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Organic loading rate: A promising microbial management tool in anaerobic digestion

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ABSTRACT

This study investigated the effect of changes in organic loading rate (OLR) and feedstock on the volatile fatty acids (VFAs) production and their potential use as a bioengineering management tool to improve stability of anaerobic digesters. Digesters were exposed to one or two changes in OLR using the same or different co-substrates (Fat Oil and Grease waste (FOG) and/or glycerol). Although all the OLR fluctuations produced a decrease in biogas and methane production, the digesters exposed twice to glycerol showed faster recovery towards stable conditions after the second OLR change. This was correlated with the composition of the VFAs produced and their mode of production, from parallel to sequential, resulting in a more efficient recovery from inhibition of methanogenesis. The change in acids processing after the first OLR increase induced a shift in the microbial community responsible of the process optimisation when the digesters were exposed to a subsequent OLR increase with the same feedstock. When the digesters were exposed to an OLR change with a different feedstock (FOG), the recovery took 7d longer than with the same one (glycerol). However, the microbial community showed functional resilience and was able to perform similarly to pre-exposure conditions. Thus, changes in operational conditions can be used to influence microbial community structure for anaerobic digestion (AD) optimisation. Finally, shorter recovery times and increased resilience of digesters were linked to higher numbers of *Clostridia incertae sedis* XV, suggesting that this group may be a good candidate for AD bioaugmentation to speed up recovery after process instability or OLR increase.

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1. Introduction

In the past decade the increased importance of the renewable energy obtained from AD has raised considerable interest in the application of this technology to new feedstocks and co-digestion substrates (Zhang et al., 2012; Zhang et al., 2010). In particular, the co-digestion of glycerol waste, a by-product of biodiesel manufacture, and lipid rich wastes such oil-rich wastewater sludge, waste oil and FOG (Fat, Oil and Grease) from sewers and grease traps, are interesting model compounds and have shown potential for improving yields of methane in anaerobic digesters (Fernández et al., 2000; Palatsi et al., 2010). However, high concentrations of glycerol waste and/or FOG waste can cause process instability through sludge flocculation (resulting in biomass wash out), direct inhibition, VFAs overload, and physical fouling of equipment (Palatsi et al., 2010; Long et al., 2011). More generally, process instability can also be linked to sudden changes in feedstock composition or organic loading rate (OLR) (Akunna et al., 2007; Rincón et al., 2008). As feedstock availability can fluctuate throughout the year it is often difficult for an operator to maintain these two parameters stable (Akunna et al., 2007). It is therefore important that the effects of changes in feedstock composition and OLR on AD performance are fully understood so that management strategies to mitigate negative effects are developed (Ward et al., 2008). Operational tools for co-digestion have been developed over the years, based mainly on the optimisation and monitoring of physical parameters (Ãlvarez et al., 2010; Derbal et al., 2009). Yet, biotechnological tools, to manage the complex consortium of bacteria and archaea in the digesters are still underexploited (Ferguson et al., 2014; Tale et al., 2015). During the AD process bacterial fermentative redox pathways are used to ferment organic waste into different organic acids, which are then converted into acetate, H_2 and CO_2 for methanogens to produce methane. This final

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conversion process relies upon syntrophic relationships between fermentative microorganisms and methanogens (Demirel and Scherer, 2008; Cavaleiro et al., 2010; Lyberatos and Skiadas, 1999), thus VFAs profile and other fermentation products are fundamental in structuring both bacterial and methanogenic community involved in the process and process yields. Previous studies have shown that VFAs concentrations play an important role in structuring the methanogenic community of anaerobic digesters (Delbès et al., 2001; Griffin et al., 1998; Ryan et al., 2010; Hori et al., 2006; Karakashev et al., 2005). Ros et al. (2013) showed that gene copy numbers of *Methanosarcina* increased by 3 orders of magnitude when the concentration of acetic acid doubled to levels higher than 1600 mg l^{-1} and the Ripley ratio (VFAs over total alkalinity) increased from 0.3 to more than 0.5. A shift to *Methanosarcina* when VFAs start to accumulate in digesters, with *Methanosaeta* dominating at concentrations lower than 1 g l^{-1} VFAs, was also reported by other authors (Griffin et al., 1998; Karakashev et al., 2005). Despite the great deal of research on the influence of VFAs composition on the methanogenic community dynamics, our understanding on the bacterial community dynamics is still limited and often contradictory. For example, Delbès et al. (2001) suggested there was a correlation between high VFAs concentration and increased abundance of Clostridia while Hori et al. (2006) suggested that pH had a greater impact on the structure of the bacteria than VFAs themselves. Other studies reported factors such as feedstock composition and OLR can affect both bacterial and archaeal communities (Dearman et al., 2006; Supaphol et al., 2011; Wang et al., 2009; Xia et al., 2011) but there is little consensus on the types of effects such as decrease of bacterial richness and shifts in the community structure or bacterial densities and respiratory rates (Dearman et al., 2006; Xia et al., 2011; Zhang et al., 2010). For example, adaptation of bacterial communities to changes in OLR and feedstock during co-digestion has been shown to improve recovery times after periods of instability due to either changes in community structure (i.e. higher numbers of syntrophic propionate-oxidizing and fatty acid-beta-oxidizing bacteria) or change in physiology (McMahon et al., 2004; Palatsi et al., 2010). In this context, this study aims to shed light into the effect of changing OLR and feedstock on VFAs production and assesses whether OLR can be used as a bioengineering tool to enhance digester performance and recovery.

2. Materials and methods

2.1. Digester design and operational parameters

Nine 1 L semi-continuous stirred digesters, with a 700 ml working volume and six 5 L semi-continuous stirred digesters with a 4.5 L working volume were operated at 38 °C with a 7-day residence time (Table 1). The change in experimental scale was due to ease of management of larger scale reactors when running long-term experiments. Glycerol addition was done at both scales to guarantee data comparability (Figs. 1A and 5B). All reactors were seeded with digested sludge from a commercial Sewage Treatment digester and fed with autoclaved primary sludge and (1) biodiesel-derived Glycerol waste or (2) fat rich – FOG waste collected from a restaurant grease trap. For feeding and sampling, first it was checked that the digester was well mixed (that stirring had not been interrupted prior to feeding) then using a sterile 50 ml syringe (VWR, UK) digestate was drawn from the digester via a silicon tube permanently attached to the digester (if required the digestate was set aside for sampling); then fresh feedstock was added to the digester via the same silicon tube with 50 ml syringe, the digesters remained airtight during this process. Feedstocks of the correct concentration and mixture of co-digestants were

Table 1
Summary of experimental conditions tested.

	Digester characteristics			Initial stable period			OLR change 1 (over 1 HRT)				Recovery period 1			OLR change 2 (over 1 HRT)				Recovery period 2			
	Digester no	Digester working volume (ml)	HRT (days)	Time (days)	Feedstock	OLR ($kgVS/m^3d$)	Days of OLR changes	Feedstock	Additional feedstock concentration ($g\ l^{-1}$)	OLR 1 ($kgVS/m^3d$)	Time (days)	Feedstock	OLR ($kgVS/m^3d$)	Days of OLR changes	Feedstock	Additional feedstock concentration ($g\ l^{-1}$)	OLR 2 ($kgVS/m^3d$)	Time (days)	Feedstock	OLR ($kgVS/m^3d$)
Single OLR change using one co-feedstock	1,2,3	700	7	1–91	SL	1.4	91–93	SL+Gly	30	1.4+1.5	94–120	SL	1.4								
	10,11,12	4500	7	1–63	SL	1.4	63–67	SL+FOG	1.5	1.4+1.5	67–120	SL	1.4								
Repeated OLR changes using one or different co-feedstock	4,5,6	700	7	1–7	SL	1.4	8–14	SL+Gly	30	1.4+1.5	15–90	SL	1.4	91–93	SL+Gly	30	1.4+1.5	94–120	SL	1.4	
	13,14,15	4500	7	1–20	SL	1.4	21–25	SL+Gly	1.5	1.4+1.5	26–62	SL	1.4	63–67	SL+FOG	1.5	1.4+1.5	68–120	SL	1.4	

SL: sludge; Gly: glycerol; FOG: grease waste; HRT: hydraulic retention time; OLR: organic loading rate.

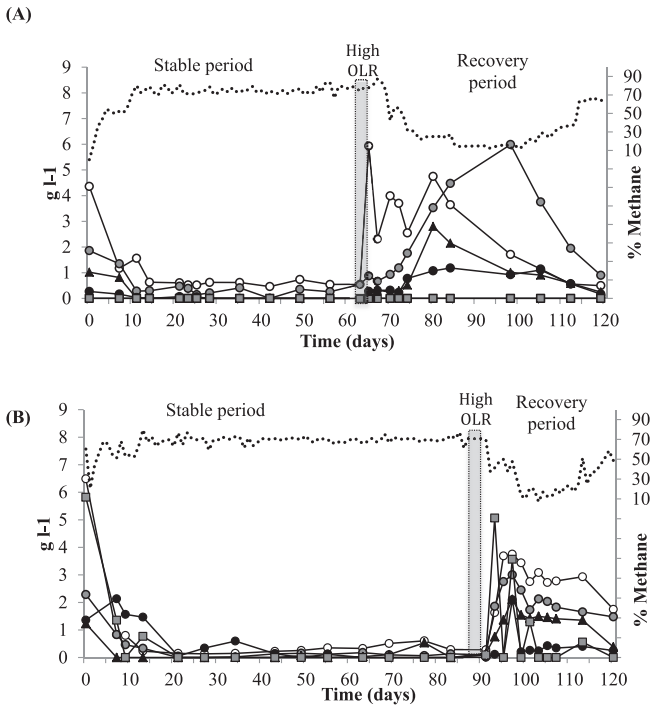


Fig. 1. VFA concentrations and methane content in digesters after one OLR change with glycerol (A) and FOGs (B). Acetic acid (white circles), propionic acid (grey circles), iso-butyric acid (black circles), n-butyric acid (black triangle) and lactic acid (grey squares). Dashed line shows methane content. Triplicate digester average, error bars are not shown to improve legibility.

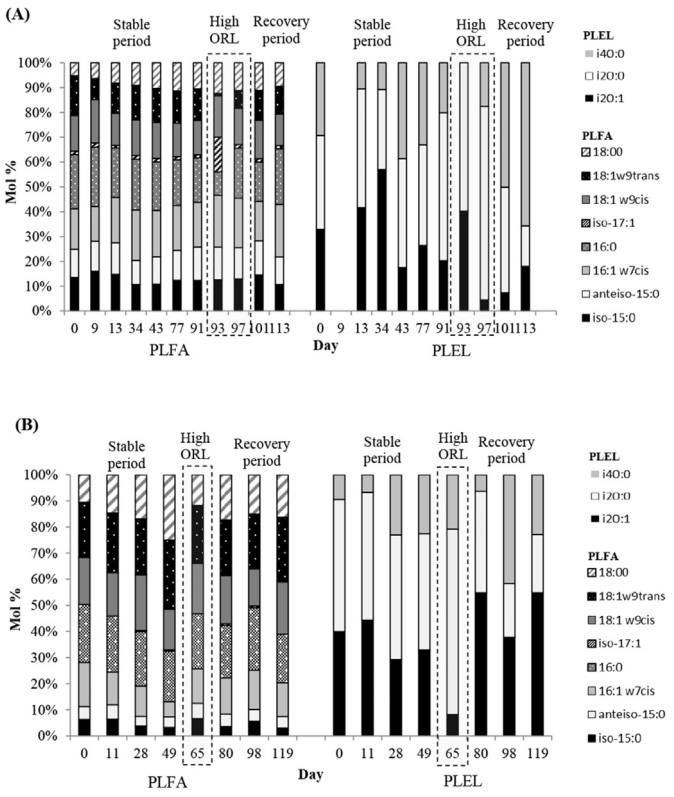


Fig. 2. Relative changes in bacterial and archea PLFA and PLEL (% of GC mass) after one OLR change with glycerol (A) and FOGs (B). x axis is not to scale and only shows days when samples were taken.

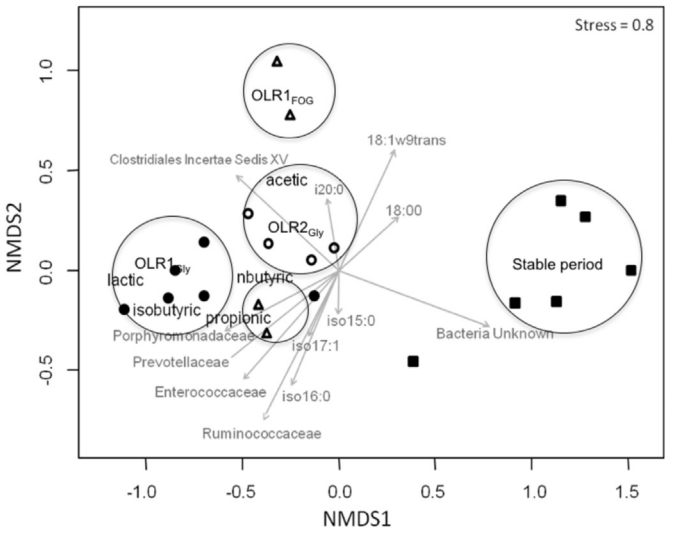


Fig. 3. VFA and microbial lipids and OTUs profiles in digesters during normal OLR (Stable period = Black squares), after one OLR increase with glycerol (OLR 1^{st}_{Gly} = Black circles) or FOG (OLR 1^{st}_{FOG} = Unfilled triangles) and after two OLR increases with glycerol (OLR 2^{nd}_{Gly} = Unfilled circles). Triplicate digester averages were used for the cluster analysis. Profile similarities based on UPGMA cluster analysis and the Bray Curtis similarity index, 2D stress was 0.8. Black text denotes predominant VFAs for digester groups. Grey arrows show vectors for microbial lipids and bacterial OTUs based on mol % or proportion of sequences, the arrow points to the direction of most rapid change in the proportion of the lipid/OTU and the length of the arrow is proportional to the correlation between the NMDS and the lipid/OUT.

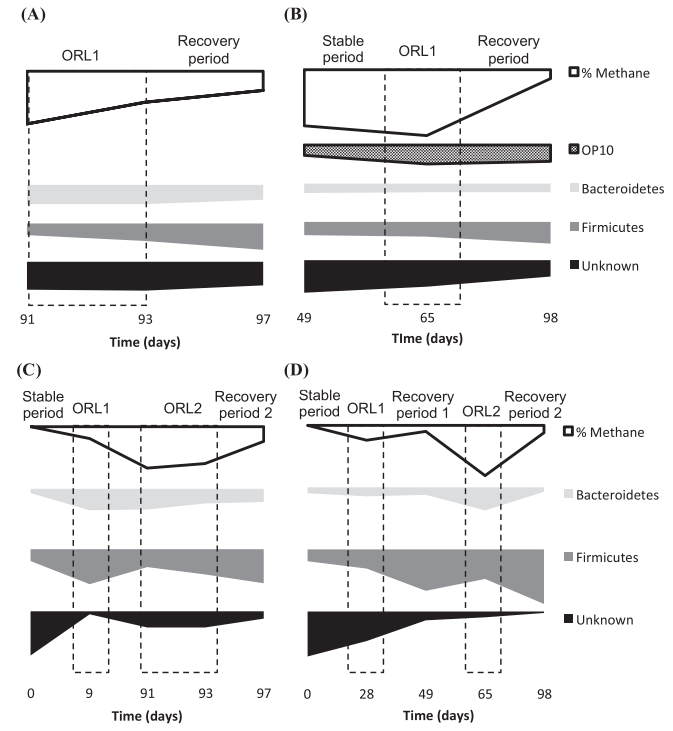


Fig. 4. Relative changes in bacterial phyla based on % of sequences assigned at phyla level and methane content in digesters after one OLR change with glycerol (A) and FOGs (B) and after two OLR change with the same feedstock glycerol (C) and with different feedstocks glycerol-FOGs (D). x axis is not to scale and only shows days when samples were taken.

prepared and mixed together in advance of feeding rather than adding co-digestants separately. Preliminary work showed that a 7

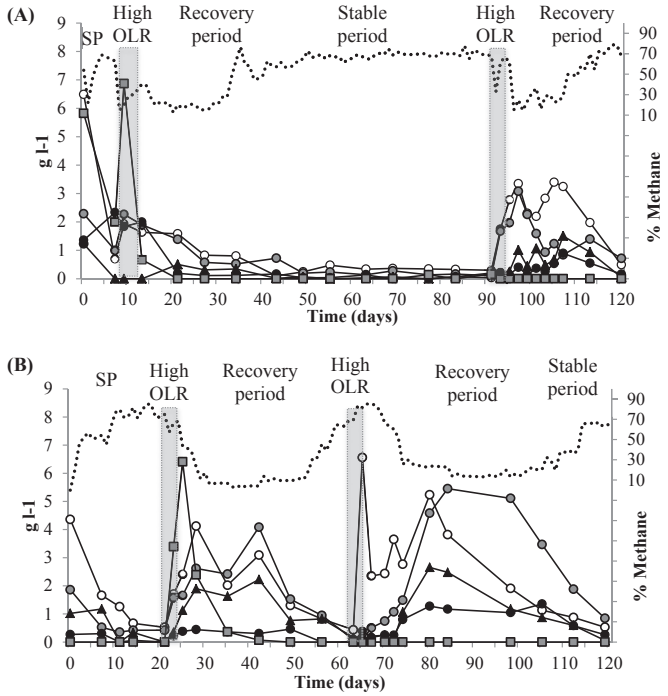


Fig. 5. VFA concentrations and methane content in digesters after two OLR change with the same feedstock glycerol (A) and with different feedstocks glycerol-FOGs (B). Acetic acid (white circles), propionic acid (grey circles), iso-butyric acid (black circles), n-butyric acid (black triangle) and lactic acid (grey squares). Dashed line shows methane content. Triplicate digester average, error bars are not shown to improve legibility.

days retention time was appropriate for stable performance and optimal reduction in volatile solids in digestate. To induce periods on unstable performance in the digesters the organic loading rate (OLR) 1.4 kg VS m^{-3} d^{-1} was increased to 2.9 kg VS m^{-3} d^{-1} for one HRT (OLR 1) and then returned to 1.4 kg VS m^{-3} d^{-1}, these OLR were selected as they were known to cause digester failure based on preliminary work (Data not presented). The change in OLR was performed using either glycerol waste (digesters 1–3) or FOG waste (digesters 10–12) in co-digestion with primary sludge. The effects of two sequential changes in OLR were also investigated using the same co-digestion substrate (glycerol waste only, digesters 4–6) or with a different co-digestion substrate (glycerol then FOG waste, digesters 13–15). Periods of co-digestion were stopped (return to feeding with primary sludge only) after methane production decreased to determine the ability of the digesters to recover after changes in substrate composition and OLR.

Biogas production was measured daily by water displacement and methane content using a SERVOPRO1400 CH4 gas analyser (Servomex, UK). VFAs analysis was carried out on 40 ml aliquot of digestate. Briefly, the aliquot was centrifuged at 5000 g for 5 min (Sorvall Legend RT+, DJB Labs, Newport Pagnel UK) and the supernatant was filtered to < 0.45 μm with a syringe filter (VWR, UK). Then 5 μl of 97% sulphuric acid was added to avoid acid degradation during storage and the sample was stored at − 20 °C until analysis. 100 μl of the sample was injected into a HPLC (535 Kontron, Bio-TEK, UK) equipped with a Bio-Rad fermentation column (Cat 125-0115) 300 × 7.8 mm maintained at 65 °C, and a UV detector at 210 nm. The mobile phase was acidified water (0.001 M sulphuric acid) with a flow rate of 0.8 ml/min. Acetic, propionic, n-butyric, iso-butyric and lactic acids were quantified using an external multilevel calibration ranging from 0.1 g l^{-1} to 5 g l^{-1}. The % error in the repeatability of measurements for each acid was 0.6, 0.8, 0.7, 1, and 3% for acetic, propionic, n-butyric, iso-butyric and lactic acids respectively. All chemicals were HPLC grade and sourced from VWR, UK.

2.2. Phospholipids (PLFA) and ether-linked isoprenoids (PLEL) analysis

For PLFA, total lipids were extracted from 40 g aliquot of freeze-dried digestate using a modified version of the Bligh-Dyer technique as described by Frostegard et al. (1991) The dried fatty acid methyl esters (FAMEs) were resuspended in 0.2 ml of hexane and analysed by gas chromatography equipped with flame ionisation detector (GC-FID Agilent Technologies 6890N) as described by Pankhurst et al. (2012). For PLEL another aliquot of the phospholipids fraction, equivalent to 40 g of the digestate was used for PLEL analysis according to the method described by Gattinger et al. (2003). The dried ether-linked isoprenoids were reconstituted in 0.2 ml of hexane and analysed by gas chromatography coupled to mass spectrometry (GCMS Agilent Technologies 6890N) according to the operating conditions described by Gattinger et al. (2003). Nonadecanoic acid methyl ester (Sigma, UK) was added as an internal standard to each sample after SPE.

2.3. 454-Pyrosequencing analysis and bioinformatics

Samples were processed for NGS by 454-Pyrosequencing on the GS FLX System (Roche) as described in Eduok et al. (2015). Sequencing resulted in a total of 11,576 reads with an average reads length of 600 bases. The obtained sequence data were then processed using the CloVR-16S 1.0 pipeline (http://clovr.org/) according to White et al. (2011) as described in Eduok et al. (2015).

2.4. Statistical analysis

All analysis was carried out in R using the specified packages (http://www.R-project.org). To test if production of VFAs after OLR increase was significantly different between treatments a linear mixed effects model was carried out using the package “nlme” significance was accepted at $p < 0.05$; digester and day were used as random effects to account for repeated measures on the same digester and as we were interested testing for significant differences between treatments rather than between days (Pinheiro et al., 2015). Non-metric multi-dimensional scaling (nMDS) ordination was carried out using the R package “Vegan 2.0–9” after Oksanen et al. (2013). The function “metaMDS” was used to group digesters based on dissimilarity in the concentration of VFAs, the obtained VFAs profiles were then linked to the microbial community using the “envfit” function for the bacterial OTUs, PLFA and PLEL lipid markers. Only bacterial markers that were significant at $p < 0.05$ were plotted on the nMDS.

3. Results and discussion

Before the onset of increased substrate addition (either glycerol or FOG) balanced anaerobic performance was evident with stable biogas production of 0.29 ± 0.08 m^3 kg^{-1} VS day $^{-1}$ and 0.27 ± 0.09 m^3 kg^{-1} VS day^{-1}, biogas methane content of 69% ± 7 and 74% ± 5.4 in the small and large digesters, respectively when the OLR was maintained at 1.4 kg VS m^{-3} d^{-1} (Fig. 1 A–B, Stable period). The average total VFAs concentration prior the change in OLR was 0.6 ± 0.2 and 0.8 ± 0.2 g l^{-1} in the small and large digesters respectively.

3.1. Effect of one OLR change on VFAs production

OLR was increased by 100% to 2.9 kg VS m^{-3} d^{-1} with glycerol waste (digesters 1–3, day 91) or FOG waste (digesters 10–12, day 63) as a co-digestion substrate (first high OLR).

After glycerol addition an immediate decrease in all parameters was observed: after 6 days biogas production decreased by 96%, methane content by 70%, alkalinity by 57% (2.8 ± 0.3 g to 1.2 ± 0.1 $CaCO_3$ l^{-1}) and pH < 6.5 (Fig. 1A). In contrast, OLR increase due to the addition of FOG waste (digesters 10–12 day 63) initially resulted in a 20% increase in biogas and a 7% increase in biogas methane content (Fig. 1B). However after day 74, the biogas production decreased by 85% and the methane content by 67% (Fig. 1B). There was also a parallel decrease in pH to <6 and alkalinity by 48% (from 3.3 ± 0.2 to 1.6 ± 0.8 g $CaCO_3$ l^{-1}).

After decrease in digester performance at day 93 for glycerol and day 67 for FOG, the OLR was returned to 1.4 kg VS m^{-3} d^{-1} allowing the digesters to recover (Recovery period) and return to conditions no longer significantly different to those recorded in the stable period (based on biogas production, *t*-test, $p < 0.05$). This took 45 ± 0 and 52 ± 1 days for the digesters exposed to glycerol and FOG, respectively.

During the OLR change with glycerol (digesters 1–3) the total VFAs concentration increased rapidly reaching 10.9 ± 2.9 g l^{-1} by day 97, while methane levels started to drop below 50% just after the addition at day 91, when total VFAs level were around 8 g l^{-1}. After day 97 there was 30% decrease in VFAs concentration, followed by a more gradual decrease between days 99 and 120 to 3.8 ± 0.1 g l^{-1} (Fig. 1A). Lactic acid was the major fermentation product in the first 3 days of glycerol co-digestion (53% of total acids on day 93) with concentrations up to 5 ± 0.6 g l^{-1} (Fig. 1A). From day 95 onwards lactic acid disappeared and acetic, propionic and butyric acids were the dominant organic acids, produced in parallel until day 120 when methane level increased above 50%, consistently making up around 50%, 35% and 25% of the VFAs fingerprint. Previous studies have reported that a major pathway for the microbial degradation of lactic acid is its conversion to propionic acid (Ren et al., 1997; Zhang et al., 2010) a well-established indicator of digester imbalance and the slowest and most sensitive process of all the VFAs degradation (Pind et al., 2003). Therefore high concentrations of lactic acid after addition of glycerol are also undesirable for methane production. Indeed as discussed by Yazdani and Gonzalez (2007) more value can be created from waste glycerol via other microbial conventions.

Production of VFAs during FOG waste co-digestion could be split into two distinct phases, days 63–73 and days 73-onwards, respectively. During the first phase total VFAs concentration increased to 5.4 ± 1.2 g l^{-1} with acetic acid as dominant organic acid produced (>70% of the total VFAs) accompanied by high biogas and methane production (Fig. 1B). Inhibitory levels of acetic acid proposed vary greatly among studies and it depends on the system under study. Concentration levels of 3–5 g l^{-1} were proposed as maximum value for optimal methane production by Holm-Nielsen et al. (2008) and Wang et al. (2009) while Ahring et al. (1995) showed that concentrations up to 6 g l^{-1} of acetate and 3 g l^{-1} of propionate caused no inhibition to the process. Ros et al. (2013) suggested that biogas methane content could be maintained above 70%, with acetic acid concentrations ranging from 1 to 2.6 g l^{-1}, when pH was maintained between 7 and 7.5. Similarly, in this study, methane content higher than 80% was observed with acetic acid concentrations greater than 4 g l^{-1} day when pH was maintained above 6.5 (day 73) and high concentrations of acetic acid resulted in exceptional methane production. After day 75 (second phase) propionic acid was the dominant VFAs (6 ± 1 g l^{-1}) and the biogas methane content decreased to less than 20% (Fig. 1B). This shift from acetic to propionic acid production indicates a shift in the metabolic function of the microbial community, probably as a consequence of the saturation of the acetate-derived methane production (Pind et al., 2003). If balance can be maintained, the high concentrations of acetic acid produced during FOG co-digestion can result in exceptional methane production (Nielsen and Ahring, 2006). However, processing of excess acetic acid into other VFAs by the bacterial community can also result in process instability. Indeed, Cavaleiro et al. (2010) suggested that LCFA degradation was the limiting step in lipid biomethanisation and bioaugmentation with synthropic bacteria accelerated LCA degradation. As shown in this study and other studies, lipid rich wastes without proper microbial management can result in digester imbalance and poor performance (Alves et al., 2009; Cirne et al., 2007; Long et al., 2011; Nielsen et al., 2007; Sousa et al., 2007, 2008). Therefore careful monitoring of acetic acid concentration, via OLR management, is suggested for taking advantage of the high biogas potential of FOG co-digestion.

3.2. Effect of one OLR change on microbial biomass and community dynamics

Both co-digestion substrates produced a 5 and 15 fold decrease of the bacterial and archaeal biomass respectively during the period when biogas production and methane content decreased. Specifically, the OLR change with glycerol caused an immediate drop in biomass and biogas production whereas the FOG waste did not induce a decrease in biomass until 17 days after the OLR change, when the biogas production decreased significantly (Supplementary material, Fig. I). This was expected for the archaeal community, the sole producers of methane, while the bacterial biomass drop was not an obvious result as bacteria can adapt and carry out other fermentation pathways, thus potentially maintaining biomass. Indeed an increase in VFAs concentration after OLR increase was observed in this study, indicating such an activity. However these fermentative pathways were clearly not sufficient to maintain bacterial biomass. The main changes in the bacterial lipid profiles were on day 93 in the digesters co-fed with glycerol, and coincided with high lactic acid production. Specifically there was an increase of the sulphur reducing bacteria (SRB) marker iso-17:1 by 10% and a decrease by 10% in the PLFA 18:1w9trans day 93 (Figs. 2A and 3). This is in contrast to the bacterial lipid profiles of the digesters co-fed with FOG, where iso-17:1 was not present at any stage and the proportions of the individual PLFA was constant during the changes in biomass. The PLFA iso-17:1 can be used as an indicator of sulphate reduction, an alternative pathway to methanogenesis when syntrophic interactions between bacteria and Archaea are inhibited (Van Den Berg et al., 1980). Hence, it is likely that iso-17:1 is related to the high concentration of lactic acid resulting from glycerol digestion (Fig. 3).

The isoprenoid i20:0 (>60 mol %) was predominant in the PLEL profiles after the OLR change with both co-feedstock (Fig. 2A and B). After the higher OLR was returned to initial values the concentration of this PLEL decreased. It is suggested that a higher proportion of this PLEL is related to changes in the degradation pathways in AD and could therefore be an indicator of imminent digester instability. In addition, all samples with a i40:0:i20:0 ratio >1 were always associated with stable or good performance whilst i40:0:i20:0 ratio <1 was mainly associated with poor biogas production.

There were similarities in the bacterial community changes of the two systems (Glycerol and FOG) during the periods when biogas methane content decreased. *Firmicutes* tripled and unknown bacterial OTUs decreased by 20% when the biogas methane content decreased during glycerol co-digestion. Similarly, a 2-fold increase of *Firmicutes* and a decrease in unknown bacteria was observed

during FOG waste co-digestion as biogas production decreased (Fig. 4A and OLRB). This is in contradiction with results reported by Rincón et al. (2008) where *Firmicutes* were dominant at lower OLR. A probable explanation for this discrepancy is the use of DGGE for their analysis, which is able to resolve only few dominant bacterial OTUs. Our results indicate that there are similarities in the microbial community response to low methane production, irrespective of feedstock/co-digestion and that a decrease in unknown OTUs and increase in *Firmicutes* OTUs were related to poor AD performance.

3.3. Effect of two OLR changes on VFAs production (same or different co-digestion feedstock)

Digesters were exposed to two changes in OLR using the same or different co-digestion feedstock (glycerol, digesters 4–6 or glycerol then FOG waste, digesters 13–15). As with digesters 1–3 the first increase in OLR with glycerol (day 7–14) caused an immediate decrease in biogas production and methane. However, the second OLR change produced significantly faster recovery (defined as no longer significantly different to pre-addition values) to initial methane content (23 ± 3 in contrast to 31 ± 1 days) and biogas production levels (30 ± 6 in contrast to 45 ± 0 days) (Fig. 5A). Analysis of the VFAs profiles showed this was related to changes in the way the feedstocks were processed and to the their production rates, with acid production rates between 40 and 60% lower after the second change in OLR compared to the first one (Fig. 6A and B). The production of acids was simultaneous during the first OLR and then switched to sequential production after the second OLR change (Fig. 6A).

The two changes in OLR also produced different amount of acetic, propionic, and lactic acid (linner mixed effects modle, $N = 3$, $p = 0.015$ and standard error $= 0.35$, $p < 0.001$ and standard error $= 0.29$, $p = < 0.001$, standard error $= 0.21$ for acetic, propionic, and lactic acid respectively) (Fig. 5A). Lactic acid was less than 6% of the total organic acid fingerprint after the second change in OLR, in contrast to >50% after the first one. In addition after the second change, there are two distinct periods of VFAs production with an initial period of propionic acid production followed by a peak in production of acetic acid after day 105. Thus it is possible that the conversion of propionic acid to acetic acid resulted in a faster recovery of digesters to stable conditions. This finding indicates that the processing of VFAs can be changed over time with multiple OLR changes and therefore more efficient recoveries from methanogenesis inhibition can be achieved.

In digesters exposed to two changes in OLR with different feedstocks (Fig. 5B) the production of VFAs was not significantly different to that previously described for digesters only exposed to one OLR change (linear mixed effects model, $p > 0.05$, $N = 3$, standard error $= 0.25$). This result further supports that a previous history of OLR increase with a different co-digestion substrate does not influence subsequent performance but instead enhance tolerance to further OLR variation. The suggested mechanisms of feedstock degradation are reported in Fig. 2A and B. The schematic clearly shows the impact of OLR on VFAs composition and its potential for process tailoring. However, it is also important to understand if this can be linked to changes in the structure of the bacterial and archaeal communities as this may lead to improved control and monitoring of the AD process during recovery after unstable performance.

3.4. Effect of two OLR changes on microbial biomass and microbial community dynamics

When using the same feedstock, the changes caused by the second OLR increase were similar to those observed after the first one (Supplementary material, Fig. I) at the exception of the bacterial PLFA 18:1w9trans between the two periods which was not

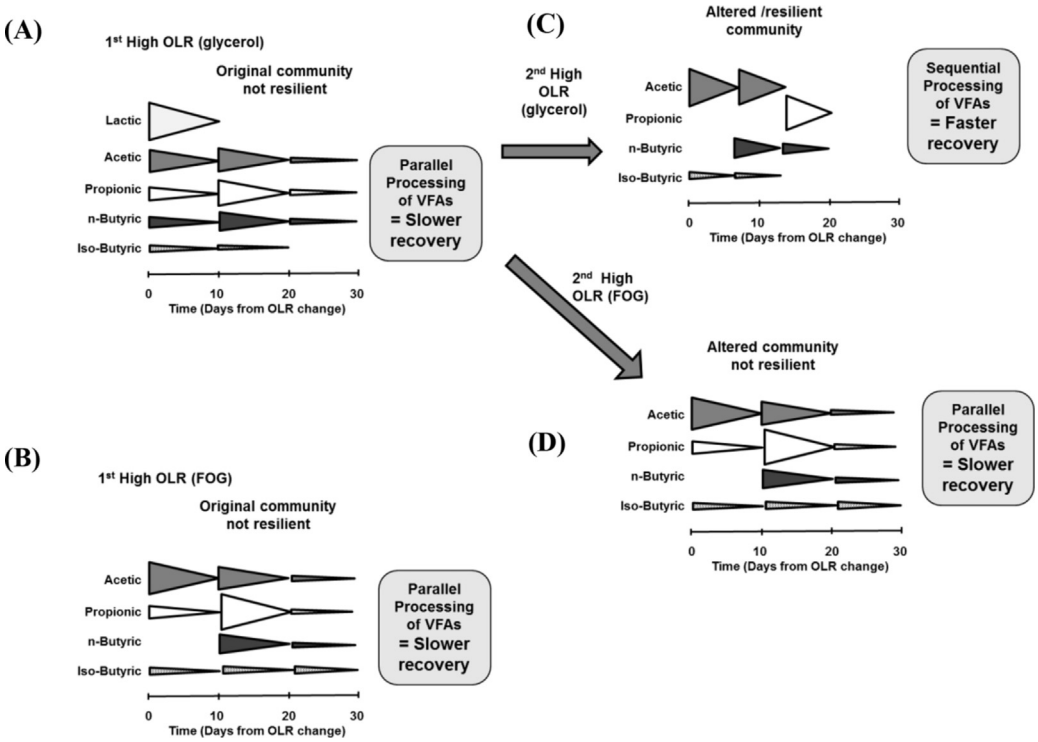


Fig. 6. Suggested schematic of glycerol and FOG degradation pathways in digesters after the first OLR change with glycerol (A) and FOGs (B) and after the second OLR change with the same feedstock glycerol (C) and with different feedstocks glycerol-FOGs (D).

present after the first addition (Fig. 7A). The other difference was observed in the archaeal community where the i40:0:i20:0 ratio returned to values higher than one in half of the time after the second glycerol addition. This suggests that the methanogenic community became resilient to OLR changes which resulted in shorter recovery times. This is in agreement with the results reported by McMahon et al. (2004), where digesters previously exposed to periods of unstable operation were more resilient to further perturbation.

When a different co-digestion substrate was used, PLFA 18:1w9cis increased 2-fold (day 98), with a corresponding decrease of the 18:1w9trans PLFA (Fig. 7B). This change in the Mol % of the 18:1w9cis/trans PLFA is related to *Bacillus* and *Clostridia* spp. and indicates that metabolic or structural shifts are taking place within these bacterial groups. These features can be linked to the faster recovery of methane production observed.

In contrast, the archaeal biomass changed as previously reported for the single OLR change (Supplementary material, Fig. I). Ratio between i40:0 and i20:0 returned to values higher than one more slowly with FOG than with glycerol; however, both archaeal and bacterial biomasses were able to recover after both OLR changes, demonstrating that multiple changes in OLR can be managed during AD without permanent damage to the microbial community.

Unknown bacterial OTUs were predominant in stable digesters, this implies that the predominant OTUs associated with optimal AD (high methane production and low VFAs concentrations) are as yet unclassified. More research is required with the specific aim of assigning function to these unknown OTUs as a complete picture of the diversity of bacteria in AD is required for AD optimisation (Fig. 4A-D).

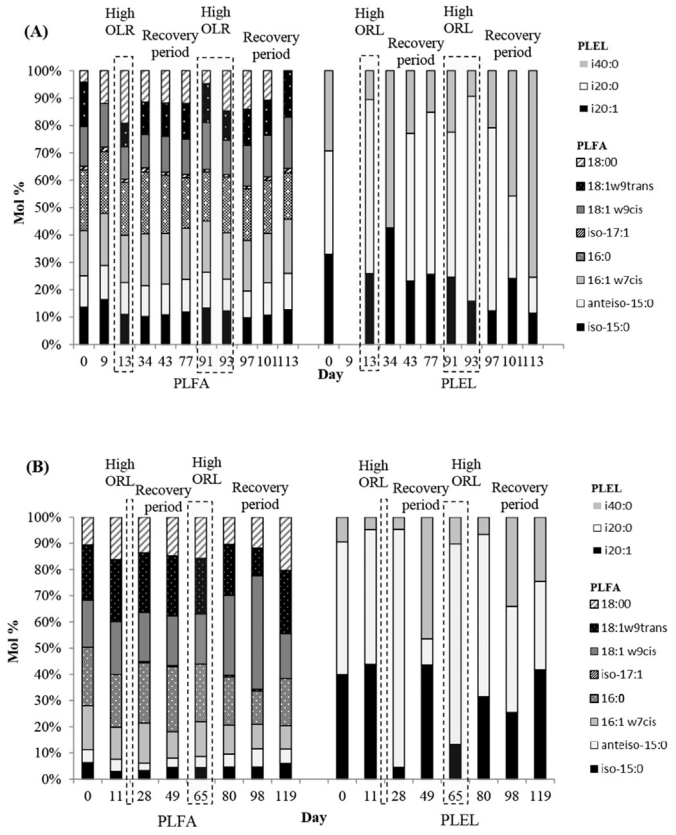


Fig. 7. Relative changes in bacterial and archea PLFA and PLEL (% of GC mass) after two OLR change with the same feedstock glycerol (A) and with different feedstocks glycerol-FOGs (B). x axis is not to scale and only shows days when samples were taken.

3.5. OLR as microbial management tool in AD optimisation: linking microbial community changes to VFAs and biogas production

The links between archaea and the concentration of VFAs are well established. However, this study provides novel insights into the relationship between bacteria and VFAs composition and how the VFAs pool can be manipulated using OLR. As bacteria are primarily involved in the production of VFAs during the acidogenic and acetogenic phases of AD, it is expected that the bacterial community structure plays a key role in regulating and controlling VFAs production and their resultant conversion into methane. Analysis of the VFAs profiles (Fig. 6A and B) showed that the same feedstock was processed differently after two changes in OLR: acids were produced in parallel after the first OLR and in sequence after the second OLR (propionic and then acetic acid). The second OLR induced a more efficient recovery from methanogenic inhibition and the microbial community was able to process higher concentrations of VFAs. The change in VFAs profile was due to a shift in the microbial population as evidenced by the lipid analysis (PLEL i40:0/i20:0 ratio and PLFA 18:1w9cis/trans ratio) and pyrosequencing (*Firmicutes* spp. increase) during changes in OLR with both co-digestion substrates. To identify links between the bacterial community and VFAs production non metric multidimensional scaling (nMDS) was carried out to group digesters with similar VFAs fingerprints and vectors for the bacterial OTUs and lipid fingerprints were fitted to the nMDS (Fig. 3). High concentration of acetic acid was correlated with *Clostridia incerta sedis* XV, and the PLEL i20:0 (a general methanogenic marker) and partially to the PLFA 18:1w9trans (*Bacilli and Clostridia* marker). The closest relative to the *Clostridia incertae sedis* XV OTU was *Cloacibacillus* genus belonging to the *Synergistaceae* family isolated from an AD pilot plant with high butyric acid concentration (FJ799129, 83%, unpublished) and identified in a wide number of anaerobic habitats (Baena et al., 2000; Diaz et al., 2007; McSweeny et al., 1993). All cultivable *Synergistaceae* so far isolated have the ability to degrade amino acids into volatile fatty acids and contribute to acidogenesis and acetogenesis via syntrophic relationships with methanogens (Baena et al., 1998; Delbès et al., 2000, 2001; Diaz et al., 2007; Menes and Muxí, 2002; Vartoukian et al., 2007). *Clostridia incertae sedis* XV has played a role in stabilising our digesters by improving degradation of VFAs and promoting methanogenesis over other pathways, as suggested in Cirne et al. (2006), who showed that hydrolysis of the lipid fraction could be improved by *Clostridium lundense* bioaugmentation. Thus bioaugmenting digesters with *Clostridia incertae sedis* XV could be a way forward for enhancing AD performance. However it is also important to point out that bioaugmentation presents some technical challenges, such as among others the isolation of the bacterial strain in the first place and then maintaining its presence in the digester over time Ferguson et al. (2014). In contrast manipulation of the process using operational tools such as OLR, have shown to be a promising alternative to bioaugmentation in AD (Briones and Raskin, 2003; Chen et al., 2012; Tale et al., 2015).

4. Conclusions

The results reported in this paper demonstrate that the response of digesters exposed to variations in OLR depends on the past operation of the reactor and that digesters previously exposed to OLR increase recover to initial values of biogas quantity and quality faster when exposed to new OLR increase with the same feedstock. They also show that tolerance to increased OLR can be built in the anaerobic digesters and generate an increase in AD performance in

terms of biogas quantity and quality, and most likely reducing biogas fluctuations correlated with feedstock variability. AD process stability is linked to the quality of the feedstock material entered in the system. Cleaner waste stream, currently going to AD, are nowadays considered a better feedstock for the production of high-value compounds rather than just for energy recovery. Understanding how to manipulate the AD microbial communities to cope with more contaminated and mixed feedstock is the only way to grow and maintain this industry. However, further studies at pilot or full scale AD are required to ascertain if this could be replicated in a real AD system.

The results also established links between the microbial composition and VFAs profiles. Analysis of the microbial communities indicated that the change in OLR induced changes in the microbial community structure, abundance and dynamics and that decreases in biogas were linked to decrease in both bacterial and archaeal biomass. The proposed schematic pathways for FOG and glycerol degradation clearly show the impact of OLR on VFAs composition and its potential for process tailoring. Once recognised the pattern of acids production (parallel or sequential) the acid excess could be mitigated and the process optimised by bioaugmentation or OLR manipulation. The results also suggest that syntrophic relationships between *Clostridia incertae sedis* XV and Archaea can result in a reduction of the concentrations of other VFAs and a more efficient methanogenesis via the acetogenic pathway. Monitoring of microbial biomass (with PLFA and PLEL) and OTUs numbers of the described bacterial families during changes could form the basis for more intelligent monitoring and control of AD.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2016.05.009.

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