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Biogas production from undiluted chicken manure and maize silage: a study of ammonia inhibition in high solids anaerobic digestion

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Abstract

The feasibility of co-digestion of chicken manure (CM) and maize silage (MS) without water dilution was investigated in 5-L digesters. Specific methane production (SMP) of 0.309 L CH₄ g⁻¹ volatile solids (VS) was achieved but only at lower %CM. Above a critical threshold for total ammonia nitrogen (TAN), estimated at 7 g N L⁻¹, VFA accumulated with a characteristic increase in acetic acid followed by its reduction and an increase in propionic

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acid. During this transition the predominant methanogenic pathway was hydrogenotrophic. Methanogenesis was completely inhibited at TAN of 9 g N L⁻¹. The low digestibility of the mixed feedstock led to a rise in digestate TS and a reduction in SMP over the 297-day experimental period. Methanogenesis appeared to be failing in one digester but was recovered by reducing the %CM. Co-digestion was feasible with CM ≤20% of feedstock VS, and the main limiting factor was ammonia inhibition.

Keywords: ammonia inhibition, chicken manure, mesophilic anaerobic digestion, methanogenic pathway, isotope tracing

1. Introduction

Anaerobic digestion (AD) is considered an attractive agri-waste management technique because the goals of pollution control, energy recovery and nutrients recycle can be achieved simultaneously (Li et al., 2014). Of particular importance is its application to the treatment of animal wastes where it can play a significant role in minimising the uncontrolled emission of methane to the atmosphere, thus reducing the impact of greenhouse gases associated with livestock management. The microbiology of the process relies on a number of interdependencies which allow a variety of complex organic materials to be converted to methane through the processes of hydrolysis, acidogenesis, acetogenesis, hydrogenogenesis and methanogenesis (Demirel & Scherer, 2008).

Chicken manure (CM) is a waste feedstock that has a high recoverable energy potential as it is easily degradable in an AD system (Wang et al., 2014). This material has a high protein content, however, which releases ammonia when hydrolysed and although this can

increase the buffering capacity of the AD process, the overall C/N ratio is only 5 to 10 (Yangin-Gomec & Ozturk, 2013). This is lower than that for manure from other farm animals, food waste, and waste active sludge (Bujoczek et al., 2000; Niu et al., 2013) and can lead to operational difficulties through inhibition of the methanogenic microflora as a result of free ammonia accumulation in the digester (Sun et al., 2015a; Yenigun & Demirel, 2013). Ammonia nitrogen is less inhibitory in its ionic form (NH_4^+) than as free ammonia (NH_3), but the partitioning between these forms is dependent on temperature and pH. For this reason ammonia inhibition has been reported at a wide range of total ammonia nitrogen (TAN) concentrations between 1500 to 7000 mg N L⁻¹ (Chen et al., 2008). It is therefore important to cite the temperature and pH conditions when reporting ammonia inhibition thresholds. There are also reports of increased tolerance as a result of bio-acclimatisation and/or the source of inoculum used (Angelidaki et al., 2003). Digester failure due to ammonia inhibition is usually a result of the accumulation of volatile fatty acids (VFA) to a point where the buffering capacity of the digester is broken and the pH falls to <6 with a corresponding loss of methane production and a reduction in the methane content of any biogas produced (Abouelenien et al., 2014; Fricke et al., 2007). Using CM as the sole feedstock will in most cases result in digester failure as concentrations of TAN and free ammonia (FA) exceed values that can be tolerated.

Many approaches have been suggested for dealing with nitrogen-rich feedstock for AD, such as acclimatisation, trace element addition, dilution, and ammonia stripping (Nie et al., 2015; Niu et al., 2013). One form of dilution is co-digestion with a low nitrogen co-substrate to achieve a favourable C/N ratio and ensure that TAN and free ammonia nitrogen (FAN)

remain below the threshold inhibitory concentrations. For animal manures this can be achieved by mixing with agricultural crops, as suggested by a number of researchers; this may also have the benefit of improving the volumetric methane production of the digester and thus its economic performance (Rajagopal et al., 2013). The co-digestion of cattle slurry with up to 50% fruit and vegetable wastes can provide a good methane yield (Callaghan et al., 2002) and an increase in methane production of 93% has been reported for chicken manure compared to its mono-digestion (Abouelenien et al., 2014). A theoretical study calculated that daily methane production could be improved approximately 1.2-fold by co-digestion of maize silage (MS) with cattle manure and CM (Yangin-Gomec & Ozturk, 2013). Co-digestion of dairy manure, CM and wheat straw were also reported to show a higher methane potential than digestion of the individual components, suggesting synergetic effects (Wang et al., 2012). A similar effect was also seen when corn stover was mixed with CM at ratios of 3:1 and 1:1 on a volatile solids (VS) basis (Li et al., 2013): this is the only study to date carried out with substrates of this type and no previous work on co-digestion of MS and CM has been reported.

CM is a mixture of bird droppings and bedding material which usually contains 25% or more dry matter and is rich in nitrogen (Abouelenien et al., 2014). MS typically contains around 30% dry matter with a higher lignocellulosic and lower nitrogen content than CM. Digestion without water addition leads to a relatively long hydraulic retention time which should ensure sufficient time for degradation of the organic matter at typical organic loading rates. Where breakdown of solids is inhibited, however, for example by ammonia toxicity, the total solids (TS) content of the digester will increase as the process approaches 'dry' anaerobic

digestion. This term is commonly applied to digesters that operate at 15–40% TS, conditions that may lead to limitations in heat and mass transfer rates (Xu & Li, 2012).

Because of the high nitrogen and solids content, co-digestion of CM and MS in their original undiluted form may be technically challenging (Rajagopal et al., 2013). Dilution with water, however, involves the additional costs of the water required and the subsequent treatment of substantial amounts of effluent (Nie et al., 2015; Niu et al., 2013). In addition, dilution itself cannot increase the biodegradability of feedstock or the fundamental efficiency of the AD system. If, on the other hand, undiluted feedstocks are used, higher ammonia and solids concentrations will occur in the digester and may interact, with the risk of multiple inhibitions increasing the potential for loss of methanogenesis and acidification. In fact inhibition of methanogenesis by ammonia has been considered as a means of promoting the production of organic acids for separation and recovery from AD systems. Digestion of high nitrogen substrates could thus form the basis of an anaerobic biorefinery with alternative end products to methane.

The purpose of the current study was thus to evaluate a multi-inhibited AD system using CM and MS as the feedstock without any water addition. The system was operated for approximately three hydraulic retention times (HRT) to establish pseudo-steady state conditions and determine the thresholds for ammonia inhibition/toxicity in these conditions. The results indicate those conditions that allow operation for biofuel production through conventional anaerobic digestion to methane, or alternatively to stimulate organic acids production through methane repression. The transition between the two states, with the possibility of recovering methane productivity from an acidified unstable digester, was also

investigated. Changes in the metabolic pathway for methane production and the cessation of methane production as ammonia concentrations increased were observed using ^{14}C isotopes. The dataset obtained is of potential use for further kinetic evaluation of the system and in optimisation of process requirements for both methane and VFA production.

2. Materials and Methods

2.1 Feedstock

The feedstock was provided by Wykey Farm, Shrewsbury, UK. The 16-week-old CM was stored outside while the maize, which had been harvested 12 months previously, was ensiled. After visual inspection and removal of any non-biodegradable contaminants the feedstock was homogenized by a macerating grinder (S52/010 Waste Disposer, IMC Limited, UK), packed into plastic storage bags and frozen at $-18\text{ }^{\circ}\text{C}$. The frozen feedstock was thawed before use and stored at $4\text{ }^{\circ}\text{C}$ for a maximum period of one week.

2.2 Inoculum

The inoculum was taken from the AD plant of Wykey Farm. This was fed on 20% CM, 70% MS and 10% triticale at a loading rate of $18\text{ tonnes day}^{-1}$, and at the time of sampling had been operating for 8 months. During this time the ammonia concentration had risen and the digester had experienced some operational difficulties, with methane concentration in the biogas falling to 41%. A series of remedial measures were undertaken in an attempt to acclimate the digester to ammonia. These included reducing the operating temperature from $41\text{ }^{\circ}\text{C}$ to $37\text{ }^{\circ}\text{C}$, increasing the recirculation rate of separated liquor, and the addition of trace elements. The inoculum taken was methanogenically active despite a history of stressed

conditions, and showed some evidence of partial acclimatisation to high ammonia concentrations. After being transported to the laboratory, the inoculum was sieved and maintained at 36 °C for 3 days before being fed.

2.3 Digester setup and operation

Four 5-L digesters each with a 4 L working volume were used in this study. These were maintained at 36 ± 1 °C and mixed by an offset bar stirrer operating continuously at a speed of 40 rpm. Each digester was fed daily and digestate was withdrawn weekly, a sample of which was used for analysis. Biogas production was recorded using tipping bucket gas counters connected to a continuous data-logging system: these were periodically calibrated by collecting the biogas discharged in Tedlar bags (Tedlar, SKC Ltd., UK) and measuring the volume in a water displacement gasometer, with reported values corrected to standard temperature and pressure (STP) of 0 °C and 101.325 kPa (Walker et al., 2009). The biogas and methane production are expressed as a 7-day average values.

Over the first 44 days of operation, the organic loading rate (OLR) was increased from 1 to 3 g VS L⁻¹ day⁻¹ with a step increase of 0.5 g VS L⁻¹ day⁻¹ every 11 days, and with the CM representing 20% of the mixture on a VS basis. The OLR was then maintained at 3 g VS L⁻¹ day⁻¹ giving an HRT of approximately 100 days depending on the ratio of the two feedstocks. The proportion of CM used in the mixed feedstock was adjusted to increase or decrease TAN concentrations in order to maintain digester stability or induce the required experimental conditions. Details of the operating conditions applied are summarised in Table 1.

2.4 Analytical methods

The TS, VS, pH, VFA and alkalinity, including total (TA), partial (PA) and intermediate

alkalinity (IA), and the biogas composition (CH_4 and CO_2) were measured according to previously reported methods (Banks et al., 2012). Total Kjeldahl nitrogen (TKN) was determined using a Kjeldahl heating block with an exhaust system (Foss Tecator 1007 Digestion System 6, Foss Analytical, Sweden). TAN was determined by distillation (Foss Tecator 1002, Foss Analytical, Sweden), and FAN was calculated according to (Nie et al., 2015). Elemental composition was quantified using an elemental analyzer (FlashEA 1112, Thermo Finnigan, Italy). The calorific value (CV) was measured by a bomb calorimeter (CAL2k, Digital Data Systems Ltd, South Africa).

2.5 Isotope tracer experiment

The pathway for methanogenesis from acetate was determined in an isotope tracer experiment, in which ^{14}C labelled [2- ^{14}C] sodium acetate ($^{14}\text{CH}_3\text{COONa}$; MP biomedical, Solon, OH, United States) was used as tracer. Digestate samples were taken between days 265–282, during which time the TAN concentrations were in the range of 6–8 g N L⁻¹. A 10 kBq of [2- ^{14}C] sodium acetate dose was used for 45 mL anaerobic cultivation broth of fresh-sampled digestate. Duplicate samples were taken to ensure reliability of the results. An HCl solution was used to maximize the release of any dissolved $^{14}\text{CO}_2$ in the broth. A 10% mix of oxygen in nitrogen was applied as the sparging gas and CO_2 , including $^{14}\text{CO}_2$, was trapped using 20 mL of 5 M NaOH solution. After passing through the first CO_2 trap, the sparging gas was passed through a furnace tube (800 ± 5 °C) packed with copper oxide catalyst where any CH_4 was oxidised to CO_2 and captured by the second trap. The relative proportion of $^{14}\text{CO}_2$ was then determined by scintillation counting by taking 1 mL of liquid sample from each trap and mixing with 15 ml of Hionic-Fluor™ scintillation cocktail

(PerkinElmer Inc., Buckinghamshire, UK). All scintillation counts were carried out on a Beckman Coulter LS6500 scintillation counter (Beckman Coulter, Inc., UK).

Measurement of the $^{14}\text{CO}_2/^{14}\text{CH}_4$ ratio was used to determine the dominant pathway for acetate utilisation. A $^{14}\text{CO}_2/^{14}\text{CH}_4$ ratio < 1 indicates that the main pathway is acetoclastic methanogenesis, while $^{14}\text{CO}_2/^{14}\text{CH}_4 > 1$ indicates that hydrogenotrophic methanogenesis is dominant (Fotidis et al., 2013).

2.6 Statistical analysis

Duncan's multiple range tests at the 5% level were applied to determine the significance of the differences in biogas production among the digesters.

3. Results and Discussion

3.1 Feedstock and inoculum characteristics

The characteristics of the feedstock and inoculum are shown in Table 2. The C/N ratios of the CM and MS were approximately 8.9 and 18.0, respectively. The C/N ratios of the mixed feedstock with 10, 20, 25 and 30% CM were 15.6, 14.0, 13.4 and 12.8 and the feedstock TKN concentrations were 7.1, 9.4, 10.6 and 11.7 g kg⁻¹ wet weight (ww), respectively. According to O'Rourke's equation (Sun et al., 2015b), the theoretical methane production of CM and of MS was 0.523 and 0.616 L CH₄ g⁻¹ VS, respectively. The TS content of the mixed feedstocks was 31.0–35.1%.

The TKN and TAN concentrations of the inoculum were high since, as noted above, this material came from an AD plant that had received a high nitrogen feedstock for several months. The pH was 7.97, the TAN concentration 3.5 g N L⁻¹, TA 18.3 g CaCO₃ L⁻¹ and the

total VFA concentration was around 3 g L⁻¹ with an IA: PA ratio of 0.37. These values indicated that when the inoculum was taken the process was still stable with the high TAN content buffering the VFA, and good gas production indicating that some acclimatisation to ammonia had occurred.

3.2 Digestion stability parameters

Ammonia. TAN, FAN total VFA and pH in R1–R4 during the experimental period are shown in Fig. 1. During stage 1 the TAN concentration in all four digesters gradually increased, reaching between 5.3-5.5 g N L⁻¹ by the end of this stage. In the second and subsequent stages TAN concentrations in the digesters began to diverge, reflecting the different proportions of CM in each feedstock (Table 2), with concentrations ranging from 6.2 to 6.8 g N L⁻¹ at the end of stage 2. In the final stage TAN rose as high as 9 g N L⁻¹ in R4 with 30% CM, 8.5 g N L⁻¹ in R3 with 25% CM and 7.4 g N L⁻¹ in R1 with 20% CM; while in R2 where the CM was reduced to 10%, TAN had fallen to 5.9 g N L⁻¹ by the end of the run.

This long-term TAN accumulation in the digesters is attributable to the high-nitrogen feedstock combined with low rates of conversion of nitrogen into biomass and limited losses into the gas phase as NH₃. It is recognised, however, that multiple factors affect the final TAN concentration in a digester (Lindorfer et al., 2012, Roberts et al., 2016) as well as its partitioning into the free and ionic forms. It is also likely that methanogens and other members of the anaerobic consortium can acclimate to ammonia, and it is therefore not surprising that inhibition of the digestion process has been reported over a wide range of TAN concentrations (Chen et al., 2008). It is generally recognised that free ammonia is more toxic to methanogens than the ionic form (Chen et al., 2008) and also that acetoclastic

methanogens are more susceptible than hydrogenotrophic methanogens. FAN concentrations of 700 to 1100 mg N L⁻¹ were reported to be capable of triggering inhibition for many types of feedstocks (Niu et al., 2013). A suggested mechanism for ammonia inhibition of biogas production is the diffusion of FAN into the cell membranes of methanogens, causing an imbalance in intra- and extracellular NH₄⁺ concentration and pH, and finally leading to a reduction or cessation of methane production (Kayhanian, 1999).

Alkalinity, VFA and pH. TA, PA, IA and IA/PA ratios in digesters R1–R4 during the four experimental stages are shown in Fig. 2, while VFA profiles are shown in Fig. 3. In stage 1 the OLR was increased over the first 44 days, and all digesters were operated under the same conditions. During this stage TA and PA increased rapidly reflecting the increase in TAN concentrations, while IA rose more slowly, and the IA/PA ratio remained stable or fell slightly (Fig. 2). By the end of stage 1 pH had stabilised at around 7.9-8.0 (Fig. 1).

At the beginning of stage 2 the %CM in digesters R2-R4 was increased as shown in Table 1. By day 115 acetic acid concentrations in all of the digesters began to increase, reaching around 2.5 g L⁻¹ by the end of stage 2 (Fig. 3). The threshold at which acetic acid concentrations appeared to increase was equivalent to a FAN concentration of 630 mg N L⁻¹ at pH 8 and 37 °C, similar to the value found by Banks et al. (2012) in the digestion of food waste. In R1-R3 with the lower %CM feedstocks, acetic acid concentrations started to stabilise then fell from around day 227, having reached maximum values of 6.2, 6.2 and 8.7 mg L⁻¹ in R1, R2 and R3 respectively. In R4, however, where the CM was increased to 30% in stage 3, the TAN concentration reached 9 g N L⁻¹. This was accompanied by a continued increase in acetic acid concentration, which reached 25 g L⁻¹ by the end of the run (Fig. 3). In

digesters R1–R3 the pattern of accumulation of acetic acid is typical of that observed when acetoclastic methanogens are inhibited and the metabolic pathway shifts to the more ammonia tolerant hydrogenotrophic methanogens, with acetic acid being oxidised to hydrogen and CO₂ (Banks et al., 2012).

Since R4 had shown signs of failure once the TAN concentration reached 8 g N L⁻¹, the %CM in the feed to R2 was decreased at the start of stage 4. This resulted in a downward trend in the TAN and in consumption of some of the acetic acid, which fell to 2 g L⁻¹ in R2 by the end of the run.

Propionic acid accumulation started shortly after the TAN concentration reached 7 g N L⁻¹ in all four digesters (Fig. 3). In R1, R2 and R3 this was accompanied by the decrease or stabilisation in acetic acid concentrations noted above, with final concentrations of propionic acid reaching 19, 16 and 32 g L⁻¹ respectively by the end of stage 4. The increase in propionic acid in R4 was also accompanied by a brief fall in acetic acid; but this was temporary and concentrations of both acids continued to rise until the end of the experiment, reaching a total VFA of 68 g L⁻¹ (Fig. 1) in which propionate and acetate were the main constituents (Fig. 3).

VFA with chain lengths greater than C3 are shown in Fig 3. In each case, the rise in concentration only began shortly after the TAN exceeded 7 g N L⁻¹ and propionic acid accumulation started to occur. The profiles in R1-R3 were similar, with the individual VFA concentrations higher at higher %CM. The dominant acid above C3 was iso-valeric, followed by iso-butyric. N-butyric concentrations also increased but then stabilised or fell, while valeric acid appeared slightly later and continued to accumulate in R1 and R3, but fell in R2 where the %CM was reduced at the start of stage 4. In R4 the pattern of accumulation was

similar to that in R1-R3 until around day 215, when the trend of decreasing n-butyric was reversed. The concentration of this acid exceeded that of iso-butyric, and finally reached around 3.2 g L^{-1} by the end of stage 4.

During stage 4 there was a fall in pH in all digesters, which was much more pronounced in R4 (Fig. 1). Changes in pH are related to alkalinity, with PA indicating the available buffering from bicarbonate and ammonia while IA represents the contribution from VFA. The ratio between IA/PA is thus often used as an indicator of stability, with sudden increases indicating a reduction in buffering capacity. During stage 4 TA fell slightly, with a more severe decrease in PA and increases in IA which were most pronounced in R4 and R3 (Fig. 2). In R3 the IA/PA ratio increased from around day 250 and pH decreased but remained above 7.3. The IA/PA ratio rose more slowly in R1 and R2, reaching around 1 by the end of the run, but with pH relatively stable at around 7.5 to 7.7. In R4 as the TAN concentration approached 9 g N L^{-1} there was a sharp rise in IA/PA, indicating the onset of instability. As the IA:PA ratio rose further, the pH dropped sharply and biogas production was seriously inhibited. Since methane production had virtually ceased there was no route for the further destruction of VFA, and the concentrations of both acetic and propionic acid rose rapidly resulting in the buffering capacity of the digester being broken and pH falling below 6.

3.3 Biogas production

Volumetric biogas production (VBP), specific methane production (SMP) and biogas methane content in R1–R4 during the experimental run are shown in Fig. 4. As can be seen, these parameters broadly reflected the changes in feedstock composition, digestate TAN

concentrations and the overall decline in stability and performance.

During stage 1 there was no significant difference in VBP, SMP or methane content between the four digesters ($p>0.05$). From day 1-44 VBP rose in line with the increase in OLR. After the OLR reached $3 \text{ g VS L}^{-1} \text{ day}^{-1}$, the average values of methane content, VBP and SMP during stage 1 were 53.8%, $1.38 \text{ L biogas L}^{-1} \text{ day}^{-1}$ and $0.309 \text{ L CH}_4 \text{ g}^{-1} \text{ VS}$, respectively.

Differences between gas parameters for different digesters began to appear in the second stage. The average VBP and SMP of R2 and R4 during stage 2 decreased to $1.24 \text{ L L}^{-1} \text{ day}^{-1}$ and $0.283 \text{ L CH}_4 \text{ g}^{-1} \text{ VS}$, respectively, which was significantly lower than in R1 ($p<0.01$); there was, however, no significant difference between R3 and R1 with average VBP and SMP values of $1.34 \text{ L L}^{-1} \text{ day}^{-1}$ and $0.303 \text{ L CH}_4 \text{ g}^{-1} \text{ VS}$, respectively. The biogas methane content during this stage ranged from 52.5-54.8% but the differences between digesters were not significant ($p>0.05$).

During stage 3 there was a marked decline in VBP and SMP, which was most severe in R4 and R3 (Fig. 4). The decline in R1 and R2 was less severe and showed signs of recovery during stage 4, particularly in R2 where the CM was reduced to 10%. At the end of stage 4 the SMP in R1 appeared to have stabilised at around $0.243 \text{ L CH}_4 \text{ g}^{-1} \text{ VS}$, $0.058 \text{ L CH}_4 \text{ g}^{-1} \text{ VS}$ lower than in stage 2. Around 60% of this difference in SMP could be accounted for by the quantity of VFA removed in the waste digestate. After the %CM in the feed to R2 was decreased, the VBP and SMP in this digester were higher than expected based on the values for R1 (Fig. 4). This may be partially explained by the continuing gradual accumulation of VFA in R1, while in R2 total VFA concentrations had stabilised (Fig. 1). VBP and SMP in

R3 and R4 continued to fall in stage 4. By day 270 SMP in R4 was close to zero indicating that methanogenesis had failed, accounting for the high accumulation of VFA.

One of the earliest indicators of digester stress was the decrease in biogas methane content. This was evident in R4 from day 157 onwards and in R3 from day 171 onwards, corresponding to the rise in total VFA at these times (Fig. 1). Conversely the reduction in %CM in R2 during stage 4 resulted in a recovery in methane content to a value close to that in R1 (Fig. 4). By the end of stage 4 the biogas methane content appeared to stabilise at around 51% and 52% in R1 and R2, respectively, only slightly below the average value of 53.7% for all digesters in stages 1 and 2.

The decline in VBP and SMP and the decrease in biogas methane content mirror the changes in other stability parameters and again clearly show the impact of changing the feed composition in such a way as to increase TAN concentrations in the digester. The TAN concentrations at which severe inhibition occurred were considerably higher than those found in a study on mesophilic mono-digestion of CM: it was reported that ammonia inhibition occurred at approximately 2.7 g TAN L⁻¹ while methane production declined by 90% at 3.5 g TAN L⁻¹, at pH values of approximately 7.7 (Wang et al., 2014). According to Niu et al. (2013), biogas production of 0.35 to 0.40 L g⁻¹ VS was achieved when the TAN concentration was lower than 5 g N L⁻¹ in the continuous mesophilic digestion of CM. With NH₄HCO₃ addition, the biogas production decreased to 0.3 L g⁻¹ VS at TAN 10 g N L⁻¹ and was totally suppressed at TAN 16 g N L⁻¹. Niu et al. (2013) also reported that reducing TAN and FAN to 4 g N L⁻¹ and 300 mg N L⁻¹ respectively by dilution and washing after extreme ammonia inhibition could recover biogas production to 0.5 L g⁻¹ VS (Niu et al., 2013). In the current

study, 10–20% inhibition of biogas and methane production occurred when the TAN exceeded 7 g N L^{-1} (FAN $\sim 600 \text{ mg N L}^{-1}$ and pH ~ 7.9) in R2–R4.

When the TAN concentrations in R3 and R4 reached 8.4 g N L^{-1} , biogas and methane production were inhibited by around 40% compared to the values in stage 1. This was accompanied by a continuing decline in biogas production in R3, even though its TAN stabilised at around 8.1 g N L^{-1} by the end of the experiment. In R4, 50% inhibition of biogas and methane production occurred when the TAN exceeded 8.8 g N L^{-1} , while methane production effectively ceased when the TAN exceeded 9 g N L^{-1} .

It should also be noted that biogas and methane productivity in R2 recovered around 35 days after its feedstock was changed to 10% CM. By then, the TAN concentration in R2 was just below 7 g N L^{-1} and the FAN and pH were approximately 600 mg N L^{-1} and 7.9, respectively. However, a fall in FAN concentration to $< 600 \text{ mg N L}^{-1}$ was not sufficient by itself to recover biogas production from an inhibited state. This was evident as methane generation in R3 and R4 did not resume, although their FAN concentrations also decreased to $< 600 \text{ mg N L}^{-1}$ by the end of stage 4. In this case, however, the reduction in FAN was brought about by the decline in pH that resulted from VFA accumulation, rather than by a decrease in TAN concentration as a result of feeding with a low nitrogen-content feedstock.

3.4 Methanogenic pathway under ammonia inhibition

The characteristic effects of TAN inhibition as seen in this set of experiments, with acetic acid concentrations first rising then falling accompanied by a rise in propionic acid, may be explained by inhibition of acetoclastic methanogens at a lower TAN concentration and the switch in metabolic pathway to hydrogenotrophic methanogenesis, which is generally

recognised to be more tolerant to free ammonia. The increase in propionic acid is a consequence of this switch, and of the resulting increase in demand for specific enzymes. Banks et al (2012) described long-term accumulation of propionic acid in food waste digesters at high TAN concentrations and concluded that this could be due to a deficiency of trace elements (TEs), such as selenium and cobalt. These TEs are necessary to synthesise the enzymes needed in syntrophic hydrogenotrophic methanogenesis: in particular for formate dehydrogenase, which is essential for the final stage in propionic acid degradation. Any accumulation of formate would result in feedback inhibition, preventing further breakdown of propionate. In long-term operation TE are washed out to the concentrations supplied in the feedstock (Banks et al., 2012), and if deficient must be added. Even with proper TE supplementation to prevent accumulation of propionic acid, however, hydrogenotrophic methanogenesis becomes severely inhibited as the TAN concentration exceeds 8 g N L^{-1} (FAN $\sim 6\text{--}700 \text{ mg N L}^{-1}$). Under these conditions propionate will again accumulate as there is no syntrophic route for its removal.

The ^{14}C labelled acetate isotope tracer experiment was conducted to determine the predominant methanogenic pathway under the high ammonia concentrations in these digesters. A counting efficiency above 90% was achieved in all samples. The $^{14}\text{CO}_2\text{:}^{14}\text{CH}_4$ ratios were 1.86 ± 0.13 , 0.71 ± 0.01 , 3.42 ± 0.12 and 3.58 ± 0.13 , respectively, corresponding to TAN concentrations of approximately 7.0, 6.0, 8.0 and 8.0 g N L^{-1} for R1–R4, respectively. The percentages of the total ^{14}C in the labelled acetate that were converted into $^{14}\text{CO}_2$ were $65.0 \pm 0.8\%$, $41.6 \pm 0.3\%$, $77.2 \pm 0.7\%$ and $78.2 \pm 0.6\%$ for R1–4, respectively. The higher the TAN concentration, the more ^{14}C in the labelled acetate that flowed into CO_2 . These results

indicated that the major methanogenic pathway in R1, R3 and R4 at this point was hydrogenotrophic methanogenesis; this was in agreement with the hypothesis that the pathway of acetate shifts from acetoclastic to hydrogenotrophic methanogenesis when ammonia inhibition occurs under mesophilic AD conditions (Karakashev et al., 2006). This also implied that a TAN concentration of approximately 7 g N L⁻¹ was the threshold for a dominantly hydrogenotrophic methanogenic pathway in this study. For R2, in which the ¹⁴CO₂:¹⁴CH₄ ratio and TAN concentration dropped below 1 and 6 g N L⁻¹ after feeding with low nitrogen feedstock, the main methanogenic pathway appeared to be acetoclastic. This indicated that acetoclastic methanogenesis was able to resume when ammonia inhibition stress was relieved in a timely manner. A similar effect was reported by Serna-Maza et al. (2014) who found that the acetoclastic pathway in a mesophilic food waste digester could be recovered when TAN concentration was reduced by ammonia stripping, even after operation over a long period.

3.5 Effect of multiple inhibitions on organic matter degradation

Digestate TS and VS content during the experimental period are shown in Fig. 5. The TS content of the inoculum was 5%, but the degree of dilution in the source digester at Wykey farm is unknown. No water was added during the experiment and the digestate TS increased, finally reaching approximately 18% in R1, 20% in R3 and >20% in R4. The final TS content in R2 was lower at 16%, in part reflecting the reduced proportion of CM added from day 185 onwards. The VS/TS ratio was similar in all digesters and appeared to have stabilised at close to 80% by day 220, well before the end of the run. VS destruction was estimated on a mass

balance basis, by calculating the mass of biogas produced based on the average weekly volume and the methane and carbon dioxide content (i.e. ignoring water vapour and other gases). In stage 1 once the OLR had reached $3 \text{ g VS L}^{-1} \text{ day}^{-1}$, VS destruction was around 74%. At the end of stage 4 this had fallen to 62% in R1 and 41% in R3, and recovered to around 72% in R2.

The results thus showed a clear decline in the degradation rate of organic matter under stressed conditions. This could be as a result of limitations on the carbon flow due to ammonia inhibition restricting the rate of methane production and hence the syntrophic reactions which lead to its formation. On the other hand, the feedstock was rich in lignocellulose which is known to reduce digestibility as it restricts the accessibility of the substrate to micro-organism, making hydrolysis the rate-limiting step (Brown et al., 2012; Sun et al., 2015b). An increase in TS and the build-up of fibrous components can reduce the heat and mass transfer capability of the digestate, which in turn may affect rates of digestion and carbon flow and could contribute to the accumulation of intermediate products. The combined effects of high nitrogen and diminishing biodegradability as a result of CM in the input feedstock mix are the prime factors leading to digester instability, and in the worst case digester failure. Reduced mass transfer between microbes and substrates has previously been implicated in low methane yields (Yang et al., 2015) as well as being attributed to the accumulation of digestion intermediates, such as ammonia and VFA.

The increase in digestate TS content also changed the rheology of the digester which effectively evolved from a 'wet' system at <10% TS to a 'dry' system where the TS approached 20%. Possible reasons for TS accumulation are discussed above but the transition

between 'wet' and 'dry' states corresponded to a TAN accumulation of more than 7 g N L^{-1} in all digesters.

A further effect of the slow down or cessation of methane production was the considerable potential for VFA production without lowering the pH to inhibitory values for acidification reactions. As a result, by monitoring and controlling both the ammonia concentration and the TS contents it may be possible to regulate methane/VFA production by combining lignocellulosic biomass and nitrogen-rich feedstock in the right proportions. The prospect of VFA production from agricultural residues and low cost carbon sources through AD is an idea worth trying because the VFA are of high commercial value. Improving the production of VFA, including acetic acid and propionate acid, could be a new direction for the development of AD technology.

4. Conclusions

Feedstock CM above 20% resulted in TAN concentrations rising above a critical threshold of $\sim 7 \text{ g N L}^{-1}$, above which VFA accumulation occurred. Methanogenesis was completely inhibited at $\text{TAN} > 9 \text{ g N L}^{-1}$. ^{14}C isotope labelling showed the predominant methanogenic pathway at high TAN was hydrogenotrophic. Reducing the proportion of CM allowed recovery of a failing digester with a reversal to a predominantly acetoclastic pathway to methane production at lower TAN concentrations. The low digestibility of the mixed feedstock led to a rise in digestate TS and a reduction in both specific and volumetric methane production.

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Figure captions

Fig. 1 TAN, FAN, total VFA concentration and pH in digesters R1–R4 during four experimental stages. Vertical dotted lines indicate the start or end of each stage.

Fig. 2 TA, PA, IA and IA:PA ratio in digesters R1–R4 during four experimental stages. Vertical dotted lines indicate the start or end of each stage.

Fig. 3 VFA profiles in digesters R1–R4 during four experimental stages. Vertical dotted lines indicate the days on which digestate TAN concentrations reached 7 g N L^{-1} .

Fig. 4 VBP, SMP and biogas methane content in digesters R1–R4 during four experimental stages. Vertical dotted lines indicate the start or end of each stage.

Fig. 5 TS, VS and VS/TS in digesters R1–R4 during four experimental stages. Vertical dotted lines indicate the start or end of each stage.

Table 1 Operating conditions for CSTRs (R1– R4)

	stage 1 (day 0–94)	stage 2 (day 95–128)	stage 3 (day 129–184)	stage 4 (day 185–297)
R1	20%CM	20%CM	20%CM	20%CM
R2	20%CM	25%CM	25%CM	10%CM
R3	20%CM	25%CM	25%CM	25%CM
R4	20%CM	25%CM	30%CM	30%CM

Table 2 Characteristics of inoculum and feedstocks

	Inoculum	CM	MS
TS (%)	5.02±0.09	49.62±0.73	28.87±0.12
VS (%)	71.58±0.65	82.48±0.46	94.74±0.35
VS (g kg ⁻¹ ww)	35.97±0.95	409.23±7.53	273.52±0.15
VS/TS ratios (ww basis)	71.6%	82.5%	94.8%
pH	7.97±0.02	N/A	N/A
TKN (mg kg ⁻¹ ww)	3751±18	27963±90	4754±115
TAN (mg kg ⁻¹ ww)	3483±43	N/A	N/A
TA (g CaCO ₃ L ⁻¹)	18.30±0.21	N/A	N/A
IA (g CaCO ₃ L ⁻¹)	12.91±0.26	N/A	N/A
PA (g CaCO ₃ L ⁻¹)	4.82±0.26	N/A	N/A
C (% , TS basis)	N/A	43.00±0.41	48.17±0.40
H (% , TS basis)	N/A	5.24±0.37	5.56±0.15
N (% , TS basis)	N/A	4.83±0.22	2.68±0.08
O (% , TS basis)	N/A	24.64±0.77	34.12±0.61
CV (MJ g ⁻¹ TS)	N/A	17.16±0.54	18.98±0.50

N/A, not available

Values are mean ± standard deviation of triplicates.