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1 **Dynamic changes in anaerobic digester metabolic pathways and microbial populations**  
2 **during acclimatisation to increasing ammonium concentrations**

3

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5

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12

13 **Abstract**

14 Transitions in microbial community structure in response to increasing ammonia  
15 concentrations were determined by monitoring mesophilic anaerobic digesters seeded with a  
16 predominantly acetoclastic methanogenic community from a sewage sludge digester.

17 Ammonia concentration was raised by switching the feed to source segregated domestic food  
18 waste and applying two organic loading rates (OLR) and hydraulic retention times (HRT) in  
19 paired digesters. One of each pair was dosed with trace elements (TE) known to be essential to

20 the transition, with the other unsupplemented digester acting as a control. Samples taken during  
21 the trial were used to determine the metabolic pathway to methanogenesis using <sup>14</sup>C labelled  
22 acetate. Partitioning of <sup>14</sup>C between the product gases was interpreted via an equation to

23 indicate the proportion produced by acetoclastic and hydrogenotrophic routes. Archaeal and  
24 selected bacterial groups were identified by 16S rRNA sequencing, to determine relative  
25 abundance and diversity. Acclimatisation for digesters with TE was relatively smooth, but OLR

26 and HRT influenced both metabolic route and community structure. The  $^{14}\text{C}$  ratio could be  
27 used quantitatively and, when interpreted alongside archaeal community structure, showed that  
28 at longer HRT and lower loading *Methanobacteriaceae* were dominant and hydrogenotrophic  
29 activity accounted for 77% of methane production. At the higher OLR and shorter HRT,  
30 *Methanosarcinaceae* were dominant with the  $^{14}\text{C}$  ratio indicating simultaneous production of  
31 methane by acetoclastic and hydrogenotrophic pathways: the first reported observation of this  
32 in digestion under mesophilic conditions. Digesters without TE supplementation showed  
33 similar initial changes but, as expected failed to complete the transition to stable operation.

34

35 **Keywords** anaerobic digestion, hydrogenotrophic methanogenesis, acetoclastic  
36 methanogenesis,  $^{14}\text{C}$  labelling, ammonia, trace elements

37

### 38 **Abbreviations**

39

40 ASV, Amplicon Sequence Variant Table; COD, Chemical Oxygen Demand; CSTR,  
41 Continuously-Stirred Tank Reactor; FAN, Free Ammonia Nitrogen; HRT, Hydraulic Retention  
42 Time; IA, Intermediate Alkalinity; OLR, Organic Loading Rate; OTU, Operational Taxonomic  
43 Unit; PA, Partial Alkalinity; SAOB, Syntrophic Acetate Oxidising Bacteria; SMP, Specific  
44 Methane Production; TAN, Total Ammonia Nitrogen; TE, Trace Element; TKN, Total  
45 Kjeldahl Nitrogen; TA, Total Alkalinity; TS, Total Solids; VBP, Volumetric Biogas Production;  
46 VFA, Volatile Fatty Acid; VS, Volatile Solids; WW, Wet Weight

47

## 48 **1 Introduction**

49

50 In digestion of low nitrogen feedstocks two pathways to methane production are utilised, with  
51 approximately 60-70% of CH<sub>4</sub> produced by direct cleavage of acetate via acetoclastic  
52 methanogenesis, and the remaining 30-40% mainly formed from H<sub>2</sub> and CO<sub>2</sub> by  
53 hydrogenotrophic methanogens (Jerris and McCarty, 1965). In a typical digester fed on  
54 municipal wastewater biosolids, the dominant methane producers are the acetoclastic  
55 *Methanosaetaceae* (Karakashev et al., 2005). When a high nitrogen feedstock is introduced to  
56 such an inoculum, *Methanosaetaceae* and other methanogens with low tolerance for ammonia  
57 may suffer inhibition. This inhibitory effect was first noted in the 1960s (McCarty, 1964), and  
58 has since been widely discussed, with several recent reviews of the topic (Rajagopal et al.,  
59 2013; Yenigun and Demirel, 2013; Jiang et al., 2019). By the late 1970s it was realised that  
60 digesters could become adapted to high ammonia concentrations (e.g. Van Velsen, 1979), and  
61 the first practical steps towards digestion of ammonia-rich feedstocks were suggested in the  
62 work of Angelidaki and Ahring (1993). The mechanism of inhibition was initially unclear, but  
63 by the mid-1980s some research had indicated hydrogenotrophic methanogens were more  
64 tolerant than acetoclastic (Koster and Lettinga, 1984). It is now considered that at high  
65 ammonia concentrations stable methanogenesis can be achieved if there is a change in the  
66 methanogenic community and pathway from acetoclastic to hydrogenotrophic methanogenesis,  
67 with syntrophic acetate oxidation occurring as an essential step (Schnürer et al., 1999).  
68 Schnürer and Nordberg (2008) applied a <sup>14</sup>C labelling technique to demonstrate this shift in  
69 pathway. This change, and its reversibility after ammonia stripping, was subsequently verified  
70 for food waste digestion (Jiang et al., 2018; Serna et al., 2014), and is further supported by the  
71 observations of Karakashev et al. (2006) and others on full-scale commercial digesters, which  
72 also indicate the importance of syntrophic acetate oxidation in establishing stable  
73 methanogenic communities of this type.

74

75 The above transition is only possible if each of the reactions can be catalysed, and the essential  
76 role of selenium in allowing acclimatisation to increasing ammonia concentrations in food  
77 waste digestion was first noted by Banks et al. (2012). This element alleviates the slow  
78 progressive accumulation of propionic acid which was characteristic both of food waste  
79 digesters (Banks et al., 2008), and of other digesters operating at high ammonia concentrations  
80 (Resch et al., 2011; Molaey et al., 2018). Selenium appears to be essential in promoting the  
81 conversion of propionic acid, which has an uneven carbon chain length, into H<sub>2</sub> and CO<sub>2</sub> via  
82 formate, and in forming the seleno-cysteine complex necessary for formate dehydrogenase  
83 production (Jones and Stadtman, 1981; Wood et al., 2003). The typical volatile fatty acid (VFA)  
84 'signature' observed in food waste digesters in response to increasing ammonia concentrations  
85 shows an initial acetic acid peak that is consumed in advance of the build-up of propionate and  
86 other longer chain VFA. This initial peak has been interpreted as resulting from the loss of  
87 acetoclastic activity: its subsequent decline is a result of the establishment of syntrophic acetate  
88 oxidation, but the accumulation of propionate suggests that syntrophic oxidation of longer  
89 chain VFA is at least partially inhibited. Eventually the buffering capacity of the digester is  
90 overcome by the acid accumulation, leading to a fall in pH and inhibition of all methanogenic  
91 activity. When the trace element requirements for both acetate and propionate oxidation are  
92 satisfied, the stable digestion of food waste at ammonia concentrations, which were once  
93 thought to be inhibitory under mesophilic conditions, can be achieved. Under thermophilic  
94 conditions this strategy of trace element addition is not effective (Yirong et al., 2015, 2017),  
95 and in this case stable operation can only be achieved by maintaining the free ammonia  
96 concentration below a critical threshold e.g. by ammonia stripping or dilution (Zhang et al.,  
97 2017a and b).

98

99 The commercial significance of understanding the process of acclimatisation to ammonia and  
100 some of the factors that regulate it has been high. It has allowed digester operators the option  
101 of using food waste from domestic and commercial sources as a high potential energy substrate  
102 without dilution. This was not always so, as the material has an intrinsically high protein  
103 content which on hydrolysis releases ammonia at concentrations previously considered  
104 inhibitory, with total ammonia nitrogen (TAN) typically in excess of 5 g N L<sup>-1</sup> promoting  
105 changes in both the pH and alkalinity of the system (Banks et al., 2011; Zhang et al., 2012). As  
106 a consequence of overcoming this limitation, anaerobic digestion of food waste is now  
107 recognised in the food waste hierarchy as the best route to recovering value after all other  
108 options for reduction and reuse have been considered; and is now used in many parts of the  
109 world for both energy production and nutrient recycling from food wastes (Banks et al., 2018).

110

111 Better understanding of factors affecting the transition to and dominance of hydrogenotrophic  
112 methanogenesis is also of value for other feedstocks and applications: for example to assist in  
113 the establishment and maintenance of stable mixed culture communities for biomethanisation  
114 of CO<sub>2</sub>. This topic has recently attracted considerable attention, as it offers a means of  
115 converting surplus renewable electricity into a storable and infrastructure-compatible fuel via  
116 electrolytic hydrogen production (Aryal et al., 2018). While many approaches focus on high-  
117 rate and pure culture systems, in situ mixed culture conversion is also of interest as a means of  
118 increasing methane yield and upgrading biogas methane content from conventional feedstocks  
119 (Luo et al., 2013a and b). Recent work showing the feasibility of upgrading biogas from  
120 multiple digesters in one (Tao et al., 2019) supports this potential for application in large-scale  
121 conventional digestion of food wastes and sludges, making more efficient use of existing  
122 capacity.

123

124 While it is now widely recognised that acclimatisation of anaerobic digesters to ammonia can  
125 occur, previous studies using  $^{14}\text{C}$  labelling to identify the associated change in methanogenic  
126 pathway have mainly reported results for full-scale commercial digesters at high and low  
127 ammonia concentrations (Schnürer et al., 1999; Karakashev et al., 2005; Fotidis et al. 2014).  
128 Only a few have attempted to observe the transition (Schnürer and Nordberg, 2008) or to  
129 compare known points in the process (Serna et al., 2014; Sun et al., 2016; Jiang et al., 2018);  
130 and none have linked the dynamic changes in pathway to changes in methanogenic population  
131 over the transition period. The  $^{14}\text{C}$  labelling technique used to demonstrate the shift in  
132 metabolic pathway is based on the splitting of the C-C bond between the methyl and carboxyl  
133 group of acetic acid, with  $\text{CH}_4$  being formed from the labelled methyl group while  $\text{CO}_2$  is  
134 formed from the carboxyl group in acetoclastic methanogenesis (Ferry, 1993). A pure  
135 acetoclastic methanogenic community will thus channel all  $^{14}\text{C}$  labelled acetate to  $^{14}\text{CH}_4$ , while  
136 a hydrogenotrophic methanogenic community forming biogas via syntrophic acetate oxidation  
137 will give an equal distribution of  $^{14}\text{CO}_2$  and  $^{14}\text{CH}_4$ . In practice the results of  $^{14}\text{C}$  labelling assays  
138 are rarely clear cut, and the ratio of the partition is often used simply as an indication of the  
139 dominant route (Fotidis et al., 2013). Jiang et al. (2018), however, derived an equation to  
140 quantify the proportion of carbon going by each route based on the ratio of attached  $^{14}\text{C}$  labels.

141  
142 The current study is the first to trial the application of this equation throughout the transition  
143 period in order to investigate its potential quantitative significance. It also presents one of the  
144 first detailed analyses of dynamic changes in the microbial population that occur during the  
145 shift from a predominantly acetoclastic population to a predominantly hydrogenotrophic  
146 pathway and population under increasing ammonia concentrations. These changes were  
147 mapped using chemical/biochemical analysis of the digestate, biofunctional information  
148 obtained from a  $^{14}\text{C}$  labelling assay, and microbial identification analysis based on 16S rRNA

149 gene sequencing. Food waste was used as the substrate for the experiments as the  
150 acclimatisation process has been previously demonstrated (Banks et al., 2012): using  
151 Selenium as the 'key' to turn on the shift in pathway allowed a population transitioning to  
152 hydrogenotrophic metabolism through syntrophic acetate oxidation to be compared to one  
153 where this transition could not be achieved due to blockage of at least part of the metabolic  
154 pathway attributed to metallo-enzyme deficiencies.

155

## 156 **2 Materials and methods**

157

### 158 2.1 Inoculum and substrate

159

160 The inoculum was taken from a mesophilic anaerobic digester treating municipal wastewater  
161 biosolids in Millbrook, Southampton, UK.

162

163 Source separated food waste was collected from Otterbourne waste transfer station in  
164 Hampshire, UK. After collection, the material was manually sorted to remove a small  
165 proportion of oversized or unbiodegradable items such as plastic bags, garden rubbish and large  
166 bones or seeds. The material was homogenized using a S52/010 macerating grinder (Imperial  
167 Machine Company Ltd, UK), then mixed thoroughly, packed into 4-L plastic containers and  
168 frozen at -20 °C. Before use, the frozen food waste was thawed at ambient temperature then  
169 stored at 4 °C and used within a short period.

170

### 171 2.2 Digesters and semi-continuous digestion

172



173 Semi-continuous digestion was carried out in 4 no. 5-L continuously-stirred tank reactors  
174 (CSTR). These were constructed in PVC with a top flange to which a top plate was secured by  
175 stainless steel bolts, with a closed-pore neoprene gasket to provide a gas-tight seal. The top  
176 plate was fitted with a gas outlet and a feed port sealed with a rubber bung. A DC motor  
177 mounted on the top plate was coupled to an asymmetric bar stirrer through a draught tube with  
178 a gas-tight compression seal. Digester contents were continuously stirred at 40 rpm, with  
179 digestate removed from a 15 mm diameter outlet port at the base of the digester. Temperature  
180 was maintained at 35 +/- 0.5 °C by water circulating through an external heating coil. Gas  
181 production was measured continuously by the alternate filling and discharging of a calibrated  
182 cell, with each discharge logged via a labjack (labjack Ltd, UK) computer interface. Gas  
183 counter calibration was checked twice per week using gas-impermeable bags connected to the  
184 gas counter outlet, with gas volumes measured in a weight-type displacement gasometer  
185 (Walker et al., 2009). All gas volumes are reported as dry gas at a standard temperature and  
186 pressure of 0 °C and 101.325 kPa.

187

188 The four digesters were fed over a period of 180 days on the source separated food waste. Two  
189 digesters (M1 and M2) were fed at an organic loading rate (OLR) of 3 kg VS m<sup>-3</sup> day<sup>-1</sup>, and  
190 the other two (M3 and M4) at 5 kg VS m<sup>-3</sup> day<sup>-1</sup>. Feed was added daily and digestate removed  
191 once per week to maintain a working volume of 4 L. The digesters were monitored on a weekly  
192 basis for pH, TAN, alkalinity, total and volatile solids and VFA concentrations.

193

194 M2 and M4 were supplemented once per week with a trace element (TE) solution to maintain  
195 an additional working concentration in the digestate of the following elements (mg L<sup>-1</sup>): Cobalt  
196 1.0, Nickel 1.0, Molybdenum 0.2, Selenium 0.2, Tungsten 0.2, based on Banks et al. (2012).  
197 No TE solution was added to M1 or M3.

198

### 199 2.3 Analytical methods

200

201 Total solids (TS) and volatile solids (VS) determination was carried out in accordance with  
202 Standard Method 2540 G (APHA, 2005). pH was measured using a Jenway 3010 meter (Bibby  
203 Scientific Ltd, UK) with a combination glass electrode, calibrated in buffers at pH 4, 7 and 9.2.  
204 Alkalinity was measured by titration according to Standard Method 2320B (APHA, 2005).  
205 TAN was determined according to Standard Method 4500-NH<sub>3</sub> B and C (APHA, 2005). Total  
206 Kjeldahl Nitrogen (TKN) was determined as TAN after acid digestion of the material. Soluble  
207 Chemical Oxygen Demand (COD) was determined after filtration through a 0.45 µm syringe  
208 filter (Merck Millipore, SLCR033NS) followed by centrifugation (Eppendorf 5417 C/R,  
209 Eppendorf, Hamburg Germany) at 5000 rpm for 5 min, according to according to Environment  
210 Agency (2007). Samples for VFA analysis were prepared by centrifugation at 13,000 g for 30  
211 min, and the supernatant was acidified to 10% (v/v) with formic acid. VFA concentrations were  
212 measured using a Shimadzu GC-2010 gas chromatograph (Shimadzu, UK) with a flame  
213 ionization detector and a capillary column (SGE Europe Ltd, UK) and Helium as the carrier  
214 gas. The GC was calibrated with a standard solution containing acetic, propionic, iso-butyric,  
215 n-butyric, iso-valeric, valeric, hexanoic and heptanoic acids, at three dilutions to give  
216 individual acid concentrations of 50, 250 and 500 mg L<sup>-1</sup> respectively. Biogas composition was  
217 quantified using a Varian Star 3400 CX gas chromatograph (Varian Ltd, UK). The GC was  
218 fitted with a Hayesep C column and used argon as the carrier gas at a flow of 50 mL min<sup>-1</sup> with  
219 a thermal conductivity detector. The GC was calibrated with a standard gas containing 35%  
220 CO<sub>2</sub> and 65% CH<sub>4</sub> v/v (BOC, UK).

221

### 222 2.4 Radio-isotope labelling experiments

223

224 A Carbon-14 tracer technique was used to determine the metabolic pathway for methane  
225 production. Each sample was mixed with anaerobic medium as described in Jiang et al. (2018)  
226 in the ratio of 1:2 v/v. 10 KBq of  $^{14}\text{CH}_3\text{COONa}$  (MP biomedical, USA) was added into 45 mL  
227 of the sample/medium mixture and incubated in 119 mL crimp-top serum bottles at 37 °C for  
228 48 hours.  $\text{CO}_2$  and  $\text{CH}_4$  produced in the headspace were separately collected in alkali traps  
229 containing 20 mL of 1 M NaOH solution: before collection, the  $\text{CH}_4$  was oxidised to  $\text{CO}_2$  in a  
230 tube furnace consisting of a heating block containing an embedded quartz tube (6.2 mm OD, 4  
231 mm ID, 180 mm length, H. Baumbach & Co Ltd, UK) packed with copper (II) oxide. The  
232 furnace operating temperature was regulated at  $800 \pm 5$  °C using a temperature controller  
233 (Omega DP7004, UK). After collection, 0.4 mL of NaOH solution from each alkali trap and  
234 0.4 mL of the sample/medium mixture (after centrifugation) were added to 3.6 mL Gold Star  
235 multi-purpose liquid scintillation cocktail (Meridian 56 Biotechnologies Ltd, UK) and counted  
236 in a PerkinElmer 2450 MicroBeta<sup>2</sup> liquid scintillation counter (PerkinElmer Life and  
237 Analytical Sciences, USA).

238

239 The proportion of methane generated by acetoclastic and hydrogenotrophic routes was  
240 estimated according to Equation 1 based on Jiang et al. (2018)

241

$$242 \quad P_a = 1 / ({}^{14}\text{CO}_2 / {}^{14}\text{CH}_4 + 1) \text{ Equation (1)}$$

243

244 Where  $P_a$  is the proportion of methane produced via acetoclastic methanogenesis  
245 and  ${}^{14}\text{CO}_2$  and  ${}^{14}\text{CH}_4$  are the volumes of labelled carbon dioxide and labelled methane,  
246 respectively, produced from acetate labelled on the methyl group.

247

248 2.5 16S rRNA sequencing

249

250 *DNA extraction*

251 The microbial pellet was separated from the supernatant by centrifugation at 8,000 x g for 10  
252 minutes at 4 °C. The Power Soil DNA (MOBIO) extraction protocol was applied to 200 mg of  
253 pelleted biomass according to the manufacturer's recommendations.

254

255 *PCR based analysis*

256 Metagenomic DNA (50 ng) extracted from the digester samples (n = 53) was used directly to  
257 amplify 16S rRNA genes using primers containing Illumina adapters designed to cover the V4  
258 region (S-D-Arch-0519-a-S-15 = CAGCMGCCGCGGTAA, S-D-Bact-0785-b-A-18  
259 TACNVGGGTATCTAATCC). PCR reactions were carried out in 50 µL volumes containing  
260 200 µM of dNTPs, 0.5 µM of each primer, 0.02 U Phusion High-Fidelity DNA Polymerase  
261 (Finnzymes OY, Finland) and 5x Phusion HF Buffer containing 1.5mM MgCl<sub>2</sub>. The following  
262 PCR conditions were used: initial denaturation at 98 °C for 2 min, followed by 25 cycles  
263 consisting of denaturation (98 °C for 5 sec), annealing (52 °C for 30 sec) and extension (72 °C  
264 for 30 sec) and a final extension step at 72 °C for 5 min. The expected amplicon size for 16S  
265 rRNA product was approximately 280 bp. The amplified fragments were purified with  
266 Agencourt AMPure XP (Beckman Coulter, UK). The quantity of purified PCR products was  
267 analysed by Qubit fluorometer (Life Technologies, USA).

268

269 *Illumina sequencing of 16S rRNA tags*

270 Illumina libraries were prepared using a Nextera XT kit, following the company's  
271 recommendations for 16S PCR amplicon barcoding, clean up and libraries normalisation. All  
272 indexed libraries were quantified using a Qubit fluorometric system, diluted to 4 nM and mixed

273 in equal volumes of uniquely barcoded samples. Pooled libraries and PhiX control were  
274 denatured with freshly-made 0.2 N NaOH, diluted to 5 pM with hybridization buffer and mixed  
275 together in the ratio 3.3:1 v/v. Samples were heated at 96 °C for 2 min and cooled for 5 min  
276 then immediately loaded on a MiSeq v3 cartridge for 300 bp sequencing in both directions.  
277 The completed run was demultiplexed with Illumina's Casava software.

278

### 279 *Data analysis*

280 The Illumina-sequenced paired-end fastq files that had been split (or “demultiplexed”) by  
281 sample and from which the adapters had been removed were used for the data analysis using  
282 the DADA2 (version 1.6.0) pipeline (Callahan et al., 2016). Non-biological nucleotides  
283 (primers) were trimmed from fastq raw data using the FastX-Toolkit (Hannonlab, Cold Spring  
284 Harbor Laboratory, NY, USA). The forward and reverse reads were filtered by truncating at  
285 250 and 200 bp respectively with the default parameters at maxEE=2, truncQ=5, maxN=0. The  
286 error rate was determined using the DADA2 parametric error model (err). The pair-end reads  
287 were merged to obtain the full denoised sequence and dereplicated prior to building an  
288 amplicon sequence variant table (ASV). The sequence table is a matrix with rows  
289 corresponding to (and named by) the samples, and columns corresponding to (and named by)  
290 the sequence variants. Chimeric sequences were identified and removed from the final  
291 operational taxonomic unit (out) table. The taxonomy of each OTU was assigned using  
292 DADA2 package with a native implementation of the naive Bayesian classifier method.  
293 DADA2 formatted reference database Silva version 132 (Quast et al., 2012) was used for this  
294 purpose. Detailed R script to data analysis is provided in Supplementary Information section  
295 S1. Subsequent visualization and statistical analysis used Prism7 (GraphPad Software, San  
296 Diego, CA).

297

298 It should be noted that this technique does not distinguish between live and dead organisms,  
299 and may also identify undegraded fragments of 16S rRNA from the latter. The significance of  
300 this for the study of a transition period is briefly discussed in Supplementary Information S3.

301

### 302 **3 Results and discussion**

303

#### 304 3.1 Feedstock and inoculum properties

305

306 The inoculum was taken from a mesophilic anaerobic digester receiving a feed of co-settled  
307 primary and secondary sewage sludge at a municipal wastewater treatment plant. Its  
308 characteristics were typical of material from this type of digester, with a pH around 7.5, TAN  
309  $1.5 \text{ g N kg}^{-1}$  wet weight (WW), total VFA concentrations  $< 100 \text{ mg COD L}^{-1}$ , and total  
310 alkalinity (TA) around  $7.5 \text{ g CaCO}_3 \text{ kg}^{-1}$  WW. The characteristics of the food waste are shown  
311 in Table 1 and are also typical of this type of material (Banks et al., 2018). At the applied OLR  
312 of  $3$  and  $5 \text{ g VS L}^{-1} \text{ day}^{-1}$  the corresponding average hydraulic retention times (HRT) in the  
313 digesters were respectively 69 and 41 days.

314

#### 315 3.2 Digester operation and performance

316

317 Figure 1 shows changes in key digestion stability parameters over the duration of the  
318 experiment. In M3, the more highly loaded of the two digesters not receiving trace elements,  
319 there was a rapid accumulation of VFA from day 55 onwards (Figure 1a) reaching  $9.6 \text{ g COD}$   
320  $\text{L}^{-1}$  by day 70. On day 72 feeding was suspended for 5 days, during which the VFA  
321 concentration fell slightly. After feeding was re-started the total VFA concentration increased  
322 again, and by day 83 had reached  $18.2 \text{ g COD L}^{-1}$ . At this point the pH dropped sharply to 7.3

323 (Figure 1b), the intermediate alkalinity (IA) increased, partial alkalinity (PA) fell and the IA/PA  
324 ratio rose to 1.05 (Figure 1c-e). A similar pattern was seen in M1, the lower-loaded digester  
325 without TE supplementation; but, as expected due to the longer HRT, the onset was delayed  
326 until around day 110. Total VFA concentrations in M1 increased from day 70 and plateaued at  
327 around 1.5 g COD L<sup>-1</sup> (Figure 1a), before rising again from day 97 to reach 15.9 g COD L<sup>-1</sup>  
328 on day 126, by which point the IA/PA had risen to 1.46 and the pH fell to 7.37.

329

330 In both cases the onset of VFA accumulation corresponded to the TAN concentration reaching  
331 around 3.6 g N kg<sup>-1</sup> WW (Figure 1f), in good agreement with the results of a previous study  
332 using a similar food waste (Yirong et al. 2017). This behaviour has also been reported by other  
333 researchers: once a threshold TAN concentration is exceeded, inhibition of acetoclastic  
334 methanogenesis occurs, resulting in accumulation of first acetic acid and then longer-chain  
335 VFA, due to product-induced inhibition of acetogenesis (Karakashev et al., 2006; Schnürer and  
336 Nordberg, 2008). In M1 the VFA primarily consisted of acetic acid, although small amounts  
337 of longer chain VFA appeared after day 70 and the propionic acid concentration reached 0.7 g  
338 L<sup>-1</sup> by day 126. In M3 propionic acid had reached 1.9 g L<sup>-1</sup> by day 83.

339

340 In M2 and M4, the digesters with TE supplementation, total VFA concentrations also began to  
341 rise on days 70 and 55 respectively when TAN reached around 3.6 g N kg<sup>-1</sup> WW; but then fell  
342 from their peak values of 4.5 and 8.5 g COD L<sup>-1</sup> to < 0.5 g COD L<sup>-1</sup> by days 90 and 126 in M2  
343 and M4, respectively. This was despite the continuing increase in TAN, which reached final  
344 values of 5.3 and 5.4 g N kg<sup>-1</sup> WW in M2 and M4, respectively. Because of the high TAN and  
345 the relatively low VFA concentrations, the pH in these digesters slowly increased from 7.6 to  
346 8.0 over the experimental period. The IA/PA ratios remained relatively stable at around 0.4

347 throughout the trial period, apart from small increases during the transient VFA peaks (Figure  
348 1e).

349

350 Digestate solids content and VS/TS ratios (Figure 1g) were similar in pairs of digesters at the  
351 same OLR, with the slightly higher values in M3 and M4 probably reflecting an increase in  
352 microbial biomass at the higher OLR of 5 g VS L<sup>-1</sup> day<sup>-1</sup>. Soluble COD content also showed  
353 reasonable agreement for digesters at the same OLR (Figure 1h) until VFA accumulation  
354 occurred in the unsupplemented digesters. VFA typically represented less than 20% of the  
355 soluble COD, which averaged around 8 g L<sup>-1</sup> higher in M4 than in M2 from day 77 onwards.  
356 These results support previous observations on the relatively high concentration of soluble  
357 microbial products and extracellular polymeric substances present in food waste digesters,  
358 which are a cause of poor dewaterability (Lü et al., 2015).

359

360 The volumetric biogas production (VBP, in litres of biogas per litre of digester working volume  
361 per day) and the specific methane production (SMP, in litres of CH<sub>4</sub> per g of feedstock VS  
362 added) are shown in Figure 2. In the TE-supplemented digesters M2 and M4 these were  
363 relatively consistent throughout the experimental period, apart from for short periods  
364 corresponding to the temporary increase in VFA after the TAN concentration in each of these  
365 digesters exceeded 3.6 g N kg<sup>-1</sup> WW. The average SMP was around 0.45 L CH<sub>4</sub> g<sup>-1</sup> VS, and  
366 the VBP was between 2.0-2.5 L L<sup>-1</sup> day<sup>-1</sup> at the lower OLR (M2) and 3.5-4.0 L L<sup>-1</sup> day<sup>-1</sup> at the  
367 higher OLR (M4). In digesters M1 and M3 without TE supplementation, both SMP and VBP  
368 both showed a progressive decline after the threshold TAN concentration was reached, and  
369 then a rapid fall once the buffering capacity of the system was overcome and the pH dropped  
370 sharply. These digesters were considered to be at the point of final failure by days 127 and 83  
371 corresponding to around 1.8 and 2.0 HRT respectively, and feeding was stopped at this point.



372

373 Digesters M2 and M4 were operated for 180 days, with samples for final analysis of most  
374 monitoring parameters taken on day 174. A period of 3 HRT is normally considered necessary  
375 to achieve steady -state operation, based on washout of around 95% of the digester's initial  
376 contents. M4 with a HRT of 41 days ran for 4.2 HRT in total but M2 only achieved 2.5 HRT,  
377 corresponding to approximately 98 and 92% displacement of the initial digester contents,  
378 respectively. The final values for M2 are thus not fully representative of steady-state conditions,  
379 although stabilisation of key values is evident in Figure 1 and 2. Since the focus of this study  
380 is specifically on the transition period, however, this was not considered to be a major issue.

381

382 The performance of all digesters thus demonstrated the patterns consistently observed on  
383 previous occasions for this type of feedstock (Banks et al., 2012; Zhang et al., 2017), with TE  
384 supplementation once again shown to be essential in enabling stable operation at the applied  
385 loadings and resulting TAN concentrations in each case.

386

387 3.3 Changes in microbial community and metabolic function in response to increasing  
388 TAN concentrations

389

390 3.3.1 Classification of microbial taxa

391

392 Amplicon sequencing of 16S rRNA genes from the digesters resulted in 9.68 million pair-end  
393 reads (n = 60, average = 182,577, standard error of mean [s.e.m] = 6,028) of which 5.56 million  
394 were retained after quality control trimming and removal of chimeric reads (see Supplementary  
395 Information Table S2). The initial OTU table contained 2,046 individual bacterial and archaeal  
396 OTUs. OTUs where the number of reads across all the samples was lower than 10 (so-called

397 singletons) were removed, resulting in 378 OTUs remaining for final analysis. The archaeal  
398 communities were exclusively classified to families within the Euryarchaeota, mainly  
399 *Methanosarcinaceae*, *Methanosaetaceae*, and *Methanobacteriaceae*. The evolution of these is  
400 discussed below. Changes in bacterial communities are briefly discussed in the Supplementary  
401 Information section S2.

402

### 403 3.3.2 Response to increasing TAN in lower loaded, TE-supplemented digester M2

404

405 Figure 3a shows archaeal community structure, TAN and acetic acid concentrations and  $^{14}\text{C}$   
406 ratios in digester M2. Until day 70 only a small number of Archaea were observed, and  
407 meaningful analysis of the relative abundance was not possible. By day 70 the TAN  
408 concentration was  $3.6 \text{ g N kg}^{-1} \text{ WW}$  (FAN  $\sim 0.25 \text{ g N kg}^{-1} \text{ WW}$ ) and acetoclastic  
409 *Methanosaetaceae* were the dominant family, making up 96.4% of the observed Archaea. The  
410  $^{14}\text{C}$  ratio was 0.25, indicating that methane production was mainly by the acetoclastic route. As  
411 noted earlier, from day 70 onwards there was a small increase in the acetic acid concentration,  
412 which remained steady at around  $1 \text{ g L}^{-1}$  until day 104. At this point the TAN concentration in  
413 M2 had reached  $4.3 \text{ g N kg}^{-1} \text{ WW}$  (FAN  $\sim 0.41 \text{ g N kg}^{-1} \text{ WW}$ ), and a sharp increase in acetic  
414 acid concentration was seen. *Methanosaetaceae* were still dominant in the archaeal community  
415 (92.5%), however, and the  $^{14}\text{C}$  ratio was 0.15, confirming that acetoclastic methanogenesis was  
416 still the primary route to methane formation.

417

418 The acetic acid concentration continued to rise, reaching  $3.7 \text{ g L}^{-1}$  on day 118 and remaining  
419 around this value until day 126. This accumulation was accompanied by a gradual increase in  
420 TAN, which had reached  $4.6 \text{ g N kg}^{-1} \text{ WW}$  by day 118. The relative abundance of  
421 *Methanosaetaceae* fell in this period and the proportion of hydrogenotrophic

422 *Methanobacteriaceae* began to increase, reaching 41% of the archaeal community by day 126.  
423 The  $^{14}\text{C}$  ratio also rose to 0.72 by day 126, indicating that over half of methane production was  
424 now by the hydrogenotrophic route.

425

426 From day 140 onwards the *Methanobacteriaceae* were the dominant family, accompanied by  
427 a slight increase in the relative abundance (up to 2%) in the relative abundance of other  
428 Euryarchaeota, while the proportion of *Methanosaetaceae* fell. By day 160 the relative  
429 abundance of *Methanobacteriaceae* was 68% compared to 18-22% *Methanosaetaceae*, with a  
430  $^{14}\text{C}$  ratio of 3.53 indicating a strongly hydrogenotrophic pathway to methane production. Based  
431 on Equation 1, these results indicated that between days 118 and 146 hydrogenotrophic  
432 methane production had increased from 14% to 77% of the total.

433

434 It was also noted that on day 133 there was a small recovery in the relative abundance of  
435 *Methanosaetaceae*, corresponding to the sharp fall in acetic acid concentration, which then  
436 remained around  $1 \text{ g L}^{-1}$ .

437

438 3.3.3 Response to increasing TAN in higher loaded, TE-supplemented digester M4

439

440 Figure 3b shows the archaeal community structure, TAN and acetic acid concentrations and  
441  $^{14}\text{C}$  ratios in digester M4. By day 55 the TAN concentration had reached  $3.6 \text{ g N kg}^{-1} \text{ WW}$ .  
442 Until this point the acetoclastic *Methanosaetaceae* were dominant, with a relative abundance  
443 of around 96% (Figure 3b), and the  $^{14}\text{C}$  ratio of  $< 0.3$  indicated predominantly acetoclastic  
444 methane formation. Between days 55 to 77 the relative abundance of *Methanosaetaceae* fell,  
445 while the *Methanobacteriaceae* increased from 6 to 42%. At this point, the trends in population  
446 appeared similar to the early stages of transition in M2 between days 104 and 126 (Figure 3a);

447 but with the difference that acetic acid concentrations remained low and no significant change  
448 was observed in the  $^{14}\text{C}$  ratio, which was still 0.14 on day 70.

449

450 From day 77 there was a very sharp increase in the acetic acid concentration, which reached 7  
451  $\text{g L}^{-1}$  by day 90. This was accompanied by an increase in TAN from 4.0 to 4.4  $\text{g N kg}^{-1}$  WW,  
452 passing through the value at which the acetic peak had appeared in M2. On days 83 and 90  
453 there was a temporary recovery in *Methanosaetaceae* and a corresponding fall in the relative  
454 abundance of *Methanobacteriaceae*. The acetic acid concentration remained high until day 97,  
455 however, when a large increase in *Methanosarcinaceae* was observed, up to 54% of the  
456 archaeal population. Between days 90-97 the  $^{14}\text{C}$  ratio rose from 0.22 to 0.46, indicating that  
457 the switch in pathway from dominantly acetoclastic towards hydrogenotrophic methanogenesis  
458 had begun. This transition continued, with the relative abundance of *Methanosarcinaceae*  
459 increasing from 8% on day 90 to 93% on day 160. The  $^{14}\text{C}$  ratio also rose, peaking at 2.56 on  
460 day 146 before falling slightly in the final two weeks of operation. The TAN concentration in  
461 this final period was around 4.9  $\text{g N kg}^{-1}$  WW (FAN  $\sim 0.5\text{-}0.8$   $\text{g N kg}^{-1}$  WW), and  
462 *Methanosarcinaceae* continued to dominate the community, with *Methanobacteriaceae* at  
463 around 6% and other Euryarchaeota accounting for around 1%.

464

### 465 3.3.4 Comparison of responses at different loading rates and HRT

466

467 The operating protocols adopted for M2 and M4 were the same except that the loading rate was  
468 higher and the HRT consequently shorter in M4 than M2. Both digesters showed a transition  
469 to predominantly hydrogenotrophic methanogenesis that was triggered at a threshold TAN  
470 concentration of around 4.3  $\text{g N kg}^{-1}$  WW; and both exhibited a characteristic increase in acetic  
471 acid concentration, followed by a fall which could be attributed to the establishment of

472 syntrophic acetate oxidation. Both digesters also showed a period where there was a near-linear  
473 increase in the  $^{14}\text{C}$  ratio. In M2 this occurred between days 118-146, with a slope of  $0.117 \text{ day}^{-1}$   
474 ( $R^2 = 0.9635$ ,  $n = 5$ ,  $p < 0.005$ ); while in M4 it occurred between days 90-146, with a slope  
475 of  $0.043 \text{ day}^{-1}$  ( $R^2 = 0.9960$ ,  $n = 9$ ,  $p < 5 \times 10^{-9}$ ). T-testing indicated that the two slopes were  
476 significantly different ( $p < 0.0005$ ), confirming that the transition in M4 was slower than in  
477 M2, and the final  $^{14}\text{C}$  ratio was also lower.

478

479 Both M2 and M4 started with archaeal communities dominated by the acetoclastic  
480 *Methanosaetaceae*; but the final community compositions were different, with  
481 *Methanobacteriaceae* being dominant in M2 and *Methanosarcinaceae* in M4. Yet it at first it  
482 appeared as though the same route would be followed in both digesters as there was an initial  
483 increase in hydrogenotrophic *Methanobacteriaceae* from day 55 to 77 in M4, similar to that in  
484 M2 between days 104 to 126. In both M2 and M4 this was likely to have been in response to  
485 the increase in TAN concentration, leading to inhibition of acetoclastic methanogenesis and a  
486 temporary rise in acetic acid. In M2 at lower OLR the increase in acetic acid was less rapid,  
487 and its decline probably occurred as a result both of the establishment of syntrophic acetate  
488 oxidising bacteria (SAOB) and of a partial recovery in acetoclastic activity, as supported by  
489 the observation of a temporary increase in *Methanosaetaceae* at the expense of  
490 *Methanobacteriaceae* before the latter became fully established as the dominant methanogenic  
491 family. A similar recovery in *Methanosaetaceae* was also observed in M4 between days 83 -  
492 90, but the rise in acetic acid was more rapid and the total accumulation greater. The peak value  
493 of  $7 \text{ g L}^{-1}$  acetic acid in M4 is outside the optimal range for *Methanosaetaceae*, which are  
494 known to have a high acetate affinity linked to their ability to apply different systems for  
495 activation, electron transfer and energy conservation (Smith and Ingram-Smith, 2007;  
496 Westerholm et al., 2016). *Methanosarcinaceae* were favoured in M4, and their ability to utilise

497 a broad spectrum of substrates for methanogenesis including acetate, H<sub>2</sub> and CO<sub>2</sub>, methanol  
498 and methylamines (Thauer, Kaster et al., 2008; Liu, 2010) may have contributed towards this:  
499 the fall in acetate concentration in M4 only occurred after a sharp increase in their relative  
500 abundance. *Methanosarcinaceae* have recently been reported as being dominant in digestion  
501 trials conducted at very high ammonia concentrations and relatively short HRT, using the  
502 organic fraction of municipal solid waste (Yan et al., 2019) and cattle slurry with macroalgae  
503 (Tian et al., 2018) as feedstocks: the results of the current work support this observed behaviour  
504 and suggest HRT may have a role in promoting it. Neither of these previous studies carried out  
505 <sup>14</sup>C determination, but Yan et al. (2019) suggested a potential shift by the dominant  
506 *Methanosarcina soligelidi* to hydrogenotrophic metabolism while Tian et al. (2018) interpreted  
507 an increase in the abundance of SAOB as indicating significant hydrogenotrophic activity.

508

509 It is clear that establishment of a SAOB population plays an essential role in the transition from  
510 acetoclastic to hydrogenotrophic methanogenesis. Westerholm et al. (2016) noted, however,  
511 that the doubling time for syntrophy between SAOB and hydrogenotrophic methanogens can  
512 range from 9-78 days, and may also involve a lag phase before inception: one batch study  
513 reported 49-54 days and 62-70 days for the initiation of syntrophy in thermophilic and  
514 mesophilic digesters, respectively (Hao et al., 2017). The longer retention time in M2 could  
515 thus have been a major contributor to the smooth transition in this digester, as the rate of  
516 increase in TAN was lower and the onset of acetic acid accumulation occurred much later than  
517 in M4. This would have allowed a longer period for the build-up of an SAOB population in  
518 M2, responding to the competitive availability of acetic acid and facilitating a transition to  
519 hydrogenotrophic methanogenesis mediated through the *Methanobacteriaceae*. M4 had a  
520 shorter HRT, reached the inhibitory TAN threshold earlier than M2, and the higher organic  
521 loading led to higher acetic acid production. Any or all of these factors may have caused or

522 contributed to a delay in the establishment of a SAOB population in M4; thus promoting  
523 *Methanosarcinacea*, with its greater metabolic versatility, as the dominant methanogenic  
524 community member in this case.

525

526 The slower increase in the  $^{14}\text{C}$  ratio and the lower final value in M4 compared to M2 indicate  
527 that a significant fraction of methanogenesis in M4 was still via the acetoclastic route, despite  
528 the very marked shift in the dominant population. These results therefore strongly suggest that  
529 the *Methanosarcinaceae* present are generating methane by both pathways simultaneously,  
530 with different species in the community or perhaps even different individuals within one  
531 species utilising different metabolic routes. A recent study based on metatranscriptomics has  
532 shown *Methanosarcina thermophila* simultaneously performing acetoclastic,  
533 hydrogenotrophic, and methylotrophic methanogenesis in acetate-fed thermophilic digesters  
534 (Zhu et al., 2020): the current trial provides additional evidence for this simultaneous multi-  
535 trophic metabolism and is the first time this has been reported in conventional mesophilic  
536 digestion.

537

538 It should be noted that conditions during the  $^{14}\text{C}$  labelling assay do not fully replicate those in  
539 the digester, and this could potentially influence the outcome of the assay. For example, the  
540 presence of anaerobic medium in the serum bottle provides dilution, which may reduce the  
541 TAN concentration in an ammonia-rich digestate below the inhibitory threshold. In this case  
542 some revival of acetoclastic methanogenesis could occur, particularly if members of the  
543 community are already capable of utilising both metabolic routes. A major switch in metabolic  
544 pathway or community structure appears unlikely within the relatively short 48-hour assay  
545 period; but this aspect should be considered in future studies using this type of approach. In the  
546 current study the TAN concentrations of M2 and M4 were similar from day 112 onwards, but

547 only M4 had a population dominated by the metabolically versatile *Methanosarcinaceae*. This  
548 dominance was maintained consistently from day 97 onwards, representing 93% or more of  
549 the archaeal population in the last four weeks of operation.

550

551 In the last part of the experimental period both M2 and M4 showed residual concentrations of  
552 acetate in the range 0.5 - 1.5 g L<sup>-1</sup> (as acetic acid), combined with high pH and TAN  
553 concentrations. In these conditions the dominant species produced in acetogenesis is acetate  
554 and the proportion present as molecular acetic acid, which is the form taken up by microbial  
555 biomass, will be very low (Wilson et al., 2012). In M4 this residue may thus partly reflect the  
556 relatively low affinity of *Methanosarcinaceae* for acetate (Smith and Ingram-Smith, 2007; De  
557 Vrieze et al., 2012). In both digesters, however, the dominant metabolic route in this period  
558 was hydrogenotrophic. Under standard conditions, the oxidation of acetic acid to CO<sub>2</sub> and H<sub>2</sub>  
559 is a non-spontaneous reaction. For it to occur the ratio of the relative amounts of products to  
560 reactants in the digester must be very low. This may have contributed to the presence of a  
561 detectable acetic acid concentration, at least during this adaptation period. Despite differences  
562 in their community structure and metabolic carbon flow, these conditions were achieved in  
563 both M2 and M4, and both digesters showed a successful transition to accommodate the  
564 selective pressure of an elevated TAN concentration.

565

566 In contrast, digesters M1 and M3 both failed to make such a transition and suffered VFA  
567 accumulation and inhibition of methanogenesis. This was as expected, and confirms once again  
568 the importance of trace elements in enabling syntrophic acetate oxidation coupled with  
569 hydrogenotrophic methanogenesis. M1 and M3 were only sampled twice for 16S rRNA  
570 amplicon sequencing, on days 70 and 126 for M1 and days 55 and 83 for M3. The number of  
571 OTUs for these digesters was not significantly different from those for M2 and M4, with 195



572 and 185 OTUs in M1 and M3 respectively. The relative abundance of bacteria in each case was  
573 also similar to that for the equivalent digester with TE addition on the same date  
574 (Supplementary Information Figure S1). M1 showed the start of a similar transition in  
575 community to that seen in M2, with the relative abundance of *Methanosaetaceae* falling from  
576 94.1% to 54.5% and of *Methanobacteriaceae* rising from 4.6% to 36.4% from day 70 to day  
577 126, although the proportion of other Euryarchaeota was slightly higher in M1 on day 126 at  
578 6.1%. On day 55 relative abundance of *Methanosaetaceae* was slightly lower in M3 at 80.9%,  
579 compared to 92.9% in M4, with the difference mainly represented by additional  
580 *Methanobacteriaceae* in M3; but by day 83 the two digesters were similar with  
581 *Methanosaetaceae* remaining dominant. The initiation of a change in the population structure  
582 was therefore visible, especially in M1; but without TE supplementation this transition could  
583 not be completed. This observation supports the view that a lack of vital metallo-enzymes  
584 caused by trace element deficiencies is likely to be the primary reason for failure in these  
585 conditions (Banks et al., 2012), rather than any differences in methanogenic population. There  
586 was thus no opportunity for establishment of a stable alternative pathway mediated by more  
587 ammonia-tolerant methanogens, and as expected both digesters without TE supplementation  
588 failed as a result of inhibition of acetoclastic methanogenesis.

589

590 The unsupplemented digester M1 showed a small rise in acetic acid concentration to around 1  
591 g L<sup>-1</sup> between day 77 - 97 which was nearly identical to that also seen in M2 (Figure 1a). The  
592 reason for this is unknown, but may have been related to the increasing pH in this period linked  
593 with the rising TAN concentrations, and the consequent effects on acetic acid speciation and  
594 availability. If it is assumed that the COD value of food waste is approximately 1.4 g COD g<sup>-1</sup>  
595 VS, however, and that 70% of this is converted to acetic acid in the acetogenesis stage, then  
596 the residual concentration of 1 g L<sup>-1</sup> represents only around 2% of the estimated daily

597 production; thus indicating that there was no major metabolic blockage whichever  
598 methanogenic route was being utilised at this point. Neither M3 nor M4 showed a distinct step  
599 of this type, although a slow rise in acetic acid concentrations was visible before the main peak.

600

### 601 3.3.5 Semi-quantification of metabolic functionality using $^{14}\text{C}$ equation

602

603 Figure 4a shows the estimated proportion of methane generated by the acetoclastic route in M1  
604 - M4, based on Equation 1. The acetoclastic contribution in M1 and M3 without TE  
605 supplementation remained consistently high and close to the values seen in M2 and M4 (TE  
606 supplemented) on the same dates, until monitoring of M1 and M3 was stopped. In M2 and M4  
607 the equation-derived values show a strong trend in the dominant pathway, as expected since  
608 these reflect the changes in the  $^{14}\text{C}$  ratio in each case.

609

610 The  $^{14}\text{C}$  ratio has previously been mainly regarded as a qualitative indicator, with values in  
611 excess of 1 taken as indicating a dominantly hydrogenotrophic pathway (Fotidis et al., 2013).  
612 The derivation of the relationship in Equation 1 is itself based on a number of assumptions and  
613 simplifications, including the importance of the affinity of the micro-organisms involved for  
614 lighter isotopes, and the proportion of dissolved  $\text{CO}_2$  present (Jiang et al., 2018). The current  
615 study, however, is one of the first to look in detail at the period of transition from a  
616 predominately acetoclastic to a predominantly hydrogenotrophic community; the strong trends  
617 seen for digesters M2 and M4 in Figure 4 support the view that the  $^{14}\text{C}$  ratio and the values  
618 obtained from the equation are at least semi-quantitative, and can be used to compare the  
619 proportion of each metabolic route being utilised in digesters operating under similar  
620 conditions.

621

622 During the trial it was noted that the proportion of labelled acetate remaining in the sample at  
623 the end of the  $^{14}\text{C}$  assay was approximately constant in all four digesters from day 14-40, but  
624 rose in digesters M2 and M4 towards the end of the experiment (Figure 3b). This increase  
625 began earlier in M4 than M2 but the final value was higher in M2, mirroring the transition to  
626 hydrogenotrophic methanogenesis in each case. A possible explanation for these observations  
627 could be that the rate of conversion of acetic acid by the syntrophic acetate oxidation pathway  
628 is considerably lower than for acetoclastic methanogenesis (Schnürer et al., 1999); thus as the  
629 proportion of methane produced by the hydrogenotrophic route increases, the amount of  
630 labelled acetate converted in the fixed-duration assay decreases and the residue increases. If  
631 this explanation is correct, it provides another indicator of the transition in methanogenic  
632 pathway which is independent of the  $^{14}\text{C}$  ratio. Figure 4c shows the residual labelled acetate as  
633 a proportion of the total amount added, plotted against the estimated proportion of methane  
634 produced by the acetoclastic route (calculated from Equation 1 and based on the  $^{14}\text{C}$  ratio), for  
635 the whole experimental period for digesters M2 and M4 operating at OLR 3 and 5 g VS L day<sup>-1</sup>  
636 respectively. Regression analysis showed reasonably strong relationships (M2  $R^2 = 0.7491$ ,  $n$   
637  $= 20$ ; M4  $R^2 = 0.8179$ ,  $n = 19$ , with  $p < 10^{-6}$  in each case), while t-testing confirmed that the  
638 slopes of -0.73 for M2 and -0.62 for M4 were not significantly different ( $p > 0.3$ ), despite the  
639 different OLR and HRT in these two digesters. These results provide evidence of a clear  
640 relationship between two independent measures of the degree of transition between pathways,  
641 and further support the view that values obtained from Jiang's equation are at least semi-  
642 quantitative in nature and can be used to compare digesters operating under different conditions.  
643

644 The  $^{14}\text{C}$  ratio and the values obtained from Equation 1, in conjunction with the relative  
645 abundance of the dominant methanogenic families, provided a clear picture of the occurrence  
646 and duration of the critical transition period. In M2 this occurred over 28 days compared to 56

647 days in M4, equivalent to 0.4 and 1.3 HRT for the respective digesters. It is evident that the  
648 transition is much more strongly influenced by the change in digester conditions than by  
649 washout of microbial populations. The time required for transition is comparable with that for  
650 the rise in VFA which indicated kinetic uncoupling of VFA and methane production in M1 and  
651 M3. In both cases the change was quite rapid, especially in comparison with the very long slow  
652 build-up of propionic acid previously observed in reactors operating on similar food waste  
653 feedstocks at lower OLR (Banks et al., 2011, 2012).

654

655 Previous researchers have used batch studies to determine the metabolic pathways and  
656 dominant species in anaerobic systems at very high ammonia concentrations. Fotidis et al.  
657 (2013) and Tian et al. (2019) both found *Methanosarcinaceae* to be dominant or near-dominant  
658 in acclimated batch cultures, with a strongly acetoclastic metabolic pathway ( $^{14}\text{C}$  ratio  $< 0.1$  at  
659  $3 \text{ g N L}^{-1}$  or above in mesophilic conditions, corresponding to 90% or more acetoclastic  
660 methanogenesis according to Equation 1). These results at first sight may appear to conflict  
661 with those of the current study. Fotidis et al. (2013) also tested non-acclimated inoculum,  
662 however, and noted that in the mesophilic system an elevated ammonia concentration resulted  
663 in a change from acetoclastic to hydrogenotrophic metabolism with a  $^{14}\text{C}$  ratio of 4 or more,  
664 which corresponds to over 80% hydrogenotrophic methanogenesis based on Equation 1. In the  
665 current study comparative results from the  $^{14}\text{C}$  assay were not available after day 155, but it is  
666 intriguing to note that in the last two weeks of operation of M4 the  $^{14}\text{C}$  ratio fell to below 1.8  
667 (Figure 2), indicating an increase in acetoclastic methanogenesis with over one third of  
668 methane production now coming from this route. During this period the abundance of  
669 *Methanosarcinaceae* was unchanged, thus suggesting a further shift by this versatile family to  
670 a preferred metabolic pathway. The current work makes it clear that OLR and HRT play an  
671 important role in determining the transition to a dominant community and pathway in digesters

672 fed on real organic feedstocks; and thus may partially explain some of the otherwise apparently  
673 conflicting results in the literature on batch and semi-continuous systems at high ammonia  
674 concentrations (Fotidis et al., 2013; Hao et al., 2017; Yan et al., 2018).

675

676 Regression analysis was also conducted between the estimated percentage of methane  
677 produced by the acetoclastic route and the relative abundance of dominant families during the  
678 transition periods. The results for *Methanosaetaceae*, declining from dominance in the  
679 respective transition periods, are shown in Figure 4d and indicate reasonably strong  
680 relationships in both M2 (days 118-146; n = 5, p < 0.05) and M4 (days 97-146, n = 8, p <  
681 0.005). Similar but inverse relationships between the percentage of methane produced by the  
682 acetoclastic route and the relative abundance of the increasing hydrogenotrophic population  
683 were also found in these periods for *Methanobacteriaceae* ( $R^2 = 0.732$ , p = 0.06) in M2 and  
684 *Methanosarcinaceae* ( $R^2 = 0.760$ , p < 0.005) in M4. Despite the fact that the 16S rRNA  
685 technique may include material from inactive microbes, the genetic analysis thus showed  
686 relatively good agreement with the  $^{14}\text{C}$  analysis, providing added confidence in the results from  
687 both methods, the applicability of Equation 1, and the overall interpretation of the transition  
688 period data. Observation of changes during a transition period can thus support and  
689 complement previous studies reporting steady-state values from digesters in operation under  
690 different conditions.

691

#### 692 **4 Conclusions**

693

694 The pattern of change in the chemical characteristics of the digesters in response to increasing  
695 ammonia concentrations was typical of that previously reported when using source segregated  
696 food waste as feedstock. Addition of trace elements was essential to prevent rapid accumulation

697 of VFA at an otherwise inhibitory ammonia concentration, as demonstrated in previous studies.  
698 As expected, at the loadings used the initial peak in VFA was sufficient to cause a fall in pH  
699 and in specific methane production in unsupplemented digesters. The work confirmed that  
700 where trace elements were added, the digestion process was resilient to the rise in ammonia  
701 concentration and transitioned smoothly from a predominantly acetoclastic to a predominantly  
702 hydrogenotrophic metabolic route. This change could be monitored using the  $^{14}\text{C}$  labelling  
703 technique, and under the lower loaded conditions at a higher HRT the gradual change in  $^{14}\text{CO}_2$   
704  $/^{14}\text{CH}_4$  ratio reflected the increasing predominance of hydrogenotrophic *Methanobacteriaceae*  
705 within the archaeal community. The slower rate of transition and the lower final  $^{14}\text{CO}_2/^{14}\text{CH}_4$   
706 ratio seen at the higher loading and shorter HRT, coupled with the strong predominance of  
707 *Methanosarcinaceae* in the archaeal community, indicated that both acetoclastic and  
708 hydrogenotrophic pathways were being used by members of this group, which is known to  
709 have metabolic capability for both routes. This is the first time evidence of such simultaneous  
710 multi-trophic behaviour has been reported in conventional anaerobic digestion. It can be  
711 concluded that the make-up of the archaeal community and the dominant metabolic pathway  
712 is not solely due to relative tolerance to ammonia, and can be influenced by other factors  
713 including OLR or HRT or both. Monitoring of the transition also gave strong support to the  
714 idea that analysis of the  $^{14}\text{CO}_2/^{14}\text{CH}_4$  ratio can be used to provide an estimate of the proportion  
715 of methane produced by the acetoclastic and hydrogenotrophic routes which is at least semi-  
716 quantitative in nature, thus enabling comparisons between digesters working under different  
717 operating conditions. The results also provide an insight into how the archaeal population could  
718 be manipulated to increase hydrogenotrophic activity, which may have a useful purpose in  
719 future applications such as microbial  $\text{CO}_2$  reduction.

720

721 **Data accessibility**

722 Raw, unprocessed fastq data were deposited to the European Nucleotide Archive and can be  
723 accessed from PRJEB30275 study.

724

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729

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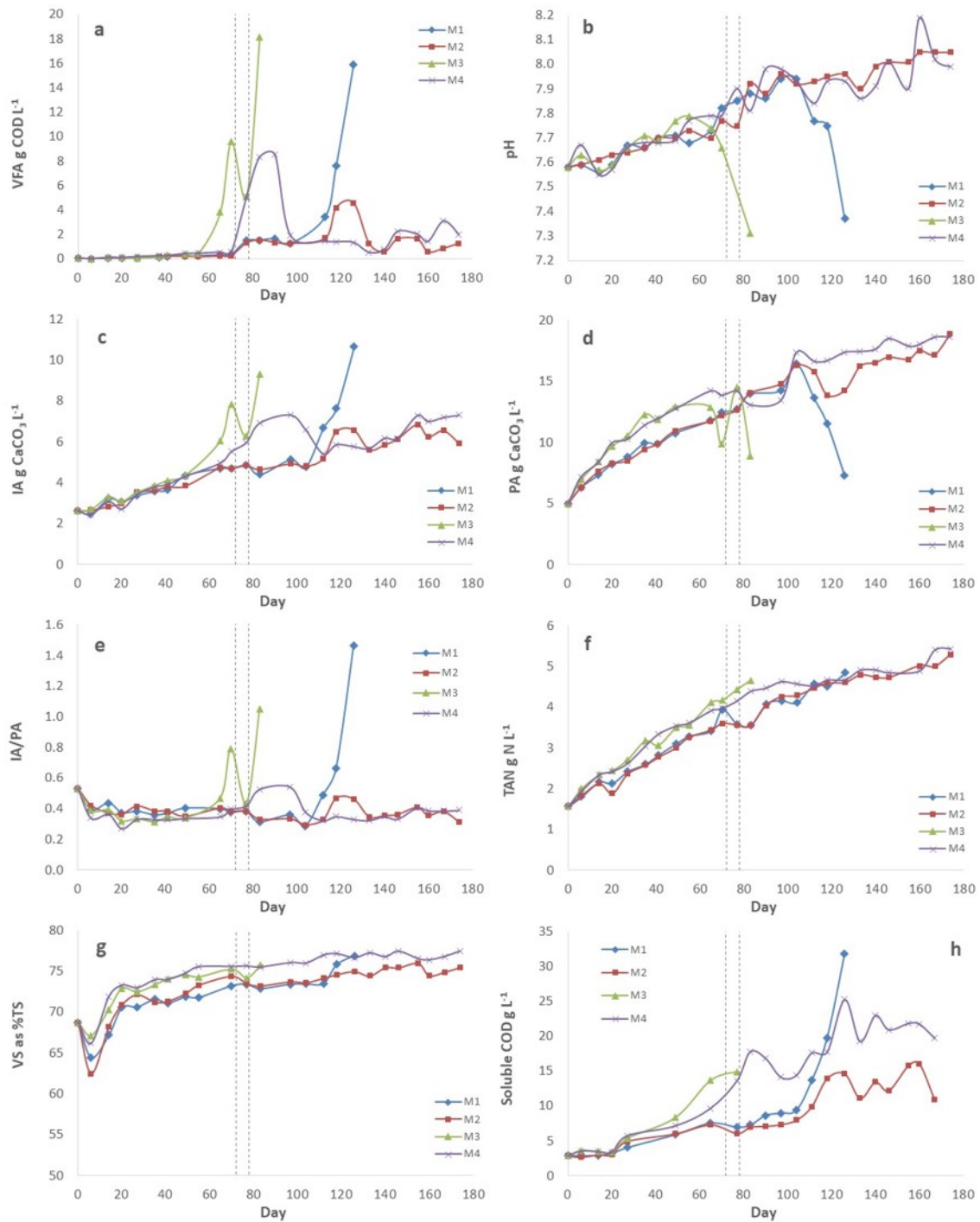
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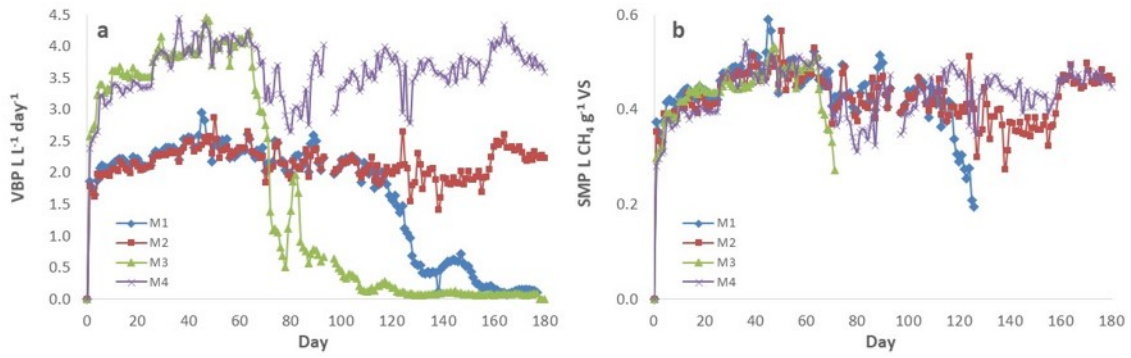
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878 **Figures and Tables**

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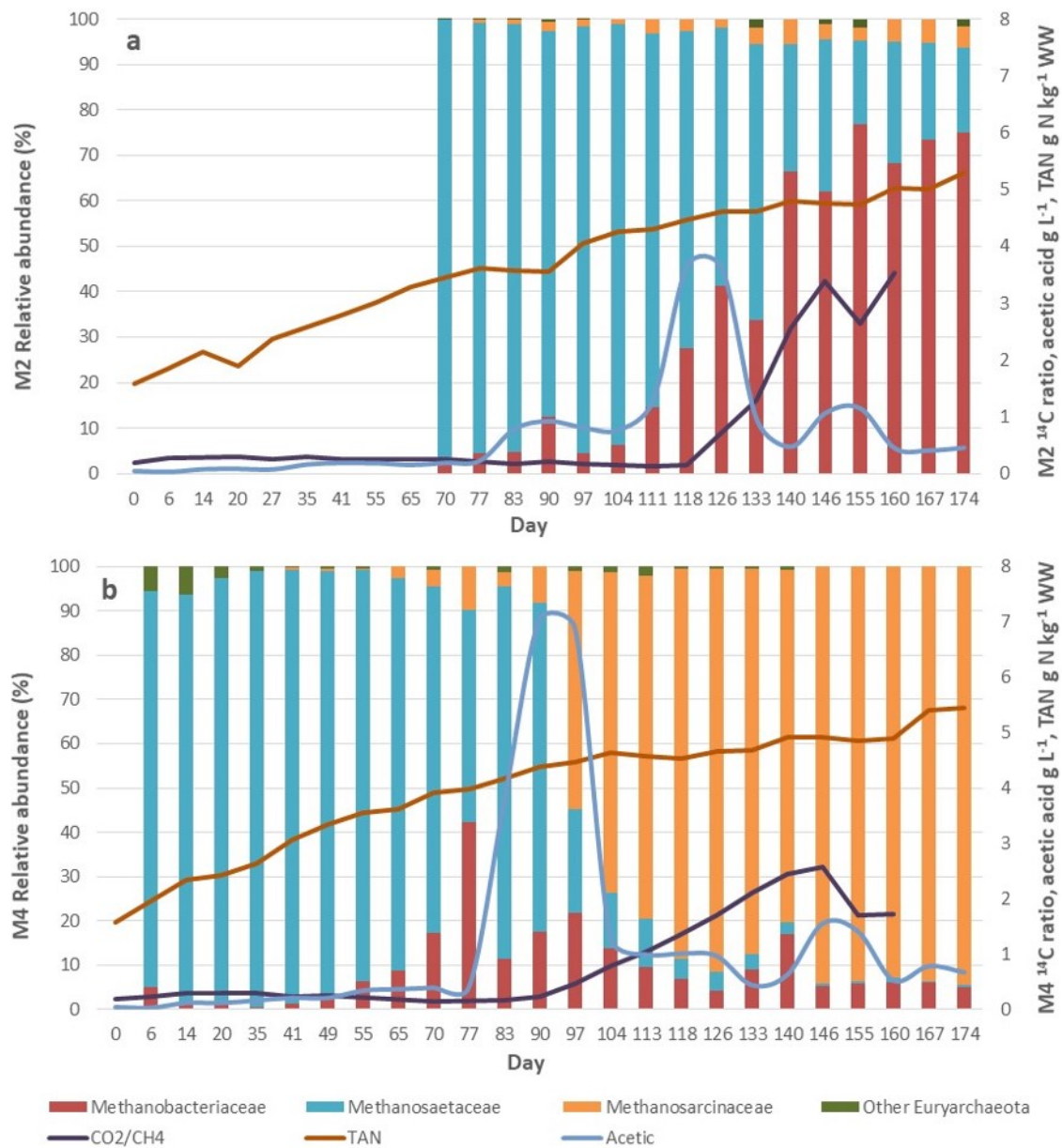


**Figure 1** Selected monitoring parameters for all digesters during experimental period: (a) VFA, (b) pH, (c) IA, (d) PA, (e) IA/PA, (f) TAN, (g) VS/TS and (h) soluble COD. Vertical dotted lines indicate period when M3 was not fed.



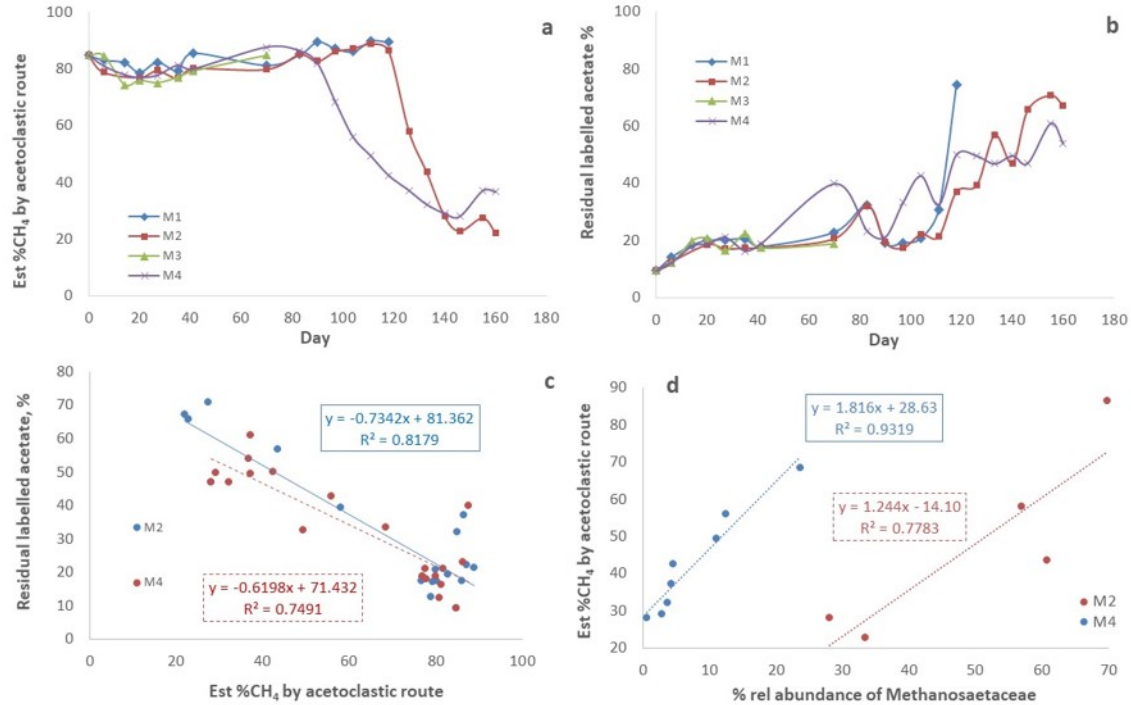
**Figure 2** Gas production for all digesters during experimental period: (a) VBP, (b) SMP.

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**Figure 3** Relative abundance of archaeal groups,  $^{14}\text{C}$  ratio, TAN and acetic acid concentrations in digesters: (a) M2 and (b) M4 during the experimental period

882



**Figure 4** Results based on for  $^{14}\text{C}$  assay and Equation 1: (a) Estimated proportion of  $\text{CH}_4$  produced by acetoclastic route for digesters M1-4 against time; (b) residual labelled acetate in  $^{14}\text{C}$  assay for digesters M1-4 against time; (c) Estimated proportion of  $\text{CH}_4$  produced by acetoclastic route versus residual labelled acetate in  $^{14}\text{C}$  assay for digesters M2 and M4; (d) Estimated proportion of  $\text{CH}_4$  produced by acetoclastic route versus relative abundance of *Methanosaetaceae* during transition periods in digesters M2 and M4

883

884 **Table 1** Physicochemical characteristics of foodwaste

Parameter	Units	Value	SD
Total solids	g TS $\text{kg}^{-1}$ WW	238.5	$\pm$ 1.2 <sup>a</sup>
Volatile solids	g VS $\text{kg}^{-1}$ WW	206.8	$\pm$ 2.1 <sup>a</sup>
TKN	g N $\text{kg}^{-1}$ WW	7.0	$\pm$ 0.12



Elemental C	% of TS	50.28	±	0.75
Elemental H	% of TS	6.37	±	0.07
Elemental N	% of TS	3.68	±	0.09
Carbohydrate	g kg <sup>-1</sup> VS	492.1	±	12.5
Lipid	g kg <sup>-1</sup> VS	197.0	±	2.2
Crude protein	g kg <sup>-1</sup> VS	211.2	±	3.7
Calorific value	MJ kg <sup>-1</sup> TS	22.0	±	0.06
	MJ kg <sup>-1</sup> VS	25.4	±	0.06

885 

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SD = standard deviation for triplicate samples unless noted.

886 <sup>a</sup> 8 samples

887