Effects of low temperature thermal pretreatments in solubility and co-digestion of waste activated sludge and microalgae mixtures

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Abstract

A low temperature thermal pretreatment was applied to three different ratios of waste activated sludge (WAS) and microalgae mixtures to increase their solubility. The performance of three organic flocculants was assessed in order to select the best flocculant for previous microalgae harvesting. The effect of the following variables in the solubilisation were evaluated: ratio of the mixture of WAS and (flocculated and non-flocculated) microalgae (50:1, 25:1 and 10:1, in v:v basis), incubation time (24, 48 and 72 h) and temperature (37 and 60°C). A cationic polymer of diallyl dimethyl ammonium chloride free of acrylamide was selected for microalgae flocculation due to its high harvesting efficiency (95%). In pretreatments at 37°C, effect of temperature and time in the solubilisation of the mixtures was negligible. However, results showed a high increase in solubilisation pretreatments at 60ºC using both flocculated and non-flocculated microalgae in the mixtures. Initial soluble chemical oxygen demand in the mixtures containing WAS and flocculated microalgae was higher in comparison with that in the mixtures of the same ratio without previous flocculation of the microalgae. According to the results, the optimal pretreatment conditions were incubation of the mixtures during 24 h at 60°C in a 25:1 (WAS:microalgae, in v:v basis) mixing ratio. Thus, these optimal mixtures using flocculated microalgae and non-flocculated microalgae were chosen to assess the effect of the pretreatment in the biogas yield. The anaerobic co-digestion of the selected mixtures indicated a lack of proportionality between the soluble chemical oxygen demand attained during the pretreatment and the methane yield obtained (51 and 34% lower in comparison to the control, respectively). This behaviour could be explained by the high organic matter consumption in the low temperature thermal pretreatment before the anaerobic co-digestion, which could have been fostered by the enzymatic activity of the sludge.

Keywords: Flocculation, Thermal pretreatment, Solubilisation, Methane production, Sludge, Algae.

1. Introduction

Nowadays, the integration of microalgae-based systems in Water Resource Recovery Facilities (WRRF, a.k.a. WWTP) is more frequently evaluated as a promising alternative for wastewater treatment and resource recovery. Microalgae biomass generated by those systems could constitute an innovative source of biofuels and bioproducts [1,2].

Anaerobic digestion (AD) is the main technique used for waste activated sludge (WAS) treatment and stabilization in WWTP facilities, reducing its volume and providing a carbon neutral source of energy. Biogas obtained by AD is the only biofuel characterized for employing the whole organic content of the biomass for energy production [3]. Valorisation of microalgae biomass could be done by the simultaneous treatment of both resources, using microalgae as a co-substrate to WAS for biogas production in an anaerobic co-digestion process (AcoD). Co-digestion of two or more substrates increases the organic loading, while it could create a synergism between the substrates improving methane yield [4]. Diverse studies reported the co-digestion of WAS and microalgae [5–8]. On the other hand, there are some full-scale facilities capable of coupling co-digestion of filamentous algae and WAS, however published data are scarce [9]. Using microalgae as co-substrate to WAS in AD process improve the C/N ratio [1], enhance the dewaterability of the digestate, and provide alkalinity and micronutrients to the process [2,10]. Several advantages emerge from this scenario: wastewater treatment and nutrient recovery by microalgae-based systems, as well as, waste stabilization, energy production and reduction of greenhouse emissions.

In AD, multiple microorganisms are involved in the degradation of complex organic matter to biogas through hydrolysis, acidogenesis, acetogenesis and methanogenesis processes. The first and the latter phases are known as the main rate limiting steps in AD of WAS and microalgae biomass [11,12]. During hydrolysis, extracellular enzymes from hydrolytic bacteria convert complex organic polymers such as lipids, carbohydrates and proteins into soluble monomers namely fatty acids, monosaccharides and amino acids, respectively, in a slow reaction [13].

Due to the specific properties of both substrates, WAS and microalgae are characterized to have a low methane yield. Secondary sludge or waste activated sludge results from the biological wastewater treatment and consists in a floc structure composed by aggregates of microorganisms, attached particulate organic matter and inorganic particles [14]. The flocs are

surrounded by polymeric network consisting of extracellular polymeric substances (EPSs) containing polysaccharides, humic acids, lipids, proteins, etc. creating a barrier that retains compounds within this matrix [15]. Bacteria as single cells or colonies are the main microorganisms forming the floc, along with other microorganisms such as fungi, protozoa, cyanobacteria, algae, metazoan and archaeal populations [16]. In addition to the hard and resistant cell wall of these microorganisms, EPS provides protection to the floc [15]. The low organic degradation efficiency of WAS (ca. 30 – 50%) is attributed to its complex composition, usually requiring a pretreatment to improve its biodegradability [17].

Concerning microalgae, two main issues are needed to be addressed in valorisation of microalgae by means of AD: their biochemical composition, as well as, their cell wall complexity, both are strain specific [18]. Cell wall of the majority of photosynthetic organism contributes to regulate environmental interactions and contains mainly two biopolymers, namely sporopollenin and algaenan, recalcitrant macromolecules that resist acetolysis and hamper the release of organic matter from microalgae through bacterial degradation and chemical hydrolysis [19,20]. Microalgae cell wall is a rigid structure resistant to hydrolysis, representing a boundary for microalgae organic matter release [21]. Hence, to avoid a limited biomethane production, a pretreatment of both substrates is required for organic matter freed into the soluble phase as a prior step to their AcoD.

Pretreatments of WAS prior to AD contribute to disaggregate the flocs structure, disintegrate EPSs, release cell content, enhancing the kinetic and overall performance of the process [22]. High temperature thermal pretreatment of secondary sludge has been studied with the aim of accelerating hydrolysis and enhancing biomass biodegradability by disruption of chemical bonds in cell wall, releasing of intra- and extracellular biopolymers to the soluble phase [23,24]. Thermal pretreatment at high temperature has a positive impact in biogas generation, volume reduction, dewatering and improves the quality of bio-solid obtained after the AD [25,26]. Conversely, this pretreatment could fail to afford a positive energy balance due to energy requirements, especially when the pretreatment is implemented at high pressure [27,28]. A promising alternative to high temperature pretreatments are low temperature thermal pretreatments (< 100°C), effectively applied to secondary sludge as a biological-enzymatic pretreatment, since thermophilic conditions

promote the activity of thermophilic hydrolytic bacteria [23,27,29]. It represents a technically and economically feasible pretreatment that accomplish a synergetic effect of temperature and hydrolytic freed enzymes in a simple operation process, without the need of catalysts [29].

Regarding microalgae, chemical, mechanical, enzymatic and thermal pretreatments have been studied for cell wall disruption, increasing organic matter solubilisation and availability for anaerobic biodegradation [3,30]. Those pretreatments pursue cell membrane disintegration with the subsequent release of organic compounds, followed by their solubilisation. Pretreatments to improve microalgae anaerobic digestibility should preserve its organic matter content, along with avoiding the generation of inhibitory products that could disrupt AD process [31]. Numerous pretreatments were assessed employing pure microalgae species, however pretreatment effect on microalgae rely mainly on the microalgae species involved and the pretreatment conditions. Microalgae-based systems employed in WRRF are predominantly constituted by microalgae along with a polyculture of microorganisms [32,33]. Usually, thermal pretreatments studies applied to microalgae cultures are performed between 55 and 180°C from some minutes to several hours (15 min - 24 h) and can be executed under pressure [34–37]. In contrast, energy consumption is demanded to reach those temperature and pressure conditions. Mendez et al. [3] performed thermal pretreatment of microalgae at high temperature and short time (120ºC, 40 min) increasing methane production by 93%; nonetheless energy required in the pretreatment was 4-fold higher than the energy obtained by the anaerobic digestion. Pretreatments that require less energy input such as low temperature thermal pretreatment are a sustainable alternative [12]. The main advantages reported for those pretreatments are the generation of monomers and more soluble substrates by accelerating organic matter hydrolysis rate, the reduction in energy consumption achieving a positive energy balance, and the scalability of the technology [19,27].

According to the aforementioned, the influence of low temperature thermal pretreatments in WAS or microalgae as mono-substrates for AD has been studied by some authors [27,29,38,39]. Nonetheless, few studies analyse the effect of the same pretreatment when WAS and microalgae are mixed. For instance, Arias et al. [6], reported a thermal pretreatment (55°C, 7.5 h) of a mixture composed by microalgae and activated sludge (50% of each substrate, on a VS basis), obtaining a solubilization ratio of 21% and a higher methane yield (2-fold) when the mixture is co-digested

(in comparison with microalgae as sole substrate). Thus, incubating the mixtures of WAS and microalgae in a pre-digestion step could increase bacteria hydrolytic activity enhancing biomasses solubilisation.

The aim of this work is to assess the effectiveness of a low temperature thermal pretreatment (at 37 and 60°C) of three different ratios of WAS and microalgae mixtures in solubility of biomasses, as well as in the methane yield of the co-digested mixtures. Additionally, considering the relative low density of microalgae in water (0.5 to 5 g L^{-1} dry weight) [40], microalgae flocculation was also assessed as a former step to the low temperature thermal pretreatment of the mixtures.

2. Materials and methods

2.1. Substrates

WAS and microalgae proceed from a winery company (Miguel Torres S.A.). Photosynthetic algae cultures include predominantly *Scenedesmus almeriensis* [41] and *Chlorella vulgaris* species (microscopically identified). Microalgae were grown in batch mode in 9 L pilot photobioreactors (PBRs) located in an outdoor greenhouse at 25 ± 2°C. PBRs were fed with grey wastewater and mixing was provided by air bubbling. Microalgae employed in the pretreatment were collected during the stationary growth phase.

WAS proceed from the industrial WWTP of the winery company with a typical activated sludge treatment. In order to define a proper ratio of the WAS:microalgae mixture to pretreat, microalgae generation in the pilot plant and its potential enlargement were considered. As a result, three different ratios of WAS:microalgae mixture were selected: 50:1, 25:1, and 10:1 in volume basis (v:v). Employed substrates are characterized in Table 1.

Table 1

Characteristics of waste activated sludge and microalgae employed in the mixtures. Results are given as mean ± standard deviation (n = 3).

Parameter	WAS	Microalgae
Total solids (g L^{-1})	11.9 ± 1.8	1.2 ± 0.4
Volatile solids (g L^{-1})	8.5 ± 1.7	0.8 ± 0.2
Total suspended solids (g L^{-1})	6.0 ± 2.0	0.3 ± 0.1
Volatile suspended solids (g L^{-1})	5.0 ± 1.6	0.3 ± 0.1
pH	7.2 ± 0.2	8.7 ± 1.2
Soluble chemical oxygen demand (mg L^{-1})	161.5 ± 24.7	59 ± 17.1

2.2. Pretreatment experimental set-up

A schematic representation of the experimental set-up is presented in Fig. 1. WAS and microalgae biomasses mixtures were pretreated in batch mode at two low temperatures, 37 and 60°C, during 24, 48 and 72 h under continuous orbital stirring at 100 rpm. Three different ratios of WAS and microalgae were studied: 50:1, 25:1 and 10:1 (v:v basis). As shown in Table 2, pretreatments for the mixtures employing non-flocculated microalgae were evaluated during 3 days at 37°C (Mixtures A, B and C) and 60°C (Mixtures D, E and F). Pretreatments were performed at 37 and 60°C, since these temperatures correspond to the mesophilic and thermophilic conditions of the anaerobic digestion processes, respectively. According to the solubilisation achieved in these experiments, further pretreatments of the mixtures employing flocculated microalgae were assessed during 3 days at 60°C (Mixtures G, H and I). Triplicates of the mixtures were prepared in 1 L Erlenmeyer flasks covered with a cotton plug. 500 mL of WAS and the corresponding volume of flocculated or non-flocculated microalgae were placed inside the flasks. Pretreatments at 37°C were performed in an orbital shaker inside a controlled temperature chamber, while pretreatments at 60°C were done in a controlled temperature shaker. Controls for each mixture (Mixtures J, K and L using non-flocculated microalgae; and mixtures M, N and O using flocculated microalgae) were performed in duplicates at room temperature (20°C). All flasks were under constant agitation.

In mixtures G, H and I, microalgae were first flocculated using three organic synthetic flocculants provided by Derypol, S.A. (Barcelona, Spain). Flocculant A consist in a cationic polymer of diallyl dimethyl ammonium chloride (polyDADMAC) free of acrylamide, flocculant B is a cationic copolymer of acrylamide, while flocculant C is a cationic polysaccharide obtained from chitosan. Flocculant A and B are frequently employed in WWTP to reduce sludge volume and improve its dewaterability. Flocculant C is applied for turbidity reduction in the winery industry. These flocculants were used in two doses according to the instructions of the manufacturer: flocculant A (250 and 375 mg L^{-1}), flocculant B (500 and 750 mg L^{-1}) and flocculant C (750 and 1000 mg L^{-1}). Flocculation was carried out in triplicate in a jar-test device (Flocculator SW1 from Stuart Scientific, Staffordshire, UK) using 200 mL of microalgal suspension, according to a procedure previously described [42]. The optimal flocculant and its dose were selected according to the harvesting efficiency achieved, calculated as follows, in Eq. (1):

$$\text{Harvesting efficiency } (\%) = \frac{(\text{TSS}_\text{i} - \text{TSS}_\text{f})}{\text{TSS}_\text{i}} * 100 \quad \text{(Eq. 1)}$$

Where TSS_i is the initial total suspended solids and TSS_f is the final total suspended solids in the supernatant.

Table 2

Identification of the mixtures according to the ratio of the mixture and temperature of the pretreatment.

Waste activated sludge:Microalgae ratio (v:v)	Pretreatment			Control	
	37°C	60°C	Microalgae flocculation → 60°C	20°C	Microalgae flocculation → 20°C
50:1	A	D	G	J	M
25:1	B	E	H	K	N
10:1	C	F	I	L	O

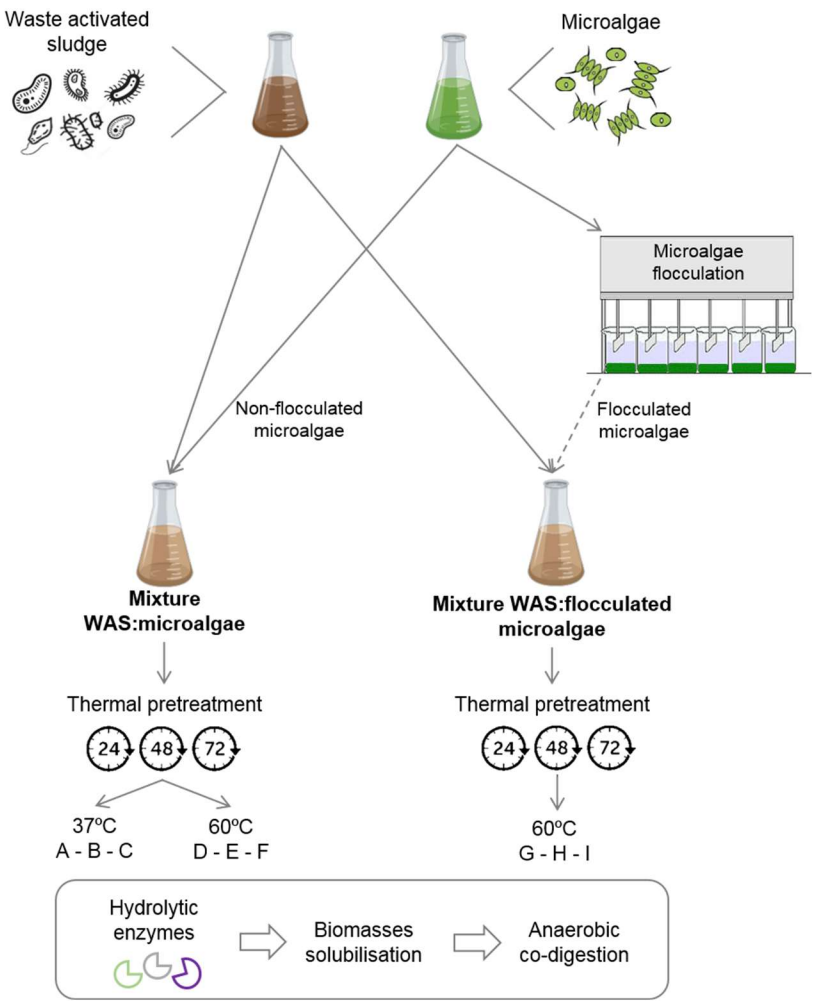


Fig. 1. Schematic representation of the experimental set-up. A, B, C, D, E, F, G, H and I are waste activated sludge (WAS) and microalgae mixtures identified in Table 2.

(1.5-column fitting image)

2.3. Biomethane potential test (BMP)

Batch tests were used to measure the methane production obtained from the AcoD of the selected mixtures in order to evaluate their digestibility after the thermal pretreatment. Anaerobic batch tests were performed based on the procedure described in Martín-González et al. [43], taking into consideration recommendations from Angelidaki et al. [44] and Holliger et al. [45]. BMP tests were performed in a controlled temperature chamber at 37 ± 1°C, using 900 mL stainless steel bottles as reactors, with a working volume of 600 mL. An inoculum to substrate ratio of 2:1 on a volatile solids-basis [35,43] was employed in the assays, using 250 mL as fixed inoculum volume.

Reactors were filled up to the working volume using tap water, then the reactor bottles were closed using a cap with an adapted valve for manometric gas measurement and purged with N_2 gas to remove oxygen. Later, were incubated at 37°C. Blank reactors, containing only inoculum and water, were used to provide information about the background methane production of the inoculum, and reference reactors with crystalline cellulose were used to verify the quality of the inoculum. Bottles were manually shacked before pressure measurement. A manometer was used to measure biogas production in the headspace volume of the bottles according to its generation, up to the depletion of the biogas production. Accumulated volumetric biogas production was calculated considering the pressure increase in the headspace volume, expressed in standard temperature and pressure conditions (273.15 K, 1.0135 bar). Net biogas production was calculated subtracting the background methane production of the inoculum (blank) from the gross methane production of the substrate reactors. Periodically, samples were taken from the reactors to analyse biogas composition by gas chromatography. All BMP tests were performed in triplicates and results are expressed as mean ± standard deviation. Digestate from a sludge anaerobic digester of a municipal WWTP was used as fresh inoculum. Due to its content of easily biodegradable organic matter, the inoculum was pre-incubated at 37 ± 1°C along 15 days to deplete its organic content.

2.4. Analytical methods

The effect of hydrolytic bacteria from WAS in the solubilisation of organic matter of WAS and microalgae mixtures, was analysed by means of sCOD and volatile suspended solids (VSS). Samples were filtered using glass microfiber filters (GF/A, Whatman). Organic matter solubilisation was evaluated by means of sCOD of the filtrate using COD kits (Hach Lange). TSS and VSS were calculated to assess organic matter conversion according to procedure described in Standard Methods [46]. pH was determined by a Crison pHmeter.

Biogas composition (carbon dioxide and methane content) was analysed using a gas chromatograph (Hewlett Packard 5890, Agilent Technologies, Mississauga, Canada) equipped with a thermal conductivity detector and a Supelco Porapack Q column (3 m x 3.2 mm) (Pennsylvania, USA). Helium was the carrier gas (338 KPa), and the oven, injector and detector

temperatures were 70, 150 and 180ºC, respectively. Injection of the samples was done with a 100 µL syringe (VICI PS Syringe A-2, 0.74 mm x 0.13 mm x 50.8 mm).

Volatile fatty acids concentrations (VFA) were determined by a Dionex 3000 ultimate high-performance liquid chromatography (Barcelona, Spain) equipped with a UV/visible detector. The chromatographic separation was performed in an ICE-COREGEL 87H3 column (7.8 x 300 mm, Transgenomic, USA), heated at 40ºC, employing 0.006 mM of H_2SO_4 as a mobile phase at a flow rate of 0.5 mL min^{-1}. Samples were previously centrifuged (10 min, 8000 rpm, Beckman Coulter, Avanti J20 XP) and then filtered by 0.45 µm nylon syringe filters. The VFA content was determined by UV spectroscopy (210 nm).

2.5. Kinetic model of methane production

The modified Gompertz equation [47] (Eq. 2) was employed to model the biomethane production and calculate kinetic parameters.

$$P_{net}(t) = P_{max}.\exp\left\{-\exp\left[\frac{R_{max}.e}{P_{max}}(\lambda - t) + 1\right]\right\} \quad \text{(Eq. 2)}$$

In Eq. 2, $P_{net}(t)$ is the net cumulative methane yield (NmL CH_4 g^{-1} VS) at time t, P_{max} is the methane yield potential (NmL CH_4 g^{-1} VS), R_{max} is the maximum daily methane production rate (NmL CH_4 g^{-1} VS d^{-1}), t is the digestion time, and λ represents the lag phase (d).

The hydrolysis rate was determined according to a simplified model assuming a first order kinetic as shown in Eq. 3 [47]:

$$B = B_0.[1 - \exp(-K_H.t)] \quad \text{(Eq. 3)}$$

B is the cumulative methane yield (mL CH_4 g VS^{-1}), B_0 is the methane yield potential (mL CH_4 g VS^{-1}), t is the digestion time (d), and K_H is the hydrolysis rate (d^{-1}) [22]. Kinetic analyses were performed using the software Matlab R2015a (The MathWorks Inc., Natick, MA, USA).

3. Results and discussion

3.1. Flocculation assays

Assuming that partial coagulation through natural processes could take place, such as biological growth and physical mixing [48], only flocculants were employed for microalgae harvesting.

Polymeric flocculants agglomerate small flocs into bigger ones, creating larger, compact and denser aggregates that settle faster in comparison with the flocs obtained by coagulation, improving their removal form water [49].

Microalgae flocculation and later thermal pretreatment of the flocculated microalgae and WAS mixture was applied. As can be seen from Fig. 2, flocculant A reached the highest harvesting efficiency (95 ± 6%, at the lower dose), followed by flocculant B (94%, at the higher dose) and flocculant C (58 ± 9%, at the lower dose). The effectiveness and performance attained by the polyDADMAC flocculant was similar to the reported in other studies [50,51]. Hence, flocculant A at 250 mg L^{-1} was selected for microalgae flocculation, before been mixed with WAS in the mixtures G, H and I; and their respective controls (mixtures M, N and O).

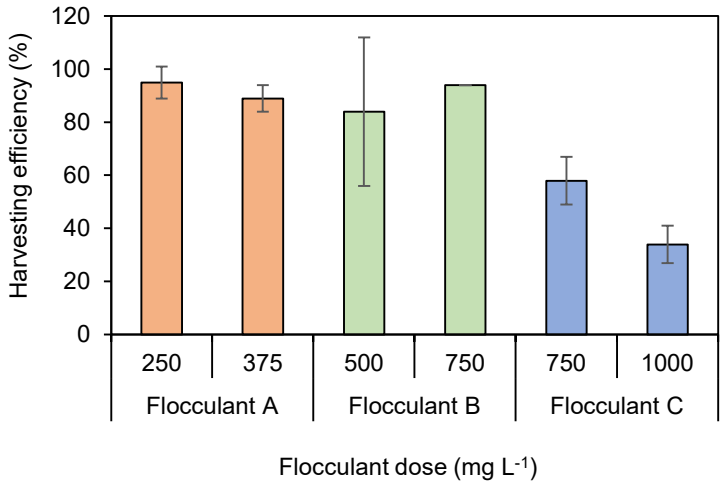


Fig. 2. Harvesting efficiency (%) of microalgae suspension after the flocculation with flocculant A, B and C at their respective doses (mg L^{-1}). Results are given as mean ± relative standard deviation (n = 3).

(single-column fitting image)

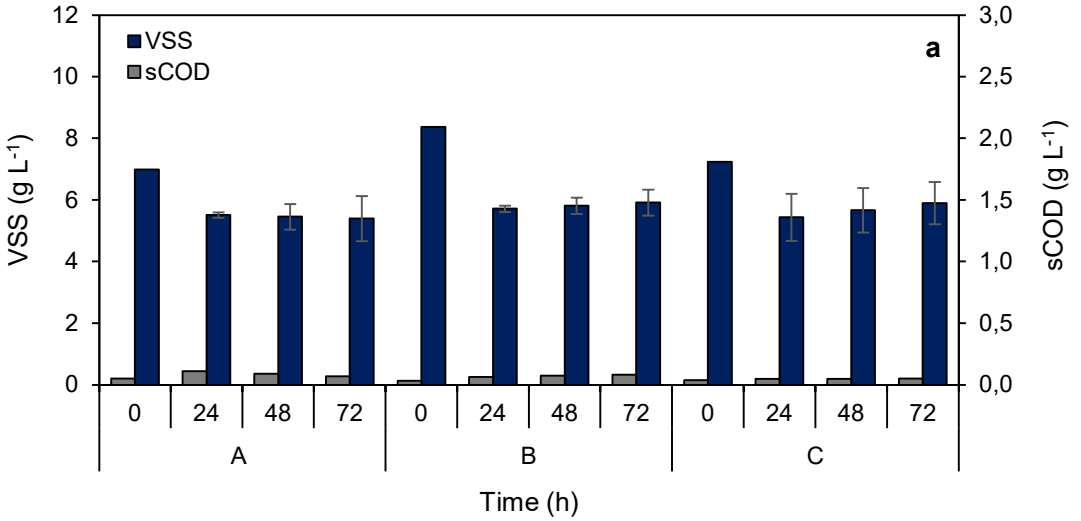
3.2. Low temperature thermal pretreatment

Activated sludge was mixed with microalgae at three different ratios and incubated at 37 and 60ºC during 24, 48 and 72 h, with the aim of assessing the combined effect of temperature and incubation time in the enzymatic activity of the WAS and the further solubilisation of the mixture.

Previous studies performing high temperature thermal pretreatment defined temperature as the main parameter determining biomass disintegration and further anaerobic biodegradability, over exposure time [25,36]. In contrast to high temperature pretreatment, time seemed to have a main influence in WAS disintegration at low temperatures [27,52,53]. This fact could be associated to the exposure time required for the hydrolysis considering the diversity of hydrolytic bacteria and microalgae structure and composition, ranging from hours to few days [54]. In this work, the solubilisation of the mixtures was evaluated along three days with the aim of providing enough contact time between hydrolytic bacteria from the sludge and the biomass from the mixture.

Enzymatic pretreatment to enhance biomass hydrolysis could be performed using commercial exogenous enzymes or endogenous enzymes from microorganisms. Higher sCOD is released when using enzymatic cocktail rather than single enzymes in the pretreatment [31,55]. A promising strategy to overcome microalgae cell wall resistance is bacterial bioaugmentation employing bacterial cultures from different low-cost substrates, such as WAS, that contain a diversity and quantity of active microorganisms providing a constant source of in-situ or endogenous enzymes [21,29,56]. Particle and colloidal wastes in activated sludge are degraded by both endoenzymes and exoenzymes (esterases, aminopeptidases, lipases, glucosidases, etc.) generated by bacteria, transforming complex organic matter into soluble low-molecular weight compounds assimilated by microorganisms to be used as energy and carbon sources [57–59]. Sources of exoenzymes in sewage sludge could be the sewage effluent, the activated sludge via cell autolysis and enzymes excreted by cells [58]. It could be assumed that hydrolytic exoenzymes could be mostly found on the EPS matrix surrounding activated sludge flocs [60]. Those enzymes could hydrolyse microalgae cell wall with the subsequent release of microalgae intracellular organic matter and further increase methane production [1,2,10]. Moreover, it is assumed that once microalgae cells are damaged by a certain pretreatment, their content might be available to be degraded by hydrolytic enzymes [36], and then the organic content could get inside the bacteria cell wall for its further digestion by endoenzymes [57]. In view of that, prior hydrolysis of the substrates is desirable to let them available for subsequent bacterial and archaeal transformation into biogas [31].

As shown in Fig. 3a (also shown in Table S1 Supplementary material (SM)), minor changes were detected in sCOD and VSS during the pretreatment at 37°C. For mixture A, sCOD achieved its maximum value after 24 h pretreatment, increasing 2.2-fold, and decreased to 1.8 and 1.4-fold after 48 and 72 h pretreatment, respectively. The same parameter showed a gradually growth along time for mixture B, reaching a maximum 2.3-fold increase by 72 h pretreatment. On the other side, in mixture C sCOD increase 1.2-fold after 24 h and remains slightly constant during the treatment. For 37°C pretreatment, time effect over the three ratios of the mixtures was similar in comparison with the same mixtures without thermal pretreatment (Mix J, K and L, Fig. 3c), suggesting that this temperature is not enough to increase organic matter solubilisation. This result is coherent with Prorot et al. [28], suggesting that temperatures above 50ºC are required for solubilisation of WAS.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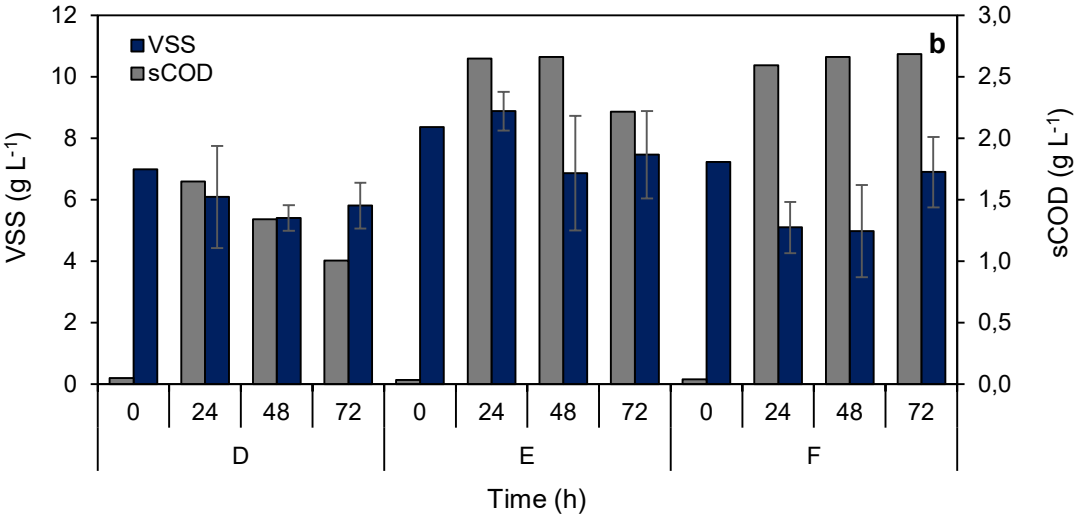
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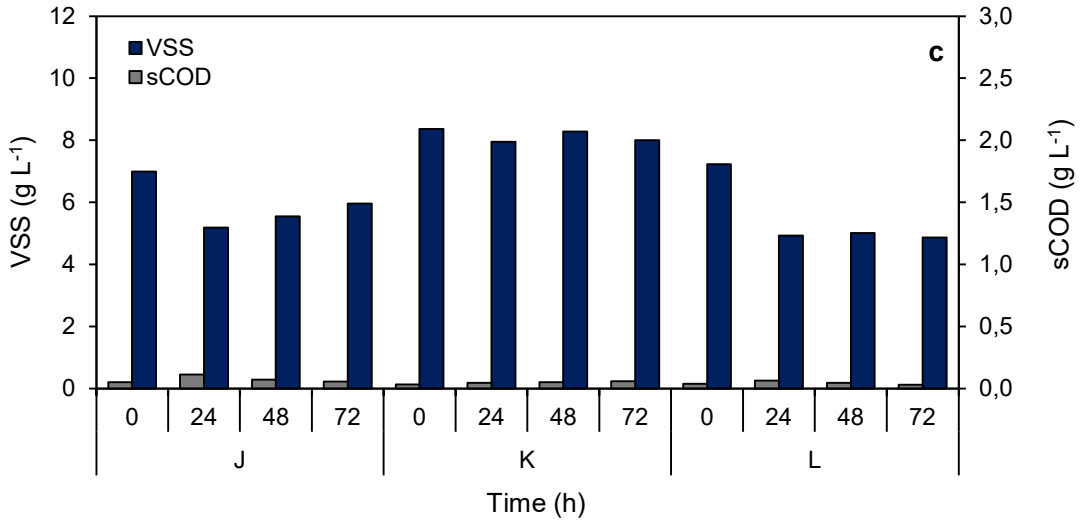

Fig. 3. Volatile suspended solids (VSS) and soluble chemical oxygen demand (sCOD) evolution during the pretreatment for mixtures containing WAS and non-flocculated microalgae (ratios are expressed in v:v basis). a) Mixtures A (50:1), B (25:1) and C (10:1) pretreated at 37°C; b) Mixtures D (50:1), E (25:1) and F (10:1) pretreated at 60°C; and c) Mixtures J (50:1), K (25:1) and L (10:1) without thermal pretreatment (control at 20°C). For VSS, results are given as mean ± standard deviation (n = 3).

(2-column fitting image)

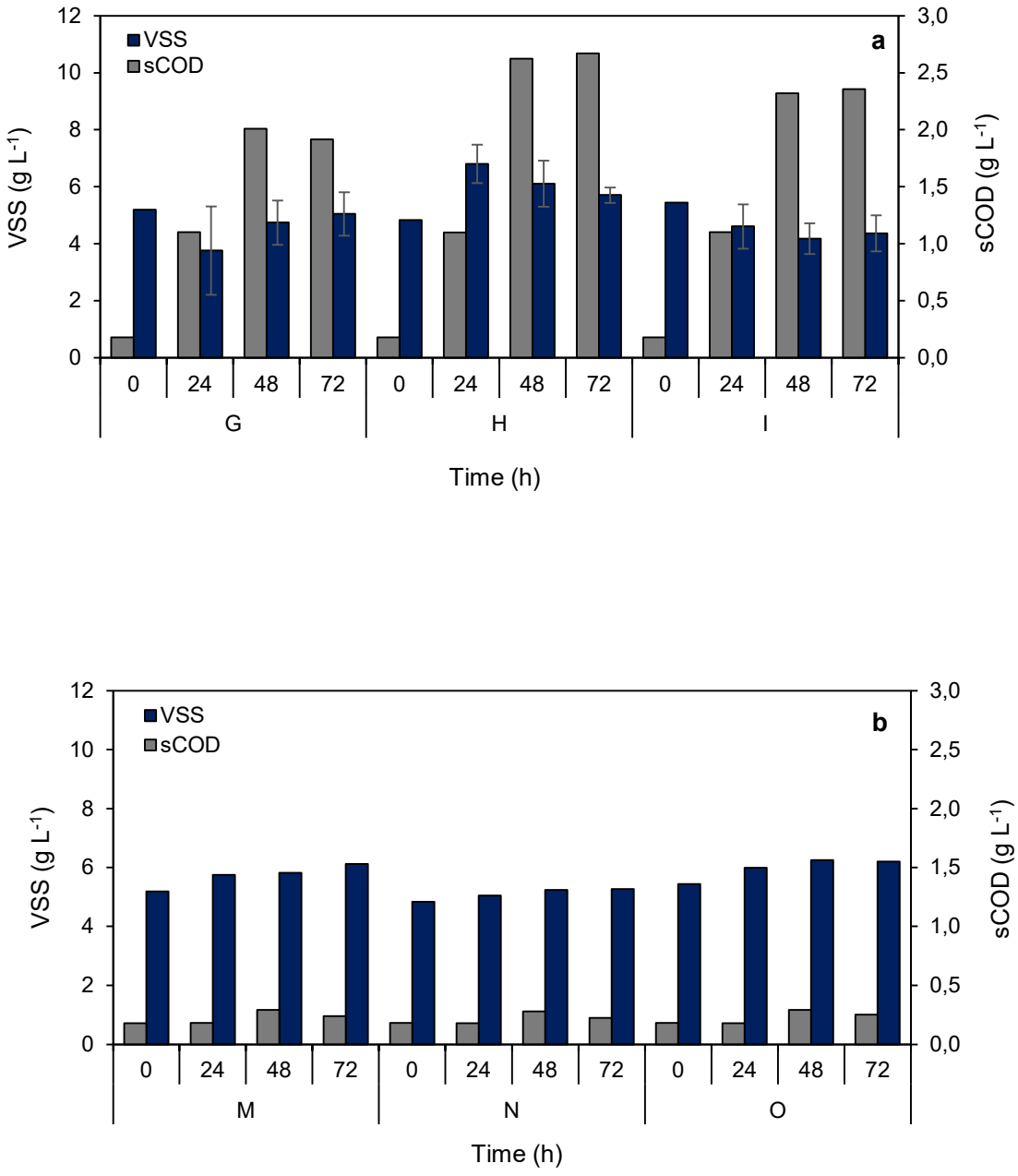

Fig. 4. Volatile suspended solids (VSS) and soluble chemical oxygen demand (sCOD) evolution during the pretreatment for mixtures containing WAS and flocculated microalgae (ratios are expressed in v:v basis). a) Mixtures G (50:1), H (25:1) and I (10:1) pretreated at 60°C; and b) Mixtures M (50:1), N (25:1) and O (10:1) without thermal pretreatment (control at 20°C). For VSS, results are given as mean ± standard deviation (n = 3).

(2-column fitting image)

Significant variations took place in mixtures pretreated at 60°C (mixtures D, E and F) using non-flocculated microalgae (Fig. 3b), indicating that solubilisation increase proportionally with temperature. Afterwards 24 h treatment, the sCOD rise to 33, 74 and 65-fold for mixtures D, E and F, respectively. For mixture E, the sCOD continued relatively constant up to 48 h, while for mixture F it was maintained practically steady from 24 h up to the end of the treatment. This rise in sCOD differed remarkably from control mixtures and could be associated with the enhancing effect of thermophilic temperature (60°C) in the activity of hydrolytic bacteria from activated sludge, fostering organic solids solubilisation [61]. During this aerobic process, a variety of aerobes and facultative anaerobes microorganisms contribute simultaneously to substrate degradation [57]. Furthermore, the higher sCOD at 60°C could be explained by the increase of ammonium concentration (released from the hydrolysis of nitrogen-containing organic molecules) leading to the accumulation of free ammonia at higher levels, which might benefit WAS and microalgae disintegration [62,63]. Nonetheless, at 72 h pretreatment, COD solubilisation was lower (20 and 62-fold) for mixtures D and E, respectively, showing that sCOD tends to decrease at longer incubation times. It could be hypothesized that once complex molecules were degraded by hydrolysis microorganisms to soluble compounds readily bioavailable, organic matter might be further oxidized by aerobic microorganisms using molecular oxygen as final electron carrier, obtaining CO_2, water and cellular growth [29]. Influence of time in sCOD appear to be intensive during the first 24 h, with afterwards lesser variations. In this sense, Alzate et al. [35] verified that after 24 h pretreatment of a microalgae-bacteria consortia, aerobic degradation of the organic matter was larger. In earlier studies *Scenedesmus* sp. was pretreated at 70 and 90ºC during 3 h, and variations in sCOD along time were associated to different stages in organic matter degradation with specific chemical reactions taking place at that temperature [53].

Regarding the control mixtures at 20ºC employing non-flocculated microalgae (Fig. 3c), temperature have a minor effect in sCOD rise. sCOD increased by 2.3, 1.4 and 1.7-fold after 24 h pretreatment for mixtures J, K and L, respectively (see Table S1 in SM). However, for mixtures J and L, longer incubation time tended to diminish sCOD. As a general behaviour for all control mixtures, VSS diminished at 72 h pretreatment.

The initial values of sCOD in the mixtures G, H and I containing WAS and flocculated microalgae (Fig. 4a) were 3.6, 5 and 4.5-times higher than the mixtures of the same ratio without previous flocculation of the microalgae (mixtures A to F, Table S1 in SM). The same applied to control mixtures M, N and O (Fig. 4b). Higher initial solubilisation might be associated with the faster biodegradation of organic matter and the organic flocculant during the previous harvesting treatment. During the first day, sCOD increase in the same proportion (around 6.1 times) for the three mixtures G, H and I. At 48 h, solubilisation was almost doubled in the three mixtures, keeping stable until the 72 h. The control mixtures incubated at 20ºC employing flocculated microalgae (M, N and O, Fig. 4b) shown constant sCOD values up to the 24 h, increasing solubilisation ca. 1.5 times after two days pretreatment. Later, solubilisation showed a slightly tendency to diminish.

Mixtures E and F (pretreated at 60ºC with non-flocculated microalgae) achieved a higher solubilisation after 24 h and remained almost stable up to 48 h. On the contrary, mixtures H and I (pretreated at 60ºC with flocculated microalgae) attained higher solubilisation at 48 h and it continued stable until the end of the pretreatment. Considering that sCOD along time was lesser in mixtures D and G, it could be argued that solubilisation increase proportionally with higher microalgae proportion in the mixture.

Moreover, when comparing both pretreatments (with and without previous microalgae flocculation step) after 72 h at 60ºC, a higher increase in sCOD was achieved when non-flocculated microalgae were used (20-fold, 62-fold and 67-fold for mixtures D, E and F, respectively). Since initial sCOD values are higher when employing flocculated microalgae in the mixtures, sCOD rise after 72 h pretreatment is lower (11-fold, 15-fold and 13-fold for mixtures G, H and I, respectively).

Although there are several studies that evaluate the impact of thermal pretreatments in WAS or microalgae substrates, few previous studies analyse the effect of low temperature thermal pretreatment when WAS and microalgae are mixed. Regarding microalgae, *Chlorella* sp. and *Scenedesmus* sp. biomass are mainly composed by proteins (52%), followed by carbohydrates (16%) and a minor amount of lipids (9%) [64]. Despite of the minor fraction of lipids in those algae [53], lipids possess a higher energy content in comparison with proteins and carbohydrates, but a slow hydrolysis rate [10]. Besides, cell wall composition is strain-specific: species of genus *Chlorella* possess a hemicellulose-cellulose cell wall, while *Scenedesmus* present a complex and

more resistant cell wall integrated by several layers containing sporopollenin, restraining the hydrolysis step and further methane conversion [53,65]. According to Ometto et al. [19] only microalgae thermal pretreatments at temperatures higher than 165°C could achieve their complete solubilisation. However, other authors studied the auto-hydrolysis pretreatment of *Chlorella* sp. and *Scenedesmus* sp. at 50°C during 24 and 48 h, attaining 17% and 5.7% rise in sCOD after 48 h incubation, respectively [66]. Results from other authors verify that pretreatments at low temperature can attain important rise in solubilisation [67].

Concerning the secondary sewage sludge, Appels et al. [27] stated that thermal pretreatment of sludge at 70°C during 60 min just enhanced sCOD by 3 times. Prorot et al. [28] analysed the effect of low temperature thermal treatments (65 to 95ºC, 20 min) on the floc structure of WAS, determining that macro-flocs were disaggregate but not complete broken. It could be presumed that a low temperature thermal pretreatment performed during short times could led to lower WAS disintegration and COD solubilisation. For this reason, in this study the incubation of the mixtures during longer time was tested.

The results obtained in soluble organic matter availability after treatments of the six mixtures at 60°C (mixtures D to I) were significant. The optimal time and temperature conditions that increase solubility of the mixture were identified for assess the improvement in biogas yield. Pretreatment during 24 h at 60°C for mixtures ratio of 25:1 were selected as the optimal conditions for the low temperature thermal pretreatment of WAS and microalgae mixtures. In this sense, mixture E (using non-flocculated microalgae) and H (employing previously flocculated microalgae) were selected to assess the effect of the pretreatment in the biogas yield.

3.3. WAS and microalgae co-digestion

BMP tests were performed for mixture E and H obtained in previous experiments (Table 3). Additionally, biogas production was compared with a control mixture without thermal pretreatment (Fig. 5a), employing the same ratio of WAS and non-flocculated microalgae. After 52 days, a methane yield of 153.6 ± 1.3 NmL CH_4 g VS^{-1} was obtained in control reactors, representing 34% (101.7 ± 13.8 NmL CH_4 g VS^{-1}) and 51% (75.8 ± 3.5 NmL CH_4 g VS^{-1}) more production in comparison with mixture H and E, respectively (Fig. 5b). During the first 10 days of the digestion,

the methane production by mixtures E and H was similar, later mixture H showed a slightly increment. Methane content in biogas (around 72 - 77%) was similar to the reported values for the anaerobic digestion of only microalgae (69-75%), pointing out an optimal biomass conversion into methane and the maintenance of the energy content of the biogas [10,27]. Additionally, the pH of the mixture after anaerobic digestion remains around 7 and 7.2, indicating that steady conditions were reached. The concentration of VFAs remaining in all reactors after the AcoD was low, indicating that the cease in biogas production at day 25 was not linked to VFAs accumulation.

Table 3

Conditions obtained from the pretreatments to assess the anaerobic co-digestion of the waste activated sludge:microalgae mixtures.

Mixture Id.	Mixture ratio (waste activated sludge:microalgae)	Microalgae	Pretreatment conditions	Soluble chemical oxygen demand increment (X-fold)
E	25:1	Non-flocculated	60°C, 24 h	74.1
H	25:1	Flocculated	60°C, 24 h	6.1
Control	25:1	Non-flocculated	20°C, 24 h	1.4

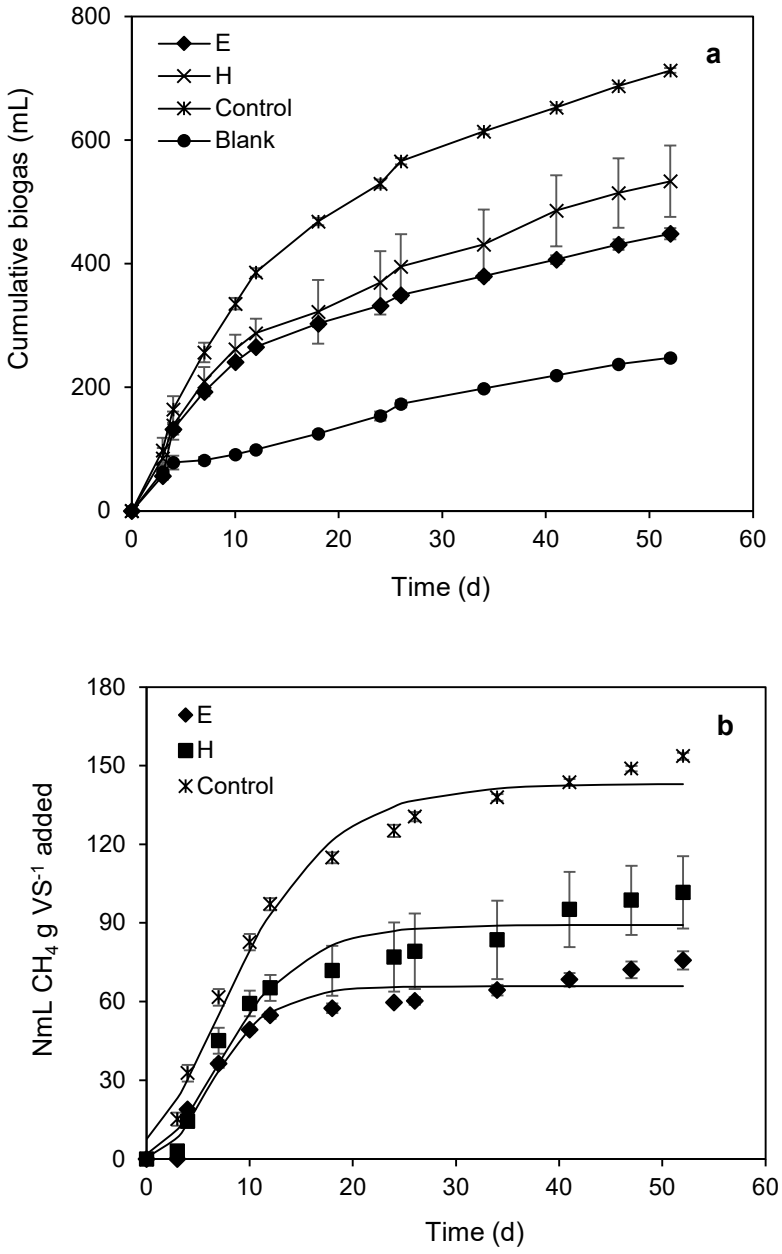

Fig. 5. a) Cumulative biogas production from mixtures (25:1) E (waste activated sludge:non-flocculated microalgae), H (waste activated sludge:flocculated microalgae), control (waste activated sludge:non-flocculated microalgae) and blank reactor. b) Net methane production from mixtures E, H and control. Dots represent experimental data and curves are data estimated by Gompertz model. Results are given as mean ± standard deviation (n = 3).

(single-column fitting image)

The hydrolysis constant calculated from Eq. (3) for the studied mixtures varied among 0.07 and 0.11 d^{-1} (Table 4), being slightly higher when non-flocculated microalgae were used in the mixture. This values are in accordance with the K_H values determined by other authors for pure microalgae species, such as the hydrolysis rate constant of 0.1 d^{-1} found for *Chlorella vulgaris* [3], and 0.09 d^{-1} for *Botryococcus braunii* [68]. The latter authors found similar hydrolysis constant when co-digesting a mixture of 75% of WAS and 25% of lipid-spent microalgae *Botryococcus braunii* (K_H= 0.07 d^{-1}). Lee et al. [13] determined the hydrolysis rate in the co-digestion of several ratios of WAS and microalgae using a modified first-order kinetic model, showing that a ratio of 90% of WAS and 10% of microalgae achieved the highest hydrolysis coefficient (K_H=0.14 d^{-1}); while as microalgae proportion in the mixture increased more than 25%, the hydrolysis rate tended to diminish (K_H= 0.07 – 0.12 d^{-1}). This reduction could be attributed to the microalgae cell wall that hampers hydrolysis.

Table 4

Parameters after anaerobic digestion of mixtures E, H and control. Results are given as mean ± standard deviation (n = 3).

Mixture Id.	pH	Volatile fatty acids (mg L^{-1})			Mean CH_4 content (%)	Experimental net CH_4 (NmL)	Experimental net CH_4 yield (NmL CH_4 g VS^{-1})	P_{max} (NmL CH_4 g VS^{-1})	R_{max} (NmL CH_4 g VS^{-1} d^{-1})	λ (d)	r^2	K_h (d^{-1})
		Acetic acid	Butyric acid	Valeric acid								
E	7.2 ± 0.1	n.d.	2.4	111.8	77	167.7 ± 7.8	75.8 ± 3.5	65.9 ± 2.4	6.8 ± 1.4	2.0 ± 0.9	0.96	0.11 ± 0.03
H	7.0 ± 0.2	4.0	1.9	69.1	72	225.2 ± 30.5	101.7 ± 13.8	89.3 ± 3.7	6.9 ± 1.4	1.7 ± 1.3	0.95	0.08 ± 0.02
Control	7.0 ± 0.1	n.d.	4.0	n.d.	73	340.1 ± 2.8	153.6 ± 1.3	143.1 ± 3.6	8.4 ± 0.9	0.5 ± 0.8	0.98	0.07 ± 0.01

n.d.= not detected.
P_{max}= methane yield potential.
R_{max}= maximum daily methane production rate.
λ= lag phase.
K_h= hydrolysis rate.

The Gompertz model fits suitably the experimental data. The lag phase (λ) determined by the model was ca. 2 days for mixtures E and H, while it was 0.5 d for the control. A longer lag phase could be a sign that the substrate content was not straightforwardly hydrolysed, indicating that microorganisms from the inoculum might need more time to acclimate to the substrates obtained after the pretreatment, lengthen the beginning of the methane production. Carvajal et al. [29] detected an inhibition effect that generates a delay of 0.6 and 2.4 d after the pretreatment of WAS at 55°C for 12 and 24 h, respectively. However, this delay is lower than lag phases obtained by Olsson et al. [5] in the co-digestion of different proportions of wet and dry microalgae mixtures with sewage sludge at 37°C, ranging from 2.1 to 13.7 d.

From these results, it can be inferred that conditions that enhance solubility did not generate a proportional increase in methane yield. After the thermal pretreatment, solubilized organic matter might be rapidly consumed by active microorganisms from the sludge as a carbon source, lowering the organic matter content available to be anaerobically degraded for energy recovery. In coherence with this results, other authors stated that treating the sludge under thermophilic conditions and a high oxygen content, foster organic matter consumption by aerobic microorganisms from the WAS, being no longer available for methane production in the next anaerobic digestion process [29].

Table 5 shows previous reported results on sCOD and methane production increase after a low temperature thermal pretreatment of microalgae, WAS and WAS-microalgae mixtures. In the case of pure microalgae cultures, some authors achieved an increase in sCOD but not in methane production when pretreating *Chlorella vulgaris* biomass at 50°C for 24 h [66]. In addition, these authors observed an increase in sCOD after a thermoalkaline pretreatment of *Chlorella* and *Scenedesmus* sp. using NaOH at 50°C, followed by a low methane production enhancement. They attributed this behaviour to the release of soluble cell wall exopolymers rather than intracellular organic matter release after cell wall rupture. Likewise, González-Fernández et al. [53] observed no disruption of the cell wall of *Scenedesmus* biomass after thermal pretreatment at 70ºC, associating soluble organic matter rise (4 times higher) to the detachment of exopolymers from the microalgae cell wall. In this sense, the low biogas production in the mixtures E and H could be in concordance with results attained by these authors, despite the fact that microalgae

consortia rather than pure cultures were employed in the present work. Results achieved by different authors regarding solubility and methane improvement after thermal pretreatment are in some way controversial. Alzate et al. [35] pretreated a microalgae mixture as well as microalgae-bacteria consortia at 55ºC for 12 and 24 h. When a microalgae mixture was employed, sCOD increased (11%) after 24 h, but methane productivity diminished (8%); while in the case of microalgae-bacteria consortia, both solubility and methane productivity increased (29% and 5%, respectively) which could be associated to the hydrolytic enzymes excreted by bacteria. Other authors pretreated different species of microalgae at low temperature (55, 75 and 95ºC) during 15 h, obtaining an increase in methane production in consistency with biomass solubilisation [67]. However, they determined that around 50% of the organic matter was not anaerobically degraded. In the case of mixtures E and H, anaerobic biodegradability was 78 and 76%, respectively. This fact endorses the previous hypothesis that soluble organic matter was consumed after the thermal pretreatment, while left organic content was converted to methane.

Some authors studied the co-digestion of WAS and microalgae without previous pretreatment, reporting a synergetic effect at mesophilic conditions that boost biogas production [5,13]. Thorin et al. [69] stated an average methane production of 317 ± 101 NmL g VS^{-1} from the co-digestion of 29 WAS and microalgae (pure cultures and polycultures) mixtures at mesophilic conditions. They outline a mean methane production of 304 ± 114 NmL g VS^{-1} for ten different activated sludges, meanwhile for thirteen different microalgae strains (pure cultures and polycultures) the generation was of 258 ± 106 NmL g VS^{-1} (high standard deviation is associated to the variety of biomass composition from the microalgae and WAS). On the other hand, Wang et al. [2] performed the co-digestion of WAS and microalgae (*Chlorella* sp.) mixtures containing 0, 4, 11, 41 and 100% microalgae, obtaining a rather lower methane production for all the mixtures in comparison to WAS digested as a sole feed. However, they obtained a methane production of the mixtures 73 to 79% higher when comparing with anaerobic digestion of only microalgae. Therefore, an increase in solubilisation degree does not directly enhance methane productivity and is coherent with our results.

Table 5

Solubility and methane production enhancement after low temperature thermal pretreatment applied to microalgae, waste activated sludge and waste activated sludge-microalgae mixtures.

Substrates	Thermal pretreatment conditions	Solubility increase after the pretreatment (in soluble chemical oxygen demand basis)	Methane production (NmL CH_4 g VS^{-1})*	Methane yield increase after the pretreatment*	Reference
Microalgae					
Scenedesmus sp.	70ºC 3 h	4-fold	85 (in COD basis)	1.1-fold	[53]
	90ºC 3 h	4.4-fold	170 (in COD basis)	2.2-fold	[53]
Chlorella sp.	50ºC 24 h	16.2%	-	0%	[66]
	50ºC 24 h 5% w/w NaOH	18.2%	-	13%	[66]
Scenedesmus sp.	50ºC 24 h	5.7%	-	0%	[66]
	50ºC 24 h 5% w/w NaOH	16.8%	-	20%	[66]
Microalgae mixture (40% *Chlamydomonas*, 20% *Scenedesmus*, 40% unknown microalgae)	55ºC 24 h	11%	252 ± 5	- 8%	[35]
Microalgae (mainly *Microspora*)-bacteria consortia	55ºC 24 h	29%	266 ± 2	5%	[35]

Scenedesmus sp.*, Coelastrum* sp.	80ºC 3 h	13%	280 ± 9	11%	[39]
Microalgal (*Chlamydomonas*, *Chlorella*, *Ankistrodesmus*, *Monorraphidium*, *Scenedesmus* and diatoms *Nitzchia*) and bacterial biomass	55ºC 15 h	400%	124.6 ± 3.3	13%	[67]
	75ºC 15 h	1048%	160.4 ± 0.7	53%	[67]
	95ºC 15 h	1188%	169.9 ± 3.7	60%	[67]
WAS					
WAS	70ºC 1 h	2.9-fold	22.4	1.4%	[27]
	90ºC 1 h	25.6-fold	240.4	11-fold	[27]
Polyacrylamide flocculated WAS	75ºC pH 11 17.5 h	-	210.8	2-fold	[70]
Textile dying sludge	60ºC 1 h	0.2-fold	82.9	2%	[24]
	70ºC 1 h	1.7-fold	130.5	61%	
	90ºC 1 h	4.2-fold	156.4	93%	
WAS-microalgae mixtures					
Raw sludge-*Chlorella* sp.	No pretreatment	-	116 ± 3	-	[71]
WAS (75%)-lipid-spent *Botryococcus braunii (25%)* (in VS basis)	No pretreatment	-	374 ± 13	-	[68]

Undigested sewage sludge (63%)-*Scenedesmus* and *Chlorella vulgaris* (37%) (in VS basis)	No pretreatment	-	408 ± 16	-	[5]
WAS-*Chlorella* sp. 4% (in VS basis)	No pretreatment	-	257	-	[2]
WAS-*Chlorella* sp. 11% (in VS basis)	No pretreatment	-	254	-	[2]
WAS-*Chlorella* sp. 41% (in VS basis)	No pretreatment	-	260	-	[2]
WAS (79%)-Micractinium sp. (21%) (in VS basis)	No pretreatment	-	236	-	[7]
WAS (79%)-*Chlorella* sp. (21%) (in VS basis)	No pretreatment	-	253	-	[7]
Mixture E	60ºC 24 h	74-fold	75.8 ± 3.5	-51%	This study
Mixture H	60ºC 24 h	6-fold	101.7 ± 13.8	-34%	This study

(*) Mesophilic conditions were considered.

Besides, organic matter solubilised after the thermal pretreatment could be later oxidized to complex molecules [66] with low and/or slow anaerobic digestibility. Sapkaite et al. [25] applied a thermal hydrolysis pretreatment to a WAS from a municipal WWTP showing a 36% rise in solubility at 170°C during 10 min and 140°C during 50 min. Nevertheless, methane production decreased after 30 min pretreatment and 160°C, indicating that solubilisation achieved did not led to higher anaerobic digestibility due to the presence of soluble but non-biodegradable substances (melanoidins) generated at high temperature pretreatment, inhibiting the anaerobic digestion and reducing the methane yield. Those recalcitrant compounds are created by the Maillard reaction by non-enzymatic chemical reactions of sugars and proteins during thermal hydrolysis of sludge at temperatures higher than 140°C [26] or during extended pretreatment time in low temperature thermal pretreatments (<100°C) [14], hindering the degradation of other organic substances [15]. Likewise, minor methane yield in mixtures E and H may be attributable to the presence of low biodegradable compounds.

Regarding the influence of the flocculant in the AD, it is known the negative impact of cationic polyacrylamide accumulation in the WAS after flocculation, affecting the sludge disintegration, as well as, the overall efficiency of the AD process as a consequence of its degradation metabolites [72,73]. However, Liu et al. [70] reported an important rise in methane potential of polyacrylamide-flocculated WAS after a thermal-alkaline pretreatment (75°C, pH 11), indicating that the pretreatment distorted the structure of the flocculant, improving its degradation by anaerobic microorganisms. In this work a flocculant was used, and the higher methane production in mixture H in comparison to mixture E could be related with the favourable effect of the thermal pretreatment in the anaerobic degradation of the flocculant. On the other hand, the high sCOD achieved during the thermal pretreatment at 60°C for mixture E might have generated a higher ammonium release and free ammonia accumulation, thus inhibiting methanogenesis [63,74]. This fact could explain the lowest methane yield obtained in WAS and non-flocculated microalgae mixture.

In brief, results in this study indicate that at major sCOD achieved (mixture E > mixture H > control), methane production decreases (mixture E < mixture H < control). The absence of proportionality between organic matter solubilisation after the thermal pretreatment (determined

by sCOD) and organic matter digestibility (determined by methane production) could be ascribed to: 1) a faster consumption of bioavailable compounds as a result of the higher enzymatic activity of thermophilic bacteria, tending to diminish the organic content available for the anaerobic stage; 2) the release of soluble microalgae cell wall exopolymers that might increase sCOD with no influence in methane production; 3) the conversion of solubilized organic matter into complex molecules with scarce anaerobic biodegradability; and 4) the inhibition of methanogens due to toxicity of ammonia released. According to Wang et al. [63] and bearing in mind that in our study the thermal pretreatment at 60°C did not enhance the methane yield of the mixtures, it might be interesting to study the fermentative hydrogen production, since hydrogen consumers could have been inhibited due to the high free ammonia concentration.

4. Conclusions

Low temperature thermal treatments were applied to WAS and microalgae mixtures as a previous step to anaerobic digestion. Microalgae harvesting showed good clarification results when a cationic polymer of diallyl dimethyl ammonium chloride free of acrylamide was used as an organic flocculant. Higher solubility of WAS and microalgae mixtures was achieved after the pretreatment at 60°C in comparison with the pretreatment at 37ºC, indicating that solubilisation increases proportionally with temperature. Initial sCOD in the mixtures containing flocculated microalgae was higher than in the mixtures of the same ratio without previous flocculation of the microalgae. Besides, the effect of longer incubation time of the mixtures (72 h) in their solubilisation was tested. When mixtures with non-flocculated microalgae were pretreated at 60°C, influence of time in sCOD was intensive during the first 24 h, with lower later variations. Regarding to WAS:microalgae ratios, in general solubilisation increases proportionally with higher microalgae volume in the mixture. Treatment at 60°C during 24 h for mixtures ratio 25:1 using flocculated and non-flocculated microalgae were selected as the optimal pretreatment conditions to assess its effect on the methane potential. However, the anaerobic digestibility of the high solubilised mixtures was not enhanced after the pretreatment. The lack of proportionality between the solubilisation of the mixture (sCOD) and further methane production evidence the need of identifying other parameters to determine the effect of the pretreatment in the co-digestion. Furthermore, as methane production may have been inhibited, it could be interesting to study the

fermentative hydrogen production of both substrates. The larger methane yield obtained for the WAS and flocculated microalgae mixture could be associated with the positive effect of the low temperature thermal pretreatment in the degradation of the flocculant by anaerobic microorganisms. Nonetheless, the hydrolysis rate was higher when co-digesting the pretreated WAS and non-flocculated microalgae.

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Author contribution

Romina Avila: experimental work, data interpretation, discussion of results and writing the main manuscript. Teresa Vicent and Paqui Blánquez: conception and experimental design, supervision of the experimental work, review and edition of the manuscript. Elvira Carrero, Eudald Crivillés and Mercè Mercader: experimental design and discussion of results.

All the authors have read and approved the manuscript.

Conflict of interest

The authors declare no competing financial interests.

Statement of informed consent, human or animal rights

Not applicable.

Appendix A. Supplementary material

Supplementary data related with this article can be found at Supplementary Material link.

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