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# Evaluation of different strategies to produce biofuels from *Nannochloropsis oculata* and *Chlorella vulgaris*

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## A B S T R A C T

The lipid extraction using hexane and methanol:hexane increased the biodegradability of *Nannochloropsis oculata* by 36% and 24% respectively. Moreover, hexane increased the methane production from raw microalgae, from  $253 \pm 11$  to  $313 \pm 9$  mL<sub>CH<sub>4</sub></sub>/g<sub>VS</sub>. Methanol:hexane did not affect the methane production, which yielded  $254 \pm 10$  mL<sub>CH<sub>4</sub></sub>/g<sub>VS</sub>, mainly due to the significant changes in the biomass composition.

On the other hand, the lipid extraction failed to increase the biodegradability of *Chlorella vulgaris*, which resulted around 44% for raw and lipid-extracted microalgae. The methane productions were  $219 \pm 6$ ,  $202 \pm 1$  and  $200 \pm 4$  mL<sub>CH<sub>4</sub></sub>/g<sub>VS</sub> from raw and pre-treated microalgae using hexane and methanol:hexane respectively.

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

The substrate to inoculum ratio influenced raw *N. oculata* digestion. At 1:1 VS<sub>substrate</sub>:VS<sub>inoculum</sub>, the methane production throughout the first days decreased but not the ultimate methane production. *C. vulgaris* digestion was unaffected, probably due to the biomass characteristics.

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

## 1. Introduction

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

solid waste and sewage sludge, has gained more attention for processing microalgae in recent years [2,6]. Another driving force behind AD is the possibility of recycling nutrients for microalgae cultivation; microalgae can uptake nutrients from the aqueous phase recovered after digestion [5,10,11].

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

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

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

## 2. Materials and methods

### 2.1. Materials

#### 2.1.1. Microalgae, inoculum and sewage sludge

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

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

### 2.2. Experimental procedure

#### 2.2.1. Pre-treatment with organic solvents: lipid extraction

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

#### 2.2.2. Anaerobic digestion experiments

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

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

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

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

### 2.3. Analytical techniques

#### 2.3.1. Substrate characterisation

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

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

#### 2.3.2. Products characterisation

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

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

$$\ln \frac{B_0 - B}{B_0} = -k_h \cdot t \quad (1)$$

where B is the cumulative methane yield at the time t (units:  $\text{mL}_{\text{CH}_4}/\text{g}_{\text{VS}}$ ),  $k_h$  ( $\text{days}^{-1}$ ) is the first order hydrolysis constant and  $B_0$  ( $\text{mL}_{\text{CH}_4}/\text{g}_{\text{VS}}$ ) is the ultimate methane production. The values of  $k_h$  and  $B_0$  were determined in MS Excel 2007® using the Solver tool, by minimising the residual sum of squared errors between the experimental data and the data predicted by the model.

The theoretical methane potential was calculated based on the relative fractions of lipid, protein and carbohydrate in the substrates, and assuming the specific methane yields of  $1014\text{ mL}_{\text{CH}_4}/\text{g}_{\text{VS}}$ ,  $496\text{ mL}_{\text{CH}_4}/\text{g}_{\text{VS}}$ ,  $415\text{ mL}_{\text{CH}_4}/\text{g}_{\text{VS}}$  for lipid, protein and carbohydrate respectively [26]. The biodegradability was then calculated considering the measured and the theoretical methane production, Eq. (2):

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

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

## 3. Results and discussion

### 3.1. The influence of the SIR on anaerobic digestion

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

The SIR did not affect the ultimate methane production from *N. oculata* and *C. vulgaris*; the differences were smaller than 3% in the experiments using the same microalgae species. On the contrary, the  $k_h$

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

Parameter	Inoculum	Sludge	<i>Nannochloropsis oculata</i>			<i>Chlorella vulgaris</i>		
			Raw <sup>(a)</sup>	Hex <sup>(b)</sup>	Met:Hex <sup>(c)</sup>	Raw <sup>(a)</sup>	Hex <sup>(b)</sup>	Met:Hex <sup>(c)</sup>
TS (g/L)	14.8 ± 0.1	33.2 ± 0.1	37.0 ± 0.0	–	–	32.8 ± 0.4	–	–
VS/TS	0.63	0.83	0.90	0.88	0.88	0.81	0.76	0.74
COD (mg O <sub>2</sub> /L)	13600 ± 280	42000 ± 400	51000 ± 900	–	–	35500 ± 300	–	–
C:N	–	13.88	5.36	–	–	7.44	–	–
Proteins (g/100g <sub>VS</sub> )	–	–	60.9 ± 3.4	67.6 ± 4.9	85.9 ± 2.1	48.7 ± 4.0	49.6 ± 1.5	73.8 ± 3.3
Lipids (g/100g <sub>VS</sub> )	–	–	21.9 ± 0.4	13.8	–	6.5 ± 0.6	3.4	–
Carbohydrates (g/100g <sub>VS</sub> )	–	–	16.7 ± 1.5	18.7 ± 1.8	14.1 ± 2.2	41.2 ± 0.7	42.5 ± 1.5	26.2 ± 2.4

<sup>a</sup> Suspension in deionised water.

<sup>b</sup> Pre-treated using hexane.

<sup>c</sup> Pre-treated using methanol:hexane.

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

Microalgae were observed under a light microscope before and after digestion. Fig. 1a shows *N. oculata* after thawing. Microalgae cells with a similar appearance were recovered from digestate at the end of the experiment (Fig. 1b). Regarding *C. vulgaris*, Fig. 1c reveals the presence of microalgae aggregates before digestion. The aggregates originated during microalgae drying were also visible after digestion (Fig. 1d). The resistance of several *Chlorella* species to anaerobic digestion and the presence of intact cells after AD were reported by others [28]. *Chlorella* species presents stronger cell walls than *Nannochloropsis*, requiring powerful pre-treatment to enhance the biodegradability [3]. Furthermore, *C. vulgaris* aggregates may hamper the interaction with microorganisms.

The pH at the end of the experiments was close to neutrality, ranging 7.35–7.68. Ammonia concentration ranged 677–836 mg/L, whereas 1500 mg/L is considered as threshold for inhibition [2]. Inhibition during

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

Substrate	<i>N. oculata</i>	CH <sub>4</sub> mL <sub>CH<sub>4</sub></sub> /g <sub>VS</sub> $k_h^{(a)}$ (days <sup>-1</sup> )	SIR		
			1:4	1:2	1:1
			278 ± 3 0.31 (0.91)	275 ± 4 0.35 (0.93)	282 ± 9 0.19 (0.99)
	<i>C. vulgaris</i>	CH <sub>4</sub> mL <sub>CH<sub>4</sub></sub> /g <sub>VS</sub> $k_h^{(a)}$ (days <sup>-1</sup> )	229 ± 12 0.36 (0.98)	223 ± 4 0.36 (0.99)	222 ± 3 0.37 (0.71)

<sup>a</sup> In brackets, the values of R<sup>2</sup>.

AD results in VFA accumulation and decrease in pH [2]; however, the concentrations of VFA were under the detectable limit of the analysis, indicating no signs of inhibition. Biogas composition was analysed periodically and the methane content was between 71%–74%; there was not a relationship between the methane content and the substrate or the SIR.

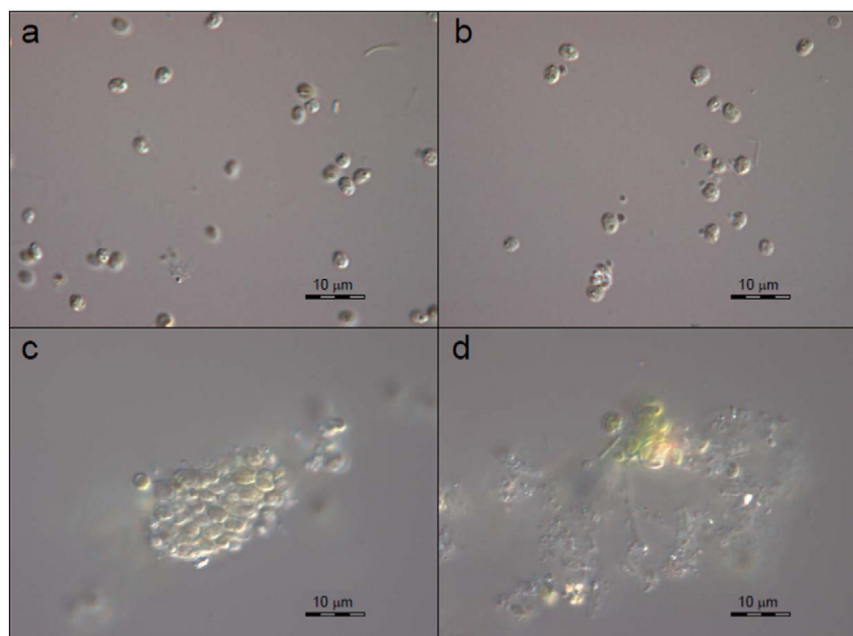
The results indicate that the SIR is important parameter in anaerobic digestion and its effects over anaerobic digestion depend on the substrate characteristics. For the AD of microalgae, the SIR evaluation may prevent anaerobic digestion failure mainly due to the high protein content. Based on the results,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

### 3.2. Microalgae pre-treatment and biodiesel production

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

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

On the other hand, the biodiesel composition was unaffected by the solvents. The biodiesel profile for *N. oculata* showed high content of C16:0, C16:1 and C20:5 using both solvents. A similar profile was reported by Balasubramanian et al. using the same polar mixture [16].



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

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

### 3.3. Anaerobic digestion of pre-treated microalgae

#### 3.3.1. *Nannochloropsis oculata*

The methane production curves from raw and pre-treated *N. oculata* are presented in Fig. 3a. It stands out the curve obtained from the microalgae pre-treated using hexane; the methane yield was considerably higher than the yield obtained from raw microalgae. The ultimate methane production from the raw *N. oculata* was  $253 \pm 11$  mL<sub>CH<sub>4</sub></sub>/g<sub>VS</sub>, similar to the value reported in the literature [2]. The pre-treatment with hexane increased the methane production until  $313 \pm 9$  mL<sub>CH<sub>4</sub></sub>/g<sub>VS</sub>; whereas the polar mixture seemed not to influence the methane production.

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

The  $k_h$  was calculated (Eq. (1)) for the different substrates. It resulted in  $0.40 \text{ day}^{-1}$  ( $R^2 = 0.97$ ) for raw microalgae, and  $0.31 \text{ day}^{-1}$  ( $R^2 = 0.99$ ) and  $0.21 \text{ day}^{-1}$  ( $R^2 = 0.99$ ) for the pre-treated microalgae using hexane and methanol:hexane respectively. These values indicated that

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

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

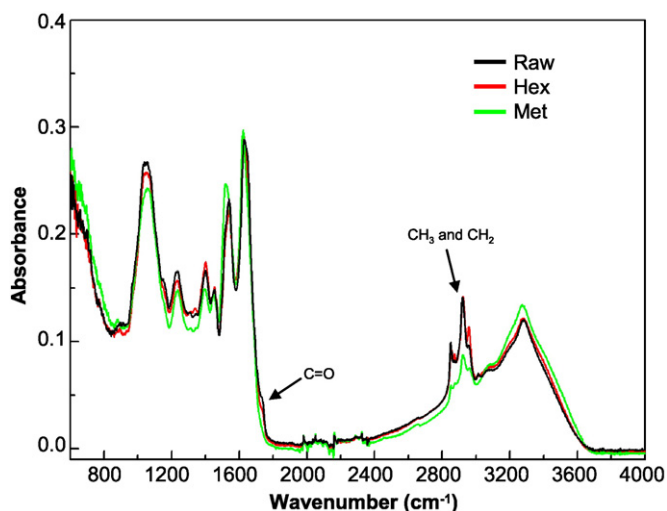


Fig. 2. FTIR spectra of raw *N. oculata* (raw) and pre-treated, using hexane (Hex) and methanol-hexane (Met).

the pre-treatment decreased the methane production throughout the first days of the experiment. However, all reactors had exceeded 80% of the ultimate methane production by the 8th day, thus most of the substrate was degraded at that time. Since the same SIR was set in all the reactors, the decrease in  $k_h$  can not be attributed to a stress response

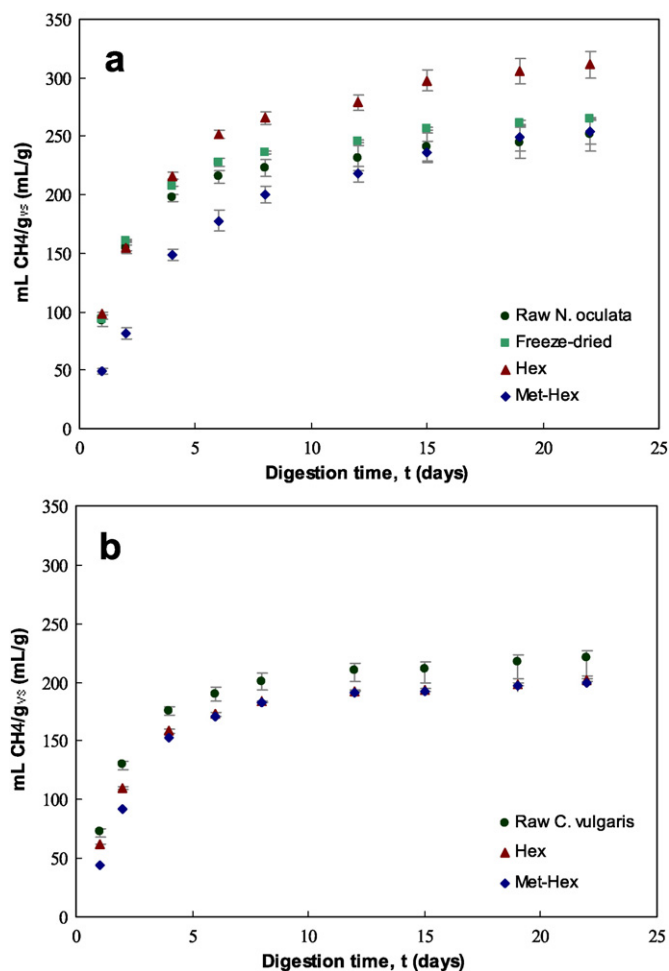


Fig. 3. The methane production curves from a) *N. oculata* and b) *C. vulgaris*. Symbols: ● raw, ■ freeze-dried and pre-treated, using ▲ hexane and ◆ methanol-hexane. Batch reactors at 33 °C and SIR of 1:2 VS<sub>substrate</sub>:VS<sub>inoculum</sub>.

as it was explained in Section 3.1. In this case, the values indicated that raw microalgae were easily digested than pre-treated ones. Different compounds were extracted in the pre-treatment, thus different compounds remained in the pre-treated microalgae. Possibly the pre-treatment extracted some easily-degradable components or it changed the characteristics of some of them, resulting in slow degradation.

Regarding the effects of drying, the methane production yielded  $265 \pm 3$  mL<sub>CH<sub>4</sub></sub>/g<sub>VS</sub> after freeze-drying. The methane production curve was similar to the curve for raw microalgae (Fig. 2a), which indicated the absence of adverse effects. Adverse effects are mainly attributed to microalgae heating in the drying process [22,33], but heating was avoided during *N. oculata* drying.

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

### 3.3.2. *Chlorella vulgaris*

The methane production curves from raw and pre-treated *C. vulgaris* are presented in Fig. 3b. The methane production yielded  $219 \pm 6$  mL<sub>CH<sub>4</sub></sub>/g<sub>VS</sub> from raw microalgae and decreased around 8–9% after the pre-treatment. Contrary to *N. oculata*, both solvents negatively affected the methane production from *C. vulgaris*. The theoretical methane production barely decreased after the pre-treatment due to the low lipid content in raw *C. vulgaris* (Table 1); thus the biodegradability resulted in 44% for raw microalgae and 42% for the pre-treated microalgae. The analysis of  $k_h$  confirmed that the pre-treatment does not accelerate the digestion of microalgae;  $k_h$  were  $0.39$  day<sup>-1</sup> ( $R^2 = 0.99$ ) for raw microalgae, and  $0.36$  day<sup>-1</sup> ( $R^2 = 0.99$ ) and  $0.32$  day<sup>-1</sup> ( $R^2 = 0.99$ ) for the microalgae pre-treated using hexane and methanol:hexane respectively. As observed during *N. oculata* AD, part of the easily-degradable components may be extracted during the pre-treatment, decreasing  $k_h$ .

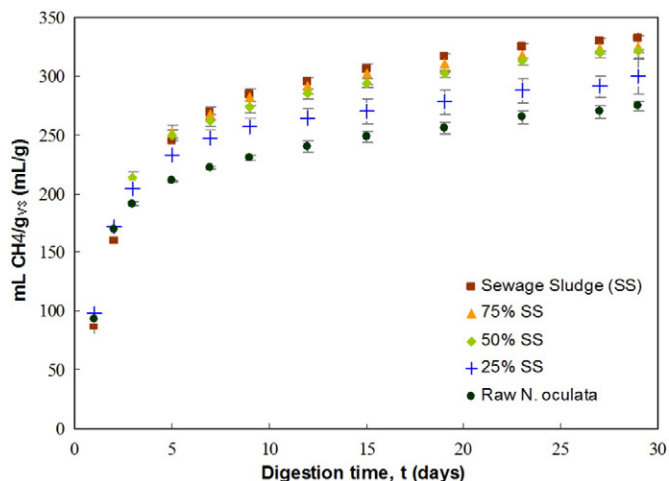
The analysis performed at the end of the experiment confirmed that all digesters operated at optimum conditions and exhibited no signs of inhibition. The biogas composition ranged between 69% and 74% CH<sub>4</sub> in all reactors.

### 3.4. Anaerobic co-digestion of microalgae and sewage sludge

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

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

The C:N during *N. oculata* digestion was 5.36, far from the range 20–25 considered as optimal for AD [18,19,22]. The C:N in sewage sludge was 13.88, thus the addition of sewage sludge did not increase the C:N significantly. The synergistic effects observed during the Aco-D of microalgae and sewage sludge or other co-substrates have been attributed to the nutrients provided to microorganisms, more than to the C:N balance [19,22]. Regarding the Aco-D of *Nannochloropsis* species, the synergetic effects were also attributed to the essential elements supply or an enhanced alkalinity [18]. In the present experiments, the addition



**Fig. 4.** The methane production curves from sewage sludge and *N. oculata* co-digestion. Symbols: ● raw microalgae, +25% sewage sludge ◆ 50% sewage sludge, ▲ 75% sewage sludge and ■ 100% sewage sludge. Batch reactors at 33 °C and SIR of 1:2 VS<sub>substrate</sub>:VS<sub>inoculum</sub>.

of synthetic medium containing micronutrients, vitamins and trace metals [26] may hide the benefits of the Aco-D. The lack of synergy may be also a consequence of the microalgae characteristics [22,23].

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

#### 4. Conclusions

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

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

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

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