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Halophytes as Feedstock for Biogas Production: Composition Analysis and Biomethane Potential of *Salicornia* spp. Plant Material from Hydroponic and Seawater Irrigation Systems

Aadila Cayenne [1], Ariel E. Turcios [2], Mette Hedegaard Thomsen [3], Rui Miranda Rocha [4], Jutta Papenbrock [2,*] and Hinrich Uellendahl [1,*]

[1] Faculty of Mechanical and Process Engineering and Maritime Technologies, Flensburg University of Applied Sciences, Kanzleistr. 91-93, D-24943 Flensburg, Germany; aadila.cayenne@hs-flensburg.de

[2] Institute of Botany, Leibniz University Hannover, Herrenhäuserstr. 2, D-30419 Hannover, Germany; a.turcios@botanik.uni-hannover.de

[3] Department of Energy Technology, Aalborg University, Niels Bohrs Vej 8, 6700 Esbjerg, Denmark; mht@energy.aau.dk

[4] Riasearch, Lda., Cais da Ribeira de Pardelhas nº 21, 3870-168 Murtosa, Portugal; ruirocha@riasearch.pt

[*] Correspondence: papenbrock@botanik.uni-hannover.de (J.P.); hinrich.uellendahl@hs-flensburg.de (H.U.); Tel.: +49-511-762-3788 (J.P.); +49-461-805-1293 (H.U.)

Abstract: The halophyte plant species *Salicornia europaea* and *Salicornia ramosissima* were investigated for their potential to serve as a substrate for biogas production. *Salicornia europaea* was cultivated in hydroponic systems under varying salt concentrations (0, 10, 20, and 30 g/L NaCl), while *S. ramosissima* was grown in greenhouse farming with aquaculture effluent irrigation. The biomethane potential of the two halophyte feedstocks was determined through batch experiments, and correlations to the plant biochemical composition were investigated. Ash and mineral content of *S. europaea* was correlated to the increasing salt concentration used for plant cultivation in hydroponic systems. No indication of inhibition of the anaerobic digestion process was detected for sodium concentrations of up to 2400 mg/L in the anaerobic batch-test assays. The highest biomethane yield of *S. europaea* of 250 mL CH_4/gVS was obtained when grown under 20 g/L NaCl and up to 300 mL CH_4/gVS for *S. ramosissima*. By concentrating the dry matter content, the biomethane yield per ton of feedstock could be increased from 24 m^3 CH_4/t of the fresh halophyte plant to 74 m^3 CH_4/t by fractionation into a pulp fraction and to 149 m^3 CH_4/t by drying of the plant at room temperature for 1 week.

Keywords: biomethane potential; biodegradability; anaerobic digestion; halophyte biomass

1. Introduction

Halophytes have the ability to thrive optimally in high salt conditions and possibly irrigate using saline water by sustaining favorable biomass or seed yields and eventually assuring food security in land areas affected by salinity. For this reason, halophytes and their residues from food production are potential candidates also as bioenergy crops for saline environments that, due to their perennial life cycle, permit harvesting for many years without reseeding and maintaining high biomass yields with relatively low lignocellulosic content [1–3]. According to the findings of Glen et al. [4], members of the *Salicornia* genus can be cultivated for food, fodder/forage, medicinal, and as oilseed crops, with the most productive species yielding 13 to 25 tons/ha of dry matter using seawater irrigation and on average 2 tons/ha of seeds over a 200-day developing cycle, comparable to conventional crops. Therefore, based on the extent of their diversity, salt-tolerant plant species can be considered a valuable feedstock for the production of bioenergy through anaerobic digestion (AD).

Anaerobic digestion of complex organic matter encompasses a sequence of conversion processes, from hydrolysis to methanogenesis, that is carried out by a consortium of

microorganisms that operate in synergy to produce methane (CH_4) and carbon dioxide (CO_2). The accumulation of intermediate products such as volatile fatty acids (VFA) depends largely on the balance of the activity of the different microbial groups, while the formation of other compounds such as hydrogen sulfide (H_2S), ammonia, depends on, e.g., the sulfur and protein content of the biomass [1,5]. The AD process is firmly established for the conversion of organic waste such as agricultural residues, food waste, and animal slurry and of energy crops such as cereals, maize, and clover and their residues and other organic materials into biogas/biomethane [6–8]. However, limited research has been carried out on AD of halophytic biomass for biogas production.

One unambiguous challenge encountered in the AD of halophytic biomass is associated with the high salinity within the cells and tissues of the biomass as well as of the saltwater attached to the plant material. More specifically, the potential and possible inhibitory effect of halophyte biomass as a substrate for the AD process is dependent on the content of carbohydrates, proteins, fats and lignin, and salt, namely the salts of sodium (Na), potassium (K), calcium (Ca), magnesium (Mg) and phosphorous (P) [2,9]. According to literature, the main inhibiting element, Na, of the light earth metals has been reported to cause strong inhibition of the methanogens at mesophilic conditions at concentrations of 8000 mg/L of anaerobic medium [10–12]. Likewise, the presence of some secondary metabolites in biomass feedstocks can potentially hinder the activity of biomass-degrading microbes, to differing degrees, during the AD process [13,14]. As reported in previous studies, halophytic plant material contains a variety of phytochemicals [15–17], in particular phenolic compounds, which are considered antimicrobial and can probably cause inhibition in the production of biomethane [10]. Nevertheless, the composition of halophytic biomass differs significantly based on the genotype, specific species, life cycle status, mode of cultivation (whether using a hydroponic system, greenhouse seawater irrigation system, or naturally grown), and climate conditions [1].

The aim of this study was to determine the biomethane potential (BMP) of the halophyte plant material and to investigate correlations of the BMP to the plant composition. In order to identify thresholds for salt inhibition and the influence of other plant compounds, the experimental BMP values are compared to the theoretical biomethane potential (TBMP) derived from elemental analysis and are correlated to the ash, sodium, and lignin content of the halophyte plant material. Based on the thresholds for salt inhibition, the maximum admixing ratios of halophyte plant material in co-digestion with other non-saline biomass such as manure or sewage sludge are calculated.

2. Materials and Methods

2.1. Halophyte Plant Material and Residues Used for the Biogas Potential Tests

The halophytic biomass used in this study was the above-ground plant part, collected from different cultivation conditions and under varying saline conditions.

Salicornia europaea was cultivated in hydroponic systems at the Institute of Botany, Leibniz University Hannover (LUH), Germany, with variations in NaCl concentrations (10, 20, 30 g/L NaCl) in the nutrient solution as well as under non-saline conditions. Each experimental unit consisted of eight plants per container, with three replicates per treatment. Polypropylene containers (L400 × W300 × H175 mm) with a capacity of 16 L were used. Each container had 13 L of modified Hoagland solution, containing 606 mg/L KNO_3, 944 mg/L $Ca(NO_3)_2 \cdot 4H_2O$, 230 mg/L $NH_4H_2PO_4$, 246 mg/L $MgSO_4 \cdot 7H_2O$, 3.73 mg/L KCl, 1.55 mg/L H_3BO_3, 0.34 mg/L $MnSO_4 \cdot H_2O$, 0.58 mg/L $ZnSO_4 \cdot 7H_2O$, 0.12 mg/L $CuSO_4 \cdot 5H_2O$, 0.12 mg/L $MoNa_2O_4 \cdot 2H_2O$, and 9.16 mg/L Fe-EDDHA (0.56 mg Fe/L). The water was aerated constantly by small compressors and one air stone in the middle of each tank (Eheim, Deizisau, Germany). The hypocotyl was fixed with soft foam in 35 mm holes. The water level was adjusted constantly in each tank with tap water to compensate the evapotranspiration. The halophytes were cultivated in a greenhouse at temperatures of 35/21 °C during day/night. A total of 14 h of artificial light was provided (sodium vapor lamps, SON-T Agro 400W, Philips, Amsterdam, The Netherland). Light intensity ranged

from 65 µmol/m^2 s to 740 µmol/m^2 s depending on the season, the time of the day, and the weather conditions. The plants were harvested after 5 weeks in the hydroponic cultures. To avoid further modification of the biomass composition and to facilitate the transport and storage of the biomass, the plant material was dried at 60 °C for 48 h prior to composition and BMP analysis. For scaling-up experiments, these drying conditions can be used as the reference standard conditions. However, it is important to note that both dry and fresh biomass can be used for anaerobic digestion.

Salicornia ramosissima was cultivated in greenhouse conditions, under natural photoperiod, at Riasearch (RSR) facilities in Murtosa, Portugal. Prior to germination of locally harvested seeds, freshwater was used to maintain the soil moisture. The seeds were sown in a 2 g/m^2 density and irrigated twice daily utilizing a mixture of the recirculating aquaculture system (RAS) effluents, with salinity around 18 g/L NaCl, and freshwater, in order to keep soil salinity around 12 g/L NaCl. The aquaculture effluents were from shrimp (*Penaeus vannamei*) and European seabass (*Dicentrarchus labrax*) cultivation. After a growth phase of approximately 5 months, *S. ramosissma* biomass was harvested after it began to flower. The estimated green biomass production was around 1.85 kg/m^2 per year. The fresh green succulent plants were harvested, and the roots discarded. After using the green tips for food production, the plants were divided in two groups. The whole green plants were packed in styrofoam boxes with cold packs and shipped for approximately 48 h to Flensburg University of Applied Sciences (FUAS) for composition and BMP analysis. At FUAS, a fraction of the *S. ramosissima* whole green plant samples was separated into a pulp and a juice fraction by wet-pressing in a juice extractor (Green Star GS-2000, Tribest, Anaheim, California, USA). In Portugal, the mature plants were dried on mesh trays in the shade, for 1 week, at room temperature (min. 18 °C, max. 28 °C), simulating the large-scale drying in a greenhouse without additional heat supply. The plants were moved on the trays daily in order to prevent the development of fungus and to accelerate the drying process. The dried mature plants were packed in styrofoam boxes and shipped at room temperature to FUAS.

2.2. Biomass Composition Analysis

The total solids (TS), volatile solids (VS), and ash content of the halophyte plant biomass were determined according to standard methods DIN EN 12880 and DIN EN 12879, respectively [18,19]. The TS content of the substrates was determined by drying at 105 °C until constant weight (approximately 24 h). The VS content was measured by subsequent incineration of the TS at 550 °C for 3 h in a muffle furnace (M104, Thermo Fisher Scientific Corporation, Waltham, MA, USA).

2.2.1. Content of Carbohydrates, Lignin, and Proteins

Carbohydrates and Klason lignin (acid-insoluble lignin) of the substrates were analyzed according to the standard protocols developed by the National Renewable Energy Laboratory (NREL) [20]. The dried (>85% TS), milled and sieved (1 mm screen) plant material was subjected to strong acid hydrolysis (SAH) using 72 w/w% sulfuric acid (H_2SO_4) for 1 h at 30 °C, followed by diluting the acid to 4% and autoclavation at 121 °C for 1 h. A mixture of glucose, xylose, and arabinose was used as spike solution. The hydrolysate from SAH was filtered, and Klason lignin content was measured as the difference between the ash content and weight of insoluble residue. The monomeric sugars glucose, xylose, and arabinose released in the hydrolysate were analyzed using a Hitachi Chromaster High-Performance Liquid Chromatography (HPLC, Hitachi High-Tech Corporation, Tokio, Japan), mounted with a Macherey Nagel (Düren, Germany) Nucleogel Sugar 810 H column and reflective index (RI) detector at 63 °C using 5 mM H_2SO_4 as the eluent (mobile phase) with a 0.4 mL/min flowrate.

To analyze for easily dissolvable sugars in the liquid fraction after fractionation of *S. ramosissima* the liquid sample was subjected to weak acid hydrolysis (WAH) by digesting

with 8 $w/w\%$ H_2SO_4 at 121 °C with subsequent analysis of the three monomeric sugars using HPLC.

Crude protein content was determined based on the nitrogen content by a nitrogen-to-protein conversion factor of 6.25.

2.2.2. Elemental Analysis

The elemental analysis of carbon (C), hydrogen (H), nitrogen (N), and sulfur (S) contents was performed using a CHNS-O Thermo Scientific FlashSmart elemental analyzer with BBOT (2,5-Bis(5-tert-butyl-benzoxazol-2-yl)thiophene) as standard. The oxygen (O) content was determined by assuming C + H + N + S + O + ash = 99.5% (on TS basis) [21–23].
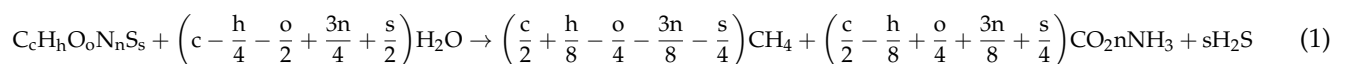
2.2.3. Mineral Content in Substrates and Inoculum

The mineral content of the plant material with respect to Na, K, Ca, Mg, and P was analyzed by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) (iCAP 6000 ICP Spectrometer, Thermo Fisher Scientific Corporation, Waltham, MA, USA), and the procedure was carried out as described by Weese et al. [24]. Briefly, about 38 mg of fine powder from each sample was incinerated for 8 h in a muffle furnace (M104, Thermo Fisher Scientific Corporation, Waltham, MA, USA). After cooling the samples to room temperature, 1.5 mL of nitric acid (diluted 1:3, Rotipuran® Supra 69%) was added. After 10 min, 13.5 mL of ultrapure water was added. The solution was filtered (0.45 µm pore size, Carl Roth) and stored in vials at 4 °C before final analysis. An empty vial was treated in parallel with the samples and later used as a blank. The mineral content of juice fraction of *S. ramosissima* was calculated based on the TS content as a difference between the whole plant and pulp fraction post fractionation.

2.3. Theoretical BMP and Biodegradability

The TBMP was calculated based on the elemental composition C, H, O, N, and S of the halophyte plant material and on the extended reaction equation Equations (1) and (2) proposed by Boyle [25]. This equation includes nitrogen and sulfur to calculate the production of ammonia and hydrogen sulfide. The general chemical formula $C_cH_hO_oN_nS_s$ was determined from the elemental analysis. The TBMP was hinged on the subsequent assumptions [26]:

- optimal microbial condition, constant temperature, and ideal mixing, leading to complete anaerobic degradation of the organic matter content (no differentiation of biodegradable and non-biodegradable compounds);
- input substrate organic matter consists only of the elements C, H, O, N and S, and gaseous products resulting from anaerobic digestion are CH_4, CO_2, NH_3, and H_2S.

$$C_cH_hO_oN_nS_s + \left(c - \frac{h}{4} - \frac{o}{2} + \frac{3n}{4} + \frac{s}{2}\right)H_2O \rightarrow \left(\frac{c}{2} + \frac{h}{8} - \frac{o}{4} - \frac{3n}{8} - \frac{s}{4}\right)CH_4 + \left(\frac{c}{2} - \frac{h}{8} + \frac{o}{4} + \frac{3n}{8} + \frac{s}{4}\right)CO_2 nNH_3 + sH_2S \quad (1)$$

$$\text{TBMP (mL } CH_4/gVS) = \frac{22400\left(\frac{c}{2} + \frac{h}{8} - \frac{o}{4} - \frac{3n}{8} - \frac{s}{4}\right)}{12c + h + 16o + 14n + 32s} \quad (2)$$

The anaerobic biodegradability (BD_{CHONS}) was determined by the ratio of the cumulative methane yield from experimental BMP batch assay to TBMP, as shown in Equation (3).

$$BD_{CHONS}(\%) = \frac{BMP}{TBMP} \times 100 \quad (3)$$

2.4. Experimental Biomethane Potential (BMP)

The BMP of the different halophyte samples was determined using the Automated Methane Potential Test System II (AMPTS II, Bioprocess Control Sytems, Lund, Sweden). The AMPTS II is equipped with pressure and temperature sensors that are used to normalize the produced gas volume at standard temperature and pressure (STP). All batch fermentation assays were conducted in 500 mL flasks in triplicates under mesophilic conditions 37 ± 2 °C according to German Standard, VDI 4630 using an inoculum to substrate ratio (ISR) ≥ 2 (on VS basis) and a TS concentration in the batch assay of ≤10% to ensure sufficient mass transfer [27].

Inoculum for the biomethane potential (BMP) tests was sludge taken from an anaerobic digester of the Wastewater Treatment Plant (WWTP) in Flensburg, Germany, operating at mesophilic conditions (38–42 °C). The fresh inoculum was incubated at 37 ± 2 °C for up to 5 days to minimize the production of background gas and influence of the blanks prior to inoculation. The TS and VS concentration of the inoculum was 2.0 wt% (weight percent) TS and 60 wt% VS of TS, respectively.

The batch tests were operated until the daily gas production was less than 1 vol% of the accumulated gas production on three consecutive days. Since the inoculum also contains biodegradable material, the gas yield of blank samples only containing inoculum was deducted from the gas produced from the batches containing substrate and inoculum. Microcrystalline cellulose was used as a reference to evaluate inoculum activity and its ability to degrade cellulosic polymers.

3. Results and Discussion

3.1. Salt Content of Halophyte Plant Material

The TS, VS, and ash content of the different *Salicornia* samples used in this study are shown in Table 1. The results of the ash content show that the content of inorganics is relatively high for halophytes, ranging from 22 wt% to 52 wt% of TS in the plant materials, similar to the findings for halophytic plants [17,28–31]. After green fractionation of the halophyte plants, a major part of the minerals is found in the juice fraction, resulting in ash content of 58 wt% of TS in the juice, while the ash content in the separated fibers is only 12 wt% of TS.

Table 1. TS, VS and ash content of *Salicornia* samples in wt% of fresh mass (FM) and wt% of TS, standard deviation of triplicates in parentheses. For *Salicornia europaea* the biomass values after drying of the fresh plant material are given.

Halophyte Species	TS	VS	VS	Ash Content
	wt% FM	wt% FM	wt% TS	wt% TS
Salicornia europaea				
0 g/L NaCl	97.2 (0.02)	76.1 (4.09)	78.3 (4.2)	21.7 (4.2)
10 g/L NaCl	98.2 (0.1)	58.7 (1.46)	59.8 (1.49)	40.2 (1.49)
20 g/L NaCl	98.6 (0.14)	49.3 (1.3)	50.0 (1.36)	50.0 (1.36)
30 g/L NaCl	98.7 (0.18)	48.0 (2.97)	48.6 (3.10)	51.4 (3.1)
Salicornia ramosissima				
Whole (green) plant	11.8 (0.55)	8.1 (0.39)	68.6 (0.31)	31.4 (0.31)
Pulp fraction	30.1 (1.45)	26.6 (1.47)	88.2 (0.65)	11.8 (0.65)
Juice fraction	7.1 (0.03)	3.0 (0.03)	41.9 (0.30)	58.1 (0.30)
Dry mature plant	69.6 (0.9)	49.8 (0.94)	71.5 (0.85)	28.5 (0.85)

For the plant samples of *S. europaea* cultivated at different NaCl concentrations, the ash content increases for the halophytes cultivated at 10 and 20 g/L NaCl, but not as much for higher NaCl concentrations (Table 1).

The same tendency can be seen for the Na content measured in the plant tissue of *S. europaea* (Table 2), which increases from 68.0 mg/gTS under non-saline conditions to 190 mg/gTS for 20 g/L NaCl, but no further for 30 g/L NaCl in the culture medium. This

indicates that higher NaCl concentration in the culture medium leads to a higher NaCl uptake in the plant tissue, only up to 20 g/L NaCl in the culture medium, which shows that the plant is able to regulate its salinity level to maintain a certain salt concentration in their cells and tissues to sustain its water potential gradient and osmotic pressure required for water intake [1].

Table 2. Mineral content of *Salicornia* samples in mg/gTS, standard deviation of triplicates in parentheses.

Halophyte Species	Na	K	Ca	Mg	P
	mg/gTS	mg/gTS	mg/gTS	mg/gTS	mg/gTS
Salicornia europaea					
0 g/L NaCl	68.0 (0.27)	36.0 (0.28)	36.0 (0.21)	2.1 (0.01)	4.7 (0.06)
10 g/L NaCl	126 (0.87)	24.0 (0.03)	23.1 (0.10)	1.7 (0.01)	4.6 (0.13)
20 g/L NaCl	190 (0.85)	15.8 (0.07)	5.3 (0.03)	0.8 (0.01)	3.1 (0.03)
30 g/L NaCl	191 (7.16)	15.7 (0.17)	4.5 (0.04)	0.7 (0.01)	2.9 (0.02)
Salicornia ramosissima					
Whole (green) plant	118 (2.42)	13.5 (0.11)	6.7 (0.03)	1.9 (0.01)	4.4 (0.08)
Pulp fraction	46.2 (0.93)	5.0 (0.08)	7.5 (0.04)	0.9 (0.01)	2.4 (0.04)
Juice fraction	197 *	22.7 *	5.9 *	3.0 *	6.5 *
Dry mature plant	60.5 (0.23)	27.8 (0.05)	3.8 (0.01)	1.5 (0.01)	4.8 (0.02)

* calculated based on fractionation of whole plant into pulp and juice fraction.

The concentrations of K, Ca, Mg, and P, on the other hand, decreased with the higher NaCl concentration used for cultivation (Table 2), indicating that the higher osmotic pressure is counterbalanced by a lower uptake of these ions. In this research, the increasing content of Na in the cultivating conditions of *S. europaea* and plant material could have caused low uptake of K owing to the antagonism and synergism between the elements [1,32]. Likewise, the Ca content was significantly suppressed at salinity higher than 10 g/L NaCl cultivating condition.

The content of Na, Mg, and P content of *S. ramosissima* cultivated in a greenhouse equipped with seawater irrigation was similar to the respective concentrations of *S. europaea* cultivated in the hydroponic system at varying salt concentrations. The concentrations of K and Ca were, however, much lower in *S. ramosissima*. Applying fractionation of the fresh succulent material into juice and pulp fraction showed that the mineral content in the pulp fraction was except for Ca, less than 50% of the content in the whole green plant. Furthermore, the majority of the mineral elements are left in the juice fraction post fractionation pretreatment. The mass balance and the separation of the different minerals, C, N, S, and lignin, are shown in Figure 1. Compared to the fractionation of red clover and clover grass by Santamaria-Fernandez et al. [33], the separation of C and N was very similar to the fractionation of red clover (60% and 53% in pulp fraction) while after fractionation of red clover the percentage of total mass, K and P was much higher in the pulp fraction (40%, 43%, and 53%) and of S much lower (52% in pulp fraction).

In the dry mature *S. ramosissima* plant residues, the concentration of Na and Ca was 50% lower compared to the whole fresh plant, while the K concentration was about twice, indicating an increased uptake of K at this plant growth stage [32].
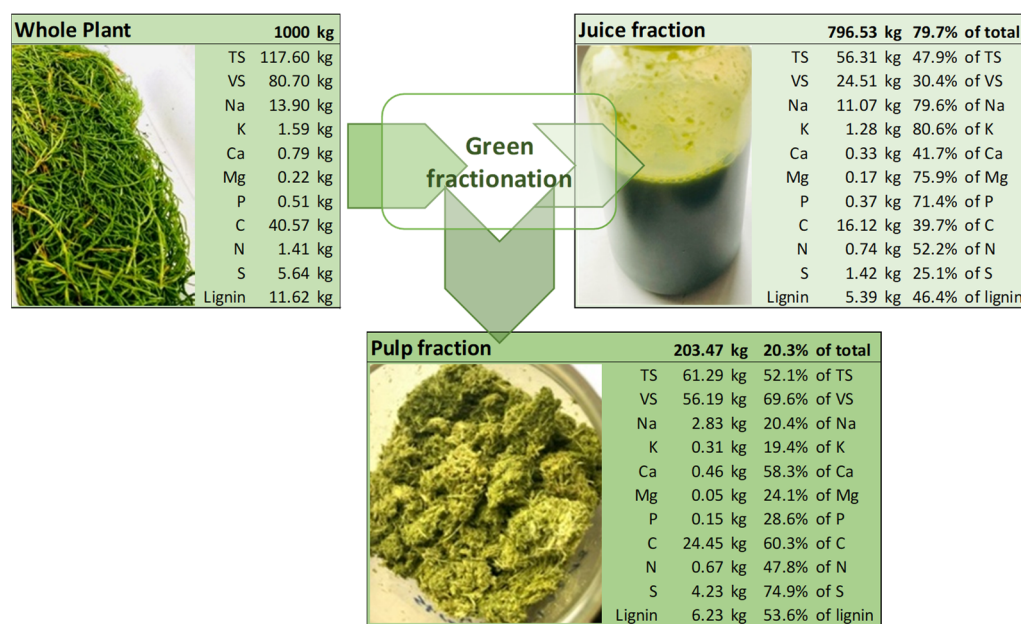
Figure 1. Mass balance of green fractionation of *S. ramosissima* whole plant biomass into pulp and juice fraction in mass per 1000 kg of whole plant input and % of mass found in juice and pulp fraction.

3.2. Content of Carbohydrates, Lignin and Proteins

The content of total sugars, acid-insoluble lignin, and proteins in the samples is shown in Table 3. The total sugars are the sum of the monomeric sugars analyzed by strong acid hydrolysis, i.e., glucose, xylose, and arabinose, while the protein content is based on the N content determined by the elemental analysis (Table 4). Of the three analyzed sugars, based on the total sugar content, 57% glucose, 33% xylose, and 10% arabinose were generally present in all Salicornia samples, which is in accordance with the analyses of Salicornia bigelovii [17,31,34]. The carbohydrate and lignin content of *S. europaea* was lower, while the protein content was higher compared to the *S. ramosissima* samples. The low sugar content measured in the *S. europaea* samples is not consistent with the content of organic matter in the dry matter of 49–79 wt% VS of TS (Table 1). This may indicate either the presence of another monomeric sugar that was not analyzed or a general deviation of the strong acid hydrolysis analysis of samples with high ash content.

Table 3. Total sugars, lignin, and protein content of *Salicornia* samples in g/100 gTS, standard deviation of triplicates in parentheses.

Halophyte Species	Total Sugars	Klason Lignin	Crude Protein
	g/100 gTS	g/100 gTS	g/100 gTS
Salicornia europaea			
0 g/L NaCl	9.8 (0.21)	4.6 (0.53)	26.9
10 g/L NaCl	10.6 (0.54)	7.9 (0.31)	24.0
20 g/L NaCl	12.9 (0.61)	6.1 (0.51)	18.2
30 g/L NaCl	8.7 (0.43)	6.7 (0.25)	18.9
Salicornia ramosissima			
Whole (green) plant	45.1 (5.89)	9.9 (0.54)	7.2
Pulp fraction	58.6 (3.89)	10.2 (0.32)	7.0
Juice fraction	14.1 (0.21)	9.6 *	nd
Dry mature plant	45.9 (2.78)	15.3 (0.46)	5.5

* calculated based on fractionation of whole plant into pulp and juice fraction; nd = no data.

Table 4. Elemental analysis, C/N ratio and overall composition of *Salicornia* samples, standard deviation of triplicates in parentheses.

Halophyte Species	C	H	N	O	S	C/N Ratio	Elemental Composition
			wt% TS			(*wt/wt*)	$C_cH_hN_nS_sO_o$
Salicornia europaea							
0 g/L NaCl	29.3 (0.8)	4.3 (0.1)	4.3 (0.03)	33.1 (0.9)	5.6 (0.1)	6.8	$C_{14}H_{25}N_2S_1O_1$
10 g/L NaCl	28.1 (0.4)	3.8 (0.1)	3.8 (0.1)	18.0 (0.5)	5.1 (0.1)	7.3	$C_{15}H_{24}N_2S_1O_7$
20 g/L NaCl	22.0 (1.4)	2.9 (0.2)	2.9 (0.1)	21.9 (0.9)	3.9 (0.1)	7.6	$C_{15}H_{24}N_2S_1O_{11}$
30 g/L NaCl	20.9 (0.3)	2.7 (0.1)	3.0 (0.04)	20.1 (0.2)	3.8 (0.3)	6.9	$C_{15}H_{23}N_2S_1O_{11}$
Salicornia ramosissima							
Whole (green) plant	34.5 (0.5)	4.1 (0.1)	1.2 (0.1)	23.6 (0.6)	4.8 (0.1)	30.0	$C_{35}H_{50}N_1S_2O_{18}$
Pulp fraction	39.9 (0.2)	5.5 (0.1)	1.1 (0.02)	34.3 (0.2)	6.9 (0.1)	35.6	$C_{42}H_{69}N_1S_3O_{27}$
Dry mature plant	36.5 (0.2)	5.1 (0.03)	0.9 (0.04)	22.1 (0.4)	6.5 (0.1)	41.4	$C_{39}H_{63}N_1S_3O_{22}$

The difference in protein content between the two Salicornia species may be due to the difference in the growth stage at the time of harvesting. *Salicornia ramosissima* was harvested at the maturity stage with a higher lignification and, therefore, higher lignin and lower protein content. In contrast, *S. europaea* was harvested in the vegetative growth phase, not yet lignified with a highly active metabolism and, therefore, higher protein content.

For *S. ramosissima*, the sugar content was highest in the pulp fraction and similar in the dry mature plant and the whole green plant. The lignin content was higher in the dry, mature plant compared to all other samples, indicating that lignification increases as the plant matures. For *S. europaea* cultivated at different salinity, the sugar and lignin content generally increased while the protein content decreased with increasing NaCl concentration used for hydroponic cultivation. This inverse relationship between salinity and protein content of halophytic biomass was previously confirmed for Tripolium pannonicum and Chenopodium quinoa [29,30].

3.3. Elemental Composition of Organic Matter

The results of the elemental analysis of C, H, N, O, and S were used to derive the overall composition formula of the organic matter (VS) of the halophyte material and the C/N ratio (Table 4). The TBMP of the different halophyte samples calculated from the composition formula can be seen in Table 5. Furthermore, the sum of the elements C, H, N, O, and S reflects the wt% VS of TS, as shown in Table 1, of the substrates.

Table 5. Experimental BMP, TBMP and BD_{CHONS} of *Salicornia* samples in mL CH_4/gVS, m^3 CH_4/tFM and %, standard deviation of triplicates in parentheses.

Halophyte Species	BMP	TBMP	BMP	BD_{CHONS}
	mL CH_4/gVS	mL CH_4/gVS	m^3 CH_4/tFM	%
Salicornia europaea				
0 g/L NaCl	215 (6.3)	316	164	68
10 g/L NaCl	218 (9.8)	466	128	47
20 g/L NaCl	248 (14.3)	347	122	71
30 g/L NaCl	226 (6.3)	348	109	65
Salicornia ramosissima				
Whole (green) plant	293 (6.7)	497	23.6	62
Pulp fraction	280 (12.2)	442	74.4	63
Juice fraction	265 (8.3)	nd	7.8	nd
Dry mature plant	299 (13.8)	547	149	55

nd = no data.

The C content of *S. europaea* (20–30 wt% of TS) was much lower than for *S. ramosissima* (34–40 wt% of TS), which is in accordance with the lower total sugars measured (Table 3), but in contrast with the similar concentration of VS of TS measured for the two Salicornia species (Table 1).

Due to the significant difference in C and N content of the two Salicornia species, the C/N ratio of *S. europaea* (7–8) was much lower than for *S. ramosissima* (30–41), probably due to harvesting at different physiological stages, *S. europaea* at a relatively early stage and *S. ramosissima* at a mature stage, rather than dependent on characteristics of the different species or growing conditions. According to the findings of Amon et al. [35], the optimum C/N range for a stable AD process is between 10–30, which was found to be below the minimum for *S. europaea* and above the maximum for *S. ramosissima*. Therefore, AD of *S. europaea* can result in a high release of ammonia, which may hinder the activity of methanogenic bacteria leading to the accumulation of VFA. On the contrary, utilizing *S. ramosissima* will eventually cause N deficiency in the entire microbial community, which may lead to decreasing methane production when digested as mono-substrate, based on reported research [35,36]. Consequently, to achieve an optimal C/N ratio, co-digestion is suggested with a carbon-rich substrate such as grass or straw for *S. europaea* and for *S. ramosissima* with a protein-rich substrate such as cow manure or lipid-rich sources. Likewise, Chen et al. [37] showed that co-digesting the halophyte Spartina alterniflora with cow manure lowered the C/N ratio, which ultimately improved degradation with lower VFA content and higher biomethane yields of 7–44%.

The results for S in Table 4 show that the sulfur content of *S. ramosissima* ranges between 5–7% TS, whereas for *S. europaea* 4–6% TS. The sulfur concentration of the *S. europaea* samples was lower with the increasing salinity of the culture medium. Compared to the composition analysis of red clover and clover grass by Santamaria-Fernandez et al. [33], the sulfur concentration per kgTS was 25 to 45 times higher in the halophyte samples. This difference in S content should be considered for the selection of feedstock for biogas production since anaerobic degradation of S-containing compounds leads to the formation of H_2S with its toxic and corrosive effects.

3.4. Biomethane Potential of Halophytic Substrates

The course of the BMP batch assays is shown for *S. europaea* in Figure 2 and for *S. ramosissima* in Figure 3. Table 5 shows the final methane yields of all samples compared to the calculated TBMP based on the composition analysis.

The course of the experimental BMP assay shows a relatively rapid degradation for all *S. europaea* samples with a digestion duration of less than 20 d (Figure 2). For *S. ramosissima*, the degradation generally takes longer, especially for the dry mature plant material, indicating a higher content of less degradable organic matter (Figure 3).

The final methane yields of *S. ramosissima* (280–299 mL CH_4/gVS) were, however, higher than the BMP of *S. europaea*, which were in the range of 200–250 mL CH_4/gVS. The biodegradability values are accordingly higher for *S. ramosissima* compared to *S. europaea*. The highest final yield for *S. europaea* of 248 mL CH_4/gVS was found for the plant cultivated at 20 g/L NaCl.

The similar methane yield of the pulp fraction compared to the whole green plant of *S. ramosissima* indicates a similar degradability of the pulp fraction. However, the longer digestion time of more than 30 d indicates a slower hydrolysis process due to the lignocellulosic content (Figure 3).

Compared to methane yields of non-halophyte biomass, the measured values are lower than of green grass biomass and similar to the yields of lignocellulosic biomass. Previous literature reported an average specific methane yield of 250–450 mL CH_4/gVS for maize silage, 200–250 mL CH_4/gVS for straw, and 300–450 mL CH_4/gVS for grass [7,8].
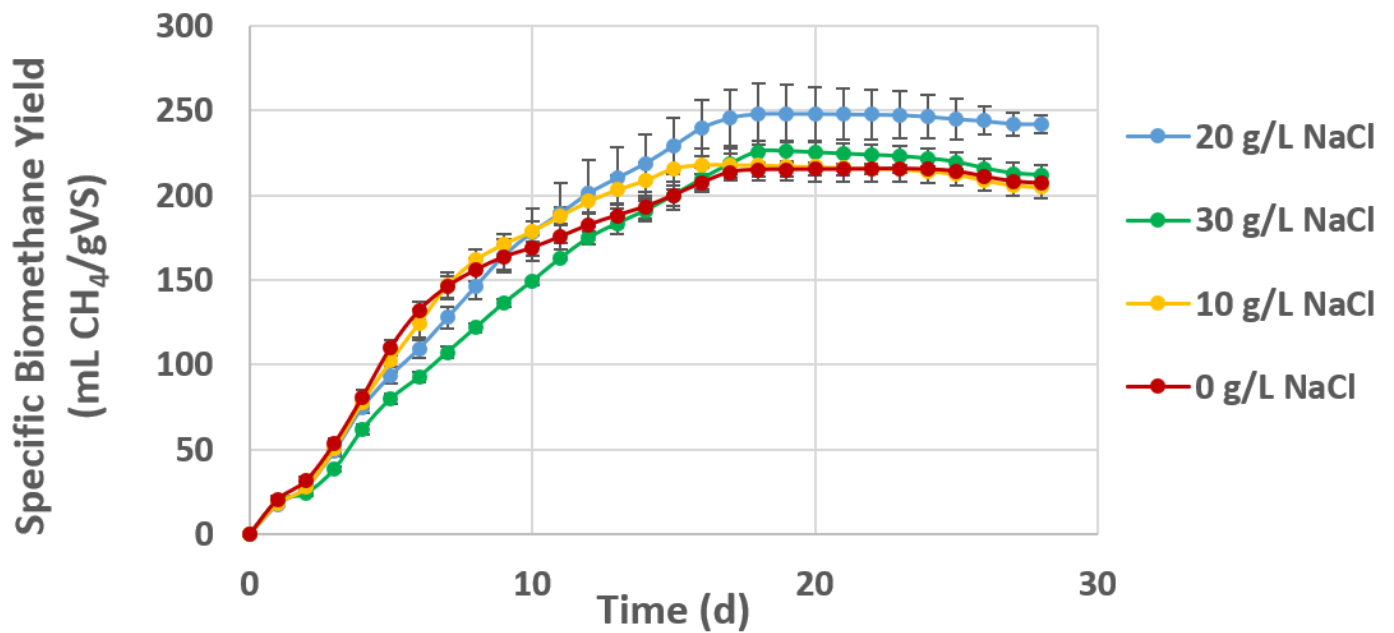
Figure 2. Cumulative BMP of *S. europaea* cultivated under hydroponic conditions at different salinity concentrations (0, 10, 20 and 30 g/L NaCl). Values of each curve represent the average biomethane yield and standard deviation of triplicate BMP batch assays.
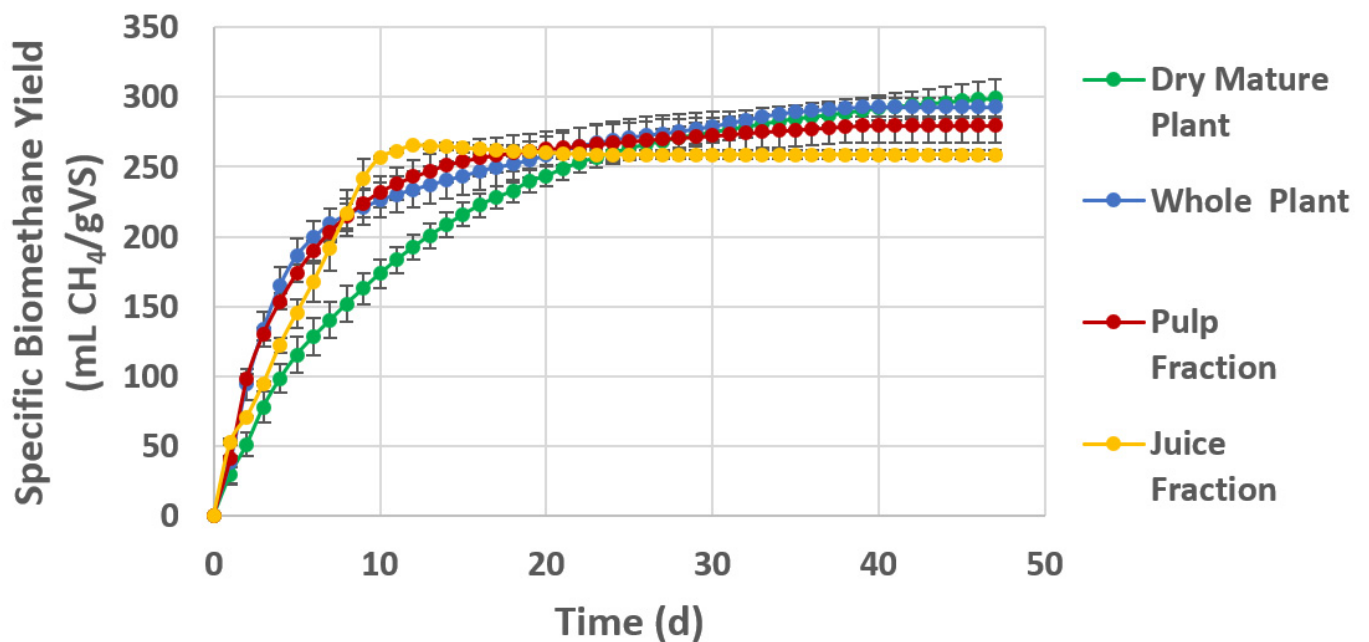

Figure 3. Cumulative BMP of *S. ramosissima* cultivated through saline water irrigated greenhouse farming as whole green plant, dry mature plant and pulp and liquid fraction after fractionation. Values of each curve represent the average biomethane yield and standard deviation of triplicate BMP batch assays.

The biomethane yields per ton of FM of the halophyte feedstock were 110–164 m^3 CH_4/tFM for the dried samples of *S. europaea* and 23.6, 74.4, 7.8, and 149 m^3 CH_4/tFM for the whole green plant, pulp fraction, juice fraction and dry mature plant of *S. ramosissima*, respectively. This indicates that drying and fractionation of a pulp fraction of the halophyte plant material increase the methane yield per ton by a factor of 3–7. For the different dried samples of *S. europaea*, it can be seen that the higher ash content of the samples cultivated

at higher NaCl concentrations leads to a lower methane yield per ton of the feedstock even though the specific methane yield per gVS is higher.

The lower values of BMP can be due to the recalcitrance nature of lignin present in the substrates as this composite structural constituent of biomass physically shields the available carbohydrates from degrading enzymes to be converted to fermentable sugars, whereas the TBMPs are based on complete anaerobic degradation.

The relatively high TBMP values of the dry mature *S. ramosissima* are presumably due to higher lignin content that is characterized by a lower oxygen/carbon ratio. The anaerobic biodegradability of the organic matter content of the halophyte biomass based on elemental content varied from 47–71% for *S. europaea* and 55–63% for *S. ramosissima* substrates.

3.5. Mineral Concentration in Batch Tests

An overview of the mineral concentration in the BMP batch-test set-up of the mixture of halophyte plant material and inoculum is shown in Table 6. The mineral content in the batch assay was calculated based on the sum of the TS added per liter in the test set-up from the inoculum and substrate samples. The Na content in the batches of *S. europaea* grown at different salinities increased from 814 mg/L for the samples cultivated under non-saline conditions to 2458 mg/L for the samples cultivated at 30 g/L NaCl. The different fractions of *S. ramosissima* showed the highest Na content of 2349 mg/L in the juice fraction, then 1434, 887, and 675 mg/L in the whole green plant, dry mature plant, and pulp fraction, respectively.

Table 6. Mineral concentration in the BMP batch test set-up of mixtures of *Salicornia* samples and inoculum in mg/L.

Halophyte Species	Na	K	Ca	P	Mg
	(mg/L)	(mg/L)	(mg/L)	(mg/L)	(mg/L)
Salicornia europaea					
0 g/L NaCl	814 (34.41)	282 (17.87)	433 (18.24)	87.7 (2.45)	24.1 (1.07)
10 g/L NaCl	1508 (63.49)	255 (12.04)	398 (11.96)	97.5 (2.41)	25.2 (0.87)
20 g/L NaCl	2358 (36.74)	200 (3.06)	240 (1.42)	88.0 (0.71)	18.3 (0.18)
30 g/L NaCl	2458 (96.67)	207 (7.94)	233 (2.66)	87.3 (1.58)	17.2 (0.37)
Salicornia ramosissima					
Whole (green) plant	1434 (12.44)	158 (1.41)	246 (1.09)	95.4 (0.56)	27.1 (0.21)
Pulp fraction	675 (7.46)	69.4 (0.80)	238 (1.51)	72.2 (0.47)	15.8 (0.15)
Juice fraction	2349 *	267 *	245 *	126 *	40.0 *
Dry mature plant	887 (8.89)	282 (3.80)	218 (0.93)	97.8 (0.76)	23.1 (0.22)
Inoculum					
Inoculum control	344	33.3	184	55.1	9.5

* calculated based on fractionation of whole plant into pulp and juice fraction.

The presence of these different elements in the anaerobic process is necessary for microbial development. In particular, sodium is vital for methanogenic bacteria and archaea, but in high concentrations can lead to inhibition [29]. The development of mesophilic anaerobes is beneficial with Na content between 100–200 mg/L [7,8]. McCarty [11] investigated the inhibitory effect of Na contents and reported that sodium is considered the main inhibiting element of light earth metals with moderate inhibitory effects ranging between 3500–5500 mg/L and strong inhibition at 8000 mg/L to the methanogens under mesophilic conditions.

In the conducted experiments, *S. europaea* cultivated in non-saline conditions had the highest Ca content of 433 mg/L and 246 mg/L in the whole green *S. ramosissima* plants, which were both slightly above the optimal condition for methanation of acetic acid of

200 mg/L but much below the lower limit of the moderate inhibitory concentration of 2500–4000 mg/L reported by Kugelman and McCarty [12]. However, on the contrary, Jackson-Moss et al. [38] reported no inhibitory effect on AD at 7000 mg Ca/L, with the majority of the calcium content found in the effluent. Furthermore, the potassium concentration in the anaerobic BMP test set-up was below the 400 mg K/L threshold, whereas magnesium content was much lower than the optimum of 720 mg Mg/L for anaerobic bacteria [10].

3.6. Correlation of Mineral and Lignin Content and BMP

An overview of the ash, Na, and lignin content and the biomethane yield of the different samples of *S. europaea* and *S. ramosissima* is given in Figure 4. The correlations of ash, Na, and lignin content and experimental BMP of the different samples of *S. europaea* and *S. ramosissima* are shown in Figure 5 with the equations and statistical R^2 factors of the respective linear regression lines.

For the different samples of *S. europaea*, the biomethane yield increased with increasing salinity of the plant material resulting in Na concentrations in the batch assays up to 2358 mg Na/L. These findings are in accordance with the results of Turcios et al. [29], who reported the lowest methane yield of *T. pannonicum* of 301 mL CH_4/gVS cultivated under non-saline conditions with a sodium concentration of 929 mg/L and the highest methane yield of 347 mL CH_4/gVS grown at 30 g/L NaCl with a sodium content of 1988 mg/L. The lower methane yield of *S. europaea* cultivated at 30 g/L NaCl in the present study may give an indication that a Na concentration of 2400 mg Na/L is a limit for inhibition of the AD process. Accordingly, there is a moderate linear correlation of Na and ash content to BMP of these samples, which is expressed by R^2 values below 0.58 (Figure 5). Furthermore, for the plant and pulp samples of *S. ramosissima*, the biomethane yield increases with higher Na-concentrations in the batch set-up. Only for the juice fraction of *S. ramosissima* with a Na concentration in the batch assay of 2349 mg Na/L the biomethane yield is much lower. This non-linear correlation can also be seen in the correlations of ash and Na content and BMP of the *S. ramosissima* samples with R^2 values below 0.45 (Figure 5).
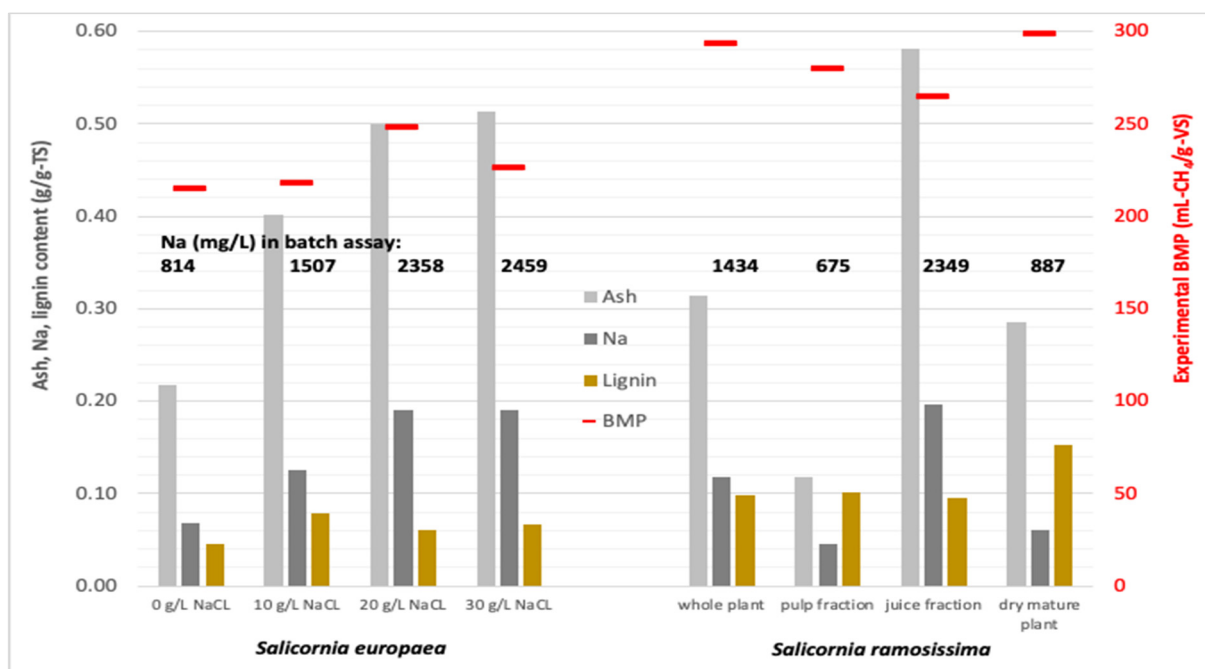

Figure 4. Experimental BMP and ash, Na and lignin content of the different samples of *S. europaea* and *S. ramosissima*.
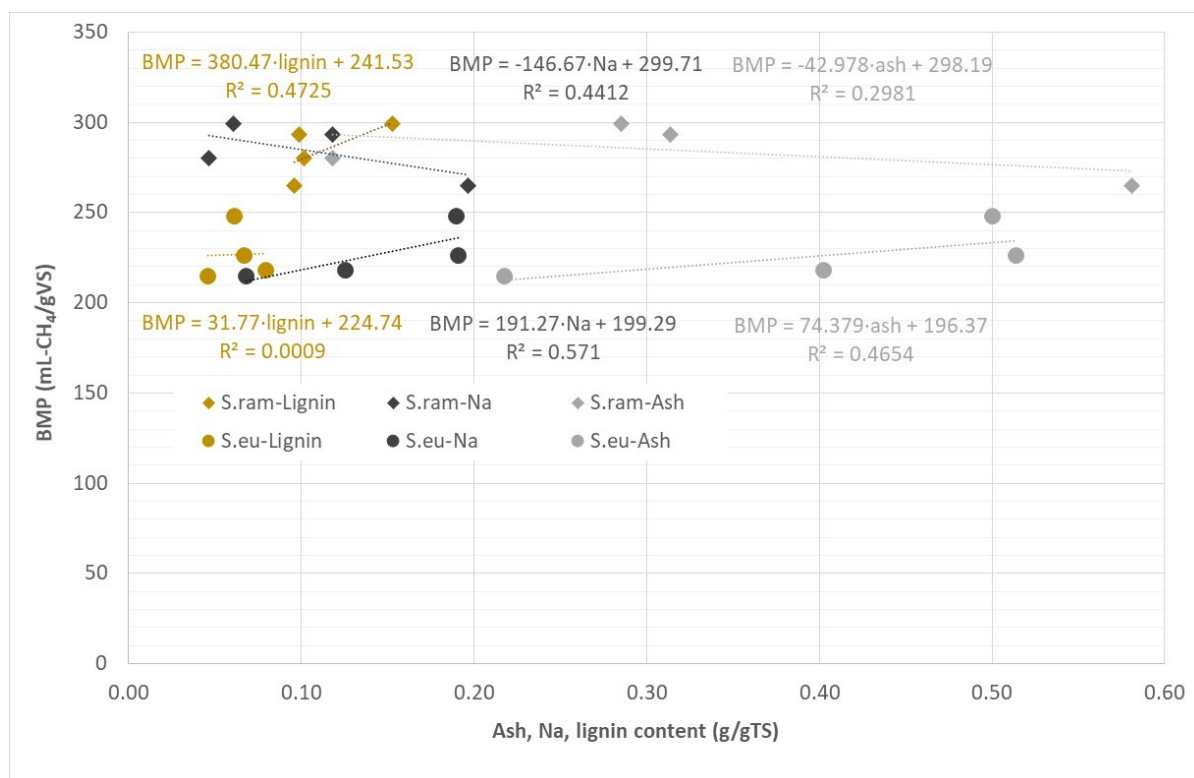
Figure 5. Correlations of ash, Na and lignin content and experimental BMP of the different samples of *S. europaea* (S.eu) and *S. ramosissima* (S.ram), including equations and R^2 factors of linear regressions.

These findings indicate that AD of the investigated Salicornia plants was not inhibited by sodium concentrations below 2400 mg Na/L. This means that halophyte plant material should be added only in limited amounts in co-digestion with other non-saline substrates such as manure or sewage sludge. The amount of halophyte material that can be added to 1 kg of the non-saline substrate until the threshold value of 2400 mg/L is reached is dependent on the TS content and the Na concentration per TS. From these values, it can be calculated that in co-digestion with 1 ton of a substrate with a low sodium concentration of 350 mg Na/L (like the inoculum used), 11–32 kg of *S. europaea* dry matter plant material or 51.5 kg of dried mature plant or 178 kg of the fresh plant or pulp fraction of *S. ramosissima* may be added until the sodium concentration of 2400 mg/L is reached (Table 7).

With respect to a possible correlation between biomethane yield and lignin content, it can be seen that *S. europaea* grown at 10 g/L NaCl had a relatively low biomethane yield and contained the highest lignin content of these samples of 7.9 g/100 gTS. For the other samples of *S. europaea*, however, the biomethane yield was not correlated to the lignin content, which was rather similar. Furthermore, for the *S. ramosissima* samples, no correlation between the biomethane yield and the lignin content can be detected since the dry mature plant of *S. ramosissima* with the highest lignin content of 15.3 g/100 gTS had the highest biomethane yield. This indicates that the lignin content of the halophyte plant material is too low to have a significant effect on the degradation of this biomass.

Overall, the influence of salt and lignin content on the biomethane potential cannot be clearly allocated. For the Na content, a threshold for AD process inhibition could be estimated, while the lignin concentration of the halophyte plant material was obviously too low to have a clear effect on the degradation. Furthermore, antagonism and synergism may occur between different salt ions as well as organic compounds of the substrates. According to Chen et al. [10], for example, combinations of cations induce antagonisms advantageous to that of an individual cation. It was reported that a mixture of Na and K or Mg and Na achieved a methane yield 10% greater as compared to the yield of solely Na.

Table 7. Limit of *Salicornia* material that can be added in co-digestion with 1 ton (t) of non-saline substrate to reach Na concentration of 2400 mg Na/L, based on TS concentration of the material and Na concentration in g/kg in the BMP batch-test set-up.

Halophyte Material	TS	Na	Limit of Material Added to 1 T Non-Saline Substrate
	% FM	g/kg	kg
Salicornia europaea			
0 g/L NaCl	97.2	68	32.2
10 g/L NaCl	98.2	126	17.0
20 g/L NaCl	98.6	190	11.1
30 g/L NaCl	98.7	191	11.0
Salicornia ramosissima			
Whole (green) plant	11.8	118	178.2
Pulp fraction	30.1	46	178.1
Juice fraction	7.1	197 *	178.3
Dry mature plant	69.6	61	52.6

* calculated based on fractionation of whole plant into pulp and juice fraction.

4. Conclusions

In the present study, the biomethane potential of the halophyte plants *S. europaea* and *S. ramosissima* was analyzed and correlated to their biomass composition, focusing on the content of carbohydrates, lignin, proteins, and minerals. A higher NaCl concentration used in the culture medium in a hydroponic system for *S. europaea* showed an increase in Na content of the plant tissue while the concentration of K, Ca, Mg, and P decreased with increasing NaCl concentration used for the cultivation. The highest methane yield of 248 mL CH_4/gVS was found for *S. europaea* cultivated at 20 g/L NaCl. The methane yield of *S. ramosissima* cultivated in a greenhouse with seawater irrigation was generally higher, with yields of up to 300 mL CH_4/gVS for both the whole green plant, pulp, and juice fraction. In general, the anaerobic digestion process showed no signs of inhibition at final sodium concentrations of up to 2400 mg Na/L with methane yields similar to the yields of conventional grass crops. Up to this sodium concentration, a moderate correlation between sodium content and biomethane yield was identified. Furthermore, no clear correlation between the relatively low lignin content and the biomethane yield could be found in the samples. The results show that the halophyte crop *Salicornia* either cultivated in hydroponic systems or in a greenhouse equipped with seawater irrigation, can be utilized as a feedstock for biogas production in co-digestion with a non-saline feedstock. It can be estimated that up to 10–30 kg of dry matter, 50 kg of dried mature plant material, or up to 178 kg of whole green plant or separated pulp fraction of halophytes can be added to 1 ton of non-saline feedstocks without causing inhibition of the process. Attention must be paid to the 25–45 times higher sulfur content in halophyte plant material at least compared to grass biomass that may cause significant H_2S production during the biogas process. Although this higher sulfur content did obviously not hinder the anaerobic digestion process in the present study, H_2S has to be removed from the biogas to avoid corrosion in subsequent technical applications.

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