.5
5	9	1.5
5	12	1.5
5	19	1.5

3.3.1 Hydrodynamic conditions

The fluid mechanical conditions in the OFR-SPC can be controlled with both oscillatory Reynolds (Re_O) and Strouhal (St) numbers, both being essential to monitor the hydrodynamic conditions in the OFR. The Reynolds number describes the intensity of mixing in the column, and can be determined as defined in Eq. 3.1:

$$Re_O = \frac{2\pi f x_o \rho D}{\nu} \qquad \text{Eq. 3.1}$$

where f is the oscillation frequency (in Hz), x_O the oscillation amplitude (in m), ρ the fluid density (in $kg \cdot m^{-3}$), D the internal diameter (in m) and v the fluid viscosity (in $kg \cdot m^{-1} \cdot s^{-1}$).

The Strouhal number describes the effective eddy propagation and is defined by Eq. 3.2:

$$St = \frac{D}{4\pi x_o} \quad \text{Eq. 3.2}$$

3.3.2 k_La determinations

For mass transfer coefficient (k_La) determination, initially the CO_2 present in the liquid phase was removed by bubbling N_2. When the dissolved CO_2 concentration reached a stable value (close to zero), the CO_2:N_2 gas mixture was fed into the column. At this moment the CO_2 mass transfer process, from bubbles to the liquid, begins and continues until CO_2 concentration in the liquid reaches the saturation. Dissolved CO_2 concentration values were measured online using a pCO_2 mini fibre optic CO_2 transmitter (PreSens, Germany) and directly recorded in the computer, through the corresponding software. The dissolved CO_2 variation with time, *t*, was obtained, and k_La was calculated according to the following procedure. Taking into account the mass balance for CO_2 mass transfer in the liquid, represented in Eq. 3.3:

$$\frac{dC}{dt} = k_La \cdot (C^* - C) \quad \text{Eq. 3.3}$$

being *C* and C^* the CO_2 concentration in the liquid and CO_2 solubility, respectively (Ferreira et al., 2015). Assuming that the liquid phase is homogeneous, and the initial CO_2 concentration is C_0, one can integrate the previous equation into Eq. 3.4:

$$\ln(C^* - C) = \ln(C^* - C_0) - k_La \cdot t \quad \text{Eq. 3.4}$$

The volumetric mass transfer coefficient can then be determined by plotting *ln(C*-C)* against time. CO_2 solubility was measured experimentally for each run. The dissolved CO_2 concentration during the aeration phase has two different zones: a first one with a high mass transfer zone, where CO_2 concentration rises rapidly, and a second one, where CO_2 concentration is close to the saturation and the mass transfer rate starts decreasing. By determining the slope in the linear zone of the aforementioned plot, it is possible to obtain the k_La value (Ferreira et al. 2015).

3.3.3 Mean chord size determinations

To analyse if the variations in k_La were due to variations in k_L or *a*, the mean chord size of the gas bubbles entering the reactor were also measured. For this, a phase optical probe optoelectronic module (A2 – Photonic Sensors, France) connected to a computer to record the results was used. This method consisted in having the probe drown in the culture medium and when the sensor goes through a bubble, it measures the time required by the bubble to pass through the sensor and determines bubbles' size. This method can only measure chord sizes, not diameters because it is not possible to guarantee that all the bubbles are spherical and pass in the sensor exactly at bubble centre.

3.4 Microalgal growth experiments

3.4.1 Microalgal growth in the OFR

The different experiments were made in the system presented in Figure 3.2 and the conditions tested are shown in Table 3.2.

Figure 3. 2 - Experimental setup.

Table 3. 2 - Experimental conditions tested to evaluate microalgal growth

CO_2 concentration (% v/v)	Amplitude, x_o (mm)	Frequency, f (Hz)
0.04	19	1.5
1	19	1.5
1	12	1.5
1	9	1.5
1	6	1.5
2	9	1.5
2	4	1.5

The air flow was regulated by two precision gas flow controllers (Alicat scientific, United States), one for air-K and other for CO_2, being the flow in the OFR-SPC constant in the different experiments. The flow was chosen to be 0.5 vvm (volume of gas per working volume per minute), value typically used in microalgal cultures (Maeda et al. 1995). Accordingly, taking into account the volume of culture medium used in this OFR-SPC (approximately 70 mL), the gas flow rate used in this reactor was 35 $mL \cdot min^{-1}$. CO_2 in the system was provided by a CO_2 canister, with the percentage of carbon dioxide in the air phase varying according to the different experiments.

The temperature was regulated by a water jacket and a thermostatic bath, and maintained at 25 ºC, because, according to different studies, optimum growth rate for *C. vulgaris* is obtained for temperatures between 25 and 30 ºC (Kessler 1985). The light was provided continuously by a fluorescent lamp, being average light intensity at the surface of the reactor approximately 23.15 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (values measured using a photo-radiometer dataloger HD2102.2, Delta Ohm, Italy).

3.4.2 Growth monitoring

Growth conditions were monitored (three times a day, with time intervals between analysis of approximately three hours) through pH and temperature measurements, using a C6010 (Consort, Belgium) pH meter. The temperature was measured to make sure that this parameter was being properly controlled by the water jacket and the thermostatic bath, so that the temperature did not achieve values that could be harmful for microalgal cells. pH measurement was performed to: (i) keep track of the concentration of carbonates in the medium, in order to make sure that there was enough dissolved CO_2 to provoke microalgal growth; (ii) make sure that pH did not vary to values that could inhibit microalgal growth; and (iii) have an idea about the photosynthetic efficiency of the cultured cells, as the pH in the culture medium tends to increase as CO_2 is assimilated by microalgae.

To monitor and evaluate microalgal growth, samples were collected with the same periodicity of pH and temperature measurements (three times a day, with a time interval between sampling of approximately three hours) to measure OD_{680}. The measurements were performed in duplicate using a Genesis 10 UV Scanning (Thermo Scientific, United States) spectrophotometer and distilled water was used as blank. Selection of the 680 nm wavelength was based on the absorption spectrum determined for *C. vulgaris*, which had a peak of maximum absorption in the referred wavelength (as shown in Figure 3.3).

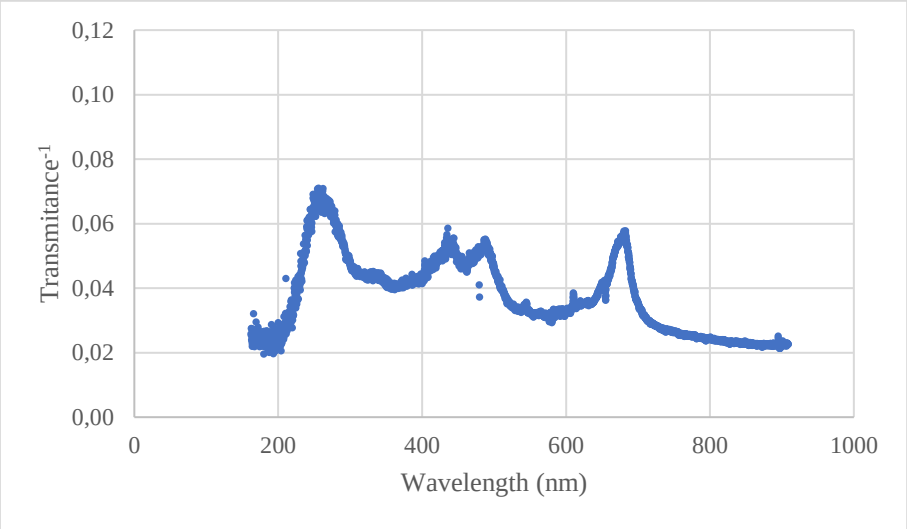

Figure 3. 3 - Absorption spectrum determined for *C. vulgaris*.

The relationship between OD_{680} and biomass concentration, in mg $DW \cdot L^{-1}$ was determined by linear regression (Annex A.1). For this, a dense microalgal inoculum was submitted to different dilutions and these samples were used for OD_{680} and biomass concentration measurements. OD_{680} measurements were performed as previously referred. Regarding biomass concentration in terms of ash free dry weight (X, in mg $DW \cdot L^{-1}$), it was determined by drying 10 mL of each dilution for 24 h at 105 °C in previously dried and weighed porcelain crucibles, until a constant weight was obtained (A). The dried biomass was then oxidized at 550 °C for 2 h in a muffle and re-weighed (B). The mass loss obtained after biomass oxidation divided by the samples' volume (V) gives the ash free dry weight biomass concentration, as represented in Eq. 3.5:

$$X(\text{mg DW} \cdot \text{L}^{-1}) = \frac{A - B}{V} \qquad \text{Eq. 3.5}$$

With biomass concentration values, specific growth rates and average biomass productivities were determined for each culturing condition. Specific growth rates were determined according to Eq. 3.6 (Feng et al. 2012):

$$\mu(\text{d}^{-1}) = \frac{\ln(X_2) - \ln(X_1)}{t_2 - t_1} \qquad \text{Eq. 3.6}$$

being X_1 and X_2 biomass concentrations (in mg $DW \cdot L^{-1}$), at times t_1 and t_2 (in d), which correspond to the beginning and end of the exponential growth phase, respectively.

Average biomass productivities were calculated according to Eq. 3.7 (Feng et al. 2012; Jacob-Lopes et al. 2009):

$$P(\text{mg DW} \cdot \text{L}^{-1} \cdot \text{d}^{-1}) = \frac{X_f - X_i}{t_f - t_i} \qquad \text{Eq. 3.7}$$

being X_f and X_i the biomass concentrations (in mg $DW \cdot L^{-1}$), at times t_f and t_i (in d), which correspond to the end and beginning of the cultivation period, respectively.

3.4.3 Nutrients concentration

To evaluate nutrients uptake during microalgal growth in the OFR-SPC, quantification of nitrate and phosphate in the culture medium was performed. Samples for analysis were collected on a daily basis. To determine both nitrate and phosphate concentrations, the collected samples were centrifuged at 12000 rpm for 10 min (in an Eppendorf 5424 centrifuge, Eppendorf, Germany) and the supernatants were stored at 4 °C until being analysed.

Nitrate concentration was determined according to Standard Methods (APHA 1999). Firstly, the supernatants were diluted 20 times in distilled water and the solutions were filtered using 0.22-

µm cellulose acetate syringe filters (Orange Scientific, Belgium). After filtration, absorbance of the samples was measured at 220 nm using a quartz cuvette in a T60 UV/VIS spectrophotometer (Oasis Scientific, United States). Distilled water previously filtered was used as blank. The calibration curve (Annex A.2) was determined by preparing $NaNO_3$ standards with concentrations ranging from 5 to 500 $mg \cdot L^{-1}$ and submitting them to the same procedure as the analysed samples. Samples were analysed in duplicates (Collos et al. 1999).

Phosphate quantification was performed by measuring absorbance at 820 nm of a phosphomolybdate complex formed by reaction of inorganic phosphate with ammonium molybdate. In this method, 225 µL of each supernatant was pipetted into 1.5-mL tubes and then 525 µL of the reaction mix was added. The reaction mix was prepared by adding 1 part of reagent 1 (10% w/v ascorbic acid) to 6 parts of reagent 2 (0.42% w/v $(NH_4)_2MoO_4 \cdot 4H_2O$ in 1 N H_2SO_4). The samples were then incubated at 37 °C for 1 h and the absorbance was measured at 820 nm in a Genesis 10 UV Scanning Spectrophotometer (Thermo Scientific, United States). The blank was measured by repeating the procedure using distilled water. To determine the calibration curve (Annex A.3), standards of KH_2PO_4 with concentrations ranging from 1 to 60 $mg \cdot L^{-1}$ were submitted to the same procedure. Samples were analysed in duplicates (B. Lee et al. 2009).

In addition, it was also determined the average removal rate and the efficiency of the nutrient removal. The average removal rate was determined with Eq. 3.8:

$$\text{Average Removal Rate } (\text{mg} \cdot \text{L}^{-1} \cdot \text{d}^{-1}) = \frac{\text{C}_\text{i} - \text{C}_\text{f}}{\text{t}} \qquad \text{Eq. 3.8}$$

being C_i and C_f the initial and final concentrations of said nutrient in the culture medium, in $mg \cdot L^{-1}$, respectively, and t the cultivation time, in d.

The efficiency of the nutrient removal was determined with Eq 3.9:

$$\text{Removal Efficiency } (\%) = \frac{\text{C}_\text{i} - \text{C}_\text{f}}{\text{C}_\text{i}} \times 100 \qquad \text{Eq. 3.9}$$

4. Results and discussion

4.1 Mass transfer experiments

4.1.1 Hydrodynamic conditions

To evaluate the hydrodynamic conditions of the OFR-SPC used in this study, both the oscillatory Reynolds and Strouhal numbers were determined. Both depend on the oscillation amplitude and frequency, but not on the CO_2 concentration. Table 4.1 shows the values of the oscillatory Reynolds number and Strouhal number determined when oscillation amplitude varied. Oscillatory Reynolds number and Strouhal number determined for different oscillation frequencies and a fixed amplitude of 9 mm are presented in Table 4.2.

Table 4. 1 - Oscillatory Reynolds and Strouhal numbers determined for different oscillation amplitudes, maintaining an oscillation frequency of 1.5 Hz

Amplitude (mm)	Reynolds number	Strouhal number
4	602	0.318
6	903	0.212
9	1354	0.141
12	1806	0.106
19	2859	0.067

Table 4. 2 - Oscillatory Reynolds and Strouhal numbers determined for different oscillation frequencies, maintaining an oscillation amplitude of 9 mm

Frequency (Hz)	Reynolds number	Strouhal number
1.0	903	0.141
1.5	1354	0.141
2.0	1806	0.141
3.0	2709	0.141

According to Table 4.1 almost all oscillation amplitudes result in a laminar flow (Re_O<2300), except for an amplitude of 19 mm, where turbulent flow takes place. Likewise, according to Table 4.2, almost all oscillation frequencies tested result in a laminar flow, except for a frequency of 3 Hz. On the other side, the Strouhal number decreases with an increase in oscillation amplitude, and do not depend on the frequency. Grobbelaar (1994) tested the effect of the turbulence (and consequently Reynolds number) in a tubular reactor and found that the biomass productivity increases in this reactor until a Reynolds number of 6000. Moheimani (2013) obtained good values of biomass productivity in a bag PBR with a Reynolds number of 1500. So, according to

these studies, it is expected to achieve increased biomass productivities in experiments operating at higher Reynolds number. For this, it is necessary to increase the oscillation amplitude or frequency. According to the hydrodynamic conditions determined for the OFR-SPC, it is expected to obtain increased *C. vulgaris* growth with 9 mm of amplitude and 3 Hz of frequency, or 19 mm of amplitude and 1.5 Hz of frequency.

4.1.2 k_La determination

To evaluate the variation in the gas-liquid mass transfer created by the variation of several parameters, such as oscillation amplitude and frequency and CO_2 concentration in the inlet stream, k_La determinations were performed. CO_2 saturation curves obtained in these determinations can be found in Annex B. k_La values determined for different oscillation frequencies (maintaining an amplitude of 9 mm and a CO_2 fraction of 1% v/v) are presented in Figure 4.1. On the other hand, Figure 4.2 shows the variation in k_La observed for different amplitudes and CO_2 concentrations in the inlet stream.

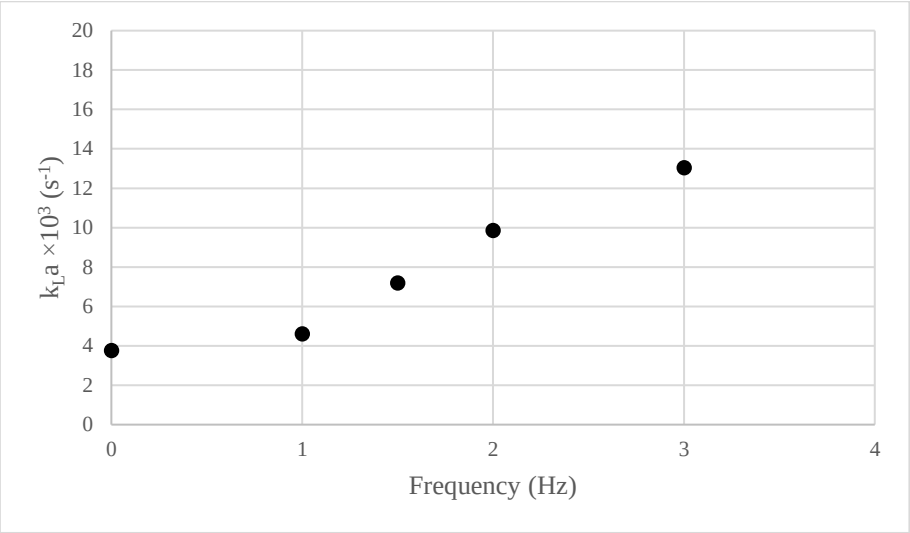

Figure 4. 1 - Mass transfer coefficient (k_La) variation with oscillation frequency. Operational conditions: Q – 35 mL·min^{-1} (0.5 vvm); Amplitude – 9 mm; % CO_2 in the inlet stream – 1% (v/v); Inoculum concentration – 164 mg DW·L^{-1}.

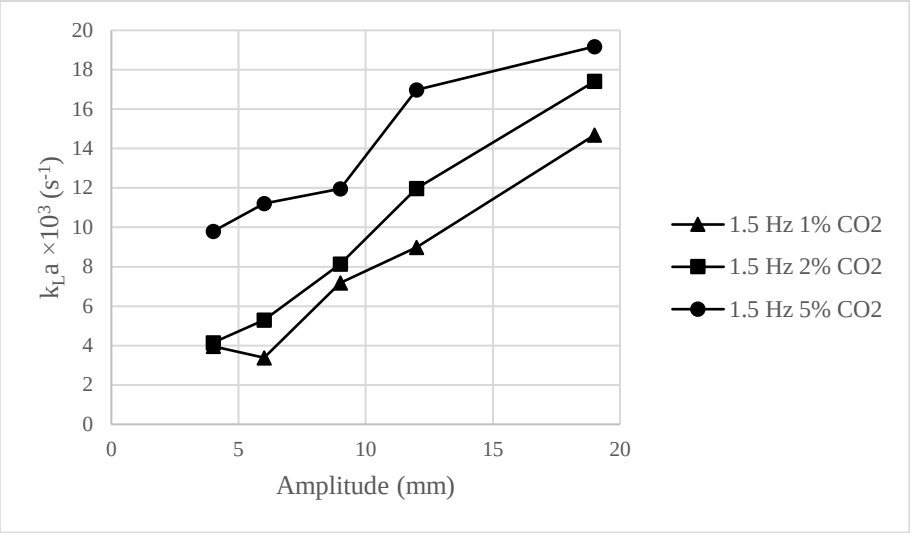

Figure 4. 2 - Mass transfer coefficient (k_La) variation with oscillation amplitude for different CO_2 concentrations in the inlet stream. Operational conditions: Q – 35 mL·min^{-1} (0.5 vvm); Frequency – 1.5 Hz; Inoculum concentration – 164 mg DW·L^{-1}.

With the analysis of Figure 4.1, it is possible to observe that the mass transfer coefficient increases with an increase in oscillation frequency, and that this increase is almost linear, with the exception of the value obtained without agitation, which was slightly lower than the one obtained for the lowest oscillation frequency tested (1 Hz). These results indicate that varying the oscillation frequency between 0 and 1 Hz would not lead to significant variations in the k_La.

The analysis of Figure 4.2 shows that an increase in amplitude provokes an increase in k_La, for all different CO_2 concentrations measured, although there was a decrease in mass transfer coefficient from 4 to 6 mm in the case of experiments performed with 1% (v/v) of CO_2. However, this decrease can be assumed as measurement errors, because both are smaller amplitudes, close to each other, and it was expected that both would result in similar k_La values. Moreover, more measurement errors were expected when working with a CO_2 concentration of 1% (v/v) because these conditions are very close to the detection limits of the equipment used (operation range of the used CO_2 probe is between 1 and 25% v/v CO_2).

In addition, according to the same figure, an increase in CO_2 concentration in the air mixture also provokes an increase in mass transfer, for all amplitudes tested. The effect of increasing the CO_2 concentration from low (1% or 2% v/v) to high (5% v/v) values is more pronounced for the lowest amplitudes tested: for the amplitudes of 4 and 6 mm, k_La values duplicate between the CO_2 concentrations of 2 and 5% (v/v). These results indicate that for lower oscillation amplitudes, when CO_2 concentration increases, the mass transfer coefficient is mainly influenced by CO_2 concentration rather than amplitude. On the other hand, the differences between the lowest CO_2 concentrations (1 and 2% v/v) are not very significant (for the whole range of amplitudes tested) because the difference between these concentrations is too small to provoke high differences in k_La values.

These results are similar to ones obtained in other studies. Ferreira et al. (2015) reported that the oxygen mass transfer coefficient increases greatly with both oscillation frequency and amplitude: (i) from values of 10.5×10^{-3} s^{-1} with 1 Hz to 18.8×10^{-3} s^{-1} with 3.0 Hz, in an experiment performed at an oscillation amplitude of 9 mm; and (ii) from 10.5×10^{-3} to 20.9×10^{-3} s^{-1} for experiments performed at 1.0 Hz, with oscillation amplitudes of 9 and 19 mm, respectively. With this study, the authors concluded that oscillation amplitude has higher influence on k_La values because this parameter controls the length of the eddy generated in the column, which strongly influences bubbles' behaviour in the OFR-SPC. Similar results were obtained in this study. In the experiments performed at 1% (v/v) CO_2 and an oscillation amplitude of 9 mm, when oscillation frequency increased from 1.5 to 3.0 Hz, k_La values increased from 7.18×10^{-3} to 13.0×10^{-3} s^{-1}. On the other hand, when oscillation frequency was maintained at 1.5 Hz and amplitude increased from 9 to 19 mm, k_La values obtained were 7.18×10^{-3} and 14.7×10^{-3} s^{-1}, respectively. CO_2 mass transfer coefficients in the same order of magnitude were determined by several authors for different reactor configurations. Y. Shen et al. (2009) reported values of 2.5, 50, 75 ($\times10^{-3}$) s^{-1} for raceway ponds, flat panel PBRs and tubular PBRs, respectively. Fernandes et al. (2014) reported values between 7 and 40 ($\times10^{-3}$) s^{-1}, for superficial gas velocities between 1 and 8 ($\times10^{-3}$) $m\cdot s^{-1}$, in a split cylinder airlift PBR.

4.1.3 Mean chord size determination

During k_La determination experiments, the mean chord size of the bubbles was also evaluated, to determine whether the specific interfacial area, *a*, varied or not. It was not possible to measure bubbles' diameter, which means that it was not possible to determine the specific interfacial area. Accordingly, it is not possible to say exactly how liquid-side mass transfer coefficient, k_L, and specific interfacial area, *a*, influence the k_La. One can only infer that a variation in mean chord size is also related to a variation in bubbles' diameter and, consequently, to a variation in the specific interfacial area. It is important to highlight that it is not very correct to conclude this, because from one test to another, the chords can be measured in different parts of the bubble, which means that two bubbles with the same size can be characterised by different chord sizes. However, if a high number of bubbles is measured, it is relatively safe to assume that a higher mean chord size corresponds to bubbles with higher diameters.

Although it is not possible to have a full characterisation of the bubbles, these measurements allowed to see if the mass transfer variation obtained in the tested conditions was due to a variation in the specific interfacial area or not. It is not correct to say, even if there is a variation due to *a*, that the variation occurs exclusively by changes in this parameter because: (i) the values of *a* were not determined; and (ii) the variation of the k_L was not measured. Table 4.3 shows the values of mean chord size measured in the different oscillation frequencies. Table 4.4 shows the values of mean chord size measured in the different oscillation amplitudes. The variation of the mean chord

size with CO_2 concentration was not measured because the airflow was maintained constant (35 $mL \cdot min^{-1}$) and the effect of changing from 1 to 5% (v/v) CO_2 could be ignored.

Table 4. 3 - Mean chord size variation with frequency. Operational conditions - Q – 35 $mL \cdot min^{-1}$(0.5 vvm); Amplitude – 9 mm; % CO_2 in the inlet stream – 1% (v/v); Inoculum concentration – 164 mg $DW \cdot L^{-1}$

Amplitude, x_o (mm)	Frequency, f (Hz)	Mean chord size (mm)
0	0	5.08
9	1.0	7.20
9	1.5	5.24
9	2.0	4.17
9	3.0	2.55

Table 4. 4 - Mean chord size variation with amplitude. Operational conditions - Q – 35 $mL \cdot min^{-1}$(0.5 vvm); Frequency – 1.5 Hz; % CO_2 in the inlet stream – 1% (v/v); Inoculum concentration – 164 mg $DW \cdot L^{-1}$

Amplitude, x_o (mm)	Frequency, f (Hz)	Mean chord size (mm)
0	0	5.08
4	1.5	9.23
6	1.5	8.26
9	1.5	5.24
12	1.5	4.16
19	1.5	3.41

According to Table 4.3, a higher oscillation frequency leads to a lower mean chord size, because the increase in oscillation frequency provokes an increase in agitation and hence, the maintenance of small bubbles along the OFR-SPC. Likewise, according to Table 4.4, there is a decrease in mean chord size with the oscillation amplitude, which can be explained by the increased agitation promoted by higher amplitudes that difficult the aggregation of small bubbles into a bigger one and result in lower mean chord sizes.

Other studies also came to similar results, with Ferreira et al. (2017) concluding that the increase in oscillation (both frequency and amplitude) results in a reduction in bubbles' size and an increase in bubble residence time, with the consequent increase in the interfacial area.

4.2 Microalgal growth experiments

Microalgal growth in the OFR-SPC was performed to evaluate if the improvements in CO_2 mass transfer achieved in this reactor were favourable to microalgal growth and consequent CO_2 uptake. In addition, different operational conditions (oscillation amplitude and CO_2 concentration in the inlet gas) were evaluated, in order to infer about the most adequate conditions for microalgal

biomass production. The oscillation amplitudes evaluated in this study were 4, 6, 9, 12 and 19 mm and CO_2 concentrations evaluated were 0.04, 1 and 2% (v/v).

4.2.1 Biomass growth

To evaluate if this system provides the adequate conditions for microalgal growth, a first experiment with oscillation amplitude and frequency of 19 mm and 1.5 Hz, respectively, was performed using atmospheric air (with a CO_2 concentration of ≈0.04% v/v) in the gas inlet at a flow rate of 30-40 mL·min^{-1} (≈0.05 vvm). The growth curve obtained in these conditions is shown in Figure 4.3.

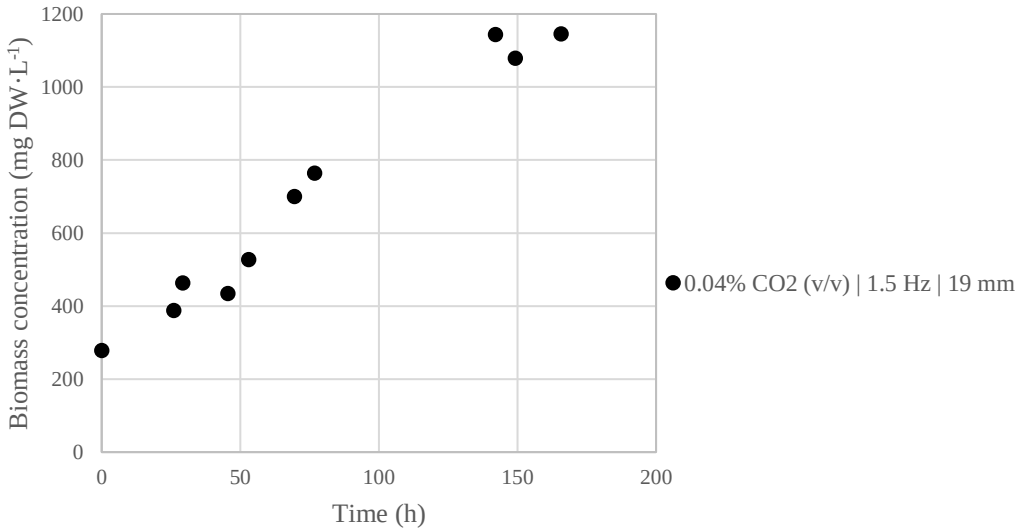

Figure 4. 3 – Growth curve obtained for *C. vulgaris* in the experiment performed with atmospheric CO_2 (≈0.04% v/v), gas flow rate of 30-40 mL·min^{-1} and oscillation amplitude and frequency of 19 mm and 1.5 Hz, respectively.

According to Figure 4.3, an increase in biomass concentration was observed during the experiment. It is important to note that, although in some moments there was a decrease when comparing with the previous value, this decrease is not significant, and can be attributed to measurement errors. There is growth during the first 24 hours (biomass concentration increased approximately 100 mg·L^{-1} in this period), which means that either there was not an adaption phase, or this one was very short. There was growth until 140 hours after the beginning of the experiment, with the biomass concentration growing approximately four times in this period. Afterwards, the stationary growth phase began and there was not a high variation in biomass concentration, maybe due to the depletion of nutrients required for microalgal growth. Therefore, with these results, one can conclude that it is possible to produce microalgae in this kind of bioreactor (OFR-SPC), even without an additional supply of CO_2. In addition, with an optimised set of parameters, this reactor can be a viable alternative to grow *C. vulgaris*. However, it is still necessary to evaluate its feasibility at an industrial scale. In a similar time scale, Gonçalves et al.

(2016) obtained a biomass concentration of 474 mg DW·L^{-1}, when growing batch cultures of *C. vulgaris* in 500-mL flasks supplied with atmospheric air at 0.5 vvm.

Taking into account the positive results obtained in this experiment, in terms of microalgal biomass production, to further improve biomass production and CO_2 uptake, experiments with increased CO_2 concentration (1% v/v) were performed. Initial conditions tested for this concentration were the same as for atmospheric CO_2 concentration: oscillation amplitude and frequency of 19 mm and 1.5 Hz, respectively, and gas flow rate of 35 mL·min^{-1} (0.5 vvm). Subsequent experiments were performed in the same conditions, but at gradually decreased oscillation amplitudes (12, 9 and 6 mm). Growth curves obtained in these experiments are shown in Figure 4.4 and pH and temperature values measured throughout the cultivation time are presented in Tables C.1-C.3 from Annex C (the pH and temperature for the experiment with 19 mm of amplitude was not measured).

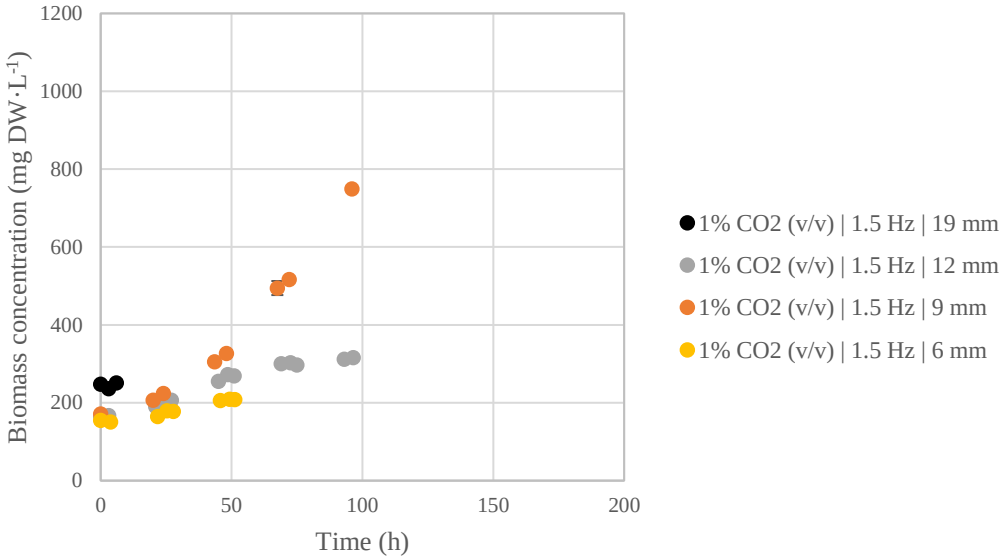

Figure 4. 4 - Growth curves obtained for *C. vulgaris* in the experiments performed with 1% (v/v) of CO_2, flow rate of 35 mL·min^{-1} (0.5 vvm), oscillation frequency of 1.5 Hz and variable amplitudes of 19, 12, 9 and 6 mm.

With the analysis of the Figure 4.4, one can see that in the experiments performed with an amplitude of 19 mm, *C. vulgaris* did not grow during the first day of culturing. In addition, after 24 h, microalgal cells present in the culture medium were in the form of flakes and most of them were settled at the bottom of the reactor, which limited microalgal growth in these conditions. For this reason, it was not possible to carry on the experiments. Figure 4.5-A shows a microscopic photograph of *C. vulgaris* flakes formed in these conditions and Figure 4.5-B shows microalgal cells settled at the bottom of the reactor. Aggregates formation in these conditions may be related to the high agitation observed in these conditions, in combination with an increased CO_2 concentration in the culture medium. Similar results were obtained by Abbott et al. (2015). A

solution to this problem is to increase the bubble size, which can be done by lowering the amplitude of the system, as demonstrated in Table 4.4. In the experiments performed with a lower amplitude (12 mm), an increase in biomass concentration was observed in the 96 h of cultivation period, with biomass concentration reaching a value of 316 mg $DW \cdot L^{-1}$. Although biomass concentration increased for 70 h, meaning that these conditions are better than previous ones, this increase was not as high as the one obtained in the experiments conducted with atmospheric air, which might be related to the formation of flakes observed after 70 h of culturing. Accordingly, it is possible to conclude that using an oscillation amplitude of 12 mm promoted microalgal growth, but further improvements should be attained, in order to avoid the achievement of the stationary growth phase due to flocs formation. Taking into account these results, the following experiments were performed using an oscillation amplitude of 9 mm. In these conditions, there was a constant increase in biomass concentration. For this reason, biomass concentration at the end of the cultivation period was much higher than the initial one (approximately four times higher). During these experiments, flakes formation was not observed, contrasting with the results obtained for higher oscillation amplitudes with the same CO_2 concentration. In these conditions, the decrease in amplitude resulted in a lower oscillation and in the formation of larger air bubbles, which, although create a lower gas-liquid mass transfer, also provoke less risks of flocculation. To further evaluate if microalgal biomass production could be enhanced by another decrease in oscillation amplitude, experiments with 6-mm oscillation amplitude were also performed. Growth curves obtained in these conditions show that there was a constant increase in biomass concentration within the cultivation period. However, biomass concentrations achieved were lower than those obtained for the amplitude of 9 mm. This might be related with the lower agitation and, consequently, lower gas-liquid mass transfer and CO_2 available for autotrophic growth observed for this oscillation amplitude. In these experiments, formation of flakes was not detected, which is in accordance with the lower agitation experienced in these conditions. With these results, one can conclude that *C. vulgaris* growth with 1% (v/v) CO_2 and oscillation frequency of 1.5 Hz is possible for an amplitude range of 6-12 mm. However, the best results for microalgal growth, both in terms of biomass production and non-ability for flakes formation, was obtained for the amplitude of 9 mm.

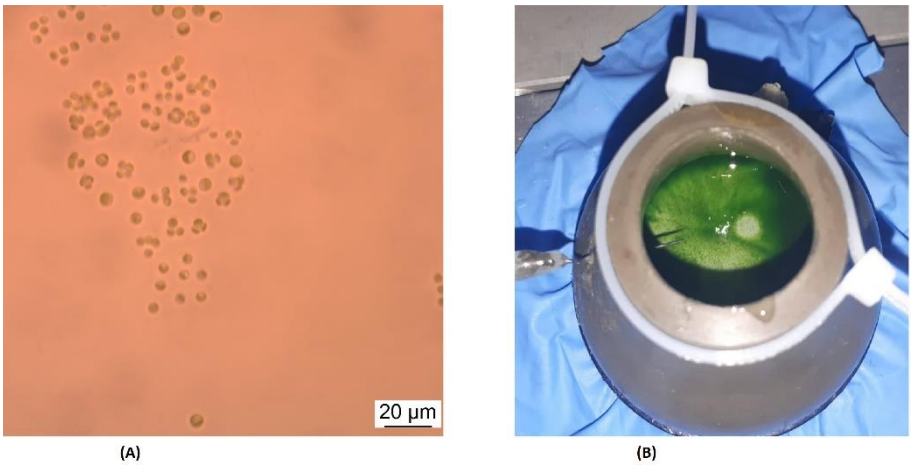

Figure 4. 5 – Flocculation occurred during the test with 1% (v/v) of CO_2, 19 mm of amplitude and 1.5 Hz of frequency; A – microscopic image of flakes present in the culture; B – image of flakes of microalgal cells settled at the bottom of the reactor.

Once optimization of microalgal growth in the OFR using 1% (v/v) CO_2 was performed and since the pH of the cultures in these experiments increased throughout the cultivation period, the possibility of increasing CO_2 concentration to 2% (v/v) was evaluated. For this, the optimal conditions obtained with 1% (v/v) CO_2 were reproduced at a higher concentration: oscillation amplitude and frequency of 9 mm and 1.5 Hz, respectively and gas flow rate of 35 $mL \cdot min^{-1}$ (0.5 vvm). Since these conditions promoted flakes formation, a further decrease in oscillation amplitude (to 4 mm) was evaluated. Figure 4.6 presents the growth curves obtained for *C. vulgaris* in these conditions. pH and temperature values measured in these cultures are presented in Tables C.4 and C.5 from Annex C.

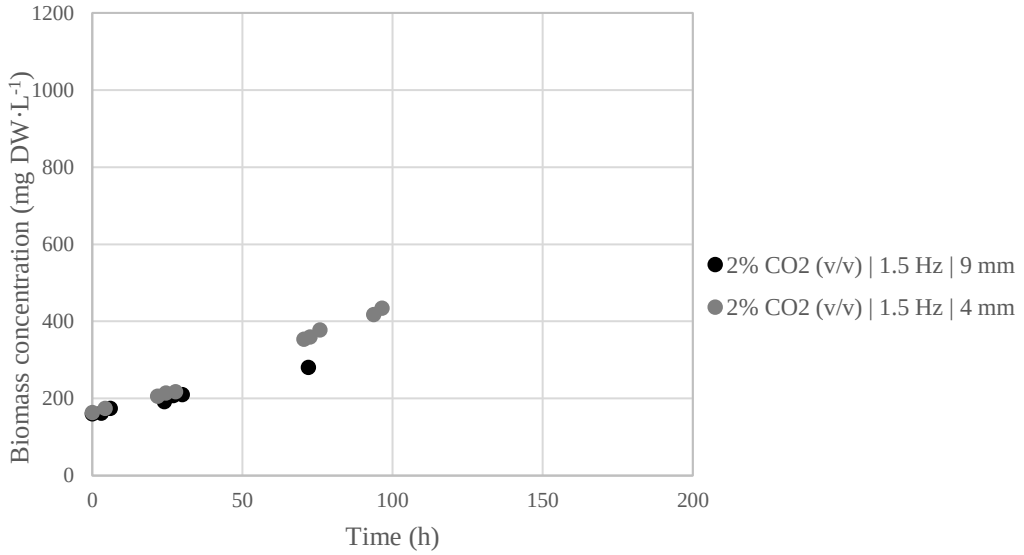

Figure 4. 6 - Growth curves obtained for *C. vulgaris* in the experiments performed with 2% (v/v) of CO_2, gas flow rate of 35 $mL \cdot min^{-1}$ (0.5 vvm), oscillation frequency of 1.5 Hz and variable amplitudes of 9 and 4 mm.

According to Figure 4.6, during the experiments conducted with 2% (v/v) CO_2 and 9 mm of oscillation amplitude, microalgal growth was not very high (after 72 h of culturing, biomass concentration only increased two times). One reason for the lower microalgal growth observed in these conditions may be the formation of flakes that occurred between 30 and 72 h, resulting in microalgal settling and in a reduction in the OD_{680} measured in the last experimental point. With these results, one can conclude that these conditions are not the most suitable for *C. vulgaris* growth. These results also confirm the hypothesis that the tendency for flakes formation increases as the CO_2 concentration increases, maintaining the oscillation conditions constant. In addition, for higher CO_2 concentrations, flakes formation can be avoided by decreasing agitation and promoting the formation of larger bubbles. This was done in the following experiment, where the oscillation amplitude was reduced to 4 mm. In these conditions, a constant increase in biomass concentration was observed throughout the cultivation time. However, biomass concentrations achieved in these conditions were not very high (434 mg $DW \cdot L^{-1}$), which may be due to the low amplitude used in this experiment, which provokes a low gas-liquid mass transfer and thus a low CO_2 concentration in the culture medium to be used by *C. vulgaris* in autotrophic growth. Due to the low agitation promoted in these conditions, flakes formation did not occur, which means that the lower biomass concentrations achieved in these conditions can be exclusively attributed to the low CO_2 mass transfer experienced in these conditions.

For the conditions evaluated in this study, both specific growth rates and average biomass productivities were determined, to infer about growth kinetics. The only exception was the determination of specific growth rate for the experiments conducted with 1% (v/v) CO_2 and oscillation amplitude of 19 mm, which was not possible because there was no microalgal growth. Table 4.5 shows the specific growth rates and average biomass productivities determined.

Table 4. 5 – Specific growth rates and average biomass productivities determined for *C. vulgaris* under the different operational conditions evaluated

%CO_2 (v/v)	Amplitude, x_o (mm)	Frequency, f (Hz)	μ (d^{-1})	P (mg $DW \cdot L^{-1} \cdot h^{-1}$)
0.04	19	1.5	0.239	5.23
1	19	1.5	ND	0.477
1	12	1.5	0.163	1.57
1	9	1.5	0.369	6.02
1	6	1.5	0.140	1.04
2	9	1.5	0.210	1.66
2	4	1.5	0.243	2.81

μ - specific growth rate; P - average biomass productivity; ND - not determined.

Table 4.5 shows that for 0.04% (v/v) CO_2, specific growth rates obtained were 0.239 d^{-1} and average biomass productivities were 5.23 mg $DW \cdot L^{-1} \cdot h^{-1}$. In cultures performed at 1% (v/v) specific growth rate values ranged between 0.140 and 0.369 d^{-1} and average biomass productivities varied between 0.477 and 6.02 mg $DW \cdot L^{-1} \cdot h^{-1}$. Finally, experiments conducted with a CO_2 concentration of 2% (v/v) resulted in specific growth rates of 0.210 and 0.243 d^{-1} and average biomass productivity values of 1.66 and 2.81 mg $DW \cdot L^{-1} \cdot h^{-1}$. These results are in accordance with the biomass concentration results present in Figures 4.3-4.6, with the highest biomass productivities achieved in the experiments with 0.04% (v/v) CO_2 / 19 mm / 1.5 Hz and 1% (v/v) CO_2 / 9 mm / 1.5 Hz. On the other hand, the lowest values were obtained in the experiment conducted with 1% (v/v) CO_2 / 19 mm / 1.5 Hz, where microalgal growth was not detected due to the occurrence of flocculation after 24 hours of the beginning of the experiment. As for microalgal growth, both specific growth rates and biomass productivities increased with an increase in amplitude, until reaching an optimum value, where these values start decreasing with further increase in oscillation amplitude.

There is a large number of studies focusing on *C. vulgaris* growth in different PBRs. For example, Fernandes et al. (2014) obtained a maximum volumetric biomass productivity of 31.25 mg $DW \cdot L^{-1} \cdot h^{-1}$ in a split cylinder airlift PBR, with a CO_2 concentration of 2% (v/v) (0.5 vvm), and superficial gas velocity of 0.0044 $m \cdot s^{-1}$, with a constant temperature of 30 ºC and an initial biomass concentration of 50 mg $DW \cdot L^{-1}$. Sadeghizadeh et al. (2017) obtained a specific growth rate of 0.2246 d^{-1} in an airlift PBR with 2% (v/v) of CO_2, using superficial air velocity of 1.6×10^{-3} $m \cdot s^{-1}$, and maintaining the culture at a constant temperature of 30 ºC. Chang et al. (2018) obtained a maximum biomass productivity of 10 mg $DW \cdot L^{-1} \cdot h^{-1}$ and an average biomass productivity of 6.25 mg $DW \cdot L^{-1} \cdot h^{-1}$ in a membrane PBR, using 5% (v/v) of CO_2 (0.3 vvm), a temperature of 25 ºC and an initial concentration of 100 mg $DW \cdot L^{-1}$. Zhu et al. (2019) obtained biomass productivity values of 14.30 mg $DW \cdot L^{-1} \cdot h^{-1}$ in a batch reactor, fed with 2% (v/v) of CO_2 (0.3 vvm). Although some of the reported values are higher than the highest values obtained in this study, the majority is close to the values obtained under the best operational conditions, however is important to refer that the operational conditions in the previously referred studies are not the same as the ones used in this study, and therefore one cannot compare both with high accuracy. Accordingly, it is possible to conclude that the OFR-SPC showed great potential in terms of mass transfer and biomass productivities, meaning that operational conditions can be further optimised to improve the obtained results.

4.2.2 Nutrients concentration

Throughout microalgal growth experiments, nutrients concentration in the culture medium was determined to evaluate if these nutrients were supplied in concentrations that do not limit microalgal growth and evaluate the performance of these cultures for nutrients uptake. Nutrients

monitored in this study were nitrate and phosphate, sources of two of the most important macronutrients (nitrogen and phosphorus) for the autotrophic growth of microalgae. This determination was performed for all the experiments, except in the experiments performed with atmospheric CO_2 concentration and in the experiments performed with 1% (v/v) CO_2 and an amplitude of 19 mm. Experiments performed with atmospheric CO_2 were exclusively used as control, to evaluate if the OFR-SPC could be used as a platform for microalgal growth. In the other experiments, nutrients concentration was not determined because no microalgal growth was observed in these conditions. The concentration of these nutrients was determined through spectrophotometric methods, with the calibration curves determined for both present in Figures A.2 and A.3 form Annex A. Figures 4.7 and 4.8 show the variation in concentration of nitrate for tests with 1 and 2% (v/v) CO_2, respectively. Likewise, Figures 4.9 and 4.10 show the concentration of phosphate in the experiments conducted with CO_2 concentrations of 1 and 2% (v/v), respectively.

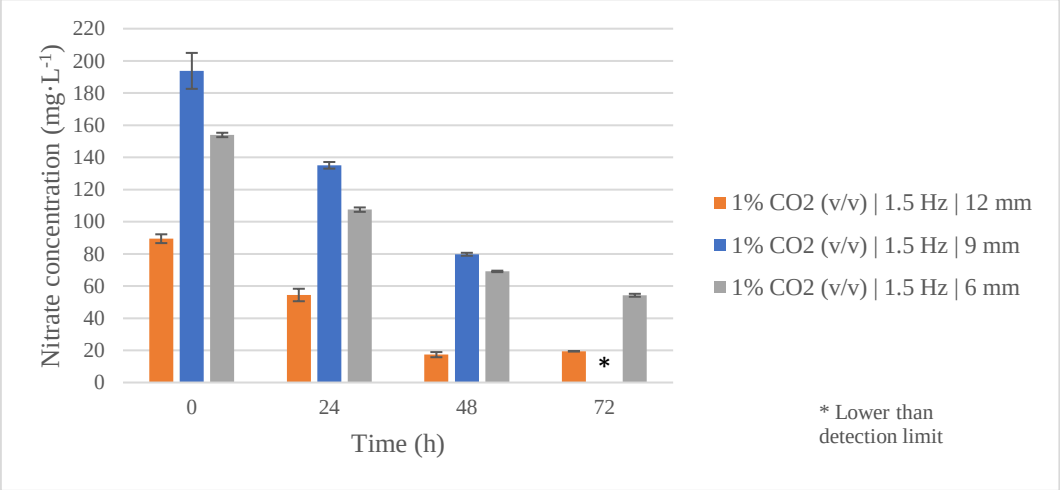

Figure 4. 7 - Nitrate concentration determined throughout the cultivation period in the experiments performed with 1% (v/v) of CO_2.

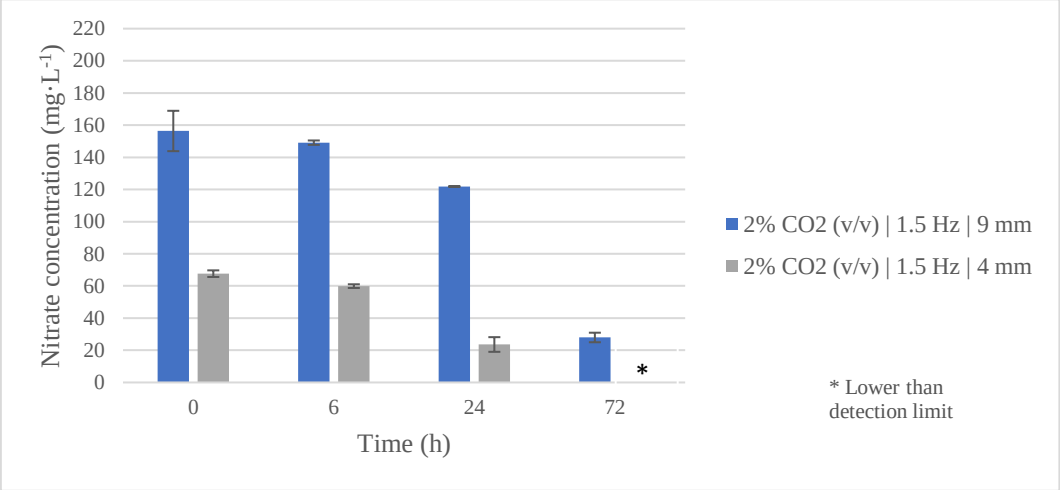

Figure 4. 8 - Nitrate concentration determined throughout the cultivation period in the experiments performed with 2% (v/v) of CO_2.

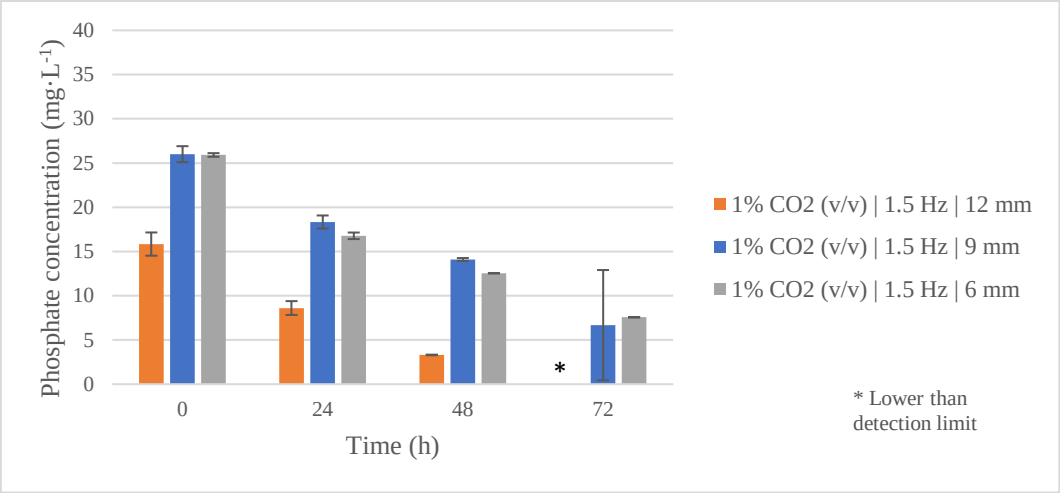

Figure 4. 9 – Phosphate concentration determined throughout the cultivation period in the experiments performed with 1% (v/v) of CO_2.

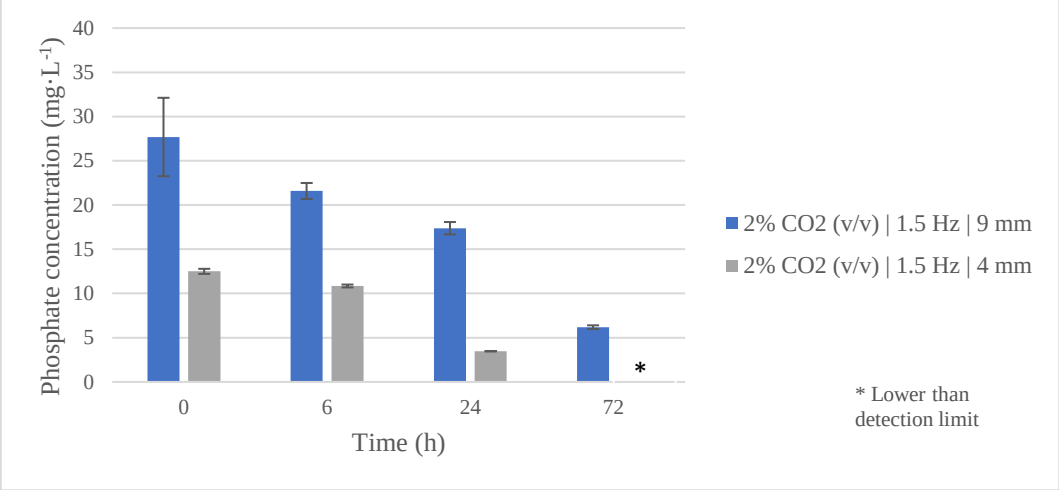

Figure 4. 10 - Phosphate concentration determined throughout the cultivation period in the experiments performed with 2% (v/v) of CO_2.

With the analysis of previous figures, one can see that both in the test with an amplitude of 4 mm and 2% (v/v) of CO_2 and in the test with 12 mm of amplitude and 1% (v/v) of CO_2, the initial concentration of both compounds was considerably lower than the other tests, and this is probably due to errors during the preparation of either the stock solutions or the culture medium. Apart from this, analysis of these data shows that there is a constant reduction in the concentration of nitrate and phosphate, which is related to the consumption of these nutrients in autotrophic growth. In cultures performed at 1% (v/v) CO_2 and an amplitude of 6 mm, nitrate concentrations decreased from 154 to 54.2 $mg \cdot L^{-1}$, and in cultures with 1% (v/v) CO_2 and an amplitude of 12 mm decreased from 89.4 to 19.4 $mg \cdot L^{-1}$. Similarly, for cultures supplied with 2% (v/v) CO_2 and an oscillation amplitude of 9 mm, nitrate concentrations decreased from 156 to 28.0 $mg \cdot L^{-1}$. In the other experiments, final concentrations of nitrate were almost negligible, since the values determined in these conditions were lower than the detection limit of the method, which

corresponds to 15.5 $mg \cdot L^{-1}$. In the case of phosphate concentration, these values decreased from 26.0 to 6.67 $mg \cdot L^{-1}$ in cultures with 1% (v/v) CO_2 and an amplitude of 6 mm, from 25.9 to 7.56 $mg \cdot L^{-1}$ in cultures performed with 1% (v/v) CO_2 and an amplitude of 9 mm and from 27.7 to 6.19 $mg \cdot L^{-1}$ in the cultures aerated with 2% (v/v) CO_2, with an oscillation amplitude of 9 mm. As for nitrate concentrations, phosphate concentration determined in the end of the other cultures was almost negligible, since values determined were below the detection limit of this method (1.97 $mg \cdot L^{-1}$).

Tables 4.6 and 4.7 show the values of removal efficiencies and average removal rates determined for nitrate and phosphate, respectively.

Table 4. 6 – Nitrate removal efficiencies and average removal rates obtained for the different microalgal growth experiments

Test	Removal Efficiency (%)	Average Removal Rate ($mg \cdot L^{-1} \cdot h^{-1}$)
1% CO_2 | 1.5 Hz | 12 mm	78.3	0.972
1% CO_2 | 1.5 Hz | 9 mm	>99	2.69
1% CO_2 | 1.5 Hz | 6 mm	64.8	1.38
2% CO_2 | 1.5 Hz | 9 mm	82.1	1.78
2% CO_2 | 1.5 Hz | 4 mm	>99	0.940

Table 4. 7 – Phosphate removal efficiencies and average removal rates obtained for the different microalgal growth experiments

Test	Removal Efficiency (%)	Average Removal Rate ($mg \cdot L^{-1} \cdot h^{-1}$)
1% CO_2 | 1.5 Hz | 12 mm	>99	0.220
1% CO_2 | 1.5 Hz | 9 mm	74.4	0.269
1% CO_2 | 1.5 Hz | 6 mm	70.8	0.255
2% CO_2 | 1.5 Hz | 9 mm	77.6	0.299
2% CO_2 | 1.5 Hz | 4 mm	>99	0.173

According to the results from Table 4.6, the highest nitrate removal efficiencies (approximately 100%) and removal rates (2.69 $mg \cdot L^{-1} \cdot h^{-1}$) were obtained in the experiments with 1% (v/v) CO_2, 9 mm of amplitude and 1.5 Hz of frequency, which agrees with the results obtained for microalgal growth. In the case of phosphate, the same conditions were also favourable, with removal efficiencies of 74% and average removal rates of 0.269 $mg \cdot L^{-1} \cdot h^{-1}$ (Table 4.7). On the other hand, although having the lowest removal rate for both nutrients, the test with 2% CO_2, 4 mm of amplitude and 1.5 Hz of frequency resulted in almost 100% removal of both nutrients,

which can be explained by the lower initial nutrients concentration supplied in this experiment. In addition, these conditions resulted in promising values of specific growth rate and average biomass productivities, just after the results obtained with 1% (v/v) CO_2 and an oscillation amplitude of 9 mm. Accordingly, these results suggest that if the initial nutrients concentration was higher, the experiments could have resulted in even higher biomass productivities. The higher biomass concentrations together with high nutrients removal efficiencies constitute very promising results that can lead to other applications of microalgae, namely the possibility of using wastewater for microalgal growth. In addition to produce microalgal biomass, the use of wastewaters also promotes treatment of said waters, which leads to further advantages, both environmental (the use of freshwater can be avoided) and economic (less costs regarding the acquisition of nutrients for microalgal growth).

5. Conclusions and future research

5.1 General conclusions

The k_La in an OFR-SPC increased with an increase in oscillation amplitude, being this the most influent parameter in k_La variation. An increase in oscillation frequency also leads to an increase in k_La, mainly frequencies above 1 Hz, meaning that increasing the agitation of the OFR-SPC is a good way to increase the gas-liquid mass transfer. Increasing the CO_2 concentration in the airflow is also an alternative to increase the mass transfer coefficient, mainly when working with higher amplitudes (9 mm or higher).

An increase in amplitude results in a decrease in chord size of the bubbles, because the higher amplitude provokes higher agitation, which leads to a decrease in bubble size. For the same reason, an increase in frequency also results in a decrease in chord size.

The best results of microalgal growth were obtained in the experiments with 1% (v/v) of CO_2, 9 mm of amplitude and 1.5 Hz of frequency. In these conditions biomass concentration increased approximately four times, with this test having a specific growth rate of 0.369 d^{-1} and an average biomass productivity of 6.02 mg $DW \cdot L^{-1} \cdot h^{-1}$.

In addition, one can see that an increase in oscillation amplitude would lead to an increase in microalgal growth, while the agitation is not high enough to provoke flocculation. When flocculation starts to occur, an increase in oscillation amplitude or frequency would lead to worse results in microalgal growth, because this phenomenon would occur earlier and that would result in less time for microalgae to grow before they start to flocculate, which is not desirable.

On the other side, an increase in CO_2 concentration would lead to the start of the flocculation at lower amplitudes. In opposition, an increase in CO_2 concentration leads to the possibility of growing microalgae at lower amplitudes. In this study better growth was obtained with 9 mm and 1% (v/v) of CO_2 when compared with the one obtained with atmospheric air and 19 mm. Similarly, biomass productivity achieved with 4 mm and 2% (v/v) of CO_2 was twice the value obtained with only 1% (v/v) of CO_2 and a slightly higher amplitude (6 mm).

There was a constant decrease in nutrients (nitrate and phosphate) concentrations during the experiments, with removal efficiencies exceeding 70% for both nutrients in all studied conditions after 72 hours. Additionally, average removal rates obtained in the OFR-SPC ranged between 0.940 and 2.69 $mg \cdot L^{-1} \cdot h^{-1}$, for nitrate, and between 0.173 and 0.299 $mg \cdot L^{-1} \cdot h^{-1}$, for phosphate.

5.2 Future Research

In this study, it was assessed several different growth conditions. However, it is still necessary to do more experiments with different operational conditions, in order to obtain a better efficiency of the system.

It is necessary to study the growth of *C. vulgaris* in an OFR-SPC with different pairs of oscillation amplitude and CO_2 concentration, in order to optimise the process. It is also necessary to test the feasibility of growth of said microalgae with slightly higher CO_2 concentrations in the airflow. The possibility of using higher CO_2 concentrations would lead to the possibility of using lower amplitudes and frequencies, which would consequently lead to a reduction in the energy input and operational costs of the OFR-SPC. In addition, it is necessary to test the influence of the oscillation frequency in microalgal growth in this kind of reactor and optimise this operational condition together with CO_2 concentration and amplitude.

It is also necessary to test the possibility of using the OFR-SPC to saturate with CO_2 a certain volume of a culture medium, which afterwards would be continually fed to a reactor, to improve the efficiency of CO_2 usage by the microalgae in that reactor. It was already done a preliminary test to access this possibility, where approximately 80 mL of two cultures with same initial conditions were put to grow in 100-mL glass flasks, one of them previously saturated with 1% (v/v) CO_2 in the OFR-SPC and the other used as control. In annex D, the growth curves obtained in this preliminary experiment are presented (Figure D.1). The values of temperature and pH measured in the saturated and non-saturated cultures are presented in Tables D.1 and D.2, respectively. Although the difference in biomass growth between the previously saturated culture and non-saturated one was not high, further experiments should be performed to determine if this method is feasible or not.

Taking into account the flocculation that occurs with higher oscillation amplitudes and frequencies, it is necessary to test the possibility of using this phenomenon to harvest the biomass after the production phase, by increasing the oscillation in the OFR-SPC. This would promote aggregates formation, thus contributing to the development of an efficient harvesting method, without the need of increasing much the operational costs of the system.

Finally, it is also important to test the possibility of using an OFR-SPC to both microalgal growth and wastewater treatment, namely nutrient removal for wastewater. The use of wastewater to microalgal growth is a better alternative both environmental and economical, because it removes pollutants from wastewater and reduces the requirements for clean water to microalgal growth, as well as reduces the costs of production, namely the costs associated with nutrient acquisition. However, to use this reactor to treat wastewater it is necessary to achieve values of concentration below the limits defined by EU in Directive 1991/271/EEC, 1991 and Directive 1998/15/EC, 1998. According to these Directives, the limits for effluent discharge per population

equivalent (PE) are: (i) 15 mg $N \cdot L^{-1}$ (10 to 100 thousand PE) or 10 mg $N \cdot L^{-1}$ (more than 100 thousand PE) for total nitrogen, or a minimum percentage of reduction of 70-80%; and (ii) 2 mg $P \cdot L^{-1}$ (10 to 100 thousand PE) or 1 mg $P \cdot L^{-1}$ (more than 100 thousand PE) for total phosphorus, or a minimum percentage of reduction of 80% (Directive 1998/15/EC 1998; Directive 1991/271/EEC 1991). The values of concentration and reduction of nutrients measured in the experiments were very close to the legal limits for discharge, but further improvements are required to use the OFR-SPC for both microalgal biomass production and nutrients removal.

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Annex A

A.1 Calibration curve of optical density *vs* biomass concentration

Figure A.2 shows the calibration curve of absorbance measured at 680 nm *versus* biomass concentration, in mg DW·L^{-1}.

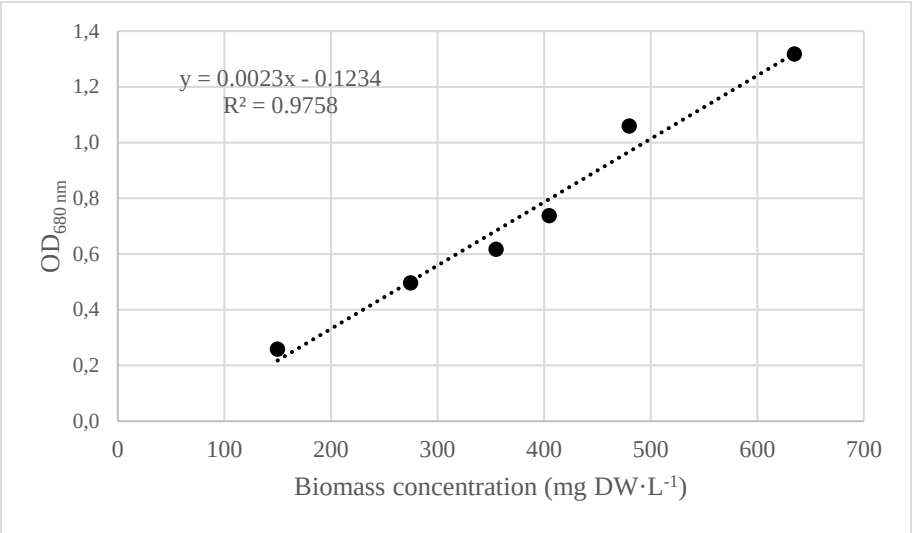

Figure A. 1 - Biomass calibration curve.

A.2 Calibration curve for nitrate quantification

Figure A.2 shows the calibration curve of absorbance measured at 220 nm *versus* nitrate concentration, in mg·L^{-1}.

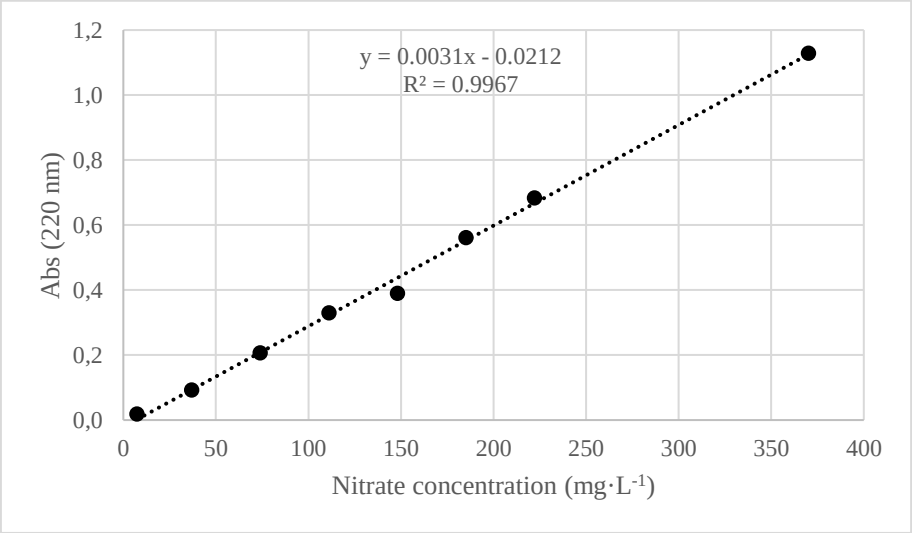

Figure A. 2 - Nitrate calibration curve.

A.3 Calibration curve for phosphate quantification

Figure A.3 shows the calibration curve of absorbance measured at 820 nm *versus* phosphate concentration, in $mg \cdot L^{-1}$.

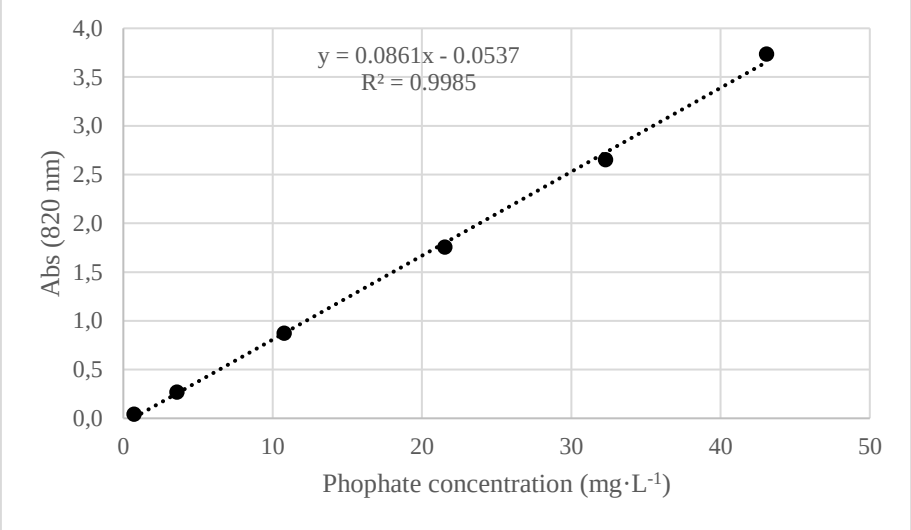

Figure A. 3 - Phosphate calibration curve.

Annex B

The following figures (figures B.1 to B.19) show the variation in CO_2 concentration in time during the saturation period, measured by the CO_2 probe and used in the k_La determinations.

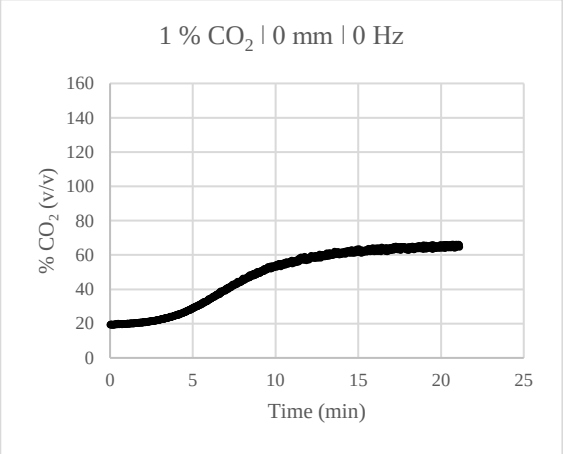

Figure B. 1 - Saturation without agitation.

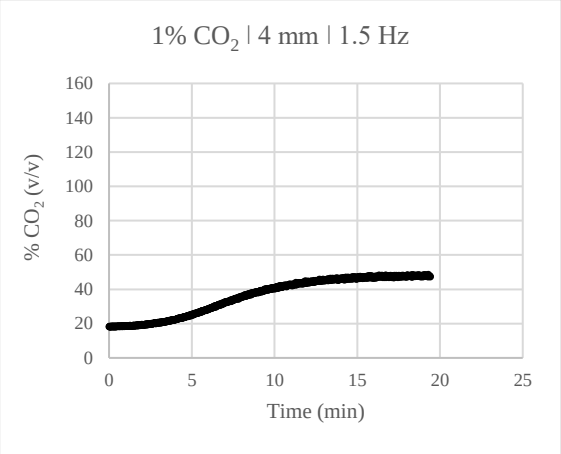

Figure B. 2 - Saturation with 1% (v/v) of CO_2, 4 mm and 1.5 Hz.

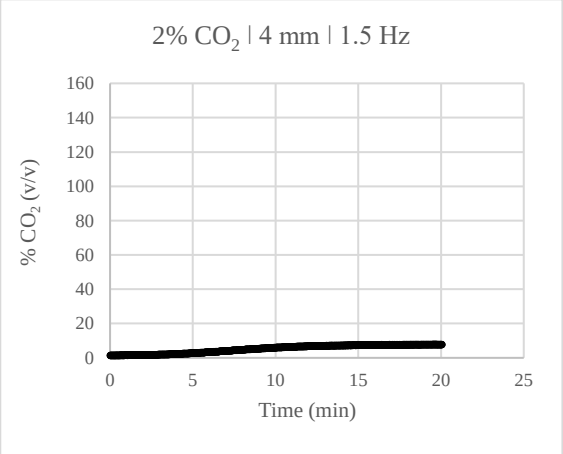

Figure B. 3 - Saturation with 2% (v/v) of CO_2, 4 mm and 1.5 Hz.

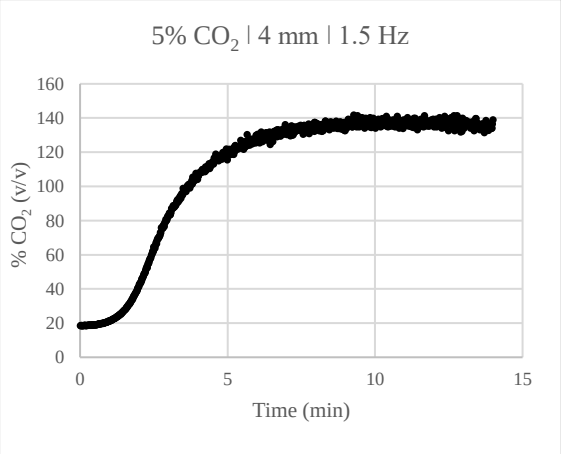

Figure B. 4 - Saturation with 5% (v/v) of CO_2, 4 mm and 1.5 Hz.

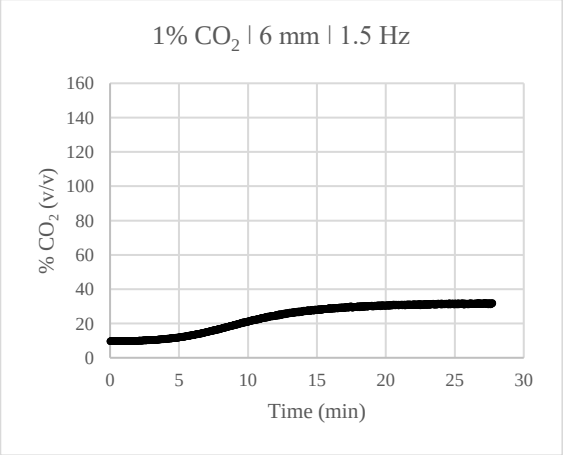

Figure B. 5 - Saturation with 1% (v/v) of CO_2, 6 mm and 1.5 Hz.

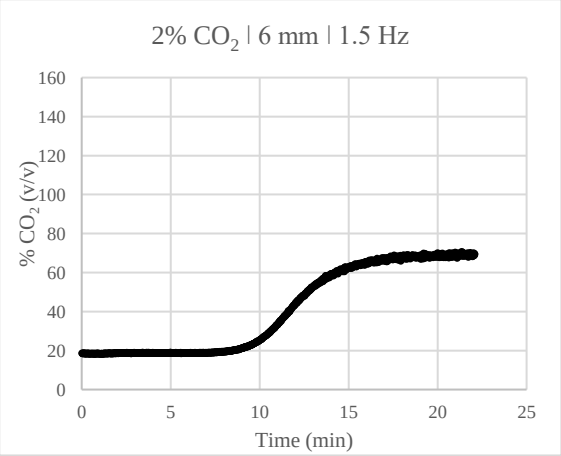

Figure B. 6 - Saturation with 2% (v/v) of CO_2, 6 mm and 1.5 Hz.

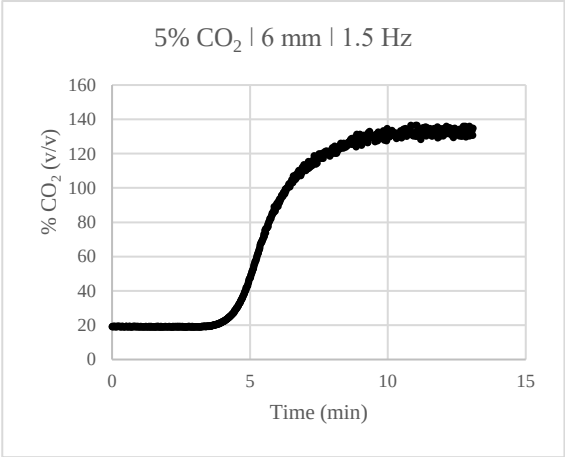

Figure B. 7 - Saturation with 5% (v/v) of CO_2, 6 mm and 1.5 Hz.

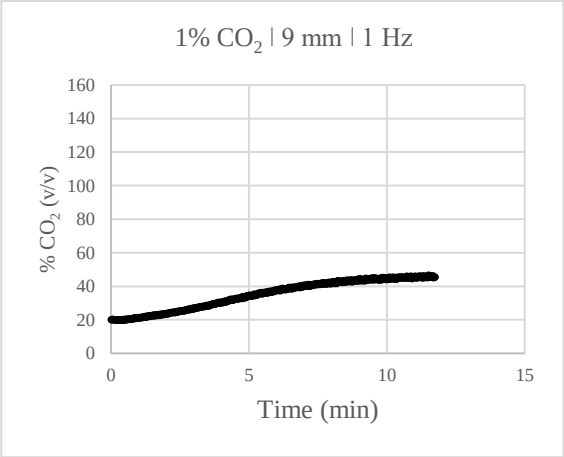

Figure B. 8 - Saturation with 1% (v/v) of CO_2, 9 mm and 1 Hz.

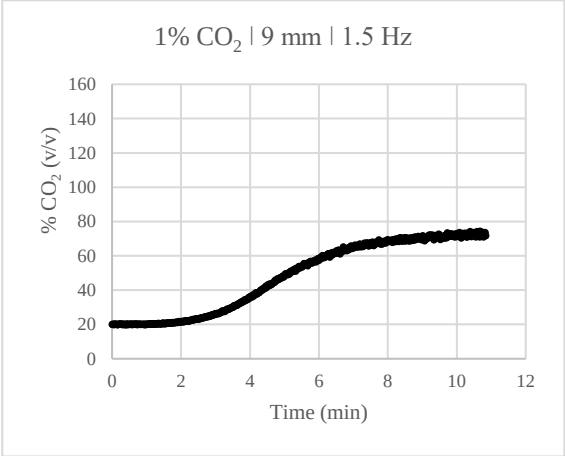

Figure B. 9 - Saturation with 1% (v/v) of CO_2, 9 mm and 1.5 Hz.

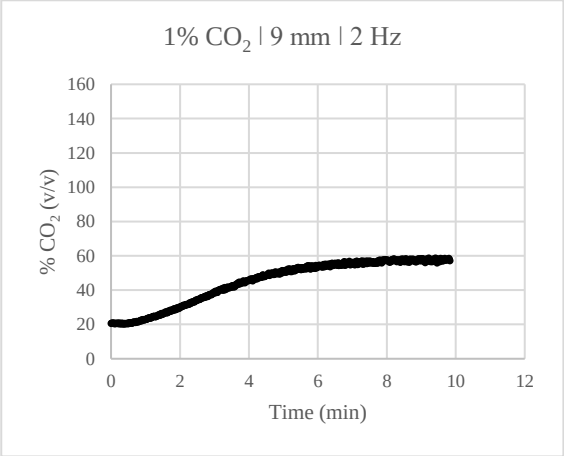

Figure B. 10 - Saturation with 1% (v/v) of CO_2, 9 mm and 2 Hz.

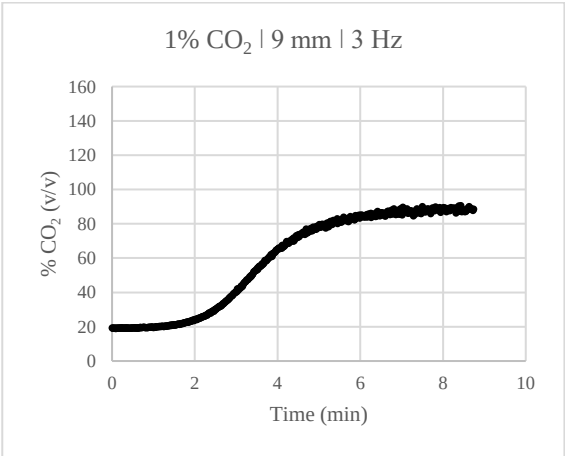

Figure B. 11 - Saturation with 1% (v/v) of CO_2, 9 mm and 3 Hz.

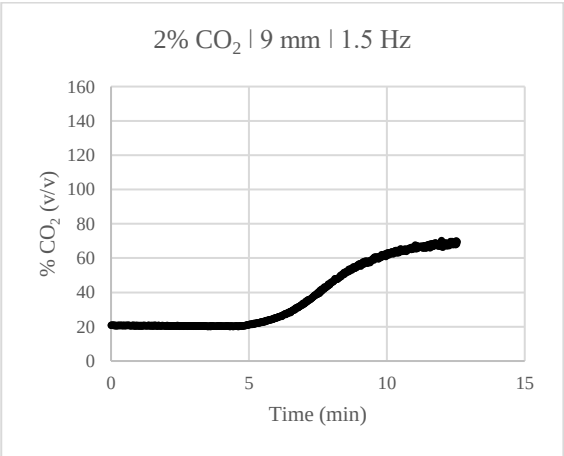

Figure B. 12 - Saturation with 2% (v/v) of CO_2, 9 mm and 1.5 Hz.

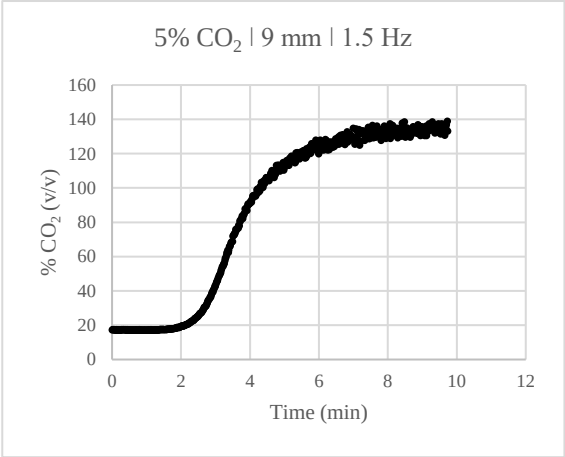

Figure B. 13 - Saturation with 5% (v/v) of CO_2, 9 mm and 1.5 Hz.

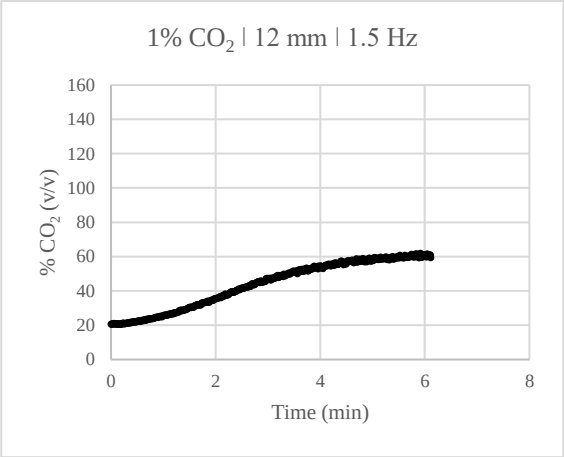

Figure B. 14 - Saturation with 1% (v/v) of CO_2, 12 mm and 1.5 Hz.

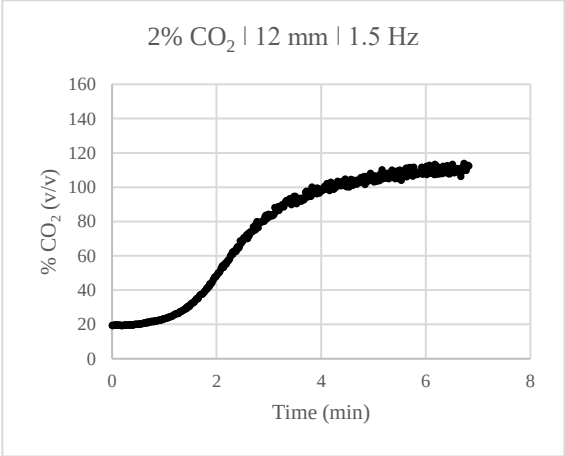

Figure B. 15 - Saturation with 2% (v/v) of CO_2, 12 mm and 1.5 Hz.

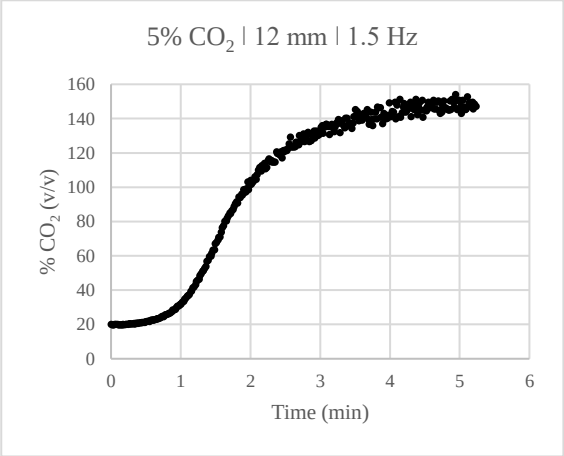

Figure B. 16 - Saturation with 5% (v/v) of CO_2, 12 mm and 1.5 Hz.

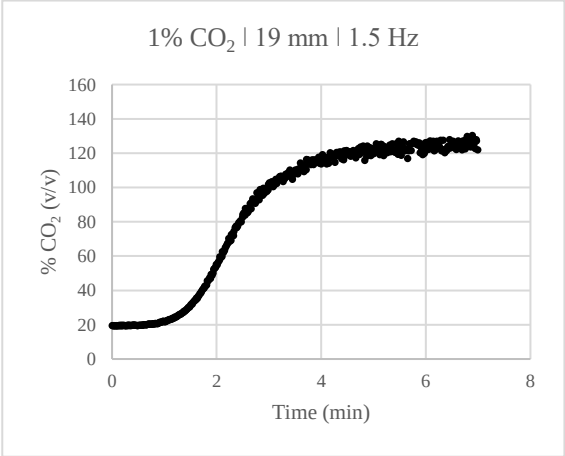

Figure B. 17 - Saturation with 1% (v/v) of CO_2, 19 mm and 1.5 Hz.

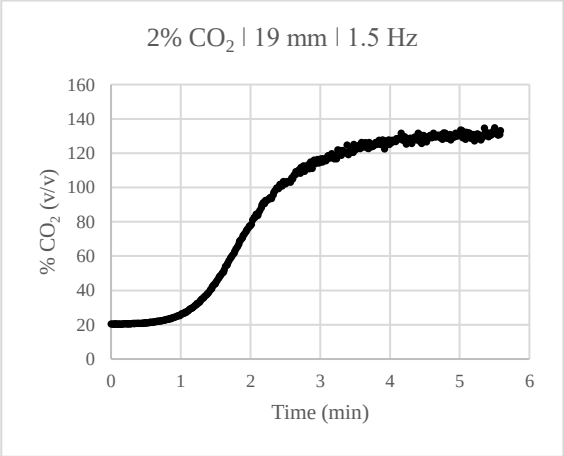

Figure B. 18 - Saturation with 2% (v/v) of CO_2, 19 mm and 1.5 Hz.

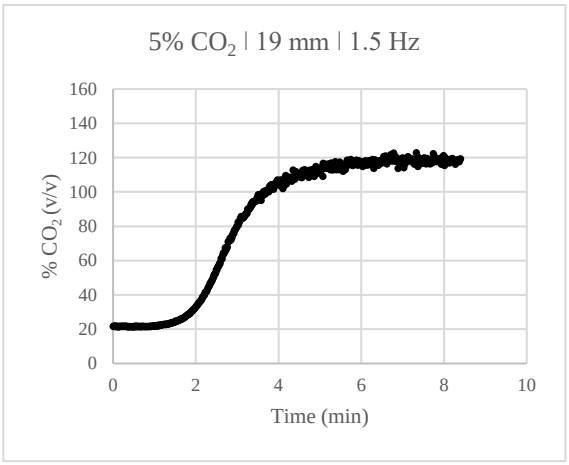

Figure B. 19 - Saturation with 5% (v/v) of CO_2, 19 mm and 1.5 Hz.

Annex C

In this section it is shown the pH and temperature measured in the various experiments involving *C. vulgaris* growth.

C.1 Test with CO_2 of 1% (v/v) and 12 mm of amplitude

Table C.1 shows the values of pH and temperature measured in the experiments with 1% (v/v) of CO_2 and 12 mm of amplitude.

Table C. 1 - pH and temperature determined in the experiments with 1% (v/v) of CO_2 and oscillation amplitude of 12 mm

Time (h)	pH	Temperature (ºC)
0	6.45	23.2
3	6.92	27.1
21	7.12	25.8
24.5	7.15	26.3
27	7.32	25.2
45	7.27	24.5
48.5	7.28	24.1
51	7.29	24.4
69	7.21	25.9
72.5	7.26	24.3
75	7.30	25.0
93	7.29	24.8
96.5	7.33	24.4

C.2 Test with CO_2 of 1% (v/v) and 9 mm of amplitude

Table C.2 shows the values of pH and temperature measured in the experiments with 1% (v/v) of CO_2 and 9 mm of amplitude.

Table C. 2 - pH and temperature determined in the experiments with 1% (v/v) of CO_2 and an oscillation amplitude of 9 mm

Time (h)	pH	Temperature (ºC)
0	6.71	27.3
20	6.99	23.8
24	6.98	24.1
43.5	7.22	24.1
48	7.23	23.9
67.5	7.35	24.0
72	7.22	24.2
96	6.85	24.2

C.3 Test with CO_2 of 1% (v/v) and 6 mm of amplitude

Table C.3 shows the values of pH and temperature measured in the experiments with 1% (v/v) of CO_2 and 6 mm of amplitude.

Table C. 3 - pH and temperature determined in the experiments with 1% (v/v) of CO_2 and an oscillation amplitude of 6 mm

Time (h)	pH	Temperature (ºC)
0	6.50	24.5
3.75	6.83	25.6
21.75	7.03	25.9
25.25	7.02	25.5
27.75	7.00	26.3
45.75	7.18	25.7
49.25	7.23	25.6
51.25	7.23	26.2

C.4 Test with CO_2 of 2% (v/v) and 9 mm of amplitude

Table C.4 shows the values of pH and temperature measured in the experiments with 2% (v/v) of CO_2 and 9 mm of amplitude.

Table C. 4 - pH and temperature determined in the experiments with 2% (v/v) of CO_2 and an oscillation amplitude of 9 mm

Time (h)	pH	Temperature (ºC)
0	6.37	24.2
3	6.48	24.7
6	6.48	24.6
24	6.61	24.5
27	6.64	24.2
30	6.63	24.6
72	6.91	24.5

C.5 Test with CO_2 of 2% (v/v) and 4 mm of amplitude

Table C.5 shows the values of pH and temperature measured in the experiments with 2% (v/v) of CO_2 and 4 mm of amplitude.

Table C. 5 - pH and temperature determined in the experiments with 2% (v/v) of CO_2 and an oscillation amplitude of 4 mm

Time (h)	pH	Temperature (ºC)
0	6.12	26.9
4.25	6.16	25.5
21.75	6.54	24.3
24.5	6.56	24.6
27.75	6.45	25.1
70.5	6.74	24.6
72.5	6.75	24.9
75.75	6.78	25.6
93.75	6.76	25.7
96.5	6.80	25.6

Annex D

In this section, it is shown the results obtained in the test of the saturation of the culture with CO_2 and further growth in shots. Tables D.1 and D.2 show the values of pH and temperature measured in this test, for the saturated and unsaturated cultures, respectively.

Table D. 1 - pH and temperature determined in the experiment with previous saturation of the culture with CO_2

Time (h)	pH	Temperature (ºC)
0	6.50	21.9
4	8.73	19.9
6	9.24	21.0
24	10.25	18.4
28	8.80	21.4
30	9.07	22.2
48	9.80	19.3
53	9.41	19.4
55	9.50	20.1
72	9.81	19.3

Table D. 2 - pH and temperature determined in the experiment without previous saturation of the culture with CO_2

Time (h)	pH	Temperature (ºC)
0	7.37	21.3
4	9.01	20.2
6	9.41	20.7
24	10.11	18.1
28	8.74	21.0
30	9.01	21.7
48	9.71	19.3
53	9.36	19.1
55	9.44	19.6
72	9.70	18.7

Figure D.1 shows the values of biomass concentration obtained in this experiment, for both the culture previously saturated and the culture not saturated.

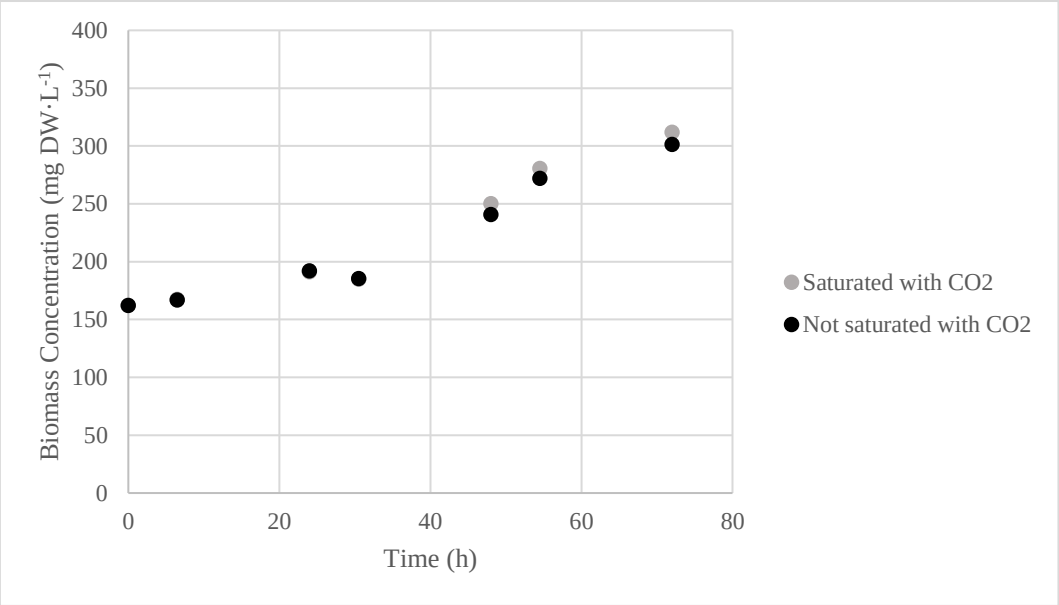

Figure D. 1 - Growth curves obtained for *C. vulgaris* in the experiments in glass flasks, with and without previous saturation of the culture with 1% (v/v) of CO_2 in the OFR-SPC.