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PhD Thesis

STRATEGIES TO ENHANCE MICROALGAE ANAEROBIC DIGESTION IN WASTEWATER TREATMENT SYSTEMS: PRETREATMENTS AND CO-DIGESTION

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A la meva filla Núria.

ABSTRACT

Microalgae-based wastewater treatment systems are promising solutions to shift the paradigm from wastewater treatment to energy and resources recovery. In these systems, microalgae assimilate nutrients and produce oxygen, which is used by bacteria to biodegrade organic matter improving water quality. Moreover, microalgae biomass can be harvested and reused to produce biofuels or other non-food bioproducts. In this context, anaerobic digestion is one of the most consolidated and well-known technologies to convert organic waste generated in a wastewater treatment plant into bioenergy. However, microalgae anaerobic digestion is generally limited by their resistant cell walls, which lead to low methane potential (degradation extent) and conversion rate (degradation speed). Also, microalgae have high protein content, which can lead to ammonia nitrogen inhibition during the anaerobic digestion process.

This PhD thesis aims to overcome these drawbacks and enhance microalgae anaerobic digestion by combining different strategies. On one hand, the bioconversion process can be improved by applying a pretreatment before anaerobic digestion. Pretreatment methods disrupt or weaken the structure of microalgae cell wall, making the intracellular content more bioavailable and improving microalgae anaerobic biodegradability. Also, anaerobic co-digestion (i.e. the simultaneous digestion with two or more substrates) can contribute to: improve microalgae anaerobic digestion performance; increase methane production; reduce the risk of ammonia inhibition and enhance synergies between substrates (nutrients composition, rheology, etc.). In addition, co-digestion can lead to economic benefits, since several wastes can be treated together in the same facility.

Firstly, co-digestion of microalgae from high rate algal ponds (HRAPs), used as secondary treatment of urban wastewater, and primary sludge produced in the same treatment process has been investigated. Results have shown that the most suitable option to anaerobically digest microalgae from HRAPs would be the co-digestion with primary sludge at a 20-day hydraulic retention time (HRT), that leads to higher methane production (63% increase). Moreover, the energy assessments conducted according to these results have revealed that microalgae co-digestion with primary sludge is a sustainable solution for energy recovery in microalgae-based wastewater treatment systems, since it might produce between 3.5 and 4.5-fold the energy consumed during the anaerobic digestion process. Finally, potential reuse of microalgae digestates in agriculture has been investigated, including their co-digestion with primary sludge. All microalgae digestates have presented organic matter, macronutrients as well as organic and ammonium nitrogen content suitable for their reuse in agriculture as soils amendment. However, the digestate produced by microalgal biomass and primary sludge co-digestion was proven to be the one which has presented less phytotoxicity.

Besides, co-digestion of microalgal biomass with storable agricultural wastes (i.e. wheat straw) has been assessed. As for microalgae, wheat straw anaerobic digestion is limited by hydrolysis step due to its lignocellulosic structure. Thus, their co-digestion has being investigated after a simultaneous thermo-alkaline pretreatment applied to both substrates. Results have shown that when microalgae were co-digested with wheat straw (50% microalgae and 50% wheat straw on a VS basis) at 20-day HRT, the methane yield increased from 0.12 L CH_4/g VS to 0.21 L CH_4/gVS (77% increase). On the other hand, the pretreatment has only increased the methane yield by 15% compared to the untreated substrates co-digestion (0.24 L CH_4/g VS). Thus, the co-digestion of microalgae and wheat straw was proven to be a suitable strategy to improve microalgae methane production even without the pretreatment.

Microalgae-based system can be also integrated in conventional activated sludge system (e.g. as a tertiary treatment). In this case, waste activated sludge (WAS) is an abundant waste that can be used as co-substrate. In this PhD thesis, microalgae and WAS co-digestion was investigated after applying a simultaneous autohydrolysis pretreatment at 55 °C to improve microalgae biodegradability by promoting inherent enzymes release from WAS. Results showed that microalgae solubilisation was not improved by the simultaneous pretreatment with WAS. This means that WAS enzymes have not been effective at disrupting microalgae cell walls. However, WAS

co-digestion (80% WAS and 20% microalgae on a VS basis) after pretreatment increased microalgae mono-digestion methane yield by 130%.

RESUM

Els sistemes de tractament d'aigües residuals amb microalgues són solucions tecnològiques que permeten canviar el paradigma del tractament d'aigües residuals a la recuperació d'energia i recursos. En aquests sistemes, les microalgues assimilen nutrients i produeixen oxigen que utilitzen els bacteris per a la biodegradació de matèria orgànica, millorant així la qualitat de l'aigua. A més, la biomassa de microalgues es pot recol·lectar i reutilitzar per produir biocombustibles. En aquest context, la digestió anaeròbia és una de les tecnologies més establertes que permeten convertir els residus orgànics generats en una depuradora en bioenergia.

No obstant això, la digestió anaeròbia de microalgues està generalment limitada per la seva resistent paret cel·lular, i per aquest motiu presenten un baix potencial de metà i una baixa taxa de degradació (velocitat de degradació). A més, les microalgues tenen un elevat contingut en proteïnes, fet que pot conduir a la inhibició per amoníac durant el procés de digestió anaeròbia.

Aquesta tesi doctoral pretén millorar la tecnologia de la digestió anaeròbica combinant l'aplicació de pretractaments amb la codigestió. Mentre que els pretractaments actuen per alterar o debilitar l'estructura de la paret cel·lular de les microalgues, permetent que el contingut intracel·lular sigui biodisponible, la co-digestió (és a dir, la digestió simultània amb dos o més substrats) pot contribuir a millorar el rendiment de la digestió de les microalgues augmentant el potencial de metà, diluint compostos inhibidors o fomentant sinergies entre substrats (composició de nutrients, reologia, etc.). A més, la codigestió pot generar beneficis econòmics derivats del tractament de diversos residus en una única instal·lació.

En primer lloc, s'ha investigat la codigestió de les microalgues procedents de llacunes d'alta càrrega (LLAC), utilitzades com a tractament secundari per a aigües residuals urbanes, i fangs primaris, que es produeixen en el mateix procés de tractament. Els resultats obtinguts indiquen que l'opció més adequada per digerir microalgues és amb la codigestió amb fang primari en un temps de retenció hidràulica (TRH) de 20 dies. Els balanços energètics duts a terme d'acord amb aquests resultats han mostrat que l'energia produïda és fins a 4 vegades l'energia consumida durant la digestió anaeròbica. Finalment, s'ha investigat la possible reutilització dels efluents de la digestió de microalgues en l'agricultura (inclosa la seva codigestió amb fang primari). Tots els digestats de microalgues han presentat propietats adequades per se utilitzats com esmena de sòls agrícoles, tot i que l'efluent procedent de la codigestió ha presentat la menor fitotoxicitat.

Complementàriament, s'ha avaluat la codigestió amb residus agrícoles que puguin ser emmagatzemables (palla de blat). Com passa amb les microalgues, la digestió anaeròbia de palla de blat està limitada per l'hidròlisi a causa de la seva estructura lignocel·lulosica. Per tant, la seva codigestió ambles microalgues també s'ha investigant després d'un pretractament simultani a tots dos substrats (termoalcalí). Quan les microalgues s'han co-digerit amb palla de blat, el rendiment del metà ha augmentat des de 0,12 m^3 CH_4/kgVS fins a 0,21 m^3 CH_4/kgVS (augment del 77%), mentre que el pretractament només ha augmentat el rendiment del metà en un 15% en comparació amb la codigestió dels substrats no tractats (0,24 m^3 CH_4/kgVS). Així, s'ha demostrat que la codigestió de les microalgues i la palla de blat és una estratègia adequada per millorar substancialment la producció de metà de les microalgues fins i tot sense l'ús del pretractament.

Per últim, s'ha investigat la codigestió de microalgues i fangs biològics després d'aplicar un pretractament simultani a ambdós substrats d'autohidròlisi (55 °C). L'objectiu d'aquesta estratègia és millorar la biodegradabilitat de les microalgues per mitjà de l'alliberament d'enzims inherents als fangs. Tot i que en els assajos s'ha vist que els enzims alliberats pels fangs no han estat eficaços degradant la paret cel·lular de les microalgues, la codigestió amb els fangs biològics després del pretractament ha permès permet augmentar la producció de metà de les microalgues fins a un 130%.

PREFACE

The current thesis is framed within the context of two Spanish National Projects: the DIPROBIO project "Algal biomass production and digestion from wastewater" (CTM2012-37860), financed by the Spanish Ministry of Science and Innovation; and the project entitled FOTOBIOGAS "Biogas production from microalgae-bacteria grown in closed photobioreactors for wastewater treatment (CTQ2014-57293-C3-3-R), financed by the Spanish Ministry of Economy and Competitiveness.

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LIST OF CONTENTS

ACRONYMS AND ABBREVIATIONS

AD	Anaerobic digestion
AcoD	Anaerobic co-digestion
BMP	Biochemical methane potential
CH	Carbohydrates
CHs	Soluble carbohydrates
C/N	Carbon to Nitrogen ratio
COD	Chemical oxygen demand
CODs	Soluble chemical oxygen demand
CST	Capillary suction time
CSTR	Continuous stirred tank reactor
HRAP	High rate algal pond
HRT	Hydraulic retention time
HPLC	High-performance liquid chromatograph
EC	Electric conductivity
EOC	Emerging organic contaminant
GC	Gas chromatograph
GI	Germination index
GrI	Growth index
M	Microalgae / microalgal biomass
Mp	Pretreated microalgae
NH_4^+-N	Ammonium nitrogen
OLR	Organic loading rate
PBR	Photobioreactor
PPCP	Pharmaceuticals and personal care products
PS	Primary sludge
TKN	Total Kjeldah nitrogen
TKNs	Soluble total Kjeldah nitrogen
TN	Total nitrogen
TOC	Total organic carbon
TS	Total solids
VFA	Volatile fatty acids
VS	Volatile solids
WAS	Waste activated sludge
WASp	Pretreated waste activated sludge
WEOC	Water extractable organic carbon
WEOM	Water extractable organic matter
WS	Wheat straw
WSp	Pretreated wheat straw
WWTP	Wastewater treatment plant

1 STATE OF THE ART

* This chapter is based on the article **Co-digestion strategies to enhance microalgae anaerobic digestion: A review.** Solé-Bundó, M., Passos, F., Romero-Güiza, M., Ferrer, I., Astals, S. Submitted.

1.1 Introduction

The development of integrated microalgae-based facilities, so-called microalgae biorefineries , has attracted a great deal of attention from both academia and industry (Chew et al., 2017; Subhadra and Edwards, 2011; Trivedi et al., 2015). Microalgae biorefineries combine the production of biofuels (e.g. biodiesel, bioethanol) and value-added products (e.g. pigments, proteins, omega-3,6). Thus, they go one-step beyond the "third-generation biofuels" concept, which only aims at the production of biodiesel or bioethanol from microalgae. Moreover, in biorefineries, microalgae cultivation costs can be reduced by using wastewater streams as nutrient source; achieving the dual goal of wastewater treatment and value-added chemicals production (Craggs et al., 2014; Gupta et al., 2016; Wang and Park, 2015).

Anaerobic digestion (AD) is a microbiological process able to transform organic matter into renewable energy (biogas). It has been pointed out as a key technology to maximize microalgae resource recovery (Andersson et al., 2014; Peng and Colosi, 2016; Tijani et al., 2015; Uggetti et al., 2014). This technology is also appropriate to treat microalgae residues from the extraction of metabolites and reduce costs associated with their treatment and disposal (Ehimen et al., 2011; Ramos-Suárez and Carreras, 2014; Subhadra and Edwards, 2011; Zhang et al., 2013). Additional benefits of treating microalgae or microalgae residues via anaerobic digestion are the mobilization of nutrients (N and P) and the release of CO_2 through biogas combustion/upgrading, which can be recycled for microalgae cultivation (González-González et al., 2018; Toledo-Cervantes et al., 2016; Ward et al., 2014). However, microalgae AD is generally limited by their resistant cell wall, which lead to low methane potential (degradation extent) and conversion rate (degradation speed), and by the risk of ammonia nitrogen inhibition.

Pretreatment methods may be applied to disrupt or weaken the structure of microalgae cell wall, allowing the intracellular content to become more bioavailable, hence improving microalgae anaerobic biodegradability (extent and rate). Microalgae pretreatments (without co-products recovery) have been reported to increase microalgae methane yield up to 100% (Mahdy et al., 2015; Schwede et al., 2013b).

However, this increase in methane yield may not always compensate the pretreatment implementation and operational costs, especially if it involves techniques that demand high electricity input (Passos et al., 2014b). Indeed, the co-production of value-added products and biogas can contribute to improve biorefineries profitability, since there is more profit recovered from the co-products (Milledge and Heaven, 2014; Peng and Colosi, 2016; Trivedi et al., 2015). Furthermore, microalgae residues after co-products extraction have shown an increase in anaerobic biodegradability when compared to raw microalgae, since the extraction step behaves as a pretreatment, promoting cell wall disruption and organic matter solubilization. For example, Ramos-Suárez and Carreras (2014) observed an increase in *Scenedesmus* sp. methane yield from 140 to 272 and 212 $mLCH_4/gVS$ after the extraction of proteins and lipids, respectively; while Parimi et al. (2015) reported a methane yield increase from 181 to 254 $mLCH_4/gVS$ from protein spent *Spirulina platensis*. Even if upstream processing increases microalgae's anaerobic biodegradability, microalgae and microalgae residues are generally characterized by low methane yields (~100 to 250 $mLCH_4/gVS$) and degradation rates (~0.12 day^{-1}) when compared to traditional anaerobic digestion substrates, such as sewage sludge (~350 $mLCH_4/gVS$,~0.30 day^{-1}), animal manure (~350 $mLCH_4/gVS$, ~0.15 day^{-1}) and food waste (~550 $mLCH_4/gVS$, ~0.50 day^{-1}) (Astals et al., 2013; Braguglia et al., 2018; Dębowski et al., 2017; Gunaseelan, 1997; Li et al., 2018; Nasir et al., 2012; Ramos-Suárez and Carreras, 2014; Raposo et al., 2012).

A key issue for microalgae and microalgae residues anaerobic digestion is the risk of ammonia nitrogen inhibition, typically associated with a low carbon-to-nitrogen (C/N) ratio. Ammonia nitrogen is a potential inhibitor of the AD process that is released during the biodegradation of nitrogenous organic matter (e.g. proteins, amino acids, urea and nucleic acids) (Nghiem et al., 2017; Panpong et al., 2015). In this manner, microalgae biodegradability could be improved by different strategies, as selecting microalgae strains, tuning cultivation conditions and/or using pretreatments (Córdova et al., 2018; Passos et al., 2015b, 2014b). However, a high protein content and low C/N ratio is common across all microalgae species, especially when they grow in a medium with high concentration of nutrients like wastewater. The risk of ammonia inhibition limits the maximum organic loading rate (OLR) at which a microalgae digester can be operated, with an OLR around 2 $gVS/(L_r \cdot day)$ being the observed OLR threshold prior clear evidence of process inhibition (Ehimen et al., 2011; Herrmann et al., 2016; Rétfalvi et al., 2016; Yen and Brune, 2007; Zhong et al., 2013). The OLR threshold is a critical constraint for

microalgae and microalgae residues anaerobic digestion feasibility, requiring (i) longer hydraulic retention times (HRT), i.e. larger digester volume or (ii) lower influent organic matter concentration; either way the resulting in low volumetric methane yields ($LCH_4/(L_r \cdot day)$) which may compromise the economic feasibility of microalgae AD.

Anaerobic co-digestion (AcoD) is the simultaneous digestion of two or more substrates. It is a well-established and cost-effective option to overcome the drawbacks of mono-digestion and improve the economic feasibility of AD plants (Mata-Alvarez et al., 2014; Thorin et al., 2017). The main advantages of AcoD include increased OLR, higher methane production, dilution of inhibitory compounds, synergies between substrates (nutrients composition, rheology, etc.) and economic advantages derived from treating several wastes in a single facility (Nghiem et al., 2017; Panpong et al., 2015). Microalgae and microalgae residues have been successfully co-digested with different co-substrates such as sewage sludge, animal manure, food waste, energy crops, crops residues, glycerol, paper waste and fat, oil and grease (FOG). Ideal co-substrates for microalgae are highly biodegradable carbon-rich substrates, which boost methane production without increasing the nitrogen load (Mata-Alvarez et al., 2014). Additionally, microalgae can also be used as co-substrate in biogas plants. For instance, (Schwede et al., 2013a) explored the possibility of substituting pig manure by microalgae as source of alkalinity, macro- and micronutrients in corn silage anaerobic digestion.

1.2 Scenarios of microalgae biorefineries coupling anaerobic co-digestion

So far, multiple microalgae anaerobic co-digestion mixtures and scenarios have been investigated, with different microalgae species and growing purposes (e.g. production of value-added products, wastewater treatment and nutrient removal). They have been co-digested with a wide range of co-substrates types and availability. Indeed, the main criteria for selecting a co-substrate has been the election of organic wastes produced in the same facility.

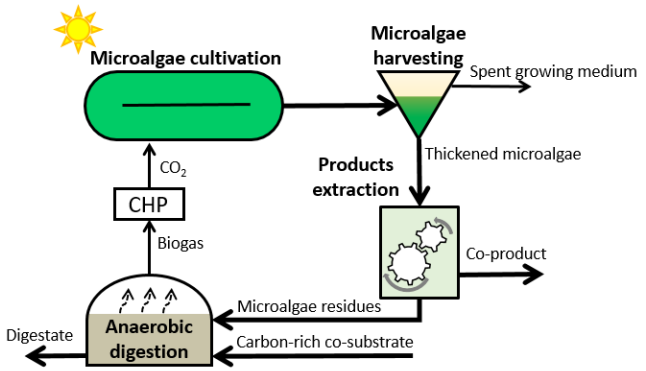
Several microalgae biorefinery scenarios incorporating anaerobic co-digestion may be found in the literature, including (Fig. 1.1):

- **Value-added product(s) biorefinery** (Fig. 1.1A): microalgae are cultivated to extract certain macromolecules (like lipids or proteins) and obtain value-added products (like pigments, omega-3,6). Microalgae residues are then co-digested with an external carbon-rich co-substrate (Astals et al., 2015; Parimi et al., 2015; Ramos-Suárez and Carreras, 2014). CO_2 from biogas combustion can be recycled for microalgae cultivation. In this case, biosecurity may restrict the use of AD supernatant for microalgae cultivation depending on the value-added product use.

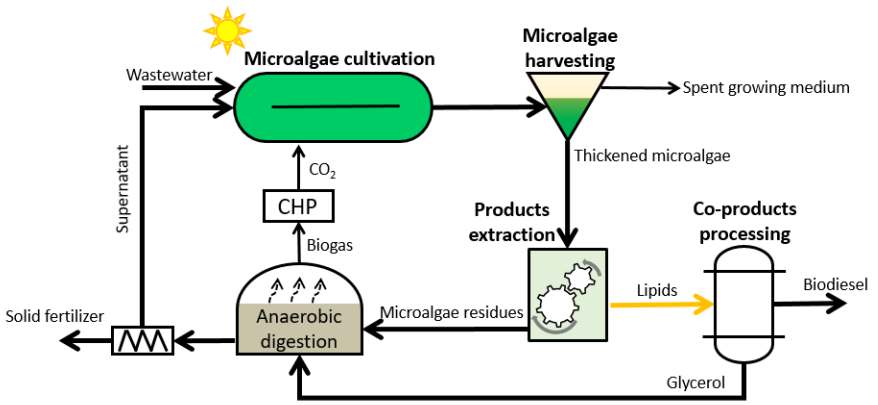
- **Biodiesel biorefinery** (Fig. 1.1B): lipid spent microalgae are co-digested with glycerol, a by-product of lipids transesterification for biodiesel production (Ehimen et al., 2009; Ehimen et al., 2011; Ramos-Suárez and Carreras, 2014; Santos-Ballardo et al., 2015). Anaerobic digestion supernatant and CO_2 from biogas combustion can be recycled for microalgae cultivation.

- **Secondary treatment in wastewater treatment plants** (Fig. 1.1C): a microalgal pond is used for municipal wastewater treatment and harvested.

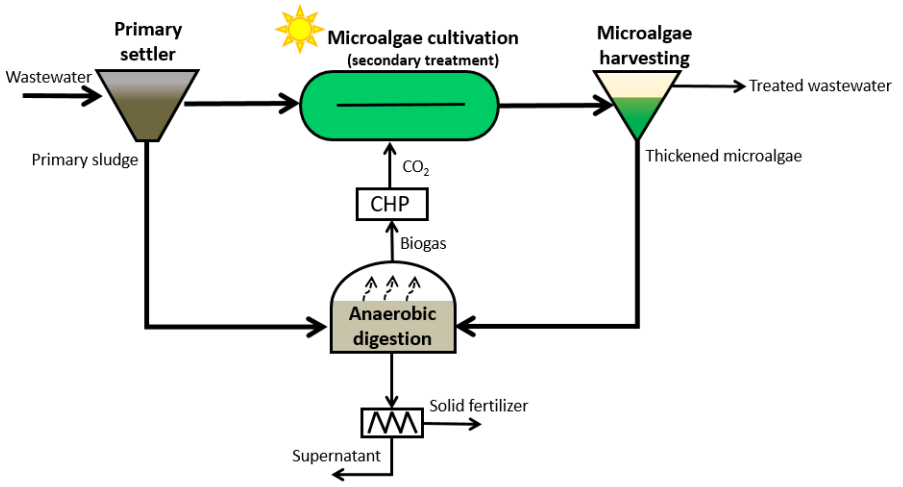
A


Microalgae cultivation
Microalgae harvesting
Spent growing medium
Thickened microalgae
Products extraction
CO_2
CHP
Biogas
Co-product
Microalgae residues
Digestate
Anaerobic digestion
Carbon-rich co-substrate

B


Microalgae cultivation
Microalgae harvesting
Wastewater
Spent growing medium
Thickened microalgae
Supernatant
CO_2
CHP
Products extraction
Co-products processing
Biogas
Lipids
Biodiesel
Solid fertilizer
Anaerobic digestion
Microalgae residues
Glycerol

C


Primary settler
Microalgae cultivation
(secondary treatment)
Microalgae harvesting
Wastewater
Treated wastewater
Primary sludge
Thickened microalgae
CO_2
CHP
Biogas
Anaerobic digestion
Solid fertilizer
Supernatant

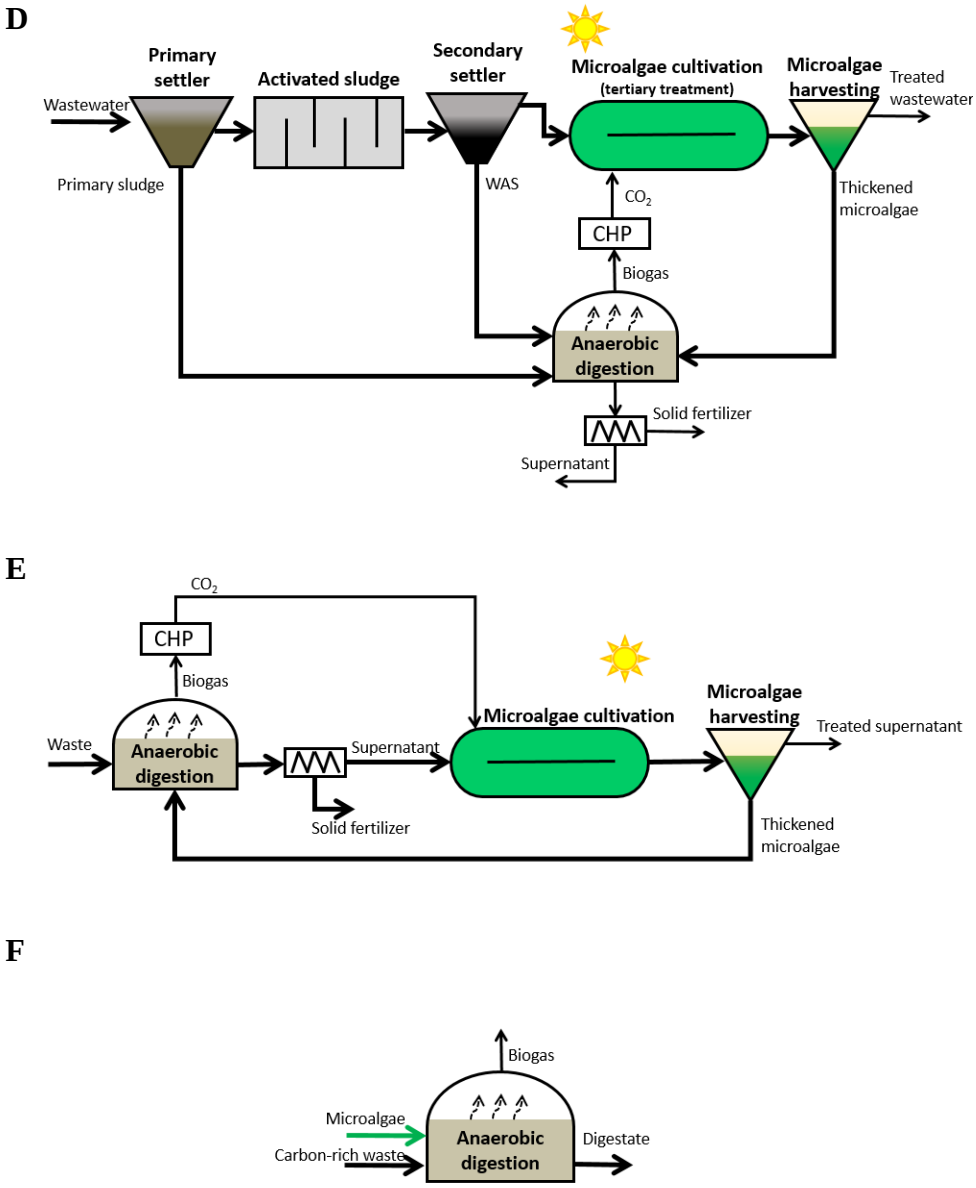


Figure 1-1 Most common scenario for microalgae anaerobic co-digestion: (A) high-value products biorefinery, (B) biodiesel biorefinery, (C) algae pond as secondary treatment in a wastewater treatment plant, (D) algae cultivation as tertiary treatment in a wastewater treatment plant, (E) algae pond to treat anaerobic digestion supernatant, and (F) addition of microalgae cultivated outside an existing facility

microalgae are co-digested with primary sludge (Hlavínek et al., 2016; Mahdy et al., 2015; Passos et al., 2017)

- **Tertiary treatment in wastewater treatment plants** (Fig. 1.1D): a microalgae photobioreactor follows the activated sludge unit to remove nutrients from the secondary effluent and improve the final effluent quality (Arias et al., 2018; Peng and Colosi, 2016; Yuan et al., 2012).

- **Anaerobic digestion supernatant treatment** (Fig. 1.1E): a microalgal pond is used to remove nutrients from the anaerobic digestion supernatant and harvested microalgae are used as co-substrate. This approach has been studied to decrease the nutrient content of the return stream in municipal wastewater treatment plants (WWTP)(Garoma and Nguyen, 2016; Hidaka et al., 2017; Olsson et al., 2014; Rusten and Sahu, 2011; Wang and Park, 2015; Yuan et al., 2012) or improve the effluent quality of animal manure anaerobic digesters (Astals et al., 2015; González-Fernández et al., 2011; Mahdy et al., 2017; Wang et al., 2016; Zhang et al., 2013).

- **Microalgae as co-substrate in biogas plants** (Fig. 1.1F): microalgae cultivated outside the biogas plant is used to improve digesters' performance (El-Mashad, 2013; Formagini et al., 2014; Schwede et al., 2013a). Moreover, microalgae taken from microalgae blooms (Miao et al., 2014; Zhao and Ruan, 2013; Zhong et al., 2013; Zhong et al., 2012) are added as co-substrate to anaerobic digesters in order to treat microalgae and reduce their environmental impact.

1.3 Co-digestion of microalgae and sewage sludge

Sewage sludge is the most studied co-substrate for microalgae AD so far. This may be explained by the amount of research dealing with the cultivation of microalgae in wastewater treatment plants, either as secondary treatment (Fig. 1.1C), tertiary treatment (Fig 1.1D) or anaerobic digestion supernatant treatment (Fig. 1.1E) (Hidaka et al., 2017; Peng and Colosi, 2016; Sahu et al., 2013). Indeed, microalgae ponds are a well-known technology for wastewater treatment (Craggs et al., 2014; Salerno et al., 2009), which eases the adoption of microalgae cultivation systems in WWTP.

On the one hand, the integration of microalgae cultivation as tertiary treatment and anaerobic digestion supernatant treatment aims at improving nutrients removal (N and P) from wastewater, while generating an additional co-substrate for sewage sludge (primary and waste activated sludge) AcoD. The cultivation of microalgae on anaerobic digestion supernatant has special interest since it has the potential to: (i) reduce the nutrient load of the return side-stream, which represents up to 20% of the WWTP nutrient load; (ii) mitigate greenhouse gases emissions by using CO_2 from biogas combustion/upgrading for microalgae growth and; (iii) generate significant amounts of microalgae as onsite co-substrate, which lowers the uncertainty about co-substrate availability and seasonality (Escalante et al., 2016; Mata-Alvarez et al., 2014). Nonetheless, the supernatant may need to be pretreated and/or diluted to reduce the presence of inhibitory compounds for microalgae growth and improve light transmittance (Muñoz and Guieysse, 2006; Sahu et al., 2013; Yuan et al., 2012). On the other hand, microalgae-based WWTPs, where microalgae ponds (i.e. high rate algal ponds (HRAPs)) are used as secondary treatment, stand as a low-energy wastewater treatment system for regions with sufficient surface area and solar radiation (Craggs et al., 2014; Passos et al., 2017). In HRAP, microalgae grow in symbiosis with heterotrophic bacteria responsible of organic matter biodegradation. Thus, harvested biomass consists of a mix community of microalgae, bacteria and protozoa forming flocs (Gutiérrez et al., 2016a). In this scenario, microalgae from the HRAP are co-digested with primary sludge from the primary treatment.

Microalgae and sewage sludge co-digestion is not a new concept, since the first published study dates from 1983, when Samson and LeDuy (1983) co-digested *Spirulina maxima* with three different wastes, including sewage sludge. However, the number of papers dealing with this topic has grown exponentially over the last few years alongside the growing interest on microalgal-derived biofuels. Most of these studies have been carried out using batch assays, so-called biochemical methane potential (BMP) tests, under mesophilic conditions (Table 1.1). Nevertheless, a few studies have researched the performance of this mixture in lab-scale continuous systems such as continuous stirred tank reactors (CSTR) (Table 1.2). The main differences between these studies lie in the microalgae strain, sewage sludge composition (primary and/or waste activated sludge) and the proportion of each co-substrate.

Most of the BMP-based studies analysed a wide range of proportions between both co-substrates. Mahdy et al. (2015), Neumann et al. (2015), Beltran et al., (2016), Garoma and Nguyen (2016) and Lee et al. (2017) tested the co-digestion of different microalgae species and WAS (25, 50 and 75 %). The same mixture range was tested by Mahdy et al. (2015) for primary sludge, by Olsson et al. (2014) and Caporgno et al. (2015) for sewage sludge and by Lu and Zhang (2016) for septic sludge. Exploring a wide range of proportions between microalgae and sludge is important since the production of microalgae shows a strong seasonality, and depends on the photobioreactor design and wastewater composition. For instance, (Passos et al., 2017) explored the feasibility of a microalgae-based wastewater treatment plant (similar to Fig. 1.1C) and calculated that the proportion between microalgal biomass and primary sludge would be around 30/70% and 60/40% (VS-basis) in winter and in summer, respectively. Similarly, Peng and Colosi (2016) performed a life cycle assessment on the implementation of a microalgae pond as tertiary treatment (similar to Fig. 1.1D) and estimated that proportion between microalgae and sewage sludge would be between 5/95% and 20/80% (VSS-basis). Therefore, mixtures where microalgae represent less than 50% may better represent WWTP scenarios. As detailed in Table 1, Yuan et al. (2012), Wang et al. (2013), Olsson et al. (2014) and Peng and Colosi (2016) focused on mixtures with low microalgae proportion. Finally, Wágner et al. (2016) studied the possibility of using bacterial biomass from an enhanced biological phosphorus removal system (similar to WAS) as bioflocculant for microalgae harvesting and subsequent anaerobic co-digestion. According to the authors, using 10g of bacterial biomass/ g of microalgae reduced the polymer dosing by 40%.

Although the methane yield of sewage sludge is affected by multiple factors, in general the WAS methane yield is similar to microalgae (around 180-320 mL CH_4/gVS) and significantly lower than primary sludge (~400 mLCH_4/gVS), which is a readily degradable organic matter. Also, BMP tests results show that the methane yield of microalgae and sludge (primary and/or WAS) AcoD is proportional to the amount of microalgae and sludge in the mixture. However, some authors have reported synergies (increased methane yield compared to the proportional one) of up to 25% when co-digesting microalgae and sewage sludge (Beltran et al., 2016; Wágner et al., 2016; Wang et al., 2013). Regarding synergisms, in most cases the improved methane yield is not significant if the methane yield uncertainty was taken into account Thorin et al. (2017). As far as full-scale plants are concerned, minor methane yield improvements due to synergisms would be masked by natural variations of the co-substrates load, composition and biodegradability.

Although microalgae and sludge co-digestion has primarily focused on the methane yield, the feasibility of the process is also linked to the kinetics of the AcoD limiting step (Bala and Satter, 1990; Gaddy et al., 1974). The anaerobic digestion of particulate substrates like microalgae is limited by the hydrolysis rate. The first-order constant rates range between 0.03 and 0.24 day^{-1} (average of 0.12 day^{-1}); which is at the lower end of the first-order constant rates reported for sewage sludge (Astals et al., 2013; Da Silva et al., 2018). With the exception of Wágner et al. (2016), publications comparing the degradation kinetics of microalgae and sewage sludge mono-digestion and co-digestion observed a 20 – 50% increase of the degradation kinetics under co-digestion conditions (Beltran et al., 2016; Lee et al., 2017; Neumann et al., 2015). The reasons behind the kinetics improvement under co-digestion conditions remain unexplored and call for further research, since they open the door at reducing the treatment time and reactor's size, alternatively improving the digestate stabilization. It should be noticed that BMP tests apparent degradation kinetics are partly influenced by the inoculum characteristics (De Vrieze et al., 2015; Koch et al., 2017). In this regard, Beltran et al. (2016), Lee et al. (2017) and Wágner et al. (2016) used digested sewage sludge as inoculum, while Neumann et al. (2015) used granular biomass from a UASB reactor. Digested sewage sludge is the inoculum recommended by Raposo et al. (2012) and Holliger et al. (2016) when adapted inoculum is not available. The correlation between the degradation kinetics observed in BMP tests and continuous reactors is a topic of current research and discussion within the anaerobic community.

Table 1-1 Summary of microalgae co-digestion with sewage sludge in BMP tests.

Microlgae	Co-substrate	Mixture ratio	T (°C)	Methane yield (mL CH_4/g VS)	Improvement[1] (%)	Reference
Chlorella sp.	WAS	41:59 (TS)	37	468	23	Wang et al., 2013
Chlorella sp. and *Scenedesmus* sp.	Sewage Sludge	37:63 (TS)	37	408	n.d.	Olsson et al., 2014
		12:88 (TS)	55	408	n.d.	
Isochrysis galbana	Sewage Sludge	25:75 (VS)	33	413[2]	- 8	Caporgno et al., 2015
Selenastrum capricornutum	Sewage Sludge	50:50 (VS)	33	392[2]	9	
Chlorella vulgaris	WAS	50:50 (COD)	35	90.6[3]	- 4	Mahdy et al., 2015
		75:25 (COD)		107.4[3]	6	
Chlorella vulgaris (pretreated[4])	Primary Sludge	50:50 (COD)	35	282.8[3]	16	
		75:25 (COD)		293.4[3]	13	
Lipid-spent *Botryococcus braunii*	WAS	75:25 (VS)	35	393	7	Neumann et al., 2015
Micractinium sp.	WAS	21:79 (VS)	37	236	0	Wang and Park, 2015
Chlorella sp.	WAS	21:79 (VS)	37	253	5	
Chlorella sorokiniana.	WAS	25:75 (VS)	37	442	26	Beltran et al., 2016
		50:50 (VS)		380	12	
		75:25 (VS)		354	1	
Scenedesmus quadricauda.	WAS	25:75 (VS)	35	172	- 14[4]	Garoma and Nguyen 2016

Microlgae	Co-substrate	Mixture ratio	T (°C)	Methane yield (mL CH_4/g VS)	Improvement[1] (%)	Reference
		49:51 (VS)		222	12[4]	
		76:24 (VS)		207	8[4]	
Chlorella sp.	Septic Sludge	50:50 (VS)	35	547[1]	84	Lu and Zhang, 2016
Chlorella sorokiniana. and *Scenedesmus* sp.	Anaerobic waste sludge[5]	9:91 (VS)	37	560	28	Wágner et al., 2016
	Aerobic waste sludge[5]	9:91 (VS)	37	400	11[4]	

[1] The methane yield improvement is calculated as the product summation of each substrate methane yield, considering the co-substrates proportion and their experimental methane yield in mono-digestion; [2] Expressed as mL biogas/g VS; [3] Expressed as mL CH_4/g COD; [4] 120 ºC for 40 min; [5] Statistically not significant; [6] From an enhanced biological phosphorous removal system (EBPR); [7] at 55 ºC for 8h. n.d. = not defined

Table 1-2. Summary of microalgae co-digestion with sewage sludge with lab-scale reactors.

Microalgae	Co-substrate	Mixture ratio	Operation	T (°C)	Working volume (L)	OLR (g VS /L·day)	HRT (days)	Methane Yield (m^3 CH_4/kg VS)	Reference
Spirulina maxima	Sewage sludge	50:50 (VS)	Continuous	35	1.5	3.9	20	0.36	Samson and LeDuy, 1983
Algal biommass[1]	Primary sludge	92.5:7.5 (VS)	Batch	36	22	9.6[2]	-	0.17[3]	Hlavínek et al., 2016
Scenedesmus sp.	Sewage sludge	25:75 (VS)	Semi-continuous[4]	35	0.45	0.5[5]	15	0.39[5]	Peng and Colosi, 2016
		50:50 (VS)						0.33[5]	
Algal biommass[1]	Sewage sludge	25:75 (VS)						0.51[5]	
		50:50 (VS)						0.44[5]	
Chlorella sp.	Sewage sludge	4-15:96-85 (v/v)	Continuous	55	10	n.d.	28	0.4	Hidaka et al., 2017

[1] Native algae collected from a WWTP; [2] Expressed as g VS/L; [3] Expressed as m^3 biogas/kg VS; [4] Feeding every 48 hours; [5] Expressed as VSS.
n.d. = not defined

Despite the higher methane production, the implementation of anaerobic co-digestion in a WWTP has a direct impact on other key factors, such as the supernatant nutrient content, digestate dewaterability, biosolids quality and biogas composition (e.g. H_2S); all of them directly affecting the WWTP economic and environmental impacts (Arnell et al., 2016; Puyol et al., 2016). The impact of a co-substrate on digestate dewaterability, biosolids stability and amount of biosolids to be handled are of particular importance, since they affect the volume of biosolids to be transported outside the WWTP, as well as the digestate management opportunities (Jensen et al., 2014; Yuan et al., 2012).

Regarding the digestate dewaterability, Yuan et al. (2012) reported that co-digesting 5 and 15% of *Spirulina platensis* with WAS improved the digestate dewaterability when compared to WAS alone. Nonetheless, in the same study, the digestate dewaterability was worsened when 5 and 15% of *Chlorella* sp. were co-digested with WAS (Yuan et al., 2012). Conversely, Wang et al. (2013) reported that the anaerobic co-digestion of *Chlorella* sp. and WAS improved the digestate dewaterability at low *Chlorella* sp. proportions (4 and 11% on a TS basis), but worsened it at higher proportions (41% *Chlorella* sp.). However, these results should be carefully interpreted since the dewaterability was measured on digestates obtained from BMP tests. In a BMP test, the properties of the digestate are mostly controlled by the inoculum properties rather the added co-substrates properties (Astals et al., 2015; Herrmann et al., 2016). Moreover, all previous studies evaluated digestate dewaterability by determining the capillarity suction time (CST), likely due to its simplicity and affordability. However, the CST is a proxy parameter for dewaterability, since it does not resemble the actual dewatering process and it fails to predict the solids concentration of dewatered cake (To et al., 2016). Future research should complement CST with other dewaterability methods such as thermo-gravimetric (Kopp and Dichtl, 2001), filtration-centrifugation (Higgins et al., 2014) and/or rheology analysis (Örmeci, 2007; Ruiz-Hernando et al., 2015; To et al., 2016).

Finally, the circular economy paradigm, along with the cradle-to-cradle concept, call for production systems where wastes become by-products (Puyol et al., 2016). Therefore, beyond biogas production, AD plants need to find suitable management and disposal solutions for the digestate to enhance AD plants feasibility (Alburquerque et al., 2012; Astals et al., 2012). Agricultural reuse is regarded as the best option to recycle the nutrients contained in the digestate (Alburquerque et al., 2012; Mata-Alvarez et al., 2014). However, this can only be done when the digestate

quality fulfils the legal quality requirements. To the best of our knowledge, the suitability of microalgae digestate for agricultural reuse has yet to be determined (i.e. concentration of nutrients, heavy metals, pathogens, phytotoxicity and organic matter stability assessment).

1.4 Co-digestion of microalgae and animal manure

Animal manure (i.e. pig, cattle, and poultry) and microalgae co-digestion has received less attention than other substrates, like sewage sludge. However, although the relatively low C/N ratio of both substrates, which increases the risk of ammonia inhibition, the possibility of recovering nutrients, improving the effluent quality and producing an onsite co-substrate through microalgae cultivation makes manure and microalgae co-digestion worth investigating. Even more when Mahdy et al. (2017), who co-digested *Chlorella vulgaris* and cattle manure, showed that anaerobic biomass could be acclimated to tolerate free ammonia and total ammonical nitrogen (TAN) concentrations up to 650 mgNH_3-N/L and 3.8 gTAN/L, respectively.

Most of the animal manure and microalgae co-digestion research has been carried out in BMP tests, pig manure being the most studied (Table 1.3). The BMP test is a suitable analytical method to understand the interaction between substrates occurring during co-digestion. However, a BMP test is not the most indicated method to assess the impact of inhibitors (e.g. free ammonia), since they get diluted by the inoculum (Astals et al., 2015; Herrmann et al., 2016). Regarding the co-substrates interaction, González-Fernández et al. (2011), Astals et al. (2015) and Wang et al. (2016) observed that co-digesting microalgae with pig manure increased microalgae anaerobic biodegradability to different extents. An improvement of the methane yield (compared to the proportional one) was also obtained by Prajapati et al. (2014) and Mahdy et al. (2017) when co-digesting microalgae and cattle manure, and by Meneses-Reyes et al. (2017) and Li et al. (2017) when co-digesting microalgae and poultry manure (Table 1.3). Prajapati et al. (2014) and Mahdy et al. (2017) attributed the synergic effect to the improved C/N ratio, while Li et al. (2017) attributed it to the N/P ratio. Although the C/N ratio is the most reported parameter to explain the synergies occurring during anaerobic co-digestion, synergism could not always be linked to the C/N ratio (Astals et al., 2015; González-Fernández et al., 2011; Meneses-Reyes et al., 2017). In this regard, Astals et al. (2015) hypothesized that synergism was due to the addition of specific microbes from pig manure, since other factors previously used to explain co-

Table 1-3. Summary of microalgae co-digestion with animal manure

Microalgae	Co-substrate	AD operation	Mixture ratio	T (°C)	Methane yield (mL CH_4/g VS)	Improve-ment[1] (%)	Reference
Chlorella vulgaris and *Scenedesmus obliquus*	Swine manure	BMP	50:50 (COD)	35	220	15	González-Fernández et al., 2011
Chroococcus sp.	Cattle dung	BMP	1:1 (VS)	36	292	70	Prajapati et al., 2014
Scenedesmus sp.	Swine manure	BMP	30:70 (VS)	37	n.d.	n.d.	Astals et al., 2015
Chlorella sp.	Swine manure	BMP	6:94 (VS)	35	348	11	Wang et al., 2016
		CSTR	10:90 (VS)	35	190	0[2]	
Chlorella 1067	Chicken manure	BMP	20:80 (VS)	35	239	31	Li et al., 2017
Chlorella vulgaris (pretreated [3])	Cattle manure	BMP	80:20 (VS)	55	431	10	Mahdy et al., 2017
		Continuous	80:20 (VS)	37	351	n.d.	
Chlorella vulgaris	Chicken litter and glycerol	BMP	30:3:67 (TS)	37	131	15.7[4]	Meneses-Reyes et al., 2017

[1] The methane yield improvement is calculated as the product summation of each substrate methane yield, considering the co-substrates proportion and their experimental methane yield in mono-digestion; [2] No significant differences were observed when compared to swine manure mono-digestion; [3] Enzymatic pretreatment based on protease; [4] Compared to mono-digestion of chicken litter. n.d. = not defined

digestion synergisms (e.g. micro- and macronutrients, C/N ratio, ammonia inhibition, alkalinity) were unlikely to occur under the trialed experimental conditions. The impact of incoming microbes (microbes arriving with the substrate) on anaerobic (co-) digestion microbial community and performance is a topic of current discussion and research.

Due to BMP tests limitations, continuous experiments are required to better assess the benefits and constraints of co-digesting microalgae and animal manure. However, to the best of our knowledge, only two research studies have reported the operation of continuous anaerobic digesters co-treating microalgae and animal manure, both of them under mesophilic conditions (Table 1.3). Specifically, Wang et al. (2016) co-digested *Chlorella* sp. and pig manure (10/90% VS-basis) at a HRT of 21 days and an OLR around 1.4 gVS/(L_r·day), while Mahdy et al. (2017) co-digested *Chlorella vulgaris* and cattle manure (80/20% VS-basis) at a HRT of 23 days an OLR of 2.1 gVS/(L_r·day). These differences on manure source and OLR resulted in quite different pH, TAN and NH_3 concentrations. However, both studies showed that co-digesting *Chlorella* sp. with manure was technically feasible and that the digester methane yield was not significantly affected by the addition of a co-substrate when compared to the control reactor.

1.5 Co-digestion of microalgae and agro-industrial products and wastes

Agro-industrial waste streams are characterized by a high C/N ratio, which can lead to AD performance issues primarily associated with poor alkalinity and/or deficit of macro- and micro-nutrients (Romero-Güiza et al., 2016; Schwede et al., 2013a). Therefore, co-digesting microalgae with agro-industrial wastes has been suggested as an option to overcome mono-digestion limitations (Fernández-Rodríguez et al., 2014; Mata-Alvarez et al., 2014). Another advantage is that microalgae can be cultivated using marginal soil in rural areas where other suitable co-substrates are not available (Neumann et al., 2015; Schwede et al., 2013a). Conversely, agro-industrial wastes can be used as co-substrates in microalgae digesters, in order to increase the OLR and methane yield without increasing (or even diluting) the nitrogen concentration.

Microalgae have been co-digested with a wide range of agro-industrial wastes and products, including crops wastes (e.g. corn silage, corn stover, wheat straw), energy crops (e.g. switchgrass, *Opuntia maxima*), waste paper/sludge, olive mill waste, fat oil and grease (FOG) and glycerol. Most of the studies focused on improving the AD performance by balancing the C/N ratio, since agro-industrial wastes present relatively high C/N ratios (>45), while microalgae present relatively low C/N ratios (< 12). Table 1.4 and 1.5 summarize the studies co-digesting microalgae and agro-industrial waste in BMP tests and in continuous reactors, respectively.

Fig. 1.2 illustrates the improvement of the methane yield depending on the C/N ratio for a wide range of microalgae and agro-industrial wastes co-digestion. The methane yield improvement is obtained by comparing the experimental co-digestion value with the theoretical one, based on the experimental mono-digestion values. The latter is calculated considering the co-substrates proportion and their experimental methane yield in mono-digestion (Beltran et al., 2016; Zhen et al., 2016). Positive values (>10%) indicate synergism (i.e. the mixture produces more methane than expected), while negative values (<10%) indicate antagonism (i.e. the mixture

produces less methane than expected). Values between -10% and 10% are considered neutral (neither synergistic nor antagonistic) in order to account for the uncertainty around measured methane yields and the propagation of multifarious analytical errors.

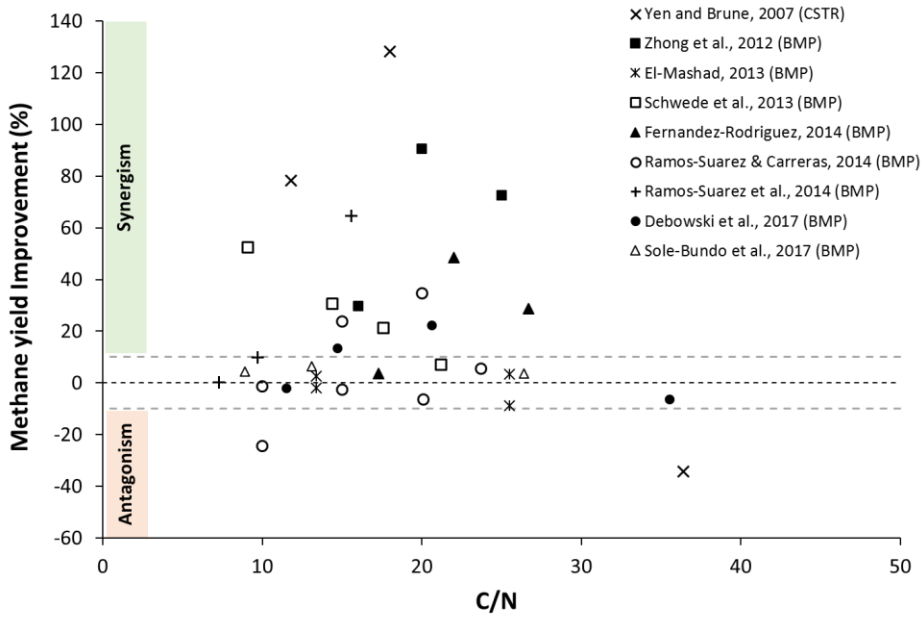


Figure 1-2 Methane yield improvement vs. C/N ratio during microalgae and agro-industrial waste co-digestion.

As shown in Fig.1.2, most studies target mixtures with C/N ratios ranging between 15 and 30, which falls into the optimum range for successful AD performance (Ehimen et al., 2011; Fernández-Rodríguez et al., 2014). However, both neutral responses and synergisms are observed within this C/N range. Given the variability of methane yield improvements for a given C/N ratio, it is clear that optimizing the co-substrate dosage based on the C/N ratio is an oversimplification. The C/N ratio is a proxy for macronutrients availability, ammoniacal nitrogen concentration and/or system alkalinity. However, it does not consider other important factors such as substrate biodegradability, secondary risk of inhibition and micronutrients. Thus, the long legacy of using the C/N ratio as key factor to explain the synergisms and antagonisms occurring during anaerobic co-digestion has caused an overlook of the actual mechanisms behind such phenomena.

Table 1-4. Summary of microalgae co-digestion with agro-industrial wastes in BMP tests.

Microalgae	Co-substrate	Mixture ratio	C/N	T (°C)	Methane yield (L CH_4/kg VS)	Improvement[1] (%)	Reference
Chlorella (Biodiesel production waste)	Glycerol	67:3 (v/v)	>15	37	267 [2]	4-7	Ehimen et al., 2009
Taihu blue algae	Corn straw	n.d.	20	35	325	62	Zhong et al., 2012
Spirulina platensis	switchgrass	33:67 (VS)	13	35	198	- 2	El-Mashad, 2013
		33:67 (VS)	13	50	236	3	
Nannochloropsis salina	Corn silage	14:86 (v/v)	21	37	660 [3]	15	Schwede et al., 2013a
	Corn cob mix	25:75 (v/v)	18		610 [3]	17.6	
Dunaliella salina	Olive mill solid waste	50:50 (VS)	22	35	285	48	Fernández-Rodríguez et al., 2014
Scenedesmus sp	Opuntia maxima	25:75 (VS)	16	37	234	65	Ramos-Suárez et al., 2014a
Scenedesmus sp extracted aminoacid biomass	Paper sludge	74:26 (VS)	20	37	173	35	Ramos-Suárez and Carreras, 2014
Scenedesmus sp	Opuntia maxima	10:90 (VS)	24	37	166	7	
Scenedesmus sp extracted lipid biomass	Residual glycerine	88.9:11.1 (VS)	7	37	255	n.d.	

Microalgae	Co-substrate	Mixture ratio	C/N	T (°C)	Methane yield (L CH_4/kg VS)	Improvement[1] (%)	Reference
Spirulina platensis	Pretreated [4] switchgrass	50:50 (TS)	n.d.	50	354	10	El-Mashad, 2015
B. braunii	Glycerol	90:10 (VS)	n.d.	37	430	9	Neumann et al., 2015
Nannochloropsis gaditana extracted lipid biomass	Cellulose	50:50 (VS)	11	37	286	n.d.	Barontini et al., 2016
Hydrolized algae residues	Corn stover	50:50 (VS)	46	35	186	n.d.	Yue et al., 2016
Chlorella sp. and *Scenedesmus* sp.	*Sida hermaphrodita*	40:60 (VS)	21	35	352	56	Dębowski et al., 2017

[1] The methane yield improvement is calculated as the product summation of each substrate methane yield, considering the co-substrates proportion and their experimental methane yield in mono-digestion; [2] Expressed in TS basis; [3] Expressed as mL biogas/g VS; [4] 1% (w/v) NaOH at 50 ºC for 12h. n.d. = not defined

Table 1-5. Summary AcoD of microalgae biomass and agro-industrial wastes under mesophilic continuous operation.

Microalgae	Co-substrate	Mixture ratio	C/N	OLR (g VS /L·day)	HRT (day)	Methane Yield (mL CH_4/g VS)	Reference
Mixed *Scenedesmus* sp. and *Chlorella* sp.	Waste paper	n.d.	7	1	10	0.90	Yen and Brune, 2007
		n.d.	13	2	10	0.24	
		n.d.	18	3	10	0.28	
		50:50 (VS)	23	4	10	0.32	
		n.d.	27	5	10	0.14	
Lipid extracted *Nannochloropsis salina* waste	Oil waste	33:67 (VS)	13	2	40	0.45	Park and Li, 2012
				4	20	0.12	
				6	13	> 0.1	
Lipid extracted *Nannochloropsis salina* waste	Oil waste	50:50 (VS)	11	2	40	0.40	
				4	20	0.54	
				6	13	> 0.1	
Lipid extracted *Nannochloropsis salina* waste	Oil waste	67:33 (VS)	8	2	40	0.38	
				4	20	0.28	

Microalgae	Co-substrate	Mixture ratio	C/N	OLR (g VS /L·day)	HRT (day)	Methane Yield (mL CH_4/g VS)	Reference
				6	13	> 0.1	
Nannochloropsis salina	Corn silage	14:86 (v/v)	31	2	n.d.	1.0-1.5 [2]	Schwede et al., 2013a
Nannochloropsis salina (pretreated [1])	Corn silage			2	n.d.	1.5-1.8 [2]	
				4	n.d.	1.8-2.0 [2]	
				5	n.d.	2.2-fail [2]	
Scenedesmus sp	*Opuntia maxima*	25:75 (VS)	16	2	30	0.21	Ramos-Suárez et al., 2014a
				4	15	0.29	
				6	10	0.20	
Scenedesmus sp	*Opuntia maxima*	25:75 (VS)	16	2	40	0.32	
				4	20	0.30	
				5	15	0.31	
				7	12	0.28	

[1] 120ºC for 2h; [2] Expressed as m^3 biogas/m_r^3·day; [3] 10% CaO (TS) at 75 ºC for 24h. n.d. = not defined

In relation to this, Yen and Brune (2007) observed that adding NH_4Cl to decrease the waste paper C/N ratio from 2000 to 21.5 was not enough to explain the synergism occurring during microalgae and waste paper co-digestion (Fig. 1.2). The authors hypothesized that microalgae improved waste paper anaerobic digestion by balancing the C/N ratio and providing a range of essential micronutrients. Herrmann et al. (2016) co-digested *Spirulina platensis* with three distinct carbon-rich substrates (i.e. barley straw, beet silage and brown seaweed) in a separate CSTR each. They also observed that the C/N ratio should not be the only parameter to consider when optimizing co-digestion mixtures. Besides the digester treating only *Spirulina platensis*, the other three CSTRs were fed with the co-digestion mixture that provided a C/N ratio of 25 (i.e. 15% barley straw, 45% beet silage and 55% brown seaweed on a VS-basis). Herrmann et al. (2016) reported that the reactor digesting *Spirulina platensis* was inhibited (substantial decrease of the methane yield) when the OLR increased from 1 to 2 gVS/(L_r·day), whereas the CSTRs co-digesting barley straw, beet silage and brown seaweed were inhibited when the OLR was subsequently increased to 3, 4 and 5 gVS/(L_r·day), respectively. As the maximum OLR for stable AD operation increased together with the co-substrate proportion, Herrmann et al. (2016) that the difference in performance was linked to the occurrence of ammonia inhibition rather than the C/N ratio itself.

Synergisms associated to microalgae anaerobic co-digestion have also been linked to other parameters more difficult to quantify and monitor than the C/N ratio ot the macronutrients availability. For instance, (Schwede et al., 2013a) claimed that the micronutrients (i.e. Co, Mo, Ni, Na) supplemented by *Nannochloropsis salina* were one of the key factors preventing digestion failure when the OLR was increased to 4.7 gVS/(L_r·day). Indeed, micronutrients (e.g. Co, Mo, Fe, Ni and Se) are well-known cofactors in numerous enzymatic reactions involved in the biochemistry of methane formation (Romero-Güiza et al., 2016; Schattauer et al., 2011). Yen and Brune (2007) results may also indicate that the observed increase in cellulase activity (enzyme that catalyzes cellulose hydrolysis) was partly related to the supplementation of micronutrients by microalgae. However, Zhong et al. (2013) did not observe an improvement of cellulose activity when *Microcystis* sp. was co-digested with corn straw, as cellulase activity decreased as the corn straw proportion in the mixture decreased. The role of micronutrients and enzymes activity on anaerobic (co-) digestion performance is a research topic that warrants further investigation.

Although most studies have emphasized possible synergisms between substrates, more attention should be given to inhibition/antagonism phenomena occurring during anaerobic co-digestion, since they are clear indicators of constraints associated to the co-digestion of a particular co-substrate. In practice, co-substrate selection and dose are primarily controlled by the availability and occurrence of secondary inhibition phenomena (e.g. salinity, heavy metals, ammoniacal nitrogen, volatile fatty acids (VFA), long chain fatty acid (LCFA), biogas H_2S concentration) (Arnell et al., 2016; Chen et al., 2008; Long et al., 2012; Mata-Alvarez et al., 2014; Nghiem et al., 2017; Rodriguez-Verde et al., 2018; Xie et al., 2016). For instance, the addition of microalgae into a digester could increase the heavy metals concentration in the digestion media, which may not only impact the AD performance, but also the possibility of reusing the digestate on land (Ramos-Suárez and Carreras, 2014). In the same way, the addition of microalgae grown on brackish or brine water can increase the concentration of Na^+ and other cations (e.g. Ca^{2+}, K^+ and Mg^{2+}) in the digestion media, all of them well-known inhibitors of the AD process. Na^+ and K^+ concentrations may also be increased when crude glycerol, a by-product of biodiesel production, is used as co-substrate in a microalgae digester; although the main limitation when using crude glycerol as co-substrate is linked to the accumulation of propionate (Jensen et al., 2014). Similarly, the risk of LCFA inhibition limits the dose of FOG as co-substrate (Long et al., 2012; Mata-Alvarez et al., 2014; Park and Li, 2012). Finally, it is worth highlighting that antagonisms occurring during co-digestion are more difficult to detect and quantify than synergisms. This is because (i) the impact of inhibitors and intermediate metabolites in BMP testing is diluted, and (ii) long operation time and a certain co-substrate loading rate may be required prior an inhibitor reaches its inhibitory concentrations

1.6 The role of the pretreatments and product extraction on the microalgae co-digestion

The pretreatment of microalgae has been largely investigated since microalgae low anaerobic biodegradability (extent and rate) is one of the major bottlenecks of microalgae anaerobic digestion (Carrere et al., 2016; Jankowska et al., 2017; Passos et al., 2014b). Microalgae pretreatment prior to its anaerobic co-digestion has also been used to improve both microalgae biodegradation (hydrolysis) rate and methane yield (Mahdy et al., 2017, 2015; Schwede et al., 2013a). Therefore, some references can be found combining the application of a pretreatment prior to microalgae co-digestion (Astals et al., 2015; Schwede et al., 2013a). Regarding the effect of the pretreatment on the anaerobic co-digestion, these studies revealed that the synergies due to co-digestion were less significant when combined with a pretreatment. This is mainly attributed to the fact that the pretreatment itself significantly accelerates the kinetics of the process, so the effects of the co-digestion were less discernible than for untreated substrates (Astals et al., 2015). And the other way around, the pretreatment effect is less evident upon microalgae co-digestion than mono-digestion. Even if the pretreatment is successful, depending on the energy consumption, the energy balance may not always be positive, i.e. the pretreatment may require more resources than those recovered from the additional methane production (Passos et al., 2014b). In this case, it would not be worth pretreating the biomass. Microalgae pretreatment (out of the scope of this literature review) has been extensively reviewed by Passos et al. (2014) and Jankowska et al. (2017).

A more suitable approach may be to pretreat microalgae as a necessary step to recover value-added compounds (e.g. lipids, proteins, antioxidants, pigments) and biodegrade microalgae residues trough anaerobic digestion (Milledge and Heaven, 2014; Safi et al., 2014a). Interestingly, several authors have reported that the methane yield of microalgae residues is between 20 and 100% higher than the methane yield of raw microalgae (Astals et al., 2011; Barontini et al., 2016; Keymer et al., 2013; Mahdy et al., 2015; Prajapati et al., 2014a; Ramos-Suárez and Carreras, 2014). This is mainly because extraction step behaves as a pretreatment. However, as highlighted by Astals

et al. (2015), the recovery of value-added products will reduce the amount of microalgae diverted to AD and, consequently, methane yields cannot be used to directly compare the amount of methane that will be produced in each scenario. Finally, a factor that is not always taken into account is that microalgae pretreatment also increases microalgae hydrolysis rate, which further contributes improving the methane yield of a continuous AD system.

The anaerobic co-digestion of microalgae residues after lipid and/or protein extraction with a range of co-substrates is discussed in the following subsections.

1.6.1 Co-digestion of lipid-spent microalgae with glycerol and other co-substrates

The anaerobic co-digestion of lipid-spent microalgae and glycerol (by-product of biodiesel production) has been investigated by several researchers (Ehimen et al., 2009, 2011; Neumann et al., 2015; Ramos-Suárez and Carreras, 2014; Santos-Ballardo et al., 2015). The integration of biodiesel production from microalgal lipids and the anaerobic co-digestion of by-products is a biorefinery approach that aims at making the process more economically feasible by (i) maximising the energy recovery from microalgae; (ii) reducing the amount of residues to be managed; and (iii) reusing the nutrients released during the AD and the CO_2 from biogas combustion for microalgae cultivation (Fig. 1.1B).

Ehimen et al. (2009), who produced biodiesel from *Chlorella* sp. (oil fraction of 27%) using both conventional (via solvent extraction) and in-situ transesterification, calculated a maximum yield of 0.028 g of glycerol per g (dry) of *Chlorella* sp. or 0.038 g of glycerol per g (dry) of lipid-spent *Chlorella* sp. The co-digestion BMP tests carried out using this relative quantity showed that glycerol addition increased the methane yield by 4% and 7% when co-digested with in-situ and conventional lipid-spent microalgae, respectively. These values are in agreement with those obtained when the experimental methane yield of the in-situ (0.27 L CH_4/g TS) and conventional (0.22 L CH_4/g TS) lipid-spent microalgae are combined with the glycerol theoretical methane yield (0.426 L CH_4/g TS). Combining the glycerol maximum yield (0.038 g of glycerol per g (dry) of lipid-spent *Chlorella sp.*) with a hypothesised volatile-to-total solids (VS/TS) ratio of 0.8 for the lipid-spent *Chlorella* sp., it is shown that the addition of glycerol would only represent a ~5% increase of the digester OLR (VS-basis). In a subsequent study, Ehimen et al. (2011) evaluated the feasibility of co-

digesting lipid-spent *Chlorella* sp. with glycerol in continuous digesters under several treatment conditions (i.e. HRT, OLR, C/N ratio and temperature). The addition of glycerol to increase the C/N ratio from 5.4 (mono-digestion) up to 12.4 improved the methane yield from 0.19 to 0.30 L CH_4/g VS. However, when the glycerol dose was further increased to reach a C/N of 24.2, there was a reduction of the methane yield linked to the accumulation of VFA. It is worth highlighting that the amount of glycerol needed to increase the C/N ratio from 5.4 to 12.4 is much higher than the glycerol generated from microalgal lipids transesterification, being the literature average 0.03 g of glycerol per g (dry) of lipid-spent microalgae.

The results obtained by Ramos-Suárez and Carreras (2014) co-digesting lipid-spent *Scenedesmus* sp. with crude glycerol showed the same trend as Ehimen et al. (2009, 2011). On the one hand, the methane yield of the mixture with the relative proportion between lipid-spent microalgae and glycerol (0.0235 g of glycerol per g (VS) of lipid-spent *Scenedesmus* sp.) did not show any significant difference compared to the methane yield of lipid-spent microalgae alone. This is likely due to the small amount of glycerol in the mixture. On the other hand, larger amounts of glycerol (11% VS-basis) were able to increase the methane yield; but when the glycerol concentration was further increased (29% VS-basis) the test showed clear signs of inhibition. From Ehimen et al. (2011) and Ramos-Suárez and Carreras (2014) results, it can be concluded that a lipid-spent microalgae digester was capable of accepting all crude glycerol produced during the biodiesel production and still showed capacity to accept other suitable co-substrates. This organic and volumetric loading spare capacity could be used to digest other waste and further improve the biorefinery economic feasibility.

Besides glycerol, lipid-spent microalgae have been co-digested with other carbon-rich wastes such as FOG (Park and Li, 2012), food waste leachate (Yun et al., 2016), and cellulose Barontini et al. (2016). Also, lipid-spent microalgae have been co-digested with nitrogen-rich co-substrates, such as waste activated sludge (Neumann et al., 2015), pig manure (Astals et al., 2015) and poultry litter (Meneses-Reyes et al., 2017). Most of these studies have been carried out using BMP tests and results already showed that the co-digestion of lipid-spent microalgae with nitrogen-rich wastes was not antagonistic. Therefore, the co-substrate loading rate and subsequent methane production improvement will depend on the (i) AD plant capacity, (ii) co-substrate availability and biodegradability, (iii) secondary inhibitors, and (iv) the impact of the co-substrates on supernatant and digestate quality. However, as previously discussed,

most of these factors can only by reliably evaluated in continuous experiments. Park and Li (2012), who operated the continuous co-digestion of lipid-spent *Nannochloropsis salina* and FOG, observed that the addition of FOG allowed to increase the OLR from 2 to 3 gVS/(L_r·day) whereas the control reactor (microalgae residues only) was inhibited when the same OLR change occurred; likely due to ammonia inhibition. The co-digester was inhibited when the OLR was subsequently increased to 4 gVS/(L_r·day); likely due to LCFA inhibition. Park and Li (2012) results showed that there was a clear synergy between *Nannochloropsis salina* and FOG since microalgae provided alkalinity and nutrients while FOG boosted the methane production and diluted ammonia concentration. However, Park and Li (2012) results also showed that there was a risk associated with the addition of a co-substrate, particularly when a certain threshold is surpassed. The benefits and constraints of using FOG as co-substrate have already been discussed by Long et al. (2012) and Mata-Alvarez et al. (2014).

1.6.2 Co-digestion of protein-spent microalgae

The anaerobic co-digestion of protein-spent microalgae has received less attention than the co-digestion of lipid-spent microalgae. This is likely due to (i) the past few years' interest on the production of microalgal-derived biodiesel (Andersson et al., 2014; Ward et al., 2014) and (ii) the lower production costs and higher nutritional value obtained when the whole microalgal biomass is used as feed source (Bleakley and Hayes, 2017; Hayes et al., 2017). However, protein hydrolyzates have several applications in the food and drink industry (e.g. sport drinks) and the fermentation industry (Ramos-Suárez and Carreras, 2014). Additionally, the extraction of proteins would reduce the risk of ammonia inhibition associated with microalgae anaerobic digestion.

To the best of our knowledge, only Ramos-Suárez and Carreras (2014) and Astals et al. (2015) have studied the anaerobic co-digestion of protein-spent microalgae. Ramos-Suárez and Carreras (2014) co-digested protein-spent microalgae with paper sludge and *Opuntia maxima*; while Astals et al. (2015) co-digested protein-spent microalgae with pig manure. Although both studies used *Scenedesmus* sp., the method used to release the protein was different since Ramos-Suárez and Carreras (2014) used an enzymatic pretreatment and Astals et al. (2015) used free nitrous acid (chemical pretreatment). Both studies observed that the extraction of protein

significantly increased microalgae's methane yield from 140 to 273 $mLCH_4/gVS$ (Ramos-Suárez and Carreras, 2014) and from 163 to 222 $mLCH_4/gVS$ (Astals et al., 2015). However, Astals et al. (2015) also showed that protein extraction reduced by 54% the amount microalgae diverted to anaerobic digestion, while lipid extraction only reduced it by 14%. Since microalgae typically have a larger proportion of protein than lipid (González-González et al., 2018), the need to implement anaerobic co-digestion in order to reach an OLR that makes an AD plant economically feasible is even more important when protein-spent microalgae is used.

2 OBJECTIVES AND OUTLINE OF THE THESIS

2.1 Main objective and outline of the thesis

2.1.1 Main objective

In view of the state of the art, microalgae anaerobic digestion presents several limitations that can be overcome. Thus, this PhD thesis aims to assess possible strategies to improve microalgae anaerobic digestion in wastewater treatment systems. To this end, co-digestion of microalgae with appropriate substrates is preferentially investigated. Moreover, the combination of the co-digestion with the application of pretreatments to microalgae or to both co-substrates before their co-digestion is also evaluated. The selection of the pretreatment is according to each co-substrate properties. Regarding the co-digestion, wastes produced in wastewater treatment plants (WWTP) (i.e. primary sludge and waste activated sludge) are preferred. Complementary, co-digestion with storable agricultural wastes (i.e. wheat straw) is also assessed.

2.1.2 Outline of the thesis

Among the seven chapters presented in this document, the experimental part of the thesis covers Chapters 3 and 4, which address the co-digestion of microalgae with WWTPs byproducts (primary sludge and waste activated sludge), and Chapter 5, which is focused on the co-digestion of microalgae with agro-industrial wastes (wheat straw).

Chapter 3 is dedicated to the co-digestion of microalgae with primary sludge. In all the experiments in this chapter, microalgae were harvested from high rate algal ponds (HRAP) used as secondary treatment for urban wastewater. In this context, primary sludge is another waste produced together with microalgae during the same process. But there is still a lack of knowledge on their co-digestion, especially in continuous reactors. Thus, the co-digestion of both microalgae and primary sludge was investigated in Section 3.1. To achieve higher microalgae biodegradability levels, the co-digestion was first investigated after applying a thermal pretreatment to microalgae. The optimal pretreatment conditions were selected following the

recommendations of a previous PhD thesis (Passos, 2014). However, results in Section 3.1 concluded that the anaerobic co-digestion of microalgae with primary sludge could be successful even without applying a pretreatment. Then, co-digestion of untreated microalgae with primary sludge was further investigated. It also comprised the study of the occurrence of some emerging organic contaminants in microalgae and primary sludge and their removal during their anaerobic (co-) digestion. Finally, this chapter also approached the possible reuse of microalgae digestates for agricultural purposes. To this end, an extended characterization of digestate properties (from microalgae anaerobic digestion and in co-digestion with primary sludge) was performed (Section 3.3).

Then, Chapter 4 addresses the co-digestion of microalgae when used as tertiary treatment in WWTPs. In such a case, microalgae came from a closed photobioreactor that treated secondary effluent and were co-digested with WAS. As a novelty, their co-digestion was investigated after applying a simultaneous autohydrolysis pretreatment, given WAS characteristics, to improve microalgae biodegradability (Section 4.1).

Chapter 5 is focused on the co-digestion of microalgae with agricultural wastes (i.e. wheat straw). Wheat is a widespread crop which straw is a storable carbon-rich waste and has potential as microalgae co-substrate. However, its lignocellulosic composition limits the hydrolysis step during its anaerobic digestion. Thus, the use of a pretreatment before the anaerobic digestion is recommended. In this context, microalgae and wheat straw could simultaneously be pretreated before their co-digestion. To optimize the process, an alkaline pretreatment with lime (CaO) was first evaluated on microalgae (Section 5.1). The optimal condition was then applied and investigated with wheat straw co-digestion (Section 5.2).

Chapter 6 consist of a general discussion that summarizes and extends all the knowledge generated over the whole study. Finally, in Chapter 7 the main conclusions that can be extracted from this work are presented.

2.2 Specific objectives

Accordingly, the specific objectives of this PhD thesis can be defined as:

2.2.1 Microalgae co-digestion with primary sludge (Chapter 3)

O.1.1. To evaluate the effect of the co-digestion of primary sludge on microalgae biodegradability in terms of the process kinetics and final methane yield in Biochemical Methane Potential (BMP) tests (Section 3.1).

O.1.2. To evaluate the effect of the microalgae thermal pretreatment on their co-digestion with primary sludge in terms of the process kinetics and final methane yield in BMP tests (Section 3.1).

O.1.3. To assess anaerobic digestibility of thermally pretreated microalgae in co-digestion with primary sludge by means of continuous mesophilic lab-scale reactors (Section 3.1).

O.1.4. To assess anaerobic digestibility of microalgae in co-digestion with primary sludge by means of continuous mesophilic lab-scale reactors (Section 3.2).

O.1.5. To evaluate the occurrence of some emerging organic contaminants in microalgae and primary sludge and their removal during their anaerobic (co-)digestion (Section 3.2).

O.1.6. To characterize digestates from microalgae anaerobic digestion and co-digestion with primary sludge to determine their suitability for agricultural reuse (Section 3.3).

2.2.2 Microalgae co-digestion with waste activated sludge (WAS) (Chapter 4)

O.2.1. To evaluate the effect of a simultaneous autohydrolysis pretreatment to microalgae and WAS on volatile solids solubilization to select best pretreatment conditions (Section 4.1).

O.2.2. To evaluate the effect of a simultaneous autohydrolysis pretreatment followed by the co-digestion of WAS on microalgae biodegradability in terms of the process kinetics and final methane yield in BMP tests (Section 4.1).

2.2.3 Microalgae co-digestion with wheat straw (Chapter 5)

O.3.1. To evaluate the effect of a thermo-alkaline pretreatment on microalgae in terms of organic matter and macromolecules solubilization and methane yield increase in BMP tests to select best pretreatment conditions (Section 5.1).

O.3.2. To evaluate the effect of the co-digestion of wheat straw on microalgae biodegradability in terms of the process kinetics and final methane yield in BMP tests (Section 5.2).

O.3.3. To evaluate the effect of a simultaneous pretreatment followed by the co-digestion of wheat straw on microalgae biodegradability in terms of the process kinetics and final methane yield in BMP tests (Section 5.2).

O.3.4. To assess anaerobic digestibility of microalgae in co-digestion with wheat straw, with and without a simultaneous thermo-alkaline pretreatment, by means of continuous mesophilic lab-scale reactors (Section 5.2).

3 CO-DIGESTION OF MICROALGAE WITH PRIMARY SLUDGE

* This chapter is based on the following articles:

Strategies to optimize microalgae conversion to biogas: co-digestion, pretreatment and hydraulic retention time. Solé-Bundó, M., Salvadó, H., Passos, F, Garfí, M., Ferrer, I. Submitted.

Co-digestion of microalgae and primary sludge from wastewater treatment systems: effect on biogas production and emerging contaminants removal. Solé-Bundó, M., Garfí, M., Matamoros, V., Ferrer, I. In prepatation.

Assessing the agricultural reuse of the digestate from microalgae anaerobic digestion and co-digestion with sewage sludge. Solé-Bundó, M., Cucina, M., Folch, M., Tàpias, J., Gigliotti, G., Garfí, M., Ferrer, I., 2017. Science of the Total Environment 586, 1–9. doi:10.1016/j.scitotenv.2017.02.006

3.1 Co-digestion of thermally pretreated microalgae with primary sludge

This study aims at optimizing the anaerobic digestion (AD) of biomass in microalgal-based wastewater treatment systems. It comprises the co-digestion of microalgae with primary sludge, the thermal pretreatment (75 °C for 10h) of microalgae and the role of the hydraulic retention time (HRT) in anaerobic digesters. Initially, a batch test comparing different microalgae (untreated and pretreated) and primary sludge proportions showed how the co-digestion improved the AD kinetics. The highest methane yield was observed by adding 75% of primary sludge to pretreated microalgae (339 mL CH_4/g VS). This condition was then investigated in mesophilic lab-scale reactors. The average methane yield was 0.46 m^3 CH_4/kg VS, which represented a 2.9-fold increase compared to pretreated microalgae mono-digestion. Conversely, microalgae showed a low methane yield despite the thermal pretreatment (0.16 m^3 CH_4/kg VS). Indeed, microscopic analysis confirmed the presence of microalgae species with resistant cell walls (i.e., *Stigioclonium* sp. and diatoms). In order to improve their anaerobic biodegradability, the HRT was increased from 20 to 30 days, which led to 50% methane yield increase. Overall, microalgae AD was substantially improved by the co-digestion with primary sludge, even without pretreatment, and increasing the HRT enhanced the AD of microalgae with resistant cell walls.

3.1.1 Introduction

Algal biofuels call for low-cost technologies for being competitive with fossil fuels. In this context, microalgae cultivation in wastewater reduces freshwater and nutrients consumption, while providing sanitation. Microalgal-based wastewater treatment systems consist of open ponds (e.g., high rate algal ponds (HRAPs)) capable of removing organic matter without aeration in the biological reactor, as for conventional activated sludge systems. Indeed, heterotrophic bacteria use the oxygen released through microalgae photosynthesis. The biomass grown in the ponds is then harvested to obtain a clarified effluent. Harvested biomass can be valorized as an

organic fertilizer (Arashiro et al., 2018) or to produce bioenergy, being anaerobic digestion (AD) the most straightforward technology for this purpose (Uggetti et al., 2017; Ward et al., 2014).

However, microalgae AD is limited by their resistant cell wall, which hampers the conversion into methane (González-Fernández et al., 2012). Thus, the application of pretreatment methods to damage or weaken the microalgae cell wall increases the bioavailability of intracellular contents to anaerobic microorganisms (Jankowska et al., 2017; Passos et al., 2014b). Even so, some pretreatments might result in higher costs (e.g., chemicals or biological products) or energy requirements (e.g., thermal or mechanical techniques) than the benefits obtained by implementing the pretreatment step (energy gain). This is a relevant aspect when choosing the most appropriate pretreatment for each substrate (Carrere et al., 2016). In this sense, microalgae thermal pretreatment at low temperature (<100 °C) has shown a promising energy balance (Passos and Ferrer, 2014).

In addition, the high nitrogen content (i.e., low C/N ratio) of microalgae can lead to methanogens inhibition due to ammonia toxicity during the AD process (Ehimen et al., 2011; Herrmann et al., 2016). To overcome this issue, possible solutions include the reduction of protein levels in microalgae biomass by culturing them in low nitrogen media or the use of ammonia tolerant anaerobic inoculum (Magdalena et al., 2018; Mahdy et al., 2017). More commonly, the co-digestion (i.e., the simultaneous digestion of two or more substrates) of microalgae with other carbon-rich biomass has been proposed to reduce the ammonia concentration levels in the reactors while increasing the organic loading rate (OLR) (Jankowska et al., 2017). In such a case, co-substrates obtained near or at the same treatment plant are preferred to avoid transport costs (Mata-Alvarez et al., 2014). This strategy could be easily implemented in microalgal-based wastewater treatment plants (WWTPs), where harvested microalgal biomass could be co-digested with primary sludge from primary settlers. Indeed, primary sludge is more readily digestible and has less protein content than microalgae (Mahdy et al., 2015), so it could enhance microalgae biodegradability while increasing the OLR. To the best of our knowledge, only a few studies have evaluated the co-digestion of microalgae with primary sludge and always in batch tests (Hlavínek et al., 2016; Mahdy et al., 2015). Given that some benefits were pointed out (e.g., methane yield increase), these results should better be validated in continuous reactors.

The aim of this study is to optimize the AD process in WWTPs based on HRAP. Thus, the co-digestion of primary sludge from primary settlers and harvested microalgal biomass from HRAP (hereafter called microalgae) was investigated in both batch and continuous reactors. Moreover, a thermal pretreatment at 75 °C for 10 h was applied to microalgae and the HRT of anaerobic digesters was increased to evaluate their effect on the microalgae methane yield. Microscopic analyses were used to help understanding how microalgae were degraded during the pretreatment and AD process. Finally, an energy assessment of each studied scenario was calculated to attest the viability of full-scale application.

3.1.2 Materials and methods

3.1.2.1 Substrates origin and characteristics

Microalgal biomass (hereafter called microalgae) used in this study consisted of a microalgae bacteria consortia grown in a pilot raceway pond that treated wastewater from a municipal sewer, as described by (Passos et al., 2015b). Microalgae was harvested from secondary settlers and gravity thickened in laboratory Imhoff cones at 4 °C for 24 h. The pilot plant was located at the laboratory of the GEMMA research group (Barcelona, Spain).

The thickened primary sludge and the digested sludge used as inoculum in both assays (BMP and continuous reactors) came from a municipal WWTP near Barcelona. The inoculum was collected before each assay set up while the primary sludge was periodically collected (every 3 weeks) and stored at 4 °C before use.

Throughout the operation of the continuous rectors, harvested and thickened by gravity microalgae presented an average concentration of 3.7% TS and 2.7% VS, while primary sludge had average values of 4.6% TS and 3.4% VS. To keep digesters fed with the same OLR, all substrates were diluted to achieve 2.5% VS.

3.1.2.2 Pretreatment performance

Thermal pretreatment of microalgae was carried out in glass bottles with a total volume of 250 mL and liquid volume of 150 mL. Bottles were placed in an incubator under continuous stirring at a constant temperature of 75 °C for 10 hours. For the continuous performance, microalgae were collected and pretreated once a week. Pretreated biomass was then stored at 4 °C before use.

3.1.2.3 Biochemical Methane Potential (BMP) tests

BMP tests were used to study the anaerobic biodegradability of co-digestion of primary sludge and microalgae with and without thermal pretreatment (Fig. 3.1). To this end, three proportion conditions were tested: i) 25% of microalgae and 75% of primary sludge, ii) 50% of microalgae and 50% of primary sludge and, iii) 75% of microalgae and 25% of primary sludge; all in VS basis. In addition, for all conditions, microalgal biomass was also thermally pretreated as previously described.

Substrate to inoculum (S/I) ratio was 0.5 g COD/g VS, according to (Arias et al., 2018). After adding the proper amount of both substrates and inoculum, serum bottles (160 mL) were filled with distilled water up to 100 mL, flushed with Helium gas, sealed with butyl rubber stoppers and incubated at 35 °C until biogas production ceased. Accumulated biogas was measured with a manometer (GMH 3161 Greisinger, Germany) and the methane content in biogas was periodically analyzed by gas chromatography. A blank treatment was used to quantify the amount of methane produced by the inoculum alone. Each condition was performed in duplicate, whereas the controls (only microalgae, pretreated microalgae and primary sludge) and blank were performed in triplicate.

Figure 3-1. BMP tests.

3.1.2.4 Continuous anaerobic digestion performance

Microalgae anaerobic (co-)digestion was performed and monitored using two lab-scale reactors (2 L), with an effective volume of 1.5 L (Fig. 3.2). Reactors were operated under mesophilic conditions (37 ± 1 °C) by implementing an electric heating cover (Selecta, Spain). Constant mixing was provided by a magnetic stirrer

(Thermo Scientific). Reactors were operated on a daily feeding basis, where the same volume was purged from and added to digesters using plastic syringes.

During a first period, one of the digesters utilized pretreated microalgae and operated as control while the second one simulated a co-digester and received pretreated microalgae (25% VS) and primary sludge (75% VS). Both reactors were operated at an HRT of 20 days and were considered to be under steady-state after 2,5 HRTs. Afterwards, anaerobic digestion performance was further monitored during 2 complete HRTs (~6 weeks). During second period, HRT was increased up to 30 days. One reactor was still fed with pretreated microalgae while the second one received untreated microalgae as a control. They were also considered to be under steady-state after 2,5 HRTs and anaerobic digestion performance was further monitored during next 2 complete HRTs (~8,5 weeks). The total operation period of the digesters was 225 days.

Biogas production was measured by the water displacement method and the methane content was periodically analyzed by GC. The volume of the produced biogas was adjusted to the standard temperature (0 °C) and pressure (1 atm) condition (STP).

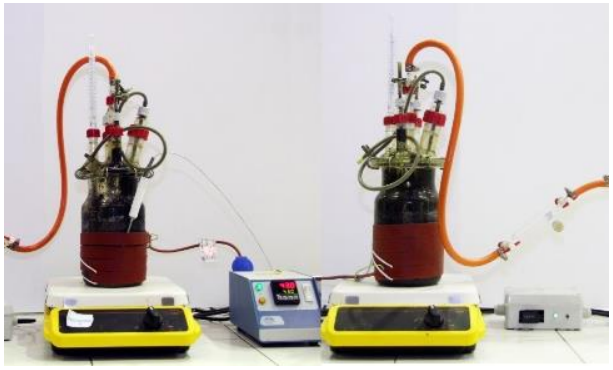


Figure 3-2. Lab-scale continuous reactors

3.1.2.5 Microscopic observations

Microalgae identification was periodically carried out during the continuous reactors performance. For their examination it was used an optic microscope (Motic BA310E, China), equipped with a camera (NiKon DS-Fi2) using the software NISElements Viewer. Microalgae genus were identified from classical specific literature (Palmer, 1962; Bourelly, 1966).

To prove the effect of the thermal pretreatment and the AD process on microalgae population, four sampling campaigns were conducted. The samples collected on each campaign were i) untreated microalgae; ii) thermally pretreated microalgae; iii) effluent (digestate) from untreated microalgae AD and iv) effluent (digestate) from pretreated microalgae AD. From these samples, microalgae species were identified and two of the most abundant were quantified (*Chlorella* sp. and diatoms). For their quantification, each well homogenized sample were examined by bright and contrast phase microscopy using a Zeiss microscope Axioskop 40. In each subsample, *Chlorella* sp. and diatoms were counted in vivo at 100 and 400 magnification using coverslides of 20 mm side (Salvadó et al., 2004). Previous to the counting, the aggregated flocs of these unicellular species were broken down by means of an ultrasound technique (Abzazou et al., 2015).

3.1.2.6 Analytical procedures

The TS and VS analysis was done according to the Standard Methods (Association. et al., 2005). Quantification of total COD concentrations was performed according to the closed reflux colorimetric method outlined by Standard Methods (Association. et al., 2005). TKN was determined by titration after a mineralization step performed by a BUCHI 370-K distillator/titrator. The concentration of the ammonium nitrogen ($N\text{-}NH_4^+$) was measured according to the method by Solorzano (Solorzano, 1969). pH was determined with a Crison Portable 506 pH-meter and dewaterability was evaluated by means of the capillary suction time (CST) test (Triton Electronics Ltd.). Volatile fatty acids (VFA) concentrations in continuous flow digesters were measured once a week by injecting 1 μL of each sample, once centrifuged (4200 rpm for 8 min) and filtered (0.2 μm), into an Agilent 7820A GC after sulphuric acid and diisopropyl ether addition. The GC was equipped with an auto-sampler, flame ionization detector and a capillary column (DP-FFAB Agilent 30 m x 0.25 mm x 0.25 μm), and operated at injector and detector temperatures of 200 and 300 °C, respectively, with helium as carrier gas.

Biogas composition was determined by calculating the percentage of methane and carbon dioxide in the digesters headspace. Gases were measured by means of a GC (Thermo Finnigan) equipped with a thermal conductivity detector (TCD) (Hayesep packed column). The carrier gas was helium and injector/detector/oven temperatures of 150, 250, 35 °C, respectively. Methane percentage from BMP was measured in each sampling while in continuous-flow reactors was quantified twice a week.

3.1.2.7 Statistics and kinetic data analysis

The statistically significant effects of independent variables were evaluated via multi-factor analysis of variance (ANOVA) considering 95% confidence level ($\alpha = 0.05$) using R Statistics Software.

To evaluate the kinetics of the process from BMP tests, experimental data was adjusted to a first-order kinetic model (Eq. 3.1.1) by the least square method.

$$B=B_0 \{1-\exp[-k \cdot t]\} \qquad \text{(Eq. 3.1.1)}$$

where, B_0 stands for the methane production potential (ml CH_4/gVS), k is the first order kinetic rate constant (day^{-1}), B is the accumulated methane production at time t (ml CH_4/gVS) and t is time (day).

The error variance (s^2) was estimated by the following equation (Eq. 3.1.2):

$$s^2 = \frac{\sum_1^i (y_i - \hat{y}_i)^2}{N-K} \qquad \text{(Eq. 3.1.2)}$$

where y_i is the experimental value, $\hat{y}_i$ is the value estimated by the model, N is the number of samples and K is the number of model parameters.

3.1.2.8 Energy assessment calculations

The theoretical energy balance of full-scale reactors was estimated from experimental data, considering flow rates of 10-25-100 m^3/day, which correspond to the target of a medium-size WWTP. Electricity and heat requirements for microalgae pretreatment and anaerobic digestion were calculated according to (Passos and Ferrer, 2014).

Input heat was calculated as the energy required to heat influent biomass from ambient temperature (Ta) to digestion temperature (Td), according to Eq 3. The density (ϱ) and specific heat (γ) of microalgae and primary sludge were assumed to be the same as those of water, 1000 kg/m^3 and 4.18 kJ/kg·°C, respectively. Heat losses through the reactor wall were considered and the heat transfer coefficient (k) was assumed to be 1 W/m^2·day. The reactor wall surface area was calculated from the reactor useful volume, considering a 2:1 diameter to height ratio; while the reactor bottom and top were not accounted for.

$$Ei,heat = \varrho \cdot Q \cdot \gamma \cdot (Td - Ta) + k \cdot A \cdot (Td - Ta) \cdot 86.4 \qquad \text{(Eq. 3.1.3)}$$

where Ei,heat: input heat (kJ/day); ϱ: density (kg/m^3); Q: flow rate (m^3/day); γ: specific heat (kJ/kg·°C); Td: anaerobic digestion temperature (37 °C); Ta: ambient temperature (20 °C); k: heat transfer coefficient (W/m^2·°C); A: surface area of the reactor wall (m^2).

When thermal pretreatment is involved, heat recovery is considered. Input heat was calculated as the energy required to heat influent biomass from Ta to pretreatment temperature (Tp), subtracted by the heat recovered when cooling down biomass from Tp to Td (Eq. 4). Heat would be recovered by means of a heat exchanger, with an efficiency φ of 85%.

$$Ei,heat = \varrho Q\gamma \cdot (Tp - Ta) - \varrho Q\gamma \cdot (Tp - Td) \cdot \varphi + kA \cdot (Td - Ta) \cdot 86.4 \qquad \text{(Eq. 3.1.4)}$$

where Ei,heat: input heat (kJ/day); ϱ: density (kg/m^3); Q: flow rate (m^3/day); γ: specific heat (kJ/kg·°C); Td: anaerobic digestion temperature (37 °C); Ta: ambient temperature (20 °C); Tp: pretreatment temperature (75 °C); φ: heat recovery efficiency (85%); k: heat transfer coefficient (W/m^2·°C); A: surface area of the reactor wall (m^2).

Furthermore, input electricity for anaerobic digestion was estimated as the energy required for biomass pumping and reactor mixing, which were assumed to be 1800 kJ/m^3 and 300 kJ/ m$^3_{reactor}$ day, respectively (Eq. 3.1.5):

$$Ei,electricity = Q \cdot \theta + V \cdot \omega \qquad \text{(Eq. 3.1.5)}$$

where Ei,electricity: input electricity (kJ/day); Q: flow rate (m^3/day); θ: electricity consumption for pumping (kJ/m^3); V: useful volume (m^3); ω: electricity consumption for mixing (kJ/m$^3_{reactor}$·day).

The energy output of the process was calculated from the methane production rate of each reactor, according to Eq 6. The lower heating value of methane (ξ) was assumed to be 35 800 kJ/m^3 CH_4. An efficiency of 90% on energy conversion was considered (η).

$$Eo = P,CH_4 \cdot \xi \cdot V \cdot \eta \qquad \text{(Eq. 3.1.6)}$$

where Eo: output energy (kJ/d); P,CH_4: methane production rate (m^3 CH_4/ $m^3_{reactor}$·day); ξ: lower heating value of methane (kJ/ m^3 CH_4); V: useful volume (m^3); η: energy conversion efficiency.

Finally, results were expressed as energy balance (ΔE) and energy ratio (Eo/Ei). The energy balance was calculated as the difference between the energy output and energy input (heat and electricity) (Eq. 7), while the energy ratio was calculated from the energy output over the energy input (heat and electricity) (Eq. 3.1.8).

$$\Delta E = Eo - (Ei,heat + Ei,electricity) \quad \text{(Eq. 3.1.7)}$$

$$Eo/Ei = Eo/(Ei,heat + Ei,electricity) \quad \text{(Eq. 3.1.8)}$$

3.1.3 Results

The co-digestion of microalgae and primary sludge at different proportions was initially studied by means of Biochemical Methane Potential (BMP) tests (Section 3.1.3.1.1). Subsequently, two continuous lab-scale anaerobic reactors were run in parallel (Table 3-1). During the first period, the co-digestion of pretreated microalgae with primary sludge was investigated (Section 3.1.3.1.2). During the second one, microalgae mono-digestion (with and without pretreatment) at longer HRT was compared (Section 3.1.3.2.1), including a microscopic analysis (Section 3.1.3.2.2).

Table 3-1. Experimental conditions during the mesophilic AD in lab-scale reactors.

	Period I (HRT= 20 days)	Period II (HRT= 30 days)
Digester 1	25% VS pretreated[1] microalgae + 75% VS primary sludge	Untreated microalgae
Digester 2	Pretreated[1] microalgae	Pretreated[1] microalgae

[1] 75°C for 10h.

3.1.3.1 Improving microalgae anaerobic digestion by co-digestion with primary sludge and thermal pretreatment

3.1.3.1.1 Anaerobic co-digestion of microalgae and primary sludge in batch tests

The co-digestion of microalgae with primary sludge was evaluated at different proportions (25, 50 and 75% of microalgae, on a volatile solids (VS) basis) (Table 2). Additionally, in some trials microalgae were pretreated at 75 °C for 10h in order to solubilize the biomass and enhance the anaerobic digestion rate and extent (Passos and Ferrer, 2014). Indeed, the microalgae methane yield was increased by 62% (from 90 to 146 mL CH_4/g VS) and the first-order kinetics constant (k) by 128% (from 0.07 to 0.16 day^{-1}) after the pretreatment (Table 3.2). However, primary sludge showed the highest methane yield (380 mL CH_4/gVS) and faster kinetics (k= 0.24 day^{-1}) as compared to untreated and pretreated microalgae. This is due to the nature of primary sludge, which is more readily digestible than microalgae.

Table 3-2. Ultimate methane yield (mean values ± standard deviation) and first-order kinetics constant (k) (error variance (S^2) represented in brackets) obtained in the BMP test.

Trial	Methane yield (ml CH_4/gVS)		First-order kinetics (k) (day^{-1})	
	Experimental values [1]	Calculated values [2]	Experimental values [1]	Calculated values [3]
Microalgae (M)	90 ± 2	-	0.07 (≤30)	-
75% M + 25% PS [4]	133 ± 6	162	0.27 (≤74)	0.16 (70)
50% M + 50% PS [4]	216 ± 1	234	0.28 (≤80)	0.20 (88)
25% M + 75% PS [4]	291 ± 9	306	0.27 (≤108)	0.23 (113)
Pretreated Microalgae (Mp)	146 ± 6	-	0.16 (≤ 75)	-
75% Mp + 25% PS [4]	183 ± 2	204	0.25 (≤ 85)	0.20 (72)
50% Mp + 50% PS [4]	249 ± 17	262	0.28 (≤ 99)	0.22 (82)
25% Mp + 75% PS [4]	339 ± 2	320	0.25 (≤ 150)	0.23 (107)
Primary Sludge (PS)	378 ± 4	-	0.24 (≤ 162)	-

[1] Experimental data from BMP tests; [2] Theoretical values calculated as the sum of the ultimate methane yield of each substrate mono-digestion times their proportion in the trial; [3] Values obtained from the curves that represent the theoretical values calculated as the sum of the ultimate methane yield of each substrate mono-digestion times their proportion in the trial over time; [4] volatile solids basis.

However, the co-digestion of microalgae with primary sludge substantially improved the anaerobic digestion kinetics (k = 0.25-0.28 day^{-1}) as compared to mono-digestion trials. Also, when comparing the experimental values of kinetics from co-digestion trials with those values calculated from the theoretical curves obtained as the sum of mono-digestion experimental values (Table 3.2), the experimental k value was always higher than the theoretical one. This means that mixing both substrates accelerated the AD process, as already observed in other cases (Beltran et al., 2016; Neumann et al., 2015). This could contribute to reduce costs by decreasing the digesters hydraulic retention time (HRT) and thus their volume. Still regarding the kinetics, no differences were observed between pretreated and untreated trials, since microalgae and primary sludge co-digestion without pretreatment already improved by far the anaerobic digestion rate. On the other way around, the pretreatment itself had already accelerated the kinetics of the process, so the effects of the co-digestion resulted less discernible than for untreated substrates (Astals et al., 2015; Solé-Bundó et al., 2017c).

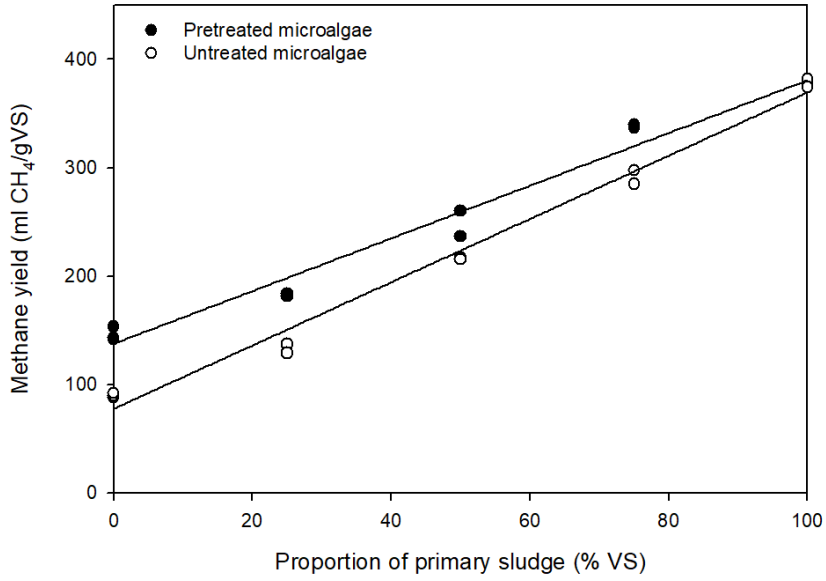


Figure 3-3. Correlation between the methane yield and the primary sludge proportion added to untreated and pretreated microalgae.

Otherwise, the higher the proportion of primary sludge, the higher the methane yield (Fig. 3.3), being 339 mL CH_4/gVS the highest methane yield achieved with the co-digestion of 75% primary sludge and 25% pretreated microalgae. These findings

suggest that there was no synergic effect with respect to the ultimate methane production when co-digesting both substrates.

3.1.3.1.2 Anaerobic co-digestion of microalgae and primary sludge in lab-scale reactors

The best co-digestion condition (25-75% VS of thermally pretreated microalgae and primary sludge) from BMP tests was thereafter compared to the mono-digestion of thermally pretreated microalgae in lab-scale reactors (Table 3.3). During the whole experimental period, both reactors were operated with an OLR around 1.2 kg VS/m^3·day, given the concentration of VS in microalgae harvested and thickened by gravity (around 4 % TS and 2.5 % VS) and the HRT (20 days).

In the co-digestion reactor the average methane yield was 0.46 m^3 CH_4/kg VS, which represented a 2.9-fold increase as compared to pretreated microalgae mono-digestion (0.16 m^3 CH_4/kg VS). Also the methane production rate increased, from 0.20 to 0.53 m^3 CH_4/m^3·day. Despite this important increase in methane yield and methane production rate, the average VS removal was not so different (34.3% for co-digestion vs. 27.9% for mono-digestion). A possible reason for this is that primary sludge had higher lipid content than microalgae, which are mainly composed by proteins. Indeed, a previous study that investigated similar microalgae and primary sludge (they came from the same HRAP and WWTP, respectively) quantified lipids in 45% and 24% VS for sludge and. microalgae, respectively, and proteins in 29% and 58% VS (Solé-Bundó et al., submitted). Comparing the methane potential of both macromolecules, lipids can achieve 1.014 m^3 CH_4/kgVS and proteins only 0.851 m^3 CH_4/kgVS (Sialve et al., 2009). Therefore, the conversion potential of primary sludge to methane is higher than microalgae, as already observed in the BMP tests. The methane yield of the co-digestion reactor was higher than that obtained co-digesting sewage sludge with *Spirulina maxima* (50% VS each) at 20 days of HRT (0.36 m^3 CH_4/kgVS) (Samson and LeDuy, 1983), and similar to that obtained co-digesting *Scenedesmus* sp. or native microalgal biomass (25% VS) with sewage sludge (75% VS) at 15 days of HRT (0.39 and 0.51 m^3 CH_4/kgVS, respectively) (Peng and Colosi, 2016).

Concerning the stability of digesters, pH values were stable during the whole period, ranging from 7.35 to 7.55 (Table 3.3). Regarding the ammonium concentration, the highest value was observed in the mono-digestion reactor with pretreated microalgae (1.1 g N-NH_4/L) due to a higher protein release during the AD process. This value is close to the threshold which has resulted in AD inhibition (Rajagopal et al., 2013).

Table 3-3. Biogas production, solids removal, influent (substrate) and effluent (digestate) characteristics from untreated or thermally pretreated microalgae AD and co-digestion with primary sludge in lab-scale reactors. Mean ± standard deviation.

	Period I		Period II	
	Microalgae,p	Co-digestion	Microalgae	Microalgae,p
Operational conditions				
HRT (days)	20	20	30	30
OLR (kg VS/m^3·day)	1.21 ± 0.06	1.17 ± 0.09	0.85 ± 0.01	0.81 ± 0.02
Biogas production				
Methane production rate (m^3 CH_4/m^3·day)	0.20 ± 0.05	0.53 ± 0.29	0.12 ± 0.08	0.19 ± 0.07
Methane yield (m^3 CH_4/kg VS)	0.16 ± 0.05	0.46 ± 0.27	0.14 ± 0.07	0.24 ± 0.07
Methane content in biogas (% CH4)	66.2 ± 2.62	71.7 ± 0.9	67.6 ± 1.6	69.5 ± 1.7
Removal efficiency				
TS removal (%)	16.6 ± 4.1	19.0 ± 1.7	26.2 ± 3.7	18.6 ± 1.7
VS removal (%)	27.9 ± 1.9	34.3 ± 2.4	36.2 ± 2.5	39.5 ± 3.7
Influent characteristics				
TS [% (w/w)]	3.87 ± 0.28	4.13 ± 0.29	2.87 ± 0.16	2.67 ± 0.27
VS [% (w/w)]	2.47 ± 0.17	2.38 ± 0.15	1.58 ± 0.06	1.45 ± 0.11
VS/TS (%)	64 ± 3	58 ± 3	56 ± 2	55 ± 2
COD (g O_2/L)	42.0 ± 6.7	42.9 ± 7.7	26.6 ± 1.6	25.2 ± 1.8
TKN (g/L)	n.a.	n.a.	2.4 ± 0.1	2.3 ± 0.1
N-NH_4 (g/L)	0.16 ± 0.07	0.13 ± 0.06	0.06 ± 0.01	0.26 ± 0.06

	Period I		Period II	
	Microalgae,p	Co-digestion	Microalgae	Microalgae,p
Effluent characteristics				
pH	7.55 ± 0.15	7.30 ± 0.08	7.35 ± 0.11	7.55 ± 0.08
TS [% (w/w)]	3.49 ± 0.34	3.53 ± 0.18	2.87 ± 0.16	2.67 ± 0.27
VS [% (w/w)]	1.77 ± 0.09	1.62 ± 0.11	1.58 ± 0.06	1.45 ± 0.11
VS/TS (%)	51 ± 3	46 ± 2	56 ± 2	55 ± 2
COD (g/L)	30.9 ± 2.1	29.0 ± 3.0	26.6 ± 1.6	25.2 ± 2.1
N-NH_4 (g/L)	1.1 ± 0.2	0.6 ± 0.1	0.7 ± 0.1	0.8 ± 0.1
VFA (mg COD/L)	124 (<756[1])	44 (<757[1])	0 (< 0[1])	130 (<596[1])
CST (s)	982 ± 61	290 ± 11	795 ± 71	919 ± 21

[1] Maximum value achieved. p=pretreated

Therefore, if reactors had been operated at higher OLRs, inhibition for ammonia toxicity may have occurred. Conversely, co-digestion with primary sludge reduced the ammonium concentration in the digester to 0.6 g $N\text{-}NH_4$/L. In this case, the OLR could have been increased without approaching the ammonia inhibition threshold. VFA concentrations were also very low in both reactors (Table 3.3).

Finally, an important aspect for the digestate management and final disposal is its dewaterability. While the digestate from thermally pretreated microalgae digestion presented a poor dewaterability (CST value of 982 s), the results were consistently improved by the co-digestion with primary sludge (CST value of 290 s). In this sense, the co-digestion substantially improved the effluent dewaterability since primary sludge has less affinity for water than microalgae.

3.1.3.2 Effect of the thermal pretreatment on microalgae anaerobic digestion

3.1.3.2.1 Anaerobic digestion of thermally pretreated microalgae in lab-scale reactors

As previously discussed, microalgae showed a low methane yield despite the thermal pretreatment (0.16 m^3 CH_4/kg VS). In order to improve their anaerobic biodegradability, the digester HRT was increased from 20 to 30 days. In parallel, another digester with untreated microalgae was operated as control. During this period, the methane production rate of pretreated microalgae increased by 58% (from 0.12 to 0.19 m^3 CH_4/ m^3·day) and the methane yield by 71% (from 0.14 to 0.24 m^3 CH_4/kgVS) as compared to control (Table 3.3). Accordingly, the VS removal also increased from 36.2 to 39.5% (Table 3.3).

Regarding the ammonium concentration, it was higher in the pretreated reactor digestate than in the control (0.8 g $N\text{-}NH_4$/L vs. 0.7 g $N\text{-}NH_4$/L), suggesting a higher protein solubilization in the case of pretreatment. However, as a result of increasing the HRT, the OLR decreased from 1.2 to 0.8 kg VS/m^3·day. Consequently, the $N\text{-}NH_4$ concentration in the reactor was reduced in comparison with the previous period at 20 days of HRT (0.8 vs. 1.1 g $N\text{-}NH_4$/L).

The methane yield increase observed in this study is in agreement with the results obtained by Passos and Ferrer (2014), who reported an increase of 70% after applying a thermal pretreatment at 95 °C for 10h to similar microalgae species. However, different conclusions regarding the effect of the thermal pretreatment on microalgae can be found in the literature. For instance, no significant effect was observed after

a pretreatment at 70 °C for 3h to *Scenedesmus* sp., but same pretreatment at 90 °C enhanced the anaerobic biodegradability of *Scenedesmus* sp. from 22 to 48% in BMP tests (C. González-Fernández et al., 2012) Other authors found no influence of the thermal pretreatment, but did find an effect of the thermochemical pretreatment which increased methane yield by 40% in some microalgae species (Bohutskyi et al., 2014). Indeed, the effect of the thermal pretreatment highly depends on the microalgae species and the conditions applied, so a pilot-scale evaluation of the pretreatment performance is required before scaling-up.

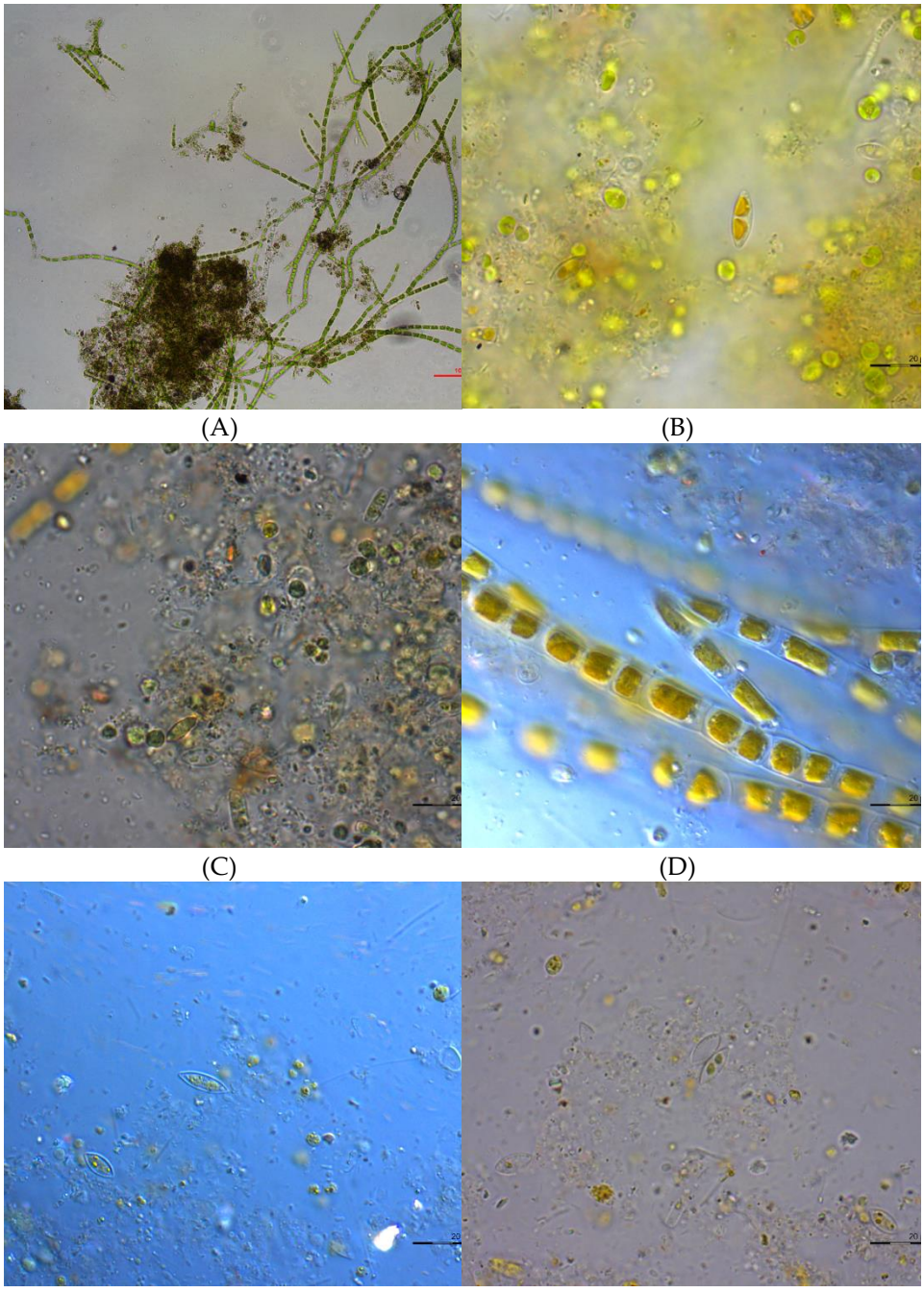
In terms of digestate dewaterability, both the untreated and thermally pretreated microalgae showed a poor dewaterability, with higher CST values (795 and 919 s, respectively) than the co-digestion reactor (290 s).

3.1.3.2.2 Microscopic analysis

Microalgae were periodically characterized by optical microscopy over the whole experimental period. Qualitative results showed how microalgal biomass was flocculated. The main green microalgae species belonged to the genus *Chlorella* and *Stigeoclonium*, along with diatoms (Fig. 3.4A and B). These microalgae species remained predominant during the whole period, although the relative abundance varied over time, which is common in open ponds treating wastewater (Passos et al., 2015b).

After the thermal pretreatment, microalgae clearly appeared to be less pigmented than fresh microalgae and most of the cells were dead (Fig. 3.4C and D). Also, in the pretreated sample, a higher amount of amorphous material was found, because of organic matter release. However, most of the cell walls were found unbroken. This was especially the case for diatoms (Fig. 3.4C) and *Stigeoclonium* sp (Fig. 3.4D), which presented a higher resistance to the pretreatment. Indeed, other authors concluded that the thermal pretreatment was not able to break microalgae cell walls but it did damage or weaken them (Ometto et al., 2014; Passos et al., 2014a).

To further evaluate the effect of the thermal pretreatment on microalgae AD, microscopic images from the digestate of pretreated microalgae (Fig. 3.4F) were compared to those from the digestate of untreated microalgae (Fig. 3.4E). In this manner, it was possible to elucidate whether pretreated cells were more accessible



(A) (B)

(C) (D)

(E) (F)

Figure 3-4 Microscopic images of microalgae before (A-B) and after (C-D) thermal pretreatment along with the digestates from untreated microalgae AD (E) and thermally pretreated microalgae AD (F) at a HRT of 30 days.

to methanogens, even if cell walls were not lysed after the pretreatment step. A higher amount of particulate substances was observed in the untreated microalgae digestate (Fig. 3.4E), although entire microalgae cells were found in both digestates even after 30 days of digestion.

Next, a quantitative analysis was conducted by counting the two most abundant microalgae species, *Chlorella* sp. and diatoms, in the influent and effluent (Fig. 3.5). This analysis confirmed the qualitative results. While the amount of *Chlorella* sp. individuals was reduced by the thermal pretreatment, no significant differences were observed for diatoms. Indeed, both of them present a resistant cell wall, but their characteristics and composition differs. On the one hand, *Chlorella* sp. has mainly a carbohydrate-based cell wall, and carbohydrates solubilization can be boosted by the thermal pretreatment (Solé-Bundó et al., 2017a). On the other hand, diatoms have a siliceous-based cell wall, which resists the effect of temperature.

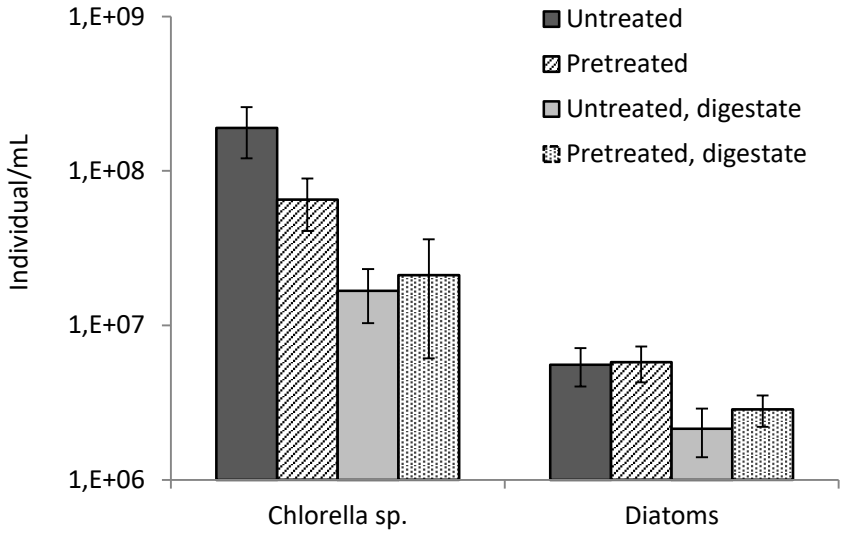


Figure 3-5 *Chlorella* sp. and diatoms counting in the influents (untreated; pretreated) and effluents (untreated digestate; pretreated digestate) during period II. Mean values and standard deviation are represented.

In spite of this, both microalgae species were partially removed during the AD process according to digestate counting. While *Chlorella* experimented around one logarithmic unit removal, a much lower removal efficiency was observed for diatoms, leading to a higher diatoms relative abundance in the digestates. Comparing both *Chlorella* and diatoms abundance in untreated and pretreated microalgae digesters, no

significant differences were found. Even so, the pretreated microalgae digester showed a higher methane yield and VS removal. This may be because, although having same quantity of entire cells, those cells that were attacked by microorganisms were more degraded in pretreated microalgae reactor.

3.1.3.3 Effect of the HRT on microalgae anaerobic biodegradability

The effect of the HRT can be evaluated by comparing the results on pretreated microalgae AD obtained in both periods (at 20 and 30 days of HRT). When the HRT was increased to 30 days, the methane yield of pretreated microalgae increased by 50% (from 0.16 to 0.24 m^3 CH_4/kg VS) compared to that obtained at 20 days of HRT (Table 3.3, Fig. 3.6). Indeed, the VS removal was also higher with a HRT of 30 days (39.5%) as compared to 20 days (27.9%).

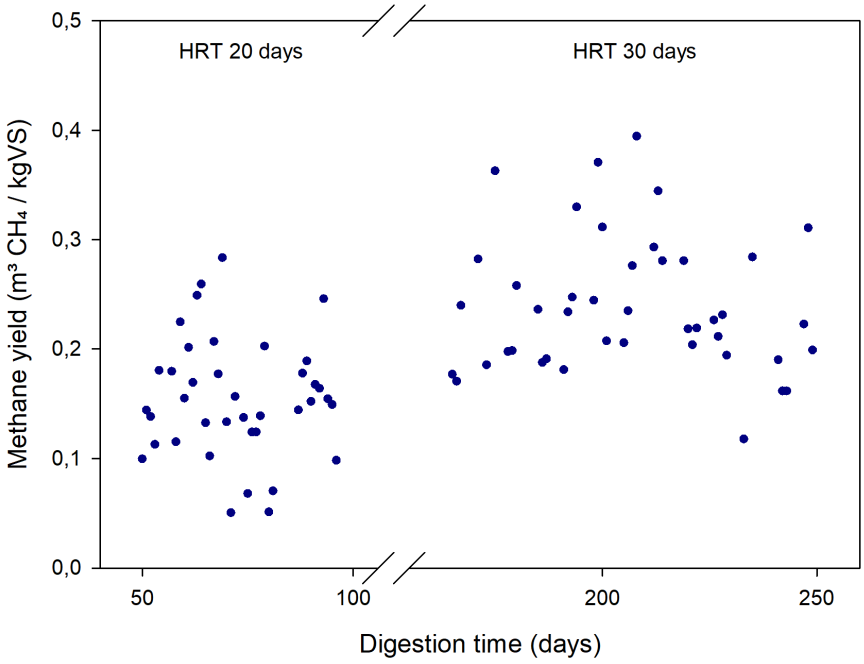


Figure 3-6 Daily methane yield of thermally pretreated microalgal biomass for the two studied periods: Period I at HRT of 20 days and Period II at HRT of 30 days.

Although one expected benefit of applying a pretreatment is the kinetics improvement and thereby a reduction of the HRT (Carrere et al., 2016), the methane yield increase reported in this study was still significant when he HRT was increased

from 20 to 30 days. Thus, operating microalgae digesters at moderate HRTs seems appropriate, even if applying pretreatments. As discussed in the previous section, the thermal pretreatment weakened the microalgae cell wall but without completely lysing and releasing all intracellular material. Therefore, increasing the HRT enhanced the chance for microorganisms to access microalgae intracellular material through their weakened or damaged cell wall. These results are in agreement with previous studies. For instance, applying a thermal pretreatment to microalgae did not show any significant differences with a HRT of 15 days., but it increased the methane yield by 72% with a HRT of 20 days (Passos and Ferrer, 2014). It has been suggested that the operation of digesters at high sludge retention times (SRT) promotes the presence of low growth-rate microorganisms and increases the hydrolytic potential of the system (Greses et al., 2018). Comparing a thermophilic continuous stirred tank reactor working at 50 days of HRT (and SRT) with an anaerobic membrane bioreactor (AnMBR) with a SRT of 70 days, higher microbial diversity could be found in digesters working at higher HRT system (Greses et al., 2018).

3.1.4 Discussion

Results have shown how the co-digestion with primary sludge can substantially improve the microalgae mono-digestion, by increasing the methane yield, decreasing the ammonia concentration which may enable increasing the OLR, and improving the digestate dewaterability.

This study assessed different proportions of primary sludge and microalgae in batch tests, and the best one in semi-continuous lab-scale reactors. The truth is that in full-scale microalgal-based WWTPs, this proportion would change over the year. Indeed, the microalgal biomass production shows a strong seasonality (Passos et al., 2015b), depends on the HRAPs operation conditions, influent characteristics, etc. (Passos et al., 2017). These factors determine not only the amount but also the microalgae species in the system (Gutiérrez et al., 2016a; Passos et al., 2015b). And the microalgae species also affect the anaerobic digestion rate and extent, depending especially on the characteristics of the cell wall (Passos et al., 2015b). Overall, the implementation of anaerobic digesters in HRAPs plants involves working with different proportions of microalgae and primary sludge, and different microalgae species over the year. All these factors should be considered when it comes to sizing an AD plant integrated to a HRAP system. For instance, if the proportion of primary sludge is expected to be high, the biogas production is also expected to be high, and

the operation of the digesters should be feasible at 20 days of HRT. However, if the proportion of microalgae is expected to be high, then it is necessary to assess the most appropriate strategy to follow (increasing the HRT and/or applying a pretreatment).

In this study the thermal pretreatment increased the microalgae methane yield, but not as much as expected due to the presence of microalgae species with hardly degradable cell walls (i.e., *Stigioclonium* sp. and diatoms). However, when the reactors were operated at longer HRT (30 days), the methane yield of pretreated microalgae increased considerably (from 0.16 to 0.24 m^3 CH_4/kg VS). When considering these alternatives, different issues should be addressed. Firstly, the balance between the energy requirements vs. the energy gain of the pretreatment step. Secondly, the increase of volume, surface area and costs resulting from an increased HRT.

Consequently, an energy assessment was carried out by scaling-up the results of the lab-scale reactors during both experimental periods (I: co-digestion vs. pretreated microalgae mono-digestion at 20 days of HRT; II: pretreated vs. untreated microalgae at 30 days of HRT). Flow rates between 10-100 m^3/day were considered (Table 3.4).

Table 3-4. Results of the energy assessment for the co-digestion and pretreated microalgae mono-digestion at 20 days of HRT; and for the untreated and pretreated microalgae mono-digestion at 30 days of HRT, with different flow rates (Q=10, 25 and 100 m^3/day). Ei (i.e., energy input) and Eo (i.e., energy output).

	Period I						Period II					
	Microalgae,p			Co-digestion			Microalgae			Microalgae,p		
Q (m^3/day)	10	25	100	10	25	100	10	25	100	10	25	100
Ei (GJ/day)	1.2	2.8	10.5	1.0	2.3	8.6	1.0	2.3	8.5	1.2	2.9	11.0
Eo (GJ/day)	1.3	3.2	12.9	3.4	8.5	34.2	1.4	3.4	13.5	1.8	4.6	18.4
ΔE= Eo-Ei (GJ/day)	0.1	0.5	2.4	2.5	6.3	25.3	0.4	1.1	5.0	0.6	1.7	7.3
Eo/Ei (-)	1.1	1.2	1.2	3.5	3.7	4.0	1.4	1.5	1.6	1.5	1.6	1.7

The assessment compared the energy required to apply the pretreatment (if any) and anaerobic digestion (Ei) with the energy obtained through the biogas produced in each case (Eo). In this way, when the energy ratio (Eo/Ei) is higher than 1, there is

an energy gain. As can be seen in Table 3.4, this value was higher than 1 in all scenarios, meaning that the energy balance was always positive. However, the best results were obtained with the co-digestion of microalgae and primary sludge (energy ratio between 3.5-4). This means that the energy produced with the co-digestion is at least 3.5-fold the energy consumed. Regarding the thermal pretreatment, it also showed an energy gain in all cases. However, the energy ratio increased from 1.1-1.2 to 1.5-1.7 by increasing the HRT from 20 to 30 days. When comparing the energy gain with untreated and pretreated microalgae at the same HRT of 30 days, the results are really similar (from 1.4-1.6 to 1.5-1.7). Bearing in mind the investment and operation costs of the pretreatment, it would not be worth it in terms of energy production, and only if other benefits like hygenisation were considered.

To sum up, the most suitable option to anaerobically digest microalgae from HRAPs would be the co-digestion with primary sludge at a 20-day HRT if the proportion of sludge was high, and at 30 days if the proportion of microalgae was high. The energy gain could be used to cover the energy demand of the WWTP, moving towards energy neutral WWTPs (Passos et al., 2017).

3.2 Co-digestion of microalgae with primary sludge: an extended analysis including emerging contaminants removal

Microalgal-based wastewater treatment plants are conceived as low cost and low energy consuming systems. The operation of these plants involves the management of primary sludge and microalgal biomass. The aim of this study was to analyse the anaerobic co-digestion of both by-products in terms of biogas production and emerging organic contaminants removal. The co-digestion of 25% microalgae and 75% primary sludge (on volatile solids basis) was investigated in continuous reactors and compared to microalgae mono-digestion at a hydraulic retention time of 20 days. Results showed how the co-digestion enhanced the anaerobic digestion of microalgal biomass, since primary sludge is a more readily biodegradable substrate, which led to higher methane production (65% increase) and reduced the risk of ammonia toxicity. Regarding the emerging organic contaminants, it was observed that musk fragrances (galaxolide and tonalide) and triclosan showed the highest abundance on primary sludge (0.5-25 μg/g TS), whereas caffeine, methyl dihydrojasmonate and triphenyl phosphate were barely detected on both substrates (<0.1 μg/g TS). The removal of these contaminants was compound-depending and ranged from no removal to up to 90%. Nevertheless, results showed that microalgae mono-digestion resulted in a higher removal of selected contaminants than the co-digestion with primary sludge.

3.2.1 Introduction

Microalgal-based wastewater treatment systems, such as high rate algal ponds (HRAPs), are low cost technologies that remove organic matter and nutrients from wastewater thanks to the symbiosis between microalgae and bacteria. Indeed, microalgae release oxygen through photosynthesis, which is used by heterotrophic bacteria for organic matter degradation. Since aeration is not needed in these systems, they can replace conventional activated sludge systems reducing energy consumption associated with wastewater treatment. Moreover, microalgae biomass can be harvested and digested or co-digested with other substrates, such as primary sludge

from the primary treatment settlers, in order to produce bioenergy (Iyovo et al., 2010).

The co-digestion of both primary sludge and microalgae in the same reactor could enhance the anaerobic digestion performance while easing the management of these by-products (Mata-Alvarez et al., 2014). This strategy may improve microalgae anaerobic digestion rate and extent by increasing the carbon to nitrogen ratio (C/N) and reducing the risk of ammonia toxicity, due to the high content of proteins in microalgae cells (Magdalena et al., 2018). In addition, co-digestion may promote macro and micro-nutrient equilibrium, balance moisture content, optimize the organic loading rate and dilute possible inhibitory compounds produced from the anaerobic digestion process (Astals et al., 2015; Herrmann et al., 2016; Schwede et al., 2013a). Indeed, a previous study dealing with co-digestion of the cyanobacteria *Spirulina maxima* (50%) and sewage sludge (50%) in continuous reactors at 20 days hydraulic retention time (HRT), reported a methane yield increase of 2.1-fold compared to cyanobacteria alone. The authors also identified a synergy when mixing both substrates due to the increase of C/N ratio (Samson and LeDuy, 1983). So far, most research has been conducted co-digesting microalgae with waste activated sludge (WAS) or sewage sludge, while only a few studies tested microalgae co-digestion with primary sludge in batch test (Hlavínek et al., 2016; Mahdy et al., 2015). A 15% increase in microalgae methane yield was reported after primary sludge co-digestion in BMP tests (Mahdy et al., 2015). To the best of our knowledge, co-digestion of microalgal biomass grown in wastewater and primary sludge in continuous reactors has not been explored yet.

Besides biogas, anaerobic digestion process may give place to a stabilized digestate, which can be applied as fertilizer in agriculture (Solé-Bundó et al., 2017b). In this context, the occurrence of emerging organic contaminants (EOCs), such as pharmaceuticals and personal care products (PPCPs), in urban wastewater sludge is an important issue to be addressed (Matamoros et al., 2012). It is known that conventional systems (e.g. activated sludge systems) are generally not designed to treat these contaminants and that sludge contains considerable high amount of hydrophobic compounds such as musk fragrances or triclosan (log Kow>4) (Clarke and Smith, 2011). Hence, sludge reuse in agriculture is a potential source of crop exposure to these compounds (Macherius et al., 2012). In this regard, different approaches have been used to remove these contaminants from sewage sludge, such as anaerobic digestion or sludge treatment reed bed (STRB) systems. For instance,

Carballa et al. (2007) found that musk fragrances were removed around 60-70% after anaerobic digestion, but other authors showed no removal under similar conditions (Clara et al., 2010). On the other hand, Chen et al. (2009) observed that these compounds are barely removed in STRBs after one year of incubation, and suggested to increase it from two to more than three years. Nevertheless, studies which assessed the presence and removal of EOCs in anaerobic reactors degrading microalgal biomass are still missing.

The aim of this study was to evaluate the co-digestion of microalgal biomass and primary sludge, both by-products of a pilot-scale microalgae-based WWTP. For this, the co-digestion of 25% microalgae and 75% primary sludge (volatile solids (VS) basis) was investigated in continuous reactors and compared to the anaerobic digestion of microalgae alone. Furthermore, an energy assessment was carried out to determine the scalability of this technology. Finally, EOCs were analysed before and after the anaerobic digestion process in order to study their presence and fate.

3.2.2 Material and Methods

3.2.2.1 Substrates and inoculum

The experimental set-up was located at the laboratory of the GEMMA research group (Universitat Politècnica de Catalunya·BarcelonaTech, Spain) (Fig. 3.7). Microalgal biomass, hereafter called microalgae, was harvested from a pilot HRAP (0.5 m^3; 1.5 m^2) treating wastewater from the municipal sewer of Barcelona. The HRAP received the primary effluent of a settling tank (7 L; 0.9 h of HRT) and was used as secondary treatment unit. Microalgae were harvested from a secondary settler (9 L; 9 h of HRT) and thickened by gravity in laboratory Imhoff cones at 4 ºC for 24 hours. A detailed description of the wastewater treatment system operation and performance may be found elsewhere (Passos et al., 2015b). Microalgae species were periodically identified over the semi-continuous reactors operation using specific literature (Palmer, 1962). The optical microscope (Motic BA310E, China) used was equipped with a camara MRc5, using the software Axioplan LE.

Primary sludge and digested sludge used as inoculum for digesters start-up came from a municipal WWTP located nearby. The primary sludge was periodically collected (every 3 weeks) after thickening and stored at 4 °C before use. The inoculum was taken from a mesophilic digester.

Figure 3-7. Experimental high rate algal pound (laboratory of the GEMMA research group, Barcelona)

3.2.2.2 Digesters operation

The anaerobic co-digestion of microalgae with primary sludge was evaluated in two lab-scale reactors (2 L), with a useful volume of 1.5 L. The co-digestion of 75% primary sludge and 25% microalgal biomass (VS basis) and the anaerobic mono-digestion of microalgal biomass (control) were simultaneously investigated. Reactors were operated under mesophilic conditions (37 ± 1 ºC) by implementing an electric heating cover (Selecta, Spain). Constant mixing was provided by a magnetic stirrer (Thermo Scientific). Reactors were operated at an HRT of 20 days and were considered to be under steady-state after three HRTs. Afterwards, anaerobic digestion performance was further monitored during 2 complete HRTs (~6 weeks). The total experimental period of the digesters was 100 days.

The reactors were operated on a daily feeding basis. The same volume was purged from and added to digesters using plastic syringes (75 mL). Biogas production was measured by water displacement and methane content was periodically analysed by GC (Solé-Bundó et al., 2017c). To keep digesters fed with the same OLR, the reactors feeding was prepared once a week with a VS content of 4%. To adjust the solids concentration, distilled water was used when necessary.

3.2.2.3 Energy balance calculations

The theoretical energy balance of full-scale reactors was estimated from experimental data, considering a flow rate of 10 m^3/day and a useful volume of 200 m^3 for 20 days HRT. Electricity and heat requirements for microalgal biomass pretreatment and anaerobic digestion were calculated according to Passos and Ferrer (2014).

Input heat was calculated as the energy required to heat influent biomass from ambient temperature (Ta) to digestion temperature (Td), according to Eq. 3.2.1. The density (ϱ) and specific heat (γ) of microalgal biomass were assumed to be the same as those of water (i.e. 1000 kg/m^3 and 4.18 kJ/kg·°C, respectively). Heat losses through the reactor wall were calculated assuming the heat transfer coefficient (k) equal to 1 W/m^2·day. The reactor wall surface area was calculated from the reactor useful volume, considering a 2:1 diameter to height ratio. The reactor bottom and top were not accounted for.

$$Ei,heat = \varrho \cdot Q \cdot \gamma \cdot (Td - Ta) + k \cdot A \cdot (Td - Ta) \cdot 86.4 \qquad \text{(Eq. 3.2.1)}$$

where Ei,heat: input heat (kJ/d); ϱ: density (kg/m^3); Q: flow rate (m^3/day); γ: specific heat (kJ/kg·°C); Td: anaerobic digestion temperature (37 °C); Ta: ambient temperature (20 °C); k: heat transfer coefficient (W/m^2·°C); A: surface area of the reactor wall (m^2).

Furthermore, input electricity for anaerobic digestion was estimated as the energy required for biomass pumping and reactor mixing, which were assumed to be 1800 kJ/m^3 and 300 kJ/m$^3_{reactor}$·day, respectively (Eq. 3.2.2).

$$Ei,electricity = Q \cdot \theta + V \cdot \omega \qquad \text{(Eq. 3.2.2)}$$

where Ei,electricity: input electricity (kJ/d); Q: flow rate (m^3/day); θ: electricity consumption for pumping (kJ/m^3); V: useful volume (m^3); ω: electricity consumption for mixing (kJ/m$^3_{reactor}$·day).

The energy output of the process was calculated from the methane production rate ($P_{,CH4}$) of each reactor (control microalgae and co-digestion), according to Eq. 3.2.3. The lower heating value of methane (ξ) was assumed to be 35 800 kJ/m^3 CH_4. An efficiency of 90% on energy conversion was considered (η).

$$Eo = P_{,CH4} \cdot \xi \cdot V \cdot \eta \qquad \text{(Eq. 3.2.3)}$$

where Eo: output energy (kJ/d); $P_{,CH4}$: methane production rate mixing (m^3 CH_4/m$^3_{reactor}$·day).; ξ: lower heating value of methane (kJ/m^3 CH_4); V: useful volume (m^3); η: energy conversion efficiency (%).

Finally, results were expressed as energy balance (ΔE) and energy ratio (Eo/Ei) for both reactors (control microalgae and co-digestion). The energy balance was calculated as the difference between the energy output and energy input (heat and electricity) (Eq. 3.2.4), while the energy ratio was calculated by dividing the energy output by the energy input (heat and electricity) (Eq. 3.2.5).

$$\Delta E = Eo - (Ei,heat + Ei,electricity) \quad \text{(Eq. 3.2.4)}$$

$$Eo/Ei = Eo/(Ei,heat + Ei,electricity) \quad \text{(Eq. 3.2.5)}$$

3.2.2.4 Analytical procedures

Physical-chemical parameters of the influent and effluent of both reactors were determined as follows: temperature was monitored daily; pH was neither controlled nor regulated, but determined twice a week with a Crison Portable 506 pH-meter; the concentration of TS, VS, and total Kjeldhal nitrogen (TKN) were determined according to Standard Methods (APHA, 2005) and N-ammonium ($N\text{-}NH_4$) according to Solorzano method (Solorzano, 1969) on a weekly basis; Volatile fatty acids (VFA) concentrations in continuous flow digesters were measured once a week by injecting 1 μL of each sample, once centrifuged (4200 rpm for 8 min) and filtered (0.2 μm), into an Agilent 7820A GC after sulphuric acid and diisopropyl ether addition; the GC was equipped with an auto-sampler, flame ionization detector and a capillary column (DP-FFAB Agilent 30 m x 0.25 mm x 0.25 μm), and operated at injector and detector temperatures of 200 and 300 ºC, respectively, with helium as carrier gas.

Biochemical composition of microalgae and primary sludge was analysed by three samplings distributed throughout the experiment. Carbohydrate content was determined by a phenol–sulphuric acid method after acid hydrolysis and measured by spectrophotometry (Spectronic Genesys 8). Protein content was determined from the TKN, using a TKN/protein conversion factor of 5.95 (González López et al., 2010). Lipid content was determined by the Soxhlet extraction method (APHA, 2005). Values were expressed as percentage of lipids, carbohydrates and proteins over the VS content.

Biogas composition was calculated by measuring the percentage of methane and carbon dioxide in the reactor headspace using a GC equipped with a thermal conductivity detector (TCD) (Trace GC Thermo Finnigan with Hayesep packed

column). The injector/detector/oven temperatures were 150, 250, 35 °C, respectively. Helium gas was used as carrier.

The analytical mythology and quality parameters for the determination of EOCs (caffeine, methyl dihydrojasmonate, triphenyl phosphate, galaxolide, tonalide and triclosan, ibuprofen, naproxen) in the microalgae and sludge samples are described elsewhere (Matamoros et al., 2015).

3.2.2.5 Emerging organic contaminants data analysis

Concentrations of the selected EOCs were analysed for the feedstock (microalgae and primary sludge) and for the two digestates during a period of six weeks (weekly integrated samples).

The percentage of each contaminant removed in the anaerobic reactors was calculated according to the following mass balance (Eq. 3.2.6):

$$C_{remov}(\%) = \frac{C_{in} \cdot TS_{in} \cdot V_{in} - C_{out} \cdot TS_{out} \cdot V_{out}}{C_{in} \cdot TS_{in} \cdot V_{in}} \cdot 100 = \frac{C_{in} \cdot TS_{in} - C_{out} \cdot TS_{out}}{C_{in} \cdot TS_{in}} \cdot 100 \quad \text{(Eq. 3.2.6)}$$

where C_{remov} is the removal of the contaminant in %, C_{in} and C_{out} are the concentrations of the contaminant in the affluent and effluent, respectively, expressed as ng contaminant/gTS; TS_{in} and TS_{out} are the total solids concentration of the influent and effluent, respectively, expressed as gTS/L and Vin = Vout is the daily influent/effluent volume feeding the reactors, expressed in L.

3.2.2.6 Statistics and data analysis

The effect of the methane production rate and yield was determined by was determined by the ANOVA test using R 3.0.1 software (The R Foundation for Statistical Computing). $\varrho = 0.01$ was set as the level of statistical significance.

3.2.3 Results

3.2.3.1 Biogas production from microalgal biomass and primary sludge anaerobic co-digestion under continuous flow conditions

3.2.3.1.1 Substrates characterization

Microscope examination showed that microalgae were mainly composed of *Chlorella* sp. Microalgae individuals formed flocs, which facilitated their settling and

harvesting. An average concentration of 5.4 g TS/ 100g was achieved after settling and thickening. Biochemical analysis indicated that microalgae were mainly composed of proteins (58%), followed by lipids (24%) and carbohydrates (15%) (Fig. 3.8). These values are in accordance with those reported in the literature for *Chlorella* species (Safi et al., 2014b).

In contrast, primary sludge had higher amount of lipids (45%), followed by proteins (29%) and carbohydrates (12%) (Fig. 1). Other studies reported similar protein content but higher amount of carbohydrates than of lipids (Jimenez et al., 2013; Mahdy et al., 2014a). This was attributed to the high content of fibers in sludge. Indeed, primary sludge composition is highly variable and depends on many factors, such as wastewater source and characteristics, pretreatment and primary treatment steps design. Since the sludge investigated in this study presented high lipids content, a high methane potential was expected (Sialve et al., 2009). However, possible inhibition due to long-chain fatty acids (LCFA) must be considered (Cirne et al., 2007). Apart from its composition, primary sludge also differs from microalgae in its structure. While *Chlorella* sp. has a complex structure characterized by resistant cell walls, primary sludge is formed by colloidal organic matter which can be easily converted into biogas.

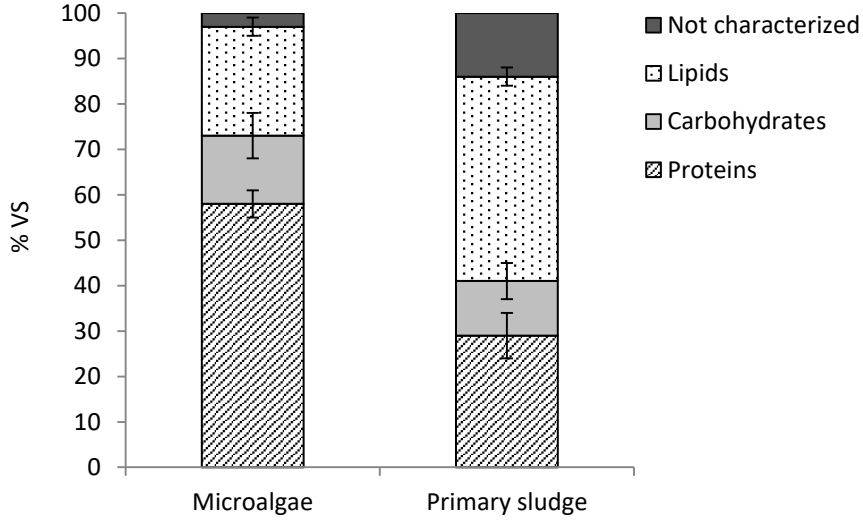


Figure 3-8. Microalgae and primary sludge composition as function of the volatile solids.

3.2.3.1.2 Reactors performance

Continuous co-digestion of 25% VS microalgae and 75% VS primary sludge and microalgae anaerobic mono-digestion (control) at 20 days of HRT were performed in lab-scale reactors during 100 days. In the case of co-digestion, the average methane yield was 0.33 m^3 CH_4/kg VS, which represented 65% increase as compared to microalgae mono-digestion (0.20 m^3 CH_4/kg VS) and the methane production rate increased from 0.38 to 0.63 m^3 CH_4/m^3·day (Table 3.5). Consistently, the VS and COD removal in microalgae digester was 25 and 31%, respectively. On the other hand, co-digestion digester achieved removal efficiencies of 47 and 53%, respectively. Thus, primary sludge co-digestion enhanced the anaerobic digestion of microalgae, leading to higher substrate biodegradability and biogas production. This is mainly because primary sludge is a more readily degradable carbon rich substrate. In addition, synergetic effects due to substrates co-digestion could have contributed to enhance their biodegradability, as already reported by other authors. For instance, Olsson et al. (2014) observed a 23% increase in methane yield when 63% of sewage sludge was co-digested with 37% of microalgae slurry in BMP compared to sewage sludge alone. Also, a kinetics increased by 116% was observed when lipid-spend microalgae residue was co-digested with waste activated sludge (Neumann et al., 2015). Furthermore, higher methane yields were achieved when co-digesting microalgae with primary sludge in batch experiments (5-10%) in comparison with the theoretically calculated methane yield of each substrate (Mahdy et al., 2015).

In the present study average methane yield of microalgae mono-digestion was high (0.20 m^3 CH_4/kg VS) compared to previous studies. This might be mainly due to the microalgae species. Indeed, it has been proven that microalgae biodegradability is highly specie-dependent (Mussgnug et al., 2010; Passos et al., 2015b). For instance, *Chlorella* is more easily degraded compared to other species grown in wastewater systems which are characterized by a more resistant cell walls, such as *Stigeoclonium* sp., *Oocystis* sp. or diatoms. Indeed, a previous study that performed the anaerobic digestion of a mixed culture of *Chlorella* sp., *Monoraphidium* sp. and diatoms grown in the same HRAP as this work, showed much lower average methane yield compared to that obtained in this study (0.12 m^3 CH_4/kg VS vs. 0.20 m^3 CH_4/kg VS, respectively) (Solé-Bundó et al., 2017c). Moreover, lower methane yield compared to that one obtained in the present study was also observed after applying a thermal pretreatment (Passos and Ferrer, 2015, 2014). Indeed, methane yields were 0.17 m^3 CH_4/kg VS for *Oocystis* sp. (Passos and Ferrer, 2015) and 0.18 m^3 CH_4/kg VS for a mix composed by *Monoraphidium* sp. and *Stigeoclonium* sp. (Passos and Ferrer, 2014).

Therefore, the predominance of *Chlorella* species in HRAP help increasing energy production from microalgae biomass.

Table 3-5. Biogas production from microalgal biomass with and without co-digestion. Mean values ± standard deviation.

Parameter	Control Microalgae	Co-digestion
Operational conditions		
HRT (days)	20	20
OLR (kg VS/m^3·day)	1.91 ± 0.27	1.89 ± 0.26
Biogas production		
Methane production rate (m^3 CH_4/Lr·day)	0.38 ± 0.10	0.63 ± 0.13 [a]
Methane yield (m^3 CH_4/kg VS)	0.20 ± 0.04	0.33 ± 0.05 [a]
Methane content in biogas (% CH_4)	65.5 ± 1.4	66.5 ± 1.5
Removal efficiency		
TS removal (%)	19.3 ± 4.4	38.9 ± 1.6 [a]
VS removal (%)	25.1 ± 4.1	46.8 ± 1.6 [a]
COD removal (%)	30.8 ± 8.8	53.2 ± 13.6 [a]
Influent		
pH	6.8 ± 0.3	6.0 ± 0.4
TS [% (w/w)]	5.4 ± 0.3	5.2 ± 0.3
VS [% (w/w)]	4.0 ± 0.1	3.9 ± 0.3
VS/TS (%)	73.8 ± 4.0	75.8 ± 3.2
COD (g/L)	67.7 ± 12.3	72.6 ± 12.6
TKN (g/L)	4.4 ± 0.4	2.8 ± 0.3
N-NH_4 (mg/L)	80 ± 40	88 ± 32

Parameter	Control Microalgae	Co-digestion
VFA (mg COD/L)	1026 ± 404	2962 ± 569
CST (s)	197 ± 5	178 ± 17
CST (s/g TS)	4 ± 0	3 ± 0
Effluent		
pH	7.5 ± 0.3	7.4 ± 0.3
TS [% (w/w)]	4.5 ± 0.2	3.2 ± 0.1
VS [% (w/w)]	2.9 ± 0.2	2.1 ± 0.1
VS/TS (%)	64.9 ± 2.7	65.6 ± 3.2
COD (g/L)	47.4 ± 8.3	32.9 ± 7.8
TKN (g/L)	4.4 ± 0.2	2.6 ± 0.0
N-NH_4 (mg/L)	1340 ± 160	744 ± 97
VFA (mg COD/L)	269 ± 174	156 ± 137
CST (s)	1575 ± 75	274 ± 56
CST (s/g TS)	35 ± 2	8 ± 2

[a] Stand for significantly higher values between paired columns ($\rho = 0.01$)

The OLR was as high as 1.9 kg VS/m^3·day, due to the high concentration of TS in the harvested biomass. Even if high values of OLR can lead to higher methane production rates, they can increase N-NH_4 concentrations in the digesters, causing inhibition. In this study, the N-NH_4 concentrations in the digestate was 1.3 and 0.7 g N-NH_4/L for microalgae mono-digestion and co-digestion, respectively (Table 3.5). Some authors have reported ammonium toxic concentrations of 1.7 g/L (Schwede et al., 2013b) or even 1.5 g/L when working at high pH (Rajagopal et al., 2013). In this study, N-NH_4 concentrations of microalgae digester were close to these values, being 2 times higher than that observed for co-digestion with primary sludge. However, digesters performance were stable during the whole experimental period, with an average pH of 7.4 - 7.5 for microalgae digestion and co-digestion, respectively

(Table 3.5). Also, VFA average values in digesters effluents were 269 mg HAc/L (microalgae) and 156 mg HAc/L (co-digestion). These values were much lower than the value established as threshold for a proper anaerobic digestion performance (e.g. 1.5 g HAc /L) (Boe et al., 2010). Although ammonia inhibition was not detected in this study, co-digestion with primary sludge may also enhance reactors stability, since it reduces ammonium concentration which can lead to inhibition. Indeed, the anaerobic digestion of *Chlorella vulgaris* at an OLR of 2.1g VS/L·day, achieved such a high toxic value (4.4 g N-NH_4/L) (Mahdy et al., 2017). However, in such a case, they received a previous protease enzymatic pretreatment which lead to higher ammonium release.

Regarding digestates dewaterability, lower values of CST were observed for microalgae co-digestion with primary sludge compared to microalgae mono-digestion (1575s vs. 274s, respectively), showing that the former significantly improved digestate dewaterability.

3.2.3.1.3 Energy considerations

The energy assessment of microalgae anaerobic digestion with and without primary sludge co-digestion (Table 3.6) was carried out considering the experimental results obtained from the continuous reactors (Tables 3.5). Since energy balances (ΔE) were calculated by subtracting the energy input (heat and electricity) to the energy output (biogas production), positive values indicate energy surplus. As can be seen in Table 3.6, energy gains were observed in both cases, achieving values of 1.6 and 3.2 GJ/day of net production for control and co-digestion reactor, respectively. Also, the ratio E_o/E_i indicated that microalgae anaerobic digestion generated 2.7-fold the energy applied. In the case of co-digestion, this ratio increased up to 4.5-fold. Considering the transformation of this potential energy to electricity by means of cogeneration with an electricity conversion efficiency of 35%, 151 and 307 kWh can be provided daily for control and co-digestion, respectively. Passos et al. (2017) estimated in 140 kWh/day the electricity demand of a HRAP with a similar biomass flow rate (15-55 m^3/day). Therefore, both configurations can supply the energy demand of the whole system. However, with the co-digestion, there is an energy surplus that can be sold back to the grid.

In view of these results, it can be concluded that anaerobic digestion is a key technology for the energy recovery in microalgal-based WWTPs, especially if microalgae is co-digested with primary sludge. In this regard, it is worthy to note that

the amount of microalgae produced in these systems depends on the climate and can vary during the year. A previous study which considered the same pilot-scale HRAP, showed that microalgae production during a year may vary from 3 g of suspended solids (SS)/m^3 in winter (minimum value) to 23 g SS/m^3 in summer (maximum value) (Passos et al., 2015b). On the other hand, the amount of primary sludge produced depends only on the characteristics of the influent wastewater and were defined as constant throughout the year. Therefore, the proportion of microalgae and primary sludge can vary throughout the year, from 70% to 30% VS of microalgae (Passos et al., 2017). Thus, the results of the energy balances obtained in this study should be taken as approximate values only.

Table 3-6. Energy assessment of microalgal biomass anaerobic digestion with and without co-digestion with primary sludge

Parameter	Control Microalgae	Co-digestion
E_i (GJ/day)	0.90	0.90
$E_{i,heat}$ (GJ/day)	0.82	0.82
$E_{i,electricity}$ (GJ/day)	0.08	0.08
E_o (GJ/day)	2.45	4.06
E_o/E_i	2.7	4.5
$\Delta E = E_0 - E_i$ (GJ/day)	1.55	3.16

3.2.3.2 Emerging organic contaminants fate and removal

3.2.3.2.1 Occurrence of EOCs

The compounds were selected in bases to their high concentration levels found in raw wastewater and sludge samples (Yang et al., 2016). Among the 8 analysed EOCs, only 6 were detected in the microalgal or sewage sludge samples (Fig. 3.9). The concentration of compounds in sludge samples ranged from non-detectable to 25,000 ng/g TS. Galaxolide, tonalide and triclosan were the most abundant (>500 ng/g TS) in agreement with the fact that they were the most hydrophobic ones (log Kow>4). The concentration of these musk fragrances in sludge samples were similar to those found in the sludge from conventional WWTPs (Bester, 2004; Gonzalez-Gil et al., 2016; Kupper et al., 2004). Other compounds such as caffeine, triphenyl

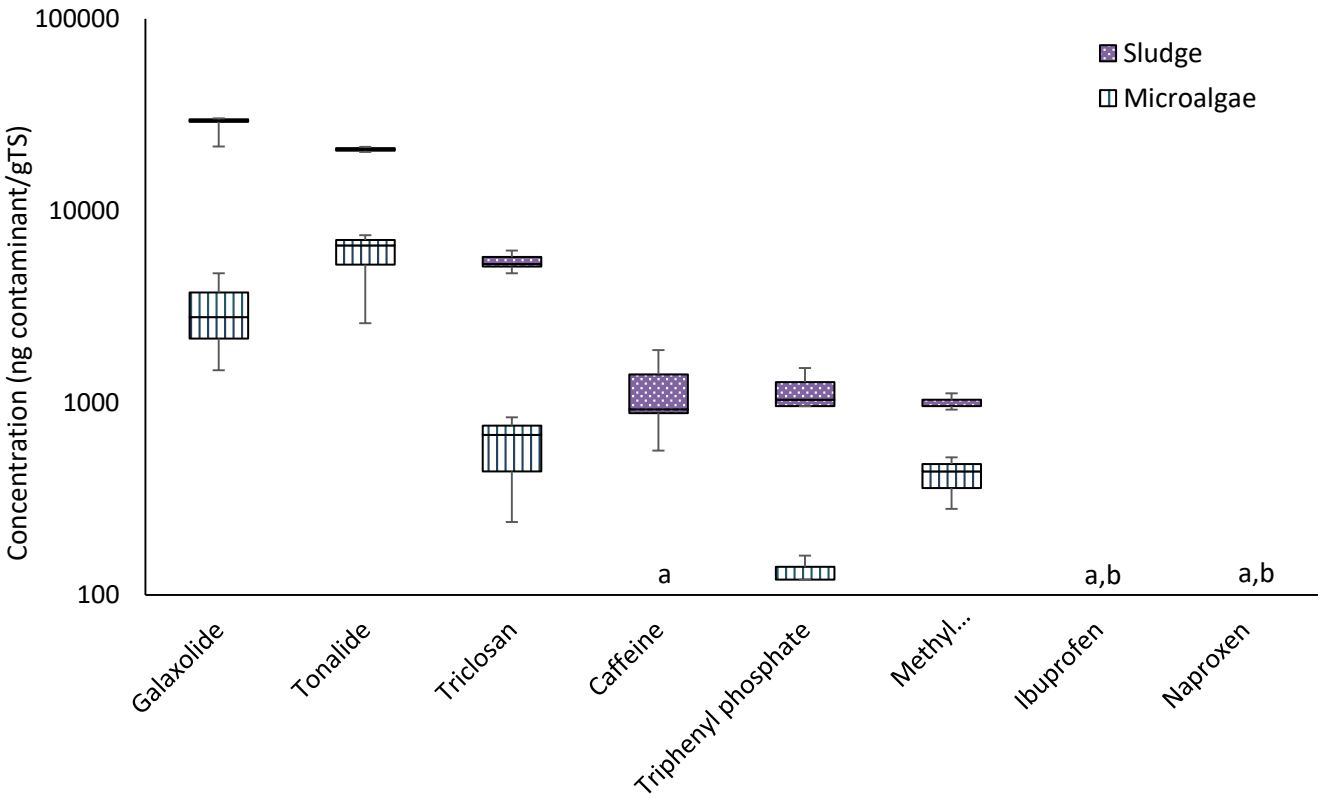


Figure 3-9. Occurrence of eight emerging contaminants on microalgae and primary sludge substrates.

a: values < limit of detection for microalgae; b: values < limit of detection for sludge

phosphate and methyl dihydrojasmonate were also found, but at much lower concentration (<100 ng/g TS). These compounds are usually detected at very high concentrations in raw wastewater, but since they are hydrophilic their interaction with the organic matter is low. For instance caffeine has been detected in raw wastewaters up to 300 μg/L (Buerge et al., 2003).

The concentration of EOCs in the non-digested samples was lower in the microalgae than sludge samples. This was due to the fact that sludge is originated from primary treatment where the concentration of these compounds is higher, whereas microalgae is originated from a secondary treatment. In this regard, it is important to notice that since the most abundant compounds are hydrophobic they tend to adsorb onto the organic matter and suspended solids, which are predominantly retained during the primary treatment. This was in accordance with previous studies that observed low concentration of these compounds in microalgae biomass from a HRAP in comparison with sludge from a conventional WWTP (Matamoros et al., 2015).

3.2.3.2.2 Removal of PPCPs during AcoD

Table 3.7 shows the removal efficiency of selected ECOs during the digestion of microalgae and sewage sludge respectively. The removal for the compounds identified in all samples (i.e. galaxolide, tonalide, triclosan, and methyl dihydrojasmonate) ranged from no removal to 90%. The compounds which occurred at the highest concentration showed the lowest removal efficiency due to their recalcitrance to biodegradation (Gonzalez-Gil et al., 2016). Methyl dihydrojasmonate and caffeine showed higher removal efficiencies than musk fragrances and triclosan, which is in agreement with the high biodegradability for these compounds already observed in WWTPs (Schaider et al., 2017). Kupper et al. (2006), which carried out a mass balances in a Swiss sludge monitoring network, reported that galaxolide and tonalide were reduced by 50% during sludge anaerobic digestion. Carballa et al. (2006) observed average removal of these compounds during mesophilic and thermophilic digestion, ranging between 60% and 70% (Table 3.7). On the contrary, Clara et al. (2011) reported no or only slight removal during sludge anaerobic digestion. The low efficiencies of the anaerobic digestion on the musk fragrances removal observed in this study are also in agreement with previous studies which observed that anaerobic digestion resulted in a lower removal of musk fragrances than aerobic digestion (Guerra et al., 2015).

Table 3-7. Concentration of EOC of the influents and effluents of the anaerobic digesters and their EOC removal.

	Control Microalgae			Co-digestion		
EOC	Influent (ng/g TS)	Effluent (ng/g TS)	Removal (%)	Influent (ng/g TS)	Effluent (ng/g TS)	Removal (%)
Galaxolide	2,791 ± 1,002	2,273 ± 675	32	18,836 ± 3,122	33,190 ± 7,955	-10
Tonalide	5,748 ± 1,941	4,057 ± 1,236	41	11,271 ± 617	17,376 ± 3,885	3.7
Triclosan	576 ± 232	417 ± 180	39	3,580 ± 406	5,940 ± 1,757	-3.7
Methyl dihydrojasmonate	37 ± 13	26 ± 9	41	47 ± 6	22 ± 5	71
Caffeine	< LOD	< LOD	-	81 ± 29	< LOD	> 92
Triphenyl phosphate	14 ± 4	< LOD	-	75 ± 18	43 ± 23	64

Note: LOD = limit of detection

The average removal of EOCs was of 38% and 15% for microalgae digestion and co-digestion, respectively. The higher EOCs removal in microalgae digestion might be due to the better biodegradation of EOCs due to microalgae chemical composition. This may suggest that bacteria grown under such condition will be more effective for removing EOCs, but other conclusions cannot be disregarded.

3.2.4 Conclusions

This study analysed the anaerobic co-digestion of primary sludge and microalgae, which represent the by-products of microalgal-based wastewater treatment systems. The mesophilic co-digestion of 25% microalgae and 75% primary sludge (on volatile solids basis) was investigated in continuous reactors and compared to microalgae mono-digestion at a hydraulic retention time of 20 days. Results showed that co-digestion enhanced the anaerobic digestion of microalgal biomass, since primary sludge is a more readily degradable carbon rich substrate, leading to higher methane production (65% increase), while reducing the risk of ammonia toxicity. Moreover, the occurrence and fate of the most common emerging organic contaminants was evaluated. Musk fragrances (galaxolide and tonalide) and triclosan showed the highest abundance (0.5-25 μg/gTS). On the other hand, caffeine, methyl dihydrojasmonate and triphenyl phosphate were barely detected (<0.1 μg/g dry weight). The removal of these contaminants was compound-depending and ranged from no removal to 90%. Nevertheless, results showed that microalgae mono-digestion resulted in a higher removal of selected contaminants than the co-digestion with primary sludge.

3.3 Assessing the agricultural reuse of the digestate from microalgae co-digestion with sewage sludge

Microalgae anaerobic digestion produces biogas along with a digestate that may be reused in agriculture. However, the properties of this digestate for agricultural reuse have yet to be determined. The aim of this study was to characterise digestates from different microalgae anaerobic digestion processes (i.e. digestion of untreated microalgae, thermally pretreated microalgae and thermally pretreated microalgae in co-digestion with primary sludge). The main parameters evaluated were organic matter, macronutrients and heavy metals content, hygenisation, potential phytotoxicity and organic matter stabilisation. According to the results, all microalgae digestates presented suitable organic matter and macronutrients, especially organic and ammonium nitrogen, for agricultural soils amendment. However, the thermally pretreated microalgae digestate was the least stabilised digestate in comparison with untreated microalgae and co-digestion digestates. In vivo bioassays demonstrated that the digestates did not show residual phytotoxicity when properly diluted, being the co-digestion digestate the one which presented less phytotoxicity. Heavy metals contents resulted far below the threshold established by the European legislation on sludge spreading. Moreover, low presence of *E. coli* was observed in all digestates. Therefore, agricultural reuse of thermally pretreated microalgae and primary sludge co-digestate through irrigation emerges a suitable strategy to recycle nutrients from wastewater.

3.3.1 Introduction

Microalgae-based wastewater treatment systems represent a cost-effective alternative to conventional activated sludge systems. The major advantage is that mechanical aeration is not required, since oxygen is provided by microalgae photosynthesis. Moreover, microalgae cultures are capable of removing nutrients (N, P) from wastewater by means of different mechanisms, such as assimilation or precipitation (Rawat et al., 2011). Furthermore, these systems can also combine wastewater treatment and bioenergy production if harvested microalgal biomass is downstream

processed. In particular, anaerobic digestion is one of the most well-known processes to valorise organic waste generated in a wastewater treatment plant. Over the last decades, several studies on biogas production from microalgae have been carried out (Uggetti et al., 2017). They have demonstrated that some microalgae species have a resistant cell wall, which may hamper their bioconversion into methane. Microalgae cell wall disruption could be enhanced by applying pretreatment methods, being the most suitable those pretreatments with low energy demands (Passos et al., 2014b). Besides, in the context of microalgae grown in wastewater, co-digestion of microalgae with sewage sludge is a profitable strategy, since the sludge is generated in the same process chain (Uggetti et al., 2017). This could optimise waste management and increase the organic loading rate of the digester (Mata-Alvarez et al., 2014).

Apart from biogas, microalgae anaerobic digestion also produces a digestate that can be reused in agriculture. Even though several studies have pointed out the necessity of recycling nutrients through digestate reuse to improve the sustainability of biogas production from microalgae (Collet et al., 2011), the properties of microalgae digestate for agricultural reuse have yet to be characterised. In general, anaerobic digestates have proper chemical properties for agricultural reuse (Rowell et al., 2001). For instance, they are rich in ammonia nitrogen, readily available for plant uptake, and other macronutrients such us phosphorus and potassium (Teglia et al., 2011a). However, depending on digestates properties, their reuse could be more addressed to improve or maintain the physico-chemical or biological properties of soils (soil amendment) or to boost the plants growing (fertilisers). In the first case, digestates with high organic matter, organic carbon and organic nitrogen content are preferred, while digestates with important mineral fractions have a higher potential for application as fertiliser (Nkoa, 2014).

Anaerobic digestion is often designed to achieve the maximum energy production, leading to a low stabilisation of the organic matter of the feedstock. As a consequence, digestates may be characterised by a high labile organic matter content and, thus, their agricultural reuse may face agronomic and environmental issues. In fact, it is known that by adding low-stabilised organic matter the soil microbial activity may be excessively stimulated. Indeed, it can produce high CO_2 fluxes from the soil, soil oxygen consumption with sequential nitrogen losses, and phytotoxicity phenomena (Pezzolla et al., 2013; Abdullahi et al., 2008). In addition, the digestate composition can highly vary depending on the feedstock or anaerobic digestion operating conditions. Even the application of a pretreatment on the feedstock

previous to anaerobic digestion can influence the final composition of the digestate (Monlau et al., 2015a). Thus, the characterisation of a digestate before evaluating its potential applications is convenient.

When characterising new digestates, particular attention should be addressed to the macronutrients content, potential phytotoxicity and stabilization of the organic matter. *In vivo* bioassays are useful to assess the potential phytotoxicity (José Antonio Alburquerque et al., 2012; Zucconi et al., 1985). The quantification of CO_2 emissions and the water extractable organic matter (WEOM) in digestate amended soils are suitable strategies to assess organic matter stabilization (Pezzolla et al., 2013; Said-Pullicino and Gigliotti, 2007). On the other hand, land application of anaerobic digestates may also introduce physical, chemical and biological contaminants into soils which may be up-taken by crops and endanger their long-term agricultural activity (Nkoa, 2014). For instance, European legislation on sewage sludge spreading (EC Directive 86/278/CEC) mainly regulates the heavy metals content in digestates to avoid their accumulation in amended soils. However, a more recent European Directive draft (2003/CEC) also proposes restrictions on the occurrence of bio-accumulative organic compounds and their hygenisation before being spread on soils. Consequently, the presence of these contaminants in digestates should be assessed if they are going to be reused in agricultural soils.

The aim of this study was to characterise for the first time the quality of microalgae digestates for agricultural reuse. To this end, the effluents from three different anaerobic digesters fed by untreated microalgae, thermally pretreated microalgae and thermally pretreated microalgae in co-digestion with primary sludge were analysed. The main parameters evaluated were organic matter, macronutrients and heavy metals content, hygenisation, potential phytotoxicity and organic matter stabilisation.

3.3.2 Material and Methods

3.3.2.1 Digestate origin and sampling

The microalgal biomass used in this study consisted of a microalgae-bacteria consortia grown in a pilot raceway pond that treated wastewater from a municipal sewer, as described by (Passos et al., 2015b). Microalgal biomass was harvested from secondary settlers and gravity thickened in laboratory Imhoff cones at 4 ºC for 24 hours. The pilot plant was located at the laboratory of the GEMMA research group (Barcelona, Spain). According to optic microscope examinations (Motic BA310E,

equipped with a camera NiKon DS-Fi2), predominant microalgae were *Chlorella* sp. and diatoms (Fig. 3.10).

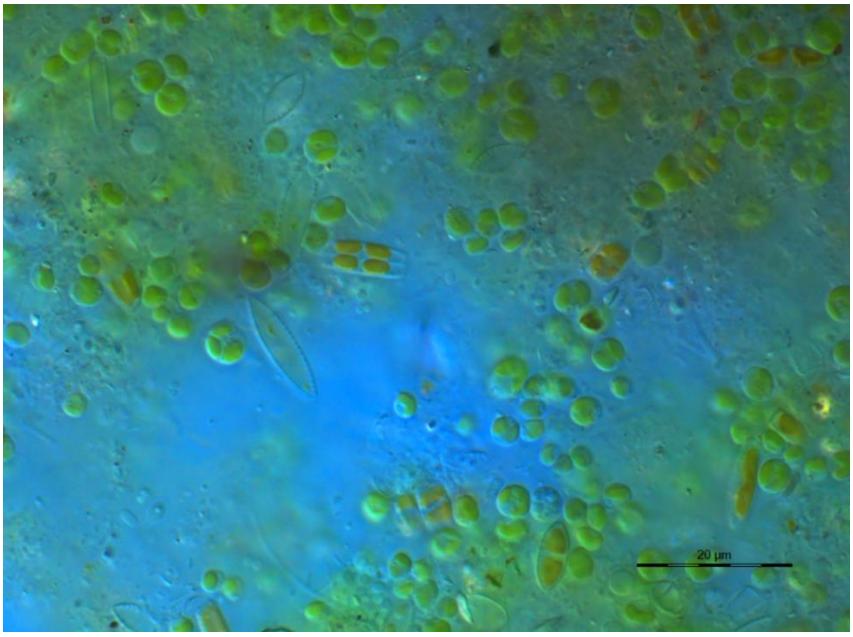


Figure 3-10 Microscopic image of microalgal biomass mainly composed by Chlorella sp. and diatoms.

In order to improve microalgae biodegradability, a part of the harvested and thickened biomass was thermally pretreated at 75 ºC for 10h, as suggested by Passos and Ferrer (2014). The pretreatment of microalgal biomass was carried out in glass bottles with a total volume of 250 mL and a liquid volume of 150 mL, which were placed in an incubator under continuous stirring at 75 ºC for 10h. Untreated (control) and pretreated microalgae were digested in lab-scale reactors under mesophilic conditions. Furthermore, the anaerobic co-digestion of pretreated microalgal biomass with primary sludge (25%-75% VS, respectively) was also evaluated. The thickened primary sludge was collected in a municipal wastewater treatment plant near Barcelona.

Thus, the following effluents from microalgae anaerobic digestion were analysed:

- Digester 1 (D1): Microalgal biomass;
- Digester 2 (D2): Thermally pretreated microalgal biomass;

- Digester 3 (D3): Co-digestion of pretreated microalgal biomass and primary sludge.

Anaerobic reactors (1.5 L) were operated on a daily feeding basis, where same volume was purged from and added to digesters using plastic syringes. Operation conditions of the reactors and feedstock characteristics are shown in Table 3.8. Digestate samples were analysed weekly over a period of 11 weeks of stable reactors operation. Physico-chemical properties were analysed during 11 weeks (n=11) while macronutrients and pathogens were analysed during the last 6 weeks (n=6) and the heavy metals during the 3 last weeks (n=3).

Table 3-8. Main parameters of the anaerobic digestion and feedstock properties.

	Digester 1 (D1): Microalgae	Digester 2 (D2): Pretreated microalgae	Digester 3 (D3): Co-digestion
Operation conditions			
Temperature (ºC)	36.2 ± 1.1	36.6 ± 1.8	35.7 ± 1.8
OLR (gVS/L.day)	0.83 ± 0.04	0.82 ± 0.02	0.83 ± 0.01
HRT (days)	30	30	30
Feedstock			
Composition (% VS)	100 % M	100 % Mp	25 % Mp + 75% PS
TS (%)	3.9 ± 0.4	3.7 ± 0.3	3.7 ± 0.4
VS (%)	2.5 ± 0.2	2.4 ± 0.1	2.4 ± 0.1
VS/TS (%)	66 ± 5	66 ± 6	66 ± 8
COD (g/L)	43.4 ± 8.1	44.0 ± 7.0	48.1 ± 8.0

Note: M= microalgal biomass; Mp= pretreated microalgal biomass, PS= primary sludge. Pretreatment conditions: 75ºC, 10h.

3.3.2.2 Digestate characterisation

3.3.2.2.1 Physicochemical properties and macronutrients

Total solids (TS), volatile solids (VS), total chemical oxygen demand (COD) and total Kjeldahl nitrogen (TKN) were analysed according to Standard Methods (APHA, 2005). Ammonium nitrogen (NH_4^+-N) was measured according to the Solorzano

method (Solorzano, 1969). Volatile fatty acids (VFA) concentrations were measured by injecting 1 μL of centrifuged (4200 rpm for 8 min) and filtered samples (0.2 μm) into an Agilent 7820A GC after sulphuric acid and diisoprppyl ether addition. The GC was equipped with an auto-sampler, flame ionization detector and a capillary column (DP-FFAB Agilent 30 m x 0.25 mm x 0.25 μm), and operated at injector and detector temperatures of 200 and 300ºC, respectively, with helium as carrier gas. Electric conductivity (EC) was determined with a Crison EC-Meter GLP 31+ and pH with a Crison Portable 506 pH-meter. Total organic carbon (TOC) and total nitrogen (TN) were measured using an automatic analyser (aj- Analyzer multi N/C 2100S). TOC was analysed with an infrared detector (NDIR) according to combustion-infrared method of Standard Methods (APHA, 2005) by means of catalytic oxidation at 800 ºC using CeO_2 as catalyst. Following, a solid-state chemical detector (ChD) was used to quantify TN as NOx. Phosphorous was determined by means of Olsen-P modified method (Watanabe and Olsen, 1965). Ca^{+2} and Mg^{+2} were analysed by EDTA titrimetric method after ammonium acetate extraction (1N at pH 7), while Na^{+} and K^{+} were determined by flame photometric method after ammonium acetate extraction (1N at pH 7) (MAPA, 1994). Dewaterability was evaluated by means of the capillary suction time (CST) test (Triton Electronics Ltd.).

3.3.2.2.2 Heavy metals

In order to determine the heavy metals concentration, samples were dried at 100 ºC during 24h. After HCL-HNO_3 (3:1, v/v) digestion (200ºC, 15 min) of dry digestate, Cd, Cr, Cu, Hg, Ni, Pb and Zn were determined by inductively coupled plasma mass spectrometry (ICP-MS) (Perkin Elmer Elan 6000).

3.3.2.2.3 *Pathogens*

Escherichia coli (*E. coli*) was determined according to Standard Methods (APHA, 2005). The *E. coli* ChromID™ Coli (COLI ID-F) used in this study was supplied by Biomérieux and the culture medium was m-coliBlue24® from Difco.

3.3.2.3 Organic matter stabilisation

3.3.2.3.1 Soil incubation procedure

Organic matter stabilisation from digestates was evaluated through a microcosm soil experiment. Fresh digestates were used to amend an agricultural soil (soil chemical characterization not shown), using a digestate dose according to the limits prescribed by the European Nitrates Directive (91/676/CEC) for the protection of

groundwater against pollution caused by nitrates. Specifically, digestate application doses were calculated to apply 170 kg N ha^{-1}. 200g of soil (dry matter) were amended and placed in an incubation chamber (20 ± 2 °C) for 30 days at 70% of the water holding capacity.

3.3.2.3.2 CO_2 emissions evaluation

CO_2 emissions resulting from the organic matter mineralization were measured after 0, 2, 5, 8, 12, 20 and 30 days of amending, using an alkaline-trap and subsequent titration. At the same time, 10 g (fresh weight) of soil were collected and air-dried for the WEOM determination.

3.3.2.3.3 Water extractable organic matter determination

The WEOM was analysed both in the digestates and amended soils. Fresh digestate samples were centrifuged at 4,200 rpm for 6 min and filtered through a 0.45 μm membrane filter (GVS). Soil WEOM was extracted from the dry soil samples with deionised water (solid to water ratio of 1:10 w/w) for 24 h. The suspensions were then centrifuged at 4,200 rpm for 6 min and filtered through a 0.45 μm membrane filter. Water Extractable Organic Carbon (WEOC) concentration in the filtrates was then measured by an automatic analyser (Analytic Jena-Analyzer multi N/C 2100S) and the WEOM was calculated according the following equation (Pribyl, 2010):

$$WEOM = WEOC \cdot 2.0 \qquad \text{(Eq. 3.3.1)}$$

3.3.2.4 Potential phytotoxicity

3.3.2.4.1 Seed germination bioassay

To evaluate the germination index (GI), a modified phytotoxicity test employing seed germination was used (Zucconi et al., 1985). Pure digestates together with three dilutions (0.1 %, 1 % and 10 % v/v in deionised water) were used as germination media. A filter paper placed inside a 9 cm diameter Petri dish was wetted with 1 mL of each germination solution and 10 *Lepidium sativum* L. seeds were placed on the paper. 100% deionised water was used as a control. Five replicates were set out for each treatment. The Petri dishes, closed with plastic film to avoid moisture loss, were kept in the dark for 2 days at 20 °C. After the incubation period, the number of germinated seeds and the primary root length were measured. The GI was expressed as a percentage of the control.

3.3.2.4.2 Plant growth bioassay

To evaluate the influence of digestate on plant biomass accumulation, a modified phytotoxicity test employing plant growth was used (Alburquerque et al., 2012). Plastic seedbeds made of 12 cells (50 mL/cell with a drainage hole in the bottom) were used for the experiment, after filling them with commercial perlite (2-3 mm diameter). Seedbeds were placed 24 h in a vessel (20x15x5 cm) containing 500 mL of deionised water to reach the saturation of the substrate. Then, 5 seeds of *Lepidium sativum* L. were sown in each cell. After the 3 days needed for the germination and seedlings occurrence, 32 seedlings were left in each seedbed and deionised water was replaced by 500 mL of the digestate dilutions to be tested (0.1 %, 1% and 10% v/v). Pure digestates were not tested in this case, since no germination was observed in the germination test. One seedbed was used as a control, leaving 100% deionised water as growth media. During all the experiment, the vessels were placed in environmental controlled conditions (25 ± 2 °C, daily photoperiod of 14 h). At the end of the experiment, after 10 days from the replacement of the growth media, seedlings survived were harvested and their total dry mass (TS) was determined after drying at 105 °C. The growth index (GrI) was calculated for each digestates as the percentage of the control (distilled water). The whole experiment was replicated three times.

3.3.3 Results and Discussion

3.3.3.1 Physico-chemical characterisation

All the digestates analysed presented low dry matter content (~3% TS) (Table 3.9) and can be considered as liquid products. To ease their management, these digestates could be directly spread on soils in nearby areas. However, if transportation/distribution was required, a dewatering process to reduce the moisture content would be recommended. If we look at the CST measurements, which estimate the ability of each digestate to release water (Gray, 2015), we can see how microalgae digestates presented poor dewaterability (25 and 28 s·L/gTS·L for D1 and D2, respectively), while these results were consistently improved by the co-digestion of primary sludge (8 s·L/gTS·L) (Table 3.9). This is due to the higher dewaterability of primary sludge digestate with respect to microalgae digestate.

On the other hand, the measured pH presented slightly-alkaline values in all digestates (>7.0). Among them, pretreated microalgae digestate (D2) presented the highest pH value, which can be attributed to the higher concentration of NH_4^+-N released from proteins during the thermal pretreatment (Passos and Ferrer, 2014).

However, all pH values are compatible with the common pH on soils and therefore, their application should not affect the soil pH.

Other factors that may cause an impact on soils after digestate spreading are the EC and VFA's content, since phytotoxicity effects have been correlated to both parameters (José Antonio Alburquerque et al., 2012; Di Maria et al., 2014). Although EC was moderate in all digestates (5.9-8.2 dS/m), the digestate from the co-digestion showed the lowest value. Consequently, it would cause less impact on soil. Besides, all digestates showed low VFA's concentrations (Table 3.9). Again, the lowest value was found in the co-digestion digestate (10 mg COD-eq/L). This indicates that the anaerobic digestion process results in a more stabilised digestate when pretreated microalgae are co-digested with the primary sludge.

3.3.3.2 Organic matter and fertiliser properties

The three digestates had moderate organic content due to organic matter mineralization during the anaerobic digestion process. While the two microalgae digestates presented a similar VS/TS ratio of 53-54%, the percentage of organic matter in the co-digestion digestate was lower (47%) due to the higher mineralization of primary sludge, which is a more readily biodegradable substrate than microalgae. In fact, the percentage of organic matter in digestates is highly dependent on the type of substrate and the operating conditions of anaerobic reactors (Monlau et al., 2015b). For instance, Teglia et al. (2011a) compared digestates from different origins and found that digestates from agri-food industries showed higher organic matter content than digestates from sewage treatment plants. The results obtained in this study are in accordance with those from similar microalgae anaerobic digestion processes (Passos and Ferrer, 2014, 2015).

Several studies have shown that anaerobic digestates can be as effective as mineral fertilisers (Nkoa, 2014). To assess the fertiliser properties of the microalgae

Table 3-9. Main physico-chemical properties and organic matter of the three microalgae digestates analysed (mean ± standard deviation; n=11, except for TOC and TN (n=3)).

Parameter	Units	Digestate D1: Microalgae	Digestate D2: Pretreated microalgae	Digestate D3: Co-digestion
pH	-	$7.35^{a} \pm 0.11$	$7.55^{b} \pm 0.08$	$7.30^{a} \pm 0.15$
EC	dS/m	$7.0^{b} \pm 0.7$	$8.2^{a} \pm 0.3$	$5.9^{c} \pm 0.4$
TS	g/g, %	$3.0^{a} \pm 0.1$	$2.9^{a} \pm 0.2$	$3.0^{a} \pm 0.2$
VS	g/g, %	$1.6^{b} \pm 0.1$	$1.5^{b} \pm 0.1$	$1.4^{a} \pm 0.1$
VS/TS	%	$54^{b} \pm 2$	$53^{b} \pm 1$	$47^{a} \pm 2$
COD	g/L	$26^{a} \pm 2$	$25^{a} \pm 2$	$24^{a} \pm 1$
TOC	g/L	7.6 ± 0.1	6.4 ± 0.0	6.1 ± 0.1
TN	g/L	2.4 ± 0.0	2.2 ± 0.1	1.9 ±0.1
C/N	-	3.17	2.98	3.27
VFA	mgCOD-eq/L	$100^{a} \pm 138$	$270^{a} \pm 365$	$10^{a} \pm 25$
CST	s	$795^{b} \pm 71$	$919^{b} \pm 122$	$272^{a} \pm 21$
	s·L/gTS	$25^{b} \pm 3$	$28^{b} \pm 4$	$8^{a} \pm 1$

[a,b,c] letters indicate a significant difference between digestates at a level of $p < 0.05$ after Tuckey's test.

Note: TS= total solids, VS= volatile solids, COD= chemical oxygen demand, TOC= total organic carbon, TN= total nitrogen, C/N= Carbon-Nitrogen ratio, VFA= volatile fatty acids, CST= capillary suction time

Table 3-10. Macronutrients characterisation of the three digestates analysed (mean ± SD, n=6).

Parameter	Units	Digestate D1: Microalgae	Digestate D2: Pretreated microalgae	Digestate D3: Co-digestion
TKN	gN/L	$2.4^{a} \pm 0.1$	$2.3^{a} \pm 0.1$	$1.7^{b} \pm 0.0$
	gN/kg TS	$79.8^{a} \pm 4.0$	$80.6^{a} \pm 2.2$	$56.0^{b} \pm 1.1$
NH_4^+-N	gN/L	$0.7^{b} \pm 0.1$	$0.8^{b} \pm 0.1$	$0.5^{a} \pm 0.1$
NH_4^+-N/TKN	%	30.9	33.8	32.5
P	gP_2O_5/L	$0.25^{b} \pm 0.02$	$0.27^{b} \pm 0.02$	$0.21^{a} \pm 0.03$
	gP/kg TS	$3.6^{b} \pm 0.3$	$3.9^{b} \pm 0.2$	$3.2^{a} \pm 0.5$
K	gK_2O/L	$0.17^{b} \pm 0.03$	$0.19^{b} \pm 0.02$	$0.08^{a} \pm 0.03$
	gK/kg TS	$4.8^{b} \pm 0.8$	$5.2^{b} \pm 0.7$	$2.2^{a} \pm 1.0$
Ca	gCaO/L	$0.43^{a} \pm 0.13$	$0.37^{a} \pm 0.10$	$0.54^{b} \pm 0.07$
	gCa/kg TS	$10.2^{a} \pm 3.1$	$8.9^{a} \pm 2.4$	$13.4^{b} \pm 1.7$
Mg	gMgO/L	$0.18^{a} \pm 0.09$	$0.21^{a} \pm 0.09$	$0.17^{a} \pm 0.10$
	gMg/kg TS	$3.6^{a} \pm 1.8$	$4.2^{a} \pm 1.8$	$3.6^{a} \pm 2.0$
Na	gNa_2O/L	$0.40^{b} \pm 0.05$	$0.38^{b} \pm 0.06$	$0.32^{a} \pm 0.03$
	gNa/kg TS	$10.0^{b} \pm 1.3$	$9.4^{b} \pm 1.4$	$8.1^{a} \pm 0.8$

^{a,b} letters indicate a significant difference between digestates at the level of $p < 0.05$ after Tuckey's test

Note: TKN= total Kjeldahl nitrogen

digestates, the macronutrients content was here evaluated (Table 3.10). The main nutrient present in all digestates was nitrogen. Even so, the nitrogen content of microalgae digestates (both untreated and thermally pretreated) was significantly higher than the co-digestion digestate (39-42%), showing values of 80 g/kg TS and 56 g/kg TS, respectively. Microalgae digestates presented similar nitrogen values compared to those from farm-byproducts that are frequently applied as nitrogen suppliers on soils (Alburquerque et al., 2012; Zucconi et al., 1985). Moreover, the nitrogen content was much higher than the common values found in sewage sludge digestates (36-40 g/kg TS) (Di Maria et al., 2014; Gell et al., 2011), even in the co-digestion digestate. The highest concentration of NH_4^+-N was found in the pretreated microalgae digestate. However, the NH_4^+-N/TKN ratio only varied from 30.9 to 33.8% among all digestates, presenting all of them a similar soluble mineral nitrogen fraction. This means that the organic nitrogen fraction is predominating in all digestates, so they should be used as soil amendment rather than fertiliser (Teglia et al., 2011b). As expected, the digestates also showed low C/N ratios around 3 (Table 3.10). These values are within the typical range for other digestates as sewage sludge, poultry slurry or pig slurry (José Antonio Alburquerque et al., 2012; Gutser et al., 2005). Unfortunately, with low C/N ratios, N is present in excess and it can be lost by ammonia volatilization or leaching (Bernal et al., 2009). In order to increase the carbon content in microalgae digestates, they could be co-digested with other carbon rich substrates, like waste paper (Yen and Brune, 2007).

Moderate quantities of P and K^+ were also found in all the digestates (Table 3.10). P content was slightly higher in microalgae digestates (D2 and D3) compared to the digestate obtained by the co-digestion (3.6-3.9 and 3.2 g P/kg TS, respectively). On the other hand, the content of K^+ of the microalgae digestates was 2-fold higher compared to the digestate obtained by the co-digestion (4.8-5.2 and 2.2 g K/kg TS, respectively). Conversely to nitrogen, no significant differences were found between P and K^+ contents of microalgae and sewage sludge digestates. In particular, literature reported values from 2.2-3.0 g K/kg TS and 3.2-3.8 g P/kg TS in sewage sludge digestates (Di Maria et al., 2014; Gell et al., 2011; Tambone et al., 2010), which fall within the range of the co-digestion digestate analysed in the present study. Ca^{2+}, Mg^{2+} and Na^+ presented similar concentrations in all the cases. This can be attributed to the composition of the wastewater treated in both systems where microalgae and primary sludge were obtained, which came from the same water source. The content of salts should be carefully analysed when applying the digestates to the soils to avoid their salinization, especially the presence of Na^+ (Daliakopoulos et al., 2016).

On the whole, microalgae digestates could especially contribute to nitrogen supply on soils. However, with a moderate NH_4^+-N/TKN ratio (<35%) their use should be addressed as soil amendment rather than direct biofertiliser. Indeed, the digestates nutrients content was lower than those recommended by the standards of European countries that have regulated the commercial uses of liquid fertilisers (EC 2003/2003). Conversely, their organic matter content and their high mineral and organic nitrogen content make them suitable for land spreading. Nonetheless, the stability of organic matter and potential toxicity of digestates must be taken into account, along with their potential risks on soil contamination. These issues are analysed and discussed in the following sections.

3.3.3.3 Stabilisation of the organic matter

Figure 3.11A shows the CO_2 emissions measured from the digestate amended soils studied in the microcosm experiment. Whereas the control (un-amended soil) showed moderately constant emission rates throughout the incubation period, the addition of digestates increased the CO_2 fluxes with respect to the control, particularly in the first days after amendment. Similar results were obtained by other authors after amending soils with anaerobic digestate and compost (Alluvione et al., 2010; Pezzolla et al., 2013). The highest emission rates were observed immediately after applying the digestates for the soils treated with pretreated microalgae (D2) and co-digestion (D3) digestates (230 and 245 $mgCO_2/kg_{dm}\cdot day$, respectively). CO_2 emissions decreased steadily over time, reaching constant values similar to the control ones within 13 days. Conversely, the soil treated with unpretreated microalgae (D1) showed a different behaviour, whose highest value was observed after 2 days from the amendment (170 $mgCO_2\ kg_{dm}\cdot day$). Besides, cumulative net CO_2 emissions at the end of the incubation period increased in the following order: D1 < D3 < D2 (Table 3.11). Considering the amount of organic carbon added to the soil with the microalgae digestates (Table 3.11), higher fluxes of CO_2 were expected from D1 and D3 amended soils. However, the highest cumulative CO_2 emissions were detected for the soil amended with thermally pretreated microalgae, indicating that the organic matter of this digestate was less stabilised than the organic matter of the other digestates (D1 and D3). This is in accordance with the fact that D1 and D3 also showed lower biodegradability in the soil than D2. It can be deducted from the values of C-mineralization, expressed as the % of the added

Table 3-11. Carbon mineralization rate from digestate amended soils after 30 days of incubation (mean ± standard deviation, n=3).

Parameter	Units	Digestate D1: Microalgae	Digestate D2: Pretreated microalgae	Digestate D3: Co-digestion
Total N [1]	mg/L	2.4 ± 0.1	2.2 ± 0.1	1.9 ± 0.2
Application dose	mL	13.0	14.3	16.6
TOC_{added}	Mg	98.1	92.0	101.1
WEOM	mg/L	1335.9	892.3	790.5
WEOM added	mg	17.4	12.8	13.1
Net CO_2 *emission*	mg-C	21.2 ± 1.9	47.1 ± 2.1	30.7 ± 2.6
TOC_{added} *mineralised*	%	21.6 ± 1.7	51.2 ± 6.7	30.4 ± 5.2

[1] total N values used for the dosage calculation

Note: TOC= total organic carbon, WEOM= water extractable organic matter

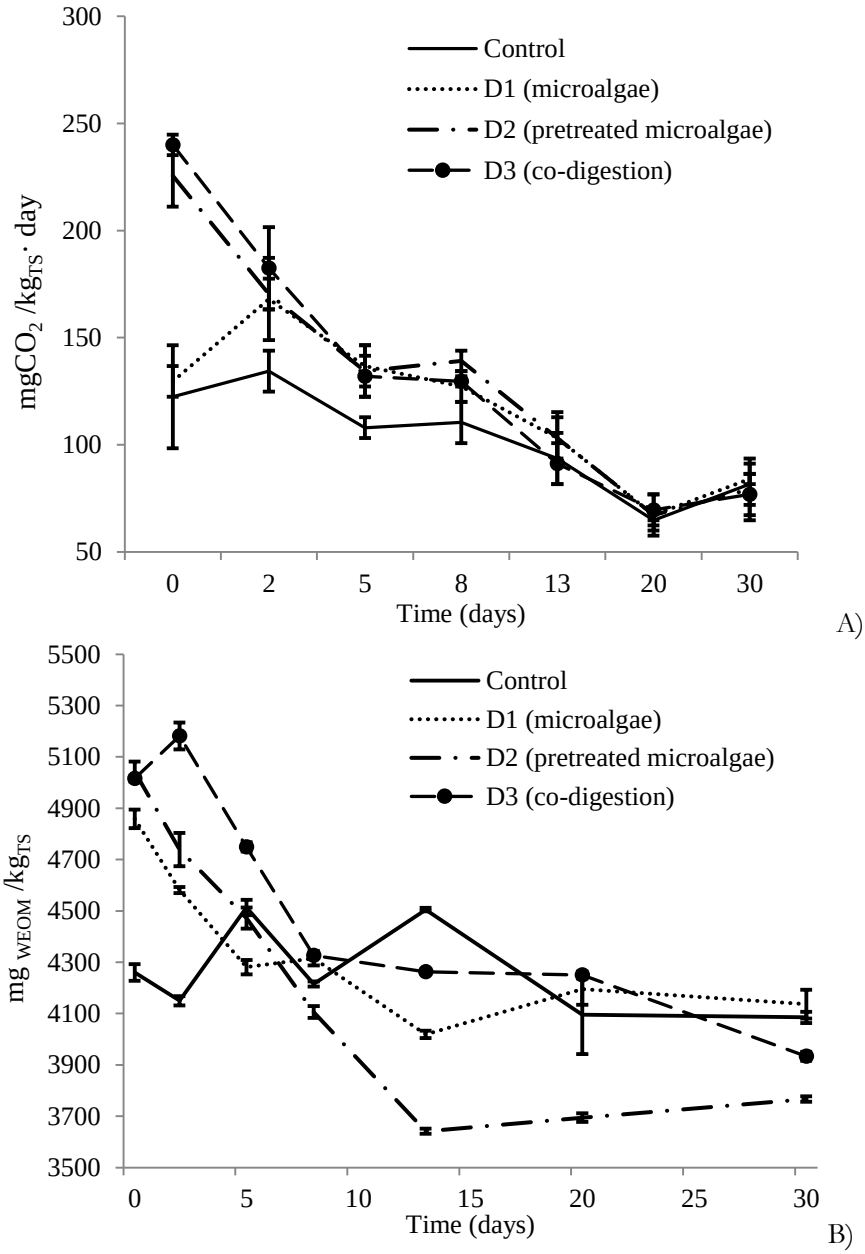


Figure 3-11 (A) CO_2 emissions from microalgae-derived digestates amended soil (mean ± standard deviation, n=3); (B) Water extractable organic matter content in microalgae-derived digestates amended soil during the incubation period (mean ± standard deviation, n=3).

TOC that was mineralised at the end of the incubation (Table 3.11). The lower stabilisation of pretreated microalgae digestate with respect to the other digestates could be attributed to the different anaerobic digesters operations. For instance, comparing the anaerobic digestion of untreated and thermally pretreated microalgal biomass, higher NH_4^+-N and VFA concentrations were found in the latter (Passos and Ferrer, 2014). As a consequence, the digestate from thermally pretreated microalgae could be less stabilised and could show higher soluble organic matter content that can be quickly mineralized in the soil. On the other hand, the co-digestion with primary sludge could also reduce the NH_4^+-N and VFA concentrations in the reactors. The addition of easily degradable substances to the soil implies the consumption of soil oxygen that, in some circumstances, can lead to anoxic conditions, fermentation processes and to the production of phytotoxic substances (Wu et al., 2000). Stability-dependent respiration rates were reported by various authors for soils amended with organic materials (Sánchez-Monedero et al., 2004). Most of them also observed CO_2 emissions peaks in the first few days after amendment with an intensity related to the contents of WEOM and microbial biomass. In fact, it is well known that organic amendment can change the amount and quality of dissolved organic matter present in the soil solution (Chantigny, 2003). As WEOM is an easily available organic matter fraction for soil microorganisms, it has important implications on microbial activity and soil respiration. Moreover, Said-Pullicino et al., (2007) have shown that the soluble organic matter fraction of organic amendments tends to decrease with organic matter stabilisation.

Figure 3.11B shows the time course of the WEOM in the digestate amended soils. Digestate application enhanced significantly ($p < 0.05$) the concentration of WEOM in the treated soils with respect to control during the first days after amendment. Following, the WEOM concentration showed a clear decreasing trend during the incubation period due to the soil microbial respiration. While D1 and D3 amended soils showed a decrease of WEOM content to the control level, in the D2 amended soils the WEOM mineralisation appears to be stronger and lead to a final content significantly lower ($P < 0.05$) than the control soils. The WEOM behaviour observed in the D2 amended soils and the low biodegradability showed by D1 and D3 appear to be in contrast with the WEOM concentrations in the microalgae-derived digestates (Table 3.11). In fact, D1 showed a higher content of WEOM with respect to D2 and D3. Therefore, it can be assumed that the labile organic matter of D2 was characterized by a low stability due to the thermal pretreatment of the microalgae biomass that was responsible for the solubilisation of labile and reactive organic

compounds. As a consequence, the application of the thermal pretreated microalgae digestate to the soil can lead to the *priming effect*, with strong short-term changes in the turn-over of soil organic matter after the application of low stabilized organic amendments (Kuzyakov et al., 2000).

In all the amended soils, the strongest WEOM mineralization appeared to be concluded after 13 days from the application, similarly to what was observed for the CO_2 emissions. As already demonstrated by Pezzolla et al. (2013), when an organic amendment is applied to soil, WEOM is strictly related to the soil CO_2 emission rates. In the present work, this fact was confirmed by the correlation between the soil respiration rates of all the soil samples and their WEOM contents. Indeed, a high positive correlation was found ($y = 1.5313x - 2655.5$) to be significant ($r = 0.7750$) at $p < 0.05$ ($n = 28$). In the last two weeks of incubation a constant trend was observed for the WEOM content in the amended soils. This behaviour can be explained considering the dynamic equilibrium that occurs between the consumption of WEOM due to the mineralization and the release of WEOM by the soil microorganism during their hydrolytic activity (Rochette and Gregorich, 1998).

In the light of the results obtained, it appears clear that pretreated microalgae digestate is less recommendable for soil application than the other digestates due to the low stabilisation of its soluble organic matter. Indeed, untreated microalgae and co-digestion digestates spreading lead to a lower impact on soil system and higher benefits for the environment and the agriculture.

3.3.3.4 Evaluation of the potential phytotoxicity of digestates

Phytotoxicity effects are often found in anaerobic digestates due to the high contents in soluble salts, NH_4^+-N and low weight organic compounds (i.e. volatile fatty acids, phenols) (José Antonio Alburquerque et al., 2012). In this study, the GI was used to evaluate the digestates phytotoxicity by applying different concentrations of digestate (100 %, 10 %, 1% and 0.1 %) and comparing the germination of cress seeds (*Lepidium sativum* L.) to a control (100 % of deionised water) (Fig. 3.12).

The results showed that no germination was detected for any pure digestate. Thus, the GI of pure digestates (0 %) indicates that they cannot be spread on agricultural

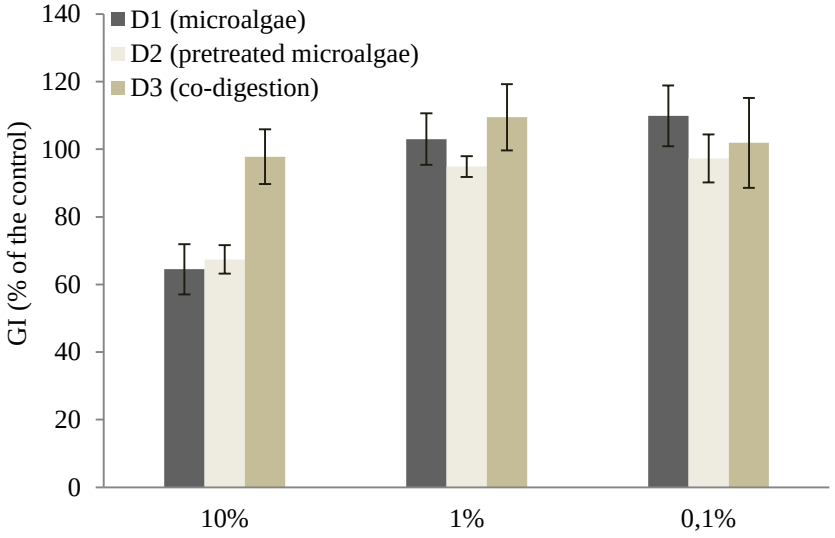


Figure 3-12 Effects of microalgae digestates and their dilutions on the germination index (GI) of cress (Lepidium sativum L.) (mean ± standard deviation, n=5). GI was 0 % for all the pure (100 %) digestates

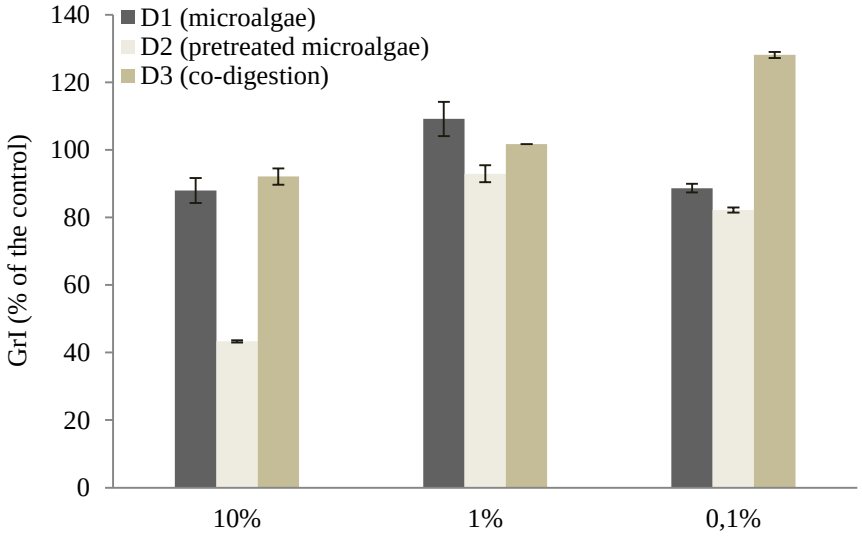


Figure 3-13 Effects of microalgae digestates and their dilutions on the growth index (GrI) of cress (*Lepidium sativum* L.) (mean ± standard deviation, n=5).

soils without dilution or a stabilisation post-treatment process. For instance, a composting post-treatment would produce a compost where phytotoxic compounds, still abundant in anaerobic digestates and responsible of the absence of germination (Abdullahi et al., 2008), can be reduced. Conversely, positive results in the germination assays were found for digestate dilutions. Untreated and pretreated microalgae digestates (D1 and D2, respectively) gave a similar GI trend, showing the highest GI for the 0.1% dilution (109.9% and 97.3%, respectively). At this dilution (0.1%), the highest GI was observed for D1, probably due to the lower content of ammonia nitrogen with respect to D2 (Table 3.9). In both cases, the lowest GI value was observed at 10 % dilution. On the contrary, no significant differences were observed between 1% and 0.1% dilutions, when values close to the control were achieved. It means that the largest phytotoxic potential was removed at 1% dilution. Concerning D3, there were no significant ($p < 0.05$) differences for the GI between dilutions of 10%, 1% and 0.1% (GI of 97.8%, 109.5% and 101.9% respectively), meaning that the phytotoxicity effect of the microalgae digestate was reduced through the co-digestion. Indeed, co-digestion processes are known to be more advantageous than mono-digestion ones due to a dilution effect of inhibitory compounds, among other factors (Tritt, 1992).

Moreover, the effect of digestates dilutions (10%, 1% and 0.1%) on the biomass production of cress (*Lepidium sativum* L.), expressed as GrI, were evaluated (Fig. 3.13). Concerning D1, no significant ($p < 0.05$) phytotoxic effect was detected on the production of biomass. Conversely, D2 showed a strong reduction of GrI at the highest concentration tested (10%), which is probably due to the high content of ammonium nitrogen of D2 (Table 3.10). At lower concentrations (1%, 0.1%), the GrI of D2 increased due to the dilution of the phytotoxic compounds. For both D1 and D2, the 1% dilution which showed a significantly higher ($p < 0.05$) GrI than the 0.1% dilution. As shown for other plants, low level of phytotoxicity can lead to a normal growth, or even higher than the un-stressed control, due to the genetic adaptability of the plants (Wang et al., 2015). This phenomena may be responsible of the GrI behaviours in D1 and D2. Nevertheless, the best performance in the plant growth bioassay was obtained from D3. Thus, co-digestion process appears to be the most suitable process for the reduction of phytotoxicity as already showed by the results obtained from the GI bioassay. Concerning the GrI determination, 10% and 1% dilutions of D3 did not show significant differences with respect to the control, showing the absence of residual phytotoxicity. When diluted at 0.1%, D3 showed plant nutrient, growth stimulant or even phytohormone-like effects (José Antonio

Alburquerque et al., 2012) that lead to a significant increase of the GrI ($p < 0.05$) with respect to the control (128.1%).

In the present work, NH_4^+-N, VFA and EC of the digestates were found to be significantly ($p < 0.05$) and negatively correlated both to GI and GrI, as expected from what described in literature (Alburquerque et al., 2012; Zucconi et al., 1985). Statistical models used in this evaluation are described in Table 3.12.

In light of what was found in the germination and growth bioassays, agricultural application of the microalgae-derived digestates through dilution in the irrigation water would be the most suitable option, as the digestate would be diluted before coming in contact with seeds and plants. Moreover, dilution could also avoid salts and heavy metal concentration in the soil (Moral et al., 2005). Co-digestion digestate appeared to be the most suitable for agricultural reuse. In fact, it would require less water for dilution and, thus, it would be a more concentrated organic fertiliser. Moreover, the co-digestion digestate was the only one that did not show residual phytotoxicity; conversely it showed stimulating properties in the *in vivo* assays.

Table 3-12. Linear regression equations (y = mx + q) calculated for selected parameters of the digestates (n=11).

Y	x	m	q	r
$N\text{-}NH_4^+$	*GI*	-0.0073	0.7254	0.9054*
VFA		-0.6728	67.351	0.9301*
EC		-0.0067	6.7041	0.9572*
$N\text{-}NH_4^+$	*GrI*	-0.0068	0.6826	0.8691*
VFA		-0.6270	63.0660	0.8862*
EC		-0.0628	6.2935	0.9156*

Note: GI= Germination Index, GrI= Growth Index, VFA= volatile fatty acids, EC= electric conductivity. *: significant at $p < 0.05$

3.3.3.5 Potential risks of digestates: heavy metals and pathogens

In order to assess the potential risks of soil contamination after digestate spreading, the occurrence of heavy metals and the presence of pathogens (*E. Coli*) were evaluated.

Regarding the digestate hygenisation, low *E.coli* presence was found in all digestates (Table 3.13), below the threshold values proposed by the EU Directive draft on spreading sludge on land (less than $5 \cdot 10^5$ colony forming units per gram of wet weight of treated sludge) (2003/CEC). Moreover, it is noteworthy that thermal pretreatment improved the hygenisation leading to absence of *E.coli* in the digestate. In fact, according to the EU draft, the combination of thermal pretreatment and anaerobic digestion can be considered as an advanced sludge treatment.

Table 3-13. *Escherichia coli* content (CFU/ml) in microalgae digestates (mean ± standard deviation; n=6).

Digestate	Mean	Maximum value
D1 (microalgae)	39.8	316.2
D2 (pretreated microalgae)	0.0	Absence
D3 (co-digestion)	25.1	199.5

Concerning heavy metals, their concentrations in the three digestates were lower than the threshold established by the sludge European Directive (EC directive 86/278/CEC), and also by the even more restrictive EU Directive draft (2003/CEC) (Table 3.14). Although all digestates presented appropriate heavy metal contents for soil application, special attention should be paid to the co-digestion digestate because of its high Zn content that is originated from the primary sludge. This is a particularity of the wastewater treatment plant where the primary sludge was collected, since they receive wastewater from industries generating high Zn concentration in their effluents. With regards to the microalgae digestate, despite microalgae ability for assimilating metals (Suresh Kumar et al., 2015), no significant heavy metal concentrations increase was found in microalgae digestates (D1 and D2) compared to the mixture with the primary sludge (D3) (Table 3.14).

Table 3-14. Concentration of heavy metals in microalgae digestates (mean ± standard deviation, n=3).

Parameter	Units	Digestate D1: Microalgae	Digestate D2: Pretreated microalgae	Digestate D3: Co-digestion	Limit values[1]	Limit values[2]
Cd	mg/kg TS	2.2 [a] ± 1.9	2.7 [a] ± 0.3	8.6 [a] ± 5.4	20-40	10
Cu	mg/kg TS	584 [a] ± 108	593 [a] ± 100	491 [a] ± 23	1000-1750	1000
Pb	mg/kg TS	47 [a] ± 3	49 [a] ± 1	221 [b] ± 112	750-1200	750
Zn	mg/kg TS	637 [a] ± 53	592 [a] ± 9	2202 [b] ± 135	2500-4000	2500
Ni	mg/kg TS	104 [a] ± 9	127 [a] ± 9	101 [a] ± 5	300-400	300
Cr	mg/kg TS	69 [a] ± 2	75 [a] ± 14	127 [b] ± 9	-	1000
Hg	mg/kg TS	2.0 [a] ± 0.5	1.7 [a] ± 0.6	<1.1 [a] ± 0.2	16-25	10

[1] Limit values according to current European legislation (EC directive 86/278/CEC); [2] Limit values according to the European draft (2003/CEC)

[a,b] letters indicate a significant difference between digestates at the level of $p < 0.05$ after Tuckey's test.

3.3.4 Conclusions

Agricultural reuse of the digestate from microalgae anaerobic digestion and co-digestion with primary sludge appears to be a promising solution towards zero waste generation in microalgae-based wastewater treatment systems. All microalgae digestates considered in this study presented organic matter and macronutrients content, especially organic and ammonium nitrogen, suitable for agricultural soils amendment. However, the thermal pretreated digestate presented a higher concentration of easily consumable organic carbon that can be mineralized on soil producing environmental impacts. Conversely, untreated microalgae and co-digestion digestates appeared to be more stabilised. *In vivo* bioassays demonstrated that the digestates did not show residual phytotoxicity when properly diluted, being the co-digestion digestate the one which presented less phytotoxicity. Furthermore, it showed interesting stimulant properties for plants. Heavy metals contents resulted far below the threshold established by the European legislation on sludge spreading. Low presence of *E.coli* was observed in all digestates. In addition, the thermal pretreatment improved the hygenisation obtaining absence of *E. coli* in the digestate. In this context, agricultural reuse of thermally pretreated microalgae and primary sludge co-digestate through irrigation emerges as a suitable strategy to recycle the nutrients and organic matter in agriculture.

4 CO-DIGESTION OF MICROALGAE WITH WASTE ACTIVATED SLUDGE

* This chapter is based on the article:

Integrating microalgae tertiary treatment into activated sludge systems for energy and nutrients recovery from wastewater. Arias, D., Solé-Bundó, M., Uggetti, E., Garfí, M., García, J., Ferrer, I., 2018. Bioresource and Technology, 247, 513-519. DOI: 10.1016/j.biortech.2017.09.

4.1 Co-digestion of microalgae with activated sludge after a simultaneous autohydrolysis co-pretreatment

In this study, microalgae digestate and secondary effluent were used to grow microalgae in a tertiary wastewater treatment, and then, the biomass was co-digested for biogas generation. The potential biogas production of the cultivated microalgae and waste activated sludge were determined in batch tests. To improve their biodegradability, a novel method combining their co-digestion with activated sludge after a simultaneous autohydrolysis co-pretreatment was evaluated. After the co-pretreatment, the methane yield increased by 130 % compared to raw microalgae. Thus, integrating microalgae tertiary treatment into activated sludge systems is a promising and feasible solution to recover energy and nutrients from waste, improving wastewater treatment plants sustainability.

4.1.1 Introduction

Until now, wastewater treatment plants (WWTPs) were mainly conceived for removing contaminants and organic matter, and were designed and managed to protect human and environmental health (Muga and Mihelcic, 2008). However, the increasing water scarcity forces the need for new technological solutions with low cost and low energy demand (Chisti, 2008). To transform a conventional wastewater treatment system into a self-sustainable process it is necessary to shift from the current model towards a new one in which wastewater treatment systems will become a low energy processing industry, able to generate marketable products rather than wastes. For this reason, special efforts have been made recently to increase energy and resource recovery from wastewater by producing valuable byproducts (e.g. biofuels) from WWTPs.

Under this scenario, nature-based treatment solutions, such as microalgae-based systems, are conceived as a breakthrough to a new model for wastewater treatment (Pittman et al., 2011). Indeed, such systems are able to reuse nutrients from wastewater and other wastes (i.e. digestate from anaerobic digestion) in order to grow

microalgae biomass which can be used as bioenergy feedstock (Uggetti et al., 2014a). However, the alternative of recycling microalgae digestate has been poorly explored. The main concern in the use of digestate as nutrient for microalgae growth is the elevated ammonium content. Though, this inconvenience may be solved by diluting it with another low strength waste effluent (i.e. secondary effluent from wastewater treatment).

Considering small-medium conventional WWTPs based on the activated sludge process with anaerobic digestion for waste activated sludge (WAS) treatment, a microalgae photobioreactor (PBR) could be introduced as a tertiary treatment in order to improve the treated water quality and increase the biogas production (Fig. 4.1). Indeed, the microalgae biomass produced in the PBR could be co-digested with waste activated sludge from the conventional plant. In such a case, their co-digestion could improve the methane productivity and the hydrolysis efficiency compared to each substrate mono-digestion, increasing the bioenergy recovery efficiency of the plant (Zhen et al., 2016).

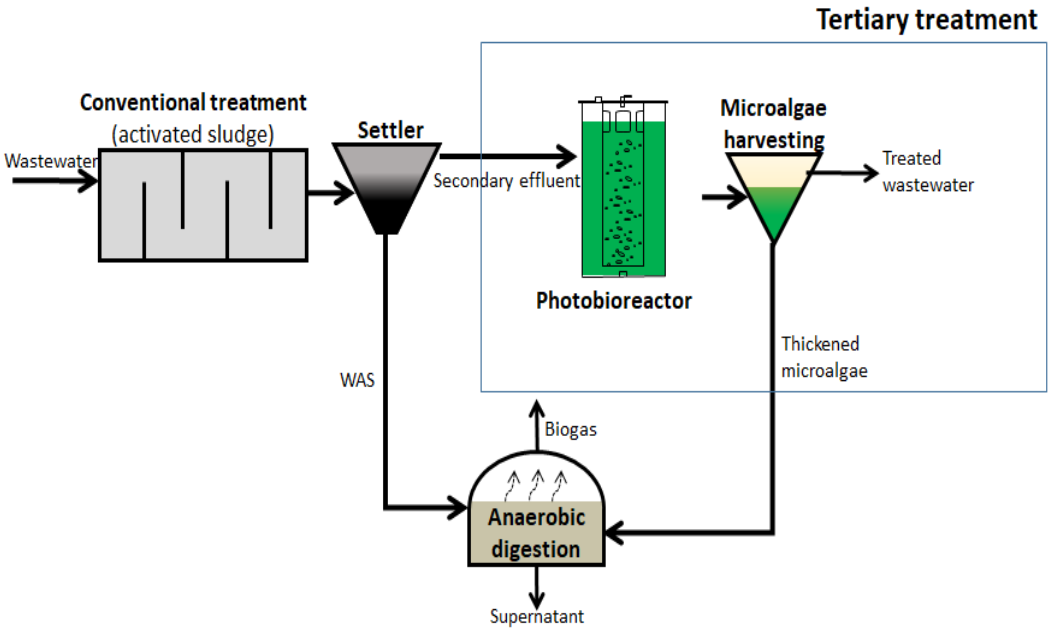


Figure 4-1 General scheme of the system proposed in this study.

In fact, recent investigation has reported higher methane yield and/or rate when microalgae and WAS are co-digested (Beltran et al., 2016; Neumann et al., 2015). Besides, WAS has inherent enzymes inside its extracellular polymeric substances (EPS) which are released after a thermal pretreatment at 55ºC resulting in autohydrolysis of WAS (Carvajal et al., 2013). Hence, the co-pretreatment and subsequent co-digestion of microalgae and WAS may improve the hydrolysis. Moreover, the digestate from the anaerobic digestion could be reused as a source of nutrients for microalgae biomass growth together with the secondary effluent. In this way, the quality of treated wastewater would be improved, as compared to conventional biological systems, and the digestate would be treated while increasing the concentration of nutrients for microalgae growth. Therefore, the objective of this research was to quantify the methane yield of harvested microalgae biomass co-digested with waste activated sludge after an autohydrolysis pretreatment.

4.1.2 Methodology

4.1.2.1 Experimental set-up

Experiments were carried out at the laboratory of the GEMMA Research Group (Barcelona, Spain). Microalgae were grown in a closed cylindrical photobioreactor (30L) (Fig. 4.2). The PBR was fed with microalgae uncentrifuged digestate diluted in secondary effluent from a pilot high rate algal pond (HRAP) treating municipal wastewater. The latter came from a pilot system treating municipal wastewater which comprised a primary settler, a high rate algal pond (HRAP) and a secondary settler (Gutiérrez et al., 2016b). The digestate was obtained from lab-scale anaerobic digesters (1.5 L) that produced biogas from microalgae biomass harvested from the HRAP. A detailed description of the anaerobic digesters and HRAP may be found in Passos et *al.* (2015).

4.1.2.2 Photobioreactor operation

A mixed microalgae culture obtained from a pilot high rate algal pond was utilized as inoculum to start-up the photobioreactor. This inoculum consisted of a community of microalgae, bacteria, protozoa and small metazoan, specifically dominated by the microalgae genus *Chlorella* sp.*, Scenedesmus* sp. and *Stigeoclonium* sp. The closed photobioreactor was located indoors and consisted of a cylindrical vessel made of polymethyl methacrylate with a working volume of 30 L. The mixed liquor was stirred by means of an air sparger placed at the bottom of the photobioreactor, at a flow of 10 L/min and a pressure of 0.034 MPa using a 105 W air compressor (model ACQ-

012, JAD, China). The photobioreactor design and operation characteristics may be found elsewhere (Arias et al., 2017). The culture in the photobioreactor was in continuous operation alternating light:dark periods of 12 h. During the illuminance period, light was supplied by an external lamp (600W, Sunmaster, USA) placed at 80cm in front of the photobioreactor, providing 19,000 lux (289 μmol/m^2s). The temperature of the culture along the experimental period ranged from 25 to 29 ºC.

Figure 4-2. Lab-scale photobioreactor

The photobioreactor was fed once a day (semi-continuously) with microalgae digestate diluted in secondary effluent at a ratio of 1:50, and operated at 8 days of hydraulic retention time (HRT) and solids retention time (SRT). The dilution ratio of 1:50 was performed in order to decrease the ammonium ($N-NH_4^+$) content to concentrations below 10 mg/L in the photobioreactor influent. The physico-chemical characterization of the digestate and secondary effluent used as influent for microalgae growth in the photobioreactor is shown in Table 4.1.

Table 4-1. Composition of the wastewater used as photobioreactor feedstock.

Parameter	Digestate	Secondary effluent	Photobioreactor influent [a]
pH	-	-	7.9 ± 0.3
TSS (g/L)	13.4 ± 8.5	[b]	0.26 ± 0.17
VSS (g/L)	12.3 ± 6.5	[b]	0.24 ± 0.13
Alkalinity(mg $CaCO_3$/L)	-	-	153 ± 38.4
CODs (mg O_2/L)	122.8 ± 25.9	18.3 ± 5.5	141.1 ± 36.1
N–NH_4^+ (mg/L)	459 ± 166.5	0.21 ± 0.84	9.17 ± 3.33
N-NO_2^- (mg/L)	<LOD [c]	1.44 ± 0.69	1.53 ± 0.91
N-NO_3^- (mg/L)	<LOD [c]	15.94 ± 4.94	15.94 ± 4.94
TIN	-	-	26.64 ± 3.06
P-PO_4^{3-} (mg/L)	<LOD [c]	2.18 ± 0.87	2.18 ± 0.87

TIN: Total Inorganic Nitrogen

[a]Photobioreactor influent prepared by diluting the digestate in secondary effluent (1:50 ratio).

[b]TSS and VSS in the secondary effluent presented values <0.03 g/L.

[c] LOD: Limit of Detection.

4.1.2.3 Biochemical methane potential assay

4.1.2.3.1 Substrates and inoculum

The microalgae biomass used in the biochemical methane potential (BMP) assays was collected from the photobioreactor effluent after stable operation. At the time, the microalgae biomass was clearly dominated by *Scenedesmus* sp. Harvested biomass was settled for 1 day, and then thickened for 3h to reach the target total solids (TS) concentration of 2.8 %. This procedure was performed at 5ºC to preserve microalgae properties.

WAS was used as co-substrate for *Scenedesmus* sp digestion. It was obtained from a secondary settler of a conventional WWTP (Barcelona, Spain). WAS had a TS and VS content of 1.8 % and 1.3 %, respectively. It was stored at 5 ºC until use.

Mesophilic digested sludge from the same WWTP (Barcelona, Spain) was used as inoculum for BMP assays and was stored at 5 ºC until use.

4.1.2.3.2 Autohydrolysis pretreatment: preliminary solubilisation assay

A preliminary solubilisation assay was carried out in order to determine the optimal contact time for the autohydrolysis pretreatment. The assay was performed at 55 ºC in order to activate WAS enzymes (Carvajal et al., 2013).

The autohydrolysis pretreatment was carried out in four glass bottles with a total volume of 250 mL and liquid volume of 200 ml each. Bottles were placed in a heater under mild continuous mixing using multi magnetic stirrers at a constant temperature of 55 ºC. Trials were prepared with microalgae and WAS alone (controls) and with mixtures of microalgae and WAS at different proportions: 50 % microalgae + 50 % WAS and 80 % microalgae + 20 % WAS (on a VS basis).

Time course of biomass solubilisation was analysed from the solubilisation curves defined by the solubilisation ratio (S) obtained at increasing exposure times. The solubilisation ratio was defined as follows:

$$S = \frac{VSs}{VS} \cdot 100 \qquad \text{(Eq. 4.1.1)}$$

where S is the solubilisation ratio expressed as a percentage, VSs is the soluble volatile solids concentration and VS refers to the total volatile solids concentration.

In order to compare the experimental data of the microalgae and WAS mixtures with the expected solubilisation ratio without substrates interaction, the theoretical solubilisation ratio was calculated using the following equation:

$$S_{calc} = f_A \cdot S_A + f_{WAS} \cdot S_{WAS} \qquad \text{(Eq. 4.1.2)}$$

where S_{calc} is the calculated solubilisation ratio expressed as a percentage, f_A and f_{WAS} refer to the proportion of microalgae and WAS content in each solubilisation trial, respectively, and S_A and S_{WAS} are the experimental solubilisation ratio of microalgae and WAS tested alone, respectively.

4.1.2.3.3 Microalgae and WAS co-digestion BMP assays

BMP tests were carried out in order to determine the methane yield and rate (k) of co-digestion trials with microalgae and WAS, after an autohydrolysis pretreatment. The pretreatment was applied simultaneously to both substrates, taking into account the results of the preliminary solubilisation assay in terms of exposure time (Section 3.4.2.3.2). Three conditions were tested: i) 20 % of microalgae and 80 % of WAS, ii) 50 % microalgae and 50 % of WAS and iii) 80 % of microalgae and 20% of WAS (on a VS basis). The mono-digestion of each substrate (with and without pretreatment) was also performed as control.

All experimental trials were prepared in triplicate with a substrate to inoculum (S/I) ratio of 0.5 g CODVS/g VS according to Passos et al. (2013). A blank trial without substrate was used to quantify the amount of methane produced by the inoculum. After adding the proper amount of both substrates and the inoculum, serum bottles (160 mL) were filled with distilled water up to 100 mL, flushed with Helium gas, sealed with butyl rubber stoppers and incubated at 35 ºC until biogas production ceased.

A first-order kinetic model (Eq. 4.1.3) was applied to assess the performance and the kinetics of (co-)digestion assays.

$$B = B_0 \cdot [1 - \exp(-k \cdot t)] \qquad \text{(Eq. 4.1.3)}$$

where B represents the cumulative methane production (mL CH_4/gVS), B_0 is the final methane production (mL CH_4/gVS), k refers to the first-order kinetic constant ($days^{-1}$) and t is time (days).

The pair of experimental data (B,t) was adjusted by the least square method using the SOLVE function from Excel. This allowed the determination of parameters k and B_0 of each co-digestion assay.

Furthermore, experimental data obtained by each co-digestion mixture was compared to theoretical values calculated from microalgae and WAS specific methane productions (Eq. 4.1.4):

$$BMP_{calc} = f_A \cdot BMP_A + f_{WAS} \cdot BMP_{WAS} \quad \text{(Eq. 4.1.4)}$$

where BMP_{calc} is the calculated BMP, f_A and f_{WAS} refer to the percentage of microalgae and WAS content in each trial, respectively, and BMP_A and BMP_{WAS} are the experimental methane yield of microalgae and WAS mono-digestions, respectively.

4.1.2.4 Analytical procedures

The total volatile solids (VS) and soluble volatile solids (VSs) were analysed according to Standard Methods (APHA AWWA-WPCF, 2001). The soluble fraction was obtained after biomass centrifugation (UNICEN20, 4200 rpm, 8min, 20 ºC) followed by filtration via glass-fiber filters (0.45 μm).

The cumulative biogas production was determined from the pressure increase in the headspace volume of the bottles measured with a manometer (GMH 3161 Greisinger, Germany). The methane content in biogas was periodically analysed by gas chromatography, using a chromatograph with a thermal conductivity detector (Trace GC Thermo Finnigan with Hayesep packed column) and injector/detector/oven temperatures were 150, 250, 35 ºC, respectively, using helium gas as carrier.

4.1.3 Results and discussion

4.1.3.1 Autohydrolysis pretreatment effect on biomass solubilization

The effect of the autohydrolysis pretreatment was initially evaluated by the biomass solubilisation increase (Fig. 4.3). WAS reached the highest solubilisation ratio (25.7 %) and microalgae the lowest (11.4 %). In view of the results, microalgae showed to be less biodegradable than WAS due to the resistant structure of their cell wall. case in particular, *Scenedesmus* has been reported to have a complex multilayer cell wall (Tukaj and Bohdanowicz, 1995).

The results obtained in this study are in accordance with those obtained by Mahdy et al., (2015), who observed higher solubilisation rates with WAS than microalgae after a thermal pretreatment at 120 ºC for 40 min. Besides, similar solubilisation rates for WAS were obtained by Carvajal et al. (2013) (25 % for proteins and 21 % for

carbohydrates), who studied how inherent enzymes of WAS were released by applying a thermal pretreatment at 55 ºC.

Considering the mixed substrates, at the end of the assay the solubilisation ratios were 21 % and 15 % for the mixtures with 50 % and 80 % of microalgae, respectively. Indeed, the solubilisation ratio decreased proportionally to the concentration of WAS decrease (R^2=0.95). This proportionality was confirmed by comparing experimental data with theoretical solubilisation ratios, calculated from Eq. 4.1.2. This means that there was no co-pretreatment effect, since microalgae solubilisation was not improved by pretreating it together with WAS. Therefore, inherent enzymes of WAS released during the autohydrolysis pretreatment were not effective at disrupting microalgae cell wall.

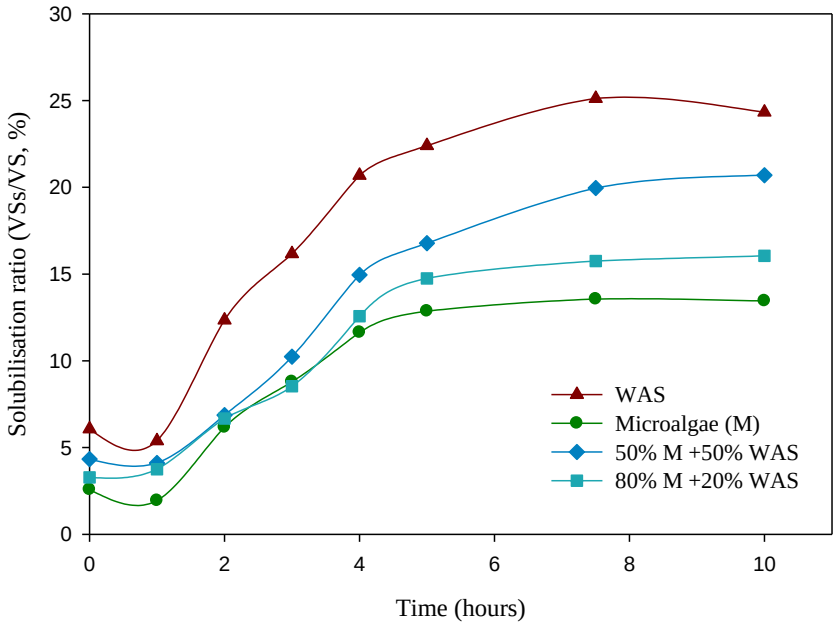


Figure 4-3 Solubilisation ratio over the solubilisation assay (10 h).

Note: M= microalgae; WAS= waste activated sludge.

Finally, figure 4.3 shows that all assays reached an asymptote by the end of the assay, meaning that solubilisation ratio increase was stabilised by that time. An increase on the contact time would not entail a significant increase of substrate solubilisation, whereas it would increase the amount of energy needed for the pretreatment. Therefore, 7.5 hours was selected as the optimum contact time for the autohydrolysis

pretreatment prior to biochemical methane potential assays. This is in accordance with our previous studies which showed that a contact time of 8 hours was the optimum when pretreating microalgae at low temperature (Passos et al., 2013).

4.1.3.2 Biochemical methane potential of pretreated microalgae and WAS co-digestion

The anaerobic co-digestion BMP assays lasted 41 days (Fig. 4.4). Regarding the pure substrates, WAS showed the highest methane yield (139 mL CH_4/g VS) while microalgae presented the lowest (82 mL CH_4/g VS) (Table 7.1). Nonetheless, after the pretreatment, microalgae presented a higher increase with respect to WAS. Indeed, the pretreatment applied to microalgae increased the methane yield by 64 %, achieving a value of 134 mL CH_4/g VS. On the other hand, pretreated WAS showed a production of 204 mL CH_4/g VS, which represents an increase of 47 %. These results are in accordance with the literature highlighting the importance of microalgae pretreatment, since their resistant cell wall hampers microalgae hydrolysis and anaerobic fermentation (Passos et al., 2014b). Particularly, *Scenedesmus* sp. has a complex rigid cell wall which makes even more difficult the accessibility of enzymes to the substrate during the digestion process (C. González-Fernández et al., 2012).

The cumulative methane yields of the co-digestion trials were 187 mL CH_4/g VS, 162 mL CH_4/g VS and 132 mL CH_4/g VS for the mixtures of WAS with 20 %, 50 % and 80 % of microalgae, respectively. In order to detect potential co-digestion synergies, the theoretical methane yields were calculated according to Eq. 4.1.4. The results showed neither positive nor negative synergies between substrates, meaning that the co-digestion did not improve microalgae anaerobic biodegradability. The lack of WAS enzymes effect on *Scenedesmus* sp. cell wall disruption, or the low C/N ratio might be responsible for the lack of synergies. These results are in agreement with Costa et al. (2012), who studied the co-digestion of macroalgae species (*Ulva* and *Gracilaria*) with WAS without any pretreatment. Additionally, Neumann et al. (2015) studied the co-digestion of *Botryococcus braunii* and WAS and synergies were neither identified. On the contrary, Wang et al. (2013) observed 23 % increase in biogas production when co-digesting *Chlorella* sp. and WAS, with 41 % of microalgae. Despite *Chlorella* sp. has a rigid cell wall due to its high content of cellulose, the co-digestion with WAS enhanced the hydrolysis.

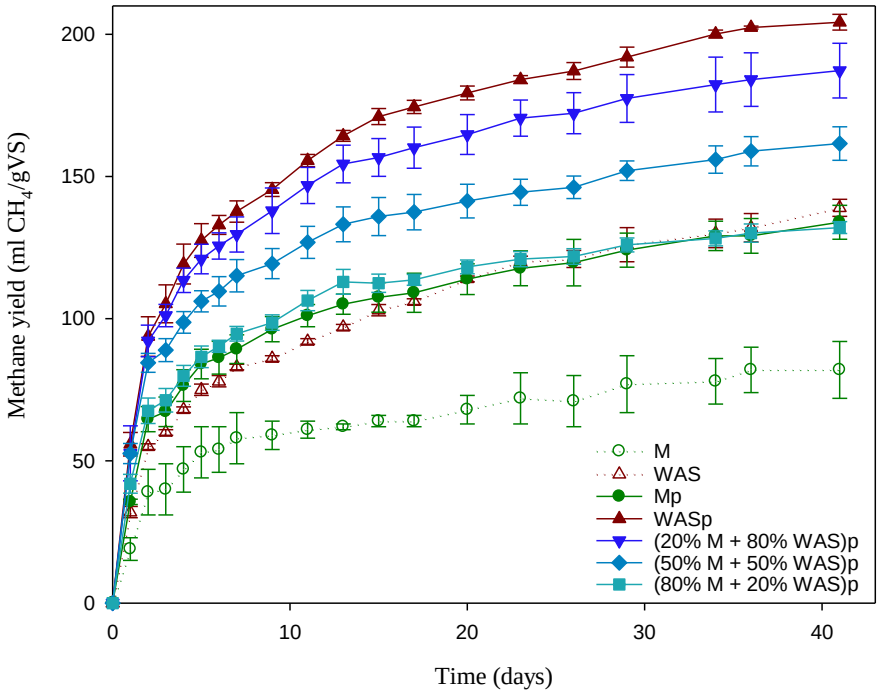


Figure 4-4 Cumulative methane yield (mg CH_4/g VS) over the biochemical methane potential assays with Scenesdesmus sp. and WAS (co-digestion and mono-digestion). Symbols represent the mean value and standard deviation.

Note: M= microalgae; WAS= waste activated sludge; p = pretreated

The methane content in biogas of each co-digestion assay was periodically measured (Table 4.2). Results showed no differences among trials. Thus, the methane content was independent of the ratio between co-digestion substrates (Caporgno et al., 2015) and it was neither affected by the autohydrolysis pretreatment nor by the co-digestion.

Moreover, the methane production rate was also analysed through the apparent kinetic constant (k) of the first-order experimental model, as defined in Eq. (4.1.3). Table 4.2 shows that substrates without pretreatment had the lowest values of k (0.16 $days^{-1}$ and 0.17 $days^{-1}$ for microalgae and WAS, respectively), whereas pretreated substrates increased their kinetic constants up to 0.27 $days^{-1}$ and 0.25 day^{-1} for microalgae and WAS, respectively. Thus, a significant increase of the production rate (69 % for microalgae and 47 % for WAS) was observed by applying the pretreatment.

Table 4-2. Experimental results and data analysis at the end of the biochemical methane potential assays.

	Methane yield	% CH_4	k
	mg CH_4/g VS	%	day^{-1}
Microalgae (M)	82 ± 10	63.3 ± 0.1	0.16
WAS	139 ± 3	63.9 ± 0.8	0.17
(M)p	134 ± 6	64.0 ± 0.1	0.27
(WAS)p	204 ± 3	63.5 ± 0.3	0.25
(20 %M+80 %WAS)p	187 ± 9	64.0 ± 0.4	0.29
(50 %M+50 %WAS)p	162 ± 6	64.3 ± 0.9	0.32
(80 %M+20 %WAS)p	132 ± 2	64.6 ± 0.7	0.30

p = pretreated

Moreover, the co-digestion trials showed higher kinetic constants (0.29 $days^{-1}$, 0.32 $days^{-1}$ and 0.30 $days^{-1}$ for 20 %, 50 % and 80 % of microalgae content co-digestions) as compared to the mono-digestions. This evidenced how the co-digestion of microalgae and WAS can improve the mono-digestion of both substrates. Costa et al. (2012), Neumann et al. (2015) and Wang et al. (2013) agreed that co-digestion of microalgae and WAS improved the kinetic constant despite having different conclusion in terms of the final methane yield. This result was considered the main advantage of the studied microalgae and WAS co-digestion, as it may reduce the time needed for reaching the highest biogas production. This means that lower hydraulic retention times, hence smaller digesters could be used, reducing the costs.

4.1.3.3 The approach of recycling nutrients in a bioenergy producing system

This study highlights the viability of integrating an algae-based tertiary wastewater treatment system in a conventional WWTP that includes both processes: activated sludge and anaerobic digestion. This short term study also offers an alternative to the recycling use of digestate.

Although the reuse of digestate as biofertiliser can promote a sustainable biogas production (Solé-Bundó et al., 2017b), this substrate can be combined with secondary effluents as an alternative substrate to produce microalgal biomass. Additionally, this process could improve the treatment of remaining nutrients from secondary effluents

and taking advantage of the nutrients contained in the digestate. Considering the promising results here included, further studies based in long term conditions are recommended. This approach would involve a promising opportunity to close the biorefinery loop, accomplishing a sustainable and self-supporting use of resources and reducing disposal costs and environmental impacts.

4.1.4 Conclusions

Microalgal anaerobic digestate diluted with secondary wastewater was an effective source of nitrogen and phosphorus for microalgae growth in a photobioreactor. This biomass, mainly composed by *Scenedesmus* sp., supported a low methane yield (82 ml CH_4/gVS) that was improved by 130% after an autohydrolysis co-pretreatment and co-digestion with waste activated sludge. Thus, integrating microalgae tertiary treatment into activated sludge systems is a promising and feasible solution to recover energy and nutrients from waste, improving wastewater treatment plants sustainability.

5 CO-DIGESTION OF MICROALGAE WITH AGRO-INDUSTRIAL WASTES

* This chapter is based on the articles:

Enhancement of microalgae anaerobic digestion by thermo-alkaline pretreatment with lime (CaO*).* Solé-Bundó, M., Carrère, H., Garfí, M., Ferrer, I., 2017. Algal Research 24, 199–206. doi:10.1016/j.algal.2017.03.025

Anaerobic co-digestion of microalgal biomass and wheat straw with and without thermo-alkaline pretreatment. Solé-Bundó, M., Eskicioglu, C., Garfí, M., Carrère, H., Ferrer, I., 2017. Bioresource Technology 237, 89–98. DOI10.1016/j.biortech.2017.03.151

5.1 Optimization a thermo-alkaline pretreatment with lime to microalgae

The aim of this study was to evaluate for the first time the effect of a thermo-alkaline pretreatment with lime (CaO) on microalgae anaerobic digestion. The pretreatment was carried out by adding different CaO doses (4 and 10%) at different temperatures (room temperature (25ºC), 55 and 72ºC). The exposure time was 4 days for pretreatments at 25ºC, and 24h for pretreatments at 55 and 72ºC. Following, a biochemical methane potential test was conducted with pretreated and untreated microalgae. According to the results, the pretreatment enhanced proteins solubilisation by 32.4% and carbohydrates solubilisation by 31.4% with the highest lime dose and temperature (10% CaO and 72°C). Furthermore, anaerobic digestion kinetics were improved in all cases (from 0.08 to 0.14 day-1 for untreated and pretreated microalgae, respectively). The maximum biochemical methane potential increase (25%) was achieved with 10% CaO at 72°C, in accordance with the highest biomass solubilisation. Thus, lime pretreatment appears as a potential strategy to improve microalgae anaerobic digestion.

5.1.1 Introduction

Over the last decades, the feasibility to obtain biogas from microalgae has been proved. However, some microalgae species can present a low biodegradability due to the complex structure of their cell walls. This fact may hamper the hydrolysis step (González-Fernández et al., 2012). For that reason, some pretreatment techniques have been evaluated to improve both the microalgae anaerobic biodegradability and the kinetics of the process (González-Fernández et al., 2012; Passos et al., 2014b). The most studied methods have been mechanical and thermal pretreatments, which may increase the biomass solubilisation, methane yield and methane production rate. Nevertheless, energy balances are not always positive, since some of these pretreatments have a high energy demand (Passos et al., 2014b). Thus, pretreatments which require minimal energy input, such as low-temperature, biological and

chemical methods, have recently been gaining interest (Passos et al., 2016; Passos and Ferrer, 2014).

Chemical pretreatments consist of adding acids (acid pretreatment) or bases (alkaline pretreatment) under different conditions (e.g. different temperatures and exposure times). First applications of alkaline pretreatments were found to improve the biodegradability of lignocellulosic biomass due to their effectiveness at breaking ester bonds between lignin and polysaccharides (Monlau et al., 2013) and partially solubilising hemicelluloses and celluloses to a lower extent (Monlau et al., 2012). Although microalgae do not contain lignin, some benefits have also been reported in the application of an alkaline pretreatment to microalgae. Indeed, Mahdy et al. (Mahdy et al., 2014a) reported that both organic matter solubilisation and methane yield increased by applying an alkaline pretreatment. In addition, while an acid pretreatment of microalgae only increased carbohydrate solubilisation, an alkaline pretreatment enhanced the solubilisation of both proteins and carbohydrates (Mendez et al., 2013). Moreover, the combination of thermal and alkaline pretreatments applied to different microalgae species was more effective than alkaline or thermal pretreatments applied separately (Bohutskyi et al., 2014). The combination of temperature and alkali pretreatments has been tested at low (<100 °C) and high (>100 °C) temperatures. However, it has been demonstrated that high temperatures may lead to the production of refractory organic compounds or inhibitory intermediates generated through intramolecular reactions (i.e. Maillard reactions) (Stuckey and McCarty, 1984). Therefore, the use of lower temperatures might be more appropriate.

To date, the most used alkali for microalgae pretreatment is NaOH, although a recent study also analysed the effect of KOH, Na_2CO_3 and NH_4OH (Kassim and Bhattacharya, 2015). However, some environmental and economic drawbacks should be considered when applying these chemicals. In particular, NaOH increases the concentration of Na^+ in digestates, which is known to be inhibitory to methanogens (Feijoo et al., 1995) and could be harmful for soil upon digestate agriculture reuse (Solé-Bundó et al., 2017b). On the other hand, NH_4OH may not be recommended for microalgae, as their high nitrogen content combined with the addition of NH_4OH could inhibit anaerobic digestion (Yenigün and Demirel, 2013). Concerning KOH, it is more expensive than other alkalis. Conversely, lime ($Ca(OH)_2$ or CaO) is more environmentally friendly and cheaper (Ramirez et al., 2013). In particular, lime is around 1.5 and 4-fold less expensive than NaOH and KOH, respectively. Lime

pretreatment has already been tested on lignocellulosic biomass (i.e. wheat straw or sunflower stalks), showing a significant increase in biomass solubilisation and methane yield (Monlau et al., 2013, 2012). To the best of our knowledge, no studies have assessed the effect of lime pretreatment on microalgae anaerobic digestion.

The aim of this study is to evaluate and determine the best pretreatment conditions (alkali dose and temperature) for a thermo-alkaline pretreatment of microalgae with lime (CaO) by means of biomass solubilisation and methane production analysis.

5.1.2 Material and Methods

5.1.2.1 Microalgal biomass

Microalgae used in this study were harvested from a pilot raceway pond (17 m^3) located at the INRA-LBE facilities (Narbonne, France) (Fig. 5.1), which treated synthetic wastewater based on the composition tested by Bracklow et al. (2007) (Bracklow et al., 2007). A detailed description of the system can be found in Hreiz et al. (2014) (Hreiz et al., 2014). Microalgal biomass, which consisted of a mixed culture of microalgae and bacteria, was harvested by membrane concentration followed by gravity settling (24h at 4 ºC). Microalgae species were identified by optical microscopy (Olympus BX53).

Figure 5-1. Experimental high rate algal pound (INRA-LBE, Narbonne)

5.1.2.2 Microalgae pretreatment

Thermal and thermo-alkaline pretreatments of microalgal biomass were carried out in glass bottles of 160 mL containing 27.62 g of microalgal biomass with a concentration of 14.5 g VS/L. In order to assess the best pretreatment condition, two lime (Akdolit® Q90; purity ≥ 92%) doses were tested: 4 and 10% CaO on a TS basis, based on the common doses used when applying this pretreatment (Liang et

al., 2013). According to the literature, lime pretreatment requires long exposure times, ranging from several days to weeks, which can be reduced by increasing temperature (Ramirez et al., 2013). For this reason, the following combinations of temperature and exposure time were tested: 4 days at room temperature (25 °C) and 24 h at 55 and 72 °C. After adding lime, bottles were closed and incubated with constant agitation. All conditions were compared with control trials (without lime): microalgae stored for 4 days at 4 °C, and microalgae exposed to 25 °C for 4 days and 55 and 72 °C for 24h.

Each pretreatment condition was performed in five different bottles. Later, three of them were used in the biochemical methane potential (BMP) test (triplicates) (Section 4.1.2.3) and the rest were devoted to all analysis (Section 4.1.2.4). As far as the pretreatment at room temperature is concerned, 4 extra bottles were used in order to monitor the pH (duplicates), and the gas pressure and composition inside the bottles (duplicates).

5.1.2.3 Biochemical methane potential tests

Methane potentials of untreated and pretreated microalgae were tested by means of BMP tests. Each condition was performed in triplicate. The inoculum was granular sludge from a mesophilic digester which treated the effluent of a sugar factory. The sludge was diluted with distilled water to reach a concentration of 60 g TS/L and 47.6 g VS/L. Then, it was kept under anaerobic conditions at 35 °C with continuous stirring until use.

In order to avoid biomass loss during the experimental process, the test was carried out using the same glass bottles as the pretreatment. As already mentioned, each bottle contained 4 g VS/L of microalgae. The substrate to inoculum ratio (S/I) was 1 g VS substrate / g VS inoculum. Macronutrients, oligoelements and buffer solutions were added providing 360 mg NH_4-N/L, 118 mg PO_4-P/L, 37.1 mg Mg/L, 42.3 mg Ca/L, 5.6 mg Fe/L, 1.24 mg Co/L, 0.28 mg Mn/L, 0.25 mg Ni/L, 0.24 mg Zn/L, 0.09 mg B/L, 0.23 mg Se/L, 0.15 mg Cu/L, 0.04 mg Mo/L and 2.6 g $NaHCO_3$/L. Bottles were filled with distilled water up to 100 mL, flushed with nitrogen gas, sealed with butyl rubber stoppers and incubated at 35 ºC until biogas production ceased.

Accumulated biogas production was measured with a manometer (LEO 2, Keller) while biogas composition (CH_4, CO_2, N_2, O_2, H_2) was analysed by means of a gas

chromatograph (Clarus 580, PerkinElmer) equipped with RtQBond and RtMolsieve columns coupled to a thermal conductivity detector (TCD). The carrier gas was argon, and the temperatures of the injector, detector and oven were 250, 150 and 60°C, respectively.

A blank treatment was used to quantify the amount of methane produced by the inoculum. The net biogas production was calculated by subtracting the blank results to each trial.

5.1.2.4 Analytical methods

Microalgal biomass was characterised by the concentration of TS, VS and total chemical oxygen demand (COD), following APHA Standard Methods (Association. et al., 2005). Biomass macromolecular composition was expressed in terms of percentage of proteins, carbohydrates and lipids over the VS content. Proteins were calculated by multiplying the total Kjeldahl nitrogen (TKN) by 5.95 (López et al., 2010), and TKN was titrated using a Buchi 370-K after mineralisation of samples. The total carbohydrate content (CH) was analysed by the phenol-sulphuric method (DuBois et al., 1956) after acid hydrolysis. The lipid content was determined after heptane extraction (ASE®200, DIONEX).

The liquid fraction from each pretreatment was analysed for soluble COD (CODs), TKN (TKNs) and CH (CHs) as described before. Soluble sugars were also quantified by High Performance Liquid Chromatography (HPLC) coupled to refractometric detection (Waters R410) after mild acid hydrolysis (Sluiter and (U.S.), 2008). Chemicals were separated by an Aminex HPX-87H column (300 x 7.8mm, Biorad) equipped with a protective precolumn (Microguard cation H refill catbridges, Biorad). The eluting solution was 2 mM H_2SO_4, the flow rate was 0.3 ml/min, the column temperature was 45 °C and the refractive index detector (Waters 2414) worked at 45 °C to quantify sugars. All physico–chemical analyses were performed in triplicate.

5.1.2.5 Solubilisation rates and biomass loss calculation

Biomass solubilisation was evaluated by the soluble to total COD, CH and TKN ratios using the following equations (Eq. 5.1.1-5.1.3):

$$COD\ solubilised\ (\%) = \frac{(COD_s)_p}{(COD)_0} \cdot 100 \qquad \text{(Eq. 5.1.1)}$$

$$CH\ solubilised\ (\%) = \frac{(CH_s)_p}{(CH)_0} \cdot 100 \qquad \text{(Eq. 5.1.2)}$$

$$TNK\ solubilised\ (\%) = \frac{(TNK_s)_p}{(TNK)_0} \cdot 100 \qquad \text{(Eq. 5.1.3)}$$

where sub-indexes refer to pretreated (p) and untreated (0) biomass.

The biomass loss after pretreatment was calculated in terms of COD loss according to Eq. 5.1.4, where $(COD)_p$ is the total COD concentration of pretreated samples and $(COD)_0$ is the total COD concentration of untreated microalgae (control).

$$COD\ losses\ (\%) = \frac{(COD)_p - (COD)_0}{(COD)_0} \cdot 100 \qquad \text{(Eq. 5.1.4)}$$

5.1.2.6 Solubilisation rates and biomass loss calculation

In order to evaluate the kinetics of the process, experimental data from BMP tests was adjusted to a first-order kinetic model (Eq.5.1.5) by the least square method.

$$B = B_0 \cdot \{1 - exp[-k \cdot (t - \lambda)]\} \qquad \text{(Eq. 5.1.5)}$$

where, B_0 stands for the methane production potential (ml CH_4·/gVS), k is the first order kinetic rate constant (day^{-1}), B is the accumulated methane production at time t (ml CH_4/gVS), t is time (day) and λ represents the lag phase (day).

The error variance (s^2) was estimated by the following equation:

$$s^2 = \frac{\sum_1^i (y_i - \hat{y}_i)^2}{N - K} \qquad \text{(Eq. 5.1.6)}$$

where y_i is the experimental value, $\hat{y}_i$ is the value estimated by the model, N is the number of samples and K is the number of model parameters.

5.1.2.7 Statistical analyses

Linear regressions were fit to find the relationship between solubilisation and explanatory variables (i.e lime dose, temperature). Differences among experimental conditions for the methane yield were determined by the ANOVA and Tukey tests.

Differences were considered significant at p values below 0.05. All statistical analyses were performed using R 3.0.2 software.

5.1.3 Results and discussion

5.1.3.1 Microalgal biomass characteristics

Microscope examination showed that the predominant microalgae were *Chlorella* sp. and *Scenedesmus* sp. (Fig. 5.2). Both genus are characterised by a resistant cell wall which hampers their biodegradability, especially in the case *Scenedesmus* which has a complex multilayer cell wall (Tukaj and Bohdanowicz, 1995).

Figure 5-2 Microscopic image of microalgal biomass mainly composed of *Chlorella* sp. and *Scenedesmus* sp.

Biochemical analysis indicated that microalgae biomass was mainly composed of proteins (52%), followed by carbohydrates (16%) and lipids (9%) (Table 5.1). These results are in accordance with the literature (Dong et al., 2016). Carbohydrates were mainly constituted by glucose and xylose (48 and 39% of the total carbohydrates, respectively). This is in agreement with previous studies which found a similar

carbohydrate composition in *Chlorella sorokiniana* and *Scenedesmus almeriensis* (Hernández et al., 2015).

Table 5-1. Biochemical composition of microalgal biomass (mean ± standard deviation).

Parameter	Value
TS (g/L)	17.8 ± 0.1
VS (g/L)	14.5 ± 0.1
COD (g O_2/L)	23.5 ± 0.2
Carbohydrates (% VS)	16.3 ± 0.5
Proteins (% VS)	52.0 ± 0.5
Lipids (% VS)	8.8 ± 0.0
Ash (%)	18.4 ± 0.9

5.1.3.2 pH monitoring over lime pretreatment

pH is an important parameter in alkaline pretreatments, as alkaline conditions must be ensured during the whole pretreatment process. For that reason, pH was measured before and after applying the pretreatment with lime. While untreated microalgae showed a pH of 8.1, this value increased to 11.9 and 12.4 when 4 and 10% CaO was added, respectively. However, the final pH decreased after 4 days of alkaline pretreatment at room temperature and after 24h of thermal and thermo-alkaline pretreatment (Table 5.2).

Concerning the alkaline pretreatment, pH values achieved at the end of the pretreatment were very low (7.6 and 8.1 with 4 and 10% CaO, respectively). These results were unexpected, since lime was applied to induce alkaline conditions during the whole pretreatment. To further investigate the pH drop, the lime pretreatment at room temperature was repeated measuring the pH and gas content in the bottles over time (Fig. 5.3). As can be observed in figure 5.3, after the first 20-30 hours the pH decreased and then it stabilised at similar values as those obtained during the thermal pretreatment without lime (pH = 7.3 ± 0.3). The same graph also shows that the CO_2 content increased over time. This can be explained by the presence of heterotrofic bacteria in the microalgal biomass, which release CO_2 as a result of organic matter biodegradation. The higher the dose of lime, the lower the CO_2 concentration in the gas phase, especially at the beginning of the pretreatment when

CO_2 increase was moderate (even null for 10% CaO). This fact suggests that CO_2 was dissolved, decreasing the pH. Hence, the alkaline pretreatment of this type of biomass at room temperature only makes sense with contact times below 24 h.

Regarding the thermo-alkaline pretreatment at 55 and 72 ºC, higher final pH values were achieved as compared to the alkaline one (8.8 for 4% CaO and 11.9 for 10% CaO) (Table 5.2), even though they showed a pH decrease at the end of the pretreatment. On the other hand, thermally pretreated samples presented a slight pH decrease with respect to untreated microalgae (7.71 and 7.78 at 55 and 72 ºC, respectively). In this case, the decrease could be attributed to a certain acidification caused by organic matter biodegradation. The same evidence was detected after pretreating the macroalga *Palmaria palmata* with 4% NaOH, when the pH decreased from 11.3 to 9.3 and 9.9 after 24 h at 70 and 85 °C, respectively (Jard et al., 2013). Nonetheless, in comparison with the alkaline pretreatment at room temperature, mild temperatures enhanced alkaline conditions during the pretreatment.

Table 5-2. Pretreatment conditions and final pH achieved after the pretreatment.

Trial	Pretreatment conditions			Final pH
	Temperature (°C)	Contact time (h)	CaO dose (% TS)	
Untreated microalgae	-	-	-	8.06
Room temp.	25	96	0	8.12
Room temp. + 4% CaO	25	96	4	7.55
Room temp. + 10% CaO	25	96	10	8.09
55 ºC	55	24	0	7.71
55 ºC + 4% CaO	55	24	4	8.85
55 ºC + 10% CaO	55	24	10	11.92
72 ºC	72	24	0	7.78
72 ºC + 4% CaO	72	24	4	8.82
72 ºC + 10% CaO	72	24	10	11.91

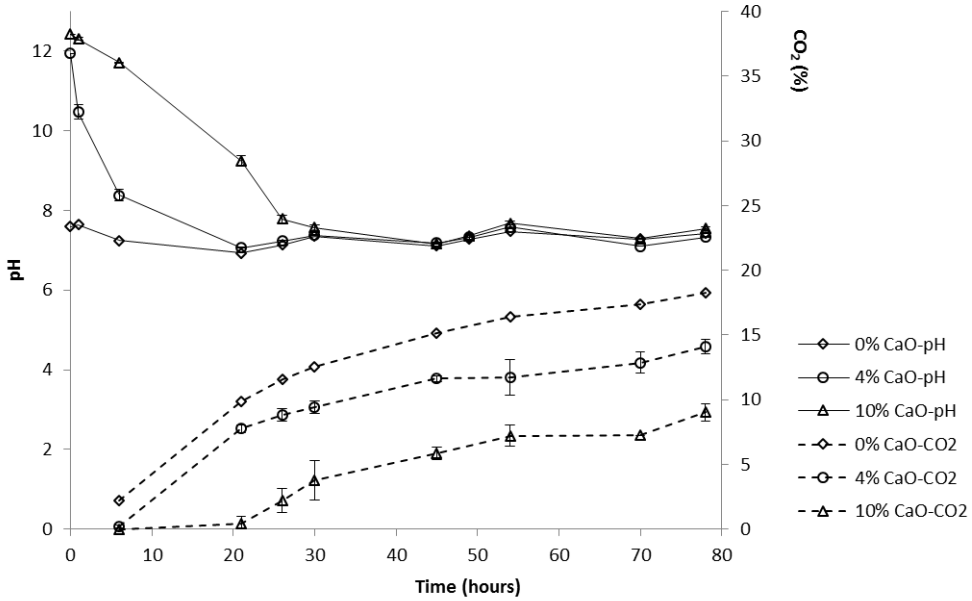


Figure 5-3 pH and CO2 measured in the bottles after addition of 0, 4 and 10% CaO at room temperature.

5.1.3.3 Effect of the pretreatment on microalgal biomass solubilisation and biomass loss

5.1.3.3.1 Organic matter solubilisation

Thermal and thermo-alkaline pretreatments enhanced organic matter solubilisation under all pretreatment conditions (Fig. 5.4). Indeed, the soluble to total COD ratio increased by 10-25%, depending on the pretreatment condition. Moreover, the addition of lime enhanced biomass solubilisation under all temperatures assayed. The highest soluble COD values were observed for the thermo-alkaline pretreatment with 10% CaO at 55 and 72°C (20 and 25% CODs, respectively).

Similar results were observed in a previous study that analysed COD solubilisation after applying NaOH at mild temperature (50 °C) to different microalgae species (Mahdy et al., 2014a). They obtained values of 16-20% of COD solubilised when pretreating *Chlorella* sp. and 4-18% for *Scenedesmus* sp. The authors attributed such a low COD solubilisation to the fact that the tested pretreatments were unable to break down microalgae cell walls. Hence, soluble COD increase seemed to be caused by exopolymers release rather than intracellular material. Higher COD solubilisation was

observed by applying NaOH to *Chlorella* sp. and autoclaving at 120°C, achieving up to 81% CODs (Bohutskyi et al., 2014). This shows how higher solubilisation can be achieved by combining alkaline pretreatment with high temperatures as compared to mild temperatures.

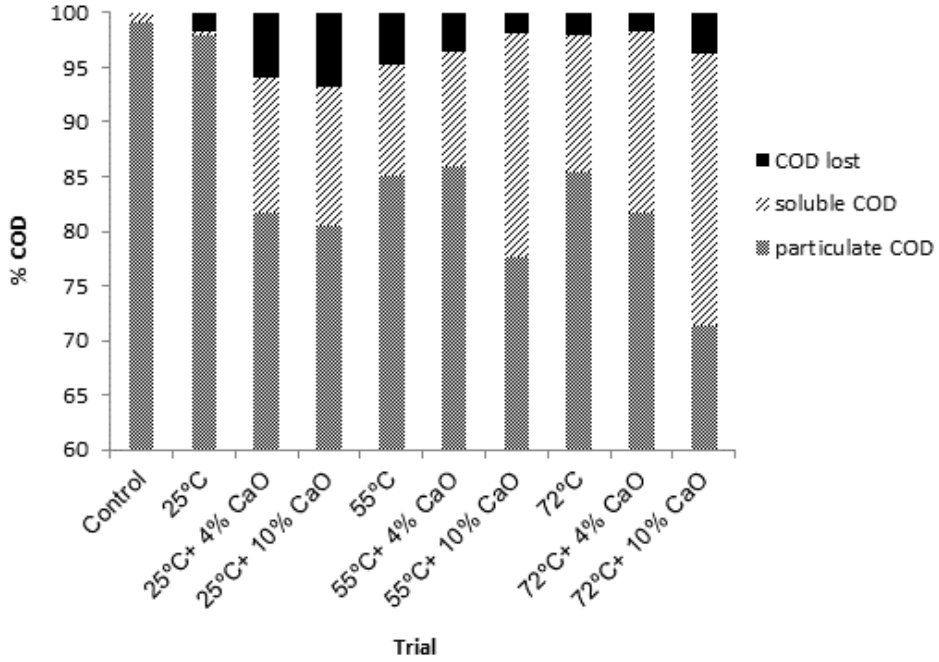


Figure 5-4 COD fractions after thermo-alkaline pretreatment, expressed as % of the total initial COD of untreated microalgae. Soluble fractions were calculated according to Eq. 5.1.1; particulate fractions were calculated as the difference between total COD and soluble COD; and removed COD fractions were calculated according to Eq. 5.1.4. Mean values (relative error < 2%).

5.1.3.3.2 Biomass loss during the pretreatment

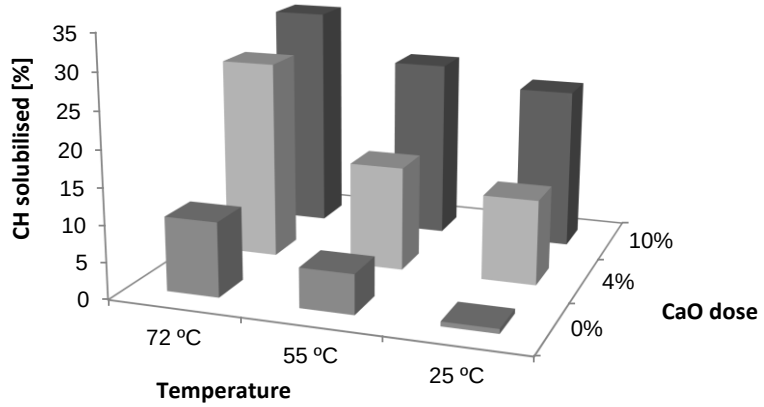
During the pretreatment step biomass loss should be minimised not to reduce the methane potential. In this study, biomass loss was expressed as the total COD removed during the pretreatment (Eq. 5.1.4) and the values were low (< 7%). As can be observed in figure 5.4, organic matter loss was the highest (between 6-7%) after alkaline pretreatment at room temperature. This was due to the fact that alkaline conditions were not preserved during the whole pretreatment (Table 5.2). Thus, biomass solubilisation by the pretreatment enhanced the consumption of readily biodegradable organic matter by heterotrophic bacteria. On the contrary, in the pretreatments at mild temperatures (55, 72 ºC), lime addition contributed to avoid

organic matter biodegradation (except for the sample pretreated at 72 °C with 10% CaO). In that case, thermal effects prevailed over biological ones.

5.1.3.3.3 Carbohydrate and protein solubilisation

CH and proteins are the main macromolecules of microalgae biomass (Table 5.1). In addition, CH are the main constituents of microalgae cell wall, which hampers microalgae hydrolysis. In order to evaluate the effect of the pretreatment on both macromolecules, CH and TKN (which is directly related to proteins) contents in the liquid phase were analysed after each pretreatment (Fig. 5.5 and 5.6).

A)



B)

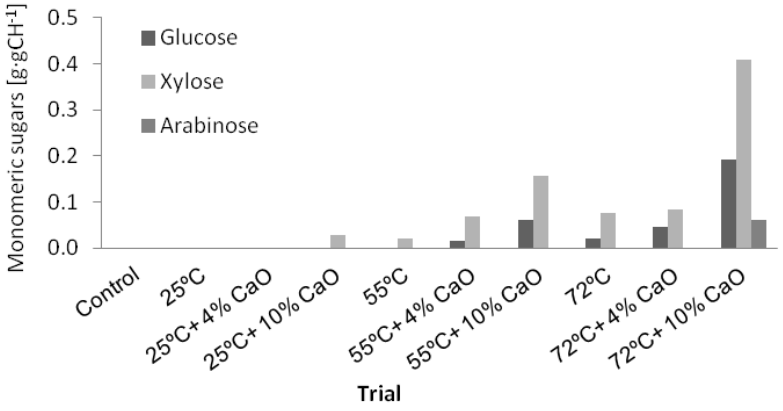


Figure 5-5 Carbohydrates solubilised (CHs) expressed as percentage over the total carbohydrates (CH) (Eq. 4.2.2) (A) and main sugar monomers solubilised (B) after each pretreatment. Mean values (relative error < 2%).

According to the results, CH solubilisation increased with temperature and lime dose (from 5% of solubilised CH for samples pretreated at room temperature with 4% CaO to 31% for samples pretreated at 72°C with 10% CaO). In fact, the combination of alkali and temperature could induce cellulose swelling, increasing the internal surface area and reducing the degree of crystallinity and polymerization (Kumar et al., 2009). Moreover, the hydrolysis of CH may occur through a variety of reactions induced by lime, including the disruption of H-bonds and saponification of intermolecular ester bonds in cellulose and hemicelluloses and crosslinking hemicellulose with other polymeric components (Ramirez et al., 2013). Indeed, carbohydrate release after thermo-chemical pretreatment of microalgae has already been reported (Hernández et al., 2015; Mahdy et al., 2014a).

However, the comparison of alkali and acid pretreatments showed how alkaline hydrolysis cleaved intermolecular linkages between complex polysaccharides and fibbers and other polymeric compounds, but only acid hydrolysis was able to break down complex carbohydrates into simple sugars (Hernández et al., 2015). Opposite to (Mahdy et al., 2014a), who observed low COD solubilisation (4-20%) attributed to exopolymers release, in the current study, the high COD and CH solubilisation (>30%) observed with the highest lime dose and temperature (10% CaO and 72ºC) could not only be attributed to exopolymers release but also other structural macromolecules. Indeed, the soluble fraction of different structural sugar monomers (i.e. glucose, xylose and arabinose) was also analysed (Fig. 5.5B). The goal was to verify if carbohydrates released during the pretreatment came not only from intracellular material but also from structural carbohydrates from the cell wall. The results showed a substantial increase in glucose and xylose after the pretreatment at the highest temperature and lime dose (72ºC and 10% CaO). Moreover, arabinose release was only detected in that case. Such a significant sugar release could be attributed to the cell wall damage, since the cell wall of the studied microalgae species is constituted by these monomeric sugars (Aikawa et al., 2015; Chakraborty et al., 2013).

Regarding proteins, there was no direct correlation between their solubilisation and the lime dose (Fig. 5.5). For the pretreatment at room temperature, the percentage of solubilised TKN was the highest with the lowest lime dose (17.2 and 12.9% with 4 and 10% CaO, respectively). Taking into account that the pH decreased after lime addition at room temperature (Table 5.2), it seems that the biological degradation of proteins prevailed over the chemical one. Thus, at room temperature the lowest lime

dose favoured the biological degradation of organic matter and consequently its solubilisation. A different behaviour was observed at 55 and 72ºC (Fig. 5.5), at which thermo-chemical effects prevailed over biological ones. Nevertheless, the highest soluble TKN fraction (32%) was reached with the most severe pretreatment condition (10% CaO and 72ºC).

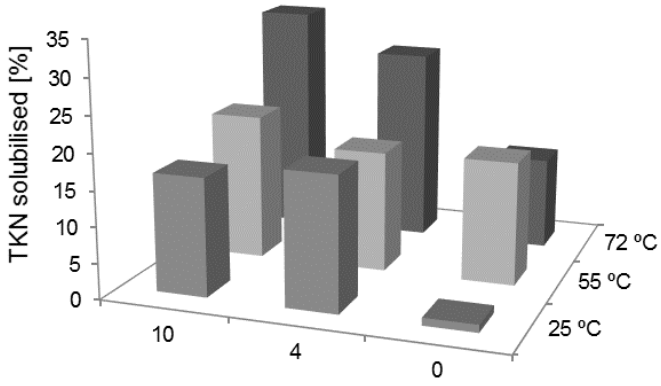


Figure 5-6 Soluble TKN (TKNs) after each pretreatment expressed as percentage over the TKN (Eq. 8.3). Mean values (relative error < 2%).

In conclusion, the use of alkali mainly enhanced protein solubilisation, while the combination of alkali and temperature was required to solubilise carbohydrates. This is in accordance with the literature. For instance, Mendez et *al.* (2013) found that proteins prevailed over carbohydrates solubilisation when *Chlorella* was subjected to alkaline conditions (Mendez et al., 2013). Similarly, Yang et *al.* (2011) concluded that protein solubilisation of lipid-extracted microalgal biomass was influenced by NaOH addition while carbohydrate solubilisation was not (Yang et al., 2011).

5.1.3.4 Effect of the pretreatment on the methane production

To evaluate the effect of pretreatments on the methane production, both methane production rate and extent were evaluated in BMP tests.

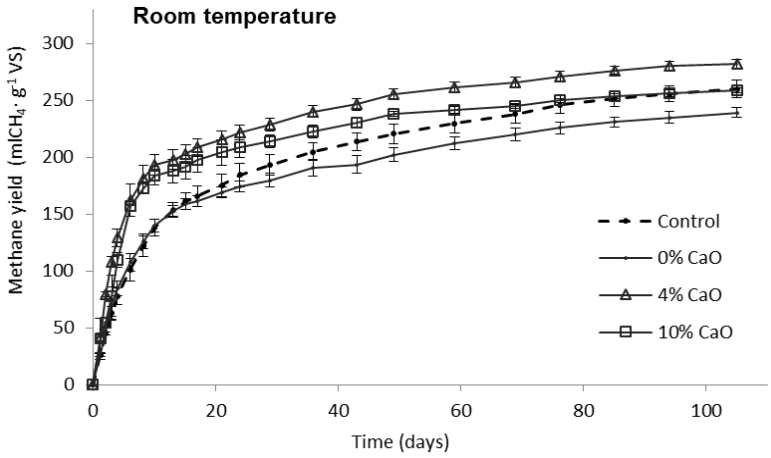
5.1.3.4.1 Biochemical methane potential increase with the pretreatment

Fig. 5.7 shows the cumulative methane yield obtained after 105 days of assay, while Table 5.3 reports the final methane potential achieved for each pretreatment condition. It should be notice that the methane yield is referred to the initial VS of

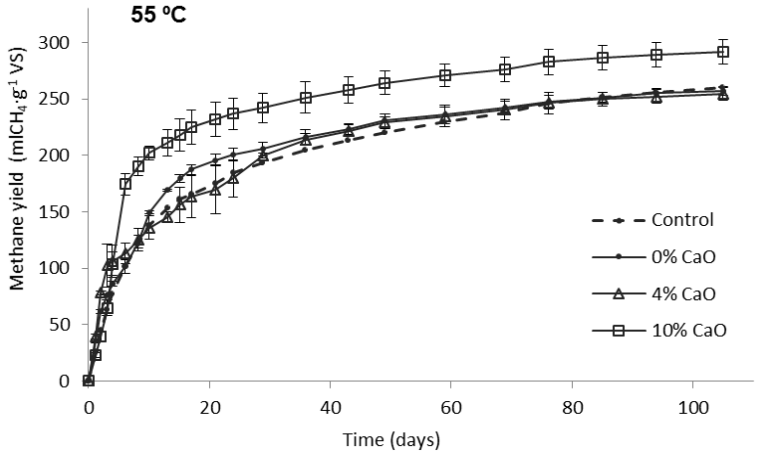
Table 5-3. Final methane yield and methane content obtained in BMP tests for each pretreatment condition (mean ± standard deviation).

Trial	Methane yield (mL CH_4·/g VS_{Rm}*)	Methane content (%)	Methane yield increase (%)	Methane loss (mL CH_4/gVS)	Methane yield increase considering methane loss (%)
Untreated microalgae	260 ± 8	67.2 ± 0.6	-	-	-
Room temperature	239 ± 5	67.5 ± 0.5	-8.0	10.3	-4.0
Room temperature + 4% CaO	282 ± 4	70.0 ± 1.0	8.4	29.7	19.8
Room temperature + 10% CaO	259 ± 2	75.5 ± 2.8	-0.5	39.9	14.9
55 ºC	257 ± 4	69.8 ± 0.7	-1.0	28.1	9.8
55 ºC + 4% CaO	255 ± 6	69.7 ± 0.3	-2.1	21.5	6.2
55 ºC + 10% CaO	292 ± 11	77.3 ± 1.8	12.2	11.2	16.5
72 ºC	230 ± 7	71.4 ± 0.5	-11.6	12.3	-6.8
72 ºC + 4% CaO	287 ± 4	74.3 ± 0.5	10.3	10.6	14.3
72 ºC + 10% CaO	325 ± 12	77.9 ± 0.6	25.0	22.1	33.5

*Rm= raw microalgae



A)



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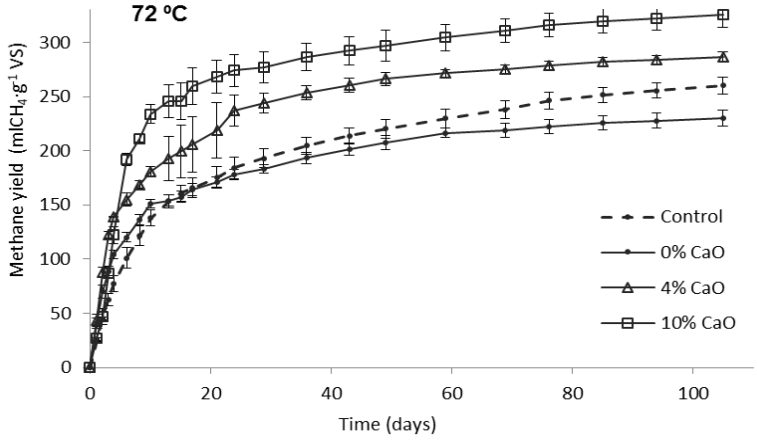


C)

Figure 5-7 Cumulative methane yield of chemically pretreated microalgae at room temperature (a) and thermo- chemically pretreated microalgae at 55 °C (b) and 72°C (c) with 0, 4 and 10% CaO

untreated microalgae. In Table 5.3, the methane yield increase is compared to the methane yield increase considering methane potential losses resulting from organic matter losses during the pretreatment step. To do so, COD losses (Eq. 5.1.4) were converted into methane losses

The results show how untreated microalgae produced 260 mL CH_4/gVS, which is in accordance with reported methane yields for *Chlorella* sp. (189-403 mL CH_4/gVS) and *Scenedesmus* sp. (240-287 mL CH_4/gVS) (Ward et al., 2014). Some samples presented a similar methane yield after the pretreatment (i.e. 10% CaO at 25 ºC; 0% and 4% CaO at 55 ºC), while in others the methane yield increased by 10% (i.e. 4% CaO at 25 and 72 ºC; 10% CaO at 55 ºC). The most significant methane yield increase (25%) was achieved by the pretreatment with 10% CaO at 72 ºC (325 mL CH_4/gVS). This methane yield increase is even higher (> 33% increase) if the biomass loss during the pretreatment step is taken into account. The highest methane production can be attributed to the highest solubilisation of both carbohydrates and proteins after the thermo-chemical pretreatment (Fig. 5.5 and 5.6), and to the release of sugar from the cell wall, namely glucose, xylose and arabinose (Fig. 5.5B). Accordingly, the methane production increase may have resulted from the cell wall damage after the pretreatment with 10% CaO at 72ºC. Similar results were obtained by pretreating *Chlorella* sp. and *Scenedesmus* sp. with 5% NaOH at 50 °C increasing the methane yield by 17 and 20%, respectively (Mahdy et al., 2014a). Comparing the lime pretreatment with others, similar methane yield increase (29%) was achieved by applying a thermal pretreatment at 120 °C on *Chlorella* sp. and *Scenedesmus* sp. culture (Cho et al., 2013) and a low-temperature pretreatment at 80 °C on *Chlorella vulgaris* (11–24%) (Kinnunen and Rintala, 2016). Regarding mechanical pretreatments, lower values were obtained by applying ultrasounds (6-15%) (Cho et al., 2013) but higher improvements were found with other mechanical pretreatments (i.e. milling) on *Acutodesmus obliquus* (51%) (Gruber-Brunhumer et al., 2015). Comparing the effect of lime for each tested temperature, two different trends were observed. For thermally pretreated samples, the higher the dose of lime, the higher the methane yield (increasing from 257 to 292 ml CH_4/gVS at 55 °C and from 230 to 325 ml CH_4/gVS at 72 °C). Conversely, the pretreatment at room temperature presented the highest methane yield with 4% CaO (282 ml CH_4/gVS). These results are consistent with the higher protein solubilisation obtained with 4% CaO compared to 10% CaO, and also with the higher biomass loss of the pretreatment with 10% CaO. According to the results, the thermo-alkaline pretreatment had more effect in terms of biomass solubilisation than methane production. Indeed, it has been shown that organic

matter solubilisation can increase significantly more than the methane yield of several microalgae species (Bohutskyi et al., 2014; Cho et al., 2013). Nevertheless, with the most severe condition (10% CaO at 72 ºC) not only biomass solubilisation but also the final methane yield was improved.

5.1.3.4.2 Kinetics improvement with the pretreatment

All the pretreatments improved the kinetics of the process as shown by the first order kinetic constant (*k*) (Table 5.4). While untreated microalgae showed the lowest *k* (0.08 day^{-1}), *k* values increased to 0.09-0.14 day^{-1} when biomass was pretreated. In general, the higher the lime dose, the higher the *k*. This kinetics enhancement was attributed to organic matter solubilisation after the pretreatment. Altogether, no correlation between the percentage of COD solubilised and the kinetic rate constant was found (R^2=0.136). However, since alkaline and thermo-alkaline pretreatments presented different behaviours in terms of macromolecules solubilisation and methane production, the correlation was analysed separately. By doing so, higher correlation coefficients were found (R^2=0.985 and R^2=0.779 for the alkaline and thermo-alkaline pretreatments, respectively).

The kinetics improvement could be responsible for the higher methane production rate during the first days of the BMP test (Fig. 5.7). To ease comprehension, the methane yield increase for each pretreatment condition with respect to untreated microalgae at days 10, 21 and 36 was compared (Fig. 5.8). As can be observed in figure 5.8, alkaline and thermo-alkaline pretreatments presented different behaviours. Once again, higher values were obtained with 4% CaO for the alkaline pretreatment at room temperature and 10% CaO for all thermo-alkaline pretreatments

5.1.4 Conclusions

This study evaluated the effect of a thermo-alkaline pretreatment with lime on microalgal biomass anaerobic digestion. The pretreatment increased proteins and carbohydrates solubilisation up to 32.4% and 31.4%, respectively. Consequently, anaerobic digestion kinetics were also improved (the first order kinetic rate constant increased from 0.08 to 0.14 day^{-1}). The pretreatment with the highest lime dose (10% CaO) and temperature (72 °C) showed both the highest macromolecules solubilisation (31-32%) and the highest biochemical methane potential increase (25%). Bearing in mind that lime is not toxic and that it is less expensive than other chemicals (e.g. NaOH), the use of lime could also contribute to reduce pretreatment

Table 5-4. Kinetic parameters obtained from Eq.5.1.5. Estimated error variance (S^2) of each fitting calculated from Eq. 5.1.6.

Trial	λ (day)	Bo (ml CH_4/gVS)	k (day^{-1})	S^2
Untreated microalgae	0.00	238	0.08	173
Room temperature	0.00	214	0.10	209
Room temperature + 4% CaO	0.00	255	0.14	325
Room temperature + 10% CaO	0.00	237	0.14	201
55 ºC	0.00	240	0.09	132
55 ºC + 4% CaO	0.00	236	0.09	456
55 ºC + 10% CaO	1.17	271	0.12	261
72 ºC	0.00	209	0.12	274
72 ºC + 4% CaO	0.00	265	0.12	398
72 ºC + 10% CaO	1.17	305	0.13	223

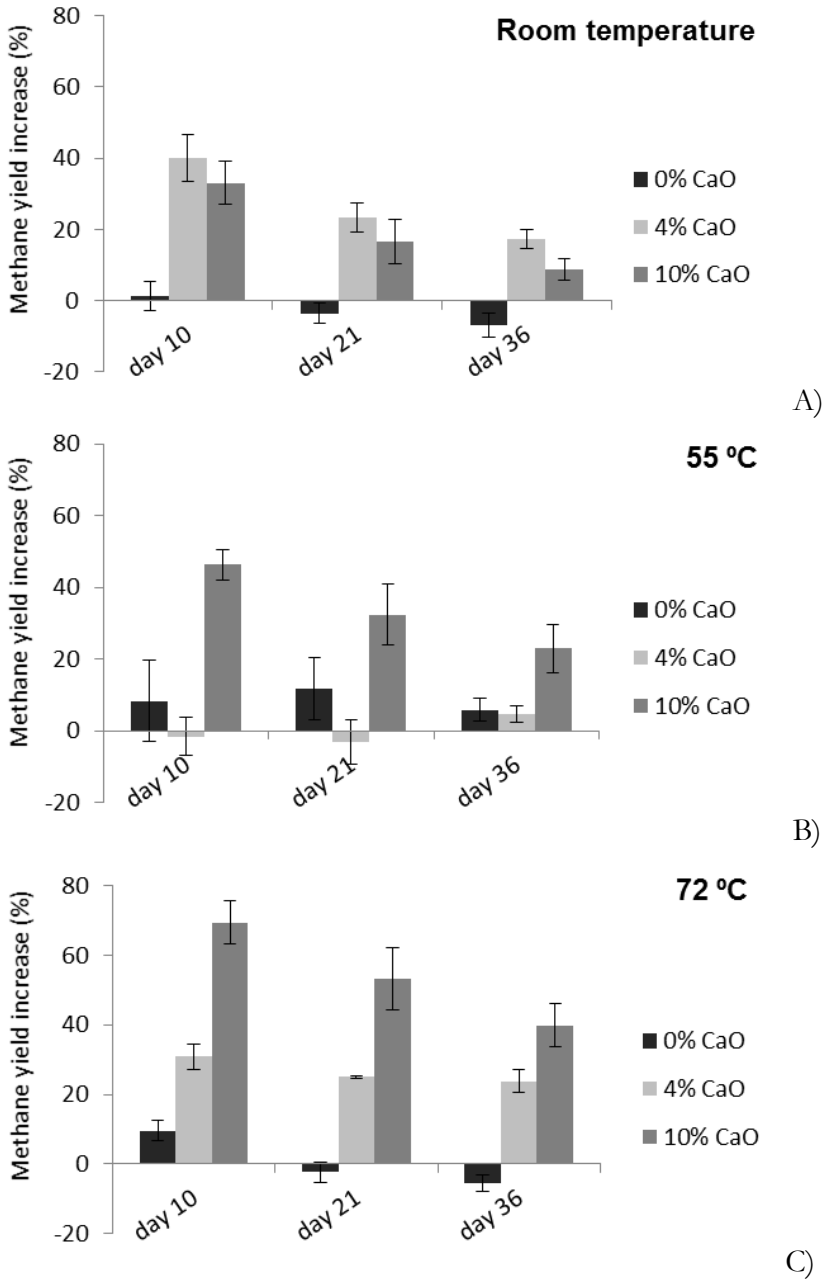


Figure 5-8 Methane yield increase of pretreated samples at room temperature (A), 55 ºC (B) and 72 ºC (C) with respect to untreated microalgae (control) after 10, 21 and 36 days of BMP assay.

costs and potential environmental impacts. Nevertheless, the application of the best pretreatment condition should be further investigated in continuous reactors to estimate the energy balance and economic cost of the process.

5.2 Anaerobic co-digestion of microalgal biomass and wheat straw

This study aimed at analysing the anaerobic co-digestion of microalgal biomass grown in wastewater and wheat straw. To this end, Biochemical Methane Potential (BMP) tests were carried out testing different substrate proportions (20-80, 50-50 and 80-20%, on a volatile solid basis). In order to improve their biodegradability, the co-digestion of both substrates was also evaluated after applying a thermo-alkaline pretreatment (10% CaO at 75 ºC for 24h). The highest synergies in degradation rates were observed by adding at least 50% of wheat straw. Therefore, the co-digestion of 50% microalgae - 50% wheat straw was investigated in mesophilic lab-scale reactors. The results showed that the methane yield was increased by 77% with the co-digestion as compared to microalgae mono-digestion, while the pretreatment only increased the methane yield by 15% compared to the untreated mixture. Thus, the anaerobic co-digestion of microalgae and wheat straw was successful even without applying a thermo-alkaline pretreatment.

5.2.1 Introduction

In order to overcome the world's major challenges of freshwater shortage and energy crisis, carbon- and energy-neutral wastewater treatment processes are urgently needed. Towards this goal, algae-based wastewater treatment plants (WWTPs) offer many advantages over the conventional WWTPs with activated sludge process for carbon (C) and biological nutrient removal processes for nitrogen (N) and phosphorus (P) treatment. Microalgae are capable of using inorganic N, P in the wastewater along with CO_2 and produce biomass and oxygen through photosynthesis in the presence of sunlight. The oxygen produced by microalgae can be utilized by heterotrophic bacteria within the flocs for organic C removal which reduces the energy requirement of wastewater treatment and provides CO_2 for microalgae (Rawat et al., 2011). Furthermore, excess algal biomass from the wastewater treatment process can be digested/co-digested in anaerobic digesters (Golueke et al., 1957; Ward et al., 2014) for organic matter reduction and methane-

rich biogas recovery prior to land application as soil amendment (Solé-Bundó et al., 2017b).

Despite the aforementioned advantages, there are barriers to accomplish sustainable, large-scale, algae-based WWTPs incorporating anaerobic digestion. First of all, volatile solids (VS) removal of microalgal biomass grown in wastewater is limited to 21–36% in continuously-fed anaerobic digesters at a hydraulic retention time (HRT) range of 15–20 days with specific methane yields of 0.10–0.18 L/ g VS (Passos and Ferrer, 2014). The low conversion yield to methane is attributed to the nature of the cell structure in microalgae, which is mostly composed of organic compounds with low biodegradability that creates resistance to hydrolysis during anaerobic digestion. Furthermore, as the type of predominant species in microalgal biomass and their growth rates are quite seasonal depending on wastewater characteristics and availability of sunlight, the amount, characteristics and biodegradability of algal biomass are changing throughout the year (Passos et al., 2015b).

In the last 10 years, many pretreatment technologies have been investigated to break apart the complex structure of microalgae and make organics within the cell walls bioavailable to acid/methane formers to increase methane yields. A review by Passos et al. (2014) revealed that thermal (< 100 °C, atmospheric pressure), hydrothermal (> 100 °C, gradual pressure release), and steam explosion (> 100 °C, sudden pressure release) pretreatments of different microalgae species (some grown in wastewater) resulted in a wide range of improvements in methane yields (-13 to 220%). In general, pretreatments achieving high temperature (110 – 170 °C) and pressure (1 - 6.4 bar) via steam injection/explosion or hydrothermal ways achieved superior solubilization/methane yield results (Alzate et al., 2012). However, energy assessments rarely pointed out a feasible full-scale application unless microalgal biomass was concentrated (i.e. > 8% TS) prior to pretreatment (Passos and Ferrer, 2015). Mechanical pretreatments (i.e. ultrasound, microwave, high-pressure homogenization) were found less microalgae strain-dependent but required high energy input (i.e. 132 – 529 MJ/kg dry mass) (Lee et al., 2012). There are only a few studies reported on chemical (acid or alkali) and thermo-chemical pretreatment of different microalgae species so far with the latter, in general, achieving better results in terms of solubilization/methane yield (Bohutskyi et al., 2014; Solé-Bundó et al., 2017a). Similar pretreatments, mostly with NaOH or $Ca(OH)_2$ in a wide range of combinations (0.5 -30% w/w, 15 – 160°C, 10 min – 48 h), were previously tested and reported as effective in breaking ester bonds between lignin and polysaccharides and

improving both hydrogen/methane production from a variety of lignocellulosic substrates (Monlau et al., 2013). However, controversial results were also obtained for thermo-chemical pretreatment of microalgae. For example, among chemical (4 M H_2SO_4 at pH = 2, 4 M NaOH, pH = 10), thermal (120^{o}C for 20 or 40 min) and a combination of the aforementioned pretreatments tested, thermally pretreated (120 ^{o}C, 40 min) *Chlorella vulgaris* produced the highest methane yield which was attributed to the formation of inhibitory substances during the chemical and thermo-chemical pretreatments (Mendez et al., 2013). More research is needed to identify/quantify inhibitors to optimize thermo-chemical pretreatment of microalgae.

Another bottleneck of microalgal biomass digestion is significantly lower (~6) than optimum C/N ratio (15-30) (Weiland, 2010) of microalgae which may lead to ammonia toxicity to methanogens (Yen and Brune, 2007). One remedy to this problem is co-digestion of microalgal biomass with commonly available, carbon-rich substrates such as paper waste (Yen and Brune, 2007) or lignocellulosic waste (i.e. wheat straw, sorghum, maize) (Rétfalvi et al., 2016). Paper and lignocellulosic wastes can also benefit from moisture and nutrient content of microalgae when co-digested. If a low-cost pretreatment method, effective for both microalgae and lignocellulosic waste, could be identified, co-digestion of pretreated microalgae and/or the co-substrate could enhance both the rate and extent of digestion with a more favourable energy balance. Therefore, the main objective of this study was to evaluate, for the first time, the improvement of the microalgae anaerobic digestion by adding wheat straw. Moreover, thermo-alkaline pretreatment of microalgae with wheat straw was assessed under both batch and continuous flow mesophilic anaerobic co-digestion. Thermo-alkaline pretreatment (10% CaO, 72 °C, 24 h) was selected based on the previous literature that optimized pretreatment conditions for microalgal biomass digestion (Solé-Bundó et al., 2017a). Although these conditions were optimized for microalgae, literature review indicated that these conditions were also found effective for wheat straw pretreatment (Monlau et al., 2013).

5.2.2 Materials and Methods

Batch experiments were conducted at INRA –LBE (Narbonne, France), while continuous flow reactors were operated at GEMMA – UPC (Barcelona, Spain). This necessitated changes in characteristics of inoculum and analytical methods which are outlined below.

5.2.2.1 Biochemical methane potential (BMP) assays

5.2.2.1.1 Microalgal biomass and lignocellulosic biomass

Microalgal biomass was grown in a pilot-scale high-rate algal pond (HRAP) equipped with a paddle wheel for mixing and had an effective volume of 470 L. HRAP was located outdoors at the laboratory of the GEMMA research group and utilized natural sunlight. The domestic wastewater was first treated in a primary settling tank (effective volume of 7 L, HRT of 0.9 h) and then fed to HRAP under an HRT of 8 days. Upon treatment, effluent from HRAP was sent to a secondary clarifier (9 L, HRT of 9 h) where microalgal biomass was harvested. In order to increase TS concentration to around 2.8 ± 0.1% TS (w/w), microalgal biomass was further thickened in bench-scale Imhoff cones at 4°C for 24 h. Microscopic examination of biomass indicated that the predominant microalgae specie was *Chlorella sp*. although *Monoraphidium sp.* and diatoms were also observed.

Wheat straw, grown in France (48°50´18´´N, 4°13´54.5´´E), was used as lignocellulosic agricultural biomass. It was processed using a cutting mill, and was further sieved to have a particle size range of 400 μm - 1 mm (Fig. 5.9).

Figure 5-9. Wheat straw after milling and sieving.

5.2.2.1.2 Anaerobic inoculum

The inoculum used was granular sludge from a mesophilic upflow anaerobic sludge blanket (UASB) reactor treating wastewater from a sugar factory in France. Prior to setting up BMP assays, the inoculum was placed in a 5 L glass closed vessel and

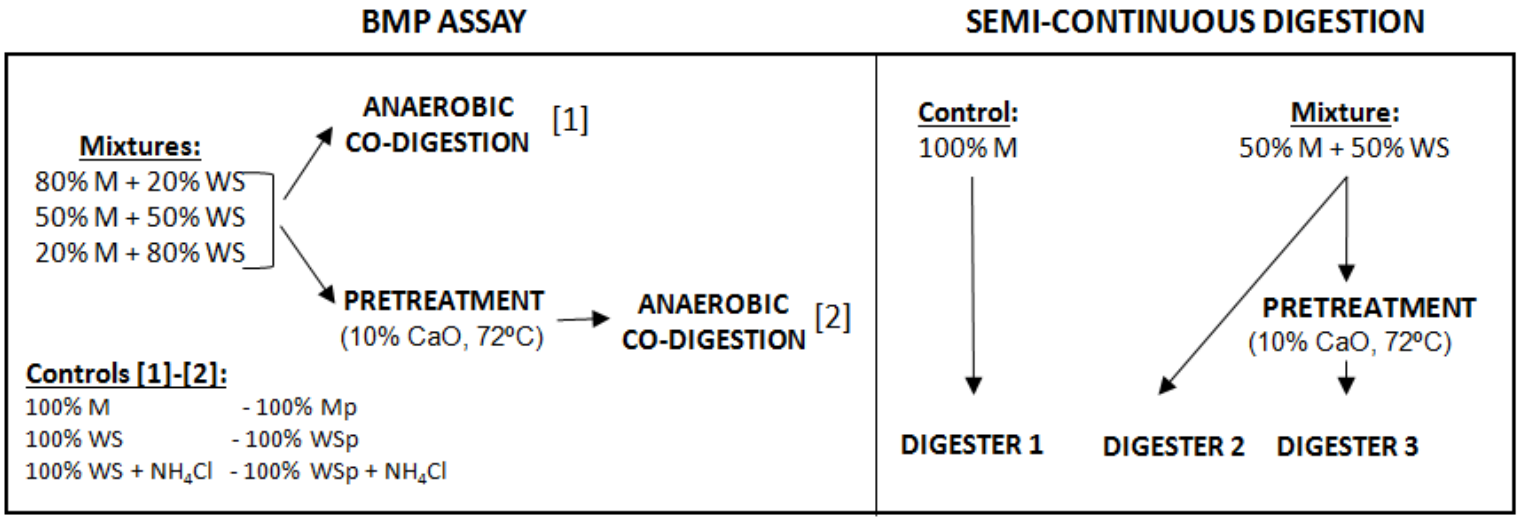


Figure 5-10. Experimental set-up.

Note: M= microalgae; Mp= pretreated microalgae; WS= wheat straw; WSp= pretreated wheat straw

mixed to break apart the granules under endogenous anaerobic conditions (35°C for 5-7 days) to reduce non-specific biogas generation. The inoculum contained TS and VS concentrations of 2.93 ± 0.04 and 2.55 ± 0.03% (w/w), respectively. It had a maximum specific methanogenic activity of 33 ± 2 mL CH_4/g VS/day, as measured by degrading 1.3 ± 0.3 g/L of ethanol as chemical oxygen demand (COD).

5.2.2.1.3 Thermo-alkaline pretreatment

Thermo-alkaline pretreatment of microalgal biomass and wheat straw was conducted in glass BMP bottles, with total and effective volumes of 160 and 100 mL, respectively. Microalgal biomass and/or wheat straw were first added to the bottles according to figure 5.10. The bottles were sealed with septa/aluminium caps and kept in an oven (set to 72 °C) for 24 h without mixing after addition of CaO in dry form (10 g CaO/100 g TS of substrate). Distilled water was added in different amounts to bottles to ensure that all pretreatments were performed at the same TS concentration.

5.2.2.1.4 BMP assay set-up

BMP assays were conducted in the same bottles as the thermo-alkaline pretreatment. Upon completion of thermo-alkaline pretreatment, the bottles were cooled down to ambient temperature (~20°C), and the pH of the substrates in the bottles were measured. To prevent accumulation of volatile fatty acids (VFAs) during digestion, each bottle was added 5.2 ml of buffer solution prepared at 2.6 g $NaHCO_3$/L concentration. To be able to see the effect of C/N ratio balancing in the co-digested BMPs, the assays were conducted without external nutrient addition. However, considering the risk of not being able to digest wheat straw without nutrient addition, additional bottles were set-up with wheat straw / pretreated wheat straw and 1.7 ml of NH_4Cl solution at 0.5 g/L concentration as controls.

A total of 39 bottles (including triplicates and blanks) were operated to assess the BMP performance. Each bottle contained substrate (single or co-substrates) concentration of 4 g VS/L. The amount of the substrate and inoculum added to each bottle was calculated considering the food/microorganism (F/M) ratio of 1 gVS/gVS. In the co-digested BMP bottles displayed in figure 5.10, 20, 50 and 80% represented VS weight percentages of microalgal biomass or wheat straw in the total substrate concentration (i.e. 4 g VS/L) in the bottles. Finally, the bottles were filled up to 100 mL with distilled water and nitrogen gas was purged to each bottle to remove residual oxygen. Upon sealing the bottles with septa/caps, the excess pressure caused during the purging was released by puncturing the septa with a

needle. The digesters were then located on a shaker (at 90 rpm) in a temperature controlled room at 37 °C. Accumulated gas pressure in the bottles was measured with a digital manometer (LEO 2, Keller, Switzerland), while biogas composition was analysed by a gas chromatograph (GC).

5.2.2.2 Continuous flow digestion

5.2.2.2.1 Microalgal and lignocellulosic biomass

Microalgal biomass was obtained from the same HRAP system described for BMP assays (section 4.2.2.1.1) and thickened using the same methodology. Throughout the operation of the continuous flow digesters, TS and VS concentrations of microalgal biomass changed in ranges of 2.6-3.0% and 1.8-2.4%, respectively. The lignocellulosic substrate had identical characteristics described for BMP assays (section 4.2.2.1.2). Microalgae and wheat straw were co-digested by 50-50% on VS basis, according to previous BMP assay results.

5.2.2.2.2 Anaerobic inoculum

Anaerobic mesophilic digested sludge from a municipal WWTP (Barcelona, Spain) was used to inoculate the semi-continuously fed digesters. The inoculum contained TS and VS concentrations of 2.14 ± 0.01 and 1.31 ± 0.01% (w/w), respectively

5.2.2.2.3 Thermo-alkaline pretreatment

Thermo-alkaline pretreatment of microalgal biomass and wheat straw was conducted together in the same glass bottle, with total and effective volumes of 250 and 150 mL, respectively. Microalgal biomass and/or wheat straw were added to the bottles according to Table 3. The bottles were kept in an oven (set to 72 °C) for 24 h under continuous stirring after addition of CaO in dry form (10 g CaO/100 g TS of substrate). Distilled water was added in different amounts to bottles to ensure that all pretreatments were performed at the same TS concentration.

5.2.2.2.4 Reactor set-up

Microalgae anaerobic digestion performance was monitored using three bench-scale reactors (2 L), with an effective volume of 1.5 L. One of the digesters utilized untreated microalgal biomass and operated as control. The second one simulated a co-digester and received untreated microalgae and wheat straw. The third reactor was fed with thermo-alkaline pretreated microalgal biomass and wheat straw

Reactors were operated under mesophilic conditions (37 ± 1°C) by implementing an electric heating cover (Selecta, Spain). Constant mixing was provided by a magnetic stirrer (Thermo Scientific). Reactors were operated on a daily feeding basis, where the same volume was purged from and added to digesters using plastic syringes (50 mL). Reactors were operated at an HRT of 20 days and were considered to be under steady-state after three complete HRTs. Afterwards, anaerobic digestion performance was further monitored during 2 complete HRTs (~6 weeks). The total operation period of the digesters was 106 days. Biogas production was measured by the water displacement method and the methane content was periodically analysed by GC. The volume of the produced biogas was adjusted to the standard temperature (0 °C) and pressure (1 atm) condition (STP).

5.2.2.3 Analytical procedures

The TS/VS analysis was done according to the Standard Methods (APHA, 2005). Quantification of total and soluble (< 0.45 μm) COD concentrations were performed according to the closed reflux colorimetric method outlined by Standard Methods (APHA, 2005). Except for the raw wheat straw samples, all pretreated and untreated substrates and co-substrates were freeze dried (for a minimum of 3 days, at -69°C, 0.25 atm) before structural carbohydrates, lignin, protein and lipid content quantification. Determination of cellulose, hemicelluloses and Klason lignin in raw/pretreated wheat straw were measured using a strong acid hydrolysis method adapted from Sluiter et al. (2008). Raw or freeze-dried samples (100 mg) were first hydrolysed with H_2SO_4 (72%) in capped/mixed test tubes at 30°C for 1 h, then diluted to reach a final acid concentration of H_2SO_4 (4%) and kept at 120°C for 1 h. Upon cooling, the tube content was filtered via glass-fibber filters (0.45 μm) to separate insoluble residue, which was placed in a crucible/dried at 100°C for 24 h to yield Klason lignin content. The liquid fraction obtained after filtration was further filtered via 0.2 μm and analyzed by a high-performance liquid chromatograph (HPLC) equipped with a refractive index detector (Waters R410/Waters 2414) for structural carbohydrates (i.e. glucose, xylose and arabinose). Target compounds were separated by an Aminex HPX-87H column (300 x 7.8 mm, Bio-Rad) placed after a protective precolumn (Microguard cation H refill catbridges, Bio-Rad). The eluting solution was 0.005 mM H_2SO_4**,** and the flowrate, column/detector temperatures were 0.3 mL/min, 45°C, respectively. TKN was determined by titration after a mineralization step performed by a BUCHI 370-K distillator/titrator. Total organic carbon (TOC) was measured using an automatic analyser (aj- Analyzer multi N/C 2100S). TOC was analysed with an infrared detector (NDIR) according to

combustion-infrared method of Standard Methods (APHA, 2005) by means of catalytic oxidation at 800ºC using CeO_2 as catalyst. The concentration of the ammonium nitrogen ($N\text{-}NH_4^+$) was measured according to the method by Solorzano (1969). pH was determined with a Crison Portable 506 pH-meter.

Biogas composition in BMP bottles was conducted by measuring the percentage of methane, oxygen, nitrogen, hydrogen, and carbon dioxide in the digester headspace using a GC (Clarus 580, Perkin Elmer) equipped with a thermal conductivity detector (TCD) and RtQBond/RtMolsieve columns. The carrier gas was argon and injector/detector/oven temperatures of 250, 150, 60ºC, respectively. Methane percentage from continuous-flow reactors were quantified twice a week with a similar GC/TCD configuration (Trace GC Thermo Finnigan with Hayesep packed column) with injector/detector/oven temperatures were 150, 250, 35 ºC, respectively, using helium gas as carrier.

Volatile fatty acids (VFA) concentrations in continuous flow digesters were measured once a week by injecting 1 μL of each sample, once centrifuged (4200 rpm for 8 min) and filtered (0.2 μm), into an Agilent 7820A GC after sulphuric acid and diisopropyl ether addition. The GC was equipped with an auto-sampler, flame ionization detector and a capillary column (DP-FFAB Agilent 30 m x 0.25 mm x 0.25 μm), and operated at injector and detector temperatures of 200 and 300ºC, respectively, with helium as carrier gas.

5.2.2.4 Statistics and kinetic data analysis

The statistically significant effects of independent variables were evaluated via multi-factor analysis of variance (ANOVA) considering 95% confidence level ($\alpha = 0.05$) using R Statistics Software.

In order to evaluate the kinetics of the process from BMP tests, experimental data was adjusted to a first-order kinetic model (Eq. 5.2.1) by the least square method.

$$B = B_0 \cdot \{1 - exp[-k \cdot t]\} \qquad \text{(Eq. 5.2.1)}$$

where, B_0 stands for the methane production potential ($\text{ml } CH_4/\text{gVS}$), k is the first order kinetic rate constant (day^{-1}), B is the accumulated methane production at time t ($\text{ml } CH_4/\text{gVS}$) and t is time (day).

The error variance (s^2) was estimated by the following equation:

$$s^2 = \frac{\sum_1^i (y_i - \hat{y}_i)^2}{N-K} \qquad \text{(Eq. 5.2.2)}$$

where y_i is the experimental value, $\hat{y}_i$ is the value estimated by the model, N is the number of samples and K is the number of model parameters.

5.2.3 Results and Discussion

5.2.3.1 Thermo-alkaline pretreatment of microalgae and wheat straw

Several studies have recommended the application of pretreatments on microalgae and wheat straw in order to enhance their bioconversion into methane. While microalgae resistant cell wall can be damaged by different pretreatment methods (Passos et al., 2014b), lignocellulosic biomass delignification followed by hemicelluloses and cellulose hydrolysis can also be enhanced by applying pretreatments (Croce et al., 2016). Therefore, a thermo-alkaline pretreatment with CaO was tested on both substrates before their anaerobic digestion/co-digestion. The simultaneous application of a pretreatment on both substrates may reduce the operation costs and ease their management in full-scale plants. The pretreatment conditions were 10% CaO at 72 °C for 24 h, based on a previous study that evaluated the addition of different CaO doses at different temperatures on microalgae (Solé-Bundó et al., 2017a). The study concluded that these conditions lead to the highest levels of carbohydrate and protein solubilization (up to 32 and 31%, respectively). Moreover, 25% methane yield increase compared to untreated microalgae was obtained in BMP tests (Solé-Bundó et al., 2017a). In contrast, the methane yield increase achieved by the thermo-alkaline pretreatment in the present study was 9% (Table 5.5). Although the methane yield of raw microalgae was similar in both cases (260 ml CH_4/g VS in Solé-Bundó et al. and 264 ml CH_4/g VS in this study), the methane yield achieved after applying the same pretreatment was slightly lower in the latter (325 ml CH_4/g VS *vs.* 287 ml CH_4/g VS). This difference may be attributed to the characteristics of the microalgae culture. In the first one the mixed culture was predominated by *Chlorella* sp. and *Scenedesmus* sp., while in the second one it was mainly predominated by *Chlorella* sp. and contained some diatoms and *Monoraphidium*

Table 5-5. Ultimate methane yield obtained in the BMP assay (mean values ± standard deviation; n=3) and first-order kinetics (k) obtained from Eq.8.1. (the error variance (S2) of each fitting (Eq. 8.2) is represented in brackets).

Substrates	C/N*	Methane yield, ml CH_4/g VS				First-order kinetics, day^{-1}			
		Experimental values [a]		Calculated values from mono-digestions [b]		Experimental values [a]		Calculated values from mono-digestions [c]	
		Untreated	Pretreated	Untreated	Pretreated	Untreated	Pretreated	Untreated	Pretreated
Control Microalgae	7.4	264 ±3	287 ±9	-	-	0.085 (175)	0.133 (205)	-	-
80% Microalgae + 20% Wheat Straw	8.9	279 ±6	289 ±15	267 ± 3	290 ± 7	0.079 (114)	0.150 (186)	0.075 (199)	0.131 (188)
50% Microalgae + 50% Wheat Straw	13.1	289 ±3	299 ±15	271 ± 5	295 ± 6	0.071 (80)	0.150 (159)	0.062 (224)	0.127 (166)
20% Microalgae + 80% Wheat Straw	26.4	289 ±4	315 ±7	276 ± 7	300 ± 6	0.067 (55)	0.142 (172)	0.051 (236)	0.124 (147)
Control Wheat Straw	95.4	279 ±9	304 ±7	-	-	0.045 (240)	0.122 (136)	-	-
Control Wheat Straw + NH_4Cl	-	280 ±9	303 ±7	-	-	0.049 (61)	0.125 (157)	-	-

* C/N = TOC/TKN

[a] Values obtained from experimental data in BMP assay

[b] Values calculated as the sum of the final methane yields produced for each substrate mono-digestion: ((pretreated) wheat straw/(pretreated) microalgae).

[c] Values obtained from the curves that represent the sum of the individual ((pretreated) wheat straw /(pretreated) microalgae) methane yields produced over the time

sp. It is well known that the methane production from microalgal biomass is highly species-dependent, and not only governed by its biochemical composition but also by their cell structure (Bohutskyi et al., 2014). Comparing the effect of this pretreatment with that obtained by applying other technologies or methods, a moderate effect was here observed. For example, Passos et al. (2015) reported 72% methane yield increase by applying a thermal pretreatment at 95 °C for 10 h. Similarly, an enzymatic pretreatment with carbohydrolase and protease showed 55% methane production enhancement on *Chlorella vulgaris* (Mahdy et al., 2014b). Although 9% methane yield increase would not justify the pretreatment costs, an important first-order kinetic constant increase was obtained after the pretreatment (from k = 0.085 to 0.133 day^{-1}). This can have an impact on the continuous anaerobic digestion typically operated at 20-30 days of HRT.

Compared to microalgae, wheat straw showed a slightly higher methane yield (279 ml CH_4/g VS) but considerably slower kinetics (k = 0.045 day^{-1}) (Table 5.5). Since wheat straw has a very high C/N ratio (~95), the deficit of nitrogen may actually limit the final methane yield obtained in BMPs. Thus, the same wheat straw supplemented by NH_4Cl was also tested (Table 5.5). When both BMP assays were compared, results showed no significant differences between the methane yields (p-value= 0.926). Concerning the kinetics, when NH_4Cl was added, only a slight increment in the first-order kinetic constant was obtained (from k = 0.045 day^{-1} to 0.049 day^{-1}). This suggests that microorganisms were in fact using the nitrogen from the digested sludge used as inoculum. Therefore, the methane yield of the wheat straw itself was not underestimated, and wheat straw without NH_4Cl could be used as control for the co-digestion analysis in the following sections.

Conversely to microalgae, the pretreatment conditions used in this study were not optimized for wheat straw. However, according to Carrere et al. (2015), alkaline pretreatments are promising techniques to enhance the anaerobic digestion of lignocellulosic biomass. Indeed, the application of these pretreatments and their effects have extensively been reported. The main idea is to increase the accessibility and solubility of cellulose and hemicelluloses by facilitating delignification. According to the literature, wheat straw is characterized by having high carbohydrate polymer content (cellulose and hemicelluloses) and relatively low lignin content (Croce et al., 2016). The wheat straw used in this study was composed by 32% cellulose, 29% hemicelluloses and 23% lignin. This composition is coherent with the literature (Barakat et al., 2015). In order to study the effect of the pretreatment on the wheat

straw structure, its chemical composition was evaluated before and after pretreatment (Table 5.6). Slight lignin removal (9%) and more notorious hemicelluloses removal (25%) were observed. Consequently, an increase of soluble sugars was also observed (from 2.8 to 8.4%). However, the celluloses content was not reduced. This is in accordance with most of the literature that evaluated the effect of an alkaline or thermo-alkaline pretreatment on lignocellulosic biomass. However, the level of delignification or hemicelluloses removal varies among them. For instance, Reilly et al. (2015) applied 7.4% of $Ca(OH)_2$ for 42 h to wheat straw obtaining low delignification but 30% hemicelluloses removal. On the other hand, Sambusiti et al. (2013) applied 10% NaOH at 100ºC on wheat straw and obtained a higher decrease of lignin (53%). Considering these results, it can be concluded that $Ca(OH)_2$ is not as effective as NaOH, although the pretreatment effectiveness also depends on the substrate. Furthermore, the application of temperature during the pretreatment may facilitate delignification. For example, Monlau et al. (2012) achieved up to 30% lignin removal by applying 4% $Ca(OH)_2$ at 55ºC for 24 h on sunflower stalks. Although sunflower stalks composition is similar to that of wheat straw, higher lignin removal was achieved by applying the pretreatment on stalks.

Table 5-6. Chemical composition of wheat straw, before and after the thermo-alkaline pretreatment. Mean values ± standard deviation of triplicates.

	Wheat straw	Pretreated wheat straw
TS (%)	93.5 ± 0.1	94.2 ± 0.9
VS (%)	89.4 ± 0.1	84.8 ± 0.8
VS/TS (%)	95.6 ± 0.0	87.8 ± 0.3
Lignin (%, VS)	23.0 ± 0.4	21.0 ± 0.2
Cellulose (%, VS)	32.5 ± 0.2	32.1 ± 0.6
Hemicellulose (%, VS)	28.8 ± 0.2	21.7 ± 0.2
Soluble sugars[a] (%, VS)	2.8 ± 0.4	8.4 ± 0.0
Acetate (%, VS)	3.8 ± 0.1	3.4 ± 0.2

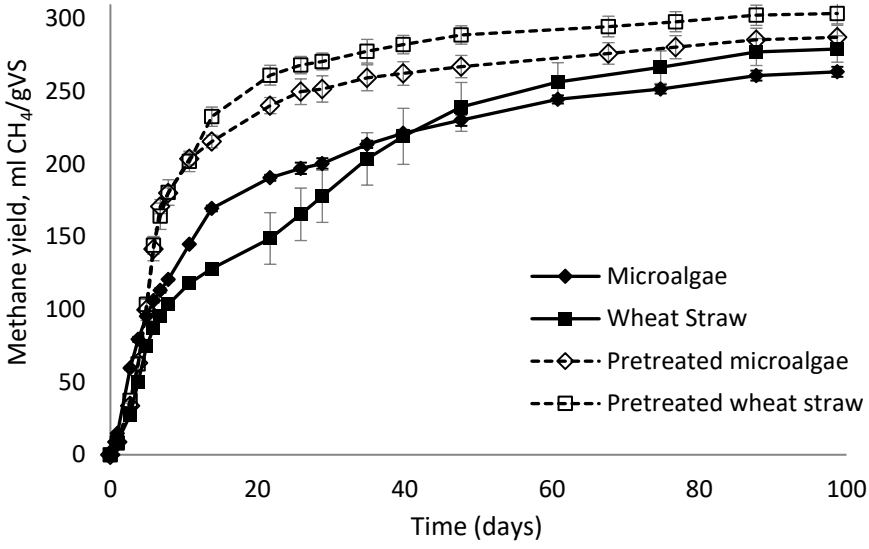
[a] Glucose, xylose, ramnose, arabinose, succinate, glycerol and acetate

Regarding the methane yield, BMP assays showed 9% increase for pretreated wheat straw compared to the untreated substrate. This is a moderate increase as compared to other studies on alkali pretreatment of lignocellulosic substrates. For example,

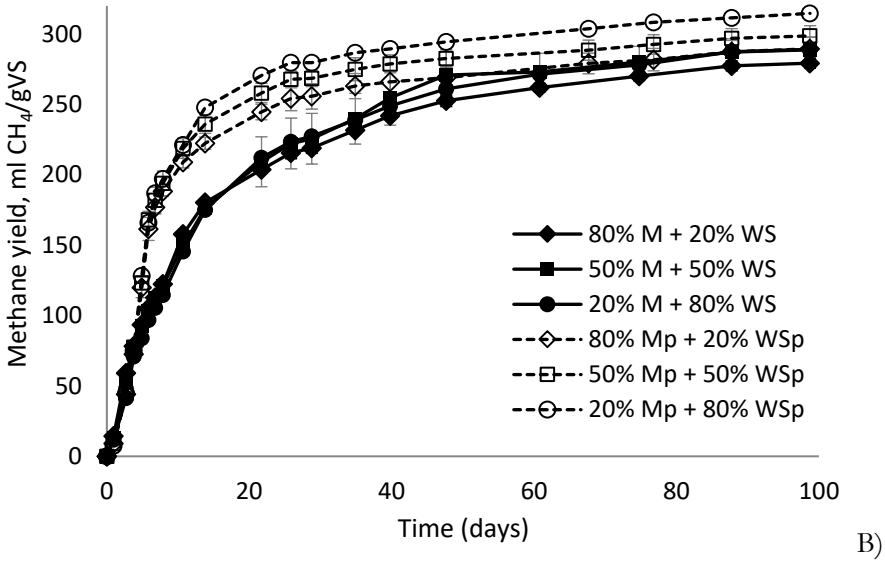
Monlau et al. (2012) reported 26% increase by pretreating sunflower stalks with 4% $Ca(OH)_2$ at 55ºC for 24 h. And significantly higher values (67% increase) were obtained by Sambusiti et al. (2013) by pretreating wheat straw with 10% NaOH at 100ºC. Nevertheless, the kinetics were clearly accelerated when the pretreatment was applied (k constant increased from 0.045 to 0.122 day^{-1}) (Table 5.5). Kinetics improvement for pretreated wheat straw was even higher than for pretreated microalgae, especially during the first 50 days of the assay, as it can clearly be seen in figure 5.11A. This can indeed improve the bioconversion process in continuous reactors, so that higher efficiencies could be obtained. Moreover, the application of this pretreatment when microalgae and wheat straw are co-digested should present more benefits than when these substrates are digested alone due to their complementary characteristics.

5.2.3.2 Co-digestion performance in BMP tests

Microalgal biomass is characterized by its high nitrogen content, which can limit the substrate utilization during anaerobic digestion. On the contrary, wheat straw mono-digestion can present a deficit of nitrogen due to its high C/N ratio. For that reason, wheat straw has traditionally been co-digested with nitrogen-rich manures (Liu et al., 2015), since both substrates can be easily found in agricultural areas. However, microalgae biomass is an emerging source that offers an alternative for co-digestion with carbon-rich substrates. Therefore, anaerobic co-digestion of microalgae and wheat straw can perform better than the individual anaerobic mono-digestion performances. To evaluate this, the anaerobic co-digestion of three different mixtures of microalgae and wheat straw was compared in BMP assays: 80-20%, 50-50% and 20-80% of microalgae and wheat straw, respectively (VS basis) (Table 5.5; Fig. 5.11B). According to section 9.3.1., the pretreatment on both substrates enhance their anaerobic digestion, especially regarding the kinetics Thus, the same proportions were also tested with pretreated substrates (Table 5.5; Fig. 5.11B). The C/N ratios resulting from the mixtures are shown in Table 5.5. Whereas the mixture with 20% wheat straw still presented a low ratio (C/N= 9), the other proportions (50 and 80% wheat straw) showed values close to 15-30 (C/N= 13 and 26, respectively), suggested as optimal for anaerobic digestion (Weiland, 2010).



A)



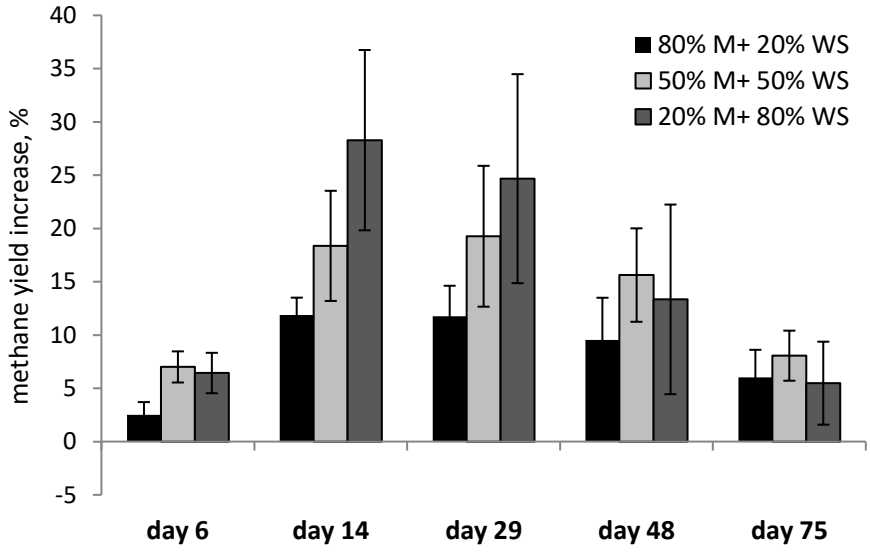
B)

Figure 5-11 Cumulative methane yield of raw microalgae and wheat straw (controls) and with a thermo-alkaline pretreatment (10% CaO at 72°C for 24 h) (A) and their anaerobic co-digestion (80-20%VS; 50-50%VS and 20-80%VS, respectively) with untreated and preatreated substrates (B).

Note: M= microalgae; Mp= pretreated microalgae; WS= wheat straw; WSp= pretreated wheat straw

The existence of synergies due to co-digestion can be studied by means of BMP tests. BMPs can show whether the final methane yield of the mixtures is actually higher than the methane yield expected as the sum of the methane yield of each substrate (mono-digestion) and / or whether the kinetics improve when the substrates are co-digested. In order to determine if the kinetics of the process was improved by the co-digestion, the first-order kinetic constant was calculated according to Eq. 5.2.1 for the BMP curves obtained with the co-digestion (Fig. 5.11B) and for the expected curves calculated with the values obtained from the mono-digestion of each substrate (data not shown). Both the ultimate methane yield and first-order kinetic constant are reported in Table 5.5. As can be observed almost all the experimental methane yields obtained with co-digestion were slightly higher than those expected from the mono-digestion calculations (1-6% methane yield increase). Since this slight increase is similar to BMB assay systematic error (~5%), no conclusive results can be stated regarding the final methane yield increase. In fact, most of the studies that have analysed the co-digestion of different substrates in BMP assays did not find significant methane yield increase (Astals et al., 2014; Neumann et al., 2015). Moreover, in the studies that did report a methane yield increase, the values obtained were relatively low. For instance, Schwede et al. (2013a) reported about 7% and 9% increase when the marine microalga *Nannochloropsis salina* was co-digested with corn silage and corn-cob-mix, respectively. Nevertheless, the main consistent finding among these studies is that the process kinetics was improved (Astals et al., 2014; Neumann et al., 2015; Ramos-Suárez et al., 2014). Indeed, kinetics improvement was also observed in this experiment by comparing the first-order kinetic constants (Table 5.5). The highest increase (31%) was found with the highest proportion of wheat straw when the pretreatment was not applied, since it showed a slower degradation.

In order to provide an insight into the kinetics analysis, a comparison was made between the methane yield increase of the BMPs with co-digestion and the expected values from the BMPs with single substrates (mono-digestion) over time (Fig. 5.12). This figure shows how the methane yield increases were significant during the early days of the experiment. However, when the substrates were not pretreated, synergies could be observed for more than 75 days, with methane yield increases up to 25% for around 14 to 29 days (Fig. 5.12A). As far as pretreated substrates are concerned, this effect became insignificant after 6 days (Fig. 5.12B). These results suggest that synergies due to co-digestion took place in both cases, but it was less significant when the biomass was pretreated. This can be attributed.



A)

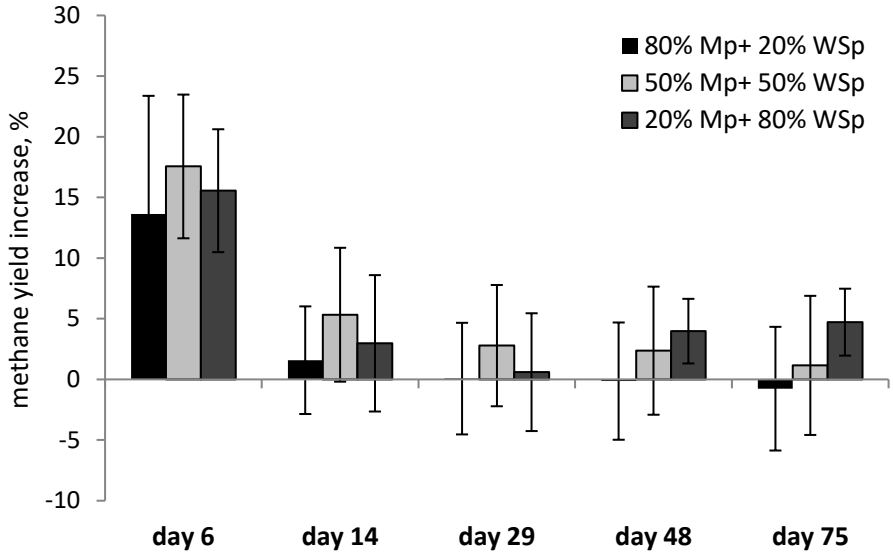


Figure 5-12 Methane yield increase of co-digested samples with respect to calculated values proportional to mono-digested substrates (microalgae and wheat straw) without pretreatment (A) and with thermo-alkaline pretreatment (10% CaO at 72°C for 24 h) (B) after 6, 14, 29, 48 and 75 days of BMP assay.

B)

Note: M= microalgae; Mp= pretreated microalgae; WS= wheat straw; WSp= pretreated wheat straw

to the fact that the pretreatment itself significantly accelerates the kinetics of the process, so the effects of the co-digestion are less discernible than for untreated biomass. Finally, significant differences among substrate proportions could also be observed with untreated substrates. Higher improvements were observed with 50 and 80% wheat straw, corresponding to C/N ratios of 13 and 26, respectively, especially during the first 30 days of assay (Fig. 5.13). This is in accordance with other studies that found higher synergies when the C/N values were close to 20. For instance, Yen and Brune (2007) suggested an optimum C/N of 20-25 for the co-digestion of algal sludge and waste paper, and Hassan et al. (2016) reported the C/N of20 for co-digestion of wheat straw and chicken manure. However, no significant differences in methane yield increase were found among C/N ratios when biomass was pretreated. Nonetheless, the information provided by BMP tests is limited, and these results should be complemented with a continuous digestion performance

5.2.3.3 Continuous anaerobic co-digestion of microalgae and wheat straw

Co-digestion of 50-50% VS of microalgal biomass and wheat straw was thereafter tested in laboratory-scale continuous reactors. This proportion corresponds to the lowest quantity of wheat straw required to obtain the highest synergistic impact on the co-digestion, according to the results obtained in the BMP assay. The co-digestion was simultaneously performed for both untreated (digester 2) and pretreated biomass (10% CaO, 72 °C, 24 h) (digester 3). Also, a reactor treating microalgal biomass as sole substrate was performed as control (digester 1). During the whole experimental period, all reactors were operated with an organic loading rate (OLR) around 1 g VS/L·day and an HRT of 20 days (Table 5.7). Weekly average methane yield from each reactor during the steady state period is shown in figure 5.12.

The methane yield of untreated microalgal biomass was 0.12 L CH_4/g VS, with a VS removal around 25%. When microalgae were co-digested with wheat straw, the methane yield increased to 0.21 L CH_4/g VS (77% increase), with a VS removal around 36%. In fact, the methane production rate and yield were significantly higher for the co-digestion reactor in comparison with the control (Table 5.7). Bearing in mind that the BMP of untreated microalgae and wheat straw were similar, and that the kinetics of the wheat straw was significantly lower than that of microalgae, advantageous results were obtained with their co-digestion in continuous flow. One of the explanations in agreement with literature is the C/N balance achieved by the co-digestion. However, there are other benefits of the co-digestion that can improve

the bioconversion process. For instance, Yen and Brune (2007) demonstrated that the co-digestion of algal sludge with waste paper increased the cellulose activity of the digester as compared to the individual algal sludge digestion. On the other hand, Tsapekos et al. (2017) also demonstrated that the co-digestion of manure and lignocellulosic biomass modified and increased the methanogenic activity in the reactor as compared to manure mono-digestion. With regards to pretreated substrates, their co-digestion showed the best performance with a methane yield of 0.24 L CH_4/g VS and a VS removal around 49%. This represents 102% methane yield increase with respect to microalgae mono-digestion and 15% increase compared to the untreated substrates co-digestion (Table 5.7).

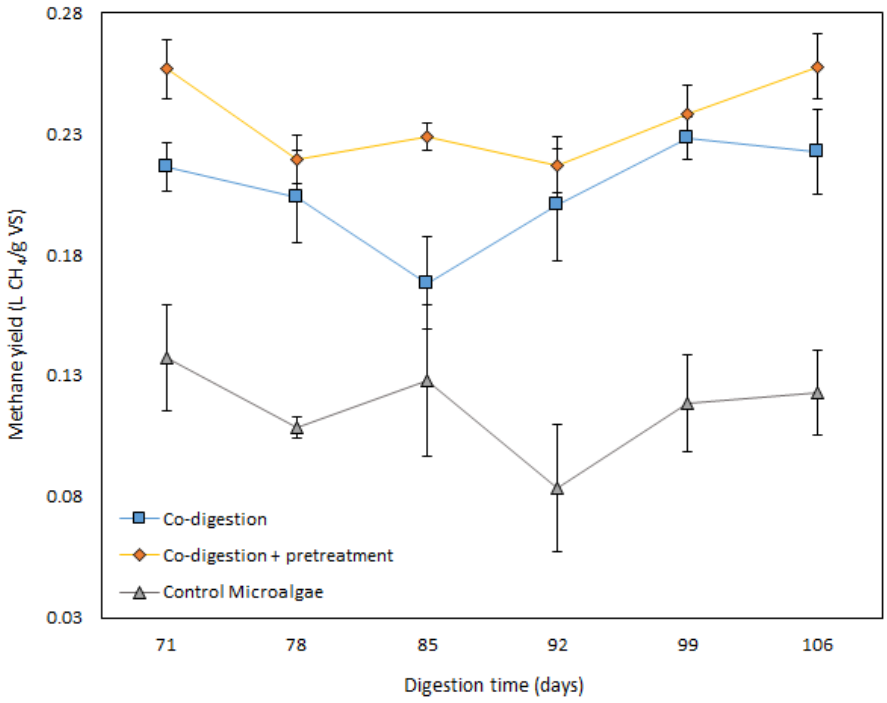


Figure 5-13 Steady-state weekly average methane yields of untreated microalgae (control), untreated microalgae and wheat straw co-digestion (50-50%) (co-digestion) and thermo-alkaline pretreated microalgae and wheat straw co-digestion (50-50%) (co-digestion+pretreatment) obtained in continuous reactors.

Table 5-7. Influent and digested biomass characteristics from microalgae continuous anaerobic digestion (control) and co-digestion with wheat straw (50-50% VS), with and without thermo-alkaline pretreatment (10% CaO at 72°C for 24 h). Mean ± standard deviation of 6 samples from steady-state.

Parameter	Digester 1: Control Microalgae	Digester 2: Co-digestion	Digester 3: Co-digestion + pretreatment
Operation conditions			
HRT (days)	20	20	20
OLR (kg VS/m^3 d))	1.12 ± 0.07	1.04 ± 0.03	0.97 ± 0.02
Influent composition			
pH	7.06 ± 0.14	6.82 ± 0.10	12.04 ± 0.18
TS [% (w/w)]	2.74 ± 0.14	2.39 ± 0.14	2.70 ± 0.11
VS [% (w/w)]	2.10 ± 0.10	2.06 ± 0.12	1.97 ± 0.16
VS/TS (%)	79.8 ± 3.0	86.2 ± 1.7	71.9 ± 5.7
C/N (-)	4.7 ± 0.4	13.7 ± 2.1	12.8 ± 2.0
N-NH_4 (mg/L)	28 ± 8	15 ± 5	44 ± 9
Effluent composition			
pH	7.51 ± 0.27	7.17 ± 0.18	7.49 ± 0.16
TS [% (w/w)]	2.32 ± 0.13	1.75 ± 0.06	1.79 ± 0.04
VS [% (w/w)]	1.65 ± 0.08	1.36 ± 0.04	0.98 ± 0.03
VS/TS (%)	70.8 ± 0.9	78.1 ± 1.1	54.5 ± 0.8
N-NH_4 (mg/L)	304 ± 25	160 ± 39	199 ± 59
VFA (mg COD/L)	<LOD	<LOD	<LOD
Removal efficiency			
TS removal (%)	18.0 ± 2.7	33.1 ± 5.1	35.4 ± 1.5
VS removal (%)	26.3 ± 5.2	37.6 ± 2.8	48.3 ± 2.9
Biogas production			
Methane production rate (L CH_4/L·d)	0.14 ± 0.02	0.21 ± 0.03	0.23 ± 0.02
Methane yield (L CH_4/g VS)	0.12 ± 0.02	0.21 ± 0.03	0.24 ± 0.02
Methane content in biogas (% CH_4)	67.8 ± 0.3	61.8 ± 2.1	67.0 ± 0.7

Concerning the stability of digesters, pH values were stable during the whole period, ranging from 7.2 to 7.5 (Table 5.7). Although a high pH value (pH=12) of the pretreated effluent was obtained as a consequence of the CaO addition, the pH in digester 3 was nearly neutral (pH = 7.5). Therefore, a good buffer capacity of the digester and substrate dilution may have enabled the operation of the digester without the necessity of externally adjusting the pH. The same fact was reported by Monlau et al. (2015) for continuously-fed digesters with an alkaline pretreated substrate at pH=11 at a similar OLR (1.5 g VS/L·day). Regarding the ammonium concentration, the highest value was observed in the digester treating microalgae as sole substrate. The reactor effluent exhibited around 300 mg $N\text{-}NH_4$/L and 76 mg $N\text{-}NH_3$/L (according to (Emerson et al., 1975)), which is below toxic concentrations of 1.7 g $N\text{-}NH_4$/L (Schwede et al., 2013b). This is due to the fact that reactors were operated under a very low OLR. In case of increasing this OLR, the ammonium and ammonia concentrations in the reactor would increase and therefore it would have consequences on the stability of the digester. Nevertheless, when wheat straw was added, the ammonium concentration decreased around 2-fold for the untreated substrates and 1.5-fold for the pretreated ones (Table 5.7). VFAs were not detected in any digester effluent (Table 5.7). This is again a consequence that the reactors were working at low OLRs and no inhibitions were detected. It is important to highlight that the OLR was fixed by the VS concentrations obtained from low-cost microalgae harvesting (settling and thickening). In fact, Passos and Ferrer (2015) evaluated the anaerobic digestion of microalgae biomass obtained from a similar process and almost no presence of VFAs was detected in the reactors. When wheat straw was added (digesters 2 and 3), dilution of the substrate was necessary to keep the same VS concentrations as the microalgae sole substrate, with the same OLR as the microalgae reactor (digester 1). This allowed for comparison among the three reactors. However, in a full-scale operation, the co-digestion of microalgae with wheat straw could lead to increase the digesters OLR.

Overall, the methane yield obtained from microalgae and wheat straw co-digestion, weather pretreated or not, was significantly higher than that obtained from microalgae mono-digestion. By comparing the results from digesters 2 and 3, a low improvement was observed. Only a moderate methane yield increase of 15% was found due to the pretreatment. Although this value is higher than that obtained in the BMP assays (4%), the energy surplus obtained from the methane production increase would not compensate the energy requirements and chemical costs to perform the pretreatment step. Indeed, the study carried out by Passos and Ferrer

(2014) concluded that 33% methane production increase was necessary to achieve a neutral energy balance when microalgae biomass was pretreated at 75 °C for 10 h. On the contrary, the co-digestion of microalgae and wheat straw presents some advantages. For example, the addition of wheat straw increases the efficiency of the reactor, mainly due to the improvement of the C/N balance. Also, it allows increasing the OLR of the digestion by avoiding the stability problems that microalgae mono-digestion can present (inhibition due to high $N\text{-}NH_4$). For example, Herrmann et al. (2016) demonstrated that while the anaerobic digestion of the microalgae *Arthisoira platensis* was stable at a low OLR of 1 g VS/L·day, their co-digestion with a carbon-rich substrate (brown seaweed) achieved an OLR up to 4 g VS/L·day. Another advantage of co-digesting microalgae and wheat straw without any pretreatment is that the only additional energy required is related to wheat straw milling. In this study, a milled wheat straw between 400 and 1 mm was used. However, for a more efficient performance, an optimization of the milling would be recommended. On the other hand, one of the most limiting costs associated to the co-digestion is the transport of the co-substrates from their origin to the digestion plant (Mata-Alvarez et al., 2014). For that reason, the wheat crop area should be located nearby the digestion plant.

5.2.4 Conclusions

This study showed how microalgae and wheat straw co-digestion improved either mono-digestion in BMP assays. Higher improvements were obtained with untreated microalgae and wheat straw mixtures of 50-50% and 20-80%, with C/N ratios of 13 and 26, respectively. The co-digestion of 50-50% microalgae and wheat straw in lab-scale reactors increased the methane yield by 77% compared to microalgae mono-digestion, while the pretreatment only increased the methane yield by 15% compared to the untreated substrates co-digestion. Thus, the co-digestion of microalgae and wheat straw was successful even without the thermo-alkaline pretreatment.

6 CONCLUSIONS

In this PhD, different strategies to improve microalgae anaerobic digestion in wastewater treatment systems have been investigated. This mainly comprises the co-digestion of microalgae with appropriate substrates combined (if necessary) with a pretreatment.

The conclusions have been separated in three main blocks, where each block corresponds to each co-substrate investigated. This division is in accordance with the structure of the thesis to facilitate the identification of each conclusion with the starting objectives.

6.1 Microalgae co-digestion with primary sludge

The effect of the co-digestion of primary sludge on microalgae biodegradability in terms of the process kinetics and final methane yield has been evaluated in Biochemical Methane Potential (BMP) tests. The conclusions are:

- Primary sludge showed the highest methane yield (380 mL CH_4/gVS) and faster kinetics (k= 0.24 day $^{-1}$) as compared to untreated microalgae. (90 mL CH_4/gVS and k=0.07 day $^{-1}$).
- The higher the proportion of primary sludge, the higher the methane yield. The highest methane yield was observed by adding 75% of primary sludge to microalgae (291 mL CH4/g VS).
- There was no synergic effect with respect to the ultimate methane production when co-digesting both substrates.

- The co-digestion of microalgae with primary sludge substantially improved the anaerobic digestion kinetics (k =0.25-0.28 day^{-1}) as compared to mono-digestion trials.

The effect of the microalgae thermal pretreatment (75 °C for 10h) on their co-digestion with primary sludge in terms of the process kinetics and final methane yield has been evaluated in BMP tests. The conclusions are:

- With thermal pretreatment, microalgae methane yield was increased by 62% (from 90 to 146 mL CH_4/g VS) and the first-order kinetics constant (k) by 128% (from 0.07 to 0.16 day^{-1})
- The highest methane yield was observed by adding 75% of primary sludge to pretreated microalgae (339 mL CH_4/g VS).
- After co-digestion, no differences were observed between pretreated and untreated trials regarding the kinetics.

The anaerobic co-digestion of 25% thermally pretreated (75 °C for 10h) microalgae with 75% primary sludge has been assessed in continuous mesophilic lab-scale reactors at 20 day-HRT. The conclusions are:

- Microalgae mono-digestion was substantially improved by the co-digestion with primary sludge. With co-digestion, the average methane yield was 0.46 m^3 CH_4/kg VS, which represented a 2.9-fold increase compared to pretreated microalgae mono-digestion (0.16 m^3 CH_4/kg VS).
- The energy assessment revealed that the energy produced with the co-digestion was at least 3.5-fold the energy consumed.
- No ammonia inhibition was detected during microalgae anaerobic digestion process. However, co-digestion with primary sludge reduced the ammonium concentration in the digester from 1.1 g N-NH_4/L to 0.6 g N-NH_4/L. This let reactors working at higher OLR without the risk of ammonia inhibition.
- Co-digestion substantially improved the effluent dewaterability, showing lower CST values (290 s).than in microalgae mono-digestion (982 s).
- Microalgae showed a low methane yield despite the thermal pretreatment (0.16 m^3 CH_4/kg VS). This was mainly attributed to the presence of microalgae species with hardly degradable cell walls (i.e., *Stigioclonium* sp. and diatoms).
- Microscopic analysis showed that thermal pretreatment weakened the microalgae cell wall but without completely lysing and releasing microalgae intracellular material.

- Methane yield of microalgae mono-digestion increased by 50% when HRT was increased from 20 to 30 days (from 0.16 to 0.24 m^3 CH_4/kg VS). The energy assessment concluded that increasing the HRT is preferred over pretreatment to improve the anaerobic digestion of microalgae with resistant cell walls.

The anaerobic co-digestion of 25% microalgae with 75% primary sludge has been assessed in continuous mesophilic lab-scale reactors at a 20-day HRT. The conclusions are:

- Microalgae mono-digestion was substantially improved by the co-digestion with primary sludge. With co-digestion, the average methane yield was 0.33 m^3 CH_4/kg VS, which represented a 65 % increase compared to microalgae mono-digestion (0.20 m^3 CH_4/kg VS).
- No ammonia inhibition was detected during microalgae anaerobic digestion process. However, co-digestion with primary sludge reduced the ammonium concentration in the digester from 1.3 g N-NH_4/L to 0.7 g N-NH_4/L, thus, the risk of ammonia toxicity.
- Microalgae and primary sludge had different biochemical composition. Microalgae were mainly composed of proteins (58%), followed by lipids (24%) and carbohydrates (15%) and primary sludge had higher amount of lipids (45%), followed by proteins (29%) and carbohydrates (12%).
- Microalgae methane yield was favoured by the predominance of *Chlorella* sp.

The occurrence of some emerging organic contaminants in microalgae and primary sludge was analysed and their removal during their anaerobic (co-)digestion was evaluated. The conclusions are:

- Musk fragrances (galaxolide and tonalide) and triclosan showed the highest abundance (0.5-25 μg/g TS), whereas caffeine, methyl dihydrojasmonate and triphenyl phosphate were barely detected (<0.1 μg/g TS).
- The attenuation of these contaminants was compound-depending and ranged from no removal to up to 90%.
- Microalgae digestion resulted in a better removal of selected contaminants than primary sludge digestion.

Digestates from microalgae anaerobic digestion and co-digestion with primary sludge were characterize to determine their suitability for agricultural reuse. The conclusions are:

- All microalgae digestates (untreated and pretreated microalgae and pretreated microalgae in co-digestion with primary sludge) presented suitable organic matter and macronutrients, especially organic and ammonium nitrogen, for agricultural soils amendment.
- The thermally pretreated microalgae digestate was the least stabilised digestate in comparison with untreated microalgae and co-digestion digestates.
- Co-digestion digestate was the one which presented less phytotoxicity.
- Heavy metals contents resulted far below the threshold established by the European legislation on sludge spreading. Moreover, low presence of E. coli was observed in all digestates.
- Agricultural reuse of thermally pretreated microalgae and primary sludge co-digestate through irrigation emerges the most suitable strategy to recycle nutrients from wastewater.

6.2 Microalgae co-digestion with waste activated sludge (WAS)

The effect of a simultaneous autohydrolysis pretreatment (at 55 ºC) to microalgae and WAS was evaluated in terms of volatile solids solubilization to select best pretreatment condition. The conclusions are:

- WAS reached the highest solubilisation ratio (25.7 % VS) and microalgae the lowest (11.4 % VS).
- There was no co-pretreatment effect, since microalgae solubilisation was not improved by pretreating it together with WAS. Therefore, inherent enzymes of WAS released during the autohydrolysis pretreatment were not effective at disrupting microalgae cell wall.
- Solubilisation ratios of co-digested samples reached an asymptote by 7.5 hours. This value was selected as the optimum contact time for the autohydrolysis

The effect of a simultaneous autohydrolysis pretreatment followed by the co-digestion of WAS on microalgae biodegradability was evaluated in BMP tests in terms of the process kinetics and final methane yield. The conclusions are:

- Microalgae, mainly composed by *Scenedesmus* sp., supported a low methane yield (82 ml CH_4/gVS) while WAS showed a higher methane yield (139 mL CH_4/g VS).

- The pretreatment applied to microalgae increased the methane yield by 64%, achieving a value of 134 mL CH_4/g VS while pretreated WAS showed a production of 204 mL CH_4/g VS, which represents an increase of 47 %.
- There was no synergic effect with respect to the ultimate methane production when co-digesting both substrates.
- Co-digestion trials showed higher kinetic constants (0.29 $days^{-1}$, 0.32 $days^{-1}$ and 0.30 $days^{-1}$ for 20 %, 50 % and 80 % of microalgae content co-digestions, respectively) as compared to the mono-digestions (0.27 $days^{-1}$ for pretreated microalgae and 0.25 day^{-1} for pretreated WAS).

6.3 Microalgae co-digestion with wheat straw

The effect of a thermo-alkaline pretreatment (4 and 10% TS of CaO at 25, 55 and 72°C) on microalgae was evaluated in BMP tests in terms of organic matter and macromolecules solubilization and methane yield increase. The conclusions are:

- The pretreatment increased proteins and carbohydrates solubilisation up to 32.4% and 31.4%, respectively.
- Anaerobic digestion kinetics were also improved by pretreatment. The first order kinetic rate constant increased from 0.08 to 0.14 day^{-1}.
- The pretreatment with the highest lime dose (10% CaO) and temperature (72 °C) showed both the highest macromolecules solubilisation and the highest biochemical methane potential increase (25%). This was selected as optimal pretreatment condition.

The effect of the co-digestion of wheat straw on microalgae biodegradability in terms of the process kinetics and final methane yield was evaluated in BMP tests. The conclusions are:

- Wheat straw showed a slightly higher methane yield (279 ml CH_4/g VS) but considerably slower kinetics ($k = 0.05\ day^{-1}$) than microalgae (264 ml CH_4/g VS and $k = 0.09\ day^{-1}$).
- Microalgae and wheat straw co-digestion improved either mono-digestion in BMP assays. Higher improvements were obtained with microalgae and wheat straw mixtures of 50-50% and 20-80%, with C/N ratios of 13 and 26, respectively.
- Almost all the experimental methane yields obtained with co-digestion were slightly higher than those expected from the mono-digestion calculations (1-6% methane yield increase). Since this slight increase is similar to BMB assay

systematic error (~5%), no conclusive results can be stated regarding the final methane yield increase.

- All co-digestion trials showed higher kinetic constants as compared to the mono-digestions.

The effect of a simultaneous pretreatment (10% CaO at 72°C for 24 h) followed by the co-digestion of wheat straw on microalgae biodegradability was evaluated in BMP tests in terms of the process kinetics and final methane yield. The conclusions are:

- The methane yield achieved after applying the pretreatment was 287 ml CH_4/g VS for microalgae (9% increase) and 304 ml CH_4/g VS for wheat straw (9% increase).
- The kinetics were clearly accelerated when the pretreatment was applied (k constant increased from 0.045 to 0.122 day^{-1}).
- Slight lignin removal (9%) and more notorious hemicelluloses removal (25%) were observed after thermos-alkaline pretreatment to wheat straw.
- Synergies due to co-digestion were less significant when the biomass was pretreated.

The anaerobic digestibility of microalgae in co-digestion with wheat straw, with and without a simultaneous thermo-alkaline pretreatment (10% CaO at 72°C for 24 h), was assessed in continuous mesophilic lab-scale reactors. The conclusions are:

- The methane yield of untreated microalgal biomass was 0.12 L CH_4/g VS. When microalgae were co-digested with wheat straw (50% VS), the methane yield increased to 0.21 L CH_4/g VS (77% increase), while the pretreatment only increased the methane yield by 15% compared to the untreated substrates co-digestion (0.24 L CH_4/g VS). Thus, the co-digestion of microalgae and wheat straw was successful even without the thermo-alkaline pretreatment.
- When wheat straw was added, the ammonium concentration decreased around 2-fold for the untreated substrates and 1.5-fold for the pretreated ones. Thus, the co-digestion of microalgae with wheat straw could lead to increase the digesters OLR.

6.4 Final remarks

Thesis final remarks are the followings:

- **Kinetics improvement in BMPs.** All co-digestion trials showed higher kinetic constants as compared to the mono-digestions, even not always

substantially balanced substrate C/N ratio. This can be explained as co-digestion may also promote other benefits as macro and micro-nutrient equilibrium, balance moisture content and dilute possible inhibitory compounds produced from the anaerobic digestion process.

- **Pretreatment effect.** In general, when combining a pretreatment with co-digestion, the effects of the co-digestion resulted less discernible than for untreated substrates. This can be explained because the pretreatment itself had already accelerated the kinetics of the process and made substrates more available for microorganisms. However, co-digestion of substrates without any pretreatment can enhance substantially the anaerobic digestion process. Therefore, the use of pretreatments in that context can result less attractive than in mono-digestion performances.
- **Co-digestion with primary sludge in continuous reactors.** Co-digestion of microalgae with primary sludge, both by-products of microalgal-based wastewater treatment systems, substantially enhanced the anaerobic digestion of microalgal biomass, since primary sludge is a more readily degradable carbon rich substrate, leading to higher methane production (from 65% to 2.3-fold increase), while reducing the risk of ammonia toxicity. The most suitable option is the co-digestion with primary sludge at a 20-day HRT if the proportion of sludge is high and at 30 days if the proportion of microalgae is high. To increase HRTs is preferred over to apply a thermal pretreatment.
- **Co-digestion with wheat straw in continuous reactors.** Co-digestion of microalgae and wheat straw (50-50% VS) at 20-day HRT is successful even without the thermo-alkaline pretreatment. The methane yield has increased by 77% as compared to microalgae mono-digestion. As wheat straw is a storable substrate, with high TS content (> 90%), this strategy can lead increase digesters OLRs without any risk of ammonia inhibition.
- **Co-digestion with WAS.** Co-digestion of microalgae and WAS showed moderate biodegradability. Methane yields ranged from 82 to 139 mL CH_4/gVS without pretreatment, and from 134 to 204 mL CH_4/gVS after autohydrolysis pretreatment. Inherent enzymes of WAS released during the autohydrolysis pretreatment were not effective at disrupting microalgae cell wall, although WAS co-digestion (80% VS) after pretreatment increased microalgae mono-digestion methane yield up to 130%.

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CURRICULUM VITAE

Maria Solé Bundó was born in La Bisbal del Penedès (Tarragona, Catalonia), in 1987. She obtained her degree in Civil Engineering, specialisation in Environmental Engineering (2013) at the Universitat Politècnica de Catalunya ·BarcelonaTech. Her master thesis evaluated the effect of the microwave pretreatment on microalgae. She then continued her PhD research on the anaerobic digestion of microalgae at the same University in 2014.

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ALERA (local actions in water reuse) (2015- 2016), financed by Pyrénées-Méditerranée Euroregion, call for projects: Efficacité et sobriété dans l'usage des ressources (eau ou énergie) 2013. http://wp.granollers.cat/alera/

DIPROBIO - Algal biomass production and digestion from wastewater (2014- 2015) (CTM2012-37860), financed by the Spanish Ministry of Science and Innovation, coordinated by the Group of Environmental Engineering and Microbiology (GEMMA-UPC)

BIOALGAS - Biogas production from algae biomass produced in high rate ponds for wastewater treatment (2012-2014) (CTM2010-17846), financed by the Spanish Ministry of Science and Innovation, coordinated by the Group of Environmental Engineering and Microbiology (GEMMA-UPC)

Stays in internationally recognized research centres

Laboratoire de Biotechnologie de l'Environnement (LBE); French National Institute for Agricultural Research (INRA), Narbonne, France. 6 month from June to December 2015.

Others

Organising committee of the 10th International Society for Environmental Biotechnology (ISEB) Conference, 1st-3rd June 2016 Barcelona, Spain.

Reviewer of the following journals: Algal Research, Science of the Total Environment, Waste and Biomass Valorization, Waste Management and Water Science and Technology.