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RESEARCH ARTICLE

Process stability and microbial community composition in pig manure and food waste anaerobic co-digesters operated at low HRTs

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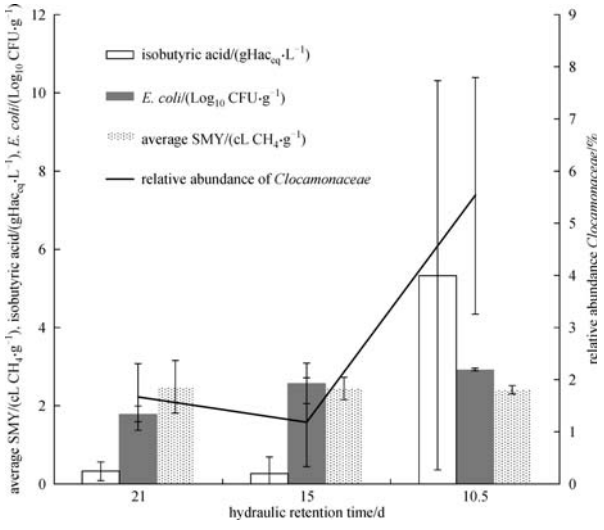
HIGHLIGHTS

- Reducing HRT to 10.5 days caused shifts in acidogenic population & VFA accumulation.
- VFA-oxidizing bacteria were key in process stability when HRT was 10.5 days.
- Reducing HRT to 10.5 days reduced substrate utilization.
- Pathogen removal was not achieved when HRT was < 21 days.

GRAPHIC ABSTRACT



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ABSTRACT

This study assessed the effects of reducing hydraulic retention times (HRTs) from 21 days to 10.5 days when anaerobically co-digesting pig manure and food waste. Continuously stirred tank reactors of 3.75 L working volume were operated in triplicate at 42°C. Digester HRT was progressively decreased from 21 to 15 days to 10.5 days, with an associated increase in organic loading rate (OLR) from 3.1 kg volatile solids (VS)·m^{-3}·day^{-1} to 5.1 kg VS·m^{-3}·day^{-1} to 7.25 kg VS·m^{-3}·day^{-1}. Reducing HRT from 21 days to 15 days caused a decrease in specific methane yields and VS removal rates. Operation at a HRT of 10.5 days initially resulted in the accumulation of isobutyric acid in each reactor. High throughput 16S rRNA gene sequencing revealed that this increase coincided with a shift in acidogenic bacterial populations, which most likely resulted in the increased isobutyric acid concentrations. This may in turn have caused the increase in relative abundance of *Clocamonaceae* bacteria, which syntrophically degrade non-acetate volatile fatty acids (VFAs) into H_2 and CO_2. This, along with the increase in abundance of other syntrophic VFA oxidizers, such as *Spiorchatetes,* suggests that VFA oxidation plays a role in digester operation at low HRTs. Reducing the HRT to below 21 days compromised the ability of the anaerobic digestion system to reduce enteric indicator organism counts below regulatory limits.

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1 Introduction

Co-digesting manures with carbon- rich organic wastes has increased in popularity over the past 10 years [1]. The

increasing prevalence of such co-digestion systems means that many biogas plants now operate with a changing multi-substrate feedstock [2]. This poses some challenges in terms of optimization of biogas plant operation. Substrate utilization is typically limited by hydrolysis [3] and therefore the hydrolysis rate of the substrate being treated defines the minimum hydraulic retention time (HRT) required to achieve a target level of substrate degradation [4] in a given biogas plant.

Manures are characterized by their low hydrolysis rates and low biomethane potential (BMP). Co-digesting organic wastes (such as food wastes (FW)) with manure typically results in high substrate hydrolysis rates and high BMPs [3], allowing for digester operation at higher organic loading rates (OLR). As co-digestion systems are typically continuously stirred tank reactors (CSTRs), the mixing rates of manure and other feedstocks is constrained by the need to maintain digester total solids (TS) contents below ~ 15% [5]. In the case of plants designed for operation with feedstocks with lower hydrolysis rates (such as manure and energy crops), additional capacity exists for HRT to be reduced and OLR to be increased when a rapidly hydrolysable substrate, such as FW, is added. This can increase digester throughput and maximize volumetric methane yields. Most commercial mesophilic biogas plants operate with HRTs within the range of 20–50 days, however plants operating with a HRT as low as 10 days have been reported [2].

Operating at shorter HRTs can, however, have negative effects in terms of digester process stability; low HRTs can result in washout of slow-growing methanogenic and syntrophic microorganisms key to anaerobic digestion (particularly in agricultural biogas plants where hydrogenotrophic methanogenesis plays a key role [6]). This in turn can lead to a drop in pH and build-up of volatile fatty acids (VFAs) within the reactor, which can result in irreversible failure of digesters. In addition low HRTs may result in greater methane emissions from digestate and therefore reduce the environmental sustainability of the biogas plant.

The advent of high throughput DNA sequencing has resulted in a greater understanding of the effects that changes in digester operating conditions, such as temperature, substrate composition [7] and reactor configuration [5] can have on key microbial communities. Observing shifts in specific microbial populations in response to such changes has paved the way for the development of biomarkers for shifts in process stability and biochemical pathways [8], despite the fact that microbial populations in anaerobic digester systems change dynamically even when operating conditions are not changed [9]. Thus far, there does not appear to have been any assessment of the effects that operating CSTR-type systems at low HRTs may have on the composition of microbial populations. Assessing changes in the microbial profile of digesters as HRT is decreased stepwise can allow for identification of causes of reactor instability, and may identify key microbial species required for stable anaerobic digestion at low HRTs.

Operating at low HRTs may also have negative effects on pathogen removal rates in digesters, due to the short retention times and high dilution rates. This in turn can have consequences for digestate disposal. In the EU, on-farm biogas plants undertaking co-digestion of manure with animal by-products such as FW are required to comply with the Animal By-Products Regulation (EU Commission Regulation (EU) No 142/2011). This regulation requires that digestate must be negative for *Salmonella* in every 25 g tested and have less than 1000 CFU·mL^{-1} *E. coli* or *Enterococcus*. Therefore, it is important to know whether operation at low HRT when co-digesting manure and treated FW will have any impact on compliance with these regulations.

Analysis of the effect of co-digestion of manure and FW at low ($<$ 20 days) HRTs merits attention as, while it may improve the methane yields achievable from the co-digestion of pig manure (PM) and FW, it may have negative effects on substrate utilization, process stability and pathogen removal.

The aim of this study was therefore to identify how CSTR-type reactors operated when HRTs were reduced from 21 days to 10.5 days, in terms of both process stability and digestate quality, using established empirical techniques and advanced high-throughput DNA sequencing technology. The specific objectives of this study were to assess how reducing HRT from 21 days to 10.5 days would affect 1) specific and volumetric methane yields (SMY and VMY); 2) reactor operation stability; 3) digestate pathogen content and post-methane production potential; and 4) digester microbial community composition.

2 Materials and methods

2.1 Substrates

The PM used in this study was taken from beneath the slatted unit of a local pig farm in Galway, Ireland. To ensure that the microbial population of the PM sampled mirrored that of manure generated and stored in Ireland, PM was sampled every 4 weeks and, upon entering the laboratory, it was stored at 11°C (the average annual temperature in Ireland) until use. It was essential to ensure a fresh, microbially representative PM was used in the experiment so as to assess the pathogen removal efficacy of the reactors. The PM volatile solids (VS) content was diluted to 4.35% (the average VS content of Irish pig manure [10]); in order to ensure organic loading rates (OLR) were kept constant within each HRT condition. The FW used was sampled weekly from the brown bins of 5 local residences. Upon arrival to the laboratory, the FW samples were combined and subsampled using the method

described by Browne et al. [11]. After subsampling, the FW was blended via a food processor (Russell Hobbs 500W 18087 Blender, UK) to a particle size of < 20 mm. At this point the FW was placed in autoclaved bags and underwent sanitization at 121°C for 15 min. via laboratory autoclave (LTE Scientific, UK). This was undertaken in order to simulate the operation of small farm-scale biogas plants, where the EU Animal By-Products Regulation (EU Commission Regulation (EU) No 142/2011) requires the sanitization of any non-farm sourced Category 3 animal by-products entering the anaerobic digester. After autoclaving, the FW was stored at −20°C and defrosted as required. The inoculum used during digester start-up was sourced from three 10 L laboratory-scale anaerobic digesters which had been operating for a period of 180 days, co-digesting PM and FW at a HRT of 21 days.

The PM and autoclaved FW samples were analyzed for pH, total chemical oxygen demand (TCOD), soluble chemical oxygen demand (SCOD), TS, VS, NH_4-N, alkalinity and VFA. NH_3 concentrations were calculated using pH and NH_4-N measurements as per Anthonisen et al. [12].

$$NH_3 = \frac{NH_4^+ + 10^{pH}}{10^{pH} + e^{\frac{6344}{(273+t)}}}, \quad (1)$$

where, t is the temperature, °C.

The average characteristics of the PM, FW and inoculum used in this experiment are presented in Table 1.

2.2 Experimental design

Three CSTRs were used during this experiment. Each had an effective volume of 3.75 L and was heated to 42°C through water jackets which were connected to a thermostatically controlled water bath. Mixing was undertaken for 1 h every day via mechanical stirrers. Each reactor was operated identically throughout the experiment. After the addition of inoculum, each reactor was flushed with N_2 in order to ensure that anaerobic conditions were established.

This experiment consisted of 3 phases; a start-up phase consisting of a period operating at a HRT of 21 days (from day 0 to 28), a transitional phase operating at a HRT of 15 days (day 29 to 54) and a phase where HRT was reduced to 10.5 (from day 55 to 85). The corresponding organic loading rate (OLR) at these periods was 3.1, 5.1 and 7.25 g $VS \cdot L^{-1} \cdot day^{-1}$, respectively.

As the inoculum used in this study was taken from digesters operating for 180 days treating a mixture of PM and FW (on average 60% to 40% on a VS basis) at a HRT of 21 days, it was highly acclimated to the substrates being used in this experiment. Therefore a shorter start-up period (1.3 turnovers of HRT) can be justified. This is supported by both the observed digester stability in terms of VS removal, methane yields and VFA concentrations (Section 3.1), and the DNA sequencing data which illustrates the similarities between the microbiome of the inoculum and the digestate generated after 28 days (Appendix Fig. A1). The decrease in HRT from 15 to 10.5 days after 1.67 turnovers of HRT was undertaken as SMY, VMY, digestate VS content and VFA concentrations appeared to have stabilized. Similarly the conclusion of the experiment on day 85 was chosen as VMY, SMY and VFA concentrations appeared to have stabilized after 2.8 turnovers of HRT. While this does not guarantee that reactor conditions had returned to a pseudo steady-state, the stability of VFA concentrations and pH indicate that the methanogenic and

Table 1 Characteristics of pig manure, food waste and inoculum used in this experiment

parameter	pig manure[a)]	food waste[a)]	inoculum[a)]
pH	7.29±0.05	5.26±0.13	7.85±0.09
TCOD /($g \cdot L^{-1}$)[b)]	48.2±1.1	431±1.7	59±1
SCOD /($g \cdot L^{-1}$)[b)]	18.5±0.5	58.5±2.3	5.5±0.7
TS /(% fresh weight)	5.8±0.5	25.8±7.2	3.81±0.07
VS /(% fresh weight)	4.35±0.3	20.3±5.7	2.69±0.04
NH_4-N /($g \cdot L^{-1}$)[b)]	2.6±0.6	0.69±0.6	2.4±0.03
alkalinity /($g \cdot L^{-1}$)[b)]	2.3±0.7	0.17±0.3	5.3±0.1
total volatile fatty acids /($g \cdot L^{-1}$ acetic acid equivalents (HAc_{eq}) [b)]	8.3±3.4	7.8±2.3	2.1
acetic /($g \cdot L^{-1}$ HAc_{eq})	3.0±1.2	1.1±0.9	0.32
propionic /($g \cdot L^{-1}$ HAc_{eq})	1.4±0.3	n.d.	n.d.
isobutyric /($g \cdot L^{-1}$ HAc_{eq})	0.3±0.1	6.7±1.4	1.73
butyric /($g \cdot L^{-1}$ HAc_{eq})	1.7±1.0	n.d.	n.d.
isovaleric /($g \cdot L^{-1}$ HAc_{eq})	0.74±0.1	n.d.	n.d.
valeric /($g \cdot L^{-1}$ HAc_{eq})	1.1±0.8	n.d.	n.d.

Notes: a) Average of 4 measurements±standard deviation; b) values reported for FW presented in $g \cdot kg^{-1}$; n.d. denotes not detected

hydrolytic microbial populations within the reactor were in balance.

The feed to each reactor was comprised of 55% FW and 45% PM on a VS basis. This mixing ratio was chosen as it maximized the proportion of FW entering the system (thereby maximizing methane yields) while ensuring that TS concentrations remained below 10% in the reactor (thereby avoiding issues of reactor clogging and insufficient mixing). The addition of feedstock was undertaken every weekday, with the amount added and removed adjusted to ensure that the target HRT was achieved on a weekly basis.

2.3 Analytical methods

Mass flow meters were used to measure daily biogas volumes (OMEGA, USA). Gas chromatography (7809A, Agilent Technology, USA) was used to measure biogas methane content as described by Xie et al. [1].

Digestate was sampled and analyzed on a weekly basis on the same day (in order to ensure that data points were not confounded by weekday feeding regime effects) until day 50, after which samples were taken every 2–4 days. pH was immediately measured via a pH meter (WTW, Germany). Prior to analysis for alkalinity, NH_4-N and VFA concentrations, 5 cm^3 of sample was centrifuged (Model 2-15, Sigma, Germany) at 15000 $r \cdot min^{-1}$ for 15 min. The supernatant was then filtered through 0.45 μm cellulose nitrate membrane filter paper (Sarstedt, Germany). Total solids, VS, alkalinity, Total COD and soluble COD of the substrates and inoculum were measured according to Standard Methods [13]. NH_4-N and alkalinity concentrations were measured using a nutrient analyzer (Konelab, Thermo Clinical Labsystems, Vantaa, Finland). VFA concentrations were measured via high performance liquid chromatography (HPLC, Agilent 1200, Agilent Technology, USA) as described by Xie et al. [1].

To quantify substrate utilization efficiency, the average theoretical SMY of the substrate mixture was calculated using the method suggested by Batstone and Rodríguez [14]: 1 g COD can theoretically be converted to 350 mL CH_4. Therefore multiplying the COD/VS ratio by 350 will result in the theoretical maximum possible SMY, and the substrate utilization efficiency can be calculated by dividing observed SMYs with this value.

The post methane production potential (PMP) of the digestate generated by each reactor was analyzed on samples taken on days 25, 53 and 70, when the HRT was 21, 15 and 10.5 days respectively. Digestate was placed in a 0.5 L conical flask with a butyl-rubber stopper at 11°C, in order to simulate methane emissions expected from digestate storage in Ireland. The flask contents were purged with N_2 for 5 min prior to stoppering. Biogas was collected in 1 L ALTEF gas sampling bags (Restek Corporation, USA). After 50 days of incubation, provided that weekly biogas generation rates were negligible, the volume of biogas collected in the gas bag was measured via glass gas syringe, and the biogas methane content was measured via gas chromatography.

To assess potential changes in indicator organism counts in the digestate as HRT was decreased, digestate samples were taken on day 28 when HRT was 21 days, day 47 when HRT was 15 days, and days 72 and 82 when HRT was 10.5 days. Feedstock from days 28 and 82 were also sampled. These samples were immediately analyzed for the presence of *E. coli* and *Enterococcus*. Each sample underwent serial dilution in maximum recovery diluent (MRD) (Oxoid, UK) as required and was pour plated in duplicate. The following media and incubation conditions were used; chromoCult tryptone bile X-glucuronide (CTBX) agar (Merck, USA) with incubation at 37°C for 24 h for *E. coli*, kanamycin azide aesculin (KAA) agar (Applichem, Germany) with incubation at 45°C for 18 h for enterococci. After incubation, all colonies were counted, averaged and expressed in colony forming units $(CFU) \cdot g^{-1}$ of sample. The indicator organism count of samples was expressed as the average of the indicator organism counts measured for each sample analyzed. All digestate and feedstock samples outlined above (25 g) were also analyzed for the presence of *Salmonella* according to the International organization for standardisation (ISO) 6579:2007 (Amendment 1: Annex D) method [15].

2.4 High throughput 16S rRNA gene sequencing

Five cm^3 of digestate was sampled from each reactor on day 28 when the HRT was 21, day 47 when the HRT was 15 and twice when the HRT was 10.5 (days 72 and 84). Samples were immediately snap-frozen in liquid N_2 and stored at −80°C. Samples were then crushed to a fine powder under liquid N_2 via pestle and mortar, and the powder re-frozen at −80°C. Six hundred mg of sample then underwent repeated bead beating and a column purification DNA extraction process. The quality of extracted DNA was assessed on a 1% agarose gel. DNA was quantified by heating each sample at 52°C for 2 min, mixing and analyzing in triplicate on the Nanodrop 1000 spectrophotometer.

Modified 16S Illumina adapter fusion primers were used to generate amplicon libraries. The primers were CaporasoNexF5′TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG[GTGCCAGCMGCCGCGGTAA]3′ and CaporasoNexR5′GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG[GGACTACHVGGGTWTCTAAT]3′. The primer sequence outside the square brackets are partial Illumina adapters. The primer sequences inside the square brackets bind to the hypervariable (V4) region of the 16S rRNA gene in bacteria and Archaea and are derived from the 16S binding sites of primers previously described by Caporaso et al. [16]. PCR was conducted using 20 ng of

digestate DNA as a template and Kapa HiFi Hotstart ReadyMix (Kapa Biosystems, UK) according to the manufacturer's instructions. Thermocycling conditions were: one cycle of 95°C for 3 min, then 26 cycles of 95° C for 30 s, 55°C for 30 s, 72°C for 30 s, followed by one cycle of 72°C for 5 min. QIAquick PCR purification kits (Qiagen, UK) were used to purify libraries. The purity and quantity of PCR products were analyzed via the Nanodrop 1000. Two unique 8 bp indices were added (one index at the 5′ end of the amplicon and the other at the 3′ end) to each amplicon in a subsequent round of PCR using primers from the Illumina Nextera XT indexing kit. PCR was undertaken with 5 uL of each amplicon as a template and Kapa HiFi Hotstart ReadyMix (Kapa Biosystems). PCR conditions for this second round of PCR were: one cycle of 95°C for 3 min, then 8 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, followed by one cycle of 72°C for 5 min. Indexed libraries were then purified using the Qiagen MinElute PCR Purification Kit (Qiagen, UK), eluted in 18 μL of buffer EB, and measured on the Nanodrop1000. All libraries were pooled and gel-purified to remove primer dimers via QIAquick gel extraction kit (Qiagen). Residual agarose was removed with a QIAquick purification kit. After these purification steps, the libraries were again measured for purity and quantity on the Nanodrop 1000. Pooled libraries were diluted and denatured according to the Illumina MiSeq library preparation guide. Amplicon library (6 pM) was spiked with 30% denatured and diluted PhiX Illumina control library version 3 (12.5 pM). Sequencing run was performed on an Illumina MiSeq using 500 cycle MiSeq reagent kits (version 2).

An in-house perl script was used to undertake de-multiplexing of sequence reads. Trim Galore was used to trim and filter sequence adaptor contamination from raw sequences. Read pairs were subsequently merged into a single sequence. An in-house perl script was used to carry out a size selection of 254 bp±20 bp. All reads were combined into a single data set for analysis via QIIME. OTU identification was undertaken using the open reference calling method within QIIME, using a combination of *de novo* and reference based methods [17]. Sequences were clustered into individual OTUs using a default similarity level of 97%, with a single representative sequence from each clustered OTU used to align to the Greengenes database (version: gg_13_5) [17]. A default AN RDP Classifier was used to classify the taxonomy of each OTU, using a minimum confidence cut-off of 0.8. Any OTUs with < 100 sequences across all samples were excluded from analysis. Rarefaction curves (presented in Appendix Fig. A2) illustrate that a high degree of coverage of the microbial composition was achieved.

Quantitative PCR of bacterial DNA was undertaken on the DNA extracted as described above in order to assess the effects of reducing HRT on total bacterial DNA concentrations. This was performed using the Roche 480 Lightcycler platform in a manner similar to that described by Fouhy et al. [18]. A calibration curve using 10^9 to 10^2 copies 16S rRNA$\cdot\mu L^{-1}$ was established. Values were then converted to copies 16S rRNA$\cdot g^{-1}$. The settings used to quantify total bacterial numbers were: 95°C for 3 min followed by 45 cycles of 95°C for 10 s, 60°C for 20 s and 72°C for 1 s followed by melting curve analysis of 95°C for 5 s, 65°C for 1 min, and 97°C continuously and a final cooling at 40°C for 10 s. Each samples contained 7.2 μL of PCR-grade water, 0.4 μL of the forward primer F1 (5′-ACTCCTACGGGAGGCAGCAG), 0.4 μL of the reverse primer R1 (5′-ATTACCGCGGCTGCTGG), 2 μL of a 1 in 10 dilution of extracted DNA, and 10 μL of SYBR green (Roche Diagnostics, West Sussex UK). Samples, negative controls (where PCR-grade water was used instead of DNA) and standards were run in triplicate.

2.5 Statistical analysis

To assess the effect of HRT on methane yields and VS removal, the weekly averages of volumetric methane yield (VMY), specific methane yield (SMY) and % VS removed measured at each HRT were compared via repeated measures ANOVA. To compare the PMP, *E. coli* and *Enterococcus* counts (log-transformed) measured at each sampling point, one-way ANOVA followed by the Bonferroni method was used to identify significant differences. All statistical analyses were carried out using SPSS v22.0 (IBM, USA).

Principal coordinate analysis (PCoA) was undertaken to illustrate the effect of changing HRT had on the similarity of the composition of microbial communities in the digester (in terms of changes in OTU abundances). It was undertaken using EMPeror software provided by Department of Computer Science, University of Colorado at Boulder [19].

Two diversity indices were used to assess the effect of changing HRT on microbial community diversity and richness. The Shannon diversity index was calculated using the following formula

$$H = -\sum_{i}^{s}(p_i * \ln\ P_i), \tag{2}$$

where i is the proportion of each species, and $_{\mathrm{Pi}}$ is the total number of species.

Chao 1 species richness was calculated using the following formula

$$S_{est} = S_{obs} + \frac{(f1)^2}{2 * f2}, \tag{3}$$

where S_{est} is the estimated number of species, S_{obs} is the observed number of species, $f1$ is the number of taxa represented by a single read, and $f2$ is the number of taxa represented by a double read.

3 Results and discussion

3.1 Reactor performance

Figure 1 (a) illustrates that SMYs decreased (P = 0.034) and VMYs increased (P = 0.024) as HRT decreased (and OLR increased). Figure 1(b) illustrates that a marked increase in VFA concentrations occurred as HRT was decreased from 15 to 10.5 days. The SMY measurements presented in Fig. 1(a) mirrored closely the VS removal trends observed (Fig. 1 (c)); a decrease was observed as HRT was decreased (P = 0.004). This, allied with the increase in PMP values observed as HRT was decreased, indicates that a reduction in the level of substrate utilization occurred as HRT was reduced from 21 to 10.5 days. The substrate utilization efficiency, on average, decreased from 0.46 when the HRT was 21 days to 0.32 when HRT was 10.5 days. Data provided by Dennehy et al. [3] suggests that under optimal batch conditions, PM and unautoclaved FW co-digestion at a 60/40 ratio can achieve substrate utilization efficiencies of up to 0.82. Longer HRTs and lower OLRs result in a greater level of substrate utilization, with the rate of utilization defined by the rate limiting factor of the process, typically hydrolysis [20].

From day 64, when VMYs peaked at 1728 L $CH_4 \cdot m^{-3} \cdot d^{-1}$ (after HRT was reduced to 10.5 days) until

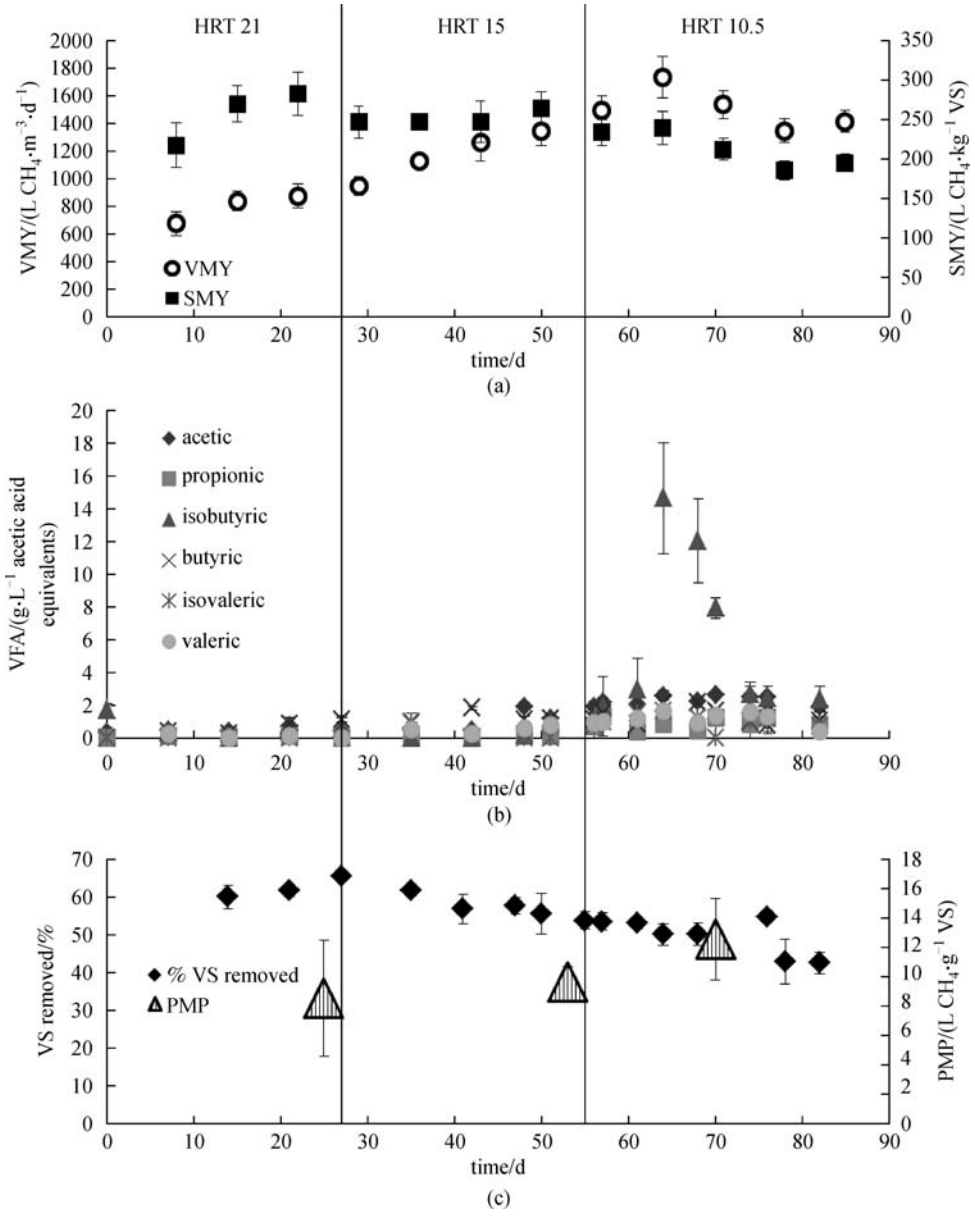

Fig. 1 Average specific methane yield (SMY), volumetric methane yield (VMY) (a), VFA concentrations (b), and post methane production potential (PMP) and % VS removed (c), measured throughout the experiment as HRT was reduced. Values are the mean of three replicates with error bars representing standard deviations

day 78, SMY and VMY values decreased markedly. After this period VMY, SMY and VFA concentrations appeared to stabilize, indicating that the process had begun to recover from the unstable conditions. While the average % VS removed continued to decrease between day 78 and 82, the change was not significant (*P*-value of 0.4562). The gradual reduction in substrate utilization from day 64 to day 78, and the stabilizing period thereafter, indicate the trends in SMY and VMY can be explained by changes in hydrolysis rates.

The accumulation of isobutyric acid in the reactors from day 61 to 64 (Fig. 1 (b)) indicates that the decoupling of acetogenesis and hydrolysis may have been responsible for the drop in VMY and SMY observed from day 64 onwards. The reason that SMYs did not drop extensively despite very high VFA concentrations is due to the high pH and high buffering capacity in the system (see Fig. 2). This meant that VFA accumulation did not result in a pH drop below 7, and therefore did not result in complete process failure. The work of Franke-Whittle et al. [21] shows that, particularly for highly buffered systems, there is no general threshold for VFA inhibition, as each system, depending on pH and buffering capacity (and the presence of salts and other anions) are vastly different. Nevertheless, pH and alkalinity did decrease due to this increase in VFA. These are signs of process instability. Isobutyric acid concentrations decreased from day 64 and appeared to stabilize from day 75 onwards. Assessment of any changes that occurred in the digester microbial community as HRT was reduced to 10.5 days may identify the specific reasons for the increase and subsequent decrease in isobutyric acid concentrations.

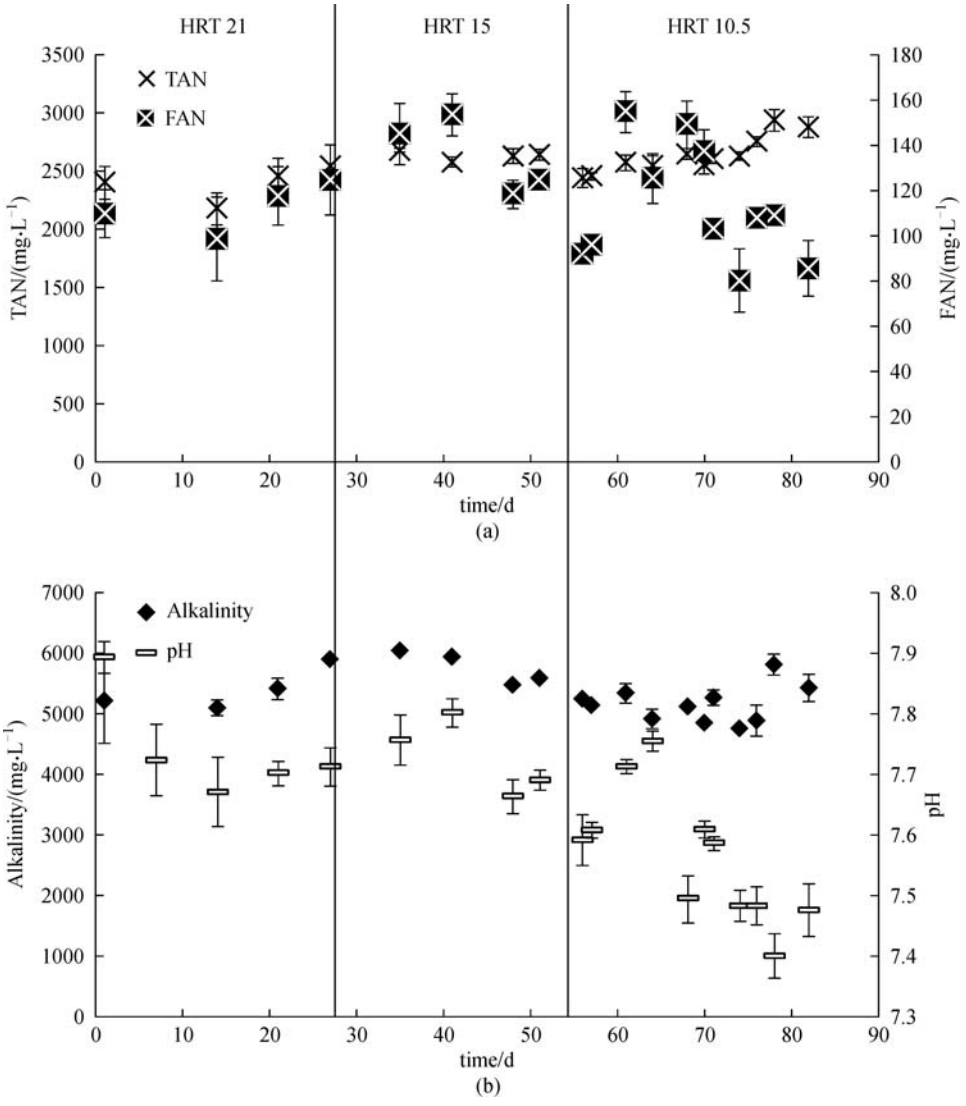

Fig. 2 Average NH_4-Nand NH_3 (a), pH and alkalinity (b) in each reactor as HRT was decreased. Values are the mean of three replicates with error bars representing standard deviations

3.2 Microbial community analysis

Results of the 16S rRNA gene sequencing of the digestate samples taken throughout this experiment revealed that the minimum number of sequence reads per sample was 81000, resulting in an average of 583±10 OTUs per sample.

3.2.1 Sample clustering and microbial diversity indices

The principal coordinate analysis (PCoA) plot presented in Fig. 3 illustrates that changing HRT had an effect on microbial communities in the digester, with samples from each respective HRT clustering together. PC1 could explain 67.42% of the variation observed in microbial communities. While the two samples taken at the HRT of 10.5 days (on days 72 and 84) clustered to some extent, there are some indications of differences between the two sample points.

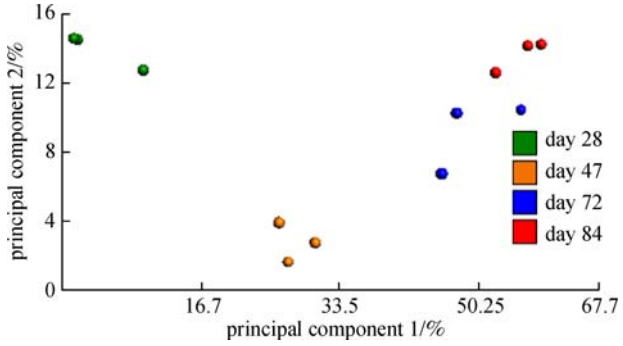

Fig. 3 Principal component analysis (PCoA) plot of samples taken from the reactors throughout the experiment

Changes in the number of observed species, Chao 1 species richness and the Shannon index were slight throughout the experiment (Appendix Fig. A3). In general, a small increase in these richness and diversity indices was observed between day 28 and 47, followed by a decrease on day 84. This may be indicative of a period of ecological succession as HRT was decreased to 15 (day 47) and 10.5 days (day 72), with new species progressively displacing established species and exploiting new ecological niches created by the reduction in HRT until a climax community was established, at which point species diversity decreased.

3.2.2 Changes in bacterial relative abundance

Figure 4 presents the taxonomic breakdown of the bacterial communities observed within each of the three reactors at each sampling point. Quantitative PCR of bacterial DNA found on average 1.28×10^{10} bacterial DNA copies per g of digestate and this did not change significantly as HRT was reduced from 21 to 10.5 days (see Appendix Fig. A4). Similarly, Maspolim et al. [22] found that decreasing HRT from 30 to 12 days had no clear effect on the concentration of bacterial and methanogen 16S rRNA reads.

Bacteria comprised between 87% and 93% of the observed microbial community within the digesters. While De Vrieze et al. [9] highlights that dynamic changes in digester microbial populations occur even when there are no changes in digester operating conditions, a response to the progressive reduction in HRT was seen for several phyla, families and genera in the present study.

A decrease in *Firmicutes* (the most abundant phylum; P = 0.037) and an increase in *Bacteroidetes* (second most abundant phylum; P = 0.009) relative abundance was observed as the HRT was decreased. Previous studies have suggested that a decrease in the ratio of *Firmicutes* to *Bacteroidetes* (both of which are associated with hydrolytic and acidogenic stages of anaerobic digestion) can be an indicator of reactor instability [23]. *Chloroflexi* decreased in relative abundance as HRT decreased, while *WWE1* and *Actinobacteria* increased in relative abundance, particularly as HRT was decreased to 10.5 days.

At family and genus level, it is clear that the reduction in *Chloroflexi* at phylum level can be attributed largely to a reduction in genus *T78* of the family *Anaerolineae*. *Anaerolineae* generally exhibit a slow growth rate and are associated with degradation of carbohydrates and amino acids [24]. This slow growth rate may explain the observed reduction (P = 0.009) in *T78* relative abundance as HRT decreased. While *T78* is uncultured it has previously been found in anaerobic digesters treating wastewater treatment sludge [25]. While *T78* decreased in relative abundance, *Actinomyces*, of the phylum *Actinobacteria*, increased. *Actinomyces* is a fermentative bacterium that has been suggested to be a cause of foaming in anaerobic digesters treating sewage sludge [26]. Therefore, its presence may be a sign of potential reactor instability.

An increase in the relative abundance of the genus *Coprococcus* (phylum *Firmicutes)* was observed as HRT decreased (P = 0.015), in particular at day 72 (when isobutyric acid concentrations increased). *Coprococcus* has been found to degrade carbohydrates and amino acids into a range of VFA's (butyric, propionic, acetic and formic acids) [27]. An increase in the abundance of *Paludibacter* , a genus which has been found to produce propionate and acetate [28], was also observed as HRT was decreased to 10.5 days. These observations, along with the increase in the abundance of *Actinomyces* are indicative of a shift in acidogenic populations. It is possible that the shift in the acidogenic system may have indirectly caused the increase in isobutyric acid observed between days 61 and 74 by: 1) producing isobutyric acid directly; 2) producing isobutyric acid indirectly via an intermediate organism isomerising the butyric acid produced; and 3) producing formate. Formate has been shown to disrupt syntrophic degradation of butyric and isobutyric acids, by inhibiting the reversible isomerisation of isobutyric acid to butyric

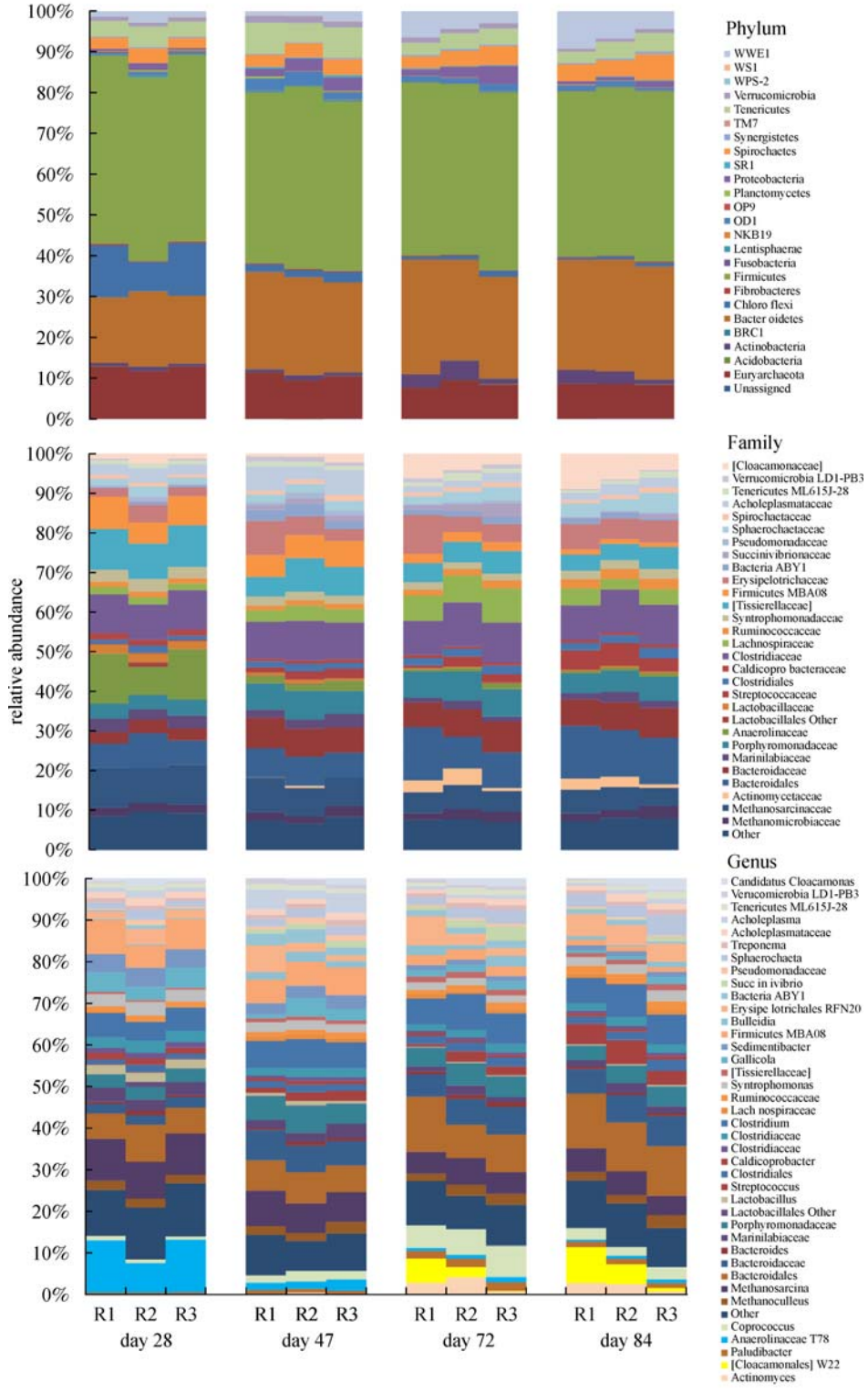

Fig. 4 Microbial relative abundance within each reactor at each sampling point at phylum, family and genus level. Genera and families with relative abundance < 1% at any point during the experiment were grouped in the "Other" category

acid, when formate is present at concentrations above 0.001 mol·L^{-1} [29]. This would result in isobutyric acid accumulation, as isomerisation to butyric acid is an intermediate step in syntrophic isobutyric acid degradation [30].

The reduction in HRT, and the accumulation of isobutyric acid, appeared to affect syntrophic VFA oxidizers, such as genus *W22* and *Candidatus Cloacanomas* of family *Cloacamonaceae* (which are responsible for the increase in abundance observed for the candidate phylum *WWE1*). *W22* tended to increase as HRT decreased (P = 0.09), particularly as isobutyric acid began to accumulate. These genera have been commonly found in anaerobic digesters, and while *W22* is uncultured, the *Cloacamonaceae* family has been found to ferment amino acids and syntrophically oxidize butyric and propionic acid [31] into H_2, CO_2 and acetate. Therefore, they may have played a key role in the observed reduction in isobutyric acid concentrations. The increase in relative abundance of the phylogenetically similar *Spirochaetes* (a phylum containing several species identified as syntrophic acetate oxidising bacteria (SAOB)s [32]) as HRT decreased suggests that an overall increase in the activity of the syntrophic VFA oxidation pathway occurred as HRT was decreased.

3.2.3 Changes in Archaeal relative abundance

As Fig. 5 illustrates, Archaea comprised between 13% and 7% of the microbial community observed across all data points. Reducing HRT resulted in a reduction in the overall relative abundance of Archaea. In contrast to the bacterial populations, the composition of the archaeal community was remarkably stable throughout the experiment due to their specialized function in terms of methanogenesis, less functional redundancy is likely to be present and stability is maintained by resilience of a small number of established organisms rather than shifts to alternative genera [9] particularly when the methanogenic pathways remain unchanged.

Figure 5 illustrates that that hydrogenotrophic methanogens dominated the archaeal populations observed. The facultative hydrogenotroph, *Methanosarcina,* was the dominant genus observed throughout the experiment. It is very commonly found in biogas plants and, as it can utilize hydrogenotrophic, aceticlastic and methylotrophic pathways for methanogenesis, it can operate over a wide temperature range (mesophilic and thermophilic) and in the presence of high concentrations of FAN [8]. The reduction in *Methanosarcina* relative abundance (P = 0.009) was the cause of the overall decrease in archaeal abundance as HRT decreased. This may be due to the reduced niche for facultative aceticlastic methanogenesis (due to the apparent increased role of hydrogenotrophic methanogenesis) when HRT was reduced to 10.5 days. Sun et al. [33] showed that while *Methanosarcina* was found at a relatively high abundance in reactors where SAOB activity was high, it was generally less dominant than in reactors where aceticlastic methanogenesis prevailed.

Methanoculleus which was the 2nd most abundant methanogen, was not affected by the decrease in HRT (P = 0.868). It is an obligate hydrogenotroph and is very commonly found in digesters treating nitrogen-rich substrates, such as manures [5]. The fact that the abundance of *Methanoculleus* remained stable as HRT decreased indicates that *Methanoculleus* growth rates were sufficiently rapid to be unaffected by HRTs as low as 10.5 days,

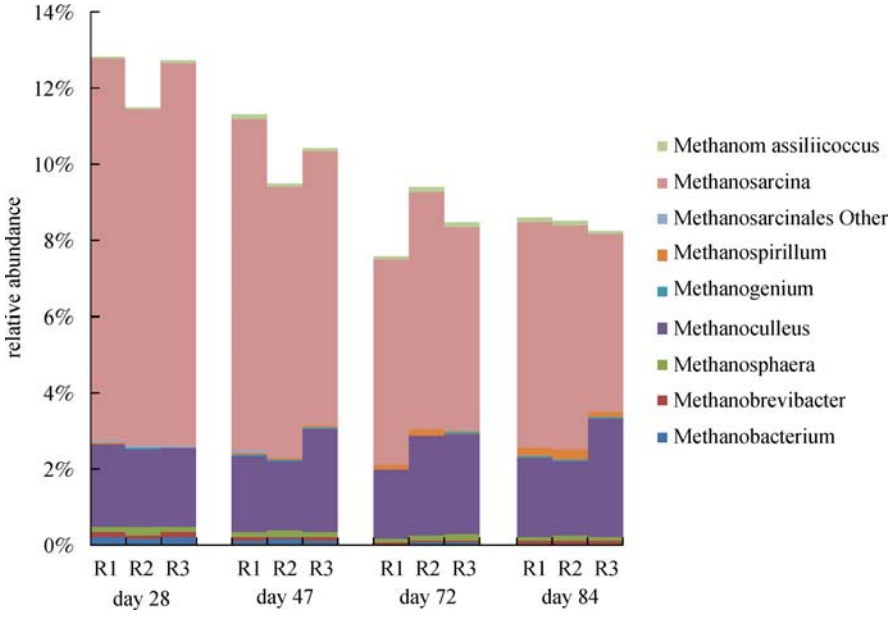

Fig. 5 Relative abundance of Archaea within each reactor at each sampling point at genus level

and were unaffected by the increase in syntrophic VFA oxidation, as discussed in Section 3.2.2.

3.3 Digestate biosafety

Figure 6 presents the average counts of enteric indicator organisms found in the digestate and feedstock during this experiment. As FW was autoclaved prior to being fed to the digesters, the enteric indicator organisms found in the feedstock were attributed to the PM. One-way ANOVA of counts vs HRT revealed increases in the counts of both *E. coli* ($P = 0.002$) and *Enterococcus* ($P < 0.001$) in the digestate as HRT was decreased.

The Bonferroni procedure was performed in order to assess the significance of the changes observed between data points. For *E. coli*, this analysis indicates that while decreasing HRT resulted in significant increases in counts as HRT is decreased from 21 to 10.5 days, the counts observed at HRTs of 15 and 10.5 days were not significantly different from each other or from the feedstock count. For *Enterococcus*, Bonferroni analysis found that while decreasing HRT from 21 days to 15 days significantly increased *Enterococcus* counts, the counts found at the HRT of 15 days were similar to those found at a HRT of 10.5 days and in the feedstock. *Enterococcus* counts measured on day 72 were lower than those found in the feedstock ($P = 0.009$), despite there being no significant difference between counts at day 82 and those in the feedstock. Increases in VFA combined with a drop in pH have been found to reduce the survival rates of indicator organisms in anaerobic digesters [34], and therefore the spike in VFA concentrations and concurrent drop in pH observed on day 72 may explain this drop in *Enterococcus* survival.

The effect of increasing HRT from 11 to 25 days on *E. coli* counts has been studied previously [35], with regulatory acceptable levels of *E.coli* removal being achieved at a HRT of 18 days. In the present study anaerobic digestion did not achieve a high level of *Enterococcus* removal at HRTs < 21 days, or of *E. coli* removal at HRTs < 15 days. From an operational perspective, the high *Enterococcus* counts present in the digestate at HRTs of < 21 days means that the digestate would not meet the standards set out in the EU animal by-products regulations (< 1000 CFU·g^{-1} for *E.coli* and *Enterococcus*) and would therefore require further treatment before disposal. Each digestate sample was also tested for the presence of *Salmonella*, with no *Salmonella* being found in any of the digesters at any point throughout the experiment.

4 Conclusions

Decreasing HRT to 10.5 days resulted initially in a drop in SMYs and VMYs and a rapid increase in isobutyric acid concentrations. This increase in isobutyric acid may have been caused by the shift in relative abundance of acidogenic bacteria. The increase in the relative abundance of the family *Cloacamonaceae* may have played a role in the subsequent reduction in isobutyric acid concentrations, as it oxidised non-acetate VFAs to acetate, CO_2 and H_2. This, along with the increase in the abundance of other syntrophic VFA oxidizers such as *Spiorchatetes* suggests

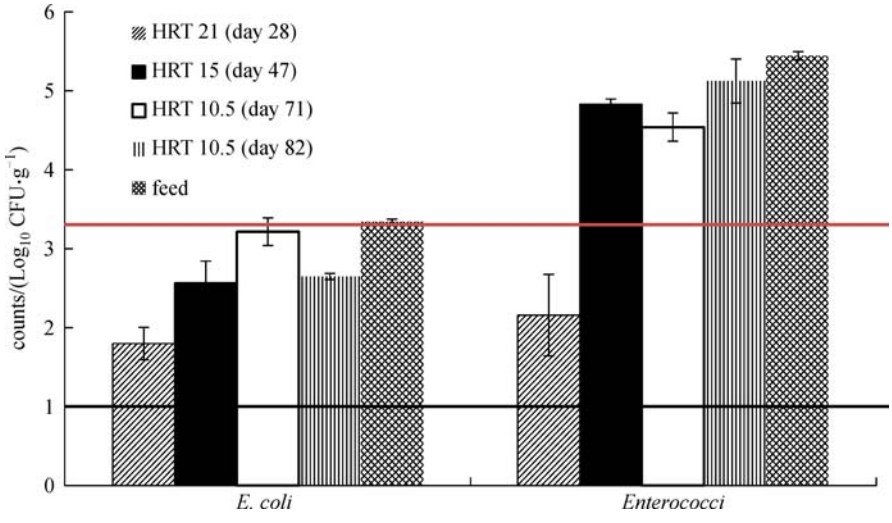

Fig. 6 *E. coli* and *Enterococcus* counts in digestate throughout the experiment, as well as in the feed on days 28 and 82. Values are the mean of three replicates with error bars representing standard deviations. Bars representing the count for the same bacteria and for the same sample type sharing a common letter are not significantly different ($P > 0.05$), as measured by the Bonferroni method. *represents the EU animal by-products regulation limit of < 1000 CFU·mL^{-1} for *E. coli* or *Enterococcus* in digestate prior to land application. LOD represents limit of detection

that syntrophic VFA oxidation should play a role when CSTRs treating PM and FW at a mixing ratio of 45%/55% were operated at low HRTs. Reducing HRT below 21 days compromised the ability of the anaerobic digestion system to reduce the concentrations of enteric indicator organisms to acceptable levels. In turn, this resulted in the digestate failing to comply with the EU animal by-products regulations.

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