Effects of ammonia on propionate degradation and microbial community in digesters using propionate as a sole carbon source

Ying Li[a,c,d,e] , Yue Zhang [b] , Xiaoying Kong[a], Lianhua Li[a] , Zhenhong Yuan[a,d,e], Renjie Dong [c] Yongming Sun[a *]

[a]*GuangZhou Institute of Energy Conversion, Chinese Academy of Sciences, Guangzhou, PR China*

[b]*Faculty of Engineering and the Environment, University of Southampton, Southampton, UK*

[c]*College of Agronomy and Biotechnology, China Agricultural University, Beijing, PR China*

[d]*Key Laboratory of Renewable Energy, Chinese Academy of Sciences, Guangzhou, PR China*

[e]*Guangdong Provincial Key Laboratory of New and Renewable Energy Research and Development, Guangzhou, PR China*

Abstract

BACKGROUND: Propionate accumulation may lead to digester failure. This study aimed to investigate the effect of ammonia, a metabolic product of protein, on propionate degradation. The shift of microbial community was also investigated.

RESULTS: Propionate accumulated over the experimental period in the reactor with a total ammonia nitrogen (TAN) concentration of 2.5 g N L^{-1}, as a result the digester failed after 4 hydraulic retention times (HRT) at an organic load rate (OLR) of 0.8 g propionic acid (HPr) L^{-1} d^{-1}. The average HPr degradation rate was below 54% during the fourth HRT, while >97 % of the degraded HPr was converted to methane. The reactor without ammonia stress did not experience HPr accumulation and OLR was increased stepwise to 1.2 g L^{-1} d^{-1} at the 8th HRT. The average HPr degradation rate and methane recovery rate of this reactor in the last HRT was 99% and 74%, respectively. According to the shifts of microbial community, acetoclastic methanogen was more vulnerable to ammonia than hydrogenotrophic methanogen.

CONCLUSION: TAN concentration of 2.5 g N L^{-1} inhibited propionate degradation more severely than methanogenesis. The loss of the abundance of *Clostridiaceae* and *Syntrophobacter* might be the main reason for the poor performance under ammonia stress.

* Correspondence to: Y Sun, No. 2 Nengyuanlu, Tianhe District, Guangzhou 510640, PR China. E-mail address: sunym@ms.giec.ac.cn

Keywords: Ammonia inhibition, propionate degradation, propionate-oxidizing bacteria, microbial community

INTRODUCTION

Anaerobic digestion (AD) is a well-accepted technology for simultaneous organic waste management and renewable energy production. Despite these benefits, poor operational stability still prevents AD process from being widely applied, especially when wastes rich in protein or other organic nitrogen compounds, such as food waste, manure, fermentation industry residues or fish processing residues, are used as feedstock. This stability problem is mainly caused by ammonia inhibition and volatile fatty acid (VFA) accumulation.[1-6]

Although ammonia is an important buffer in the AD process and the nitrogen it provides is an essential nutrient for microorganisms, at high concentrations it is one of the most common inhibitors encountered during anaerobic digestion. The same as organic overloading, the presence of ammonia is a common reason for accumulation of VFAs, which at high concentrations can cause the deterioration of digester performance. [7,8] The accumulation of propionate, together with high acetate concentration, is considered to be the major problem in digesters with high ammonia concentrations. [9] A wide range of inhibiting ammonia concentrations have been reported in the literature, ranging from 1.7 to 14 g-N L^{-1},[10] or even as high as 19 g-N L^{-1},[11] which cause a 50% reduction in methane production. The wide range of inhibitory ammonia concentrations are considered to be affected by pH, temperature, substrates, inocula, acclimation periods and solids retention time.[12] The impact of ammonia concentration on the acetate degradation pathway is of great interest, which has been studied based on microbial community structure and predominant methanogenic pathway in anaerobic digesters exposed to different ammonia concentrations.[13-15] One suggested reason for this impact is that different microbial groups have different concentration thresholds of ammonia toxicity on their microbial activity or cell growth.[16, 17] For example, many studies on microbial population diversity of anaerobic digestion systems revealed that syntrophic acetate degradation via acetate oxidizers converting acetate to H_2/CO_2 and hydrogenotrophic methanogens dominated over acetoclastic methanogenesis under ammonia stress conditions,[18-21] while the opposite has also been observed.[22] The synergetic stress from acetic acid and ammonia was also studied and it was concluded that the combined effect resulted

in different inhibition for acetoclastic and hydrogenotrophic methanogenesis and syntrophic acetate oxidation, leading to dominant pathway shift differently depending on acetate concentration and ammonium level.[14]

Compared with acetic acid, propionic acid shows greater inhibitory effect on microbial biomass and high concentrations of this VFA species usually cause severe problems in AD processes.[23] Although alternative pathways exist,[24] the syntrophic propionic acid degradation to acetic acid and hydrogen by acetogens coupled with acetic acid and hydrogen removal via methanogenesis is believed to be the major route for its degradation in digesters with low sulphate content.[25] This dominant propionic acid oxidation pathway, however, is the most thermodynamically unfavourable reaction for VFA degradation, and occurs only when the partial pressure of hydrogen is low, at 10 Pa.[26,27]

Considering the double stress from both high concentrations of propionate and ammonia encountered during the high-rate organic nitrogen-rich wastes digestion process, the effect of ammonia on propionate degrading needs further investigation, especially for continuous reactors. The inhibitory effect of either propionic acid or ammonia has usually been studied separately,[28-30] while their combined effect on microbial community structure and pathway shift is less well addressed. Therefore, this study aimed to investigate the impact of ammonia on propionate degradation, methane production, microbial populations and methanogenic pathways during continuous anaerobic digestion using propionate as feedstock.

MATERIAL AND METHODS

Inoculum

The inoculum was taken from an anaerobic digester treating biosolids (Millbrook Wastewater Treatment works, Southampton, UK). The time between collection and inoculation was 1 day during which the biosolids digestate was maintained at 37℃. Before use it was sieved through a 1 mm mesh to remove grit and other solids.

Experimental set-up

The experiment was carried out in two 2 L CSTR-type reactors, R1 and R2, with 1.5 L working volume, which were initially inoculated with sieved biosolids digestate, with headspace

flushed with a N_2:CO_2 gas mixture (mixed in 80:20 ratio v/v). They were then operated semi-continuously at 36 ± 1℃ in an orbital shaking incubator operating at 100 rpm. An identical hydraulic and solids retention time of 15 days was maintained by removing 100 mL of reactor content and replacing it with 100 mL of feed once per day under anaerobic condition. Sodium propionate was chosen as the sole substrate and the initial loading was set at 0.5 g propionic acid L^{-1} d^{-1}. The loading was elevated to 0.625 g L^{-1} d^{-1} from day 16 onwards for both reactors and that of the reactor with lower ammonia concentration was step-wise increased further during the course of the experiment. The volume of the feed was made up by nutrient medium. The nutrient medium for reactor 1 (R1) contained the following [mg L^{-1}]: NH_4Cl [400]; $MgSO_4 \cdot 6H_2O$ [250]; KCl [400]; $CaCl_2 \cdot 2H_2O$ [120]; $(NH_4)_2HPO_4$ [80]; $FeCl_3 \cdot 6H_2O$ [55]; $NaHCO_3$ [5000]; and the trace element salts (*i.e.* $CoCl_2 \cdot 6H_2O$, $NiCl_2 \cdot 6H_2O$, $MnCl_2 \cdot 4H_2O$, $CuCl_2 \cdot 2H_2O$, $AlCl_3 \cdot 6H_2O$, $ZnCl_2$, $Na_2WO_4 \cdot 2H_2O$, H_3BO_3, Na_2SeO_3 and $Na_2MoO_4 \cdot 2H_2O$) [each at 0.5].[31] The composition of nutrient medium solution for reactor 2 (R2) was the same as that for reactor 1, except that ammonium chloride concentration was increased to maintain the concentration of total ammonia nitrogen (TAN) in R2 at 2.5 g-N L^{-1}. The pH of the feed solution was maintained at 7.5 using HCl (1 mol L^{-1}) for pH adjustment. Gas sampling bags (Tedlar, SKC Ltd, UK) were used to collect biogas produced.

Analytical methods

Total solids (TS) and volatile solids[32] were measured using Standard Method 2540 G. pH was determined using a Jenway 3010 meter (Bibby Scientific Ltd, UK) with a combination glass electrode calibrated in buffers at pH 7.0 and 9.2 (Fisher Scientific, UK). Alkalinity was measured by titration with 0.25 N H_2SO_4 to endpoints of pH 5.7 and 4.3, allowing calculation of total (TA), partial (PA) and intermediate alkalinity (IA). TAN was determined in accordance with Standard Method 4500-NH_3 B and C. VFA were quantified in a Shimazdu GC-2010 gas chromatograph (Shimadzu, Milton Keynes, UK), using a flame ionization detector and a capillary column type SGE BP-21. Biogas volume was measured using weight gasometer and corrected to standard temperature and pressure (STP) of 0 °C, 101.325 kPa.[33] Methane (CH_4) and carbon dioxide (CO_2) were determined using a Varian star 3400 CX Gas Chromatograph, calibrated with a mixed gas standard of 65% (v/v) CH_4 and 35% (v/v) CO_2. Hydrogen (H_2) was analyzed by Varian CP-3800

Gas Chromatograph using 1% of H_2 for calibration.

Metagenomic DNA isolation and amplification

DNA extraction was performed using the Fast DNA SPIN Kit for Soil (QBIOgene Inc., Carlsbad, CA, USA), according to the manufacturer's instructions. DNA quality was assessed using gel electrophoresis (1% agarose) and DNA concentrations were determined using a Qubit Fluorometer (Thermo, USA).

The extracted DNA sample was then handled according to the protocol of the genomic DNA sample preparation kit (Illumina). The DNA fragmentation was firstly performed using Covaris S2 Ultrasonicator, and the DNA fragments were then processed by end reparation, A-tailing, adapter ligation, DNA size-selection. PCR reaction and products purification based on Illumina Miseq2×300 instructions. For archaea, the 16S rRNA genes were amplified through three rounds of PCR. The primers for the first round were 340F (5'-CCCTAYGGGGYGCASCAG-3') and 1000R (5'- GGCCATGCACYWCYTCTC-3'). Then the PCR products were used as templates for a second PCR with 349F (5'-CCCTACACGACGCTCTTCCGATCTN (barcode) GYGCASCA GKCGMGAAW-3') and 806R (5'-GACTGGAGTTCCTTGGCACCCGAGAATTCCAGGACTA CVSGGGTATCTAAT-3'), and the third round PCR amplified with Illumina Nested primers. The bacteria 16S rRNA genes were amplified through two rounds of PCR. First, using 341F (5'-CCCTACACGACGCTCTTCCGATCTG (barcode) CCTACGGGNGGCWGCAG -3') and 805R (5'-GACTGGAGTTCCTTGGCACCCGAGAATTCCAGACTACHVGGGTATCTAATCC3').
The PCR products were then used as templates for a second PCR with Illumina Nested primers.

Whole genome pyrosequencing analysis

For sequencing, a library consisting of approximate 170 bp fragments was constructed. The base-calling pipeline (version Illumina Pipeline-0.3) was used to process the raw fluorescence images and call sequences, and a sequencing depth of 3.0 Gb reads was applied for the sample metagenomic datasets. The metagenomic reads were trimmed using two criteria, *i.e.* a minimum quality score of 30 and a minimum read length of 35 bp, and ambiguous nucleotides were not accepted. The two criteria adopted for overlapping were: (1) at least 20 nt length of the overlap region was required, and (2) at most two mismatches were allowed. Based on all the annotation

source databases used by MG-RAST, taxonomic profiles were calculated by Best Hit classification at the E-value cutoff of 10^{-5} with minimum alignment length of 50 bp. The distribution of taxonomic domains, phyla, orders, families and genus for the annotations was analyzed in detail. Sequences have been deposited in the National Center for Biotechnology Information (NCBI) BioProject under the accession number PRJNA362546.

RESULTS and DISCUSSION

Digestion performance and mass balance

Figure 1 compares the propionic acid degradation performance of two reactors with different TAN concentrations: TAN in R1 was less than 200 mg N L^{-1} after two retention times due to the washout effect, but that in R2 was increased to around 2500 mg N L^{-1} by NH_4Cl supplementation. As Fig. 1 shows, almost no propionic acid accumulated in R1 during the whole experimental period except a small peak at 1680 mg L^{-1} appeared in the 5th retention time when OLR increased to 0.80 g L^{-1} d^{-1}. In contrast to R1, the concentration of the substrate kept increasing in R2 from day 20, and gradually flattened over at around 7000 mg L^{-1} during the beginning of 5th retention time with an OLR of 0.8 g L^{-1} d^{-1}, indicating around 75% of daily propionate input was not degraded at that point. Although acetic acid appeared in the effluent of R1 around the same time that propionic acid started to accumulate in R2, that was temporary and only resulted in a slightly lowered methane production rate of R1 (Fig. 1) for around one retention time. For methane production, the fluctuation of volumetric methane production (VMP) of R1 reflects the two occurrences of VFA accumulation and subsequently degradation. VMP of R1 was up to around 0.50 L L^{-1} d^{-1} when OLR reached 1.2 g L^{-1} d^{-1} at the last stage of the experiment, indicating 80% of methane potential of propionic acid recovered. VMP of R2 was the same as R1 at the beginning of the experiment around 0.23 L L^{-1} d^{-1}, but it reduced to 50% at the end of the third HRT (day 45) with 5% remaining when it was terminated (day 66). The average methane percentage of R1 was 77%, higher than 72% of R2, while carbon dioxide percentage of R2 was slightly higher than that of R1. No hydrogen was detected in both reactors. Although R2 had higher TAN concentration, pH of R1 was higher than that of R2 due to the persistent high VFA concentration in R2. The same reason applied to the lower ratio of IA to PA in R1 compared to that of R2.

The mass balance was conducted based on the average value of each HRT (The average performance characteristics were calculated with statistical analysis) as shown in Table 1. Propionic acid degradation rate of R1 was above 91% during each HRT. The methane recovery rate from HPr input was above 70% except in HRT 3 when HAc was accumulating. In R2, the propionic acid degradation rate kept decreasing from 100% at HRT 1 to 53% at HRT 4 under ammonia stress, and as a result the methane recovery rate declined (from 86%-11%) simultaneously if it was calculated from the total propionate input. The methane recovery rate, however, from the degraded propionate in R2 during four HRTs was much higher due to no clear HAc and H_2 accumulation. This indicated that TAN inhibited propionate degradation more severely than the methanogenesis step.

The above results suggest that a semi-continuous CSTR reactor fed with propionate as sole carbon source was not able to maintain stable performance under the test conditions: organic loading 0.625 g propionic acid L^{-1} d^{-1}, hydraulic and solid retention time 15 days, and total ammonia nitrogen 2.5 g L^{-1}. It is apparent that ammonia inhibited the phase of propionate oxidizing directly in R2 because the propionate accumulated over the experimental period, whereas the acetate produced from propionate degradation seemed to be converted to biogas promptly. Although the inhibitory effect of ammonia is often demonstrated to mainly influence the phase of methanogenesis,[34,35] propionic acid was observed as one of the dominant VFAs accumulated during ammonia stress in digesters fed with protein rich materials.[36] The different dominant VFA species is thought to be related to the loading rate and how much propionic acid was produced as intermediate product, along with other operational and environmental parameters.

Microbial community structure

For a better understanding of the microbial community structure of the bioreactors, the domains of Bacteria and Archaea have been analyzed at different taxonomic levels using high-throughput sequencing analysis. Analyses were carried out at day 0, day 60 and day 120 for R1 and day 0, day 30 and day 60 for R2.

Archaeal community shifts

For Archaeal community structure shifts in both reactors, the relative abundance of archaeal 16S rRNA gene at the order and genus levels is shown in Fig. 2. For the biomass of R1, a significant difference was found for the dominant order during the whole experimental period (Fig. 2(A)). The relative abundance of *Methanosarcinales* increased from 54% in raw sludge to 90% on day 60 and up to more than 99% on day 120. However, the members of *Methanomicrobiales* decreased from 39% to 9% on day 60 and less than 1% on day 120. It indicates that the groups of *Methanosarcinales* were responsible for methane production from propionate without ammonia stress. *Methanosarcinales* is the only order that could mediate methane production from acetate.[37] *Methanosaeta* are strict acetoclastic methanogens, which was found to be the main genus found in the order (Fig. 2(B)). For the biomass of R2, *Methanosarcinales* and *Methanomicrobiales* were the two dominant orders among all samples tested. The predominant orders shifted in the opposite way compared with R1. The relative abundance of *Methanosarcinales* reduced from 54% to 44%, and the *Methanomicrobiales* increased from 39% to 54% due to the exposure to ammonia. *Methanomicrobiales*, being a group of hydrogenotrophic methanogens, are known to have higher tolerance to ammonia.[28,38] It should also be noted that the dominant genus in the order *Methanomicrobiales* changed during the course of the experiment (Fig. 2(B)). The members of genus *Methanospirillum* increased from 9% to 38%, indicating they tolerated ammonia and grew better than *Methanosphaerula. Methanospirillum* were also found to be dominant groups in the reactors containing nitrogen-rich manure.[39]

Bacterial community shifts

More than 20000 bacterial reads for each sample were obtained, and the number of operational taxonomic units (OTUs) per sample ranged from 262 to 387. *Proteobacteria* (28-65%) and *Firmicutes* (19-50%) were identified as the two most dominant phyla in all samples (Fig. 3(A)). In addition, sequences belonging to *Chlorflexi*, *Bacteroidetes*, *Synergistetes*, *Actinobacteria* and 24 other phyla were detected in some of the samples, but at low occurrence (below 13% for each sample). The abundance of *Proteobacteria* increased in both reactors during the entire digestion process no matter if ammonia was intentionally overdosed or not. In contrast to *Proteobacteria*, *Firmicutes* increased in R1 (without ammonia stress) from 34% to 50% but decreased in R2 (with ammonia stress) from 34% to 19%. These results suggest that the bacteria

belonging to *Firmicutes* were more sensitive to ammonia than *Proteobacteria*, although *Firmicutes* can produce endospores which can resist dehydration and extreme environments even at high VFA and ammonia.[40]

Within the *Proteobacteria*, the main class shifted from *Gammaproteobacteria* on day 0 to *Betaproteobacteria* at the end of the experiment in both reactors (Fig. 3(B)). This was in line with other observations[41] where *Betaproteobacteria* was also found to be the most dominant group in propionate-, butyrate- and acetate-utilizing microbial communities.

Within *Firmicutes* phylum, *Clostridia* were the dominant class in R1 which increased from 27% on day 0 to 53% on day 120. In contrast, *Clostridia* lost its dominant position and its percentage decreased from 27% to 9% in R2, and the sequences belonging to the classes *Bacilli* instead increased. *Clostridia* are well-known in fermenters associated with a high rate of hydrolysis and VFA fermentation. *Clostridiaceae* was the dominant family within class *Clostridia* in R1, which were identified to live in a syntrophic relationship with methanogenic *Archaea* when degrading long chain fatty acids (LCFA).[8] These microorganisms are commonly proton-reducing acetogenic bacteria that require syntrophic interaction with H_2-utilizing methanogens and acetoclastic methanogens.[42,43] Although there was no direct evidence shown that members of *Clostridia* were capable of propionate degrading, the abundance difference of *Clostridia* between R1 day 120 and R2 day 60 indicted that *Clostridia* might have a function closely related to syntrophic propionate degradation.

In addition, it should be noted that *Proteobacteria* and *Firmicutes* have been repeatedly reported as the main phyla in various anaerobic digesters fed with complex substrates such as cattle manure, sludge or straw and cow manure.[44-46] The sole carbon source in this study was propionate which was much simpler than in these studies. The diversity of microbes was greater than expected. The contributions of the various microbes and their relevance to propionate fermentation still could not be clearly worked out, although it is plausible that they played a role of endogenous digestion of biomass in reactors.

Functional groups for propionate degradation

Based on the results of high throughput sequence reads, a summary of the propionate-oxidizing bacteria (POB) and methanogens in biomass of both reactors is provided in

Table 2. According to previous study,[47] the sequences from all samples affiliated to 13 genera with the ability of propionate utilization, covering propionate degrading syntrophs and non-syntrophs, but with a low relative abundance (below 2% of all the bacteria for each sample). Previously identified as specific syntrophic LCFA degrading bacteria,[48] *Syntrophomonas* groups were dominant propionate-oxidizing bacteria in the inoculum, but their percentage decreased dramatically in both digesters. In the later stage of both reactors, *Syntrophobacter* groups, as sulfate reducers, are capable of degrading propionate in syntrophic association with methanogens, were the main propionate oxidizers. The role of *Syntrophobacter* was investigated in the past, for instance one study reported a correlation between increase of *Syntrophobacter* species and the degradation of propionate.[49] The genera of *Smithella*, *Syntrophorhabdus*, *Syntrophus*, *Desulforhabdus* and *Desulfonispora* were detected only in the biomass with ammonia stress (R2). A relatively high proportion (59%) of sequences was identified to genus *Desulfobulbus* on day 60 in R2. *Desulfobulbus* is a genus that grows with propionate and sulfate, but it was not able to grow with propionate in syntrophy with methanogens.[47]

For methanogens, methanogenic archaea detected in the inoculum of both reactors were mainly *Methanosaeta*, *Methanosphaerula* and *Methanospirillum*. The relative abundance of genus *Methanosaeta* increased by 67% in R1 (from 54% to 90%) but decreased by 19% in R2 with ammonia stress (from 54% to 44%) throughout the process of propionate degradation. In contrast, although the genus *Methanospirillum* belonging to the order *Methanomicrobiales* was negligible over the experimental period in R1 (from 9% to <1%), it increased by 320% in R2 with ammonia stress (from 9% to 38%). These results tended to confirm the previous studies that *Methanosaeta*, which is an exclusive acetoclastic methanogen, was more vulnerable to ammonia than hydrogenotrophic methanogen.[11] Genus *Methanospirillumis*, a hydrogenotrophic methanogen that uses H_2/CO_2 and formate as substrate, was reported to be a syntrophic partner of *Syntrophobacter*, *Syntrophomonas*, *Smithella* and *Pelotomaculum* in degrading propionate.[50] The opposite shift of acetoclastic methanogens and hydrogenotrophic methanogens in R1 and R2 suggests again that acetoclastic methanogenesis dominated over hydrogenotrophic methanogenesis without ammonia stress, however, hydrogenotrophic methanogenesis coupled with acetoclastic methanogenesis was the main metabolic pathway under ammonia stress conditions.

According to Equation (1),[51] only around 10 mg of propionic acid can be oxidized daily using

$MgSO_4 \cdot 6H_2O$ (250 mg L^{-1}) with 100 mL daily feeding, which was much less than propionate degraded in both reactors.

$$4CH_3CH_2COO^- + 3SO_4^{2-} = 4CH_3COO^- + 4HCO_3^- + H^+ + 3HS^- \quad (1)$$

Therefore, sulphate reduction was not the dominant propionate consumption pathway for the stage with *Syntrophobacter* or *Desulfobulbus* as dominant group in each reactor. There should have some H_2-utilizing microbes if *Syntrophobacter* or *Clostridiaceae* was responsible for propionate consumption in the syntrophic pathway association with methanogens. Under ammonia stress condition, *Methanospirillum* was the syntrophic partner for propionate degrading. However, for propionate digestion without ammonia stress, further study is needed to determine whether hydrogenothrophic methanogens were H_2-scavenging microorganisms with low abundance or whether other propionate conversion pathways existed in which H_2 was not produced (e.g. propionic acid converted to butyric and acetic acid).

CONCLUSION

Results obtained from this study demonstrated that the ammonia inhibitory effect had stronger impact on propionate degradation than methanogenesis in a propionate-fed digester.

The direct inhibition of ammonia on propionate oxidizing phase resulting in low efficiency of propionate conversion was the main reason for poor digestion performance. The loss of populations of *Clostridiaceae* and *Syntrophobacter* was responsible for the dropping propionate degradation and lower methane production under ammonia stress condition. The finding of rich bacterial diversity underlines that the micro-ecology in anaerobic digestion is much more complex than expected, even when fed with a sample substrate: this needs more study to better appreciate complex microbial networks. Acetoclastic methanogens dominated over hydrogenotrophic methanogens without ammonia stress in this research, while hydrogenotrophic methanogens, which were more tolerance to ammonia, coupled with acetoclastic methanogens were the main methanogenic archaea under ammonia stress conditions.

Acknowledgements

Thanks are due to the Science and Technology Planning Project of Guangdong Province (2015B020215011) and EU FP7 ECOFUEL project (246772).

The authors would like to thank Dr Sonia Heaven and Prof Charles Banks at the University

of Southampton for hosting the lead author's research.

Data supporting this study are openly available from the University of Southampton repository at http://dx.doi.org/10.5258/SOTON/D0017'

References

1. Fisgativa H, Tremier A and Dabert P, Characterizing the variability of food waste quality: A need for efficient valorisation through anaerobic digestion. *Waste Manage* **50**:264–274 (2016).
2. Kong X, Wei Y, Xu S, Liu J, Li H, Liu Y and Yu S, Inhibiting excessive acidification using zero-valent iron in anaerobic digestion of food waste at high organic load rates. *Bioresource Technol* **211**:65-71 (2016).
3. Banks CJ, Chesshire M, Heaven S and Arnold R, Anaerobic digestion of source-segregated domestic food waste: performance assessment by mass and energy balance. *Bioresource Technol* **102**:612-620 (2011).
4. Wu SB, Ni P, Li JX, Sun H, Wang Y F, Luo H Z, Dach J and Dong RJ, Integrated approach to sustain biogas production in anaerobic digestion of chicken manure under recycled utilization of liquid digestate: Dynamics of ammonium accumulation and mitigation control. *Bioresource Technol* **205**:75-81 (2016).
5. SerranoA, Siles JA, Gutierrez MC and Martin MA, Optimization of Anaerobic Co-digestion of Strawberry and Fish Waste. *Applied Biochem and Biotechnol* **173**:1391-1404 (2014).
6. Kovacs E, Wirth R, Maroti G, Bagi Z, Rakhely G and Kovacs KL, Biogas Production from Protein-Rich Biomass: Fed-Batch Anaerobic Fermentation of Casein and of Pig Blood and Associated Changes in Microbial Community Composition. *Plos One* **8**:e77265 (2013).
7. Aboudi K, Jose Alvarez-Gallego C and Isidoro Romero-Garcia L, Biomethanization of sugar beet by product by semi-continuous single digestion and co-digestion with cow manure. *Bioresource Technol* **200**:311-319 (2016).
8. Regueiro L, Lema JM and Carballa M, Key microbial communities steering the functioning of anaerobic digesters during hydraulic and organic overloading shocks. *Bioresource Technol* **197**: 208-216 (2015).
9. Westerholm M, Muller B, Isaksson S and Schnurer A, Trace element and temperature effects on microbial communities and links to biogas digester performance at high ammonia levels. *BiotechnolBiofuels* **8**:1-19 (2015).
10. Chen Y, Cheng JJ and Creamer KS,. Inhibition of anaerobic digestion process: a review. *Bioresour Technol* **99**: 4044-4064 (2008).
11. Poirier S, Desmond-Le Quemener E, Madigou C, Bouchez T and Chapleur O, Anaerobic digestion of biowaste under extreme ammonia concentration: Identification of key microbial phylotypes. *Bioresource Technol* **207**:92-101 (2016).
12. Yenigün O and Demirel B,. Ammonia inhibition in anaerobic digestion: A review. *Process Biochem* **48**:901-911 (2013).
13. Muller B, Sun L, Westerholm M and Schnurer A, Bacterial community composition and fhs profiles of low- and high-ammonia biogas digesters reveal novel syntrophic acetate-oxidising bacteria. *Biotechnol Biofuels* **9**:1-18 (2016).
14. Lu F, Hao L, Guan D, Qi Y, Shao L and He P, Synergetic stress of acids and ammonium on the shift in the methanogenic pathways during thermophilic anaerobic digestion of organics. *Water Res* **47**:2297-2306 (2013).

15. Zhang C, Yuan Q and Lu Y, Inhibitory effects of ammonia on methanogen mcrA transcripts in anaerobic digester sludge, *FEMS Microbiol Ecol* **87**:368-377 (2014).
16. Rajagopal R, Masse DI and Singh G, A critical review on inhibition of anaerobic digestion process by excess ammonia. *Bioresource Technol* **143**:632-641 (2013).
17. Angelidaki I and Ellegaard L, Codigestion of manure and organic wastes in centralized biogas plants - Status and future trends. *Appl Biochem Biotechnol* **109**:95-105 (2003).
18. Schnurer A, Zellner G and Svensson BH, Mesophilic syntrophic acetate oxidation during methane formation in biogas reactors. *FEMS Microbiol Ecology* **29**:249-261 (1999).
19. Schnurer A and Nordberg A, Ammonia, a selective agent for methane production by syntrophic acetate oxidation at mesophilic temperature. *Water Sci Technol* **57**:735-740 (2008).
20. Westerholm M, Dolfing J, Sherry A, Gray ND, Head IM and Schnuerer A, Quantification of syntrophic acetate-oxidizing microbial communities in biogas processes. *Environ Microbiol Reports* **3**:500-505 (2011).
21. Kato S, Sasaki K, Watanabe K, Yumoto I and Kamagata Y, Physiological and Transcriptomic Analyses of the Thermophilic, Aceticlastic Methanogen Methanosaeta thermophila Responding to Ammonia Stress. *Microbes Environ* **29**:162-167 (2014).
22. Fotidis IA, Karakashev D, Kotsopoulos TA, Martzopoulos GG and Angelidaki I, Effect of ammonium and acetate on methanogenic pathway and methanogenic community composition. *FEMS Microbiol Ecol* **83**:38-48 (2013).
23. Yuan H and Zhu N, Progress in inhibition mechanisms and process control of intermediates and by-products in sewage sludge anaerobic digestion. *Renew Sustain Energy Rev* **58**:429-438 (2016).
24. de Bok FAM, Stams AJM, Dijkema C and Boone DR, Pathway of propionate oxidation by a syntrophic culture of Smithella propionica and Methanospirillum hungatei. *Appl Environ Microbiol* **67**:1800-1804 (2001).
25. Ariesyady HD, Ito T, Yoshiguchi K and Okabe S, Phylogenetic and functional diversity of propionate-oxidizing bacteria in an anaerobic digester sludge. *Appl Microbiol Biotechnol* **75**:673-683 (2007).
26. Tatara M, Makiuchi T, Ueno Y, Goto M and Sode K, Methanogenesis from acetate and propionate by thermophilic down-flow anaerobic packed-bed reactor. *Bioresource Technol* **99**:4786-4795 (2008).
27. RamK D, Chandra KW, Syed IA, Dev VK and Lokendra S, Effect of propionate toxicity on methanogenesis of night soil at phychrophilic temperature. *Bioresource Technol* **87**:299-303 (2003).
28. Moestedt J, Muller B, Westerholm M and Schnurer A, Ammonia threshold for inhibition of anaerobic digestion of thin stillage and the importance of organic loading rate. *Microbial Biotechnol* **9**:180-194 (2016).
29. Wagner AO, Reitschuler C and Illmer P, Effect of different acetate:propionate ratios on the methanogenic community during thermophilic anaerobic digestion in batch experiments. *Biochem Eng J* **90**:154-161 (2014).
30. Franke-Whittle IH, Walter A, Ebner C and Insam H, Investigation into the effect of high concentrations of volatile fatty acids in anaerobic digestion on methanogenic communities. *Waste Manage* **34**:2080-2089 (2014).
31. Tale VP, Maki JS, Struble CA and Zitomer DH, Methanogen community structure-activity relationship and bioaugmentation of overloaded anaerobic digesters. *Water Res* **45**:5249-5256 (2011).

32. Svehla P, Bartacek J, Pacek L, Hrncirova H, Radechovsky J, Hanc A and Jenicek P, Inhibition effect of free ammonia and free nitrous acid on nitrite-oxidising bacteria during sludge liquor treatment: influence of feeding strategy. *Chem Papers* **68**:871-878 (2014).
33. Walker M, Zhang Y, Heaven S and Banks C, Potential errors in the quantitative evaluation of biogas production in anaerobic digestion processes. *Bioresour Technol* **100**:6339-6346 (2009).
34. Hansen KH, Angelidaki I and Ahring BK, Anaerobic digestion of swine manure: Inhibition by ammonia. *Water Res* **32**:5-12 (1998).
35. Garcia ML and Angenent LT, Interaction between temperature and ammonia in mesophilic digesters for animal waste treatment. *Water Res* **43**:2373-2382 (2009).
36. Zhang Y and Banks CJ, Co-digestion of the mechanically recovered organic fraction of municipal solid waste with slaughterhouse wastes. *Biochem Eng J* **68:**129-137 (2012).
37. Kendall MM and Boone DR, The Order Methanosarcinales in Dworkin M. in *The Prokaryotes*, ed. By Falkow S, Rosenberg E, Schleifer KH and Stackebrandt E. Springer, New York, pp. 244-256 (2006).
38. Sun L, Muller B, Westerholm M and Schnurer A, Syntrophic acetate oxidation in industrial CSTR biogas digesters. *J Biotechnol* **171**:39-44 (2014).
39. Kampmann K, Ratering S, Baumann R, Schmidt M, Zerr W and Schnell S, Hydrogenotrophic methanogens dominate in biogas reactors fed with defined substrates. *System Appl Microbiol* **35**:404-413 (2012).
40. Niu Q, Kubota K, Qiao W, Jing Z, Zhang Y and Yu-You L, Effect of ammonia inhibition on microbial community dynamic and process functional resilience in mesophilic methane fermentation of chicken manure. *J Chem Technol Biotechnol* **90**:2161-2169 (2015).
41. Ariesyady HD, Ito T and Okabe S, Functional bacterial and archaeal community structures of major trophic groups in a full-scale anaerobic sludge digester. *Water Res* **41**:1554-1568 (2007).
42. Palatsi J, Illa J, Prenafeta-Boldu FX, Laureni M, Fernandez B, Angelidaki I and Flotats X, Long-chain fatty acids inhibition and adaptation process in anaerobic thermophilic digestion: batch tests, microbial community structure and mathematical modelling. *Bioresource Technol* **101**:2243-2251 (2010).
43. Sousa DZ, Pereira MA, Smidt H, Stams AJM and Alves MM, Molecular assessment of complex microbial communities degrading long chain fatty acids in methanogenic bioreactors. *FEMS Microbiol Ecol*,**60**:252-265 (2007).
44. Luo G, Fotidis I A and Angelidaki I, Comparative analysis of taxonomic, functional, and metabolic patterns of microbiomes from 14 full-scale biogas reactors by metagenomic sequencing and radioisotopic analysis. *Biotechnol Biofuels* **9**:1-12 (2016).
45. Campanaro S, Treu L, Kougias PG, De Francisci D, Valle G and Angelidaki I, Metagenomic analysis and functional characterization of the biogas microbiome using high throughput shotgun sequencing and a novel binning strategy. *Biotechnology for Biofuels* **9**: (2016).
46. Sun L, Pope PB, Eijsink VGH and Schnurer A, Characterization of microbial community structure during continuous anaerobic digestion of straw and cow manure. *Microbial Biotechnol* **8**:815-827 (2015).
47. Worm P, Koehorst JJ, Visser M, Sedano-Nunez VT, Schaap PJ, Plugge CM, Sousa DZ and Stams AJM, A genomic view on syntrophic versus non-syntrophic lifestyle in anaerobic fatty acid degrading communities. *Biochim Biophys Acta-Bioenergetics* **1837**:2004-2016 (2014).
48. Ziels RM, BeckD AC, Marti M, Gough HL, Stensel HD and Svensson BH, Monitoring the

dynamics of syntrophic beta-oxidizing bacteria during anaerobic degradation of oleic acid by quantitative PCR. *Fems Microbiol Ecol* **91**:1-13 (2015).

49. Moertelmaier C, Li C, Winter J and Gallert C, Fatty acid metabolism and population dynamics in a wet biowaste digester during re-start after revision. *Bioresource Technol* **166**:479-484 (2014).
50. Felchner-Zwirello M, Winter J and Gallert C, Interspecies distances between propionic acid degraders and methanogens in syntrophic consortia for optimal hydrogen transfer. *Appl Microbiol Biotechnol* **97**:9193-9205 (2013).
51. David RB and Marvin PB, Propionate-Degrading Bacterium, Syntrophobacter wolinii sp. nov. gen. nov., from Methanogenic Ecosystems. *Appl Environ Microbiol* 40:626–632 (1980).

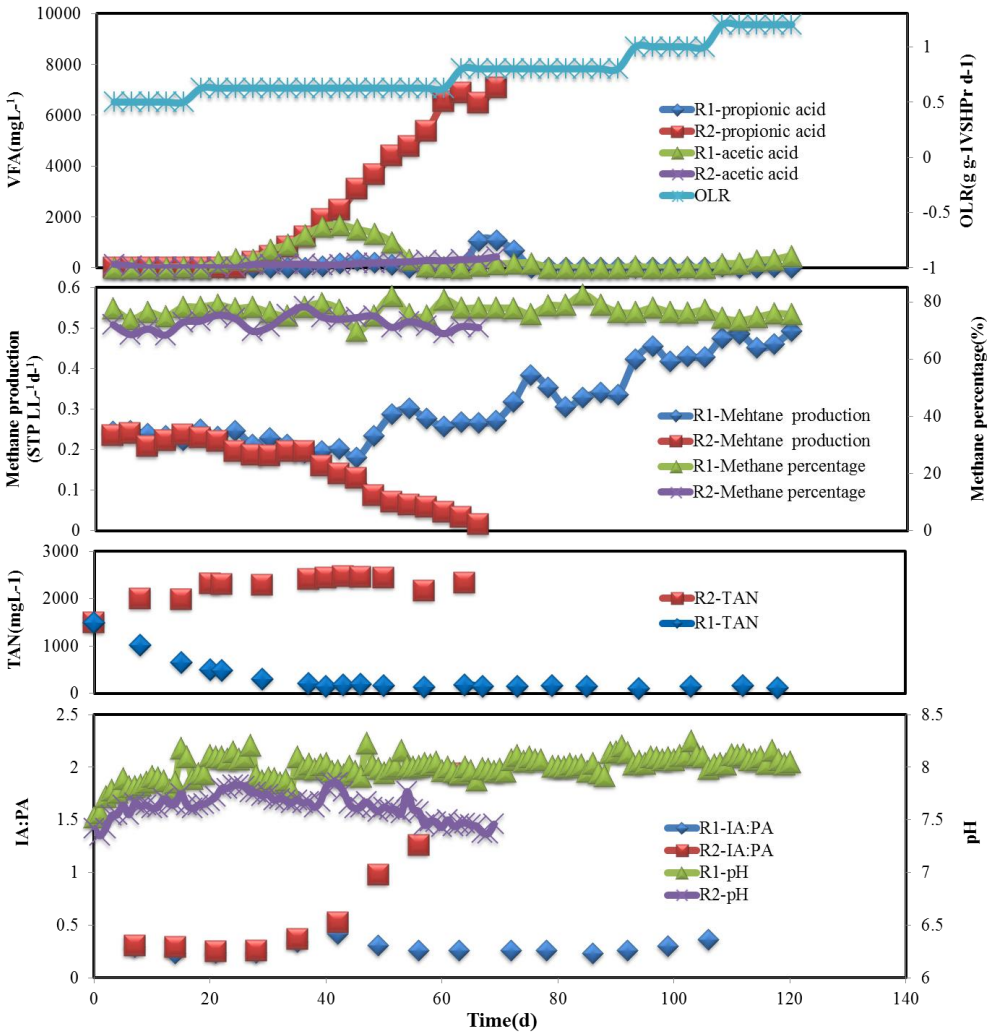

Figure 1. Propionic acid degradation performance of two reactors (R1 without ammonia stress, R2 under 2.5 g L^{-1} ammonia stress).

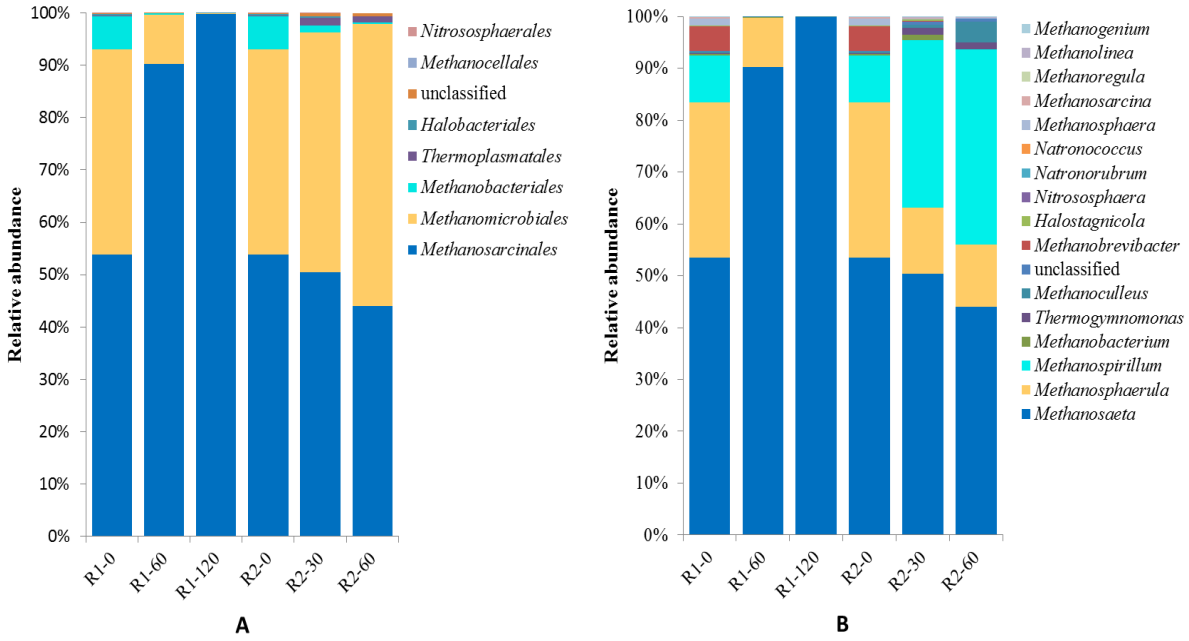

Figure 2. Relative abundance of archaea 16S rRNA gene at the order level (A) and genus level (B) in R1 at day 0, 60 and 120 (shown as R1-0, R1-60 and R1-120) and R2 at day 0, 30 and 60 (shown as R2-0, R2-30 and R2-60).

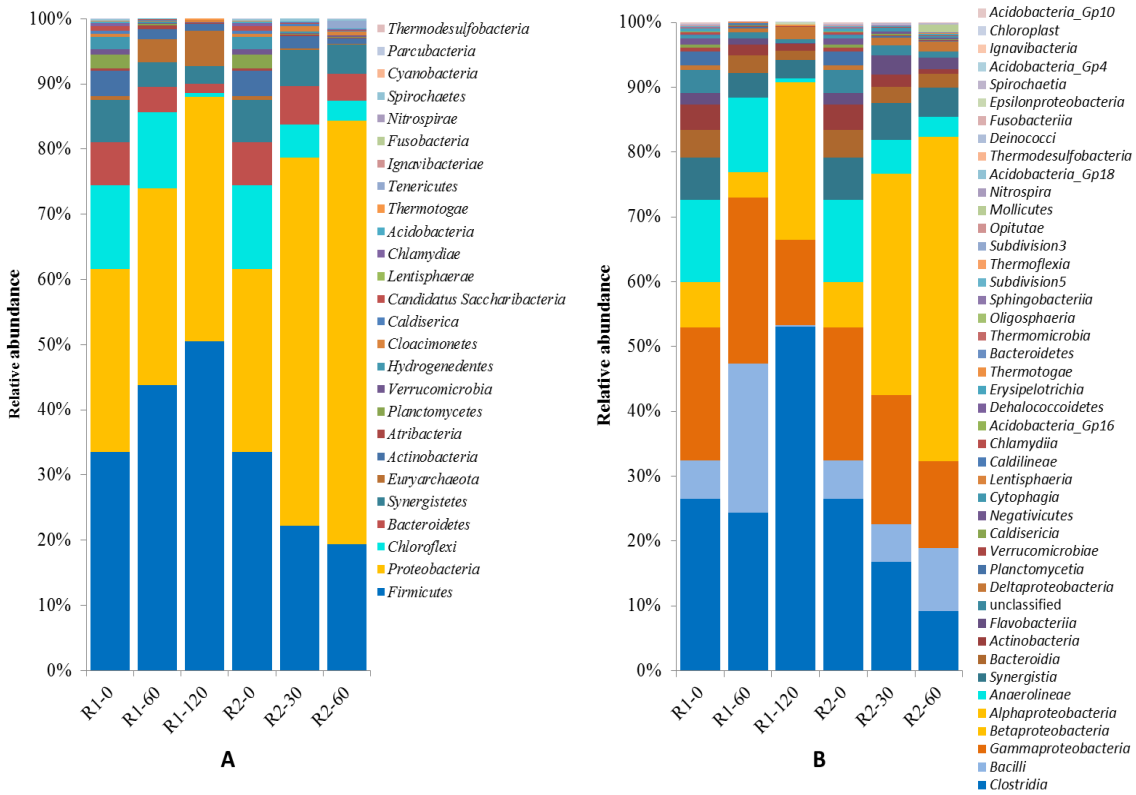

Figure 3. Relative abundance of bacteria 16S rRNA gene at the phylum level (A) and class s level (B) in R1 at day 0, 60 and 120 (shown as R1-0, R1-60 and R1-120) and R2 at day 0, 30 and 60 (shown as R2-0, R2-30 and R2-60).

Table 1. Digestion performance during different experimental periods

Period	OLR ($g\ L^{-1}\ d^{-1}$)	[a]Total HPr Input (g)	Total HPr accumulation (g)	Total HAc accumulation (g)	[b]HPr degradation rate (%)	[b]CH_4 recovery rate from HPr input (%)	[b]CH_4 recovery rate from degraded HPr (%)
R1							
HRT1	0.50	11.25	0.00	0.06	100.00 (0.00)	89.63 (3.39)	99.32 (0.82)
HRT2	0.625	14.07	0.01	1.41	99.92 (0.16)	70.56 (4.42)	87.02 (13.43)
HRT3	0.625	14.54	0.56	3.16	96.06 (3.70)	57.46 (5.00)	72.42 (13.38)
HRT4	0.625	14.17	0.04	0.19	99.73 (0.61)	81.77 (8.03)	98.49 (3.37)
HRT5	0.80	20.07	2.06	0.41	91.41 (14.89)	65.96 (18.02)	97.49 (1.45)
HRT6	0.80	18.00	0.00	0.00	100.00 (0.00)	78.63 (4.19)	100.00 (0.00)
HRT7	1.00	22.50	0.00	0.16	100.00 (0.00)	84.80 (10.16)	99.06 (1.36)
HRT8	1.20	27.16	0.16	1.05	99.37 (0.77)	73.97 (2.89)	95.00 (2.37)
R2							
HRT1	0.50	11.25	0.00	0.22	100.00 (0.00)	85.86 (4.94)	97.43 (5.75)
HRT2	0.625	14.72	0.81	0.23	94.66 (7.33)	58.43 (9.55)	98.16 (3.86)
HRT3	0.625	19.76	6.24	0.30	68.85 (8.67)	35.14 (10.26)	98.13 (1.33)
HRT4	0.625	25.50	11.94	0.50	53.40 (6.45)	10.83 (3.55)	97.52 (0.76)

a: HPr input contains daily HPr input as propionate and the accumulated HPr from the last HRT.

b: Values are expressed as mean values with the standard deviation shown in parentheses.

Table 2. Comparision of the dominant functional groups for propionate degradation and methane production

Groups (genus)	Relative abundance					
	R1-Day0	R1-Day 60	R1-Day120	R2-Day 0	R2-Day30	R2- Day 60
propionate-oxidizing bacteria[a]						
Syntrophobacter	7%	61%	99%	7%	39%	8%
Syntrophomonas	67%	11%	<1%	67%	23%	12%
Smithella	2%	<1%	<1%	2%	9%	7%
Syntrophorhabdus	10%	<1%	<1%	10%	8%	6%
Syntrophaceticus	3%	11%	<1%	3%	1%	1%
Syntrophothermus	5%	3%	<1%	5%	1%	1%
Syntrophobotulus	<1%	1%	1%	<1%	<1%	<1%
Syntrophus	<1%	<1%	<1%	<1%	1%	<1%
Desulfobulbus	4%	<1%	<1%	4%	13%	59%
Pelotomaculum	<1%	11%	<1%	<1%	2%	4%
Desulfonauticus	<1%	<1%	<1%	<1%	1%	2%
Desulforhabdus	<1%	<1%	<1%	<1%	1%	<1%
Desulfonispora	1%	<1%	<1%	1%	<1%	<1%
Total POB[b]	<1%	<1%	1.8%	<1%	1.5%	1.7%
Acetoclastic methanogens[c]						
Methanosaeta	54%	90%	>99%	54%	53%	44%
Hydrogenotrophic methangens[c]						
Methanospirillum	9%	<1%	<1%	9%	32%	38%
Methanosphaerula	30%	9%	<1%	30%	10%	12%

a: The relative abundance accounts for total propionate-oxidizing bacteria in each sample.

b: The relative abundance accounts for all bacteria in each sample.

c: The relative abundance accounts for archaea in each sample.