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ANAEROBIC DIGESTION AS A TOOL FOR IMPROVING ENERGETIC YIELD OF MICROALGAE BASED BIODIESEL

DOCTORAL THESIS IN FULFILLMENT OF THE REQUERIMENTS FOR THE DEGREE DOCTOR OF SCIENCES IN NATURAL RESOURCES

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"ANAEROBIC DIGESTION AS A TOOL FOR IMPROVING ENERGETIC YIELD OF MICROALGAE BASED BIODIESEL"

Esta tesis fue realizada bajo la supervisión del director de Tesis Dr. DAVID ALEJANDRO JEISON NUÑEZ, perteneciente al Departamento de Ingeniería Química de la Universidad de La Frontera y es presentada para su revisión por los miembros de la comisión examinadora.

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... dedicada a Dios (lo más preciado en mi vida)

... y a mis Padres (quienes me enseñaron a apreciarlo)

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Summary and outline of this Thesis

Microalgae are considered a promising feedstock of biomass for the production of biofuels. The capacity of some strains to accumulate lipids makes them an interesting alternative for biodiesel production. The anaerobic digestion of the spent (lipid-extracted) biomass has been proposed as a way of increasing energy yield and sustainability of bioenergy production from microalgae. Anaerobic digestion would produce biogas, but also would provide conditions for nutrient recovery. Thus, nitrogen recovery is expected to consider that nitrogen is reduced to soluble ammonium in anaerobic digestion microalgae. Moreover, considering that microalgae present a high protein content and ammonium inhibition in anaerobic reactor, it is expected that in substrate containing high protein content, the recovery of ammonium from anaerobic reactor will cause a double benefit: On one hand, generating a rich nitrogen fraction, which can be recycled to microalgae culture and, on the other hand, avoiding ammonium inhibition. In order to achieve this proposal, membrane filtration is presented as a process able to recover a rich ammonium fraction anaerobic reactor, hence, it will reach the double benefit already commented.

The outline of this thesis begins with a general introduction. In Chapter I, a general description of global problem and proposal of anaerobic digestion of spent microalgae for recovery energy is outlined. Moreover, the hypothesis and general/specific objectives of this thesis are presented.

Chapter II presents a literature review about anaerobic digestion of microalgae. In this chapter, the feasibility of anaerobic digestion using microalgae as substrate is discussed, considering factors such as microalgae features (composition, cell wall, degradability) and anaerobic digestion operation (ammonia and salt inhibition, pre-treatment and biogas upgrading).

In Chapter III biogas production from anaerobic digestion of spent microalgae was evaluated through BMP tests. The biogas production obtained from BMP tests was used in order to calculate how energy form total microalgae can be recovered through anaerobic digestion and energetic contribution of energy produced as biogas in a global microalgae biodiesel process.

In Chapter IV membrane filtration system was proven as harvesting process. The membrane performance filtering a microalgae culture was evaluated considering factors such as biomass concentration and cross-flow velocity. The increases in membrane resistance by cake formation and fouling were observed and the causes of these behaviors were proposed. Moreover, energetic requirement for membrane filtration was computed and compared with other harvesting processes

In Chapter V continuous operation of mesophilic and thermophilic reactors degrading spent microalgae were followed-up and compared. The reactor performance was measured considering biogas production and presence of inhibitors. Assays carried-out showed that hydrolysis is a limiting step in anaerobic digestion. Moreover, nitrogen recovery through membrane filtration system coupled to mesophilic anaerobic reactor was measured characterizing permeate fraction obtained in filtration system. Finally, Chapter VI presents a general discussion and conclusions for anaerobic digestion from spent microalgae. In addition, future directions are addressed in order to investigate and evaluate some aspects that will provide a more realistic vision about feasibility of anaerobic digestion of spent microalgae.

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CHAPTER I.
General Introduction

1.1 General introduction

Energetic crisis is the main world issue, which is caused by both increase of world population (higher energetic consumption) and fossil fuel depletion. Renewable energies not only are presented as a solution for energetic crisis, but also allow mitigating environmental impacts caused by exclusive older fossil energy. In fact, during 2010 about 19% of worldwide energy consumption came from renewable sources such as wind, solar, geothermal and biomass (REN21, 2010). Bioenergy, the energy produced from biomass, is the most important source of renewable energy nowadays. Indeed, 70% non-conventional renewable energy is based on biomass (REN21, 2010).

Microalgae, the common denomination for a broad group of photosynthetic prokaryotes and eukaryotes, have been considered as a promising feedstock of biomass for the production of "third generation" biofuels. This consideration is based on advantages of microalgae over traditional land-based crops, highlighting the ability of certain types of microalgae to accumulate lipids and its oil productivity 10 times higher than high-yielding oil crop (oil palm) (Chisti, 2007; Deng *et al.*, 2009; Mata *et al.*, 2010; Weyer *et al.*, 2010) , a higher CO_2 fixation efficiency, which is expected to mitigate atmospheric CO_2 increase (Amin, 2009; Brennan and Owende, 2010; Mutanda *et al.*, 2011) and the possibility of cultivation on non-arable land areas, reducing land competition for human consumption (Mussgnug *et al.*, 2010; Stephens *et al.*, 2010). From these advantages, most current efforts to take advantage of microalgae as a source of bioenergy have been directed to biodiesel production. Despite the advantages above mentioned, there is concern when biodiesel production from microalgae is considered; a potentially low energetic yield expected from current technology (Chisti, 2007; Sialve *et al.*, 2009; Scott *et al.*, 2010; Stephens *et al.*, 2010). Indeed, a negative energetic balance has been calculated for biodiesel process from microalgae, and in this sense, the large production costs have been associated with harvesting and drying process, which are energetically demanding (Lardon *et al.*, 2009; Scott *et al.*, 2010). Moreover, a new waste, spent microalgae, is generated, therefore, new associated costs with disposal and management of this residue will be generated, affecting industrial application of biodiesel process.

These drawbacks can be overcome if energy from spent microalgae (waste) is generated in an associated process to biodiesel production from microalgae. Thus, anaerobic digestion of

spent microalgae will contribute to biodiesel production from microalgae so a dual energy benefit is reached: on one hand, no or little energy is required for waste stabilization, and on the other hand, an energy-rich end-product is generated: biogas. Therefore, anaerobic digestion of spent microalgae may represent an important energetic input to biodiesel process, contributing in energetic yield of global process. In relation to anaerobic digestion from spent microalgae, few studies have reported drawbacks in anaerobic digestion from microalgae. Despite this, the drawbacks have been indicated, highlighting possible low performance associated with degradability of cell wall, effect of drying on methane production and ammonium inhibition associated with high relative fraction of protein in spent microalgae (Sialve *et al.*, 2009; Ehimen *et al.*, 2011; González-Fernández *et al.*, 2012). In relation to drying step, results of microalgae anaerobic digestion have indicated a negative effect on dried microalgae before lipid extraction for biodiesel production (Kinnunen and Rintala, 2010; Mussgnug *et al.*, 2010).Moreover, as above mentioned, drying step increases energetic consumption of biodiesel production process. Thus, results found may contribute greatly from an energetic point of view. Another drawback associated with anaerobic digestion of microalgae is ammonium inhibition, which can be expected considering low C/N ratio and high protein content present in spent microalgae (Becker, 2004). Anaerobic degradation of these residues is expected to generate a high ammonium concentration that may cause inhibition of anaerobic microbial consortia, especially methanogenic bacteria (Angelidaki and Ahring, 1993; Chen *et al.*, 2008; Sialve *et al.*, 2009). In addition, high ammonium concentration may affect biogas quality, therefore, ammonium can be stripped towards gas phase (Sialve *et al.*, 2009). One way to overcome the drawback caused by ammonium inhibition is the possibility of co-digestion in order to provide an optimal C/N ratio for anaerobic digestion process (Yen and Brune, 2007; Ehimen *et al.*, 2011). Thus, a higher C/N ratio co-substrate should be mixed with spent microalgae in order to increase anaerobic digestion yield. This strategy results more attractive if the fact that some co-substrate can stimulate enzymatic synthesis is considered, hence, hydrolysis and degradability increase (Yen and Brune, 2007; Sialve *et al.*, 2009). Besides, co-digestion can dilute certain toxic compounds, which will allow decreasing of concentration under toxic/inhibition conditions (Sialve *et al.*, 2009).

Another solution to improve anaerobic digestion when ammonium inhibition is evidenced may be the extraction of this nutrient through membrane filtration system in anaerobic digestion. Anaerobic membrane bioreactors (AnMBR) represent a combination between anaerobic reactor

and membrane filtration system (micro-ultra filtration) coupled to this reactor. Membrane filtration system has biomass retention as objective (Jeison *et al.*, 2007). Total biomass retention provided by membrane filtration system allows an operation at high cell concentrations. In addition, AnMBR systems can favor specific microorganism retention (Brindle and Stephenson, 1996; Ben Aim and Semmens, 2003) and generate a permeate fraction free from cells. The latter make elimination of post treatment steps possible (Jeison *et al.*, 2007), in case that recirculation of treated water is of interest.

In context of anaerobic digestion of spent microalgae, AnMBR reactor presents benefits: On one hand, filtration system will increase biomass retention time which will enhance microalgae degradation, hence, nutrient release. On the other hand, as already discussed, during anaerobic digestion process, most nitrogen present in substrate (mainly as proteins) is converted into ammonium. Membrane filtration system coupled to an anaerobic reactor will generate an ammonium rich permeate that could be recycled back to the photobioreactor as nitrogen source. In addition, this recirculation would also prevent inhibition effects, enabling stable digestion step by constantly removing ammonia from the anaerobic reactor.

1.2 Hypothesis

Anaerobic digestion of oil-extracted microalgae will improve the biodiesel production process by increasing energetic yield as a result of biogas production.

1.3 General goal

To evaluate the contribution of anaerobic digestion to a biodiesel production process from microalgae biomass.

1.4 Specific goals

- To evaluate the energetic potential of biogas produced during anaerobic digestion of oil-extracted microalgae.
- To determine nutrients (nitrogen and phosphorus) release during anaerobic digestion of oil-extracted microalgae.

CHAPTER II.
Challenges for cost-effective microalgae anaerobic digestion: A review.

Published as book chapter:

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2.1 Introduction

Microalgae, the common denomination for a broad group of photosynthetic prokaryotes and eukaryotes, are characterized by an efficient conversion of the solar energy into biomass. They are a promising feedstock for the production of third generation biofuels for several reasons:

1. Microalgae photosynthesis allows biological CO_2 fixation, which is expected to mitigate atmospheric CO_2 increase (Amin, 2009; Brennan and Owende, 2010; Mutanda *et al.*, 2011).

2. Microalgae are 10 – 50 times more efficient than plants in terms of CO_2 fixation (Wang *et al.* 2008). Thus, microalgae can fix 1.83 tonnes of CO_2 per 1 tonne of produced microalgae (Chisti, 2007).

3. Microalgae can be produced on non-arable areas such as lakes, oceans or deserts, thus reducing competition with food production (Mussgnug *et al.*, 2010; Stephens *et al.*, 2010). This advantage is a key factor when energy supply is considered in desert zones near oceans.

4. Some microalgae can grow under saline conditions, which strengthen the use of microalgae as feedstock for biofuel production in desert zones near the ocean when freshwater supply is not feasible.

Most of current efforts to take advantage of microalgae as a source of bioenergy are directed to biodiesel production, considering the ability of certain types of microalgae to accumulate lipids under controlled culture conditions. Microalgae biodiesel produced from microalgae lipids also presents technical advantages compared to lignocellulosic biomass based biodiesel. Biodiesel from microalgae has a higher calorific value (30 and 29 MJ/kg for *C. protothecoides* and *Microcystis aeruginose*, respectively) and lower viscosity and density than plants-based biodiesel (Costa and de Morais, 2011). However, the biodiesel yield from algae is rather low compared to biodiesel from lignocellulose energy (Chisti, 2007; Sialve *et al.*, 2009; Scott *et al.*, 2010; Stephens *et al.*, 2010). Indeed, with current technology, a negative energy balance was calculated by Lardon *et al.* (2009) when evaluated biodiesel production from *C.*

vulgaris, considering biomass drying and further lipid extraction by solvents. During biodiesel production from microalgae, energy consumption associated with culture mixing and pumping, lipid extraction, nutrients addition, drying is of particular importance (Scott *et al.* 2010). Indeed, Lardon *et al.* (2009) estimated that the necessary energy consumption for drying was near 85% of the total energy consumption in a biodiesel production process from microalgae. Another drawback of biodiesel process is associated with the microalgae cultivation step, as nutrient requirements are 55-111 times higher than for e.g. rapeseed cultivation (Halleux *et al.*, 2008). Under these conditions, biodiesel production from microalgae may not be energetically and environmentally sustainable (Sialve *et al.*, 2009; Ras *et al.*, 2011).

2. 2 Microalgae as a source of biogas

Biogas production through anaerobic digestion is an established technology where a wide variety of residues can be used as substrate. In 2011, 8.760 anaerobic digesters were reported in Europe (IEA, 2011). The contribution of this technology to the reduction of carbon emissions, green energy and green gas policies has generated intense interest, especially over the past decade.

When considering biogas production from microalgae two alternatives can be conceived: Microalgae biodiesel production and further anaerobic digestion of microalgae residues for biogas production (Process 1, Figure 1A) and anaerobic digestion of whole microalgae with biogas as sole biofuel (Process 2, Figure 1B).

2.2.1 Process 1: Biodiesel production and subsequent biogas production from spent microalgae.

Two principal drawbacks are identified when biodiesel production from microalgae is considered: high nutrients requirements for microalgae growth and low energy efficiency of biodiesel production process. Anaerobic digestion may contribute to overcome such limitations, by enabling nutrients recovery and biogas production when spent microalgae after lipid extraction is used as substrate. This is based on the fact that biogas can be used as source of

renewable energy and that during anaerobic digestion process, nitrogen and phosphorus may be recovered, creating opportunities for their reuse as nutrients. Theoretical energy contribution of anaerobic digestion is presented in Figure 1A, assuming microalgae content of lipids, proteins and carbohydrates to be 30, 45 and 25%, respectively. Figure 1A shows that an energy yield of 11MJ per kilogram of gross microalgae is reached when biodiesel production is considered. If oil extracted microalgae is used as substrate in anaerobic digestion process, methane produced would have a maximum theoretical contribution of 17MJ per kilogram of gross microalgae (thermal). Such value has been computed assuming carbohydrate and protein methanogenic potentials of 0.415 and 0.851 L CH_4/kg VS, respectively (Angelidaki and Sanders, 2004). If the latter thermal energy is transformed into electricity, a maximum energy yield of 5.5 MJ per kilogram of gross microalgae would be achieved (assuming a conversion efficiency of 32%). Thus, a substantial increase in energy yield could be theoretically achieved, representing a considerable contribution to biodiesel sustainability and economic feasibility. Energy contained in biogas can be used for both anaerobic digestion and trans-esterification reactor heating. Electricity obtained via co-generation can be used for different purposes such as photobioreactor mixing, microalgae harvesting and drying (Harun *et al.*, 2010; Razon and Tan, 2011). Neumann et al. (2011) evaluated energy contribution of biogas production in Process 1 for *Botryococcus braunii* with 30% lipid content. The latter study considered a nutrient recovery step through membrane liquid/solid separation from anaerobic digestion reactor and heptane evaporating step in order to recovery this solvent. Biogas production could theoretically contribute with close to 50% of the overall energy yield of Process 1.

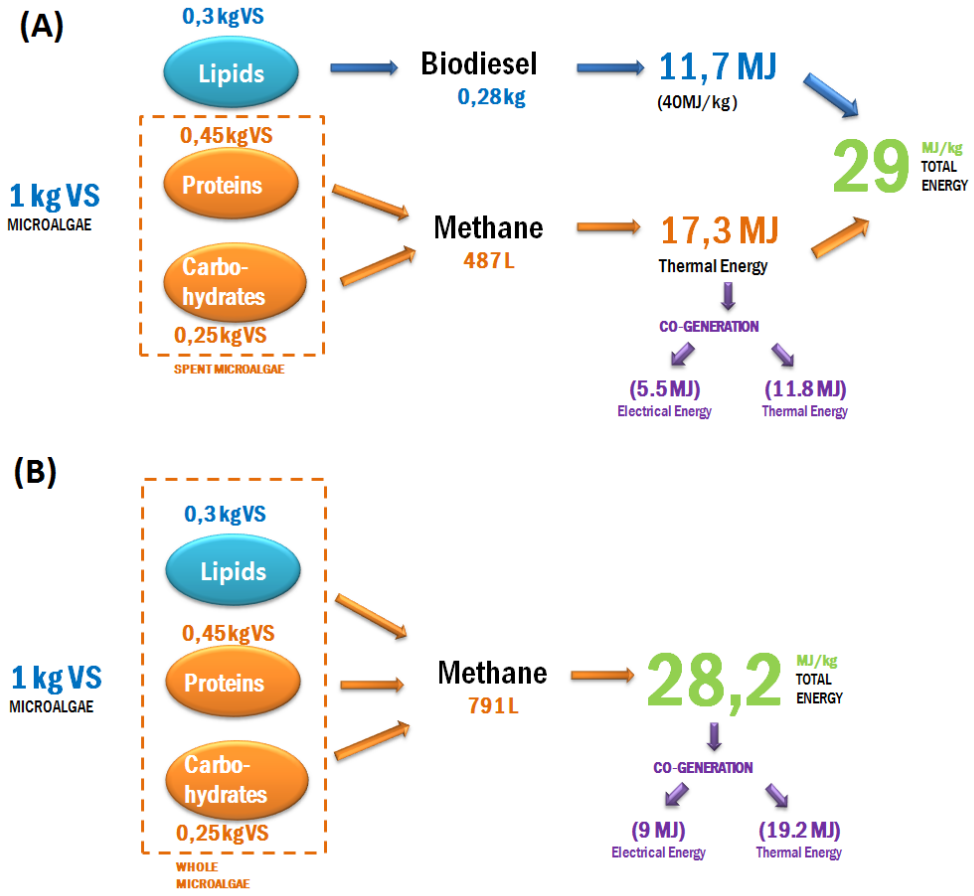

Figure 1. Energy potential of microalgae considering: (A) Biodiesel production and further anaerobic digestion of microalgae residues for biogas production or (B) Anaerobic digestion of whole microalgae only for biogas production.

2.2.2 Process 2: Biogas production from whole microalgae.

Another alternative to recover energy from microalgae consists of methane production from whole microalgae. In such process, all organic matter (proteins, carbohydrates and lipids) present in microalgae biomass would be converted into methane and carbon dioxide, without considering biodiesel production (De Schamphelaire and Verstraete, 2009; Dousková *et al.*, 2010; Zamalloa *et al.*, 2011). Several advantages are recognized when energy production from whole microalgae through biogas generation is considered: Biogas productions involves high energy yields, biogas production would not require microalgae biomass drying (it involves wet fermentation), biogas can be used to produce heat and electricity through co-generation,

microalgae cultures can be used for biogas upgrading (i.e. CO_2 biosequestration), microalgae species not capable of accumulating lipids may be also used as feedstock. Moreover, co-digestion with other types of biomass such as solid or liquid wastes is feasible. Anaerobic digestion of algal and microalgae biomass has been previously studied by some researches (Vergara-Fernández *et al.*, 2008; De Schamphelaire and Verstraete, 2009; Mussgnug *et al.*, 2010; Zamalloa *et al.*, 2011). Figure 1B shows the energy potential of Process 2, in which whole microalgae is used as substrate in order to produce biogas. In this estimation, all energy is produced as methane, which allows theoretical maximum energy recovery of 27 MJ per kg of volatile solids of microalgae (8.6MJ of electricity and 18.4 MJ of heat, if co-generation is considered). The lower operational energy demands for biogas production, compared with biodiesel together with biogas, makes Process 2 very promising for energy recovery.

2.3 Anaerobic digestion of microalgae

Reports of the anaerobic digestion of microalgae go back to the fifties when Golueke et al. (1957) was one of the first authors studying the feasibility of sunlight energy conversion to methane by algae sunlight fixation followed by biomass anaerobic fermentation. In this early study, 0.5 m^3 of biogas was obtained per volatile kg of algal biomass, with methane content 63%. More than two decades later, Nair *et al*. (1983) reported a lower yield, close to 0.22 m^3/kg VSS, at loading rate 1.7 kg/m^3·d. Despite those early reports, biogas production from algae and microalgae has not yet been widely researched (Foree and McCarty, 1970; Samson and Leduy, 1983; Tarwadi and Chauhan, 1987; Vergara-Fernández *et al.*, 2008; De Schamphelaire and Verstraete, 2009; Mussgnug *et al.*, 2010; Zamalloa *et al.*, 2011).

2.3.1 Choosing microalgal culture for direct biogas production

The ideal microalgae specie for a maximum biogas production would be that presenting:

- thin or no cell wall
- large cells
- high growth rate in non-sterile media

- high resistivity against natural contaminants
- carbohydrate-based cell wall.

Of the above mentioned factors, the quality of cell wall is crucial for anaerobic digestion of algae. This is because cell walls are hard to degrade biologically and their presence avoids contact of anaerobic bacteria with the readily degradable content of algal cells. Therefore, the influence of cell wall presence is described in detail in the following text.

2.3.1.1 Composition of algal cell wall

The Cell wall in microalgae represents 12-36% of total cell mass (cell wall weight/cell weight) in different microalgae (Table 1). Microalgae cell wall is composed mainly of carbohydrates and proteins which represent 30-75% and 1-37% of cell wall, respectively. Other compounds found in microalgal cell wall are uronic acid, glucosamine, hidroxyproline, proline, sporopollenin, carotenoids and another resistant biopolymers (Punnett and Derrenbacker, 1966; Domozych *et al.*, 1980; Blumreisinger *et al.*, 1983; Brown, 1991; Brown and Jeffrey, 1992; Abo-Shady *et al.*, 1993).

In relation to carbohydrates in microalgae cell wall, neutral sugars, cellulose and hemicelluloses are the main components. Blumreisinger *et al.* (1983) studied five different microalgae in relation to carbohydrate composition in cell wall, obtaining a prominent neutral sugar component. Composition of cellulose and hemicelluloses has ranged between 6-17% and 18-32% for microalgae studied in other researches carried out by Abo-Shady *et al.* (1993) and Domozych *et al.* (1980), respectively. On the other hand, Northcote *et al.* (1958) reported contents of cellulose near to 45% in cell wall of *Chlorella pirenoidosa*. Unlike these researches, Loos and Meindl (1982) found no presence of cellulose in cell wall of *Chlorella fusca.* In relation to proteins, peptides, proline and hidroxyproline are the main components. According to Punnett and Derrenbacker (1966), the cell wall of six different microalgae consisted of peptides (simple amino acid composition) but it contained no protein. In addition, this research revealed the existence of proline in the cell wall of *Chlorella vulgaris* and hidroxyproline in the cell wall of *Chlorella pyrenoidosa* and *Scenedesmus obliquous*.

Table 1.Cell wall composition of microalgae.

Microalgae	Cell Wall	Cell Wall composition (%)			References
	(% w/w)	Carbohydrates	Protein	n.d.*	
Chlorella vulgaris (F)	20.0	30.00	2.46	67.54	(Abo-Shady *et al.* 1993)
Chlorella vulgaris (S)	26.0	35.00	1.73	63.27	(Abo-Shady *et al.* 1993)
Kirchneriellalunaris	23.0	75.00	3.96	21.04	(Abo-Shady *et al.* 1993)
Klebsormidium flaccidum	36.7	38.00	22.60	39.40	(Domozych *et al.* 1980)
Ulothrix belkae	25.0	39.00	24.00	37.00	(Domozych *et al.* 1980)
Pleurastrum terrestre	41.0	31.50	37.30	31.20	(Domozych *et al.* 1980)
Pseudendoclonium basiliense	12.8	30.00	20.00	50.00	(Domozych *et al.* 1980)
Chlorella saccharophila	-	54.00	1.70	44,30	(Blumreisinger *et al.* 1983)
Chlorella fusca	-	68.00	11.00	20.00	(Blumreisinger *et al.* 1983)
Chlorella fusca	-	80.00	7.00	13.00	(Loos & Meindl 1982)
Monoraphidium braunii	-	47.00	16.00	37.00	(Blumreisinger *et al.* 1983)
Ankistrodesmus densus	-	32.00	14.00	54.00	(Blumreisinger *et al.* 1983)
Scenedesmus obliquos	-	39.00	15.00	46.00	(Blumreisinger *et al.* 1983)

* not determined.

2.3.1.2 Degradability of algal cell wall

Although methane yield is dependent on microalgae composition (Sialve *et al.*, 2009), the resistance of cell wall is considered to be the limiting factor for the anaerobic digestion of microalgae (Afi *et al.*, 1996; Chen and Oswald, 1998). The kinetics of anaerobic digestion is highly dependent on the degradability of the given microalgae species (Sialve *et al.*, 2009). Mussgnug *et al.* (2010) studied the methane production from six different microalgae, obtaining from 287 to 587 mL CH_4/ g VS. The low levels of methane yield were related to low cell degradation and high amount of indigestible residues. According to these results, easily degradable microalgae had no cell wall or a protein-based cell wall not containing cellulose/hemicellulose. In batch tests with low methane yields, intact cell walls of microalgae were found with light microscopy in this study. Thus, the intracellular content was not available

for efficient digestion. The presence of biopolymers resistant to anaerobic degradation has been reported in the outer cell wall of microalgae species such as *Botryococcus braunii* (Templier et al., 1992; Banerjee et al., 2002). Moreover, microalgae degradability is related to cell wall structures containing these resistant biopolymers. Some microalgae have a protective tri laminar outer wall called tri laminar sheath (*TLS*), which hinders efficient microalgae degradation (Derenne *et al.*, 1992). Thus, higher *TLS* resistance to degradation reported by Derenne *et al.* (1992) for microalgae *B. braunii* has been associated to the presence of sporopollenin-like biopolymers (Kadouri *et al.*, 1988; Derenne *et al.*, 1992). Other indigestible compound found in microalgae cell wall is algaenan, which has been reported as non-hydrolysable resistant biopolymer composed of polyether linked long-chain (up to C36) n-alkyl units (Gelin *et al.*, 1997; Blokker *et al.*, 1998; Gelin *et al.*, 1999; Simpson *et al.*, 2003).

2.3.1.3 Source of methane in microalgae

Many authors have related methane yield from microalgae to their composition (Sialve *et al.*, 2009; Mairet *et al.*, 2011; González-Fernández *et al.*, 2012; Mairet *et al.*, 2012), especially with the content of lipids, carbohydrates and proteins. However, the experimental data collected from literature do not show strong correlation between lipids, carbohydrates and proteins found in various algal species and the methane yield obtained by various authors (Figure 2). Angelidaki and Sanders (2004) presented theoretical methane yields from proteins, carbohydrates and lipids of 0.50, 0.42 and 1.01 L/g VS, respectively (Figure 3). Even when these values are used for calculation of the potential methane yield from various algal species, no strong correlation can be found (Figure 2d, e and f). Theoretically, lipids content has the biggest influence on methane yield, but as lipids are usually not the mayor source of methane (Figure 2), the correlation between lipids content and methane yield is still rather vague (Figure 2). These facts clearly show that the ratio between various macromolecules is not the most important parameter determining the actual methane yield from algae. As it was mentioned before, content of inert organic matter (e.g. cell wall) would play more important role (González-Fernández *et al.*, 2012). These findings show that plain composition of algal biomass indeed cannot be the main factor while choosing the best algal strain for methane production. Biomass production rate and the content of cell-

walls will be of higher importance. Moreover, environmental conditions such as the salinity of available water source must be taken into account.

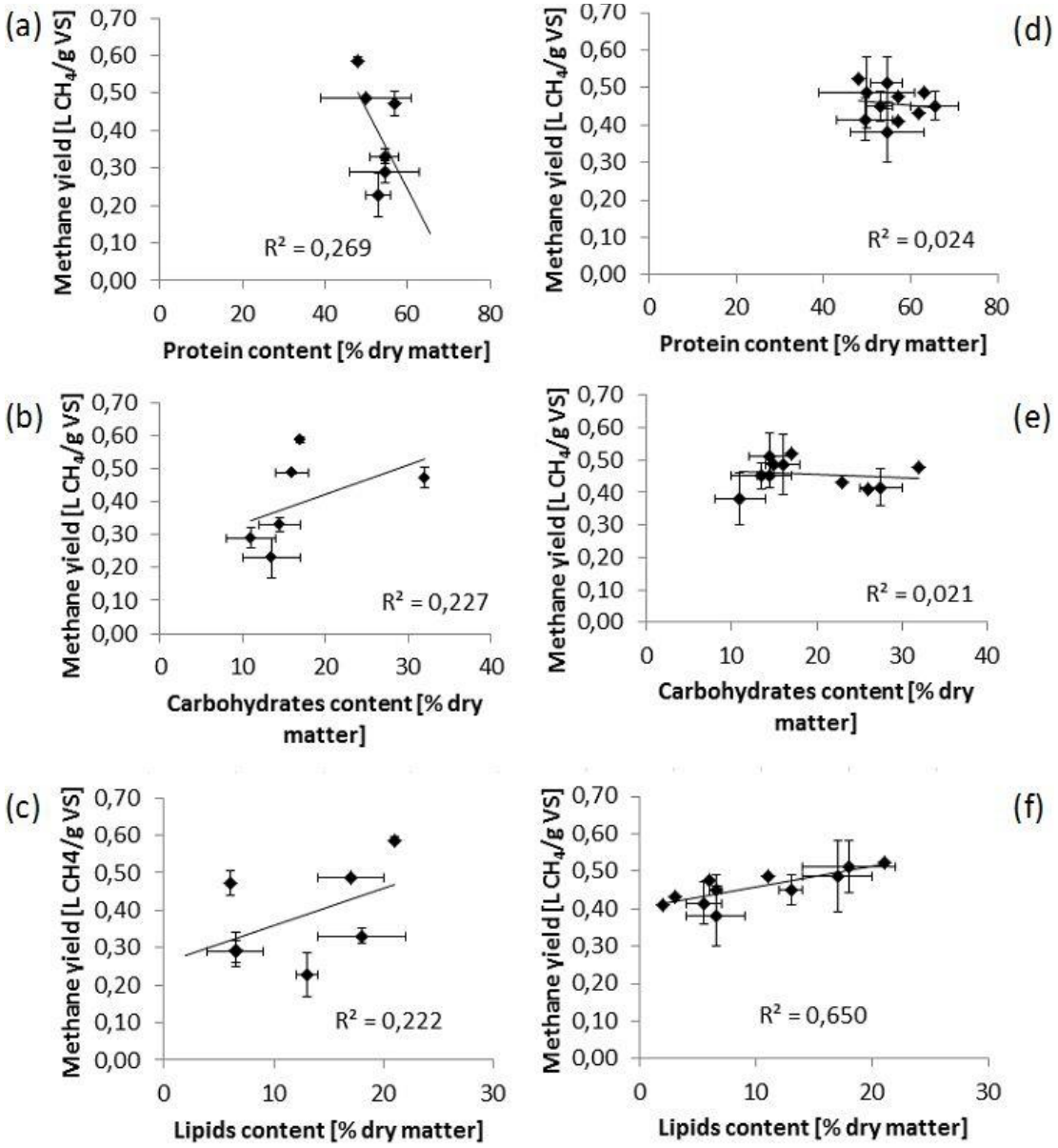

Figure 2.Dependence between methane yield from microalgae and their lipids, carbohydrates and proteins content. Each data point represents one algae species while the error bars show the range found in the literature. Figures (a), (b) and (c) show experimentally obtained methane yields, figures (d), (e) and (f) represent theoretical methane yield for the given algae composition calculated according to Angelidaki and Sanders (2004). Data were extracted from multiple authors (Becker, 2007; Griffiths and Harrison, 2009; Sialve *et al.*, 2009; Mairet *et al.*, 2011; González-Fernández *et al.*, 2012; Mairet *et al.*, 2012)

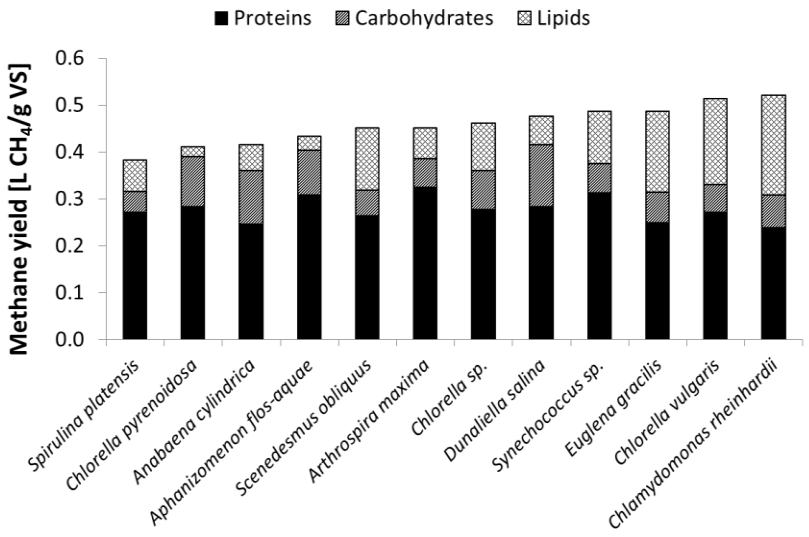

Figure 3. Potential methane yield from proteins, carbohydrates and lipids present in various algae species calculated according to Angelidaki and Sanders (2004). The data on proteins, carbohydrates and lipids content in algae were extracted from Becker (Becker, 2007), Sialve (2009), Griffiths and Harrison (2009) and González-Fernández *et al*. (2012).

2.3.2 Pre-treatment

In order to overcome limitation caused by cell wall degradability, which is necessary to access the intracellular content, cell disruption (pre-treatment) has been pointed out as an important contributor in order to enhance anaerobic digestion efficiency. As mentioned above, cell wall degradability affects both Processes 1 and 2. However, in Process 1, cell wall degradability should not be as critical as in Process 2 since lipid extraction itself may be considered a pre-treatment step.

There are different pre-treatment techniques applied to microalgae, which can be classified as enzymatic, chemical and mechanical treatments. Mechanical pre-treatment include autoclaving, homogenizers, microwaves and sonication, which increases the availability of organic matter (Angelidaki and Ahring, 2000).Chemical pre-treatment will increase availability of compounds resistant to anaerobic hydrolysis due to the enhanced disintegration (Bonmatí *et al.*, 2001). Chemical pre-treatment can be classified as acid or alkaline treatment. An increase in soluble hemicellulose present in cell wall is expected when alkaline pre-treatment is used(Abo-Shady *et al.*, 1993). Thus, chemical pre-treatment is suitable when microalgae cell wall is rich on

hemicelluloses. Also, enzymatic pre-treatment has been used in order to attack cell wall and improve compounds extraction from microalgae. Enzymatic pre-treatment with α-amilase, amylo-glucosidase and cellulase have shown a positive effect on cell wall hydrolysis (Choi *et al.*, 2010; Fu *et al.*, 2010). Fu *et al.* (2010) reported a 62% increase in cell wall hydrolysis, when *Chlorella sp.* was pretreated by immobilized cellulase. Few studies report the effect of cell disruption pre-treatment in anaerobic digestion (Samson and Leduy, 1983; Chen and Oswald, 1998). Samson and Leduy (1983) reported an increase of 78% in soluble COD when algae *Spirulina maxima* was mechanically pretreated (sonication and mechanical disintegration). However, no increase in methane yield was observed.

Finally, two considerations should be taken into account when cell disruption pre-treatment is evaluated in the context of anaerobic digestion: On one hand, energy consumption associated with pre-treatment should be low in order to avoid a negative contribution to the energy balance of anaerobic digestion process. On the other hand, contribution to the biodegradability of the given substrate should be a response variable when the effect of pre-treatment on anaerobic digestion is evaluated. In other words, some pre-treatment techniques increase solubility of organic matter but do not increase its biodegradability.

2.3.3 Inhibiting factors related to anaerobic digestion

Figure 1B shows the energy potential when microalgae are used as substrate in order to produce biogas. In this estimation, total energy is produced as methane, which allows a theoretical maximum energy recovery of 27MJ per kg of volatile solids of microalgae. As in Process 1, part of energy produced will be spent for supplying the energy necessary for microalgae harvesting and up-concentration, photobioreactor mixing, photobioreactor and anaerobic reactor heating, etc. The theoretical estimations of energy production from anaerobic digestion presented in this review have been so far computed considering 100% of microalgae biodegradability and high performance of anaerobic digestion. However, an energy production lower than ideal can be expected when limiting factors in anaerobic digestion process are considered. For this reason, this book chapter examines different limiting factors of anaerobic digestion, which are necessary to overcome in order to improve performance of this process.

2.3.3.1 Ammonium inhibition

Ammonium is presented as protonated form (NH_4^+) and deprotonated form (NH_3, ammonia). The latter is considered to be the specie responsible for the inhibition of anaerobic digestion, due to its permeability through cell membrane (de Baere *et al.*, 1984). There are several mechanism by which ammonia will act as inhibitor of anaerobic bacteria among which are intracellular pH changes, increase in energy requirements for maintenance and inhibition of specific enzymes (Wittmann *et al.*, 1995). Several factors determining ammonia concentration in anaerobic reactor has been reported, but substrate concentration is a major one (Sialve *et al.*, 2009). Distribution of total ammonia between protonated and deprotonated forms strongly depends on factors such as pH and temperature. At high pH values ammonium gets deprotonated forming toxic ammonia (NH_3) (Borja *et al.*, 1996). Its inhibitory effect can result in volatile fatty acids accumulation due to a decrease in methanogenic activity, which generates a decrease in pH and ammonia concentration (Chen *et al.*, 2008). This interaction may generate an inhibited steady-state, in which the process remains stable despite inhibition (Angelidaki and Ahring, 1993; Angelidaki *et al.*, 1993). Temperature is another variable that determine NH_4^+/NH_3 ratio, which is directly related to the increase of ammonia fraction and thus, inhibition level (Braun *et al.*, 1981; Angelidaki and Ahring, 1994).

Microalgal biomass can be expected to have low C/N ratio due to the high protein content in microalgae (Becker, 2007). Then, anaerobic degradation of these residues is expected to generate a high ammonium concentration that may cause inhibition of anaerobic microbial consortia, especially methanogenic bacteria (Angelidaki and Ahring, 1993; Chen *et al.*, 2008). In addition, high ammonium concentration may affect biogas quality since ammonia can be stripped into gas phase (Sialve *et al.*, 2009). During anaerobic digestion of oil extracted microalgae (Process 2 on Figure 1), ammonia inhibition is expected to be especially of concern, since oil extraction will decrease C/N ratio. Figure 4 shows an estimation of the effect of substrate concentration on free ammonia levels in a hypothetical anaerobic digestion reactor. Estimation was calculated considering protein content reported by Becker (2007), operation pH value 8, temperature 35º C, ammonia conversion 90% and total lipid extraction efficiency. Figure 4 shows that inhibitory ammonia concentrations will develop whenever solids concentration exceeding

2% are applied during the anaerobic digestion step. This result was evaluated considering free ammonia inhibition at 100 mg/L NH_3 (dotted line in Figure 4).

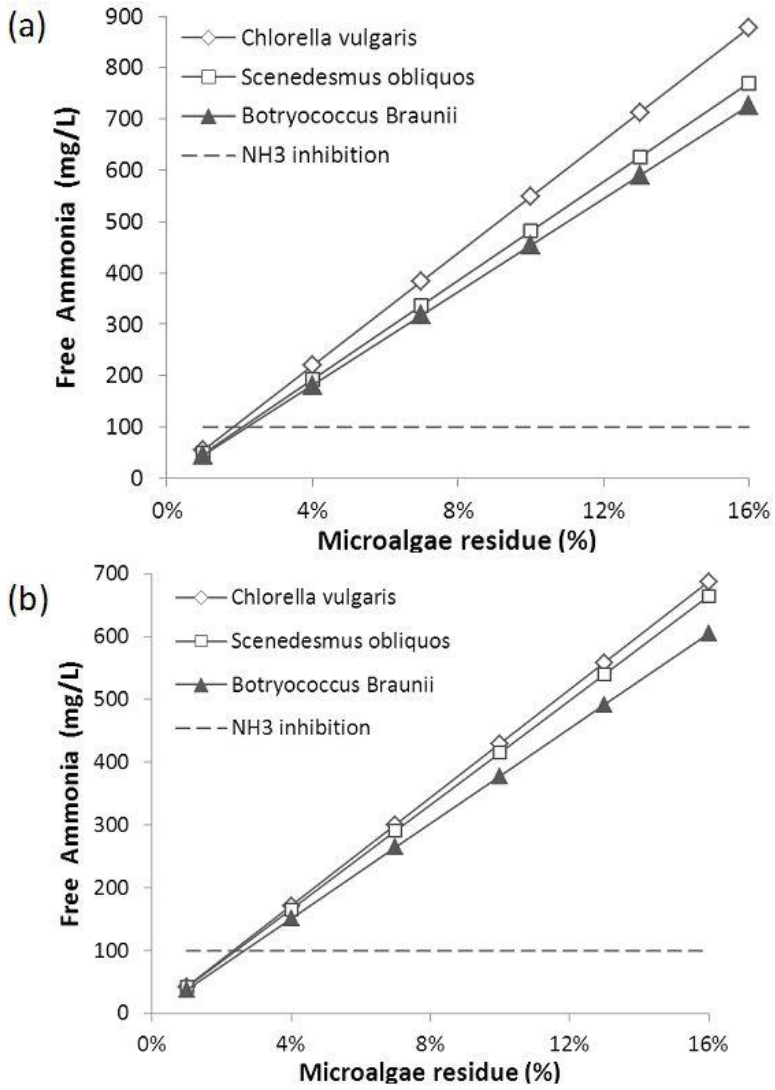

Figure 4. Estimation of free ammonia concentration on anaerobic digestion reactor from substrate level of feedstock, considering (A) processes 1,Biodiesel production and subsequent biogas production from spent microalgae and (B) process 2, Biogas production from whole microalgae.

Results shown in Figure 4 indicate that either anaerobic digestion has to be performed at very low levels of solids concentration, or mechanism for ammonia removal must be implemented. It has to be reminded that Figure 4 assumes 90% of conversion of proteins. Lower protein conversions will reduce the chances of ammonia inhibition. However it is clear that this

phenomena needs to be addressed if high rate digestion of microalgae is of interest.

One way to overcome this drawback is the possibility of co-digestion in order to provide an optimal C/N ratio for anaerobic digestion process (Yen and Brune, 2007; Ehimen *et al.*, 2011). Thus, a higher C/N ratio co-substrate should be mixed with microalgae in order to increase anaerobic digestion yield. This strategy is more attractive considering the fact that some co-substrate can stimulate enzymatic synthesis and, hence, increase hydrolysis and degradability (Yen and Brune, 2007). Also, co-digestion can dilute toxic compounds decreasing their concentration below toxic/inhibition levels (Sialve *et al.*, 2009).

2.3.3. 2 Salt inhibition

Salt inhibition is expected to be relevant when saline microalgae are used as substrate for biogas production. In those locations where freshwater is not abundant or available, saline microalgae may be of interest, if cultivation takes place close to the sea. In those situations, salinity may even be higher than sea water when open pounds are used, as a result of water evaporation. If biomass is not diluted with fresh water after harvesting, downstream processes such as anaerobic digestion may need to deal with the salinity present in the biomass.

At low concentrations, sodium is essential for methanogenic bacteria. Probably, it is due to its role in ATP formation or NADH oxidation (Dimroth and Thomer, 1989). Sodium concentration ranges 100-350mg/L have been reported as beneficial for mesophilic methanogenic growth (McCarty and McKinney, 1961; Patel and Roth, 1977). Although moderate concentrations can stimulate bacteria growth, excessive amounts of salt reduce growth rate, and can cause severe inhibition or toxicity (Soto *et al.*, 1991). Moreover, high salt levels can cause dehydration in bacteria due to osmotic pressure (de Baere *et al.*, 1984; Yerkes *et al.*, 1997).

Different levels of saline tolerance in anaerobic bacteria have been reported (Lefebvre and Moletta, 2006). Easily degradable substrates seem to increase salt tolerance, most likely as a result of higher energy availability to cope with the energetic requirements of salt tolerance mechanism (Xiao and Roberts, 2010). Rinzema *et al*. (1988) found non acetoclastic methanogenic activity at 16 g/L of sodium concentration. The concentration that generated 50% of activity reduction (IC50) was 10 g/L and no bacteria adaptation after 12 weeks was observed.

Similar saline tolerance was observed by Liu and Boone (1991). Feijoo *et al*. (1995) analyzed sodium inhibition for anaerobic bacteria from different reactors. A high tolerance in anaerobic bacteria from reactor treating wastewater under salinity conditions was observed, which was interpreted as consequence of bacteria adaptation. IC50 value for these bacteria was 16.3 g Na^+/L and entire inhibition was observed at 21 g Na^+/L. Several reports indicate that biomass acclimation may significantly increase the activity under saline conditions (Soto *et al.*, 1991; Omil *et al.*, 1995; Chen *et al.*, 2008; Kimata-Kino *et al.*, 2011). However, reports are also available where no or little acclimating was observed (Aspé *et al.*, 1997). Then, selection rather than adaptation is likely to be the mechanism to provide high activity when big changes in salinity are imposed, requiring the presence of salinity-tolerant microorganisms in the inoculum (Gebauer, 2004). It is indeed a common practice to use inoculums containing sources of saline resistant microorganisms, such as marine sediments (Xiao and Roberts, 2010).

2.3.4 Biogas upgrading

Many biogas applications such as vehicle use, household distribution and electricity production, require some level of biogas upgrading to remove impurities or to increase methane content. CO_2 removal is a key factor in order to obtain a higher calorific value of biogas. Processes such as solvent absorption, activated carbon adsorption and membrane filtration have been used for CO_2 removal (Kapdi *et al.*, 2005; Makaruk *et al.*, 2010; Ryckebosch *et al.*, 2011). Photosynthetic microorganisms such microalgae can also be used to remove CO_2 from biogas. Microalgae cultures are regarded as an interesting tool for carbon dioxide capture from gases such as flue gases from boilers, combustion engines or thermal power plants. This would not only alleviate impact of CO_2 emissions on the environment, but it would also reduce the cost of microalgae production (Doucha *et al.*, 2005; Ryu *et al.*, 2009). Stabilization ponds have been already recognized as potential CO_2 scrubbers due to their (micro-)algae growth (Shilton *et al.*, 2008). Several authors have reported the successful growth of microalgae using flue gases. Negoro *et al.* (1993) reported productivities similar to those using pure CO_2, and showed that growth was barely influenced by the content of SO_X and NO_X contained in flue gases. Similar results were obtained by Hauck *et al.* (1996) who found no inhibition of *Chlorella sp.* by the

levels of NO_X typically contained in flue gases. Doucha *et al.* (2005) reported 50% of flue gas decarbonization when working with a photobioreactor. In this study, 4.4 kg of CO_2 was needed for the production of 1 kg of dried algal biomass. Conde *et al.* (1993) achieved biogas purification in laboratory experiments up to methane content of 97% with algae grown on synthetic nutrient medium. Mandeno *et al.* (2005) achieved CO_2 reduction from 40 to less than 5% using synthetic biogas, observing little transfer of oxygen to the biogas, so explosive methane/oxygen mixtures would not be formed. Similar results in terms of CO_2 reduction were obtained by Travieso *et al*. (1993) working with real biogas. Several microalgae species such as *Chlorococcum littorale*, *Chlorella sp*., *Chlorella sp*. UK001, *Chlorella vulgaris*, *Chlorella kessleri*, *Scenedesmus obliquus, Spirulina sp., Haematococcus pluvialis* or *Botryococcus braunii* have shown high levels of tolerance to high partial pressures of CO_2 (Wang *et al.,* 2008; Brennan and Owende, 2010). Mass transfer of carbon dioxide from gas to liquid phase is dependent on several factors highlighting chemical balance in microalgae media, pH and flow pattern of reactor in which culture is growing (Kumar *et al.,* 2010). However, no full scale installations are under operation with this concept.

Available publications do not report negative effects of high methane partial pressures over microalgae cultures. Moreover, Meier et al. (2011) reported no inhibition effect when exposing a culture of *N. gaditana* to atmospheres containing methane up to 100%.

Hydrogen sulphide is present in biogas at low concentrations although its treatment should be considered. Some studies have reported a hydrogen sulphide decrease after biogas is upgraded in microalgae culture (Conde *et al.*, 1993; Heubeck *et al.*, 2007; Sialve *et al.*, 2009). Most likely, hydrogen sulphide removal should be attributed to relative high solubility in growth medium (Conde *et al.*, 1993; Sialve *et al.*, 2009). Solubilised hydrogen sulphide can be easily oxidized into sulphate due to oxygen presence in growth medium.

2.4 Conclusions

Microalgal biomass is a promising substrate for renewable energy production. In this book chapter, direct anaerobic digestion without previous biodiesel extraction was shown to be the most promising method of energy production from microalgae. Lipids used for biodiesel production can also serve as a rich source of biogas with energetic efficiency higher than when microalgae are used for subsequent biodiesel and biogas production. The higher energy efficiency is given mostly by the simple technology with low energy demand used for methane production. These benefits combined with the possibility of CO2 and nutrients recycling from the anaerobic effluents make anaerobic digestion the best technology for renewable energy production from microalgae.

CHAPTER III.
Anaerobic digestion as a tool for resource recovery from a biodiesel production process from microalgae

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3.1 Introduction

Fulfilling growing energy demands of modern societies, respecting and preserving the environment, has become a great challenge. Renewable energies are expected to play a key role in this process. In fact, during 2012 about 19% of worldwide energy consumption came from renewable sources such as wind, solar, geothermal and biomass (REN21, 2013). Bioenergy, the energy produced from biomass, is nowadays the most important source of renewable energy. In fact, during 2012, 50% of non-conventional renewable energy were based on biomass.(REN21, 2013).

Microalgae, the common denomination for a broad group of photosynthetic prokaryotes and eukaryotes, have been considered as a promising feedstock of biomass for the production of "third generation" biofuels. This is the result of some potential advantages over traditional land-based crops:

- High oil productivity of certain species, which can be10 times higher than high-yielding oil crops like oil palm.(Chisti, 2007; Deng *et al.*, 2009; Mata *et al.*, 2010; Weyer *et al.*, 2010)
- High CO_2 fixation efficiency, which can be used as a tool to mitigate CO_2 emissions. (Amin, 2009; Brennan and Owende, 2010; Mutanda *et al.*, 2011)
- The possibility of cultivation on non-arable land areas, reducing land competition with food production.(Mussgnug *et al.*, 2010; Stephens *et al.*, 2010)

Currently, most of the efforts to take advantage of microalgae as a source of bio-energy have been directed to biodiesel production. However, concern exists regarding the energetic yield of biodiesel production from microalgae, using current technologies.(Chisti, 2007; Sialve *et al.*, 2009; Scott *et al.*, 2010; Stephens *et al.*, 2010) Indeed, some authors had calculated a negative energetic balance, with important energetic requirements associated to harvesting and drying steps.(Lardon *et al.*, 2009; Scott *et al.*, 2010)

Different strategies had been proposed in order to improve the energetic yield of microalgae based biodiesel, such as the optimization of microalgae cultivation processes,

valorization of glycerol as a heterotrophic source of carbon, maximization of triglycerides accumulation through metabolic engineering, application of direct trans-esterification processes, microalgae cultivation using wastewaters and the implementation of anaerobic digestion of spent microalgae (Chinnasamy *et al.*, 2010; Scott *et al.*, 2010; Patil *et al.*, 2011). The anaerobic digestion of the residual biomass seems to be one of the most promising alternatives, due to the energy recovery in the form of biogas, and the potential re-use of the nutrients release during digestion (Sialve *et al.*, 2009). Few studies have evaluated the energetic contribution of anaerobic digestion in the biodiesel production process from microalgae. However, these studies have indicated that a considerable part of total energy contained in the biomass can be recovered if anaerobic digestion of spent microalgae is applied (Harun *et al.*, 2010; Ehimen *et al.*, 2011; Razon and Tan, 2011).

The aim of this work was to determine potential benefits of including anaerobic digestion of oil extracted microalgae, as a part of an integrated biofuels production process (biodiesel and biogas). Potential energy and nutrients recovery through anaerobic digestion were determined, and their impact over a hypothetical biodiesel production process was evaluated. Two microalgae species, *Botryococcus braunii* and *Nannochloropsis gaditana* were considered. *B. braunii* is a freshwater microalga and represents a promising species due to its high oil accumulation capacity, and a lipid profile suitability for trans-esterification (Sydney *et al.*, 2011; Ashokkumar and Rengasamy, 2012). *N. gaditana* is a seawater microalgae with important lipid content and high productivity, (Mata *et al.*, 2010; Yen Chen *et al.*, 2011) which represents an interesting alternative when microalgae is to be cultivated in areas close to the sea.

3.2 Materials and Methods

3.2.1 Microalgae biomass

Microalgae *Botryococcus braunii* race A and *Nannochloropsis gaditana* were supplied by Universidad de Antofagasta, Chile. Oil extracted microalgae (spent microalgae) was produced using a Soxhlet extraction unit, using petroleum ether as solvent, at a solvent-biomass ratio of 10:1. Oil extraction was performed operating Soxhlet unit for 16 hours.

3.2.2 Biomethane potential tests

Bio-methane potential tests (BMP) were carried-out in order to evaluate potential energy recovery through produced biogas, and nutrients release. Spent microalgae *B. braunii* and *N. gaditana* were used as substrate.BMP was determined in 600mL vials, containing 400 mL of media. Assays were done in triplicate and performed at 35 °C. An initial substrate concentration of 5 g/L of volatile solids (VS) was applied. Anaerobic granular biomass from a full scale UASB reactor treating brewery wastewater was used as inoculum. Anaerobic biomass/substrate ratio was 1:1, expressed as VS. Medium was supplemented with yeast extract (200mg/L), sodium bicarbonate (5 g/L)and macronutrients:NH_4Cl (65 mg/L), KH_2PO_4 (18.5mg/L), $CaCl_2 \cdot 2H_2O$ (4mg/L), $MgSO_4 \cdot 7H_2O$ (5.7mg/L). Methane production was determined based on the evolution of pressure and composition of the gas contained in the headspace. BMP was computed considering produced methane and VS content of spent microalgae. Total ammonium nitrogen and phosphate were determined in the liquid phase by the end of the BMP assays. Endogenous biogas production and release of nutrients from anaerobic biomass was determined by blank assays containing only inoculum.

3.2.3 Analytical Methods

Lipid, protein and ash content were determined according to Avila (2011). Carbohydrates were weighting by difference. Total solids (TS) and VS were measured according to Standard Methods (1998). Chemical oxygen demand (COD) was measured through HACH kit. Ammonium and phosphate were determined through HACH kits (salicylate method 10031 and TNT 844 respectively). Soluble protein was determined according to Lowry (1951). Pressure into vials headspace was measured through Cole-Parmer pressure transducer model 206 (-14,7 - 15 PSIG). Biogas composition was determined by gas chromatography, using a thermal conductivity detector (Clarus 580, Perkin Elmer). High calorific value (HCV) of biomass was determined in a LECO AC500 calorimeter.

3.3 Results and Discussion

3.3.1 Biochemical methane potential of lipid extracted microalgae

3.3.1.1 Microalgae characterization

Table 1 shows chemical composition of both lipid extracted microalgae used during the present study. For both species, close to 50% of the biomass was composed by protein. *B. braunii* presented high ash content, closed to 25%. *N. gaditana*, on the other hand presents a higher carbohydrate proportion. Similar COD/VS ratios were found for both species, around 1.6 g/g, indicating a high potential for biogas production. Based on the COD content of each algae, maximum theoretical methane yields can be estimated at 632 and 644 mL CH_4/g VS for *B. braunii* and *N. gaditana*, respectively. These values have been computed considering a theoretical methane yield of 395 mL CH_4/g COD (computed from stoichiometric methane oxidation at 35° C).

Table 1. Spent microalgae characterization (*B. braunii* and *N. gaditana)*

	Spent B. braunii	*Spent N. gaditana*
Proteins (%)	46.04	47.68
Lipids (%)	2.71	2.93
Carbohydrates (%)	22.26	37.45
Ash (%)	23.91	10.78
Phosphorus (%)	0.76	1.17
SV/ST (g/g)	0.746	0.82
COD/SV (g/g)	1.6	1.63

3.3.1.2 BMP determination

Figures 1 and 2 present the evolution of BMP assays of lipid extracted *B .braunii* and *N. gaditana*, respectively. Observed methane yields were 407 and 450 mL CH_4/g VS, respectively. These values are high considering those reported in literature, which are in the range 90-450 mL CH_4/g VS (Sialve *et al.*, 2009; Ehimen *et al.*, 2011; Frigon *et al.*, 2013; Alzate *et al.*, 2014). Differences in reported BMP values may be expected as a result of different microalgae

composition, which is likewise influenced by growth conditions (Sialve *et al.*, 2009). Observed BMPs were 64 and 70% of the maximum theoretical methane yield estimated based on COD/VS ratio, for *B. braunii* and *N. gaditana*, respectively. This result is an indicator of a high biodegradability of the microalgae studied in this research.

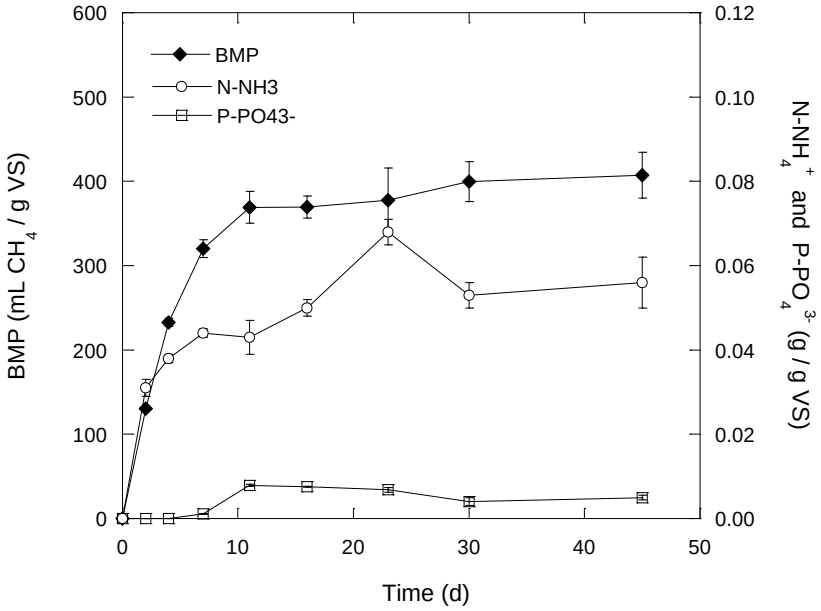

Figure 1: Methane production and nutrients release during BMP tests of spent microalgae *B. Braunii*. Bars indicate standard deviation between triplicate.

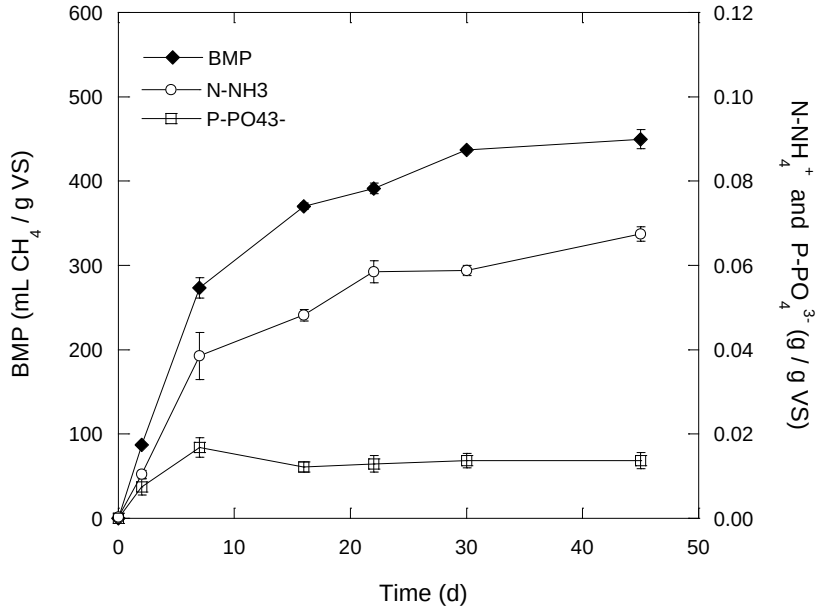

Figure 2: Methane production and nutrients release during BMP tests of spent microalgae *N. gaditana.* Bars indicate standard deviation between triplicate.

3.3.1.3 Total and recovered energy from spent microalgae

Figure 3 presents the energy potential for *B. braunii* and *N. gaditana,* derived from HCV analysis, theoretical methane potential based on COD, and actual observed methane production based on BMP tests. Values of energy potential through methane production were evaluated considering an hypothetical combustion of this gas, considering a calorific value of 35,6 MJ/m^3 CH_4 (Sialve *et al.*, 2009). The difference between values coming from HCV and those based on COD are most likely associated with the presence of non-readily oxidizable compounds in the spent microalgae. Reported values in literature for HCV for microalgae biomass range between 21 and 36 MJ/kg TS (Table 2). HCV values observed in this study are in the lower range of those presented in Table 2, since they represent the calorific value of lipid-extracted microalgae. Lipids are characterized by high calorific values, so variable lipid content of microalgae can at least partially explain the diversity of the values reported in Table 2.

Anaerobic digestion of spent microalgae was able to recover, as methane, 56 and 61% of total energy contained in the biomass (determined by HCV) of spent *B. braunii* and *N. gaditana,* respectively (Figure 3). McGinn *et al.*(2012) reported 64-67% of energy recovery through anaerobic digestion of *Scenedesmus sp.* The non recovered energy is associated with the presence of non-biodegradable organic matter. In this sense, several studies have identified the presence of resistant biopolymers in the outer cell wall of *B. braunii* (Templier *et al.*, 1992; Banerjee *et al.*, 2002). Even, these biopolymers can represent close to 10% of biomass dry weight (Kadouri *et al.*, 1988).

Table 2. Calorific value of different microalgae.

Microalgae	*Calorific Value (MJ/kg TS)*	*Reference*
Spent *B. braunii*	19.5 (26.1 MJ/ kg VS)	This research
Spent *N. gaditana*	21.6 (26.4 MJ/ kg VS)	This research
Botryococcus braunii	35.6	(Liu *et al.*, 2012)
	54.7	(Talukdar *et al.*, 2013)
Chlorella vulgaris	18.0	(Illman *et al.*, 2000)
Chlorella vulgaris (low N)	23.0	(Illman *et al.*, 2000)
Chlorella emersonii	21.0	(Scragg *et al.*, 2002)
Chlorella emersonii (low N)	24.0	(Scragg *et al.*, 2002)
Scenedesmus sp.	22.5-23.3	(McGinn *et al.*, 2012)
	23.5	(Talukdar *et al.*, 2013)
Scenedesmuso bliquus	20.2	(Talukdar *et al.*, 2013)
Haematococcus pluvialis	25.8	(Talukdar *et al.*, 2013)

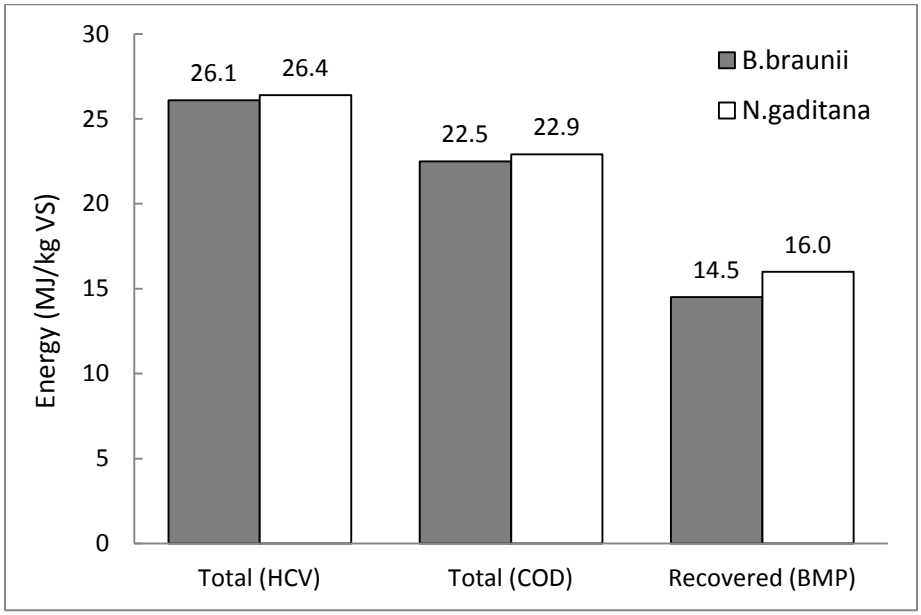

Figure 3. Total and recovered energy of spent microalgae *B. braunii* and *N. gaditana*.

3.3.2 Nutrient release during anaerobic digestion of spent microalgae

Ammonia nitrogen release during BMP tests was 0.056 and 0.067 g N-NH_3/g TS for *B. braunii* and *N. gaditana*, respectively (Figure 1 and 2). These values correspond to 76 and 88% of

the initial nitrogen content of the spent microalgae. A high level of ammonia release is normally related with a high level of protein degradation. Other authors have reported nitrogen release levels of 59-69 % (Rösch *et al.*, 2012; Alcántara *et al.*, 2013) for *C.sorokiniana*. A total nitrogen balance after 45 days of digestion can be made:

$$N_{total} = N_{N-NH_3} + N_{soluble\ protein} + N_{non-degraded\ biomass}$$

Where, $N_{N\text{-}NH_3}$ corresponds to nitrogen released as ammonia, $N_{soluble\ protein}$ corresponds to nitrogen present in the liquid phase that was not converted into ammonia, and $N_{non\text{-}degraded\ biomass}$ corresponds to protein that was not hydrolyzed and remained associated with the suspended solids. Balance neglects the assimilation of nitrogen by anaerobic biomass, considering the low expected nitrogen yield. The balance shows that, for spent *B. braunii*, 76% of the nitrogen was released as ammonia, 16% ended as soluble protein and 8% remained as non-hydrolyzed biomass.

Phosphate release could also be indicative of comprehensive degradation of the microalgae, since phosphorus can be found in intracellular compounds, membrane phospholipids of organelles, as well as nucleic acids (Richmond, 2004). Thus, its release may be indicative of the degradation of both extracellular and intracellular content. Phosphorus present as phosphate in the liquid phase at the end of the BMP test reached values of 0.0050 and 0.014 g P/g VS for *B. braunii* and *N. gaditana* respectively (Figure 1 and 2). These values correspond to phosphorus release of 82 and 117%, respectively. It is unclear the reason why phosphorus release of *N. gaditana*, exceeded 100%. This may be the result of an extra phosphorous source not taken into account.

The capacity of anaerobic digestion for releasing nutrients, turn it into an interesting alternative as a resource recovery technology, since recovered nutrients can be reused for example in the cultivation of algae. This would reduce nutrients requirements and would contribute to the sustainability of microalgae production.

3.3.3 Potential biogas contribution to biodiesel production process

Figure 4 schematically represents energy flow through a potential process of production of biodiesel and biogas from *B. braunii* and *N. gaditana*. Figure 4 data was computed based on 1kg VS of microalgae, before lipid extraction. Table 3 presents the conditions used in the evaluation. Only close to 15% of the energy contained in the microalgae would be transferred into biodiesel (Figure 4). This is the result of the low content of neutral lipids in microalgae oil (Doan *et al.*, 2011). Biodiesel production was evaluated considering that microalgae lipids contain 50% of easily methyl-able neutral lipids (Halim *et al.*, 2012).

Anaerobic digestion would be potentially able to recover approximately 40 % of total energy contained in the microalgae. Latter value is in the order of those reported by theoretical studies (Sialve *et al.*, 2009; Harun *et al.*, 2010), involving biodiesel and biogas production from microalgae. Thus, anaerobic digestion may be considered a key process for improving energetic yield when producing biofuels from microalgae.

Table 3. Parameters to calculate energy distribution of microalgae *B.braunii* and *N.gaditana*.

Parameter	*B.braunii*	*N.gaditana*	Reference
BIODIESEL			
Lipid content in total microalgae (% of VS)	24.4	24.6	Proximate analysis
[1]*Lipid extraction efficiency (%)*	88.5	89.6	This research
Methyl-able fraction of neutral lipids (%)	50		(Halim *et al.*, 2012)
Biodiesel yield (g biodiesel/g methyl-able lipids)	0.95		(Azócar *et al.*, 2010)
Lipid heat combustion lipids (kcal/g)	9		
Biodiesel heat combustion (MJ/ kg biodiesel)	40		(Costa and de Morais, 2011)
BIOGAS			
[2]*Fraction of spent microalgae (%)*	79.16	78.65	This Research
BMP (mL CH_4/ g VS) (From this research)	407	450	This research
Methane heat combustion (MJ/m^3 CH_4)	35.6		(Sialve *et al.*, 2009)

[1]Computed based on initial and final lipid content in microalgae.
[2]Computed considering mass balance of total microalgae and extracted lipid.

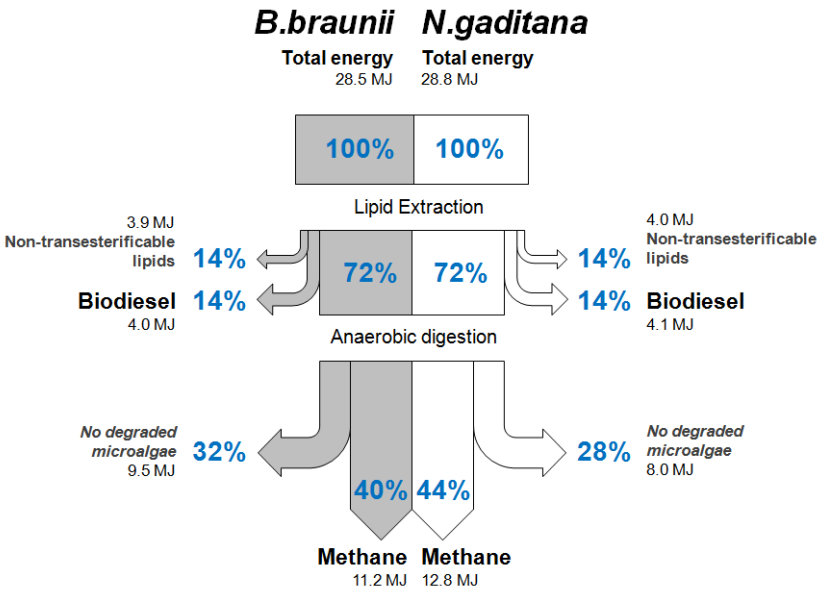

Figure 4. Energetic distribution of *B. braunii* and *N. gaditan*a through biodiesel and anaerobic digestion processes. Results were calculated considering 1kg VS calculation basis.

Biogas production from spent microalgae could be then considered as a source of energy supporting biodiesel requirements, whether indeed biodiesel is considered as the main economical product. In order to test this hypothesis, an energy balance was set, considering what could be referred to as a classical biodiesel production scheme from microalgae. Then, the potential contribution of biogas was evaluated, comparing methane productivity and energy requirements of the biodiesel production process. Figure 5 presents a schematic representation of the considered process. The following assumptions were made during this analysis:

I. Microalgae is grown and harvested for biodiesel and biogas production.

II. An hypothetical 1000m^2area and 20 cm depth (200m^3) raceway was considered as calculation basis. Volumetric biomass productivity (P_x) was assumed to be 0.10 and 0.13 kg/m^3·d for *B .braunii* and *N. gaditana*, respectively (Pulz, 2001; Chisti, 2007; Brennan and Owende, 2010). Electricity demands of raceway operation are derived from the paddle wheel operation (culture mixing) and a water pump (see Table 4).

III. Microalgae are harvested using a decanter centrifuge, which concentrates microalgae from 0.5 to 75 g/L. Then microalgae are dried until 4% of moisture.

IV. Dried microalgae are oil extracted using hexane as solvent. Lipid content of microalgae and extraction efficiency is that presented in Table 3. Energy demands in this process are derived from stirring and heating for solvent recovery. Residual microalgae from this process, i.e. spent microalgae is further used for biogas production.

V. Lipids obtained from microalgae are converted into biodiesel through a trans-esterification process, considering both biodiesel yields and methyl-able fraction presented in Table 3. Energy demands during this step are associated with stirring and heating.

VI. In order to produce biogas, an anaerobic digester was considered, working at organic load rate (OLR) of 2 g/Ld and hydraulic retention time (HRT) of 30 days. BMPs obtained in this research were used in order to calculate methane production. COD/VS and VS/TS ratios were obtained from Table 1. Energy demands for anaerobic digestion are derived from heating and stirring reactor, according to Table 4.

VII. Finally, produced biogas is used for combined heat and power generation. Electrical and thermal efficiency were considered to be 40 and 45%, respectively.

VIII. Both electrical and thermal energy generated from biogas is used in order to mitigate energy demands of the process as showed in Figure 5.

Table 4. Parameters to calculate energy demands and energy production of microalgae *B.braunii* and *N.gaditana* growth in raceway pond.

RACEWAY POND	***B.braunii***	***N.gaditana***	**Reference**
Volume (m^3)	200	200	
Biomass Productivity ($kg/m^3 \cdot d$)	0.10	0.13	(Brennan and Owende, 2010; Pruvost *et al.*, 2011; Yen Chen *et al.*, 2011; Chisti, 2013)
Flow rate (m^3/d)	40	52	Mass balance
Biomass concentration (g TS/L)	0.5	0.5	
Operation time (d/year)	365	365	
❶ *Paddle wheel (W/m^3)*	1	1	(Slade and Bauen, 2013)
❷ *H20 pump (kW_eh/m^3)*	0.077	0.077	(Chiaramonti *et al.*, 2013)
HARVESTING			
Concentration factor	150	150	(Molina Grima *et al.*, 2003)
Final concentration (g TS/L)	75	75	Mass balance
Centrifuged flow (m^3/d)	0.27	0.35	Mass balance
❸ *centrifuge (kW_eh/m^3)*	1	1	(Molina Grima *et al.*, 2003)
DRYING			
Final humidity (%)	4	4	
Flowdried (kg/d)	20	26	Mass balance
❹ *Drying (kW_{th}/m^3)*	92.98	92.98	calculated
OIL-EXTRACTION			
Methyl-able fraction (kg/d)	2.53	5.42	calculated
❺ *Heating ($kW_{th}h/kg$ biodiesel)*	6.22	6.22	(Lardon *et al.*, 2009)
❻ *Stirring (kW_eh/kg biodiesel)*	2.3	2.3	(Lardon *et al.*, 2009)
TRANS-ESTERIFICATION			
Biodiesel flow (kg/d)	1.20	2.57	calculated
❼ *Heating ($kW_{th}h/kg$ oil)*	0.72	0.72	(Razon and Tan, 2011)
❽ *Stirring (kW_eh/kg oil)*	0.0297	0.0297	(Razon and Tan, 2011)
ANAEROBIC DIGESTION			
Spent microalgae flow (kg/d)	17.47	20.58	calculated
BMP (mL CH_4/g VS)	407	450	This research
HRT (d)	30	30	
OLR (kg/m^3-d)	2	2	
Volume reactor (m^3)	10.43	13.75	calculated
Cp microalgae (MJ/kg -°C)	4.2		
Heat losses (%)	10		
ΔT° (°C)	15		
❾ *Heating ($kW_{th}h/m^3$)*	-	-	Heat and mass balance
CHP PLANT			
Electrical efficiency (%)	40	40	(Zamalloa *et al.*, 2011)
Thermal efficiency (%)	45	45	(Zamalloa *et al.*, 2011)

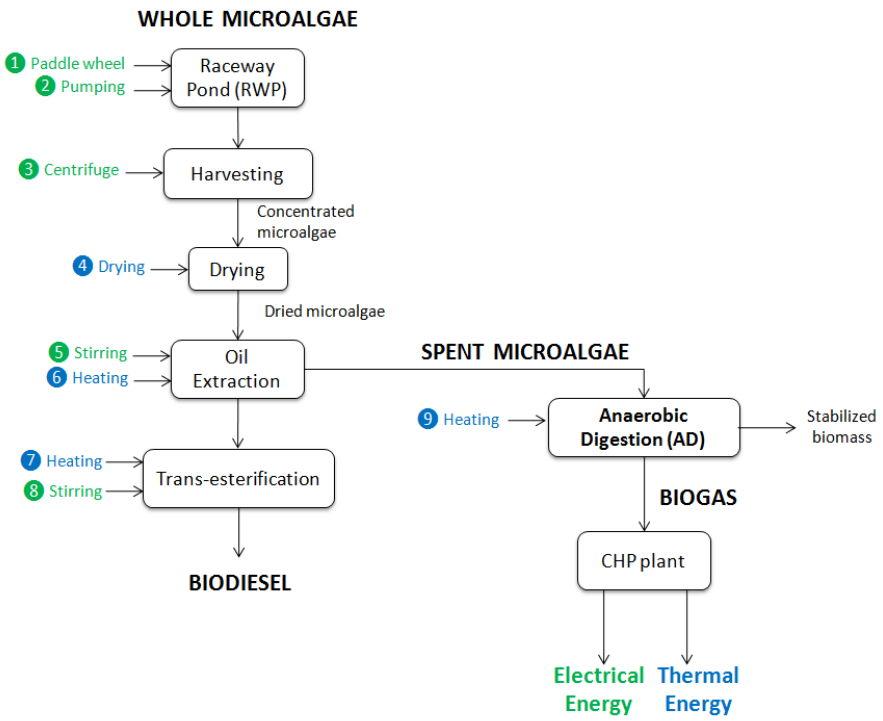

Figure 5. Scheme of process used for calculation of energy production and demands of microalgae *B. braunii* and *N. gaditana* growing in raceway pond.

Table 5 shows that neither electrical nor thermal energy produced by biogas co-generation could fully supply energy demands of the considered microalgae-biodiesel production process. This is the result of the high energy demand of the decanter used for microalgae harvest, which actually would consume close to 85% of electrical power demands. Co-generation using biogas would produce close to 50% of the overall electricity needs. It is clear that biofuel production from microalgae could only be feasible if low energy harvesting methods are developed, as has also been stated by other authors (Lardon *et al.*, 2009; Scott *et al.*, 2010).

Recovered thermal energy would potentially account for close to 75 - 80% of total heat requirements. However, the analyzed scenario does not considered any thermal recovery actions, so it represents a better case scenario. Most of the thermal energy demand is related with biomass drying. Process enabling other types of drying may be then interesting, or the use of wet biomass for direct trans-esterification (Patil *et al.*, 2011; Hidalgo *et al.*, 2013; Kumar *et al.*, 2014).

Table 5. Onsite energy production and demands in raceway growth for microalgae *B. braunii* and *N. gaditana*.

ELECTRICAL ENERGY	*B. braunii* $kW_eh/year$	*N. gaditana* $kW_eh/year$
Produced (co-generation)	+7,659	+10,964
❶ Paddle wheel	-350	-456
❷Pumping (Harvesting)	-1,124	-1,461
❸Decanter	-14,600	-18,980
❺Stirring (lipid extraction)	-1,008	-2,162
❽Stirring (Trans-esterification)	-27	-59
Balance	-9,451	-12,154

THERMAL ENERGY	$kW_{th}h/year$	$kW_{th}h/year$
Produced (co-generation)	+8,616	+12,334
❹ Spray-dryer	-8,343	-10,846
❻Heating (lipid extraction)	-2,726	-5,846
❼Heating (Trans-esterification)	-664	-1,425
❾Heating (Anaerobic digestion)	-123	-145
Balance	-3,240	-5,926

3.3.4 Biofuels from microalgae versus solar power generation

Energy contained in biodiesel or biogas is indeed solar energy, which was fixed thanks to the photosynthesis process performed by microalgae. Therefore, microalgae, as other photosynthetic organisms like crops can be considered as tools for capturing solar power. It may be then interesting to compare the production of microalgae biofuels with a thermo-solar power plant in terms of the efficiency for solar capture and transformation. Both processes end with different energetic products, liquid and gas biofuels on one hand, and electric power on the other. The simple comparison of gross power may not be fully adequate, since biofuels can be derived to other uses other than electric power, which may represent a market advantage. However, comparison is useful in order to put the production of microalgae based biofuels into perspective.

Comparison was made based on the performance of Gemasolar plant (Seville-Spain), which operates with 110GWh of annual electrical production in 185 ha of solar field (Burgaleta

et al., 2012). Figure 6 shows that total theoretical chemical energy fixed by microalgae (TCEF) would be 2497 kWh/ha·d, assuming a photosynthetic efficiency of 5% (Acién *et al.*, 2012) and 2172 kWh/m^2·year of direct normal irradiation registered in Écija, Spain, (where Gemasolar plant is located) (Amadei *et al.*, 2013). The effective potential chemical energy fixed as biomass grown in raceway pond (TCEF-RWP) was evaluated, using reported microalgae productivity (Table 4). Also, energy contained in biodiesel and biogas produced from these microalgae can be estimated based on the energy flow depicted in Figure 4. Data is presented in Figure 6, where values for biogas and biodiesel have been computed using combustion energy of both biofuels, and do not include the energy requirements for microalgae and biofuels production. If co-generation of produced biogas is considered, around 200-300 kWh/ha·d would be produced, close to 13-18% of the power provided from Gemasolar plant.

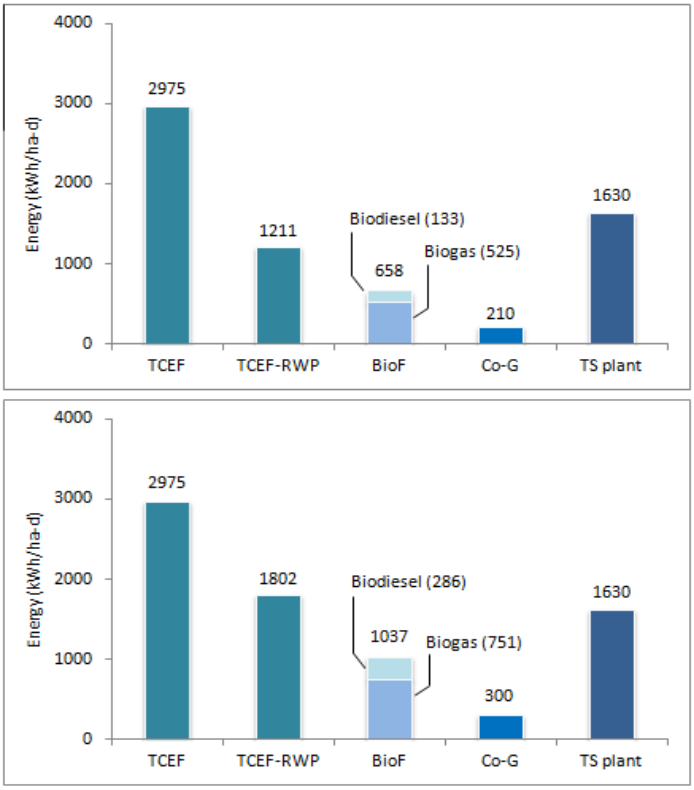

Figure 6. Comparison between energy fixed by microalgae, produced as biofuels and thermo-solar energy for *B. braunii* (a) and *N. gaditana* (b). TCEF= Total chemical energy fixed by microalgae (5% of total solar energy available), TCEF-RWP= real TCEF evaluated based on reported productivities of raceway pond, BioF= energy contained in biodiesel and biogas produced from microalgae, Co-G=electrical energy produced from biogas co-generation and TS plant =electrical energy produced in Thermo-solar plant (GEMASOLAR- Écija, Spain).

Results show that a solar power plant like the one used for comparison, can indeed be much more efficient in collecting and transforming solar energy, than a microalgae biofuel production process, considering traditional process. It seems clear then that substantial advances in process design or technology development are needed in order to make microalgae biofuels feasible. Such advances should be related with neutral lipid enhancement in low-cost microalgae cultures, study of low-demand process for harvesting and drying, biodiesel yield improve, extraction of add-value compounds (secondary metabolites).

3.4 Conclusions

The results of the present work highlight the existence of a high energy and nutrient recovery potential from spent microalgae after oil extraction for biodiesel production purposes. BMP tests indicate that close to 450 mL CH_4/ g VS could be produced from spent microalgae (*B. braunii* and *N. gaditana)*, which represents over 60% of the energy measured as calorific value. Indeed, more energy would be recovered in the form of methane, than that the one contained in the form of biodiesel. Nitrogen and phosphorus would also be released during digestion, and could then be re-used for microalgae cultivation. Sustainable and energetically efficient biofuel (biodiesel and biogas) production from microalgae requires the development of processes with low energy demand. This is specially the case of harvesting and drying. Thus, research efforts should continue in order to decrease energetic consumption of these process and thus, increasing energetic yield biofuel production.

CHAPTER IV.
Membrane filtration as harvesting process for Chlorella sorokiniana and Nannochloropsis gaditana

Paper sent to *Separation and Purification Technology*.

4.1 Introduction

During the last few years, microalgae has gathered growing interest as a source of biomass for different production processes. Nowadays, microalgae are considered as potential raw material for food production (human and animal), as purifying agents for wastewaters (Pittman *et al.*, 2011), or as source of bioproducts and/or biofuels (de la Noue and de Pauw, 1988). Indeed, most of the attention microalgae have received is the result of their potential as feedstock for biodiesel production. This is the result of the capacity of some species to accumulate lipids, and a high biomass productivity when compared to land based crops. Microalgae biomass has also been proposed as a potential carbon sequester, due to their high rate of CO_2 capture, which is even superior of that of plants (Wang *et al.*, 2008). Then, microalgae producing facilities may be coupled to industrial activities involving high CO_2 emissions, such as thermo power plants, reducing carbon footprint and generating valuable biomass.

Despite the great potential of microalgae cultures as biomass source, extensive full-scale implementation still needs to face some technological challenges. One of the first issues when conceiving microalgae production is how to provide a simple, reliable and energy-efficient harvesting method. In fact, 20-30% of the cost for producing microalgae biomass is normally associated with this process (Molina Grima *et al.*, 2003). Moreover, it has been estimated that harvesting represents 90% of equipment costs in open growth cultures (Amer *et al.*, 2011).

Harvesting is an energy demanding process since it has to deal with diluted cultures (up to 0.5 g/L in raceway ponds and 0.3-2 g/L in photo-bioreactors), so a high volumetric reduction is required. Moreover, microalgae are small in size and have a density similar to that of water (Milledge and Heaven, 2013). A broad variety of techniques for microalgae harvesting have been proposed. Some of them are centrifugation, settling, flocculation/coagulation, flotation and filtration (Molina Grima *et al.*, 2003; Uduman *et al.*, 2010; Milledge and Heaven, 2013). Membrane filtration has also been presented as a promising harvesting process (Gerardo *et al.*, 2014). Indeed, membrane filtration has been proposed not only as an alternative for microalgae harvesting, but also for a wide range of bio-refinery processes such as recovery, recycling, purification and extraction of bio-fuels, by-products, culture medium and nutrients (Abels *et al.*, 2013; Gerardo *et al.*, 2014). The development of membrane technologies during the last 3 decades has produced relevant improvements in membrane manufacturing and has steadily

decrease membrane costs (Churchouse and Wildgoose, 1999; Zhang *et al.*, 2010). This has enabled extensive application of membrane separation techniques, even in fields where their use may have been considered unlikely some time ago, such as municipal and industrial wastewater treatment (Brindle and Stephenson, 1996; Marrot *et al.*, 2004).

In fact, membrane filtration has been described as a more efficient, economic and environmentally friendly process than other separation techniques for microalgae, such as centrifugation or thermal drying (Rickman *et al.*, 2012; Hwang and Lin, 2014). Indeed, membrane filtration is characterized by a complete biomass retention, does not need the addition of chemicals and can be easily scale-up (Zhang *et al.*, 2010; Bhave *et al.*, 2012; Bilad *et al.*, 2013). Despite the reported advantages of membrane filtration, the main drawback is flux reduction as result of membrane fouling (Ahmad *et al.*, 2012; Javadi *et al.*, 2014). Operational flux is indeed a key parameter, since it determines membrane requirements.

Within membrane-based separation processes, cross-flow filtration is a widely used operation strategy, in which suspension being filtered flows tangentially to the membrane (Ahmad *et al.*, 2012). Microalgae are kept in suspension and shear stress provided by cross-flow velocity reduces cake formation and membrane fouling (Torres *et al.*, 2011; Ahmad *et al.*, 2012). From an energetic point of view, requirements of cross-flow filtration are associated with the trans-membrane pressure required for permeate collection and the energy for pumping the suspensions through the membrane filtration system (Ríos *et al.*, 2012).

This research was focused on the evaluation of membrane filtration as harvesting process for microalgae cultures, with emphasis on the determination of potential energy requirements. Considering the growing market of membranes for wastewater treatment, tubular membranes developed and marketed for this industry were used, based on their availability and lower costs when compared with membrane products developed for other purposes.

4.2 Materials and methods

4.2.1 Microalgae growth

Two microalgae were used in this research: *Nannochloropsis gaditana* and *Chlorella sorokiniana*. *N. gaditana* was supplied by Antofagasta University (Antofagasta, Chile), where was grown in 1 m^3 batch reactors with saline water under autotrophic conditions. This microalga was harvested by centrifugation and frozen for storage until it utilization. In order to carry-out filtration assays, microalga was re-suspended in saline water (35 g /L of sea salt). *C. sorokiniana* was grown in 20 L batch photo-bioreactors at 22°C, under constant illumination through fluorescent tubes (400 $\mu E/m^2 \cdot s$). The M8a media was used for this culture (Table 1). The reactors were aerated in order to provide CO_2 and for stirring.

Table 1: Composition of culture media M8a

Nutrient	Concentration in Media (mg/L)
KH_2PO_4	740
$Na_2HPO_4 \cdot 2H_2O$	260
$MgSO_4 \cdot 7H_2O$	400
$CaCl_2 \cdot 2H_2O$	13
KNO_3	3000
EDTA ferric sodium salt	116
$NA_2EDTA \cdot 2H_2O$	37.2
H_3BO_3	0.0618
$MnCl_2 \cdot 4H_2O$	13
$ZnSO_4 \cdot 7H_2O$	3.2
$CuSO_4 \cdot 5H_2O$	1.83

4.2.2 Filtration assay at constant cross-flow velocity

Concentration assays were carried out in a filtration module fitted with a single tubular ultra-filtration membrane (X-flow, NORIT). Membrane had a pore size of 30 (nm), and had an internal diameter and length of 5 mm and 0.35 m, respectively. The setup is represented in Figure

1. Microalgae suspensions were circulated through the membrane module at a constant cross-flow velocity (v_s) of 1.5 m/s, using a progressive cavity pump (Moyno), controlled by a variable frequency drive. Permeate was collected by means of a peristaltic pump (Masterflex), that provided the required transmembrane pressure (TMP). TMP was determined by a pressure transducer located in the permeate line. Permeate pump was automatically controlled in order to provide a flux enabling a 25% increase in TMP during each filtration cycle, i.e. that TMP at the end of filtration cycle was about 1.25 times the TMP at the beginning of the cycle. In order to achieve this, at the end of each cycle, flux was either decrease or increased 5 $L/m^2 \cdot h$, depending on the recorded TMP increase. Filtration cycles were 10 minutes long. After each filtration cycle, a 1 min back-flush was performed. Sensors and actuators were connected to a PC running LabView (National instruments) using a CompaqDAQ data acquisition hardware (National Instruments).

Membrane was chemically cleaned after each concentration experiment. Chemical cleaning was performed applying 30 min of oxidative cleaning, using NaOCl in order to provide a free chlorine concentration of 500 mg/L. The membrane was chemically cleaned in the same membrane module. Filtration resistance was determined before and after each cleaning procedure, recording the TMP during filtration of clean water at different fluxes.

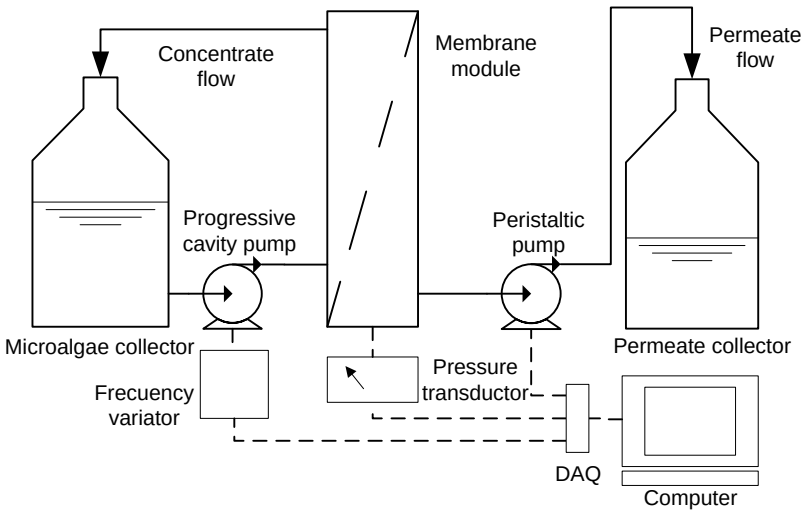

Figure 1. Set-up of membrane system for concentration assays.

4.2.3 Effect of solid concentration and cross-flow velocity over the critical flux and fouling rate.

The effects of biomass concentration and the cross flow velocity (v_s) (independent variables) over critical flux and fouling rate (responses) were evaluated. Biomass concentration was expressed as volatile solids (VS). The setup presented in Figure 1 was used for this purpose, with the only modification that permeate was sent back to the microalgae collector, in order to maintain a constant biomass concentration. Critical flux was determined using the flux step method (Torres *et al.*, 2011). Flux steps of 5 $L/m^2 \cdot h$ were used. The fouling rate was defined as the rate of TMP increase over time, evaluated at a flux 7.5 $L/m^2 \cdot h$ over critical flux. Experiments were arranged using surface response methodology (3 levels factorial design) (Montgomery *et al.*, 2001). Experimental design is shown in Table 2. This design was randomized in order to minimize error associated with operation of filtration system. ANOVA analysis was carried out in order to determine the significance of the independent variables over the studied effects, and their interaction. The discrimination of non-significant parameters of the second order model was carried out with a forward analysis (Montgomery *et al.*, 2001).

Table 2. Experimental design for critical flux assays.

	Chlorella sorokiniana		*Nannochloropsis gaditana*	
	v_s (m/s)	VS (g/L)	v_s (m/s)	VS (g/L)
1	0,59	10	0,71	10
2	0,59	30	0,71	30
3	0,59	50	0,71	50
4	1,18	10	1,53	10
5	1,18	30	1,53	30
6	1,18	50	1,53	50
7	1,77	10	2,43	10
8	1,77	30	2,43	30
9	1,77	50	2,43	50
10	1,18	30	1,53	30
11	1,18	30	1,53	30
12	1,18	30	1,53	30

4.2.4 Calculation of energy requirements

Energy requirements of a potential full-scale filtration process were theoretical determined. For this purpose, a commercial filtration module was considered, operating under the same conditions as those tested experimentally. A membrane module Compact 33 (X-Flow NORIT, Netherlands) was considered, using 6 filtration units in series (length: 3 m per module, internal diameter of tubes: 5,2mm). The required power for providing the desired cross-flow velocity in the membrane tubes was determined. Energy for permeate collection was considered negligible. The friction factor was estimated to calculate the head-losses. The Darcy-Weisbach factor was calculated as a function of the Reynolds number using the expressions showed in the equations (1), (2) and (3) (Vatankhah):

$$(1)\quad f\left(Re \leq 2100, \frac{\varepsilon}{D}\right) = \frac{64}{Re}$$

$$(2)\quad f\left(2100 < Re < 4000, \frac{\varepsilon}{D}\right) = Lineal\ interpolation : f\left(2100, \frac{\varepsilon}{D}\right) and \left(4000, \frac{\varepsilon}{D}\right)$$

$$(3)\quad f\left(Re > 4000, \frac{\varepsilon}{D}\right) = \frac{1}{\left(0.8686 * \ln\left(\frac{0.4599 * Re}{(G-0.2753)\left(\frac{G}{G+0.9741}\right)}\right)\right)^2}$$

where G is defined as:

$$G = 0.124 \cdot Re\left(\frac{\varepsilon}{D}\right) + \ln(0.4599 \cdot Re)$$

The relation between volatile solids (VS) and viscosity was experimentally determined. This relation was used to relate Reynolds number with VS. This relation is needed since VS increases during membrane concentration, so does the friction factor and therefore the power requirements for constant cross-flow. Once that f factor is determined, consumed energy in membrane process can be computed considering equation (4):

$$(4)\ C_E = \sum_{i=1}^{n} \frac{F_s * P_i * Q_i * \Delta t_i}{\eta_{pump} * V_{Cul}}$$

Where:

C_E = Consumed energy (kW·h/m^3)
n = Total filtration time (s)
F_s = Safety factor for pressure drop (1.5)
Q = Tangential flow to membrane (m^3/s)
Δt = Variation in filtration time (s)
V_{Cul} = Microalgae suspension volume (m^3)
$\boldsymbol{\eta_{pump}}$ = pumping energetic efficiency (0.6)

The energy required to achieve a certain concentration was then computed, using the expressions described above, using the relation of flux and VS determined in the filtration experiments.

4.2.5 Analytical procedures

The VS concentration was determined according to Standard Methods (APHA, 1998). The dynamic viscosity of microalgae suspensions were determined in an AND sv-10/100 series sine-wave vibro viscometer, at 20 °C. The particle size distribution of microalgae suspensions was measured using SALD-3101 particle analyzer (Shimadzu). Microalgae suspensions were also analyzed using a flow cytometer FACs Canto II with two lasers (488 nm and 633 nm). Relative particle size, complexity and the chlorophyll emission spectra of 10,000 events were determined for each sample. Microalgae suspensions were also observed using an optical microscope Axio Scope A1, Zeiss-Germany.

4.3 Results and Discussions

4.3.1 Membrane filtration as harvesting process

Figure 1 shows concentration profiles for *N. gaditana* and *C. sorokiniana*, at a constant cross-flow velocity of 1.5 m/s. The initial biomass concentration for *N. gaditana* and *C. sorokiniana* were 2 and 0.85 g VS/L, respectively. Final biomass concentration for *N. gaditana* was almost twice that for *C. sorokiniana.* Therefore, concentration factors were 21 and 27, respectively. As already commented, flux was automatically controlled during this assay in order to enable a 25% increase of TMP during each 10 min filtration cycle. Therefore, concentration was performed at a flux over the critical value. Both microalgae showed a similar concentration behavior. As biomass concentration increased in time (Figure 2b), flux decreased (Figure 2a).

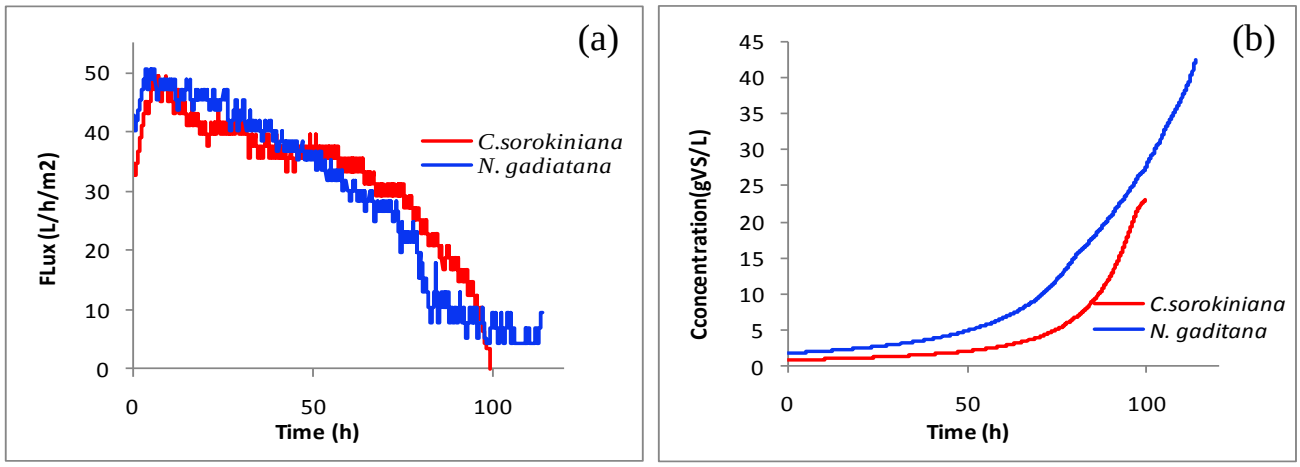

Figure 1. Concentration experiments by membrane filtration for *N. gaditana* and *C. sorokiniana*. (a) Filtration flux over time; (b) biomass concentration over time.

In both cases the flux progressively decreased until it reached values below 10 L/m^2·h. A similar behaviour has been reported by Ahmad *et al.* (2012) and Javadi *et al.* (2014), who evidenced a rapidly flux decline at the beginning of filtration at constant TMP until flux stabilization occurred. Observed flux decrease is attributed to phenomena such as cake formation, concentration-polarization and fouling (Ahmad *et al.*, 2012). During each filtration cycle, resistance increase was mainly reversible, i.e. was mostly removed by the applied back-flush cycles. However, resistance progressively built-up after consecutive filtration cycles. Resistance

of the membrane before and after concentration process, and after chemical cleaning were determined. These values enabled the evaluation of several partial resistances, by the end of concentration process (Figure 2). Removable resistance (R_r) is defined as the one that can be removed by the applied chemical cleaning. Non-removable resistance (R_{Nr}) is that remaining after applied cleaning, minus membrane resistance (R_m). Figure 2 presents these resistances. It has to be noticed that Figure 2 does not consider reversible resistance, i.e that resulting from reversible cake layer formation that could be removed by the application of consecutive back-flush cycles. Figure 2 shows that applied chemical cleaning procedure did not restore membrane permeability to its original value (R_m). Indeed R_{Nr} was similar in magnitude for both microalgae. R_r was higher for *C. sorokiniana*, most likely as a result of the higher concentration factor achieved for this algae. This means that a higher amount of permeate flowed through the membrane, increasing chances for membrane fouling.

Ahmad (2012) reported that during the microfiltration of *Chlorella sp.* although internal fouling was relevant, the predominant resistance at the end of filtration was by cake deposition. Moreover, fouling during microalgae filtration has been strongly related to exo-polysacharides (EPS) excretion by microalgae and debris (Morineau-Thomas *et al.*, 2002; Rossi *et al.*, 2008), which may be associated with surface velocity causing shear stress. In this sense, Javadi (2014) reported an increase of EPS excretion in membrane filtration of *Chlorella sp.* operated when surface velocities increased until 0.4 m/s.

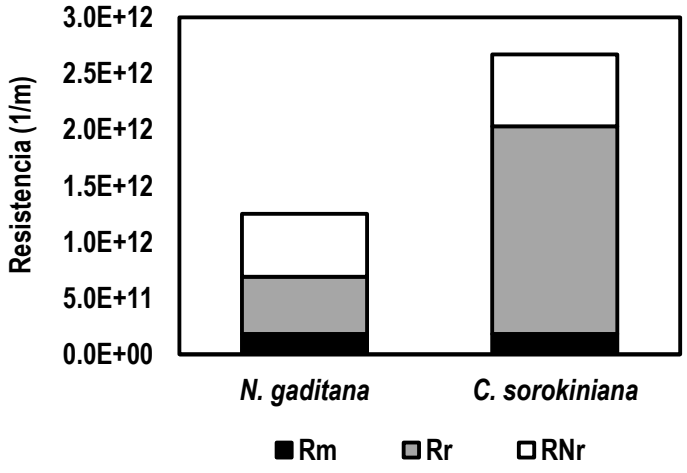

Figure 2. Partial resistances resulting from concentration experiments presented in Figure 1 for *N.gaditana* and *C. sorokiniana*. R_m, R_r and R_{Nr} stands for membrane, removable and non-removable resistances.

Microalgae cultures used in this study were not axenic. This will be the case for full-scale microalgae production, since cultivation of pure strains is at least unlikely. Presence of bacteria was detected in both cultures, by direct microscopic observation. Presence of bacteria may have contributed to the observed flux, considering their smaller particle size and that reversible cake formation was observed during each filtration cycle. Indeed, particle back-transport mechanism during cross-flow filtration are a strong function of particle size (Belfort *et al.*, 1994; Altmann and Ripperger, 1997). Moreover, it has been shown that a minor fraction of a suspension can determine the behaviour of the complete filtration system (Torres *et al.*, 2011). Figure 3 presents particle size distribution of the microalgae suspensions before and after the concentration process described in Figure 1. For *N. gaditana* 10% of the particles presented particle sizes was in the range 3-4 µm and 90% where 4 to 7 µm. By the end of filtration assays, a small increase in the presence of particles below 4 µm was observed. In the case of *C. sorokiniana* the reduction in particle size was more evident, as can be seen in Figure 3b. Decrease in particle size may be the result of deflocculation or cell disintegration, as a result of the shear stress that the pumping and cross-flow regime imposes.

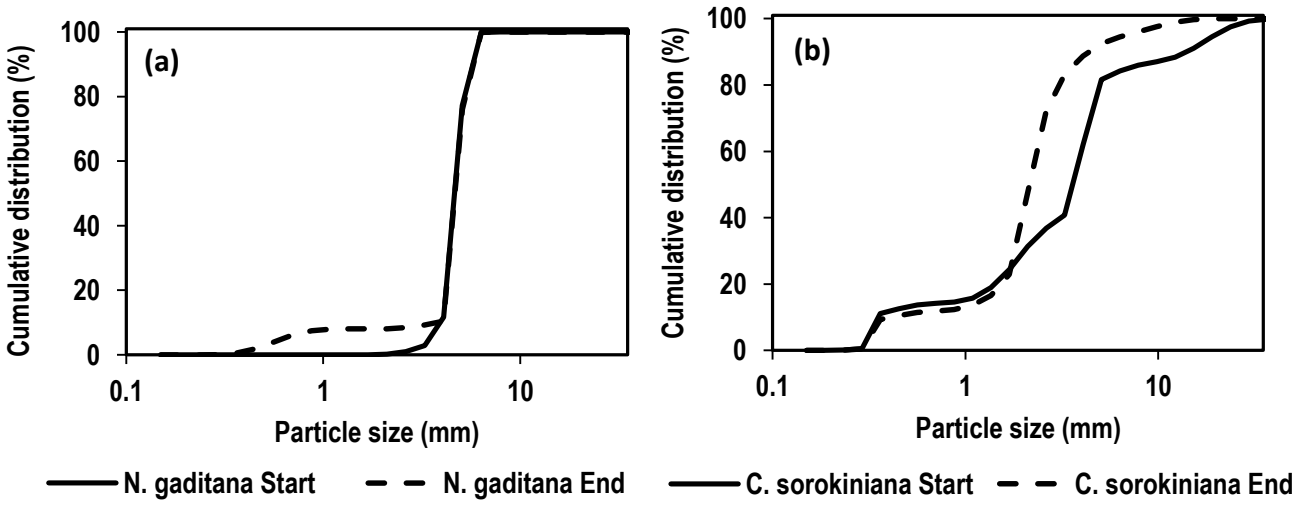

Figure 3. Particle size distribution before and after filtration assays for *N. gaditana* (a) and *C. sorokiniana* (b).

Direct microscopic observation revealed a heavy presence of bacteria. However, particle size analysis failed to identify a considerable fraction of particles in the size range normally associate with bacteria (around 1 μm). As a way to complement particle size analysis performed by laser diffraction, microalgae suspensions were analysed by flow cytometry. Results are presented in Figures 4 and 5, which present relative complexity versus relative size before and after the concentration process, for *N. gaditana* and *C. sorokiniana*. Natural fluorescence have been used as a tool to identify microalgae in the plots. Microalgae is presented by color dots (blue for *N. gaditana*, red for *C. sorokiniana*). The rest of the events are most likely associated with bacteria or debris. Figures 4 and 5 clearly shows there is a great number of events that failed to be classified as microalgae, and that this number considerably increased after concentration process. This is most likely the result of the shear stress imposed by cross-flow filtration. Non-microalgae events showed a lower relative particle size. Considering the relation between back-transport phenomena and particle size, this increase in the proportion of small particles certainly had an effect over filtration, and may be one of the factors responsible for the decrease in flux observed in Figure 2. Pumping driven shear stress have been already identified as a negative factor for microalgae membrane filtration. Babel (2010) and Ladner (2010) observed decreasing particle size and organic matter release which was related by the authors to shear stress.

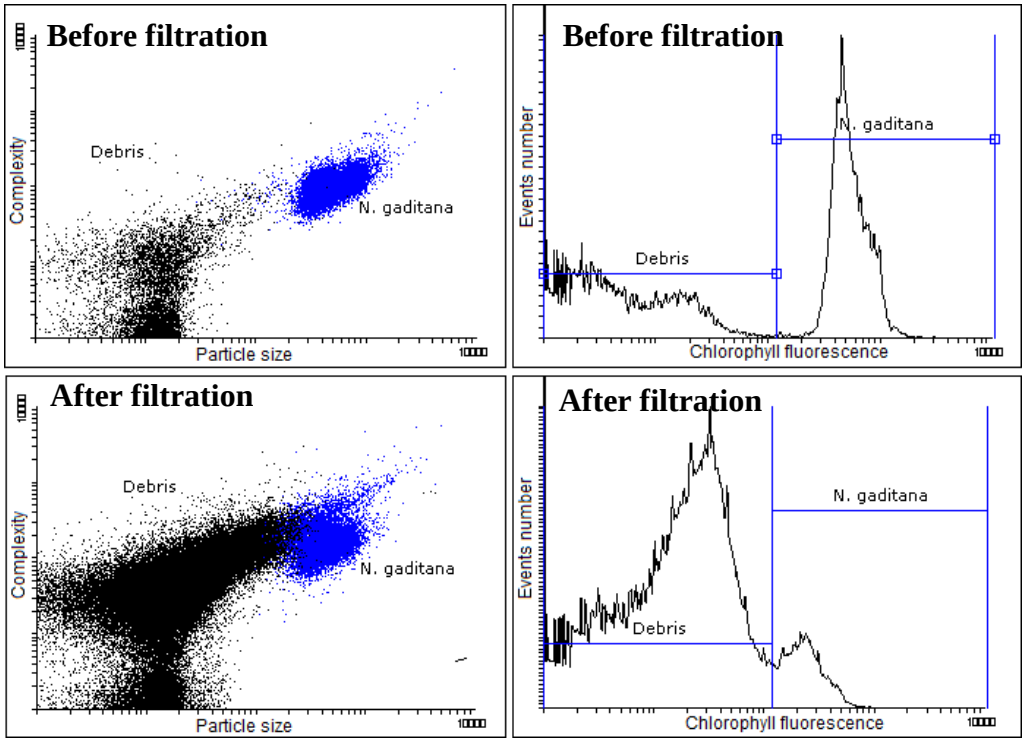

Figure 4. Flow cytometry analysis for *N. gaditana* suspensions. Complexity versus particle size and auto-fluorescence intensity are presented for before and after (B1 and B2) filtration assays.

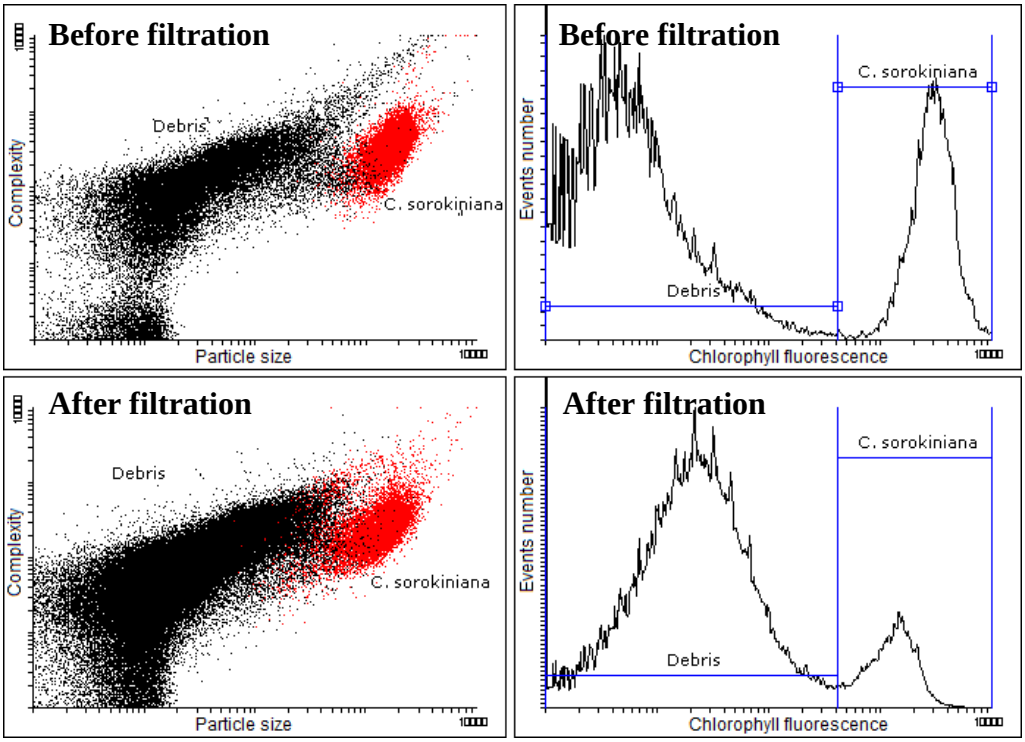

Figure 5. Flow cytometry analysis for N. gaditana suspensions. Complexity versus particle size and auto-fluorescence intensity are presented for before and after (B1 and B2) filtration assays.

4.3.2 Energy requirements for membrane filtration

Based on the results presented in Figure 1, energy requirements for microalgae concentration were determined. Figure 6 shows the energy per volume of initial microalgae culture, required to achieve a particular concentration. Figure shows that required energy increases quickly at concentrations below 5 g/L. At low concentrations, high quantities of permeate needs to be extracted in order to increase concentration, which requires longer operational times, increasing the energetic requirements derived from pumping. This effect decreases when solid concentration increases, because suspension volume is lower. Consumed energy during filtration process were 0.57 and 0.49 kWh/m^3 for *N.gaditana* and *C.sorokiniana*, respectively. As already mentioned, final biomass concentration for *N.gaditana* was almost twice that for *C. sorokiniana* and concentration factors were 21 and 27, respectively. These values are in the range of those reported by Bhave *et al.*(2012) who found energetic requirements ranging between 0.3 and 0.7 kWh/m^3 using tubular microfiltration membranes. Bilad *et al*. (2013) reported energetic consumption for submerged membranes in the range of 0.2 - 0.3 kWh/m^3, for concentration factors from 5 to 15 (biomass concentration < 4 g VS/L). The computed energy requirements in this research are in the range of those for other harvesting processes, such as vacuum flotation (0.2 kWh/kg) (Barrut *et al.*, 2013), centrifugation (0.3 - 1 kWh/m^3) and frontal filtration (0.1 - 5.9 kWh/m^3) (Molina Grima *et al.*, 2003).

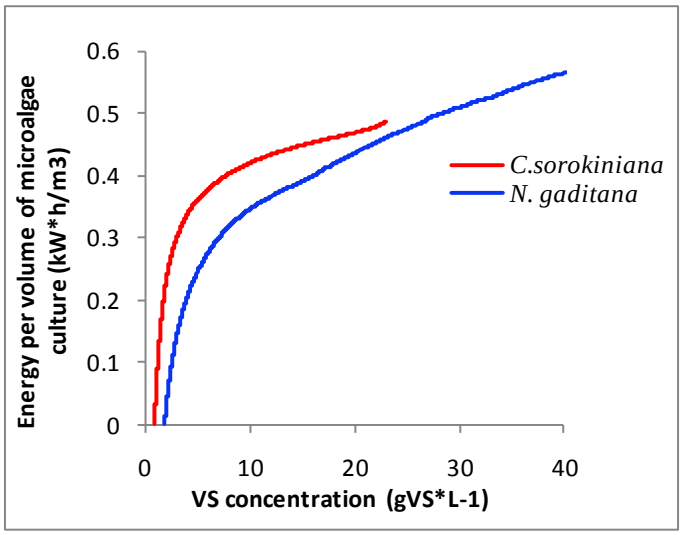

Figure 6. Calculation of consumed energy for microalgae *C. sorokiniana* and *C.reinhardtii CW-704* at two surface.

Figure 6 suggest that membrane filtration may not be an energy-efficient harvesting process for microalgae, at least for the conditions tested in this research. Energy requirement is in the range of traditional alternatives, but with concentration factors that cannot be considered high. Moderated levels of flux and the diluted nature of microalgal cultures may be the main factors hindering application of membrane filtration for microalga harvesting. However, membrane filtration may make sense as a post-concentrating step when being applied after an initial low-energy harvesting process such as settling, flocculation or flotation. These three separation processes are indeed characterized by low energy requirements. However, they usually provide low concentration factors, producing concentrates of only few grams per litre. Membrane filtration may then be an option to further concentrate these concentrates. In order to further study such option, the effect of cross flow velocity and solids concentration (in the range 10-50 g VS/L) over flux was studied for *N. gaditana* and *C. sorokiniana.*

Figure 7 shows the effect of VS and v_s on critical flux and fouling rate, for *C. sorokiniana* and *N. gaditana*. The analysis of variance (ANOVA) indicates that for both microalgae the relation between critical flux and v_s was linear, i.e. quadratic terms of the second order model were not significant (α=0.05). Moreover, VS effect over critical flux was not found to be significant, as was also the case of the interaction between v_s and VS for *C. sorokiniana*. found to was also not significant for both algae, as was also the case of VS in the case of *N. gaditana*. Then, for *C. sorokiniana* and *N.*gaditana the models after the elimination of non-significant parameters, are:

$$J_c = 5.41667 + 25.42373 \cdot v_s - 0.16667 \cdot VS$$

$$J_c = -4.89583 + 34.37500 \cdot v_s - 9.97143 \times 10^{-17} \cdot VS$$

In the case of the fouling rate, its relation with the studied factors showed to be of first order (α=0.05). Linear models after elimination of quadratic terms are:

$$F = -0.24386 + 0.088372 \cdot v_s + 0.027751 \cdot VS - 0.014264 \cdot v_s \cdot VS$$

$$F = -0.031814 + 0.018906 \cdot v_s + 7.05473 \times 10^{-3} \cdot VS - 3.16438 \times 10^{-3} \cdot v_s \cdot VS$$

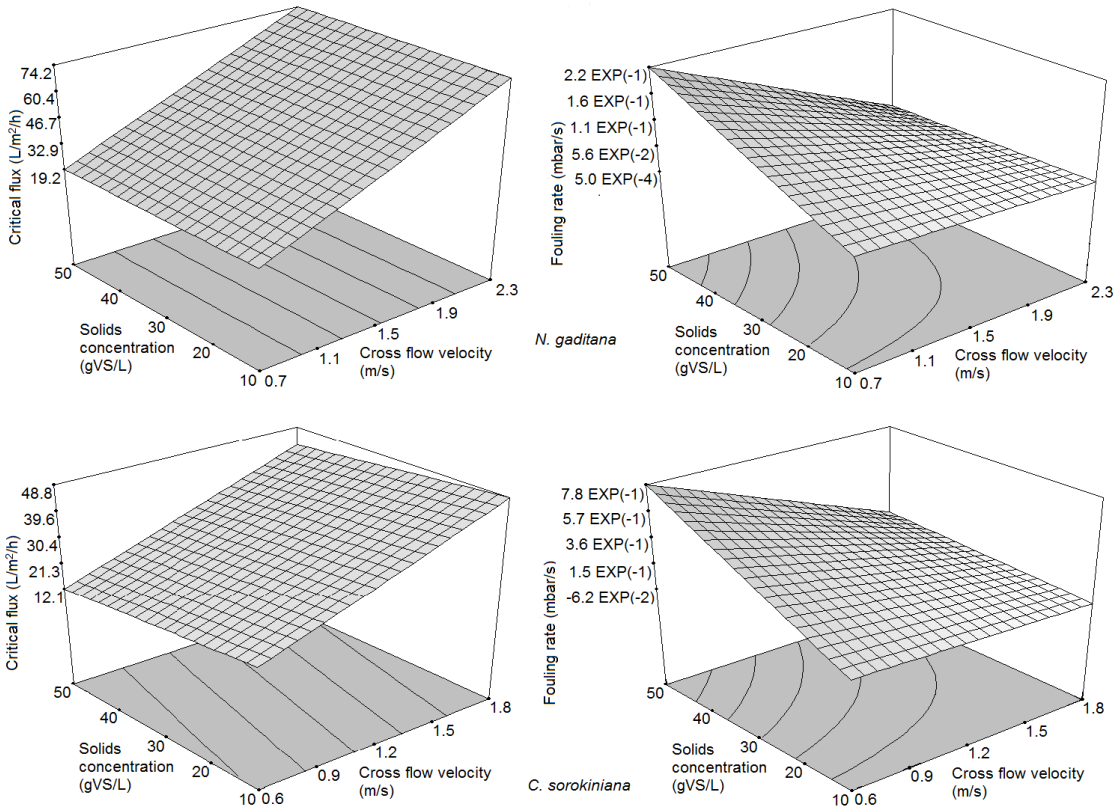

Figure 7. Effect of VS and v_s on Critical flux and fouling rate for *N. gaditana and C. sorokiniana* using surface respond methodology. Graphs represent the behaviour predicted by the models, after elimination of the non-significant parameters by forward analysis (using α=0.05).

ANOVA analysis showed that predictive models of critical flux and fouling rate are able to explain the over 80% of the response variables for both microalgae. Results show that v_s is the main parameter governing critical flux. However, even though VS had no or little effect over critical flux, it did influenced fouling rate, so high levels of solids may not affect at a high extent the flux at which cake layer begins but it affects its rate of formation.

Under the conditions tested, observed fluxes were in the range 20-27 and 10-50 $L/m^2 \cdot h$ for *N. gaditana* and *C. sorokiniana*, respectively. Such flux levels may be considered high enough to enable the use of membrane filtration as a post-concentrating step for settling or flotation. However, these results were obtained by means of short term filtration assays. Further research needs to be done to confirm if under long-term operation observed fluxes will prevail.

4.4 Conclusions

The critical flux decreased in filtration assays as result of increase in total resistance. Although part of resistance caused by cake formation and fouling was removed, an important fraction of fouling was evidenced, which blocked pores and hence, increased resistance and decreased flux.

The pumping of culture microalgae in membrane filtration assay generated shear stress which caused for both microalgae a reduction in particle size distribution. Moreover, at filtration of *C. sorokiniana* a initial bacterial concentration was elucidated.

Energy requirements in membrane filtration are rapidly increased in diluted cultures due to high media volume to be filtrated which is traduced to large times of pumping operation. At biomass concentration over 10 g VS/L, membrane performance and energetic requirements are not dependant on biomass concentration, suggesting that membrane filtration can be used as post-harvesting process including a pre-harvesting process as flocculation or sedimentation.

CHAPTER V.
Operation of Mesophilic and Thermophilic anaerobic reactors for biogas production from spent microalgae N.gaditana

5.1 Introduction

Currently, most of the efforts to take advantage of microalgae as a source of bio-energy have been directed to biodiesel production. Despite the advantages above mentioned, there is concern related to a potentially low energetic yield in the biodiesel-from-microalgae production process using current technologies (Chisti, 2007; Sialve *et al.*, 2009; Scott *et al.*, 2010; Stephens *et al.*, 2010). Indeed, some authors have calculated a negative energetic balance, with the largest production costs associated with harvesting and drying steps (Lardon *et al.*, 2009; Scott *et al.*, 2010)

In this scenario, different strategies have been proposed in order to improve the energetic yield of the process. These ones are oriented to the optimization of light delivery to the culture, use of the residual glycerol as a heterotrophic source of carbon, maximization of triglyceride accumulation through nutrient supplementation and metabolic engineering, use of direct trans-esterification (avoiding the drying of the biomass), culture in wastewater and implementation of anaerobic digestion or other energy recovery processes from the spent microalgae biomass (Chinnasamy *et al.*, 2010; Scott *et al.*, 2010; Patil *et al.*, 2011). The anaerobic digestion of the residual biomass seems to be one of the most promising strategies, due to the energy recovery in the form of biogas, the potential re-use of the released nutrients in the microalgae culture and the fact that anaerobic digestion can be used to stabilize the waste biomass and avoid other costs related to its disposal and management (Sialve *et al.*, 2009).

Few studies have evaluated the energetic contribution of anaerobic digestion in the biodiesel production process from microalgae. However, these studies have indicated that a considerable part of total energy contained in the biomass can be recovered if anaerobic digestion of spent microalgae is applied.(Harun *et al.*, 2010; Ehimen *et al.*, 2011; Razon and Tan, 2011). Thus, anaerobic digestion of spent microalgae will be evaluated in this thesis.

In anaerobic digestion, thermophilic operation is an established technology which operates at optimal temperature of 55°C. The main advantage is related to that thermophilic digestion presents a degradation rate higher than mesophilic digestion. This advantage is traduced in low HRT and small reactors. Other advantages are related to high patogen destruction, hydrolysis step improve, increase of VFA production (Buhr and Andrews, 1977; Kardos *et al.*,

2011). Thus, in this report methane production from spent microalgae under mesophilic and thermophilic conditions will be evaluated and compared.

5.2 Materials and Methods

5.2.1 Mesophilic and thermophilic BMPs

Microalga *Nannochloropsis gaditana* was harvested from raceway pond (supplied by Antofagasta University). Lipid extraction was carried-out with a soxhlet extraction unit using solvent mixture (hexane/acetone 3:1 v/v) for 8h. Lipid content was determined gravimetrically. Spent microalgae obtained from lipid extraction process were dried at 75°C and 105°C in order to evaluate effect of drying on anaerobic digestion. Bio-methane potential tests (BMP) were performed under mesophilic and thermophilic conditions for three substrates: Total microalgae dried at 105°C (without lipid extraction) (M1), spent microalgae dried at 75°C (M2) and spent microalgae dried at 105°C (M3). This assays were carried-out according to Torres *et al.* (2014). All BMP tests were carried-out in triplicate.

5.2.2 Mesophilic and thermophilic Anaerobic reactors

Two lab-scale continuous anaerobic bioreactors (1L) degrading spent microalga *N.gaditana* were operated in order to evaluate mesophilic (35° C) and thermophilic (55° C) conditions. A Filtration unit was coupled to each anaerobic reactor for nitrogen recovery (Figure 1). Reactors were operated during a period of 120 days, maintaining a OLR of 0,5 gCOD/L·d and hydraulic retention time (HRT) of 30 days. Stage 1**:**Reactor was fed with mix (wine - peptone - starch), Stage 2**:**Reactor was fed with mix (wine - starch), Stage 3**:** Reactor was fed with spent microalgae M2, Stage 4: Reactor was fed with spent microalgae M3, Stage 5: Reactor was re-fed with spent microalgae M2. Reactor was monitored through measuring of produced methane, biogas composition, pH, total and volatile solids, soluble COD, carbohydrates, ammoniacal nitrogen and VFA.

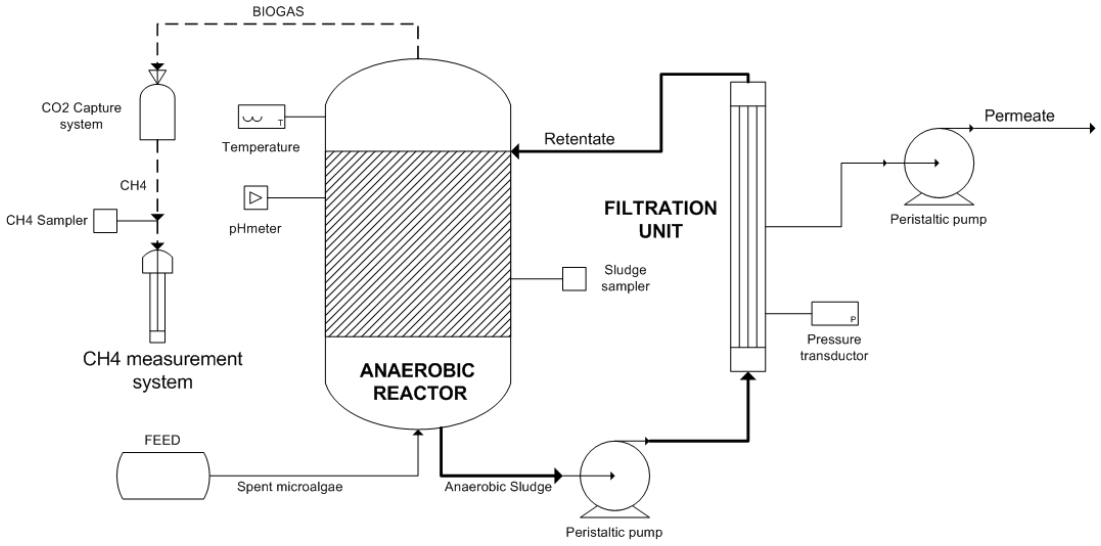

Figure 1. Setup of Mesophilic and thermophilic AD reactors.

5.2.3 Hydrolytic, Acidogenic and methanogenic activities

The activities involved in anaerobic digestion consortium were determined in batch assays. Hydrolytic (starch) (HA_C), hydrolytic (peptone) (HA_CP), Acidogenic (AA_C) and Methanogenic (MA_C) activity was evaluated According to Soto *et al. (1991).* Substrate for each activity was starch, peptone, glucose and VFA mixture (1:1:1 COD ratio for acetic, propionic and butyric acid), respectively. All activities were carried-out in triplicate.

5.2.4 Nitrogen recovery through membrane filtration

Membrane performance and nitrogen recovery were evaluated after stage 5 in mesophilic anaerobic reactor fed with spent microalgae M2 (*see Section 5.2.2).* In this stage a membrane module (single tubular ultra-filtration membrane X-flow, NORIT, pore size: 30nm, length: 39cm and internal diameter: 8mm) was operated during 30 days in order recover nitrogen through filtration of sludge. Permeate fraction was collected by means of peristaltic pump. Trans-membrane pressure (TMP) was determined measuring the pressure in the membrane module, using a pressure sensor. The retentate and permeate fraction was recycled to anaerobic reactor in order to maintain solid concentration.

Membrane performance was evaluated through permeability determination according to equation (1):

$$(1)\ Permeability = \frac{J}{TMP} = \frac{1}{\mu \cdot R_T}$$

Where *J* is the membrane flux , *TMP* is the trans-membrane pressure, μ is permeate viscosity and R_T is the total resistance to filtration. Critical flux determination was evaluated with flux steps of 2 $L/m^2 \cdot h$, according to Jeison and van Lier (2007).

Membrane recovery was evaluated comparing concentrations in soluble fraction of reactor and permeate sample collected from membrane module. COD, carbohydrates, proteins, $N\text{-}NH_3$, NO_3^-, PO_4^{3-} and VFA was measured for these samples. Rejection for every component was computed according to equation (2):

$$(2) \qquad R = \left(1 - \frac{C_p}{C_s}\right) \cdot 100$$

Where, C_p and C_s are the permeate and soluble reactor concentrations. Soluble fraction was obtained when sample was filtered to 0.45μm and permeate fraction is the product of membrane filtration.

5.2.5 Analytical procedures

The COD, total solids and volatile solids were measured according to APHA (1998). The pH was determined through pH meter Orion star A121.Carbohydrates was measured through Dubois *et al*. (1956).Volatile fatty acids (VFA) was measured through gas chromatography (GC-FID). Total ammonia nitrogen was measured through colorimetric method. (HACH KIT TNT 343). Ammonia concentration was computed considering total ammonia nitrogen and pH. Glucose was measured through reducing sugar DNS method. Starch was measured as difference between carbohydrates concentration and reducing sugar concentration, according to Soto *et al.* (1991). Pressure into vials headspace was measured through Cole-Parmer pressure transducer model 206 (-14,7 - 15 PSIG). Methane composition was measured through gas chromatography (GC-TCD). Viscosity of permeate was measured through viscosimeter (AND vibro viscosimeter SV-10) and distribution of particle size was measured through laser diffraction particle size

analyser (SALD-3101). The concentration common inhibitors (Table 3) was determined through atomic absorption spectrophotometry (APHA 3500 B Flame emission).

5.2.6 Statistical analysis

For BMP tests a one-way ANOVA analysis was computed in order to compare significant differences of substrate M1, M2 and M3. When a significant difference between substrates was found, post-hoc Tukey test was computed in order to compare means of substrates. Effect of mesophilic/thermophilic conditions for every substrate was analyzed through independent samples t-student. All these analyses were computed using statistical software SPSS19. For all analyses a significance level value of 5% ($\propto = 0{,}05$) and N=3 was used.

5.3 Results and Discussion

5.3.1 Microalgae Characterization

Table 1 shows the proximate composition for microalgae M1, M2 and M3, where as it is obvious, the fat content is lower in M2 and M3 than in M1. As a result, protein and ash proportions are higher. Considering the high protein content in all samples of *N. gaditana*, it is expected a high ammonia release into reactor, which, as already discussed, may cause inhibition of methanogenic bacteria. Also, the low proportions of crude fibers (i.e. lignin and cellulosic components) and ashes suggest that few amounts of these hardly biodegradable or inert compounds will be accumulated into reactor. Although sodium in *N. gaditana* is expected (saltwater microalgae), concentrations in all samples will indicate no inhibition under the operational conditions.

Microalgae characterization was complemented with miscroscopic observation. Figure 2 shows confocal microscopic for M2 and M3, where microalgae were stained with a non-specific cellulose staining (Calcofluor), which reveals cell wall integrity. Figure 2 shows no cell disintegration for samples M2 and M3, i.e. cell integrity was maintained, indicating that lipid extraction method used was not acting as pre-treatment. SEM microscopy in Figure 3 shows that lipid extraction cause biomass agglomeration, forming clusters structures. Moreover, even though lipid extraction does not seem to cause cell disintegration, it clearly affects the shape and the structure of the surface of microalgae cells. In fact, Figure 3 shows that lipid extracted microalgae (M2A, M3A) are more agglomerated than (M1A). Moreover, difference between images M2 and M3 indicates that clearly drying temperature after lipid extraction plays an important role in algae agglomeration. As already mentioned, cell structure was affected, which is clearly observed in M3B, where cracks and wrinkles appear on the microalgae surface. An explanation for this could be that, during the oil extraction, the solvents have to diffuse through the cell membrane and wall, extracting phospholipids present in the membrane and possibly also other cell wall components (Wurdack, 1923; Abo-Shady *et al.*, 1993).

Table 1. Results of the proximal analysis of microalgae *N. gaditana* samples M1 (dried at 105 ºC), M2 (oil extracted and dried at 75 ºC) and M3 (oil extracted and dried at 105ºC)

Component	Composition by algae group (%)		
	M1	M2	M3
Moisture	0.82	4.02	2.56
Total fats	9.31	6.38	6.01
Protein	48.33	52.54	52.85
Crude fiber	2.44	1.94	2.2
Ash	15.76	18.08	19
Carbohydrates	23.34	16.59	17.38
Calorific value (1)	370.47	337.99	335.01
Sodium (2)	1856.6	1922.6	2075.6

(1) Value in Kcal/100g
(2)Value in mg/100g

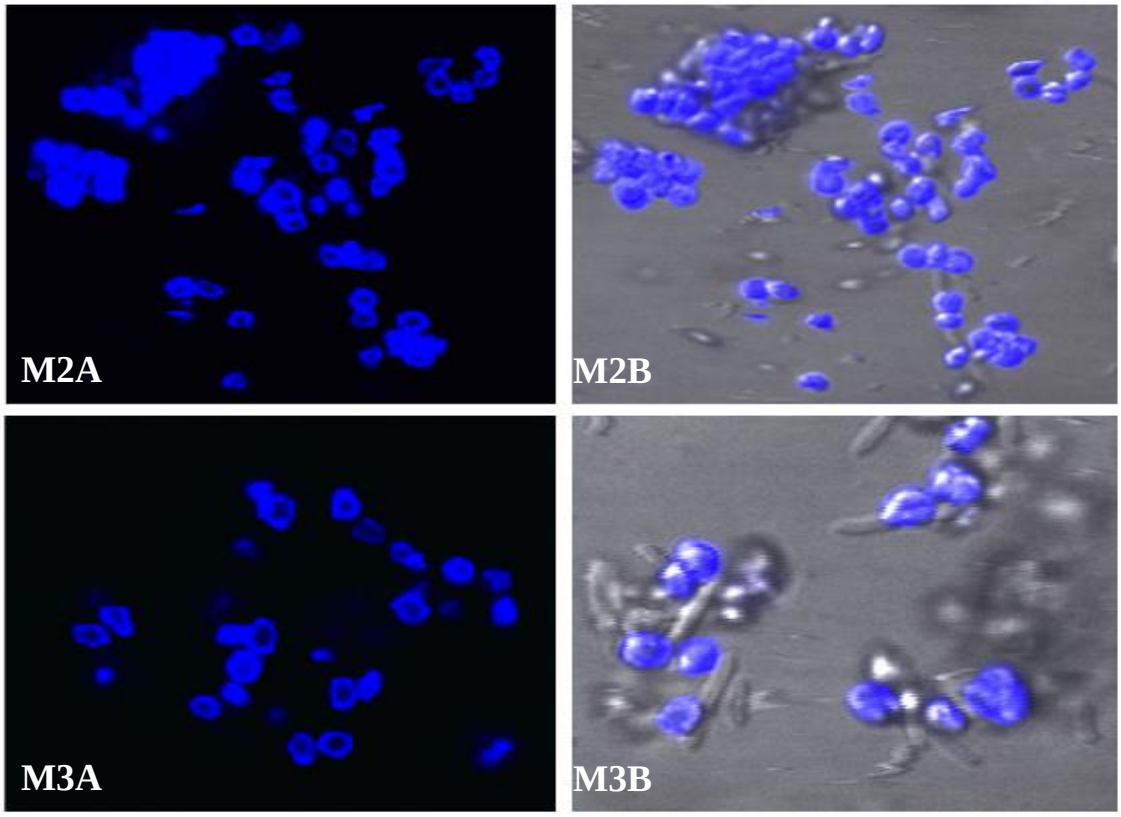

Figure 2. Confocal images of spent *N. gaditana* M2 (oil extracted and dried at 75 ºC) and M3 (oil extracted and dried at 105 ºC). Cells were stained using calcofluor. Images "A" show the blue cellulose fluorescence after staining and "B" merge blue fluorescence and general background.

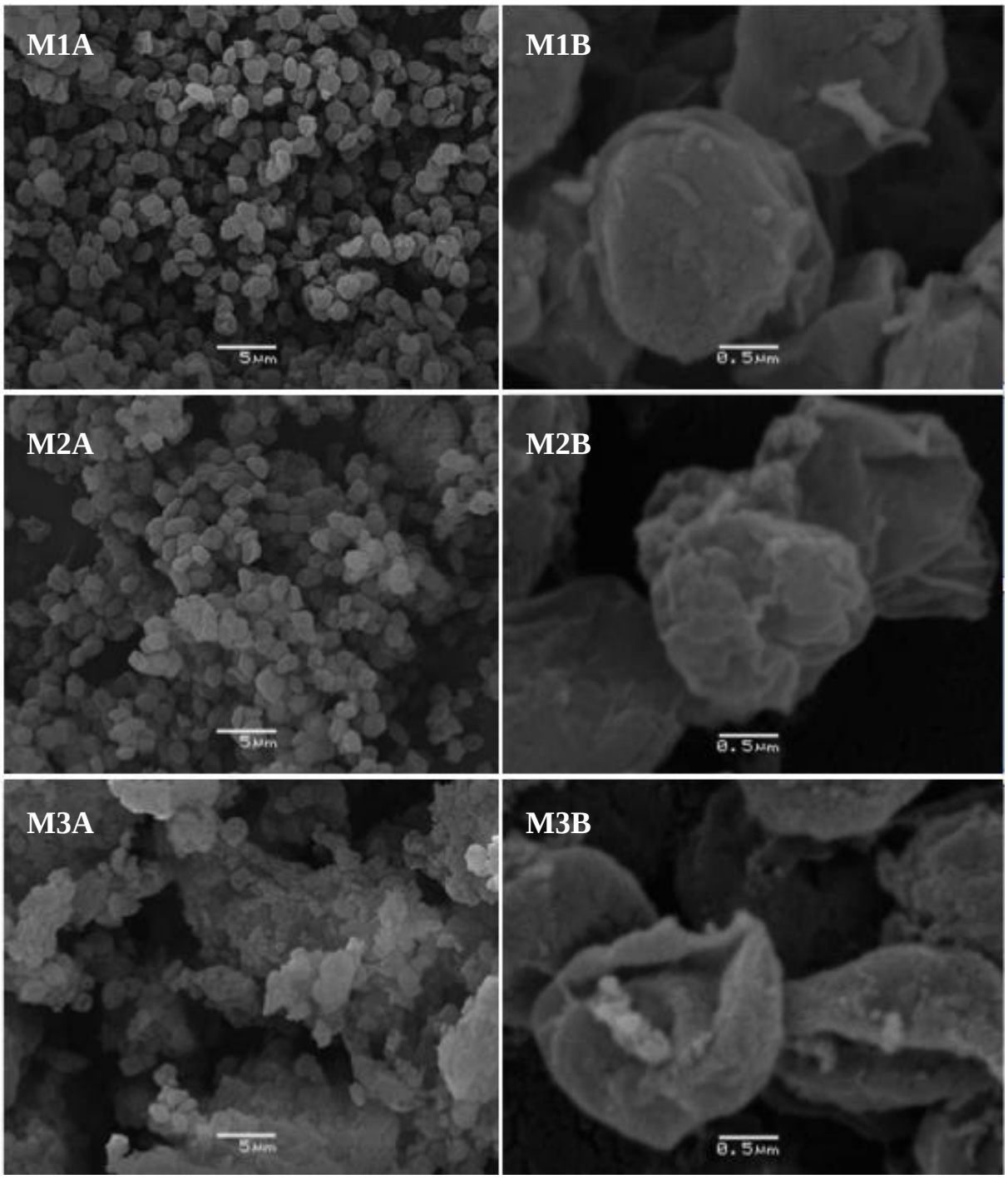

Figure 3. Images of *N. gaditana*, M1 (dried at 105 ºC), M2 (oil extracted and dried at 75 ºC) and M3 (oil extracted and dried at 105 ºC) obtained by SEM.

The plots in Figure 4 present the side-scattered light (SSC-A), the forward-scattered light (FSC-A) and the presence of alpha phycocyanine like compounds (accessory pigment to chlorophyll) (APC-A). From a practical point of view, SSC-A, FSC-A and APC are proportional to cell granularity (complexity), cell size and chlorophyll content (measured at wavelengths higher than 633 nm), respectively (Hyka *et al.*, 2013). Based on these properties, photosynthetic microorganisms like microalgae can be identified from bacteria. *N. gaditana* cells are more complex and bigger than regular bacteria and other sources of pollution, so they appear in the upper right corner of the plots. The other events are smaller microorganisms or particles present in the samples. Comparing M1 and M2 plots, it is clear that the samples present similar characteristics. Although there are slightly more particles (events) with small size in M2, the microalgae group is still clearly defined, suggesting that no significant cell disintegration occurs during lipid extraction. Moreover, Figure 4 M1B and M2B show similar levels of chlorophyll fluorescence, which may also be an indicator for cell integrity, leading again to the conclusion of no cell disintegration occurring during the oil-extraction process.

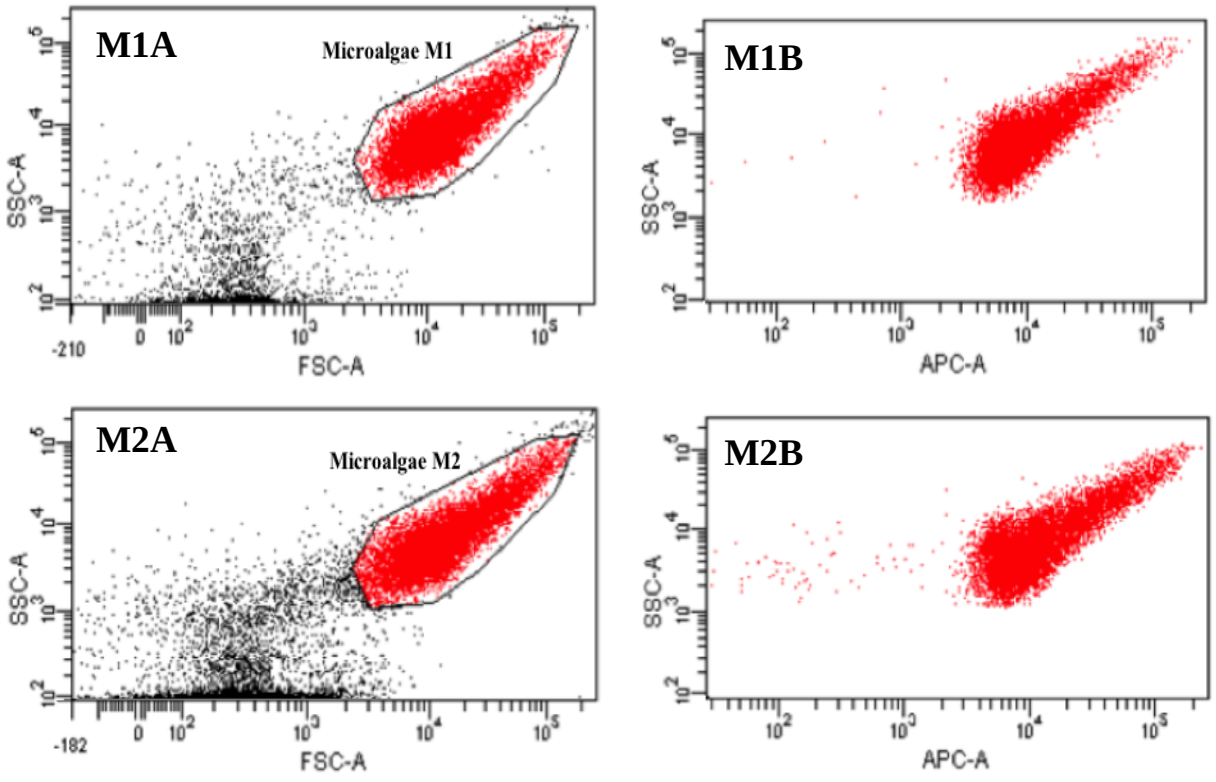

Figure 4. Flow cytometry results corresponding to algae M1 (dried at 105 ºC) and M2 (oil extracted and dried at 75 ºC). The dot-plots distinguish the microalgal populations (gated

regions) from the contamination according to their size (FSC), granularity (SSC) and chlorophyll content (APC)

5.3.2 Biomethane potential tests

Results presented in Figure 5(I) shows BMPs for total microalgae and spent microalgae. One-way ANOVA analysis for mesophilic conditions revealed no significant differences for substrate M1, M2 and M3. Unlike this result, significant differences for substrate M1, M2 and M3 were found under thermophilic conditions, being BMP of M3 the highest methanogenic potential. Unlike our expectations, results showed that for each substrate, mesophilic digestion produced more methane than thermophilic conditions. In fact, it is known that a higher solubility is reached increasing temperature; hence, higher availability of substrate for anaerobic consortia. Differences between experimental and expected results for thermophilic conditions may be associated with sludge adaptation so that thermophilic consortium has not been pre-adapted to substrate (microalgae) unlike mesophilic sludge, which was previously used at microalgae anaerobic degradation. It is worthy to notice that according to statistical analysis of one-way ANOVA and Tukey test, no significant differences in BMPs were found for spent microalgae dried at 75°C (M2) and dried at 105°C (M3). Thus, from an energetic point of view spent microalgae can be dried at 75° C without observing significant differences in BMPs. The pH measurements at final time of these assays showed values of 7,2 - 7,3 for mesophilic conditions and 7,5 - 7,6 for thermophilic conditions. Thus, these pHs were found within range of values for methanogenic optimum conditions (6,5 - 7,5). Measurements of N-NH_3 at the end of tests showed ammonia concentrations for mesophilic and thermophilic conditions of 6 -11 mg NH_3/L and 12 - 15 mg NH_3/L, respectively. These values were found lower than typical IC_{50} values of 80 - 100 mg NH_3/L (Chen *et al.*, 2008), hence no ammonia inhibition should be expected. Figure 5(II) shows biodegradability of substrate computed as fraction of organic matter that is effectively reduced into methane (experimental BMP / theoretical BMP). An important result is found based on result of one-way ANOVA tests, which shows that for mesophilic and thermophilic conditions, there are significant differences between total and spent microalgae. This result indicates that lipid extraction can act as pre-treatment increasing availability of substrate, hence, biodegradability.

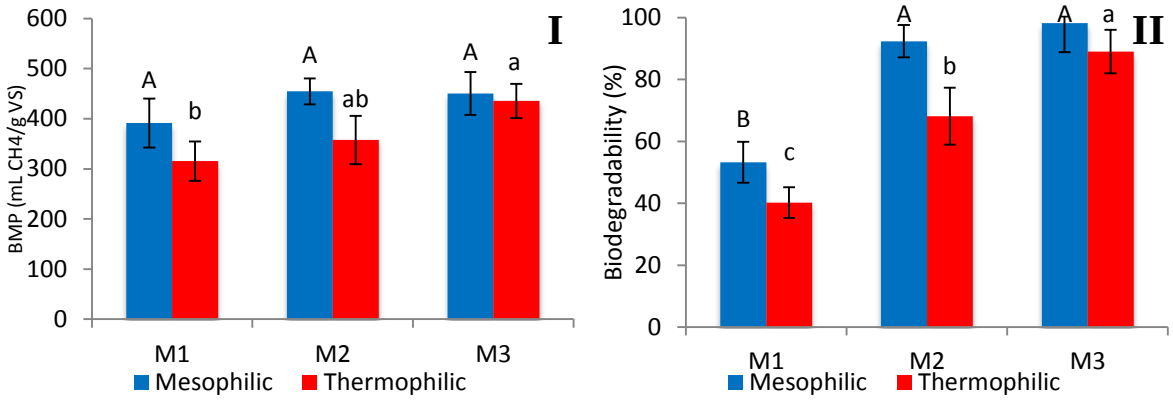

Figure 5. BMP value (I) and Biodegradability (II) of substrates M1, M2 y M3 for mesophilic (35°C) and thermophilic (55°C) conditions. Bars represent standard deviation.

In order to evaluate distribution of organic matter in batch test, COD balances were computed considering total organic matter (added substrate), organic matter reduced to methane, soluble organic matter (measured as soluble COD) and particulate organic matter (computed as difference). Results in Table 2 show that there is a fraction of organic matter that although it was solubilised, it was not degraded into methane, corresponding to values up to 10% and 15% for mesophilic and thermophilic conditions, respectively. Particulate fraction in COD balance reveals that there is organic matter that was not hydrolyzed, hence, not available for methane production, which in particular case of total microalgae was near 40-50% for mesophilic and thermophilic conditions. Unlike results of particulate fraction in M1, this fraction decreases considerably in spent microalgae M2 and M3 supporting beneficial effect of lipid extraction process on biodegradability. Thus, lipid extraction improved hydrolysis step reducing particulate organic matter, hence, increasing biodegradability of substrate.

Table 2. COD balance for BMP tests.

COD %	Mesophilic BMPs			Thermophilic BMPs		
	M1	M2	M3	M1	M2	M3
Methane	53	93	98	40	68	89
Soluble	6	4	8	11	15	13
Digestate	41	3	-7	48	16	-2

5.3.3 Continuous anaerobic bioreactors

5.3.3.1 Reactors operation

The performance of the two bioreactors during the five operation stages (described in *section 5.2.2*) is presented in Figure 6, where the methane production is indicated as a percentage of the maximum theoretical production (computed with the theoretical methane COD). By the time operation with synthetic substrate ended (day 42), a methane production close to 80% of the theoretical value was achieved.

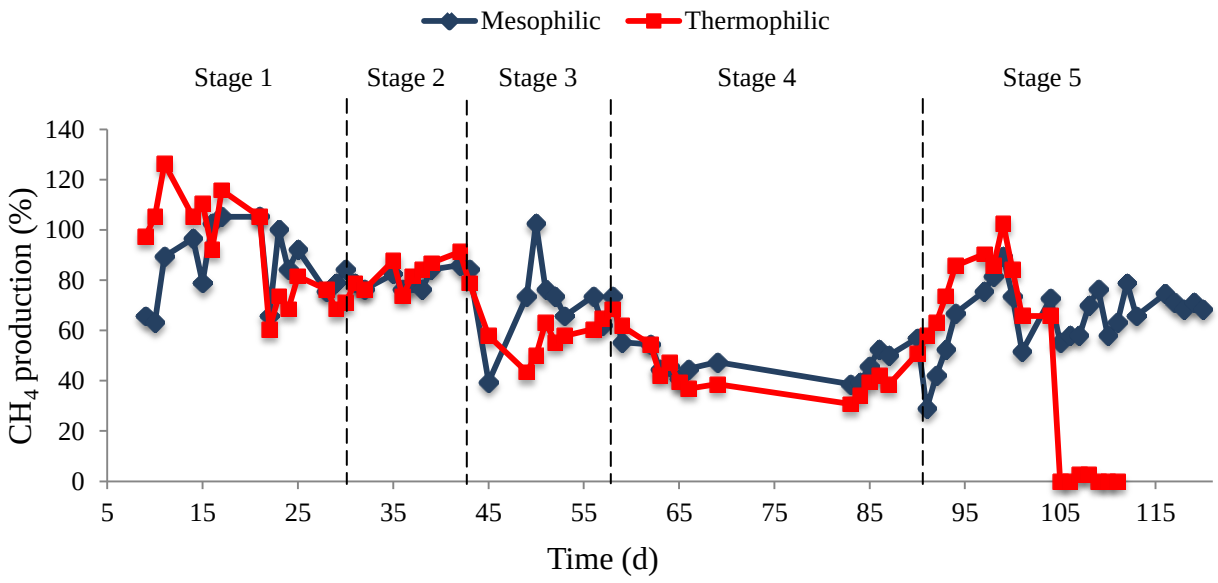

Figure 6. Methane production as a percentage of the maximum theoretical generation (computed with the theoretical methane COD) during the operation period for mesophilic (35 ºC) and thermophilic (55 ºC) reactors. OLR of 0.5 g COD·L^{-1}·d^{-1} and HRT of 30 d.

After 42 days of operation, the feed was changed to lipid-extracted microalgae M2 (Stage 3). As consequence, the biogas production decreased to 40% during the first few days. Then, biogas generation started to recover, reaching values of around 60% at the end of this stage. This decrease in the biogas production clearly corroborates that microalgae is a complex substrate for AD in continuous reactors. During stage 4 (days 58 to 90), microalgae M3 (dried at 105 ºC) was used as substrate, causing a sudden decrease in the biogas production, reaching values as low as 30% for the thermophilic reactor. This result is somehow inconsistent with the data extracted from the BMPs, where M2 and M3 showed no difference in the biogas production at mesophilic conditions (at thermophilic conditions, M3 even yielded more biogas). An explanation of why M3 produces less biogas than M2 in the continuous reactors may be the biomass agglomeration observed in Figure 2, which may hinder microalgae degradability. The last 7 days of Stage 3, an increase in the biogas generation occurred in both reactors, although it decreased again the last day in the mesophilic reactor. During the last operation period (Stage 5), the reactors were fed again with microalgae M2, observing an increase of the biogas production, getting stable in values around 60% (in accordance to the results obtained during stage 3). This clearly proves that M2 leads to the highest methane production. It is worthy to notice that at day 104 of operation, a power failure caused a series of problems that ended with the NaOH solution used for CO_2 absorption entering the thermophilic reactor. This caused a considerable increase in the pH, inhibiting the microorganisms. Even thought the system was brought again to neutral pH, the activity was not restored and operation had to be interrupted.

In general terms, no significant differences can be found between the biogas productions of both reactors. Therefore, from an energetic point of view, the mesophilic reactor would be preferable. However, as it has already been mentioned, thermophilic sludge acclimation may play an important role and thus, longer experiments should be carried out. In order to evaluate whether in thermophilic operation there was sludge acclimation, BMP test was carried-out two months after starting operation with microalgae M2. the identical BMP value and behavior (data not shown) indicated that two months was not a necessary time for sludge adaptation.

The obtained methane yields (around 290 $mL \cdot g^{-1}$ VS for M2 and 180 $mL \cdot g^{-1}$ VS for M3) are in accordance with others reports (Golueke *et al.,* 1957; Kinnunen *et al.,* 2014) .The OLR applied during reactor operation was 0.5 g $COD \cdot L^{-1} \cdot d^{-1}$, which can be considered low when compared to the values of this parameter set by these authors.

Figure 7 shows that TAN concentrations increased with time for both reactors. This is mainly due to protein degradation. However, the concentrations of the inhibitory nitrogen form (FAN) were fairly constant and lower than 40 mg/L, below the general inhibitory range of 50-150 mg NH_3/L. The FAN levels may have been slightly higher in the thermophilic reactor due to the influence of temperature in the ammonia equilibrium. Although the slightly higher TAN concentrations in the thermophilic reactor suggest more intense algae degradation under these conditions, as methane productions are similar for both reactors, this cannot be absolutely asserted.

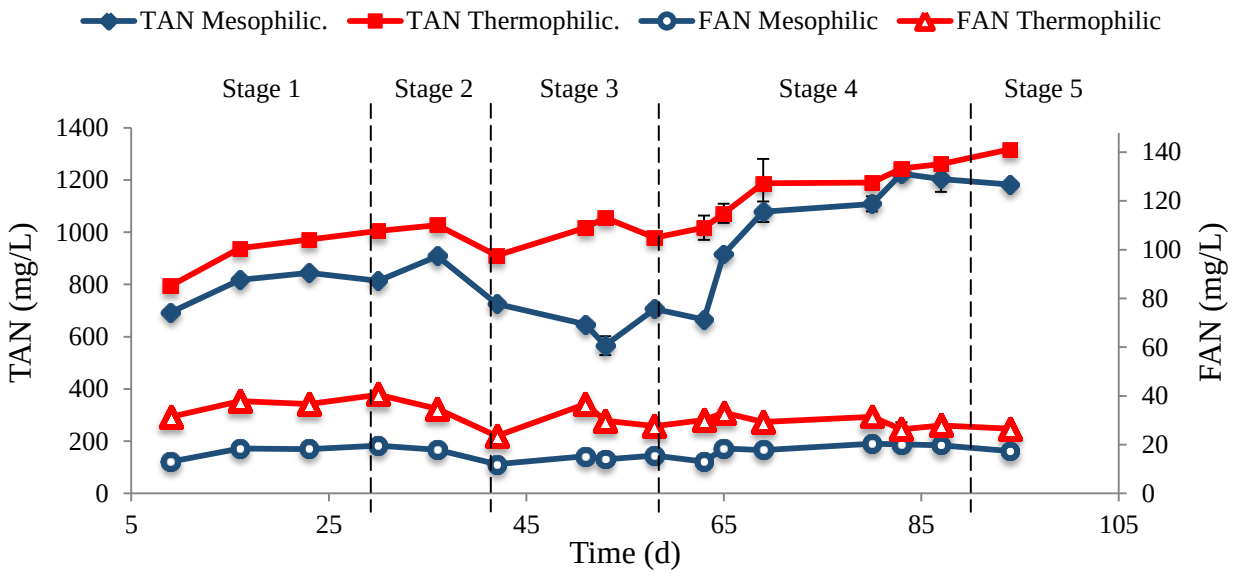

Figure 7. TAN and FAN concentrations during the operation period for mesophilic (35 ºC) and thermophilic (55 ºC) reactors.

The soluble COD (Figure 8A) was maintained at low concentrations (<1.5 g/L) for the mesophilic reactor throughout the whole operation period. On the other hand, the sCOD in the thermophilic system was kept always above 2 g/L reaching values close to 4 g/L at the end of the fourth HRT. This pronounced COD accumulation could be caused by a better hydrolysis of the substrate under thermophilic conditions, by a lower degradability of soluble compounds, or due to a combination of both. Soluble proteins and carbohydrates concentrations are presented in Figures 8B and 8C, where low values found indicate that no accumulation of these organic compounds existed, suggesting a high biodegradability in both reactors. Thus, it is also proved

that the hydrolysis step required to obtain these compounds rather than their conversion to methane is the rate-limiting step of the AD process. The same reasons explaining the higher sCOD in the thermophilic reactor when compared to the mesophilic one are applicable to the higher carbohydrate concentration observed. Moreover, the VFA concentration was maintained at values under 10mg/L (data not shown) indicating no VFA inhibition in methanogenic bacteria.

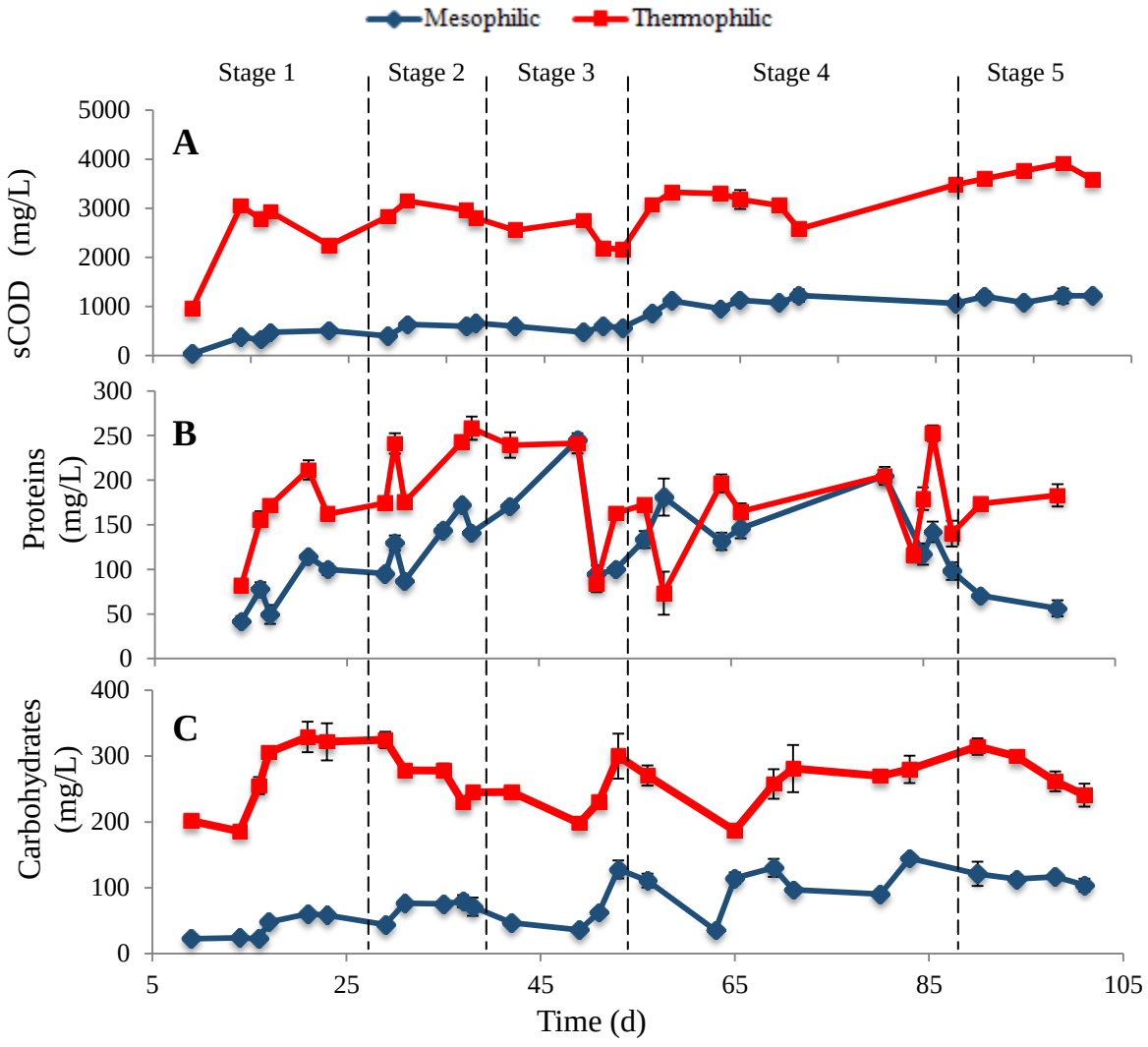

Figure 8. The sCOD (A), proteins (B) and carbohydrates (C) concentrations during the operation period for mesophilic (35 ºC) and thermophilic (55 ºC) reactors.

Comparing sCOD in the reactors with the sum-up of soluble carbohydrates, soluble proteins and VFAs, it can be observed that there is a fraction of the sCOD that is not composed by these organics. This COD is attributed to soluble microbial products (SMPs) other than the soluble carbohydrates and proteins measured. SMPs are the pool of organic compounds released into the solution from substrate metabolism and biomass decay (Aquino and Stuckey, 2008). These compounds, produced during bacteria metabolism, are not intermediates of the biogas production process. Example are humic acids, nucleic acids, polysaccharides, exocellular enzymes or structural components (Kunacheva and Stuckey, 2014). They can affect the steady state operation in a continuous bioreactor and they have been identified as most of the COD in effluents from aerobic and anaerobic biological systems (Janga *et al.*, 2007; Aquino *et al.*, 2009; Mesquita *et al.*, 2010). The temperature has an important influence on SMP formation (Feng *et al.*, 2008). As cell growth and death are faster under thermophilic conditions, more SMPs are generated under these conditions, explaining the higher sCOD in the thermophilic bioreactor. Moreover than the SMPs, fragments of cell parts (i.e. cell walls, etc.) may have passed through the 0.45 μm filters used to determine the sCOD, slightly overestimating this value.

5.3.3.2 Potential inhibitors

In order to discard a possible inhibition during the bioreactors operation, all the main inhibitors known for AD were measured. Table 3 shows the concentrations of these compounds at operation day 91. Ammonia and VFA concentrations were already presented, so their values are not shown here. In can be concluded from the results that with the followed procedure, no residual solvent (hexane and acetone) was present in the algal biomass. Moreover, all the HMs measured were far below the inhibitory limits. However, the sodium concentration was found to be much higher than that of other alkali metals such as K^+ or Ca^{2+}. This is perfectly logical, considering that a marine algae is being used as substrate. Although no inhibitory concentrations were observed, it must be mentioned that particular attention must be paid to FAN and Na^+ when operating reactors at higher solids concentrations, since these compounds showed values closer to those providing inhibition of the anaerobic consortium.

Table 3. Concentrations of common AD inhibitors in the supernatant of the reactors after 91 days of operation and inhibition values adapted from Angelidaki and Ahring (1992), Batstone *et al*, (2000) and Appels *et al*, (2008).

Compound	Concentration (mg/L)		
	Mesophilic (35°C)	Thermophilic (55 ºC)	Inhibition values
Ca^{2+}	6.6	12.1	2,500 - 4,000[(2)]
Cr^{6+}	<0.03	<0.03	10[(2)]
Cr^{3+}	<0.03	<0.03	200 - 250[(3)]
Cu^{2+}	0.7	0.6	0.5[(3)]
Mg^{2+}	4.6	11.8	1,000 - 1,500[(2)]
Ni^{2+}	2.3	3.4	30[(3)]
K^{+}	710	360	2,500 - 4,500[(2)]
Na^{+}	1740	1640	3,500 - 5,500[(2)]
Zn^{2+}	1.4	0.8	1[(3)]
S^{2-}	0.6	0.6	200[(2)]
Hexane	nd [(1)]	nd [(1)]	-
Acetone	nd [(1)]	nd [(1)]	-

[(1)] Non-detectable: concentration below detection limits of the measuring procedures.
[(2)] Moderately inhibitory concentration.
[(3)] Strongly inhibitory concentration.

5.3.3.3 Hydrolytic, acidogenic and methanogenic activities

Specific methanogenic (MAc), acidogenic (AAc) and hydrolytic activities of carbohydrates (HAc) and proteins (HAcP) were determined. The corresponding activities are presented in Table 4, where the values of the HAc are quite low when compared to literature (Soto *et al.*, 1993). Results show low rates for both sludges, suggesting that the hydrolysis of carbohydrates could be a main issue for AD with the current inocula. Significant differences exist between both HAcs, with higher values under thermophilic conditions. This suggests that carbohydrate hydrolysis may be favored at these temperatures. The values of the HAcPs are similar to those obtained for Hacs with carbohydrates, indicating that protein hydrolysis is also a slow step AD. As oil-extracted *N. gaditana* has around 50% of protein, this process (together with cell disintegration) points to be the rate-limiting step. No significant differences were found between both inocula.

Table 4. Results of the activity assays at mesophilic (35 ºC) and thermophilic (55 ºC) conditions

	Activities (gCOD/gVS·d)			
	HAc	HAcP	AAc	MAc
Mesophilic	0.074 ± 0.009	0.254 ± 0.064	5.897 ± 0.302	0.121 ± 0.009
Thermophilic	0.264 ± 0.043	0.358 ± 0.069	12.186 ± 1.132	nd [(1)]

The values of AAcs and MAcs were low when compared to the literature (Soto *et al.*, 1993; Hutñan M., 1999). This can be caused due to an insufficient reaction period. However, results showed that significant differences between the AAcs exist. Also, they suggest that acidogenesis and methanogenesis are not the rate-limiting steps for AD. In order to compare the hydrolytic performance of mesophilic and thermophilic conditions, analyzing the activities of the exo-enzymes responsible for protein and carbohydrate hydrolysis is of great interest. The activities of extracellular lipase and protease were measured according to Kim et al. (Kim *et al.*, 2012). The problem found was that in the supernatant from the reactors containing the enzymes, too much product from enzymatic degradation was present (tyrosine and maltose for protease and amylase activities, respectively). Because of that, the amount of products formed during the experiments was not significant and it was not possible to obtain representative values of the enzymatic activities. However, by measuring directly the amount of enzymatic products in the reactors, it is possible to have an idea of the hydrolysis performance. Therefore, the tyrosine concentrations in the supernatant of the bioreactors were measured. For the mesophilic bioreactor, concentrations of 1.128 ± 0.141 $\mu Moles \cdot mL^{-1}$ were found, while for the thermophilic system, significantly higher concentrations of 5.694 ± 0.266 $\mu Moles \cdot mL^{-1}$ were obtained. The obtained results suggest that the protein hydrolysis occurs at a higher rate under thermophilic conditions.

5.3.3.4 Qualitative analysis of microalgae degradation during anaerobic digestion

Samples taken from the reactors after more than 100 days of operation were studied using SEM and FC. The objective was to determine qualitatively if differences on the degradation of microalgae existed between thermophilic and mesophilic conditions. Comparing the pictures shown in Figure 9, it can be clearly observed that, while in the mesophilic sludge a great number of intact microalgal cells can be found, in the thermophilic sludge very few of them are visible. This fact clearly points towards a more exhaustive cell disintegration under thermophilic conditions. The graphs presented in Figure 10 shows great differences between the event distribution of mesophilic and thermophilic sludge. The plots in Figure 10A show that the thermophilic sludge presents a much broader range of event size when compared to the mesophilic sample. More complex events of a smaller size exist in the thermophilic sample, suggesting a more pronounced microalgae lysis. Besides, it can be observed in Figure 10B that a greater number of events maintain red chlorophyll fluorescence in the mesophilic image. That indicates that more non-lysed cells are present in the mesophilic sample. Thus, the results obtained with SEM and FC support each other, clearly pointing towards a more effective cell destruction under thermophilic conditions.

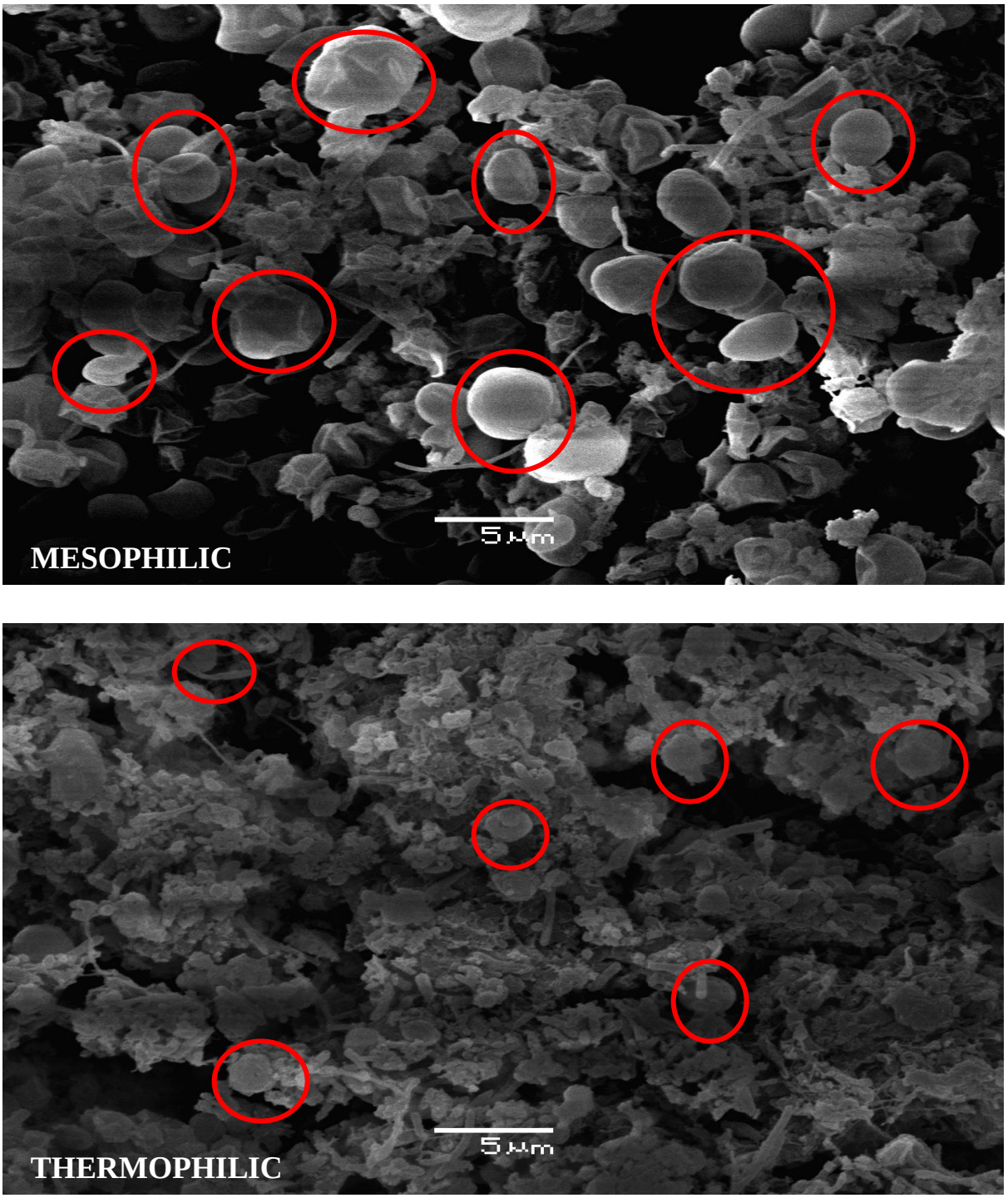

Figure 9. SEM images of mesophilic (35 ºC) and thermophilic (55 ºC) sludge samples taken from the reactors after 107 days of operation. *N. gaditana* cells are circled in red.

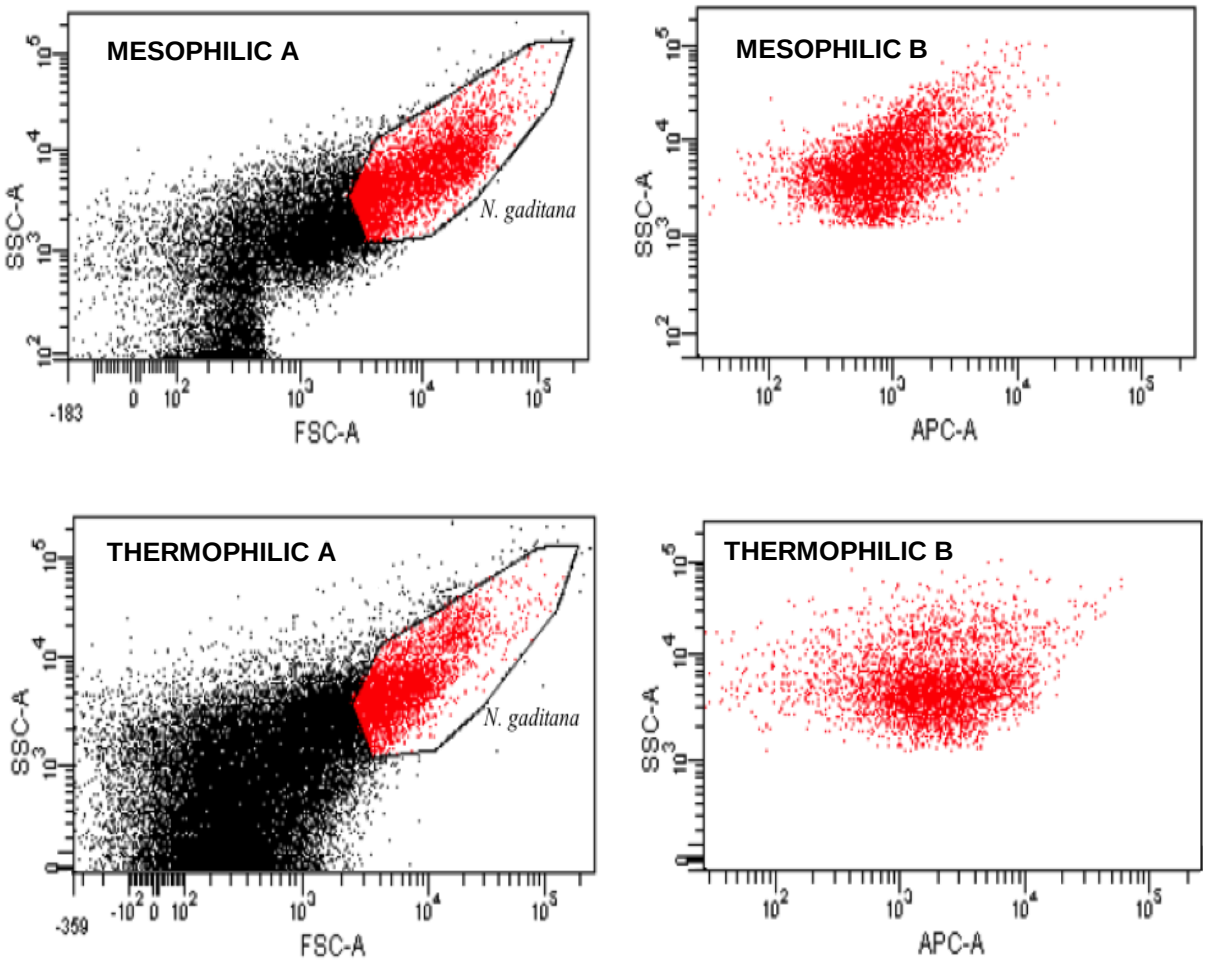

Figure 10. Flow cytometry results corresponding to mesophilic (35 ºC) and thermophilic (55 ºC) sludge samples taken from the reactors after 105 days of operation. The dot-plots distinguish the microalgal populations (gated regions) from bacteria according to their size (FSC), granularity (SSC) and chlorophyll content (APC)

5.3.3.5 Nutrient recover through membrane filtration

In order to recovery nutrients from anaerobic digestion, membrane filtration system was coupled to anaerobic mesophilic reactor at day 127 and was operated by 30 days. The membrane performance was evaluated in Figure 11 where permeability membrane was measured. Initial permeability was measured filtering water, which represents the maximal permeability of system. As expected, permeability decreases as consequence of initial and

typical membrane fouling maintaining a permeability value close to 0.3 L/m^{2}·h·mbar at constant flux value of 10 L/m^{2}·h.

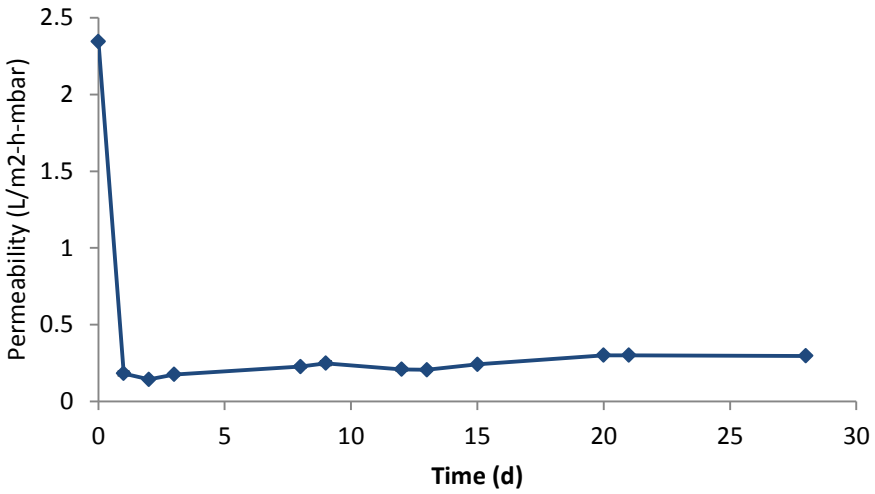

Figure 11. Permeability for membrane filtration system coupled to anaerobic mesophilic reactor.

In Figure 12 result of critical flux determination was showed indicating that as flux was increased, a no constant TMP value was observed. The critical flux computed from result in Figure 12 was 17 L/m^{2}·h. In practical terms, critical flux indicates that membrane can be operated at values under critical flux no suffering membrane fouling, and that, on the contrary, fouling is observed when membrane is operated over critical flux value. Therefore, permeability decrease was not observed as consequence of operating at values under critical flux.

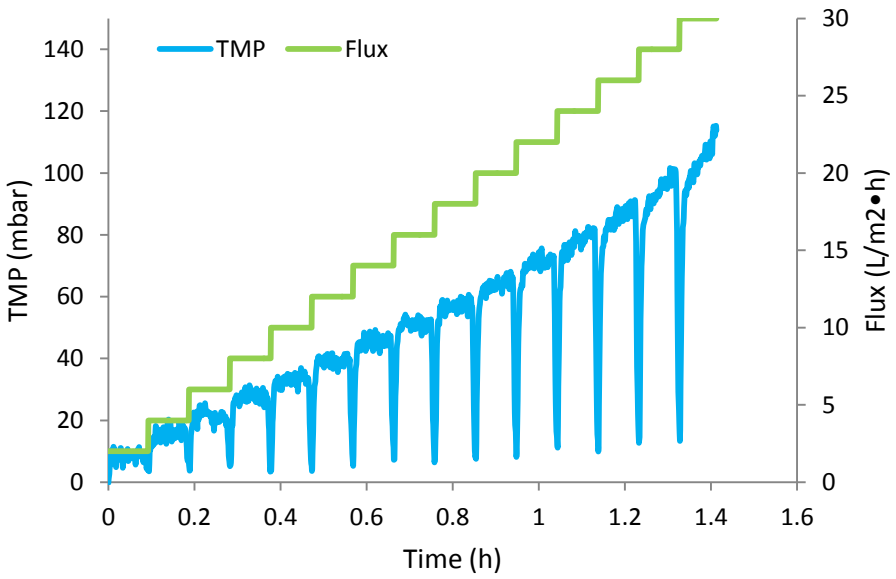

Figure 12. Critical flux determination for membrane filtration system coupled to anaerobic mesophilic reactor.

In order to evaluate nutrient recovery in membrane filtration, the concentration of soluble COD, proteins, carbohydrates, TAN, phosphate, NO^{3-} and VFA was measured in sample of both anaerobic sludge and membrane permeate. As already indicated in materials and methods section, anaerobic sludge was filtered at 0.45 µm and membrane permeate was the product of filtration at 30nm (membrane pore size). Figure 13A shows that for all components evaluated lower concentrations in permeate were found. As already showed in Figures 7 and 8A, high concentrations of soluble COD and TAN were observed, which was associated with SMPs and ammonium reduced as result of protein degradation, respectively. The nitrate and phosphate were found in concentration lower than TAN, reaching values over 200 mg/L. Figure 13B shows the rejection percentage, where the VFAs presented the lowest reject, indicating that almost all VFA in reactor cross the membrane. On the contrary, carbohydrates and proteins reached the highest rejects, being 34 and 42%, respectively. In other words, 66 and 58% of carbohydrates and proteins are able to cross membrane. This result can be explained based on both molecular weight of protein/carbohydrates and molecular weight cut-off of ultrafiltration (≥ 20kDa). In this sense, average molecular weight of microalgae protein has been reported 10 -60kDa

(Schwenzfeier *et al.*, 2011; Ursu *et al.*, 2014), even with protein fractions under 670kDa (Ursu *et al.*, 2014). Also, soluble protein in range of 10 -50 kDa has been reported for oil-extracted *Nannochloropsis spp.(Gerde et al., 2013)*. In the same way, it is concluded that rejected carbohydrate fraction in membrane filtration have a molecular weight higher than 20kDa. These result confirmed hydrolysis limiting step for protein and carbohydrates, so that 42 and 34% of concentrations, respectively, have high molecular weight unlike hydrolysis products which have low molecular weight such as amino-acids in general (75 - 200Da), tyrosine (181Da), glucose (180Da), etc.

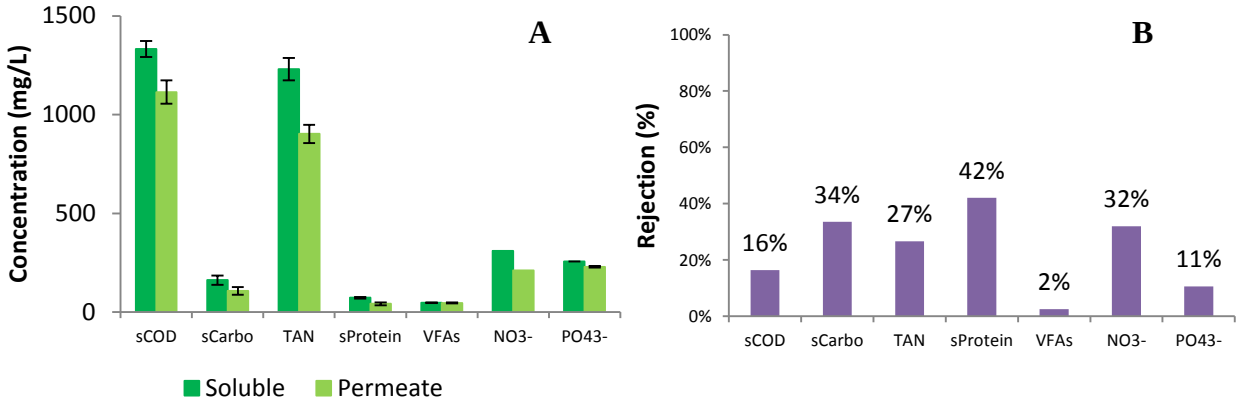

Figure 13. Soluble and permeate concentrations (A) and Rejection (B) for membrane filtration in anaerobic mesophilic reactor degrading spent *N.gaditana*.

It is worthy notice that although TAN and nitrate concentration have molecular weight much lower than ultra-filtration membranes (18 and 62Da, respectively *versus* ≥ 20kDa), reject values were not insignificant, reaching values of 27 and 32%. No explanation for this behavior has been found, so that whether it is result of salt formation (such as struvite), it salt has a molecular weight close to 0.8kDa, which in soluble form will cross membrane. Anyway, if nitrogen conversion efficiency (88% - chapter 3) and membrane reject for nitrogen (27%) are considered, a global nitrogen recovery efficiency can be computed, which reached to 55%, i.e. that 55% of nitrogen fed into reactor was recover in permeate fraction, which equals to 46.6kg N/Ton microalgae. It is worthy to notice that this value for continuous reactor can be less that computed so that nitrogen conversion of batch test was taken into account.

Thus, through membrane filtration coupled to anaerobic digestion is possible for the recovery of nutrients (ammonium, nitrate and phosphate) plus organic matter (SMPs, VFA, carbohydrates and proteins), which can be recycled to microalgae culture. Moreover, although reactors in this research were not inhibited by ammonium, membrane filtration coupled to anaerobic reactors will allow the decrease of ammonium concentration maintaining it at non inhibitory values. In relation to membrane permeate as source of nutrients for microalgae culture, there are some aspects that should be taken into account:

- The presence of organic matter in permeate fraction may benefit bacterial contamination into microalgae culture, which will hinder process such as microalgae cultivation for specific compounds (food, pharmaceutical, pigments) or biodiesel production from microalgae (decrease in oil yield).
- The presence of organic matter can benefit mixotrophic growth. In this sense, glucose from carbohydrate and acetate from VFA present in permeate will act as organic sources of carbon for microalgae growth (Mata *et al.*, 2010; Yen Chen *et al.*, 2011; Girard *et al.*, 2014)
- Although in permeate fraction the sodium concentration was not measured, it is expected that this ions was not rejected, its concentration being close to 1.7g/L (Table 3). Obviously, presence of sodium in permeate fraction was associated to microalgae used in this research (seawater microalgae).
- Should be considered that total ammonium in permeate fraction can be not available for microalgae growth due to ammonia stripping, which is dependent on pH and it effect is significant at pH values over 8. In this sense, pH increase is a common behavior caused by strong photosynthetic activity, which consumed dioxide carbon increasing pH value (Molinuevo-Salces *et al.*, 2010). Moreover, Nitrate concentration present in permeate fraction offer other nitrogen source for microalgae, which may be the most important source when stripping occurs.

Finally, it was evaluated the energetic requirements of membrane filtration process and whether energy produced in anaerobic reactor through biogas production is able to supply membrane requirements. These values were computed considering the operation of a 1000m^3 anaerobic reactor. Methodology for both biogas production and membrane

requirements were based on used in chapter 3 and 4, respectively. Considering a OLR of 0.5g/L·d and a biogas production of 50% obtained in chapter 5 for lab-scale anaerobic reactor, a methane production of 95 m^3/d was obtained. In the same way that in chapter 3, electricity generation through co-generation was considered, obtaining an electrical energy production of 376 kWh/d. The energy requirements for a membrane filtration system composed by a module containing 2 units (X-flow Norit - Compact 33) was computed considering critical flux obtained in this research (17 L/m^2·h) and permeate ratio (0.02m^3 permeate/m^3 reactor). Results obtained was 33 kWh/d, which corresponds to 9% of electrical energy generated. Thus, biogas production in mesophilic reactor is able to supplying energetic requirements of membrane filtration system.

5.4 Conclusions

- Lipid extraction process acts as pre-treatment enhancing biodegradability.
- Pre-adaptation of inoculum plays an important role in BMP performance.
- Hydrolysis is a limiting step in BMP test for total microalgae.

CHAPTER VI.
General discussion, concluding remarks and future directions

6.1 General Discussion

To date, research on anaerobic digestion of microalgae has been mostly reported as BMP tests and reports of continuous lab-scale anaerobic reactor is scarce. In this sense, results showed in this thesis indicate a high biogas production in BMP tests but a lower biogas production was found when continuous anaerobic reactor was operated. Moreover, some report that have been addressed in energy calculations considers biogas production values taken from BMP tests, which leads to overestimated energetic calculations.

The effect of oil-extraction on biogas production has not been well reported and explained. Some reports indicates that oil-extraction may act as pre-treatmeant improving methane production. Although results in this thesis indicates the positive effect of oil-extraction on methane production, a different conclusion may be obtained so that the effect of oil-extraction is associated with the type of solvents and others factors as oil-extraction parameters such as type of system, extraction time, temperature, ratio solvent/microalgae, etc. Moreover, the choice of solvent must be taken considering both oil- extraction efficiency and inhibitory effect on anaerobic consortia, which determines the anaerobic reactor performance.

As a collateral issue, the biogas energetic production computed in chapter III reopen the debate about what type of energy to be produced, so that in this research energetic recovery for biodiesel was only 14%, unlike biogas which reached 44-47%. In this way, Sialve *et al.* (2009) computed that ,from an energetic point of view, biodiesel production make sense when lipid concentration is $\geq$ 30-40% and conversely, biogas production should be produced for lower lipid concentrations. Moreover, an important parameter not taken into account is methyl-able fraction of neutral lipids, which indicates the trans-esterificable lipid fraction.

According to energetic calculations reported in the last years, it is clear that harvesting is the most energetic demanding process in microalgae refinery. Results in this thesis showed that membrane technology can be applied but it energetic requirements was highly influenced by initial concentration due to operation with diluted cultures ($\leq$ 0.5 g TS/L). Moreover, cake formation and membrane fouling are factors determining membrane

performance and influenced by particle size. In this sense, as already commented in chapter IV, reports have determined that a poor membrane performance is observed by the presence of a particle fraction ≤ 1 μm. This fact is very important if open raceways pond for biofuel production are considered, where bacterial contamination is unavoidable. Thus, feasibility of membrane technology as harvesting process may be compromised at industrial scale.

In relation to thermophilic anaerobic digestion, it has been reported as process enhancing organic matter degradation, and thus methane production compared with mesophilic operation. The key factor in this process is temperature, which favours kinetic reactions, specially hydrolysis step. On the contrary, a drawback for thermophilic operation is energy necessary for maintaining temperatures ranging in 50 - 60° C. In this sense, calculations computed in chapter III showed that close to 1% of thermal energy produced by biogas co-generation is necessary in order to supply heating in anaerobic reactor and considering thermophilic operation, this value is lower than 5%, indicating that from an energetic point of view thermophilic operation is feasible.

The thermophilic reactor evaluated in this thesis did not show an increase in methane production as a result of a improved hydrolysis step. In this sense, results in this thesis showed that protein and carbohydrates hydrolysis are limiting step. It should be noticed that this result is very important whether protein content in microalgae is considered, which can easily reaches values close to 50%.

An important factor to consider is sludge acclimation, which needs long times. In this thesis no differences in biogas production were observed after that two months of sludge adaptation. This fact emphasized that long continuous operation in anaerobic reactor must be performed in order to adapt anaerobic consortia to substrate.

Finally, membrane operation coupled to anaerobic digestion showed to be a feasible way in order to recover nutrients, that can be recycled to microalgae culture which will reduce nutrients cost and hence, improving feasibility of microalgae refinery. Moreover, it should be taken into account that cultivation of seawater microalgae may cause potential problems such as salt inhibition in anaerobic reactor and inorganic fouling in membrane.

6.2 Concluding remarks

Chapter III:

- Biogas production through anaerobic digestion recovered about 44 and 47% of energy contained in total microalgae *B. braunii* and *N. gaditana*, respectively. This result proved that spent microalgae can be considered as source of energy for biogas production. In this sense, result in this thesis showed that biogas production from spent microalgae can supply about 50 and 75-80% of electrical and thermal energy requirements, respectively, in a global microalgae refinery concept for producing biodiesel and biogas. Thus, it is clear that anaerobic degradation of spent microalgae can improve global energetic yield of biodiesel production.

Chapter IV:

- Energetic requirements of membrane system as harvesting process are strongly influenced by biomass concentration at low concentration (<10 g VS/L) which is due to that high quantities of media must be filtered, increasing pumping time and hence, consumed energy. At high concentration (10 - 50 g VS/L), cross-flow velocity is the predominant factor affecting flux and hence, energy requirements. Thus, membrane filtration is proposed as a post-concentring process which can be preceded by low energy demanding process such as flotation, flocculation/ coagulation.
- The membrane performance was affected by both cake formation as fouling. Result in this thesis indicates that presence of bacterial contamination is responsible of membrane performance.

Chapter V:

- Mesophilic and thermophilic anaerobic reactor showed a similar biogas production and performance, which was contrary to expected. Results showed that although a high celular lysis was observed in thermophilic reactor, biogas production was not increased. Moreover, hydrolysis step in anaerobic consortia was found as limiting step, determining reactor performance.

- Membrane filtration system coupled to anaerobic digestion is a feasible technology in order to recover nutrients from anaerobic reactor, which can be recycled to microalgae culture. Permeate fraction rich in ammonium, nitrate and phosphate also contains an important fraction of organic matter, which can hinder microalgae culture due to organic matter can supporting bacterial contamination.

6.3 Future directions outlined from this thesis

Undoubtedly, performance of anaerobic reactor under long-term continuous operation must be evaluated considering potential inhibitors related with microalgae culture (salt inhibition) and solvent for lipid extraction. In this sense, it is important to paid attention on salt and ammonium inhibition which can become important parameters in operation of anaerobic reactor treating seawater microalgae.

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