Can microbial ecology help improve biogas production in AD?

Robert M. W. Ferguson[a], Frédéric Coulon[b], Raffaella Villa[b]†

[a]School of Biological Sciences, University of Essex, Wivenhoe Park, Colchester CO4 3SQ, UK

[d]Department of Environmental Science and Technology, Cranfield University, Cranfield, MK43 0AL, UK.

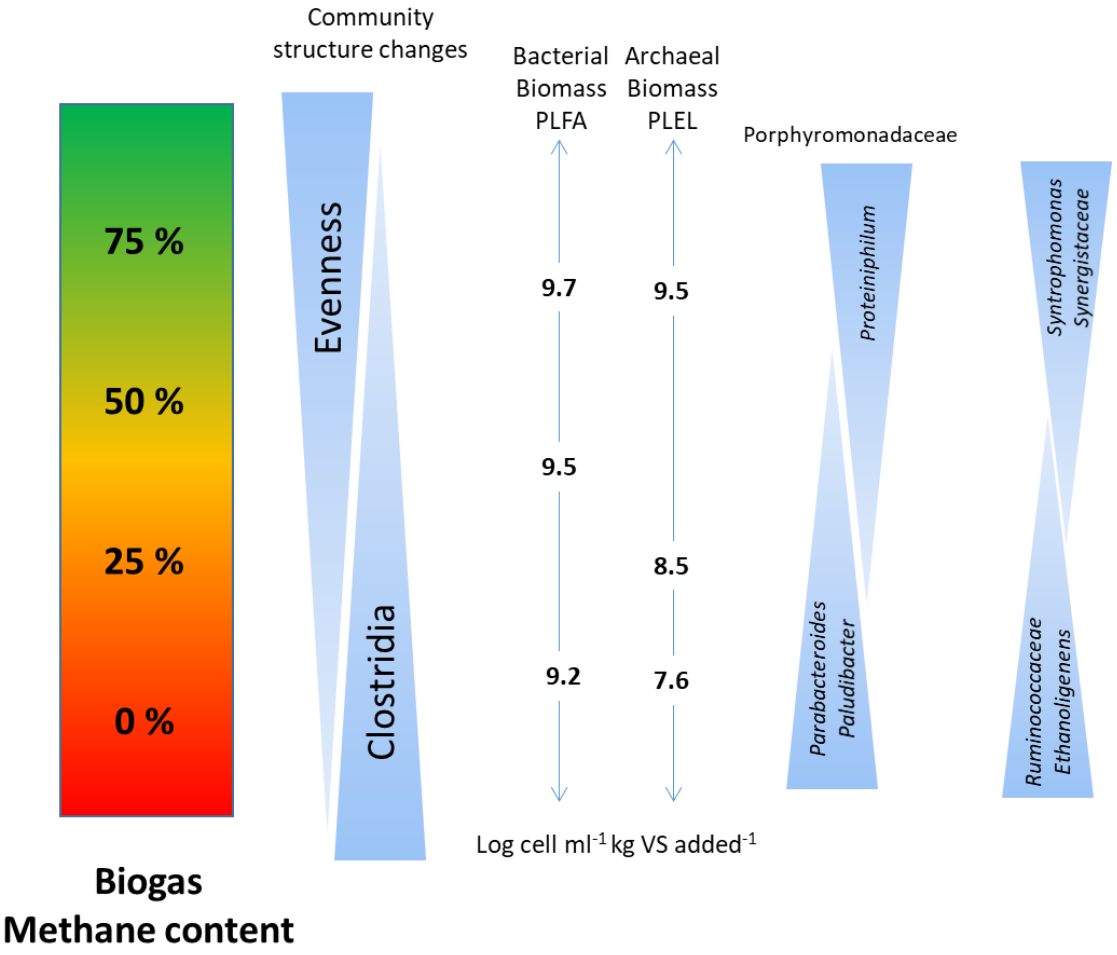
Running Head: Microbial ecology in AD

†Address correspondence to Raffaella Villa, r.villa@cranfield.ac.uk

Keywords: FOGs, Glycerol, Synergistaceae, Ruminococcaceae, Veillonellaceae, Next-generation sequencing.

Highlights

- Irrespective of the feedstock, changes in bacterial community could be related to digester performance
- Reactors with > 60 % biogas methane content had a more even distribution of bacterial diversity
- Methane content < 30 % correlated to a 50 % increase in Firmicutes (Ruminococcaceae)
- Methane content > 60 % correlated to unidentified operational taxonomic units (OTUs) and Synergistaceae



Abstract: 454-pyrosequencing and lipid fingerprinting were used to link anaerobic digestion (AD) process parameters (pH, alkalinity, volatile fatty acids (VFAs), biogas production and methane content) with the reactor microbial community structure and composition. AD microbial communities were subjected to stress conditions by changing digestion substrates and organic loading rates. 454-pyrosequencing analysis showed that methane content and pH were significantly and positively correlated with community evenness, regardless of the substrate digested. In AD, microbial communities with more even distributions of diversity are able to use more parallel metabolic pathways and have greater functional stability; hence they are more capable of adapting and responding to disturbances. A decrease in methane content to less than 30 % was always correlated with a 50 % increase of Firmicutes sequences (particularly in operational taxonomic units (OTUs) related to Ruminococcaceae and Veillonellaceae). Whereas digesters producing higher methane content (above 60 %), contained a high number of sequences related to Synergistetes and unidentified bacterial OTUs. Finally, lipid fingerprinting demonstrated that, under stress, the decrease in archaeal biomass was higher than the bacterial one, and that archaeal Phospholipid etherlipids (PLEL) levels were correlated to reactor performance. These results demonstrate that across a number of parameters (lipids, alpha and beta diversity, and OTUs) knowledge of the microbial community structure can be used to predict, monitor, or optimise AD performance.

1. Introduction

Anaerobic digestion (AD) is a widely implemented technology for the treatment of wastewater and organic mixed solid wastes. Notwithstanding this, poor anaerobic digester performance and system failure are still common issues. Most of these problems originate from inadequate operational and process control and a lack of understanding of the dynamics of the microbial processes taking place in the digesters (Leitao, van Haandel, Zeeman, & Lettinga, 2006). Plant management is mainly achieved through the monitoring of the physicochemical parameters rather than the biological ones. However, there is a general consensus among the scientific community that in-depth understanding of the AD microbial communities and their ecology is vital to optimise and adequately manage the process (Ferguson et al. 2014; Rittmann et al. 2006). Developments in culture independent molecular methods have led to a number of studies analysing the microbial communities in AD reactors, both at laboratory (Ferguson et al. 2016; Goux et al. 2015; Vanwonterghem et al. 2015) and at full-scale (Werner et al. 2012; Valentin-Vargas et al. 2012). Most of these studies demonstrated that the microbial ecology of AD is highly diverse and dynamic. Unstable communities have been observed in digesters with stable performance and functional redundancy renders it difficult to formulate any generic trends/relationships between microbial community response and digester performance (Fernández et al., 1999; Goux et al., 2015; X. Wang et al., 2011). Recently, studies of the microbial ecology of wastewater anaerobic digesters showed that it was possible to link digester performance with fundamental ecological parameters such as community evenness, as well as ecological theories such as the biogeography model, the species-area relationships and the taxa-time relationships (Wells et al. 2011; Valentin-Vargas et al. 2012; Werner et al. 2012). Therefore,

as discussed by a number of authors, the possibility of integrating the engineering of anaerobic digesters with microbial ecological theory is now a genuine prospect (Rittmann et al. 2006; Vanwonterghem et al. 2014).

In this context, there are still a number of key problems that need to be addressed; in particular and the relationship between AD performance and microbial community structure (alpha and beta diversity) and the consistency of these correlations. In short, to what extent do deterministic or stochastic processes determine the structure of microbial communities in AD. If stochastic processes dominate then unique functionally redundant microbial communities will exist in different digesters, making prediction of AD based on microbial community impossible. However, if deterministic processes dominate it will be possible predict species turnover and hence use this to monitor and predict AD performance (Måren, Kapfer, Aarrestad, Grytnes, & Vandvik, 2018). The syntrophic relationships involved in methanogenic degradation of most organic substrates in AD mean that species co-occurrence will be relatively even, and that species with similar ecological requirements will respond in similar ways (Schink, 2002). This means that it is probable that monitoring based on the presence of certain phylogenetic or functional groups should be possible, if we first gain a deeper understanding of the AD microbial community.

Molecular based lipid fingerprinting and PCR-based 454-pyrosequencing analyses were carried out to investigate the microbial community structure, biomass and dynamics in digesters running under different conditions (with varying co-digestion substrates and changing organic loading rate (OLR)). Molecular based lipid fingerprinting analysis provided insights into the microbial biomass changes and microbial community structure in the digesters. Whereas, 454-pyrosequencing was used to gain detailed phylogenetic

information on both the dominant and minor important members of the microbial community.

2. Methods

2.1 Digester operational parameters

Laboratory-scale semi-continuous digesters consisted of 1-L borosilicate glass bottles with a 700 ml working volume and 5-L bottles with a 4.5-L working volume maintained at 38 °C using a water bath. All reactors were seeded with digested sludge from a commercial Sewage Treatment digester (in a ratio of 30:70 %) and fed with autoclaved primary sludge three times a week to achieve a retention time of 7 days and an organic loading rate (OLR) of 1.4 kg VS m^{-3} d^{-1}. A different organic waste (glycerol or fat rich – FOG waste collected from a restaurant grease trap) was used to induce periods of unstable performance in the digesters (see table 1 for details of feedstocks). Glycerol or FOG was added to the autoclaved primary sludge to increase the OLR from 1.4 kg VS m^{-3} d^{-1} to 2.9 for one hydraulic retention time (HRT = 7 days) and then returned to 1.4 kg VS m^{-3} d^{-1}. These OLRs were selected as they were known to cause digester failure based on our preliminary work. All the reactors were run for more than 130 days (18-20 HRT) depending on the substrate. The effects of one or two sequential changes in OLR were investigated using the same feedstock (glycerol - glycerol) or with a different feedstock (glycerol - FOG waste). Feedstock and feeding regimes are those reported previously (Ferguson et al. 2016).

2.2. Biogas production, methane concentration and physicochemical characterisation

Gas production was measured daily by water displacement in a glass column (150 x 5 cm) and volumes corrected to standard atmospheric conditions. Methane content was

115 measured using a SERVOPRO1400 CH_4 gas analyser (Servomex, UK) according to
116 manufacturer recommendations. pH and alkalinity were measured according to standard
117 APHA methods (APHA 1989).

118 **2.3. Volatile fatty acids analysis**

119 A 40 ml aliquot of the digestate was centrifuged at 5000 g for 5 min and the supernatant
120 was filtered to < 0.45 µm with a syringe filter (Eduok, Ferguson, Jefferson, Villa, & Coulon,
121 2017). 5 µl of 97 % sulphuric acid was added (to avoid acid degradation during storage) and
122 the sample was stored at – 20 °C until analysis. 100 µl of the sample was injected into a
123 HPLC (535 Kontron, Bio-TEK, UK) equipped with a Bio-Rad fermentation column (Cat 125-
124 0115) 300 x 7.8 mm maintained at 65ºC, and a UV detector at 210 nm. The mobile phase
125 was 0.001 M sulphuric acid in HPLC grade water with a flow rate of 0.8 ml/min. Acetic,
126 propionic, n-butyric, iso-butyric and lactic acids were quantified using an external multilevel
127 calibration ranging from 0.1 g l-1 to 5 g l-1. The % error in the repeatability of
128 measurements for each acid was less than 4 %.

129 **2.4. Phospholipids (PLFA) and ether-linked isoprenoids (PLEL) analysis**

130 For PLFA, total lipids were extracted from 40 g aliquot of freeze-dried digestate using a
131 modified version of the Bligh-Dyer technique as described by Frostegård, et al. (1991). The
132 dried fatty acid methyl esters (FAMEs) were resuspended in 0.2 ml of hexane and analysed
133 by gas chromatography equipped with flame ionisation detector (GC-FID Agilent
134 Technologies 6890N) as described by Pankhurst et al. (2012). FAMEs were identified by
135 comparison of retention times with the 26 bacterial acid methyl ester (BAME) mix standard

(SUPELCO, Sigma, UK). Nonadecanoic acid methyl ester (Sigma, UK) was added (24.44 µg ml-1) as an internal standard to each sample after solid phase extraction (SPE).

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. The taxonomic affiliations are summarised in TS1. Gram-positive bacteria were represented by the series of iso and anteiso branched saturated PLFA. Gram-negative bacteria were represented by cyclopropane, hydroxyl and monounsaturated PLFA. The 16:0 straight chain PLFA has been previously demonstrated as an ubiquitous bacterial marker (Piotrowska-Seget and Mrozik 2003). The PLFA 18:2w9cis and 18:1w7trans used as markers for clostridia. The PLEL i20:0 was used as a marker for the *Euryarchaeota*, i20:1 as a marker of the aceticlastic methanogens belonging to *Methanosarcina* and i40:0 as a marker for hydrogenotrophic methanogens belonging to *Methanobacterium*, *Methanococcus*, *Methanopyrus*, and *Methanothermus* (Gattinger et al. 2002).

2.5. 454-pyrosequencing analysis and Bioinformatics

The microbial diversity and dynamics of the digesters was investigated by extracting total genomic DNA from 200 mg wet weight digestate samples using a MoBio Power Soil kit (MO BIO Laboratories, Inc, UK). Samples were then processed for NGS by 454-Pyrosequencing on the GS FLX System (Roche) as described in Eduok et al. (2015) using the following primers:

158 for amplification of the bacterial 16S rRNA gene PCR primers were adapted for 454 amplicon
159 sequencing by attaching the M13 adapter (italics) to the target forward primer M13-16S-IA-
160 FL (5'-*CACGACGTTGTAAAACGA*CCATGCTGCCTCCCGTAGGAGT-3'), whereas the 25-mer Lib-L
161 specific sequence adapter B (italics) was followed by the reverse template specific primer
162 sequence 16S-IA-RL (5'-*CCTATCCCCTGTGTGCCTTGGCAGTC*TCAGAGAGTTTGATCCTGGCTCAG -
163 3'). For amplification of the archaeal 16S rRNA gene PCR primers ARC-344F (5'-
164 *CACGACGTTGTAAAACGA*ACGGGGYGCAGCAGGCGCGA) and ARC-915R (5'-
165 *CCTATCCCCTGTGTGCCTTGGCAGT*CTCAGGTGCTCCCCCGCCAATTCCT- 71 3') where used; and
166 were adapted for 454 sequencing as described above. To multiplex the samples unique 10-
167 mer barcode sequences were included in the M13 adapter.

168 The sequences obtained were processed as described in Dumbrell *et al.* (2017). Denoising of
169 454-Pyrosequencing amplicons was carried out by the sequencing provider using
170 AmpliconNoise (Quince, Lanzen, Davenport, & Turnbaugh, 2011). The obtained sequence
171 data were then processed in QIIME using Biolunix version 8; Sequences with > 6 ambiguous
172 bases, a homopolymer run of > 6, a read length of < 300 or > 800, and a quality score < 25
173 were excluded (Caporaso et al., 2010; Ferguson, Gontikaki, Anderson, & Witte, 2017; Field
174 et al., 2006). After quality control there were 19,633 reads. *De novo* operational taxonomic
175 units (OTUs) were picked using Usearch (with *de novo* chimera removal) at 97 % percent
176 similarity using the script pick_otus.py, 2137 OTUs were identified (Edgar, 2010; Edgar,
177 Haas, Clemente, Quince, & Knight, 2011). A representative sequence for each OTU was then
178 identified with pick_rep_set.py and used to assign taxonomy with using assign_taxonomy.py
179 and the default parameters and the Green Genes reference taxonomy (13_8_99) (McDonald
180 et al. 2012; Werner et al. 2012; Wang et al. 2007). Cumulative sum scaling was used to

normalise the OTU table and account for differing sampling depth using the QIIME script normalize_table.py (Paulson, Stine, Bravo, & Pop, 2013)

2.6. Statistical analysis

Statistical analysis was carried out in R (v 3.2.0) and cited packages (R Development Core Team, 2015). Analysis of variance (ANOVA) was used to test for significant differences between digester group means (e.g. lipid biomass and alpha diversity metrics) significance was accepted at $P < 0.05$. To investigate patterns of beta diversity in the digesters a distance matrix using the Bray-Curtis method was calculated in Vegan 2.3.0 (Bray & Curtis, 1957; Oksanen et al., 2015). Permutational multivariate analysis of variance using distance matrices (PERMANOVA) was used to determine if the microbial communities were significantly different for the 6 digester groups (Anderson, 2001). Generalized additive models (GAMs) were used to correlate physicochemical parameters to the microbial community (e.g. pH, biogas methane content, biogas production, acetic acid concentration, and propionic acid concentration) with significance accepted at $P < 0.05$ (Oksanen, 2013). To test for significant changes in OTU abundance between digester groups the QIIME script group_significane.py was used to carry out a Kruskal-Wallis test. Ecological indexes were calculated as: Shannon-weaver index (*H'*), Simpsons index (*D*) and Pielou's evenness (*J*).

3. Results

3.1. Reactors performances: VFAs, alkalinity, biogas and methane production

Reactor performances over time and detailed results of the analysis have been reported previously (Ferguson et al. 2016). For the purpose of this work methane percentages were averaged across each HRT and reported in Figure 1 (a-d). The figures clearly show the

periods of low methane production following an increase in OLR. At times of 'balanced' anaerobic performance, when the OLR was maintained at 1.4 kg VS m^{-3} $d^{-}1$, biogas production was around 0.28 m^3 kg^{-1} VS day $^{-}1$ and methane content around 75 %. When organic loading rate was increased to 2.9 kg VS m^{-3} d^{-1} biogas production dropped to around 1 m^3 kg^{-1} VS day $^{-}1$ and methane content was lower than 30 % for a period of one or more HRTs, depending on the digester history. For example, reactors that received two OLR change with the same feedstock (Gly-Gly; Figure 1b) returned to 'balanced' conditions much faster after the second change (3HRTs vs 4HRTs; Figure 1a and 1b). VFA content, alkalinity, pH and VFA/alkalinity ratio changed as expected with changes in methane content (table 2).

In order to identify whether there were any general trends, correlation between microbial community structure and dynamics and digesters performance, the digesters were grouped according to the methane content of their biogas. Overall 6 groups were identified which further related to VFA production, alkalinity and VFA/Alkalinity ratio (table 2).

3.2. Microbial lipid fingerprinting (PLFA and PLEL)

Many of the bacterial lipid markers (PLFA) were associated to *Actinobacteria*, low GC Gram positive bacteria, CFB, *δ-Proteobacteria*, *Bacillus*, and *Clostridia* (table 3). There were relatively little changes in the contribution of many of the individual PLFAs to the total fingerprint at different biogas methane content. The only PLFAs that varied between the digester groups were the PLFA18:1w9cis and 18:1w9trans, used as marker for clostridia, (Table 3). The trans oleic acid (18:1w9trans) doubled in concentration when methane content was ≥ 60 % in comparison to digesters with a methane content ≤ 20 %. In contrast, the cis oleic acid (18:w9cis) doubled in digesters with a methane content ≤ 30 %. This finding

225 suggests changes in the community structure and/or metabolic function of the associated
226 bacterial groups (Anaerobes/CFB group). No change in the ratio of cyclopropyl to mono-
227 unsaturated fatty acids (cy17:0/16:1ω7c and cy19:0/18:1ω7c) was observed suggesting that
228 the microbial community of the digesters were not experiencing significant stress conditions
229 despite varying the OLR of the digesters (Frostegård et al. 2011). The PLFA 18:2w6,9 which is
230 associated with fungi made up approximately 3.5 for mol % in all digesters conditions. Fungi
231 are not often considered in studies of the microbial communities in AD and their potential
232 role in AD remains to date unresolved. It is possible that they could play a role in cellulose
233 digestion as this has been observed in cattle rumen, therefore fungi could be a key
234 hydrolytic group in AD (Bauchop & Mountfort, 1981).

235 The archaeal biomass, calculated from PLEL as number of cells ml^{-1} kg^{-1} VS added, was
236 always correlated with methane content for all the digestion substrates (sludge, sludge and
237 glycerol and sludge and FOG) at $P < 0.001$ (Figure 2b). The digesters with biogas production
238 higher than 0.2 m^3 kg^{-1} VS day^{-1} had an average archaeal biomass of 9.1 ± 0.4 log cells ml^{-1}
239 kg^{-1} VS added, in contrast digesters with biogas production < 0.2 had average archaeal
240 biomass of 8.3 ± 0.4 log cells ml^{-1} kg^{-1} VS added. In contrast, no clear relationship between
241 the bacterial biomass, calculated from PLFA as number of cells $ml^{-1}kg^{-1}$ VS added, and biogas
242 methane content was observed (Figure 2a). Thus, archaeal biomass is a key parameter in
243 biogas quality, this is not surprising as the methanogens do not have metabolic pathways
244 available other than methane production (Garcia et al. 2000). In contrast the bacterial
245 biomass growth is not restricted to methane production; bacteria are more resilient and are
246 able to switch to other pathways when methane production is inhibited. Indeed, the high
247 concentration of VFA and changes in VFA profiles to longer chain VFA would suggest that

248 bacteria have switched to other fermentation pathways (Table 2). These results are
249 consistent with previous research that showed that increasing biogas production is
250 correlated with distinct changes in lipid fingerprinting, which could be used to monitor AD
251 performance (Schwarzenauer & Illmer, 2012).

252 **3.3. DNA based molecular analysis (454-pyrosequencing)**

253 Over 75 % of the archaeal diversity was dominated by Euryarchaeota, especially the genus
254 *Methanosarcina* and to a lesser extent (only 1%) to the genus *Methanobrevibacter*. Further
255 to this, 20 % of the OTUs identified were related to unidentified Archaea and a small
256 number (< 2 %) of members belonging to Thermoprotei, a class of the Crenarchaeota. Only
257 three OTUs accounted for 54 % of all sequences, and no other OTUs had greater than 3 % of
258 sequences. The closest matches to these OTU are all from the genus *Methanosarcina* which
259 is the most metabolically diverse methanogenic genus and has also been shown to be the
260 most stress resilient (Karakashev et al. 2005; Vavilin et al. 2008). It is therefore unsurprising
261 that this group should dominate in all conditions analysed.

262 A total of 19,363 bacterial sequences were clustered into 2137 OTUs. The rank abundance
263 curve revealed that 17 % of the total OTUs were comprised of > 5 sequences (Figure S1).
264 This indicates that there was a core group of approximately 15 % of OTUs present across the
265 whole data set (figure 3). The OTUs were assigned to Bacteroidetes (21 %) Firmicutes (16 %)
266 Synergistetes (6.8 %) Proteobacteria (5.6 %) and unknown (45 %) (figure 4). Predominance
267 of these bacterial phyla and a high number of unidentified OTUs is consistent with results
268 reported in other studies using NGS techniques, the high number of unknown OTUs
269 indicates that a large amount of the diversity in AD is poorly described in reference

databases (Schlüter et al. 2008; Kröber et al. 2009; Lee et al. 2012; Werner et al. 2012; Rivière et al. 2009).

3.3.1 Alpha diversity. Bacterial OTU richness was 134 ± 83 across all samples, however there was no significant difference in OTU richness between the digester performance groups (ANOVA, $F_{5,31}$ = 0.95, P = 0.5). Shannon-weaver index (*H'*) did slightly increase from 2.6 ± 0.3 to 2.8 ± 0.1 between group 1 (0-20 % methane) and 6 (> 70 % methane) however, this was not significant (ANOVA, $F_{5,31}$ = 0.99, P = 0.43). In contrast there were significant increases for "*D*" Simpsons index (ANOVA, $F_{5,31}$ = 2.3, P = 0.006) and "*J*" Pielou's evenness (ANOVA, $F_{5,31}$ = 1.7, P = 0.02) with increased biogas methane content across the digester groups. This is clearly shown in Figure 5 and it indicates that the better performing digesters (in terms of biogas production and methane content) had more even communities. A number of studies have reported that improved digester performance was related with higher community evenness (Wittebolle et al. 2009; Merlino et al. 2012; Werner et al. 2012). Community evenness is particularly important in a system such as AD, as it signposts equitable distribution between the various AD functional groups; this enables the community to fully exploit all metabolic pathways, as well as the co-metabolic pathways, which are known to play an important in AD performance (Hashsham et al., 2000). Further to this, communities with uneven distributions of diversity tend to be dominated by groups of microorganisms specialised to the current conditions, when exposed to external changes (e.g. pH) they are unable to adapt to rapidly and require long recovery times.

3.3.2 Beta Diversity. Links between the bacterial community structure and physiochemical parameters were further demonstrated by PERMANOVA. The results showed a significant shift in the bacterial community structure between the pre-defined digester groups (table 2)

293 (PERMANOVA, $F_{5,31}$ = 2.7, P = 0.001 and, R = 0.31), specifically there was a significant
294 correlation between the community structure and methane content and biogas production
295 (GAMs P = 0.017 r^2 = 0.21, and P = 0.01, r^2 =0.25 for methane content and biogas production
296 respectively). In contrast there was no significant effect of individual reactor (PERMANOVA,
297 $F_{5,31}$ = 2.7, P = 0.3, and r^2 = 0.31). Further understanding of the key taxonomic groups and
298 OTUs involved is required to develop this into a predictive framework for optimising AD.

299 **3.4. Relationships between methane content and bacterial community**

300 Overall Bacteroidales dominated in all digesters and all conditions making up approximately
301 20 % of the community (Figure 4). The phyla Bacteroidetes and Proteobacteria, which
302 comprised at the order level mainly of Bacteroidales and the Betaproteobacteria order
303 Burkholderiales remained at fairly constant levels in all conditions, ranging from 17-25 %
304 and 5-7.6 % respectively. For optimising AD performance, it is important to understand if
305 there are core groups of bacteria that are important for good performance (high biogas
306 production, methane content, and stable production) and also to identify those that are
307 associated with the worst performance. To do this the six groupings defined earlier in
308 section 3.1, table 2 were further consolidated into three groups defined as low, medium,
309 and high biogas methane content (0-30, 31-60, and 61-85 %). A Kruskal–Wallis one-way
310 analysis of variance was then carried out to identify OTUs correlated with these
311 performance groups (Figure 6).

312 **3.4.1. Dominant OTUs at low methane content.** A number of Firmicutes OTUs (12),
313 including OTUs related to the families Ruminococcaceae, Lachnospiraceae, Clostridiaceae,
314 Lactobacillaceae, Acidaminococcaceae, and Veillonellaceae were significantly associated

315 with digesters with biogas methane content < 30 %. Firmicutes, and in particular Clostridia
316 are an important fermentative group in AD; and indeed as a key AD group they are common
317 to AD systems (Nelson et al. 2011). Most of the increase in Fermicutes was down to two
318 families, Ruminococcaceae and Veillonellaceae, which made up approximately 7 % the
319 community each in the bioreactors with the lowest biogas methane content and production
320 (group 1). Digesters with low methane concentration also had high concentration of VFA
321 (Table 2). Other studies have also shown Ruminococcaceae to be associated with poor AD
322 performance (Tian, Cabrol, Ruiz-Filippi, & Pullammanappallil, 2014; Vanwonterghem et al.,
323 2015). Tentative exploration of the metabolic capabilities of the Firmicutes OTUs (by looking
324 at the closest matches to the OTU sequence in BLASTn) revealed that the ones identified in
325 this study are probably acidogens, with the capability to produce longer chain fatty acids
326 such as butyric, propionic, lactic, and valeric acid. For example, *Butyricicoccus* can convert
327 acetic acid into butyric acid, directly competing with methanogens. The other major phyla in
328 the low group were Bacteroidetes, mainly represented by the Porphyromonadaceae and
329 Prevotellaceae families (Figure 6). The exact role of Prevotellaceae in AD is unknown but the
330 closest matches to the representative sequences for the OTUs were both isolated from
331 rumen and related to acidogenic bacteria (Ramšak et al., 2000; Whitford, Forster, Beard,
332 Gong, & Teather, 1998). Matching these OTUs using BLASTn (Altschul, Gish, Miller, Myers, &
333 Lipman, 1990) also suggested that they could be acidogens, again capable of producing a
334 wide range of VFA. In summary, there was a significant association between fermentative
335 long chain fatty acid producing bacteria and digesters with low biogas methane content; as
336 described previously the production of long chain fatty acids in AD reduces pH and

337 undermines the syntrophic relationships between bacteria and archaea the support
338 methane production (Ferguson et al., 2016).

339 **3.4.2. Dominant OTUs at high methane content.** The OTUs correlated with the high-
340 methane content group diverged at the family level from those in the low-methane one.
341 The phylum Synergistetes showed a marked increase from 1 % to 18 % as biogas methane
342 content and production increased (Figure 4). The phylum Synergistetes can produce a range
343 of organic acids that can be processed by other bacteria, or produce substrates such as
344 acetic acid and hydrogen that are directly used by methanogens; indeed it has been shown
345 that the range of substrates they use and produce is enhanced by co-culture with
346 methanogens (Baena et al., 2000). Syntrophic relationships between bacteria and
347 methanogens are required for stable AD (McMahon et al. 2004; Hattori. 2008; Stams and
348 Plugge 2009). For example, the closest match to the *Aminobacterium* OTU found in this
349 study was *Aminobacterium colombiense,* which has been detected in biogas reactors in
350 other studies and can produce acetic acid from amino acids and hydrogen; significantly
351 these functions are enhanced in via syntrophic associations with methanogens (Chertkov et
352 al., 2010). Also, *Proteiniphilum* OTUs were identified that were matched to a strain isolated
353 from a USAB reactor which can enhance rates of propionic acid conversation into methane
354 (via acetic acid) when added to a syntrophic propionate-degrading co-culture
355 (*Syntrophobacter sulfatireducens* and *Methanobacterium formicicum*) (Chen & Dong, 2005).

356 **4. Discussion**

357 **4.1. How can microbial community structure and dynamics information be used to**
358 **monitor and optimise AD?**

359 The microbial communities in AD are often treated as a black box and there is a general
360 perception amongst AD operators that optimisation will not be achieved through an
361 improved understanding of the microbial ecology. In this study, consistent shifts in the
362 structure of the microbial communities were observed with increase in biogas methane
363 content, regardless of the feedstock used. Such information can help to develop new
364 strategies for monitoring and optimising AD process, and further assist AD operators to
365 predict unstable digester performance.

366 **4.1.1. Predicting performance.** Results presented here show that there are general and
367 consistent relationships between performance and microbial community structure.
368 However, further research is needed as other authors have shown that microbial
369 communities in different digesters diverge over time; even when those digesters are under
370 stress, which you might expect to cause communities to converge due to selection pressure
371 (Goux et al. 2015; Werner et al. 2012). As counterpoint to this other studies (including ours)
372 have found that deterministic processes dominate over stochastic in AD microbial
373 communities, supporting microbial monitoring as a viable tool for AD (Vanwonterghem et
374 al. 2014; Vanwonterghem et al. 2015). It is probable that the answer falls somewhere in
375 between. For example, despite finding that communities in AD were unique to individual
376 digesters, Werner et al. (2012) was still able to show links between community structure
377 and function that were common to all digesters; and Goux *et al,* (2015) was able to find
378 predictive shifts in the archaeal component of the microbial community. It may therefore
379 be possible to monitor AD performance based on these OTUs without needing a full shotgun
380 sequencing analysis. This could conceivably be done with portable qPCR machines or even
381 loop mediated DNA amplification (LAMP) which can identify specific bacteria, without the

need for DNA extraction, in under an hour (Notomi et al., 2000). Indeed LAMP has been used to identify Ebola in remote locations in Guinea, we therefore think that its use for an AD plant is not beyond the realms of possibility (Kurosaki et al., 2016). We were also able to show consistent links between digester function and microbial community structure, but the stochastic element to the assembly of AD microbial communities needs to be taken into account. There will be inconsistencies between digesters, and decisions will need to be made considering a wide range of microbial and physiochemical parameters, including past knowledge of the specific digester. We therefore suggest a combined molecular approach using lipid fingerprinting and DNA based technologies could be employed to provide process monitoring in AD by application of existing technology. However take-up of these technologies for monitoring AD has been slow, a great deal of development and collaboration between industry and research is required for this becomes a realistic prospect.

4.1.2. Bioaugmentation and AD optimisation. The core groups of bacteria specific to particular levels of performance revealed a number of unique OTUs in digesters with high biogas methane content (Figure 4 and 6). This information can further contribute to AD optimisation via bioaugmentation. Although it has been demonstrated in principle (Enright et al. 2009; Guo et al. nd; Schauer-Gimenez et al. 2010; Tale et al. 2011; Westerholm et al. 2012), bioaugmentation is logistically challenging; the bacteria need to be isolated and cultivated in sufficient quantity and finally there is no guarantee that the community will take hold in the digester. As an alternative it has previously been shown that changes in digester performance can be used to optimise the community to improve recovery from process imbalance (Goux et al. 2015; McMahon et al. 2004; McMahon et al. 2007; Stroot et

al. 2001; Ferguson et al. 2016). Ultimately a vast quantity of knowledge needs to be collected on the relationship between community structure, function, and process control in AD so that operators can take full advantage of the possibility of process manipulation as a means of control for AD.

5. Conclusions

The results clearly demonstrate a relationship between the community structure and the performance of AD. There were consistent increases in Clostridia, specifically Ruminococcaceae and Veillonellaceae, in digesters with low biogas methane content; and an increase in the numbers of Synergistetes in those with high methane content. A statistically significant correlation between community evenness and AD performance was also demonstrated, highlighting that a more equitable distribution of diversity in AD is related to higher methane production, possibly due to improved balance between the functional groups present.

It was also demonstrated that lipid fingerprinting, due to its ability to detect changes in biomass, is a valuable companion to sequence based analysis, or even on its own as a monitoring tool. Pyrosequencing analyses of multiple digester conditions in this study also revealed that a large proportion of sequences could not be assigned to taxonomic affiliations even at the phylum/class levels. This highlights that further work is required to fully understand the identity and function of the microbial diversity present in AD.

References

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*(3), 403–10.

http://doi.org/10.1016/S0022-2836(05)80360-2

Anderson, M. J. (2001). A new method for non-parametric multivariate analysis of variance. *Aust. Ecol.*, *26*(1), 32–46. http://doi.org/10.1111/j.1442-9993.2001.01070.pp.x

Baena, S., Fardeau, M. L., Labat, M., Ollivier, B., Garcia, J. L., & Patel, B. K. (2000). Aminobacterium mobile sp. nov., a new anaerobic amino-acid-degrading bacterium. *International Journal of Systematic and Evolutionary Microbiology*, *50*(1), 259–264. http://doi.org/10.1099/00207713-50-1-259

Bauchop, T., & Mountfort, D. O. (1981). Cellulose Fermentation by a Rumen Anaerobic Fungus in Both the Absence and the Presence of Rumen Methanogens. *Applied and Environmental Microbiology*, *42*(6), 1103–1110.

Bray, J. R., & Curtis, J. T. (1957). An Ordination of the Upland Forest Communities of Southern Wisconsin on JSTOR. *Ecological Monographs*, *27*(4), 325–349.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., … Knight, R. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*, *7*(5), 335–336. http://doi.org/10.1038/nmeth.f.303

Chen, S., & Dong, X. (2005). Proteiniphilum acetatigenes gen. nov., sp. nov., from a UASB reactor treating brewery wastewater. *International Journal of Systematic and Evolutionary Microbiology*, *55*(Pt 6), 2257–61. http://doi.org/10.1099/ijs.0.63807-0

Chertkov, O., Sikorski, J., Brambilla, E., Lapidus, A., Copeland, A., Glavina Del Rio, T., … Klenk, H.-P. (2010). Complete genome sequence of Aminobacterium colombiense type strain (ALA-1). *Standards in Genomic Sciences*, *2*(3), 280–9.

http://doi.org/10.4056/sigs.902116

Dumbrell, A. J., Ferguson, R. M. W., & Clark, D. R. (2017). Microbial Community Analysis by Single-Amplicon High-Throughput Next Generation Sequencing: Data Analysis -- From Raw Output to Ecology. In T. J. McGenity, K. N. Timmis, & N. Balbina (Eds.), *Hydrocarbon and Lipid Microbiology Protocols: Microbial Quantitation, Community Profiling and Array Approaches* (pp. 155–206). Berlin, Heidelberg: Springer Berlin Heidelberg. http://doi.org/10.1007/8623_2016_228

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics (Oxford, England)*, *26*(19), 2460–1. http://doi.org/10.1093/bioinformatics/btq461

Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C., & Knight, R. (2011). UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics*, *27*(16), 2194–2200. http://doi.org/10.1093/bioinformatics/btr381

Eduok, S., Ferguson, R. M. W., Jefferson, B., Villa, R., & Coulon, F. (2017). Aged-engineered nanoparticles effect on sludge anaerobic digestion performance and associated microbial communities. *Science of The Total Environment*, *609*, 232–241. http://doi.org/10.1016/j.scitotenv.2017.07.178

Eduok, S., Hendry, C., Ferguson, R. M. W., Martin, B., Villa, R., Jefferson, B., & Coulon, F. (2015). Insights into the effect of mixed engineered nanoparticles on activated sludge performance. *FEMS Microbiology Ecology*, *91*(7), fiv082. http://doi.org/10.1093/femsec/fiv082

469 Enright, A. M., O'Flaherty, V., Aelst, A. C. van, & Lens, P. N. L. (2009). Bioaugmentation of
470 UASB reactors with immobilized Sulfurospirillum barnesii for simultaneous selenate
471 and nitrate removal. *Applied Microbiology and Biotechnology*, *83*(2), 377–388.

472 Ferguson, R. M. W., Coulon, F., & Villa, R. (2016). Organic loading rate: A promising microbial
473 management tool in anaerobic digestion. *Water Research*, *100*, 348–356.
474 http://doi.org/10.1016/j.watres.2016.05.009

475 Ferguson, R. M. W., Gontikaki, E., Anderson, J. A., & Witte, U. (2017). The Variable Influence
476 of Dispersant on Degradation of Oil Hydrocarbons in Subarctic Deep-Sea Sediments at
477 Low Temperatures (0-5 °C). *Scientific Reports*, *7*(1), 2253.
478 http://doi.org/10.1038/s41598-017-02475-9

479 Ferguson, R. M. W., Villa, R., & Coulon, F. (2014). Bioengineering options and strategies for
480 the optimization of anaerobic digestion processes. *Environmental Technology*, *3*(1), 1–
481 14. http://doi.org/10.1080/09593330.2014.907362

482 Fernández, A., Huang, S., Seston, S., Xing, J., Hickey, R., Criddle, C., & Tiedje, J. (1999). How
483 stable is stable? Function versus community composition. *Applied and Environmental*
484 *Microbiology*, *65*(8), 3697.

485 Field, D., Tiwari, B., Booth, T., Houten, S., Swan, D., Bertrand, N., & Thurston, M. (2006).
486 Open software for biologists: from famine to feast. *Nature Biotechnology*, *24*(7), 801–3.
487 http://doi.org/10.1038/nbt0706-801

488 Frostegård, A., Tunlid, A., & Baath, E. (2011). Use and misuse of PLFA measurements in soils.
489 *Soil Biology and Biochemistry*, *43*(8), 1621–1625.

Frostegård, Å., Tunlid, A., & Bååth, E. (1991). Microbial biomass measured as total lipid phosphate in soils of different organic content. *Journal of Microbiological Methods*, *14*(3), 151–163. http://doi.org/10.1016/0167-7012(91)90018-L

Garcia, J.-L., Patel, B. K. C., & Ollivier, B. (2000). Taxonomic, Phylogenetic, and Ecological Diversity of Methanogenic Archaea. *Anaerobe*, *6*(4), 205–226.

Gattinger, A., Günthner, A., Schloter, M., & Munch, J. C. (2003). Characterisation of Archaea in Soils by Polar Lipid Analysis. *Acta Biotechnologica*, *23*(1), 21–28.

Gattinger, A., Schloter, M., & Munch, J. C. (2002). Phospholipid etherlipid and phospholipid fatty acid fingerprints in selected euryarchaeotal monocultures for taxonomic profiling. *FEMS Microbiology Letters*, *213*(1), 133–139.

Goux, X., Calusinska, M., Lemaigre, S., Marynowska, M., Klocke, M., Udelhoven, T., … Delfosse, P. (2015). Microbial community dynamics in replicate anaerobic digesters exposed sequentially to increasing organic loading rate, acidosis, and process recovery. *Biotechnology for Biofuels*, *8*(1), 122. http://doi.org/10.1186/s13068-015-0309-9

Guo, J., Wang, J., Cui, D., Wang, L., Ma, F., Chang, C.-C., & Yang, J. (n.d.). Application of bioaugmentation in the rapid start-up and stable operation of biological processes for municipal wastewater treatment at low temperatures. *Bioresource Technology*, *101*(17), 6622–6629.

Hashsham, S. A., Fernández, A. S., Dollhopf, S. L., Dazzo, F. B., Hickey, R. F., Tiedje, J. M., & Criddle, C. S. (2000). Parallel Processing of Substrate Correlates with Greater Functional Stability in Methanogenic Bioreactor Communities Perturbed by Glucose. *Applied and*

Environmental Microbiology, *66*(9), 4050–4057.

Hattori., S. (2008). Syntrophic Acetate-Oxidizing Microbes in Methanogenic Environments. *Microbes and Environments*, *23*, 118–127.

Karakashev, D., Batstone, D. J., & Angelidaki, I. (2005). Influence of Environmental Conditions on Methanogenic Compositions in Anaerobic Biogas Reactors. *Appl. Environ. Microbiol.*, *71*(1), 331–338. http://doi.org/10.1128/aem.71.1.331-338.2005

Kröber, M., Bekel, T., Diaz, N. N., Goesmann, A., Jaenicke, S., Krause, L., … Schlüter, A. (2009). Phylogenetic characterization of a biogas plant microbial community integrating clone library 16S-rDNA sequences and metagenome sequence data obtained by 454-pyrosequencing. *Journal of Biotechnology*, *142*(1), 38–49.

Kurosaki, Y., Magassouba, N., Bah, H. A., Soropogui, B., Doré, A., Kourouma, F., … Yasuda, J. (2016). Deployment of a Reverse Transcription Loop-Mediated Isothermal Amplification Test for Ebola Virus Surveillance in Remote Areas in Guinea. *Journal of Infectious Diseases*, *214*(suppl 3), S229–S233. http://doi.org/10.1093/infdis/jiw255

Lee, S. H., Kang, H. J., Lee, Y. H., Lee, T. J., Han, K., Choi, Y., & Park, H. D. (2012). Monitoring bacterial community structure and variability in time scale in full-scale anaerobic digesters. *J Environ Monit*, *14*(7), 1893–1905.

Leitao, R. C., van Haandel, A. C., Zeeman, G., & Lettinga, G. (2006). The effects of operational and environmental variations on anaerobic wastewater treatment systems: A review. *Bioresource Technology*, *97*(9), 1105–1118. http://doi.org/http://dx.doi.org/10.1016/j.biortech.2004.12.007

532 Måren, I. E., Kapfer, J., Aarrestad, P. A., Grytnes, J.-A., & Vandvik, V. (2018). Changing
533 contributions of stochastic and deterministic processes in community assembly over a
534 successional gradient. *Ecology*, *99*(1), 148–157. http://doi.org/10.1002/ecy.2052

535 McDonald, D., Price, M. N., Goodrich, J., Nawrocki, E. P., DeSantis, T. Z., Probst, A., …
536 Hugenholtz, P. (2012). An improved Greengenes taxonomy with explicit ranks for
537 ecological and evolutionary analyses of bacteria and archaea. *The ISME Journal*, *6*(3),
538 610–618. http://doi.org/10.1038/ismej.2011.139

539 McMahon, K. D., Martin, H. G., & Hugenholtz, P. (2007). Integrating ecology into
540 biotechnology. *Energy Biotechnology / Environmental Biotechnology*, *18*(3), 287–292.

541 McMahon, K. D., Zheng, D., Stams, A. J. M., Mackie, R. I., & Raskin, L. (2004). Microbial
542 population dynamics during startup and overload conditions of anaerobic digesters
543 treating municipal solid waste and sewage sludge. *Biotechnology and Bioengineering*,
544 *7*, 823–834.

545 Merlino, G., Rizzi, A., Villa, F., Sorlini, C., Brambilla, M., Navarotto, P., … Daffonchio, D.
546 (2012). Shifts of microbial community structure during anaerobic digestion of agro-
547 industrial energetic crops and food industry byproducts. *Journal of Chemical*
548 *Technology & Biotechnology*, *87*(9), 1302–1311. http://doi.org/10.1002/jctb.3784

549 Nelson, M. C., Morrison, M., & Yu, Z. (2011). A meta-analysis of the microbial diversity
550 observed in anaerobic digesters. *Bioresource Technology*, *102*(4), 3730–9.
551 http://doi.org/10.1016/j.biortech.2010.11.119

552 Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., & Hase, T.

(2000). Loop-mediated isothermal amplification of DNA. *Nucleic Acids Research*, *28*(12), E63. http://doi.org/10.1093/nar/28.12.e63

Oksanen, J. (2013). Multivariate Analysis of Ecological Communities in R: vegan tutorial. Retrieved April 20, 2015, from http://cc.oulu.fi/~jarioksa/opetus/metodi/vegantutor.pdf

Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., Hara, R. B. O., … Wagner, H. (2015). Vegan: Community Ecology Package. R package version 2.3-2. https://CRAN.R-project.org/package=vegan. http://doi.org/10.4135/9781412971874.n145

Pankhurst, L. J., Whitby, C., Pawlett, M., Larcombe, L. D., McKew, B., Deacon, L. J., … Coulon, F. (2012). Temporal and spatial changes in the microbial bioaerosol communities in green-waste composting. *FEMS Microbiology Ecology*, *79*(1), 229–239. http://doi.org/10.1111/j.1574-6941.2011.01210.x

Paulson, J. N., Stine, O. C., Bravo, H. C., & Pop, M. (2013). Differential abundance analysis for microbial marker-gene surveys. *Nature Methods*, *10*(12), 1200–2. http://doi.org/10.1038/nmeth.2658

Quince, C., Lanzen, A., Davenport, R. J., & Turnbaugh, P. J. (2011). Removing noise from pyrosequenced amplicons. *BMC Bioinformatics*, *12*(1), 38. http://doi.org/10.1186/1471-2105-12-38

R Development Core Team. (2015). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org.

574 Ramšak, A., Peterka, M., Tajima, K., Martin, J. C., Wood, J., Johnston, M. E. A., … Avguštin, G.
575 (2000). Unravelling the genetic diversity of ruminal bacteria belonging to the CFB
576 phylum. *FEMS Microbiology Ecology*, *33*(1), 69–79. http://doi.org/10.1111/j.1574-
577 6941.2000.tb00728.x

578 Rittmann, B. E., Hausner, M., Löffler, F., Muyzer, G., Okabe, S., DB, O., … Wagner. (2006). A
579 vista for microbial ecology and environmental biotechnology. *Environ Sci Technol*,
580 *40*(4), 1096–1103.

581 Rivière, D., Desvignes, V., Pelletier, E., Chaussonnerie, S., Guermazi, S., Weissenbach, J., …
582 Sghir, A. (2009). Towards the definition of a core of microorganisms involved in
583 anaerobic digestion of sludge. *The ISME Journal*, *3*(6), 700–14.
584 http://doi.org/10.1038/ismej.2009.2

585 Schauer-Gimenez, A. E., Zitomer, D. H., Maki, J. S., & Struble, C. A. (2010). Bioaugmentation
586 for improved recovery of anaerobic digesters after toxicant exposure. *Water Research*,
587 *44*(12), 3555–3564.

588 Schink, B. (2002). Synergistic interactions in the microbial world. *Antonie van Leeuwenhoek*,
589 *81*(1/4), 257–261. http://doi.org/10.1023/A:1020579004534

590 Schlüter, A., Bekel, T., Diaz, N. N., Dondrup, M., Eichenlaub, R., Gartemann, K.-H., …
591 Goesmann, A. (2008). The metagenome of a biogas-producing microbial community of
592 a production-scale biogas plant fermenter analysed by the 454-pyrosequencing
593 technology. *Journal of Biotechnology*, *136*(1–2), 77–90.

594 Schwarzenauer, T., & Illmer, P. (2012). PLFA profiles for microbial community monitoring in

595 anaerobic digestion. *Folia Microbiologica*, *57*(4), 331–333.
596 http://doi.org/10.1007/s12223-012-0136-3

597 Stams, A. J. M., & Plugge, C. M. (2009). Electron transfer in syntrophic communities of
598 anaerobic bacteria and archaea. *Nat Rev Micro*, *7*(8), 568–577.

599 Stroot, P. G., McMahon, K. D., Mackie, R. I., & Raskin, L. (2001). Anaerobic codigestion of
600 municipal solid waste and biosolids under various mixing conditions 1. Digester
601 performance. *Water Research*, *35*(7), 1804–1816.

602 Tale, V. P., Maki, J. S., Struble, C. A., & Zitomer, D. H. (2011). Methanogen community
603 structure-activity relationship and bioaugmentation of overloaded anaerobic digesters.
604 *Water Research*, *45*(16), 5249–5256.
605 http://doi.org/http://dx.doi.org/10.1016/j.watres.2011.07.035

606 Tian, Z., Cabrol, L., Ruiz-Filippi, G., & Pullammanappallil, P. (2014). Microbial ecology in
607 anaerobic digestion at agitated and non-agitated conditions. *PloS One*, *9*(10), e109769.
608 http://doi.org/10.1371/journal.pone.0109769

609 Valentin-Vargas, A., Toro-Labrador, G., & Massol-Deya, A. A. (2012). Bacterial Community
610 Dynamics in Full-Scale Activated Sludge Bioreactors: Operational and Ecological Factors
611 Driving Community Assembly and Performance. *PLoS ONE*, *7*(8), e42524.

612 Vanwonterghem, I., Jensen, P. D., Dennis, P. G., Hugenholtz, P., Rabaey, K., & Tyson, G. W.
613 (2014). Deterministic processes guide long-term synchronised population dynamics in
614 replicate anaerobic digesters. *The ISME Journal*, *8*(10), 2015–28.
615 http://doi.org/10.1038/ismej.2014.50

Vanwonterghem, I., Jensen, P. D., Ho, D. P., Batstone, D. J., & Tyson, G. W. (2014). Linking microbial community structure, interactions and function in anaerobic digesters using new molecular techniques. *Current Opinion in Biotechnology*, *27*, 55–64. http://doi.org/10.1016/j.copbio.2013.11.004

Vanwonterghem, I., Jensen, P. D., Rabaey, K., & Tyson, G. W. (2015). Temperature and solids retention time control microbial population dynamics and volatile fatty acid production in replicated anaerobic digesters. *Scientific Reports*, *5*, 8496. http://doi.org/10.1038/srep08496

Vavilin, V., Qu, X., Mazéas, L., Lemunier, M., Duquennoi, C., He, P., & Bouchez, T. (2008). Methanosarcina as the dominant aceticlastic methanogens during mesophilic anaerobic digestion of putrescible waste. *Antonie van Leeuwenhoek*, *94*(4), 593–605. http://doi.org/10.1007/s10482-008-9279-2

Wang, Q., Garrity, G. M., Tiedje, J. M., & Cole, J. R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology*, *73*(16), 5261–7. http://doi.org/10.1128/AEM.00062-07

Wang, X., Wen, X., Yan, H., Ding, K., Zhao, F., & Hu, M. (2011). Bacterial community dynamics in a functionally stable pilot-scale wastewater treatment plant. *Bioresource Technology*, *102*(3), 2352–2357.

Wells, G. F., Park, H. D., Eggleston, B., Francis, C. A., & Criddle, C. S. (2011). Fine-scale bacterial community dynamics and the taxa-time relationship within a full-scale activated sludge bioreactor. *Water Res*, *45*(17), 5476–5488.

637 Werner, J. J., Knights, D., Garcia, M. L., Scalfone, N. B., Smith, S., Yarasheski, K., … Angenent,
638 L. T. (2012). Bacterial community structures are unique and resilient in full-scale
639 bioenergy systems. *Proceedings of the National Academy of Sciences of the United*
640 *States of America*, *108*(10), 4158–4163.

641 Werner, J. J., Koren, O., Hugenholtz, P., DeSantis, T. Z., Walters, W. A., Caporaso, J. G., … Ley,
642 R. E. (2012). Impact of training sets on classification of high-throughput bacterial 16s
643 rRNA gene surveys. *The ISME Journal*, *6*(1), 94–103.
644 http://doi.org/10.1038/ismej.2011.82

645 Westerholm, M., Leven, L., & Schnuer, A. (2012). Bioaugmentation of Syntrophic Acetate-
646 Oxidizing Culture in Biogas Reactors Exposed to Increasing Levels of Ammonia. *Applied*
647 *and Environmental Microbiology*, *78*(21), 7619–7625.
648 http://doi.org/10.1128/aem.01637-12

649 Whitford, M. F., Forster, R. J., Beard, C. E., Gong, J., & Teather, R. M. (1998). Phylogenetic
650 Analysis of Rumen Bacteria by Comparative Sequence Analysis of Cloned 16S rRNA
651 Genes. *Anaerobe*, *4*(3), 153–163.
652 http://doi.org/http://dx.doi.org/10.1006/anae.1998.0155

653 Wittebolle, L., Marzorati, M., Clement, L., Balloi, A., Daffonchio, D., Heylen, K., … Boon, N.
654 (2009). Initial community evenness favours functionality under selective stress. *Nature*,
655 *458*(7238), 623–626.

656

Table 1. Summary of feed and seed stock composition. Triplicate average error bars show standard deviation.

Characteristic	Unit	Seed	Primary Sludge	Co-digestion		
Co-digestion substrate				Glycerol waste + PS		FOGs waste + PS
Co-digestant concentration	g l^{-1}			30	50	1.5
pH		7.73 ± 0.005	7.09 ± 0	7.40 ± 0.04	7.43 ± 0.03	7.53 ± 0.1
TS*	%	4.59 ± 0.46	1.46 ± 0.56	2.34 ± 0.43	2.53 ± 1.65	2.33 ± 1.36
VS**	% of TS	63.17 ± 0.04	65.93 ± 0.13	88.38 ± 2.31	91.66 ± 3.42	97/82 ± 1
sCOD***	g l^{-1}	237 ± 0.65	43.0 ± 1.45	84.46 ± 0.97	115.65 ± 0.62	141.43 ± 3
Alkalinity	g l^{-1} $CaCO_3$	5.5 ± 0.5	2.5 ± 0.7	2.3 ± 0.1	2.4 ± 0.6	2.1 ± 1.2

*TS = total solids, **VS = volatile solids, ***sCOD soluble chemical oxygen demand

667 **Table 2.** Summary of the key physico-chemical parameters of the digesters.

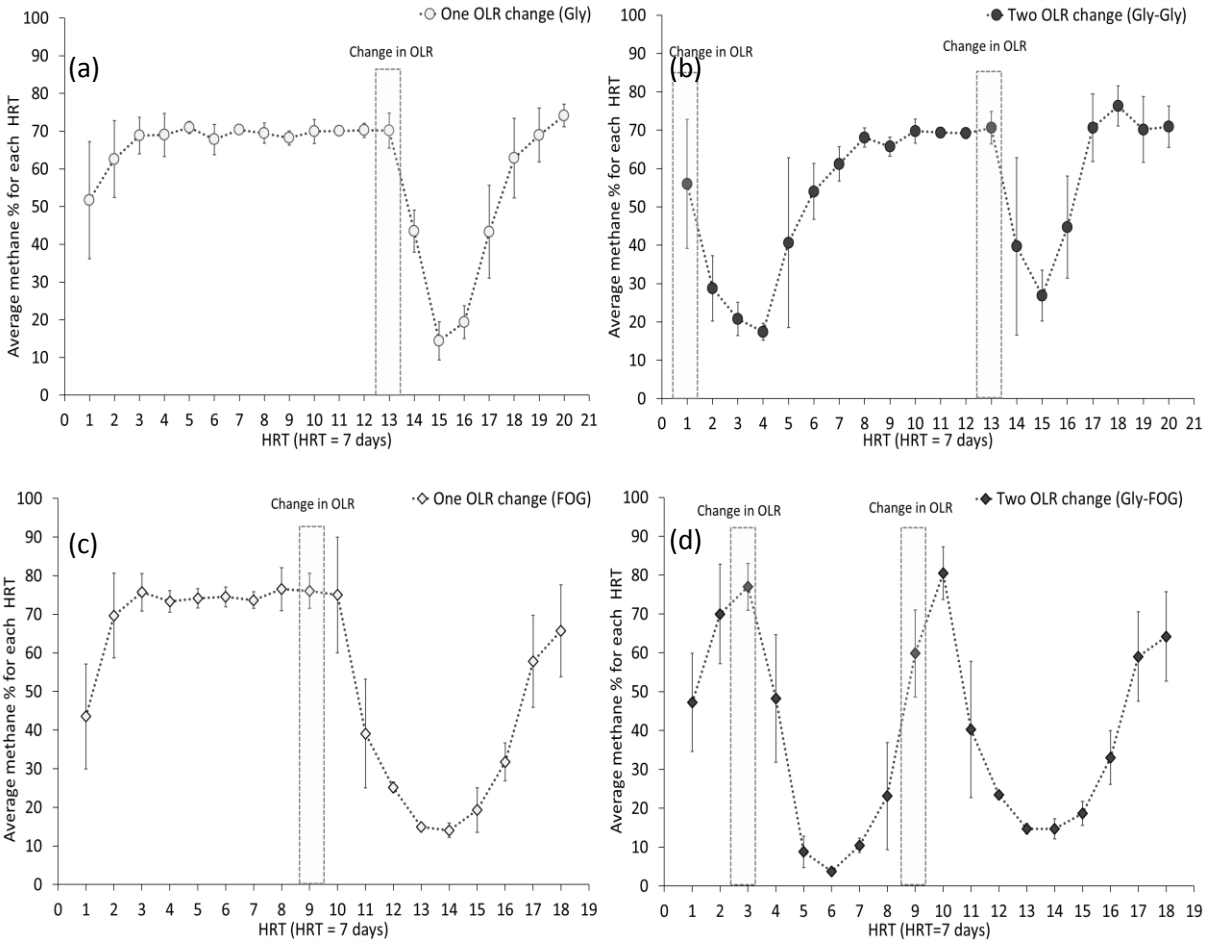
Digester group	No. of samples	CH_4	pH	Alkalinity	Volatile fatty acids						V/A ratio*
					Total	Acetic	Propionic	Butyric		Lactic	
								Iso-	n-		
		%		$gCaCO_3\ l^{-1}$	$g\ l^{-1}$	$g\ l^{-1}$	$g\ l^{-1}$	$g\ l^{-1}$	$g\ l^{-1}$	$g\ l^{-1}$	
1	7	0 – 20	5.8	1	8 ± 2	2 ± 1	4± 1	1.1 ± 2	0.8 ± 2	1.3 ± 2	8 ± 3
2	7	21- 30	5.9	1.1	6.3 ± 1	2.6 ± 1	1.9 ± 1	0.9 ± 1	0.7 ± 0.6	0.7 ± 1	5.6 ± 1
3	3	31- 45	6.5	1.3	6.4 ± 3	2.7 ± 1	1.8 ± 1	1.0 ± 1	0.7 ± 0.4	1.6 ± 1	5.4 ± 0
4	5	46 – 60	6.9	2.2	2.6 ± 2	0.9 ± 1	0.7 ± 1	0.3 ± 1	0.2 ± 0.3	0.1 ± 1	0.9 ± 0
5	9	61- 70	7.2	2.4	1.8 ± 1	0.6 ± 1	0.7 ± 1	0.2 ± 1	0.3 ± 0.3	0.5 ± 0	1.1 ± 0
6	5	> 70	7.3	3.0	2.2 ± 2	0.3 ± 0	0.7 ± 0.9	0.0 ± 0	0.04 ± 0	0.0 ± 0	0.4 ± 0

668 *Ratio of total VFA/total alkalinity

669 **Table 3.** Summary of PLFA and PLEL (mol %) in digesters with varying biogas methane
670 content. Taxonomic affiliations given in supplementary table 1. superscript numbers are
671 standard deviation.

Lipid	**Digester group (% methane)**					
	0 – 20	21 - 30	31 - 45	46 - 60	61 - 70	> 70
11:00	1.7 ± 0.3	1.5 ± 1.4	1.5 ± 1.4	0.8 ± 0.2	0.6 ± 0.4	0.4 ± 0.3
12:00	0 ± 0	0 ± 0.2	0 ± 0.2	0 ± 0	0 ± 0.1	0.1 ± 0
13:00	0.1 ± 0	0.2 ± 0	0.2 ± 0	1 ± 0	0.1 ± 0	0.1 ± 0.1
14:00	3.6 ± 0	3.6 ± 0.2	3.6 ± 0.2	4.2 ± 0	2.8 ± 0.1	2.7 ± 0.1
15:00	1.6 ± 0	1.6 ± 0	1.6 ± 0	1.6 ± 0	1.4 ± 0	1.5 ± 0
16:00	16.8 ± 0	16.3 ± 0	16.3 ± 0	16.7 ± 0	15.8 ± 0	16.8 ± 0.4
17:00	0.6 ± 0.4	0.5 ± 2.3	0.5 ± 2.3	0.6 ± 0.8	0.5 ± 0.7	0.4 ± 0.9
18:00	7.6 ± 0.2	7.7 ± 4	7.7 ± 4	8.8 ± 1.2	10.3 ± 2.5	10.7 ± 3.2
20:00	0.4 ± 0.3	0.4 ± 2.9	0.4 ± 2.9	0.3 ± 1.7	0.4 ± 2.6	0.3 ± 2.9
iso-15:0	9.3 ± 0.1	8.7 ± 0.9	8.7 ± 0.9	9.5 ± 0.2	8.5 ± 0.4	8.4 ± 0.5
a-15:0	8.5 ± 0	7.9 ± 0.7	7.9 ± 0.7	9.8 ± 0.4	8.7 ± 0.6	8.5 ± 0.6
iso-16:0	1.8 ± 0	2.2 ± 2.4	2.2 ± 2.4	1.2 ± 0.4	1 ± 0.5	1.4 ± 1.1
iso-17:1	1 ± 0.4	0.9 ± 0.7	0.9 ± 0.7	1 ± 2.9	1.2 ± 4.5	0.9 ± 4
cyc-17:0	0.2 ± 0	0.2 ± 4.3	0.2 ± 4.3	0 ± 3.1	0.4 ± 5.1	0.4 ± 3.3
cyc-19:0	0.4 ± 0	0.4 ± 0.5	0.4 ± 0.5	0.4 ± 0.2	0.3 ± 1	0.3 ± 0.3
16:1 w7cis	14.2 ± 0	12 ± 0	12 ± 0	16.3 ± 0	14.5 ± 0.3	14.1 ± 0.3
18:2 w6cis	15.3 ± 0	14.7 ± 0.3	14.7 ± 0.3	12.5 ± 0.1	12.9 ± 0.2	13.1 ± 0.2
18:1 w9cis	5.4 ± 0	5.5 ± 0.4	5.5 ± 0.4	4.1 ± 0	2.4 ± 0.5	2.2 ± 0.6
18:1w9trans	6.9 ± 4.3	9.8 ± 0.6	9.8 ± 0.6	10.8 ± 0.3	12.8 ± 1.7	12.9 ± 1.3
18:2w6,9	4 ± 0.2	3.8 ± 3.2	3.8 ± 3.2	0 ± 1.9	4.1 ± 2.9	3.5 ± 4.2
2OH-10:0	0 ± 1.7	0.1 ± 3.2	0.1 ± 3.2	0 ± 3.2	0 ± 3.4	0 ± 3.4
2OH-12:0	0 ± 0.6	0 ± 1.8	0 ± 1.8	0 ± 1.9	0.1 ± 2	0.1 ± 2.9
3OH-12:0	0 ± 0	0 ± 0.2	0 ± 0.2	0 ± 0.1	0 ± 0.1	0.2 ± 0.1
i20:1	33.5 ± 10.1	21.8 ± 17.3	21.8 ± 17.3	21.2 ± 11.9	34.5 ± 13.2	26.8 ± 12.1
i20:0	40.3 ± 20.6	60.2 ± 25.4	60.2 ± 25.4	34.3 ± 27.2	48.7 ± 15.6	47.6 ± 22.5
i40:0	26.1 ± 15.6	18.1 ± 16.5	18.1 ± 16.5	44.5 ± 29.9	16.8 ± 10	25.5 ± 20.3

672

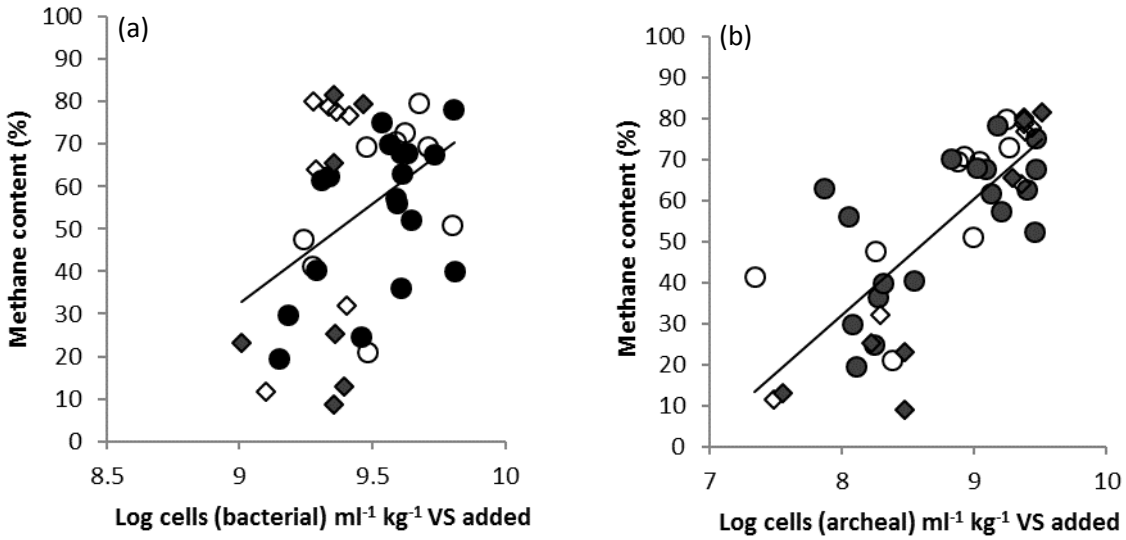


673

674 **Figure 1** Methane evolution over the four experimental settings. Reactors were fed at OLR
675 1.4 kg VS m^{-3} d^{-1} during stable periods with primary sludge (PS). OLR was increased to 2.9 kg
676 VS m^{-3} $d^{-}1$ for a whole HRT with different co-digestant at different times: (a) one OLR
677 increase with glycerol; (b) two OLR increase with glycerol; (c) one OLR increase with FOG; (d)
678 two OLR increase first with glycerol and then with FOG.

679

680



681

682 **Figure 2** Scatter plot of bacterial biomass (left panel) and archaeal biomass (right) against
683 methane content in all conditions tested. White circles: one OLR change (Gly); black circles:
684 two OLR change (Gly-Gly); white diamonds: one OLR change (FOG); black diamonds: two
685 OLR change (Gly-FOG). The solid line represents the linear regressions both are significant at
686 $P < 0.01$ and with R^2 of 0.6 for archaea and 0.2 for bacteria.

687

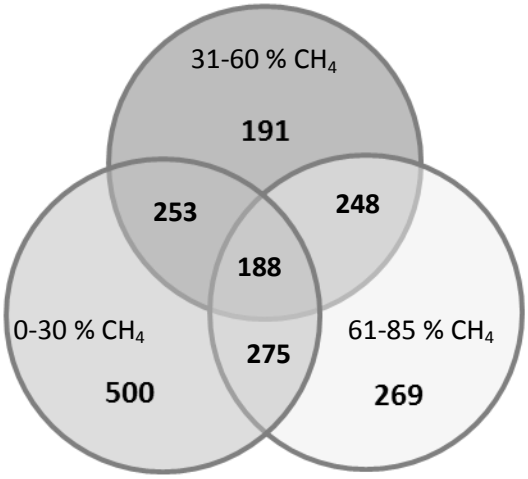
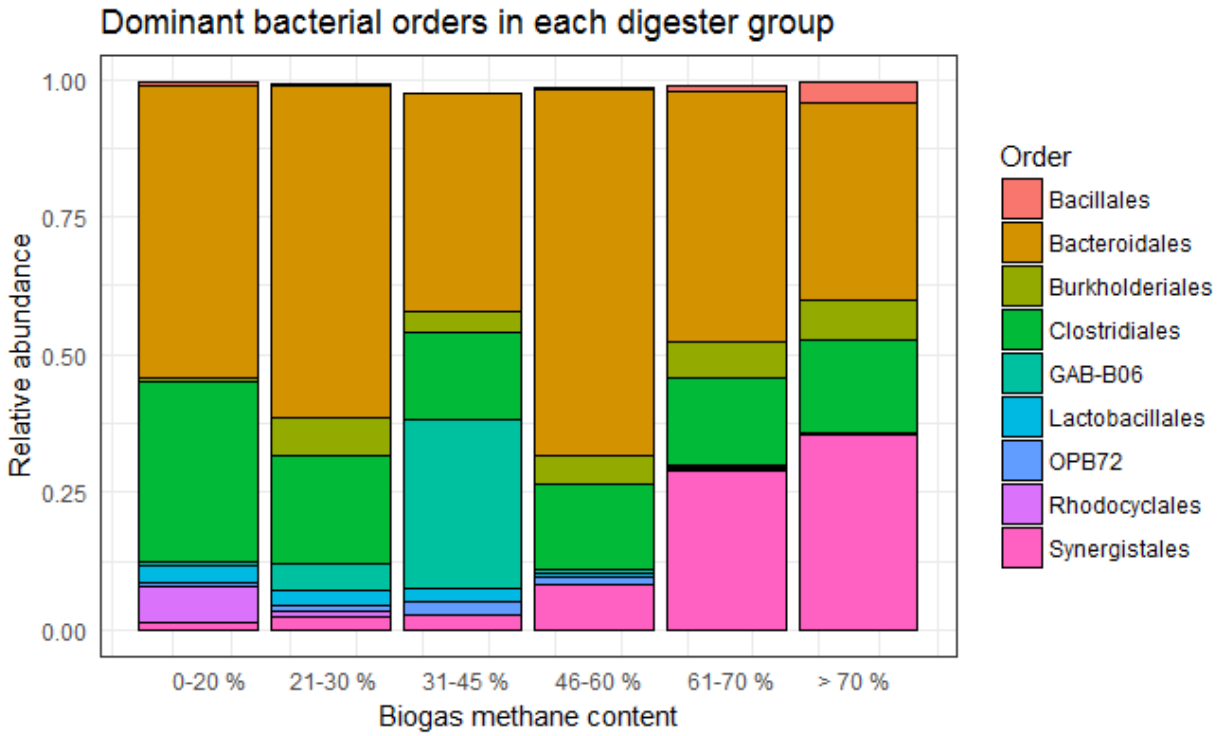


Figure 3. Venn Diagram showing number of unique and shared OTUs in digesters with 0-30 %, 31-60 % and 61-85 % methane content.



706

707 **Figure 4.** Relative proportions of the dominant orders based on the number of sequences
708 assigned to that taxonomic group.

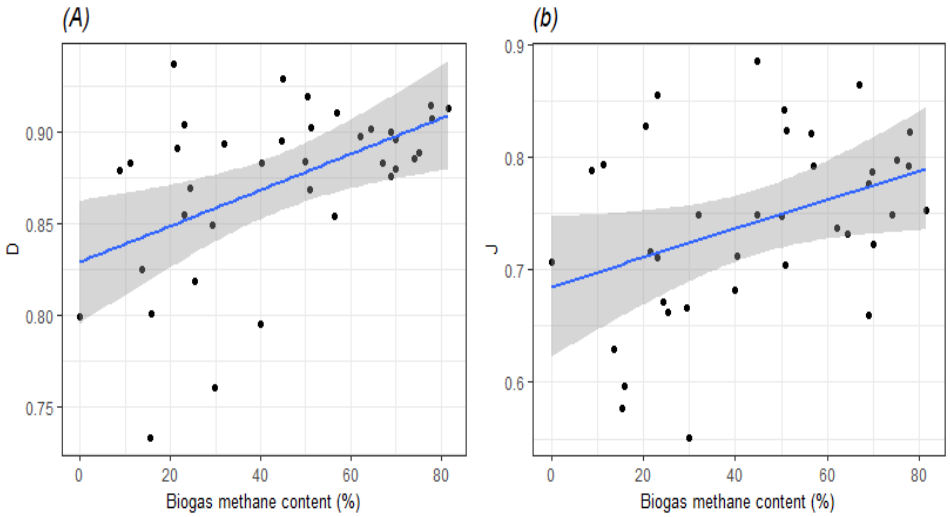
709

710

711

712

713

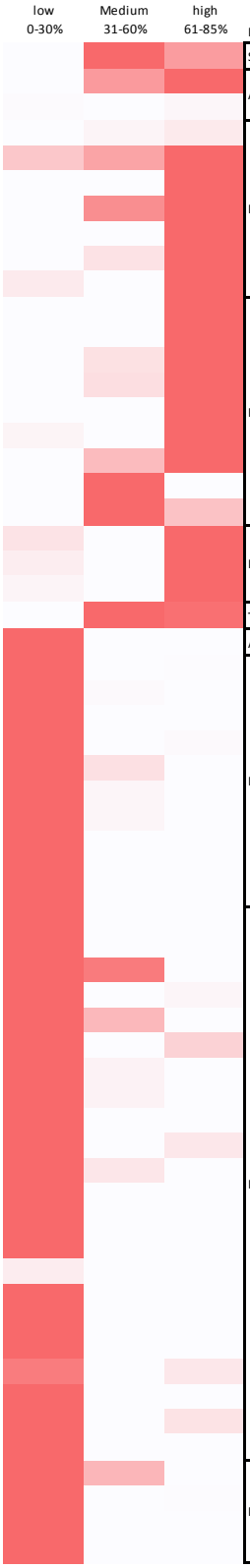


714

715 **Figure 5.** Scatter plot of Simpsons diversity index (a) and Pielou's evenness index (b) against

716 % methane. (Blue lines represent linear regression between data points and shaded area are

717 95 % confidence intervals of the predicted model).



P value	low 0-30%	Medium 31-60%	high 61-85%	Phylum	Class	Order	Family	Genus
0.026				Synergistetes	Synergistia	Synergistales	Synergistaceae	*Aminobacterium*
0.034				Armatimonadetes	Armatimonadetes gp2	Formerly phylum OP10		
0.019								
0.032				Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidales incertae sedis	*Phocaeicola*
0.013							Porphyromonadaceae	*Petrimonas*
0.037								
0.018								*Proteiniphilum*
0.037								
0.004								
0.041					Cytophagia	Cytophagales	Flammeovirgaceae	*Aureibacter*
0.037				Firmicutes	Clostridia	Clostridiales	Clostridiaceae	*Anaerovirgula*
0.037							Clostridiales Incertae SedisXIII	*Anaerovorax*
0.003								*Fervidicola*
0.001							Clostridiales IncertaesedisXI	*Sedimentibacter*
0.037								*Tepidimicrobium*
0.001							Gracilibacteraceae	*Lutispora*
0.046							Ruminococcaceae	*Acetanaerobacterium*
0.024								*Saccharofermentans*
0.019							Syntrophomonadaceae	*Pelospora*
0.046				Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	*Malikia*
0.043					Deltaproteobacteria	Myxococcales	Sorangiineae	*Polyangiaceae*
0.033					Gammaproteobacteria	Chromatiales	Halothiobacillaceae	*Thiofaba*
0.037				Thermotogae	Thermotogae	Thermotogales	Thermotogales incertae sedis	*Oceanotoga*
0.028				Actinobacteria	Coriobacteridae	Coriobacteriales	Coriobacterineae	*Olsenella*
0.034				Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	*Butyricimonas*
0.035								*Paludibacter*
0.028								*Parabacteroides*
0.035								
0.017							Prevotellaceae	*Hallella*
0.007								*Prevotella*
0.007								
0.010								*Xylanibacter*
0.028								
0.010								
0.028				Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*
0.028					Clostridia	Clostridiales	Clostridiaceae	*Geosporobacter*
0.018								
0.015								*Oxobacter*
0.013								*Proteiniclasticum*
0.049								
0.008							Lachnospiraceae	*Sporobacterium*
0.008								
0.028							Ruminococcaceae	*Anaerotruncus*
0.037								
0.004								*Butyricicoccus*
0.028								
0.010								
0.010								
0.028								*Faecalibacterium*
0.028								
0.010								
0.010								*Oscillibacter*
0.016								
0.028								
0.007					Negativicutes	Selenomonadales	Acidaminococcaceae	*Phascolarctobacterium*
0.037							Veillonellaceae	*Selenomonas*
0.016				Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	*Acidovorax*
0.034						Rhodocyclales	Rhodocyclaceae	*Azospira*
0.028					Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*
0.028								

Figure 6. Relative number of sequences assigned to dominant OTUs that varied significantly according to methane content of the digester groups. (Darker grey indicates relative increase in numbers within that OTU).