Co-Digestion of *Salix* and Manure for Biogas: Importance of Clone Choice, Coppicing Frequency and Reactor Setup

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Abstract: Animal manure represents a major source of renewable energy that can be converted into biogas using anaerobic digestion. In order to most efficiently utilize this resource, it can be co-digested with energy dense, high biomethanation potential feedstocks such as energy crops. However, such feedstocks typically require pretreatments which are not feasible for small-scale facilities. We investigated the use of single-stage and the sequential co-digestion of comminuted but otherwise non-pretreated *Salix* with animal manure, and further investigated the effects of coppicing frequency and clone choice on biomethanation potential and the area requirements for a typical Swedish farm-scale anaerobic digester using *Salix* and manure as feedstock. In comparison with conventional single-stage digestion, sequential digestion increased the volumetric and specific methane production by 57% to 577 NmL L^{-1} d^{-1} and 192 NmL (g volatile solids (VS))$^{-1}$, respectively. Biomethanation potential was the highest for the two-year-old shoots, although gains in biomass productivity suggest that every-third-year coppicing may be a better strategy for supplying *Salix* feedstock for anaerobic digestion. The biomethane production performance of the sequential digestion of minimally pretreated *Salix* mirrors that of hydrothermally pretreated hardwoods and may provide an option where such pretreatments are not feasible.

Keywords: anaerobic digestion; co-digestion; energy crops; manure; *Salix*

1. Introduction

Anaerobic digestion (AD) is an energy-production and waste-management method whereby organic matter is metabolized by complex microbial communities into biogas, consisting mainly of CH_4 and CO_2 gases. Biogas can be used for heat and power generation or upgraded to vehicle fuel. AD was the fastest growing bioenergy-producing sector in Europe between 2005 and 2015, and its continued growth may help the EU attain its goals of increased bioenergy use and reduced greenhouse gas (GHG) emissions [1]. The largest potentials for increasing biogas production are estimated to come from agricultural residues and energy crops, in both the EU [2] and Sweden [3].

Animal manure makes up a large part of the potential for biogas production from agricultural residues, with the realistic potential in the EU estimated at 16 billion m^3 [4]. In addition to producing renewable energy, AD also reduces GHG emissions from manure storage, making its use as feedstock especially attractive as a means of meeting climate targets. However, volumetric biogas production from manure alone is relatively low owing to the high moisture content and relatively low biomethanation

potential (BMP) of the material. The co-digestion of manure with complementary feedstocks, such as lignocellulosic crops, may increase volumetric biogas production by allowing a higher organic loading rate (OLR) without compromising the hydraulic retention time (HRT) [5].

Whereas most lignocellulosic energy crops being investigated as AD substrates are herbaceous (see [6] for an overview of the field), woody biomass from *Salix* spp. (willows) may provide an attractive alternative. *Salix*, cultivated in short-rotation coppice (SRC) systems, is high yielding while requiring relatively low energy inputs in planting, agrochemicals, tilling, and harvesting. In comparison to annual and herbaceous bioenergy crops, *Salix* therefore provides a higher net energy return [7,8], as well as lower production costs both in relation to the area and energy content [9]. Importantly, management inputs, primarily fertilization, have been shown to greatly affect the GHG economy of biogas plants when using energy crops as substrates [10], making *Salix* an attractive alternative also from a climate perspective. Furthermore, the use of *Salix* as an AD substrate has been estimated to provide a larger climate cooling effect compared to direct combustion, albeit at the expense of energy return [11].

Biomass from lignocellulosic bioenergy crops is typically recalcitrant to the microbial processes required for converting it into biogas, more so in woody than in herbaceous species in part due to their more rigid structures and generally higher lignin contents [12]. Harvest timing may also affect the recalcitrance of woody species, with older material being less amenable to degradation [13], although the topic is poorly studied. More frequent coppicing of *Salix* stands would incur higher costs and energy inputs, and it is thus important to understand what tradeoffs in recalcitrance may be incurred by less frequent coppicing.

In order to overcome the inherent recalcitrance of lignocellulosic crops, the use of hydrothermal or thermochemical pretreatment methods is frequently suggested [14]. However, such pretreatment methods may not be financially feasible for small-scale operations, such as many farm-scale biogas plants [15]. Biomass recalcitrance in non-pretreated *Salix*, as it relates to AD, has been shown to primarily affect methane production rates rather than potentials [16], suggesting that an increased HRT may provide an effective alternative to pretreatment. Both biomass recalcitrance and biomass productivity greatly affect the economics of a *Salix*-fed AD unit: recalcitrance dictates specific methane yields whereas the biomass productivity determines the yield per ha and thus the land required to feed a specific reactor setup. With these traits being influenced by both ancestry and management [17], the choice of clone and management strategy may affect the viability of such a venture.

The aim of this study was to investigate the possibilities of using *Salix* biomass in manure-based biogas plants. Specifically, we investigated single-stage and sequential co-digestion anaerobic digestion processes with regard to process performance and digestate quality, and moreover, evaluated the influence of clone choice and coppicing frequency on the biogas yields and area requirements of those systems.

2. Materials and Methods

For an overview of the experimental procedures of this work, see Figure 1.

2.1. Field Trial and Substrates

A field trial was established 13 May 2011 in Uppsala, Sweden (58°81′ N, 17°66′ E), on a postglacial heavy clay soil [18]. The site was cultivated twice with a rotivator, first in autumn 2010 and a second time the day before planting. The trial was planted, using 20 cm-long stem cuttings, in a double row design at a density of 20,000 cuttings ha^{-1}. The trial was a split-plot design and consisted of three blocks, each with three main plots representing different harvest regimes that at final harvest gave shoots of one, two and three years of age. Within each main plot there were six monoclonal subplots, 6 m^2 in size and each containing 12 plants. Two border rows were planted around the experimental field in order to minimize edge effects. The *Salix* clones used were 78183 (*S. viminalis*), 78195 (*S. viminalis*), Björn (*S. schwerinii* × *S. viminalis*), Jorr (*S. viminalis*), Olof (*S. viminalis* × (*S. schwerinii* × *S. viminalis*)), and Tora (*S. schwerinii* × *S. viminalis*). The field trial was fertilized with N:P:K 21:4:7 in late spring 2012 (60 kg N ha^{-1}) and in late spring 2013 (66 kg N ha^{-1}). During the years 2012 and 2013, all the stools within one subplot from each block was cut back.
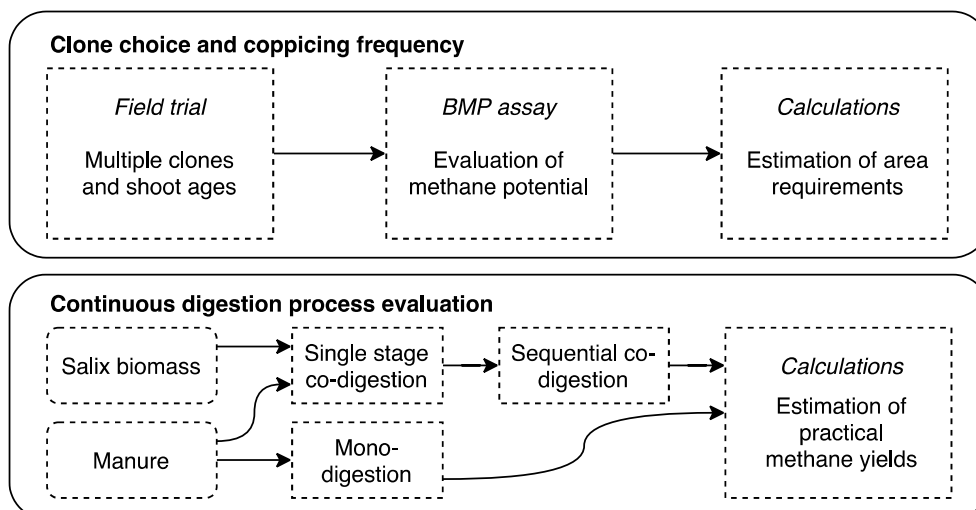
Figure 1. Overview of the experimental steps in this work. BMP: biomethanation potential.

In early April 2014, before leaf development, the entire field trial was harvested by hand, with each block now containing 1, 2, and 3 year-old (yo) shoots. All the shoots of each stool were cut 10 cm above ground, and the weight of each stool was recorded individually. The material from each clone was pooled by shoot age, and size reduced using a compost chipper (MTD90 Chipper Shredder). This material was used for biomethanation potential (BMP) assays (Section 2.2). For the co-digestion experiment, 4 yo shoot biomass from the clone Tora, cultivated on a clay loam soil (59°97′ N, 17°57′ E) was used together with cattle manure.

2.2. Biomethanation Potential Assay

A BMP assay was performed on the material from the field trial. The BMP assay was performed according to commonly used methods [19], using inoculum from a wastewater treatment plant in Uppsala, Sweden. The substrates were evaluated in triplicate, with triplicate cellulose and inoculum controls. To each bottle, 2.1 g substrate and 4.2 g inoculum was added (on a VS basis), giving an inoculum:substrate ratio of 2:1. With a total liquid volume of 700 mL, the substrate organic load was 3 g L^{-1}. The volume of the bottles was 1120 mL, leaving 420 mL as a headspace volume. BMP vessels were incubated at 37 °C on an orbital shaker. Biomethane production was evaluated using pressure measurements and gas chromatography. Gas volumes were normalized to 0 °C and 1 atm, and referred to as normalized mL (NmL) below.

2.3. Continuous Digestion Experiments

2.3.1. Single-Stage Continuous Digestion

The performance of *Salix* as a co-digestion substrate was evaluated in a continuous digestion experiment. Three 8-l continuous stirred-tank reactors (CSTRs; Belach Bioteknik, Stockholm, Sweden) with 5 l culture volume were inoculated with inoculum from a wastewater treatment plant in Uppsala, Sweden. One manure control and two *Salix*/manure co-digestion (1:1 on VS basis) reactors were evaluated. Hydraulic retention times (HRT) over the entire period were 34–38 days, with a target organic loading rate (OLR) of 1.5 g VS L^{-1} day^{-1} for the manure control and 3.0 g VS L^{-1} day^{-1} for the *Salix* co-digestion reactors. Reactors were operated at 37 °C and 90 rpm for 266 days, with the OLR being increased over the first 80 days (approx. 2 HRT), and running at the target OLR for 185 days (5 HRT). The reactors were fed with substrate 6 days per week. The pH, volatile fatty acid (VFA) production, gas production, and methane content were monitored regularly throughout the process.

2.3.2. Sequential Continuous Digestion

To enable a more thorough decomposition of the *Salix* biomass, a second continuous digestion experiment was performed with the reactors in sequence, that is, the second reactor was fed only with digestate from the first reactor. For this experiment, one *Salix*/manure reactor was operated for each stage. Other parameters were the same as for the single-stage co-digestion experiment. Both reactors in the sequential setup were operated at the same HRT, giving a total HRT of 68–76 days for the entire system. This experiment was performed in immediate succession with the previous experiment, ensuring an already adapted inoculum. The sequential continuous digestion experiment lasted for 169 days.

2.4. Chemical Analyses

The substrates and digestates were characterized for total solids (TS), volatile solids (VS), and ash content by drying at 105 °C and incineration at 550 °C. Total nitrogen and ammonium nitrogen were analyzed according to ISO 13878 [20], total carbon according to ISO 10694 [21], and the contents of P, K, Mg, Ca, Na and S were analyzed as described in [22]. Elemental analyses were performed by Agrilab AB (Uppsala, Sweden). VFA and methane contents were analyzed using HPLC and GC, respectively, as described in [23].

2.5. Calculations and Statistical Analyses

Biomass productivities per clone and shoot age were calculated using the equation:

$$P_{odt} = \frac{\frac{FW}{12} \times 20000}{1000 \times c} \tag{1}$$

where P_{odt} is the biomass productivity in oven dried tons (odt) ha^{-1} y^{-1}, FW is the fresh weight in kg of the 12 plants in the subplot, and c is the shoot age. Productivities were then evaluated using a mixed model specified by the formula:

$$Y_{ijk} = \mu + \alpha_i + \gamma_k + \eta_{ik} + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ijk}$$

where Y_{ijk} is the estimated productivity (in odt ha^{-1} y^{-1}) for shoot age i, clone j, and block k; μ is the general intercept, α_i is the fixed effect of shoot age, γ_k the fixed effect of block, η_{ik} the whole-plot error, i.e., the random interaction effect of block and shoot age, β_j the fixed effect of clone, $(\alpha\beta)_{ij}$ the fixed interaction effect of clone and shoot age, and ε_{ijk} the split-plot error. The model was fitted using the function lmer from the package lme4 version 1.1-21 [24] in R version 3.6.3 [25], and the Tukey-corrected differences between the clones and shoot ages were calculated using the package emmeans version 1.4.7 [26]. Differences in the BMP values were evaluated using ANOVA using the function aov, with clone and shoot age as independent variables, and the Tukey-corrected differences between clones and shoot ages evaluated using emmeans. The pH values of the digesters were evaluated by ANOVA with digester as the independent variable.

Average values for the specific methane production and volumetric production in the continuous digestion experiments were calculated using data points beginning at 1 HRT after reaching the target OLR and up to the end of the experiment.

For calculating the area requirements and process yields, two illustrative scenarios were considered, both with digester volumes of 1000 m^3 (typical for Swedish farm-scale biogas plants [27]). In Scenario A, the parameters were set to resemble the continuous experiment described in this study: co-digestion using 50% *Salix* and 50% cattle manure on a VS basis and a total OLR of 3 kg VS m^{-3}. In scenario B, the parameters were chosen to reflect a lower requirement of *Salix* biomass; 25% Salix is used together with 75% cattle manure on VS basis, with a total OLR of 2 kg VS m^{-3} day^{-1}. The organic load from

manure was the same in both scenarios, 1.5 kg VS m^{-3} day^{-1}. Specific methane potential (SMP) for Scenario B was calculated by first solving the following equation for SMP_{Salix}:

$$SMP_{total} = \frac{1.5 \times SMP_{manure} + 1.5 \times SMP_{Salix}}{3} \qquad (2)$$

where SMP_{total} is the SMP of the continuous co-digestion experiment, SMP_{manure} is the SMP of the manure-only continuous digestion experiment, and SMP_{Salix} is the estimated SMP of *Salix* VS in this system. The SMP of Scenario B could then be estimated using the following equation:

$$SMP_{ScenarioB} = \frac{1.5 \times SMP_{manure} + 0.5 \times SMP_{Salix}}{2} \qquad (3)$$

The estimated volumetric methane production for Scenario B was calculated using the same method, substituting the volumetric production values for SMP.

For estimating the gross energy content of the methane produced, the specific heat of combustion was used (37.03 MJ/m^3 CH$_4$).

3. Results and Discussion

3.1. Field Experiment Biomass Yields

In order to estimate the area requirements and expected biomethane yields on an area basis, the shoot biomass of each plant was weighed at harvest. The biomass yields (least-squares means) ranged from 2.5 to 8.7 odt ha^{-1} year^{-1} (Figure 2), with significant effects of clone ($p_{clone} = 3.5 \times 10^{-7}$; see Table S1 for the ANOVA table), although the Tukey-corrected *p*-values for most individual clone–shoot age combinations were not statistically significant. However, when comparing across clones but within shoot ages, the clones Tora, Björn, and Olof had significantly higher biomass yields than the other clones (Table S2). For the per-subplot harvest data, see Supplementary File 2.
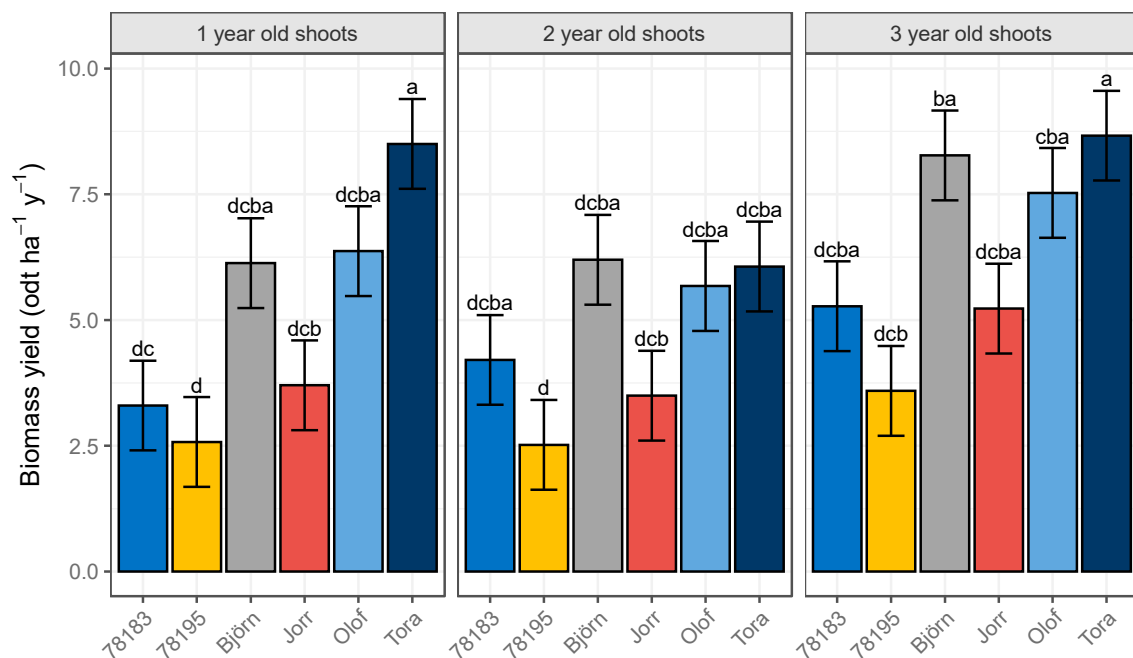

Figure 2. Least-squares means of the biomass productivity (odt ha^{-1} y^{-1}) per clone and shoot age at harvest. Error bars indicate standard error. Shared letters indicate statistically non-significant differences between pairs across clones and shoot ages.

Biomass yields obtained in this study are reflective of those typically reported in the literature, with the highest yields being similar to the best reported yields from commercial plantations [28]. Plant survival in the field experiment was high, with on average 97.1% survival. The clone Björn had the lowest survival rate, 93.5%.

3.2. Biomethanation Potentials

Biomethanation potentials, BMP, differed between clones and shoot ages (p_{clone} = 0.04; $p_{shoot\ age}$ = 5.2×10^{-13}). Tukey-corrected comparisons revealed that 2 yo shoots had higher BMP values than 1 and 3 yo shoots ($p < 0.0001$, with increases of 33% and 23%, respectively), and that 3 yo shoots had a higher BMP than 1 yo shoots ($p = 0.027$; +9%). Regarding clones, the only statistically significant comparison was between the clones Olof and Jorr, with the latter having a higher BMP ($p = 0.016$; +16%). See Table 1 for the BMP values, Figure 3A for biomethanation curves, and Table S3 for the ANOVA table.
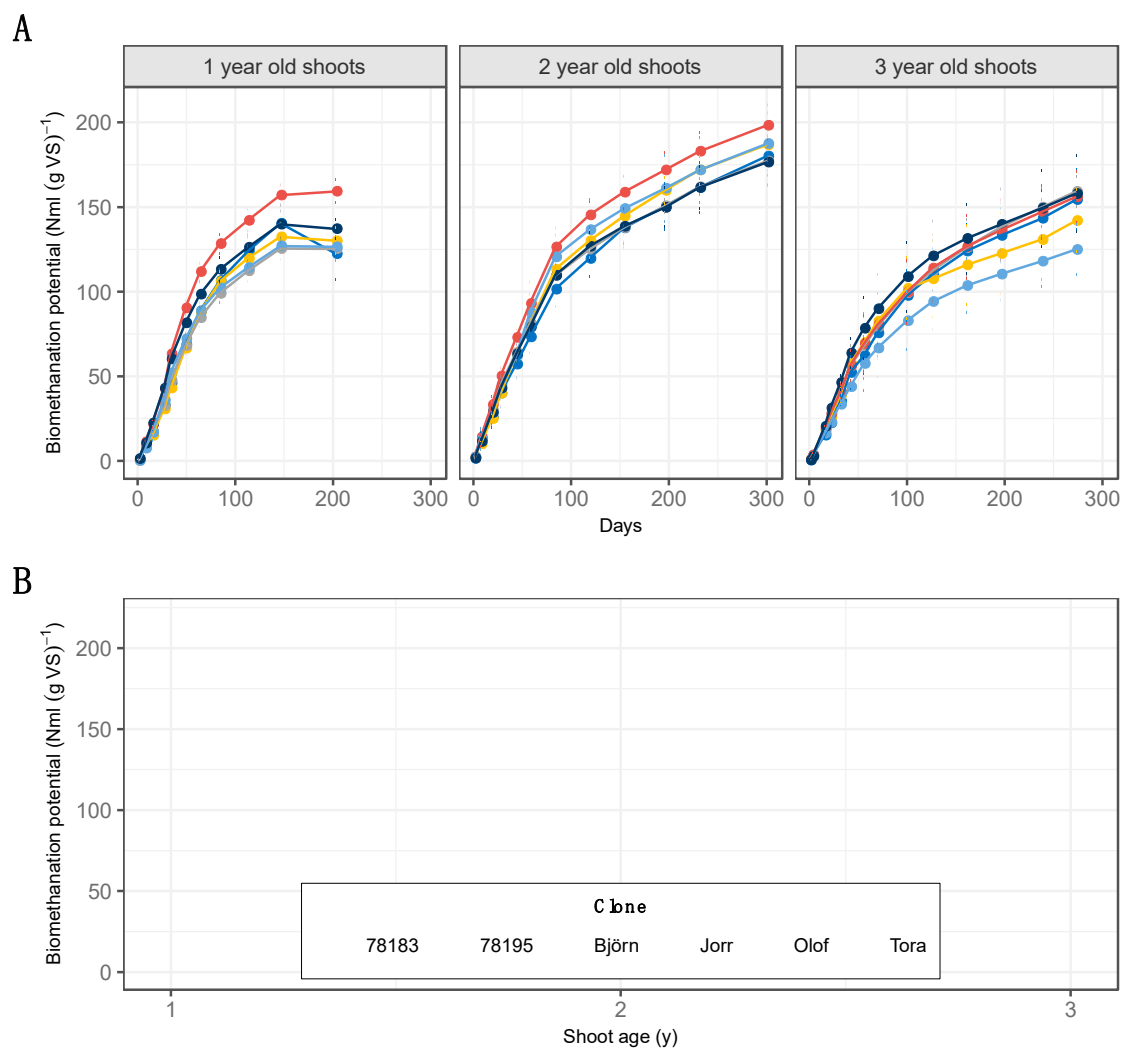

Figure 3. Biomethane production curves of the clones at different shoot ages (**A**), and the biomethanation potential vs. shoot age (**B**). The line in (**B**) illustrates the inverse-U shape of the biomethanation potentials depending on coppicing frequency. VS: volatile solids.

Bark proportion in willow stands is inversely proportional, in a quadratic relationship, to the stem diameter measured at a 55 cm height, reaching a constant value of 0.2 at around 20 mm in diameter [29]. Due to a lower content of cellulose, and higher contents of lignin and extractives [30], bark would have a lower BMP than wood. The inverse-U shape of the BMP curve (Figure 3B), with lower values for the

1 and 3 yo shoots, could be explained by the interaction of metabolizable energy content and biomass recalcitrance. In the young shoots, the metabolizable energy content is low due to a higher proportion of bark, whereas in the older shoots, biomass recalcitrance becomes a more important determinant of BMP. Biomass recalcitrance is a complex trait, only partly determined by chemical composition [31], and there may be several reasons for the increased recalcitrance of older shoots. Healey and coworkers observed that biomass from older eucalypts had a decreased lignin syringyl:guaiacyl (S:G) ratio and lignin content, compared to younger biomass [13]. Although lignin content is generally positively correlated with biomass recalcitrance, lignin S:G ratio may be a comparably more influential factor, and biomass recalcitrance in older shoots was indeed higher in the aforementioned study. However, whether these results are applicable to short-rotation coppice wood remains an open question, and contradicting results on lignin content and S:G ratio have been reported in poplar [32]. To our knowledge, there has been no similar study on age-related compositional and structural differences, and their effects on recalcitrance, in *Salix*.

Table 1. Biomethanation potentials (NmL $(g\ VS)^{-1}$; standard deviation in parentheses) of the *Salix* clones at different harvest ages.

Clone	Shoot Age		
	1	2	3
78183	140.5 (±10.9)	180.2 (±11.6)	154.4 (±17.9)
78195	132.3 (±8.4)	186.8 (±8.5)	142.0 (±18.4)
Björn	125.8 (±7.0)	177.2 (±5.6)	159.2 (±9.0)
Jorr	159.3 (±6.8)	198.3 (±12.2)	156.0 (±15.1)
Olof	127.6 (±3.3)	187.5 (±18.2)	125.0 (±14.7)
Tora	139.8 (±7.9)	176.5 (±13.5)	158.0 (±22.4)

3.3. Co-Digestion of Salix and Manure

A *Salix*/manure co-digestion experiment was carried out using both a single-stage digestion, and a two-stage sequential digestion where the digestate from the first-stage reactor was fed into a second-stage reactor. Biomethanation potentials were 225.7 (±13.0) NmL $(g\ VS)^{-1}$ for manure and 120.5 (±11.4) NmL $(g\ VS)^{-1}$ for the 4 yo *Salix*. The single-stage co-digestion was operated in duplicate. All the processes were stable with low levels of VFAs (values being $< 1\ g\ L^{-1}$ throughout the entire experimental period) and with an average pH of 7.8, with no differences observed between the reactors, neither between duplicates, single-stage or sequential setups (ANOVA, $p = 0.22$). Using *Salix* as co-digestion substrate did not impair mixing, and there was no caking. The substrate of the single-stage digesters had a TS of 13.2% (of which 88.6% was VS), and a C/N (total C/total N) ratio of 14.2 (Table 2). Substrate TS, VS, and C/N ratio was comparably lower in the second-stage digester, reflecting the further conversion of organic matter into methane. A substantial fraction of the organic N was converted after the first stage of digestion, resulting in a comparably higher level of soluble NH_4^+-N in the substrate of the second-stage digester.

The continuous digestion experiments were performed over 435 days, with both reactors running as single-stage reactors for the first 266 days. Volumetric methane production in the single-stage co-digestion process (Figure 4) was 367 NmL $L^{-1}\ d^{-1}$, a 39% increase over the volumetric production from manure alone (264 NmL $L^{-1}\ d^{-1}$). However, the average SMP of the single-stage co-digestion was 122 NmL $(g\ VS)^{-1}$, a reduction from the 176 NmL $(g\ VS)^{-1}$ observed for the manure control (Table 3). The lower SMP is indicative of a lower conversion of organic matter in the co-digestion system, which is expected due to the recalcitrant nature of *Salix* biomass and thus its lower BMP value. Still, considering the measured BMP values of the individual substrates, the obtained SMP values for both processes were on a similar level and corresponded to approximately 70% of the methane potential. This suggests that the lower SMP was caused by a comparably lower BMP value of *Salix* and that the overall degradation in the mono and co-digestion processes were on a similar level.

The average methane contents were 54% for the single-stage co-digestion, 52% for the second-stage digester in the sequential experiment, and 58% for the manure control.

Table 2. The compositional parameters of substrates used in the single stage and sequential co-digestion experiments. TS: total solids; VS: volatile solids.

Parameter	Manure	*Salix*	Single Stage [a]	Sequential [b]
TS (% of wet weight)	8.2	47.7	13.2	10.5
VS (% of wet weight)	6.7	46.7	11.7	9.1
VS (% of TS)	81.7	97.9	88.6	86.7
Total N (g kg^{-1})	4.5	2.4	4.2	4.8
Organic N (g kg^{-1})	2.1	2.4	2.1	1.6
NH_4^+-N (g kg^{-1})	2.4	0.0	2.1	3.3
Total C (g kg^{-1})	38	215	60	50
C/N (TotC/TotN)	8.4	88.7	14.2	10.4
Total P (g kg^{-1})	0.51	0.15	0.46	0.52
K (g kg^{-1})	4.2	0.5	3.9	3.8
Mg (g kg^{-1})	0.51	0.15	0.48	0.50
Ca (g kg^{-1})	1.3	1.8	1.5	1.5
Na (g kg^{-1})	0.1	0.0	0.07	0.06
S (g kg^{-1})	0.5	0.15	0.39	0.38

[a] Values in this column are calculated from the individual components in a 1:1 VS mixture. [b] Values refer to second-stage digester in the sequential experiment, i.e., the digestate produced from the first-stage digester.
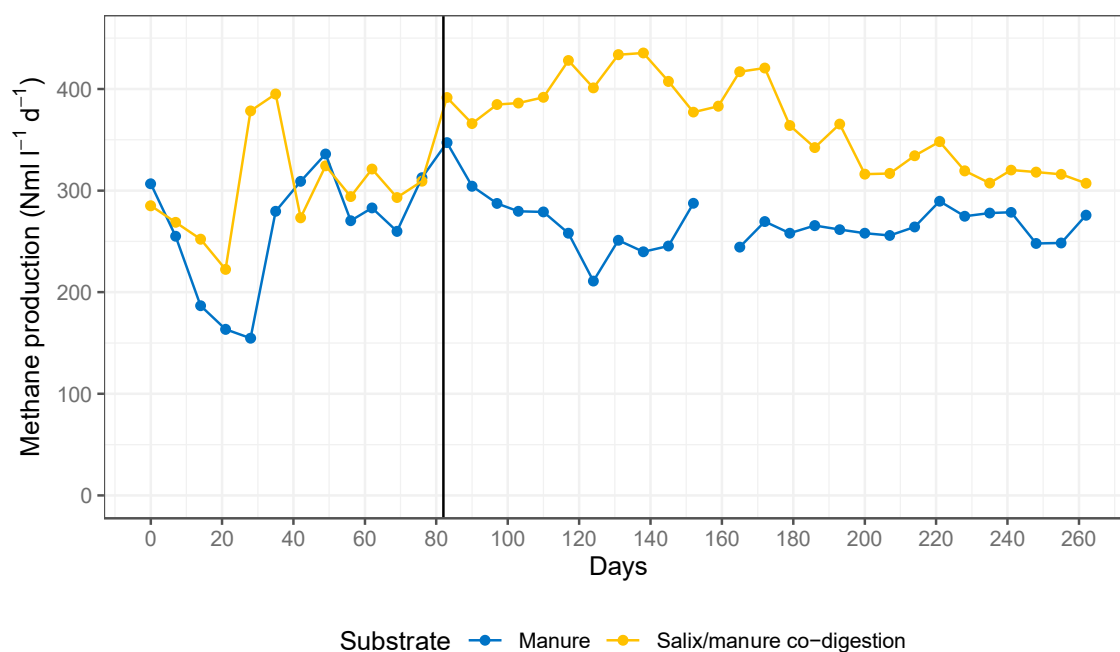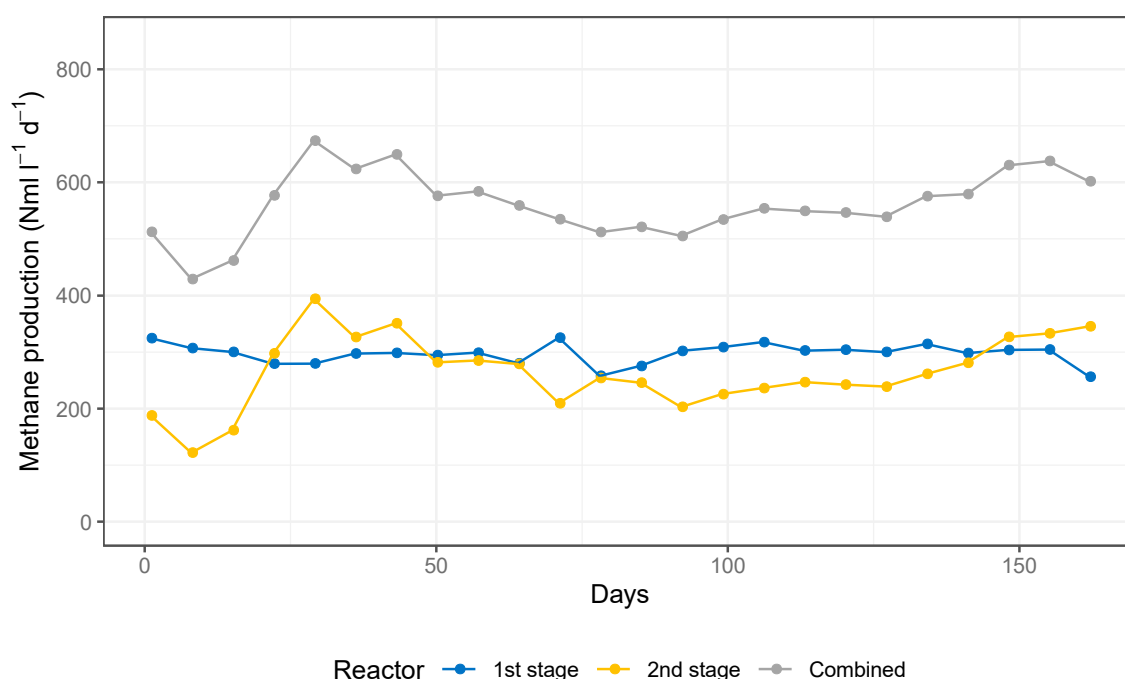

Figure 4. Volumetric methane production (weekly averages) for the single stage continuous digestion processes. The vertical line indicates where the target organic loading rate (OLR) was reached. Data are missing for the manure reactor at day 159 due to a gas leak.

After day 266, the digestate from the first co-digestion reactor was used as a substrate for the second reactor, simulating a two-stage sequential digestion process. In this sequential process, including both reactors, both the SMP and volumetric production was increased by 57% compared to the single-stage co-digestion (Figure 5). In comparison with the manure control, SMP was increased by 9% to 192 NmL (g VS)$^{-1}$, and the volumetric production was increased by 119% to 577 NmL L^{-1} d^{-1} (Table 3). This final SMP value for the co-digestion setup was even slightly above the excepted values based on the BMP measurement (173.1 NmL (g VS)$^{-1}$), possibly indicating a beneficial effect of co-digestion [33].

Table 3. Process performance parameters for the single-stage and sequential co-digestion experiments and the manure control. VS: volatile solids.

Parameter	Co-Digestion		
	Manure	**Single Stage**	**Sequential**
Specific methane production (NmL (g VS)$^{-1}$)	176	122	192
Volumetric methane production (NmL L^{-1} d^{-1})	264	367	577
OLR (g L^{-1} d^{-1})	1.5	3.0	3.0 [a]
HRT (d, average)	36	36	36 + 40 [b]

[a] OLR for the first stage digester. [b] The second value represents the average hydraulic retention time (HRT) of the second-stage digester.

Figure 5. Volumetric methane production (weekly averages) for the sequential *Salix* co-digestion process. Days are relative to the start of the sequential digestion experiment.

Comparing the methane yields (volumetric and specific) from this experiment with practical data reveals the importance of reactor configuration when using highly recalcitrant biomass for anaerobic co-digestion. Average volumetric production for Swedish farm-scale co-digestion reactors is reportedly 530 and 630 NmL L^{-1} d^{-1} depending on the source of manure (cattle and pig, respectively, using a variety of co-digestion substrates) [27]. Compared with the single-stage digestion described in the present study, the reported values are 44–72% higher. This observed difference is probably caused by the type of co-substrate, which in Swedish farm-scale biogas plants often consists of different types of food waste (household waste, grease separator fat, and slaughterhouse waste). These materials have a higher biomethane potential compared to *Salix*, often in the range of 300–500 NmL (g VS)$^{-1}$. However, the volumetric production of the sequential co-digestion experiment with *Salix* (577 NmL L^{-1} d^{-1}) was similar to those reported in the aforementioned overview.

Only a few studies have reported on the performance of pretreated hardwoods in anaerobic co-digestion. SMP values from the co-digestion of manure and steam-pretreated *Salix* biomass have been found to be 216.6 NmL (g VS)$^{-1}$ in batch (1:1 VS ratio) [34], and 215.2 NmL (g VS)$^{-1}$ (2:3 *Salix*:manure ratio; maximum weekly average), with a volumetric production of 645 NmL L^{-1} (maximum weekly average) in continuous digestion [35]. In a batch study on NaOH-pretreated eucalyptus wood chips, a SMP of 234.88 NmL (g VS)$^{-1}$ was reported [36]. These values are all similar to those reported in

the current study (Table 3). Although the co-digestion SMP is dependent on the SMP of the manure used (the range of SMP for manure in the aforementioned studies was 157–245 NmL (g VS)$^{-1}$), which complicates direct comparisons between studies, the similarity of the values found in the present study with those from earlier published reports suggest that the performance of sequential co-digestion of non-pretreated *Salix* biomass is comparable to that of single-stage co-digestion of hydrothermally or thermochemically pretreated hardwoods.

Overall, the results demonstrated that the sequential co-digestion of non-pretreated Salix biomass can provide methane yields similar to that of pretreated biomass, although at the expense of an increased HRT. Hydrothermal and thermochemical pretreatment systems are complex, often use hazardous chemicals requiring neutralization and disposal, and require significant capital expenditure to implement. The high costs are due to the requirements for heat, pressure, and/or corrosion resistant materials, as well as for additional equipment such as steam generators, storage and neutralization tanks, etc., and thus require the processing of substantial amounts of biomass in order to be financially viable [15,37,38]. Sequential anaerobic digestion systems, on the other hand, are of low technological complexity and cost, and the technology is already commonplace [39]. Whether non-pretreated Salix biomass is an appropriate co-digestion feedstock is likely to depend on numerous factors including biomass yield, distance to the field, availability of harvesting equipment, price of electricity, and fertilizer cost, all of which need to be evaluated on a case-by-case basis. A thorough technoeconomic analysis may be able to ascertain the relative importance of these and other variables, although to our knowledge such an analysis is yet to be published. Furthermore, the effects on digestate quality, especially as it relates to heavy metal accumulation, warrants further study. Finally, operational parameters such as HRT, OLR, and process temperature may significantly affect the performance of Salix co-digestion, although their effects were not evaluated in the present work.

3.4. Effect of Co-Digestion on Digestate

An important co-product of AD is the digestate, which is typically used as fertilizer. Compared to the manure control, the digestate from the co-digestion processes had higher TS and VS contents, with other values being similar (Table 4). Compared to the undigested manure (Table 2), the digestate from all the investigated processes had a higher level of plant available ammonium nitrogen (30–50%), which is positive for the value of the digestate [40]. The addition of extra carbon via *Salix* did not significantly change the overall degradation of proteins (organic N). The major part of the organic nitrogen was degraded in the first stage process while carbon seemed to be more efficiently degraded in the second stage. The high degree of decomposition in the sequential digestion process was illustrated by the carbon content in the sequential digestion digestate, which was equal to that of the single-stage manure-only process, whereas it was markedly increased in the single-stage co-digestion digestate. Co-digestion digestates containing recalcitrant carbon, such as that from lignocellulosic materials, can be used to increase soil carbon contents [40] and possibly improve other soil properties depending on the soil type [41].

The value of digestate as fertilizer is assessed by its content of nutrients and organic matter, but factors such as the content of heavy metals and hygienic parameters are also of critical importance [42]. Including *Salix* in addition to manure as a co-substrate for biogas production is not likely to impact the hygienic parameters of the digestate, however, it is important to consider the well known ability of this plant to take up metals from the soil [43], potentially causing a significant increase in the metal content in the digestate as compared to manure mono-digestion. However, the risk of a high content of metals in the above-ground biomass may be minimized by selecting a low metal cultivation site, as well as by using a *Salix* clone with low phytoremediation capacity, such as Tora [43] which was used in the present study.

Table 4. Compositional analysis of digestates originating from a pure manure digestion process and the *Salix*/manure co-digestion processes. TS: total solids; VS: volatile solids.

Parameter	Co-Digestion		
	Manure	Single Stage [a]	Sequential
TS (% of wet weight)	6.2	10.5	6.1
VS (% of wet weight)	4.7	9.1	5.3
VS (% of TS)	75.3	86.7	86.9
Total N (g kg^{-1})	5.0	4.8	4.3
Organic N (g kg^{-1})	1.5	1.6	1.4
NH_4^+-N (g kg^{-1})	3.5	3.3	2.8
Total C (g kg^{-1})	26	50	26
C/N (TotC/TotN)	5.2	10.4	6.1
Total P (g kg^{-1})	0.58	0.52	0.53
K (g kg^{-1})	4.4	3.8	4.3
Mg (g kg^{-1})	0.52	0.50	0.50
Ca (g kg^{-1})	1.3	1.5	1.3
Na (g kg^{-1})	0.13	0.06	0.17
S (g kg^{-1})	0.42	0.38	0.37

[a] Values are the means of the two digesters.

3.5. Area Requirements and Estimated Yields from Co-Digestion of Salix in Farm Scale Digesters

Based on biomass yields from the field experiment and the data from the continuous digestion experiments, the area required for supplying a farm-scale biogas plant with *Salix* for co-digestion could be estimated, as well as the resulting biogas yields. Two scenarios were considered, using either 50% or 25% *Salix* on a VS basis for co-digestion (Section 2.5). Area requirements for both scenarios were influenced to a large degree by biomass yields, primarily due to clone choice (Figure 6). In Scenario A, the area required for producing *Salix* differed by a factor of 3.7 between the lowest and highest yielding clone/harvest frequency combinations. For the highest-yielding clones Tora, Björn, and Olof, the mean area required to supply *Salix* for Scenario A was 63 ha y^{-1}, at 3 years coppicing frequency.
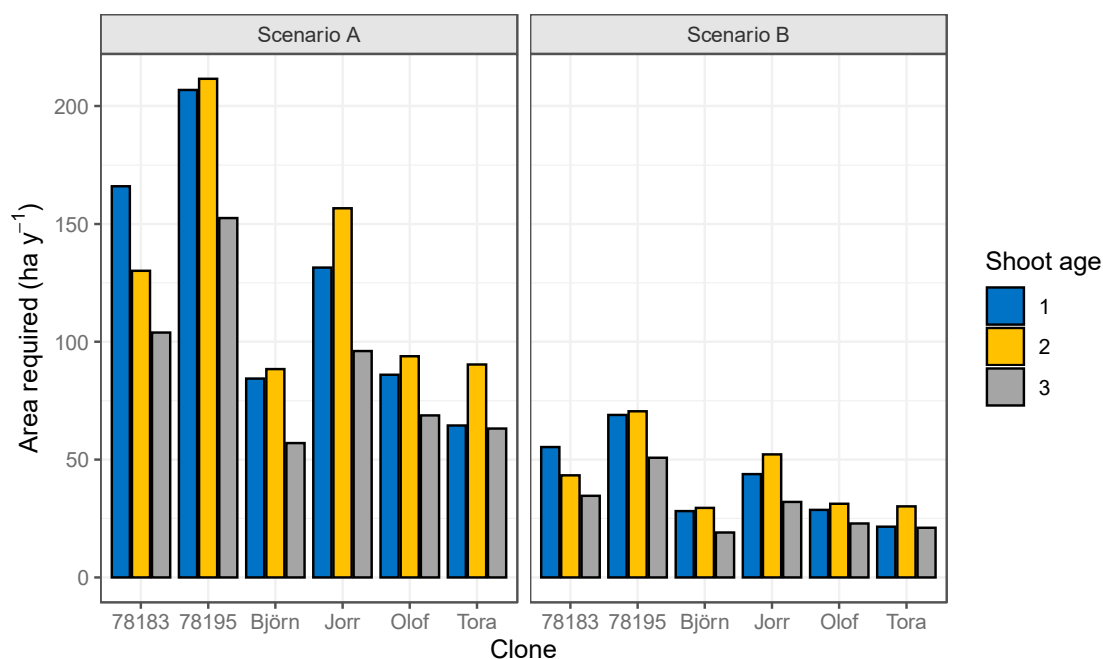

Figure 6. Area required for supplying *Salix* biomass to a 1000 m^3 co-digestion digester at 3 kg m^{-3} OLR with 50% *Salix* VS (Scenario A), and 2 kg m^{-3} OLR with 25% *Salix* VS (Scenario B). Shoot age refers to the age of the above-ground biomass at harvest.

In Scenario B, the mean required area for every-third-year coppicing was 21 ha y^{-1} for the high-yielding clones at three years coppicing frequency. Although the relative area requirements between the lowest and highest yielding clones were the same in both scenarios, absolute values differed to a lesser degree, as shown in Figure 6. For reference, a 21 ha *Salix* plantation would be considered large by Swedish standards, with the largest commercial plantation included in a 2015 survey being 40.6 ha [28].

As described in Section 3.3, the sequential *Salix* co-digestion system increased the volumetric methane production compared to the manure-only system, especially in the sequential digestion modality. These data were generated for a system similar to that of Scenario A, although according to our calculations, this would also hold true for Scenario B (Table 5). In Scenario A, the volumetric production was increased by 119% compared to the manure-only system. In Scenario B, the volumetric production could be increased by 59% over the manure control. The estimated yearly methane production from the sequential digestion system would be 210,700 and 153,800 m^3 for Scenarios A and B, respectively, representing increases of 114,200 and 57,300 m^3, respectively, compared to the manure control. The yearly gross energy output of the systems would amount to 7.8 and 5.7 TJ y^{-1} for Scenarios A and B, respectively.

Table 5. Biogas production parameters for the two scenarios.

	Manure Control	Scenario A		Scenario B	
		Single Stage	Sequential	Single Stage	Sequential
Specific methane production (NmL $(g\ VS)^{-1}$)	176	122	192	149	184
Volumetric methane production (NmL $L^{-1}\ d^{-1}$)	264	367	577	316	421
Yearly methane production for 1000 m^3 digester ($m^3\ y^{-1}$)	96,500	134,200	210,700	115,400	153,800
Gross energy yield for 1000 m^3 digester (TJ y^{-1})	3.6	5.0	7.8	4.3	5.7
OLR ($g\ L^{-1}\ d^{-1}$)	1.5	3.0	3.0 [a]	2.0	2.0 [a]
HRT (d, average)	36	36	36 + 40 [b]	36	36 + 40 [b]

[a] OLR for the first stage digester only. [b] The second value represents the average HRT of the second-stage digester.

4. Conclusions

Lignocellulosic energy crops considered for use as feedstock for biogas production in co-digestion systems are often herbaceous, owing to the lower recalcitrance of these species compared with woody crops. However, SRC willow (*Salix*) provides several advantages over herbaceous crops in terms of energy return, production costs, and effects on the climate. In the present study, several aspects related to using *Salix* biomass in anaerobic co-digestion, with a focus on farm-scale operations, were investigated.

The results illustrated that per-hectare biogas yields from *Salix* were mainly a function of biomass yield, not biomass recalcitrance. While 2 year-old shoots provided slightly higher BMP values compared to 3 year-old shoots, it is possible that the increased costs and energy inputs, and the lower biomass yields associated with more frequent coppicing, would offset the potential gains in methane production. High-yielding *Salix* clones would require 21 ha y^{-1} for supplying biomass for a 1000 m^3 reactor supplemented with 3:1 (VS) manure:*Salix* at an organic load of 2.0 g VS L^{-1} day^{-1}. Plant breeding of *Salix* with the aim to develop new clones with high and sustainable biomass production is a continuous process. Comparing older clones (in this study 78183 and 78195) with more recently released commercial clones, such as Tora, the improvement in biomass productivity gained through breeding is large and significant. Further productivity increases are likely to make *Salix* an even more attractive crop for biogas production.

Sequential co-digestion of comminuted but otherwise non-pretreated *Salix* biomass with cattle manure (1:1 VS) yielded a specific methane production comparable to values reported for single-stage co-digestion of hydrothermally pretreated *Salix* biomass. Volumetric methane production for the same system was close to the average reported for Swedish farm-scale co-digestion plants, and a considerable improvement over manure mono-digestion. Still, the co-digestion of *Salix* caused a slight reduction in specific methane potential. However, by using a sequential digester setup, the recalcitrance of willow biomass could largely be overcome, obviating the use of hydrothermal pretreatment when using this

crop as a co-digestion feedstock. This may allow smaller AD plants, such as many farm-scale facilities, to utilize *Salix* instead of herbaceous crops as a co-digestion substrate.

References

1. Scarlat, N.; Dallemand, J.-F.; Fahl, F. Biogas: Developments and perspectives in Europe. *Renew. Energy* **2018**, *129*, 457–472. [CrossRef]

2. European Biomass Association. A Biogas Road Map for Europe. Available online: http://www.aebiom.org/IMG/pdf/Brochure_BiogasRoadmap_WEB.pdf (accessed on 10 July 2020).

3. Lönnqvist, T.; Silveira, S.; Sanches-Pereira, A. Swedish resource potential from residues and energy crops to enhance biogas generation. *Renew. Sustain. Energy Rev.* **2013**, *21*, 298–314. [CrossRef]

4. Scarlat, N.; Fahl, F.; Dallemand, J.-F.; Monforti, F.; Motola, V. A spatial analysis of biogas potential from manure in Europe. *Renew. Sustain. Energy Rev.* **2018**, *94*, 915–930. [CrossRef]

5. Hagos, K.; Zong, J.; Li, D.; Liu, C.; Lu, X. Anaerobic co-digestion process for biogas production: Progress, challenges and perspectives. *Renew. Sustain. Energy Rev.* **2017**, *76*, 1485–1496. [CrossRef]

6. Monlau, F.; Barakat, A.; Trably, E.; Dumas, C.; Steyer, J.-P.; Carrère, H. Lignocellulosic Materials into Biohydrogen and Biomethane: Impact of Structural Features and Pretreatment. *Crit. Rev. Environ. Sci. Technol.* **2013**, *43*, 260–322. [CrossRef]

7. Börjesson, P.I.I. Energy analysis of biomass production and transportation. *Biomass Bioenergy* **1996**, *11*, 305–318. [CrossRef]

8. Boehmel, C.; Lewandowski, I.; Claupein, W. Comparing annual and perennial energy cropping systems with different management intensities. *Agric. Syst.* **2008**, *96*, 224–236. [CrossRef]

9. De Wit, M.; Faaij, A. European biomass resource potential and costs. *Biomass Bioenergy* **2010**, *34*, 188–202. [CrossRef]

10. Lijó, L.; Lorenzo-Toja, Y.; González-García, S.; Bacenetti, J.; Negri, M.; Moreira, M.T. Eco-efficiency assessment of farm-scaled biogas plants. *Bioresour. Technol.* **2017**, *237*, 146–155. [CrossRef]

11. Ericsson, N.; Nordberg, A.; Sundberg, C.; Ahlgren, S.; Hansson, P.-A. Climate impact and energy efficiency from electricity generation through anaerobic digestion or direct combustion of short rotation coppice willow. *Appl. Energy* **2014**, *132*, 86–98. [CrossRef]

12. Li, C.; Sun, L.; Simmons, B.A.; Singh, S. Comparing the Recalcitrance of Eucalyptus, Pine, and Switchgrass Using Ionic Liquid and Dilute Acid Pretreatments. *BioEnergy Res.* **2013**, *6*, 14–23. [CrossRef]

13. Healey, A.L.; Lupoi, J.S.; Lee, D.J.; Sykes, R.W.; Guenther, J.M.; Tran, K.; Decker, S.R.; Singh, S.; Simmons, B.A.; Henry, R.J. Effect of aging on lignin content, composition and enzymatic saccharification in Corymbia hybrids and parental taxa between years 9 and 12. *Biomass Bioenergy* **2016**, *93*, 50–59. [CrossRef]

14. Chundawat, S.P.S.; Beckham, G.T.; Himmel, M.E.; Dale, B.E. Deconstruction of Lignocellulosic Biomass to Fuels and Chemicals. *Annu. Rev. Chem. Biomol. Eng.* **2011**, *2*, 121–145. [CrossRef] [PubMed]

15. Teghammar, A.; Forgács, G.; Sárvári Horváth, I.; Taherzadeh, M.J. Techno-economic study of NMMO pretreatment and biogas production from forest residues. *Appl. Energy* **2014**, *116*, 125–133. [CrossRef]

16. Ohlsson, J.A.; Harman-Ware, A.E.; Sandgren, M.; Schnürer, A. Biomass Recalcitrance in Willow Under Two Biological Conversion Paradigms: Enzymatic Hydrolysis and Anaerobic Digestion. *BioEnergy Res.* **2019**, *311*, 1–11. [CrossRef]

17. Weih, M.; Hansson, P.-A.; Ohlsson, J.A.; Sandgren, M.; Schnürer, A.; Rönnberg Wästljung, A.C. Sustainable production of willow for biofuel use. In *Achieving Carbon-Negative Bioenergy Systems from Plant Materials*; Saffron, C., Ed.; Burleigh Dodds Science Publishing: Cambridge, UK, 2020; pp. 1–36. [CrossRef]

18. Olsson, M.; Samils, B. *Site Characterization at Energy Forest Production*; Department of Forest Soils, Swedish University of Agricultural Sciences: Uppsala, Sweden, 1984.

19. Schnürer, A.; Bohn, I.; Moestedt, J. Protocol for Start-Up and Operation of CSTR Biogas Processes. In *Hydrocarbon and Lipid Microbiology Protocols*; McGenity, T., Timmis, K., Nogales, B., Eds.; Springer: Berlin/Heidelberg, Germany, 2016; Volume 44, pp. 171–200.

20. ISO 13878. *Soil Quality—Determination of Total Nitrogen Content by Dry Combustion (Elemental Analysis)*; International Organization for Standardization (ISO): Geneva, Switzerland, 1998.

21. ISO 10694. *Soil Quality–Determination of Organic and Total Carbon after Dry Combustion (Elementary Analysis)*; International Organization for Standardization (ISO): Geneva, Switzerland, 1998.

22. Westerholm, M.; Hansson, M.; Schnürer, A. Improved biogas production from whole stillage by co-digestion with cattle manure. *Bioresour. Technol.* **2012**, *114*, 314–319. [CrossRef]

23. Westerholm, M.; Roos, S.; Schnürer, A. Syntrophaceticus schinkii gen. nov., sp. nov., an anaerobic, syntrophic acetate-oxidizing bacterium isolated from a mesophilic anaerobic filter. *FEMS Microbiol. Lett.* **2010**, *309*, 100–104. [CrossRef]

24. Bates, D.; Mächler, M.; Bolker, B.; Walker, S. Fitting Linear Mixed-Effects Models Using lme4. *J. Stat. Softw.* **2015**, *67*, 1–48. [CrossRef]

25. R Core Team. *R: A Language and Environment for Statistical Computing*; R Foundation for Statistical Computing: Vienna, Austria, 2020.

26. Lenth, R. *Emmeans: Estimated Marginal Means, AKA Least-Squares Means*, R package version 1.4.7. 2020. Available online: https://cran.r-project.org/web/packages/emmeans/index.html (accessed on 24 July 2020).

27. Ahlberg-Eliasson, K.; Nadeau, E.; Levén, L.; Schnürer, A. Production efficiency of Swedish farm-scale biogas plants. *Biomass Bioenergy* **2017**, *97*, 27–37. [CrossRef]

28. Mola-Yudego, B.; Díaz-Yáñez, O.; Dimitriou, I. How Much Yield Should We Expect from Fast-Growing Plantations for Energy? Divergences Between Experiments and Commercial Willow Plantations. *BioEnergy Res.* **2015**, *8*, 1769–1777. [CrossRef]

29. Adler, A.; Verwijst, T.; Aronsson, P. Estimation and relevance of bark proportion in a willow stand. *Biomass Bioenergy* **2005**, *29*, 102–113. [CrossRef]

30. Serapiglia, M.J.; Cameron, K.D.; Stipanovic, A.J.; Smart, L.B. Analysis of Biomass Composition Using High-Resolution Thermogravimetric Analysis and Percent Bark Content for the Selection of Shrub Willow Bioenergy Crop Varieties. *BioEnergy Res.* **2009**, *2*, 1–9. [CrossRef]

31. McCann, M.C.; Carpita, N.C. Biomass recalcitrance: A multi-scale, multi-factor, and conversion-specific property. *J. Exp. Bot.* **2015**, *66*, 4109–4118. [CrossRef] [PubMed]

32. Sykes, R.; Kodrzycki, B.; Tuskan, G.; Foutz, K.; Davis, M. Within tree variability of lignin composition in Populus. *Wood Sci. Technol.* **2008**, *42*, 649–661. [CrossRef]

33. Mata-Alvarez, J.; Dosta, J.; Romero-Güiza, M.S.; Fonoll, X.; Peces, M.; Astals, S. A critical review on anaerobic co-digestion achievements between 2010 and 2013. *Renew. Sustain. Energy Rev.* **2014**, *36*, 412–427. [CrossRef]

34. Estevez, M.M.; Linjordet, R.; Morken, J. Effects of steam explosion and co-digestion in the methane production from Salix by mesophilic batch assays. *Bioresour. Technol.* **2012**, *104*, 749–756. [CrossRef]

35. Estevez, M.M.; Sapci, Z.; Linjordet, R.; Schnürer, A.; Morken, J. Semi-continuous anaerobic co-digestion of cow manure and steam-exploded Salix with recirculation of liquid digestate. *J. Environ. Manag.* **2014**, *136*, 9–15. [CrossRef]

36. Li, R.; Tan, W.; Zhao, X.; Dang, Q.; Song, Q.; Xi, B.; Zhang, X. Evaluation on the Methane Production Potential of Wood Waste Pretreated with NaOH and Co-Digested with Pig Manure. *Catalysts* **2019**, *9*, 539. [CrossRef]

37. Lynd, L.R.; Liang, X.; Biddy, M.J.; Allee, A.; Cai, H.; Foust, T.; Himmel, M.E.; Laser, M.S.; Wang, M.; Wyman, C.E. Cellulosic ethanol: Status and innovation. *Curr. Opin. Biotechnol.* **2017**, *45*, 202–211. [CrossRef]

38. Eggeman, T.; Elander, R.T. Process and economic analysis of pretreatment technologies. *Bioresour. Technol.* **2005**, *96*, 2019–2025. [CrossRef]

39. Hopfner-Sixt, K.; Amon, T.; Bodiroza, V.; Kryvoruchko, V.; Milanovic, D.; Zollitsch, W.; Boxberger, J. Biogas Production from Agricultural Raw Materials: Characteristic Values for Assessing Material and Energy. *Landtechnik* **2006**, *61*, 148–149.

40. Alburquerque, J.A.; de la Fuente, C.; Bernal, M.P. Chemical properties of anaerobic digestates affecting C and N dynamics in amended soils. *Agric. Ecosyst. Environ.* **2012**, *160*, 15–22. [CrossRef]

41. Eich-Greatorex, S.; Vivekanand, V.; Estevez, M.M.; Schnürer, A.; Børresen, T.; Sogn, T.A. Biogas digestates based on lignin-rich feedstock—Potential as fertilizer and soil amendment. *Arch. Agron. Soil Sci.* **2017**, *64*, 347–359. [CrossRef]

42. Stürmer, B.; Pfundtner, E.; Kirchmeyr, F.; Uschnig, S. Legal requirements for digestate as fertilizer in Austria and the European Union compared to actual technical parameters. *J. Environ. Manag.* **2020**, *253*, 109756. [CrossRef]

43. Pulford, I.D.; Riddell-Black, D.; Stewart, C. Heavy Metal Uptake by Willow Clones from Sewage Sludge-Treated Soil: The Potential for Phytoremediation. *Int. J. Phytoremediat.* **2002**, *4*, 59–72. [CrossRef]