Biomass and Bioenergy 161 (2022) 106478

Contents lists available at ScienceDirect

Biomass and Bioenergy

journal homepage: www.elsevier.com/locate/biombioe

# Serial anaerobic digestion improves protein degradation and biogas production from mixed food waste

Ebba Perman [a,b], Anna Schnürer [a,b,*], Annika Björn [b,c], Jan Moestedt [b,c,d,**]

[a] Department of Molecular Sciences, Swedish University of Agricultural Sciences, Box 7015, Uppsala, SE-750 07, Sweden
[b] Biogas Research Center, Linköping University, Linköping, SE-581 83, Sweden
[c] Department of Thematic Studies - Environmental Change, Linköping University, Linköping, SE-581 83, Sweden
[d] Tekniska verken i Linköping AB (publ.), Box 1500, Linköping, SE-581 15, Sweden

ARTICLE INFO

*Keywords:*
Biogas
Anaerobic digestion
Serial digestion
Food waste
Protein degradation

ABSTRACT

Optimization of the biogas generation process is important to achieve efficient degradation and high methane yield, and to reduce methane emissions from the digestate. In this study, serial digester systems with two or three biogas reactors were compared with a single reactor, with the aim of improving degree of degradation and methane yield from food waste and assessing adaptation of microbial communities to different reactor steps. All systems had the same total organic load (2.4 g VS/(L d)) and hydraulic retention time (55 days). Serial systems increased methane yield by >5% compared with the single reactor, with the majority of the methane being obtained from the first-step reactors. Improved protein degradation was also obtained in serial systems, with >20% lower outgoing protein concentration compared with the single reactor and increasing $NH_4^+$-N concentration with every reactor step. This resulted in separation of high ammonia (>384 mg $NH_3$-N/L) levels from the main methane production, reducing the risk of methanogen inhibition. *Methanosarcina* dominated the methanogenic community in all reactors, but increases in the hydrogenotrophic genera *Methanoculleus* and *Methanobacterium* were observed at higher ammonia levels. Potential syntrophic acetate-oxidizing bacteria, such as MBA03 and *Dethiobacteraceae*, followed the same trend as the hydrogenotrophic methanogens. Phylum Bacteroidota family *Paludibacteraceae* was highly abundant in the first steps and then decreased abruptly, potentially linked to an observed decrease in degradation in the last-step reactors. Nevertheless, the results indicated a trend of increasing relative abundance of the potentially proteolytic genera *Proteiniphilum* and *Fastidiosipila* with successive reactor steps.

## 1. Introduction

Production of biogas through anaerobic digestion (AD) is a stepwise degradation process of organic material, carried out by a complex microbial community in an anaerobic environment [1]. Biogas production has several benefits, *e.g.*, it is a sustainable treatment method for organic wastes and the methane in biogas is a renewable energy carrier that can be used as vehicle fuel or for production of electricity and heat [2]. Moreover, during the AD process, mineral nutrients in the wastes are concentrated and the resulting digestate can be used as biofertilizer, thereby contributing to recycling of nutrients and reduced use of artificial fertilizer [3].

In order to reach the full potential of AD in terms of economic and environmental benefits it is important to obtain a high degree of degradation of the organic material. Many previous studies have investigated different strategies to improve degradation and efficiency of various AD processes, including both management and technological approaches. So far, a lot of effort has been put on materials rich in lignocellulose, difficult to degrade and thus typically giving low efficiency of the biogas process [4]. However, several recent publications have reported that also degradation of proteins in AD can be inefficient and result in high levels of residual protein, representing an unexploited potential of methane as well as ammonium-nitrogen ($NH_4^+$-N), in the outgoing digestate [5–8]. Even so, less effort in the scientific literature has been devoted to understanding conditions giving efficient degradation of proteins.

* Corresponding author. Department of Molecular Sciences, Swedish University of Agricultural Sciences, Box 7015, Uppsala, SE-750 07, Sweden.
** Corresponding author. Biogas Research Center, Linköping University, Linköping, SE-581 83, Sweden.
*E-mail addresses:* anna.schnurer@slu.se (A. Schnürer), jan.moestedt@tekniskaverken.se (J. Moestedt).

https://doi.org/10.1016/j.biombioe.2022.106478
Received 23 November 2021; Received in revised form 21 April 2022; Accepted 1 May 2022
Available online 18 May 2022
0961-9534/© 2022 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

Different types of food waste, *e.g.* source sorted organic household waste and residues from food industry, can vary greatly in its chemical composition, but generally has high protein and fat concentrations as well as a relatively low content of complex carbohydrates [9]. Fat and protein are energy-rich, so food waste has high biomethane potential and, owing to a low level of lignocellulose, are relatively easy to degrade [9]. AD of food waste even needs to be managed carefully, as fast degradation can cause accumulation of volatile fatty acids (VFA) and a subsequent drop in pH. Additionally, high levels of protein can lead to inhibitory levels of the fermentation product ammonia ($NH_3$), which is toxic to the microbial community and especially the methanogenic community [9,10]. Nevertheless, proteins have been shown to represent the major fraction of non-degraded macromolecules in the digestate [8].

Previous studies have evaluated degradation of recalcitrant structures by replacing a single reactor with a main methanogenic reactor followed by a methanogenic post-digester that collects residual gas potential. This has been successful for agricultural residues with high levels of recalcitrant lignocellulosic structures; both for manure [11], and crop residues, where serial reactors improved conversion of cellulose and hemicellulose compared with a single reactor [12]. In the present study we hypothesize that a serial reactor setup, in comparison to a single reactor, could enable improved degradation of food waste, and specifically proteins, in the same way as previous studies on serial digestion have shown an improved degree of degradation of recalcitrant lignocellulosic substrates [11,12]. In line with this hypothesis, Nordell et al. [13] achieved improved protein degradation in sewage sludge upon addition of a post-digester step to the process. During serial digestion, the process can be set up in different ways in regard to hydraulic retention time (HRT) and organic loading rate (OLR). For food waste, previous studies have mainly applied a high load and short retention time in the first reactor with the aim to obtain microbial phase separation [14]. Separation of hydrolysis/acidogenesis and methanogenesis steps can be a way of improving the degradation of food waste and many studies have shown promising results in terms of process stability, high methane yields, volatile solid (VS) reduction, and potential to extract additional products, such as VFA or hydrogen ($H_2$) gas, from one of the process stages [9,14–17]. However, as mentioned above, serial digestion can also be applied without phase separation and with methanogenesis in all reactors, a setup that remains to be investigated for processes operated with food waste.

In the current study, we are evaluating a setup with serial methanogenic reactors for biogas production from food waste. The main aims of this study were to investigate the possibility for improved process performance and degradation of recalcitrant structures, *e.g.* proteins, compared with a single reactor. If successful, this approach could give both higher methane yield and reduce the risk of methane emissions from the digestate. An additional aim was to investigate the microbial community structure in the different reactor steps. It has been shown that stage separation of the AD process leads to differences in the microbial communities in the different reactor steps, with enrichment of hydrolytic/acidogenic species in the first reactor [15,18]. However, the effect of a serial digester setup on the microbial community structure in different reactor steps has not yet been studied, nor how this is coupled to the degree of degradation of different molecular structures, *i.e.*, proteins, lipids, and carbohydrates. For the study, an industrial-scale biogas plant using food waste from households, in co-digestion with slaughterhouse waste and industrial organic waste, was used as a model for an experimental lab-scale setup. Systems with one, two, and three serial reactors, all with the same total HRT, were operated and compared with regard to efficiency and process performance. The industrial-scale plant has already access to several reactor tanks which, based on the result from this study, easily could be reorganized in a way that optimizes the overall process performance, without need for construction of new digesters.

## 2. Material and method

### 2.1. Experimental setup

Three systems of laboratory-scale reactors were set up (see graphical abstract): a one-step system with a single reactor (A1), a serial system with two reactors (B1 and B2), and a serial system with three reactors (C1, C2, and C3). In the two- and three-step systems, the first reactors were fed with substrate, while the other reactor/s were fed the outgoing digestate from the previous reactor step. Inoculum for the reactors was collected from a full-scale co-digestion plant in Linköping, Sweden, that runs a two-step completely-stirred tank reactor (CSTR) process (total active volume 17,100 $m^3$). The full-scale process is operated at 42 °C and 35 days HRT in the first step and 41 °C and 20 days HRT in the second step. For the laboratory-scale reactors A1, B1, C1, and C2, the inoculum used was collected from the first reactor step of the biogas plant, while the inoculum for B2 and C3 was collected from the second reactor step. The co-digestion plant receives food waste from households (50% of incoming wet weight), organic industrial residues (25%), and slaughterhouse waste (25%) and is fed an average total OLR of 4 kg VS/($m^3$ d) to the first-step reactor. The first-step reactors in this lab-scale study (A1, B1, C1) were fed substrate with a VS content of 13.3% retrieved from a hygienization tank at the co-digestion plant (temperature 70 °C). This substrate was collected on a single occasion and frozen in batches of 5 L until thawing and use. A process additive (Kemira Oyj, Helsingborg, Sweden, developed and patented by Tekniska verken i Linköping AB (publ.)) containing iron ($Fe^{2+}$/$Fe^{3+}$), cobalt ($Co^{2+}$), nickel ($Ni^{2+}$) and hydrochloric acid (HCl, <0.5%) was added in the substrate, as in the full-scale plant. The dose was set to suppress $H_2S$ levels to <50 ppmv in the biogas and supply the reactor microorganisms with extra trace elements (0.5 mg/kg $Co^{2+}$ and 0.2 mg/kg $Ni^{2+}$).

The digesters at the full-scale co-digestion plant were used as the basis for the experimental design in lab-scale, in terms of relative reactor volumes, HRT, OLR, and process temperatures. The reactor experiment was performed with CSTRs (developed and patented by Tekniska verken [19]) with an active volume of 3.3–9.1 L and operated at 41–42 °C (Table 1). For practical reasons, the initial steps (B1 and C1) were over-dimensioned in comparison with the following steps, in order to allow excess volume for sampling. Initially the total HRT was set to 55 days in all systems, however after 21 days of operation these values had to be slightly changed to compensate for the volume reduction from production of biogas. The mass of gas produced, calculated using the ideal gas law, was estimated to be 11–12% of ingoing substrate volume. Thus, to reach correct volume relationships between the reactors, the HRT of B2 and C2 was adjusted from 20 to 23 days and from 19 to 21 days, respectively (Table 1). In the results, the gas production presented was normalized to the relative volume of the reactors (Table 1). The reactors were fed semi-continuously once per day, 7 days per week, and the volume was adjusted 5 days per week. The reactors were operated for 203 days in total. The process parameters presented here are average values of measurements taken after day 174, unless otherwise stated, after three complete HRTs for all three systems.

**Table 1**
Process parameters for the laboratory-scale test reactors operating in mono-digestion or in series with two or three reactors.

| System | Reactor | HRT (days) | OLR (g VS/(L/d)) | Reactor volume (L) | Temperature (°C) | Relative volume (%) |
|---|---|---|---|---|---|---|
| One-step | A1 | 55 | 2.43 | 9.1 | 42 | 100 |
| Two-step | B1 | 35 | 3.82 | 9.1 | 42 | 64 |
| | B2 | 23 | | 4 | 41 | 36 |
| Three-step | C1 | 23 | 5.74 | 9.1 | 42 | 42 |
| | C2 | 21 | | 6.3 | 42 | 35 |
| | C3 | 12 | | 3.3 | 41 | 23 |

### 2.2. Analytical methods

Volumetric gas production was measured online with a Ritter milligas counter (MGC-10, Ritter, Waldenbuch, Germany) and methane concentration was determined with a gas sensor (BlueSens, Herten, Germany). Gas volume was normalized for standard temperature and pressure (273.2 K and 1.01325 bar). Gas composition ($CH_4$, $CO_2$, $H_2S$, $H_2$, $O_2$) was further analyzed using a Biogas 5000 device (Geotech Instruments, Coventry, UK). The VFA content was analyzed with a Clarus 550 gas chromatograph (PerkinElmer, Waltham, MA, USA) with a packed Elite-FFAP column (PerkinElmer, USA) for acidic compounds [20]. Total $NH_4^+$-N was analyzed as the sum of $NH_4^+$-N (aq) + ammonia-nitrogen ($NH_3$-N) (aq), by distillation (Kjeltec 8200, FOSS in Scandinavia, Sweden) in acidic solution ($H_3BO_3$). The $NH_4^+$-N concentration was then determined by titration with HCl (Titro 809, Metrohm, Herisau, Switzerland) according to the Tecator method for Kjeltec ISO 5664. Kjeldahl-nitrogen was determined using the same procedure and equipment as $NH_4^+$-N, with the exception that the samples were pre-treated with $H_2SO_4$ and then heated to 410 °C for 1 h. The pH was measured with a potentiometric pH meter at 25 °C, using a Hamilton electrode (WTW Inolab, Houston, TX, USA). Lignin content was measured using standardized method Tappi T 222 (acid hydrolysis/gravimetrical extraction), at MoRE Research Örnsköldsvik AB, Sweden. Bound and complexed sugar concentrations (xylose, mannose, glucose, galactose and arabinose, with measurement uncertainty of 15%, 10%, 5%, 10% and 10% respectively) were measured using SCAN-CM 71:09 (GC-MS), also at MoRE Research Örnsköldsvik AB, Sweden. Concentrations of hemicellulose and cellulose were estimated from the sugar composition. Raw fat concentration was measured using method NMKL 160 mod. (acid hydrolysis/gravimetric extraction) (measurement uncertainty 30%) at Eurofins Food & Feed Testing Sweden, Lidköping, Sweden. Protein concentrations were measured at three time-points (day 148, 169, and 199) and calculated according to equation (1). Total solids (TS) and volatile solids (VS) were measured according to Swedish standard methods (1981, SS028113). Samples for macromolecule analyses (lignin, sugars and fat) were taken twice (day 95 and 193). Since the process was stable, samples were homogenous, and substrate composition was the same throughout the experiment, these samplings were considered to be representative for the processes.

$$\text{Raw protein} = (\textit{Kjeldahl-N} - \textit{NH}_4^+\textit{-N}) \bullet 6.25 \quad (1)$$

### 2.3. RMP and methane production rate determination

Batch tests were performed to investigate the residual methane potential (RMP) of the different digestates (taken at day 203) and to evaluate the degradation rate of the key substrates: cellulose (crystalline cellulose, Macherey-Nagel GmbH & Co. KG, Germany), fat (refined rapeseed oil, Di Luca & Di Luca AB, Sweden), and protein (gelatin, Haugen-Gruppen AB, Sweden). For this, triplicate 300 mL portions (containing 9.6, 10.2, 8.4, 11.4, 9.0 and 8.4 g VS in A1, B1, B2, C1, C2, and C3, respectively) of each digestate were added to individual bottles (594 mL) and methane production was measured during incubation at 38 °C, using an automatic methane potential test system, AMPTS II (Bioprocess Control, Lund, Sweden). RMP was also measured for digestates without substrate and for digestates with added substrate (cellulose, fat, or protein) to determine how well and at what rate each substrate was digested, using 1.8 g of substrate (6.0 g VS/L). Specific methane production from the substrates was calculated according to Ref. [21]. The experiment was run until gas production leveled off and results collected until day 25 were used. To compare the degradation rates of the added substrates, initial degradation rate was calculated using 50% of the average accumulated methane production at day 25 in digestate from reactor A1 as a cut-off value. Average methane production per day until the time-point where the cut-off value was reached was calculated for each substrate.

### 2.4. 16S rRNA gene sequencing

DNA extractions were performed on samples from all digestates, taken at day 200, and on the two inocula from the co-digestion plant in Linköping. All samples were stored at −20 °C until extraction. Extraction was done in triplicate, using the FastDNA Spin Kit for Soil (MP Biomedicals Europe) according to manufacturer's instructions, with the exceptions that aliquots of 200 μL of sample were used and an extra wash step was included to remove humic acids as described in Ref. [22]. DNA was eluted using 70 μL of water. DNA concentrations were measured using a Qubit 3.0 Fluorometer with a Qubit dsDNA BR Assay Kit (Invitrogen, Thermo Fisher Science, Waltham, MA, USA). 16S rRNA-gene amplicon libraries for bacteria and archaea were prepared from the DNA samples as described previously [23]. The concentrations of the final PCR products were measured with Qubit 3.0 Fluorometer (Invitrogen, Thermo Fisher Science, Waltham, MA, USA) and 20 ng of DNA from each sample were submitted for sequencing with the Illumina MiSeq platform at SciLifeLab in Uppsala, Sweden.

16S rRNA-gene sequences were processed according to the DADA2 pipeline tutorial (version 1.16) [24], using the DADA2 package (version 1.16.0) in R (version 4.0.2). Primer and adapter sequences were removed from the raw data using Cutadapt [25]. For bacteria, parameters for filtering and trimming the reads to minimize error rates were selected using FIGARO [26]. Trimming sites 236 and 173 for forward and reverse reads, respectively, were selected and maximum number of expected errors was set to 1. For archaea, trimming sites 220 and 200 and maximum number of expected errors of 2 and 5 for forward and reverse reads, respectively, were selected. One replicate of sample C3 in the archaea dataset had few (544) reads, and was therefore removed from the subsequent analyses. Taxonomy was assigned to the sequences using the Silva reference database training set (version 138) [24,27]. The package phyloseq (version 1.32.0) was used to organize the data and visualize relative abundances of the sequences.

Bacterial communities were further analyzed using non-metric multidimensional scaling (NMDS) in R to visualize the similarity between samples from different reactors in terms of bacterial community structure, plotted together with concentrations of macromolecules (protein, fat, sugars, and lignin). To generate the distance matrix, the ordinate function in the package phyloseq was used with the Bray-Curtis calculation method. Plots were generated using the function ggplot in package ggplot2 (version 3.3.3) and macromolecule concentrations were fitted to the data using the function envfit in package vegan (version 2.5.6). Ellipses representing the 50% confidence intervals for the most abundant phyla (represented by more than three data points in the NMDS plot) were included in the NMDS plot showing the phyla, assuming multivariate normal distributions.

### 2.5. Calculation of retention time distribution

The retention time distribution (RTD) for an ideal CSTR was used here (eq. (A.1)) to indicate how the probability distribution of retention of material in the system changed when CSTRs were connected in series [28]. The RTD for a combination of CSTRs in series, with the HRTs used in this experiment (Table 1), was obtained using eq. (A.2) [28]. The probability of material leaving the system within the total HRT was calculated based on eq. (A.3), using the integrate function in R.

### 2.6. Data analysis

Statistical analyses on the results were carried out in R programming language (version 4.0.2). One sample *t*-test with confidence level 95% was used for pair-wise comparisons of daily specific methane production between the three different systems and between the three different

first-step reactors. One-way ANOVA with confidence level 95% was used for comparisons of values in the different reactors: macromolecule concentrations, $NH_4^+$-N- and $NH_3$-N-concentrations, pH, VS content, VS reduction, RMP, and methane production rates from different substrates. The t.test function was used for t-tests and the linear model (lm) followed by the anova function was used for ANOVA. Pairwise comparisons between reactors were made using the emmeans function in package emmeans (version 1.5.2.1). In case of heteroscedasticity, values were log-transformed before performing the ANOVA.

## 3. Results

### 3.1. Effects of serial AD on methane production efficiency

The reactors were operated for 203 days in total and during this time fed semi-continuously with food waste once a day. Measurements presented here were made after all systems had reached three complete HRTs (from day 174), unless otherwise stated. Specific methane production (SMP) for the complete systems was within the range 489–517 L $CH_4$/kg VS (Table 2, Figure B1). During the course of the experiment the reactors remained stable, both in terms of gas production and process parameters. VFA concentrations were below the detection limit in all reactors throughout the experiment. The average daily SMP was significantly higher ($p < 0.05$) in the serial systems than in the single reactor, reaching 5.7% and 5.2% higher in the two-step and three-step system, respectively (Fig. 1). However, there was no significant difference in SMP between the two serial systems ($p = 0.43$). Most of the methane produced from each serial system was generated in the first-step reactor (96% and 92% in B1 and C1, respectively) and small contributions were obtained from the second and third steps (Table 2). Comparing the SMP in the first-step reactors (Table 2), B1 had significantly higher methane production than both A1 and C1 ($p < 0.05$). However, SMP did not differ significantly between A1 and C1 ($p = 0.56$).

The VS reduction increased with every reactor step and was finally >80% in the two serial systems, which was a significant improvement ($p < 0.05$) in comparison with the single CSTR (79%) (Table 2). In line with this, an increase in both $NH_4^+$-N and $NH_3$-N concentrations was observed across the steps in the serial systems (Table 2). The $NH_4^+$-N and $NH_3$-N concentrations in the final digestate from the serial systems (B2 and C3) were significantly higher than those in the final digestate from A1 ($p < 0.05$).

Among the reactors, the highest methane content in the gas was obtained in reactor B1 (65%) (Table 2). The highest methane content in the three-stage system was obtained in reactor C2 (64%). Lower methane content, of around 59%, was obtained in the last-step reactors (B2 and C3).

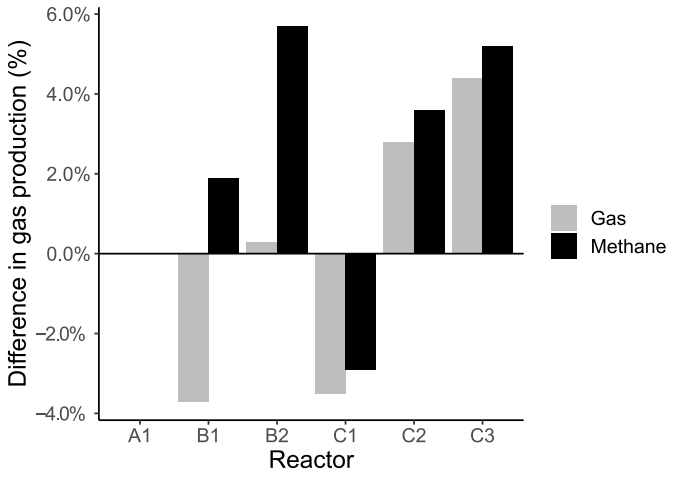


**Fig. 1.** Difference (%) in average methane and gas production from each serial reactor compared with reactor A1. Reactor A1 was operated as a single reactor, and B1 and B2 and C1, C2, and C3 in two and three steps in series, respectively. The accumulated differences for each system are shown.

### 3.2. Retention time distributions with serial reactors

To evaluate the probability of material leaving the reactor system within the HRT, the theoretical RTD was calculated for each system. The RTD values obtained illustrated the probability distribution of a pulse of an inert material added at time $t = 0$ to a reactor system with one, two, or three serial ideal CSTRs (Fig. 2). With a single reactor, the probability of material spending less time in the reactor than the HRT was 63%. In the two-step system with the same total HRT this probability was reduced to 60%, while replacing the single reactor with the three-step system reduced the probability further, to 58%. Thus increasing the number of reactors within the system, without changing the total HRT, increased the average time material spent in the reactor system.

### 3.3. Macromolecule concentrations and RMP in digestate

Concentrations of fat, lignin, and sugars (xylose, mannose, glucose, galactose, and arabinose) were measured in digestate from all reactors (sampled at day 95 and 193) (Figure C1). Measured sugar was assumed

**Table 2**
Analytical data for the digestate and gas produced from the different test reactors. Reactor A1 was operated as a single reactor, B1 and B2 as a two-step serial system, and C1, C2, and C3 as a three-step serial system. Letters indicate statistical significance (values with different letters differ significantly ($p < 0.05$)). Columns are compared separately.

| Reactor | $NH_4^+$-N (g/kg)* | $NH_3$-N (mg/kg)* | pH* | VS in digestate (%)* | VS reduction (%)* | Methane content (%)** | SMP complete system (L $CH_4$/kg VS)** | SMP first-step reactors (L $CH_4$/kg VS)** | Methane production (%)*** |
|---|---|---|---|---|---|---|---|---|---|
| A1 | 3.0 (0.1)$^{a}$ | 287 (9.5)$^{a}$ | 7.8 (0.0)$^{ab}$ | 3.2 (0.0)$^{a}$ | 78.9 (0.3)$^{a}$ | 61.8 (0.5) | 489 (24)$^{a}$ | 489 (24)$^{a}$ | 100.0 |
| B1 | 2.7 (0.1)$^{b}$ | 261 (5.6)$^{ab}$ | 7.8 (0.0)$^{ab}$ | 3.4 (0.0)$^{b}$ | 77.3 (0.3)$^{b}$ | 65.4 (0.2) | 517 (20)$^{b}$ | 499 (18)$^{b}$ | 96.3 |
| B2 | 3.4 (0.1)$^{ce}$ | 395 (6.4)$^{c}$ | 7.9 (0.1)$^{ac}$ | 2.8 (0.1)$^{c}$ | 81.0 (0.4)$^{c}$ | 59.9 (1.1) | | ND | 3.7 |
| C1 | 2.4 (0.0)$^{d}$ | 200 (24.8)$^{b}$ | 7.7 (0.0)$^{b}$ | 3.8 (0.1)$^{d}$ | 73.4 (0.4)$^{d}$ | 62.2 (0.2) | 515 (14)$^{b}$ | 475 (14)$^{a}$ | 92.3 |
| C2 | 3.3 (0.1)$^{c}$ | 384 (6.9)$^{c}$ | 8.0 (0.1)$^{c}$ | 3.0 (0.0)$^{e}$ | 79.1 (0.3)$^{a}$ | 64.2 (0.5) | | ND | 6.2 |
| C3 | 3.5 (0.1)$^{e}$ | 543 (58.5)$^{d}$ | 8.0 (0.1)$^{c}$ | 2.8 (0.1)$^{c}$ | 80.4 (0.7)$^{c}$ | 59.3 (1.8) | | ND | 1.5 |

*Mean values based on three measurements. Standard deviation within brackets.
**Mean values based on data from day 174–203. Standard deviation within brackets.
***Relative methane production, indicating how much each reactor contributed to the total amount of methane produced from each system.ND = not determined.

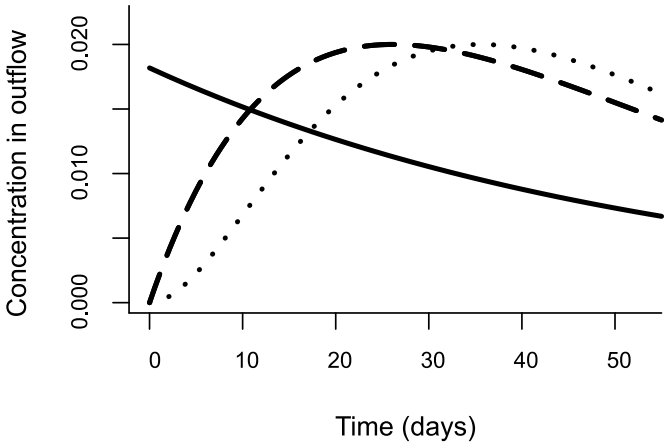


**Fig. 2.** Retention time distribution (RTD) for the three systems with one (solid line), two (dashed line), or three (dotted line) reactors in series.

to be bound as cellulose and hemicellulose. No significant differences between the reactors in concentrations of fat, lignin, or bound sugar were detected ($p \geq 0.05$). The measured concentrations were within the range 8–14, 3–5 and 2–3 g/kg for lignin, sugar, and fat, respectively (Figure C1). Among the measured macromolecules, the highest concentrations were obtained for protein (12–19 g/kg), with the lowest and highest concentration in reactor C3 and C1, respectively (Fig. 3). Comparison of protein concentrations (measured on day 148, 169, and 199) in digestate from the three different systems showed a significant ($p < 0.05$) improvement in protein degradation with the serial systems (Fig. 3). Overall, 22% and 23% lower protein concentration was obtained in the two- and three-step system, respectively, in comparison with reactor A1.

As an indicator of degradation efficiency, RMP in the digestate was measured, *i.e.*, the accumulated amount of methane produced from digestate during 25 days of incubation (Fig. 4, Figure C2). The highest RMP was obtained for digestate from reactor C1 and the lowest for digestate from C3, although levels in C3 were not significantly different from those in reactor B2 ($p = 0.81$).

Based on the macromolecule analysis, theoretical RMP in the digestate was calculated, assuming that the sugars, protein, and fat (not lignin) contributed to the RMP and assuming theoretical methane potential for these compounds of 415, 496, and 1014 L $CH_4$/kg

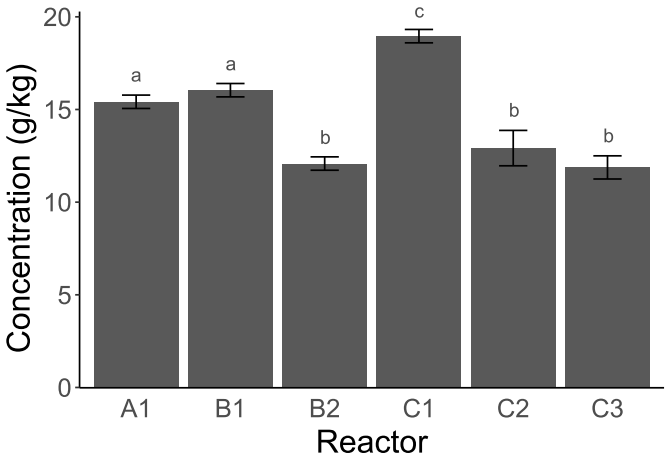


**Fig. 3.** Protein concentrations in the different reactor systems studied: A1 (single reactor), B1 and B2 (operated in series), and C1, C2, and C3 (operated in series). All reactor systems had the same total organic loading rate and hydraulic retention time (values with different letters differ significantly ($p < 0.05$)).

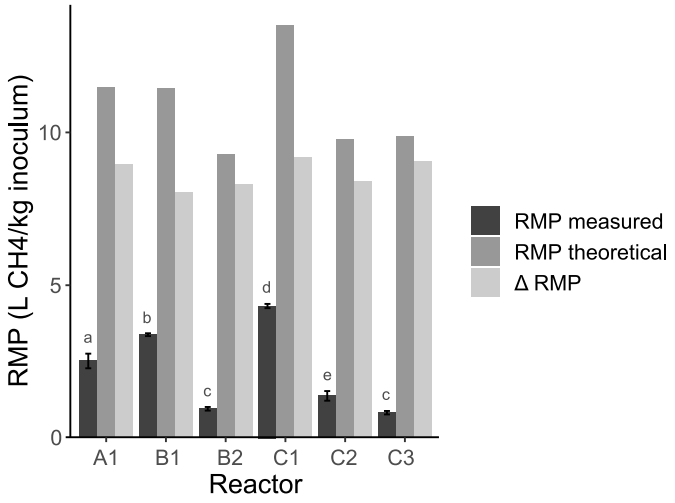


**Fig. 4.** Residual methane potential (RMP) in digestate from a single reactor (A1), reactors in a two-step system (B1 and B2), and reactors in a three-step system (C1, C2, and C3), after 25 days of incubation, measured in triplicate. Error bars represent standard deviation for the measured RMP. Theoretical RMP is based on sugar, protein, and fat concentrations in the digestates, ΔRMP represents the difference between theoretical and measured RMP. Letters indicate statistical significances (values with different letters differ significantly ($p < 0.05$)).

respectively [29]. A comparison between the theoretical and measured RMPs showed that less than 35% of the theoretical methane potential was reached in all digestates (Fig. 4). The difference between the theoretical and measured RMP was around 8–9 L $CH_4$/kg digestate for all samples (Fig. 4).

### 3.4. Conversion of protein, fat, and cellulose in digestate

Methane production from digestate after addition of protein, cellulose, and fat was evaluated in batch tests. To compare the degradation rates in the different reactors, a rate coefficient was calculated using the cut-off values (as described in section 2.3) 167 mL, 155 mL, and 442 mL for protein, cellulose, and fat, respectively (solid line, Figure C3).

For all substrates, the digestate from the first-step serial reactors (B1 and C1) had significantly higher conversion rate than the digestate from the second- and third-step reactors ($p < 0.05$) (Table 3). The most pronounced difference was seen for protein degradation, which was much higher in the tests started with digestate from C1. In general, the rates for fat and protein decreased in the order: C1 > B1 > A1 > C2 > B2 > C3. For cellulose, however, the digestate from A1 had a relatively slow conversion rate and behaved more like that from the second-step reactors (B2 and C2).

**Table 3**
Methane production rates from fat, protein, and cellulose substrates added to digestate from a single reactor (A1), reactors in a two-step system (B1 and B2), and reactors in a three-step system (C1, C2, and C3). The methane production rates are given as the average volume per day (mL $CH_4$/(g VS d)) until a cut-off time-point was reached. Methane production rates with different letters differ significantly ($p < 0.05$). Rate coefficients for each substrate are compared separately.

| Reactor | Fat | Protein | Cellulose |
|---|---|---|---|
| A1 | 113.6 (11.6)$^{a}$ | 96.2 (3.3)$^{a}$ | 35.7 (6.4)$^{ab}$ |
| B1 | 136.4 (38.7)$^{a}$ | 123.6 (46.4)$^{a}$ | 70.7 (2.3)$^{c}$ |
| B2 | 45.6 (2.0)$^{b}$ | 31.9 (7.0)$^{bc}$ | 29.5 (1.6)$^{a}$ |
| C1 | 133.9 (37.6)$^{a}$ | 208.8 (34.9)$^{d}$ | 51.5 (0.4)$^{e}$ |
| C2 | 67.5 (6.7)$^{b}$ | 50.8 (2.8)$^{b}$ | 39.3 (0.6)$^{b}$ |
| C3 | 42.1 (3.0)$^{b}$ | 21.4 (0.4)$^{c}$ | 23.3 (0.4)$^{d}$ |

### 3.5. Microbial community structure

The sequence analysis of 16S rRNA genes indicated that the archaeal community in all reactors was almost entirely dominated by the genus *Methanosarcina* (Fig. 5a). *Methanoculleus* and *Methanobacterium* were also observed at relative abundance >1% in some of the reactors. *Methanobacterium* increased in relative abundance in the second and third reactor steps in comparison with the first step. The bacterial community in the reactors was dominated mainly by the phyla Actinobacteriota, Bacteroidota, Cloacimonadota, and Firmicutes (Fig. 5b). Caldatribacteriota, Synergistota, and Thermotogota were also represented in some of the reactors, but at lower relative abundances.

The relative abundance of Bacteroidota was high in reactors B1 and C1, and decreased abruptly in the next reactor in the system. The most abundant genus within the Bacteroidota was H1, classified as a genus within the family *Paludibacteraceae* (Table 4). H1 had 23% and 31% relative abundance in B1 and C1, respectively, and this decreased to 1% and <1% in B2 and C2, respectively. Other groups showing a trend for slightly decreasing abundance over reactor steps were the family *Rikenellaceae* (phylum Bacteroidota) and genus *Sedimentibacter* (phylum Firmicutes) (Table 4).

For many taxa, a trend for increasing relative abundance over reactor steps was observed (Table 4). A representative of the phylum Bacteroidota, *Proteiniphilum*, was detected in all systems, but in highest relative abundance in reactor A1 (10%). In the serial reactor systems, the relative abundance of this genus was lower, but still with an increasing trend between reactors. A similar trend was seen for *Gallicola*, one of the dominant genera within Firmicutes, which accounted for almost 35% of the sequences in reactor A1. In the serial systems it had lower relative abundance, but it increased between reactors in both the two- and three-step systems. Other groups showing an increasing trend over reactor steps were MBA03 within the class *Limnochordia* (phylum Firmicutes), DTU014 within the class *Incertae Sedis* (phylum Firmicutes), the families *Dethiobacteraceae* and *Erysipelotrichaceae* (phylum Firmicutes), *Fastidiosipila* (phylum Firmicutes), and *Acetomicrobium* (phylum Synergistota).

One of the most highly abundant phyla in all reactor systems was Cloacimonadota, which was represented mainly by two groups; the W5 genus within the family *Cloacimonadaceae* and the W27 family within the order Cloacimonadales. W27 had relative abundance around 25% in both reactors in the two-step system and also in reactor C2. Group W5 was present at lower abundances, with the highest abundance in reactor A1, where it accounted for 4% of the sequences.

Although the two-step system in this study was operated in the same manner (but a down-scaled version) as the co-digestion biogas system used as the source of inocula, these systems were not similar in terms of bacterial community structure, suggesting a downscaling effect. Instead, the single reactor (A1) showed the highest bacterial community similarity with the original inocula from the co-digestion plant (Figure D1). Actinobacteriota was present at high relative abundances in the serial reactors, but at abundances <1% in the original inocula and in the single-step system (Table 4). The genus *Actinomyces* within Actinobacteriota was highly abundant, especially in the three-step system, comprising up to 44% of the sequences in reactor C3. Another effect of downscaling was a clear decrease in relative abundance of Thermotogota, mainly represented by *Defluviitoga tunisiensis* (Table 4).

A NMDS plot showing the bacterial phyla was created to look for clusters in relation to macromolecule concentrations (Fig. 6). The five most abundant phyla were marked with ellipses indicating the multivariate normal distribution with a confidence interval of 50%. A positive correlation was observed between high protein concentration and Actinobacteriota, Bacteroidota, and Firmicutes, and a trend for a correlation was seen between high fat concentration and a high relative abundance of Actinobacteriota and Bacteroidota. Trends for lower protein, fat, and sugar concentrations with higher abundance of Cloacimonadota, and lower lignin and sugar concentrations with higher abundance of Synergistota, were observed (Fig. 6).

## 4. Discussion

### 4.1. Degree of degradation and methane production

The SMP from the reactor systems (489–517 L $CH_4$/kg VS) was in line with previously reported values of biomethane potential for food waste [9,10]. In this study, serial digestion increased methane production by more than 5% in both the two- and three-step systems compared with a single reactor with the same total HRT (Fig. 1). This represents an increase with >25 L $CH_4$/kg VS, which would give a considerable increase in methane yield on industrial scale. For example, with the system used as the basis for this study design (total volume 17,100 $m^3$, total OLR 2.43 kg VS/($m^3$ d)), a 5% increase in SMP would increase the volumetric production with >1,000 $m^3$ $CH_4$/d (corresponding to >9.8 MWh/d [2]). Previous evaluations of serial digestion in comparison with single CSTRs have found increases in gas production of around 8–15% [12,30]. The setup in those studies was similar to that in the present study, but using agricultural waste with high lignocellulose content as substrate, which might benefit more from serial digestion than relatively easily degradable food waste. The improvement in substrate conversion obtained by serial digestion has previously been attributed to the prolonged average

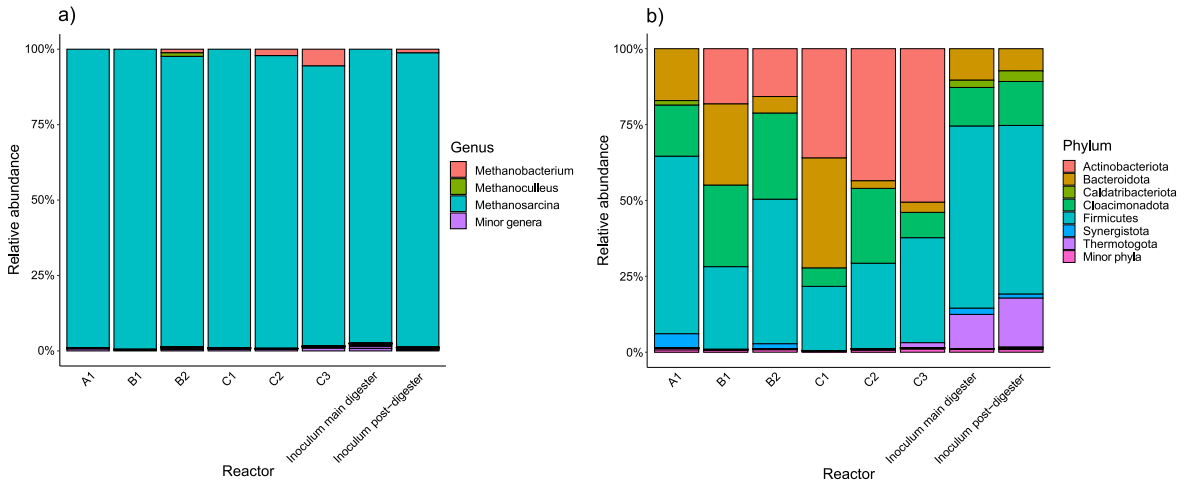


**Fig. 5.** Relative abundances of a) archaea at genus level and b) bacteria at phylum level, based on 16S rRNA-gene sequences in digestate from reactor A1 (operated as single reactor), B1 and B2 (operated in series), and C1, C2, and C3 (operated in series), and in the two inocula. 'Inoculum main digester' was used for inoculation of reactors A1, B1, C1, and C2. 'Inoculum post-digester' was used for inoculation of reactors B2 and C3.

**Table 4**
Relative abundances (%) of bacterial genera in digestate from the different reactors and the starting inocula. Genera with abundance >1% in at least one of the samples are given. Taxa with abundance <1% are grouped within "Minor taxa". Reactor A1 was operated as a single reactor, and B1 and B2 and C1, C2, and C3 in two and three reactor steps in series, respectively. 'Inoculum main digester' was used for inoculation of reactors A1, B1, C1, and C2. 'Inoculum post-digester' was used for inoculation of reactors B2 and C3.

| Phylum | Class | Order | Family | Genus | A1 | B1 | B2 | C1 | C2 | C3 | Inoculum main digester | Inoculum post-digester |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Actinobacteriota | Actinobacteria | Actinomycetales | Actinomycetaceae | Actinomyces | 0.7 | 16.7 | 13.9 | 32.7 | 38.5 | 44.0 | 0.1 | 0.2 |
| Actinobacteriota | Actinobacteria | NA | NA | Actinobacteria _cl | 0.0 | 1.4 | 1.8 | 2.4 | 4.9 | 6.3 | 0.0 | 0.0 |
| Bacteroidota | Bacteroidia | Bacteroidales | Paludibacteraceae | H1 | 5.6 | 23.0 | 1.3 | 31.2 | 0.2 | 0.4 | 1.0 | 0.3 |
| Bacteroidota | Bacteroidia | Bacteroidales | Dysgonomonadaceae | Proteiniphilum | 10.1 | 1.9 | 2.7 | 0.5 | 0.8 | 1.6 | 1.5 | 3.7 |
| Bacteroidota | Bacteroidia | Bacteroidales | Rikenellaceae | Rikenellaceae_RC9_gut_group | 0.0 | 0.5 | 0.2 | 1.8 | 0.0 | 0.1 | 1.8 | 0.2 |
| Bacteroidota | Bacteroidia | Bacteroidales | Dysgonomonadaceae | Dysgonomonadaceae _fa | 0.1 | 0.0 | 0.0 | 0.2 | 0.0 | 0.0 | 3.8 | 1.1 |
| Bacteroidota | Bacteroidia | Bacteroidales | Paludibacteraceae | Paludibacteraceae _fa | 0.7 | 1.0 | 0.1 | 1.7 | 0.0 | 0.0 | 0.2 | 0.0 |
| Bacteroidota | Bacteroidia | Sphingobacteriales | Lentimicrobiaceae | Lentimicrobium | 0.4 | 0.0 | 0.5 | 0.2 | 1.1 | 1.2 | 1.2 | 1.6 |
| Caldatribacteriota | Caldatribacteriia | Caldatribacteriales | Caldatribacteriaceae | Candidatus_Caldatribacterium | 1.4 | 0.1 | 0.8 | 0.0 | 0.2 | 0.0 | 2.4 | 3.5 |
| Cloacimonadota | Cloacimonadia | Cloacimonadales | Cloacimonadaceae | W5 | 4.0 | 1.0 | 1.2 | 0.3 | 0.5 | 0.0 | 4.1 | 4.0 |
| Cloacimonadota | Cloacimonadia | Cloacimonadales | Cloacimonadaceae | Cloacimonadaceae _fa | 0.1 | 0.8 | 0.7 | 0.8 | 1.2 | 0.0 | 0.0 | 0.0 |
| Cloacimonadota | Cloacimonadia | Cloacimonadales | W27 | W27 _fa | 12.7 | 25.1 | 26.5 | 4.8 | 22.9 | 8.2 | 8.6 | 10.2 |
| Firmicutes | Clostridia | Peptostreptococcales-Tissierellales | Peptostreptococcales-Tissierellales_fa | Alkaliphilus | 0.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.0 | 1.7 |
| Firmicutes | Clostridia | Peptostreptococcales-Tissierellales | Peptostreptococcales-Tissierellales_fa | Gallicola | 34.7 | 5.2 | 17.3 | 5.6 | 9.6 | 15.8 | 7.6 | 11.7 |
| Firmicutes | Clostridia | Peptostreptococcales-Tissierellales | Sedimentibacteraceae | Sedimentibacter | 0.3 | 0.8 | 0.1 | 0.7 | 0.1 | 0.0 | 5.4 | 1.9 |
| Firmicutes | Clostridia | Caldicoprobacterales | Caldicoprobacteraceae | Caldicoprobacter | 2.0 | 4.4 | 3.6 | 3.4 | 5.2 | 1.9 | 2.5 | 1.3 |
| Firmicutes | Clostridia | Clostridia_or | Hungateiclostridiaceae | Fastidiosipila | 5.9 | 4.9 | 6.8 | 3.0 | 3.7 | 3.9 | 11.8 | 8.3 |
| Firmicutes | Clostridia | Clostridia_or | Hungateiclostridiaceae | HN-HF0106 | 0.1 | 0.3 | 0.0 | 0.8 | 0.0 | 0.0 | 2.8 | 1.2 |
| Firmicutes | Clostridia | Peptostreptococcales-Tissierellales | Anaerovoracaceae | Anaerovoracaceae _fa | 0.4 | 0.4 | 0.3 | 0.4 | 0.0 | 0.1 | 1.4 | 1.2 |
| Firmicutes | Limnochordia | MBA03 | NA | MBA03 _or | 5.4 | 5.8 | 8.2 | 3.0 | 4.9 | 6.3 | 9.3 | 8.5 |
| Firmicutes | Limnochordia | NA | NA | Limnochordia _cl | 0.2 | 0.0 | 0.3 | 0.7 | 0.0 | 0.0 | 2.3 | 0.5 |
| Firmicutes | Incertae_Sedis | DTU014 | NA | DTU014 _or | 4.8 | 2.9 | 7.9 | 1.0 | 2.7 | 4.6 | 8.1 | 11.2 |
| Firmicutes | Dethiobacteria | Dethiobacterales | Dethiobacteraceae | Dethiobacteraceae _fa | 3.0 | 0.5 | 1.9 | 0.1 | 0.0 | 0.4 | 1.3 | 2.2 |
| Firmicutes | Bacilli | Erysipelotrichales | Erysipelotrichaceae | Erysipelotrichaceae _fa | 0.2 | 0.9 | 1.0 | 0.6 | 0.8 | 1.1 | 0.0 | 0.0 |
| Synergistota | Synergistia | Synergistales | Synergistaceae | Acetomicrobium | 4.7 | 0.7 | 1.6 | 0.0 | 0.1 | 0.4 | 2.0 | 1.2 |
| Thermotogota | Thermotogae | Petrotogales | Petrotogaceae | Defluviitoga | 0.5 | 0.0 | 0.3 | 0.0 | 0.7 | 1.7 | 11.3 | 16.2 |
| Minor taxa | | | | | 1.6 | 1.8 | 0.9 | 3.7 | 2.0 | 2.1 | 8.8 | 7.8 |

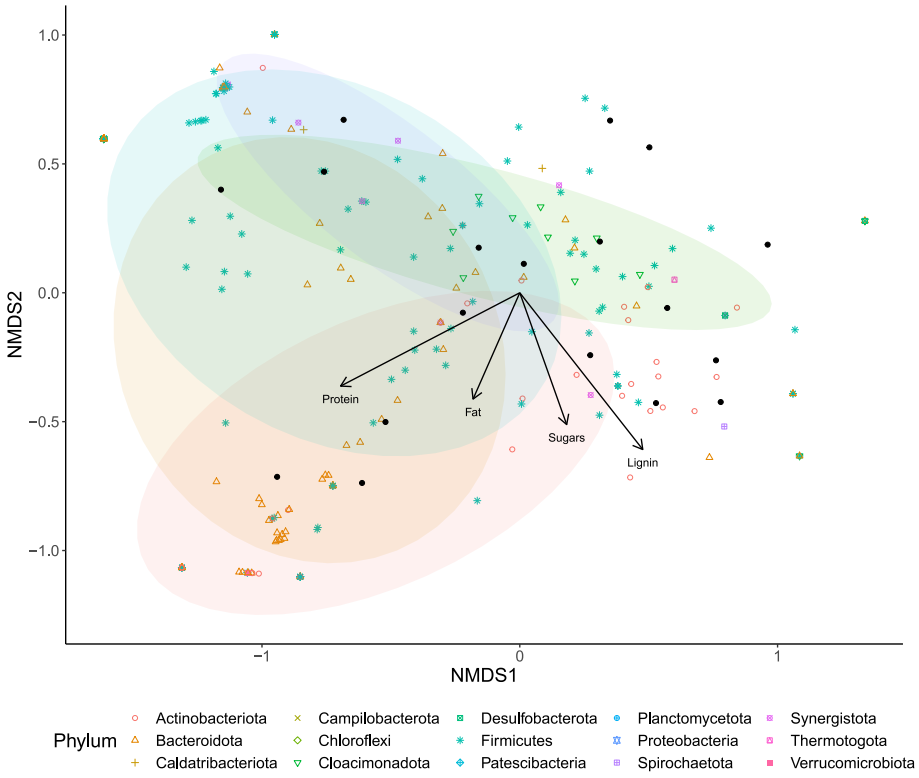


**Fig. 6.** Non-metric multidimensional scaling (NMDS) plot of bacterial phyla in digestate samples from reactors A1 (single reactor), B1 and B2 (operated in series), and C1, C2, and C3 (operated in series). Arrows indicate concentrations of macromolecules protein, fat, lignin, and bound sugars (xylose, mannose, glucose, galactose, and arabinose) in the reactors. The taxa are subset to not include unidentified phyla. Ellipses mark a multivariate normal distribution with 50% confidence interval for phyla with >3 points in the plot. Stress = 0.097.

retention of organic matter in a serial reactor system compared with a single reactor [30,31]. As shown by the theoretically calculated RTDs (Fig. 2), increasing the number of reactors in series, without changing the total HRT, delayed the exit of material from the system. In theory, a system with an infinite number of serial reactors and a sufficient total HRT could give complete degradation. In line with this, we observed a slightly greater VS reduction in the serial systems than in the single reactor (Table 2). Moreover, protein concentration was more than 20% lower after the serial systems compared with the single reactor (Fig. 3), suggesting that for food waste with recalcitrant protein, a setup with serial reactors is beneficial. Less efficient degradation in the single reactor (A1) was also demonstrated by higher RMP compared with the last steps in the serial systems (B2 and C3). Interestingly, comparisons of theoretical RMP, estimated from the macromolecule concentrations, with measured RMP in the digestates showed that approximately the same amount of undigested material remained in all digestates after the residual methane was removed (Fig. 4). This may indicate that all remaining organic material was microbial biomass that was similar in all reactors. If this was the case, the degradation was complete after the RMP test and the low RMP from B2, C2, and C3 was due to a low amount of remaining substrate, and not inactive microbial communities. However, the comparison between measured and theoretical RMP should be interpreted with care since the theoretical values were based on macromolecule concentrations, measured at a different time point as compared to the RMP. Although serial digestion improved protein degradation and VS reduction, lowered RMP in the digestates, and increased methane production, no significant differences in these parameters were observed when comparing the two serial systems. Thus for the process studied, two reactors in series were sufficient to reach higher productivity.

An important advantage of serial systems is increased average retention time of the material in the total system, but slightly higher methane production was obtained already in the first reactor in the two-step system compared with the single reactor ($p = 0.046$), despite the shorter HRT. Moreover, the SMP in reactor C1 did not differ significantly from that in A1, indicating that, compared with the single reactor, the system could be 'pressured' more by shortening the HRT and increasing the OLR, without causing disturbances and a decrease in methane production. This has previously been shown to be feasible and a way of optimizing the productivity of biogas processes [32]. The increase in OLR and shortened HRT in B1 were even slightly favorable for the SMP, possibly as a result of the lower $NH_4^+$-N concentration obtained in this reactor compared with A1 (Table 2).

### 4.2. Hydrolysis of macromolecules and microbial community structure

Methane production rates from different macromolecules indicated significantly faster methane production with inocula from the first-step reactors compared with the subsequent steps (Table 3). The first-step reactors all had the lowest $NH_4^+$-N relative concentration and were fed with fresh substrate at a higher organic load than subsequent reactors in the series, which might have contributed to more active microbial communities in these digestates. As regards macromolecule concentrations in the digestate, there were no significant differences in the concentrations of sugars, lignin, and fat between the reactors. It is probable that the majority of hydrolysis of these compounds took place already in the first reactor in the serial systems. Slightly lower methane content in the gas from the first-step reactor with the shortest HRT (C1) relative to the subsequent reactor (C2) (Table 2) could also be a sign of higher hydrolytic activity in the first step since this increases the $CO_2$ content in the gas [14]. The relative abundance of *Paludibacteraceae* H1 decreased notably after the first steps, suggesting involvement in the initial hydrolysis and acidogenesis steps in the first reactor. Members of *Paludibacteraceae* have previously been reported to utilize various sugars [33], starch [23], and potentially cellulose [34]. Although the differences in fat concentrations between the reactors were not statistically significant, an interesting trend was observed for Cloacimonadota in the NMDS plot, with higher relative abundance of this phylum at lower fat concentrations (Fig. 6). Members within this phylum may be involved in digestion of long-chain fatty acids [35], which might explain the comparatively higher relative abundance of Cloacimonadota family W27 in reactors B1, B2, and C2 (Table 4).

One of the most abundant genera in the serial systems was *Actinomyces*. The most abundant sequence classified as *Actinomyces* had 99.6% similarity with *Gleimia europaea* (NCBI, RefSeq Genome database), a sugar-fermenting bacterium [36,37] previously observed in biogas systems processing food waste [38]. In the present study, higher relative abundance of Actinomyces was observed in reactors with shorter HRT. This is in line with Feng et al. [38], who observed a sharp increase in relative abundance of *Actinomyces* upon downscaling from industrial to laboratory scale, suggesting that the genus was favored by the shorter retention time applied in the laboratory-scale reactors. Actinobacteria has been shown to be an important phylum in degradation of lignocellulosic material in compost, expressing a wide range of lignin-degrading enzymes [39]. Jaenicke et al. [40] also observed genes belonging to Actinobacteria in a biogas community degrading agricultural waste. Whether *Actinomyces* is involved in fiber degradation under the conditions maintained in reactors in the present study is unknown, but the NMDS analysis indicated higher abundance of Actinobacteriota with higher lignin and sugar concentrations (Fig. 6).

*Defluviitoga*, one of the most abundant genera (11–16%) in the inocula, is known for its ability to degrade carbohydrates [41]. It is a thermophilic genus but with the ability to grow at temperatures down to 37 °C [41], which explains its prevalence in the present study. In contrast to genus *Actinomyces,* the relative abundance of *Defluviitoga* decreased (<2%) in all reactors as compared to the inocula, suggesting a downscaling effect (Table 4). Effects of the microbial community by downscaling have been shown before and suggested to be caused by changes in feeding regimes [42,43]. Still, previous work on lab-scale processes have indicated that such changes not necessarily lead to differences in function and performance of the process [32,43].

In previous studies, inefficient protein degradation in AD has been reported at certain conditions, such as low pH [6] and high carbohydrate concentration [44,45]. It has been noted that proteins are not degraded in the acidogenic stage in phase-separated processes [15]. Nevertheless, Breure et al. [44] suggested that a phase-separation reactor setup would be beneficial for protein degradation, since it would allow carbohydrates to be acidified in a separate chamber and protein degradation could take place in the methanogenic chamber. No phase separation was used in the present study, but carbohydrate hydrolysis and acidogenesis still appeared to take place mainly in the first-step reactors, thereby creating a favorable environment for protein degradation in the subsequent reactors, with slightly higher pH and lower carbohydrate concentrations. A stepwise decrease in protein concentration across the serial systems (Fig. 3) and an increase in relative abundance of the potentially proteolytic genera *Proteiniphilum, Fastidiosipila*, and *Acetomicrobium* over reactor steps was observed. *Proteiniphilum,* within Bacteroidetes, has been described to degrade peptides and also complex carbohydrates [46–48]. *Fastidiosipila*, within Firmicutes, has previously been coupled to proteolytic activity in pure cultures and in AD systems [15,49,50]. The type species of *Acetomicrobium* (*Acetomicrobium flavidum*), a genus within Synergistota, can hydrolyze starch, casein, and tributyrin [51]. The relative increases in these groups in the second and third reactors in this study could also be a result of relatively high $NH_3$ tolerance, *e.g.*, it has been shown that *Acetomicrobium* can grow at high $NH_3$ levels [52].

Although an important proportion of protein degradation appeared to have occurred by later reactor steps, methane production from protein measured in the substrate conversion test was especially fast in reactors B1 and C1 (Figure C3, Table 3). The protein degradation in these reactors might have been performed by members within the phylum Bacteroidota classified as *Paludibacteraceae* (using the Silva database), which was highly abundant in both reactors. The most abundant of these sequences (relative abundance 20% in B1 and 25% in C1) had 100% sequence similarity with uncultured *Porphyromonadaceae* (NCBI, Nucleotide collection (nr/nt) database), whose type genus *Porphyromonas* is described as being proteolytic [53]. High relative abundance of Bacteroidota was also positively correlated with protein concentration according to the NMDS analysis (Fig. 6), possibly indicating involvement of this phylum in protein degradation in the study reactors.

In summary, one of the main trends in terms of links between microbial community structure and degradation of molecular structures was a sharp decrease in the abundance of family *Paludibacteracea* in the second-step reactors relative to the first steps. This decrease was potentially related to a comparably higher reduction of readily degradable macromolecule structures, both proteins and carbohydrates, in the first as compared to the second steps. On the other hand, there was a slight increase over reactor steps in the abundance of the potentially proteolytic genera *Proteiniphilum, Fastidiosipila*, and *Acetomicrobium*, possibly linked to the step-wise decreasing protein concentrations. Additionally, a link between Cloacimonadota family W27 to fat degradation was indicated.

### 4.3. Effect of increasing ammonia concentrations over reactor steps

The $NH_3$-N level is an important parameter in biogas processes, as methanogens are known to be inhibited at concentrations around 200–400 mg/L in mesophilic processes [54]. The toxicity depends on the process parameters, as the equilibrium between $NH_3$ and $NH_4^+$ is driven towards higher $NH_3$ levels at increasing temperature and pH. The $NH_4^+$-N concentrations were kept at relatively low levels in the first reactors (A1, B1 and C1), constantly diluted with fresh substrate, and accumulation was observed in the later reactor steps as more protein was degraded (Table 2). The pH also increased over reactor steps, creating a more toxic environment for ammonia-sensitive microorganisms in the last reactor steps compared with the first. A great advantage with the serial reactor setup used here was that the majority of the methane from each system was produced in the first reactor step (Table 2), separately from accumulation of $NH_3$-N. Nevertheless, although the $NH_3$-N concentrations were at potentially inhibiting levels in the last reactors (Table 2), the VFA concentrations did not increase, which is normally a sign of process disturbance caused by ammonia inhibition [54]. However, the OLR in the reactors with the highest $NH_3$-N levels was low and the material was represented by slowly degradable recalcitrant material, which might have reduced the risk of VFA accumulation.

As the $NH_3$-N concentration increased, there was also a slight change in methanogen community structure (Fig. 5a). The relative abundance of the hydrogenotrophic genera *Methanoculleus* and especially *Methanobacterium* increased in the later reactors in the serial systems, where the ammonia levels were higher. In general, hydrogenotrophic methanogens are more tolerant to high ammonia levels than acetoclastic species [54]. Although the abundance of hydrogenotrophic methanogens increased slightly with reactor steps, *Methanosarcina* was still the predominant genus in all reactors. This is a methanogen with a wide substrate range that can produce methane from *e.g.*, acetate, methanol, and $CO_2$ and $H_2$ [55]. The *Methanosarcina* genus is also known to be tough and have a high tolerance to stress factors such as high ammonia, low pH or high OLR [56].

The hydrogenotrophic methanogens are important to enable syntrophic oxidations of various organic acids, which is unfavorable at high partial pressure of $H_2$ [54]. A trend seen in our systems was for potential syntrophic acetate-oxidizing bacteria (SAOB) to increase in relative abundance with reactor steps, in line with the increase in hydrogenotrophic methanogens. This was possibly linked to increasing $NH_3$-N levels, since the syntrophic acetate oxidation (SAO) pathway for acetate degradation can often compete better with acetoclastic methanogenesis at high $NH_3$-N concentrations [54]. SAOB are also slow-growing, and could therefore also have been favored by the long total retention time in the last digesters in the serial systems. The orders DTU014 and the $NH_3$-tolerant MBA03 and family *Dethiobacteraceae* within Firmicutes, groups with suggested SAO activity [34,57,58], followed the trend for increasing abundance over reactor steps.

In summary, although the methanogenic community was dominated by genus *Methanosarcina*, hydrogenotrophic methanogenic genera (*Methanoculleus* and *Methanobacterium*) increased in relative abundance over reactor steps, possibly due to increasing $NH_3$-N concentration. A similar trend was observed for the suggested SAO bacterial taxa, DTU014, MBA03, and *Dethiobacteraceae*.

## 5. Conclusions

Although food waste contains relatively easily degradable material that is digested soon after it has been added to the process, this study clearly showed that serial digestion significantly can improve degradation of recalcitrant structures. Analysis of process performance showed that the serial system increased the methane yield by >5% compared with a single reactor, but with no significant differences between the two-step and three-step reactor systems. Serial systems also improved the degree of degradation, indicated by significantly lowered RMP, which both suggested a more efficient substrate utilization and proposes lowered risk for residual methane emission from the digestate.

Among macromolecules, proteins showed the highest reduction, with concentrations >20% lower after the serial systems compared with the single reactor. Microbiological analysis showed adaptations to the changing environment and substrate availability across the serial systems as well as links between the relative abundances of bacterial phyla with suggested hydrolytic and acidogenic function, with macromolecule concentrations and degradation rates. Moreover, the methanogenic community changed in relation to the $NH_3$-N concentration, also linked to relative abundances of potential SAO bacteria.

The observed positive effects of the serial setups could have derived from comparatively low $NH_3$-N concentrations in the first reactor step, lowering the risk of ammonia inhibition and associated process disturbances in the reactors where the majority of the methane is produced. The serial setup also had the advantage of retaining recalcitrant structures in the system for a longer time on average, increasing the probability of degradation and thus leading to improved yield and reduced levels of residual methane production from the digestate.

## Author contributions

Ebba Perman: Formal analysis, Investigation, Writing – Original Draft, Visualization.

Anna Schnürer: Supervision, Conceptualization, Funding acquisition, Writing - Review & Editing. Annika Björn: Funding acquisition, Investigation, Writing – Review and Editing.

.

Jan Moestedt: Conceptualization, Investigation, Resources, Data curation, Funding acquisition, Formal analysis, Supervision, Writing - Review & Editing.

## Funding sources

This study was funded by, Tekniska Verken i Linköping AB (public) and Biogas Research Center hosted by Linköping University, funded by its partners and members, Linköping University, Swedish University of Agricultural Sciences and the Swedish Energy Agency (Grant No. 35624-2).

## Data availability

16S rRNA-gene sequence data related to this article can be accessed at BioProject accession number PRJNA781440 (https://www.ncbi.nlm.nih.gov/Traces/study/?acc=PRJNA781440), at Sequence Read Archive (SRA), National Center for Biotechnology Information (NCBI).

## Acknowledgments

The authors would like to thank Tong Liu for supervision and advice during laboratory work and data processing regarding the microbiology analysis, and Yasna Calderon for laboratory work during the reactor experiment.

Tekniska verken i Linköping, Scandinavian Biogas Fuels, Biototal, and Gasum are acknowledged for their active contributions to the project through the Biogas Research Center, Sweden.

This study was supported by the Swedish Energy Agency [Grant number: 35624-2] and Tekniska verken i Linköping AB (public), and was conducted at the Biogas Research Center hosted by Linköping University, Sweden.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biombioe.2022.106478.

## References

[1] A. Anukam, A. Mohammadi, M. Naqvi, K. Granström, A review of the chemistry of anaerobic digestion: methods of accelerating and optimizing process efficiency, Processes 7 (8) (2019) 504.
[2] P. Börjesson, B. Mattiasson, Biogas as a resource-efficient vehicle fuel, Trends Biotechnol. 26 (1) (2007) 7–13.
[3] K. Möller, T. Müller, Effects of anaerobic digestion on digestate nutrient availability and crop growth: a review, Eng. Life Sci. 12 (3) (2012) 242–257.
[4] T. Sayara, A. Sánchez, A review on anaerobic digestion of lignocellulosic wastes: pretreatments and operational conditions, Appl. Sci. 9 (2019) 4655.
[5] S.S. Yekta, M. Hedenstrom, B.H. Svensson, I. Sundgren, M. Dario, A. Enrich-Prast, N. Hertkorn, A. Bjorn, Molecular characterization of particulate organic matter in full scale anaerobic digesters: an NMR spectroscopy study, Sci. Total Environ. 685 (2019) 1107–1115.
[6] T.H. Duong, K. Grolle, T.T.V. Nga, G. Zeeman, H. Temmink, M. van Eekert, Protein hydrolysis and fermentation under methanogenic and acidifying conditions, Biotechnol. Biofuels 12 (2019).
[7] R. Bevilacqua, A. Regueira, M. Mauricio-Iglesias, J.M. Lema, M. Carballa, Protein composition determines the preferential consumption of amino acids during anaerobic mixed-culture fermentation, Water Res. 183 (2020).
[8] E.-M. Ekstrand, A. Björn, A. Karlsson, A. Schnürer, L. Kanders, S.S. Yekta, M. Karlsson, J. Moestedt, Identifying targets for increased biogas production through chemical and organic matter characterization of digestate from full-scale biogas plants: what remains and why? Biotechnol. Biofuels Bioprod. 15 (2022).
[9] G. Capson-Tojo, M. Rouez, M. Crest, J.-P. Steyer, J.-P. Delgenès, R. Escudié, Food waste valorization via anaerobic processes: a review, Rev. Environ. Sci. Biotechnol. 15 (3) (2016) 499–547.
[10] C.P.C. Bong, L.Y. Lim, C.T. Lee, J.J. Klemes, C.S. Ho, W.S. Ho, The characterisation and treatment of food waste for improvement of biogas production during anaerobic digestion - a review, J. Clean. Prod. 172 (2018) 1545–1558.
[11] P. Kaparaju, L. Ellegaard, I. Angelidaki, Optimisation of biogas production from manure through serial digestion: lab-scale and pilot-scale studies, Bioresour. Technol. 100 (2) (2009) 701–709.
[12] Y. Li, C. Liu, A.C. Wachemo, H. Yuan, D. Zou, Y. Liu, X. Li, Serial completely stirred tank reactors for improving biogas production and substance degradation during anaerobic digestion of corn stover, Bioresour. Technol. 235 (2017) 380–388.
[13] E. Nordell, J. Moestedt, J. Österman, S.S. Yekta, A. Björn, L. Sun, A. Schnürer, Post-treatment of dewatered digested sewage sludge by thermophilic high-solid digestion for pasteurization with positive energy output, Waste Manag. 119 (2021) 11–21.
[14] B. Chatterjee, D. Mazumder, Role of stage-separation in the ubiquitous development of anaerobic digestion of organic fraction of municipal solid waste: a critical review, Renew. Sustain. Energy Rev. 104 (2019) 439–469.
[15] J. Moestedt, B. Müller, Y. Nagavara Nagaraj, A. Schnürer, Acetate and lactate production during two-stage anaerobic digestion of food waste driven by lactobacillus and aeriscardovia, Front. Energy Res. 8 (2020).
[16] F. Xu, Y. Li, X. Ge, L. Yang, Y. Li, Anaerobic digestion of food waste – challenges and opportunities, Bioresour. Technol. 247 (2018) 1047–1058.
[17] L.-J. Wu, A. Higashimori, Y. Qin, T. Hojo, K. Kubota, Y.-Y. Li, Comparison of hyper-thermophilic–mesophilic two-stage with single-stage mesophilic anaerobic digestion of waste activated sludge: process performance and microbial community analysis, Chem. Eng. J. 290 (2016) 290–301.
[18] Y. Liu, A.C. Wachemo, H. Yuan, X. Li, Anaerobic digestion performance and microbial community structure of corn stover in three-stage continuously stirred tank reactors, Bioresour. Technol. 287 (2019).
[19] E. Nordell, M. Karlsson, J. Moestedt, Laboratory Reactors for Anaerobic Digestion, 2011. SE 1150954-4, Sweden.
[20] S. Jonsson, H. Boren, Analysis of mono- and diesters of o-phthalic acid by solid-phase extractions with polystyrene-divinylbenzene-based polymers, J. Chromatogr., A 963 (2002) 393–400.

[21] S.D. Hafner, S. Astals, C. Holliger, K. Koch, S. Weinrich, Calculation of biochemical methane potential (BMP), Stand. BMP Methods (2020) document 200, version 1.7, https://www.dbfz.de/en/BMP. (Accessed 13 November 2020).
[22] R. Danielsson, J. Dicksved, L. Sun, H. Gonda, B. Müller, A. Schnürer, J. Bertilsson, Methane production in dairy cows correlates with rumen methanogenic and bacterial community structure, Front. Microbiol. 8 (2017).
[23] T. Liu, L. Sun, A. Nordberg, A. Schnurer, Substrate-induced response in biogas process performance and microbial community relates back to inoculum source, Microorganisms 6 (3) (2018) E80.
[24] B.J. Callahan, P.J. McMurdie, M.J. Rosen, A.W. Han, A.J.A. Johnson, S.P. Holmes, DADA2: high-resolution sample inference from Illumina amplicon data, Nat. Methods 13 (7) (2016) 581–583.
[25] M. Martin, Cutadapt removes adapter sequences from high-throughput sequencing reads, EMBnet j. 17 (2011) 10–12.
[26] M.M. Weinstein, A. Prem, M. Jin, S. Tang, J.M. Bhasin, FIGARO, An Efficient and Objective Tool for Optimizing Microbiome rRNA Gene Trimming Parameters, Cold Spring Harbor Laboratory, 2019.
[27] C. Quast, E. Pruesse, P. Yilmaz, J. Gerken, T. Schweer, P. Yarza, J. Peplies, F. O. Glöckner, The SILVA ribosomal RNA gene database project: improved data processing and web-based tools, Nucleic Acids Res. 41 (2013) D590–D596.
[28] P. Toson, P. Doshi, D. Jajcevic, Explicit residence time distribution of a generalised cascade of continuous stirred tank reactors for a description of short recirculation time, (Bypassing). Process. 7 (9) (2019) 615.
[29] I. Angelidaki, D. Karakashev, D.J. Batstone, C.M. Plugge, A.J.M. Stams, Biomethanation and its Potential, Elsevier, 2011, pp. 327–351.
[30] K. Boe, I. Angelidaki, Serial CSTR digester configuration for improving biogas production from manure, Water Res. 43 (2009) 166–172.
[31] I. Angelidaki, K. Boe, L. Ellegaard, Effect of operating conditions and reactor configuration on efficiency of full-scale biogas plants, Water Sci. Technol. 52 (1–2) (2005) 189–194.
[32] M. Westerholm, S. Isaksson, O. Karlsson Lindsjö, A. Schnürer, Microbial community adaptability to altered temperature conditions determines the potential for process optimisation in biogas production, Appl. Energy 226 (2018) 838–848.
[33] A. Ueki, Paludibacter propionicigenes gen. nov., sp. nov., a novel strictly anaerobic, Gram-negative, propionate-producing bacterium isolated from plant residue in irrigated rice-field soil in Japan, Int. J. Syst. Evol. Microbiol. 56 (1) (2006) 39–44.
[34] S. Dyksma, L. Jansen, C. Gallert, Syntrophic acetate oxidation replaces acetoclastic methanogenesis during thermophilic digestion of biowaste, Microbiome 8 (1) (2020).
[35] S. Shakeri Yekta, T. Liu, M. Axelsson Bjerg, L. Šafarič, A. Karlsson, A. Björn, A. Schnürer, Sulfide level in municipal sludge digesters affects microbial community response to long-chain fatty acid loads, Biotechnol. Biofuels 12 (1) (2019).
[36] G. Funke, N. Alvarez, C. Pascual, E. Falsen, E. Akervall, L. Sabbe, L. Schouls, N. Weiss, M.D. Collins, Actinomyces europaeus sp. nov., isolated from human clinical specimens, Int. J. Syst. Bacteriol. 47 (3) (1997) 687–692.
[37] I. Nouioui, L. Carro, M. García-López, J.P. Meier-Kolthoff, T. Woyke, N.C. Kyrpides, R. Pukall, H.-P. Klenk, M. Goodfellow, M. Göker, Genome-based taxonomic classification of the phylum Actinobacteria, Front. Microbiol. 9 (2018).
[38] X.M. Feng, A. Karlsson, B.H. Svensson, S. Bertilsson, Impact of trace element addition on biogas production from food industrial waste - linking process to microbial communities, FEMS Microbiol. Ecol. 74 (2010) 226–240.
[39] C. Wang, D. Dong, H. Wang, K. Mueller, Y. Qin, H. Wang, W. Wu, Metagenomic analysis of microbial consortia enriched from compost: new insights into the role of Actinobacteria in lignocellulose decomposition, Biotechnol. Biofuels 9 (2016) 22.
[40] S. Jaenicke, C. Ander, T. Bekel, R. Bisdorf, M. Droege, K.-H. Gartemann, S. Juenemann, O. Kaiser, L. Krause, F. Tille, M. Zakrzewski, A. Puehler, A. Schlueter, A. Goesmann, Comparative and joint analysis of two metagenomic datasets from a biogas fermenter obtained by 454-pyrosequencing, PLoS One 6 (1) (2011).
[41] W. Ben Hania, R. Godbane, A. Postec, M. Hamdi, B. Ollivier, M.-L. Fardeau, *Defluviitoga tunisiensis* gen. nov., sp nov., a thermophilic bacterium isolated from a mesothermic and anaerobic whey digester, Int. J. Syst. Evol. Microbiol. 62 (2012) 1377–1382.
[42] D.G. Mulat, H.F. Jacobi, A. Feilberg, A.P.S. Adamsen, H. Richnow, M. Nikolausz, Changing feeding regimes to demonstrate flexible biogas production: effects on process performance, microbial community structure and methanogenic pathways, Appl. Environ. Microbiol. 82 (2015) 438–449.
[43] M. Westerholm, M.D.P. Castillo, A. Chan Andersson, P. Jahre Nilsen, A. Schnurer, Effects of thermal hydrolytic pre-treatment on biogas process efficiency and microbial community structure in industrial- and laboratory-scale digesters, Waste Manag. 95 (2019) 150–160.
[44] A.M. Breure, H.H. Beeftink, J. Verkuijlen, J.G. Van Andel, Acidogenic fermentation of protein/carbohydrate mixtures by bacterial populations adapted to one of the substrates in anaerobic chemostat cultures, Appl. Microbiol. Biotechnol. 23 (3–4) (1986) 245–249.
[45] E.A. Tepari, G. Nakhla, B.M. Haroun, H. Hafez, Co-fermentation of carbohydrates and proteins for biohydrogen production: statistical optimization using Response Surface Methodology, Int. J. Hydrogen Energy 45 (4) (2020) 2640–2654.
[46] S. Chen, X. Dong, *Proteiniphilum acetatigenes* gen. nov., sp. nov., from a UASB reactor treating brewery wastewater, Int. J. Syst. Evol. Microbiol. 55 (2005) 2257–2261.
[47] S. Hahnke, T. Langer, D.E. Koeck, M. Klocke, Description of *Proteiniphilum saccharofermentans* sp. nov., *Petrimonas mucosa* sp. nov. and *Fermentimonas caenicola* gen. nov., sp. nov., isolated from mesophilic laboratory-scale biogas reactors, and emended description of the genus *Proteiniphilum*, Int. J. Syst. Evol. Microbiol. 66 (2016) 1466–1475.
[48] G. Tomazetto, S. Hahnke, D. Wibberg, A. Pühler, M. Klocke, A. Schlüter, Proteiniphilum saccharofermentans str. M3/6T isolated from a laboratory biogas reactor is versatile in polysaccharide and oligopeptide utilization as deduced from genome-based metabolic reconstructions, Biotechnol. Rep. 18 (2018), e00254.
[49] J. Cardinali-Rezende, P. Rojas-Ojeda, A.M.A. Nascimento, J.L. Sanz, Proteolytic bacterial dominance in a full-scale municipal solid waste anaerobic reactor assessed by 454 pyrosequencing technology, Chemosphere 146 (2016) 519–525.
[50] E. Falsen, M.D. Collins, C. Welinder-Olsson, Y. Song, S.M. Finegold, P.A. Lawson, Fastidiosipila sanguinis gen. nov., sp nov., a new Gram-positive, coccus-shaped organism from human blood, Int. J. Syst. Evol. Microbiol. 55 (2005) 853–858.
[51] E. Soutschek, J. Winter, F. Schindler, O. Kandler, Acetomicrobium flavidum, gen. Nov., sp. nov., a thermophilic, anaerobic bacterium from sewage sludge, forming acetate, CO2 and H2 from glucose, system, Appl. Microbiol. 5 (1984) 377–390.
[52] M. Westerholm, T. Liu, A. Schnürer, Comparative study of industrial-scale high-solid biogas production from food waste: process operation and microbiology, Bioresour. Technol. 304 (2020), 122981.
[53] H.N. Shah, M.D. Collins, Proposal for reclassification of Bacteroides asaccharolyticus, Bacteroides gingivalis, and Bacteroides endodontalis in a new genus, Porphyromonas, Int. J. Syst. Bacteriol. 38 (1988) 128–131.
[54] M. Westerholm, J. Moestedt, A. Schnurer, Biogas production through syntrophic acetate oxidation and deliberate operating strategies for improved digester performance, Appl. Energy 179 (2016) 124–135.
[55] R.A. Mah, D.A. Kuhn, Rejection of the type species Methanosarcina methanica (approved lists 1980), conservation of the genus Methanosarcina with Methanosarcina barkeri (approved lists 1980) as the type species, and emendation of the genus Methanosarcina, Int. J. Syst. Bacteriol. 34 (1984) 266–267.
[56] J. De Vrieze, T. Hennebel, N. Boon, W. Verstraete, *Methanosarcina*: the rediscovered methanogen for heavy duty biomethanation, Bioresour. Technol. 112 (2012) 1–9.
[57] S. Dyksma, C. Gallert, Candidatus Syntrophosphaera thermopropionivorans: a novel player in syntrophic propionate oxidation during anaerobic digestion, Environ. Microbiol. Rep. 11 (2019) 558–570.
[58] D. Zheng, H. Wang, M. Gou, M.K. Nubo, T. Narihiro, B. Hu, Y. Nie, Y.Q. Tang, Identification of novel potential acetate-oxidizing bacteria in thermophilic methanogenic chemostats by DNA stable isotope probing, Appl. Microbiol. Biotechnol. 103 (2019) 8631–8645.