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Biomethanation potential of macroalgae *Ulva* spp. and *Gracilaria* spp. and in co-digestion with waste activated sludge

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ABSTRACT

Biochemical methane potential of four species of *Ulva* and *Gracilaria* genus was assessed in batch assays at mesophilic temperature. The results indicate a higher specific methane production (per volatile solids) for one of the *Ulva* sp. compared with other macroalgae and for tests running with 2.5% of total solids (196 ± 9 L CH_4 kg^{-1} VS). Considering that macroalgae can potentially be a post treatment of municipal wastewater for nutrients removal, co-digestion of macroalgae with waste activated sludge (WAS) was assessed. The co-digestion of macroalgae (15%) with WAS (85%) is feasible at a rate of methane production 26% higher than WAS alone without decreasing the overall biodegradability of the substrate (42–45% methane yield). The use of anoxic marine sediment as inoculum had no positive effect on the methane production in batch assays. The limiting step of the overall anaerobic digestion process was the hydrolysis.

1. Introduction

In the seventies, the oil crisis resulted in a strong interest in the macroalgae cultivation for energy production, which was abandoned because the economic climate returned to favor fossil fuels (Briand and Morand, 1997; Chynoweth et al., 2001). Due to the recent oil crisis (with consequent increase in oil prices in international markets) and the creation of policies to mitigate greenhouse gases, after the Kyoto Protocol, the exploitation of renewable energy sources has regain considerable interest. For instance, in the European Union (EU), according with Directive 2009/28/EC, the Council of March 2007 set a binding target of 20% share of energy from renewable sources in overall EU energy consumption by 2020.

Anaerobic digestion (AD) is a process responsible for the degradation of most of the carbonaceous matter in natural environments where organic accumulation results in oxygen depletion (Appels et al., 2008). In particular, AD of energy crops and organic wastes benefits society by promoting a cleaner fuel (biogas) than fossil fuels and a bio-fertilizer (digested matter) from renewable raw materials (Appels et al., 2011).

AD of biowaste and energy crops has a role in the EU policy of energy and environment. Several research programs have investigated energy crops (aquatic and terrestrial) for methane production (Appels et al., 2011; Chynoweth et al., 2001). These programs integrated several data, including, data from crop production, harvesting, conversion to methane, and system analysis. Estimates indicate that the energy potential of marine biomass is more than 100 EJ yr^{-1}, significantly higher than the terrestrial biomass (22 EJ yr^{-1}) or municipal solid waste (7 EJ yr^{-1}) (Chynoweth et al., 2001). Furthermore, algal biomass presents high productivity rates, do not compete for land with crops, has tolerance to a wide range of temperature, salinity and nutrient concentrations, and, there is an extensive knowledge about their farming practices (Bruhn et al., 2011; Gunasselan, 1987; Hansson, 1983; Jones and Mayfield, 2011; Nielsen and Heiske, 2011; Schamm and Lehnberg, 1984). Macroalgae contain easily hydrolysable sugars and proteins; low fractions of lignin (Gunasselan, 1987; Nkemka and Murto, 2010) and high fractions of hemicellulose, which favor the enzymes accessibility to the substrate; and a good hydrolysis yield (Briand and Morand, 1997). Other advantages of aquatic biomass, such as macroalgae, over terrestrial biomass, include area availability and high growth rates (Bruhn et al., 2011; Gunasselan, 1987; Hansson, 1983).

Macroalgae have the potential of becoming a viable aquatic energy crop (Bruhn et al., 2011; Chynoweth et al., 2001; Gao and Mckinley, 1994; Hansson, 1983; Morand et al., 1990, 2006). However, the production of energy from macroalgae is still committed for reasons of economic viability (Jones and Mayfield, 2011). One possibility is the cultivation of macroalgae using nutrients from wastewater treatment plants (WWTP) and subsequent production of biogas from the cultivated biomass alone or in co-digestion with waste activated sludge (WAS) in existing digesters. Other benefits

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of co-digestion includes: dilution of the potential toxicity of any of the involved co-substrates, nutrients balance, synergistic effects on microorganisms, increasing the load of biodegradable organic matter, and higher methane yields per unit of digester volume (Cecchi et al., 1996; Nkemka and Murto, 2010). Yen and Brune (2007) reported a significant enhancement of the methane production with an addition of paper waste to algal sludge. However, using *Ulva* sp. as a co-substrate with pig manure (48%:52% w/w), no significant improvements were observed (Peu et al., 2011). Another possibility to increase the biodegradability extent and rate of macroalgae is to use an inoculum more adapted to the substrate, such as anoxic marine sediments. In fact, Schamm and Lehnberg (1984) obtained higher methane yield using marine sediment because of the specific affinity of the methanogenic archaea to the macroalgae.

Biochemical methane potential (BMP) tests are commonly used to estimate the extent of anaerobic biodegradability of substrates and the associated specific methane production (Angelidaki et al., 2009). Also, BMP assays results can provide a rough estimate of residence times required for complete digestion of a given substrate (Labatut et al., 2010).

The aim of this work was to determine the BMP of several raw macroalgae and macroalgae co-digested with WAS. The effect of using anoxic marine sediment, instead of anaerobic suspended sludge, as inoculum was also assessed. The tested macroalgae included common species that grow wild and also cultivated.

2. Methods

2.1. Substrates

The following species of macroalgae were used: *Ulva* spp. (U), including a species previously classified as *Enteromorpha* genus and hereafter identified as (UE), *Gracilaria* sp. (G), and *Gracilaria vermicullophyla* (GV).

The macroalgae *Ulva* spp., and *Gracilaria* sp. were collected in the Sapalsado aquaculture input tanks (Sado Estuary, Faralhão, Portugal), where they grow naturally. The macroalgae *G. vermiculophylla* was harvested from an experimental integrated multi-trophic aquaculture implemented in a fish monoculture in the North of Portugal (Abreu et al., 2010). To facilitate the milling process, the macroalgae were dried at 105 °C for 24 h in an oven, and then milled into pieces with less than 0.5 cm.

Activated aerobic sludges from different stages of treatment in the WWTP of Beirolas (Lisbon, Portugal) were used as co-substrate: primary sludge (PS), secondary sludge (SS) and mixed sludge (MS).

2.2. Inoculum

Digested anaerobic sludge from the WWTP reactor of Beirolas (Lisbon, Portugal), and anoxic marine sediments of the Guadiana Estuary (Portugal) were used as inocula. The digested sludge was used in the macroalgae biodegradability and co-digestion tests, while the marine sediment was used in one of the co-digestion tests to compare the two inocula performance. Both inocula were evaluated for the specific methanogenic activity and volatile solids (VS).

2.2.1. Methanogenic activity mesurements

Methanogenic activity tests were performed using the pressure transducer technique. The test involves the monitoring of the pressure increase developed in sealed vials fed with non-gaseous substrates (specific acetoclastic activity (SAA) with acetate, 0.030 M) or pressure decrease in vials previously pressurized with gaseous substrates (specific hydrogenotrophic methanogenic activity (SHMA), in the presence of H_2/CO_2 (80:20, v/v), at 1 bar). Strict anaerobic conditions were maintained. The hand-held pressure transducer was capable of measuring a pressure increase or decrease of two bar (0 ± 2202.6 kPa) over a range of −200 to +200 mV, with a minimum detectable variation of 0.005 bar. A sensing element consisting of a 2.5 mm square silicon chip with integral sensing diaphragm is connected to a digital panel meter module and the device is powered by a 7.5 V DC transformer. The basal medium used in the batch experiments, made up with distilled water, was composed of cysteine-HCl (0.5 g L^{-1}) and sodium bicarbonate (3 g L^{-1}), the pH was adjusted to 7.0 ± 7.2 with NaOH 8 N and was prepared under strict anaerobic conditions. The medium was added to the sludge (3 g VS L^{-1}) to a working volume of 12.5 mL. Blank assays were performed to discount the methane production due to residual substrate. No calcium or trace-nutrients were added. All methanogenic activity tests were performed in triplicate assays. The methanogenic activity values were corrected to the standard temperature and pressure conditions (STP).

The methane percentage in the biogas was determined by gas chromatography using a chromatograph Varian CP-4900, with a PPU column PPU (T = 80 °C) and a TCD detector (T = 110 °C). The carrier gas used was helium (150 kPa) and the injected volume was 1 mL.

2.3. Anaerobic biodegradability assays

2.3.1. Macroalgae digestion assays

Anaerobic biodegradability tests were made with the macroalgae U, G and UE to determine the biochemical methane potential and methane production rates. Batch tests were prepared in triplicate for the different macroalgae species at various percentages of total solids (TS): 1.0, 2.5, and 5.0. Also a blank was prepared in triplicate, containing only inoculum, to measure the methane production of the residual substrate, in order to account only for the methane production from the macroalgae biodegradation. The tests were carried out at mesophilic temperature of 37 °C and lasted approximately 82 days.

2.3.2. Macroalgae – sludge co-digestion assays

After the raw macroalgae biodegradability tests, several batch co-digestion assays were prepared in order to optimize the biogas production. *Ulva* sp. was chosen to test co-digestion with mixed sludge at different ratios (TS/TS): 15% U + 85% MS, 30% U + 70% MS, 60% U + 40% MS, and 80% U + 20% MS. The digestion of each substrate alone (100% U and 100% MS) was also assessed.

For the ratio 15% macroalgae and 85% WAS, different co-digestion tests were made with specific objectives, namely: (i) use *G. vermiculophylla* instead of *Ulva* sp. to test a cultivated macroalgae; (ii) use primary and secondary sludge instead of mixed sludge to test the influence of the different sludge; and, (iii) use anoxic marine sediment as inoculum instead of digested anaerobic sludge to test an inocula from a similar environment as of the macroalgae.

All the co-digestion batch assays were prepared in triplicate with 2.5% TS, and the VS of waste to VS of inoculum ratio was 2.8 ± 0.1 $g_{waste}\ g^{-1}{}_{inoculum}$. Blank assays were also prepared in triplicate for both inocula. The tests were performed at mesophilic temperature of 37 °C and lasted approximately 50 days.

2.3.3. Procedure

Anaerobic biodegradability batch assays were performed according to the directives defined in Angelidaki et al. (2009). Bottles were prepared by adding the substrate, inoculum, and basal medium containing $NaHCO_3$ (5 g L^{-1}) to a final volume of 50 mL. The pH was corrected to 7.0–7.2 using NaOH or HCl 2 M. The vials were sealed and the headspace flushed with N_2/CO_2 (80:20 v/v).

Before incubation, the medium was reduced with $Na_2S{\cdot}9H_2O$, added to a final concentration of 1 mM.

The methane accumulated in the vessels headspace was measured by gas chromatography by collecting 500 μL of sample volume with a gas-tight syringe. Methane production was corrected for STP conditions. The BMP was determined by unit of waste of VS added to each vial:

$$\mathrm{BMP} = \frac{\mathrm{COD} - \mathrm{CH_4} \times \alpha}{m\mathrm{VS}} \quad (1)$$

where BMP is the biochemical methane production (L CH_4 kg^{-1} VS), COD-CH_4 is the cumulative methane produced (kg COD-CH_4), α is the theoretical biochemical methane potential (350 L CH_4 kg^{-1} COD), and *m*VS is the amount of VS of substrate added (kg $VS_{substrate\ added}$).

The anaerobic digestion yield in terms of methane production (yield) was defined as the amount of methane produced during the assays in relation to the theoretical biochemical methane potential:

$$\mathrm{Yield} = \frac{\mathrm{COD} - \mathrm{CH_4}}{\mathrm{COD} - \mathrm{added}} \times 100 \quad (2)$$

where COD-added corresponds to the COD initially added to each vial (kg COD).

Hydrolysis was evaluated considering the percentage of solubilization ($P_{Soluble}$), which represents the percentage of the COD added to the vials that was solubilized during the anaerobic biodegradability assay, as follows:

$$P\mathrm{Soluble} = \frac{\mathrm{CODs} + \mathrm{COD} - \mathrm{CH_4}}{\mathrm{COD} \cdot \mathrm{added}} \times 100 \quad (3)$$

where *CODs* is the soluble COD present in the vials at the end of the assay (kg COD). Note that the COD transformed in biomass growth was not considered in these calculations.

2.4. Analytical Methods

Total kjeldahl nitrogen (TKN), ammonium (NH_4^+), TS and VS were measured according to standard methods (APHA, 1998). Total and soluble COD were determined using standard kits (Hach Lange, Düsseldorf, Germany). Sample filtration was performed prior to soluble COD (CODs) determination. Volatile fatty acids (VFA) were determined by HPLC (Jasco, Japan) equipped with a UV detector (210 nm) and a Chrompack column (6.5 × 30 mm^2) at 60 °C. Sulfuric acid (0.01 N) was used as mobile phase at a flow rate of 0.6 mL min^{-1}.

Methane was analyzed in a gas chromatograph (Chrompack 9001B) equipped with a TCD detector and a Porapak column. Argon was used as carrier gas (13 mL min^{-1}). The column, injector, and detector temperatures were 35, 110, and 110 °C, respectively.

The proteins content was determined based on the TKN measurement using the correction factor 6.25 (Bruni et al., 2010; Msuya and Neori, 2008). The total fat content was extracted with diethyl ether in a Soxtec System HT2 1045 extraction unit produced by Tecator (Official Method of Analysis, 2007). Carbohydrates were estimated as the remaining fraction of VS after the determination of proteins and lipids (Alvarez et al., 2010).

2.5. Data analysis

An exponential model was used to describe the progress of cumulative methane production obtained from the batch experiments (Eq. (4)):

$$M(t) = P(1 - e^{-kt}) \quad (4)$$

where *M(t)* is the methane cumulative production (mg COD-CH_4), *P* is the maximum methane production (mg COD-CH_4), *k* is the methane production rate (d^{-1}), and *t* is the time (d).

3. Results and discussion

3.1. Inocula and substrate characterization

The inocula characterization is presented in Table 1. Although no SAA was detected in the anoxic marine sediment, the SHMA was higher than in the anaerobic digested sludge.

The results from the four macroalgae (*Ulva* sp., *Enteromorpha* sp., *Gracilaria* sp., and *G. vermiculophylla*) and three WAS (primary, secondary, and mixed sludge) characterization are shown in Table 2. The nitrogen content, between 3.5% and 8.7%, may result in methanogenesis inhibition, if the appropriate measures, such as efficient dilution, are not carried out.

Table 1
Inocula characterization.

		Anaerobic digested sludge	Marine sediment
VS	g L^{-1}	15.4	29.9
SAA	g COD-$CH_{4@STP}$ g^{-1} VS d^{-1}	0.12 ± 0.02	<0.01
SHMA	g COD-$CH_{4@STP}$ g^{-1} VS d^{-1}	0.57 ± 0.13	0.72 ± 0.37

Table 2
Characterization of the different substrates (macroalgae and sludge) used in the experiments.

		Ulva sp.	*Enteromorpha* sp.	*Gracilaria* sp.	*G. vermiculophylla*	MS	PS	SS
TS	%	15.8 ± 0.7	14.7 ± 1.5	20.5 ± 2.5	20 [b]	5.6 ± 0.3	8.6 ± 0.2	3.48 ± 0.02
VS	%	Nd	Nd	Nd	Nd	3.8 ± 0.2	5.5 ± 0.2	2.56 ± 0.02
VS	% [a]	46.9 ± 2.4	43.1 ± 2.1	56.7 ± 3.1	65.3 ± 0.6	68.4 ± 0.4	63.5 ± 0.5	73.5 ± 0.2
COD	g O_2 g^{-1} $_{waste}$ [a]	0.69 ± 0.08	0.59 ± 0.05	0.63 ± 0.05	0.85 ± 0.03	1.4 ± 0.9	1.6 ± 0.2	1.2 ± 0.2
TKN	% [a]	4.1 ± 0.2	3.5 ± 0.2	7.6 ± 0.3	8.7 ± 0.2	4.3 ± 0.2	0.3 ± 0.1	0.6 ± 0.2
Proteins	% [a]	25.9 ± 1.3	22.2 ± 1.4	47.4 ± 2.1	54.5 ± 1.2	27.1 ± 1.1	2.1 ± 0.9	3.9 ± 1.1
Lipids	% [a]	0.54 ± 0.02	1.2 ± 0.1	1.6 ± 0.3	0.6 ± 0.2	2.4 ± 0.8	5.0 ± 2.7	3.5 ± 2.8
Carbohydrates	% [a]	20.5 ± 1.3	19.7 ± 1.5	7.7 ± 2.4	10.2 ± 1.0	38.8 ± 0.2	56.4 ± 1.8	66.1 ± 1.7

Nd: not determined.
[a] Dry weight.
[b] Abreu et al. (2010).

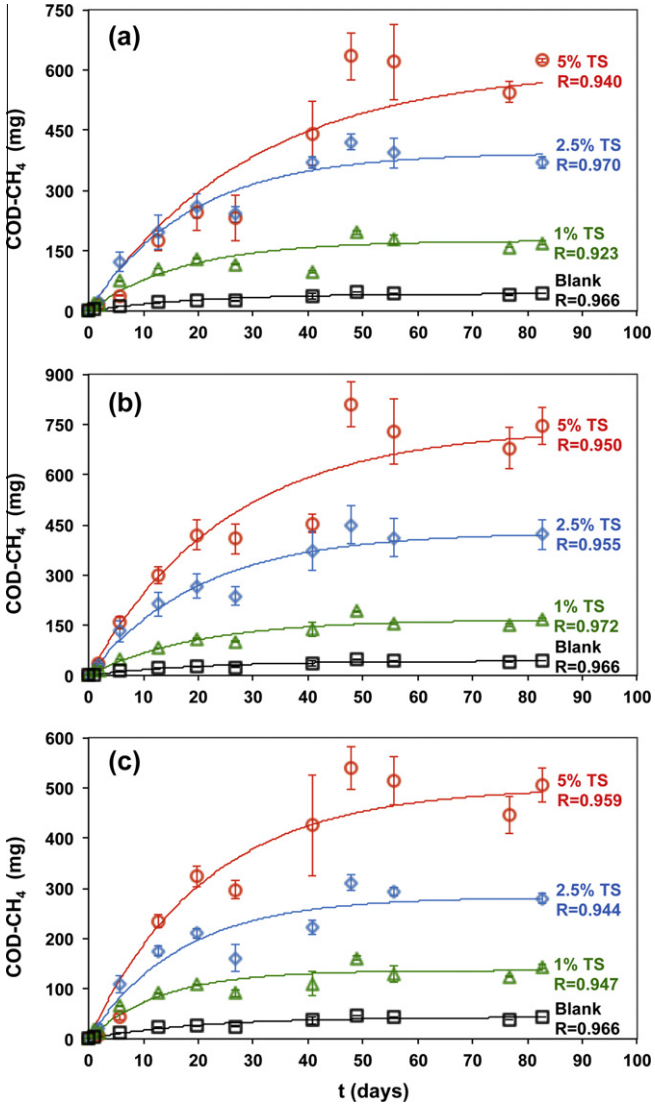

Fig. 1. Cumulative methane production (mg COD-CH_4) during anaerobic biodegradability tests with *Ulva* sp. (a), *Gracilaria* sp. (b), and *Enteromorpha* sp. (c). 0% TS (blank) (□), 1% TS (△), 2.5% TS (◇), and 5% TS (○). The symbols represent the experimental data with respective standard deviation ($n = 3$ points). The lines represent the predicted data by the exponential model.

3.2. Macroalgae biochemical methane potential

The cumulative methane profiles of the macroalgae are shown in Fig. 1. All the assays stabilized approximately after 50 days. The maximum methane production achieved was 740 ± 79 mg COD-CH_4 in the digestion with 5% TS of *Gracilaria* sp.

In Table 3 are summarized the results obtained at the end of the biodegradability tests. TKN present in the macroalgae is high, therefore is important to state that ammonium concentrations up to 1500 mg L^{-1} have no significant adverse effects on the methanogenesis, and above this value it can be inhibitory or toxic (MacCarty, 1964), being pH dependent. However, unionized ammonia inhibits methanogenesis at initial concentrations of 0.1–1.1 g N L^{-1}. In these assays, the ammonium varied between 53 and 827 mg NH_4^+–N L^{-1}, and the pH ranged from 7.23 to 7.56, therefore, despite the high nitrogen content of the macroalgae, no inhibitory levels were reached because of the high dilution used.

The biodegradability tests results (Table 3) indicate that *Ulva* sp. presents a slightly higher specific methane production in relation to *Gracilaria* sp., which in turn, was more biodegradable than *Enteromorpha* sp. The BMP obtained within this work was 196 ± 9 L CH_4 kg^{-1} VS for the *Ulva* sp., 182 ± 23 L CH_4 kg^{-1} VS for the *Gracilaria* sp., and 154 ± 7 L CH_4 kg^{-1} VS for the *Enteromorpha* sp., with 2.5% TS. However, no significant differences were observed in the assays at 1% and 5% TS, except an unexpected decrease in the specific methane yield in the assay with 1% TS of *Gracilaria* sp. These results suggest that no inhibitory levels were reached with the substrates concentrations and substrate to inoculum ratio tested. However, the methane production rate (k) was small for the assays with 5%TS probably due to the high substrate to inoculum ratio.

Although *Gracilaria* sp. contains a higher percentage of VS than *Ulva* sp., this later has higher carbohydrate content, which is favorable to methanation. According to Briand and Morand (1997), the macroalgae *Ulva* sp. is characterized by a low content of lignin and a high fraction of hemicellulose, being therefore, a suitable substrate for anaerobic digestion. Moreover, *Ulva* sp. provides cells with a thinner and simpler morphological structure, and a large surface area, making it easier to break down and digest compared to other macroalgae (Habig et al., 1984; Morand and Briand, 1999; Rigoni-Stern et al., 1990). The macroalgae *Enteromorpha* sp. had the lowest specific methane production, likely due to its lower VS content and higher refractory fraction. The methane yield (Table 3) was higher than 40% for *Ulva* sp. and *Gracilaria* sp., and around 32% for the *Enteromorpha* sp., confirming that less than 50% of the macroalgae energetic potentials are being availed and that the *Enteromorpha* sp. is the most difficult to digest.

In general, the $P_{Soluble}$ was approximately 5% higher than the methane yield (Table 3) and similar to the acidification percentage, indicating that most of the hydrolyzed material was converted to VFA. Then, almost all the VFA formed were converted to methane. The VFA analysis detected acetic, iso-butyric, and formic acids but at concentrations below 50 mg L^{-1} (data not shown). These results confirm that the methanogenesis and acidogenesis were not inhibited and the rate-limiting step of the overall AD process was the macroalgae hydrolysis. Therefore, methane yield can be improved applying pre-treatments methods (Bruhn et al., 2011;

Table 3
Experimental results obtained at the end of the macroalgae biodegradability assays.

Concentration		*Ulva* sp.			*Enteromorpha* sp.			*Gracilaria* sp.		
		1% TS	2.5% TS	5% TS	1% TS	2.5% TS	5% TS	1% TS	2.5% TS	5% TS
$VS_{waste}/VS_{inoculum}$		1.18	2.87	5.74	1.07	2.64	5.27	1.43	3.47	7.02
pH		7.41 ± 0.08	7.23 ± 0.03	7.23 ± 0.07	7.35 ± 0.17	7.54 ± 0.14	7.56 ± 0.05	7.41 ± 0.02	7.27 ± 0.06	7.53 ± 0.04
COD_s	g O_2 L^{-1}	0.27 ± 0.06	0.60 ± 0.09	3.52 ± 0.69	0.23 ± 0.04	0.98 ± 0.11	4.22 ± 1.19	0.27 ± 0.04	0.66 ± 0.03	1.43 ± 0.20
NH_4^+–N	mg L^{-1}	53 ± 65	340 ± 201	466 ± 84	121 ± 15	302 ± 115	611 ± 94	140 ± 4	528 ± 213	827 ± 152
P	mg COD-CH_4	175 ± 15	394 ± 31	606 ± 61	136 ± 17	282 ± 26	501 ± 50	167 ± 17	427 ± 49	740 ± 79
BMP	L CH_4 kg^{-1} VS	192 ± 2	196 ± 9	167 ± 13	153 ± 11	154 ± 7	148 ± 13	148 ± 5	182 ± 23	170 ± 16
Yield	%	43 ± 1	44 ± 2	38 ± 3	32 ± 2	32 ± 1	31 ± 3	38 ± 1	47 ± 6	44 ± 4
$P_{Soluble}$	%	48 ± 1	48 ± 2	49 ± 1	37 ± 2	39 ± 1	45 ± 4	43 ± 1	51 ± 6	50 ± 5
k	d^{-1}	0.058 ± 0.009	0.054 ± 0.006	0.034 ± 0.007	0.051 ± 0.006	0.049 ± 0.007	0.040 ± 0.006	0.076 ± 0.010	0.060 ± 0.008	0.047 ± 0.007

Nielsen and Heiske, 2011; Sialve et al., 2009) that increase the hydrolysis rate.

3.3. Macroalgae–sludge co-digestion

The cumulative methane production profiles during anaerobic co-digestion of *Ulva* sp. and mixed sludge are shown in Fig. 2, while Table 4 summarizes the results at the end of the biodegradability assays.

The BMP of the assays with 100% MS was 335 ± 27 L CH_4 kg^{-1} VS and in the assay with 100% U was 196 ± 9 L CH_4 kg^{-1} VS (Table 4). This difference is only due to the available organic matter (COD) in the vials. The methane yield was similar for all the assays, in the range 42–45%, indicating a similar degree of biodegradation of both substrates. A remarkable result concerns the faster production of methane during the first 15 days of experiment, even in the test with only 20% of MS, comparing with the 100% U test. The methane production rate increased significantly (2–3) times in the co-digestion assays, compared to the digestion of 100% U. The highest value of k was obtained in the co-digestion assay with 15% U and 85% MS (0.146 ± 0.037 d^{-1}).

This results indicate that the co-digestion of macroalgae with WAS seems an attractive choice, with a synergetic effect. The methane production rate of the co-digestion process, compared with the WAS digestion alone was increased 26% and the overall biodegradability was not negatively affected.

Besides the U and MS co-digestion, several tests were made changing the macroalgae species and cultivation method (U *vs.* GV), co-substrate (MS *vs.* PS or SS), and inoculum (anaerobic suspended sludge *vs.* anoxic marine sediment). The cumulative methane production profile is shown in Fig. 3 and the final results are presented in Table 5.

The BMP, of the "natural growth" macroalgae (*Ulva* sp.) and the "cultivated" macroalgae (*G. vermiculophyla*) is identical, 296 ± 19 and 294 ± 4 L CH_4 kg^{-1} VS. Also the $P_{Soluble}$ is very similar. The main difference between those macroalgae refers to the methane production rate, *i.e.*, the k in the assay with U is 1.7 times higher than in the assay with GV (Table 5). This difference is very important because it may represent, for instance, the difference between an anaerobic digester operating with a hydraulic retention time of 10 or 20 d, with all the increasing investment and operational costs that such difference involves. Therefore, the use of *Ulva* sp. seems economically more attractive.

Regarding the different sludge tested, the highest BMP was obtained with the PS, *i.e.*, 358 L CH_4 kg^{-1} VS (Table 5). This difference is explained by the higher theoretical methane potential of the PS, since the methane yield and $P_{Soluble}$ are very similar. As expected, the secondary sludge is less suitable for co-digest with macroalgae, with methane yield 10% smaller.

The last test consisted in the use of anoxic marine sediment instead of the digested anaerobic sludge. However, this alternative was not positive for the co-digestion of MS with the *Ulva* sp. since the parameters analyzed, *i.e.*, methane yield, BMP, and $P_{Soluble}$ were slightly lower than in the test with anaerobic sludge (Table 5). But

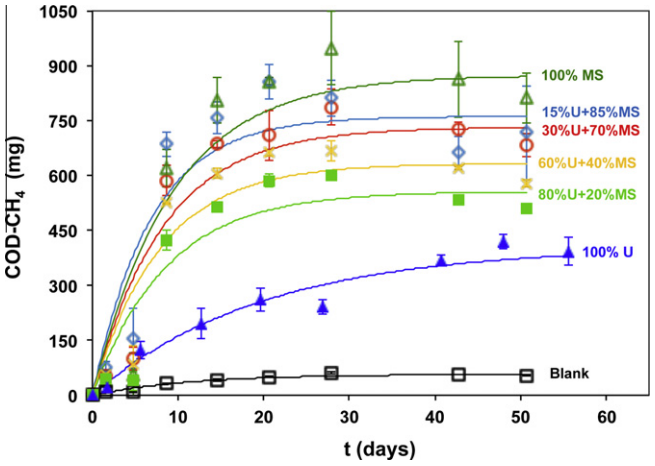

Fig. 2. Cumulative methane production (mg COD-CH_4) during anaerobic co-digestion tests with *Ulva* sp. (U) and mixed sludge (MS). Blank (□, R = 0.985), 100% U (▲, R = 0.962), 80% U + 20% MS (■, R = 0.990), 60% U + 40% MS (X, R = 0.992), 30% U + 70% MS (○, R = 0.991), 15% U + 85% MS (◇, R = 0.976), and 100% MS (△, R = 0.988). The symbols represent the experimental data with respective standard deviation (n = 3 points). The lines represent the predicted data by the exponential model.

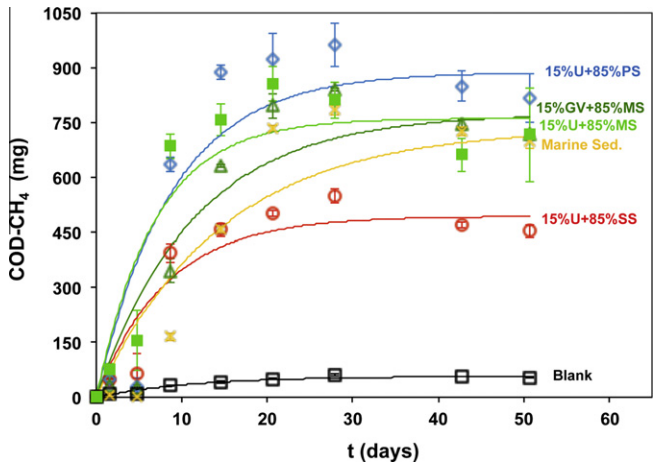

Fig. 3. Cumulative methane production (mg COD-CH_4) during anaerobic co-digestion tests with: Blank (□, R = 0.985), 15% U + 85% MS (■, R = 0.976), 15% GV + 85% MS (△, R = 0.993), 15% U + 85% PS (◇, R = 0.992), 15% U + 85% SS (○, R = 0.985), and 15% U + 85% MS and marine sediment as inoculum (X, R = 0.994). The symbols represent the experimental data with respective standard deviation (n = 3 points). The lines represent the predicted data by the exponential model.

Table 4
Experimental results obtained at the end of *Ulva* sp. (U) and mixed sludge (MS) co-digestion assays.

		100% MS	15% U + 85% MS	30% U + 70% MS	60% U + 40% MS	80% U + 20% MS	100% U
pH		7.50 ± 0.03	7.47 ± 0.06	7.46 ± 0.01	7.46 ± 0.01	7.47 ± 0.03	7.23 ± 0.03
COD_s	g O_2 L^{-1}	0.37 ± 0.01	0.48 ± 0.04	0.59 ± 0.12	0.96 ± 0.61	0.86 ± 0.07	0.60 ± 0.09
NH_4^+–N	mg L^{-1}	900 ± 80	1440 ± 490	650 ± 80	1180 ± 650	650 ± 60	340 ± 201
P	mg COD-CH_4	874 ± 94	761 ± 100	732 ± 55	632 ± 41	556 ± 41	406 ± 30
BMP	L CH_4 kg^{-1} VS	335 ± 27	296 ± 19	285 ± 19	257 ± 4	229 ± 3	196 ± 9
Yield	%	45 ± 4	42 ± 3	43 ± 3	44 ± 1	43 ± 1	44 ± 2
$P_{Soluble}$	%	46 ± 4	44 ± 3	45 ± 3	47 ± 2	47 ± 1	48 ± 2
k	d^{-1}	0.108 ± 0.027	0.146 ± 0.037	0.120 ± 0.026	0.127 ± 0.029	0.116 ± 0.028	0.051 ± 0.008

Table 5
Experimental results obtained at the end of macroalgae and sludge co-digestion assays.

Inoculum		Anaerobic digested sludge			Marine sediment	
		15% U + 85% MS	15% GV + 85% MS	15% U + 85% PS	15% U + 85% SS	15% U + 85% MS
pH		7.47 ± 0.06	7.49 ± 0.01	7.46 ± 0.01	7.59 ± 0.04	7.35 ± 0.03
COD_s	g O_2 L^{-1}	0.48 ± 0.04	1.34 ± 0.48	0.69 ± 0.32	0.37 ± 0.03	0.45 ± 0.22
NH_4^+–N	mg L^{-1}	1440 ± 490	860 ± 130	670 ± 110	890 ± 120	600 ± 30
P	mg COD-CH_4	761 ± 100	775 ± 52	888 ± 68	494 ± 40	735 ± 35
BMP	L CH_4 kg^{-1} VS	296 ± 19	294 ± 4	358 ± 2	170 ± 5	283 ± 5
Yield	%	42 ± 3	42 ± 1	43 ± 1	32 ± 1	40 ± 1
$P_{Soluble}$	%	44 ± 3	46 ± 2	45 ± 1	33 ± 1	42 ± 1
k	d^{-1}	0.146 ± 0.037	0.087 ± 0.022	0.113 ± 0.031	0.122 ± 0.028	0.068 ± 0.021

more important, the methane production rate was much lower and a lag phase of 5 d was observed. These differences may be explained by the fact that the rate-limiting step was found to be the hydrolysis. Therefore, in this context there is no real advantage in using more adapted/active inoculum.

Despite the high nitrogen content of macroalgae there was no ammonia inhibition in all co-digestion assays. It is also possible to verify by the results presented in Tables 4 and 5, *i.e.*, low soluble COD, small VFA concentration (data not show), and the similarity between the values of methane yield and $P_{Soluble}$, that the methanogenesis was not inhibited and, also in this case, the limiting step of the anaerobic digestion process was the hydrolysis.

From the point of view of a WWTP, it is possible to envisage macroalgae cultivation as an important post treatment method, where carbon dioxide and nutrients are used to the macroalgae growth, and macroalgae are subsequently used for energy production. The co-digestion of macroalgae with WAS (15% U; 85% MS) is feasible at a rate of methane production 26% higher than WAS alone. From the technical point of view, the research in the field of macroalgae AD suggests that a full-scale investment is possible, where energy and a bio-fertilizer are produced concomitantly with pollution mitigation.

4. Conclusions

The maximum specific methane production obtained was 196 ± 9 L CH_4 kg^{-1} VS of *Ulva* sp., at 2.5% TS. The limiting step of the overall anaerobic digestion process was the hydrolysis. The co-digestion of macroalgae with WAS (15% U; 85% MS) is feasible at a rate of methane production 26% higher than WAS alone without decreasing the overall biodegradability of the substrate (42–45% methane yield). From the technical point of view, the research in the field of macroalgae AD suggests that a full-scale investment is possible, where energy and a bio-fertilizer are produced concomitantly with pollution mitigation.

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