Brewery wastewater treatment by microalgae using closed bubble-column photobioreactors

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RESUMO

As microalgas aparecem como uma alternativa para as matérias-primas tradicionais para a produção de biodiesel, tais como óleos vegetais ou gorduras animais e outros produtos residuais, que estão associados a impactos económicos, sociais e ambientais. Além disso, a capacidade de elevadas produtividades em biomassa e lípidos em conjunto com a possibilidade de utilizar os nutrientes de águas residuais e CO_2 de emissões gasosas pode ajudar a reduzir os impactos ambientais e custos de cultivo. A acrescentar, tem-se o facto de a biomassa das microalgas ser utilizada para a produção de rações animais, fertilizantes, outros combustíveis e produtos químicos.

A presente tese insere-se no projecto ELAC2014/BEE0357 intitulado "GREENBIOREFINERY - Processing of brewery wastes with microalgae for producing valuable compounds". O principal objetivo do trabalho foi avaliar o crescimento e o tratamento de águas residuais duma indústria cervejeira utilizando a microalga *Scenedesmus obliquus*, em fotobiorreactores tipo coluna de bolhas, nos modos *batch* (5 L) e contínuo (6 L). Foram testados sete tempos de residência (τ) : 1,72; 2,26; 3,85; 5,58; 7,55; 9,46 e 11,80 dias.

Em relação ao estudo em *batch*, foi obtida uma concentração (AFDW) de 0,93 ± 0,01 g/L e uma produtividade em biomassa máxima e média de 156,80 ± 4,65 mg/L.dia e 87,11 ± 3,37 mg/L.dia, respectivamente. Utilizando o modo contínuo, o valor máximo de AFDW foi obtido para a taxa de diluição (D) de 0,08 dia^{-1} (τ = 11,80 dias): 0,95 ± 0,04 g/L. A taxa de diluição óptima para a produtividade em biomassa foi de 0,26 dia^{-1} (τ = 3,85 dias), atingindo um valor de 224,30 ± 22,99 mg/L.dia.

As culturas em *batch* e com D = 0.26 dia^{-1} apresentaram o tratamento da água mais eficiente. Na produção em *batch*, foi obtida uma remoção de 75% para o azoto total Kjeldahl, 40% para o fósforo e 70% para a carência química de oxigénio (CQO). Por outro lado, com taxa de diluição de 0,26 dia^{-1}, taxas de remoção de CQO e azoto ligeiramente superiores foram conseguidas (74 e 76%, respectivamente), mas a remoção de fósforo não foi tão eficiente (23%). Excepto para D = 0,58 dia^{-1} (τ = 1,72 dias), o efluente tratado mostrou valores concordantes com a legislação ambiental (decreto-lei 236/98) para descarga em águas naturais receptoras.

A biomassa produzida por *S. obliquus* apresentou um conteúdo em proteína máximo de 50.09% com D = 0,26 dia^{-1} e um mínimo de 21,15% após crescimento em modo *batch*. Com a taxa de diluição anterior foi também obtida a maior percentagem de viabilidade celular (92,33 ± 0,53%).

Palavras-chave: Fotobiorreactor, tratamento de águas residuais de cervejeira, crescimento da biomassa, *Scenedesmus obliquus.*

ABSTRACT

Microalgae seem to be an alternative to the traditional biodiesel feedstocks such as edible vegetable oils or animal fats and other residual products associated to economic, social and environmental impacts. Also, their potential high biomass and lipid productivities and the possibility of using nutrients from waste streams (e.g. wastewaters and/or CO_2 flue gas emissions) can help reduce the environmental impacts and costs of cultivation. Moreover, microalgae are a feedstock for production of feeds, fertilizers, other fuels and chemicals.

The present work was carried out within the project ELAC2014/BEE0357 entitled GREENBIOREFINERY - Processing of brewery wastes with microalgae for producing valuable compounds. The main objective of the thesis was to evaluate the growth and brewery wastewater treatment by *Scenedesmus obliquus*, in bubble-column type photobioreactors, under batch (5 L capacity) and continuous mode (6 L capacity). Seven residence times (τ) were tested: 1.72, 2.26, 3.85, 5.58, 7.55, 9,46 and 11.80 days.

Regarding the trial in batch mode, it was obtained an ash-free dry weight (AFDW) of 0.93 ± 0.01 g/L and a maximum and average biomass productivity of 156.80 ± 4.65 mg/L.day and 87.11 ± 3.37 mg/L.day, respectively. Concerning the continuous mode, the highest AFDW was obtained for the dilution rate (D) of 0.08 day^{-1} (τ = 11.80 days): 0.95 ± 0.04 g/L. The optimal dilution rate for biomass productivity was 0.26 day^{-1} (τ = 3.85 days), reaching 224.30 ± 22.99 mg/L.day.

Batch culture and continuous culture at D = 0.26 day^{-1} presented the best wastewater treatment efficiency. Batch production attained a 75% removal for total Kjeldahl nitrogen, 43% for phosphorous and 70% for chemical oxygen demand (COD). On the other hand, at D = 0.26 day^{-1}, slightly higher removal rates for COD and N were reached (74 and 76%, respectively), but the removal of P was not so efficient (23% removal). Except for the dilution rate of 0.58 day^{-1} (τ = 1.72 days), the treated water had values that are in accordance with Portuguese environmental legislation (Decree-Law 236/98) for discharge in receiving water bodies.

S. obliquus biomass presented a maximum protein content of 50.09% for D = 0.26 day^{-1} and a minimum of 21.15% for the culture in batch mode. At that dilution rate of D = 0.26 day^{-1} the biomass productivity, removal of nutrient and biomass protein content were maximum, and these results are in accordance to the highest percentage of viable cell (92.33 ± 0.53 %).

Key words: Photobioreactor, brewery wastewater treatment, biomass growth, *Scenedesmus obliquus.*

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ABBREVIATIONS AND SYMBOLS

AFDW, ash-free dry weight (g/L)

BOD, biochemical oxygen demand (mg O_2/L)

BWW, brewery wastewater

cFDA, Carboxyfluorescein Diacetate

Chl, chlorophyll

COD, chemical oxygen demand (mg O_2/L)

D, dilution rate (day^{-1})

DW, dry weight (g/L)

FAME, fatty acid methyl ester

FC, flow cytometry

μ, specific growth rate (day^{-1})

τ, residence time (day)

t_d, duplication time (day)

OD, optical density

P_x, biomass productivity (g/L day)

PBR, photobioreactor

PI, Propidium iodide

TC, total carbon (mg/L)

TN, total nitrogen (mg/L)

TOC, total organic carbon (mg/L)

TP, total phosphorous (mg/L)

X, biomass concentration (g/L)

1. OBJECTIVES

This experimental work aims to study the ability of microalgae for brewery wastewater treatment.

The first objective was to define the most suitable microalga for growth in brewery wastewater. For that, it was tested the microalgae *Chlorella vulgaris, Chlorella protothecoides*, *Scenedesmus obliquus* and *Neochloris oleoabundans* and a consortium previously isolated from an urban wastewater.

The second and the main objective was to evaluate the growth and the wastewater treatment by the chosen microalga, *Scenedesmus obliquus* (ACOI 204/07). The experiment was performed at laboratory scale using simple bubble-column type photobioreactors, which were operated in batch and continuous regimes. The influence of the dilution rate in the growth, the physiological state of the microalgal cells and the efficiency of the treatment was studied.

The last objective was to characterize the produced biomass, in terms of chlorophyll and protein content and fatty acid profile and also to evaluate the effect of the dilution rate on these aspects.

2. LITERATURE REVIEW

2.1 Microalgae: biology and diversity

Microalgae are an extremely heterogeneous group of mainly photoautotrophic organisms, that can be eukaryotic, for example green algae (Chlorophyta) and diatoms (Bacillariophyta), or prokaryotic as cyanobacteria (Cyanophyceae) (Mata *et al.,* 2010).

They are tallophytes, not having roots, stems or leaves and do not have covering of cells around the reproductive cells. Their primary photosynthetic pigment is chlorophyll *a* (Brennan & Owende, 2010).

Microalgae are present in all existing earth ecosystems, not just aquatic but also terrestrial, being capable of surviving in extreme habitats, as snowfields, hot springs and desert soil (Marques *et al.,* 2012).

Because they are key primary producers, microalgae play a vital role in the Earth's carbon cycle and are primarily responsible for 40-50% of total global photosynthetic primary production contributed by all algae (Marques *et al.,* 2012).

2.2 Microalgal biomass production

2.2.1 Microalgae nutritional modes

Microalgae can assume many types of nutritional modes (e.g. autotrophic, heterotrophic, mixotrophic) and are capable of shifting modes depending on the environmental conditions.

Photoautotrophic microalgae require only inorganic compounds such as CO_2, salts and a light energy source for growth. Photosynthesis is a key component by converting solar radiation and CO_2 absorbed by chloroplasts into adenosine triphosphate (ATP) and O_2, which is then used in respiration to produce energy to support growth (Brennan & Owende, 2010).

In the heterotrophic process, microalgae growth is independent of light energy, requiring organic carbon substrates as energy source. Comparing to autophototropic production, this system provides a high degree of growth control, needs minimal set-up costs and simpler scale-up possibilities are possible since smaller reactor surface to volume ratios can be used (due to light independence). Furthermore, heterotrophic cultivation can result in accumulation of high lipid content in cells (Brennan & Owende, 2010).

Some algae strains can combine autotrophic photosynthesis and heterotrophic assimilation of organic compounds in a mixotrophic process. Therefore, microalgae are not strictly dependent on photosynthesis, as either light or organic carbon substrates can support cell growth. Mixotrophic cultures reduces photoinhibition and improves growth rates over both autotrophic and heterotrophic cultures. Successful mixotrophic production of algae allows the integration of both photosynthetic and heterotrophic components during the diurnal cycle, that reduces the impact of biomass loss during dark respiration and decreases the amount of organic substances utilised during growth (Brennan & Owende,

2010). Many studies show the advantages of mixotrophic cultivation, over other systems. Mixotrophic cultures of microalgae (*Chlorella* sp. and *Nannochloropsis* sp.), using glucose as carbon source, produced much higher yields of biomass and lipid production than photoautotrophic and heterotrophic cultures (Cheirsilp & Torpee 2012). Other work showed that *Chlorella vulgaris* grown on sucrose with light, under mixotrophic conditions, produced the highest lipid productivity compared with other growth modes (Rattanapoltee & Kaewkannetra 2014). In an another study (Mandal & Mallick 2009), glucose-supplemented cultures of *Scenedesmus obliquus* presented higher biomass and lipid yield that cultures without supplementation.

2.2.2 Production systems

In photoauto and mixotrophic production, microalgae are grown either in open ponds or closed photobioreactor (PBR) systems. On the other hand, in heterotrophic production, microalgae grow in stirred tank bioreactors or fermenters (Mata *et al.,* 2010).

Commercial production of phototrophic microbial biomass is limited to a few microalgal species such as *Arthrospira* and *Dunaliella* and they are cultivated in open ponds (Figure 2.1), especially raceway ponds (Marques *et al.,* 2012). This is simply due to economic issues, as open-culture systems are normally less expensive to build and operate, more durable than large closed reactors (Brennan & Owende, 2010).

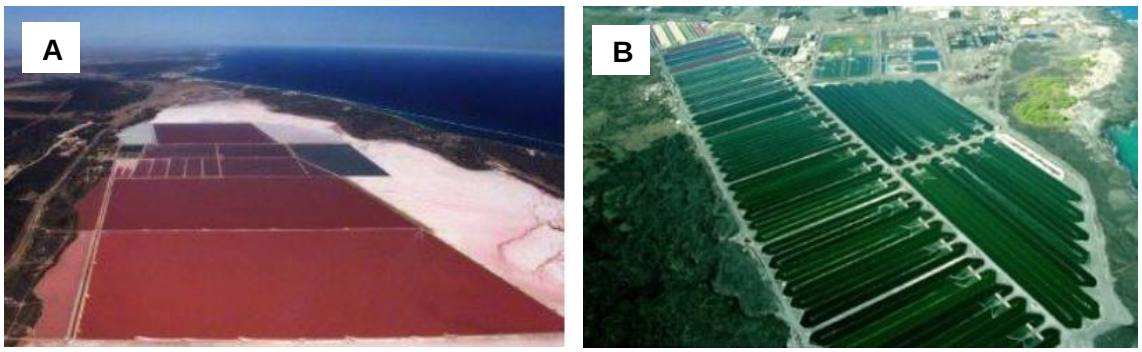

Figure 2.1. A - *Dunaliella* cultivated by Cognis at Hutt Lagoon, Australia; B - *Arthrospira* cultivated in raceway ponds by Cyanotech, Hawaii. Adapted from Marques *et al.* (2012).

Open ponds for microalgae growth is reviewed by several authors, and further information can be found in other works, as Brennan & Owende, (2010).

2.2.2.1 Photobioreactors

Closed photobioreactors have attracted much interest because they allow a better control of the cultivation conditions than open systems. With closed photobioreactors, higher biomass productivities are obtained and contamination can be more easily prevented (Ugwu *et al.,* 2008). Table 2.1 makes a comparison between PBR and ponds (Mata *et al.,* 2013).

Table 2.1. Comparison between PBRs and open pond systems (Mata *et al.,* 2013).

	PBRs	**Open ponds**
Contamination control	Easy	Difficult
Contamination risk	Reduced	High
Sterility	Achievable	None
Process and species control	Easy	Difficult
Mixing	Uniform	Poor
Operation regime	Batch, semi-continuous and continuous	Batch and semi-continuous
Area/volume ratio	High (20 – 200 m^{-1})	Low (5 – 10 m^{-1})
Investment and operations costs	High	Low
Light utilization efficiency	High	Poor
Temperature control	Uniform temperature can be achieved	Difficult
Productivity	3 – 5 times more productive	Low
Biomass concentration	3 – 5 times higher than ponds	Low
Water losses	~ ponds (depends on design)	~ PBRs
Shear stress	Low to high	Very low
O_2 inhibition	More problematic	Less problematic
Scale-up	Difficult	Difficult

PBRs can be classified according to both design and mode of operation. Many different designs have been developed, where the main categories include: flat or tubular; horizontal, inclined, vertical or spiral; and manifold or serpentine (Mata *et al.,* 2010).

Vertical column PBRs stand out because they offer the most efficient mixing with low shear stress and the highest volumetric mass transfer rates. They are low cost, compact and easy to operate. On the other hand, one of the major limitation is small illumination surface area (Ugwu *et al.,* 2008).

A gas sparger system is responsible for aeration. It is placed at the bottom of the reactor and converts the inlet gas stream into tiny bubbles, which allows for mixing, mass transfer of CO_2, and removing of the O_2 produced during photosynthesis (Sharma *et al.,* 2015). Column PBRs can be categorized into bubble column and air-lift photobioreactors (Figure 2.2).

Bubble-column reactors consist of cylindrical vessels that are less expensive and have high surface-area-to-volume ratio. They do not have moving parts and mass transfer is maintained by gas bubbling upwards from the sparger. Light is supplied from outside the column. Circulation of the liquid

from the central dark zone to the external light zone creates a differential gas flow rate which is critical for the photosynthetic efficiency (Sharma *et al.,* 2015). However, despite the high gas-liquid mass transfer efficiency, liquid-liquid and solid-liquid mass transfer aspects present some scale-up difficulties, as the gradients in the fluid phase are very significant and complex to describe (Teixeira *et al.,* 2007).

Air-lift reactors are made of a vessel with two interconnecting zones. The gas mixture flows upward to the surface from the sparger along a tube called gas riser. The down comer is where the medium flows down toward the bottom, therefore circulating within the riser and the down comer (Ugwu *et al.,* 2008). In the upper section, the bubble disintegration associated to the liquid flow contributes to a mixture so efficient that in that area the reactor acts as a stirred tank reactor. Thus, air-lifts have good mass transfer efficiency, gas-liquid as well as liquid-liquid and solid-liquid (Teixeira *et al.,* 2007).

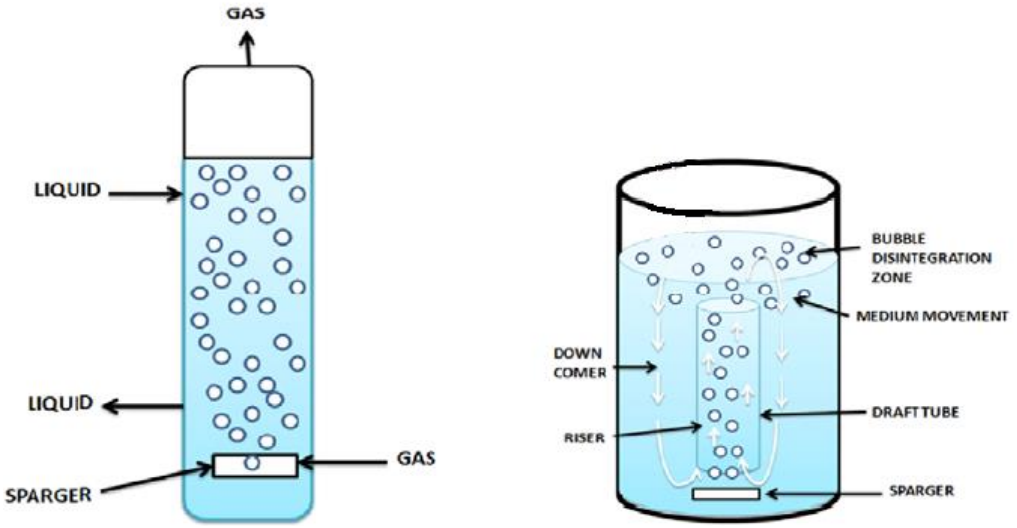

Figure 2.2. Representation of column PBRs with different air flux regime: A - bubble column and B - air-lift. Adapted from Sharma *et al.* (2015).

Other designs that receive much research attention are the tubular and flat-plate PBRs.

Tubular PBRs are very suitable for outdoor mass cultures of algae since they have large illumination surface area and fairly good biomass productivities can be reached. The largest photobioreactors are tubular (figure 2.3). The main limitations of these systems are poor mass transfer, the build-up of O_2 concentration, photoinhibition, difficult temperature control and adherence of the cells to the walls (Ugwu *et al.,* 2008).

Flat-plate PBRs have a large illumination surface area and, compared to tubular PBRs, a higher photosynthetic efficiency can be achieved and accumulation of dissolved oxygen is relatively low. However, there is some difficulty in controlling culture temperature and the possibility of hydrodynamic stress to some algal strains (Ugwu *et al.,* 2008).

Scale-up strategies are very challenging, mainly due to difficulty in maintaining optimum light, temperature, mixing, and mass transfer in larger photobioreactors. However, it can be done by

increasing the length, diameter, height or the number of compartments of the culture systems (depending on the type of photobioreactor) (Ugwu *et al.,* 2008).

Some attempts have been made to develop commercial-scale photobioreactors, but most were closed after a few months of operation. The first truly successful large-scale industrial production of microalgae in a closed photobioreactor has been accomplished in a plant built in Klötze (Germany) by Ökologische Produkte Altmark GmbH (Marques *et al.,* 2012), shown in Figure 2.3.

Figure 2.3. Chlorella sp. growing in tubular photobioreactors (Klötze, Germany) (Marques *et al.,* 2012).

2.2.3 Batch *versus* continuous operation

Figure 2.4 represents the algae growth curve in batch culture. One can distinguish five phases: (1) lag phase; (2) exponential growth phase that corresponds to the maximum growth rate under the specific conditions; (3) phase of deceleration; (4) stationary phase; (5) death phase (Coutteau 1996).

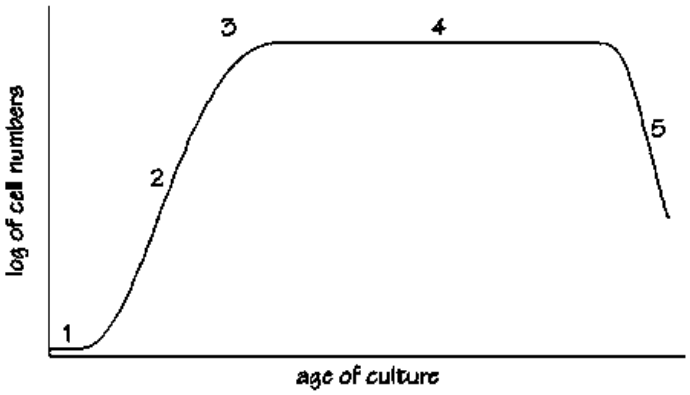

Figure 2.4. Representation of algal growth rate in batch culture. Adapted from Coutteau (1996).

PBRs can be operated in batch, semi-continuous/fed-batch and continuous mode. Continuous reactions offer increased opportunities for system investigation and analysis (Mata et al. 2010). While batch studies are critical for determining basic growth phases and characteristics of the algal species, steady-state studies are necessary to better understand and measure the specific growth parameters (Tang *et al.,* 2012).

There are several advantages of using continuous bioreactors as opposed to the batch mode (Mata *et al.,* 2010)

- Continuous bioreactors provide a higher degree of control;
- Growth rates can be regulated and maintained for extended time periods and biomass concentration can be controlled by varying the dilution rate;
- Results are more reliable and easily reproducible, due to the steady-state operation regime of continuous bioreactors.

Another important conclusion is the possibility of doubling the biomass productivity by operating in continuous mode instead of batch as described by different authors (Tang *et al.,* 2012; McGinn *et al.,* 2012).

There are also some limitations of the continuous operation (Mata *et al.,* 2010), such as:

- Relatively high cost and complexity;
- Wall growth and cell aggregation can cause wash-out or prevent optimum steady-state growth;
- The original product strain can be lost over time, if it is overtaken by a faster-growing one;
- Long growth periods increase the contamination risk.

In a chemostat, a flow of fresh medium is introduced into the culture at a predetermined rate, while culture liquid is continuously removed at the same rate to keep the culture volume constant. One of the most important features of chemostats is that microorganisms can be grown in a physiological steady-state under constant environmental conditions, where growth occurs at a constant specific growth rate. At steady state, the dilution rate (D) equals μ, the specific growth rate (Teixeira *et al.,* 2007), if the death rate is negligible.

Figure 2.5 shows the effects of D on biomass, X, and substrate, S, concentrations, as well as on productivity, P. If a dilution rate is chosen that is higher than μ_{max} (corresponding to D_{crit}), the cells cannot grow at a rate as fast as the rate with which they are being removed, so the culture will not be able to sustain itself, wash-out occurring. Biomass concentration presents almost constant values and then drastically decreases as D increases, until $D=D_{crit}$ where X is null (Teixeira *et al.,* 2007).

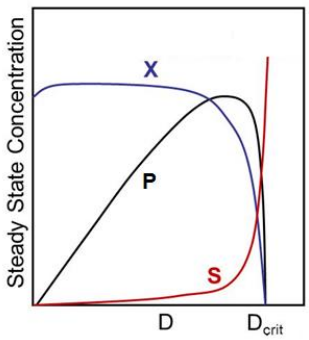

Figure 2.5. Effect of dilution rate, D, on chemostat steady-state concentrations. X= biomass, S=substrate and P=productivity. Adapted from an online presentation (http://slideplayer.com/slide/7558426/)

Studies in continuous mode can evaluate the effects of the dilution rate on several parameters. For example, a study has demonstrated the potential for continuous production of *Chlorella minutissima* and *Dunaliella tertiolecta* for the purpose of continuous lipid production from CO_2 (Tang *et al.,* 2012). The system dilution rate had a significant effect on microalgal biomass productivity, a slight effect on fatty acid profile, and no significant effect on lipid content under a steady-state condition with continuous illumination under carbon dioxide supplement conditions.

2.2.4 Cultivation parameters

There are several factors influencing algal growth: abiotic factors such as light, temperature, nutrient concentration, O_2, CO_2, pH, salinity, and toxic chemicals; biotic factors which concern pathogeny and competition by other algae and operational factors such as shear produced by mixing, and dilution rate (Mata *et al.,* 2010).

Light is the most limiting factor (except for heterotrophic cultivations), as it is required for photosynthesis. For outdoor algae production systems, this can be especially limiting due to diurnal cycles and the seasonal variations. Artificial lighting allows for continuous production, but at significantly higher energy input and cost (Brennan & Owende,, 2010)

Temperature is the second most important limiting factor, for culturing algae in both closed and open outdoor systems. Many microalgae can easily tolerate temperatures down to 15ºC lower than their optimal, but exceeding the optimum temperature by only 2–4 ºC may result in the total culture loss. Also, overheating problems can occur in closed culture systems during some hot days, when the inside of the reactor may reach very high temperatures (Mata *et al.,* 2010).

Microalgae can fix CO_2 from the atmosphere, off gases from heavy industry and dissolved carbonates such as Na_2CO_3 and $NaHCO_3$. Under natural growth conditions, microalgae assimilate CO_2 from the air (contains 360 ppmv CO_2), but most microalgae can tolerate and utilise substantially higher levels, typically up to 150 000 ppmv (Brennan & Owende, 2010). However, CO_2 leads to a reduction in the medium pH, which may inhibit the algal growth (Mata *et al.,* 2010).

Other inorganic nutrients required for algae production include nitrogen and phosphorus. While some algae species can fix nitrogen from the air in the form of NO_x the majority require it in a soluble form, with urea being the best source. Phosphorus is of lesser importance and is required in very small amounts during the algal growth cycle (Brennan & Owende, 2010).

Mixing is another important growth parameter since it homogenizes the distribution of cells, heat, and metabolites, facilitates transfer of gases and promotes the fast circulation of microalgae cells from the dark to the light zone of the reactor. On the other hand, high turbulence (due to mechanical mixing or air bubbles mixing) can damage microalgae due to shear stress (Mata *et al.,* 2010).

Common biological contaminants observed include unwanted algae, yeast, fungi, and bacteria. Cultivation of some microalgae species in raceway ponds failed, since cultures collapsed due to predation by protozoa and contamination by other algal species (Mata *et al.,* 2010).

2.3 Development of biofuel resources: potential of microalgae

The potential threat of global climate change has increased, mainly because of greenhouse gas emissions from fossil fuel usage for transport, electricity and thermal energy generation. Therefore, it has become increasingly important to adopt policies to minimize the negative effects of these emissions and to replace fossil fuels with sustainable alternatives. Biofuels are seen as real contributors to reach those goals, particularly in the short term (Brennan & Owende, 2010).

According to directive 2009/28/EC (April 23rd, 2009), the European Union requires that, by 2020, 10 percent of the energy consumed in the transport sector, in its member states, is from biofuels. The goal for 2010 was 5.75%, but it is estimated that the real value reached around 4% (OTEO 2012).

First generation biofuels have been extracted from food crops, competitively consuming food resources. To avoid this problem, the second generation biofuels use non-edible or waste vegetable oils, animal fats and wastes from agriculture, forest harvesting and wood processing. A biofuel resource is technically and economically viable if it is competitive or costs less than petroleum fuels, requires low or no additional land use, enables air quality improvement (e.g. CO_2 sequestration) and requires minimal water use. Microalgae have been recognized as an alternative, so-called third generation feedstock that could meet those conditions (Brennan & Owende, 2010; Trivedi *et al.,* 2015).

The main advantages of using microalgae for biofuels are (Brennan & Owende, 2010):

- Microalgae show a rapid growth potential combined with high oil content, being *Neochloris oleoabundans* a suitable example, reaching up to 65% on dry weigh of lipid content (Mata, Mendes, *et al.,* 2014);
- Oil productivity of microalgae cultures exceeds the yield of the best oilseed crops;
- They consume less water than terrestrial crops;
- The cultivation can be performed on non-arable land, not competing with the production of food.

Nevertheless, the use of microalgae as feedstock for the production of biofuels presents some challenges in large-scale cultivation, harvesting and conversion to useful fuels. Commercialization of microalgae biomass and biofuel production is still facing significant obstacles due to high production costs and poor efficiency (Wu *et al.,* 2012).

2.4 Applications of microalgae

Microalgae can be used as a feedstock for obtaining a number of products. These products can be divided into energy and non-energy based on their potential usage. Microalgae applications depend on its chemical composition. For example, algae rich in protein are suitable for nutrition (human or animal), carbohydrate-rich algae can be used for biofuel production and also for nutrition and lipid-rich algae are appropriate for biodiesel production. In order to give a general overview on the major

constituents of microalgae, Becker (2007) selected and compiled data of various species (Table 2.2). Applications for environmental cleaning purposes are also described.

Table 2.2. General composition (% of dry matter) of different microalgae.

Microalga	Protein	Carbohydrates	Lipids
Anabaena cylindrica	43 – 56	25 – 30	4 – 7
Aphanizomenon flos-aquae	62	23	3
Arthrospira maxima	60 – 71	13 – 16	6 – 7
Chlamydomonas rheinhardii	48	17	21
Chlorella pyrenoidosa	57	26	2
Chlorella vulgaris	51 – 58	12 – 17	14 – 22
Dunaliella salina	57	32	6
Euglena gracilis	39 – 61	14 – 18	14 – 20
Porphyridium cruentum	28 – 39	40 – 57	9 – 14
Scenedesmus obliquus	50 – 56	10 – 17	12 – 14
Spirogyra sp.	6 – 20	33 – 64	11 – 21
Spirulina platensis	46 – 63	8 – 14	4 – 9
Synechococcus sp.	63	15	11

2.4.1 Energy products from microalgae

Microalgae can be used for the production of liquid biofuels (bioethanol, biodiesel, biobutanol and bio-oil) or gaseous biofuels (biomethane, biohydrogen, syngas).

Photosynthesis is the fundamental driving force that supports all biofuel synthetic processes, converting solar energy into biomass, carbon storage products, and/or H_2 (Figure 2.6) (Beer *et al.,* 2009).

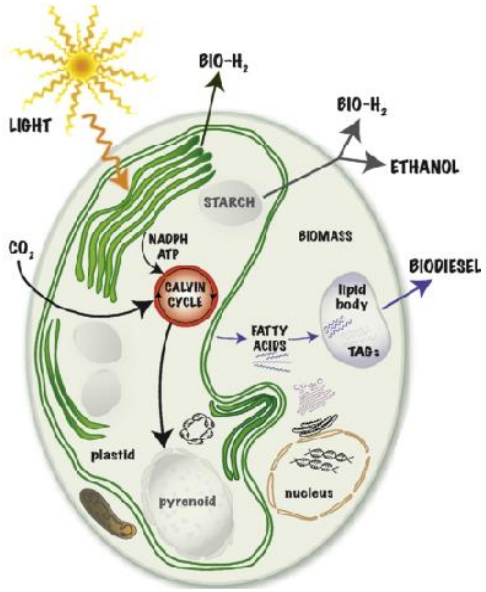

Figure 2.6. Metabolic pathways in green algae related to biofuel and biohydrogen production (Beer *et al.,* 2009).

2.4.1.1 Biodiesel

Biodiesel can be used directly in conventional diesel engines. It is a mixture of fatty acid monoalkyl esters (FAMEs), usually obtained by transesterification of oils.

Algal biodiesel has also been found to meet the International Biodiesel Standard for Vehicles (EN14214). Also, a comparison of typical properties of fossil oils with microalgae-derived biodiesel shows that the latter has a lower heating value, lower viscosity and higher density (Brennan & Owende, 2010).

Glycerol is obtained as a by-product during the transesterification process that could be used as a carbon source and converted to valuable metabolic products by using eukaryotic microorganisms such as yeast and fungi (Trivedi *et al.,* 2015) or even heterotrophic microalgae.

2.4.1.2 Other biofuels

Biogas production from microalgae is more technically viable than other fuels, but it is not yet economically feasible due to the high cost of algae biomass feed stocks. This biofuel is produced by anaerobic digestion of the biomass and consists of primarily methane and carbon dioxide (Brennan & Owende, 2010).

Another method to produce biogas is through the gasification technique. Biomass reacts with oxygen and steam to produce a mixture of gases known as syngas that consists of gases like methane, hydrogen, carbon dioxide and nitrogen. Syngas can be either directly burnt to produce energy or can be used as a fuel to run turbine engines (Brennan & Owende, 2010; Trivedi *et al.,* 2015).

Bioethanol is produced by alcoholic fermentation, converting the biomass materials which contain sugars, starch or cellulose into ethanol. Starch based biomass like microalgae require additional processing (conversion to sugars) before fermentation. *Chlorella vulgaris* is a potentially good source of ethanol due to the high starch content (Brennan & Owende, 2010).

Hydrogen is a naturally occurring molecule, which is a clean and efficient energy carrier. Microalgae possess the necessary metabolic pathways to produce hydrogen through photosynthesis (Brennan & Owende, 2010).

2.4.2 Non-energy products from microalgae

Microalgae have the potential for co-production of non-energy products like carbohydrates, proteins, and high value bioproducts, such as pigments, vitamins and polyunsaturated fatty acids (PUFAs). The most relevant microalgae that have a high commercial interest and are produced in large-scale are *Spirulina*, *Chlorella*, *Dunaliella salina* and *Haematococcus pluvialis* (Brennan & Owende, 2010).

2.4.2.1 Protein

Proteins can be used for different purposes such as animal feed, human nutrition, fertilizers, industrial enzymes, bioplastics, and surfactants (Trivedi *et al.,* 2015).

Investigations conducted so far confirm that algal biomass shows promising qualities as a novel source of protein, as the average quality for most of the algae examined is equal, sometimes even superior, when compared to conventional plant proteins (Becker 2007).

Despite its high content of nutritious protein, dried microalgae have not gained significant importance as food or food substitute yet, because of the following factors: the production costs for microalgae are still too high to compete with conventional protein sources; due to strict food safety regulations, the human consumption is limited to very few species; and the technical difficulties to incorporate the algal material into palatable food preparations (Becker 2007; Brennan & Owende, 2010).

To date, the major sales can be found in the health food market, where microalgae biomass is marketed in tablet or powder forms as food additives. *Chlorella*, *Spirulina* and *Dunaliella* are the dominant microalgae in the market (Brennan & Owende, 2010).

Regarding animal feed, the target domestic animal is poultry, because incorporation of algae into poultry rations offers the most promising prospect for their commercial use. It is estimated that about 30% of the current world algal production is sold for animal feed application (Becker 2007). Another growing market is the utilization of microalgae in aquaculture, where some of the main applications are fish feed and enhancement of the immune systems of fish. Algae such as *Chlorella*, *Spirulina* and *Scenedesmus* are used for these purposes (Brennan & Owende, 2010).

2.4.2.2 Carbohydrates

The accumulation of carbohydrates in microalgae is associated to CO_2 fixation during photosynthesis. The algal carbohydrates are mainly composed of starch, glucose, cellulose/hemicellulose, and other kinds of polysaccharides (Trivedi *et al.,* 2015). Of these, starch/glucose could be used for biofuel production, especially bioethanol and biohydrogen. On the other hand, algal polysaccharides represent a class of high-value compounds with many downstream applications in food, cosmetics, textiles, stabilizers, emulsifiers, lubricants, thickening agents and clinical drugs (Trivedi *et al.,* 2015).

2.4.2.3 High value bioproducts

Besides chlorophyll, the most relevant pigments in microalgae are phycobiliproteins and carotenoids, that are used in cosmetics and as pro-vitamins (Trivedi *et al.,* 2015). The carotenoid β-carotene, produced by *Dunaliella salina*, can be used as a food colouring agent, a source of pro-vitamin A and an additive to cosmetics (Brennan & Owende, 2010).

Microalgae are a primary source of polyunsaturated fatty acids (PUFAs) that are essential for human development and physiology. Among other things, PUFAs have been proven to reduce the risk of cardiovascular disease. Microalgal PUFA can be applied as additives for infant milk formula and

nutritional supplements. Currently, docosahexaenoic acid (DHA) is the only algal PUFA that is commercially available (Brennan & Owende, 2010).

Microalgae are also a valuable source of almost all essential vitamins such as A, B1, B2, B6, B12, C, E, nicotinate, biotin, folic acid and pantothenic acid (Trivedi *et al.,* 2015).

2.4.3 Environmental applications

The combined production of renewable energy and material resources with unique environmental applications for greenhouse gas (GHG) emissions mitigation and wastewater treatment is one of the hallmarks of microalgal research (Brennan & Owende, 2010). At large-scale applications, the use of artificial media is not viable, therefore the use of wastewater and flue gas would be highly recommended for microalgae cultivation (Trivedi *et al.,* 2015).

2.4.3.1 CO_2 mitigation

Biological CO_2 mitigation has attracted much attention in the last few years. A large volume of CO_2 is emitted from the power plants and industries into the environment. Therefore, the use of flue gas as a source of CO_2 for microalgae growth provides a very promising alternative to current GHG emissions mitigation strategies. *Chlorella* microalgae and *Scenedesmus obliquus* shows good results in CO_2 assimilation (Trivedi *et al.,* 2015; Brennan & Owende, 2010).

2.4.3.2 Wastewater treatment

Many species of microalgae are able to effectively grow in wastewater media through their ability to utilize organic carbon and inorganic N and P in the wastewater, as well as heavy metals (Brennan & Owende, 2010).

The advantages of employing microalgae in the treatment of wastewater are (Al-rajhia *et al.,* 2012; Pittman *et al.,* 2011):

- Cost effective;
- Low energy requirement;
- Efficient recycling of nutrients, as the produced biomass is useful for several applications;
- Removal of heavy metals;
- Reduction in waste sludge formation;
- Biofuel production in conjunction with wastewater treatment by microalgae is possible and seems to be the most plausible commercial application in the short term.

The use of microalgae in wastewater treatment has long been promoted, but chemical processing of wastewater or the use of activated sludge systems are the conventional treatment methods. However, microalgae are used throughout the world for wastewater treatment although on a

relatively minor scale. This is through the use of conventional stabilization ponds or high-rate algal ponds, the latter more developed and efficient (Pittman *et al.,* 2011).

There are many compounds capable of causing the pollution of a water course, mainly organic compounds and nutrients. Therefore, the treated wastewater discharge has to comply with environmental legislation. Table 2.3 presents some indicative wastewater discharge limits as are generally applied in Portugal (decree-law 236/98).

The organic load of wastewater comprises a large number of compounds, which all have at least one carbon atom. These carbon atoms may be oxidized both chemically and biologically to yield carbon dioxide. If biological oxidation is employed, the associated oxygen consumption can be assessed through a test termed the Biochemical Oxygen Demand (BOD), whereas for chemical oxidation, the correspondent oxygen consumption test is the Chemical Oxygen Demand (COD). Microalgae can consume these organic compounds, achieving high removal rates (Abdel-Raouf *et al.,* 2012).

Disposal of large quantities of nutrients (in particular N and P) into water bodies leads to the eutrophication process. Wastewaters contain adequate quantities of these nutrients for the growth of microalgae. P is particularly difficult to remove from wastewater: for most commercial wastewater processing, this nutrient is precipitated from the effluent with the use of chemicals or is converted into activated sludge by microbial activity. Treatment with microalgae have been found to be as efficient at removing P from wastewater as chemical treatment (Abdel-Raouf *et al.,* 2012; Pittman *et al.,* 2011).

Table 2.3. Emission value limits (ELV) for wastewater discharge into natural water bodies (decree-law 236/98; Portugal).

Parameter	**ELV** [(1)]
COD	150 mg O_2/L
BOD_5	40 mg O_2/L
TSS (Total suspended solids)	60 mg/L
Total N	15 mg N/L
Ammonia N	10 mg NH_4/L
Total P	0.5 – 10 mg P/L

[(1)] ELV – emission value limit, on a monthly basis

Cultivation of microalgae in these substrates helps to reduce the organics and nutrients by synthesizing new biomass and thereby preserving fresh water resources (Abinandan & Shanthakumar 2015). Being efficient in removing N, P and toxic metals the microalgae have potential to play an important remediation role particularly during the final (tertiary) phase of wastewater treatment (Pittman *et al.,* 2011).

Microalgae can treat a huge variety of wastewaters: human sewage, livestock wastes, agro-industrial wastes and industrial wastes, such as piggery effluents, effluents from food processing factories and brewery effluents. Microalgae-based system for the removal of toxic minerals such as lead, cadmium, mercury, scandium, tin, arsenic and bromine are also being developed (Abdel-Raouf *et al.,*

2012; Al-rajhia *et al.,* 2012). There is significant interest in the use of algae for remediation of industry-derived wastewaters, predominantly for the removal of heavy metal pollutants (cadmium, chromium, zinc, etc.) and organic chemical toxins (hydrocarbons, biocides, and surfactants), rather than N and P. However, due to generally low N and P concentrations and high toxin concentrations, algal growth rates are low in many industrial wastewaters. On the other hand, because of N or P limitation, lipid accumulation in the cell can reach very high concentrations (Pittman *et al.,* 2011), which is favourable for biofuel (biodiesel) production.

2.5 Microalgae based biorefinery

The main goal of a biorefinery is to integrate the production of higher value chemicals and commodities, as well as fuels and energy, optimizing the use of resources, maximizing profitability and benefits and also minimizing wastes (Trivedi *et al.,* 2015). The versatility and huge potential of microalgae for many applications can support a microalgae-based biorefinery (Gouveia 2014).

The economic viability of algae-based biofuels is still unfeasible. However, microalgae-based biorefineries could play a major role in changing economies and create many opportunities in the global microalgae business. Only the co-production of high added value products and environmental benefits, such as water bioremediation and CO_2 mitigation, could eventually compensate for the high production costs of mass microalgae cultivation and support a microalgae-based bioeconomy (Gouveia 2014).

Cross-feeding of products, co-products and power from the algal biofuel industry into the associated industries is necessary for improving resource management and minimization of the ecological footprint of the entire system In fact, this type of biorefinery should integrate several processes and related industries, such as food, feed, energy, pharmaceutical, cosmetic, and chemical (Trivedi *et al.,* 2015; Gouveia 2014). A microalgae-based biorefinery and how several industries are integrated are exemplified in Annex B.

The main bottleneck of this biorefinery approach is to separate the different fractions and guarantee a sufficient quality for a variety of end products. Thus, there is a need for mild inexpensive and low energy consumption separation techniques (Gouveia 2014).

2.6 Harvesting of microalgal biomass

Algal harvesting consists of biomass recovery from the culture medium and may contribute to 20–30% of the total biomass production cost. It can be difficult, due to low cell densities (typically in the range of 0.5 – 5 g/L) and the small size of algal cells (generally in the range of 2-40 μm) (Brennan & Owende, 2010).

In order to remove large quantities of water and process large microalgal biomass volumes, a suitable harvesting method may involve one or more steps and be achieved in several physical, chemical, or biological ways. Most common harvesting methods include sedimentation, centrifugation, filtration, ultra-filtration, sometimes with an additional flocculation step or with a combination of

flocculation–flotation (Mata *et al.,* 2010). Generally, microalgae harvesting is a two-stage process (Brennan & Owende, 2010). In the first stage a bulk harvesting is performed to achieve 2 – 7% total solids, with values of 100-800-fold for concentration factors, employing processes such as flocculation, flotation or gravity sedimentation. After that, the obtained slurry is thickened with more energy- intensive techniques.

Flocculation is used as the first step in bulk harvesting, to aggregate the microalgal cells to increase the effective particle size, easing the subsequent harvesting steps. Microalgae and cyanobacteria are coated with extracellular polysaccharides (EPS), which give them a negatively charged surface that allows flocculation or aggregation of the cells using metal cations or other flocculating agents. Multivalent metal salts like ferric chloride ($FeCl_3$), aluminium sulphate ($Al_2(SO_4)_3$) and ferric sulphate ($Fe_2(SO_4)_3$) are suitable flocculants. Also, by adjusting the pH to a range of 10-11 with NaOH, flocculation can be improved (Brennan & Owende, 2010). However, metals and chemicals, despite their positive effects on microalgal harvesting, pollute the environment when directly discharged without further treatment. Therefore, flocculation enhancement without any chemical addition is necessary. A study by Darpito *et al.* (2015), using brewery wastewater, showed that the cells cultivated in the effluent were larger than those cultivated in standard medium, increased cell agglomeration and, consequently, enhancing the autoflocculation of the microalga.

Flotation is mentioned as a potential harvesting method, particularly applied to algae strains with high lipid content, that allows the microalgae to float. However, its technical and economic viability is questioned (Brennan & Owende, 2010).

Gravity sedimentation is the most common harvesting technique for algae biomass with low value and in wastewater treatment because of the large volumes treated (Brennan & Owende, 2010).

On the other hand, for high-value products it is often recommended to use continuously operating centrifuges that can process large volumes of biomass. Despite their high energy costs, centrifuges are suitable to rapidly concentrate any type of microorganisms, which remain fully contained during recovery and can be easily cleaned or sterilized to effectively avoid bacterial contamination or fouling of the raw product (Mata *et al.,* 2010).

Conventional filtration is better suited for harvesting large microalgae. For processing low volumes and recovering small and fragile cells, membrane microfiltration and ultrafiltration are better alternatives. However, these filtration processes are more expensive because of the need for membrane replacement and pumping (Brennan & Owende, 2010).

Electrocoagulation, a non-conventional harvesting method seems to be a future trend. In a study by Gouveia *et al.* (2016), this step was performed prior to centrifugation, saving 89% of energy when compared to centrifugation alone.

2.7 *Scenedesmus obliquus*

2.7.1 Biology

Scenedesmus obliquus is a green microalga, that can be found in freshwater bodies all around the world, and even in the soil (Trainor 1998).

The most common mode of reproduction of *S. obliquus* is asexual, by the formation of autospores. Inside the parental cell wall, the mother cell undergoes from 1 to 4 successive divisions into 2 to 16 daughter cells. A motile stage in the life history of *S. obliquus* (zoospores) is a far less observed phenomenon in nature and can only be observed under stress conditions such as nitrogen deprivation (Trainor 1998; Lürling 2003).

This microalga is characterized by a high degree of phenotypic plasticity. Cells are commonly occurring in colonies as multiples of two, with two or four cells being most common. However, *S. obliquus* may produce unicells as well. It is a cosmopolitan species and is able to withstand harsh conditions, such as periods with strong grazing pressure. Under those conditions, typical phenotypes, as protective eight-celled colonies, could be produced (Lürling 2003).

The cell wall contains mainly cellulose, pectin and the polycarotenoid sporopollenin, which gives it an extremely high resistance (Trainor 1998). As all Chlorophyta, *S. obliquus* has chlorophyll *a* and *b* as photosynthetic pigments and forms starch within the chloroplast.

The cells contain a good amount of proteins (50-56 % dry matter), lipids (12-14 % dry matter) and carbohydrates (10-17 % dry matter) (Becker, 2007).

2.7.2 Environmental applications

Because of ease of cultivation, rapid growth and adaptation to the environmental conditions, *Scenedesmus obliquus* is a versatile organism for the use in domestic and industrial wastewater treatment (Hodaifa *et al*., 2008; Batista *et al*., 2014). Some published works regarding treatment of wastewater from several origins by *S. obliquus* are summarized below.

This microalga has shown extraordinary results in nutrient removal from urban wastewaters (Batista *et al*., 2015), also registering growth rates similar to those reported for a complete synthetic medium. Phosphorus removal of 98% and total ammonia removal has been achieved by Martínez *et al.* (2000) with wastewater previously submitted to secondary treatment. In a very recent study, Gouveia *et al.* (2016) achieved a maximal removals of 95, 92 and 63 % for total nitrogen, phosphorous and COD, respectively, using a vertical photobioreactor. The treated water had values that are in accordance with environmental legislation (Directive 98/15/CE) and could be discharged into natural receiving waters.

Hodaifa *et al.* (2008) studied the production of *S. obliquus* in wastewater from olive oil extraction. Despite achieving a maximum removal of 67.4% for BOD_5 (with 25% diluted wastewater) it was clear that these industrial wastewaters are not a complete medium for the culture of the microalga, showing N deficiency, which yielded low biomass productivity.

S. obliquus shows potential for brewery wastewater treatment (Mata *et al.,* 2012). In a study in shake flask, using a simulated brewery effluent, reductions of 57.5, 20.8, 56.9 % for COD, total N and total C, respectively, were attained.

Treatment of wastewaters with higher organic matter load and nutrient concentrations, such as piggery wastewaters, is also supported by *S. obliquus*. In a study, the microalga was evaluated for its ability to remove carbon, nitrogen and phosphorous from eight-fold diluted wastewater from a swine manure treatment plant (Godos *et al.,* 2010). The results from the batch biodegradation tests, the batch oxygenation tests and the continuous piggery wastewater biodegradation operation confirmed some efficiency in TOC (Total Organic Carbon) and ammonia removal and showed moderate tolerance towards ammonia.

S. obliquus can also be used to remove toxic components. The microalga was examined for degrading cyanide from mining process wastewaters (Gurbuz *et al.,* 2009) and it was observed that cyanide was degraded up to 90% after the introduction of algae into the system.

Regarding biofuels, *S. obliquus* biomass is a suitable feedstock for biodiesel, bioethanol and biohydrogen production. Lipid accumulation in this microalga was studied under various culture conditions, a lipid content of 58.3% of dry cell weight being reached, only by manipulating the nutrient status (Mandal & Mallick 2009). In the same study, biodiesel was produced containing mainly saturated and mono-unsaturated fatty acids (methyl palmitate and methyl oleate) that accounted for almost 75% of the total FAME, promoting a high oxidative stability. Bioethanol was produced from this microalga biomass by Miranda *et al.* (2012) using fermentative yeasts and biohydrogen was also produced by dark fermentation, using *E. aerogenes* and *C. butyricum* bacteria (Batista *et al.,* 2014).

S. obliquus can be used for CO_2 biofixation. In a three-stage serial tubular photobioreactor, using CO_2-enriched air, the microalga was cultivated at 30ºC and achieved biofixation rates of 28% and 14% for 6% and 12% CO_2, respectively (de Morais & Costa 2007).

Furthermore, the biorefinery concept using *S. obliquus* can be considered. It is known that this microalga has in it sufficient accumulation potential for lipids and other important functional components. For example, in the process described by Patnaik & Mallick (2015), 100 g of *S. obliquus* biomass yielded 0.06 g of β-carotene, 38 g of biodiesel, 2 g of omega-3 fatty acids, 3 g of glycerol and 17 g of ethanol, using mixotrophy as a mode of nutrition. Another biorefinery was performed with S. *obliquus* by Batista *et al*. (2015) by using urban wastewater as a culture medium to growth the microalga, with the obtained biomass being used to produce biohydrogen (Batista *et al*., 2015) and a biofertilizer, tested in *Lactuca sativa* (lettuce) seeds (Ambrosano, 2015).

2.8 Brewery industry

2.8.1 An overview

In the food and beverage industry, the brewing sector constitute an important economic segment of most countries, being beer the third most popular drink overall, after water and tea. The annual beer production in 2014 exceeded 1.96 billion hectolitres worldwide and reached almost 401 million hectolitres in Europe (Statista, 2016; Beer Institute's Brewer's Almanac, 2014). Portugal holds the 13th position regarding production in Europe, with 7.29 million hectolitres in 2014 (Beer Institute's Brewer's Almanac 2014).

Beer is obtained through alcoholic fermentation, using selected yeasts of the genera *Saccharomyces*, of wort prepared from malt cereals, to which were added hop flowers, or their derivatives, and adequate water (Brito *et al.,* 2007). Figure 2.7 shows a typical technological process. Beer production includes chemical and biochemical reactions (mashing, boiling, fermentation and maturation) and three solid-liquid separations (wort separation, wort clarification and rough beer clarification) (Fillaudeau *et al.,* 2006).

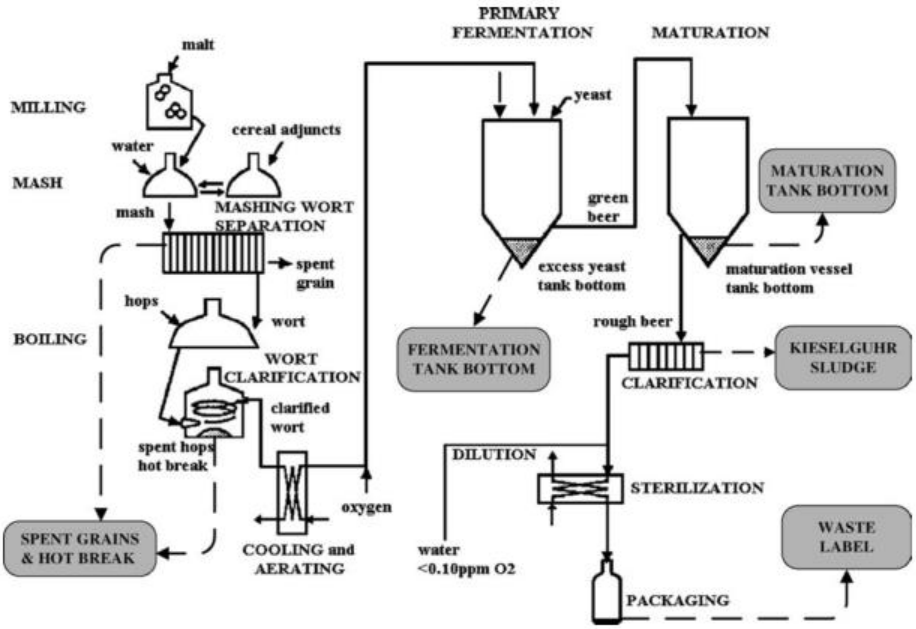

Figure 2.7. Brewing process (Fillaudeau *et al.,* 2006).

The brewing industry has shown increasing awareness for environmental protection and the need of sustainable production processes. Moreover, water management and waste disposal have become a significant cost factor and an important aspect in the brewery process, as the severity of waste management requirements in beverage industry has been increased in recent years (Fillaudeau *et al.,* 2006).

A large amount and variety of wastes are produced (Figure 2.7): water, spent grains, spent hops, surplus yeast, trub, caustic and acid cleaners, waste beer and waste label. Water and spent

grains are the most extensive problems, 3-10 hectolitres of water and 14 kg of spent malt being generated per 1 hectolitre of beer produced. However, a number of wastes are combined into a single waste flow. Washg water, waste yeast, cleaning chemicals and spoilt beer may all be discharged together, requiring common processing and often produce a sludge from solids separation (Thomas & Rahman 2006).

Since brewery spent grains are a waste from a beverage industry they have food compatible characteristics, suitable to be used in agriculture due to their high organic matter content, as animal feed or soil improver and for the production of low-value composts (Thomas & Rahman 2006). The common disposal route for brewery sludge has been landfill. However, there have been attempts to utilize this brewery by-product in an environmentally friendly manner. For example, Kanagachandran & R. Jayerantene (2006) proposed to sun-dry it and use it as organic fertilizer, as analysis revealed that the sludge contained valuable nutrients for plant growth with high water retention capacity.

A model for zero emission in the brewery industry (Pitcher 2012) is presented in Annex A. This material flow presents solutions for every by-product and waste and interconnect them, which creates a sustainable waste management scheme. Some solutions can provide products that can be used as inputs for the brewery industry or even other industries. Alga culture is pointed as one of the solutions for the wastewater, which will be discussed in section 2.1.2.1. Nevertheless, algae can also be used for organic waste treatment. For example, spent yeast can serve as a low-cost substrate for cultivation of the DHA-rich microalga, *Aurantiochytrium* sp. KRS101 as previously published by Ryu *et al.,* (2013).

2.8.2 Characterization of brewery wastewater

Because of the large amount of water used, the brewery industry discharges voluminous highly polluting effluents throughout the process. The effluent load is very similar to the water consumption load since most of it ends up as effluent, with a part of the water being disposed with the brewery by-products and a part lost by evaporation (Fillaudeau *et al.,* 2006; Driessen & Vereijken 2003).

The composition and quantity of brewery effluent depends on the process within the brewery (raw material handling, wort preparation, fermentation, filtration, CIP (clean in place), packaging, etc). For example, bottle washing results in a large wastewater volume, but it contains only a minor part of the total organics discharged. On the other hand, effluents from fermentation and filtering have a high BOD but are generally low in volume, accounting for about 3% of the total wastewater volume but 97% of the BOD. In addition, cleaning of tanks, bottles, machines, and floors produces high quantities of polluted water (Simate *et al.,* 2011).

Organic components in brewery wastewater (BWW) are generally easily biodegradable and mainly consist of sugars, soluble starch, ethanol and volatile fatty acids, which leads to a BOD/COD ratio of 0.6 to 0.7. Nitrogen and phosphorous levels mainly depend on the handling of raw material and the amount of spent yeast present in the effluent. High phosphorus content also can result from the chemicals used in the CIP unit. pH levels are determined by the amount and the type of chemicals used at the CIP units, such as caustic soda, phosphoric acid and nitric acid (Driessen & Vereijken 2003). Table 2.4 summarizes some of the main characteristics of brewery wastewater.

Table 2.4. Characterization of brewery wastewater (Driessen & Vereijken 2003).

Parameter	Values range
COD (mg/L)	2000 – 6000
BOD (mg/L)	1200 – 3600
TSS (mg/L)	200 – 1000
T(ºC)	18 – 40
pH	4.5 – 12
Nitrogen (mg/L)	25 – 80
Phosphorus (mg/L)	10 – 50

2.8.3 Brewery wastewater treatment

The discharge of untreated (or partially treated) BWW into water bodies can cause potential or severe pollution problems since the effluents contain organic compounds that require oxygen for degradation. However, if the brewery is permitted to discharge into a municipal sewer, pretreatment may be required to meet municipal bylaws and/or to reduce the load on the municipal treatment plant (Simate *et al.,* 2011).

The treatment of BWW can be done by three methods. The first is a physical method by applying physical forces to remove coarse matter, allowing the suspended materials to settle or to float; it leads to little BOD removal, and it is necessary to combined it with other methods. The second method is chemical, involving pH adjustment or coagulation and flocculation; it can aggregate small particles that are then removed more easily by physical separation. The last method is the biological process that, compared to physicochemical or chemical methods, has three advantages: the treatment technology is mature, presents high efficiency in COD and BOD removal (80 to 90%) and requires low investment cost (Simate *et al.,* 2011). Due to its importance, this type of wastewater treatment is discussed next.

2.8.3.1 Biological BWW treatment

Brewery effluents are generally treated by biological methods. Therefore, after the BWW has undergone physical and chemical pre-treatments, the wastewater can go to biological treatment (Simate *et al.,* 2011).

There are two main processes: anaerobic (without oxygen) and aerobic (with air/oxygen supply). Anaerobic treatment (digestion) is characterized by biological conversion of organic compounds (COD) into biogas (mainly methane 70-85% (v/v) and carbon dioxide 15-30% (v/v). During aerobic treatment, air (oxygen) is supplied to oxidize the BOD into carbon dioxide and water. Both processes produce new biomass (Driessen & Vereijken 2003).

Anaerobic digestion constitutes an option to treat the brewery effluent, when it is being discharged into a municipal sewer. The fact that there is virtually no biological sludge production, that requires disposal, is a key factor, as well as its ability to reduce COD, BOD and suspended solids at low

hydraulic residence time ranges (Fillaudeau *et al.,* 2006). Moreover, in the context of decreasing fossil fuel reserves, anaerobic wastewater treatment makes a brewery more independent from external fuel supply, as, in breweries, direct utilization of biogas in a boiler is possible and a current solution (Simate *et al.,* 2011).

In case of discharge into surface water bodies, the brewery has to comply with more stringent wastewater quality limits than those that can be achieved by anaerobic treatment only. Anaerobic pre-treatment followed by aerobic post-treatment will result in a positive energy balance, reduced sludge production and space saving (Driessen & Vereijken 2003).

Several types of anaerobic reactors can be applied to brewery wastewater treatment. However, the upflow anaerobic sludge blanket (UASB) reactor clearly accounts for the most usual full-scale systems (Brito *et al.,* 2007).

In the UASB reactor, the wastewater enters a vertical tank at the bottom, passes upwards through a dense bed of anaerobic sludge (granular) where the microorganisms in the sludge convert the organic compounds, releasing biogas. As the biogas rises, it carries some of the granular microbial blanket. At the top of the UASB reactor, the gas–liquid–solid-separator separates the biomass from the biogas and wastewater (Driessen & Vereijken 2003).

Even though the UASB reactors fulfilled their task very well for many years, a new generation of reactors started to become popular in the brewing industry during the late 1990's, namely the high tower reactors such as the FB (Fluidised Bed), the EGSB (Expanded Granular Sludge Bed) and the IC (Internal Circulation) reactors. Whereas the fluidised bed reactor uses fluidised carrier material for the biomass to grow on, the EGSB and IC reactors use granular anaerobic sludge, identical to that in UASB reactors. Both also have the gas–liquid–solid-separator. The IC reactor is a two-staged UASB reactor design, in which the lower UASB receives extra mixing by an internal circulation, driven by its own gas production (Driessen & Vereijken 2003).

Activated sludge is the most frequent and widely applied aerobic system for the treatment of industrial effluents. It is based on an aerated reactor with suspended flocculent aerobic biomass, mixed by aerators supplying the necessary oxygen. An external gravity clarifier separates the sludge and the decanted aerobically purified effluent is discharged, while the settled aerobic sludge is returned to the aeration basin. Excess aerobic sludge is removed, dewatered and disposed of (Driessen & Vereijken 2003).

In an airlift-reactor the water/sludge mixture is intensively circulated over internal cylinder(s) by an airlift created by the air bubbles supplied by compressors. Whereas the activated sludge system operates with flocculent aerobic sludge, this reactor operates with highly concentrated granular aerobic biomass, and it is possible to operate it with much higher volumetric loading rates. Airlift-reactors with an integrated denitrification unit (converting nitrate to nitrogen gas) have been developed and applied on brewery and malting effluent treatment (Driessen & Vereijken 2003).

Figure 2.8 presents a schematic overview of the most widely applied anaerobic and aerobic effluent treatment systems in brewery industry.

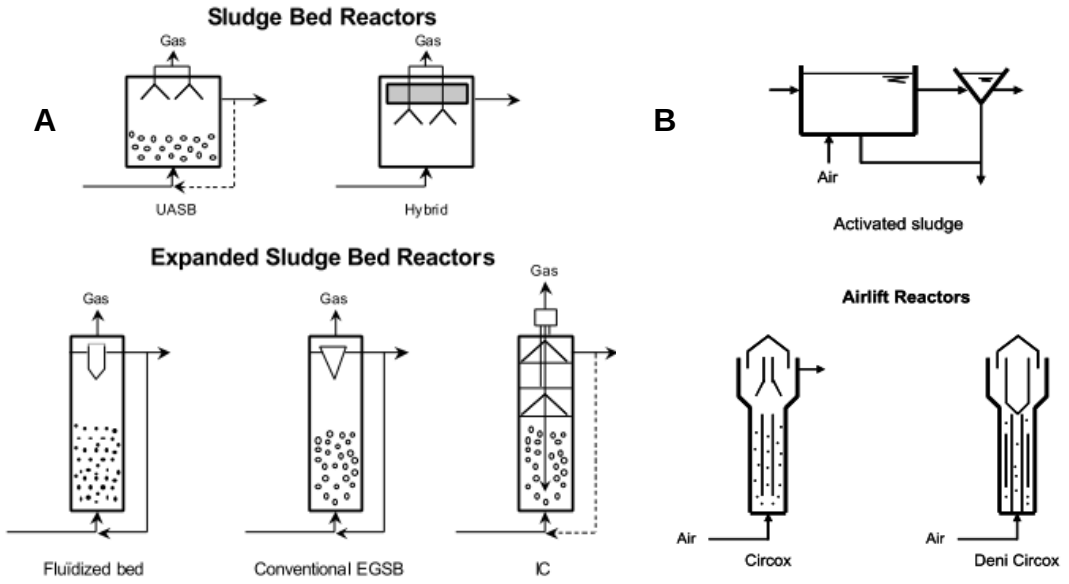

Figure 2.8. Schematic overview of the most widely applied aerobic effluent treatment systems. A – anaerobic treatment and B – aerobic treatment. Adapted from Driessen & Vereijken (2003).

2.8.3.2 BWW treatment by microalgae

Regarding the potential of using microalgae for brewery wastewater biological treatment few studies still exist. Some of the main published works over the last years are summarized in Table 2.5 and are further discussed below.

In the first work presented (Raposo *et al.,* 2010), the effluent of a brewery was used for the growth of *Chlorella vulgaris* and for a consortium obtained from the autochthonous flora of that effluent (microalgae, cyanobacteria and bacteria). The highest removal rate of nutrients was obtained using the autochthonous flora. Consortia could be advantageous since bacteria are able to use ammonia, converting it to nitrate, which would be used by the algae. Also, bacteria produce CO_2 during metabolism, which is necessary for microalgae to carry out photosynthesis. Considering the protein, fatty acid content and the absence of heavy metals in significant amounts, the biomass produced can be appropriate for use as animal feed or for biofuel production.

Mata *et al.* (2013) proved to be possible the combination of *S. obliquus* growth with brewery effluent treatment and concluded that biodiesel production from this microalga is feasible. An average lipid concentration of 0.24 g/L was obtained from the *S. obliquus* biomass cultivated in the simulated brewery effluent and biodiesel was produced with the extracted oil, containing 56.4 % of saturated esters and less than 12 % of the linolenate (unsaturated) ester.

Table 2.5. Overview of BWW treatment studies with microalgae.

Microalgae and cultivation	X_{max} (g/L)	Days of cultivation	% Removal	Reference
Chlorella vulgaris and consortium • Plastic bags V = 15 L • Industrial BWW	-	19 *(C. vulgaris)* 14 (consortium)	• COD: 15 • BOD: 27 • TN: 85–90 • TP: 54-66	(Raposo *et al.,* 2010)
Botryococcus braunii • Flask scale V=250 mL • Untreated industrial BWW	0.109	20	• Carbohydrates: 89	(Kong *et al.,* 2012)
Botryococcus braunii • Flask scale V=250 mL • Industrial BWW supplied with 0.50 g/L potassium nitrate	0.205	20	• Carbohydrates: 98 • Nitrate: 99	
Scenedesmus obliquus (SAG 276-3d) • Flask scale V=250 mL • Synthetic BWW	0.9	14	• COD: 58 • TN: 21 • TC: 57	(Mata *et al.*, 2013)
Chlorella vulgaris (UTEX-265) • Two-stage photoautotrophic–mixotrophic • Industrial BWW	3.20	1st stage: 7 2nd stage: 5	1st stage • TN, TP and TOC: >80 2nd stage • Depletion of nutrients	(Farooq *et al.,* 2013)
Chlorella vulgaris (UTEX-265) • Two-stage photoautotrophic–photoheterotrophic • Industrial BWW	3.20	1st stage: 8 2nd stage: 5	1st stage • TN, TP and TOC: >80 2nd stage • TN, TP and TOC: >70	
Chlorella protothecoides (UTEX-1806) • Flask scale V=250 mL • Anaerobically treated industrial BWW	1.88	6	• TN: 96 • TP: 90	(Darpito *et al.,* 2015)

TN is total Nitrogen, TP is total phosphorous and TC is total carbon

A cultivation system in two-stage photoautotrophic–photoheterotrophic/mixotrophic mode was adapted to maximize lipid productivity of *Chlorella vulgaris* grown in brewery wastewater (Farooq et al., 2013). In photoheterotrophic cultivation, algae require light when using organic compounds as the carbon source. Two types of BWW were used: an original effluent coming from a brewery industry (BWW #1) and the effluent from the pre-treatment of this wastewater in an anaerobic digester (BWW#2). BWW #2 was used in the first stage of cultivation.

In the course of the two-stage photoautotrophic–mixotrophic cultivation, microalga was first grown under photoautotrophic conditions for seven days and then, in the late-exponential stage, mixotrophic conditions were introduced and maintained for five days using BWW #1, glucose or glycerol as organic carbon sources. With BWW#1 as the carbon source, the residual organic carbon content in the effluent was close to the permissible discharge limit and a lipid content of 24% and 60 mg/(L.day) of lipid productivity were obtained.

In the two-stage photoautotrophic–photoheterotrophic growth mode, cells were first grown in the photoautotrophic mode for eight days, after which the culture was allowed to settle for 12 h, and then the settled biomass was grown in the photoheterotrophic mode for five days, with glucose or BWW#1 as the sole carbon sources. With 10 g/L of glucose, maximum lipid content and productivity was achieved: 42% and 108 mg/(L.day).

It was concluded that the addition of organic carbons affected the lipid compositions and increased their contents in oleic acid which is more suitable for biodiesel applications. The main FAMEs were: oleic acid (44% in total lipid), palmitic acid (20% in total lipid), linoleic acid (14% in total lipid), and linolenic acid (5% in total lipid).

In a recent study (Darpito *et al.,* 2015), the potential of *Chlorella protothecoides* cultivated in anaerobically treated BWW was investigated for its application to wastewater treatment and economical biodiesel production. The maximum total fatty acid content of the *C. protothecoides* grown in the BWW was about 36% of its dry cell weight and the main constituents were palmitic acid (26%), stearic acid (24%), oleic acid (15%), linoleic acid (15%) and linolenic acid (15%). An auto-setting test was also performed. Rapid settling due to the increased cell size and cell agglomeration in the BWW was obtained, resulting in an enhancement to nearly 80 % in harvesting efficiency within 20 min, as compared with only 4 % for biomass grown in the standard medium BG-11, providing an economic advantage in microalgal harvesting.

In another study (Mata *et al.,* 2014), not presented in Table 2.5, *Chlamydomonas* sp. was grown in a medium containing 20 % (v/v) of brewery wastewater, without sugar addition or blended with pentose sugars (xylose, arabinose or ribose) resulting from the hydrolysis of brewer's spent grains. The study was performed in flask scale (V = 1 L). Despite not showing results on nutrient or organic compounds removal by the microalga, this work evaluated the sustainability of biodiesel produced by *Chlamydomonas* sp. in this wastewater, taking into account the fuel life cycle: microalgae cultivation, harvesting, biomass processing for lipids extraction, microalgae oil transportation, biodiesel production and its final use. In terms of lipid productivity and content, better results (5.53 mg/(L.day) and 53.5% on a wet basis, respectively) were achieved when pentose sugars were not added. However, with the addition of sugars higher biomass concentrations were reached in shorter periods of time, being the maximum value 0.229 g/L for the addition of xylose. Comparing the effect of the different pentose sugars, xylose promoted the highest lipid productivity (4.86 mg/(L.day)). The best sustainability results were estimated for the culture without pentose addition, resulting in the lowest estimated life cycle energy needs (1.48 MJ/MJ biodiesel).

All of these works proved that under certain conditions microalgae growth in brewery wastewater can be successfully implemented by using its easily biodegradable contaminants as nutrients. Besides removing the organic compounds from the wastewater, the obtained microalgae biomass can be used as animal feed, as fertilizer, for the extraction of valuable compounds and even for biodiesel production.

2.8.3.3 BWW treatment for reuse

The future reuse of brewery wastewater seems to be inevitable, as water shortage has become a serious global and environmental problem. Besides that, the growing world population and industrial activities coupled with rigorous environmental requirements, lead to an increase in the cost of water. Thus, the demand for water reuse in the brewery industry is expected to increase.

The discharged wastewater from the biological treatment processes can be further treated to recycle the water back to the production process.

Various methods that may be used to treat brewery wastewater for reuse are explored by Simate *et al.* (2011) and the most promising are presented next.

Membrane filtration - The application of membrane filtration (e.g., nanofiltration and reverse osmosis) to drinking water treatment and wastewater treatment for reuse are well established. Reverse osmosis, in particular, has been shown to be an efficient and cost effective process for the treatment of brewery wastewater for reuse. For example, the Portuguese brewery company Sociedade Central de Cervejas installed a tertiary treatment process with reverse osmosis membranes, that improves the quality of the treated effluent, allowing it to be reused in the brewery's cooling towers and steam boilers. In 2014, this reuse represented a 0.3 hl/hl reduction in water consumption (30 litres less water consumed per 100 litres of beer brewed), corresponding to a 10% reduction in total brewery water consumption (SCC 2015).

Membrane bioreactor – This type of reactor combines two proven technologies: enhanced biological treatment using activated sludge and membrane filtration. Significant levels of COD removal (up to 97%) were reported (Visvanathan & Pokhrel 2003).

Combined anaerobic and aerobic treatments - Anaerobic and aerobic treatments are often combined in brewery wastewater treatment, as said before. Firstly, in the anaerobic reactor the bulk of the COD (70–85%) is converted to biogas and secondly, in an aerobic post-treatment step, up to 98% of the remaining COD and nutrients are removed.

Microbial fuel cell - This device is a combined system with anaerobic and aerobic characteristics. It is designed for anaerobic treatment by bacteria in the solution in the anode compartment, with the cathode exposed to oxygen (or to an alternative chemical electron acceptor). Electrons released by bacterial oxidation of the organic matter are transferred through the external circuit to the cathode where they combine with oxygen to form water. Recently, brewery wastewater has been treated while simultaneously generating electricity from organic matter in the wastewater (Wen *et al.,* 2010). Due to

the high COD removal levels, up to 96%, microbial fuel cells can provide a new approach for brewery wastewater treatment while offering a valuable alternative to energy generation.

2.9 Flow cytometry

2.9.1 How does it work

Flow cytometry (FC) is a real time process control strategy that can be used to monitor bioprocess development, at-line, providing single cell information (Lopes da Silva *et al.,* 2012).

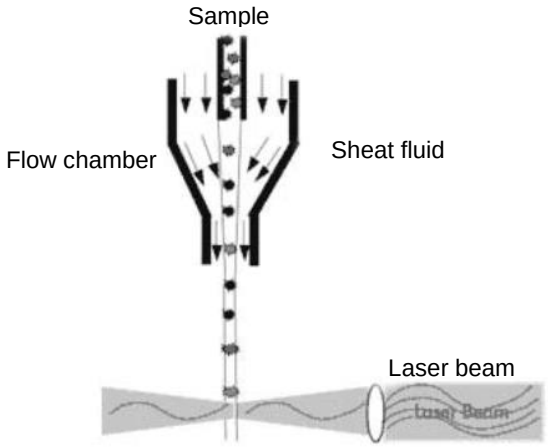

Figure 2.9. Schematic representation of the flow chamber in a flow cytometry system. Adapted from Lopes da Silva *et al. (*2004)

A flow cytometer is a five-element system: one or more light sources (typically laser light), the flow chamber, optical filters, light detectors and a processing unit. The cellular suspension is injected into the centre of a flow sheath and cells are hydro-dynamically focused in the sheath fluid, leading to an individual passing of the cells in a high-speed fluid stream through the chamber (figure 2.9), at up to 100 000 cells/s (Lopes da Silva *et al.,* 2012; Lopes da Silva *et al.,* 2004).

Figure 2.10 shows a graphical representation of the optical system of the cytometer used in this work. At the laser intercept, one or multiple laser beams illuminate the individual passing cells to measure their scattering properties or fluorescence. The laser is focused by a series of lenses. Cell detection is based in two types of scattering: forward scatter (FSC), which is measured in the plane of the beam and gives information on cell size, and side scatter (SSC), which is measured at a 90º angle with respect to the laser beam and reports on cell granularity and internal features. The fluorescence detectors, FL1, FL2, FL3 and FL4 capture fluorescence in very specific wavelengths and allow detection of specific signals.

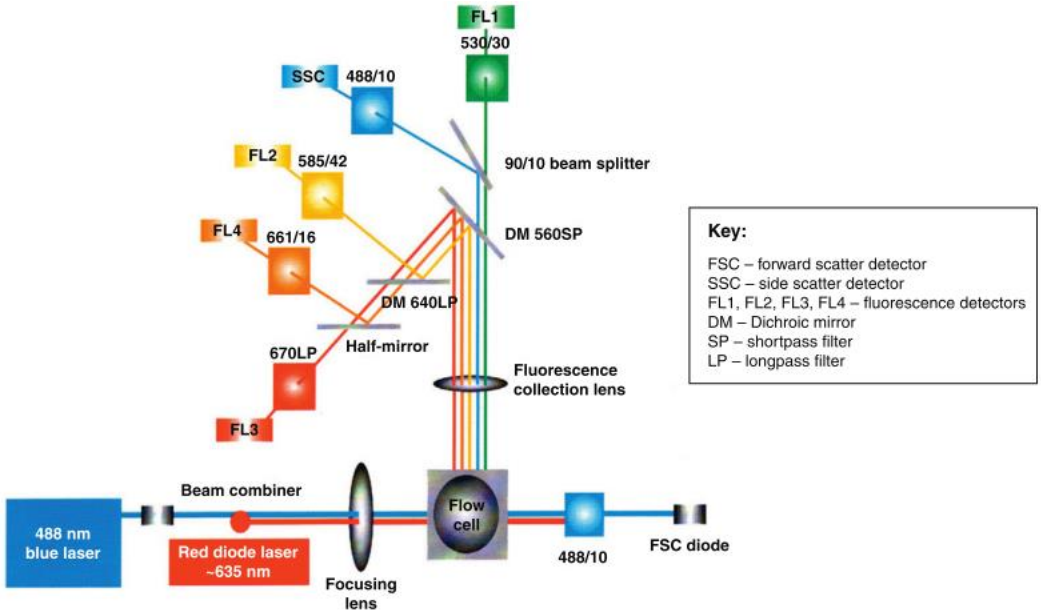

Figure 2.10. Optical diagram of the BD FACScalibur flow cytometer (Lopes da Silva *et al.,* 2012)

2.9.2 FC vs conventional methods

Contrary to conventional microbiology methods that are based on off-line analysis, FC can monitor at-line, in near real time, the development of a given bioprocess, which improves process model formulation and validation. FC has already shown a wide variety of potential applications, particularly in microbial biofuels production, ranging from single-cell to community-cell level analysis (Lopes da Silva *et al.,* 2012)

FC in combination with fluorescent staining stands out for its high accuracy and high speed, with the capacity for multi-parametric analysis of targets, as well as providing the opportunity to select (sort) cells of interest. Relative changes in cell physiological state (including biomass composition) can be detected during a cultivation process (Hyka *et al.,* 2013).

Photomultipliers tubes can detect fluorescence produced by intracellular compounds with specific fluorescence wavelengths, so certain cell components can be selectively analysed. Cells can be differentiated based on their structure and metabolic activity with FC, which is not possible with conventional microbiology methods (Lopes da Silva *et al.,* 2012). There is an extensive spectrum of fluorescent dyes/probes that are used in combination with FC, targeting specific cell compartments, molecules, or their biological functions. Some dyes bind specifically to cell molecules or components (nucleic acids, proteins and lipids) increasing their fluorescence, others accumulate in specific cell compartments or modify their properties, through specific biochemical reactions (Lopes da Silva *et al.,* 2012; Hyka *et al.,* 2013).

A flow cytometer can also be equipped for fluorescence-activated cell sorting (FACS) analysis, that enables sorting based on cellular properties indicated by a fluorescence probe (Lopes da Silva *et al.,* 2012). This technique is used in biotechnological processes for the isolation of cells with target

features (isolation of overproducers, strain improvement) or for the preparation of axenic cultures (Hyka *et al.,* 2013).

By analysing the cells individually, FC detects a variety of physiological and metabolic states, characteristic of different subpopulations (Lopes da Silva *et al.,* 2004). This provides information on the intrinsic heterogeneity of a population in a bioreactor, that cannot be assessed with the routine methods used in bioprocess development and monitoring (Hyka *et al.,* 2013).

Furthermore, by adding a microsphere standard to the sample, absolute cell counts can be obtained with FC (Lopes da Silva *et al.,* 2012), minimizing the errors associated with human counting by optical microscopy.

The use of this technique is hindered by the cost of the equipment and its maintenance as well as the requirement of skilled and trained personnel to operate the instrument. Since comparison between data from various laboratories using different FC devices is generally complex and occasionally not possible, the standardisation of methods and measurements is necessary, which is one of the most limiting disadvantages of FC (Hyka *et al.,* 2013).

2.9.3 Flow cytometry applied to microalgae

A boom in microalgal biotechnology has been evident in the last decade. Flow cytometry is described as a rapid, accurate and sensitive method with the potential to contribute to the efficient development of biotechnological processes with microalgae (Hyka *et al.,* 2013).

Single microalgal cells can be counted, examined or sorted according to their features or physiological state, such as metabolic activity, viability, composition or morphology. For control of microalgal cultivation it is important to monitor not only the increase in biomass concentration, but also changes in biomass composition. The biomass composition of microalgae is extremely variable depending on the species and cultivation conditions (Hyka *et al.,* 2013)

The main advantage of using FC to analyze microalgae is the autofluorescence of naturally occurring intracellular pigments (chlorophylls, carotenoids, sometimes also phycobilins), which are detectable as strong red or orange autofluorescence signals (>600 nm). Chlorophyll autofluorescence is a unique biomarker for microalgae, enabling the differentiation from other particles and microorganisms without being necessary to apply fluorescent probes. Nevertheless, one should pay careful attention in the selection of the dyes for the investigation of microalgae, as the autofluorescence spectrum can overlap with the red spectral range of commonly used dyes (Hyka *et al.,* 2013).

FACS is a rapid method for microalgal isolation and purification from environmental samples. Cell sorting allows an efficient differentiation and subsequent isolation of single cells based on their morphology and variations in autofluorescence. The microalgal populations are distinguishable from contaminating species due to their size (FSC), granularity (SSC) and chlorophyll content (FL3, detected as autofluorescence at >650 nm). One of the main advantages of FACS is the ability to provide axenic cultures through the removal of bacteria. Axenic cultures are required for studies where contamination with other microorganisms is undesirable, or in cultivations under heterotrophic conditions which present a higher risk of overgrowth of the microalgal culture by contaminants (Hyka *et al.,* 2013).

However, FC is limited in terms of sorting of algae cells occurring as aggregates. This limitation may be overcome by sonication of the samples in order to disrupt connections within microalgae aggregates (Lopes da Silva *et al.,* 2004).

In the present thesis, flow cytometry was used to assess the viability and enzymatic activity of the microalgae *Scenedesmus obliquus.*

Cell viability can be evaluated by detecting membrane integrity, as cell death is accompanied by the loss or reduction of that cell feature. An effective method to determine cell viability with FC is to measure the fluorescence of cells using propidium iodide (PI). This fluorescent dye is unable to penetrate intact cells, but strongly stains cells with permeabilized membranes by binding to DNA (Hyka *et al.,* 2013; Lopes da Silva *et al.,* 2012).

Enzymatic activity is a basic feature of live cells, providing information on their physiological state. Esterase activity is the most common way to measure cellular enzyme activity. It can be detected by non-fluorescent probes from the family of fluorescein diacetates (e.g. FDA, cFDA, CMFDA) that are taken up by the cell. If the cell is enzymatically active, the substrate is hydrolysed to a fluorescent compound that can be subsequently detected (Hyka *et al.,* 2013).

3. MATERIALS AND METHODS

3.1 Microalgae selection for wastewater treatment

3.1.1 Microalgae

In this study four strains of green microalgae were used, *Scenedesmus obliquus* (ACOI 204/07, Coimbra University - Culture Collection, Portugal), *Chlorella vulgaris (INETI 58, 90 LNEG_UB, Portugal)*, *Chlorella protothecoides* (strain 25, UTEX Culture Collection, Austin University, Texas, USA) and *Neochloris oleoabundans (UTEX #1185, UTEX* Culture Collection*, Austin University, Texas, USA)*, and a consortium previously isolated from an urban effluent including different genera such as *Chlorella*, *Chaetophora*, *Scenedesmus* and *Navicula*. These microalgae were maintained on standard liquid media (section 3.1.2), in an incubator (New Brunswick Scientific Co. Inc EDISON N.J USA) at 25 °C, under an agitation speed of 120 rpm and continuous illumination delivered by 4 fluorescent lamps (Philips 18 W), providing an average light intensity of 1.1 klux (measured with a Phywe Lux-meter).

3.1.2 Medium for the inoculum

The two *Chlorella* strains were maintained in an organic medium containing per litre: 1.25 g KNO_3, 1.25 g KH_2PO_4, 1 g $MgSO_4.7H_2O$, 0.11 g $CaCl_2.2H_2O$, 0.5 g $NaHCO_3$, 10 mL Fe.EDTA solution and 10 mL trace elements solution (Chu medium). The composition of the trace elements solution is the following: 286 mg/L H_3BO_4, 154 mg/L $MnSO_4.H_2O$, 22 mg/L $ZnSO_4.7H_2O$, 5 mg/L $CuSO_4$, 6 mg/L $Na_2MoO_4.2H_2O$ e 8 mg/L $CoSO_4.6H_2O$.

Scenedesmus and *Neochloris* strains and the consortium were maintained in Bristol medium containing per litre: 250 mg $NaNO_3$, 75 mg K_2HPO4, 33 mg $CaCl_2.2H_2O$, 75 mg $MgSO_4.7H_2O$, 175 mg KH_2PO_4, 25 mg NaCl, 60 mg Fe.EDTA and 10 mL trace elements solution.

All media were autoclaved at 121 °C for 20 min before inoculation. In order to prevent the precipitation of salt complexes due to the high temperatures in the autoclaving process, KH_2PO_4 and $NaHCO_3$ were autoclaved separately and mixed under aseptic conditions after cooling.

3.1.3 Effluent

The brewery effluent to be used for the growth of the microalga was collected from the anaerobic digester BIOPAQ®IC at the Vialonga factory of SCC - Sociedade Central de Cervejas e Bebidas, S.A. The effluent was stored in a refrigerating chamber at 4ºC until its use as the algae cultivation medium, after adjustment to room temperature. The effluent was characterized at the time of use (section 3.1.5.1).

3.1.4 Cultivation

Firstly, all five microalgae inocula were tested. The different strains and consortium were grown in 250 mL Erlenmeyer flasks with 100 mL effluent in an incubator, under the same conditions used in maintaining the inoculum. The flasks were closed with cotton plugs, allowing air diffusion. The inocula were microalgal cultures at the exponential phase, previously grown in the appropriate culture medium for each strain and consortium (section 3.1.2). The volume of inoculum was calculated in order to obtain an initial concentration corresponding to an OD_{540} around 0.1 measured against effluent, and the inoculation was performed in non-sterile conditions. The essays were prolonged until the stationary phase was reached.

After that, the trials were repeated in 1 L Erlenmeyer flasks and only *C. vulgaris*, *C. protothecoides* and *S. obliquus* were tested, since these strains gave the best results for growth in the effluent.

All experiments were performed in duplicate and the effluent without microalgae inoculation served as control.

3.1.5 Analytical methods and calculations

3.1.5.1 Characterization of the effluent

The wastewater portions collected from SCC were characterized in terms of pH, COD, nitrogen (ammonia and 'Kjeldahl nitrogen'') and phosphorus.

The measurement of pH was performed using a laboratory pH meter (InoLab WTW).

COD was determined according to the standard method 5220 B (APHA 1998). A 20 mL sample was mixed with the proper reagents (~0.5 g $HgSO_4$, 30 mL H_2SO_4 and 10 mL potassium dichromate solution) and the reflux occurred for 2 h at 150°C in a Bloc digester 20 P-Selecta. The mixture was diluted with distilled water to 400 mL and titrated with standard ferrous ammonium sulphate (0.25 N), using 2-3 drops of ferroin indicator.

Ammonia nitrogen was quantified by titration after a preliminary distillation step based on standard methods 4500-NH_3 B and C (APHA 1998). In a Kjeldahl tube, a 150 mL sample buffered at pH=9.5 with 25 mL of borate buffer was distilled in a Buchi Distillation Unit K-350 for 6 min. The distillate was collected in an Erlenmeyer flask containing 50 mL of boric acid indicator solution and was titrated with a stock solution of H_2SO_4 0.02 N.

The "Kjeldahl nitrogen'' was determined by a modified Kjeldahl method adapted from the standard method 4500-N_{org} B (APHA 1998). In a Kjeldahl tube, 5 mL of sample and 50 mL of digestion reagent (per liter: 134 g K_2SO_4 + 650 mL H_2O + 200 mL H_2SO_4 + 2 g HgO/25 mL H_2SO_4 6N) were added. The mixture was digested in a Buchi Digestor Unit K-424 for 4 – 4.5 h. After cooling, it was diluted with 100 mL of distilled water and distilled in the distillation unit for 6 min with 50 mL of reagent sodium hydroxide-sodium thiosulfate (per liter: 500 g NaOH + 25 g $Na_2S_2O_3.5H_2O$). The distillate was collected in an Erlenmeyer flask containing 50 mL of boric acid indicator solution and was titrated with a stock solution of H_2SO_4 0.02 N.

A commercial kit for phosphorus determination was used Phosver 3 (ascorbic acid) method using Powder Pillows (HACH, Cat. 212599) in a HACH DR/2010 spectrophotometer. This test gives the results in mg/L of PO_4^{3-} (orthophosphate), P_2O_5 (phosphorus pentoxide) and $P–PO_4^{3-}$ being the phosphorus presented in the phosphate.

All of these analyses were performed in duplicate.

3.1.5.2 Microalgae growth

The microalgae growth was monitored by measuring optical density measurements at 540 nm (OD_{540}) against water using a Hitachi U-2000 spectrometer and by determination of the dry weight (DW) after filtering 5 to 10 mL of culture through a pre-weighed GF/C 25 mm glass fibre filter (Whatman) of 1.2 μm pore size. The filter was weighted after drying overnight at 80 ºC and cooling in a desiccator. DW was calculated by dividing the weight of microalgae biomass retained in the filter by the filtered culture volume. The samples were diluted in order to ensure that the measured optical density values were lower than 1. The pH of the samples was also measured using a laboratory pH meter (InoLab WTW). To construct the growth curve, the values of ln (OD_{540} of the sample – OD_{540} of control) were plotted versus time of incubation.

Correlations between OD_{540} and ash-free dry weight (AFDW) values were established for *C. vulgaris*, *C. protothecoides* and *S. obliquus* given by the following expressions: $AFDW_{C.vulgaris}$ (g/L) = 0.5899 OD_{540} – 0.0591, with a correlation coefficient value (r^2) of 0.8939, $AFDW_{C.protothecoides}$ (g/L) = 0.7697 OD_{540} – 0.1982, with r^2 = 0.905, $AFDW_{S.\ obliquus}$ (g/L) = 0.6682 OD_{540} – 0.1447, with r^2 = 0.9063. The AFDW was determined by filtering the samples through a Whatman GF/C 25 mm filter of 1.2 μm pore size, drying the filter overnight at 105ºC, weighting, incinerating for 1h at 550ºC, and weighting again. The AFDW value was calculated by dividing the difference between the filter weights before and after incineration at 550ºC by the filtered culture volume.

For all the weightings of the filters a Mettler Toledo Ab204-5 analytical balance was used.

3.1.5.3 Determination of the specific growth rate and duplication time

The specific growth rate (μ, day^{-1}) was calculated from the growth curve of the microalgae. The exponential growth phase was identified and then μ was determined as the slope of the straight line in equation (1):

$$ln\, N = \mu t + ln\, N_0 \qquad (1)$$

where *N* is the biomass amount at time *t* within the exponential phase of growth and N_0 the biomass amount at the beginning of the exponential phase.

The doubling time (t_d, day) was calculated from equation (2):

$$t_d = ln\, 2/\mu \qquad (2)$$

3.1.5.4 Determination of biomass productivity

The maximum volumetric biomass productivity ($P_{X,max}$, g/(L.day)) was calculated from equation (3), where X_t is the final biomass concentration (g/L) at the time t (day) and X_0 the initial biomass concentration (g/L) at the initial time t_0 (day):

$$P_{X,max} = (X_t - X_0)/(t - t_0) \quad (3)$$

3.2 Production of *Scenedesmus obliquus* in brewery wastewater using bubble-column photobioreactors

3.2.1 Microalgae

In these studies *Scenedesmus obliquus* (ACOI 204/07, Coimbra University Culture Collection, Portugal) was used, since it was the microalga that showed the best results concerning the adaptation to the effluent and provided the highest biomass concentration at the end of the flask cultivation experiments.

3.2.2 Production of *S. obliquus* in batch mode

3.2.2.1 Photobioreactor

A 5 L polyethylene terephthalate (PET) bottle was used as a bubble-column photobioreactor (PBR) to grow *S. obliquus* in batch mode, indoors, in the laboratory. The PBR has a diameter of 14 cm and 34 cm in height.

3.2.2.2 Feed effluent

The microalgae culture medium for the photobioreactor was the effluent collected from the Vialonga Factory of SCC (section 3.1.3), without any supplementation or pre-treatment.

3.2.2.3 Photobioreactor operation

The inoculum was a microalgal culture grown in a 250 mL Erlenmeyer flask on Bristol medium in an incubator, as described previously in section 3.1.1, up to an optical density of OD_{540}~1.2.

The PBR was inoculated with 160 mL of inoculum culture and the working volume was completed to 4 L (dilution factor=1/25) with effluent.

An aquarium Elite air diffuser (Hagen), placed at the centre bottom of the PBR, was used for air supply and agitation. The culture was illuminated continuously with an average light intensity of 3.2 klux

(measured with a Phywe Lux-meter), supplied by 3 fluorescent lamps (Philips 36 W) assembled at one lateral side of the PBR.

The essay was conducted up to the stationary growth phase. A parallel PBR was operated as a control, in the same conditions, but only with effluent (without inoculation).

Samples were collected from the PBR 2-3 times a day (except on weekends) for OD readings to evaluate microalgae growth. At the end of the experiment, flow cytometry analyses were performed as well as determinations of chlorophyll content. A volume of culture of 1.5 L was used for microalga biomass harvesting for characterization and to evaluate the efficiency of the wastewater treatment.

3.2.3 Production of *S. obliquus* in continuous mode

3.2.3.1 Photobioreactor system

Six-L PET bottles with 14 cm diameter and 40 cm in height were used as bubble-column PBRs to grow *S. obliquus* in continuous mode in the laboratory. In order to test six different residence time values (τ),a continuous photobioreactor system was designed that consisted in six PBRs (one for each residence time), placed in parallel, associated to a 20 L polycarbonate carboy containing the feed effluent, as depicted in figure 3.1. The effluent was fed to each one of the PBRs by a silicone rubber tube attached to a glass tube immersed in the effluent in the carboy. The outlet streams were collected by overflow into plastic containers through silicone rubber tubing fixed to a side opening in each of the PBR.

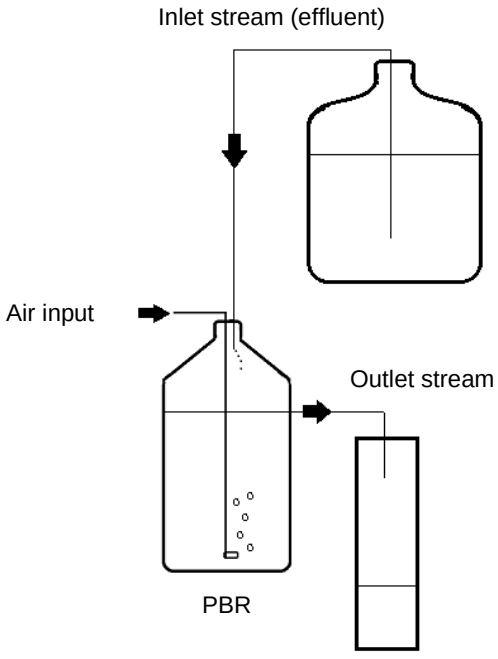

Figure 3.1. Scheme of the laboratory continuous photobioreactor system.
Only one PBR of the six is represented. Not in scale

3.2.3.2 Feed effluent

As in the batch mode the effluent collected from SCC was used as feed medium. However, in continuous mode cultivation the effluent was left to settle for 24 h in the dark at room temperature and the supernatant was then separated for characterization and use as the algal culture medium. This step was necessary to allow the settling of the suspended materials, that could cause clogging problems in the feeding tubes, since a very small diameter was necessary to guarantee the low values of feed flow rate tested in this experiment.

3.2.3.3 Photobioreactor operation

The inoculum was a microalgal culture grown in batch mode in a PBR up to an OD_{540}~3,5. At start-up, the PBRs were inoculated with 550 mL of inoculum culture each, diluted with 4 L of effluent, and were operated in batch mode. Once the culture OD started to stabilize, the operational mode was switched from batch to continuous, by starting the feeding of effluent to each photobioreactor. The feed carboy was covered with aluminium foil to prevent the light from reaching the effluent, in order to avoid the growth of photosynthetic organisms. A working volume of 5 L was maintained by overflow of the culture into the receiving containers, once the liquid reached the imposed level in the PBRs.

Microalgae growth and wastewater treatment were evaluated as a function of residence time (or dilution rate). Six τ values were tested in parallel: 2.26, 3.85, 5.58, 7.55, 9.46 and 11.80 days. A further residence time value was tested (1.72 days), in order to approach washout conditions. This last test was carried out in the PBR previously used for the τ = 3.85 days test. After the biomass harvest, the PBR was operated with additional effluent, with a fresh inoculum, in batch followed by continuous mode, as described before.

The different feeding rates were controlled manually using Hoffman tubing clamps. In order to minimize fluctuations in the flow rates, the liquid level on the carboy was maintained by adding fresh effluent every day. However, due to this manual control technique, some variations occurred, so. mean values were used in the calculations. Feed flow rates were measured by two different ways. On labour days, the volume of effluent fed to the PBR in a certain time interval was measured several times a day. On weekends, the rate was estimated by measuring the accumulated volume of the outlet stream collected during that period (assumed to be practically the same as the inlet volume, evaporation and liquid level variations in the PBR being negligible). Finally, the feed rate was calculated as the mean of all the measured values, over the PBR operational time. The residence time values were then calculated from equation (4).

$$\tau = V/\bar{F} \qquad (4)$$

where *V* the photobioreactor working volume (L) and $\bar{F}$ (L/day) is the mean value of flow rate.

The conditions of aeration, mixing and lighting were the same as those described for the batch system.

Samples were collected 2 times a day (except on weekends) from the PBRs to evaluate microalgae growth. When a steady-state was achieved, flow cytometry analyses and determination of chlorophyll content were performed on culture samples. A volume of 2-2.5 L of culture was collected to harvest the biomass for further characterization and evaluation of the efficiency of the wastewater treatment. Steady-state conditions were considered as established when three consecutive OD readings provided the same value (usually after an operational period corresponding to 3-5 turnovers or residence times) (Teixeira et al. 2007).

3.2.4 Biomass and supernatant recovery

The collected culture volume was allowed to settle for 24h at room temperature in graduated cylinders. The supernatant was recovered and filtered through 47 mm cellulose nitrate filters of 0.45 mm pore size (Sartorius). The concentrated biomass was centrifuged at 10 000 rpm for 10 min at 15ºC (Heraeus multifuge 3SR+ centrifuge, Thermo Scientific) and freeze-dried (Heto Power Dry LL3000, Thermo Scientific). Both filtered supernatant and freeze-dried biomass were kept at -18ºC, for further characterization.

3.2.5 Analytical methods and calculations

3.2.5.1 Microscopic observations

The culture samples, after the steady-state was reached, were observed under an optical microscope (Olympus BX 60) with magnifications from 40x and 100x.

3.2.5.2 Microalgae growth

In batch mode, the biomass concentration was measured by optical density readings at 540 nm (OD_{540}) and by determination of DW and AFDW as described in section 3.1.5.2.

In continuous mode, the growth of the microalga was monitored 2 times a day (except weekends) by reading the OD_{540}, in duplicate, against water. The dry weight and ash-free dry weight were also determined, as described before, on the day of inoculation and at the time when steady-state was reached (according to the OD_{540} readings). The pH of the culture was also measured on the same schedule.

The equipments for these measurements were the same as described before.

3.2.5.3 Determination of growth rates and biomass productivity

In batch mode, the specific growth rate (μ) and the maximum biomass productivity ($P_{X,max}$, g/(L.day)) were calculated as before (sections 3.1.5.3 and 3.1.5.4).

In continuous culture at steady-state, μ equals the dilution rate value (D, day^{-1}), that is defined by equation (5).

$$D = \bar{F}/V = 1/\tau \qquad (5)$$

Volumetric biomass productivity (P_X, g/(L.day)) was determined by:

$$P_X = D \times X \quad (6)$$

where X is the biomass concentration at steady-state.

3.2.5.4 Characterization of the fed wastewaters and supernatants after biomass removal

Both effluents used in batch and continuous systems were characterized as described in section 3.1.5.1. In order to evaluate the efficiency of the wastewater treatment, all the analyses performed for the effluent (pH, COD, nitrogen and phosphorus) were done on the filtered supernatants coming from PBR cultivation runs, using the same methods.

COD, nitrogen and phosphorus removal levels were calculated according to equation (7):

$$Removal\ (\%) = (C_e - C_s)/C_e \times 100\ \% \quad (7)$$

where C_e is the concentration of the measured parameter in the effluent and C_s is the concentration in the filtered supernatant.

3.2.5.5 Microalgae viability and esterase activity

Multi-parameter flow cytometry was used to assess the microalgal cell viability and enzymatic activity using a *FACSCalibur* flow cytometer (*Becton-DickinScn Instruments*, Belgium), equipped with a blue laser (488nm) and a red laser (650nm). Cytograms were analyzed with *CellQuestPro* software.

Samples from the final PBR cultures were pre-treated in an ultrasound bath for 10 seconds to tear apart possible cell aggregates. The cell suspension and added buffer volumes were adjusted to result in 300-400 events s^{-1} and the final measured volume was 500 µL.

Cell viability was determined by detecting membrane permeabilization with Propidium iodide (PI). Samples were diluted with phosphate buffer solution (PBS, pH 7.0) and stained with 4 µL of PI stock solution (Invitrogen, Carlsbad, USA). PI was excited at 488 nm and detected by FL2 channel. The viability of microalgal cells was expressed as the percentage of viable cells (non-stained cells) in the total number of cells analyzed.

Enzymatic activity was measured by detection of esterase activity with cFDA. Samples were diluted with MCI buffer, stained with 5 µL of cFDA and incubated in the dark for 40 minutes prior to analysis. cFDA was excited at 488 nm and detected by FL1 channel. The enzymatic activity of microalgal cells was expressed as the percentage of active cells (stained cells) in the total number of cells analyzed.

All FC analyses were performed in duplicate.

3.2.5.6 Quantification of chlorophyll content

Microalga culture final samples from the PBR (2 mL) were first concentrated by centrifugation for 10 min at 3900 rpm (Sigma 2-6E, Sartorius). Two mL of acetone (99.5%, Sigma-Aldrich) and glass beads were added to the tube and extraction was performed by submitting the mixture to vortex during 2 min followed by 2 min in an ice bath. This procedure was repeated three times, then the mixture was centrifuged at 3900 rpm for 20 minutes, and the supernatant collected. The extraction procedure was repeated on the recovered pellet until a colourless supernatant was obtained. All the tubes were covered with aluminium foil to prevent pigment degradation by light exposure. The total volume of the extract phases collected was quantified.

Chlorophylls *a* and *b* in the extractst (C_a and C_b) were quantified by spectrophotometry (Hitachi U-2000), measuring the absorbances (A) at 630, 647, 664 and 691 nm. The calculations were performed using the following equations (8) and (9) (Ritchie 2008):

$$C_a\ (mg/L) = -0.3319\ A_{630} - 1.7485\ A_{647} + 11.9442\ A_{664} - 1.4306\ A_{691} \quad (8)$$

$$C_b\ (mg/L) = -1.2825\ A_{630} - 19.8839\ A_{647} + 4.8860\ A_{664} - 2.3416\ A_{691} \quad (9)$$

The chlorophyll concentrations in the culture samples (Chl *a* and Chl *b*) were then calculated using the following expression (10):

$$Chl\ (mg/L) = \frac{C\ (mg/L) \times extractant\ volume\ (mL)}{sample\ volume\ (mL)} \quad (10)$$

The chlorophyll contents (Chl *a* and Chl *b*) in the algal cells (mg/g) were calculated by dividing the concentration of the chlorophylls (mg/L) by the cell dry weight (g/L) in the culture samples and total chlorophyll content was obtained by summing the values for Chl *a* and Chl *b.*

The quantification was made in duplicate for all the samples.

3.2.5.7 Biomass fatty acid composition

Fatty acid composition of microalgal biomass samples was analysed, in duplicate, by Gas Chromatography (GC). Before GC analysis, the fatty acids were transesterified by the method of Lepage & Roy (1986) with modifications.

In Pyrex tubes with Teflon liner screw caps, to 100 mg of freeze-dried microalgae 2 mL of a methanol/acetyl chloride (95:5 v/v) mixture and 0.2 mL of heptadecanoic acid in petroleum benzin 60-80ºC (5 mg/mL) as internal standard solution were added. The mixture was heated at 80ºC for 1 h and was cooled to room temperature before being diluted with 1 mL of water and 2 mL of n-heptane. The tube contents were left to stand until phase separation. The upper layer was recovered, dried over anhydrous Na_2SO_4, filtered, and collected in vials, containing the fatty acid methyl ester derivatives (FAMEs) ready for injection in the GC or to be stored at -18ºC until use.

FAMEs were analysed in a CP-3800 GC (Varian, USA) equipped with a 30-m SUPELCOWAX 10 capillary column (film 0.32 μm) with helium as carrier gas at a constant flow rate of 3.5 mL/min.

Injector and detector (flame ionization) temperatures were 250 °C and 280 ºC respectively. The split ratio was 1:50 for the first 5 min and 1:10 for the remaining analysis time. The column temperature programme started at 200 °C for 8 min, increased at 4 °C/min up to 240 °C and was held at that value for 16 min. Individual fatty acid contents were calculated as a percentage of the total fatty acids present in the sample, determined from the chromatographic peak areas.

3.2.5.8 Quantification of biomass protein content

The Kjeldahl nitrogen present in the microalgae biomass was determined by a modified Kjeldahl method described in section 3.1.5.1, using 200 mg of freeze-dried microalga. Protein content was calculated by multiplying Kjeldahl nitrogen content by the conversion factor of 5.95 (López *et al.,* 2010).

4. RESULTS AND DISCUSSION

4.1 Wastewater characterization

The composition of the wastewater used in the flask scale batch trials and in batch and continuous studies in PBRs can be seen in Table 4.1. For the continuous mode, it can be noted a lower organic load (COD) than in the effluent used for batch mode. This is due to the settling of the effluent, through which most of the suspended solids settled and were not included in the recovered supernatant. However, both nitrogen and phosphorus contents were higher. This can be explained by the fact that the two wastewaters were from different campaigns to collect the effluent from the brewery, presenting different compositions probably because of variations in the process.

Table 4.1. Composition of the brewery wastewater used in batch and continuous wastewater treatment. Results are the average of the replicates.

	N-NH_3 (mg N/L)	**Kjeldahl N (mg N/L)**	**PO_4^{3-} (mg/L)**	**P-PO_4^{3-} (mg P/L)**	**P_2O_5 (mg/L)**	**COD (mg O_2/L)**
Batch	15.40 ± 0.13	22.40 ± 0.00	20.00	6.50	15.00	354.17 ± 17.68
Continuous	21.65 ± 0.00	23.80 ± 1.98	26.80	8.70	20.10	241.67 ± 11-79

4.2 Microalgae selection for brewery wastewater (BWW) treatment

In this experiment, the main objective was the selection of the microalga, among the four strains *Chlorella vulgaris (Cv), Chlorella protothecoides* (*Cp*), *Scenedesmus obliquus* (*Sc*) and *Neochloris oleoabundans* (*No*), and the consortium (*Cons*) tested.

In the study done in 250 mL flasks, *No* and *Cons* showed the worst results regarding growth in the brewery wastewater, when compared to *Cv*, *Cp* and *Sc*. The trials lasted 18 days and the growth curves for all microalgae and the consortium are represented in Annex C. *Cv*, *Cp* and *Sc* showed good adaptability to the effluent and a rapid growth in the first 4 days. On the contrary, growth of *No* and *Cons* was only noted after 8 and 6 days, respectively. Regarding biomass production, *No* and Cons were also the ones which presented the lowest maximum dry weight (DW) values, 0.59 g/L and 0.81 g/L, respectively and *Sc* achieved the highest value, 1.56 g/L (Table 4.2).

The consortium did not show good adaptability to this wastewater, in spite of being isolated from another wastewater (urban). This behaviour could be explained by competition between the different genera present. In fact, *Chlorella* strains, which are present in the population of *Cons,* are very competitive, for example with *Scenedesmus (*also included in the consortium) and could be capable of biocidal activity against other microorganisms (Hong et al. 2014). Therefore, these microalgae compromise the growth of other microalgae and, by using part of their energy for competition, their own growth could also be compromised.

Table 4.2. Biomass accumulation of the different microalgae and the consortium in the batch growth tests in brewery effluent (250 mL flasks).

	DW_{max}* (g/L)	Day
No	0.59 ± 0.03	18
Cons	0.81 ± 0.01	18
Cv	1.24 ± 0.04	18
Cp	1.30 ± 0.09	18
Sc	1.56 ± 0.01	18

* Mean value ± standard deviation

After exclusion of *No* and *Cons*, the trials were repeated for *Cv*, *Cp* and *Sc*, but in 1 L Erlenmeyer flasks, in order to finally select the best microalga capable of BWW treatment. This experiment lasted 10 days. Growth curves and pH behaviour of the three microalgae are shown in Figure 4.1. It must be noted that one of the replicates of *Cp* changed to a brownish colour on day 7, probably due to contamination with other strains. This interfered with the OD readings, therefore the values from that point were ignored for that replicate, which was discharged.

Analysing the OD_{540} evolution, both *Chlorella* strains presented similar behaviour and growth rates: 1.14 day^{-1} for *Cv* ad 0.99 day^{-1} for *Cp* (Table 4.3. They entered the exponential phase within the first day of cultivation, reaching the stationary phase on the third day. The growth curves also showed a death phase, represented by the decrease of OD_{540}, on days 8 and 7, for *Cv* and *Cp*, respectively.

Sc also entered the exponential phase on the first day, with a growth rate of 0.58 day^{-1} (Table 4.3), but a death phase was not observed in the 10 days of cultivation. On the third day, the growth slowed down and, on day 6 showed a new rate increase, entering a second exponential phase, before entering a stationary phase on day 9.

Maximum volumetric biomass productivity values ($P_{X,max}$) were also calculated and *Cp* achieved the highest value, followed by *Cv* and *Sc* (Table 4.3).

Concerning the pH, for all microalgae its evolution followed the OD evolution, as expected. The maximum pH reached by the two *Chlorella* was very similar (9.35 for *Cv* and 9.37 for *Cp*) and *Sc* cultures presented a maximum value of 9.96, due to higher OD_{540} reached.

Despite the lower growth rates of *Sc*, when compared to to *Cv* and *Cp*, this microalga showed a more robust growth, as the algal cells maintained active growth for a longer time, not attaining a death phase within the time of the experiment, which is favorable for wastewater treatment. Therefore, this strain was selected to perform the BWW treatment experiments in the photobioreactors.

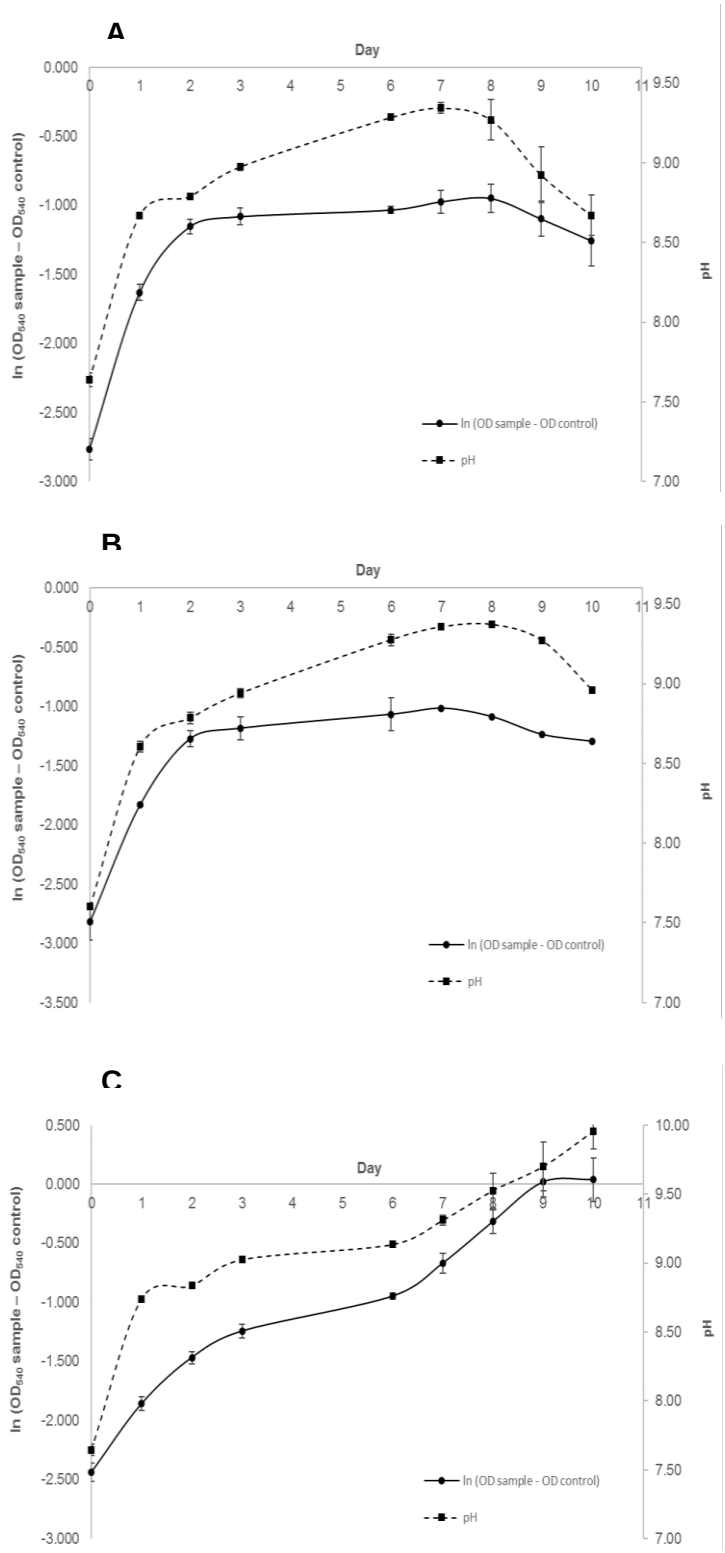

Figure 4.1. Growth curves and pH evolution for *Chlorella vulgaris* (A), *Chlorella protothecoides* (B) and *Scenedesmus obliquus* (C) cultivated in brewery wastewater in 1 L Erlenmeyer flasks.

Table 4.3. Specific growth rates (μ) and doubling times (t_d) for Cv, Cp and Sc cultivated in brewery wastewater (1 L flasks).

	μ (day^{-1})	t_d (day)	$P_{x,max}$ (mg/(L.day))
Cv	1.14 ± 0.14	0.61 ± 0.07	83.33 ± 18.86
Cp	0.99 ± 0.17	0.71 ± 0.12	122.78 ± 10.21
Sc	0.58 ± 0.21 (1)	1.28 ± 0.46 (1)	55.00 ± 3.93 (1)
	0.35 0.03 (2)	1.98 ± 0.14 (2)	22.16 ± 3.23 (2)

The data used for these calculations was obtained between day 0 and day 1, for Cv and Cp. For Sc, the first exponential phase (1) (day 0 to day 1) and the second exponential phase (2) (day 7 to day 8) were considered. Results are the average of the replicates.

4.3 Brewery wastewater treatment by *Scenedesmus obliquus* in photobioreactors in batch mode (5 L) and continuous mode (6 L)

The trial in batch mode lasted 12 days (286 hours) and ended when the algal growth reached the stationary phase. In the trial in continuous mode, 7 mean residence times (τ) were tested: 1.72, 2.26, 3.85, 5.58, 7.55, 9.46 and 11.80 days and the trial for each one ended as soon as the steady state was established.

4.3.1 Growth evaluation

All the results regarding the growth of *Sc* in the BWW using PBRs are depicted in Table 4.4.

Table 4.4. Summary of growth related results obtained (biomass concentration, biomass productivity and pH) for *Sc* grown in PBRs using brewery wastewater, under batch (5 L capacity) and continuous modes (6 L capacity). The day when cultures achieved the steady state is also depicted.

τ (day)	D (day^{-1})	day	AFDW (g/L)	P_x (mg AFDW/(L day))	pH
1.72	**0.58**	3	0.33 ± 0.00	189.45 ± 0.00	9.18
2.26	**0.44**	10	0.31 ± 0.02	138.39 ± 7.83	9.25
3.85	**0.26**	13	0.86 ± 0.09	224.30 ± 22.99	9.53
5.58	**0.18**	20	0.79 ± 0.05	141.23 ± 9.51	9.45
7.55	**0.13**	20	0.91 ± 0.23	120.91 ± 30.45	9.39
9.46	**0.11**	31	0.81 ± 0.02	85.88 ± 1.87	9.49
11.80	**0.08**	35	0.95 ± 0.04	80.51 ± 3.00	9.42
Batch mode		12	0.93 ± 0.01	87.11 ± 3.37	9.53 ± 0.01

Observing the growth curve of *Sc* in batch mode (Figure 4.2), the microalga reached its exponential growth after 45.8 h (on the second day of cultivation), with a maximum growth rate of 0.046 h^{-1} (1.10 day^{-1}) and a doubling time of 15.13 h, and the stationary phase after 214.0 h (day 9). This behaviour was similar to that seen in the 1 L scale trial. The obtained growth rate was almost 10-fold

higher than the value found by Raposo *et al.* (2010) for *C. vulgaris* grown in industrial brewery effluent in 15 L plastic bags. On the other hand, McGinn *et al*. (2012) achieved a growth rate of 1.4 day^{-1} for *Sc* using 300L internally illuminated Brite-Box photobioreactors with secondary municipal wastewater, which is not a much higher value than that achieved in this study.

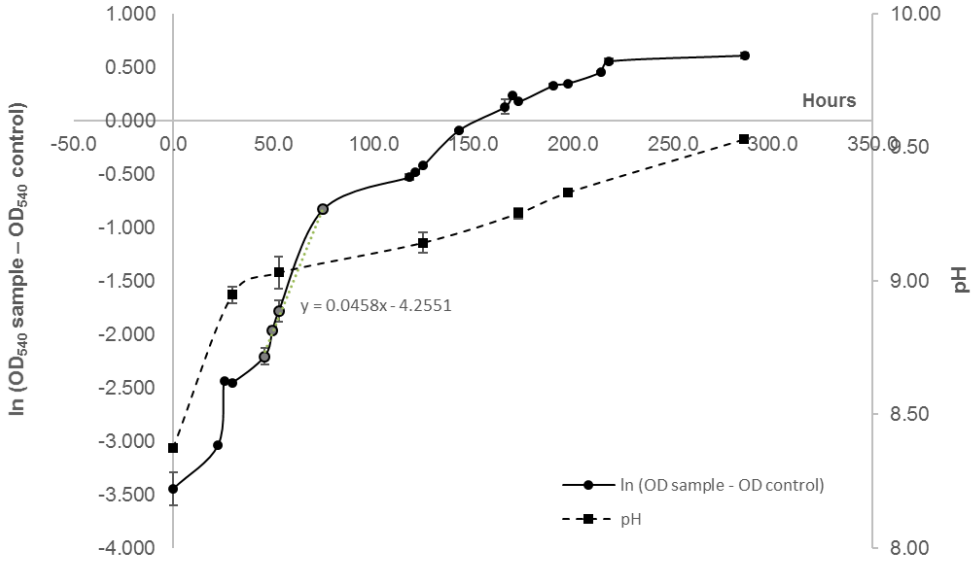

Figure 4.2. Growth curve and pH behaviour of *Sc* in brewery effluent in a 5 L PBR, in batch mode. A linear regression was applied to the exponential phase (grey data points: ●) to calculate the specific growth rate.

Still regarding the trial in batch mode, an ash-free dry weight (AFDW) of 0.93 ± 0.01 g/L and a maximum biomass productivity of 156.80 ± 4.65 mg/(L.day) were obtained. A very similar maximum biomass concentration (0.90 g/L) was obtained by Mata *et al.* (2012) for *Sc* using synthetic BWW. Furthermore, Kong *et al.* (2012) achieved a significantly smaller biomass production, using *Botryococcus brauni* in this type of wastewater. However, better results regarding biomass concentration and productivity were achieved by Darpito *et al.* (2015), for the cultivation of *C. protothecoides* in anaerobically treated BWW.

The biomass concentration evolution in time (ln OD_{540} *vs* time) for the 6 L PBR system in continuous mode is shown in ANNEX E, where the time that the microalgae cultures took to achieve its steady-state can be observed. The latter were in accordance with the literature (after 3-5 residence times). In the parallel tests, the maximum cultivation time was obtained for τ = 11.80 (35 days) and the minimum for τ = 2.26 (10 days). For τ = 1.72, the test only lasted 3 days. This trial was interrupted on that day due to a malfunction of the aeration that caused the culture to settle to the bottom of the PBR.

The growth of the microalga *Sc* was not inhibited by the relatively high pH of the brewery wastewater. In the batch study, an increase of the pH values was noted from 8.38 ± 0.01 up to the end

of the trial when it was reached a maximum value of 9.53 ± 0.01. In the continuous trials, the maximum pH value in steady-state conditions was obtained for D = 0.26 day^{-1}, the same measured for the batch mode (9.53). Furthermore, for all D values tested, the final pH values were similar, ranging from 9.18 to 9.53.

4.3.1.1 Effect of dilution rate on biomass concentration and productivity

The biomass (AFDW) concentration and productivity at steady-state, as a function of dilution rate, are shown in Figure 4.3.

AFDW is drastically reduced from 0.86 ± 0.09 to 0.31 ± 0.02 as the dilution rate increases from 0.26 to 0.44 day^{-1}. This decrease in biomass concentration is predicted in general chemostat operation literature (Teixeira *et al.,* 2007), as discussed before in the literature review (see figure 2.5). The highest biomass concentration was obtained for D = 0.08 day^{-1} (0.95 ± 0.04 g/L). Increasing biomass productivity was observed with increasing dilution rate from 0.08 to 0.26 day^{-1} and then the productivity declined from 0.26 to 0.44 day^{-1}. Therefore, the optimal dilution rate was 0.26 day^{-1}, giving a biomass productivity of 224.30 ± 22.99 mg/(L.day). This trend in the effect of D on biomass productivity was also presented by Tang *et al*. (2012), as a decline of biomass productivity was obtained for dilution rates lower and higher than the optimal value.

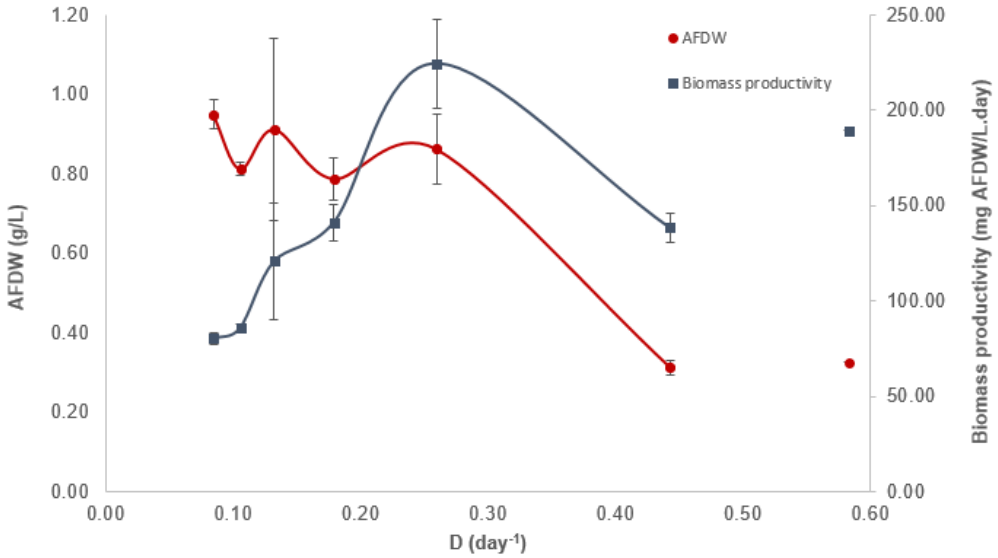

Figure 4.3. Biomass (AFDW) concentration and productivity of steady-state cultures of *S. obliquus* as a function of dilution rate in the 6 L PBR system. The data linked with a line correspond to the parallel testing of 6 residence times. The isolated points correspond to the additional, incomplete test.

After the parallel test of six dilution rates, a smaller residence time of 1.72 days (D = 0.59 day^{-1}) was tested in an isolated run, to verify if in these conditions the microalga culture would suffer washout. The results showed that the washout did not occur within the experimental time, and the obtained biomass concentration was 0.33 ± 0.00 g AFDW/L. This value is slightly higher than the result for the

highest dilution rate in the parallel test run, 0.44 day^{-1}. Moreover, the biomass productivity was surprisingly higher than expected, not following the trend observed in Figure 4.3. At the third day of operation, the settling of the biomass at the bottom of the PBR was noted, which caused improped mixing and led to the accumulation of biomass. The test was thus ended.

In batch mode, an average biomass productivity of 87.11 ± 3.37 mg/L.day (calculated from AFDW values at the beginning and end of the trial) was obtained. This value is close to that achieved for the lowest dilution rate. Furthermore, the final AFDW in batch mode is very similar to the AFDW value for that condition.

The maximum biomass productivity obtained in the continuous mode was lower, but close to the values found by McGinn *et al*. (2012): 234 and 267 mg/(L.day), using *Sc* grown in secondary municipal wastewater in two chemostats at a dilution rate of 0.75 day^{-1}.

4.3.2 Wastewater treatment evaluation

The results of the nutrient contents in the wastewater after treatment and maximal removal rates are depicted in Tables 4.5 and 4.6, respectively. The results regarding ammonia and total Kjeldahl nitrogen, phosphorus, COD and pH were compared to emission value limits (EVLs) of the Portuguese legislation (decree-law 236/98), to assess the possibility of discharging the treated effluent in a natural water body. The Kjeldahl nitrogen was considered to be equal to the total nitrogen present in the treated effluent.

Table 4.5. Characterization of the filtered supernatant, after biomass recovery, for all trials of *Sc* growth in batch mode in 5 L PBR and in 6 L PBRs in continuous mode, using brewery wastewater.

D (day^{-1})	$N\text{-}NH_3$ (mg N/L)	Kjeldahl N (mg N/L)	PO_4^{3-} (mg/L)	$P\text{-}PO_4^{3-}$ (mg P/L)	P_2O_5 (mg/L)	COD (mg O_2/L)	pH
0.08	0.56	5.60	23.20	7.60	17.40	81.20 ± 6.04	9.40
0.11	0.65 ± 0.13	5.60	24.30	7.90	18.20	108.33 ± 11.79	9.52
0.13	0.84 ± 0.13	5.60	19.60	6.40	14.70	81.20 ± 6.04	9.51
0.18	2.05 ± 0.26	9.80	22.10	7.20	16.50	81.20 ± 6.04	9.45
0.26	2.89 ± 0.66	5.60	20.50	6.70	15.30	62.50 ± 5.89	9.52
0.44	7.19 ± 0.13	8.40	23.00	7.50	17.20	104.17 ± 17.68	9.27
0.58	12.97 ± 1.72	23.80	26.80	8.70	20.00	128.21 ± 0.00	9.25
Batch mode	0.19 ± 0.00	5.60	11.20	3.70	8.40	107.41 ± 5.24	9.32
Legislation (1)	10	15	-	10	-	150	6 - 9

(1) Legislation — Decree-law no. 236/98 (PT)

Batch culture and continuous culture at D = 0.26 day^{-1} were the best conditions in terms of wastewater treatment. The batch production of *Sc* attained a 75% removal for total Kjeldahl nitrogen, 43% for phosphorous and 70% for COD. On the other hand, at D = 0.26 day^{-1}, slightly higher removal rates for COD and N were reached (74 and 76%, respectively), but the removal of P was not so efficient (23% removal).

Table 4.6. Nutrient removal rates of *Sc* cultures grown in BWW, using PBRs operated in batch and continuous modes.

	Removal rates (%)			
D (day^{-1})	**N-NH_3**	**Kjeldahl N**	**P**	**COD**
0.08	97	76	13	66
0.11	97	76	9	55
0.13	96	76	26	66
0.18	91	59	17	66
0.26	87	76	23	74
0.44	67	65	14	57
0.58	40	0	0	47
Batch mode	99	75	43	70

The culture at D = 0.58 day^{-1} was inefficient in wastewater treatment. At the end of the trial, the recovered supernatant composition was not much different from that of the untreated wastewater.

Not considering the culture at the highest dilution rate, removal of N ranged from 65 to 76%, which is much higher than the results obtained by Mata *et al.* (2010), 11 – 24%, but lower than those reported by Raposo *et al.* (2010) and Darpito *et al.* (2015), that achieved a removal rate above 85%. All the previous results regarded brewery wastewater treatment in batch mode. One has to consider that the microalgae may have been only partly responsible for the measured N consumption. Ammonia is easily removed by out-gassing to the atmosphere, due to the high pH, which shifts the equilibrium in favour of NH_3 (Martínez *et al.*, 2000). In fact, a near complete removal of ammonia was obtained for batch mode and the lowest dilution rates in continuous modes, corresponding to the higher retention time values.

Phosphorous removal rates were always lower than those observed for N removal, ranging from 9 to 43% (excluding the result for D = 0.58 day^{-1}). These results are lower than those attained by Raposo *et al.* (2010), 54 – 66%, and Darpito *et al.* (2015), more than 90%. For cultures grown under continuous mode, the removal of P was significantly lower than in batch mode.

In continuous mode, McGinn *et al*. (2012) achieved a better wastewater treatment by *Sc* using urban wastewater, with near complete N and P removal at a very short retention time (1.33 day).

Concerning COD removal, removal rates from 55 to 74% were obtained, which are in accordance with the results by Mata *et al.* (2010), wherein a maximum of 66.8% removal was achieved.

Table 4.5 showed that all the conditions, except D = 0.58 day^{-1}, presented results that met the discharge requirements in the legislation, proving that *S. obliquus* using this PBR system is sufficiently efficient to treat brewery wastewater.

Being the objective to achieve efficient wastewater treatment together with optimal biomass production, one can select the culture at D = 0.26 day^{-1} (τ = 3.85 days) to be the most favourable condition, since the maximum productivity was obtained (224.30 mg/(L.day)), the total nitrogen and COD removal rates were the highest and the phosphorus removal rate was the third best.

4.3.3 Physiological state of the microalgal cells

Flow cytometry was used to assess the viability and enzymatic activity (esterase activity) of the microalga *Sc* at the end of the cultivation runs for all conditions tested. All the results are summarized in Table 4.7 and the effect of the dilution rate on these parameters can be observed in Figure 4.4.

Table 4.7. FC analysis to assess the viability and enzymatic (esterase) activity of *Sc* grown in PBRs with BWW.

D (day^{-1})	% Viable cells	% Enzymatically active cells
0.08	88.24 ± 0.15	52.43 ± 0.98
0.11	85.77 ± 0.02	55.79 ± 3.99
0.13	85.49	75.72
0.18	90.95	65.39
0.26	92.33 ± 0.53	49.47 ± 3.82
0.44	86.04 ± 0.88	42.40 ± 4.65
0.58	82.80 ± 0.18	83.42 ± 5.45
Batch mode	95.13 ± 0.82	69.12 ± 0.35

The results for the highest dilution rate (0.58 day^{-1}) are not to be directly compared to the results for the other dilutions, since this trial was not performed in the same conditions, as discussed before. However, it can be concluded that at that dilution rate, the microalga *Sc* proved to be very resistant, presenting good results in what concerns its physiological state, as most of the cells (about 80%) are intact and enzymatically active.

Regarding cell viability, the batch culture mode showed the best result (95.13 % of the cells present intact membranes). For the six tested dilution rates, the range of values obtained was narrow, from 85.49 to 92.33 % of cells with intact membranes. An increasing percentage of cell viability was found with increasing dilution rate from 0.13 to 0.26 day^{-1} and then the percentage decreased from 0.26 to 0.58 day^{-1}. The optimal dilution rate for cell viability was the same as for biomass productivity (0.26 day^{-1}).

In what concerns esterase activity, the maximum percentage of active cells was obtained for D = 0.13 day^{-1}, followed by the result for batch mode, 75.72 and 69.12 % of cells with esterase activity, respectively. A decline in the percentage of cell activity with increasing dilution rate from 0.13 to 0.44 day^{-1} can be observed, where the minimum achieved was 42.40 ± 4.65 %. However, for the two lowest dilution rates, lower percentages of enzymatically active cells (around 50%) were also found. This could be due to the age of the cultures, as it took 31 and 35 days to achieve steady-state of the cultures at D = 0.11 and 0.08 day^{-1}. Those values were considerably higher than the ones obtained for the other cultures: 3 to 20 days.

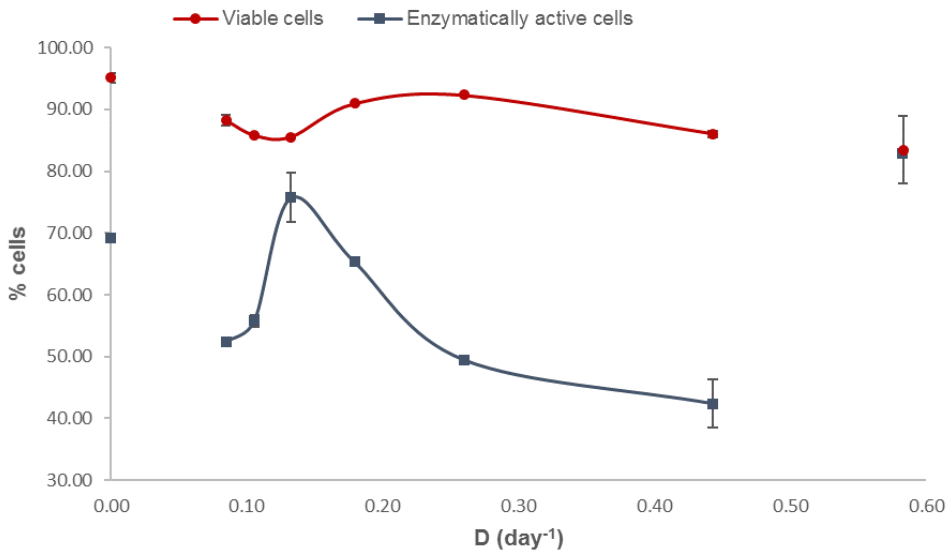

Figure 4.4. Viability and esterase activity of *Sc* cells in steady-state cultures as a function of dilution rate in the 6 L PBR system. The value obtained for the batch regime is represented by D = 0. The data linked with a line corresponds to the parallel testing of 6 dilution rates. The isolated points at D = 0.58 day^{-1} correspond to the additional, incomplete test.

Veloso *et al.* (1991) stated that the value of the Chl *a*/AFDW ratio was found to be a simple and characteristic indicator of the physiological state of an algal culture. A value of less than 1% means imminent population crash, which may be due to a predator or to a lack of nutrients. Figure 4.5 shows that all ratio values are higher than 1%. For the batch regime a Chl *a*/AFDW ratio of 1.58 ± 0.10 % was obtained and for continuous trials, higher values were obtained for the higher dilution rates, reaching 2.77 ± 0.04 % at D = 0.44 day^{-1}, and the lowest value was 1.50 ± 0.02 % for D = 0.26 day^{-1}. However, the highest value obtained was for D = 0.11 day^{-1} (3.08%), which is probably due to the high value of Chl *a* content, to be discussed next.

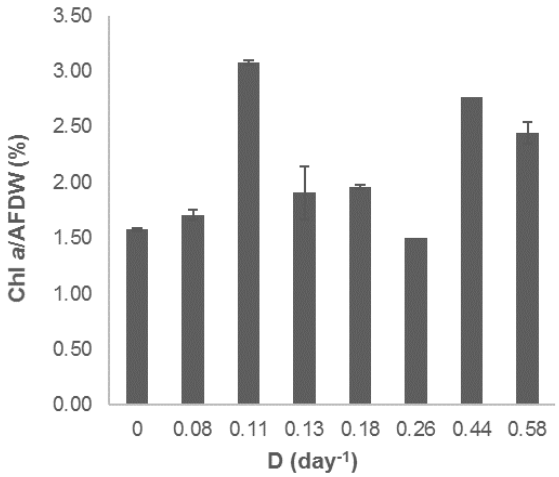

Figure 4.5. Chl *a*/AFDW ratio of steady-state cultures of *S. obliquus* in the 6 L PBR system for all dilution rates tested and in the 5 L PBR for the batch trial. The value obtained for the batch regimen is represented by D = 0.

Therefore, according to the calculated Chl *a*/AFDW ratio values, imminent population crash was not expected. Besides, microscopic observations were carried out to check for the occurrence of contaminations and assess the morphology and physiological state of the microalgae cells. For all cultures, the microalga *Sc* was mainly observed as a group of two or four cells (Figure 4.6 - A and B), showing membrane integrity. This result is in accordance with the data obtained by cell FC analysis using PI. However, for the batch culture and for the highest residence times in continuous cultures, the presence of Chlorella was noted as a contamination (Figure 4.6 - C).

Figure 4.6. Microscopic observations. A: four cells colony of *S. obliquus*, B: two cell agglomeration of *S. obliquus* and C: presence of *Chlorella.*

4.3.4 Biomass composition

The results obtained for the biochemical characterization of *Scenedesmus obliquus* biomass after the effluent treatment runs are presented in Table 4.8.

Table 4.8. *S. obliquus* biomass composition obtained after brewery wastewater treatment.

D (day^{-1})	Chl *a* (mg/L)	Chl *a+b* (mg/g)	Protein content (%)
0.08	16.22	20.53	45.37
0.11	25.03	45.71	42.40
0.13	17.42 ± 0.12	26.78 ± 2.62	37.93
0.18	15.45 ± 1.87	22.34 ± 1.27	45.28
0.26	12.91 ± 0.15	18.52 ± 0.51	50.09
0.44	8.64 ± 0.13	38.81 ± 1.17	39.90
0.58	7.94 ± 0.03	27.85 ± 1.67	37.81
Batch mode	14.76 ± 0.85	18.94 ± 0.80	21.15

Chl contents values were obtained within the range from 18.52 mg/g for D = 0.26 day^{-1} to 45.71 mg/g for D = 0.11 day^{-1}. For the cultures in the PBRs operated in continuous mode, the results were close to each other, except for the maximum (45.71 mg/g), which is higher than expected. However, it is lower than the maximum Chl content achieved by Raposo *et al*. (2010), with *C. vulgaris* grown in brewery wastewater.

The continuous mode proved to be advantageous regarding the protein content of the produced biomass, since the minimum content was obtained for the culture in batch mode (21.15%). The highest protein content (50.09 %) was found for D = 0.26 day^{-1}, which is the optimal value for biomass productivity (and cell viability). This result is within the range presented by Becker (2007) for *S. obliquus*. All the protein content values obtained in the continuous mode were higher than those reported by Batista *et al*. (2014), 20.4%, using Bristol medium, Gouveia *et al.* (2016), 32.7%, using urban wastewater, and Hodaifa *et al.* (2008), 30.8%, using wastewater from olive oil extraction.

5. CONCLUSIONS AND FUTURE WORK

Anaerobically treated brewery wastewater proved to be a low-cost culture medium, satisfying the basic nutrient requirements for microalgae growth.

Using the microalga *Scenedesmus obliquus*, the performance of the PBRs operated in batch and continuous modes was interesting, as the brewery wastewater treatment was efficient, even at low residence times (~2 days). The results were unfavourable only for the highest dilution rate tested. High removal rates were achieved for nitrogen and COD removal (up to 80%, and around 70%, respectively). However, phosphorus removal rates were lower (up to 40%). The resulting treated effluent was ready for discharge in a natural water body, in accordance to the Portuguese environmental legislation (decree-law 236/98). Thus, the brewery wastewater treatment by *Scenedesmus obliquus* can be done, combined with other treatment technologies.

The continuous cultivation mode proved to be advantageous regarding biomass productivity, and chlorophyll and protein contents in the biomass. Using this feeding mode, at selected dilution rates, the N and COD removal rates were even higher than for the batch operational mode.

The value of dilution rate was found to have a strong influence on some performance parameters, mainly on biomass productivity and esterase activity.

Taking into account wastewater treatment performance and biomass productivity, quality and cell viability, continuous culture at D = 0.26 day^{-1} is the best option.

Further research should be conducted to evaluate this PBR system for wastewater treatment by *Scenedesmus obliquus* at larger scale. Also, optimization of culture conditions to maximize biomass productivity without compromising wastewater treatment efficiency or the economic viability of the process, should be considered. Light intensity is one of the parameters that can be optimized, as continuous artificial illumination could be expensive. Furthermore, a future work line could be the study of the influence of CO_2 supplementation of the air flow on the wastewater treatment and microalgal growth performances.

Moreover, the biorefinery integration of this process should be investigated, as the downstream processing of microalgae biomass may result not just in lipids, suitable for biodiesel production, or protein, but also in a variety of high-value products.

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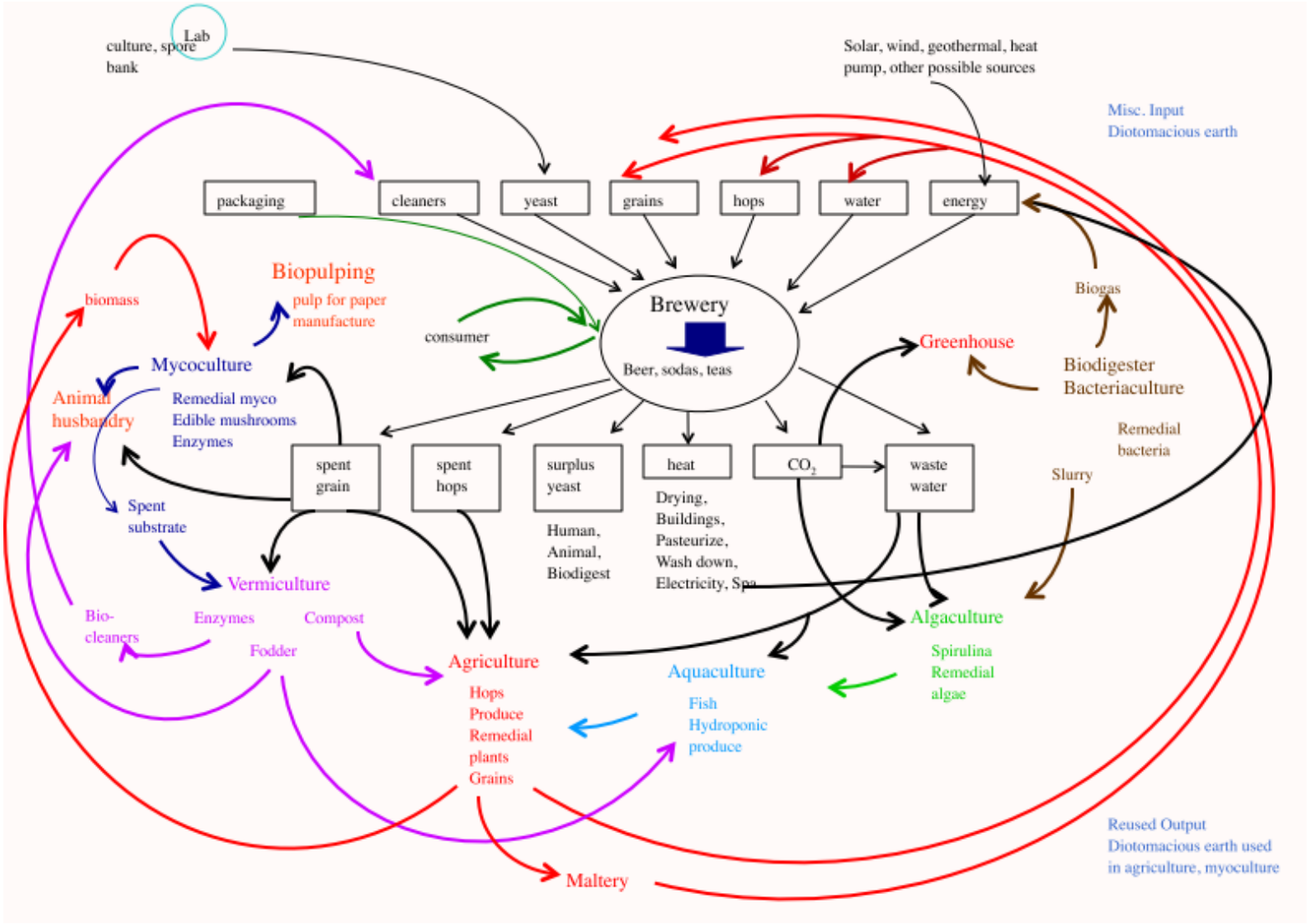
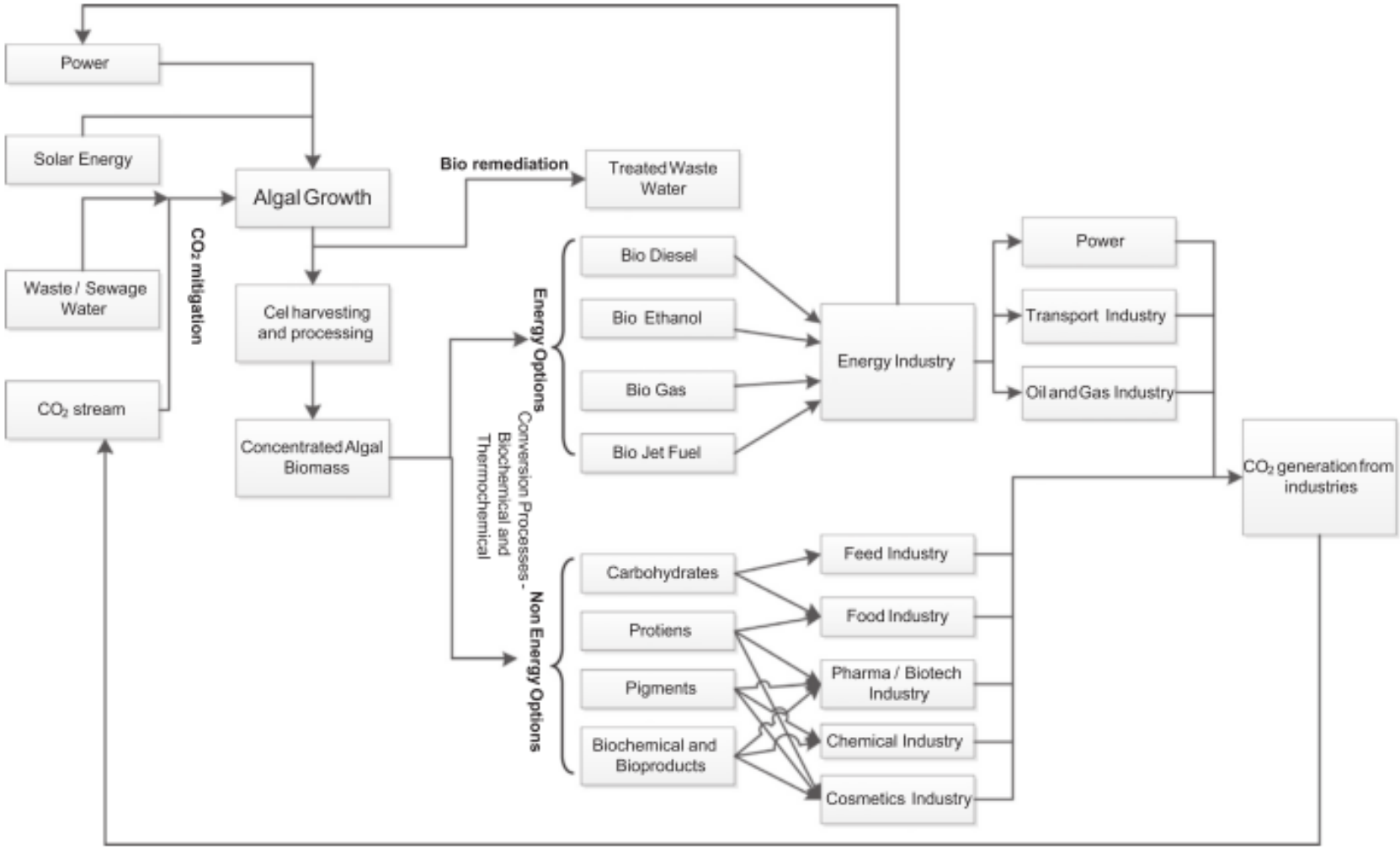
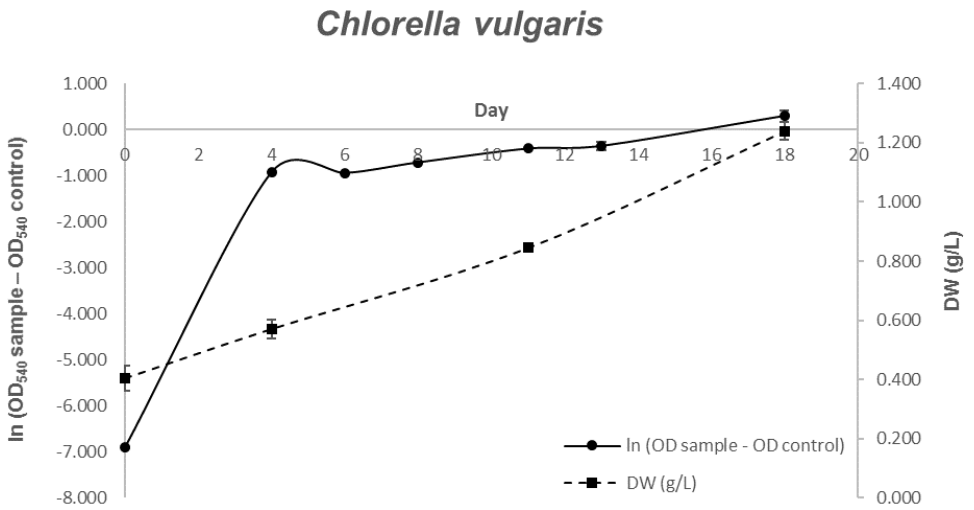
ANNEX A. Model for Zero Emission Brewery Industry Material Flow (Pitcher 2012)

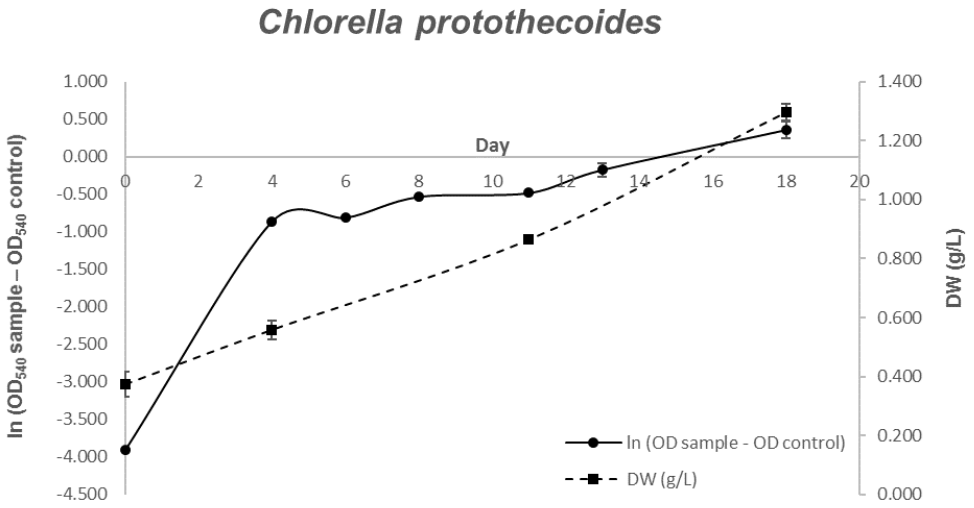


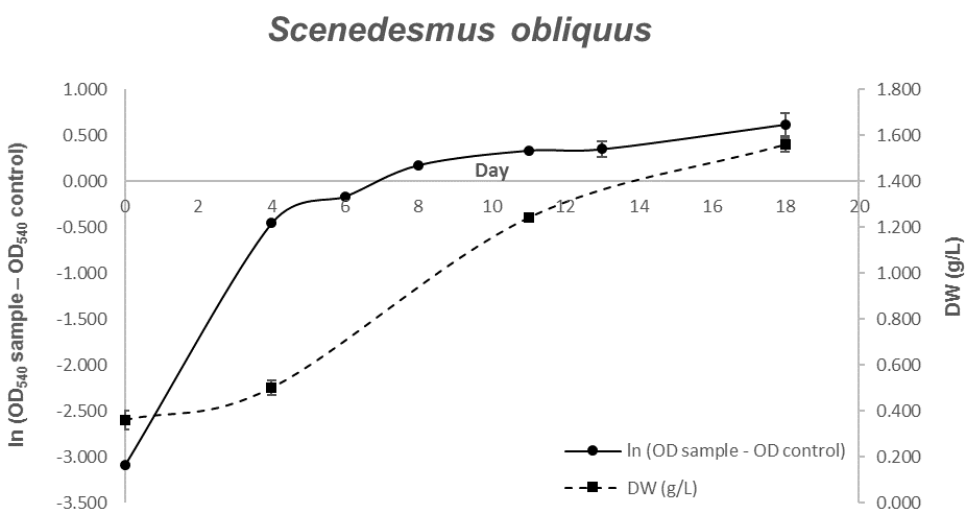
ANNEX B. Schematic flow sheet for a microalgae biorefinery (Trivedi *et al.*, 2015)

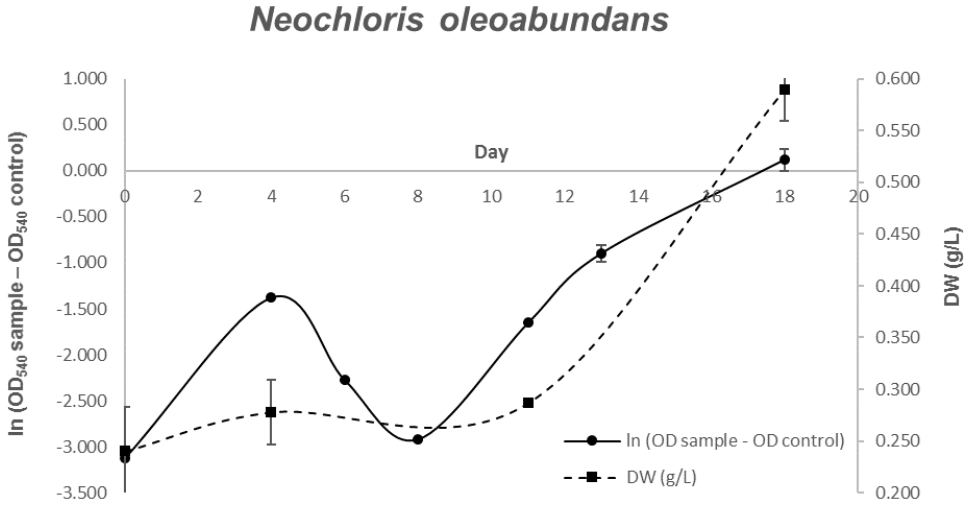


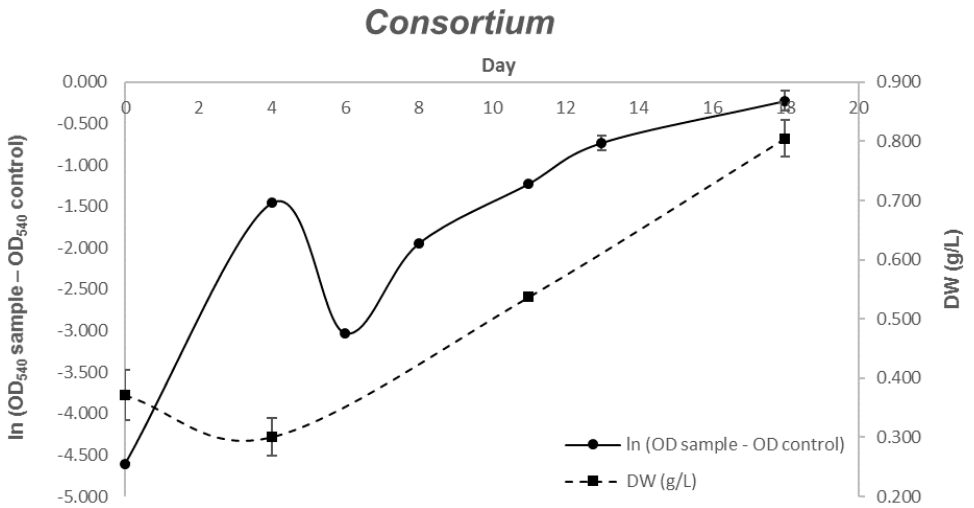
ANNEX C. Growth curves of microalgae cultivated in brewery effluent in 250 mL Erlenmeyer flasks



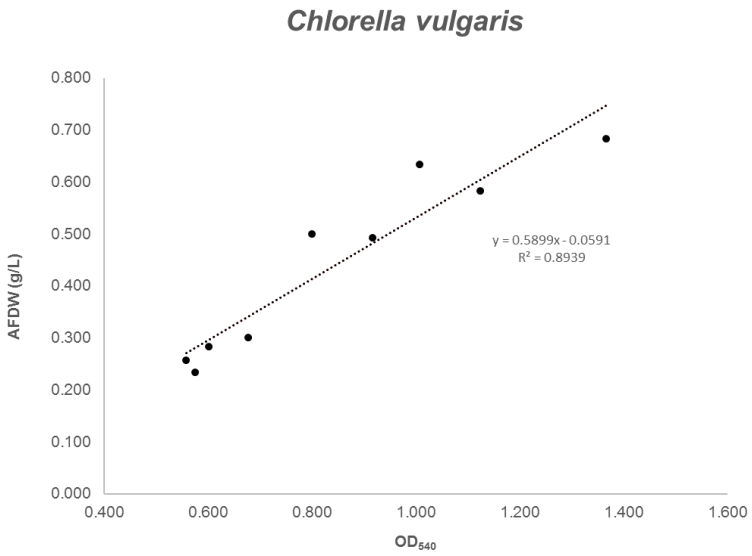


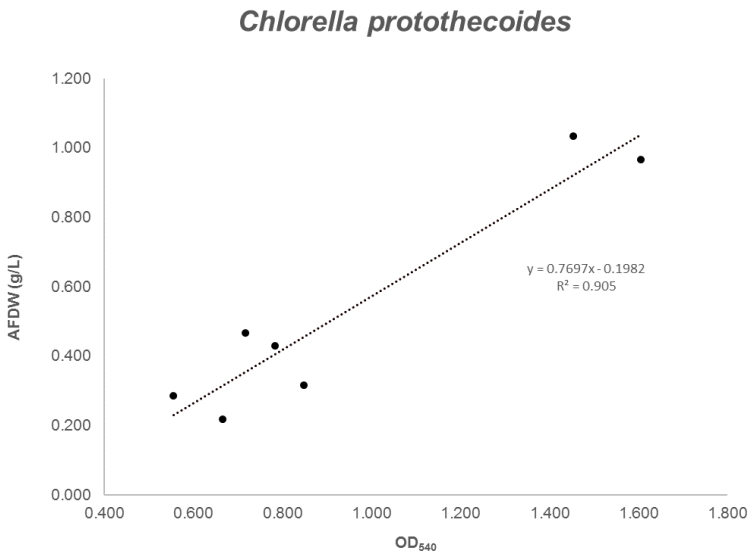

Scenedesmus obliquus
ln (OD540 sample – OD540 control)
DW (g/L)
Day
ln (OD sample - OD control)
DW (g/L)

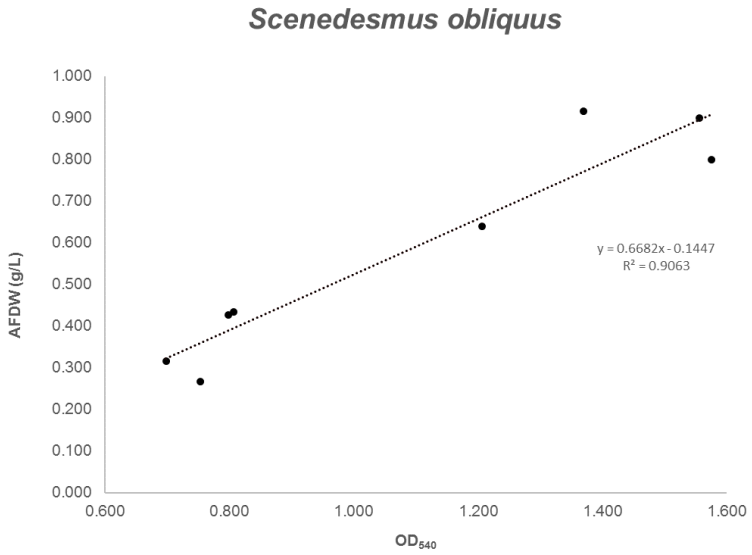

Neochloris oleoabundans
ln (OD540 sample – OD540 control)
DW (g/L)
Day
ln (OD sample - OD control)
DW (g/L)


Consortium
ln (OD540 sample – OD540 control)
DW (g/L)
Day
ln (OD sample - OD control)
DW (g/L)

ANNEX D. Correlations AFDW vs OD_{540}







ANNEX E. Variation of ln OD_{540} in continuous mode study