THE POTENTIAL OF SEWAGE SLUDGE AND MICROALGAE: "GREEN ENERGY" PRODUCTION AND ENVIRONMENT BENEFITS

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MICROALGAE CONVERSION INTO BIOFUELS: ENERGY PRODUCTION AND NUTRIENTS RECYCLING

DOCTORAL THESIS

Department of Chemical Engineering

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DEPARTMENT OF CHEMICAL ENGINEERING

CREPI RESEARCH GROUP

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I, Dr. Christophe Bengoa, associate professor in the Department of Chemical Engineering of the Rovira i Virgili University,

CERTIFY:

That the present study, entitled "MICROALGAE CONVERSION INTO BIOFUELS: ENERGY PRODUCTION AND NUTRIENTS RECYCLING", presented by Martín Pablo Caporgno for the award of the degree of Doctor, has been carried out under my supervision at the Department of Chemical Engineering of this university, and that it fulfils all the requirements to be eligible for the International Doctorate Label.

Tarragona, 14th February 2016

Dr. Christophe Bengoa

"Life is not easy for any of us. But what of that? We must have perseverance and above all confidence in ourselves. We must believe that we are gifted for something, and that this thing, at whatever cost, must be attained."

"La vida no es fácil para ninguno de nosotros. ¿y qué? Tenemos que ser perseverantes y, sobre todo, tener confianza en nosotros mismos. Debemos creer que estamos dotados para lograr algo, y que ésto, a cualquier costo, debe alcanzarse."

MARIA SKŁODOWASKA-CURIE

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Summary

Modern society consumes large amounts of energy due to the world population growth and industrialisation. Both energy production and consumption are economically, environmentally and socially unsustainable nowadays, thus require changes in the production processes. The depletion of the fossil-fuel reserves is an additional problem. The biofuels production from renewable feedstocks, such as the biodiesel production from edible oil seeds, caused a dilemma by shifted out the land from food to energy production. In this context, microalgae have arisen as low-cost and non-edible feedstocks for biofuels production.

This thesis investigates different scenarios to produce biofuels from microalgae. *Isochrysis galbana, Selenastrum capricornutum, Phaeodactylum tricornutum, Chlorella vulgaris, Chlorella kessleri, Nannochloropsis oculata* and *Nannochloropsis oceanica* are microalgae species used in the experiments. The biomass was mainly converted into two different biofuels: methane, produced by anaerobic digestion (AD), and bio-oil, produced by hydrothermal liquefaction (HTL).

The anaerobic digestion process is a widely known technology already available on large-scale; it has been used for a long time to digest sewage sludge in wastewater treatment plants (WWTPs), reducing the operating costs in these facilities. One of the main advantages of AD is the possibility to process slurries containing more than 95% water. Microalgae are characterised by high water content even after concentration, and the elimination of water is a high-energy consuming process. Furthermore, fractions of lipid, protein, and carbohydrate in microalgae are converted into methane.

The experiments described in this manuscript show that the methane production strongly depends on the species. The methane is affected by some characteristics of the microalgae such as the biochemical composition, which in turn, is affected by the cultivation conditions amongst other factors.

The experiments also evidence one of the problems associated with the high protein content in microalgae: the inhibition by ammonia. The evaluation of different amounts of substrate loaded into the reactors demonstrates that inhibition can occur at high loads. The evaluation of the temperature for digestion indicates that mesophilic conditions reduce the probability of inhibition by ammonia compared to thermophilic conditions.

The co-digestion of microalgae and sewage sludge, aimed to prevent the detrimental effects of ammonia and to increase the production of methane, reveals interesting results. Although the sewage sludge addition did not increase the methane production, the lack of inhibitory effects suggests the possibility of digesting both substrates together. The main advantage of co-digestion lies on the possibility of coupling the microalgae cultivation unit to wastewater treatment facility, recycling nutrient for microalgae cultivation and taking advantage of the oversized digesters in some WWTPs.

Another problem associated with microalgae digestion is the high resistance of the biomass to the microorganism attack. The effectiveness of the pre-treatments to increase the degradability depends on the microalgae characteristics, and there is not a direct relation between the effects of the pre-treatments on the microalgae and the methane production. For example, the ultrasonic pre-treatments can completely destroy the structures of some microalgae species and hardly increase the methane production. A novel pre-treatment using N-methylmorpholine-

N-oxide (NMMO) shows interesting results with some microalgae species, but further research is required in order to recover the NMMO after using it. The lipid extraction can act as pre-treatment for AD, and the lipids can be further converted into biodiesel. The result reveals that microalgae characteristics and type of solvent are some of the factors which affect these results.

Although biodiesel from microalgae still catches the attention of researchers, this type of biofuel is not fully investigated in this thesis. As mentioned before, the lipid-extraction process was performed as pre-treatment to improve AD. However, different scenarios were evaluated to recover energy from *Nannochloropsis* waste by AD. This thesis also includes a review of the most recent papers about AD of lipid-extracted waste.

The bio-oil is another promising biofuel from microalgae. The HTL can also process wet biomass inasmuch as the reactions take place in the aqueous medium; for this reason, it is here investigated. The temperature is a very influential parameter in HTL, which affects the yields and the composition of the different fractions obtained. The influence of this parameter is evaluated, processing *Nannochloropsis* slurry containing approximately 75% water.

Some of the disadvantages of the bio-oil from microalgae are the high viscosity and the high oxygen content, requiring additional processing to improve its quality. It has been reported that processing microalgae with organic solvents like ethanol or methanol can lead to better bio-oil quality, but this option requires the elimination of water. The addition of alcohols as co-solvents, which means processing microalgae in mixtures of water and alcohols, is studied as way to improve the bio-oil yields and quality.

Additionally, another advantage of HTL is the possibility of converting all microalgae fractions into bio-oil. The HTL of *Nannochloropsis* waste is evaluated, but the main objective of these experiments was to demonstrate that the aqueous phase after HTL is useful for microalgae cultivation.

Resúmen

La sociedad actual consume grandes cantidades de energía debido al crecimiento de la población mundial y la industrialización. Tanto la producción como el consumo de energía son económicamente, ambientalmente y socialmente insostenible hoy en día, por lo que se requieren cambios en los procesos de producción. El agotamiento de las reservas de combustibles fósiles es un problema adicional. La producción de biocombustibles a partir de materias primas renovables, tales como la producción de biodiesel a partir de oleaginosas que también son utilizadas como materias primas en la industria alimentaria, causa un dilema al desplazar tierras utilizadas para la producción de alimentos hacia la producción de energía. En este contexto, las microalgas han surgido como materia prima para la producción de biocombustibles, siendo de bajo costo y no comestibles.

En esta tesis se investigan diferentes escenarios para producir biocombustibles a partir de microalgas. *Isochrysis galbana, Selenastrum capricornutum, Phaeodactylum tricornutum, Chlorella vulgaris, Chlorella kessleri, Nannochloropsis oculata* y *Nannochloropsis oceanica* son las especies de microalgas utilizadas en los experimentos. La biomasa se convirtió principalmente en dos biocombustibles diferentes: metano, producido por la digestión anaeróbica (DA), y bio-aceite, producido por hidrolicuefacción térmica (HLT).

El proceso de digestión anaerobia es una tecnología ampliamente conocida y disponible a gran escala; se ha utilizado durante mucho tiempo para digerir lodos biológicos en las estaciones depuradoras de aguas residuales (EDAR), reduciendo sus costos de operación. Una de las principales ventajas de la DA es la posibilidad de procesar materias primas que contienen más de un 95% de agua. Las microalgas se caracterizan por un alto contenido de agua incluso después de la concentración, y su eliminación es un proceso de alto consumo energético. Además, la DA permite que tanto las fracciones de lípidos, proteínas como carbohidratos en las microalgas se conviertan en bio-combustible.

Los experimentos descritos en este manuscrito demuestran que la producción de metano depende en gran medida de la especie. El metano se ve afectado por algunas características de las microalgas tales como la composición bioquímica, que a su vez, se ve afectada por las condiciones de cultivo entre otros factores.

Los experimentos también ponen de manifiesto uno de los problemas asociados con el alto contenido de proteína en microalgas: la inhibición causada por la producción de amoniaco. La evaluación de diferentes cantidades de biomasa alimentada en los reactores demuestra que la inhibición puede producirse a elevadas cargas. La evaluación de la temperatura de digestión indica que en condiciones mesofílicas, la probabilidad de la inhibición por amoníaco se reduce en comparación con las condiciones termófilas.

La co-digestión de microalgas y de lodos de depuradora se ha evaluado con el objetivo de prevenir los efectos perjudiciales de amoníaco y para aumentar la producción de metano. Aunque la adición de lodos de depuradora no aumenta la producción de metano, la falta de efectos inhibitorios sugiere que es posible digerir ambos sustratos juntos. La principal ventaja de la co-digestión radica en la posibilidad de acoplar la unidad de cultivo de microalgas a las instalaciones de tratamiento de aguas residuales, reciclando nutrientes para el cultivo de microalgas y aprovechando el sobredimensionamiento de los digestores en algunas EDAR.

Otro problema asociado con la DA de microalgas es su elevada resistencia al ataque microbiano. La eficacia de los pre-tratamientos para aumentar la degradabilidad de las microalgas depende de las características de las mismas, y no hay una relación directa entre los efectos de los pre-tratamientos sobre la biomasa y la producción de metano. Por ejemplo, el pre-tratamientos con ultrasonidos puede destruir completamente algunas especies de microalgas y apenas aumentar la producción de metano. Un novedoso pre-tratamiento con N-methylmorpholine-N-oxide (NMMO) muestra resultados interesantes con algunas especies de microalgas, pero aún se requiere mayor investigación a fin de recuperar el NMMO después de su uso. La extracción de los lípidos de las microalgas puede actuar también como pre-tratamiento para la DA, a la vez que los lípidos pueden convertirse en biodiesel. El resultado revela que las características de microalgas y tipo de disolvente son algunos de los factores que afectan estos resultados.

A pesar que el biodiesel de microalgas sigue captando la atención de los investigadores, la producción de este biocombustible no se ha investigado con detalle en esta tesis. Como se ha mencionado anteriormente, la extracción de lípidos se ha realizado solo como un pre-tratamiento para mejorar la AD. Sin embargo, se han evaluado diferentes alternativas para recuperar energía a partir de los residuos *Nannochloropsis* mediante DA. Debido al interés en el tema, esta tesis también incluye un capítulo con una revisión (review) de los trabajos publicados más recientes sobre DA de microalgas recuperadas después de la extracción de sus lípidos.

El bio-aceite es otro tipo de biocombustible prometedor. La hidrolicuefacción térmica también puede procesar biomasa húmeda debido a que las reacciones ocurren en medio acuoso; y por ésta razón fue investigada. La temperatura es un parámetro muy influyente en el proceso, que afecta tanto los rendimientos como la composición de las diferentes fracciones obtenidas. Se evaluó la influencia de este parámetro, utilizando como biomasa *Nannochloropsis* con un contenido de aproximadamente 75% de agua.

Algunas de las desventajas del bio-aceite producido a partir de HLT de microalgas son su alta viscosidad y elevado contenido de oxígeno, que requieren un refinado adicional para mejorar su calidad. El procesamiento de microalgas con disolventes orgánicos, como etanol o metanol, puede conducir a una mejora de la calidad del bio-aceite, pero esta opción usualmente exige la eliminación del agua presente en las microalgas. La adición de alcoholes como co-disolventes, lo que significa procesar microalgas en mezclas de agua y alcoholes, se estudió como una forma de mejorar el proceso.

Finalmente, la HLT presenta otra ventaja, la posibilidad de convertir todas las fracciones de microalgas en bio-aceite. La HLT de residuos de *Nannochloropsis* se ha evaluado pero con el principal objetivo de utilizar la fase acuosa generada en el proceso para el cultivo de microalgas.

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Chapter 1

Introduction

1. The biofuels and world's energy scenario

Biofuels are usually referred to liquid or gaseous fuels, obtained by processing biomass. They are commonly classified into first, second and third generation biofuels [1]. The first generation biofuels are produced from food crops such as into biodiesel or ethanol from sugar cane, corn or wheat. The second generation biofuels utilise non-food crops and residues, or crops cultivated in lands unsuitable for food production; ethanol from cellulosic material fits in the category. Finally, the third generation biofuels denote the microalgae-based biofuels. Microalgae were previously considered as second generation biofuels due to their use of non-arable land, but they were assigned to a new category based on their capability of higher yields with lower resources. Regarding microalgae utilisation for biofuels, microalgae can be cultivated for biofuels production or the biofuels can be produced from the microalgae waste generated after recovering high-valuable compounds from microalgae. Biofuels have been recently classified taking into account the technologies used for their production, which distinguish between conventional and advanced technologies [1]. The conventional technologies are well-established processes and available on a commercial scale; this definition includes the first generation biofuels. The advanced technologies are still in the research and development stage or in demonstration stage. Table 1 summarises some of the biofuels and the current status of their production.

Table 1. Types of biofuels and current status of their production.

	Advanced biofuels			**Conventional biofuels**
	R&D	*Demonstration*	*Early commercial*	*Commercial*
Bio-ethanol		Cellulosic ethanol		Ethanol from sugar and starch crops
Diesel-type biofuels	Biodiesel from microalgae; Sugar-based hydrocarbons	BtL-diesel (gasification/ FT)	Hydrotreated vegetable oil	Biodiesel (by transesterification)
Other fuels and additives	Novel fuels	Bio-butanol; DME; Pyrolysis-based fuels	Methanol	
Biomethane	Biogas from microalgae	Bio-SG		Biogas (anaerobic digestion)
Hydrogen	Novel routes	Gasification with reforming	Biogas reforming	

Liquid biofuels Gaseous biofuels

BtL: Biomass to liquid. FT: Fisher-Tropsch DME: Dimethylether Bio-SG: Biosynthetic gas

The early ideas about biofuels production started in the late 19th century, when the Rudolf Diesel's engine was run on peanut oil [2]. However, the development of biofuels has been always influenced by the fossil fuel prices; the biofuels become unviable for transport fuels when the petrol prices decrease. In the 1970s, the production of bio-ethanol started and the commercial production of biofuels came up. The bio-ethanol production promoted the biodiesel production, which was also helped by the government policies in some countries [3].

Modern society consumes large amounts of energy. Both world population growth and industrialisation will strongly affect the energy consumption in the future. According to a report published by the U.S. Energy Information Administration (EIA), the consumption of liquid fuels is predicted to increases from 87 MMbbl/d consumed in 2010 to 98 MMbbl/d in 2020 and 119 MMbbl/d in 2040. Transports and industries will account around 92% of the global liquid fuels demand in 2040 [4]. The fossil fuel reserves depletion is a serious problem; petroleum, natural gas, and coal reserves depletion is predicted for the next 45, 60, and 120 years respectively [5].

As a consequence, the cost competitiveness of other fuels will increase, and some sectors will switch to other sources of energy if possible.

Currently, biofuels provide only around 2% of total transport fuel. In spite of this low percentage, biofuels shows the potential for growing over the coming decades; this percentage may increase to 27% in 2050 [1]. Energy production and consumption is economically, environmentally and socially unsustainable nowadays, requiring changes in the society. Figure 1 depicts some of the environmental, social and economic aspects related to biofuels [1].

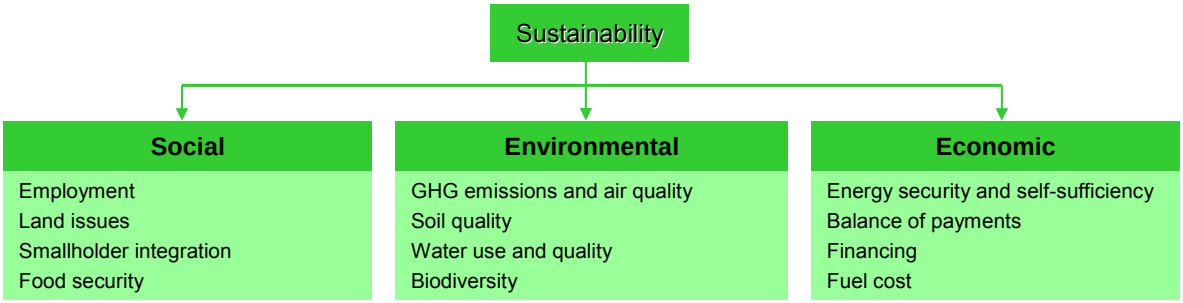

Figure 1. Environmental, social and economic aspects of biofuels production.

The policies at national and international level should establish criteria and standards to prevent or limit negative impacts from biofuel production. The negative impacts can be minimised or avoided through careful management, choice and design.

2. Microalgae

2.1. About algae

Microalgae are photosynthetic microorganisms; this means that they synthesise and accumulate large quantities of energetic organic compounds from CO_2 by using sunlight. They also need some inorganic nutrients like nitrogen, phosphorus and some species, silicon. The energy accumulated as sugars, is then converted to all the lipids, carbohydrates, and proteins necessary to make up the biomass. Since they are unicellular organisms, they do not invest in supporting structures such as roots, stems and leaves in terrestrial plants. Because algae do not consume energy for the development of supporting structures, the solar to biomass conversion yield is expected to be higher than in terrestrial plants [6,7].

Concerns about the fossil fuel dependence and the problems derived from this dependence have promoted the conversion of renewable feedstocks into biofuels. Microalgae arose as viable alternative due to several competitive advantages over the traditional feedstocks currently used [6,8-10]. Some of the advantages had been widely discussed:

- rapid growth and efficiently solar energy conversion,
- production not limited to a specific harvest period
- production in non-productive or non-arable land,
- no competition with human food supply,

- utilisation of salt water or wastewaters unsuitable for agriculture,
- contribution to mitigate the release of Green House Gas (GHG) into the atmosphere by using CO_2 from industrial process, and
- production of valuable co-products additionally to the biofuels.

Unfortunately, as observed in Table 1, the biofuels production from microalgae is still in R&D or pilot stage. There is still some distance away from maturity for microalgae conversion into biofuels, and the industrial-scale production does not currently exist [6,8-10]. The petroleum-based fuels still cover most of the market demand, thus biofuels should be competitive or cost less than petroleum fuels. In case of first generation biofuels such as biodiesel, the competition with petroleum fuels is possible thanks to government subsidies. Other limitations to overcome for industrial-scale production are directly related to both the microalgae and the biofuels production processes. Several efforts are being made to find microalgae species with high oil content and rapid growth rate, to innovate and improve oil extraction and harvesting methods, to explore the co-production of valuable fractions.

The bio-refinery concept contemplates microalgae as sources of chemicals, cosmetics and health products, and animal and human feed, additionally to their energetic value [11-13]. This new concept of microalgae exploitation includes both the co-production of biofuels and valuable products and the recycling of wastes for the culture of microalgae, maximising the value of the differences fraction in the biomass. The reduction of the costs is essential in a bio-refinery, and this can be achieved by reducing the costs associated to microalgae cultivation. Some processes for biofuels production generates by-products such as nutrients and CO_2 which can be potentially recycled for microalgae cultivation [13-17].

2.2. Production of microalgae

Microalgae production can be photoautotrophic, heterotrophic and mixotrophic [6,8-10]. The photoautotrophic production converts solar energy and CO_2 into biomass, and currently, it is the only method which is technically and economically feasible for large-scale production of algae biomass for non-energy production. From the thousands of known microalgae species, only a handful are currently available at large-scale. These species are mainly cultivated for the production of high-valuable compounds such as pigments or proteins, and some other for aquaculture. The reactors used for cultivation are open ponds and closed photobioreactor. The raceway ponds are the most commonly used; they are typically made of a closed loop, oval shaped recirculation channels between 0.2 and 0.5 m deep, and equipped with a paddlewheel in continuous operation to mix and prevent sedimentation. The CO_2 is consumed from the surface air or provided by submerged aerators (Figure 2a). The closed photobioreactors reduce the problems associated with contamination risks, thus being used for species susceptible to contamination and for high-value products in pharmaceutical and cosmetics industries. Closed systems comprise tubular, flat plate, and column photobioreactors (Figure 2b). In the closed photobioreactors, the oxygen generated can cause inhibition. On the contrary, the lack of CO_2 can cause carbon starvation and an increase in pH. For these reasons, there is a degassing zone where the culture is bubbled with air to remove the excess oxygen and provide CO_2. Photobioreactors may require temperature control, especially for cooling during daylight hours, which makes their operation more expensive.

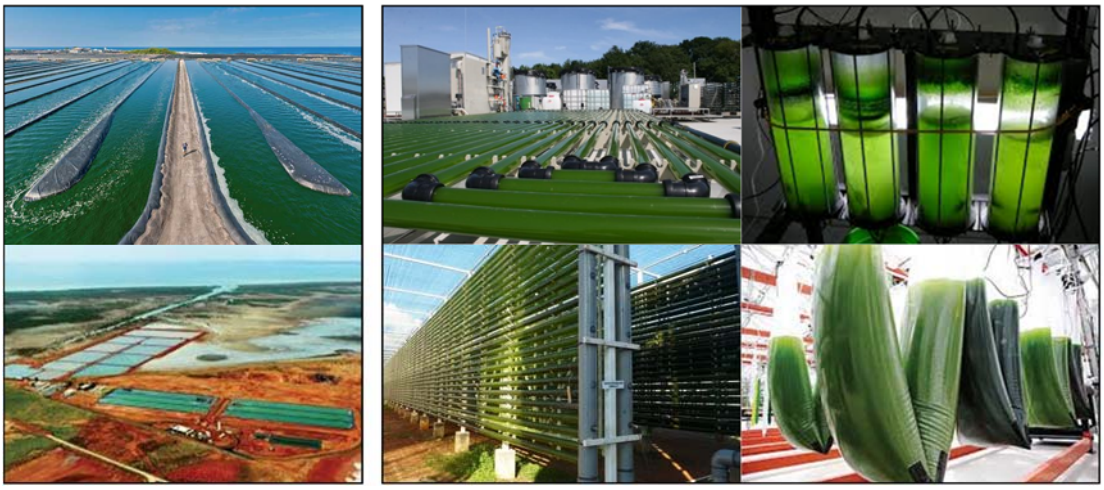

Figure 2. Microalgae production technologies at large scale; open ponds (a) and closed photobioreactors (b).

Besides phototrophic production some microalgae can be grown heterotrophically and mixotrophically. In heterotrophic production, microalgae grow in darkness using exogenous carbon sources such glucose. The reactors are usually stirred tanks; the systems provide high degree of growth control and high cell densities, reducing the harvesting costs. In mixotrophy, the light energy is not an absolutely limiting factor for growth; either light or organic carbon substrates support the growth. Growth rates of mixotrophic production are comparable to photoautotrophic production in closed photobioreactors and higher than for open pond, but are considerably lower than for heterotrophic production. However, the open ponds seem to be the most implemented growth mode for large microalgae production mainly due to their cost effectiveness; closed photobioreactors become the option for the production of high-valuable compounds [18].

3. Biofuels

Several biofuels can be produce from microalgae [6,7,19,20]. Figure 3 summarises some of the technologies studied for the conversion of microalgae into biofuels.

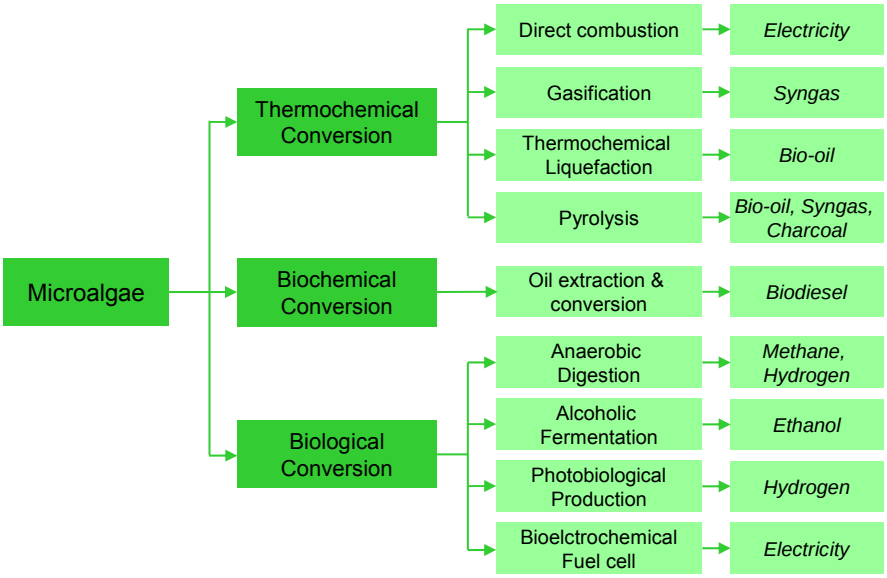

Figure 3. Different biofuels produced from microalgae.

As mentioned before, microalgae arose as potential feedstock for biodiesel production mainly derived from their possibility to accumulate more lipid than the crops currently used and their no-impact on the food markets. However, some barriers need to be overcome for scale-up the production of biofuels from microalgae:

- reduction in the cost associated to the biomass and biofuel production such as harvesting, dewatering or oil extraction for the biodiesel production,
- identification of microalgae strains with high production rates and/or oil yields,
- modification of the processes in order to have a higher net energy output, and
- integration of the algal biofuels into the fuels market.

In order to avoid a detailed explanation about the different biofuels, only the biofuels and the technologies evaluated in the present manuscript are described below: the biodiesel production by transesterification, the bio-oil by hydrothermal liquefaction and finally, the biogas by anaerobic digestion.

3.1 Transesterification: Biodiesel production

The transesterification reaction converts vegetable oils or animal fats into fatty acid methyl esters (FAMEs), using an alcohol like methanol in the presence of a catalyst. The mixture of FAMEs is known as biodiesel, a non-toxic and biodegradable biofuel which provides comparable engine performance to petroleum diesel fuel and reduces sulphur and particulate matter emissions [21].

Biodiesel is usually produced from vegetable oils by transesterification of triglycerides with methanol in the presence of basic catalyst. It can be produce from microalgae too. In microalgae, lipids are present as free fatty acids (FFAs), thus the reaction is performed with methanol, but with acid catalyst. The reactions catalysed with acid are slow, so promising catalysts are required [10]. Figure 4 shows a scheme of the reactions.

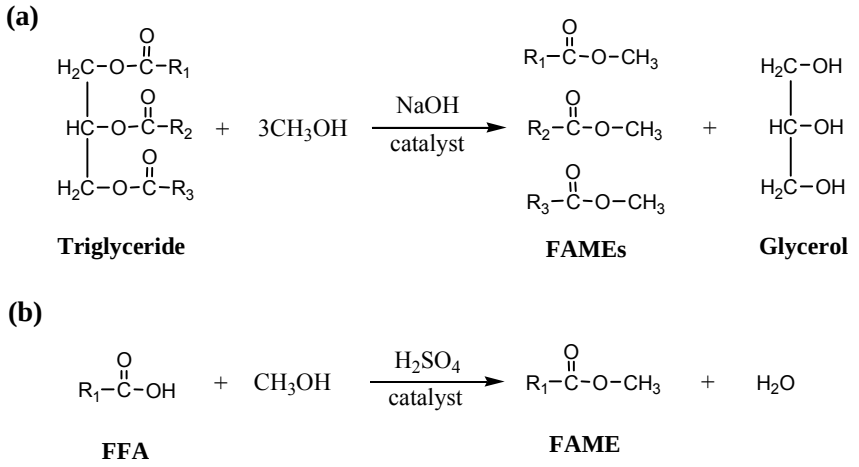

Figure 4. Transesterification of triglyceride (a) and esterification of FFA (b).

Biodiesel from microalgae could substitute the fossil fuel demand, with several advantages over the feedstocks currently used for biodiesel [22]. However, the large-scale production of biodiesel using microalgae is still economically challenging. Harvesting is still one of the major challenges in the microalgae production since it can contribute up to 30% of the total biomass production costs [23]. Large-scale process for lipid extraction is technically challenging too; the mechanical pressing equipment used for the oil extraction from biomass cause loss

of biomass and low efficiency when are used for microalgae processing. On the other hand, the extraction methods using organic solvents are efficient to extract the lipids from crops but unsuitable for microalgae. Water content is probably the main drawback; water negatively influences the lipid extraction and the subsequent conversion into biodiesel [23]. The energy required for the dewatering process can account for 85% of the total energy consumption [23]. In spite of the barriers, the biodiesel production from microalgae still gets the attention of many researchers.

Several researchers suggested that that organic solvents used for lipid extraction from microalgae could act as pre-treatment to improve AD [24,25]; in this case, biodiesel and methane production would be complementary processes. The utilisation of the microalgae waste after the lipid extraction for energy purposes could improve the energetic and economic balances; approximately 60-70% of the biomass remains as waste in the biodiesel production process [26]. Furthermore, the lipid extraction mitigates the inhibitory effect of lipid on AD [27].

3.2 Hydrothermal liquefaction: Bio-oil production

The hydrothermal liquefaction (HTL) or solvolysis is a thermochemical process which converts organic biomass into a liquid biofuel called bio-oil. Bio-oil is an energy-dense liquid, dark and viscous, produced from hydrothermal degradation of the lipids, proteins, and carbohydrates [28]; it has an energy content of 70-95% of that of petroleum and it is similar in nature to heavy crude [29].

The thermochemical processes are faster than the biochemical ones; however, they require high temperatures to produce the chemical reactions [6]. Unlike pyrolysis and gasification, HTL requires much less energy and can be carried out at relatively low temperatures. The process is carried out in a closed oxygen-free reactor pressurised using inert gases (N_2 or He) or reducing gases (H_2 or CO), temperatures between 200 ºC and 380 °C and a pressure range of 50-280 bar. The hot compressed water, at sub-/near-critical conditions, has some properties very different from water at standard conditions. The temperature increase strongly decreases the dielectric constant of water from 78.5 at ambient temperature to 13.96 at 350 ºC, becoming fairly non-polar and solubilising hydrophobic organic compounds. The ionic product increased until 10^{-12} compared to 10^{-14} at 25 ºC, thus promoting H^+ and OH^- for acid- and base-catalysed reactions such as hydrolysis [29]. A complex series of chemical reactions fragment the biomass components into small molecular weight species, then stabilised by hydrogen addition. The products can be later separated according to their boiling points, their solubility in different solvents or other properties. Besides bio-oil, other products are distributed in aqueous, gaseous, and solid phases as indicated in Figure 5.

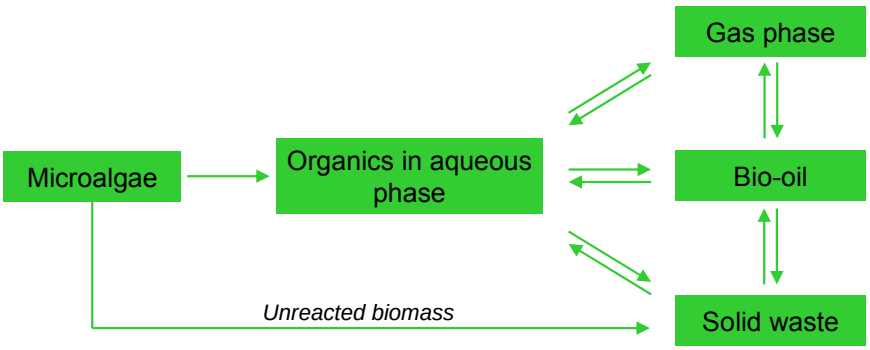

Figure 5. Scheme of microalgae HTL.

The bio-oils usually contains high amount of oxygen, partially attributed to the incorporation of water. Oxygen decreases the caloric value and the storage stability of the bio-oil. For this reason, it is interesting to reduce the oxygen content and incorporate enough hydrogen. The oxygen content is reduced by formation of CO, CO_2 or H_2O. The formation of water implies hydrogen consumption, thus the product would be coke. Due to the high oxygen content in most of the lignocellulosic biomass, CO and CO_2 formation reduces the bio-oil yields. For this reason, the process is carried out under hydrogen pressure or/and with a hydrogen donor solvent. Ethanol has been suggested as valuable solvent for HTL. The dielectric constant of ethanol is 4.20 at 240 ºC. Moreover, the critical parameters for ethanol are 374.15 ºC and 221 Bar, requiring much milder operation conditions than water (243.25 ºC, 63 Bar). Some physical and chemical properties are comparable to those of sub-critical water, thus replacing water with ethanol as the processing solvent for HTL is theoretically feasible [30]. Catalysts can also improve the process.

Although the first ideas were to process microalgae with high-lipid content, microalgae with low-lipid content have been also suggested as suitable biomass for HTL. The whole microalgae, not only the lipid fraction, can be converted into bio-oil. What is more, the microalgae waste recovered after the extraction of some valuable components can be used [31]. The lipid fraction, usually in the form of triacylglycerols (TAGs), is hydrolysed. The glycerol is converted into several compounds such as methanol, acetaldehyde, ethanol and gas products, mainly CO, CO_2 and H_2. Fatty acids have a higher thermal stability, but they can be converted into long-chain hydrocarbons. Proteins are quickly hydrolysed and further converted into carbonic acid, amines, ammonia and organic acids. After re-polymerisation, they produce long chain hydrocarbons and aromatic ring type structures like phenols or nitrogen heterocyclics such as indole or pyrrole. Carbohydrates do not directly contribute to bio-oil formation. They manly break down to polar water-soluble organics, such as organic acids (***i.e.*** formic, acetic, and lactic), aldehydes, benzenes and alcohols, all carrying a substantial amount of oxygen. The aldehyde- and benzene-type structures may further produce larger hydrocarbons in the bio-oil. Finally, the high-resistant insoluble macro-polymers called algaenans appears to yield directly to hydrocarbons (alkanes and alkenes) and alkyl-aromatics.

Several factors have demonstrated to influence the HTL of microalgae [29], some of them are summarised below:

- Biomass characteristics: composition, pre-processing and the concentration of solids, which remains still unclear.
- Temperature: sub-critical conditions usually increase the bio-oil yield, decrease the oxygen content and consequently increase the HHV. However, the nitrogen content also increases. Super-critical water mainly leads into more volatile compounds that form gas, decreasing the viscosity of the bio-oil.
- Holding time: to attain high bio-oil yields, high temperatures and low holding times are required. The optimisation of reaction time and temperature minimises the production costs.
- Catalysts: Homogeneous catalysts like Na_2CO_3 or KOH usually have a positive effect, improving bio-oil yields and decreasing the solid residues. It is attributed to the high pH, which promotes decarboxylation, and to the formation of H_2 and CO_2. However, Na_2CO_3 resulted inefficient for feedstock containing significant amount of sodium [30]. The H_2 gas subsequently acts as reducing agent. Several heterogeneous catalysts have been reported in the literature: Pd, Pt or Ru supported on

C; CoMo, Ni, Pt, Ni/SiO_2 supported on Al_2O_3; and zeolite. The knowledge generated on this topic to the present is scarce and contradictory.

Some barriers to overcome have been also identified [29]:

- difficulty to ensure constant properties and composition of biomass over time, and presence of impurities which can cause plugging of the reactor or poison the catalyst;
- solid concentration in the biomass; minimum 5% solids in the feed for a positive energy balance but concentrations higher than 15% and the difficulty to are difficult to pump into the reactor at the high pressures required;
- fouling and catalysts deactivation in continuous operation, additionally to the lack of adequate catalysts;
- corrosive characteristics of sub-critical water, which requires high-resistance materials.

3.3 Anaerobic digestion: Biogas production

The anaerobic digestion (AD) is a biological process to convert organic biomass mainly into a mixture of CH_4 and CO_2 called biogas. The mixture later upgraded to remove CO_2, H_2S, excess moisture and other impurities is called bio-methane and can be used as substitute of the natural gas. The process has been widely used for the conversion of organic waste such as sewage sludge and manures. The volume of waste is reduced, making its final disposal easy, and the biogas generated can be used for electricity and heat production. In the wastewater treatment plants (WWTPs), the benefits of digesting sewage sludge can be observed by a 50% reduction of the operating costs [32].

The process allows the conversion of slurries with more than 95% water; the sewage sludge for example can contain up to 98% water. This is one of the main advantages amongst some others for using the process to recover energy from microalgae, characterised by high water content [26,33,34]. The AD process, schematised in Figure 6, comprises four basic steps [35].

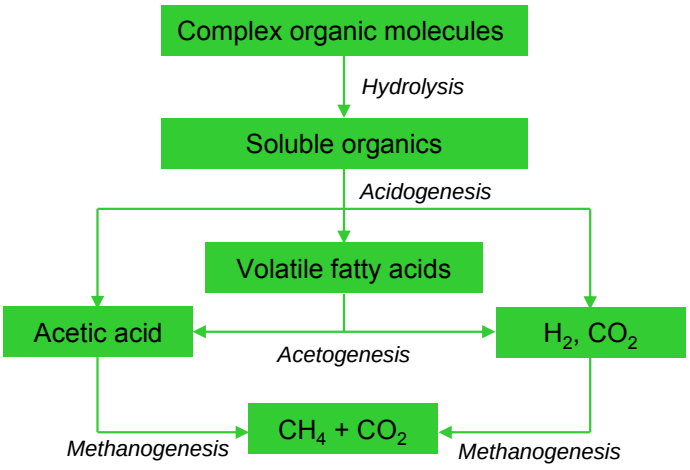

Figure 6. Scheme of the steps involved in the anaerobic digestion process.

- Hydrolysis, where complex organic molecules are hydrolysed into smaller and simpler ones: carbohydrates to monosaccharides, proteins to amino acids, and lipids to fatty acids. The step involves extracellular enzymes (hydrolases) produced by hydrolytic bacteria like *Streptococcus* and *Enterobacterium*.

- Acidogenesis, where the simple molecules are mainly converted into volatile fatty acids (formic, acetic, propionic, butyric, and pentanoic), alcohols (methanol, ethanol), aldehydes, carbon dioxide, and hydrogen; most of these products can not be directly used methanogens. Ammonia and hydrogen sulphide are generated in this phase too. The acid phase bacteria, facultative anaerobes, use oxygen creating favourable conditions for obligatory anaerobes: *Pseudomonas, Bacillus, Clostridium, Micrococcus,* or *Flavobacterium*.

- Acetogenesis, where organic acids are converted into acetic acid, hydrogen, ammonia, and carbon dioxide. Since approximately 70% of the methane is produced from acetate, this step determines the efficiency of biogas production. Some of the bacteria belong to the genera of *Syntrophomonas* and *Syntrophobacter*.

- Methanogenesis, characterised by the methane production derived from acetic acid, H_2, CO_2, and formate and methanol, methylamine, or dimethyl sulphide previously produced. Although it is promoted by microorganisms classified as *Archaea* or *Archaeabacteria*, methanogens are usually mentioned as methanogenic bacteria in the literature. Owing to the substrate used, methanogens are classified as hydrogenotrophic (they use only H_2 and CO_2), acetotrophic (they reduce methyl groups, being acetate their most important substrate) and methylotrophic (they reduce methylated compounds).

The pH, alkalinity, temperature, and hydraulic retention times are some of the parameters which affect AD. The volatile fatty acids (VFAs) can decrease the pH, optimal 6.5-8.0, and affect mainly the methanogenic bacteria. The process is controlled by the CO_2 concentration in the gas phase and the HCO_3-alkalinity of the liquid phase. The temperature influences the growth rates and the metabolisms of microorganisms and the solubility of the compounds such as ammonia, thus the overall process. The retention times indicates the average time the liquid is held in the reactor; it must allow enough time for the bacteria reproduction to avoid the washout of methanogenic bacteria or a reduced production capacity of the plant [32]. During microalgae digestion, between 10 and 30 days retention time is require as a consequence of their higher resistance [36].

Golueke et al. were the first authors working on the AD of microalgae [37]. However, the majority of the results have been reported in the last years due to the concern for the fossil fuel depletion. Unlike biodiesel, the most investigated biofuel from microalgae, the biogas production allows the conversion of microalgae containing water and the valorisation of all components presents in them. Some of the disadvantages of the AD of microalgae are [33,34,38]:

- Microalgae concentration: higher biomass concentration is mandatory to avoid the washout of the reactor. The excessive content of water can be reduced by improving the harvesting and concentration methods, still under development.

- Resistance of the cell walls: the cell walls characteristics can strongly influence the microalgae degradation. In some cases, it was observed little or even no degradation, remaining the cells almost intact after digestion. The cell walls hamper the microorganism attack, thus affecting the biodegradability. The implementation of pre-treatments is an alternative to overcome this drawback; mechanical, physical, thermal and chemical methods have been reported.

- Carbon/nitrogen ratio: It has been reported that anaerobic digestion requires a ratio close 20 to balance the C and N requirements; the ratio is below 10 in microalgae. The unbalance derives in high concentration of ammonia (NH_3) during degradation, with inhibitory characteristics for microorganisms. NH_3 is in equilibrium with ammonium (NH_4^+), less toxic for the microorganisms. The temperature and the pH increases favour the ammonia concentration, and consequently the toxicity. The anaerobic co-digestion (Aco-D) with carbon-rich substrates has been suggested as an option to avoid the inhibition caused by ammonia.

AD is one of the processes for biofuels production which allows recycling some of the by-products generated for microalgae cultivation such as nutrients present in the digestate and CO_2 [14-16]. Furthermore, it is estimated that around 30% of the volume in the digesters from WWTPs is currently available since most of them are oversized [39]. In this context, microalgae cultivation could be done in the WWTP to reduce the costs associated to the nutrient consumption, and then processed to produce energy or valuable compounds.

4. Objectives of the thesis

The overall objective of this thesis is the valorisation of microalgae for biofuels production, focusing also in the environmental benefits achieved by recycling nutrients for microalgae cultivation. The objective is schematised in Figure 7.

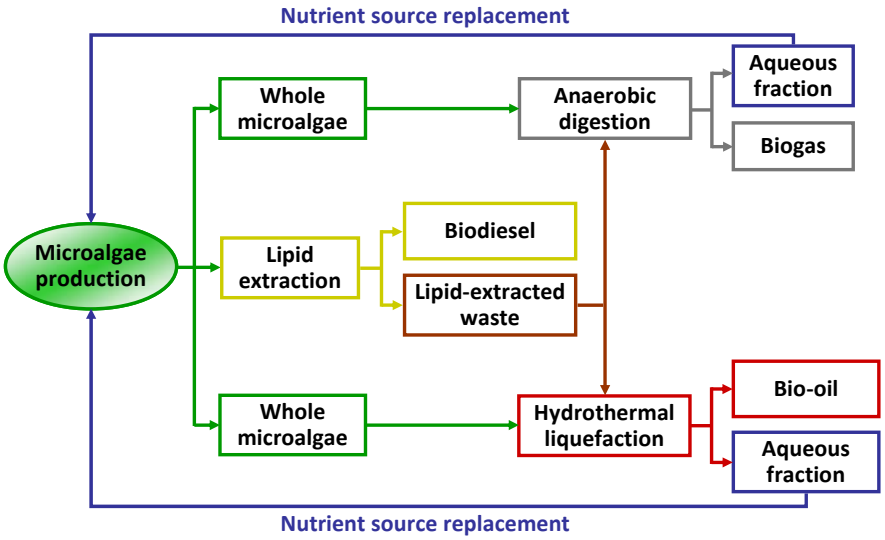

Figure 7. Scheme of the processes evaluated in this thesis.

The first part of the thesis evaluates the biomass conversion into biogas. Different microalgae species were evaluated as potential feedstocks. Regarding the operating condition in digesters, the effect of temperature and substrate loads on AD were evaluated. The lipid-extraction from microalgae and other techniques were applied as pre-treatment to improve microalgae degradability. Based on the benefits of coupling microalgae cultivation and processing in WWTP mentioned in section 2, mixtures of microalgae and sewage sludge were digested. Finally, the integration of microalgae cultivation and their valorisation was performed by using the nutrients from anaerobic digesters for microalgae cultivation.

The second part of the thesis evaluates the hydrothermal liquefaction of microalgae biomass. The whole and lipid-extracted microalgae were converted into bio-oil. Different temperature during HTL and use of organic solvents were studied to improve yields and quality of the bio-oil. As it was done in the first part of the thesis, the nutrient recycling was performed to integrate microalgae cultivation and HTL.

5. Thesis outline

This thesis is organised into ten chapters outlined below:

- Chapter 1 presents a general introduction and the objectives of this work.
- Chapter 2 investigates the influence of *Isochrysis galbana* and *Selenastrum capricornutum*, saltwater and fresh water species, on the biogas production. The microalgae were also co-digested with sewage sludge. The process was carried out under mesophilic and thermophilic conditions. The experiments were done in collaboration with IRTA institute. The results have been published in *Renewable Energy*.
- Chapter 3 investigates *Phaeodactylum tricornutum* as substrate for anaerobic digestion. This species is a promising substrate for biodiesel production, thus the lipid-extraction and the subsequently conversion of the waste into biodiesel were carried out. The whole microalgae was also digested and besides, the influence of ultrasonic pre-treatment and co-digestion with sewage sludge were evaluated. The experiments were done in collaboration with IREC institute. The results have accepted for publication in *Journal of Environmental Management*.
- Chapter 4, similar to chapter 3 evaluates the lipid-extraction and the biogas production from the lipid-extracted waste, simply digestion of the microalgae, influence of ultrasonic pre-treatment and codigestion with sewage sludge. *Nannochloropsis oculata* and *Chlorella vulgaris* have been used in the experiments. The experiments were done in collaboration with GEPEA institute. The results have been published in *Fuel Processing Technology*.
- Chapter 5 presents the results obtained during a research stay at the GEPEA group in Saint Nazaire, France. The main goal was the microalgae cultivation in wastewater. *Chlorella vulgaris*, *Chlorella kessleri* and *Nannochloropsis oculata* were cultivated in urban wastewater from the WWTP in Saint Nazaire. Afterwards, the biomass was converted into biogas and biodiesel. The results have been published in *Algal Research*.
- Chapter 6 presents the feasibility of a novel pre-treatment method to increase microalgae biodegradability, using the organic solvents N-methylmorpholine-N-oxide (NMMO). The results have been published in *Bioresource Technology*.
- Chapter 7 is a review paper; it summarises the last results obtained during the anaerobic digestion of microalgae waste after the lipid extraction due to the current interest on the valorisation of these waste. The review was accepted for publication in the special issue Biomethane, in the *Current Biochemical Engineering* journal, but not available online yet.
- Chapter 8 explores several alternatives for the valorisation of *Nannochloropsis* waste after the extraction of lipids. The alternatives cover the conversion into methane via anaerobic digestion and the

conversion into bio-oil via hydrothermal liquefaction. Moreover, the aqueous phase recovered after liquefaction was utilised as nutrient source for microalgae cultivation. The results have accepted for publication in the *ACS Sustainable Chemistry & Engineering* journal.

- Chapter 9 studies the HTL of *Nannochloropsis oceanica*. The results obtained during a research stay at the Département Systèmes Energétiques et Environnement in L'École des Mines de Nantes, France. The first part of the experiments evaluated the influence of the temperature on the distribution of products. Afterwards, the HTL was carried out adding alcohols as co-solvents to improve the process. The results have accepted for publication in *Bioresource Technology*.
- Chapter 10 presents the main conclusions of this thesis and outlines suggestions for future work in this field.

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Chapter 2

Biogas production from sewage sludge and microalgae co-digestion under mesophilic and thermophilic conditions [1]

ABSTRACT

Isochrysis galbana and *Selenastrum capricornutum*, marine and freshwater microalgae species respectively, were co-digested with sewage sludge under mesophilic and thermophilic conditions. The substrates and the temperatures significantly influenced biogas production.

Under mesophilic conditions, the sewage sludge digestion produced 451±12 mL_{Biogas}/g_{VS}. Furthermore, all digesters were fed with *I. galbana*, or mixed with sludge, resulting in an average of 440±25 mL_{Biogas}/g_{VS}. On the contrary, *S. capricornutum* produced 271±6 mL_{Biogas}/g_{VS} and in the mixtures containing sludge produced intermediate values between sludge and microalgae production.

Under thermophilic conditions, the sewage sludge digestion achieved yet the highest biogas yield, 566±5 mL_{Biogas}/g_{VS}. During co-digestion, biogas production decreased when the microalgae content increased, and for *I. galbana* and for *S. capricornutum* it reached minimum values, 261±11 and 185±7 mL_{Biogas}/g_{VS}, respectively. However, no evidence of inhibition was found and the low yields were attributed to microalgae species characteristics.

The methane content in biogas showed similar values, independently from the digested substrate, although this increased by approximately 5% under thermophilic condition.

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1. Introduction

The industrialisation process and the current population growth have had an immense impact on the environment. The demand on water and petroleum-based fuels are clear evidence of the impact on natural resources.

The wastewater treatment plants (WWTPs) have become an essential component in society to ensure necessary water supplies. A by-product produced in these facilities during the wastewater treatment process is sewage sludge; hence as demand grows so does this by-product. The final disposal of sludge is a problem in WWTPs as this can represent up to 50% of the operating cost [1]. The anaerobic digestion of the sludge is one of the most widespread stabilization processes in WWTPs, which converts sludge into a stable product and simultaneously recovers energy by biogas generation. On the other hand, WWTPs are a potential source of nutrients for microalgae growth: CO_2 generated and released in the atmosphere when biogas is burned; also nitrogen and phosphorus are present in wastewater [2], [3], [4] and [5].

Microalgae arose as a source of valuable chemical productions, but may be the main feature was as promising feedstocks for renewable biofuels. In 2008, 88% of world energy demand was supplied by fossil fuels, including oil (35%), coal (29%) and natural gas (24%) [4]. Unfortunately, coal supplies depletion is predicted by 2112 and oil and gas reserves depletion by 2042, thus a rapid transition to renewable energy is needed in the near future [6]. Biodiesel production from microalgae appeared as a solution due to the microalgae advantages over the feedstocks currently used in the biodiesel industry; however, the process scale-up is unviable nowadays without a cost-effective dewatering method. Additionally, microalgae cultivation is not simple, although they grow naturally in aquatic environments. The low cell densities required for light penetration to ensure their growth and the small size of the cells are counterproductive to the harvesting step, which can represent between 20 and 30% of the total biomass production costs [4] and [7].

The anaerobic digestion process creates an alternative for energy recovery from microalgae. The ability to process wet biomass avoids a drying step, thus reducing large amounts of energy input. Besides, all microalgae compounds can be turned into biogas and those species unsuitable for biodiesel production due to their low oil content become potential substrates [8]. During anaerobic digestion, the organic nitrogen and phosphorus initially as biomass constituents are converted into ammonium and phosphate, and can be recycled for microalgae cultivation reducing fertilizer needs [7]. Under these circumstances, the possibility of microalgae growth followed by anaerobic digestion in WWTPs is currently being assessed [9].

Since Golueke et al. [10] studied anaerobic digestion of microalgae for the first time, at the end of the 1950s, many microalgae species and process conditions have been evaluated for biogas production. However, not all species have the potential to produce high amounts of methane, and the proper selection of species and operating conditions are the key for biogas production [11]. The cell wall structure and composition, the carbon to nitrogen ratio (C/N), are crucial for microalgae degradability [8]. Cell wall resistance may act as a barrier hampering microorganisms being attacked; on some occasions, pre-treatment methods are required to improve digestibility [12] and [13]. The C/N balance, low in microalgae derived from their high protein content, increased ammonia and volatile fatty acids concentration and may potentially inhibit anaerobic digestion. Co-digestion with carbonaceous-rich waste is a method to balance this ratio and overcome this disadvantage. Sewage sludge, waste

activated sludge, waste paper and corn straw are examples of increasing biogas production from different microalgae species [14], [15], [16] and [17].

In this context, this paper attempts to evaluate biogas produced from two microalgae species: the marine species *Isochrysis galbana*, and the freshwater species *Selenastrum capricornutum*, by their co-digestion with sewage sludge. Initially, the influence of the microalgae to sludge ratio and the digestion temperature over the biogas production and its methane content was evaluated.

2. Materials and methods

2.1. Materials

2.1.1. Sewage sludge

The sludge sample consists of a primary and secondary blend, in ratio 65:35 v/v. It was collected from the municipal WWTPs in Reus (Tarragona, Spain) designed to process approximately 25,000 m^3 of wastewater daily. Sludge was received weekly and immediately stored at 4 °C in a fridge prior to use. The experiment involved the use of two different kinds of reactors, semi-continuous reactors for acclimation and batch reactors for co-digestion; the same type of sludge was utilised in both reactors. For the first reactors the maximum storage time was a week; for the second, the maximum storage time was 2 days in order to avoid major changes on its composition or properties.

2.1.2. Microalgae

Two microalgae species were used in the experiments: *Isochrysis galbana*, marine species, and *Selenastrum capricornutum*, freshwater species. Both species were provided by the Institute for Research and Technology in Food and Agriculture IRTA (San Carles de la Ràpita, Spain). For the marine species cultivation, Walne's medium [18] was prepared with filtered (0.45 μm), autoclaved seawater from Alfacs Bay (Ebro Delta, Spain). For the freshwater species cultivation, Woods Hole MBL medium [19] was prepared with deionised water, and autoclaved before inoculation. The microalgae were cultured under batch conditions in 6 L volumetric flasks. The volumetric flasks were kept in an isothermal chamber at 20±3 °C under continuous irradiance of 120-150 μmol photons m^{-2} sec^{-1}, provided by cool-white fluorescent lamps (Philips TLD 58w/865). Mixing was provided by air flow and air was enriched by 0.7% CO_2 addition. The microalgae were grown under these conditions for approximately 2 weeks, until the culture reached a plateau in terms of absorbance (680/800 nm).

2.1.3. Inoculum

From the start, inoculum was provided by the municipal WWTP in Reus (Tarragona, Spain). It consisted of digested sludge from mesophilic and thermophilic anaerobic reactors under continuous operating conditions. Although acclimation to new conditions was not strictly required, an anaerobic semi-continuous plant was set up to adapt inoculum to more stable temperatures, 33 °C and 50 °C. Four reactors (5 L each) were placed in a thermostatic bath for temperature control under magnetic stirring. Biogas production was continuously registered by volumetric gas flow meters. All reactors were daily fed with sludge, following effluent withdrawal.

Prior to the co-digestion experiments, the inoculum had previously been "degassed" by incubation at a constant temperature without feeding, to reduce the residual biodegradable organic material [20]. Observation indicated no significant methane being produced after 5 days incubation.

2.2. Experimental procedure

2.2.1. Microalgae preparation

Once microalgae were received, the biomass was collected by centrifugation using a centrifuge with a capacity of 240 mL sample (Digicen 20, Orto Alresa Centrifuges). Microalgae were recovered after 4 min at 10,304 RCF without temperature control. The supernatant was removed and only the pellet was recovered. Deionised water was added to recover the pellet and the total solid (TS) content of the microalgae suspension was around 10 g/L. The suspension was stored at 4 °C prior to use, and always used before 12 h after microalgae centrifugation finished.

2.2.2. Co-digestion procedure

The batch reactors were set up following the procedure described by Angelidaki et al. [20]. The temperature for the experiments was chosen in accordance to the temperature in the WWTP reactors, 33 °C in the mesophilic range and 50 °C in thermophilic range.

All experiments of co-digestion were conducted in 120 mL serum bottles in triplicate. To carry out the digestion, the microorganisms were provided with 50 mL "degassed" inoculum and optimal environmental conditions were assured by 10 mL anaerobic basic medium addition. The substrates were sewage sludge and microalgae, separately or mixed. Blank assays were prepared without substrate addition, and the biogas production was subtracted from the reactors fed with the substrates. The specific methanogenic activity for inoculum was determined with an initial concentration of 1 g/L acetic acid in the reactor.

Sludge and microalgae were added according to the experimental design. The total substrate amount was decided at 0.12 g volatile solids (VS) equivalent to 2-3 g_{COD}/L, which generates a measurable but not excessive biogas volume. The reactor feed was based on 100% sludge VS for sewage sludge digestion (denoted as Sludge in the figures), and subsequently 25%, 50%, 75% and 100% of the sludge VS were replaced with microalgae VS respectively (denoted as 25%, 50%, 75% and 100% in the figures). Deionised water was added to a final volume of 80 mL and the reactors were closed with a septum and an aluminium crimp. Finally, the reactors were purged with nitrogen to assure anaerobic conditions and placed into an oven.

Biogas production was volumetrically measured by liquid displacement. As a barrier solution, a saline solution consisting of 200 g/L NaCl and 5 g/L citric acid was used. Prior to measurements, the reactors were removed from the oven and left to reach room temperature. It was imperative that the room temperature be record in conjunction with the measurement of the biogas volume to provide results under standard conditions. Before returning the reactors to the oven, they were gently shaken. The experiment being considered finished when the biogas production was negligible.

2.3. Analytical techniques

Total solids (TS) and volatile solids (VS) were analysed according to standard methods 2540B and 2540E respectively [21]. The chemical oxygen demand (COD) was measured in a UV-spectrophotometer (DINKO UV-

VIS 800 spectrophotometer) according to the standard method 5220D [21]. The protein content in the microalgae was quantified by the Lowry method [22], pre-treating samples at 100 °C for 10 min with 2 N NaOH. The total sugar amount was quantified by phenol-sulphuric acid method [23]. Microalgae lipids were determined using the Bligh and Dyer method [24]. In order to assess microalgae biodegradability, the theoretical biogas potential was calculated following Buswell's equation for microalgae proteins, lipids and carbohydrates [25]. Biodegradability was expressed as measured to theoretical methane production ratio. For the carbon to nitrogen ratio (C/N), carbon was estimated by dividing the organic matter content by 1.724 and nitrogen, dividing the protein content by 6.25 [26].

Once the experiments were concluded, volatile fatty acids (VFA) were analysed in the soluble phase by using an Agilent gas chromatograph 6890 GC equipped with a flame-ionization detector (FID). The method was performed according to Application Note 228-398 [27] from Agilent Technologies online library. An ion selective electrode (ISE) was used for ammonia concentration determination (Ammonia Gas Sensing combination electrode, mod. 51927-00, HACH).The biogas composition was analysed in a gas chromatograph (Agilent gas chromatograph 6890 GC) with manual injection and a thermal conductivity detector (TCD). The separation was achieved in a porapak q 50/80 packed column 3.6 m × 6.35 mm × 0.4 mm (Agilent Part No. CP99960C), using helium as carrier gas. The injector was set at a temperature of 40 °C. The sample volume was 1 mL. The oven temperature programme started at 40 °C and after 2 min was increased by 22 °C/min to 150 °C and held for 4 min. The standard used for identification and quantification of biogas components was supplied by Carburos Metálicos S.A. It consists of a mixture of methane (60% v/v), carbon dioxide (35% v/v), hydrogen (2% v/v) and hydrogen sulphide (3% v/v). Only methane and carbon dioxide were quantified, and the results were expressed as the methane percentage in a two component mixture.

The digestate was observed by Scanning Electron Microscopy (SEM) to determine the presence of entire microalgae cells which could remain after digestion. A Jeol JSM-6400 SEM was used for this purpose. A drop of each digestate sample was deposited on the support, dried at room temperature and then coated under vacuum with a gold layer before examination.

Characteristics of the sludge, the microalgae species and the inoculum used in the experiments can be seen in Table 1.

Table 1. Inoculum and substrates characteristics.

Parameter	Inoculum	Sewage sludge	*Isochrysis galbana* (marine species)	*Selenastrum capricornutum* (freshwater species)
TS (g/L)	18.9±0.1	30.5±1.9	9.0-10.0[a]	9.0-10.0[a]
VS/TS	0.70	0.88	0.90	0.98
COD/VS	1.0±0.1	1.5±0.5	1.37	1.56
TN ($g/100g_{VS}$)	-	-	7.8	4.9
C:N	-	-	7.1	9.2
Proteins ($g/100g_{VS}$)	-	-	51.2	39.7
Lipids ($g/100g_{VS}$)	-	-	22.5	30.2
Carbohydrates ($g/100g_{VS}$)	-	-	15.4	29.1

[a] *Microalgae were diluted with distilled water after centrifugation.*

3. Results and discussion

3.1. Biogas production under mesophilic conditions

The biogas production was reported as the volume of biogas at standard conditions, 0 °C and 1 atm, per gram VS fed, mL_{Biogas}/g_{VS}. Fig. 1 shows accumulated biogas production at 33 °C for the marine species ***I. galbana***, and Fig. 2, for the freshwater species *S. capricornutum*.

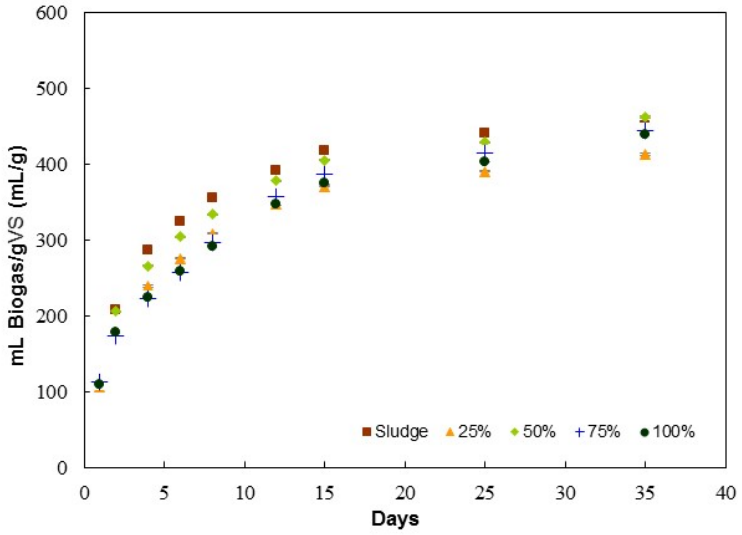

Figure 1. Marine microalgae and sludge co-digestion. Batch reactors, 33 ºC, and 35 days.

As it can be seen in Fig. 1, the biogas production between reactors did not differ significantly, either when microalgae and sludge were digested separately or mixed. After 35 days, *I. galbana* digestion produced 439±4 mL_{Biogas}/g_{VS}. This value is higher than the reported for other saltwater microalgae like *Spirulina maxima* (330 mL_{Biogas}/g_{VS}) and for *Gracilaria* species and strains (280 and 400 mL_{Biogas}/g_{VS} respectively) [28] and [29]. In the same experiment, sewage sludge digestion produced 451±12 mL_{Biogas}/g_{VS}; this value is in agreement with the values reported for sludge anaerobic digestion [1]. The comparison of microalgae and sewage sludge digestion highlights that microalgae are able to produce more or less the same quantity of biogas than sewage sludge, a substrate currently used in anaerobic digestion. In particular, ***I. galbana*** produces the same amount of biogas, demonstrating that this microalgae species is an eligible substrate for anaerobic digestion, competing with sewage sludge. This fact is confirmed by the results obtained during the anaerobic digestion of *Phaeodactylum tricornutum* in continuous mode (530 mL_{Biogas}/g_{VS}) and during the anaerobic digestion of *Arthrosira platensis* and *Dunaliella salina* in batch digesters (480 and 505 mL_{Biogas}/g_{VS} respectively) [11] and [29]. The authors suggested that anaerobic digestion constitutes the best option to recover energy from these microalgae species.

Furthermore, when a substrate mixture was fed, biogas production showed quite similar values for all experiments, independently of the sludge to microalgae ratio in the mixture. The averaged biogas production was 440±25 mL_{Biogas}/g_{VS}. The mixture with 25% microalgae produced the lower biogas yield, 413±7 mL_{Biogas}/g_{VS}. However, no evidence of unbalanced digestion conditions was found in these reactors. The yield drop barely represents 9% compared to the sludge or the microalgae yields, and may be attributed to experimental differences during gas volume measurements. Consequently, it was expected that by mixing the substrate the biogas yields would increase, but the results do not indicate synergistic effects. The literature relating to microalgae co-digestion

showed the possibility of increasing the biogas production by mixing microalgae and sludge, due to the improved C to N ratio [10] and [17]. Recently, Wang et al. [14] found that *Chlorella sp.* digestion reached a considerable low biogas yield compared to waste activated sludge (WAS) digestion, but microalgae and WAS mixtures reached biogas yields similar to WAS. Hence, suggestions were put forward that the WAS addition increases the microbial activity instead of improving the C to N ratio, thus affecting microalgae cell hydrolysis. In our experiments, microalgae digestion was not improved by WAS presence in the substrate. During the course of the experiment, it was noted that after 12 days all reactors had already exceeded 350 mL_{Biogas}/g_{VS} which represents more than 80% of the total biogas produced at the end of the experiment. Any influence of substrate mixing in the hydrolysis rate can be also dismissed.

According to Buswell's equation, conversion of proteins, lipids and carbohydrates produce 496 mL_{CH4}/g_{VS}, 1014 mL_{CH4}/g_{VS} and 415 mL_{CH4}/g_{VS} respectively [25]. Based on the *I. galbana* composition, the theoretical methane yield is 612 mL_{Biogas}/g_{VS} source and the biodegradability is 55.2%. This is a high value according to conversion degrees obtained under practical conditions for highly particulate or structural substrates, and can be explained by the microalgae species characteristics [25]. Additionally, it is fairly well known that the microalgae cell wall determines the potential conversion into biogas; *I. galbana* cells have been described as small size cells, with a lack of a cell wall which facilitates anaerobic digestion [11] and [31]. Furthermore, the change from a high saline to a non-saline environment favours microalgae disintegration [30].

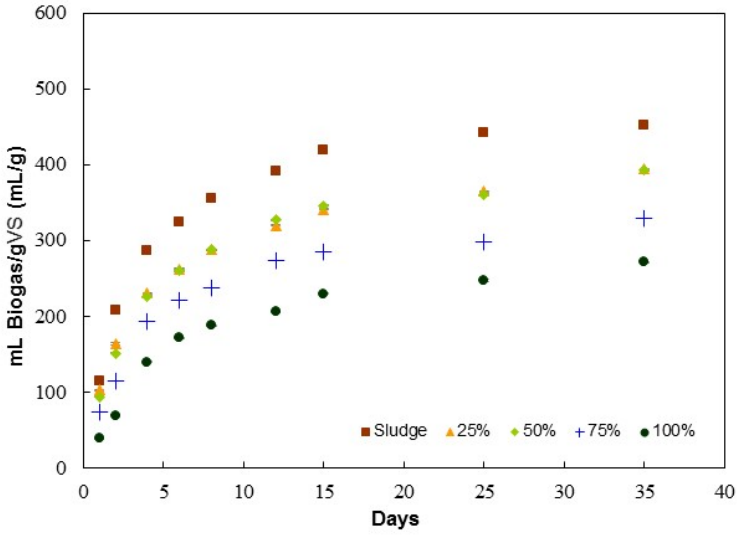

Figure 2. Freshwater microalgae and sludge co-digestion. Batch reactors, 33 ºC, and 35 days.

Barely 271±6 mL_{Biogas}/g_{VS} were produced during the experiments with *S. capricornutum*; this production is the lowest obtained under mesophilic conditions (Fig. 2). Although higher biogas productions were reported for freshwater microalgae species, similar results were observed in species characterised by carbohydrate-based cell walls, like in *S. capricornutum* [34]. Mussgnug et al. digested different microalgae species and observed that the species *Scenedesmus obliquus* and *Chlorella kessleri*, both with carbohydrate-based cell walls, showed lower biogas production and higher amount of indigestible residues than species without cell wall or with protein-based cell walls digested in the same conditions [11]. Even a lower biogas production was reported during *Scenedesmus sp.* digestion, only 75 mL_{Biogas}/g_{VS} after 35 days at mesophilic temperatures [32]. On the other side, comparison with biogas production from sewage sludge, digested in the same experiment, shows that *S. capricornutum* reached

around 60% of the biogas production from sewage sludge, 451±12 mL_{Biogas}/g_{VS}. This result differs from the saltwater species *I. galbana*, which produced more or less the same quantity of biogas than sewage sludge.

In the case of substrate mixture productions were between sludge and microalgae production. Their digestion reached 394±14 mL_{Biogas}/g_{VS}, 392±4 mL_{Biogas}/g_{VS} and 330±6 mL_{Biogas}/g_{VS} for 25%, 50% and 75% microalgae respectively. It is noticeable that the higher the amount of VS from microalgae, the lower the biogas production. Hence, substrate mixing does not increase the biogas yield of the substrates separately digested as was expected.

During the course of the experiment, the biogas production rate followed approximately the same tendency independently of the reactor feed composition (Fig. 2). The exception being reactors fed only with microalgae, which reached 76% of the total biogas produced in the experiments after 12 days digestion, all other reactors reached 80% of the total biogas produced for the same period.

S. capricornutum has a remarkable ability to accumulate lipids with a potential for biodiesel production. This characteristic also showed a positive influence in relation to the potential methane yield, due to the high methane yield of lipids [25] and [33]. The theoretical biogas potential for *S. capricornutum* was estimated at 693 mL_{Biogas}/g_{VS} source employing Buswell's equation. Biodegradability was 30.1%, a low value compared to the marine counterpart. *S. capricornutum* cell walls are thick and rigid carbohydrate-based structures, which act as strong barriers for microorganism reducing cell degradation [34]. Additionally, the carbohydrate-based cell wall species can resist degradation better than protein-based cell wall microalgae or microalgae without a cell wall [11].

The results obtained by digesting sewage sludge and microalgae mixtures were not as expected, and the biogas yields of these mixtures shows that the process occurred without synergy between substrates.

The marine microalgae digestion performs better than the freshwater species and similar to the sludge digestion under mesophilic conditions. Unfortunately, in an integrated system for wastewater treatment, microalgae cultivation and anaerobic digestion, freshwater microalgae cultivation does not require a seawater system, making system integration easier.

3.2. Biogas production under thermophilic conditions

The biogas production under thermophilic conditions during the co-digestion of *I. galbana* is shown in Fig. 3, whereas Fig. 4, shows the biogas production during the co-digestion of *S. capricornutum*. The results are given as volume of biogas at standard conditions per gram VS fed, mL_{Biogas}/g_{VS}. After 20 days, biogas production from all substrates was almost negligible, and after 27 days, biogas production from blank reactors reached the highest biogas production. The experiment was considered completed and the values obtained by day 20 were considered the final biogas production yields.

The way the temperature affected substrate digestion was completely opposite. The temperature increase had a negative influence on microalgae digestion; under thermophilic conditions, *I. galbana* and *S. capricornutum* reached 261±11 mL_{Biogas}/g_{VS} and 185±7 mL_{Biogas}/g_{VS} respectively, which represent 40.5% and 31.7% decrease compared with their biogas productions at 33 °C. Varel et al. observed the same effect during anaerobic digestion of *Spirulina maxima*, but also process instability, decreasing the biogas production and its methane content [28].

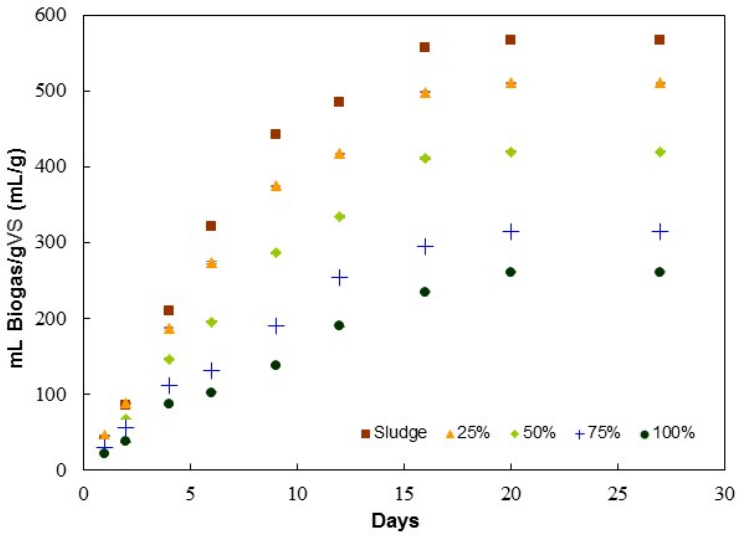

Figure 3. Marine microalgae and sludge co-digestion. Batch reactors, 50 ºC, and 27 days.

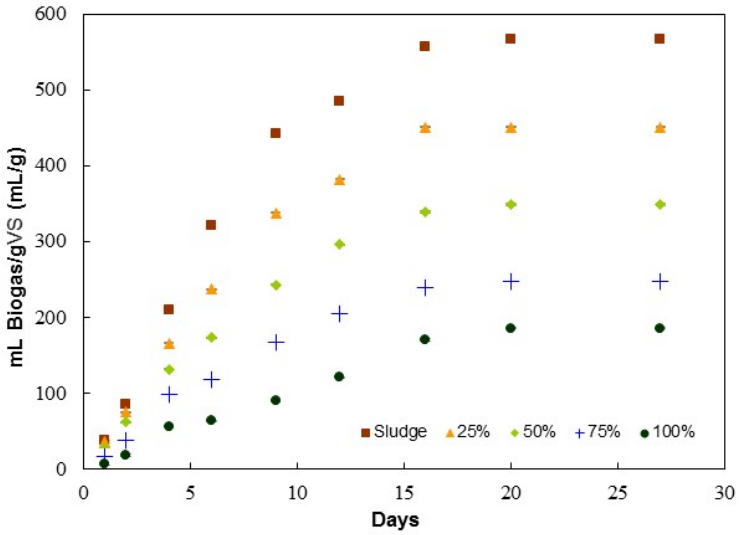

Figure 4. Freshwater microalgae and sludge co-digestion. Batch reactors, 50 ºC, and 27 days.

On the contrary, some authors reported beneficial effects of the temperature increase. For example, Golueke et al. [10] reported increases in gas production and volatile matter destruction due to a temperature increase from 35 °C to 50 °C. El-Mashad [26] observed higher biogas production from *Spirulina platensis* when the temperature changed from 35 °C to 50 °C, but the methane content was lower and the methane production resulted similar. It seems that the effects of the temperature over anaerobic digestion are related to species characteristics.

As mentioned before, the temperature affected sewage sludge digestion differently and the temperature increase improved sludge digestion. Biogas production reached 566±5 mL_{Biogas}/g_{VS}, indicating that a 25.5% more biogas was produced by increasing temperature. Consequently, the way in which the temperature affects substrates digestion intensifies the differences between biogas production from microalgae and sludge; whereas *I. galbana* and *S. capricornutum* produced around 97% and 60% of the amount of biogas produced from sludge under mesophilic conditions, under thermophilic conditions these percentages were only 46% and 33%.

In regards to the co-digestion, it can be observed that independently of the microalgae specie, experiments present similar tendencies. The higher the VS from microalgae, the lower the biogas production. Additionally, biogas

production can be estimated from the measured biogas production for both substrates individually digested. The similarity of these values to the experimental values shows no advantages of mixing substrates.

In order to determine if inhibition phenomena took place during digestion, thereby explaining the lower biogas production, ammonia and VFA concentration was determined in the reactors. The protein content may represent a high percentage of the organic matter in microalgae, and their digestion release free ammonia; high free ammonia concentration has toxic effects on microorganisms. Furthermore, the temperature increase enhances the toxic effects [8]. After digestion ends, the ammonia concentration in the reactors containing microalgae was slightly higher compared with the concentration in the blank reactors. The ammonia increase was less than 50 mg/L and ammonia concentration never exceeded 550 mg/L. These concentrations are considerably lower than 1500 mg/L, the value reported as detrimental for microorganisms [1]. VFA are intermediate compounds in anaerobic digestion and their accumulation cause process instability or failure due to methanogenic inhibition. It is reported that VFA can be toxic to methanogenic microorganisms at a concentration range of 6.7-9.0 mol/m^3 [1]. Despite VFA were not detected under mesophilic conditions, their concentration were on average 3.04±0.45 mol/m^3 under thermophilic conditions. However, this concentration is not high enough to cause methanogenic bacteria inhibition. The analysis of these results confirms that the negative influence of temperature on microalgae digestion is not related to process instability.

When compared to the mesophilic digestion, under thermophilic conditions reactors fed with microalgae needed more time to reach 80% of the total biogas, produced at the end of the experiment. Besides, a biogas increase can be observed after 8 day microalgae digestion. Sludge digestion had produced more than 80% of its total biogas production after 8 days. Before starting the experiment, anaerobic inoculum was totally acclimated to sludge, but not to microalgae. At the beginning of the experiment, microorganisms were suddenly exposed to high concentrations of microalgae and therefore an acclimation period was necessary. After this initial period, which can be seen as a lag phase, biogas production increased but after a few days it reached the final biogas yield. During co-digestion, microorganisms may degrade sewage sludge at first and later microalgae. Although the thermophilic process shows several benefits compared to the mesophilic, it is more sensitive and thus explaining the absence of lag phase under mesophilic conditions [1].

Once again, biodegradability indicates that *I. galbana* and *S. capricornutum* produced 34.9% and 21.9% of the predicted amount of biogas respectively. Similarly to digestion under mesophilic conditions, the marine species produced more biogas than the freshwater counterpart. These results may be attributed to the salinity change in the environment and the cell wall characteristics [11] and [30].

Finally, since a green colour was observed at the bottom of the reactor, digestate samples were examined in a Scanning Electron Microscopy (SEM) to determine microalgae incomplete digestion. In Fig. 5 and Fig. 6, the presence of microalgae cells after 30 days digestion can be seen. Microalgae cells were previously photographed in their own pure culture sources; their shape and size were determined, thus facilitating microalgae identification in the digestate samples. Although digestate samples from mesophilic reactors were also observed in SEM, no cells were found. The cells indicate low degree of decomposition and presence of indigestible residues, confirming that the decrease in biodegradability at higher temperatures is due to species characteristics.

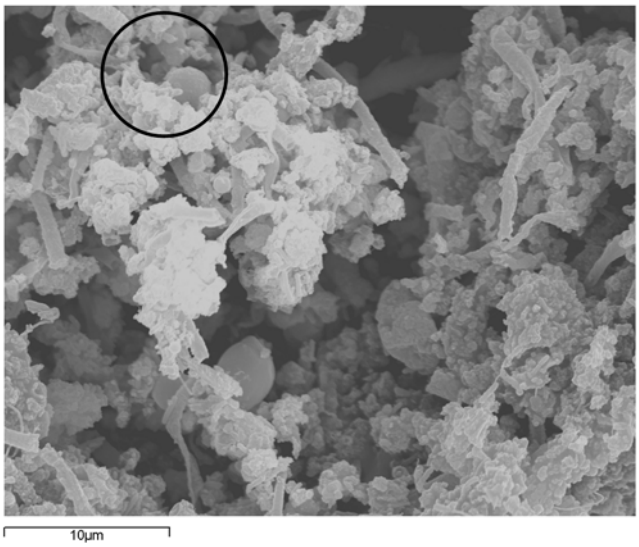

Figure 5. Marine microalgae cell presence after digestion. Batch reactors, 50 ºC, and 35 days.

Figure 6. Freshwater microalgae cell presence after digestion. Batch reactors, 50 ºC, and 35 days.

3.3. Effect of temperature digestion on biogas quality

Since biogas is mainly a methane and carbon dioxide mixture, its quality is related to the methane content among other gases. Biogas analysis showed approximately the same methane content in all reactors operating under mesophilic conditions, averaging 77±1%CH_4, independently substrate. Also under thermophilic all reactors showed a similar composition however, the average was 82±1%CH_4. Comparable results were also reported for *Scenedesmus obliqqus* and *Phaeodactylum tricornutum* digestion, under mesophilic and thermophilic conditions [30]. On the contrary, in *Spirulina maxima* and *Spirulina platensis* digestion, the higher the temperature, the lower its methane content [26] and [28]. As with biogas production yield, for the biogas composition the species characteristics determine the most suitable process conditions.

Based on biogas composition, *I. galbana* produced 338±3 mL_{CH4}/g_{VS} and 219±10 mL_{CH4}/g_{VS} at 33 °C and 50 °C respectively, whereas *S. capricornutum* produced 209±5 mL_{CH4}/g_{VS} and 152±6 mL_{CH4}/g_{VS} at 33 °C and 50 °C

respectively. The results demonstrate that mesophilic anaerobic digestion represents the best alternative to digest both microalgae species despite the higher methane content produced under thermophilic conditions. The situation is completely the opposite for sewage sludge, since thermophilic digestion increase biogas production and methane content. The amount of methane was 347±9 mL $_{CH4}$/g$_{VS}$ at 33 °C compared with the 464±4 mL $_{CH4}$/g$_{VS}$ at 50 °C. However, an energy balance should be done taking into account the heating requirements of the thermophilic process.

4. Conclusions

Microalgae and sludge co-digestion does not improve biogas yield in comparison with individual digestion of both substrates. Neither does the microalgae to sludge ratio nor does the digestion temperatures improve biogas production. However, our results highlight the feasibility of the marine species *I. galbana* as an eligible substrate for biogas production under mesophilic conditions, since it produces a similar amount of biogas to sewage sludge. On the contrary, its freshwater counterpart *S. capricornutum* produces the lowest biogas amount.

Under thermophilic conditions, the biogas production from both microalgae species decreases by 40.5% and 31.7% for *I. galbana* and *S. capricornutum* respectively, contrary to biogas production from sewage sludge which improves by 25.5%. During co-digestion, the higher the microalgae content in the reactors, the lower the biogas production.

Although the methane content increases from 77±1% to 82±1% at higher temperatures, the improvement does not compensate the biogas reduction caused by the higher temperature, except for sludge digestion.

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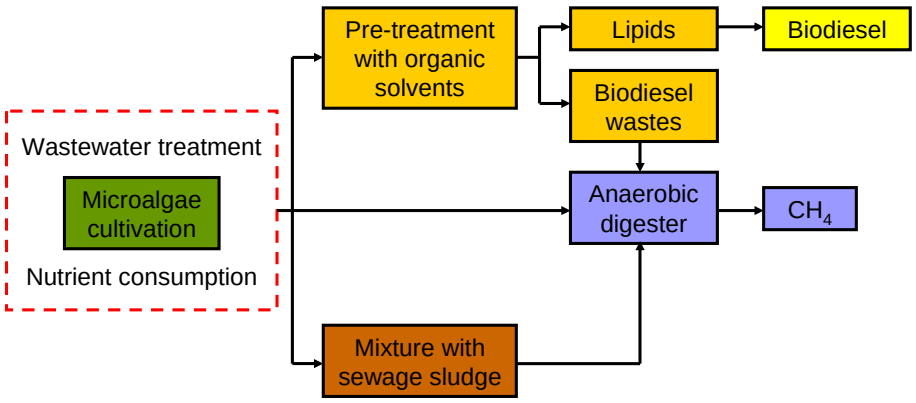
Chapter 3

Evaluation of different strategies to produce biofuels from Nannochloropsis oculata and Chlorella vulgaris [1]



ABSTRACT

The lipid extraction using hexane and methanol:hexane increased the biodegradability of *Nannochloropsis oculata* by 36% and 24% respectively. Moreover, hexane increased the methane production from raw microalgae, from 253±11 to 313±9 mL_{CH4}/g_{VS}. Methanol:hexane did not affect the methane production, which yielded 254±10 mL_{CH4}/g_{VS}, mainly due to the significant changes in the biomass composition.

On the other hand, the lipid extraction failed to increase the biodegradability of *Chlorella vulgaris*, which resulted around 44% for raw and lipid-extracted microalgae. The methane productions were 219±6, 202±1 and 200±4 mL_{CH4}/g_{VS} from raw and pre-treated microalgae using hexane and methanol:hexane respectively.

Regarding the lipid extraction yields, using methanol:hexane the yields were 4.7 and 3.7 times higher for *N. oculata* and *C. vulgaris* than using hexane. The biodiesel yields were also higher using methanol:hexane, 2.4 and 1.9 times than using hexane. However, the biodiesel composition was unaffected by the solvent.

The substrate to inoculum ratio influenced raw *N. oculata* digestion. At 1:1 $VS_{Substrate}$:$VS_{Inoculum}$, the methane production throughout the first days decreased but not the ultimate methane production. *C. vulgaris* digestion was unaffected, probably due to the biomass characteristics.

Finally, the co-digestion of microalgae and sewage sludge showed no synergy, nor inhibition.

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1. Introduction

Microalgae present extraordinary characteristics for producing renewable biofuels, such as high biomass productivity and accumulation of lipids amongst some others [1] and [2]. The genus *Nannochloropsis* and *Chlorella* are examples of promising microalgae for biodiesel and methane production [3], [4], [5] and [6]. Biodiesel has high biodegradability, low toxicity, low emission profile, and does not need major modification in engines and refuelling technology [7]; however, the biodiesel from microalgae presents economical and sustainable limitations. There are considerable efforts underway to achieve favourable energy balances and production costs, for example, trying to find cost-effective and efficient lipid extraction technologies [1]. Processes using ionic liquids [7] or wet extraction can reduce the high costs of harvesting and drying [8] and [9], but they are not large-scale processes yet. Methane is an alternative to biodiesel. The anaerobic digestion (AD) or methanisation is ideal for processing wet biomass, reducing the cost derived from microalgae drying. This well-known technology, widely used with manures, municipal organic solid waste and sewage sludge, has gained more attention for processing microalgae in recent years [2] and [6]. Another driving force behind AD is the possibility of recycling nutrients for microalgae cultivation; microalgae can uptake nutrients from the aqueous phase recovered after digestion [5], [10] and [11].

In spite of the differences, biodiesel and methane production can be complementary. The organic solvents can break the cell walls during the lipid extraction from microalgae [12] and [13], acting as a pre-treatment before AD. The lipid extraction also mitigates the inhibitory effect of high concentration of lipid on AD [14]. Hexane is one of the solvents commonly used in the commercial extraction of edible lipid, and in the extraction of omega-3 LC-PUFA from microalgae [15]; it is inexpensive and offers high efficiency and suitability for industrial processes [1]. High extraction yields have been reported using polar solvents or mixtures, but most of the mixtures contain halogenated solvents considered carcinogenic [15]. Methanol:hexane (2:3 v:v) proved to be one of the best non-halogenated polar mixtures for the lipid extraction from of *Nannochloropsis sp.* [16]. After the extraction, the AD of the microalgae generates methane which can be used for electricity and/or heat in the biodiesel production [2]. An increasing number of publications recently appeared showing the benefits of coupling both processes [17].

The anaerobic co-digestion (Aco-D) of microalgae is another favourable option to convert microalgae into methane, mainly due to the unbalanced C to N ratio (C:N) in microalgae [2], [6], [18] and [19]. Unlike AD, the co-digestion processes mixtures of substrates, resulting in a more favourable C:N. The sewage sludge is one of the most widely used co-substrates; it is generated in wastewater treatment plants (WWTP), available in large quantities and suitable for AD. The over-sized digesters in WWTP promote the utilisation of sewage sludge in co-digestion as well [20]. Sewage sludge has been co-digested with some microalgae species [21], [22] and [23], but no results were reported using *Nannochloropsis*.

The present study considers various strategies to produce methane from *Nannochloropsis* and *Chlorella* species. Firstly, the influence of the substrate to inoculum ratio (SIR) on AD was evaluated for both microalgae species. The SIR markedly affects the performance of the digesters, depending on the species and characteristics of microalgae [12], [14] and [24]. The next experiments were performed applying the lipid extraction as pre-treatment to improve AD, using hexane and methanol:hexane (2:3 v:v). The lipids recovered from the pre-treatment were

converted into biodiesel, and the influence of solvents was evaluated. Finally, the last strategy consisted in the co-digestion of microalgae and sewage sludge.

2. Materials and methods

2.1 Materials

2.1.1 Microalgae, inoculum and sewage sludge

Nannochloropsis oculata and *Chlorella vulgaris* were provided by AlgoSource's (Alpha Biotech, Asserac, France). *N. oculata* was received as frozen slurry with 28% total solids, thus stored at - 15 °C until required. *C. vulgaris* was received dried, thus stored in a desiccator. Both microalgae are hereinafter referred to as "raw" biomass.

The inoculum utilised in AD experiments consisted of digested sludge obtained from a mesophilic pilot-plant (33 °C) under semi-continuous operating conditions. Prior to setting the experiments, the inoculum was "degassed" as described in Caporgno et al. [23]. The sewage sludge utilised for feeding the pilot-plant and the batch digesters in co-digestion consisted of a primary and secondary-sludge blend (65:35 v/v), collected from the municipal WWTPs in Reus (Tarragona, Spain). This blend is representative of the sludge generated in the WWTPs.

2.2 Experimental procedure

2.2.1 Pre-treatment with organic solvents: lipid extraction

Before the pre-treatment, *N. oculata* was freeze-dried (FT33-A Freeze Drier, Armfield Inc.); *C. vulgaris* drying was unnecessary. For the pre-treatment, 2 g of dried microalgae were extracted in a Soxhlet apparatus with a reflux period of 7 h. Hexane and methanol:hexane (2:3 v:v) were utilised as solvents. The recovered lipids were converted into biodiesel, identified and quantified according to the procedure described by Olkiewicz et al. [25]. The pre-treated microalgae were anaerobically digested. Prior to AD, the biomass was left unstoppered in a hood for several hours until complete evaporation of the remaining solvent.

2.2.2 Anaerobic digestion experiments

Batch reactors were set up at 33ºC following the procedure described by Angelidaki and Sanders for the determination of methane potential [26]. The substrates differed in the experiments. The microalgae samples were re-suspended in deionised water before digestion, resulting in a solid concentration similar to sewage sludge.

The SIR experiments were performed using only raw microalgae as substrates. The SIRs were set at 1:4, 1:2 and 1:1 $VS_{Substrate}$:$VS_{Inoculum}$, where VS is the volatile solid content in substrates and inoculum.

The effects of the pre-treatment were evaluated using pre-treated and raw microalgae. Since *N. oculata* was freeze-dried before the pre-treatment, the possible effects of the drying process were evaluated using freeze-dried microalgae. The SIR was 1:2 $VS_{Substrate}$:$VS_{Inoculum}$ in all reactors.

Co-digestion was performed using mixtures of raw *N. oculata* and sewage sludge. The mixtures contained 25%, 50% and 75% sewage sludge on a VS basis. The SIR was 1:2 $VS_{Substrate}$:$VS_{Inoculum}$ in all reactors.

2.3 Analytical techniques

2.3.1 Substrate characterisation

Total solids (TS), volatile solids (VS) and chemical oxygen demand (COD) were analysed according to standard methods 2540B, 2540E and 5220D respectively [27]. Protein, carbohydrate and lipid content in raw and pre-treated biomass were quantified as described in [23]. The characteristics of the inoculum and the substrates are summarised in Table 1. The TS and COD values for raw microalgae correspond to the microalgae suspensions in deionised water used in the experiments.

Raw and pre-treated microalgae samples were analysed by Fourier Transform Infrared (FTIR) spectroscopy using a Fourier Jasco FT/IR-600 Plus spectrometer with a diamond golden gate ATR (GS10542, Specac Ltd) reflectance cell.

Table 1. Characteristics of the inoculum and the substrates used in the anaerobic digestion experiments.

Parameter	Inoculum	Sludge	*Nannochloropsis oculata*			*Chlorella vulgaris*		
			Raw[a]	Hex[b]	Met:Hex[c]	Raw[a]	Hex[b]	Met:Hex[c]
TS (g/L)	14.8±0.1	33.2±0.1	37.0±0.0	-	-	32.8±0.4	-	-
VS/TS	0.63	0.83	0.90	0.88	0.88	0.81	0.76	0.74
COD (mg_{O2}/L)	13600±280	42000±400	51000±900	-	-	35500±300	-	-
C:N	-	13.88	5.36	-	-	7.44	-	-
Proteins (g/100g_{VS})	-	-	60.9±3.4	67.6±4.9	85.9±2.1	48.7±4.0	49.6±1.5	73.8±3.3
Lipids (g/100g_{VS})	-	-	21.9±0.4	13.8	-	6.5±0.6	3.4	-
Carbohydrates (g/100g_{VS})	-	-	16.7±1.5	18.7±1.8	14.1±2.2	41.2±0.7	42.5±1.5	26.2±2.4

[a] *Suspension in deionised water.*
[b] *Pre-treated using hexane.*
[c] *Pre-treated using methanol:hexane.*

2.3.2 Products characterisation

The biogas production and its composition, and the volatile fatty acid concentration (VFA) were measured following the procedure described in [23]. The ammonia concentration was determined with an ion selective electrode (Ammonia Gas Sensing combination electrode, mod. 51927-00, HACH), an expressed as ammonium nitrogen.

The first order hydrolysis model [26] was used for hydrolysis rate calculation, equation (1):

$$\ln \frac{B_0 - B}{B_0} = -k_h \cdot t \qquad \text{(Eq. 1)}$$

where B is the cumulative methane yield at the time t (units: mL_{CH4}/g_{VS}), kh ($days^{-1}$) is the first order hydrolysis constant and B_0 (mL_{CH4}/g_{VS}) is the ultimate methane production. The values of kh and B0 were determined in MS Excel 2007® using the Solver tool, by minimising the residual sum of squared errors between the experimental data and the data predicted by the model.

The theoretical methane potential was calculated based on the relative fractions of lipid, protein and carbohydrate in the substrates, and assuming the specific methane yields of 1014 mL_{CH4}/g_{VS}, 496 mL_{CH4}/g_{VS}, 415 mL_{CH4}/g_{VS} for

lipid, protein and carbohydrate respectively [26]. The biodegradability was then calculated considering the measured and the theoretical methane production, equation (2):

$$\text{Biodegradability}(\%) = \frac{\text{measured methane production}}{\text{theoretical methane potential}} \times 100 \qquad \text{(Eq. 2)}$$

Microalgae cells before and recovered after AD were observed under light microscope to evaluate the integrity of the cells (ZEISS Axio Scope.A1, with ProgRes® SpeedXT core 3 camera).

3. Results and discussion

3.1. The influence of the SIR on anaerobic digestion

The influence of the SIR on AD was evaluated in order to determine the most suitable SIR for the experiments. The ultimate methane productions and the kh for *N. oculata* and *C. vulgaris* are listed in Table 2.

Table 2. Ultimate methane productions and kh during AD of *N. oculata* and *C. vulgaris* at different SIR.

			SIR		
			1:4	1:2	1:1
Substrate	*N. oculata*	CH_4 mL_{CH4}/g_{VS}	278±3	275±4	282±9
		kh[(a)] ($days^{-1}$)	0.31 (0.91)	0.35 (0.93)	0. 19 (0.99)
	C. vulgaris	CH_4 mL_{CH4}/g_{VS}	229±12	223±4	222±3
		kh[(a)] ($days^{-1}$)	0.36 (0.98)	0.36 (0.99)	0.37 (0.71)

[a] *In brackets, the values of* R^2.

The SIR did not affect the ultimate methane production from *N. oculata* and *C. vulgaris*; the differences were smaller than 3% in the experiments using the same microalgae species. On the contrary, the kh was affected differently by the SIR for both microalgae. For *N. oculata*, the hydrolysis rate accelerated when the SIR increased from 1:4 to 1:2 but it slowed down when the SIR increased until 1:1. The kh depends on the methane production throughout the first days of the experiment, and the SIR can considerably influence the methane production during these days [12], [14] and [24]. The substrate concentration abruptly increased from low concentration in the "degassed" inoculum to high concentration after setting up the reactors at SIR 1:1, which caused a stress response in bacteria. The kh suggests that VFA accumulated and were not efficiently converted into methane. The methane yields at the end of the experiment indicated that the VFA accumulation did not cause inhibition because of the possibility of bacteria to adapt to different environmental conditions over time [2]. However, SIR higher than 1:1, could lead to higher VFA concentration and inhibition of the process. *N. oculata* was characterised by high protein content (Table 1), which can cause inhibition by ammonia [2]; inhibition by ammonia was dismissed based on its concentration at the end of the experiment. Regarding *C. vulgaris*, the kh was unaffected by the SIR. The presence of aggregates in *C. vulgaris* reduced the interaction between microorganisms and microalgae, thus the amount of substrates readily available for microorganisms was lower than in the reactors with *N. oculata*. The SIR threshold which affects AD depends on the microalgae and their characteristics [12], [14] and [24].

Microalgae were observed under a light microscope before and after digestion. Fig. 1a shows *N. oculata* after thawing. Microalgae cells with a similar appearance were recovered from digestate at the end of the experiment

(Fig. 1b). Regarding *C. vulgaris*, Fig. 1c reveals the presence of microalgae aggregates before digestion. The aggregates originated during microalgae drying were also visible after digestion (Fig. 1d). The resistance of several *Chlorella* species to anaerobic digestion and the presence of intact cells after AD were reported by others [28]. *Chlorella* species presents stronger cell walls than *Nannochloropsis*, requiring powerful pre-treatment to enhance the biodegradability [3]. Furthermore, *C. vulgaris* aggregates may hamper the interaction with microorganisms.

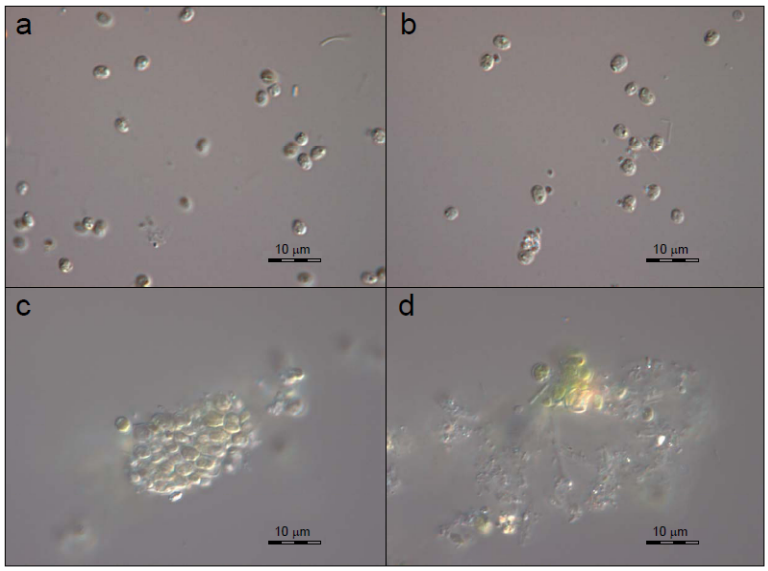

Figure 1. Light micrographs of raw *N. oculata* (a) and recovered from the digestate (b), and raw *C. vulgaris* (c) and recovered from the digestate (d).

The pH at the end of the experiments was close to neutrality, ranging 7.35-7.68. Ammonia concentration ranged 677-836 mg/L, whereas 1500 mg/L is considered as threshold for inhibition [2]. Inhibition during AD results in VFA accumulation and decrease in pH [2]; however, the concentrations of VFA were under the detectable limit of the analysis, indicating no signs of inhibition. Biogas composition was analysed periodically and the methane content was between 71%-74%; there was not a relationship between the methane content and the substrate or the SIR.

The results indicate that the SIR is important parameter in anaerobic digestion and its effects over anaerobic digestion depend on the substrate characteristics. For the AD of microalgae, the SIR evaluation may prevent anaerobic digestion failure mainly due to the high protein content. Based on the results, the SIR 1:2 $VS_{Substrate}$:$VS_{Inoculum}$ was decided for all the experiments. The presence of *C. vulgaris* aggregates suggested the need of pre-treatment before AD, thus the microalgae was not used in the Aco-D experiments.

3.2. Microalgae pre-treatment and biodiesel production

Lipid extraction and biodiesel production yields are presented in Table 3. The main purpose of the lipid extraction was the microalgae pre-treatment; although the lipids were converted into biodiesel, the feasibility of biodiesel production was not evaluated in the present studio. The microalgae obtained after the pre-treatment may represent the waste generated in a wide variety of processes employing organic solvents. The pre-treatment may offer additional advantages if high-value by-products are obtained.

Table 3. Lipid extraction yields, biodiesel yields and fatty acids composition.

Fatty acids	*Nannochloropsis oculata*		*Chlorella vulgaris*	
	Hex	Met:Hex	Hex	Met:Hex
Lipid extraction yield ($g/100g_{VS}$)	10.1±0.4	48.1±2.6	4.7±0.9	17.6±0.2
Biodiesel yield ($g/100g_{VS}$)	5.0±0.1	12.6±0.2	2.4±0.1	4.5±0.1
Biodiesel composition ($g/100g_{sample}$)				
Myristic (C14:0)	3.7±0.1	4.1±0.1	0.2±0.1	-
Palmitic (C16:0)	6.6±0.1	14.3±0.1	21.9±0.2	24.2±0.2
cis-9 Palmitoleic (C16:1)	19.8±0.2	25.0±0.1	9.2±1.0	12.1±0.1
Cis-10-heptadecenoic (C17:1)	-	-	12.1±0.1	10.4±0.1
Oleic (C18:1)	1.9±0.1	2.0±0.1	8.3±0.1	8.1±0.1
Linoleic (C18:2)	1.3±0.1	1.4±0.1	8.2±0.1	8.2±0.1
Linolenic (C18:3)	0.9±0.1	1.0±0.1	26.2±0.1	24.1±0.1
cis-11,14-Eicosadienoic (20:2)	3.2±0.1	3.4±0.1	-	-
cis-5,8,11,14-Eicosatetraenoic (20:4)	5.2±0.1	3.9±0.1	-	-
cis-5,8,11,14,17-Eicosapentaenoic (20:5)	49.9±0.1	38.7±0.4	-	-
Others	7.6±0.1	6.1±0.1	13.0±0.1	13.8±0.1

The highest lipid extraction yields were obtained using the polar mixture. From *N. oculata*, the lipid extractions yielded 48.1±2.6 and 10.1±0.4 $g/100g_{VS}$ using methanol:hexane and hexane respectively; from *C. vulgaris*, 17.6±0.2 and 4.7±0.9 $g/100g_{VS}$ respectively. The polar mixture led to the highest biodiesel yields as well; biodiesel increased from 5.0±0.1 to 12.6±0.2 and $g/100g_{VS}$ in *N. oculata* and from 2.4±0.1 to 4.5±0.1 $g/100g_{VS}$ in *C. vulgaris* using the polar mixture. It is widely known that polar solvents or their mixtures lead to higher extraction yields than the non-polar ones. Polar solvents extract polar lipids such as phospholipids and glycolipids [14], [15] and [16] and also polar components such as proteins and carbohydrates; these latter are not lipid but increase the lipid extraction yield significantly [29]. Since the major part of these polar components fails transesterification, the biodiesel yield was not as high as expected based on the lipid extraction yields (Table 2). Fig. 2 shows the spectra obtained during FTIR analyses of raw and pre-treated *N. oculata*. The spectrum after the extraction using hexane confirms the reduction in the lipid content; the absorption bands at 1700-1750 cm^{-1} characteristics of C=O groups in lipid esters and the absorption bands at 2,800-3,000 cm^{-1} characteristics of CH_2 and CH_3 groups in lipid acyl chains [30] are less intensified than in raw microalgae spectrum. These absorption bands are much lower intensified in the spectrum after the extraction using methanol:hexane.

On the other hand, the biodiesel composition was unaffected by the solvents. The biodiesel profile for *N. oculata* showed high content of C16:0, C16:1 and C20:5 using both solvents. A similar profile was reported by Balasubramanian et al. using the same polar mixture [16]. These fatty acids were also the most predominant in the biodiesel produced from the same microalgae species using ionic liquids [7]. Regarding the profile for *C. vulgaris*, it was similar to the profile reported for the biodiesel from vegetable oils [31]; C16:0, C18:0, C18:1, C18:2 and C18:3 were predominant in *C. vulgaris*, as reported by other authors [32]. When comparing the biodiesel from both microalgae species, the main difference lies in the high content of the polyunsaturated fatty acid C20:5 in *N. oculata*. The C20:5 is associated with a decrease in the oxidative stability of the biodiesel [7]

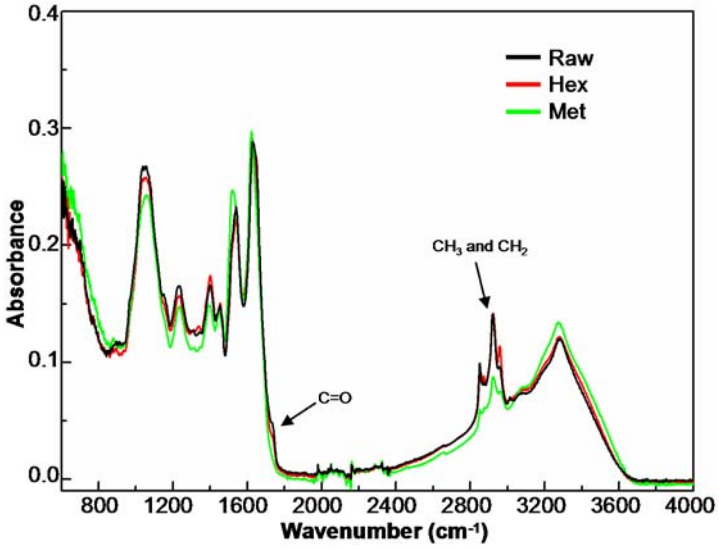

Figure 2. FTIR spectra of Raw *N. oculata* (Raw) and pre-treated, using hexane (Hex) and methanol-hexane (Met).

3.3. Anaerobic digestion of pre-treated microalgae

3.3.1. Nannochloropsis oculata

The methane production curves from raw and pre-treated *N. oculata* are presented in Figure 3a. It stands out the curve obtained from the microalgae pre-treated using hexane; the methane yield was considerably higher than the yield obtained from raw microalgae. The ultimate methane production from the raw *N. oculata* was 253±11 mL_{CH4}/g_{VS}, similar to the value reported in the literature [2]. The pre-treatment with hexane increased the methane production until 313±9 mL_{CH4}/g_{VS}; whereas the polar mixture seemed not to influence the methane production.

The biodegradability depends on both the theoretical and the measured methane production (Eq. 2), being the theoretical methane calculated from the substrates composition. The theoretical methane production significantly decreased in pre-treated microalgae; the lipid content decreased and they almost double proteins and carbohydrates in terms of theoretical methane production. However, the measured methane production from pre-treated microalgae was similar or higher than from raw microalgae. As a consequence, the biodegradability resulted in 57% and 52% for the pre-treated microalgae using hexane and the polar mixture respectively, higher than the 42% calculated for raw microalgae. Hexane degraded some compounds present in the microalgae or disrupted the cell walls, resulting in higher methane production and enhanced biodegradability [13]. The high extraction yields with polar solvents entailed significant changes in the biomass composition which affect the methane production [14,29]. These changes in composition may hide the disruption caused by the solvents; the biodegradability increased but the methane production decreased compared with raw microalgae [14].

The kh was calculated (Eq. 1) for the different substrates. It resulted in 0.40 day^{-1} (R^2=0.97) for raw microalgae, and 0.31 day^{-1} (R^2=0.99) and 0.21 day^{-1} (R^2=0.99) for the pre-treated microalgae using hexane and methanol:hexane respectively. These values indicated that the pre-treatment decreased the methane production throughout the first days of the experiment. However, all reactors had exceeded 80% of the ultimate methane production by the 8th day, thus most of the substrate was degraded at that time. Since the same SIR was set in all the reactors, the decrease in kh can not be attributed to a stress response as it was explained in section 3.1. In this case, the values indicated that raw microalgae were easily digested than pre-treated ones. Different compounds

were extracted in the pre-treatment, thus different compounds remained in the pre-treated microalgae. Possibly the pre-treatment extracted some easily-degradable components or it changed the characteristics of some of them, resulting in slow degradation.

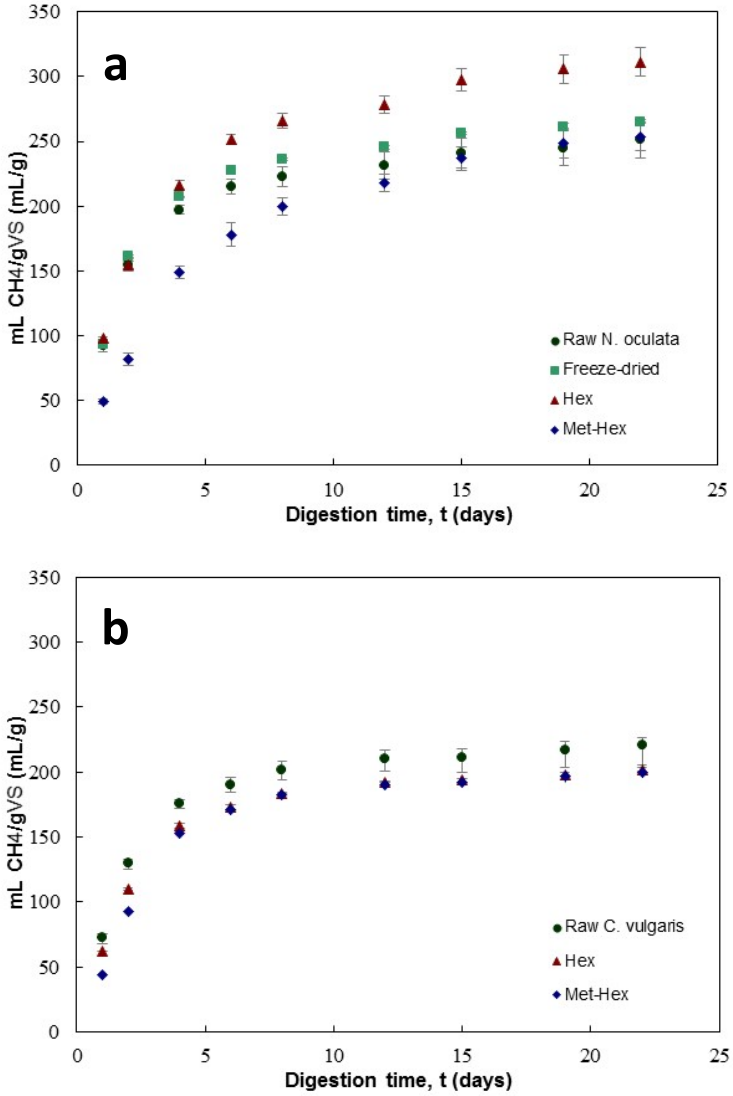

Figure 3. The methane production curves from a) *N. oculata* and b) *C. vulgaris*. Symbols: ● raw, ■ freeze-dried and pre-treated, using ▲ hexane and ♦ methanol-hexane. Batch reactors at 33ºC and SIR of 1:2 $VS_{Substrate}$:$VS_{Inoculum}$.

Regarding the effects of drying, the methane production yielded 265±3 mL_{CH4}/g_{VS} after freeze-drying. The methane production curve was similar to the curve for raw microalgae (Figure 2a), which indicated the absence of adverse effects. Adverse effects are mainly attributed to microalgae heating in the drying process [22,33], but heating was avoided during *N. oculata* drying.

The pH, ammonia and VFA concentrations at the end of the experiments confirmed that all digesters operated at optimum conditions. The digesters exhibited no signs of inhibition in spite of the solvent utilisation; the presence of residual organic solvents can entail the failure of the digester [14], [29] and [34]. Periodical analysis of the biogas composition revealed that the methane content was between 70% and 73% in all reactors.

3.3.2. Chlorella vulgaris

The methane production curves from raw and pre-treated *C. vulgaris* are presented in Figure 3b. The methane production yielded 219±6 mL_{CH4}/g_{VS} from raw microalgae and decreased around 8-9% after the pre-treatment. Contrary to *N. oculata*, both solvents negatively affected the methane production from *C. vulgaris*. The theoretical methane production barely decreased after the pre-treatment due to the low lipid content in raw *C. vulgaris* (Table 1); thus the biodegradability resulted in 44% for raw microalgae and 42% for the pre-treated microalgae. The analysis of kh confirmed that the pre-treatment does not accelerate the digestion of microalgae; kh were 0.39 day^{-1} (R^2=0.99) for raw microalgae, and 0.36 day^{-1} (R^2=0.99) and 0.32 day^{-1} (R^2=0.99) for the microalgae pre-treated using hexane and methanol:hexane respectively. As observed during *N. oculata* AD, part of the easily-degradable components may be extracted during the pre-treatment, decreasing kh.

The analysis performed at the end of the experiment confirmed that all digesters operated at optimum conditions and exhibited no signs of inhibition. The biogas composition ranged between 69% and 74% CH_4 in all reactors.

3.4. Anaerobic co-digestion of microalgae and sewage sludge

The Aco-D of *N. oculata* and sewage sludge was evaluated as an option to improve AD. Figure 4 shows the methane production curves from *N. oculata*, sewage sludge and their mixtures.

The sewage sludge and the microalgae produced the highest and the lowest methane yields respectively. The mixtures showed yields between these extremes. In fact, the methane production from the mixtures could be calculated based on the relative fractions of microalgae and sewage sludge, and their methane productions. The values obtained in this way were slightly higher than the calculated productions; however, the differences were less than 5%, being not statistically significant to suggest synergy. All the substrates were similarly degraded and the methane production by the 7th day had exceeded 80% of the ultimate methane production. These results are in agreement with the reported for other microalgae species and sewage sludge [23].

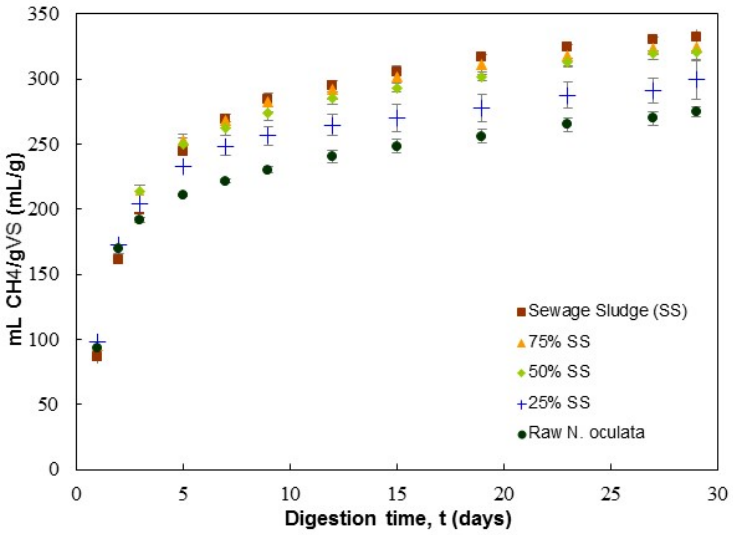

Figure 4. The methane production curves from sewage sludge and *N. oculata* co-digestion. Symbols: ● raw microalgae, + 25% sewage sludge ♦ 50% sewage sludge, ▲ 75% sewage sludge and ■ 100% sewage sludge. Batch reactors at 33ºC and SIR of 1:2 $VS_{Substrate}$:$VS_{Inoculum}$.

The C:N during digestion was 5.36, far from the range 20-25 considered as optimal for AD [18,19,22]. The C:N in sewage sludge was 13.88, thus the addition of sewage sludge did not increase the C:N significantly. The synergistic effects observed during the Aco-D of microalgae and sewage sludge or other co-substrates have been attributed to the nutrients provided to microorganisms, more than to the C:N balance [19,22]. Regarding the Aco-D of *Nannochloropsis* species, the synergetic effects were also attributed to the essential elements supply or an enhanced alkalinity [18]. In the present experiments, the addition of synthetic medium containing micronutrients, vitamins and trace metals [26] may hide the benefits of the Aco-D. The lack of synergy may be also a consequence of the microalgae characteristics [22,23].

In spite of the results mentioned above, the absence of inhibitory effects during Aco-D is favourable for a possible the integration of phycoremediation and anaerobic digestion. The integration of processes, growing microalgae for wastewater treatment and energy production, can be economically and environmentally beneficial [10,19,22]. The results demonstrate that this integration may be possible. *Nannochloropsis* species can consume nutrients from wastewaters during growth [5,35]; furthermore, digesters in WWTP are over-sized, being the non-used capacity approximately 30% [20].

4. Conclusions

The SIR reveals that AD of *N. oculata* can present inhibition if this ratio is higher than 1:1. The SIR 1:1 causes stress in the digesters, decreasing the kh, but does not affect the ultimate methane production. On the contrary, the SIR does not affect *C. vulgaris* AD as consequence of the presence of aggregates in the biomass.

Although the pre-treatment with both solvents increases *N. oculata* biodegradability, only the pre-treatment using hexane increases the methane production. On the other hand, *C. vulgaris* requires stronger pre-treatment methods. The polar solvent increases the lipid and the biodiesel yields from both species, but does not affect the biodiesel composition.

Regarding Aco-D, the C:N increase in the reactors does not affect the methane production. However, the absence of inhibitory effects suggests that *N. oculata* and sewage sludge could be digested in the same reactor.

ACKNOWLEDGEMENTS

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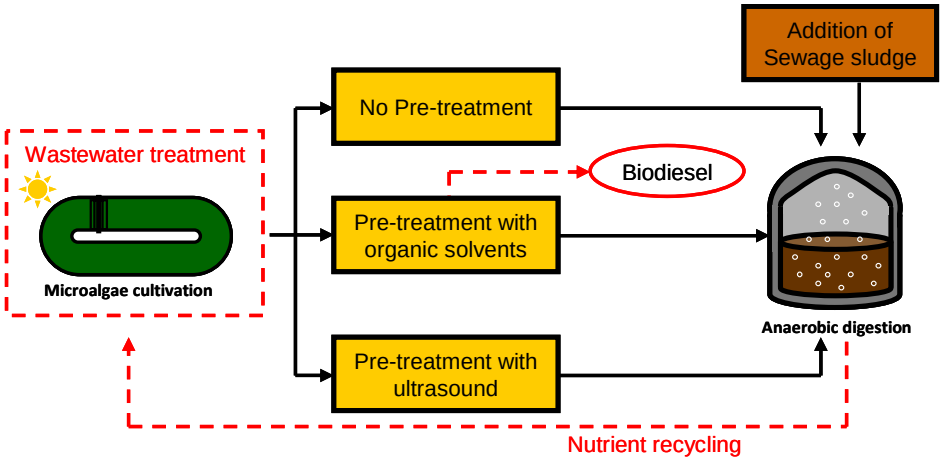
Chapter 4

Effect of pre-treatments on the production of biofuels from Phaeodactylum tricornutum [1]



ABSTRACT

Several characteristics make *Phaeodactylum tricornutum* potential candidate for biofuels production such as methane and biodiesel. For this reason, some alternatives are evaluated in this manuscript to improve the conversion of this microalgae into methane.

One of these alternatives is the addition of sewage sludge to *P. tricornutum* for anaerobic co-digestion. Although the co-digestion resulted in lack of synergy, the absence of inhibition indicated that both substrates could be co-digested under certain circumstances, for example if microalgae are cultivated for wastewater treatment purposes.

The extraction of lipids using organic solvents has been evaluated for biodiesel production but also as a pre-treatment for anaerobic digestion. The results revealed that the type of solvent influences lipid and biodiesel yields. The high polarity of the mixture methanol/hexane increased the lipid and the biodiesel yields from 10±1 to 53±2 $g_{Lipids}/100\ g_{VS}$ and from 7±1 to 11±1 $g_{Biodiesel}/100\ g_{VS}$ compared with hexane. However, none of these solvents affected the composition of biodiesel. Regarding the methane production after the extraction, it yielded 257±8 and 180±6 mL_{CH4}/g_{VS} from lipid-extracted *P. tricornutum* using hexane and methanol/hexane respectively. The methane production from the raw microalga was 258±5 mL_{CH4}/g_{VS} in the same experiment. The difference in methane production, mainly after the extraction with methanol/hexane, was a consequence of the changes in the composition of the microalgae after extraction. The extraction did not influence the biodegradability.

The ultrasonic pre-treatment prior anaerobic digestion completely disrupted the microalgae cells, but the solubilisation of the organic fraction was scarce (<9.5%). The methane production from pre-treated samples was barely 10-11% higher than the obtained from non pre-treated samples, indicating that the refractory nature of the organic fraction in *P. tricornutum* is the main obstacle for the methane production.

[1] M. Caporgno, M. Olkiewicz, C. Torras, J. Salvadó, E. Clavero, C. Bengoa, Effect of pre-treatments on the production of biofuels from Phaeodactylum tricornutum, Journal of Environmental Management. Accepted for publication.

1. Introduction

In a wide variety of microalgae species considered as promising feedstocks for renewable biofuels, the seawater *Phaeodactylum tricornutum* presents several advantages: cultivation at commercial scale, high biomass productivities and accumulation of lipids [Silva Benavides et al., 2013; Bellou et al., 2014; Song et al., 2014; Davis et al., 2015; Vinayak et al., 2015]. Unfortunately, the scarce literature about methane production from *P. tricornutum* reports that the species present low degradability during anaerobic digestion (AD) [Zamalloa et al., 2012; Frigon et al., 2014; Zhao et al., 2014].

The low degradability of microalgae is usually attributed to several causes, one of which is the low C:N. The low ratio lead AD to fail due to the high concentration of ammonia generated, but it can be overcome by mixing microalgae and carbon-reach substrates. Several substrates have been co-digested with microalgae [Schwede et al., 2013; Wang et al., 2013; Olsson et al., 2014; Prajapati et al., 2014; Caporgno et al., 2016]. Sewage sludge has shown synergy when co-digested with some microalgae species [Wang et al., 2013; Olsson et al., 2014; Mahdy et al., 2015]. The over-sized digesters in wastewater treatment plants (WWTP) [Mata-Alvarez et al., 2014] and the possibility of recycling nutrients by growing *P. tricornutum* in wastewaters [Davis et al., 2015] are additional reasons to investigate the co-digestion of this species and sewage sludge.

The low degradability can be also consequence of strong cell walls, which hamper the microorganisms attack. Pre-treatments, such as the ultrasonic, can break the cell walls, release internal compounds and increase the methane production [González-Fernández et al., 2012; Alzate et al., 2014]. However, the effects of pre-treatment on the degradability of *P. tricornutum* have not been evaluated yet. Furthermore, organic solvents used for the extraction of lipids in the biodiesel production process can act as pre-treatment increasing the degradability of the microalgae waste [Alzate et al., 2014; Ramos-Suárez and Carreras, 2014; Caporgno et al., 2016]. Although some authors reported the AD *P. tricornutum* after lipid-extraction [Frigon et al., 2014; Zhao et al., 2014], the extraction was not always performed using organic solvents. More information about the influence of the solvents is necessary.

This communication attempts to show some preliminary results about the possibility of coupling the AD of *P. tricornutum* with other processes like the sewage sludge treatment in WWTP. For this reason, the first option considers the possibility of co-digest microalgae and sewage sludge. The influence of the substrate to inoculum ratio (SIR) was evaluated for both substrates, and then, different mixtures of substrates were co-digested. Furthermore, the possibility of increasing the degradability of microalgae was evaluated by applying an ultrasonic pre-treatment at varying intensities.

A second option considers the lipid-extraction for biodiesel production and as pre-treatment for AD. The effects of different solvents on biodiesel yields and composition, and methane productions from lipid-extracted microalgae have been evaluated in these experiments.

2. Materials and methods

2.1 Materials

2.1.1 Microalgae, inoculum and sewage sludge

The marine microalgae *Phaeodactylum tricornutum* Bohlin (strain CCAP 1055/5) were obtained from the Culture Collection of Algae and Protozoa (CCAP). The cultivation was started in 200 mL culture and scaled up through 4

L cultures, using seawater (37 g/L salinity) filtered through 0.22 µm, enriched with Walne's medium [Walne, 1970] and autoclaved. Cultures were kept at 22±2 °C, illuminated (16:8 light: dark cycle) with cool daylight fluorescents (Osram L30W/865) to give an irradiance of 100-140 µE/m2s, and aerated with air. For 300 L culture, a vertical bag was used as photobioreactor. Seawater was filtered through four filter cartridges with 25, 10, 5 and 1 µm pore sizes and treated with UV light to eliminate biological contamination, and then enriched with 0,3 mL/L of the commercial fertilizer Codafol 14-6-5 and 107 µM Na2SiO3. Cultures were kept at 22±2 °C, illuminated (16:8 light: dark cycle) with cool daylight fluorescents (Philips TLD 58W/865) to give an irradiance of ca. 200 µE/m2s at the culture surface, and aerated with air. Microalgae were then concentrated to approximate 70 gTS/L in a continuous centrifuge. Microalgae were stored in a freezer at -20 ºC until utilisation.

The sewage sludge consisted of a primary and secondary-sludge blend (65:35 v/v), collected from the municipal WWTPs in Reus (Tarragona, Spain). Regarding the inoculum, it consisted of digested sludge taken from an anaerobic semi-continuous plant as described in a previous work [Caporgno et al., 2015].

2.2 Experimental procedure

2.2.1 Biomass processing

The first experiments consisted in the co-digestion of microalgae and sewage sludge. For co-digestion, mixtures of both substrates containing 25%, 50% and 75% sewage sludge on a VS basis were fed into the reactors.

In the following experiments, the lipids from microalgae were first extracted and converted into biodiesel, and the remaining microalgae was converted into methane. The lipid extraction was performed using hexane and methanol/hexane in ratio (2:3 v:v), following the procedure detailed in [Caporgno et al., 2016]. Since the microalgae were dried using freeze-drying equipment (FT33-A Freeze Drier, Armfield Inc.) prior extraction, the dried microalgae were digested to evaluate the effects of drying on AD.

The ultrasonic pre-treatment was carried out using an ultrasonic device (UP200S Hielscher Ultrasonics GmbH, Germany) at 24 kHz working frequency and 93 W ultrasonic power. The samples were disintegrated at room temperature in a water bath to avoid heating the sample. Three energy inputs were evaluated, 21 MJ/kg_{TS}, 36 MJ/kg_{TS} and 52 MJ/kg_{TS}. The Disintegration degree (Dd) was measured by the soluble COD increase:

$$Dd = \frac{(SCOD - SCOD_0) \cdot 100}{TCOD_0 - SCOD_0} \qquad \text{(Eq. 1)}$$

where SCOD is the soluble COD; $SCOD_0$ represents the values of soluble COD before the disintegration treatment; $TCOD_0$ represents the values of total COD before the disintegration treatment.

Microalgae samples were observed under a light microscope (ZEISS Axio Scope.A1, with ProgRes® SpeedXT core 3 camera) to evaluate the effects of ultrasonic pre-treatment on the microalgae cells.

2.2.2 Anaerobic digestion experiments

Batch reactors were set up at 33 ºC following the procedure described in [Angelidaki et al. 2009]. The effects of the SIR were evaluated using raw microalgae and sewage sludge as substrates; the SIRs were set at 1:4, 1:2 and 1:1 $VS_{Substrate}$:$VS_{Inoculum}$, where VS is the volatile solid content in substrates and inoculum. Based on the results, the SIR was decided at 1:2 $VS_{Substrate}$:$VS_{Inoculum}$ for the experiments using lipid-extracted and ultrasonic pre-treated microalgae.

The first order hydrolysis model was used to determine the hydrolysis constant, kh (1/day) [Caporgno et al., 2016]. The theoretical methane potential was calculated based on the biochemical composition of the substrates, and assuming the specific methane yields of 1014 mL_{CH4}/g_{VS}, 496 mL_{CH4}/g_{VS}, 415 mL_{CH4}/g_{VS} for lipid, protein and carbohydrate respectively [Caporgno et al., 2016]. The biodegradability was defined by the following equation:

$$\text{Biodegradability}(\%) = \frac{\text{measured methane production}}{\text{theoretical methane potential}} \times 100 \qquad \text{(Eq. 2)}$$

2.2.3 Analytical techniques

Total solids (TS), volatile solids (VS) and chemical oxygen demand (COD) were analysed according to standard methods 2540B, 2540E and 5220D respectively [Rice et al., 2012]. The soluble COD (SCOD) was measured following the same procedure that for COD, but the sample consisted of the supernatant after centrifugation. The biochemical composition of microalgae was determined according to the Lowry method for protein determination [Lowry et al., 1951], phenol-sulphuric acid method for sugars determination [Dubois et al., 1956] and the Bligh and Dyer method for lipids determination [Bligh and Dyer, 1959]. The characteristics of the inoculum, the sewage sludge and the microalgae are summarised in Table 1.

Table 1. Characteristics of the inoculum and the substrates.

Parameter	Sludge	Inoculum	*Phaeodactylum tricornutum*		
			Raw	Hexane	MeOH/hexane
TS (g/L)	33.1±0.3	14.5-17.4 [(a)]	66.8±0.1	-	-
VS/TS	0.82	0.63-0.64 [(a)]	0.76	-	-
COD (g O_2/L)	39.5±0.2	17.8±0.1 [(a)]	36±1.3	-	-
C:N	13.88	-	6.06	-	-
Proteins ($g/100g_{VS}$)	-	-	60±1	58 [(c)]	70 [(c)]
Carbohydrates ($g/100g_{VS}$)	-	-	19±1	18 [(c)]	30 [(c)]
Lipids ($g/100g_{VS}$)	-	-	36±1 [(b)]	25 [(c)]	0 [(c)]

[(a)] value ranges in all the experiments.
[(b)] quantified following the Bligh and Dyer method.
[(c)] approximate composition based on the raw microalgae composition and extraction yields.

The biogas production, biogas composition, volatile fatty acid and concentration (VFA) and ammonia concentration were measured as described in [Caporgno et al., 2016]. The extracted lipids were quantified gravimetrically and the converted into biodiesel and quantified as described in [Olkiewicz et al., 2014].

3. Results and discussion

3.1. Raw microalgae digestion

3.1.1 Influence of the Substrate to inoculum ratio (SIR)

The SIR influenced the AD of sewage sludge and microalgae differently, as it can be seen in Table 2. The final methane production from sewage sludge was unaffected by the SIR increase from 1:4 to 1:2, but the SIR increase up to 1:1 resulted in a high methane production. The values agree with previous results [Caporgno et al., 2015]. On the other hand, *P. tricornutum* resulted in similar methane production for all the SIR evaluated. These values are around 20-30% lower than the reported in the literature [Zamalloa et al., 2012; Frigon et al., 2014; Zhao et al.,

2014], but the differences can be attributed to differences in microalgae caused by the cultivation conditions [Silva Benavides et al., 2013] or to the differences in the inocula.

Table 2. Final methane productions and kinetic parameters for sewage sludge and microalgae at different SIR. Batch reactors at 33 ºC, 29 days.

			SIR		
			1:4	1:2	1:1
Substrate	SB	mL_{CH4}/g_{VS}	329±15	332±6	363±5
		kh[(a)] (1/days)	0.26 (0.98)	0.27 (0.98)	0.23 (0.99)
	100% Raw	mL_{CH4}/g_{VS}	278±3	275±4	282±9
		kh[(a)] (1/days)	0.31 (0.90)	0.35 (0.93)	0.19 (0.99)

[(a)] In brackets, values of R^2.

Although the highest methane production was obtained at SIR of 1:1 with both substrates, the kinetic parameter kh showed opposite results (Table 2). The kh depends on the methane production throughout the first days of the experiment, which was negatively influenced by the SIR increase. Similar effects were reported during the AD of other microalgae species under comparable conditions [Alzate et al., 2014; Zhao et al., 2014; Caporgno et al., 2016]. The decreased values of kh at high SIR can be attributed to the abruptly change in the concentration of substrate after feeding the reactors. The high substrate concentration after feeding caused a stress response in bacteria, requiring their adaptation [Caporgno et al., 2016]. There is a SIR threshold which leads to inhibition; the VFA produced during hydrolysis are not efficiently converted into methane at this SIR, the pH decreases and the methane production stops [Zhao et al., 2014]. The low kh but the high methane production at the end of the experiment (Table 2) indicate that the VFA accumulated at 1:1, but their concentration was not high enough to cause inhibition and the methane production continued afterwards. Nevertheless, SIR higher than 1:1 could lead to inhibition. Regarding sewage sludge digestion at SIR 1:1, the stress response was not observed because the inoculum was acclimatised to this substrate, as described in section 2.1.1. Based on the results, the SIR 1:2 was set in further experiments. The comparison between both substrates indicates that microalgae digestion produced more methane than sewage sludge throughout the first days of the experiment, in spite of their low methane production at the end of the experiment. Since the same amount of VS was loaded in reactors with the same SIR, these differences are caused the characteristics of the organic fraction. Microalgae have a readily degradable organic fraction easily converted into methane; nonetheless, the major part of the organic fraction resisted degradation during the experiment. On the contrary, the organic fraction from sewage required more time for degradation at the beginning, but the degradation is high.

At the end of the experiment, the pH ranged between 7.20 and 7.42. The ammonium nitrogen increased from 510 mg/L to 760 mg/L when the SIR increased from 1:4 to 1:1, but the levels were lower than the threshold for inhibition [Caporgno et al., 2016]; furthermore, VFA were not detected, confirming the absence of inhibition. The methane content on biogas, analysed several times during the experiments, ranged between 69% and 74% and it was independent of the substrate.

3.1.2. Influence of the co-digestion with sewage sludge

The methane production curves during Aco-D are shown in Figure 1a. At the end of the experiment, the highest and the lowest yields were obtained from sewage sludge and *P. tricornutum* respectively. The higher the addition of sewage sludge, the higher the methane production, but no synergy was observed during co-digestion [Caporgno

et al., 2015; 2016]. The methane production from the mixtures could be calculated based on the relative fractions of microalgae and sewage sludge, and their methane productions. These calculated productions were quite similar to the values measured in the experiments, thus they could be caused by the experimental variation. These results are in agreement with the results reported during the co-digestion of other microalgae species and sewage sludge [Caporgno et al., 2015; 2016; Mahdy et al., 2015].

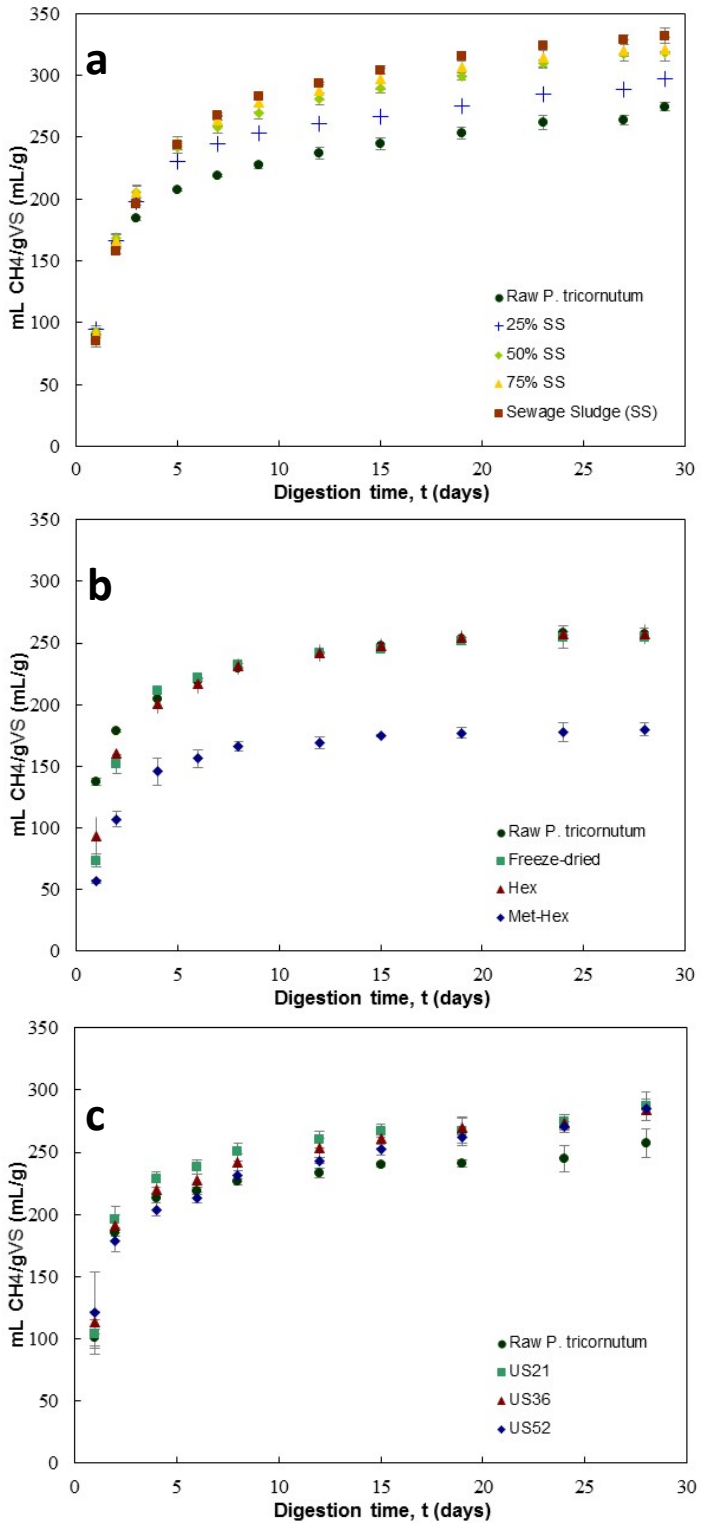


Figure 1. The methane production curves from *P. tricornutum* (a) during co-digestion with sewage sludge, (b) after lipid-extraction using hexane and methanol/hexane, and (c) after ultrasonic pre-treatment applying 21, 36 and 52 MJ/kg$_{TS}$. Batch reactors at 33ºC and SIR of 1:2 VS$_{Substrate}$:VS$_{Inoculum}$.

Regarding the kh, the highest kh was observed in reactors with microalgae and the lowest kh in reactors with sewage sludge, 0.35 (0.93) and 0.27 (0.98) respectively (in brackets the values of R2). During co-digestion, the values of kh were proportional to the composition of the mixtures. All reactors exceeded the 80% of their final methane production after the first week.

The C:N were 6.06 and 13.88 during *P. tricornutum* and sewage sludge digestion respectively (Table 1). Both substrates have the C:N ratio lower than the considered as optimal for anaerobic digestion, which is in the range 20-25 [Prajapati et al., 2014]. The addition of sewage sludge in co-digestion did not increased the C:N significantly. Opposite results are reported in the literature regarding the influence of the C:N on co-digestion; synergy was reported after mixing microalgae and substrates with low C:N [Olsson et al., 2014; Prajapati et al., 2014; Caporgno et al., 2015; 2016] but also no synergy when microalgae were mixed with carbon-reach substrates [Schwede et al., 2013]. The increased methane production in co-digestion has been attributed to a favoured nutrients availability for microorganisms [Olsson et al., 2014; Prajapati et al., 2014] or the more stability in the process too [Schwede et al., 2013]. It is evident that the C:N ratio is not be the primary agent in the *P. tricornutum* digestion. Additionally, the benefits of mixing microalgae and sewage sludge may be hidden by the addition of the synthetic medium according to the methodology or due to the characteristics of the microalgae.

On the other hand, the absence of inhibition during co-digestion suggest the possibility of coupling microalgae cultivation in WWTP for wastewater treatment and their conversion into methane, as reported by other authors [Caporgno et al., 2015; 2016; Mahdy et al., 2015]. This option become even more interesting when considering the availability of over-sized digesters in WWTP [Mata-Alvarez et al., 2014]. The sewage sludge blend utilised in the experiments represents the sludge generated in WWTP, thus minor modification would be required in the facility.

3.2. Microalgae pre-treatments before anaerobic digestion

3.2.1. Lipid extraction

Table 3 summarises the lipid and biodiesel yields, and the composition of the biodiesel obtained from *P. tricornutum* using hexane and methanol/hexane. The highest lipid extraction yields were obtained using methanol/hexane due to the polar characteristics of this mixture [Hernández et al., 2014; Ryckebosch et al., 2014]. Hexane, as other non-polar solvents, extracted mainly non-polar lipids and part of lipids remains in microalgae after the extraction (Table 1) [Frigon et al., 2014; Zhao et al., 2014]. The mixture of methanol/hexane extracts polar lipids, but some non-lipid components are extracted too. These non-lipid components increase the extraction yield but most of them fail transesterification [Ehimen et al., 2009; Hernández et al., 2014; Caporgno et al., 2016]. As can be seen in Table 1, the lipid yield obtained using methanol/hexane exceeded the lipid content in raw microalgae determined following the Bligh and Dyer method due to the presence of non-lipid components. The Bligh and Dyer method uses a polar mixture but includes the addition of water which removes the non-lipid components.

Regarding the biodiesel yields, the yield obtained using methanol/hexane was slightly higher compared to using hexane, but similar to the yield obtained from the lipids extracted with the Bligh and Dyer method (9±2 $g_{Biodiesel}/100g_{VS}$). The result demonstrates that the major part of the compounds extracted with methanol/hexane fails transesterification. On the other hand, the composition of the biodiesel was not affected by the solvents; the

fatty acid profiles were dominated by palmitic (C16:0), palmitoleic (C16:1) and eicosapentaenoic (C20:5), in agreement with the profiles reported in the literature for the same microalgae species [Silva-Benavides et al., 2013; Frigon et al., 2014].

Table 3. Lipid extraction yields, biodiesel yields and biodiesel composition.

Fatty acids	Solvent	
	Hexane	MeOH/hexane
Lipid extraction yield (gLipids/100g_{VS})	10±1	53±2
Biodiesel yield (gBiodiesel/100g_{VS})	7±1	11±1
Biodiesel composition (g/100g_{sample})		
Myristic (C14:0)	8.2±0.1	5.6±0.1
Palmitic (C16:0)	11.0±0.2	13.9±0.1
cis-9 Palmitoleic (C16:1)	35.8±0.4	34.4±0.2
Cis-10-heptadecenoic (C17:1)	5.5±0.1	6.8±0.2
Stearic (C18:0)	0.7±0.1	1.8±0.4
Oleic (C18:1)	2.8±0.1	4.9±0.3
Linoleic (C18:2)	1.5±0.1	2.5±0.1
Linolenic (C18:3)	0.4±0.1	1.0±0.1
cis-11,14-Eicosadienoic (20:2)	1.2±0.1	2.6±0.1
cis-5,8,11,14-Eicosatetraenoic (20:4)	4.3±0.1	4.4±0.4
cis-5,8,11,14,17-Eicosapentaenoic (20:5)	24.5±0.2	19.3±0.1
Others	4.1±1.2	2.8±1.6

The methane production curves from lipid-extracted microalgae are presented in Figure 1b; raw microalgae were also digested for comparison purposes. It is worth mentioning that raw microalgae produced 258±5 mL_{CH4}/g_{VS}, a bit less than during the experiments presented in section 3.1. These experiments were not performed simultaneously, thus differences can be caused by changes in the inocula used. After extraction with hexane, the methane production was similar to the obtained from raw microalgae. Comparable results were reported using hexane and supercritical CO_2 to extract lipids from *P. tricornutum* before AD [Frigon et al., 2014; Zhao et al., 2014]. Regarding the extraction using methanol/hexane, a large decrease on the methane production was observed compared to raw microalgae. Since the same amount of VS was the same in all reactors (SIR 1:2), the differences in the methane production were caused by changes in the composition and characteristics of the substrates. The microalgae composition resulted almost unaffected by hexane due to the low extraction yield, but opposed results were observed by using methanol/hexane (Table 1). The changes in the composition decrease the theoretical methane production, due to the extraction of the lipids. The biodegradability (Eq. 1) was 39%, 42% and 38% in raw microalgae and extracted with hexane and methanol/hexane respectively. The results show that the biodegradability was almost unaffected, and lipid-extracted microalgae are still difficult to digest.

As can be seen in Figure 1b, the reactors with raw microalgae had the highest production of methane throughout the first days of experiments, whereas reactors with microalgae extracted using methanol/hexane had the lowest. The extraction process can either extract easily-degradable components or slow down the degradation of some components, but further study would be necessary to confirm the effect of solvents. Regarding the effects of drying, only the methane production at the beginning of the experiment was affected (Figure 1b). Although freeze-drying

can modify microalgae composition and characteristics [Cordero-Esquivel et al., 1993], the detrimental effects of drying on AD are mainly attributed to heating [Olsson et al., 2014], which is avoided in the freeze-drying method.

The values of pH, concentration of ammonia and VFA at the end of the experiments confirmed stability in all the reactors.

3.2.2. Ultrasound

The Dd (Eq. 1) gives useful information about the organic compound solubilisation after pre-treatment. The Dd increased by 3.8%, 7.2% and 9.5% after energy inputs of 21 MJ/kg_{TS}, 36 MJ/ kg_{TS} and 52 MJ/ kg_{TS} respectively. The stronger the energy input, the higher the disintegration level; however, the solubilisation of the organic fraction was slightly affected. Figure 2 shows the changes in the microalgae structures throughout the experiment. Even after applying the lowest energy input (Figure 2c), the pictures reveal structural changes on the cells and the release of the inner content. When compared to the microalgae structure before pre-treatments (Figure 2a), the cell wall structure completely disappeared in all the pre-treated samples (Figure 2c-e). Some chloroplasts remained close, like aggregates (Figure 2c), due to the release of the inner content of the cells [González-Fernández et al., 2012]. *P. tricornutum* has an atypical weakly silicified cell wall compared with other diatoms [De Martino et al., 2007], being easily disrupted at low energy input. As it has been recently reported by Chantrasakdakul et al., some organics and polymers released during the pre-treatment may act as flocculants. The flocs constitute the pellet after centrifugation and, as a consequence, the solubilisation of organic compounds (solubilised COD or solubilised solids) can be low or decrease in spite of the high cell disruption [Chantrasakdakul et al., 2015].

As shown in Figure 1c, the methane production was unaffected by the changes in the energy input, 287±11 mL_{CH4}/g_{VS}, 284±9 mL_{CH4}/g_{VS} and 285±4 mL_{CH4}/g_{VS} were achieved after applying 21, 36 and 52 MJ/kg_{TS} respectively. These values represent barely 10% increase in the methane production over the raw microalgae used as a blank experiment (258±12 mL_{CH4}/g_{VS}). The lower-energy pre-treatment was strong enough to break *P. tricornutum* cells and to increase the methane production.

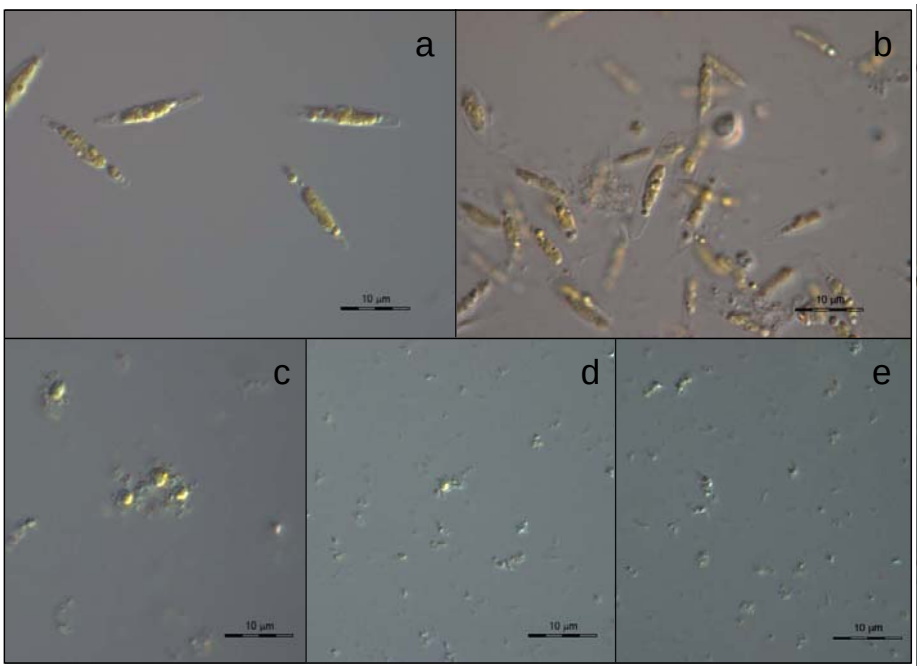


Figure 2. Light micrographs of *P. tricornutum* (a) raw, (b) recovered from digestate, and after ultrasonic pre-treatment applying (c) 21, (d) 36 and (e) 52 MJ/kg_{TS}.

The highest energy input decreased the daily methane production during the first days (Figure 1c); the first day is an exception, but it is characterised by high standard deviation in the methane production. However, no major differences were observed by the end of the experiment. The increases in the energy input can lead to re-flocculation [González-Fernández et al., 2012; Chantrasakdakul et al., 2015], reducing the methane production rate. The energy input can also affect the characteristics of the biomass, increasing the solubilisation but decreasing the methane production [González-Fernández et al., 2012; Alzate et al., 2014].

The biodegradability increased from 39% to around 43% after the pre-treatment. Some microalgae cells were recovered from reactors fed with raw microalgae; the pictures revealed that the cells had similar appearance before (Figure 2a) and after digestion (Figure 2b). Although this showed the high resistance of cells to the microorganisms attack, the degradability of pre-treated microalgae demonstrated that the low methane production is a consequence of the low degradability of the organic fraction in *P. tricornutum*.

4. Conclusions

In spite of the co-digestion of *Phaeodactylum tricornutum* and sewage sludge does not shows synergy, the absence of inhibitory effects suggest that the process can be beneficial under certain circumstances such as the microalgae cultivation for wastewater treatment or over-sized digesters in WWTP.

The anaerobic digestion of *P. tricornutum* can be also coupled to the biodiesel production process. The lipid extraction using organic solvents do not affect the biodegradability of microalgae, but it influences the lipid and biodiesel yields. The proper selection of the solvents for extraction of valuable compounds from *P. tricornutum* can affect the quality of the extract and the methane production of the waste when digested.

The ultrasonic pre-treatment confirms that the main obstacle of the *Phaeodactylum tricornutum* anaerobic digestion is the refractory nature of the organic fraction, since the pre-treatment disrupts the microalgae cells but it does not enhance the degradability.

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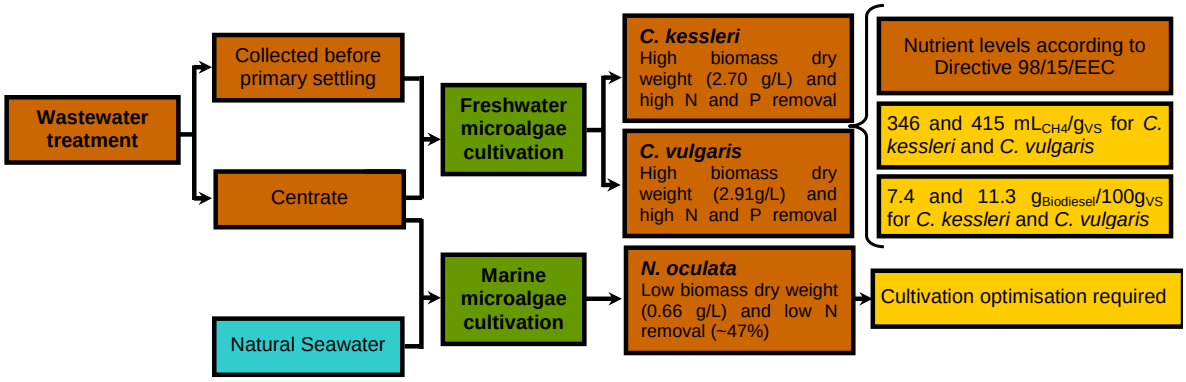
Chapter 5

Microalgae cultivation in urban wastewater: Nutrient removal and biomass production for biodiesel and methane [1]



ABSTRACT

The freshwater microalgae species *Chlorella kessleri* and *Chlorella vulgaris*, and the marine microalgae species *Nannochloropsis oculata* were cultivated in urban wastewater. The freshwater species demonstrated the possibility of growing in urban wastewater reaching high biomass production and nutrient removal when cultured in batch mode using a flat-panel airlift photobioreactor. Both microalgae species reached high biomass dry weights, 2.70±0.08 g/L and 2.91±0.02 g/L respectively, accompanied by nitrogen concentration reduction around 96% and 95%, and a phosphorous concentration reduction around 99% and 98% respectively. *N. oculata* was able to uptake nutrients from wastewater to grow but with less efficiency, indicating the need of microalgae acclimation or process optimisation to achieve high nutrient removals. During *C. kessleri* and *C. vulgaris* cultivation, the nitrogen consumption led to a progressive N-starvation process which increased the microalgae potential for biofuels production; both species produced 346±3 mL_{CH4}/g_{VS} and 415±2 mL_{CH4}/g_{VS} during anaerobic digestion, and 7.4±0.2 $g_{Biodiesel}/100g_{VS}$ and 11.3±0.1 $g_{Biodiesel}/100g_{VS}$ respectively.

[1] M.P. Caporgno, A. Taleb, M. Olkiewicz, J. Font, J. Pruvost, J. Legrand, C. Bengoa, Microalgae cultivation in urban wastewater: Nutrient removal and biomass production for biodiesel and methane, Algal Research 10 (2015) 232-239.

1. Introduction

Microalgae are able to convert solar energy and carbon dioxide into energy as a result of their photosynthetic activity; when converted into biodiesel or methane, this energy could meet the population energy needs [1]. The microalgae cultivation with energetic purposes started years ago when microalgae, able to accumulate a high amount of lipids, appeared as promising crop substitute in biodiesel production [2]. Currently, crops are the common feedstocks in the biodiesel production industry but also in the food industry, thus the crop-based biofuels production has created increased food prices [3]. A wide variety of microalgae species reach higher lipid productivities than crops [2], becoming potential substrates to alleviate the referred to "food-versus-fuel competition" [3]. Despite the efforts made, the industrial biodiesel production from microalgae is not economically viable nowadays due especially to the high costs for drying and lipid extraction among other costs [4]. On the contrary, the anaerobic digestion process converts wet biomass into methane [5], and energy is recovered not only from the lipid fraction.

The anaerobic digestion process is a widely known technology, currently used in the wastewater treatment plants (WWTPs). The sewage sludge, a waste produced during the wastewater treatment, generates high operating costs in its final disposal [5]; the anaerobic digestion process converts the sludge into a stable product with simultaneous generation of a valuable by-product such us methane. Methane utilisation contributes favourably to reduce the high operating costs generated by the final disposal of sewage sludge [5].

The nutrient level in wastewaters might be another problem in WWTPs. The water discharge with high nutrient levels cause eutrophication problems, thus the Directive 98/15/EEC establishes nutrient levels or minimum percentage of reduction before water discharge [6]. Although the nutrient concentration varies from a WWTP to another, and between the wastewater streams in the process, the stream generated during the digested sludge dewatering process always has higher nitrogen and phosphorus concentrations than in any other streams [7]. This stream, named centrate, is usually recycled for further treatment to avoid environmental problems, but it increases the WWTP costs [7]. Microalgae cultivation offers a solution to reduce the high nutrient content in centrate since microalgae consume nutrients when growing [8]. Moreover, in a waste-to-value approach, the produced biomass constitutes a by-product which could be used for various purposes, including biofuels.

Some freshwater microalgae species have already been cultured in different wastewater streams, with the purpose of reducing the nutrient concentration in wastewater or producing lipids for biofuels [7], [9], [10], [11], [12] and [13]. Similarly, seawater microalgae species have also been cultured using wastewaters despite the salinity requirements [14] and [15]. However, the centrate utilisation for microalgae cultivation is scarce; most of the studies were done using wastewater with a low nutrient content compared to the centrate. The use of centrate as nutrient medium allows coupling the wastewater treatment and the microalgae cultivation process with a minimum modification in the WWTP facilities. Wastewater treatment by microalgae cultivation is still limited, and the waste activated sludge (WAS) process is the conventional treatment in WWTP [8]. The substitution of the WAS process implies a full modification of the WWTP scheme, whereas the centrate utilisation in microalgae cultivation only needs a cultivation unit coupling.

This study analyses the possibility of coupling microalgae cultivation in WWTPs, removing nutrients from the centrate while producing biomass with energy recovery purposes. Due to the high nutrient level in the centrate

which can inhibit the cell growth, the first studies aimed to determine the most suitable media for *C. kessleri*, *C. vulgaris* and *Nannochloropsis oculata* cultivation. The microalgae species were cultivated in different dilutions of the centrate; the dilutions were carried out with wastewater before the primary settling tank for the cultivation of freshwater microalgae species, or with natural seawater for the marine microalgae species. After the culture medium screening, the microalgae species were cultured in the most suitable medium, where the nutrient removal and the biomass production were evaluated. Finally, the harvested biomass was used for methane and biodiesel production.

2. Materials and methods

2.1. Pre-treatments and characteristics of the wastewaters

Wastewater samples from the WWTP of Saint Nazaire (ACCUEIL CARENE, Saint-Nazaire, France) were used as cultured medium for microalgae cultivation; they consisted of the centrate and the wastewater from a line before the primary settling tank. For marine microalgae cultivation, natural seawater was collected from the coastal area of Saint-Nazaire in France.

Large solid particles were first removed by centrifugation and then, the samples were filtered through a 0.45 µm pore size filter to remove undesirable small particles. The total nitrogen (TN), ammonia nitrogen (NH_3-N), nitrate nitrogen (NO_3-N), nitrite nitrogen (NO_2-N), phosphate phosphorus (PO_4-P), and chemical oxygen demand (COD) were determined following the Hach DR 2800 Spectrophotometer Manual using the HACH LANGE cuvette tests and following the procedure specified for each test (Hach, 2008). The characteristics of the wastewaters and the natural seawater can be observed in Table 1.

Table 1: Characteristics of the wastewater and the natural seawater used for microalgae cultivation.

Parameter	Water for microalgae cultivation		
	Before the primary settling tank a	Centrate	Natural seawater
pH	7.42-7.79	8.2±0.1	7.7±0.1
COD (mg/L)	95-169	706±9	-
TN (mg N/L)	39-65	1233±78	n.d.
NH_4^+ (mg NH_4-N/L)	36-62	1198±81	n.d.
NH_4^-N/TN (%)	95±2	98±1	-
NO_3^- (mg NO_3-N/L)	0.1-0.9	2.34±0.11	6.2±0.1
NO_2^- (mg NO_2-N/L)	0.1-0.4	0.14±0.02	n.d
P (mg PO_4-P/L)	3.1-5.4	11.90±0.10	n.d.
Salinity (‰)	-	-	28±0.5

[a] *Parameter ranges from two different samples collected in two different opportunities.*
[n.d.] *not detected*

As can be observed, the composition of the wastewater sampled before the primary settling tank composition is given as a range rather than the average value with the standard deviation; two different samples collected in different opportunities were used in the experiments. These differences in the nutrient content were taken into account for the cultured medium preparation.

2.2. Microalgae cultivation

2.2.1. Shake flasks

Three different microalgae species were cultured. The freshwater microalgae species *Chlorella kessleri* (strain UTEX2229) and *Chlorella vulgaris* (strain CCAP211/19) were obtained from the collection of algae at the University of Nantes; and the marine microalgae species *N. oculata*, from the Alphabiotech collection (Asserac, France).

The microalgae were first inoculated in shake flasks. The freshwater microalgae species in 250 mL Erlenmeyer flask containing a modified Bold Basal Medium (BBM) and the marine microalgae species, in filtered and sterilised seawater with salinity adjusted at 25‰ and enriched with Conway medium (3 mL/L of seawater). The detailed composition of the modified BBM and Conway medium is given in Pruvost et al. [16] and [17].

2.2.2. Culture medium screening in Efficient Overproducing Screening System-Photobioreactors (EOSS-PBR).

The EOSS-PBR was especially developed for the fast screening of culture media and microalgae species in conditions representative of PBR cultivation. It consisted of six small-scale photobioreactors (bubble columns) run in parallel, each tube having a volume $Vr = 3 \cdot 10^{-5}$ m^3, an illuminated area of $SL = 0.008$ m^2 and a specific illuminated area of alight = S/Vr of 266.7 1/m. A full description of the EOSS-PBR is done in Taleb et al. [18].

The culture medium was prepared by diluting the centrate to reduce the high TN concentration, either with wastewater or with natural seawater (Table 1). Before inoculation, the culture medium was filtered through a 0.45 μm pore size filter to remove undesirable small particles. The TN concentrations in the culture medium for the freshwater microalgae species were 30 mg N/L (0.002 mol/L), 140 mg N/L (0.010 mol/L), 260 mg N/L (0.019 mol/L), 490 mg N/L (0.035 mol/L), 700 mg N/L (0.050 mol/L) and 1200 mg N/L (0.086 mol/L). For the marine microalgae species cultivation, the TN concentrations were 6 mg N/L (< 0.001 mol/L), 71 mg N/L (0.005 mol/L), 135 N mg N/L (0.010 mol/L), 265 mg N/L (0.019 mol/L), 524 mg N/L (0.037 mol/L) and 782 mg N/L (0.056 mol/L).

The pH in the culture medium was around 7.5 for the freshwater microalgae species and 8 for the marine microalgae species, according to Pruvost et al. and Taleb et al. [17] and [18]. The culture agitation was provided by continuous injection of air with 2 vol.% CO_2 at a flow rate of 3 mL/min; the incident photon flux density (PFD), by a set of 6 fluorescent white tubes was ~150 μmol/m^2·s. The temperature was regulated at 25 °C by ambient air flow. The reactor was operated in batch mode.

The microalgae growth was evaluated following the evolution of the number of cells as a function of time (t). Cell concentration N expressed as number of cells per millilitre of culture was determined under an optical microscope (Axiostar-Plus, Carl Zeiss, Germany) using Malassez counting cell. The chlorophyll fluorescence in the microalgae was observed in the microscope, using the green filter set 530-585 (BP 530-585 as exciter filter, FT 600 as chromatic beam splitter and LP 615 as barrier filter; Zeiss, Oberkochen, Germany). The algal dry weight concentration (Cx) was determined at the end of the experiment, by filtration through a pre-dried and pre-weighed glass-fibre filter (Whatman GF/F). The filters were dried for 24 h at 105 °C, cooled down in a desiccator and then weighed again. The total pigment content was determined using a spectrophotometric method, following the procedure described in [17].

2.2.3. Microalgae cultivation in flat-panel airlift photobioreactor (PBR)

Once the culture medium was defined, microalgae were cultured in batch mode using a flat-panel airlift PBR with $Vr = 10^{-3}$ m^3, and depth of culture of $Lz = 3 \cdot 10^{-2}$ m, a $SL = 8 \cdot 10^{-3}$ m^2 and a alight = 33.3 1/m. Before inoculation, the cells were centrifuged for 3 min at 6000 ×g at room temperature, and the cell pellets were washed with the decided culture medium twice before finally suspending cells.

The temperature was 25 °C, regulated by ambient air flow. The pH was monitored with a sensor inside the reactor (Mettler Toledo SG 3253) and automatically regulated by CO_2 injection, at 7.5 for the freshwater microalgae species and 8 for the marine microalgae species. The culture agitation was provided by air bubbling. A constant PFD (~100 μmol/m^2·s) was used during the experiments.

The microalgae growth and the Cx were evaluated during cultivation as described in Section 2.2.2. The different nutrient concentrations during cultivation were determined as described in Section 2.1.; the samples were taken from the reactor, centrifuged and the supernatant analysed. The evolution of proteins, carbohydrates and lipids in the biomass during cultivation was followed with infrared spectra of the microalgae cells; the spectra were obtained in a Bruker Tensor 27 FTIR spectrometer equipped with the ATR platinum module, with a deuterated triglycine sulfate detector RT-DLaTGS and OPUS v7.0.122 software (Bruker Optics, Germany). After finishing the experiment, the biomass was recovered by centrifugation and dried using freeze-drying equipment (FT33-A Freeze Drier, Armfield Inc.) for 48 h to avoid major changes on its composition. The carbohydrate content in biomass was quantified by the phenol-sulphuric acid method and protein content, by the Lowry method as described in Caporgno et al. [19].

2.3. Methane and biodiesel production

Anaerobic digestion was carried out by triplicate, in 120 mL serum bottles sealed with a septum and an aluminium crimp. The inoculum consisted of mesophilic digested sludge taken from an anaerobic semi-continuous plant; the substrates were the freeze-dried microalgae species re-suspended in deionised water. The final volume in the reactors was adjusted to 80 mL before sealing, then the reactors were purged with nitrogen to assure anaerobic conditions and placed into an oven at 33 °C; a detailed procedure is described in Caporgno et al. [19]. The amount of substrate loaded in reactors was calculated based on the organic matter added per inoculum content, the substrate to inoculum ratio was 1:2 $VS_{Substrate}$:$VS_{Inoculum}$. The biogas production and composition, and parameters after anaerobic digestion such as the volatile fatty acid concentration (VFA) were measured as described in Caporgno et al. [19]. The ammonia concentration at the end of the experiment was measured with an ion selective electrode (ISE) (Ammonia Gas Sensing combination electrode, mod. 51927-00, HACH); the alkalinity was analysed according to the standard method 2320B [20]. The theoretical methane production was calculated based on the microalgae composition, and the biodegradability of the biomass, as the fraction of the theoretical methane production reached at the end of the anaerobic digestion experiment as described in Caporgno et al. [19].

The freeze-dried microalgae biomass was converted to FAME-biodiesel by direct transesterification procedure according to Johnson and Wen [21]. The FAMEs were analysed by GC-FID according to the Agilent Application Note 228-398 using a HP-INNOWax column (19091 N-133), and a 37 component FAME standard mixture (Supelco: 47885-U) was used for calibration of the method.

3. Results and discussion

3.1. Culture medium screening in EOSS-PBR

The Fig. 1a shows the evolution of the number of cells over time for *C. kessleri*, in nutrient media containing from 30 mg N/L to 1200 mg N/L. As can be observed in Table 1, although the differences in the TN concentration in both wastewaters, most of the N content in both wastewaters is in the form of NH_4^+.

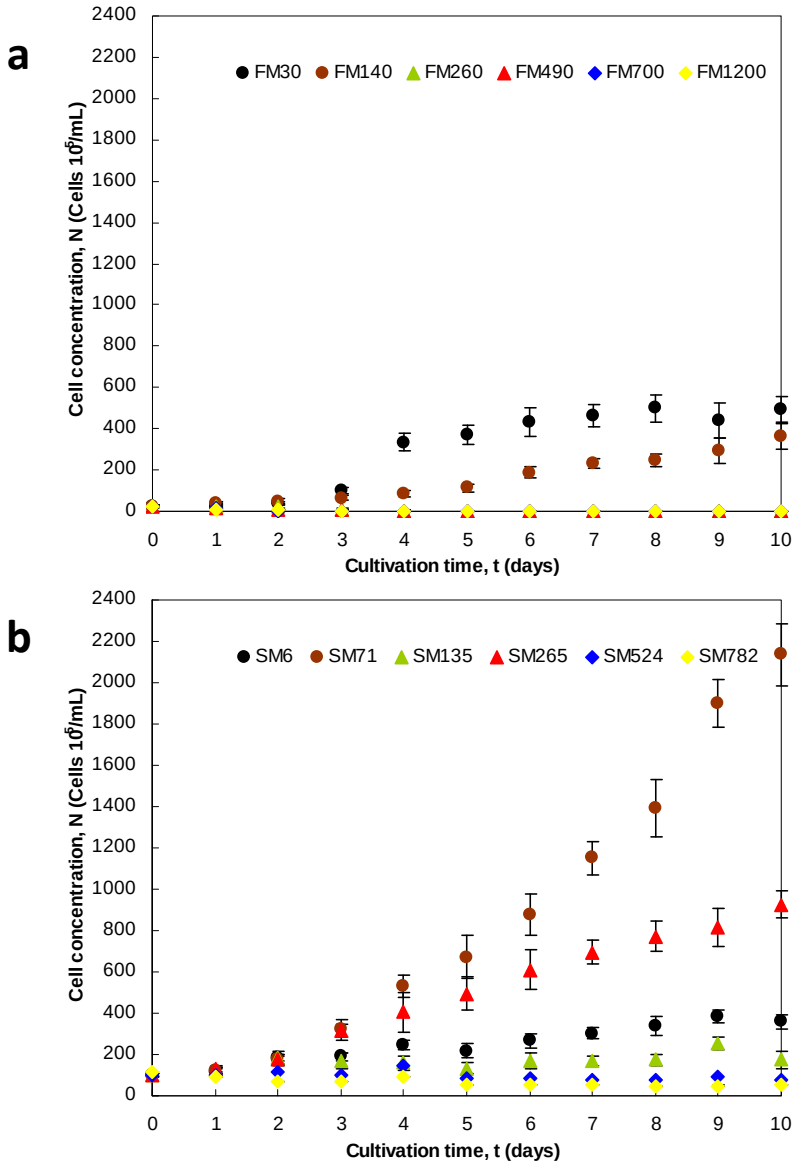


Fig. 1. Microalgae growth as the evolution of the number of cells over time for the freshwater microalgae species *C. kessleri* (a) in the culture media with 30 mg N/L, 140 mg N/L, 260 mg N/L, 490 mg N/L, 700 mg N/L and 1200 mg N/L, referred as FM30, FM140, FM260, FM490, FM700, respectively and FM1200. In part (b), the marine microalgae species *N. oculata*, in the culture media with 6 mg N/L, 71 mg N/L, 135 N mg N/L, 265 mg N/L, 524 mg N/L and 782 mg N/L, referred as SM6, SM71, SM135, SM265, SM524 and SM782, respectively.

In the nutrient medium with the lowest TN concentration, the curve follows a typical evolution for batch cultivation. There was a lag phase at the beginning, characterised by a period of physiological adjustment due to changes in the nutrient conditions. The cells adapted to the new conditions after 2 days and the growth accelerated; this is the exponential growth phase. Finally, there was no growth after day 9; this is the stationary phase. As

observed in Fig. 1, when the TN concentration increased from 30 until 140 mg N/L, the lag phase became longer and the growth rate during the exponential growth phase became lower. There was not any microalgae cell growth at a TN concentration higher than 140 mg N/L; in fact, the number of cells decreased and no cells were observed after 3 days of cultivation, suggesting inhibition of the cell growth. In the centrate, there was a high level of N in the form of ammonium, and although it can be used by microalgae as a source of N, it can also inhibit microalgae growth [8]. Microalgae cells were recovered from the bottom of the reactors, and their appearance was observed in the microscope also using the green filter for chlorophyll fluorescence observation. The cells in the media with 30 mg N/L were smaller than in the media with 140 mg N/L. This result agreed with the dry weight analysis results, which determined similar algal dry weight in both media, in spite of the higher number of cells in the media with 30 mg N/L. Regarding the microalgae recovered from the reactors with a nitrogen concentration higher than 140 mg N/L, the cells presented an absence or much less florescence intensity than that recovered from reactors with 30 and 140 mg N/L, indicating that nitrogen concentrations higher than 140 mg N/L affect the cells negatively. Comparable results were reported for *C. vulgaris* cultivation on wastewater with a high concentration of NH_4^+-N [12].

At the end of the experiment, the algal dry weight was determined in the reactors. The Cx was similar in nutrient media with 30 mg N/L and 140 mg N/L, 1.34±0.10 g/L and 1.32±0.06 g/L respectively, in spite of the differences in the number of cells. At TN concentrations higher than 140 mg N/L, microalgae cells settled and Cx was lower than 0.05 g/L, as was expected due to inhibition. The total pigment content was analysed at the end of the experiment, due to the more intense green colour observed in the reactor with a TN concentration of 140 mg N/L compared with 30 mg N/L, although the lower number of cells. The results indicated that the total pigment content was 15±0.1 μg/mL for 140 mg N/L and 1.8±0.1 μg/mL for 30 mg N/L, as was also observed elsewhere [12].

Based on the results, it was decided that a nutrient medium with a TN concentration around 140 mg N/L is the most suitable for *C. kessleri* species cultivation; this allows the usage of the highest amount of the centrate and produces a high biomass concentration. Furthermore, since similar growth evolution and biomass production was reported for *C. kessleri* and *C. vulgaris* cultivation in wastewater [13], no screening was carried out with *C. vulgaris* species and the same nutrient medium was used for both species.

Regarding the culture medium for the marine microalgae species *N. oculata*, Fig. 1b shows microalgae growth in the nutrient media containing from 6 mg N/L to 782 mg N/L. Centrate and seawater mixing affects medium salinity due to a dilution effect; the higher the amount of the centrate in the nutrient medium, the higher the TN concentration but the lower the salinity. The highest salinity was 28‰ in pure seawater with a low TN concentration due to the NO_3^- presence; the lowest was 3‰ with a TN concentration of 782 mg N/L.

As can be observed in Fig. 1b, there was not a significant microalgae growth in natural seawater due to the low nutrient content. However, a higher N content after the centrate addition was enough to increase the number of cells. In the medium with 71 mg N/L and 24‰ salinity, *N. oculata* cultivation shows good results. The evolution of the number of cells shows a lag phase during the first 2 days and then, the growth accelerates almost continuously. Finally, by the 10^{th} day, it seems that microalgae growth is close to the stationary phase. The increases in the TN concentration up to 135 and 265 mg N/L decreased the salinity of the medium, 18‰ and 12.5‰ respectively. As can be seen in the curves, the microalgae did not grow in the medium with 135 mg N/L in spite of the higher salinity of this medium, but they grew in the medium containing 265 mg N/L. In this medium,

the evolution of the number of cells followed a 2 day lag phase and an exponential growth and a stationary phase around the 8th day. These results indicated inhibition in the culture medium with 135 mg N/L; however, the inhibition could not be attributed to a high TN concentration or to a low salinity. Comparing the media containing 71 mg N/L and 265 mg N/L, the number of cells decreased by almost 50% due to the low salinity; the optimum salinity for *N. oculata* has been suggested between 22‰ and 25‰ and the changes in the medium salinity affect *N. oculata* cultivation [22]. It was reported that *Nannochloropsis sp.* grew in wastewater with 33‰ and 18.5‰ salinity (11 mg N/L and 55 mg N/L respectively), but the culture failed at lower salinities due to osmotic stress [14]. Furthermore, it was reported that *Nannochloropsis salina* grew in wastewater reaching similar productivities when the salinity was adjusted, and the TN was in a range between 80 mg N/L and 480 mg N/L [15]. Based on this, it was confirmed that the inhibition in the media with 130 mg N/L was not attributable to salinity or TN concentration in the medium. A possible reason was pH; it was reported that pH affects *Nannochloropsis sp.* significantly [23] and in the experiment, this parameter was not automatically regulated. During ammonia assimilation by microalgae, the pH of the medium decreases and may reach levels near pH 3; the pH shifts might cause growth inhibition [24].

At 71 mg N/L and 24‰ salinity in nutrient medium, the Cx was 1.07±0.07 g/L. The TN concentration increased to 265 N/L decreased the Cx to 0.48±0.02 g/L. In other culture media, the Cx was lower than 0.02 g/L, as was expected due to the inhibition observed.

Assuming that inhibition in the medium with 135 mg N/L was fortuitous, a nutrient medium with 135 mg N/L and 24‰ salinity allows using the higher amount of centrate without affecting Cx, thus these were the characteristics of the culture medium chosen for *N. oculata* cultivation.

3.2. Microalgae cultivation in flat-panel airlift PBR

3.2.1. Chlorella kessleri cultivation

The microalgae were cultured in 1 L PBR to evaluate the microalgae growth, the nutrient removal and the biomass production. Fig. 2a shows *C. kessleri* growth as the evolution of the number of cells over time in a culture medium with 130 mg N/L (0.009 mol/L), and Fig. 2d shows the N evolution as the percentage of the initial N which remained in the culture medium, and the Cx evolution in the culture. As can be observed in Fig. 2a, there was a scarce microalgae growth during the first two days but the algal dry weight slightly increased, indicating microalgae cell size increase. This cell size increase was accompanied by a TN concentration reduction from 130.0±0.3 mg N/L to 108.2±0.3 mg N/L, which represented ~16% TN consumption. After the 2nd day, the microalgae growth started a steady increase until the 7th day. At this time, the Cx in the reactor reached 1.78±0.05 g/L; the total TN consumption was ~95%, the remaining TN concentration was under the limits for the TN discharge [6]. The microalgae growth continued until it reached the stationary phase on the 9th day. Although there was not a significant TN consumption after the 7th day, the Cx reached 2.34±0.01 g/L on the 9th day and 2.70±0.08 g/L at the end of the experiment. The final TN concentration was lower than 5 mg N/L, which meant > 96% removal. Regarding P as a nutrient, the initial concentration of TP in growth medium was 5.76±0.08 mg P/L, and at the end of the experiment 0.04±0.01 mg P/L, which indicated more than 99% removal efficiency. Under the culture conditions in this experiment, *C. kessleri* showed a high efficiency at removing TN and TP; the culture conditions affect TN and TP removal differently [7]. The results indicated that *C. kessleri* cultivation can remove TN and TP without a previous acclimation step; a microalgae acclimation step was unnecessary to improve nutrient

removal as it is suggested in the literature [25]. Similar results were also reported by Arbib et al. [13]. The N/P ratio in the culture media was 23, higher than the range 6.8-10 indicated as optimal for freshwater microalgae cultivation [9]. As some other freshwater microalgae species [9], *C. kessleri* has demonstrated the ability of growing in nutrient media with an unbalanced N/P ratio.

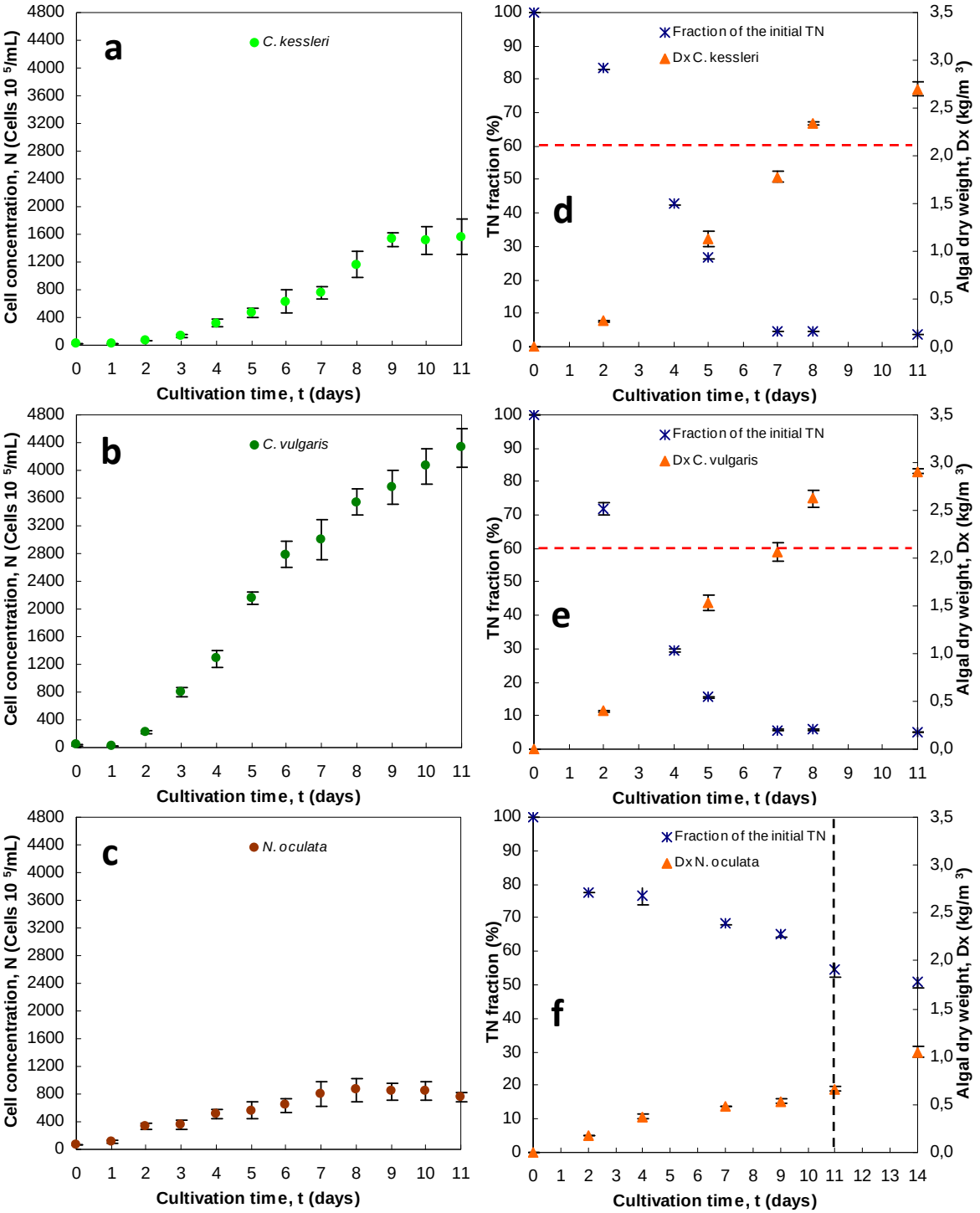


Fig. 2. In the upper part, the microalgae growth as the evolution of the number of cells over time for (a) *C. kessleri*, (b) *C. vulgaris* and (c) *N. oculata* respectively. In the lower part, the N evolution as the percentage of the initial N which remains in the culture medium and the Cx evolution during cultivation for (d) *C. kessleri*, (e) *C. vulgaris* and (f) *N. oculata* respectively.

Based on the stoichiometric needs of biomass and nutrient concentration in the medium, the maximal biomass concentration without starvation was calculated. Assuming an elemental composition of biomass equal to $CH_{1.715}O_{0.427}N_{0.148}S_{0.014}P_{0.012}$, where the C-molar mass is 23.45 g C/mol [16], and an initial TN concentration of 130 mg/L, the maximal biomass concentration without starvation resulted to 2.18 g/L. As can be seen in Fig. 2d (dashed line), this concentration was reached between the 7th and 8th days of cultivation. In the same period, it remained less than 5% of the initial TN concentration (Fig. 2b), indicating that microalgae cells may experience N-starvation.

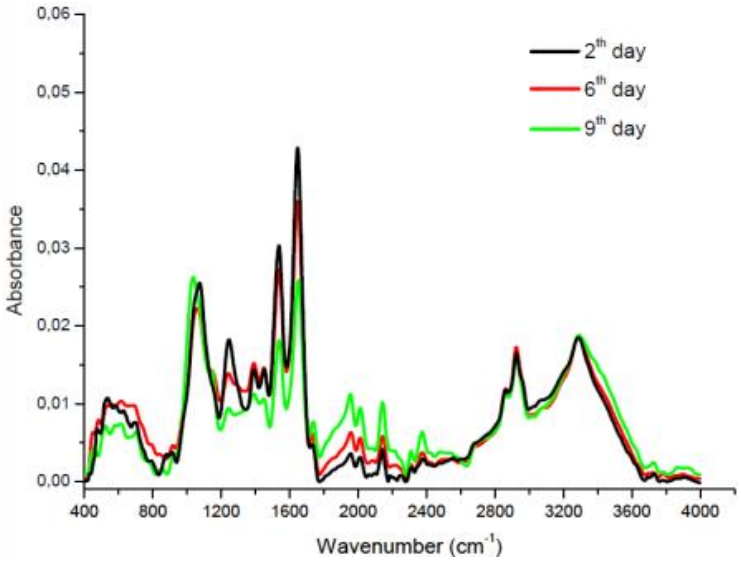


Fig. 3 indicates the microalgae spectra obtained during FTIR analyses, for biomass collected during the 2nd, 6th and 9th days, corresponding to the beginning of the exponential phase, when the TN concentration in the culture medium was ~5 mg N/L, and at the beginning of the stationary phase.

Since the lipids, protein and carbohydrates can be identified due to their characteristic groups [26], the comparison of the three spectra in Fig. 3 shows the evolution of the proteins, carbohydrates and lipids in biomass. The protein content decreased during cultivation, due to less intensified absorption bands at 1500-1700 cm^{-1} for peptide amide groups of proteins. On the contrary, the absorption bands at 1000-1200 cm^{-1}, characteristics of C-O and C-O-C groups of carbohydrates, increased. Moreover, more intensified bands at 1700-1750 cm^{-1} and 2800-3000 cm^{-1}, characteristics for lipids indicated a lipid content increase. These changes in the absorption bands indicated that the microalgae underwent a period of N-starvation; the cell division decreased progressively, the dry weight increase became lower and the lipid content triggers. These facts, visible in Figs. 2a and d and 3, were also observed in other freshwater microalgae species which experienced progressive N-starvation [16]. The results confirmed that the microalgae cultivation in wastewater follows a progressive N-starvation process, increasing the microalgae lipid content, valuable for biofuels production. The total lipid content was not analysed, and the saponifiable lipid fraction (convertible into biodiesel) was quantified instead; the saponifiable lipid content yielded 7.4±0.2 $g/100g_{VS}$. The content of proteins and carbohydrates in *C. kessleri* were 36.7±1.0 $g/100g_{VS}$ and 44.6±0.1 $g/100g_{VS}$ respectively.

3.2.2. Chlorella vulgaris cultivation.

C. vulgaris growth in a culture medium with 130 mg N/L (0.009 mol/L) can be observed in Fig. 2b as the evolution of the number of cells over time; the N evolution as the percentage of the initial N which remained in the culture medium and the Cx evolution in the culture can be observed in Fig. 2e.

Similar to *C. kessleri*, the number of cells did not change until the second day but the algal dry weight increase revealed a cells size increase. This was accompanied by a 27% consumption of the TN; the TN concentration decreased from 130.0±0.3 mg N/L to 93.4±2.5 mg N/L. From the 2nd day until the 6th day, the microalgae growth showed a steady increase. After the 6th day, the growth rate decreased and on the 11th day the culture was near the steady phase. The Cx in the reactor increased daily and it reached 2.63±0.09 g/L the 8th day, when the TN consumption was ~94% and the TN level lower than 10 mg N/L required for water discharge [6]. The subsequent number of cell increase was not accompanied by substantial Cx increase or N consumption; in fact, the Cx and the TN consumptions reached 2.91±0.02 g/L and ~95% respectively. At the end of the experiment, TP in growth medium was 0.11±0.02 mg P/L, lower than the threshold established by the European Directive (< 1 mg P/L) [6], resulting in a removal efficiency higher than 98%. The results also indicated that *C. vulgaris* grows in wastewater, even when the N/P ratio is unbalanced and higher than the indicated as optimal for freshwater microalgae cultivation [9]. The nutrients removal efficiencies were higher than the reported after *C. vulgaris* cultivation in wastewater sampled in different point of a WWTP, including centrate samples with a similar composition than in the present experiment [9]. Although similar nutrient removals were reported in wastewater mixed with glycerol, the biomass concentration reported at the end of the experiment was considerably lower [10]. Comparable nutrients removals and lower biomass production were reported during the *C. vulgaris* cultivation using centrate obtained from a digester processing cattle slurry and raw cheese whey [11].

As during *C. kessleri* cultivation, *C. vulgaris* experienced a progressive N-starvation process, which led biomass to a cell division decrease, a low dry weight increase and a total lipid content accumulation. The maximal biomass concentration without starvation resulted in 2.18 g/L [16] and it is indicated in Fig. 2e by a dashed line. This biomass concentration was reached between the 7th and 8th day of cultivation, when the TN concentration was less than the 5% of the initial concentration. After this period, the microalgae increased their lipid content. The saponifiable lipid fraction yielded 11.3±0.1 g/100g_{VS} at the end of the experiment, similar to the results reported for the same *C. vulgaris* strain cultivation under N-starvation [17]. The content of proteins and carbohydrates in *C. vulgaris* were 35.2±1.4 g/100g_{VS} and 36.2±0.3 g/100g_{VS} respectively.

A comparison between *C. kessleri* and *C. vulgaris* shows a significant difference in the number of cells. *C. kessleri* reached around 1500 • 10^5 cells/mL at the end of the experiment, whereas *C. vulgaris* reached almost three times this number (Fig. 2a and b). However, the Cx was in both species, which agrees with the larger size of the *C. kessleri* cells compared with the *C. vulgaris* cells. The higher cell size in *C. kessleri* may increase harvesting efficiency, thus being beneficial from an engineering point of view. The literature comparing *C. kessleri* and *C. vulgaris* for wastewater treatment is scarce, but both species have shown a similar biomass production when cultured under the same conditions [13]. However, the reported biomass production was lower than the 2.70±0.08 g/L and the 2.91±0.02 g/L obtained for *C. kessleri* and *C. vulgaris* respectively in the present studio.

Chlorella microalgae are usually found in wastewater treatment ponds [8], suggesting that can uptake nutrients from wastewater to grow. The species cultured in this study shows a promising future for nutrient removal in wastewaters. Moreover, the biomass cultivation makes possible the centrate utilization as nutrient medium instead of its recirculation to a previous step in the process. The biomass can be used as fertiliser, as animal feed or as biofuel feedstocks, being economically advantageous for the WWTP [8]. Moreover, the progressive N-starvation during cultivation triggers lipid accumulation in biomass, attractive for biofuels production [17].

3.2.3. Nannochloropsis oculata cultivation

N. oculata was grown in a nutrient medium with ~100 mg N/L (0.007 mol/L) and 25‰ salinity. Due to the higher TN concentration in the centrate collected for this experiment, it was possible to increase the TN concentration in the nutrient medium, keeping salinity in the optimum range [22]. The evolution of the number of cells over time can be observed in Fig. 2c. The N evolution and the Cx evolution in the culture can be observed in Fig. 2f.

Fig. 2c shows that there was not a significant lag phase at the beginning of the experiment; the number of cells increased at an almost constant rate from the beginning until the 8th day. After the 8th day, it started a stationary phase followed by a declination phase derive from the biomass precipitation observed. The stationary phase started the 8th day; the TN concentration was 67.0±0.8 mg N/L, which barely represented ~35% consumption of the initial 102.8±0.1 mg N/L. On the 11th day, the TN concentration reached 55.9±2.0 mg N/L, ~47% consumption; the TN concentration in the culture medium exceeded the limit required for water discharge [6] but to prolong the cultivation beyond did not increase the N consumption significantly. The experiment was conducted until the 14th day due to the low N consumption observed; however, the number of cells was not counted beyond the 11th day. The Cx increased at an almost constant rate from the beginning of the experiment until the end of the experiment; the 11th day, the Cx reached 0.66±0.02 g/L and on the 14th day, 1.05±0.06 g/L despite there was not any N consumption in this period. The Cx values were considerably lower compared with the Cx obtained in the freshwater microalgae cultivation (Fig. 2d and e). The TP concentration at the end of the experiment was 0.04±0.01 mg P/L, lower than the threshold established by the European Directive (< 1 mg P/L), resulting in a removal efficiency higher than 96% [6].

Nannochloropsis species have shown the capability to grow in different nutrient media varying nitrogen concentration, salinity, ph, light intensities, between some other factors [14], [15] and [23]. It has been reported that the microalgae grows without major changes in a medium with N/P ratio of 32, although the optimal ratio is 16 [27]. The initial N/P ratio was considerably higher than the optimal, around 100. The excess or lack of P influences negatively the cell division and the biomass productivity [27]; thus affecting the microalgae growth and the N consumption.

Although *N. oculata* grows in wastewater, the results suggest that the aim of the wastewater utilisation should be established in advance. If cultivation is aimed to reduce nutrient content, the freshwater microalgae species are preferable; the nitrogen level at the end of *N. oculata* cultivation still exceeds the threshold established by the European Directive for water discharge. However, if cultivation is aimed at biofuels production or valuable compound production from marine microalgae species instead, centrate can replace nutrient addition reducing the production costs. In this case, the salinity needs to be adjusted in order to obtain high biomass productivities.

3.3. Methane production from microalgae

C. kessleri and *C. vulgaris* grown in wastewater were recovered from the PBR and anaerobically digested in order to evaluate their potential as substrate for methane production. The methane production curves from *C. kessleri* and *C. vulgaris* are shown in Fig. 4.

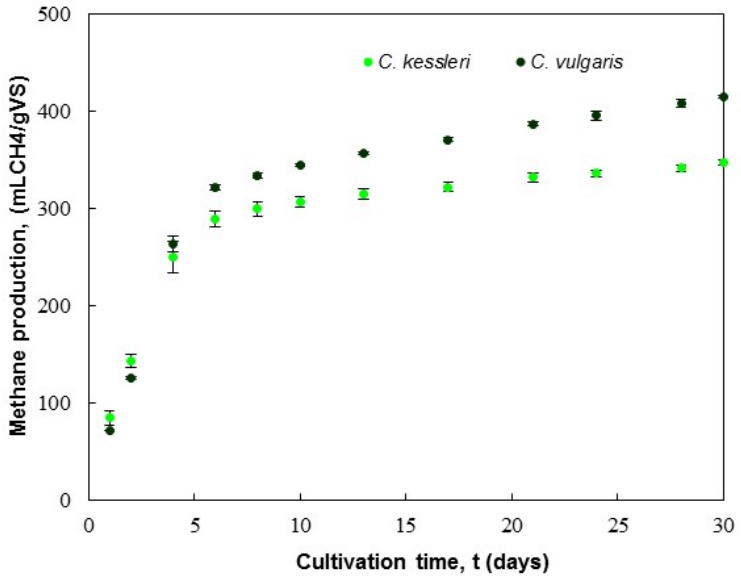


Fig. 4. Methane production curves from *C. kessleri* and *C. vulgaris* cultivated in wastewater, batch reactors and 33 °C.

The methane production was similar for both microalgae species during the first four days, becoming different after this period. On the 6th day, the methane production from *C. vulgaris* was 11% higher than from *C. kessleri*. This difference progressively increased until the end of the experiment, when it reached 20%. The final methane production from *C. kessleri* and *C. vulgaris* were 346±3 mL_{CH4}/g_{VS} and 415±2 mL_{CH4}/g_{VS} respectively. For *C. kessleri*, it has been reported 218 mL_{CH4}/g_{VS} under mesophilic conditions; for *C. vulgaris* digestion, 240 mL_{CH4}/g_{VS} and 286 mL_{CH4}/g_{VS} were reported after 28 days and 49 days mesophilic conditions respectively [4]; however, there is no literature relating to the anaerobic digestion of microalgae cultivated in wastewaters. These methane productions were clearly exceeded in the present experiment. What is more, the methane productions obtained are higher than those reported for several microalgae species [4]. Even more interesting results appear when comparing the methane production from microalgae with substrates commonly used in anaerobic digestion, such as sewage sludge, which under similar conditions produced around 350 mL_{CH4}/g_{VS} [19]. The methane production from several substrates such as municipal solid wastes, swine manure, maize silage and straw, etc., was also exceeded [4].

The theoretical methane production resulted in 533±5 mL_{CH4}/g_{VS} and 567±1 mL_{CH4}/g_{VS} for *C. kessleri and C. vulgaris* respectively. The biodegradability resulted in 65% and 66% for *C. kessleri* and *C. vulgaris* respectively, higher than the values reported for other microalgae species and similar *C. vulgaris* to the values reported for sewage sludge mesophilic digestion [4] and [19]. The values of pH, alkalinity, and concentration of ammonia and VFA were measured at the end of the experiments corroborated that the digesters operated at optimum conditions. The biogas composition, analysed several times during the experiments, indicated that the methane percentage ranged between 66% and 71% in all reactors.

The methane production from both microalgae species shows that biomass cultivation in wastewater coupled to anaerobic digesters allows wastewater treatment and energy recovery simultaneously. During the wastewater treatment process, the TN concentration reduction can lead to high lipid content in biomass; a high lipid content increases the methane production potential of the biomass.

3.4. Biodiesel production from microalgae

The FAME content in freeze-dried *C. kessleri* and *C. vulgaris* grown in wastewater was quantified, to evaluate suitability of the microalgae for biodiesel application.

The biodiesel yields obtained from *C. kessleri* and *C. vulgaris* amounted to 7.4±0.2 g/100g_{VS} and 11.3±0.1 g/100g_{VS} respectively. These results are comparable to the results obtained from *Chlorella sp.* reported elsewhere [7] and [28]. Although the biodiesel yields for both microalgae strains were not high enough for industrial biodiesel production, changes in microalgae cultivation conditions can enhance the accumulation of lipids and the biodiesel yields [29]. The biodiesel yield in *C. vulgaris* was also similar to results found during the cultivation of the same strain under N-starvation [17], confirming that the N-starvation increased the lipid content during cultivation in wastewater. Moreover, the fatty acid profiles of both microalgae strains were dominated by palmitic (C16:0), oleic (18:1), linoleic (C18:2) and linolenic (C18:3) acids; these fatty acids are the most common fatty acids contained in biodiesel produced from vegetable oils [30]. Fatty acid profile of *C. kessleri* consisted of 20.3%, 10.4%, 32.2% and 12.9% of palmitic, oleic, linoleic and linolenic acid, respectively. *C. vulgaris* contained 22.2%, 27.2%, 11.6% and 10.6% of palmitic, oleic, linoleic and linolenic acid, respectively.

Both microalgae cultivated in wastewater are suitable sources of lipids for biodiesel production, contrary to some other microalgae species with a high content of polyunsaturated fatty acids, which decreases oxidative stability of biodiesel [29] and [31]. The anaerobic digestion of microalgae wastes from biodiesel production was not evaluated. However, the idea of coupling biodiesel production and anaerobic digestion of the microalgae wastes might be a way to improve the global efficiency of biodiesel production.

4. Conclusions

The centrate from a WWTP was found interesting to supply nutrients for microalgae cultivation. The freshwater microalgae species *C. kessleri* and *C. vulgaris* removed N and P almost completely (> 95%); the progressive N-starvation derived from N consumption lead to a biomass with a high potential for methane production. Moreover, the biodiesel produced from both species showed interesting properties, indicating the possibility of producing this biofuel. Under the cultivation condition evaluated, *N. oculata* showed a low efficiency at nutrient removal; in order to obtain higher nutrient removal or higher biomass production for valuable compounds, cultivation must be optimised.

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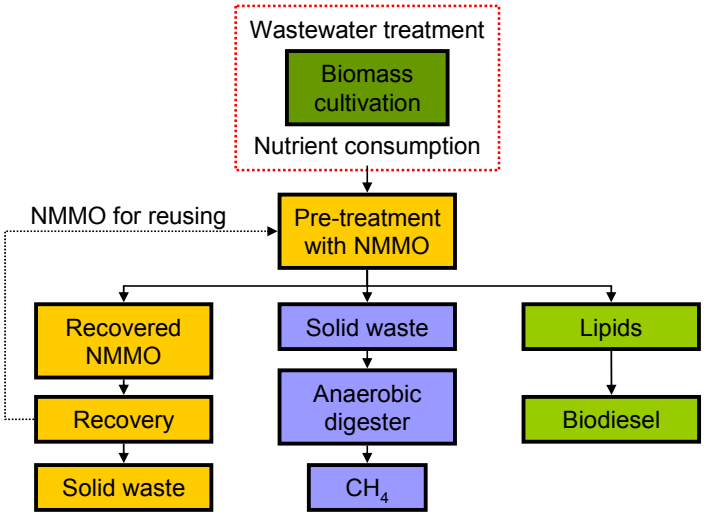
Chapter 6

A novel pre-treatment for the methane production from microalgae by using N-methylmorpholine-N-oxide (NMMO) [1]



ABSTRACT

The aim of this work was to study the effect of the solvent N-methylmorpholine-N-oxide (NMMO) to pre-treat *Nannochloropsis oculata* before the anaerobic digestion process. The results indicated that the pre-treatment affects the characteristics of the cell wall, which consequently becomes more susceptible to the microorganisms attack during anaerobic digestion. The methane production was increased by 43% after the pre-treatment, from 238±6 mL_{CH4}/g_{VS} until 339±4 mL_{CH4}/g_{VS}. On the contrary, the methane production from *Chlorella vulgaris* decreased after the pre-treatment from 251±4 mL_{CH4}/g_{VS} to 231±3 mL_{CH4}/g_{VS}. The failure on the pre-treatment was attributed to the particular characteristics of the substrate in consequence of a previous drying step.

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1. Introduction

Microalgae have the ability accumulate high amount of lipids, being promising feedstocks for biofuels (Rawat et al., 2013). Over the past years, researchers turned their attention to produce biodiesel from microalgae. Unfortunately, some challenges still required still need to be faced to scale up the biodiesel production, mainly derived from the high water content in microalgae cultures (Rawat et al., 2013). The anaerobic digestion (AD) is an alternative to produce energy from microalgae, in this case, biogas. The AD does not require the biomass drying, thus reducing the cost associated with harvesting (Kwietniewska and Tys, 2014). Moreover, AD results favourable when the biomass has low lipid content for the biodiesel production (Pragya et al., 2013). Some microalgae species such as *Chlorella sp.* and *Nannochloropsis sp.* demonstrated their suitability to grow in different wastewaters consuming nutrients, reducing the demand for fertilizer during their cultivation (He et al., 2013 and Caporgno et al., 2015b). This allows coupling the cultivation and the AD of microalgae in a wastewater treatment plant (WWTP), with environmental and economic benefits.

The organic solvent N-methylmorpholine-N-oxide (NMMO) has facilitated the AD of carbohydrates in lignocellulosic material, which is characterised by high resistance to degradation (Teghammar et al., 2014 and Kabir et al., 2014). Since the NMMO is usually used as an aqueous solution, it does not require the biomass drying. However, the amount of water affects the process (Jeihanipour et al., 2010a). This solvent is commercially used in the Lyocell fibre-production process due to the feasibility to act as solvent for cellulose at mild conditions (90-130 °C and ambient pressure) (Zheng et al., 2014). The NMMO shows low toxicity, high thermal stability, and the possibility of being recovered and reused, amongst other advantages (Jeihanipour et al., 2010a). The NMMO affects hydrogen bonds and weaken Van der Waals forces between the cellulose chain molecules, thus changing the cellulose structure (Zheng et al., 2014). Some of these changes, for example a decreased crystallinity and an increased porosity make cellulose more susceptible to AD (Jeihanipour et al., 2010a).

This paper is the first attempt to pre-treat microalgae, *Nannochloropsis oculata* and *Chlorella vulgaris*, using NMMO in order to improve the methane production.

2. Materials and methods

2.1. Materials

N. oculata and *C. vulgaris* species were provided by AlgoSource (Alpha Biotech, Asserac, France). They were cultivated in a raceway unit placed in a greenhouse with thermal control, and harvested via centrifugation. *N. oculata* microalgae was received as frozen slurry with 28% solids and stored at −15 °C. In order to facilitate the pre-treatment, the biomass was dried before the experiment. *C. vulgaris* was received dried, and the solid was stored in a dessicator.

NMMO 50% w/w in aqueous solution was concentrated to 85% as described by Jeihanipour et al. (2010a).

2.2. Microalgae pre-treatment

The pre-treatment process is described in Fig. 1. The microalgae sample, 6 g of dried biomass, was added into a round-bottom flask containing 94 g of 85% NMMO solution and placed in an oil bath at 120 °C for 3 h in atmospheric conditions. Agitation was provided by a magnetic stirrer. After the 3 h heating, 150 mL of boiling

deionised water was added to stop the process. The pre-treated microalgae were recovered by centrifugation as a solid fraction and washed with deionised water to eliminate the NMMO prior to the anaerobic digestion. The supernatant, which is the recovered NMMO, and liquid fraction from washing were collected together for further analysis. The pre-treatment was evaluated in three different opportunities.

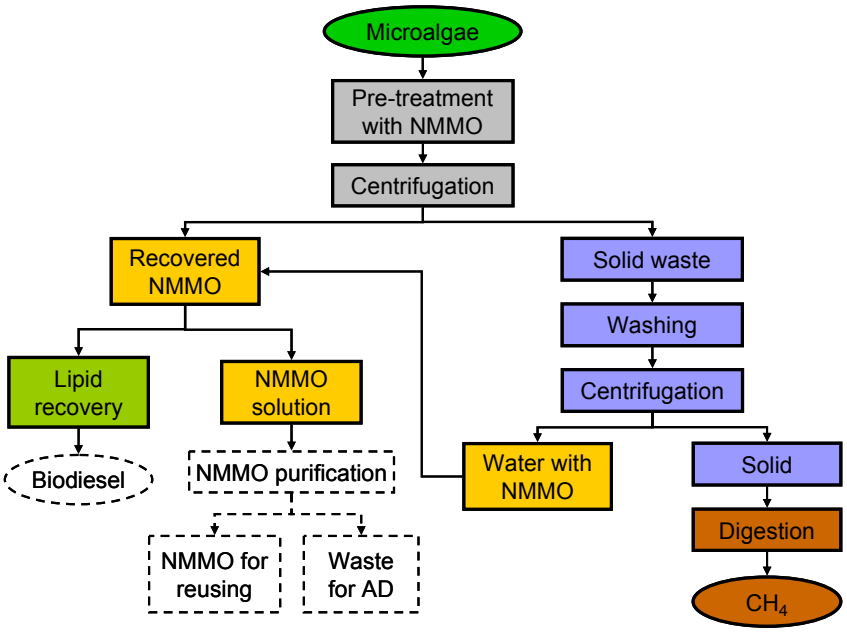

Fig. 1. Scheme of the pre-treatment process and the uses of the different products.

Microalgae samples, before and after the pre-treatment, were observed under a light microscope to evaluate their integrity The samples were also analysed by Fourier Transform Infrared (FTIR) spectroscopy to observe the changes in the protein, carbohydrate and lipid content in the biomass. Protein, carbohydrate and lipid content in raw and pre-treated samples were quantified as described by Caporgno et al. (2015a). In *C. vulgaris*, the carbohydrate content was calculated by the difference between total organic matter content and the contents of proteins and lipids.

2.3. Lipid extraction

The lipid content was analysed in the mixture containing the NMMO solutions collected after the microalgae pre-treatment and after washing, as indicated in Fig. 1. A sample of the mixture containing the NMMO was mixed with 10 ml of hexane; the hexane phase containing lipids was separated by centrifugation (3500 rpm, 10 min). This step was repeated three times. Hexane was dried under anhydrous sodium sulphate and evaporated in a rotary evaporator. For the lipid analysis in the pre-treated microalgae, a sample of microalgae was re-suspended in deionised water and acidified until pH 2 prior to the hexane addition. The results were expressed as gram of extractable lipids per gram of volatile solids in the dry microalgae used for the experiments, g/g_{VS}.

A thin-layer chromatography (TLC) was performed in order to evaluate the composition of the extracted-lipids. The lipids were dissolved in hexane and spotted on a TLC plate which was then developed in a solvent system of hexane/diethyl ether/acetic acid (60:40:1, v/v/v). Separated compounds were visualised under iodine vapour and identified by using authentic standards. The fatty acids in the extracted lipids were identified and quantified as described by Olkiewicz et al. (2014).

2.4. Anaerobic digestion

The AD experiments were performed in batch reactors at 33 °C in triplicate. The solid samples were re-suspended in deionised water for better handling. The inoculum consisted of digested sludge described in Caporgno et al. (2015a). The total solids (TS) and the volatile solids (VS) in all the substrates and in the inoculum were analysed according to standard methods as described by Caporgno et al. (2015a), and the substrate to inoculum ratio was adjusted to 1:2 $VS_{Substrate}$:$VS_{Inoculum}$ in all reactors.

The methodology for quantifying the biogas production and composition, the volatile fatty acid concentration (VFA), the ammonia concentration and the substrates biodegradability is fully described in Caporgno et al. (2015a).

3. Results and discussion

3.1. Lipid recovery after pre-treatment

Since the lipids are not solubilised in the NMMO solution due to the differences in the polarity of lipids and the NMMO solution, the released lipids can be recovered by hexane addition. The results indicated that 3.0±0.2 and 0.9±0.3 g/g_{VS} were recovered from the NMMO solution after *N. oculata* and *C. vulgaris* pre-treatment respectively. These were minor fractions considering the initial lipid content in both microalgae species (Table 1). The results indicated that the lipids were not released during the pre-treatment and remained in the pre-treated microalgae instead. Although the NMMO is usually considered as ionic liquid (IL) (Zheng et al., 2014), completely opposite results have been reported by Olkiewicz et al. (2015) using the same microalgae but different IL; the authors indicated almost complete lysis and high lipid-recovery from IL.

Table 1. Calculated by difference between difference between total organic matter content and the contents of proteins and lipids.

Parameter	Inoculum	*Nannochloropsis oculata*		*Chlorella vulgaris*	
		Raw [a]	Pre-treated [b]	Raw [a]	Pre-treated [b]
TS (g/L)	17.9±0.4	63.8±0.4	18.3±0.2	9.5±0.6	36.6±2.9
VS/TS	0.62	0.89	0.83	0.78	0.63
Lipids ($g/100g_{VS}$)	-	21.9±0.4	18.9 [c]	6.5±0.6	5.6 c
Proteins ($g/100g_{VS}$)	-	60.9±3.4	56.2±4.6	48.7±4.0	53.1±0.4
Carbohydrates ($g/100g_{VS}$)	-	16.7±1.5	23.2±0.9	41.2±0.7 [d]	37.5 [d]

[a] *Suspension in deionised water.*
[b] *Suspension in deionised water after washing.*
[c] *Calculated considering the lipid recovery from the NMMO solution.*
[d] *Calculated by difference between difference between total organic matter content and the contents of proteins and lipids.*

After the pre-treatment, cells with different appearances were distinguished (Fig. S1 in the Supplementary material); however, it was impossible to identify if the lipid fraction remained inside the microalgae cells with the naked eye. Due to the strong alkaline pH of the NMMO solution (Navard, 2012), one of the possibilities was that the lipids remains in the solid as calcium and magnesium salts, product of the reaction between the free fatty acids (FFA) and the metal ions present in the solution. Since this reaction can be reversed by acidification (Olkiewicz et al., 2014), this hypothesis was verified by the analysis of the lipid in the pre-treated microalgae after acidifying the sample until pH 2. The results indicated that 14.4±0.5 and 3.0±0.1 g/g_{VS} were recovered from *N. oculata* and *C. vulgaris* respectively after acidification of the pre-treated solid. These lipid yields represent 83% and 77% of

the total lipid recovered with hexane from *N. oculata* and *C. vulgaris* respectively, indicating that the major part of the lipid fraction remained in the solid fraction.

The TLC analysis revealed that the different lipid fractions had similar compositions. The fractions were not only FFA, but also contained monoglycerides, diglycerides and triglycerides. This refused the hypothesis about the lipids remaining as calcium and magnesium salts, and suggested that the pre-treatment modified the microalgae cell walls and facilitated the lipid extraction from the microalgae after acidification. On the other hand, the similarity in the lipid fractions revealed that the lipids recovered from the NMMO solution came from part of the biomass which was completely damaged during the pre-treatment.

The lipid fractions were converted into biodiesel. The fatty acid profile agreed with the composition reported for both microalgae species using different extraction methods (Olkiewicz et al., 2015). The *N. oculata* profile was dominated by cis-5,8,11,14,17-Eicosapentaenoic (20:5), cis-9 Palmitoleic (C16:1) and Palmitic (C16:0), whereas the *C. vulgaris* profile, by Linolenic (C18:3), Palmitic (C16:0), cis-10-heptadecenoic (C17:1), Linoleic (C18:2), Oleic (C18:1) and cis-9 Palmitoleic (C16:1).

3.2. Methane production from the pre-treated microalgae

As can be observed in Fig. 2, the methane production from the raw microalgae species resulted similar for *N. oculata* and *C. vulgaris*. Regarding the pre-treatment, it can be observed that the NMMO pre-treatment has positively affected the methane production from *N. oculata*, which increased from 238±6 to 339±4 mL_{CH4}/g_{VS} after pre-treatment, representing a 43% increase. On the contrary, *C. vulgaris* showed an 8% decrease after the pre-treatment, from 251±4 to 231±3 mL_{CH4}/g_{VS}.

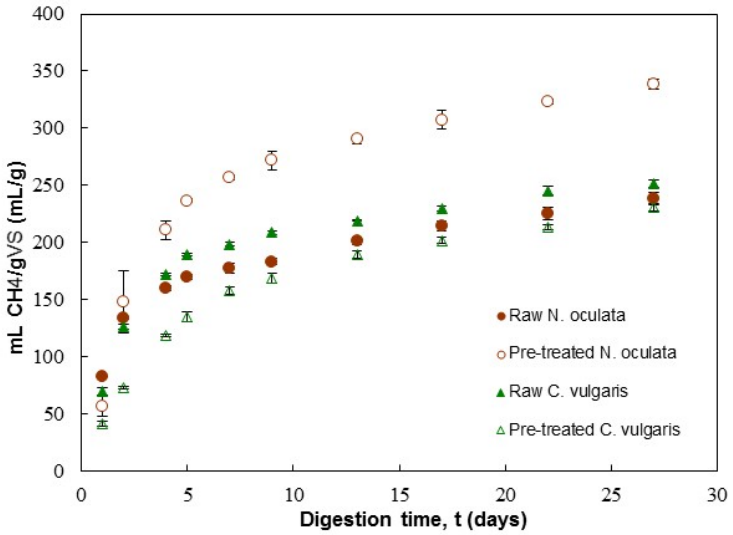

Figure 2. The methane production curves from raw and pre-treated *N. oculata* and *C. vulgaris*. Symbols: ● raw *N. oculata*, ○ pre-treated *N. oculata*, ▲ raw *C. vulgaris* and ♦ pre-treated *C. vulgaris*. Batch reactors at 33ºC.

The theoretical methane production based on the substrate compositions (Table 1) resulted in 596 and 576 mL_{CH4}/g_{VS} for raw and pre-treated *N. oculata*, and 496 and 494 mL_{CH4}/g_{VS} for raw and pre-treated *C. vulgaris* respectively. In terms of theoretical methane production, lipids almost double proteins and carbohydrates (Caporgno et al., 2015b); thus higher values resulted for *N. oculata*. Based on these values, the biodegradability

increased from 40% to 59% for *N. oculata* after the pre-treatment, and slightly decreased from 51% to 47% for *C. vulgaris*.

The pre-treatment damaged the *N. oculata* cells, remaining the lipid fraction in the cells. The lipids became then available for microorganisms, increasing the methane production drastically. The carbohydrate fraction may have also contributed to increase the methane production. The cell wall of *Nannochloropsis sp.* is characterised by a thick and multi-layered wall composed of polysaccharides (Bohutskyi et al., 2014). The addition of water to stop the pre-treatment produced the carbohydrates precipitation, which constitute part of the solid fraction recovered by filtration. The NMMO has demonstrated its feasibility to enhance the digestibility of low-digestible carbohydrates (Zheng et al., 2014); however, based on the low carbohydrate content in *N. oculata*, the methane production enhancement can be mainly attributed to the lipid fraction. In spite of *C. vulgaris* and *N. oculata* show similar cell walls composition (Bohutskyi et al., 2014), the cell aggregates originated during *C. vulgaris* drying remained partially unaffected after the pre-treatment (Fig. S1d-f). These aggregates hampered the action of the NMMO, thus the pre-treatment failed at increasing the digestibility of the low-digestible carbohydrates. The intra- and intermolecular hydrogen bonds were unaffected by the pre-treatment (Fig. S2b). Considering the higher carbohydrate fraction in *C. vulgaris* compared with *N. oculata*, the digestion of the carbohydrate fraction can contributes considerably to the final methane production. On the contrary, the lipid fraction is low in *C. vulgaris*, thus does not increase the methane production. Moreover, the lipid fraction still remained inaccessible for the microorganisms after the pre-treatment due to the microalgae aggregates. The lower methane production in pre-treated samples can derive from the minor changes in the biomass composition.

The pH ranged 7.41-7.53, the concentration of ammonium nitrogen 736-825 mg/L and VFA were not detected at the end of the experiments. These parameters corroborated that all digesters operated at optimum conditions. The biogas composition, analysed several times during the experiments, resulted in 71.3±1.9% and 71.2±2.2% CH_4 for *N. oculata* and *C. vulgaris* respectively; the pre-treatment did not influence the biogas composition.

3.3. Other considerations for economic feasibility

The pre-treatment with NMMO is high-energy consuming since the solvent is heated at 120 °C. However, all the pre-treatments reported in the literature are usually carried out at 90 °C or higher (Teghammar et al., 2014, Jeihanipour et al., 2010a, Jeihanipour et al., 2010b and Kabir et al., 2014); the reason is that the NMMO 85% in water is a solid at room temperature. The pre-treatment here proposed could be optimised either reducing the temperature and the duration of the pre-treatment or reducing the energy demand by heat-integration.

Unlike the chemical pre-treatments usually reported, this method allows recovering the NMMO for its reutilisation, being essential to make similar processes economically feasible (Jeihanipour et al., 2010b). It has been proved that the NMMO can be reused for the pre-treatment, but the characteristics of the biomass strongly affects recycling (Jeihanipour et al., 2010b and Kabir et al., 2014). The NMMO has not been reused in the present experiments; however, the 1H NMR spectroscopy analyses demonstrated the solvent stability after the pre-treatment of *N. oculata* at 120 °C for 3 h (Fig. S3).

4. Conclusions

The digestibility of *N. oculata* was successfully increased after the pre-treatment with NMMO. On the contrary, *C. vulgaris* digestibility was negatively affected; the drying step in the biomass production process influenced the digestion process more than the pre-treatment itself.

The NMMO is currently used in industrial processes and presents several advantages over some other solvents; however, the pre-treatment method requires optimisation in order to design an economically viable process. The NMMO does not decompose during the pre-treatment and could be fully recycled.

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APPENDIX A. SUPPLEMENTARY DATA

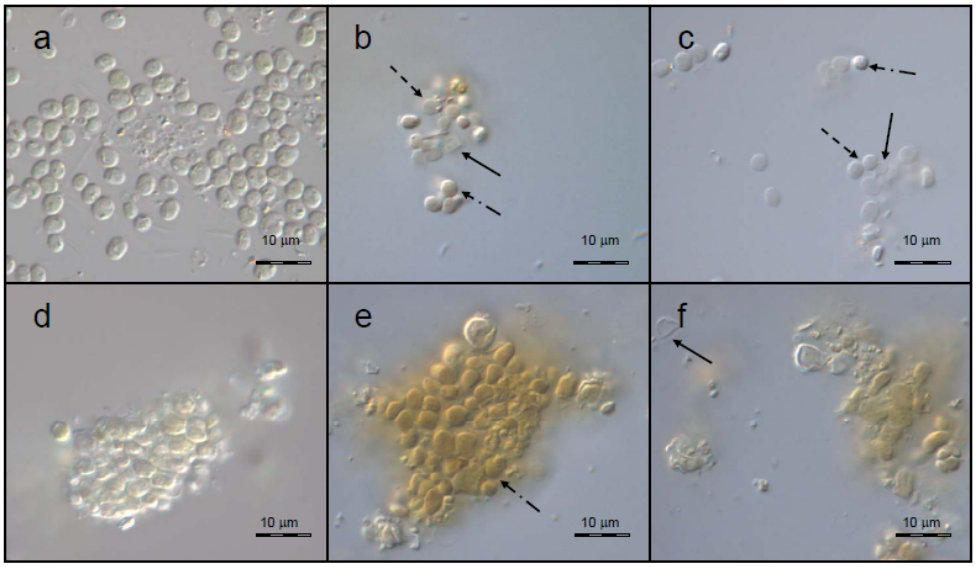

Figure S1. Light micrographs of *N. oculata* (a) raw and (b-c) pre-treated, and *C. vulgaris* (d) raw and (e-f) pre-treated.

Note: Cells with different appearances are distinguished after *N. oculata* pre-treatment (Figures S1b-c), indicated as: empty cell walls (dotted line), refringent cells which contain some internal material (dashed line) and cells with a similar appearance than before the pre-treatment (dot-dash line). Aggregates caused during *C. vulgaris* drying are present before (Figure S1d) and after the pre-treatment (Figure S1e-f). Empty cell walls are also present (Figure 2f).

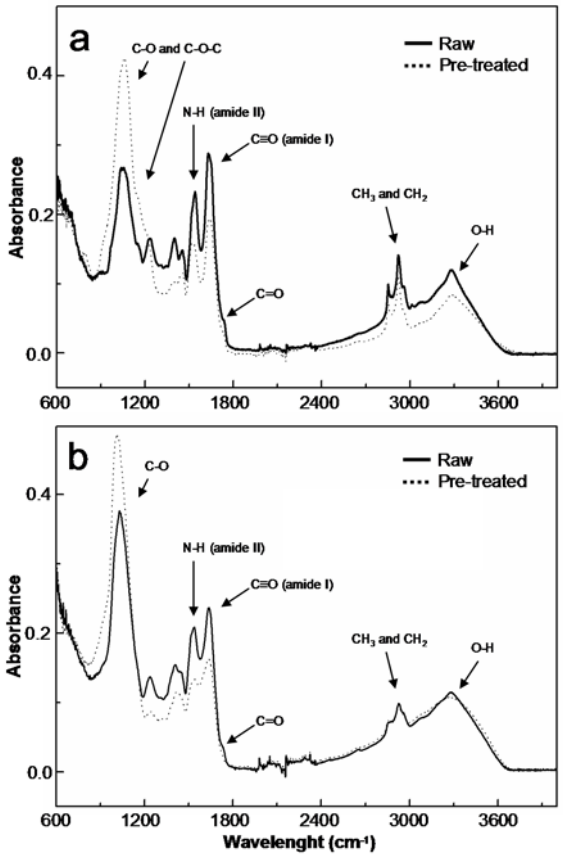

Figure S2. FTIR spectra of raw and pre-treated *N. oculata* (a) and *C. vulgaris* (b).

Note: Bands at 1,700-1,750 cm^{-1} and 2,800-3,000 cm^{-1} are characteristics of C=O groups in lipid esters and CH_3 and CH_2 in the lipid acyl chains respectively (Caporgno et al., 2015). These bands are less intensified in pre-treated *N. oculata* (Figure S2a). The bands characteristics of proteins at 1,500-1,700 cm^{-1} (Caporgno et al., 2015) are also less intensified in pre-treated samples. The bands characteristic for carbohydrates at 1,000-1,200 cm^{-1} (Caporgno et al., 2015) are stronger. The band at 3,350 cm^{-1} characteristic of O-H bonds, which indicates a weaker intra- and intermolecular hydrogen bonds (Jeihanipour et al., 2010A), is less intensified after pre-treatment.

***C. vulgaris* showed similar changes in proteins and carbohydrates after pre-treatment (Figure S2b). The change in the lipid fraction remains almost imperceptible, due to the low lipid content in the raw microalgae. The absorption band characteristic of O-H bonds remains almost unaffected after the pre-treatment.**

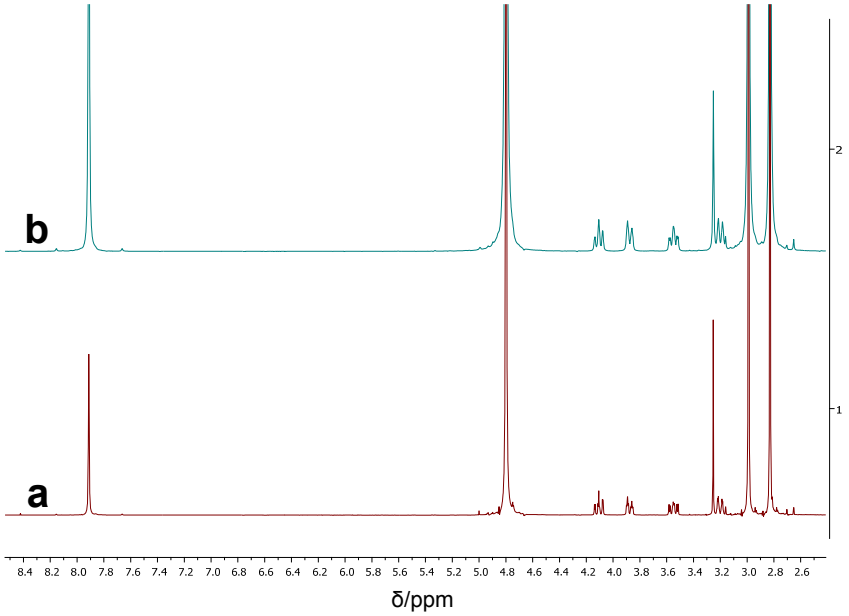

Figure S3. 1H NMR spectra (D2O) of NMMO aqueous solution: (a) fresh NMMO and (b) recovered NMMO after microalgae pre-treatment for 3 h at 120 ºC.

Note: The spectra suggest that NMMO does not decompose after using. The differences in the signal are caused by the proteins solubilised in the recovered NMMO.

Chapter 7

Anaerobic digestion of microalgae: the benefits of digesting microalgae waste [1]

ABSTRACT

The biodiesel production from microalgae seems to be a valuable alternative to meet the future energy requirements of the population; the high lipid productivity and the possibility of using no arable land and diverse sources of water provide several advantages over the current feedstocks used for biodiesel production. However, there are still some drawbacks which make the process scale-up economically unviable. The researchers have been focused on different areas of the process in order to reduce costs and increase profits; one of these areas is the additional energy recovery from the lipid-extracted waste. An increasing number of publications regarding methane production from microalgae waste have been published over the past five years; microalgae species, microalgae processing, pre-treatment application, operating condition in the digesters are some of the variables studied. This review attempts to summarise the results relative to the anaerobic digestion of lipid-extracted waste reported for more than 30 microalgae species in the last years, and some aspects which affects the process.

[1] M.P. Caporgno and C. Bengoa, Anaerobic digestion of microalgae: the benefits of digesting microalgae waste, Current Biochemical Engineering, Special Issue Biomethane. Accepted for publication.

1. Introduction

According to the projections of population growth, which are between 9 and 10 billion inhabitants by 2050, the requirements of agricultural products will almost double the current ones. The agricultural products are not only basic feedstocks for food production, but also for several products in daily life. Over recent years, agricultural products such as crops have been used for energy production, impacting on the agriculture. Since the land suitable for agriculture is scarce, in the coming years society will face problems derived from the competition for land: food production or biofuels production, food production or for nature conservation, built-up or production or conservation [1]. Basically, several changes in society habits are required to meet the future population needs.

The fossil fuel reserves depletion is problematic. Petroleum, natural gas, and coal reserves depletion is predicted for the next 45, 60, and 120 years respectively, and they represent more than 80% of the current energy demand in the world. The biofuels production has gained attention. The ethanol and biodiesel production was introduced to substitute fossil fuels for transport; however, their production had a negative impact on the agricultural markets. In contrast, biogas can be produced from inedible feedstocks [2]. The anaerobic digestion (AD) or methanisation of the current agriculture waste such as manures and crop residues, and domestic waste for example municipal organic solid waste and sewage sludge, could substitute one quarter of the current natural gas consumption [2]. Moreover, to upgrade biogas for "bio-methane" production constitutes an alternative to provide fuels for transport [3].

The initial attempts to convert microalgae into biofuels were initiated decades ago, when Golueke et al. produced methane from microalgae cultivated in a wastewater treatment plant (WWTP) during the wastewater treatment [4]. Over the past few years, microalgae arose as a potential biomass for biodiesel production due to several advantages. The microalgae show high growth rates and high lipid contents, both essential characteristics for biodiesel production. Moreover, they grow in brackish water or wastewater, and they can reduce the strains on agricultural production by using non-arable land. Unfortunately, the biodiesel production from microalgae is not yet available. The microscopic size of microalgae and the low biomass concentration in the culture complicate harvesting, which can represent between 20% and 30% of the total biomass production costs. Furthermore, the industrial-scale processes for lipid extraction from crops are unsuitable for microalgae due to microalgae moisture, and the lipid extraction from wet biomass is in the developmental stage [5]. These drawbacks motivate the AD of microalgae, which is a cost-effective technology to produce energy from wet biomass. What is more, it has been reported that AD results the best alternative for the energetic recovery when the lipid content does not exceed 40% in the biomass [6]. Nonetheless, the biodiesel production from microalgae still gathers the attention of researchers. The main efforts attempt to discover microalgae species with high lipid productivities, efficient harvesting processes, novel lipid-extraction processes from wet biomass, and any further improvement which can contribute to make the process economically viable. The energy recovery from the lipid-extracted waste (LEW) arose as an alternative due to the possibility of coupling the AD to other processes in a bio-refinery approach. Approximately 65% of the biomass remains as waste in the biodiesel production process, thus its utilisation for energy purposes may improve the energetic and economic balances. The methanisation of LEW produces energy, minimises the waste production [7] and makes possible the nutrient recycling [8]. Moreover, the lipid extraction may be beneficial since lipids are digested slowly than carbohydrates and proteins [6], and show low alkalinity and buffering capacity during digestion [7]. In spite of these advantages, the theoretical methane potential for LEW

can result considerable low when compared with the whole biomass. Lipids significantly contribute to the methane production due to their high theoretical methane potential compared with carbohydrates and proteins [6]. The higher protein content derived from the lipid extraction constitutes another drawback, since the ammonium release in the digester is high [6].

Over the past five years, an increasing number of publications regarding methane production from microalgae waste have appeared. Microalgae species, microalgae processing, pre-treatment application, operating conditions during AD and other aspects have been evaluated. However, there are no reviews in relation to this topic. This review attempts to summarise these results with reference to the AD of microalgae waste, but the main focus is on waste after the lipid extraction.

2. The anaerobic digestion of microalgae

The AD is a biological process; microorganisms breakdown biodegradable compounds, in absence of oxygen, producing a mixture composed mainly by methane and carbon dioxide called biogas. The process occurs naturally in the environment, and it has been historically used for the treatment of animal manure and sewage sludge in WWTP. The characteristics, advantages and disadvantages, and operational parameters were summarised several years ago [9].

Briefly, this complex process is promoted by different bacteria and Archaea groups and divided into four steps: hydrolysis, acidogenesis, acetogenesis/dehydrogenation, and methanogenesis. The hydrolysis is responsible for the initial attack on the polymers such as carbohydrates and proteins, fats. Most of this insoluble particulate matter is decomposed through the action of extracellular enzymes and converted into water-soluble compounds. Anaerobic species belonging to the family Streptococcus and Enterobacter are involved in this process. During acidogenesis, these water-soluble compounds are converted into short-chain organic acids (formic, acetic, propionic, butyric, and pentanoic), alcohols (methanol, ethanol), aldehydes, carbon dioxide, and hydrogen. Ammonia and hydrogen sulphide are also generated. The bacteria involved are facultative anaerobes; they consume the oxygen present favouring anaerobic conditions and the development of obligatory anaerobes microorganisms such as Pseudomonas, Bacillus, Clostridium, amongst others. Some of the compounds produced can not be used directly by methanogens, thus they are converted into acetates and hydrogen during the acetogenesis step. Bacteria of the genera of Syntrophomonas and Syntrophobacter carry out the acetogenesis. The final step, methanogenesis, is characterised by the methane production. Although it is promoted by microorganisms classified as Archaea or Archaeabacteria, it is worth mentioning that the methanogens are usually mentioned as methanogenic bacteria in the literature. Methane derives from acetic acid, H_2, CO_2, and formate and methanol, methylamine, or dimethyl sulphide previously produced. Owing to the substrate used, methanogens are classified as hydrogenotrophic (they use only H_2 and CO_2), acetotrophic (they reduce methyl groups, being acetate their most important substrate) and methylotrophic (they reduce methylated compounds). The majority of CH_4 comes from the acetic acid conversions and an insignificant part from the CO_2 reduction, thus the generated biogas is rich in CO_2 [10].

Additionally to the biogas production, digested sludge is generated in the digestion process. This second by-product still contains a solid fraction composed of indigestible organic compounds, and a nutrient-rich liquid fraction which can be used as a nutrient source for microalgae cultivation as it is further discussed.

Although microalgae digestion was first reported in the 1950s [4], the majority of studies have been reported in recent years. The utilisation of microalgae wastes in AD is even more recent. Several authors have summarised results obtained for microalgae digestion under different conditions [7,11,12]. Ward et al. provided information about the C to N ratio, the methane production and the loading rate in the digesters [11]. Similarly, Kwietniewska and Tys included some results for microalgae species cultivated under stressed-condition [7]. Finally, one of the latest reviews included the effects of several pre-treatment methods to increase the methane production from microalgae [12]. These aspects affect microalgae digestion but also microalgae waste digestion. Table 1 summarises the results published in recent years relative to the methanisation of microalgae waste. The table is comprised of: the microalgae species used in the experiments, some of the experimental conditions (the inoculum source, the temperature of digester, and the operation mode of digesters), characteristics of lipid-extraction process (solvents and processes), the pre-treatment methods, the C to N ration in the substrate and the methane production obtained. All these parameters and their influence are further discussed in detail.

3. Anaerobic digestion of lipid-extracted waste

The first attempts to digest microalgae waste from biodiesel production were reported some years ago by Ehimen et al. [13]. Later, the increasing number of publications contributed significantly to the understanding of the process. This section describes various aspects related to this topic.

3.1. The influence of the microalgae species

Several microalgae waste have been digested; some of them derived from the same species. The substrates reported in the experiments are detailed in the first column of Table 1.

Mussgnug et al. reported that species with carbohydrate-based cell walls showed low degradability, whereas those species with protein-based cell walls or species without cell walls were less resistant to AD [14]. Unfortunately, drawing general conclusions are complicated after the lipid extraction. One of the reasons being the lipid-extraction process influences the process. For example, Zhao et al. evaluated microalgae species with carbohydrate-based and silicified cell walls. Their degradability resulted in differences in spite of the similarity between some species, as a consequence of the lipid extraction [15].

Moreover, the comparison between the results reported by different authors is complicated, even though the same microalgae species is used. The results vary significantly. It became evident for example for *C. vulgaris*; the methane production differs considerably regardless of the similar conditions during digestion (Table 1) [13,15-17]. This was also observed with *Scenedesmus sp.* [19-22]. Even when the same microalgae species were used in the experiments, the cultivation conditions varied. The cultivation conditions affect the microalgae composition [23], thus the methane production. Additionally, the differences in the lipid-extraction process affect the degradability as is further explained. However, numerous authors agreed on the need of pre-treatment application to increase the degradability due to both species being characterised by strong carbohydrate-based cell walls.

The microalgae species only give an overall idea of the characteristics of the organic matter and its biodegradability.

Table 1: AD of microalgae waste published in the last years, focused on lipid-extraction waste

Microalgae species	Experimental conditions		Microalgae processing	C:N	mL_{CH4}/g_{VS}	Ref.
Chlorella sp.[1]	IA Temp.: 37 ºC Batch digester Dry biomass		Whole biomass	8.60	418.6	[13]
			LE: 1-butanol	5.60	267.5	
			LE: chloroform/methanol	5.40	negligible	
			PT: ACIST	5.60	222	
Chlorella sp.	Inoc.: IA Temp.: 35 ºC Semi-continuous digester; HRT 15 d Dry biomass	OLR: 5 Kg_{VS}/m^3	PT: ACIST	5.4	245	[16]
		OLR: 10 Kg_{VS}/m^3		5.4	240*	
		OLR: 20 Kg_{VS}/m^3		5.4	195*	
		OLR: 30 Kg_{VS}/m^3		5.4	165*	
		OLR: 40 Kg_{VS}/m^3		5.4	150*	
		OLR: 50 Kg_{VS}/m^3		5.4	120*	
Scenedesmus sp. waste	Inoc.: IDS Temp.: 37 ºC Batch digester Dry biomass	One-stage process for methane production	LE: no data PT: NaOH	10.8	322.6	[19]
		Two-stage process for hydrogen and methane production.	LE: no data PT: NaOH	10.8	393.6	
Nannochloropsis salina waste [2]	Inoc.: IDS Temp.: 37 ºC Semi-continuous digester Wet biomass	OLR: 2 $g_{VS}/L \cdot d$	LE; PT: Electric pulsation, acid hydrolysis, and extraction with hexane.	4.4	130	[30]
		OLR: 3 $g_{VS}/L \cdot d$		4.4	30*	
		OLR: 4 $g_{VS}/L \cdot d$		4.4	50*	
		OLR: 6 $g_{VS}/L \cdot d$		4.4	negligible*	
Scenedesmus sp.	Inoc.: IDS Temp.: 38 º Batch digester Dry biomass		Whole biomass	-	200*	[20]
			PT: HPTL	-	350*	
			LE: hexane	-	240*	
			LE; PT: HPTL	-	390*	
Ettlia sp. waste	Inoc.: IDS Temp.: 35 ºC Batch digester Dry biomass		LE: no data	9.5	126	[34]
			LE; PT: NaOH	9.5	80	
			LE; PT: NaOH/sonication	9.5	92	
			LE; PT: NaOH/irradiation	9.5	162	
			LE; PT: NaOH/autoclave	9.5	176	
Nannochloropsis gaditana	Inoc.: IDS Temp.: 35 ºC Batch digester Dry biomass	SIR: 0.5:1, 1:1 and 3:1 $VS_{algae}:VS_{inoc}$ SC: 10 g_{TS}/kg	Whole biomass	-	~303	[33]
			LE: ethanol	-	~327	
		SIR: 1:1 $VS_{algae}:VS_{inoc}$ SC: 10 g_{TS}/kg	Whole biomass	-	300	
			LE: ethanol	-	331	
			LE; PT: thermal hydrolysis	-	345-381	
			LE; PT: ultrasound	-	318-382	
			LE; PT: biological	-	289-321	
Nannochloropsis sp. waste	Inoc.: IDS		LE: dried process; methanol/hexane	-	194	[24]

Microalgae species	Experimental conditions		Microalgae processing		C:N	mL_{CH4}/g_{VS}	Ref.
	Temp.: 35 ºC Batch digester		PT: washing		-	171	
			LE: wet process; ethanol/hexane		-	482	
			PT: washing		-	333	
	Semi-continuous digester; 35 ºC	OLR: 0.5-2 $g_{VS}/L \cdot d$	LE: dried process		-	156-208	
	Semi-continuous digester; 35 ºC	OLR: 0.5-3 $g_{VS}/L \cdot d$	LE: dried process; PT: washing		-	128-174	
	Semi-continuous digester; 55 ºC	OLR: 1.5-2 $g_{VS}/L \cdot d$	LE: dried process; PT: washing		-	155-220	
Scenedesmus sp.	Inoc.: IA Temp.: 37 ºC Batch digester Wet biomass data		Whole biomass		5.9	140.3	
			LE: Hexane		6.1	212.3	[21]
			PT: Amino acids extraction		7.2	272.8	
Scenedesmus dimorphus waste	Inoc.: IDS Temp.: 37 ºC Batch digester Dry biomass		LE: Hexane		9	64*	[22]
		SC: 1 g/L.	LE; PT: Thermal at 100, 120 and 150 ºC.	20 min	9	76-99	
				40 min	9	88-107	
				60 min	9	99-113	
		SC: 3 g/L.	LE		9	80*	
			LE; PT: Thermal at 100, 120 and 150 ºC.	20 min	9	88-102	
				40 min	9	93-107	
				60 min	9	100-119	
		SC: 5 g/L.	LE		9	102*	
			LE; PT: Thermal at 100, 120 and 150 ºC.	20 min	9	117-142	
				40 min	9	121-150	
				60 min	9	137-163	
Chlorella vulgaris	Inoc.: IDS Temp.: 35 ºC Batch digester Dry biomass		Whole biomass		6.80	337	
			LE: hexane/isopropanol		5.51	314	
Nannochloropsis sp.			Whole biomass		7.55	357	
			LE: hexane/isopropanol		6.36	399	
Nannochloropsis salina			Whole biomass		14.87	557	
			LE: hexane/isopropanol		8.46	383	
Phaeodactylum tricornutum			Whole biomass		6.86	337	[15]
			LE: hexane/isopropanol		5.68	339	
Nanofrustulum sp.			Whole biomass		9.47	507	
			LE: methyl pentane		6.89	304	
Nannochloropsis salina	Inoc.: IDS Temp.: 35 ºC Batch digester	SIR: 10:1 $VS_{algae}:VS_{inoc}$	LE: hexane/isopropanol		8.46	86.5	
		SIR: 2:1 $VS_{algae}:VS_{inoc}$			8.46	250.5	
		SIR: 1:1 $VS_{algae}:VS_{inoc}$			8.46	248	

Microalgae species	Experimental conditions		Microalgae processing	C:N	mL_{CH4}/g_{VS}	Ref.
	LE: hexane/isopropanol (3:2 v/v)	SIR: 0.66:1 VS_{algae}:VS_{inoc}		8.46	248	
		SIR: 0.5:1 VS_{algae}:VS_{inoc}		8.46	255	
A. (Chlorella) protothecoides	Inoc.: IDS Temp.: 35 ºC Batch digester Dry biomass		Whole biomass	44.8	600	[52]
			LE: Bligh and Dyer method	22.4	385	
Isochrysis T-ISO	Inoc.: IDS Temp.: 38 ºC Batch digester Dry biomass		Whole biomass	-	188*	[27]
			LE: $SCCO_2$	-	215*	
Tetraselmis sp.			Whole biomass	-	167*	
			LE: $SCCO_2$	-	236*	
Nannochloropsis gaditana			Whole biomass	-	172*	
			LE: $SCCO_2$	-	204*	
Scenedesmus almeriensis			Whole biomass	-	204*	
			LE: $SCCO_2$	-	208*	
Nannochloropsis salina	Inoc.: IDS Temp.: 35 ºC Batch digester Dry biomass		Whole biomass	20.4	430	[53]
			LE: Hexane	8.7	140	
Chlorella vulgaris waste	Inoc.: IDS Temp.: 35 ºC Reactor: One-stage batch Dry biomass	SIR: 3:1 VS_{algae}:VS_{inoc}	LE: no data	5.3	negligible	[17]
		SIR: 2:1 VS_{algae}:VS_{inoc}		5.3	26.6	
		SIR: 1:1 VS_{algae}:VS_{inoc}		5.3	191.6	
		SIR: 0.5:1 VS_{algae}:VS_{inoc}		5.3	195.6	
	Reactor: Two-stage Batch for acidogenesis (SIR of 3:1) and Up-flow Anaerobic Sludge Blanket for methanogenesis (OLR 2-12 $g_{COD}/L \cdot d$)			5.3	319-336[3]	
Scenedesmus sp.	Inoc.: Inoc. from AD of manure Temp.: 37 ºC Batch digester Dry biomass		Whole biomass	-	160*	[28]
			PT: Protein extraction		208*	
			LE: Hexane/ethanol (3:1 v/v)		219*	
			LE; PT		204*	
Tetraselmis sp.	Inoc.: IA adapted to 7% salinity. Temp.: 37 ºC Batch digester Dry biomass		Whole biomass	-	252	[11]
			PT: Sonication		248	
			PT: Sonication and LE: Hexane		122	
Botryococcus braunii waste	Inoc.: Anaerobic granular biomass from brewery wastewater treatment. Temp.: 35 ºC Batch digester. Dry biomass		LE: petroleum ether	-	404	[36]
A. (Chlorella) protothecoides	Inoc.: IA	OLR: 1 $g_{VS}/L \cdot d$; HRT: 20 d	LE: Acetone and hexane	19.2	215*	[31]

Microalgae species	Experimental conditions		Microalgae processing	C:N	mL_{CH4}/g_{VS}	Ref.
	Temp.: 35 ºC Reactor: Semi-continuous Wet biomass	OLR: 2 g_{VS}/L·d; HRT: 20 d		19.2	180*	
		OLR: 1 g_{VS}/L·d; HRT: 40 d		19.2	255*	
		OLR: 1.5 g_{VS}/L·d; HRT: 40 d		19.2	145*	
Chlorella vulgaris	Inoc.: IDS Temp.: 33 ºC Batch digester Dry biomass		Whole biomass	-	219	[18]
			LE: hexane	-	202	
			LE: methanol:hexane (2:3 v/v)	-	200	
Nannochloropsis oculata			Whole biomass	-	253	
			LE: hexane	-	313	
			LE: methanol:hexane (2:3 v/v)	-	254	
Phaeodactylum tricornutum	Inoc.: IDS Temp.: 33 ºC Batch digester Dry biomass		Whole biomass	-	225	[26]
			LE: hexane	-	224	
			LE: methanol:hexane (2:3 v/v)	-	157	

* Values taken from graphs or calculated

3.2. The influence of the lipid-extraction process over the LEW

Researchers have reported that the lipid extraction processes influence the degradation and the methane production from the waste, but the reasons were not clearly established [20,24]. Various authors suggested cell wall disruption caused by the action of the organic solvents [20], whereas others considered that parameters such as the temperature and pressure during the extraction process, or the biomass drying caused a greater influence on degradation than the solvents themselves [24]. However, there are no doubts about the presence of a cell wall strongly affected microalgae digestion, neither that the lipid-extraction process affected the cell wall characteristics. Various characteristics of the lipid-extraction processes are summarised in Table 1.

Researchers Ehimen et al. were not only the first authors who reported microalgae waste digestion but also the first in evaluating the influence of the extraction processes over the methane production. Different extraction processes and different solvents generated waste from *C. vulgaris* with a quite similar composition; however, the methane production differed considerably. The results were attributed to the inhibition caused by chloroform, or the additional disruption caused by the sulphuric acid used in the "in situ transesterification" [13]. Although the results are not included in the report, Zhao et al. also mentioned the evaluation of different solvents to extract lipids from *Nannochloropsis sp.*. The authors also observed inhibition after using chloroform during the lipid extraction [15]. Yun et al. recently reported the inhibitory effect of chloroform, which can decrease by 30% the methane production at levels of 10 mg_{CHCl3}/L in the digester. The authors suggested co-digestion (Aco-D) or a previous step for rinsing or solvent evaporation [25]. Caporgno et al. compared the methane production from *C. vulgaris*, *N. oculata* and *P. tricornutum* waste, extracted with hexane and methanol/hexane (2:3 v/v). Although inhibition was not observed, the effects of the solvent varied between the microalgae species (Table 1) [18,26].

Characteristics of the organic solvents, for example polarity, strongly influence the methane production. Due to the polar nature of methanol, polar lipids such as phospholipids and glycolipids are extracted from microalgae but also polar compounds such proteins and carbohydrates [13]. On the contrary, a non-polar solvent such as hexane enables the extraction of mainly non-polar lipids. By mixing solvents with different polarities, polar and non-polar lipids are extracted from microalgae. As a consequence of the differences in the composition, the amount of methane varies [18]. Hernández et al. evaluated several solvents and methods for lipid extraction. Although the waste were not all digested, the differences in the lipid-extraction yields clearly evidenced the differences in the waste composition, mainly in their residual lipid content [27]. Zhao et al. reported that the residual lipid content could favourably contribute to the methane production or even inhibit the digestion process [15]. Also related to the characteristics of the organic solvents, Astals et al. concluded that the methane production may results fast or slow, if the major organic fraction in the waste is rapidly or slowly-degradable. Furthermore, the degradability of the slowly-degradable fraction determines the final methane production depending on whether or not it is digested [28]. The use of polar solvents can increase the biodiesel yields but simultaneously generate a waste with high indigestible fraction [18,26]. The influence of the solvents over the biodiesel and methane production might be evaluated before coupling both processes.

Parameters such as the temperature or the pressure during the extraction process can strongly affect the degradation. The microalgae drying decreases the methane production either by the loss of volatile solids (VS) or the changes incurred in microalgae structures [14], and drying is usually a previous step of the lipid extraction. Kinnunen et al. evaluated the methane production from *Nannochloropsis sp.* recovered after two extraction processes, using dry and wet biomass. The wet process involved high pressure and temperature, which favoured the methane production. On the contrary, drying was detrimental from digestion (Table 1) [24]. The acid utilisation in the transesterification process also affected the degradation of the waste [13]. Hernández et al. found higher organic matter degradation after the lipid extraction using SCO_2; the results were attributed to the ability of the SCO_2 process to disrupt the microalgae cell walls and break down proteins and carbohydrates into more easily degradable compounds [27].

The suitability of the microalgae waste for methane production can not be easily predicted. It is known that the polarity of organic solvents or the characteristics of the lipid-extraction processes affect the composition and/or the characteristics of the microalgae waste, thus their digestion. However, these are just some of the factors which influence the methane production.

3.3. Operating conditions affecting the anaerobic digestion of LEW

3.3.1. Temperature

The temperature in the digester determines the microorganism population in the inoculum; the predominant microorganism are psychrophilic, mesophilic and thermophilic when the operating temperature is close to 25 ºC, 35 ºC and 55 ºC respectively. Digesters usually operate at mesophilic and thermophilic ranges to increase the digestion rates. The thermophilic process has shown additional advantages over the mesophilic digestion of some substrates: higher organic matter reduction, better dewatering properties of the digestate and higher destruction of pathogens. Nonetheless, the thermophilic process is higher-energy consuming and more sensitive than the mesophilic. For example, the ammonia inhibition is stronger at a higher temperature [29]. In case of the microalgae

waste, the ammonia inhibition must be carefully controlled since the low C to N ratio leads to a high ammonia concentration in the digesters. Inhibition is further discussed in section 3.4.2.

Microalgae waste are usually digested under mesophilic conditions; experiments performed under thermophilic conditions are scarce. Kinnunen et al. evaluated the *Nannochloropsis sp.* waste digestion at 35 ºC and 55 ºC. The methane production was higher in thermophilic reactors; however, the organic matter degradation was unaffected. The high organic loading rates lead to VFA accumulation under thermophilic conditions as a consequence of the ammonia accumulation; the ammonia concentration doubled the concentration measured in the mesophilic digester [24]. Ehimen et al. also evaluated the influence of the temperature during the Aco-D of *C. vulgaris* waste and glycerol. The temperature increase from 25 ºC to 35 ºC resulted in methane production improvements between 50% and 60%. Further temperature increases caused insignificant improvements or methane production decrease [16].

Based on the reported results, the mesophilic digestion may constitute a better option than thermophilic digestion, mainly due to the lower inhibition risks.

3.3.2. Organic loading rate (OLR) and hydraulic retention time (HRT)

The OLR indicates the amount of VS fed daily into the reactor in a continuous operation; it is usually expressed in Kg_{VS}/m^3•d. A high OLR derives in VFA accumulation because the methanogens can not convert them into methane; this is known as reactor overload. On the contrary, a low ORL decreases the efficiency of the digester due to the low methane production. An optimal OLR avoids reactor overload and assures the VS conversion into VFA and then into methane; the reactor operation results efficient.

Regarding the hydraulic retention time (HRT), it indicates the average time the liquid is held in the reactor. It is calculated dividing the reactor volume by the influent flow rate in time and usually expressed in days. Similarly to the OLR, it strongly influences the reactor operation. Since methanogens reproduce slowly, the HRT must allow enough time for the reproduction. A low HRT derives in the loss of the cells with the effluent, known as washout. A high HRT reduces the production capacity of the plant, thus decreasing the process efficiency. For example, 10 days HRT is considered suitable for sewage sludge digestion [29], but microalgae require between 10 and 30 days [7] as a consequence of their higher resistance.

Ehimen et al. evaluated the influence of $5 < OLR < 50$ $kg_{VS}/m^3_{digester}$ in daily fed digesters using *C. vulgaris* waste. The highest methane production was around 245 mL_{CH4}/g_{VS} at the lowest OLR. For all the OLR evaluated, the best results were observed at HRT between 12 and 14 d [16]. The highest methane production from N. salina waste was also observed with a lower OLR (Table 2). However, co-digestion allowed the possibility of increasing the OLR [30]. Kinnunen et al. reported even better results reducing the OLR to 0.5 during *Nannochloropsis sp.* waste. Additionally, the results showed that the thermophilic digestion was sensitive to changes in the OLR conditions, showing inhibition signs at ORL of 2 [24]. Similar conclusions were reported by Bohutskyi et al. using *Auxenochlorella protothecoides* waste. The best performance and the highest specific methane production were obtained from a digester operating at the lowest OLR and the highest HRT (Table 1). The reduction in the reactor performance was caused by the inhibition due to solvents [31].

Ramos-Suárez et al. digested a different microalgae waste, amino-acid extracted *Scenedesmus sp.*. The best results were also obtained using a low OLR. However, the inoculum acclimatisation allowed increasing the OLR and reducing the HRT without affecting the reactor performance [32].

Low OLR and high HRT are suitable for high methane production from microalgae waste; however, these conditions require digesters with a large volume. The co-digestion, the inoculum acclimatisation, or any additional processing of the waste such as a solvent-removal step may significantly contribute to increase the OLR and/or reduce HRT without inhibition.

3.3.3. Substrate to inoculum ratio (SIR)

The substrate to inoculum ratio (SIR) indicates the amount of organic matter fed into a batch reactor; it is usually expressed as $g_{VSsubstrate}/g_{VSinoculum}$. It plays a similar role to the OLR in continuous reactors; a high SIR derives in VFA accumulation and can cause inhibition.

Alzate et al. were amongst the first in evaluating the influence of the SIR for *N. gaditana* waste. The final methane productions obtained at SIR of 0.5:1, 1:1 and 3:1 were almost constant, 327±2 mL_{CH4}/g_{VS} (Table 1). The only appreciable difference was observed in the methane production curves at the beginning of the experiment. The organic matter conversion into methane resulted in being inefficient at the highest SIR; however, it did not lead in VFA accumulation and inhibition [33]. Zhao et al. reported a good performance in reactors at 0.5:1, 0.66:1, and 1:1 for N. salina waste, but an inefficient organic matter conversion at 2:1[15]. This last SIR value, lower than that reported by Alzate et al. [33], is probably a consequence of the higher degradability of *N. salina*. At 10:1, the methane production and the VS reduction decreased. In the same experiments, the whole microalgae biomass was digested. The results indicated that the lipid extraction allowed increasing the SIR without decreasing the reactor efficiency, since the inhibition was caused by the high lipid content in microalgae [15]. Li et al. also observed inhibition at 2:1 with *C. vulgaris* waste, attributed to the proteins degradation (Table 1) [17].

Alzate et al. also evaluated the influence of substrate concentrations in the digester (SC) between 3 and 20 g/kg maintaining the SIR constant. This parameter provides useful information, for example, to evaluate if a pre-concentration step is required before the digester. When the concentrations were evaluated, the results indicated that the methane productions were quite similar for the SC evaluated [33]. In comparison, Sarat Chandra el at. observed significant influence when *S. dimorphus* waste were digested before and after thermal pre-treatments. The results indicated that the higher the SC, the higher the methane production [22]. However, the SC values were lower than those evaluated by Alzate et al. [33]

The SIR values depend on several factors relative to the waste, for example degradability, composition, and the presence of potentially inhibitory compounds amongst others. However, it seems that values around 2:1 and 3:1 are the most suitable for batch digesters fed with microalgae waste.

3.4. Parameters which affect the digestion of the microalgae waste

3.4.1 Microalgae degradability

The strong microalgae cell walls hamper the microorganism attack, thus affecting the biodegradability. The implementation of pre-treatments is an alternative to overcome this drawback [23]. Some authors suggested that

the lipid extraction itself acts as pre-treatment [20; however, additional pre-treatments can enhance the methane production.

The results reported by Keymer et al. evidenced the advantages of the high pressure thermal hydrolysis as a pre-treatment for *Scenedesmus sp.*. The lipid extraction increased by 10% the organic fraction solubilisation compared with the whole biomass, but the pre-treatment increased this percentage until 50%. The pre-treatment after the lipid extraction allowed almost complete solubilisation; the insoluble organic fraction after the lipid extraction was solubilised with the pre-treatment. The methane production followed the same trend (Table 1); the pre-treatment increased by 58% the methane production from the waste [20]. A thermal pre-treatment had beneficial effects on the methane production from the Scenedesmus waste. The methane production increased by between 34 and 60% after the pre-treatment at 150 ºC [22]. However, the methane production was considerably lower than that reported by Keymer et al. using the same species [20]; this was probably due to the differences in the biomass characteristics. Kinnunen et al. also observed the beneficial effects of a higher temperature and pressure, but in *Nannochloropsis sp.* waste. In this case, the higher temperature and pressure were determined by the characteristics of one of the lipid-extraction processes. These waste showed a 148% higher methane production than a waste with higher residual lipid content, generated from a process carried out at a lower pressure and temperature [24].

Alzate et al. applied ultrasonic, biological and thermal pre-treatments to *N. gaditana* waste. The results indicated no correlation between the solubilisation degree and the methane production. The ultrasonic pre-treatment increased the methane production up to 15% in spite of the low solubilisation achieved. The biological pre-treatment decreased the solubilisation and the methane production. The thermal pre-treatment showed the highest solubilisation, up to 56%, but the methane production increased by 15% maximum [33]. The relationship between the solubilisation degree and the methane production depends on several factors such as the characteristics of the pre-treatments and the microalgae waste. Sarat Chandra et al. reported considerably low solubilisation after the thermal pre-treatment of *Scenedesmus* waste but a 35% increase in the methane production [22]. Suresh et al. combined an acid and alkali addition, with ultrasound, microwave and autoclave in *Ettlia sp.* waste. The highest solubilisation, higher than 50%, was achieved at alkaline pH, thus the acid pre-treated waste were not digested. The microwave and autoclave pre-treatment increased the methane production by 29% and by 40% respectively. The production decreased by 27% after sonication in spite of the high solubilisation observed [34].

Other extraction processes are also able to improve digestion. Ramos-Suárez and Carreras digested amino-acid extracted waste from *Scenedesmus sp.*. The waste doubled the methane production obtained from the whole biomass as a consequence of the extraction process (Table 1) [32]. Similarly, Astals et al. observed higher methane production after proteins extraction from *Scenedesmus sp.*; in this case, around 30% compared with the whole biomass digestion (Table 1) [28].

The hydrogen production from microalgae prior to digestion also increased the methane production. Mussgnug et al. induced hydrogen production from *Chlamydomonas reinhardtii* via the sulphur deprivation method. After hydrogen production, the waste produced 23% more biogas than the whole microalgae. This higher production was attributed to the high concentration of easy biodegradable compounds that microalgae accumulate under deprivation conditions. Additionally, the sulphur deprivation decreases the risks of the high H_2S concentration in biogas [14]. Recently, Wirth et al. reported two-stage process for the hydrogen and methane production. Unlike Mussgnug et al. [14], the authors did not apply sulphur deprivation to generate hydrogen, simplifying and reducing

the cost of the process [35]. The hydrogen production waste produced around 260 mL_{CH4}/g_{TS}, but the whole microalgae was not digested for comparison. Yang et al. evaluated a two-stage process for the hydrogen and methane production; however, the advantage of the process lies in the utilisation of *Scenedesmus sp.* waste after the lipid extraction. The hydrogen production resulted close to the maximal and free of methane in the first stage, and during the second stage, the methane production was higher than the obtained by simply digestion (Table 1). Also the organic fraction degradation and the methane production rate were increased in the two-stage process [19].

Although various pre-treatments increase the methane production, in some cases the extraction processes can constitute a pre-treatment itself. The digester could be coupled to processes such as the extraction processes or the hydrogen production process thus avoiding additional pre-treatments. Moreover, some modifications in the processes to avoid the detrimental effects of drying could also be advantageous.

3.4.2 Inhibition effects

The low C to N ratio in microalgae, a drawback for AD [23], appears even more unfavourable in microalgae waste (Table 1). Kinnunen et al. observed unstable operation under thermophilic conditions due to the low C to N ratio in *Nannochloropsis sp.* waste [24]. However, a high methane production was obtained operating at almost double OLR after the inoculum acclimatisation. Yang et al. observed a methane production decrease after feeding the same batch digesters for three consecutive times with *Scenedesmus sp.* waste. In spite of the acclimatisation and the increased methane production rate, the final productions decreased slightly as a consequence of the ammonia accumulation [19]. The ammonia inhibition was also reported in batch digesters for *C. vulgaris* waste [17]. Ramos-Suárez et al. also observed inhibition caused by ammonia, even during *Scenedesmus sp.* waste from an amino-acid extraction process [32].

The organic solvents used in the lipid-extraction process can cause inhibition too. The detrimental effects of chloroform were the first reported by Ehimen et al. [13], but also observed by Zhao et al. using the same solvent mixture [15]. Bohutskyi et al. also observed inhibition caused by the organic solvents, but related to the residual solvent concentration in the waste [31].

Suresh et al. reported surprisingly low methane productions from alkali-sonicated *Ettlia sp.* waste, in spite of the high solubilisation degree after pre-treatment. The authors attributed these results to the high VFA accumulation derived from the pre-treatment [34].

Various cases lead to inhibition; some of them are related to the waste characteristics, and some other, to characteristics of the processes. However, there are alternatives to reduce the inhibition effect such as modifications in the process, co-digestion or inoculum acclimatisation.

3.4.3 Anaerobic co-digestion

The Aco-D of microalgae with carbon-rich substrates was first suggested as an option to avoid the inhibition caused by ammonia. The addition of carbon-reach substrates can increase the C to N ratio in microalgae waste until 10-30, considered as optimal [21,23]. The glycerol, generated during the biodiesel production, was early evaluated as co-substrate. Ehimen et al. co-digested *C. vulgaris* waste and glycerol in the same proportion both by-products are generated in the biodiesel process. The methane production increased by between 4% and 7% in batch digesters [13]. In subsequent experiments in semi-continuous mode, improvements between 20% until 61%

were observed when the C to N ratio was increased until 12.4. Further C to N ratio increases did not improve the process. The addition of glycerol increased the fraction of CO_2 in biogas, as a consequence of the high oxygen content in this co-substrate [16]. Glycerol addition was also evaluated by Ramos-Suárez and Carreras, and Newman et al. [21,36]. Ramos-Suárez and Carreras reported a maximum methane increase of 20% using *Scenedesmus sp.* waste [21]; Newman et al. reported barely 6% increase using *B. braunii* waste [36].

The Aco-D of microalgae waste showed some other benefits. Park and Li mixed lipid-rich waste composed by fat, oil, and grease with N. salina waste. The co-digestion allowed increasing the OLR from 2 until 3 $g_{VS}/L \bullet d$ in spite of both substrates showed inhibition when digested at this OLR. The mixture in equal proportions increased more than 10 times the methane production from both substrates digested separately [30]. Astals et al. reported no synergy by mixing pig manure and *Scenedesmus sp.* waste; however, the authors highlighted the benefits of mixing both substrates in a bio-refinery [28]. The addition of sludge from a WWTP to *B. braunii* waste slightly improved the digestion rate; in this case, the authors emphasised the benefits of digesting different substrates using the same facility [36].

The co-digestion has been also evaluated in microalgae waste generated after protein or amino-acid extraction from microalgae. The addition of paper waste failed to increase the methane production from *Scenedesmus sp.* waste; it was attributed to the low biodegradability of the cellulose in paper. The addition of another co-substrate considered as energy crop, Opuntia maxima cladodes, also failed [21]. Wirth et al. reported beneficial effects by mixing hydrogen-production waste with maize silage, as a consequence of the changes induced in the bacteria community [35].

The C to N ratio balance does not ensure good reactor performances; the characteristics of the co-substrate play an important role in achieving high methane productions. However, the benefits of Aco-D can not be reduced to the methane production increase. The absence of inhibitory effects allows using the same facility, coupling different processes. Moreover, Aco-D can avoid inhibition too.

4. Anaerobic digestion for nutrients recovery

The liquid fraction recovered from the digester, called centrate or digestate, is characterised by a high nutrient concentration, mainly nitrogen and phosphorus. During the sewage sludge digestion, it is recycled for further treatment to avoid environmental problems, but it increases the WWTP costs [37]. Using this stream for microalgae cultivation as a nutrient source can be beneficial [8]. Recently, Astals et al. presented a bio-refinery scheme, with a microalgae cultivation unit integrated to a treatment facility for pig manure. Regardless of the compound recover from microalgae, the microalgae waste are co-digested with the pig manure, and the centrate recycled to the microalgae cultivation unit [28].

Not all authors evaluated the nutrients recovery after the digestion of microalgae waste. Keymer at al. determined the fraction of nitrogen and phosphorous recovered from *Scenedesmus sp.* waste. These fractions resulted higher than in reactors fed with the whole microalgae. The authors determined that up to 66% of nitrogen and 39% phosphorous could be recycled [20].

Ramos-Suárez et al. designed a theoretical process for microalgae cultivation using centrate, amino-acid production from microalgae and energy recovery in a combined heat and power unit. They used experimental data

but also included several assumptions. The results indicated up to 30% reduction in the nitrogen consumption and up to 25% in the CO_2 needs. The energy savings were not determined [32].

Bohutskyi et al. published a detailed analysis for recovering nutrients. Up to 40% of the nitrogen was recovered as ammonia and between 30% and 60% of the phosphorus as orthophosphate. The concentration of macro-nutrients and micro-nutrients were affected by the operational conditions; however, between 20% and 50% of essential nutrients such as Mg, Ca and S were recycled and a lower percentage of Mn, Fe, Cu, Zn, Mo and Co. As an easy way to demonstrate the impact of the nutrients recovery, the authors estimated the nutrient consumption to produce 10% of the current liquid fuel consumption in the U.S. The process scale-up may increase by between 15% and 25% the current fertiliser consumption in agriculture, with a strong impact on the markets; however, by recycling nutrient the percentage decreased to nearly 6-16% [31].

The nutrient recovery is viable and results are economically and environmentally advantageous. The benefits depend on the biomass characteristics and the digestion process, since the process can also recycle toxic compounds.

5. Other uses for lipid-extracted waste

As it is summarised in this section, several products can be obtained from the microalgae waste.

Yang et al. focused on producing hydrogen from *Scenedesmus sp.* waste. The low biodegradability of the waste hampers the hydrogen production, as it occurs during methane production. The authors evaluated the influence of several pre-treatment methods over the microalgae waste, the inoculum pre-treatment and two-stage process for hydrogen and methane production in order to improve the process [19,38-40]. Subhash et al. also recovered hydrogen from hydrothermal pre-treated microalgae waste, as part of a bio-refinery [41]. Other authors preferred the production of liquid-biofuels, which are advantageous due to the possibility of direct combustion or upgrading. Vardon et al. demonstrated the viability of the thermo-chemical process to produce bio-oil from *Scenedesmus sp.* waste. However, the upgrading process still requires several improvements [42]. Zhu et al. simulated the process scale-up, resulting that the selling price of the liquid fuel produced from N. salina waste could compete with gasoline and diesel [43]. Wang et al. used the fast-pyrolysis process with *C. vulgaris* waste. The authors reported around 94% recovery of the energy content from the waste as bio-oil and bio-char. The bio-oil showed low oxygen content, beneficial for stability and upgrade, but high nitrogen content, detrimental for upgrading. The bio-char showed good prospects as fertilizer due to the content of N, P, K, Ca, and Mg [44].

Valuable by-products with industrial and environmental applications have also been reported. Lam et al. produced maltodextrin, with commercial applications in water soluble glues, thickening agents in food processing, and binding agents in the pharmaceutical industry [45]. Other authors reported the production of a nutrient source which can be used for microorganisms cultivation [46-48]. Zheng et al. used the nutrient source prepared to cultivate *C. vulgaris*, producing biomass with 35% lipid content and a fatty acids profile suitable for biodiesel production [47]. Sarat Chandra et al. demonstrated that *Scenedesmus dimorphus* waste are suitable absorbents. This non-conventional and low-cost adsorbent removed basic dyes from an aqueous solution, as a result of the negatively charged functional groups on the adsorbent surface [49]. Similarly, Chang et al. prepared different microporous carbon material from *Chlorella sp.* waste with a carbonisation-activation process [50]. In the aquaculture sector, Patterson et al. reported the suitability of the lipid-extracted to replace protein sources in

fishmeal. Although the whole microalgae resulted in being more nutritious, it was possible to replace up to 10% of crude protein from fishmeal and soy protein concentrate using the waste without reduction in the fish growth [51].

Methane production is not the only way to convert the microalgae waste into a valuable by-product. Depending on the characteristics of the lipid-extraction process, and other factors such as the available technology and the economic sources, several valuable products can be obtained.

6. Conclusions

The AD of the lipid-extracted waste converts the waste into a valuable by-product and can favourable contribute to the economic balance of the biodiesel production process. Similar conclusions can be drawn for other processes which generate microalgae waste, for example the amino-acid extraction processes.

The process depends on several aspects. The species characteristics and the cultivation conditions impact over the methane production, by affecting the microalgae composition and the potential methane production. The lipid-extraction process can act as pre-treatment method itself, but it can also hamper the digestion when includes a microalgae drying step or uses toxic solvents. The pre-treatment methods application can increase the methane production under certain conditions, but some pre-treatment can lead to inhibition instead.

The operating conditions in the digester affect the process: temperature, OLR and HRT in continuous processes or SIR in batch processes, can either enhance the reactor performance or lead to inhibition.

The possibility of inhibition must be carefully controlled during microalgae waste digestion; the presence of organic solvents or the high nitrogen content can cause inhibition. Although suitable operating conditions avoid the problem, a previous solvent-elimination step or Aco-D can be advantageous. The Aco-D can also enhance the digester efficiency by increasing the amount of waste converted into methane and allows coupling different processes by using the same facility.

In addition to energy production the AD reduces the waste production and offers the possibility of nutrient recycling. Both actions are economically and environmentally advantageous.

Finally, it has been shown that various valuable products can be recovered from microalgae waste; there are several options and the most suitable must be chosen.

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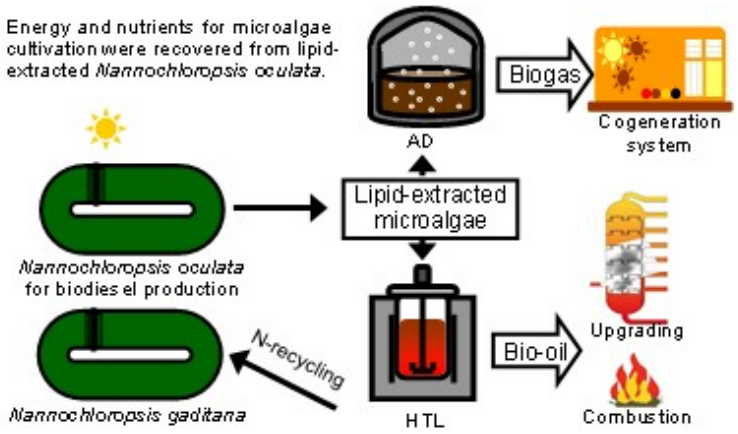
Chapter 8

Energy and nutrients recovery from lipid-extracted Nannochloropsis via anaerobic digestion and hydrothermal liquefaction [1]



ABSTRACT

The biomass, generated after the lipid extraction from *Nannochloropsis* microalgae (LEM) for biodiesel production, demonstrated their suitability for both energy and nutrients recovery. The anaerobic digestion of LEM produced a minimum of 268 mL_{CH4}/g_{VS} in different experiments. The co-digestion of LEM and sewage sludge revealed that both waste can be co-digested without inhibition, although no synergy was observed. The methane yields barely increased 10% after pre-treatments (ultrasound and ultrasound combined with alkali addition).

Regarding bio-oil production from hydrothermal liquefaction process, more than 28% of the LEM was converted into bio-oil. Moreover, the aqueous phase generated during the bio-oil production was successfully utilised as nitrogen source for microalgae cultivation.

[1] M.P. Caporgno, E. Clavero, C. Torras, J. Salvadó, O. Lepine, J. Pruvost, J. Legrand, J. Giralt, C. Bengoa, Energy and nutrients recovery from lipid-extracted *Nannochloropsis* via anaerobic digestion and hydrothermal liquefaction. ACS Sustainable Chemistry & Engineering. Accepted for publication.

1. Introduction

Over the past years, researchers turned their attention to find renewable and cost-effective feedstocks for biofuels, as a consequence of the predicted fossil-fuels reserves depletion. Microalgae utilisation as feedstock was also fostered by the "food-versus-fuel competition" derived from the biofuels production from crops [1]. Amongst the different biofuels, biodiesel still gathers the attention of researchers. The process scale-up is not economically viable nowadays [2], but increasing efforts are being made to overcome the drawbacks. For example, the valorisation of the lipid-extracted microalgae (LEM) may be beneficial for the biodiesel process. Approximately 65% of the biomass remains as waste after lipid extraction, containing some valuable compounds [3].

The anaerobic digestion (AD) is a well-known technology used for the stabilisation of the sewage sludge generated in wastewater treatment plants (WWTPs). The versatility of this process allows the treatment of a wide range of feedstocks, including microalgae. In recent years, the LEM have been also considered as promising substrates for AD [3]. The low C to N ratio in microalgae, which can lead to AD inhibition, is even lower in LEM. The anaerobic co-digestion (Aco-D) with sewage sludge is a viable alternative to balance the C to N ratio. Additionally of being widely available, sewage sludge is the second substrate utilised in co-digestion due to the over-sized digesters in WWTP [4]. The AD also offers the advantage of recycling nutrient. The aqueous phase generated during AD, rich in ammonia and phosphorous, may be used for microalgae cultivation [5,6]. On the other hand, the hydrothermal liquefaction (HTL) is a completely different technology which converts microalgae and several microalgae waste into bio-oil [7-11]. The bio-oil, a liquid fuel, can be further combusted or upgraded. Similar to AD, the HTL process generates a nutrients-rich aqueous phase (AP-HTL) which can be recycled for microalgae cultivation [12-14].

This paper is the first attempt to evaluate several processing routes for the biofuels production from LEM; the routes can be observed in the scheme presented in Figure 1.

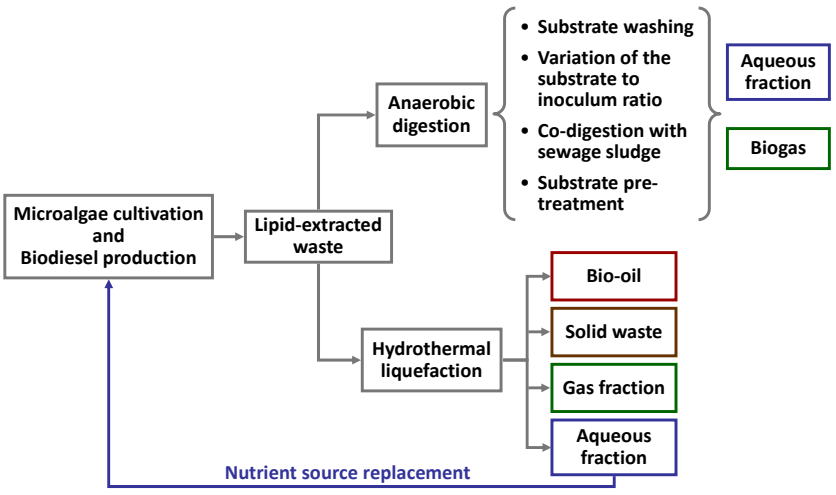


Figure 1. Scheme of the processing routes evaluated for the valorisation of the LEM.

Several options to avoid AD inhibition and to improve the methane production were evaluated. The increased protein content after the lipid extraction and the presence of sea salt in the LEM are potential causes of inhibition [3,15]. The effects of high amounts of proteins were evaluated by setting several substrate to inoculum ratios (SIR) in the experiments. Regarding sea salt, the influence of washing biomass was evaluated. The LEM were also co-

digested with sewage sludge, based on the advantages of combining both substrates as previously mentioned. The last option consisted of applying two pre-treatment methods to improve the methane production. The ultrasonic pre-treatment was chosen based on the suitability to increase the biodegradability of microalgae [16]. The combination of alkali addition and ultrasonic pre-treatment was chosen based on the benefits when applied prior to the AD of some waste [17,18].

In a completely different processing route, the LEM was used as for HTL. Although the process optimisation is not addressed in this report, the valorisation of the AP-HTL generated was evaluated as nutrients source for microalgae cultivation. The bio-oil production together with the nutrient recycling has not been reported yet.

2. Materials and methods

2.1 Materials

Lipid-extracted *Nannochloropsis oculata*, provided by AlgoSource's (Alpha Biotech, Asserac, France), was generated from a lipid-extraction process with supercritical carbon dioxide (SCCO2). The solid LEM was stored in a desiccator until required. The LEM was the substrate used for biofuels production via AD and HTL.

For the experiments where microalgae were cultivated in the aqueous phase recovered from the HTL, the species was *Nannochloropsis gaditana* Lubián, strain CCMP 1775, obtained from NCMA (National Center for Marine Algae), formerly CCMP.

The inoculum for the AD experiments consisted of digested sludge from a pilot-plant under mesophilic conditions. The reactors were operated under semi-continuous conditions and fed with a sewage sludge blend (primary and secondary sludge in ratio 65:35 v/v); the same sludge blend was utilised as substrate in the co-digestion experiments. The sewage sludge blend was collected from the municipal WWTPs in Reus (Tarragona, Spain).

2.2 Experimental procedure

2.2.1 Analytical methods

Total solids (TS), volatile solids (VS), protein and carbohydrate were analysed according to the methods described in Caporgno et al. [19]. The residual lipid in the LEM was determined by extraction of 3 g of LEM using a Soxhlet apparatus with a reflux period of 7 hours and hexane as solvent; lipids were then recovered by solvent evaporation and weighed. The TS content in the LEM was 928.8±0.7 g/kg, with VS/TS of 0.75±0.01. The organic fraction contained 61.7±4.1 % proteins, 7.6±0.1 % lipids and 25.3±2.0 % carbohydrates. The TS content and the VS/TS were 27.8±0.7 g/L and 0.76±0.01 in sewage sludge, and 17.2±1.0 g/L and 0.64±0.02 in the inoculum.

In the AD experiments, biogas production and composition, volatile fatty acid concentration (VFA), alkalinity, were determined as described in Caporgno et al. [19].

In the HTL experiments, the bio-oil was analysed by chromatography-mass spectroscopy (GC-MS). The samples were subjected to GS-MS analysis (G1099A/MSD5973) using a HP-5MS column (19091S-433) and helium as carrier gas with flow rate of 1.4 mL/min. A volume of 1 µL dichloromethane (DCM) extract was injected at 280 °C with a split ratio of 2:1. The initial temperature in the oven was 80 °C, and after 1 min, it was increased at 15 °C/min until 200 °C. Subsequently, the temperature was increased at 5 °C/min until 310 °C and held constant for 10 min. The higher heating value (HHV) in bio-oil was determined in a bomb calorimeter (Gallenkamp Ballistic

Bomb Calorimeters), using benzoic acid as a standard substance. On the other hand, the HHV of the LEM was calculated based on the elemental composition of the biomass using the Dulong's formula as described in Vardon et al. [9] due to the high content of ashes in the LEW. The ammonia nitrogen (NH_3-N), nitrate nitrogen (NO_3-N), nitrite nitrogen (NO_2-N), phosphate phosphorus (PO_4-P), and chemical oxygen demand (COD) were measured in the aqueous phase prior to the microalgae cultivation, using a HI-83099-02 bench photometer and following the procedures described in the user's manual (Hanna Instruments, 2014). The total nitrogen (TN), total carbon (TC) and total inorganic carbon (TIC) were determined in Multi NC 3100 (Analytic Jena) analyser. The gas-phase composition was analysed using the method for the biogas analysis described in Caporgno et al. [19].

Carbon, hydrogen and nitrogen content in the LEM, the bio-oil and the solid waste recovered after HTL have been analysed in an LECO TruSpec Elemental Analyser. Oxygen content was determined by difference.

In the cultivation experiments, cell growth was monitored by measuring the absorbance at 750 nm with a microplate reader (Infinite® M200 PRO, Tecan).

2.2.2 Anaerobic digestion

The batch reactors were set up following the procedure described in Caporgno et al. [19]. The substrate depended on the experiment. The effects of washing the LEM and the SIR variation were simultaneously evaluated. In the washing step, 20 g of LEM were mixed with 200 mL deionised water at room temperature, stirred 20 min and then centrifuged to recover the solid fraction; the procedure was repeated three times. Reactors with SIRs of 1:4, 1:2 and 1:1 $VS_{Substrate}$:$VS_{Inoculum}$ were prepared with different amounts of LEM, either washed or unwashed.

In the co-digestion experiments, the SIR was of 1:2 VSSubstrate:VSInoculum. Different mixtures of sewage sludge and unwashed LEM were fed, replacing 25%, 50% and 75% of the VS from LEM by VS from sewage sludge.

Two different pre-treatment methods were applied to unwashed LEM. The first one, ultrasonic pre-treatment, consisted in applying three different energy levels: 28, 48 and 55 MJ/kg_{TS} at 24 kHz working frequency and 93 W ultrasonic power (UP200S Hielscher Ultrasonics GmbH, Germany). The LEM were suspended in deionised water for the pre-treatment. The pre-treatment was carried out at room temperature and using a water bath to avoid the sample heating. The energy supplied was calculated according to the ultrasonic power, the concentration of solids in the sample and the treatment duration. The second pre-treatment consisted in combining addition of alkali and ultrasound application at low-energy level. The energy applied during ultrasonic disintegration was 17 MJ/kg_{TS}. The alkali pre-treatment consisted in adjusting the pH at 9, 11 and 13 with NaOH, and keeping the samples at room temperature for 24 h before digestion. For the pre-treatment combination, the pH in the samples was first adjusted at 9, 11 and 13, and then the ultrasonic disintegration was applied. The pH in the pre-treated samples was neutralised to pH 7 with HCl.

2.2.3 Hydrothermal liquefaction

The experiments were performed at 300 ºC, ≈10 MPa, 30 min retention time in a 1L reactor (1 Liter EZE-Seal Bolted Closure, Autoclave Engineers). The reactor was loaded with 300 g of slurry, containing 20% LEM in water (w/w), and then purged with nitrogen to remove the oxygen before heating. The different product fractions were recovered (Figure 2) and analysed. After the reactor was cooled down to room temperature, the gases were vented off and collected in gas sampling bags for further analysis. The major part of AP-HTL was removed by pouring it

into a beaker. Dichloromethane (DCM) was used as solvent to recover the bio-oil, which was adhered to the reactor walls. The DCM containing the bio-oil was separated from the remaining aqueous fraction using a separatory funnel. Both AP-HTL and DCM were vacuum-filtered through pre-weighted filters for the quantification of the amount of suspended solids. DCM was dried under anhydrous sodium sulphate and evaporated in a rotary evaporator to determine the amount of bio-oil. The AP-HTL was recovered and used for microalgae cultivation.

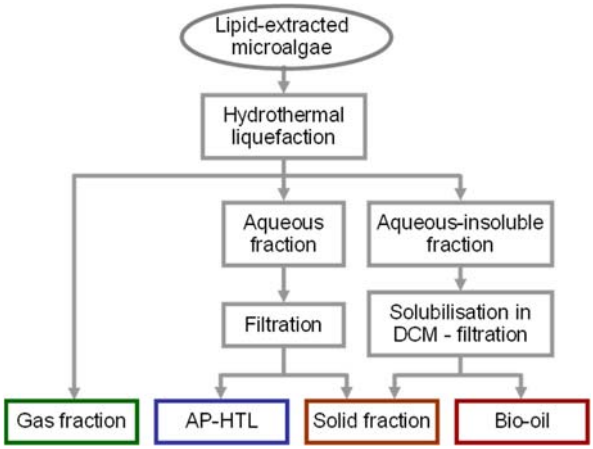


Figure 2. Procedure for the separation of the different products fraction from HTL.

For the mass balance, the products yields were defined as weight percentages relative to the raw material. The bio-oil yield accounted the DCM-soluble fraction, the AP-HTL accounted dissolved constituents remaining after the DCM extraction, the solid fraction accounted the mass of dried particulates retained after filtration, and finally, the gas-phase yield was calculated by difference.

2.2.4 Nutrients recycling

A growth experiment was performed to evaluate the feasibility of replacing the nitrogen source in the algal culture medium by the aqueous phase from HTL. N gaditana was cultivated in culture media containing different proportions of AP-HTL. Blank assays were performed with seawater enriched with a modification of Guillard's f/2 formulation [20]. Since f/2 is a rather diluted medium for biomass production purposes, nitrogen and phosphorous concentrations in this modified medium ($f/2^{+}$) were increased up to 60 mg $N\text{-}NO_3$/L and to reach a molar N:P ratio equal to 16:1. Based on the total nitrogen in AP-HTL, five different mixtures of AP-HTL and $f/2^{+}$ were prepared by the replacement of 7, 15, 30, 45 and 60 mg N/L in the $f/2^{+}$ medium by the AP-HTL addition. Media were filter sterilized through 0.2 µm.

Microalgae were cultured in sterile 6-well microplates, with a working volume of 3 mL per well and an initial microalgae density of 3·105 cells/mL. Triplicate cultures were grown in continuous agitation at 25± 1°C, under an irradiance of ca. 115 µmol photons/m2·s at the surface of the microplates, provided by daylight fluorescent lamps in a 16:8 light:dark cycle.

Microalgae growth was daily monitored by light absorbance measurements, at 750 nm, with a M200Pro Infinite Tecan microplate reader. Division rates (k) were obtained by calculating the slope of the linear least-squares regression for the $\log_2$-transformed values of the sample absorbance at 750 nm during the exponential growth phase [21].

3. Results and discussion

3.1. Anaerobic digestion

Table 1 summarises the ultimate methane productions obtained in all the digestion experiments, the kind of substrate fed and the SIR used in the reactors. It is worth mentioning that the experiments were not performed simultaneously, therefore the methane productions from LEM slightly differ in some of the experiments.

Table 1. Ultimate methane production from LEM. Batch reactors at 33ºC and 29 days digestion. Note: These experiments were not done simultaneously, thus the methane production from LEM samples differs in some of the experiments.

Experiment	Substrate	SIR	CH_4 mL_{CH4}/g_{VS}	Experiment	Substrate	SIR	CH_4 mL_{CH4}/g_{VS}
Effect of washing	Unwashed LEM	1:4	269±6	*Ultrasonic pre-treatment*	LEM	1:2	274±4
		1:2	268±17		28 MJ/kg_{TS}	1:2	269±7
		1:1	273±6		48 MJ/kg_{TS}	1:2	299±6
	Washed LEM	1:4	192±16		55 MJ/kg_{TS}	1:2	294±4
		1:2	185±9	*Combined pre-treatment: US and NaOH*	LEM	1:2	286±8
		1:1	180±11		17 MJ/kg_{TS}	1:2	289±3
Aco-D	LEM	1:2	274±4		pH 9	1:2	309±6
	25% Sludge	1:2	293±3		pH 11	1:2	300±9
	50% Sludge	1:2	319±4		pH 13	1:2	290±5
	75% Sludge	1:2	345±10		US + pH 9	1:2	290±3
	100% Sludge	1:2	360±12		US + pH 11[a]	1:2	244±4
					US + pH 13	1:2	296±3

[a] *Experimental error confirmed by analysis at the end of the experiment..*

3.1.1. LEM washing and SIR variation

The ultimate methane productions from washed and unwashed LEM indicated that the washing influenced the AD negatively. The methane production decreased between 29% and 34% after washing. The salt concentration in marine microalgae can strongly affect the methane production [15]; however, the washing removed not only salts from the biomass. The analysis of the washing water revealed that 67±1% of the solids fraction removed was constituted by organic compounds. The loss of these organics caused the significant reduction in the methane production [22]. Moreover, not only the organic fraction content was affected, but also the characteristics of the organic fraction. As mentioned 2.2.2., the same amount of substrate on VS basis was fed in all the reactors. For this reason, the lower methane production can not be attributed to the organic fraction content in the digesters. Easily-biodegradable compounds were removed by washing, thus decreasing the methane production from washed substrates.

Regarding the SIR, it was clearly demonstrated that the ratios evaluated cause no influence on the digestion of washed and unwashed LEM (Table 1). The differences in the ultimate methane production from washed LEM were less than 7%, and from unwashed LEM, less than 2%. In the literature, 1:1 is commonly considered a SIR threshold for inhibition; however, it depends on the substrate characteristics. Long chain fatty acid, hydrolysis products of lipids, lead to inhibition during the AD of microalgae with high lipid content. For this reason, the SIR can be increased in the digesters with LEM [23]. Similarly, substrates characterised by high protein content cause

inhibition by ammonia during AD [3]. Ammonia is the most probable cause of inhibition due to the high protein content in the LEM. The ammonia concentration, VFA concentration and pH amongst other parameters indicated stable operation.

In reactors with unwashed LEM, the methane production throughout the first days of the experiment was affected (Data not shown). These effects, observed at 1:1, were also reported during the AD of other LEM [23,24]. The VFA were not simultaneously converted into methane, leading to significant accumulation of VFA and methane production decrease. The VFA accumulation can lead to inhibition; however, the VFA accumulated but they were converted into methane soon afterwards. The ratio 1:1 was close to the SIR threshold for inhibition. Regarding the washed LEM, the phenomenon did not occur due to the low degradability of the organic fraction..

3.1.2. LEM co-digestion with sewage sludge

The ultimate methane production from LEM and sewage sludge co-digestion indicated no synergy by mixing both substrates. The sewage sludge and the LEM produced the highest and the lowest methane yields, 360±12 mL_{CH4}/g_{VS} and 274±4 mL_{CH4}/g_{VS} respectively. The methane productions from the mixtures were proportional to the percentage of each substrate in the mixture. Similar results were reported by Neumann et al., who highlighted that the absence of inhibition allows mixing both substrates without affecting the process operation [25]. The co-digestion with sewage sludge is even more advantageous when considering the non-used capacity of the digesters in WWTP, approximately 30% [4]. Recently, Astals et al. emphasised the benefits of integrating pig manure processing and microalgae cultivation, in spite of the absence of synergy in Aco-D [6].

3.1.3. LEM pre-treatment

The effects of the ultrasonic pre-treatment on the AD of LEM can be observed in Table 1. The lowest energy input (28 MJ/kg_{TS}) seemed to be inefficient to increase the LEM degradation, since the methane production was similar to the obtained with untreated LEM. Higher energy inputs (48 and 65 MJ/kg_{TS}) only resulted in a 7-9% increase in methane production. Similar results were reported after ultrasonic pre-treatment of *Nannochloropsis sp.* LEM [24]. The results suggest that this pre-treatment was probably unsuitable for microalgae wastes from the genus *Nannochloropsis*, probably due to the cell wall characteristics. The cell wall in the genus *Nannochloropsis* has a cellulosic inner-wall protected by an outer hydrophobic algaenan layer [26], which hampers AD degradation [27]. For this reason, powerful pre-treatment methods have been suggested for *Nannochloropsis* species [27].

In order to improve the limited degradation of the LEM, an ultrasonic pre-treatment with low-energy input was combined with alkali addition. For comparison, the effects of alkali and ultrasound were also separately tested. The first visible effects of the pre-treatment were noticed in the LEM composition. Alkali treatment caused organic matter destruction in the LEM, so that after the pre-treatment with alkali addition up to pH 13 the VS/TS ratio decreased from 0.75 to 0.62. Likewise, the combined pre-treatment decreased the VS/TS ratio down to 0.66. Similar VS/TS decreases reported during the alkali and ultrasonic pre-treatment of other substrates were attributed to the mineralization of some components [28]. Pre-treatment with alkali also increased the methane production up to 8% (Table 1), the highest methane production being obtained with the lowest NaOH dosage. The pre-treatment of undamaged microalgae with similar NaOH dosages also gave higher methane productions with lower dosages [29]. Furthermore, the highest methane production was obtained from the non pre-treated microalgae [29]. Regarding the ultrasonic pre-treatment (17 MJ/kg_{TS}), the methane production remained unchanged, as expected

considering the inefficiency of a stronger input (28 MJ/kg_{TS}) discussed at the beginning of this section. Surprisingly, the pre-treatment combination did not increase the methane production (Table 1). References regarding the AD of LEM are currently scarce, especially those focused on the effects of pre-treatments. Only Suresh et al. reported an alkali pre-treatment of LEM and its combination with ultrasonication. The authors suggested inhibition due to VFA accumulation, being the methane production negatively affected [30]. However, in the present work, the absence of inhibition was corroborated in all the reactors at the end of experiments. On the other hand, the combined pre-treatment applied to other lignocellulosic substrates did not affect the total methane production [28]. The alkaline pre-treatment affects the intermolecular linkages and functional groups of lignin, cellulose, and hemicellulose [31]. The cell walls structures are more vulnerable to the shear forces in the ultrasonic pre-treatment when the pre-treatments are combined. However, the effectiveness of the alkaline pre-treatment is considered dependent on the content of lignin in the biomass and the content of lignin in microalgae is considered low [31].

3.2. Hydrothermal liquefaction

The mass balance allows establishing the product distribution after the HTL process. The results, presented in Table 2, indicated that similar amounts of the biomass were distributed between the bio-oil, the AP-HTL, the gas and the solid fraction.

Table 2. Mass balance of HTL, and C and N recovery from the LEM.

	Bio-oil	AP-HTL	Gas	Solid fraction
Mass balance ($g/100g_{LEM}$)	28±2	24±2	27±3	21±2
C recovery (% of the C in LEW)	48±4	26±1	14±1 [a]	9±2
N recovery (% of the N in LEW)	24±2	56±1	- [b]	3±1

[a] *Only the amount of CO2 determined in the gas was considered.*
[b] *The N content in gas was not analysed.*

The bio-oil showed the highest yield, 28±2 g/$100g_{LEM}$. This yield is comparable to the obtained by processing *Scenedesmus sp.* LEM, with high-protein and low-lipid content, under the same experimental conditions [9]. Higher bio-oil yields have been obtained from *N. salina* LEM [10], but with a high amount of residual lipids (≈20 g/$100g_{sample}$). Bio-oil yields close to 45 g/$100g_{sample}$ have been recently reported from protein-extracted *Scenedesmus sp.* [11]; once again, the lipid fraction which remains after extracting the proteins (≈18.5 g/$100g_{sample}$) may contribute to the high bio-oil yield. Lipids present the highest bio-oil yield, followed by proteins and carbohydrates [12]. On the other hand, the bio-oil obtained by processing *D. tertiolecta* from β-carotene production yielded approximately 5 g/$100g_{sample}$ under operating conditions similar to the present experiments; quite severe operating conditions were required to increase the bio-oil yield [7]. The bio-oil yields obtained in the experiments here reported are also comparable with the reported in the literature, after processing from the whole *Nannochloropsis oculata*, in spite of the differences in the lipid content [12]. It worth mentioning that bio-oil yield was calculated on the dry weight basis of the LEM; the yield increases when is calculated on the dry ash-free basis due to the high content of ashes in the LEM.

Regarding the characteristics of the bio-oil, the analysis of the HHV resulted in 37.2±0.9 MJ/kg, which is in agreement with the values reported for other LEM under the same experimental conditions [9] and other

microalgae species under different HTL conditions [8,12]. The HHV was considerably low for the LEM, 17.7±0.1 MJ/kg, mainly as a consequence of the high content of ashes in the LEM. The qualitative GC-MS analysis of the bio-oil evidenced its complexity. The high protein content in the LEM led to the presence of nitrogenous compounds in the bio-oil. The main compounds can be included in the category of aromatic compounds such as indole derivatives or in the category of highly aliphatic compounds, including long chain alkanes and alkenes, free fatty acids and amide derivatives. Similar compounds have been also identified by other authors [7,9,11]. The analysis elemental of the bio-oil determined C, H, N and O percentages of 70%, 9%, 5% and 17% respectively. According to these results, the bio-oil quality is better than the reported by Vardon et al. [9] and comparable to the bio-oil quality reported by Zhu et al. [10] in terms of N and O contents. The analysis elemental of the LEM determined C, H, N and O percentages of 40%, 6%, 5% and 24% respectively. Based on these results, 48±4% of the C from the LEM was converted into bio-oil, similar to value reported by Vardon et al. [9].

The gas yields indicated that a large portion of the initial biomass was converted into gaseous products. Although the gas composition was not analysed in detail, the chromatogram revealed that the gas fraction was mainly CO_2 and small amounts of simple hydrocarbons as it was expected based on the literature [8,11]. The high content of CO_2 suggests the possibility of using the exhausted gas in the microalgae cultivation unit [32].

The yield of the solid fraction was considerably higher than the yields obtained after processing LEM under similar experimental conditions [9,11]. Comparable yields of the solid fraction were reported during the HTL of *Dunaliella tertiolecta* waste, but in this case, the bio-oil yield indicates low biomass conversion [7]. As mentioned before, the LEM here used was characterised by high ashes content, and most of the ashes contributed to increase the yield of the solid fraction. The analysis of the solid fraction revealed the presence of the major part of the inorganic fraction from the LEM, 78±3%. The analysis elemental of the solid determined C, H, N and O percentages of 78%, 12%, 4% and 6% respectively. The high percentage of C and the appearance of the solids, suggest the formation of bio-char as a consequence of the re-polymerisation and carbonisation of water-soluble compounds derived mainly from carbohydrates, due to the high temperature in the process [33]. Based on the percentages of ashes and C in the solid fraction, it can be stated that a considerable high biomass conversion was achieved during the process.

The mass balance revealed that the AP-HTL contained a high percentage of the inorganic fraction originally present in the LEM, but also organic compounds. The COD and the TOC analysis resulted in 75500 mg O_2/L and 21450 mg C/L respectively, in agreement with the concentrations reported in the literature [13,34]. The COD and TOC values indicate the presence of dissolved organic compound. Although the compounds were not identified, they may be formic acid, acetic acid, lactic acid, glycerol amongst other are some polar organic compounds which remained solubilised in water [35]. As can be seen in Table 2, 26±1% of the C from the LEM remained solubilised in the AP-HTL. Based on the high protein content, its decomposition can generate nitrogen compounds such as pyroles, indoles and phenols [12]. The nitrogen content was approximately 7000 mg N/L, and around 68% of the TN was in the form of NH_4^+. The major part of the N from the LEM remained solubilised in the AP-HTL (Table 2). The C to N ratio resulted ≈3.4 in the aqueous phase, as reported during the HTL of several microalgae species under similar experimental conditions [8,12,14]. The PO_4^{-3} was 90 mg P/L, considerably low compared with the N and C concentrations. The phosphorous distribution in the HTL products can be affected by reaction temperature and retention time, and by the reaction between the amino acids with reducing sugar [13].

As mentioned before, the optimisation of the HTL process was not studied in this manuscript. However, the production of bio-oil from LEM and the subsequent upgrading process was already reported by Zhu et al. [10]. The similarities in the composition of the biomass and the yields and characteristics of the bio-oil suggest that the HTL of lipid-extracted *N. oculata* may be also a promising alternative to produce liquid fuels which can compete with conventional fossil fuels. Moreover, the nutrient recycling can significantly contribute to improve economic aspects.

3.3. Nutrients recycling

The suitability of the AP-HTL for microalgae cultivation was evaluated. According to the nutrient analysis performed, the N:P ratio of the AP-HTL was 78:1. Such a high N:P ratio is far from the optimal N:P ratios for microalgae development [36]; this was confirmed by the low *Nannochloropsis gaditana* growth on dilutions of AP-HTL in a previous culture experiment (data not shown). Therefore, it was decided to check the suitability of the AP-HTL only as nitrogen source. With this aim, part or the entire $N\text{-}NO_3$ source of the standard medium was substituted by a volume of AP-HTL that provided with an equal amount of nitrogen. Several AP-HTL loads were tested considering that a high AP-HTL concentration could have an inhibitory effect on the culture due to toxic compounds such as amides or heterocyclic compounds [13].

Figure 3 shows N. gaditana growth curves for different loads of AP-HTL; the curves are plotted in semi-logarithmic scale for better visualisation of the exponential-growth phase. The amount of AP-HTL that accounted for the total substitution of $N\text{-}NO_3$ (HTL60) inhibited growth from the beginning, as evidenced by the reduced division rate (Table 3) and the final biomass which was 25% of the obtained in the standard medium. On the contrary, AP-HTL loads contributing up to 30 mg N (HTL30) supported growths only slightly smaller than in the standard medium. Division rates were similar or even slightly higher than in the standard medium, but at the end, final biomass (in terms of Abs750nm) was 90-77% of that obtained in standard medium, the higher the final biomass the lower the AP-HTL load.

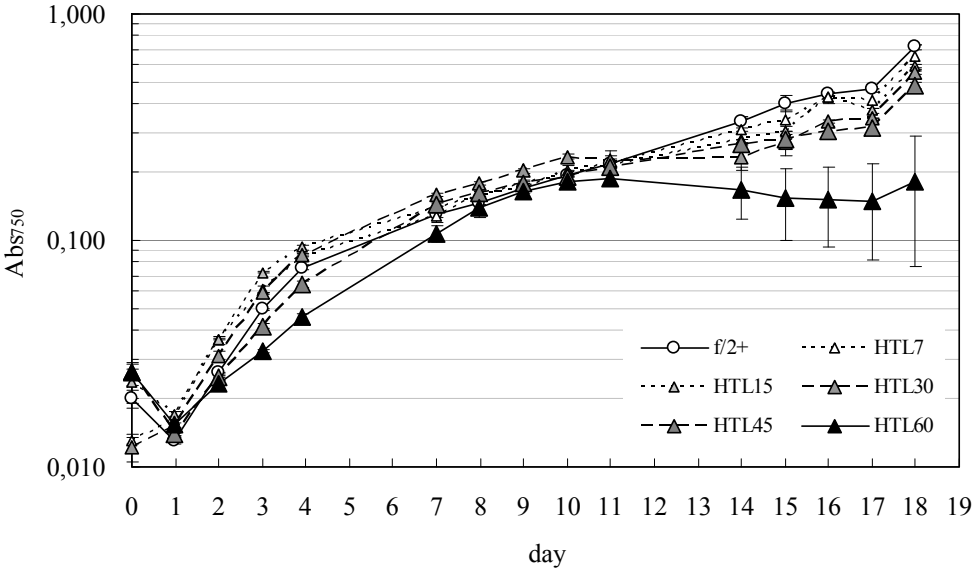


Figure 3. Growth curves in semi-logarithmic scale for *N. gaditana* in several culture media.

The applicability of an AP-HTL as microalgae culture medium depends on the concentration and form of macro- (nitrogen, phosphorus, N:P ratio) and micro- nutrients [37], the absence of harmful compounds [12,34,38], the pH

[14] and the microalgae species used [12,14]. In the present experiments, the growth inhibition at HTL60 concentrations may reflect a compound at harmful concentration rather than the form of inorganic N supplied. Although high amounts of NH_4^+ are known to be toxic [39], *N. gaditana* has demonstrated to thrive at concentrations up to 190 mg N-NH_4^+/L when the pH of the culture is controlled at 8.0 [40]. Therefore, the 60 mg·N-NH_4^+/L in HTL60 at the initial pH of 8.3 (Table 3) should not be harmful.

Table 3. N source in the culture media and summarised results obtained during cultivation.

		Media					
		f/2+	HTL7	HTL15	HTL30	HTL45	HTL60
Source of N (mg N/L)	*NO_3^- from f/2+*	60	53	45	30	15	0
	NH_4^+ from AP-HTL [a]	0	7	15	30	45	60
	Total Inorganic N	60	60	60	60	60	60
k (duplications/day)		0.95±0.03	0.97 ±0.11	1.04±0.02	0.97 ±0.07	0.79±0.02	0.54±0.02
Initial pH		8.1	7.4	7.8	8.3	8.2	8.3
Final pH		9.8±0.1	9.7±0.1	9.9±0.0	9.6±0.0	9.1±0.0	7.8±0.1

[a] *the amount of NO_3^- was negligible in the AP-HTL*

The AP-HTL contributing up to 30 mg N (HTL30) supports growth close to the maximum growth obtained with standard medium. Further experiments are needed to ascertain which other nutrients, such as iron and other micronutrients, can be substituted from standard media by AP-HTL.

4. Conclusions

This study demonstrated the valorisation of the lipid-extracted *Nannochloropsis* can be carried out producing energy and a nitrogen-source for biomass cultivation. These ways of valorisation can be beneficial for the biodiesel production process, and also for other process which extract valuable compounds from microalgae. Additionally to the possibility of converting the LEM into methane, the co-digestion with sewage sludge demonstrated the viability of the co-digestion process. The co-digestion of LEM and sewage sludge allows taking advantage of the over-sized digesters in some WWTPs. The pre-treatment of LEM before digestion resulted unsuitable for microalgae wastes, probably due to the cell wall characteristics which hamper degradation. For this reason, powerful pre-treatment methods should be evaluated to increase the biodegradability of the biomass.

Another alternative for LEM valorisation is the conversion into bio-oil. The results revealed the possibility to obtain high bio-oil yields. Moreover, recycling the aqueous phase generated during HTL to the microalgae cultivation unit, the amount of fertilisers for cultivation is reduced. The high nutrient content in the aqueous phase is problematic for water discharge, thus the microalgae cultivation can contribute to accomplish the regulation for water discharge.

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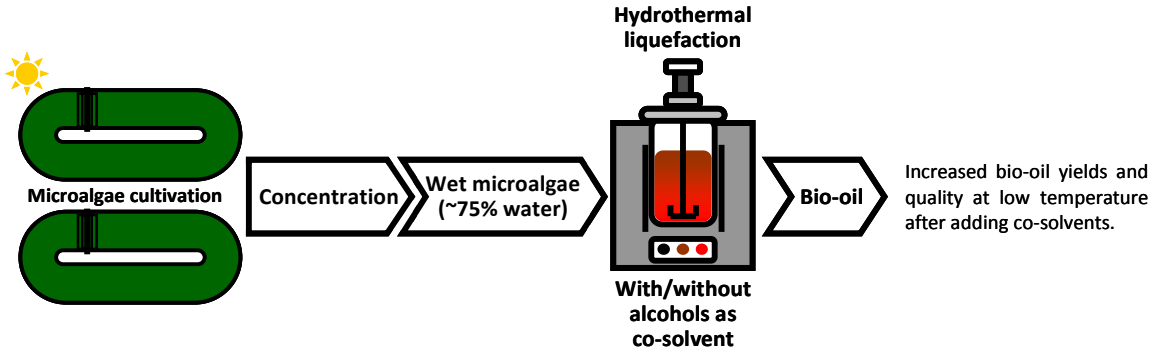
Chapter 9

Hydrothermal liquefaction of Nannochloropsis oceanica in different solvents [1]



ABSTRACT

Although the hydrothermal liquefaction is considered a promising technology for converting microalgae into liquid biofuels, there are still some disadvantages. This paper demonstrated that the bio-oil yield can be significantly improved by adding alcohols as co-solvents and carrying out the conversion at mild conditions (<250 ºC), but at the expense of a reduced bio-oil quality. By adding ethanol, the bio-oil yields obtained (up to ~60%) were comparable to the yield obtained at severe operating conditions using only water as solvent (54±2% on average), but the quality of the bio-oil was lower. However, the main advantages of the process here described lie in the utilisation of wet microalgae (~75% moisture) and alcohol concentrations which avoid both drying the microalgae and decreasing the amount of microalgae loaded in the reactor.

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1. Introduction

Microalgae are considered valuable feedstocks for biofuels; they can favourably reduce the demand of fossil fuels and simultaneously alleviate the "food-versus-fuel competition" caused by the production of first-generation biofuels [Pragya et al., 2013].

Several feedstocks characterised by high water content can be converted into a liquid fuel called bio-oil by means of hydrothermal liquefaction (HTL) [López-Barreiro et al., 2013]. The HTL is a promising technology for microalgae conversion inasmuch as the reactions take place in the aqueous medium [López-Barreiro et al., 2013], and microalgae are characterised by high water content even after concentration. The elimination of water is high-energy consuming [Pragya et al., 2013]. Another advantage of HTL is that lipid, protein, and carbohydrate fractions in microalgae are converted into bio-oil.

The influence of several processing parameters on HTL has been widely evaluated, i.e., reaction temperature, holding time, solid to liquid ratio in the biomass and presence of catalysts [Jena et al., 2011; Valdez et al., 2012; Reddy et al., 2016]. The temperature is a very influential parameter in HTL [Valdez et al., 2012; Li et al., 2014]; it affects both the yields and the characteristics of the different fractions obtained during HTL [Li et al., 2014; Gai et al., 2015]. Although opposed results have been reported about the influence of the solid to liquid ratio on the bio-oil yield [Valdez et al., 2012], economic and operational aspects may determine the most suitable value of the solid to liquid ratio. According to the literature, solid concentrations under 5% may lead to negative energy balance, whereas above 15% cause difficulty in pumping biomass into the reactor at the high pressures required [Valdez et al., 2012]. The bio-oil from microalgae is characterised by high viscosity derived from the protein content in microalgae [Guo et al., 2015]. Furthermore, the bio-oil is also characterised by high amount of oxygen, which decreases the caloric value and the storage stability [Zhang et al., 2013]. Both nitrogen and oxygen contents can be reduced either by upgrading the bio-oil [Guo et al., 2015] or adding catalysts during HTL [Pragya et al., 2013]. Recently, it has been reported that processing microalgae with organic solvents can led to better bio-oil quality; some examples are ethanol [Huang et al., 2011; Chen et al., 2012; Reddy et al., 2014; Zhang and Zhang, 2014; Peng et al., 2016], methanol [Patil et al., 2011; Sitthithanaboon et al., 2015] and others [Yuan et al., 2011; Jin et al., 2014B]. However, most of these experiments were performed in pure organic solvents or with a negligible amount of water compared to the amount of the organic solvent, which requires low-water content in microalgae. Based on the high-water content in microalgae and the increased the bio-oil production reported from some waste processed in mixtures of water and ethanol or methanol [Yuan et al., 2007; Cheng et al., 2010], processing microalgae in mixtures of solvents is a valuable alternative. Methanol has been used on an industrial for biodiesel production, and it is available at a reasonably low price; however, it is highly toxic and it is currently produced mainly from non-renewable source like natural gas [Reddy et al., 2014]. On the other hand, ethanol can be produced from renewable feedstocks [Reddy et al., 2014]. Figure 1 summarises the operating conditions applied by several authors using alcohols as co-solvents for the HTL of microalgae amongst some other substrates [Yuan et al., 2007; Cheng et al., 2010; Patil et al., 2011; Chen et al., 2012; Jin et al., 2014A; Reddy et al., 2014; Zhang and Zhang, 2014; Sitthithanaboon et al., 2015]. Processing wet microalgae with organic solvents resulted in increased bio-oil and biodiesel yields [Patil et al., 2011; Chen et al., 2012; Jin et al., 2014A; Jin et al., 2014B; Reddy et al., 2014; Zhang and Zhang, 2014], but the experiments were carried out at high temperatures [Chen et al., 2012] and high concentration of alcohols [Reddy et al., 2014]. The high concentration of alcohol was reached

by using dried microalgae [Jin et al., 2014A; Reddy et al., 2014] or loading a low concentration of wet microalgae in the reactor [Patil et al., 2011; Sitthithanaboon, 2015], being both alternatives negative from the economic and energetic point of view [Valdez et al., 2012].

The aim of this study is to investigate the HTL of *Nannochloropsis sp.* in water and in mixtures of water and alcohols. The first part of the experiments evaluates the influence of the temperature on the distribution of products, thus microalgae were processed in water between 240 ºC and 300 ºC. Afterwards, the influence of alcohols on the bio-oil characteristics was evaluated by processing microalgae in several alcohol-water mixtures, but using microalgae with high-water content, low temperature and suitable concentration of solids in the reactor.

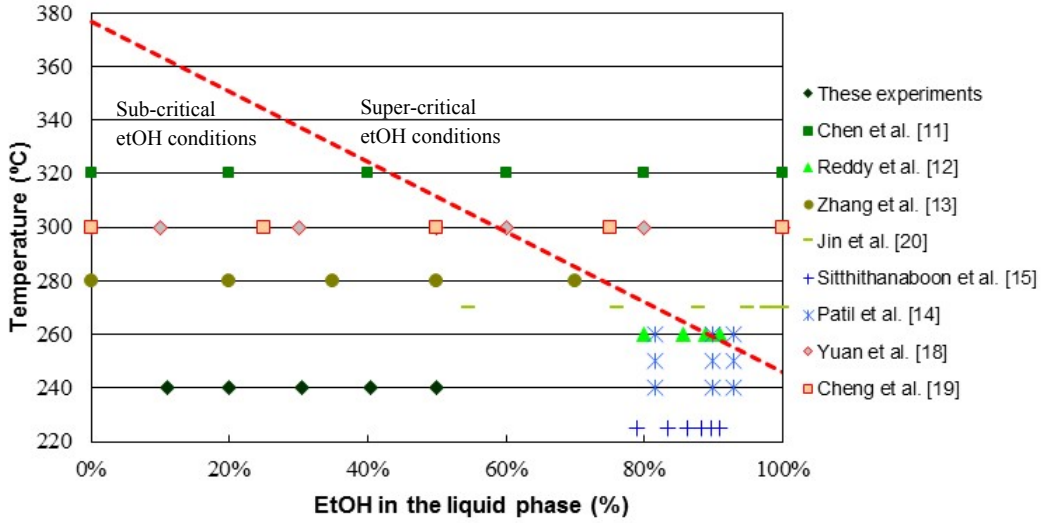

Figure 1. Summary of the operating conditions applied by several authors using microalgae and other substrates. Note: The dashed line separates the sub-critical and super-critical conditions in ethanol:water mixtures [18]. In green, experiments carried out with microalgae and ethanol:water mixtures. In blue, microalgae and methanol:water mixtures. In red, other substrates and ethanol:water mixtures.

2. Materials and methods

2.1 Materials

Nannochloropsis oceanica slurry, 28±3% lipid content (% dry weight), was provided by AlgoSource's (Alpha Biotech, Asserac, France). The frozen slurry was stored at -15 ºC until required. The total solids (TS) and volatile solids (VS) were analysed as described in [Caporgno et al., 2016A]; the TS content was 24.3±0.3% and the VS/TS 0.93±0.01.

2.2 Experimental procedure

2.2.1 Hydrothermal liquefaction

The experiments were performed with approximately 100 g of biomass in a non-stirred batch stainless steel reactor with 1L volume capacity (4593, Parr Instruments Co., Moline, IL, USA). The reactor was closed and purged with nitrogen to assure oxygen-free conditions. The temperature was increased until the desired value, and then kept constant during 30 min based on the results reported in the literature for Nannochloropsis biomass [Valdez et al., 2012; Reddy et al., 2016].

The biomass used in first part of the experiments consisted of the microalgae slurry as it was received it, without any addition of water or alcohols. The experiments evaluates the effect of the temperature on the bio-oil, thus several experiments were performed setting the reaction temperatures between 240±1 ºC to 300±3 ºC under autogenous pressure conditions. The reactor was not initially pressurised, and the pressure varied from 32 to 89 bar when the experiments were performed at temperatures from 240±1 ºC to 300±3 ºC.

In the second part of the experiments, the reaction temperature was decided around 240 ºC and kept constant during 30 min, under autogenous pressure conditions. The biomass for these experiments consisted of mixtures of alcohols and the microalgae slurry, without any addition of water. The mixtures of ethanol and microalgae slurry were prepared in order to have ethanol:water ratios of 1:10, 3:10, 4:10, 7:10 and 10:10 (w/w). Afterwards, an experiment with a methanol:water ratio of 10:10 (w/w) was performed. The reactor was not initially pressurised, and the autogenous pressure varied from 32 to 43 bar when the ration of ethanol increased.

After the 30 min at the desired temperature, the heating and isolation systems were removed from the reactor, and then the reactor was cooled down to room temperature using an external fan.

For the separation of the different products, the methodology described in a previous work was used [Caporgno et al., 2016B]. The total bio-oil, recovered using dichloromethane (DCM), was separated into light and heavy fractions by using hexane to recover the light fraction. The heavy bio-oil fraction was determined by difference between total and light bio-oil fractions. The bio-oil, aqueous products (AP) and solid waste yields were determined according to the equations 1, 2 and 3, and expressed in % w/w:

$$\text{Bio-oil yield}\,(\%) = \frac{\text{Mass of light/heavy Bio-oil}}{\text{Initial mass of microalgae}} \times 100 \qquad (1)$$

$$\text{AP yield}(\%) = \frac{\text{Mass of solids dissolved in AP}}{\text{Initial mass of microalgae}} \times 100 \qquad (2)$$

$$\text{Solid waste yield}(\%) = \frac{\text{Mass of solid residue}}{\text{Initial mass of microalgae}} \times 100 \qquad (3)$$

The number of moles of gas generated in the reactor was calculated using the ideal gas law, taking into account the pressure increase and the volume of free space in the reactor. The mass of gas was then calculated using the average molar mass of the mixture, based on the composition of the gas. The biomass conversion was determined according to the equation 4 and expressed in % w/w:

$$\text{Conversion}\,(\%) = \left(1 - \frac{\text{Mass of solid residue}}{\text{Initial mass of microalgae}}\right) \times 100 \qquad (4)$$

2.2.2 Products analysis

The amount of AP dissolved in the aqueous phase was determined following the procedures for total solids (TS) and volatile solids (VS) described in [Caporgno et al., 2016A]. The VS content was also determined in the solid waste with the same procedure.

The components in the gas fraction were determined by a gas chromatography (Two channels micro-GC Agilent technologies 3000 A) equipped with thermal conductivity detector (TCD). A molecular sieve column (molecular sieve 5Å, 10 m × 0.32 mm × 12 µm) separates O_2, N_2, CH_4, and CO, and a PLOT Q column (PLOT Q, 10 m × 0.32 mm × 10 µm) separates of CO_2 and light hydrocarbons from C_2H_n to C_4H_n.

The bio-oil samples were analysed by chromatography-mass spectroscopy (GC-MS) equipped with a capillary column (SLB-5ms, 30m × 0.25 mm × 0.25 μm) using a Perkin Elmer Turbo Mass Gold GC-MS. DCM was used as solvent. The GC oven was held at 70 °C for 1 min, heated to 180 °C at a rate of 7 °C/min, then heated to 240 °C at a rate of 12 °C/min and finally 7 min hold at 330 °C. The composition of the light bio-oil was analysed by GC-MS using the method above described but with different solvent, i.e. hexane. The constituents in the bio-oils were identified based on their retention time, and the respective mass spectra were identified from the preinstalled NIST library. The higher heating values of bio-oils (HHVs) were measured using an oxygen bomb calorimeter (6200, Parr Instrument Co., Moline, IL, USA).

The content of C, H, N and S was measured in microalgae, AP, solid waste and bio-oil using a CE Instruments Flash EA 1112 series elemental analyser.

3. Results and discussion

3.1. Influence of the temperature on HTL

Figure 2a shows the effects of the temperature on the yields of bio-oil, AP, solid waste and gas during the HTL of wet microalgae.

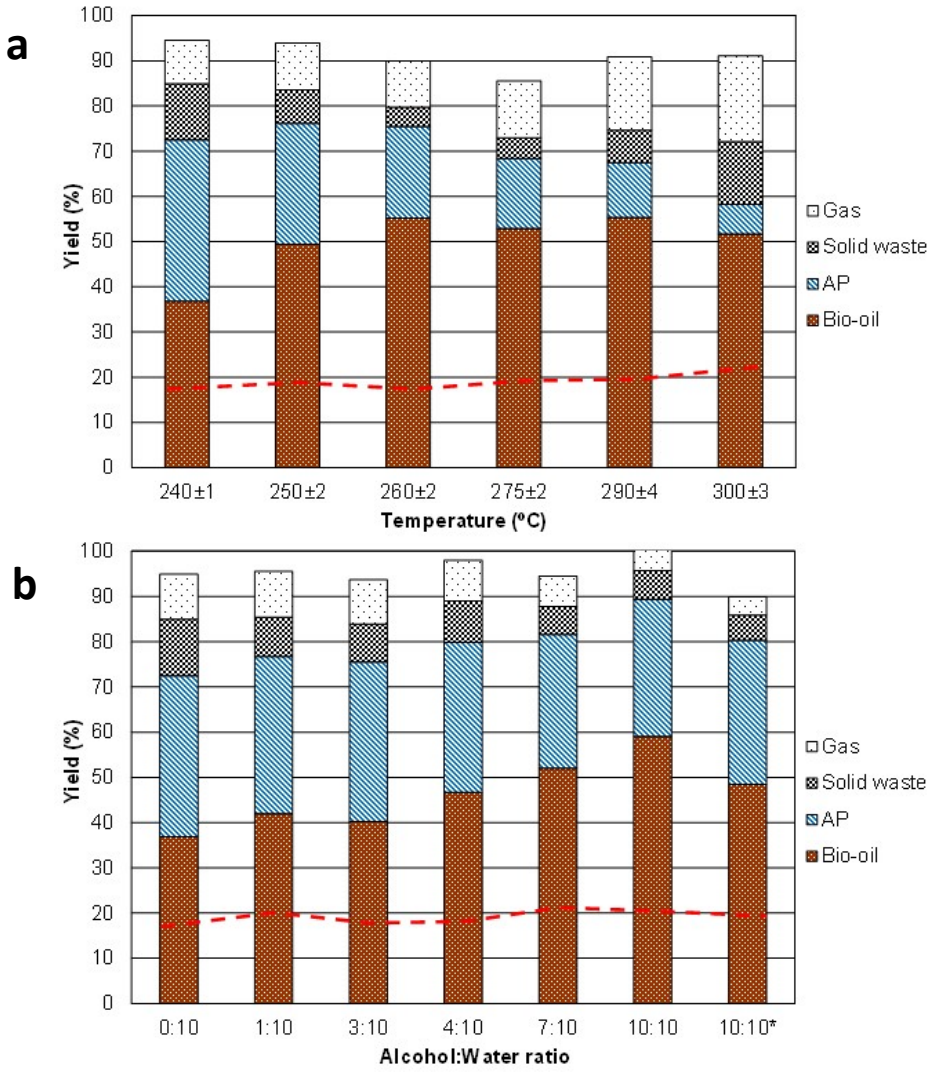

Figure 2. The effects of the temperature (a) and the addition of co-solvent (b) on the products distribution. Note: The red dashed line indicates the light fraction in bio-oil. In Figure 2 b, 10:10* represents the results obtained using methanol.

The total bio-oil yields were mainly affected when the temperature increased from 240±1 ºC to 260±2 ºC; further temperature increases did not affect the yield significantly, which averaged 54±2% between 260±2 ºC and 300±3 ºC. Focusing on the light and heavy bio-oil fractions, the results revealed that whereas the heavy fraction increased, the light fraction remained almost unaffected by the changes in the temperature. This behaviour could be a consequence of the nature of both bio-oil fractions. The light bio-oil mainly contains fatty acids and products from the decomposition of lipids [Valdez et al., 2012], which occurs at mild conditions (<250 ºC) [López-Barreiro et al., 2013]. On the other hand, the heavy bio-oil contains products generated by decomposition of carbohydrates and proteins [Valdez et al., 2012]. Since the decomposition of carbohydrates and proteins requires more severe reaction conditions [López-Barreiro et al., 2013], the fraction of heavy bio-oil was affected by the changes in temperatures [Jena et al., 2011]. Diverse results can be found regarding bio-oil yields from microalgae. The bio-oil yields in the present experiments exceed by far the yields recently reported for the HTL of *Nannochloropsis sp.* at different temperatures [Reddy et al., 2016], probably due to the high carbohydrate content in the biomass which lead to low bio-oil yields [Biller and Ross, 2011]. Other authors reported lower bio-oil yields using the same microalgae species [Tian et al., 2014; Shakya et al.,2015]. On the other hand, the bio-oil yields here presented are comparable to the results reported by using *Nannochloropsis sp*. with similar biochemical composition [Valdez et al., 2012].

Increasing temperatures promoted the conversion of the solid fraction into bio-oil, AP and gases, in accordance with the results reported for the microalgae conversion under sub-critical conditions [López-Barreiro et al., 2013]. The amount of compounds solubilised in aqueous phase was the highest at 240 ºC and gradually decreased when the temperature increased, indicating high conversion of the solubilised compounds. The highest AP decreases occurred when the temperature increased up to 260±2 ºC, which is consistent with the increases in the bio-oil yields. The biomass conversion resulted considerable high if compared with the reported in the literature [Reddy et al., 2016], where almost 40% of the initial *Nannochloropsis sp.* remained in the solid waste at temperatures between 225 ºC and 275 ºC and 30 min holding time. On the contrary, Valdez et al. achieved similar conversion at mild conditions using the same microalgae species [Valdez et al., 2012]. The biomass conversion strongly depends on the biomass characteristics.

A detailed evaluation of the characteristics of the solid revealed a brown-green hue in the filters which suggested the presence of microalgae cells in the solid waste recovered at low temperature. *Nannochloropsis* species have thick cell walls, which may require high temperatures to promote the biomass conversion [López-Barreiro et al., 2013]. The analysis revealed that further temperature increases affected the solid fraction. At 260±2 ºC, the solids represented around 4% of the biomass loaded into the reactor; these solids looked like greyish powders similar to ashes and no evidences of microalgae cells were observed. Surprisingly, the solid fraction sharply increased at 290±4 ºC and 300±3 ºC; in fact, the solid fraction reached its highest yield at 300±3 ºC. The solid recovered had dark particles mixed with greyish powders. Some dark particles were observed at 290±4 ºC, but to a lesser extent. The bio-char is generated when part of the solubilised carbohydrates undergoes to re-polymerisation and carbonisation [Tian et al., 2014; Yang et al., 2015]. The solids recovered at 290±4 ºC and 300±3 ºC were separated in two different fractions; a greyish powder which was placed in the bottom of the reactor and dark-coloured particles stuck on the reactor walls and mixed with bio-oil fractions. The increases in microalgae conversion are also evident in Table 1. The high VS/TS in solid and AP at 240±1 ºC and 260±2 confirmed the presence of unreacted microalgae in the solid fraction and the solubilisation of part of the biomass in the aqueous fraction, as

consequence of the low temperature [Shakya et al., 2015]. The higher the temperature, the lower the VS/TS in both solid and AP, which means that the organic fraction was converted into bio-oil or gas. Temperatures between 225 ºC and 250 ºC cause the thermal denaturing of proteins and their conversion into nitrogen-containing compounds in the bio-oil [López-Barreiro et al., 2013; Guo et al., 2015]. Compounds such as formic acid, acetic acid, lactic acid, glycerol, pyroles, indoles and phenols have been found in the aqueous phase elsewhere [Biller and Ross, 2011; Biller et al., 2012]. The VS/TS and the elemental analysis of both solid fractions recovered at 290±4 ºC and 300±3 ºC revealed that the greyish powder was mainly constituted by ashes from the inorganic fractions of microalgae, similar to the solid fractions recovered at 260±2 ºC and 275±3 ºC [Tian et al., 2014]. On the contrary, the dark-coloured particles (identified with the superscript b in Table 1) were mainly constituted by organic compounds rich in carbon.

Table 1. Elemental analysis of microalgae and solid and AP fractions recovered after HTL at different temperatures.

			Elemental analysis (% on total solid basis)				*VS/TS*
			N	*C*	*H*	*S*	
	Microalgae		7.3±0.1	50.3±0.2	7.6±0.1	0.3±0.2	0.93±0.01
Fraction	*Solid*	*240*	5.6±0.1	53.0±0.5	5.7±0.1	-	0.46±0.01
		250	4.6±0.1	35.0±1.0	3.8±0.1	-	0.43±0.01
		260	0.9±0.1	7.0±0.4	1.4±0.1	-	0.15±0.01
		275	0.6±0.1	7.6±0.4	1.2±0.1	-	0.15±0.01
		290	0.5±0.1	5.6±0.5	1.0±0.1	-	0.12±0.02
		290 [a]	3.0±0.1	74.7±0.3	4.1±0.2	-	0.85±0.01
		300	0.4±0.1	7.6±0.5	0.8±0.1	-	0.21±0.03
		300 [a]	3.4±0.1	77.6±0.9	4.9±0.2	-	0.94±0.01
	AP	*240*	9.8±0.3	42.9±1.1	7.1±0.2	-	0.84±0.01
		250	8.7±0.1	39.3±0.4	6.7±0.1	-	0.78±0.06
		260	5.1±0.1	29.8±0.8	4.9±0.2	-	0.65±0.05
		275	3.4±0.1	20.8±0.4	3.5±0.1	-	0.55±0.01
		290	3.6±0.1	23.0±0.6	3.5±0.1	-	0.55±0.02
		300	2.4±0.1	15.8±0.3	2.1±0.1	-	0.40±0.01

[a] *Dark particles.*

The bio-oil samples were further analysed in order to determine some of their characteristics. The HHVs were similar in all bio-oil samples; the HHV averaged 35.5±0.5 MJ/kg in the bio-oil samples produced between 240±1 ºC and 300±3 ºC, in agreement to the reported in the literature for bio-oil samples from *Nannochloropsis* [Shakya et al., 2015] and for from other microalgae species [Tian et al., 2014]. The qualitative GC-MS analysis of the bio-oil evidenced the complexity of the samples. Bio-oil samples revealed the presence of nitrogenous compounds such as pyrazine, pyridine, indole and amides, mainly generated by chemical reactions and decomposition of proteins. The content of nitrogenous compounds significantly increased when the temperature exceeded 240±1 ºC (Table 2), which is consistent with the enhanced decomposition of proteins at these temperatures [López-Barreiro et al., 2013]. Compared to the N content in microalgae, it can be observed that the HTL promoted the denitrogenation of microalgae; however, the nitrogen content in the bio-oil from microalgae is still higher than in petroleum (N/C below 0.01) [Tian et al., 2014]. The GC-MS analysis of the light bio-oil fraction showed a decreased number of peaks compared to chromatogram corresponding obtained from the heavy bio-oil fraction,

which revealed that the nitrogenous compounds constitute mainly the heavy bio-oil fraction as reported by [Valdez et al., 2012]. Some of aliphatic hydrocarbons (mainly alkanes and alkenes) and some other aromatic hydrocarbons were identified in the chromatograms. These compounds may be generated from the decomposition of lipids [López-Barreiro et al., 2013], thus they were already observed in samples produced at the lowest temperature. Their presence at high temperatures indicates stability. In a previous work, the fatty acids originally identified in this microalgae species were eicosapentaenoic (C20:5) and palmitoleic (C16:1), followed by palmitic (C16:0), eicosatetraenoic (C20:4), eicosadienoic (C20:2), oleic (C18:1) and linoleic (C18:2) [Caporgno, 2016A]. These fatty acids and their esters were also found in bio-oil samples; however, high concentration was only found in bio-oil samples obtained at 240±1 ºC. The reduced fatty acids concentration indicates that they either react or decompose, as it is reported in the literature [Shakya et al., 2015]. The identification of amides due to the conversion of oleic and linoleic acids (or their ester) may lead to the decrease in the fatty acid or esters yields. The results in Table 2 show that HTL promotes the deoxygenation of microalgae, but the O/C in bio-oil from microalgae was still high and exceeded by far the oxygen content in petroleum (O/C around 0.01) [Tian et al., 2014]. Both oxygen and nitrogen content are undesirable in bio-oil [Tian et al., 2014].

Table 2. Elemental analysis of the bio-oil recovered after HTL at different temperatures and alcohol:water ratios.

		Elemental analysis (% on total solid basis)				*H/C*	*N/C*	*O/C* [a]
		N	*C*	*H*	*S*			
		7.3±0.1	50.3±0.2	7.6±0.1	0.3±0.2	0.15	0.14	0.55
Temperature	*240*	4.8±0.1	74.5±0.5	10.1±0.1	-	0.14	0.06	0.14
	250	6.5±0.1	72.7±0.3	9.7±0.1	-	0.13	0.09	0.15
	260	6.7±0.1	74.1±0.2	9.5±0.1	-	0.13	0.09	0.13
	275	6.5±0.1	75.1±0.2	9.6±0.1	-	0.13	0.09	0.12
	290	6.0±0.1	73.8±0.3	9.4±0.1	-	0.13	0.08	0.15
	300	5.5±0.1	75.3±0.5	9.5±0.1	-	0.13	0.07	0.13
alcohol:water ratio	*1:10*	4.9±0.1	75.2±0.3	10.2±0.1	-	0.13	0.07	0.13
	3:10	5.5±0.2	74.0±0.1	10.0±0.1	-	0.14	0.07	0.14
	4:10	6.2±0.2	72.1±0.2	9.7±0.1	-	0.13	0.09	0.17
	7:10	6.6±0.2	72.2±0.5	9.7±0.1	-	0.13	0.09	0.16
	10:10	6.9±0.1	70.5±0.1	9.5±0.1	-	0.14	0.10	0.18
	10:10 [b]	6.7±0.1	72.0±0.1	9.6±0.1	-	0.13	0.09	0.16

[a] *Oxygen content calculated by difference.*
[b] *Results obtained with methanol.*

Regarding the gas fraction, the yields indicated that between 10% and 20% of the initial biomass was converted into gaseous products, corresponding these percentages to the lowest and the highest temperatures respectively. The temperature increases caused gas formation, as it has been reported in the literature for *Nannochloropsis* and other microalgae species [Valdez et al., 2012; Tian et al., 2014; Shakya et al., 2015]. Table 3 summarises the main components in the gas samples. As can be observed, the major component in all samples was CO_2. Microalgae contain high amount of oxygen, and CO_2 formation is one of the preferably ways to remove oxygen during HTL of microalgae [Valdez et al., 2012; López-Barreiro et al., 2013; Tian et al., 2014]. The percentage of CO_2 strongly decreased when the temperature increased [Brown et al., 2010], and the oxygen removal start also occurring by

O_2 and CO production. The percentages of CH_4, C_2H_n, C_3H_n and C_4H_n increased with the temperature, but making a minor contribution to the gas fraction, as observed elsewhere [Brown et al., 2010; Jena et al., 2011].

Table 3. Composition of the gas phase obtained by HTH of microalgae slurry at different temperatures.

		Gas composition (%)							
		H_2	O_2	CH_4	CO	CO_2	C_2H_n	C_3H_n	C_4H_n
Temperature	240	0.1	1.5	0.1	0.9	97.0	<0.1	<0.1	0.3
	250	0.1	1.8	0.1	0.9	96.4	0.1	0.2	0.3
	260	0.6	1.4	0.6	2.5	93.9	0.7	0.2	0.0
	275	0.5	0.6	0.5	2.0	95.5	0.4	0.4	0.1
	290	0.5	14.3	1.5	3.8	76.6	1.6	1.3	0.3
	300	0.3	9.3	2.7	2.4	75.2	3.8	1.8	4.3
Alcohol:water ratio	0:10	0.1	1.5	0.1	0.9	97.0	<0.1	<0.1	0.3
	1:10	0.1	0.2	<0.1	1.0	97.3	0.1	0.1	1.3
	3:10	0.1	0.3	<0.1	0.9	96.2	0.2	<0.1	2.4
	4:10	0.1	3.1	<0.1	0.7	93.5	0.1	<0.1	2.4
	10:10	0.1	0.8	<0.1	0.8	93.2	0.2	0.2	4.8
	10:10 [a]	0.1	8.7	0.2	1.0	89.4	0.2	<0.1	0.3

[*] *Represents the results obtained using methanol.*

3.2. Influence of the addition of alcohol on HTL

Figure 2b shows the effects of adding ethanol and methanol as co-solvents during the HTL of microalgae slurry at 240±3 ºC and 30 min holding time.

The higher the ethanol:water ratio, the higher the bio-oil yield. The effects of ethanol addition are more evident at ratios higher than 3:10. Similar to the results described in section 3.1., the increase in the bio-oil yields was caused by the increased heavy fraction, since the light fraction (red dashed line) remained almost unaffected by the changes in the ethanol content. The ethanol addition decreased the yield of the solid waste compared to HTL carried out without ethanol; in other words, ethanol addition increased the microalgae conversion. The highest solid conversion were observed at ethanol:water ratio higher than 7:10. The ethanol addition caused AP decreases too. The gas yield barely decreased at ethanol:water ratio higher than 7:10, being similar to the measured in the experiments performed without ethanol.

Increases in the bio-oil yields were also reported using wet Chlorella pyrenoidosa (approximately 70% water content) and similar ethanol:water ratios, but higher temperature and considerably long holding times (280 ºC and 120 min) [Zhang et al., 2014]. Similar results have been recently reported by increasing the ethanol:water ratio, processing Chlorella pyrenoidosa at 300 ºC and 60 min [Peng et al., 2016]. The addition of ethanol as co-solvent increased both conversion and bio-oil production from Dunaliella tertiolecta under severe operating conditions (320 ºC) [Chen et al., 2012]. Although other authors reported benefits of ethanol as co-solvent in HTL, [Yuan et al., 2007; Cheng et al., 2010; Jin et al., 2014B; Reddy et al., 2014; Zhang and Zhang 2014], most of these experiments entail high temperature or considerably high amounts of ethanol. Severe reaction conditions cause thermal denaturation of proteins and may decrease the quality of bio-oil [Guo et al., 2015], whereas high concentrations of ethanol requires dried biomass utilisation or an excessively high addition of ethanol may be used.

The main advantages of operating conditions in the present experiments are the low temperature and the utilisation of microalgae with high-water content and low concentration of ethanol.

The HHVs resulted in 37.7±0.1. 38.1±0.2. 37.9±0.2. 36.8±0.4. 36.1±0.1 and 34.6±0.4 MJ/kg for bio-oil samples obtained at ratios from 0:10 to 10:10 respectively. In spite of the higher bio-oil yields obtained when the ethanol:water ratio increases, the HHV was negatively affected. The results presented in Table 2 reveals an increased oxygen content in the bio-oil when the ethanol:water ratio increased, which is consistent with the decrease of the HHV [Tian et al., 2014]. Contradictory results are reported in the literature [Chen et al., 2012; Jin et al., 2014A; Peng et al., 2014]; however, these results may be attributed to the high temperature during HTL. The high temperatures favour the deoxygenation of bio-oil produced using ethanol as co-solvent [Peng et al., 2016]. Measured HHVs in bio-oil samples from *Nannochloropsis* are similar to the found in the present experiments [Shakya et al., 2015]. The denitrogenation was not favoured by the ethanol addition neither.

The qualitative GC-MS analysis of the bio-oil evidenced the complexity of the samples; however, it has been found larger quantities of a relatively small number of compounds than in bio-oils produced without alcohol. The main compounds identified in bio-oils were palmitic (C16:0) and palmitoleic (C16:1) acids. The esters of these acids were found in bio-oils produced with and without ethanol, which means that the acids were not efficiently converted into esters and suggests that the esterification reaction was not favoured by alcohol addition. Some authors have reported high conversion of fatty acids into esters using ethanol; however, they have either used dry microalgae [Jin et al., 2014B], high alcohol:water ratios [Chen et al., 2012; Reddy et al., 2014] or high temperatures [Chen et al., 2012; Jin et al., 2014B] to favour the esterification reactions, or they subjected the bio-oil to subsequent transesterification [Reddy et al., 2014].

The gas composition is summarised in Table 3. Once again, the major component in the gas was CO_2. The concentration of CO_2 slightly decreased when the ethanol:water ratio increased, but this reduction was not accompanied by O_2 and CO formation. On the contrary, the percentage of C_4H_n increased with the ethanol:water ratio, but making a minor contribution to the gas fraction.

The utilisation of methanol as co-solvent affected the distribution of the products obtained during HTL, as can be observed in Figure 2b. The solid waste yield was low, which indicates high biomass conversion. Although the biomass conversion and the AP yield with methanol are comparable to the obtained using the same concentration of ethanol or even a lower concentration (ethanol:water ratio 7:10), the bio-oil yield was lower that the obtained with ethanol at 10:10. Based on the light and heavy fractions in bio-oil, it is evident that methanol did not contribute at increasing the heavy bio-oil fraction. Regarding the bio-oil composition, no significant differences were found between the samples produced with ethanol or methanol, as can be observed in Table 2. The HHV resulted 36.2±0.1 MJ/kg, similar to the HHV of the bio-oil produce using ethanol in ratio 7:10, with comparable elemental composition. The main difference between ethanol and methanol as co-solvent was observed in the composition of the gas fraction; it seems that methanol favoured the deoxygenation by formation of O_2.

Comparison between ethanol and methanol as co-solvents for microalgae liquefaction has not been reported in the literature; however, these solvents have been used with woody biomass [Cheng et al., 2010]. The authors reported that both alcohols, at alcohols:water ratio of 10:10, increased the biomass conversion and bio-oil yields compared with the HTL using only water as solvent.

4. Conclusions

High biomass conversion was obtained by processing *Nannochloropsis oceanica* at mild conditions (<250 ºC). More severe operating conditions do not affect the conversion significantly, but strongly affects the distribution of products. Although the bio-oil yields gradually increases by increasing the temperature from 240±1 ºC to 260±2 ºC, they averaged 54±2% between 260±2 ºC and 300±3 ºC. The quality of the bio-oil was negatively affected.

The addition of alcohols as co-solvents allows obtaining bio-oil yield comparable to the obtained at severe operating conditions using without alcohols, but decreasing the quality of the bio-oil due to the increased nitrogen and oxygen contents.

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Chapter 10

Conclusions and future work

1. Conclusions

Based on the results here presented, it can be concluded that microalgae are promising substrates for anaerobic digestion; however, several aspects should be considered to improve the overall performance of the entire process. These aspects are listed below:

- The microalgae species: several microalgae species, marine or freshwater species, have been evaluated as substrates for anaerobic digestion in the previous chapters. The results demonstrated that the species strongly affect the methane production, but it is not possible to draw general conclusions about their utilisation. It was exposed that some characteristics of the species such as the biochemical composition, is affected by the cultivation conditions. The changes in the composition affect the methane production.

- The operating parameters in digesters: the temperature for digestion is the first parameter evaluated and it significantly influenced the process. The mesophilic digestion is preferable based on the high protein content in microalgae and the higher probability of inhibition by ammonia at thermophilic conditions. Regarding the amount of substrate loaded into the reactors, the results obtained in batch mode suggested that ammonia inhibition must be taken into consideration. The ammonia release depended on the degradability of the species and the characteristics of the biomass.

- The pre-treatments: the effectiveness of the pre-treatments depends on the microalgae characteristics. Moreover, there is not direct relation between the effects of the pre-treatments on the microalgae and the methane production; the pre-treatments like ultrasound can completely destroy the biomass structures but fail at increasing the methane production. Microalgae processing prior digestion, *i.e.* lipid extraction, can act as pre-treatment too. In this case, economic aspects concerning about the benefits for both extraction and digestion process might be assessed.

- Nutrient recycling: the possibility of recycling nutrients from the digesters to the microalgae cultivation units has the benefit of reducing the nutrients necessary for cultivation and helping to accomplish the regulation for water discharge.

- Co-digestion with sewage sludge: the possibility of co-digesting microalgae and sewage sludge is beneficial for coupling processes. As mentioned before, the nutrient required for microalgae cultivation can be recycled from anaerobic digesters. The microalgae cultivation unit can be coupled to wastewater treatment plants; thus the biomass can be digested with sewage sludge.

Regarding the hydrothermal liquefaction of microalgae, some general conclusions are drawn below:

- The temperature in the reactor strongly influences the process; the yields of the products and the composition of these products change when the temperature change. Since the processing temperature is directly related to the energy consumption, the temperature must be optimised. The optimisation process must seek high the biomass conversion and good bio-oil quality.

- The addition of co-solvents, *i.e.* alcohols, improves yields and quality of the bio-oil obtained; however other factors affect the process. Some of these factors are the characteristics of the co-solvents (the environmental impact of the solvents) and operating parameters (the dilution effects of adding co-solvents).

- Since the process converts all the components in microalgae, *i.e.* lipids, carbohydrates and proteins, the hydrothermal liquefaction can process microalgae waste obtained after the extraction of valuable compounds.
- The process also allows recycling nutrients from aqueous phase to the microalgae cultivation units. Based on this and the previous conclusion, the bio-oil production can be included as processing step in a bio-refinery.

2. Future work

As a result of the knowledge gained in this thesis, proposals for possible future work in anaerobic digestion and hydrothermal liquefaction are outlined below.

- Anaerobic digestion:
    1. Anaerobic digestion was carried out in batch mode. It may be interesting to operate in semi-continuous or continuous mode in order to evaluate the response of the inoculum to ammonia released during digestion and to salt concentration from marine microalgae species. The influence of the inoculum acclimatisation might be studied too.
    2. The nutrient recycling was only studied using wastewater from digester in wastewater treatment plant. Different nutrient sources could be evaluated: nutrients recovered after microalgae waste digestion or after co-digestion.
    3. Coupling both processes, cultivation and digestion of microalgae, allows studying the influence of several parameters on the entire process. Some of these parameters strongly related to the microalgae cultivation such as biomass concentration, cultivation under stress conditions (N-starvation). The influence of microalgae processing can be also studied, i.e. extraction of valuable compounds and pre-treatments. Finally, parameters directly related to anaerobic digestion such as different organic load rates.
- Hydrothermal liquefaction:
    1. Hydrothermal liquefaction in two steps: the first step at low temperature to produce bio-oil from the lipid fraction and to solubilise most of the nitrogenous compounds in the aqueous phase. The bio-oil can be further converted into bio-diesel by transesterification in super-critical methanol conditions without catalyst, and the aqueous phase, recycled from microalgae cultivation of to recover valuable compounds.
    2. The addition of catalyst to improve the quality of the bio-oil.
- Economical study of all the processes in order to choose the most viable alternative.

Appendix

About the author

Martín Pablo Caporgno was born in Arroyo Cabral, Córdoba, Argentina, on April 6, 1983. He obtained his B.S. degrees in Chemical Engineering in 2010 from Universidad Tecnológica Nacional (UTN) - Facultad Regional Villa María, Argentina. During his university education, he spent six months in the Engler Bunte Institut, Karlsruhe University in Germany, and then six months in the Hochschule Karlsruhe - Technik und Wirtschaft, also in Karlsruhe. He was awarded by the UTN as recognition for certain achievements during the B.S..

Afterwards, he moved to Spain and he received his M.Sc. degree in Environmental Engineering in 2011 from Rovira i Virgili University - Tarragona. He was awarded for being an outstanding student in the Master programme after receiving her Master's degree.

In October 2011 he started his Ph.D. research at the Department of Chemical Engineering of Rovira i Virgili University in Tarragona, Spain, thanks to a scholarship from the same University. In April 2012 he was awarded with a pre-doctoral contract for the University teaching staff training grants, FPU Programme of Spanish Government to continue the Ph.D. During his Ph.D., he experienced his first three months research stay at the GEnie des Procédés Environnement - Agroalimentaire laboratories (GEPEA) - Nantes University, in Saint Nazaire, France. Research topic: Microalgae cultivation using urban wastewaters. The research stay was possible thanks to the mobility scholarship received from the Spanish Government. He experienced his second three months research stay at the Département Systèmes Energétiques et Environnement - École des Mines in Nantes, France. Research topic: Hydrothermal liquefaction of microalgae.

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