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1 # Can microbial ecology help improve biogas production in AD?

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8 Running Head: Microbial ecology in AD

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11

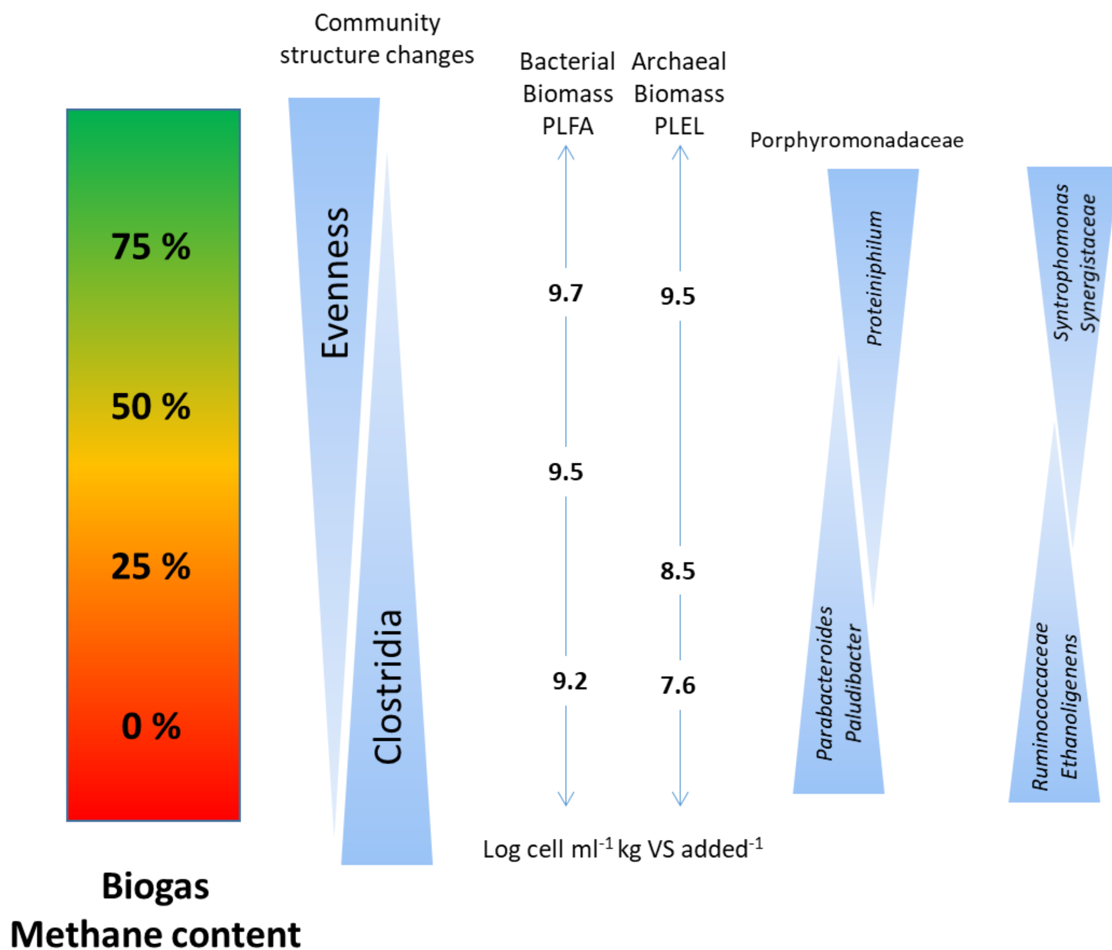
12 Keywords: FOGs, Glycerol, Synergistaceae, Ruminococcaceae, Veillonellaceae, Next-

13 generation sequencing.

14

15 **Highlights**

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



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

45

46

47 **1. Introduction**

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

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

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

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

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

95 **2. Methods**

96 **2.1 Digester operational parameters**

97 Laboratory-scale semi-continuous digesters consisted of 1-L borosilicate glass bottles with a
98 700 ml working volume and 5-L bottles with a 4.5-L working volume maintained at 38 °C
99 using a water bath. All reactors were seeded with digested sludge from a commercial
100 Sewage Treatment digester (in a ratio of 30:70 %) and fed with autoclaved primary sludge
101 three times a week to achieve a retention time of 7 days and an organic loading rate (OLR)
102 of 1.4 kg VS m^{-3} d^{-1}. A different organic waste (glycerol or fat rich – FOG waste collected
103 from a restaurant grease trap) was used to induce periods of unstable performance in the
104 digesters (see table 1 for details of feedstocks). Glycerol or FOG was added to the
105 autoclaved primary sludge to increase the OLR from 1.4 kg VS m^{-3} d^{-1} to 2.9 for one hydraulic
106 retention time (HRT = 7 days) and then returned to 1.4 kg VS m^{-3} d^{-1}. These OLRs were
107 selected as they were known to cause digester failure based on our preliminary work. All the
108 reactors were run for more than 130 days (18-20 HRT) depending on the substrate. The
109 effects of one or two sequential changes in OLR were investigated using the same feedstock
110 (glycerol - glycerol) or with a different feedstock (glycerol - FOG waste). Feedstock and
111 feeding regimes are those reported previously (Ferguson et al. 2016).

112 **2.2. Biogas production, methane concentration and physicochemical characterisation**

113 Gas production was measured daily by water displacement in a glass column (150 x 5 cm)
114 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

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

138 For PLEL another aliquot of the phospholipids fraction, equivalent to 40 g of the digestate
139 was used for PLEL analysis according to the method described by Gattinger, et al. (2003).
140 The dried ether-linked isoprenoids were reconstituted in 0.2 ml of hexane and analysed by
141 gas chromatography coupled to mass spectrometry (GCMS Agilent Technologies 6890N)
142 according to the operating conditions described by Gattinger, et al. (2003). Nonadecanoic
143 acid methyl ester (Sigma, UK) was added as an internal standard to each sample after SPE.
144 The taxonomic affiliations are summarised in TS1. Gram-positive bacteria were represented
145 by the series of iso and anteiso branched saturated PLFA. Gram-negative bacteria were
146 represented by cyclopropane, hydroxyl and monounsaturated PLFA. The 16:0 straight chain
147 PLFA has been previously demonstrated as an ubiquitous bacterial marker (Piotrowska-
148 Seget and Mrozik 2003). The PLFA 18:2w9cis and 18:1w7trans used as markers for clostridia.
149 The PLEL i20:0 was used as a marker for the *Euryarchaeota*, i20:1 as a marker of the
150 aceticlastic methanogens belonging to *Methanosarcina* and i40:0 as a marker for
151 hydrogenotrophic methanogens belonging to *Methanobacterium*, *Methanococcus*,
152 *Methanopyrus*, and *Methanothermus* (Gattinger et al. 2002).

153 **2.5. 454-pyrosequencing analysis and Bioinformatics**

154 The microbial diversity and dynamics of the digesters was investigated by extracting total
155 genomic DNA from 200 mg wet weight digestate samples using a MoBio Power Soil kit (MO
156 BIO Laboratories, Inc, UK). Samples were then processed for NGS by 454-Pyrosequencing on
157 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

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

183 **2.6. Statistical analysis**

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

198 **3. Results**

199 **3.1. Reactors performances: VFAs, alkalinity, biogas and methane production**

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

203 periods of low methane production following an increase in OLR. At times of 'balanced'
204 anaerobic performance, when the OLR was maintained at 1.4 kg VS m^{-3} $d^{-}1$, biogas
205 production was around 0.28 m^3 kg^{-1} VS day $^{-}1$ and methane content around 75 %. When
206 organic loading rate was increased to 2.9 kg VS m^{-3} d^{-1} biogas production dropped to around
207 1 m^3 kg^{-1} VS day $^{-}1$ and methane content was lower than 30 % for a period of one or more
208 HRTs, depending on the digester history. For example, reactors that received two OLR
209 change with the same feedstock (Gly-Gly; Figure 1b) returned to 'balanced' conditions much
210 faster after the second change (3HRTs vs 4HRTs; Figure 1a and 1b). VFA content, alkalinity,
211 pH and VFA/alkalinity ratio changed as expected with changes in methane content (table 2).

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

216 **3.2. Microbial lipid fingerprinting (PLFA and PLEL)**

217 Many of the bacterial lipid markers (PLFA) were associated to *Actinobacteria*, low GC Gram
218 positive bacteria, CFB, *δ-Proteobacteria*, *Bacillus*, and *Clostridia* (table 3). There were
219 relatively little changes in the contribution of many of the individual PLFAs to the total
220 fingerprint at different biogas methane content. The only PLFAs that varied between the
221 digester groups were the PLFA18:1w9cis and 18:1w9trans, used as marker for clostridia,
222 (Table 3). The trans oleic acid (18:1w9trans) doubled in concentration when methane
223 content was ≥ 60 % in comparison to digesters with a methane content ≤ 20 %. In contrast,
224 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

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

271 Rivière et al. 2009).

272 **3.3.1 Alpha diversity.** Bacterial OTU richness was 134 ± 83 across all samples, however

273 there was no significant difference in OTU richness between the digester performance

274 groups (ANOVA, $F_{5,31}$ = 0.95, P = 0.5). Shannon-weaver index (*H'*) did slightly increase from

275 2.6 ± 0.3 to 2.8 ± 0.1 between group 1 (0-20 % methane) and 6 (> 70 % methane) however,

276 this was not significant (ANOVA, $F_{5,31}$ = 0.99, P = 0.43). In contrast there were significant

277 increases for "*D*" Simpsons index (ANOVA, $F_{5,31}$ = 2.3, P = 0.006) and "*J*" Pielou's evenness

278 (ANOVA, $F_{5,31}$ = 1.7, P = 0.02) with increased biogas methane content across the digester

279 groups. This is clearly shown in Figure 5 and it indicates that the better performing digesters

280 (in terms of biogas production and methane content) had more even communities. A

281 number of studies have reported that improved digester performance was related with

282 higher community evenness (Wittebolle et al. 2009; Merlino et al. 2012; Werner et al.

283 2012). Community evenness is particularly important in a system such as AD, as it signposts

284 equitable distribution between the various AD functional groups; this enables the

285 community to fully exploit all metabolic pathways, as well as the co-metabolic pathways,

286 which are known to play an important in AD performance (Hashsham et al., 2000). Further

287 to this, communities with uneven distributions of diversity tend to be dominated by groups

288 of microorganisms specialised to the current conditions, when exposed to external changes

289 (e.g. pH) they are unable to adapt to rapidly and require long recovery times.

290 **3.3.2 Beta Diversity.** Links between the bacterial community structure and physiochemical

291 parameters were further demonstrated by PERMANOVA. The results showed a significant

292 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

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

395 **4.1.2. Bioaugmentation and AD optimisation.** The core groups of bacteria specific to
396 particular levels of performance revealed a number of unique OTUs in digesters with high
397 biogas methane content (Figure 4 and 6). This information can further contribute to AD
398 optimisation via bioaugmentation. Although it has been demonstrated in principle (Enright
399 et al. 2009; Guo et al. nd; Schauer-Gimenez et al. 2010; Tale et al. 2011; Westerholm et al.
400 2012), bioaugmentation is logistically challenging; the bacteria need to be isolated and
401 cultivated in sufficient quantity and finally there is no guarantee that the community will
402 take hold in the digester. As an alternative it has previously been shown that changes in
403 digester performance can be used to optimise the community to improve recovery from
404 process imbalance (Goux et al. 2015; McMahon et al. 2004; McMahon et al. 2007; Stroot et

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

409 **5. Conclusions**

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

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

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657 **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

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

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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

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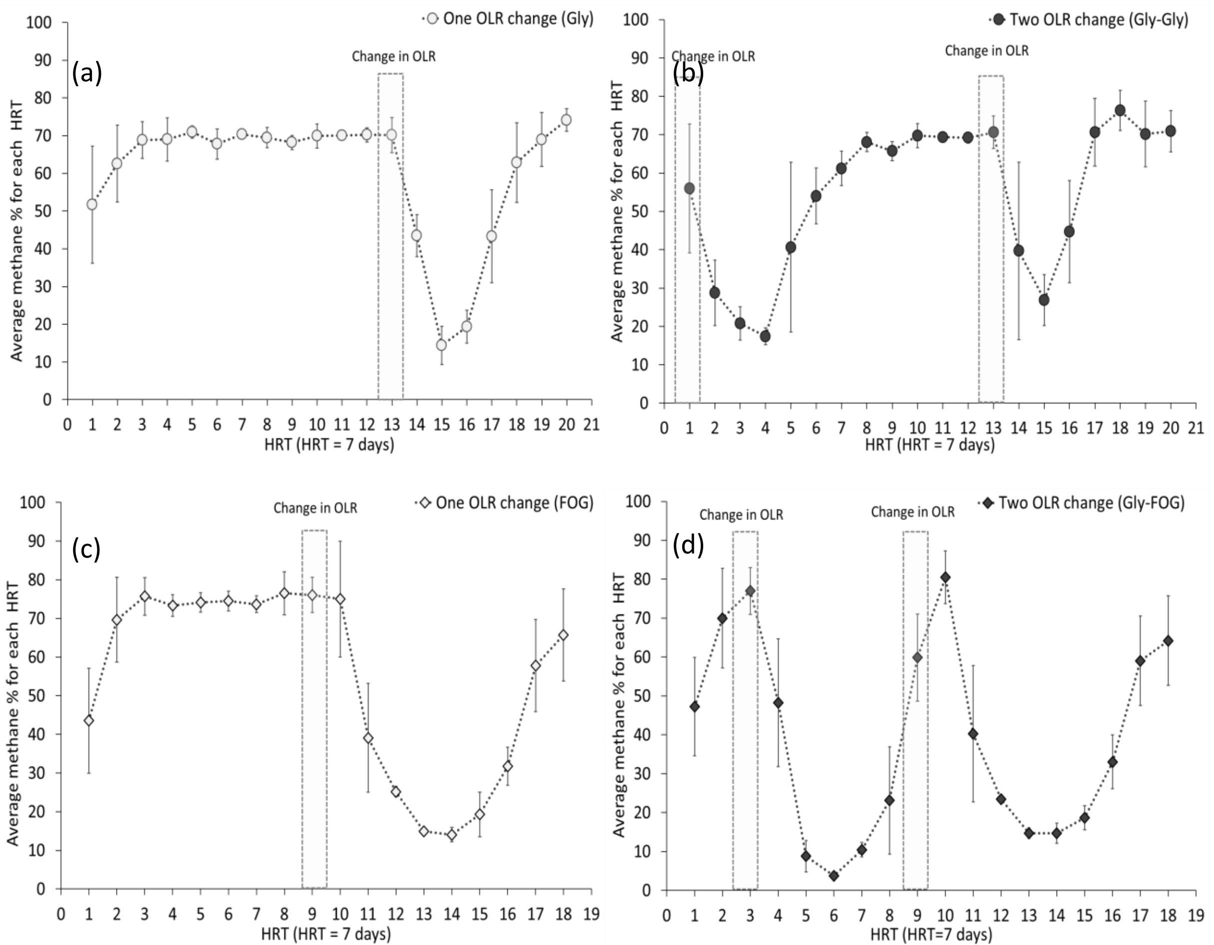

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674 **Figure 1** Methane evolution over the four experimental settings. Reactors were fed at OLR
675 1.4 kg VS m^{-3} d^{-1} during stable periods with primary sludge (PS). OLR was increased to 2.9 kg
676 VS m^{-3} $d^{-}1$ for a whole HRT with different 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.

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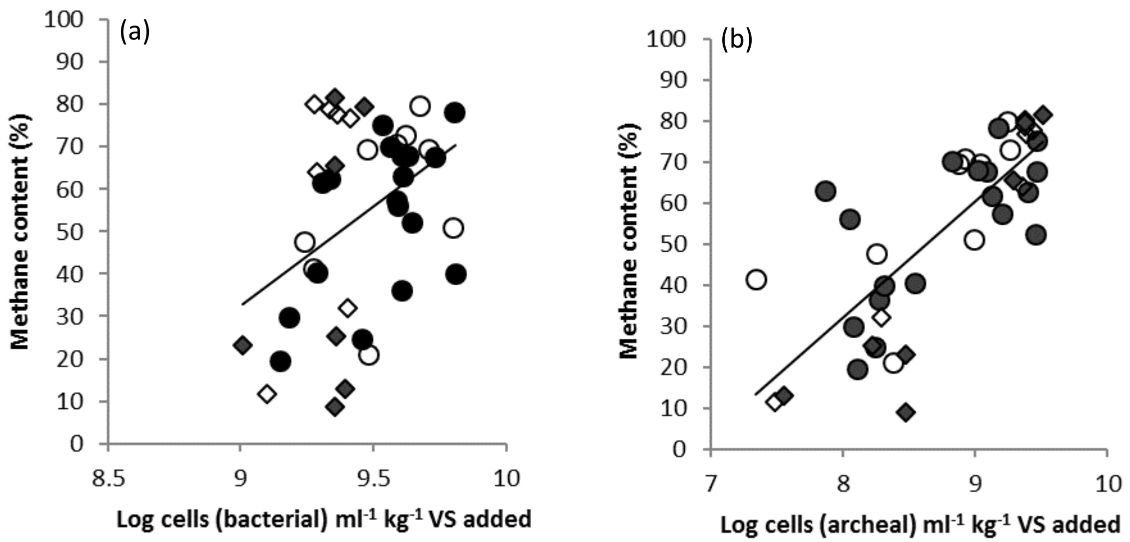

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682 **Figure 2** Scatter plot of bacterial biomass (left panel) and archaeal biomass (right) against

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.

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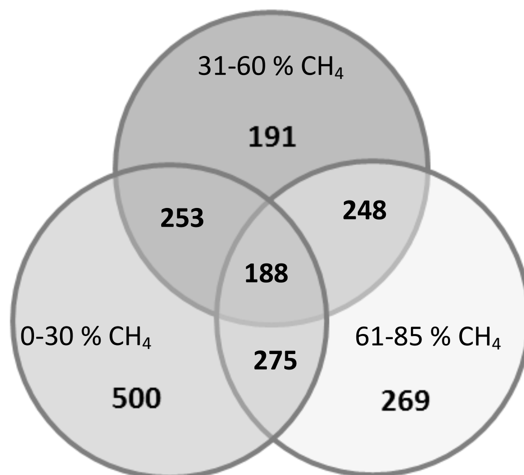

698 **Figure 3.** Venn Diagram showing number of unique and shared OTUs in digesters with 0-30

699 %, 31-60 % and 61-85 % methane content.

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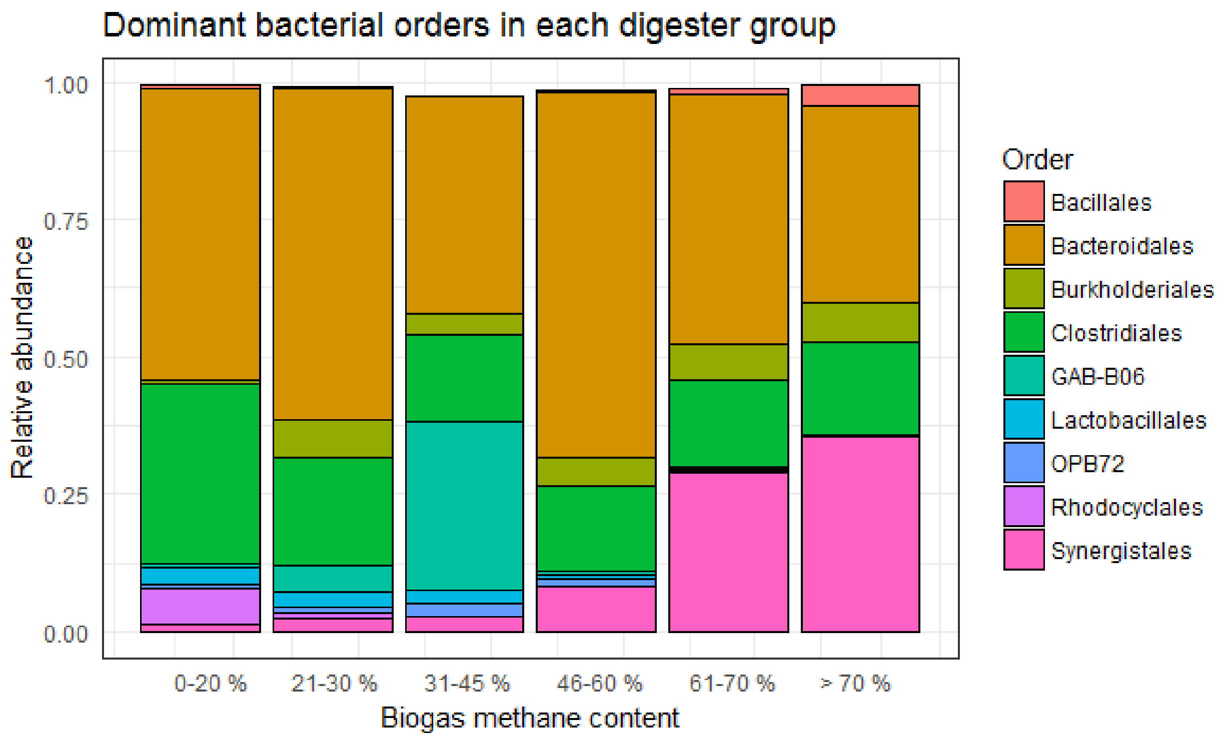

707 **Figure 4.** Relative proportions of the dominant orders based on the number of sequences
708 assigned to that taxonomic group.

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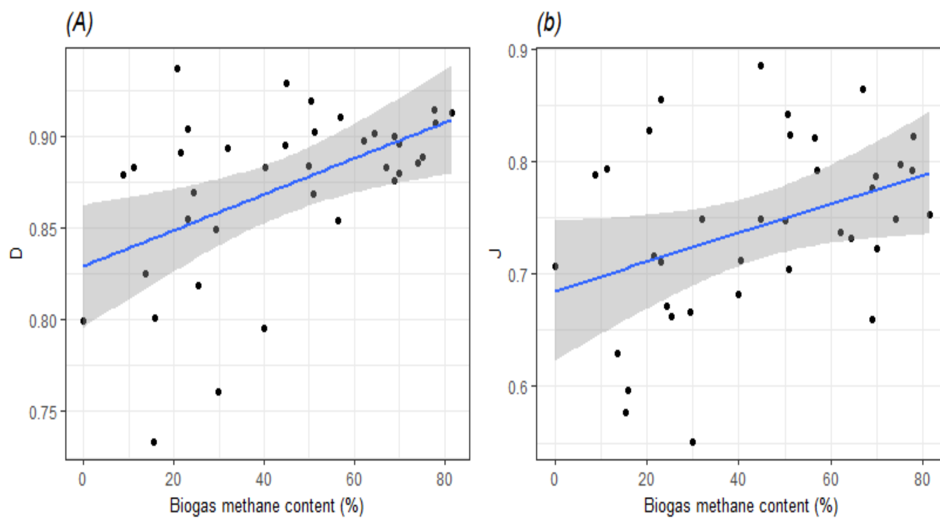

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715 **Figure 5.** Scatter plot of Simpsons diversity index (a) and Pielou's evenness index (b) against

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

717 95 % confidence intervals of the predicted model).

P value	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					
0.037					*Proteiniphilum*
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					
0.028					*Faecalibacterium*
0.010					*Oscillibacter*
0.010					
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					

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