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Abstract

Microbial communities play an essential role in the biochemical pathways of anaerobic digestion processes. The correlations between microorganisms' relative abundance and anaerobic digestion process parameters were investigated, by considering the effect of different feedstock compositions and hydraulic retention times (HRTs). Shifts in microbial diversity and changes in microbial community richness were observed by changing feedstock composition from mono-digestion of mixed sludge to co-digestion of food waste, grass clippings and garden waste with mixed sludge at hydraulic retention times (HRT) of 30, 20, 15 and 10 days. Syntrophic acetate oxidation along with hydrogenotrophic methanogenesis, mediated by *Methanothermobacter*, was found to be the most prevalent methane formation pathway, with the only exception of 10 days' HRT, in which *Methanosarcina* was the most dominant archaea. Significantly, the degradation of complex organic polymers was found to be the most active process, performed by members of *S1* (*Thermotogales*), *Thermonema* and *Lactobacillus* in a reactor fed with a high share of food waste. Conversely, *Thermacetogenium*, *Anaerobaculum*, *Ruminococcaceae*, *Porphyromonadaceae* and the lignocellulosic-degrading *Clostridium* were the significantly more abundant bacteria in the reactor fed with an increased share of lignocellulosic biomass in the form of grass clippings and garden waste. Finally, microbes belonging to *Coprothermobacter*, *Syntrophomonas* and *Clostridium* were correlated significantly with the specific methane yield obtained in both reactors.

ii

1. Introduction

The use of anaerobic digestion (AD) to treat wastewater and municipal organic waste has increased worldwide. AD is a complex biological process that converts biomass into biogas through different microbial pathways and biochemical reactions (Angelidaki et al., 1999; Appels et al., 2008; Favaro et al., 2013). One of its benefits is the recovery of biomethane, a versatile carrier of renewable energy, which can be used for electricity and heat production or as a transport fuel (Pöschl et al., 2010; Weiland, 2010). Mono-digestion of diluted substrates such as sewage sludge and manure is nowadays economically challenging because of the low energy production. Compared to mono-digestion, co-digestion of multiple substrates provides significant advantages, including a more balanced supply of nutrients, a diluting effect for toxic and inhibiting compounds and overall increased biogas production, the result of the enhanced supply of organic compounds (Mata-Alvarez et al., 2014, 2000).

Disturbances in the stability of the AD process can occur when operational parameters deviate from normal operating conditions, causing, for example, the accumulation of volatile fatty acids (VFAs) and ammonia, and a subsequent inhibition of microbial activity (Chen et al., 2008; Gerardi, 2003; Mao et al., 2015). Microbial diversity, activities and interactions can also be affected by process parameters (e.g. temperature and ammonia), which in turn affect overall AD performance (Goux et al., 2016; Lin et al., 2016). Understanding the microbial community structure and pathways in AD is thus important, to ensure the regular operation and performance of the AD process. Currently, due to technological advancements, general knowledge on AD microbial community compositions and the roles of bacteria and archaea in the degradation process is well established (Campanaro et al., 2016a; Eikmeyer et al., 2013). However, only a few studies have investigated correlations between microbial community composition and process parameters (Campanaro et al., 2016b; Luo et al., 2015; Rivière et al., 2009a).

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76   Biochemical pathways involved in the AD process are based on rather complex and diverse

77 microbial roles. Therefore, it is important to understand the effect of the microbial community's

78 composition and function with regard to the operational parameters required to operate the digester

79 at optimum conditions and maximise energy recovery. Investigations conducted on seven anaerobic

80 digesters fed with sewage sludge have revealed that the core group of bacteria common to all

81 digesters is composed of six operational taxonomical units (OTUs) related to *Chloroflexi*,

82 *Betaproteobacteria*, *Bacteroidetes* and *Synergistetes* (Rivière et al., 2009a). Sludge-based AD

83 digesters – besides strict anaerobes – contain aerobic bacteria originating from the feedstock sludge,

84 which basically consists of aerobic bacteria, and so *Chloroflexi* appear mainly in sludge-based AD

85 processes. Another study regarding sewage sludge digesters has found that the most common

86 archaeal taxa are *Methanomicrobia*, *Methanobacteria* and *Thermoplasmata* (Narihiro and

87 Sekiguchi, 2007). Microbial community variations can influence the AD process and thereby inhibit

88 or enhance the process. For example, it has been shown that the bio-augmentation of

89 hydrogenotrophic methanogen (*Methanoculleus bourgensis* MS2T) in an anaerobic digester can

90 play a significant role in overcoming ammonia inhibition (Fotidis et al., 2014). A shift in

91 methanogenic pathways and methanogenic community composition has been observed when the

92 microbial culture is exposed to increasing concentrations of acetate and ammonia (Fotidis et al.,

93 2013), while specific bacteria such as the filamentous *Microthrix* or *Nocardia* have been shown to

94 be associated with foaming incidents in biogas reactors (Kougias et al., 2014). A common feature of

95 all these studies is that they provide a snapshot of microbial community composition and activity at

96 a given time and in specific conditions. However, the response and development of microbial

97 communities to external changes in process conditions, to date, has not been reported adequately in

98 literature. This information is relevant to ensure smooth transitions when changing process

99 operations or treating specific substrates.

100   The main objective of this research was to study changes in the microbial population

101 community as a response to variations in the operation of the AD process and co-digestion of urban

102 organic waste (UOW) comprising food waste, grass clippings and garden waste with mixed sludge.

103 This was achieved by: (i) analysing the composition of the microbial community during UOW co-

104 digestion in continuously stirred tank reactors (CSTRs), operated at sequentially reduced hydraulic

105 retention times (HRTs), (ii) comparing two CSTRs fed with different UOW mixing ratios, co-

106 sewage sludge digestion, food waste, grass clippings and garden waste and (iii) analysing changes

107 in the microbial population community in terms of relative abundance and diversity, and correlating

108 these findings with reactor performance and operational process parameters.

109   **2. Materials and methods**

110 **2.1 Characterisation of input feedstock materials**

111   The feedstock materials included mixed sewage sludge, food waste, grass clippings and

112 garden waste, which were collected from several locations in Denmark, as described in Fitamo et al.

113 (2016a). The addition of UOW to existing AD operations at wastewater treatment plants (WWTPs)

114 is able to boost biogas production, and current biogas reactor facilities at WWTPs can be used in

115 this regard (Fitamo et al., 2016b). Organic feedstock was shredded into small particles with a shear-

116 shredder (ARP SC 2000) and knife mill (Wiencken 19225 and Fitzmill model D, Daso-6).

117 Individual organic waste materials were then characterised in terms of physicochemical properties

118 (e.g. total solids (TSs), volatile solids (VSs), total Kjeldahl nitrogen (TKN), lipids, VFAs, proteins,

119 total C and total N). The analytical methods are described in Fitamo et al. (2016a).

120 **2.2 Experimental set up and operation**

121 The co-digestion experiment was conducted to maximise biogas production from UOW, by adding

122 food and plant materials (garden waste and grass clippings) to existing sludge digestion at WWTPs.

123 The laboratory experimental work was carried out in two CSTRs, named R1 and R2, each with a

124   working volume of 7.5 L. The temperature was kept constant in thermophilic conditions (55°C) and

125   with hot water circulation supplied by a circular closed heating system. The co-substrates were fed

126   into the reactor via an automated feeding system, based on the organic loading rate (OLR) of the

127   reactor. The set-up was equipped with an automated stirring system and a water displacement gas-

128   metering counter to measure the amount of biogas produced. The CSTRs were operated in five

129   distinctive operational phases. Phase I aimed at establishing a baseline performance relative to

130   existing sewage sludge AD, and it included the mono-digestion of 100% mixed sludge (primary and

131   secondary sludge mixed at a 1:1 V/V ratio) in both R1 and R2, with a HRT of 30 days (HRT30).

132   After Phase I, UOW was added to the mixed sludge and fed into the reactors in fixed percentage VS

133   mixing ratios throughout Phases II to V. Reactor R1 received 10:67:16:7 and reactor R2 received

134   10:44:32:14 of sewage sludge, food waste, grass clippings and garden waste, respectively. The

135   ratios were set up in order to have high food waste in R1, while the VS share of food waste was

136   reduced but the lignocellulosic garden and clippings feedstock doubled in R2. An overview of the

137   experimental setup is provided in Table 1, which shows that the HRT was reduced stepwise, from

138   30 days (HRT30) in Phase II, to 20 days (HRT20) in Phase III, to 15 days (HRT15) in Phase IV

139   and, finally, to 10 days (HRT10) in Phase V. Phases I, II, III, IV and V lasted for about 2.5, 1.9, 1.6,

140   2.8 and 2.5, respectively. Specific methane yield, productivity, concentrations of ammonia and

141   acetate measured during the co-digestion of UOW in R1 and R2 are provided in Figure S-1 in the

142   Supporting Information (SI) (Fitamo et al., 2016a).

143   <Table 1 here>

144   **2.3 Sampling and DNA extraction**

145   Within each operational phase, duplicate reactor broth samples (10 mL) were taken from

146   both reactors once steady-state conditions were reached – this amounted to 10 samples in total.

147   Residual plant particles present in the samples were removed, using a 100 µm nylon cell strainer

148   filter. Centrifugation of the filtered samples (10,000 rpm, 10 minutes) was conducted to obtain ~1.5

149   g of cell pellet. The total microbial DNA extraction (DNA isolation and purification) was

150   performed using the PowerSoil® DNA Isolation Kit protocol (MO BIO Laboratories, Carlsbad,

151   CA) with an additional initial cleaning step by Phenol:Chloroform:Isoamyl Alcohol 25:24:1 pH 8

152   (Sigma-Aldrich, DK). The quality of the purified DNA was examined with gel electrophoresis, and

153   the DNA concentration was analysed with NanoDrop 2000 (ThermoFisher Scientific, Waltham,

154   MA).

155   **2.4 16S rRNA gene sequences**

156   The samples were sequenced by utilising the Illumina MiSeq platform at Ramaciotti Centre

157   for Gene Function Analysis, University of New South Wales (Sydney, Australia), by amplifying the

158   V4 hypervariable region of the 16S ribosomal gene RNA using 515f-806r primers and following the

159   protocol of the Earth Microbiome Project (Earth Microbiome, 2011). The raw Illumina sequence

160   data obtained in this research work were submitted to the National Centre for Biotechnology

161   Information's (NCBI) sequence read archive database (SRP078424) under the bio-project number

162   (PRJNA328964). The sequences were analysed with CLC Genomic Workbench Software (V.8.0.2),

163   equipped with a microbial genomics module plug-in as previously described (Kougias et al.,

164   2016a). OTUs were aligned using MUSCLE software (Edgar RC, Nucleic Acids Res). The

165   Maximum Likelihood Phylogenetic tree, Alpha diversity index and Beta diversity were computed

166   as described by Kougias et al., (2016). The total number of reads obtained and total OTUs with

167   corresponding taxonomy assignment for the microbial community in both R1 and R2 are reported in

168   Table 1. OTUs with 10 sequences or fewer were considered extremely rare and were discarded from

169   further analysis. Direct comparison of the microbial relative abundance between the samples was

170   performed at genus and phylum level and was calculated as a percentage of the total community for

171   each sample.

172   The classification used to make the comparison of percentage relative abundance was

173   carried out with highly abundant (> 0.5% relative abundance) and lowly abundant (between 0.01% -

174   0.5% of relative abundance) OTUs, whereas they were discarded from analysis when lower than

175   0.01%. Most of the result and discussion section focuses on the most abundant microbes in the

176   community (> 0.5% of relative abundance), while the less abundant microorganisms were

177   considered only when statistically significant. Heat maps showing the relative abundance changes

178   (fold changes), due to comparisons of different retention times and feedstock compositions, were

179   prepared with the Multiexperiment viewer (MeV 4.9.0) (Saeed et al., 2003).

180   **2.5 Statistical analysis**

181   Statistics were performed using a general linear models analysis (GLM Procedure, SAS

182   Institute, 2009). Firstly, differences in microbial abundance in the two reactors (R1 or R2) and in

183   the subsequent phases (Phases I to Phase V) were studied in a series of single-trait analyses,

184   including the reactor and the phase as effects and the abundance of each microorganism as a trait.

185   Each microbial abundance was analysed separately with the GLM. The dataset for the analysis

186   consisted in all the pairs of replicates sampled within each reactor during the different phases (data

187   structure is reported in Table 1). In order to detect the trend of microbial abundance variation with

188   respect to a change in HRT, the phase was alternatively included in the analysis as a linear,

189   quadratic or cubic covariate. The model with the most significant shapes in variation (linear,

190   quadratic or cubic; $P \leq 0.05$) for the phase effect was therefore chosen for each microbial

191   abundance.

192   Methane yield, methane content of biogas, total VFA, individual VFAs, pH, reactor productivity

193   and ammonia were then used as traits to analyse variations in the operational process parameters.

194   Reactor (R1 or R2) and phase (Phase I to V) were treated as fixed effects (e.g. traits were analysed

195   by considering if they belonged to reactor R1 or R2, or to a specific Phase, I-V), and the abundance

6

196 of each microorganism as a linear covariate. In this way, any variation in the operational process

197 parameters was considered as depending on the variation in microbial abundance. Single-trait

198 models were run, all including the same fixed factors (*fixed*) and each one considering different

199 biochemical parameter; *i* (*parameter*) as trait and a different microorganism; *k* (*microorganism*) as

200 covariate, that can be generalized as: $parameter_i = fixed + microorganism_{k:i}$. This approach was

201 used to avoid the over-parameterization of the model (i.e., to have too many parameters for the

202 number of data), and to avoid problems of overlapping variances due to the introduction of

203 microorganisms with similar variations in abundance in the same model.

204 **3. Results and discussion**

205 **3.1 General microbial community composition and diversity**

206   The phylogenetic composition of the most abundant bacteria and archaea (OTUs) in the

207 entire microbial community was established, based on the 16S rRNA gene sequence considering all

208 samples from both reactors in all of the considered phases (Figure 1). Between 90 and 96% of the

209 OTUs were classified at the phylum level, showing that the majority of the microorganisms found

210 in the reactors could be identified at the phylum level. In contrast, only 47-73% of the entire

211 community was classified at the genus level (Table 1). This shows strong diversity among the

212 samples. Further research, using advanced sequencing techniques, is needed to classify in detail any

213 unknown microbes and to understand their specific role in the complex anaerobic degradation

214 process.

215

216 < Figure 1 here >

217   In general, the bacterial community consisted of *Firmicutes*, *OP9*, *Synergistetes*,

218 *Proteobacteria*, *Bacteroidetes*, *Thermotogae*, *Dicyoglomi* and *Chrloroflexi* as the main phyla

219 (Figure 1 and Figure S-2). The predominance of phylogenetic groups such as *Firmicutes,*

220 *Proteobacteria* and *Bacteroidetes* was a result of their ability to degrade a wide range of substances

221   such as cellulose, proteins, pectin and other xenobiotic compounds (Chouari et al., 2005; Zitomer et

222   al., 2016). The only identified archaeal phylum was *Euryarchaeota* (Figure 1 and Figure S-3),

223   which is a well-known microorganism involved in biogas production. These results are comparable

224   to previous studies of dominant core microorganisms classified at the phylum level in biogas

225   reactors (Luo et al., 2015; Nelson et al., 2011; Rivière et al., 2009b; Sundberg et al., 2013).

226   Microbial community diversity between different operational reactor phases was evaluated

227   using principal coordinate analysis (PCoA), which assesses the similarities between the microbial

228   community among samples. The results of the PCoA analysis are provided in Figure 2, showing

229   that the samples were concentrated into four clusters corresponding to the individual operational

230   phases of the reactors. For both reactors, the results clearly demonstrate a shift in microbial

231   community diversity in accordance with changes in feedstock composition (AD of sewage sludge in

232   Phase I to AD of UOW in Phase II) and the HRT of the reactors (Phase II-III). Similarities and

233   differences between microbial community diversity in operational conditions could be explained

234   with PCoA, which could capture 64% of the variation of microbial communities, indicated by PCo1

235   and PCo2 as 47% and 17%, respectively.

236   During Phase I (100% mixed sludge) of the AD operation, the samples examined for both

237   R1 and R2 clustered closely when operating at HRT 30 days, as seen in Figure 2. In Phase II,

238   microbial community diversity decreased according to the PCoA and the alpha diversity (Figure S-

239   4) in both reactors, most likely because of the introduction of UOW co-substrates to the reactors

240   (the HRT of Phases I and II was the same at 30 days). This reduction in microbial community

241   diversity between Phase I and Phase II could be due to the higher amount of lipids and proteins in

242   the UOW in comparison to sewage sludge, thereby leading to inhibition of the microorganisms due

243   to the accumulation of VFAs and an increase in ammonia concentration (Fotidis et al., 2013;

244   Kougias et al., 2016b; Palatsi et al., 2010). It could also be the case that especially activated sludge

245   also contains microorganisms from the WWTP process, i.e. aerobic microaerophilic and facultative

8

246   microorganisms while urban organic waste consists of indigenous microbes (Favaro et al., 2013;

247   Kim et al., 2009). The microbial biomass in the sludge would decrease as the share of the sludge is

248   reduced in the co-digested feedstock. Moreover, the PCoA and alpha diversity showed that the

249   decrease in microbial diversity was more pronounced in R2 than in R1, in connection with the fact

250   that R1 received more food waste than R2, which instead was fed with a higher share of green

251   waste containing lignocellulosic material. This shows that the slowly degradable feedstock in R2

252   resulted in lower microbial community diversity compared to the readily degradable feedstock in

253   R1 (Figure 2 and Figure S-4) in Phase II (HRT30).

254   < Figure 2 here >

255   Keeping the feedstock composition constant, a reduction in the HRT from 30 days (Phase II)

256   to 20 days (Phase III) resulted in a shift in microbial community diversity (Figure 2) in both

257   reactors. This result could be due to the adaption of microorganisms to the new co-substrate in the

258   feedstock. However, microbial community diversity specifically increased in R2, when moving

259   from Phase II to III (Figure S-4).

260   When reducing the HRT from 20 to 15 days (Phase III to Phase IV), R1 and R2 showed

261   opposing behaviours (Figure 2), in that while R1 fed with food waste showed increased microbial

262   diversity, R2 fed with lignocellulosic material developed a more specialised microbial community.

263   Finally, in Phase V, the AD processes were operated at a very low HRT (10 days) – a drastic

264   condition that could lead to process instability and operational failure and bring the microbial

265   community to a point of imbalance. In R1, microbial community diversity decreased significantly,

266   indicating a wash out of non-adherent microbes responsible for food waste degradation (Figure S-

267   4), which are mainly present in the liquid part of the reactor. On the contrary, in R2, microbes

268   related to lignocellulosic degradation and adhering to the substrate were more resistant to the wash

269   out action.

3.2 Trends in microbial abundance variation

The relative abundance of microbes (bacteria and archaea) for each operational phase (Phase I to V) of R1 and R2 was provided in Figure 3. During Phase I, the most dominant microbes according to the taxonomy assignment at the phylum level were classified as *Firmicutes* (40-49%), *OP9* (11-13%) and *Synergistetes* (7-10%) in reactors R1 and R2 (Figure 3a). The relative abundance of *Synergistetes* and *OP9* decreased in line with decreasing HRTs. Both *Synergistetes* and *OP9* are known to ferment organic compounds (carbohydrates, organic acids) and cellulose, sugars, hemicellulose, respectively, into H_2 and acetate (Dodsworth et al., 2013).

< Figure 3 here >

Other bacteria, such as *Proteobacteria*, were abundant (11%) in Phase I (when the reactors were fed with sole-mixed sludge, MS), but they became undetectable when the reactors were fed with UOW co-substrates in Phase II, R1/30 and R2/30 (Figure 3a). Also, *Dictyoglomi* (1-5%), *EM3* (3–4%) and *Chloroflexi* (1–2%) disappeared when the substrate was changed from sludge to co-substrate (Phase I to Phase II) (MS to R1/30 and R2/30, Figure 3a), because *Chloroflexi* especially is known to come with feedstock sludge and is mainly seen in sludge digestions. These microbes were favoured in Phase I (MS), possibly because of the sludge adapting to AD, but they were less favoured compared to other microbes in the AD of UOW (Phase II - V), which could be due to the reduction in the amount of sludge in the influent. Other studies have reported that *Chloroflexi* are frequently found in digested sludge taken from waste water treatment plants (Chouari et al., 2005; Rivière et al., 2009a; Yamada et al., 2005).

On the contrary, microorganisms belonging to *Bacteroidetes* were completely absent in Phase I (MS) and were observed with high relative abundance (10.1%) in Phase II (R1/30 and R2/30, Figure 3a) and sequentially increased during Phases II to V (R1/30 to R1/10 and R2/30 to R2/10). During the AD of sludge in Phase I (MS), the relative abundance of *Thermotogae* at the

294 phylum level was 1% in R1, but this increased in subsequent operational phases with corresponding

295 values of 5%, 20%, 19% and 30% for Phases II (R1/30), III (R1/20), IV (R1/15) and V (R1/10),

296 respectively (Figure 3a). Microorganisms belonging to *Thermotogae* are known as hydrogen-

297 producing bacteria and produce acetate and CO_2 as by-products from biomass and organic waste

298 fermentation in thermophilic conditions. Similarly, an increasing trend in the relative abundance of

299 *Thermotogae* was observed in R2 (R2/30 to R2/10).

300 Regarding the archaeal community, methane-producing hydrogenotrophic *Methanothermobacter*

301 and *Methanosarcina* were the predominant and core taxa throughout the experiment (Figures 3b

302 and 3c), indicating that archaea are more independent than bacteria in response to different

303 feedstock compositions. Generally, from Phase I (MS) to Phase II (R1/30 and R2/30), the relative

304 abundance of *Euryarchaeota* increased from 2% to 9% and 7% in R1 (R1/30) and R2 (R2/30),

305 respectively (Figure 3a). On the contrary, they decreased in abundance from 3% to 0.5% (by a

306 factor of 5) and by 6% to 0.3% (by a factor of 9) in R1 (from R1/15 to R1/10) and R2 (from R2/15

307 to R2/10) when the HRT was changed from HRT15 (Phase IV) to HRT10 (Phase V) (Figure 3a),

308 thus indicating that archaea are more dependent on HRT than on feed composition. In both reactors

309 (R1 and R2), a considerable decrease in methane yield was also observed when the HRT was

310 changed from 15 days to 10 days as seen in Figure S-1 (SI), which may be due to overloading or

311 washout of *Euryarchaeota*.

312 In all phases, relative abundance of *Methanothermobacter* remained constant except in

313 Phase V (HRT10), where abundance decreased (Figure 3c, R1/10 and R2/10). The relative

314 abundance of *Methanosarcina* increased dramatically at HRT10 (Phase V, R1/10 and R2/10)

315 (Figure 3c). The genus *Methanosarcina* provides metabolic capability in both acetoclastic and

316 hydrogenotrophic methanogenesis and has also been reported to be more favourable in elevated

317 ammonia and VFA concentrations (Calli, 2005; De Vrieze et al., 2012; Staley et al., 2011).

3.3 Influence of different parameters on AD microbial community composition

3.4.1 The effect of feedstock composition

The percentage of relative microbial abundance considered in each reactor (R1 and R2) and in the different phases (Phases I to Phase V), averaged for the replicates, is shown in a heat map and also includes the fold changes of the most abundant microorganism in a steady-state condition in R1 and R2 (Figure 4). GLM analysis provided information about the significant variation in microbial abundance, due to the different UOW feedstock compositions, and to the operational phase. The core dominant genera found in both reactors were *Thermonema*, *S1* ($P \leq 0.001$), *Anaerobaculum* ($P \leq 0.05$), *Coprothermobacter* and *Methanothermobacter*, as seen in Figure 4 and Figure S-5 (SI). Species belonging to *Coprothermobacter* were identified as proteolytic anaerobic thermophilic microbes in the biogas reactors and also established syntrophy with hydrogenotrophic methanogens (Gagliano et al., 2015). Moreover, it is known that members of *Bacteroides* play a significant role in cellulose, fats and proteins degradation (Hatamoto et al., 2007; Li et al., 2013). Meanwhile, *Anaerobaculum* was found for the fermentation of organic acids and carbohydrates into acetate, hydrogen and CO_2 (Menes and Muxí, 2002).

< Figure 4 here >

Limited numbers of significant variations were found between the reactors fed with different UOW co-substrate compositions (Figure 4). Among the most abundant microbes (> 0.5% relative abundance), three OTUs classified as *Anaerobaculum*, *Thermacetogenium* and *Ruminococcaceae* were significantly more abundant (two to three times) in R2 compared to R1 ($P \leq 0.05$). *Thermacetogenium* is a thermophilic syntrophic acetate oxidising bacterium and has also been identified in the AD of kraft-pulp wastewater (Hattori, 2000). This finding confirmed that the methane production pathway was favoured by syntrophic acetate oxidation (hydrogenotrophic methanogens) in UOW co-digestion.

12

342   Other microbes, with a percentage relative abundance less than 0.5%, were significantly

343   enriched in R2 and belonged to *Porphyromonadaceae* (11 times more abundant in R2) and

344   *Clostridium* (three times more abundant in R2; P≤0.01). Members of the *Clostridium* genus are

345   known to degrade complex cellulose biopolymers (Guo et al., 2015; Nelson et al., 2011) and

346   lignocellulosic material components (Cirne et al., 2007; O'Sullivan et al., 2005).

347   *S1 (Thermotogales)* and *Thermonema*, (relative abundance (> 0.5%) decreased significantly in R2

348   compared to R1 (P≤0.05) by a factor of 2 and 1.3, respectively (Figure 4). *Thermotogales*

349   microorganisms are involved in the fermentation of substrates such as glucose, acetate, methanol

350   and starch as well as reducing elemental sulphur and sulphate (Balk et al., 2002; Feng et al., 2010).

351   R1 was enriched with carbohydrate and fat-degrading microorganisms of *Lactobacillus* (5 times;

352   P≤0.05) (Li et al., 2013). Other less abundant OTUs, such as *Exiguobacterium*, *Bacillus* and

353   *Allochromatium*, decreased in R2 compared to R1 by a factor of 6.4 and 6, respectively (Figure 4).

354   The rest of the microorganisms , apart from *Caldicoprobacter* (P≤0.05), were found in both R1 and

355   R2, irrespective of the feedstock.

356   *3.4.2 The effect of HRT*

357   Differences in microbial relative abundance, due to hydraulic retention times, were detected

358   by considering the effect of the operational phases on the abundance of each microbe. Figure 4 and

359   Figure 5, respectively reports the abundances of microbial communities in the two reactors in the

360   different phases and the changes in the relative abundance of microorganisms between phases.

361   Microbes related to the fermentation of sugars into acetate, lactate, ethanol, CO_2 and H_2, such as

362   *Thermonema*, *S1* and *Caldicoprobacter* (Bouanane-Darenfed et al., 2011), syntrophic acetate

363   oxidiser, such as *Thermacetogenium* (Hattori, 2000), and *Lactobacillus* increased by a factor of at

364   least seven (Figure 5). The GLM analysis (Figure 5) showed a significant trends for these

365   microorganisms, either linear (*Thermonema*; P≤0.01), quadratic (i.e. roughly assumed the shape of a

366   curve: *Caldicoprobacter*, *Thermacetogenium;* P≤0.001), or cubic (i.e. showing an inflection point:

367 *Lactobacillus*; P≤0.05). At HRT10 (Phase V) the community populations of *Caldicoprobacter,*

368 *Thermacetogenium* and *Lactobacillus* decreased in abundance (SI: Figure S-5), except those of

369 *Thermonema* and *S1* (Figure 6), which may be due to process inhibition resulting in a yield and

370 methane productivity drop.

371 < Figure 5 here >

372 The relative abundance of *Acinetobacter*, *Solibacillus*, *Dictyoglomus*, *Proteiniclasticum*,

373 *Exiguobacterium*, *Fervidobacterium*, *Bacillus*, *Allochromatium* and SMB53 decreased by a factor

374 of at least three in subsequent phases compared to Phase I (Figure 5 and Figure S-5 (SI)). The trend

375 of *Dictyoglomus* was linear (P≤0.01), The shape of variation for *Solibacillus* and *Proteiniclasticum*

376 was mainly linear (P≤0.05), but also a quadratic component was close to significance (P=0.06). The

377 other microorganisms had a mixed pattern of variation, with both linear and quadratic significant

378 components (P≤0.05).

379 < Figure 6 here >

380 During the AD process of Phase II to Phase V, OTUs members of *Fervidobacterium*,

381 *Bacillus*, *Allochromatium* and *SMB53* decreased in abundance, as shown in Figure 6 and Figure S-5

382 (SI). The most dominant genera in Phases II, III and IV at HRT30, HRT20 and HRT15,

383 respectively, were simple and complex sugar-fermenting bacteria (*S1*), proteolitic microorganisms

384 (*Coprothermobacter*), organic acid-degrading bacteria (*Anaerobaculum*), *Methanothermobacter* and

385 *Thermonema* ((Figure 6 and Figure S-5 (SI)). This could be due to increased OLR of UOW in the

386 feedstock. Additionally, Figure 5 shows an increasing trend of the dominant bacterial community,

387 *S1* and *Thermonema*, which could be due to higher specific growth rates surviving washouts at

388 shorter HRTs, and of a taxon belonging to *Firmicutes* (order *MBA08*; Figure S-2), another taxon

389 among the most representative. An almost decreasing trend of *Anaerobaculum* and

390 *Coprothermobacter* with respect to HRT, except for the last phase (HRT10), was also noted. The

391   methane-producing microorganism, namely *Methanothermobacter*, remained constant at HRT20

392   and HRT15 but dropped at HRT10.

393

394

395

396   **3.5 Biochemical correlation of the microbial community with AD process parameters**

397   The proper functioning of the AD process is influenced by a number of intertwined

398   microorganisms governing the complex biochemical pathways. Performance parameters measured

399   in the reactors, such as specific methane yield, methane productivity, ammonia concentration and

400   acetate (SI: Figure S-1) (reported in Fitamo et al., 2016a), were correlated with OTUs abundance.

401   The GML analysis (Table S-1, SI) produced a coefficient of linear regression for each biochemical

402   parameter-microbial abundance pair: a positive coefficient indicated that an increase in targeted

403   microbial abundance also caused an increase in the biochemical parameter under consideration,

404   whereas a negative coefficient indicated a decrease in the biochemical parameter, due to an increase

405   in microbial abundance (Table 2 and Table S-1). When comparing microbial community

406   composition with AD performance parameters (Figure S-1,SI), methane yield and productivity

407   significantly increased (P≤0.05; Table 2) when an increase in abundance variation occurred for the

408   OTUs assigned to Proteobacteria (*Acinetobacter iwoffii*, OTU: 532569; *Allochromatium*),

409   *Thermotogae* (*S1*, *Fervidobacterium*, two OTUs) and Bacteroidetes (*Thermonema*). On the other

410   hand, a significant decrease (P≤0.05; Table 2) in methane productivity and yield was observed

411   following an increase in the abundance of *Dictyoglomus*, *Fervidobacterium* (two OTUs) and in the

412   OTU 573124 belonging to *Acinetobacter*. Moreover, methane productivity and yield were

413   significantly affected by the abundance of microorganisms belonging to the phylum Firmicutes

414   (*Coprothermobacter*, *Syntrophomonas*, *Clostridium*, *Proteiniclasticum*, *Exiguobacterium*, *Bacillus*,

15

415 two OTUs *including Bacillus muralis*, *Solibacillus and SMB53*), which is mostly involved in the

416 hydrolysis of complex organic matter. Variation in the methane percentage was instead significantly

417 affected (P≤0.001) only by abundance of the OTU belonging to the phylum *Chloroflexi*, *class*

418 *Anaerolineae*, even if relatively low in abundance (P<0.05) (Table S-1), members of which may be

419 thermophilic or mesophilic, are generally ubiquitous and play an important role in the environment

420 (Yamada et al., 2006).

421   Considering the VFAs, a significant decrease in the abundance of these acids (P≤0.001;

422 Table 2), in particular in propionate (P≤0.05; Table 2), was related to an increase in

423 *Syntrophomonas* (OTU: 1110842), known to beta-oxidise saturated fatty acids to acetate or acetate

424 and propionate (Sieber, 2010). Propionate significantly decreased (P≤0.01; Table S-1) following an

425 increase in OTU 254504 belonging to the order *SHA-98* of the class *Clostridia*, phylum *Firmicutes*,

426 known to be involved in syntrophic acetate oxidation activities. The concentration of acetate

427 followed the same trend (P≤0.05) for methane yield and production, apart for *S1* (P=0.013; Table

428 2). Acetate also significantly increased when *Methanosarcina* increased (P<0.01; Table 2) and

429 seemed also to be significantly associated with the phylum *Firmicutes* (*Coprothermobacter*,

430 *Syntrophomonas*, *Clostridium*, *Solibacillus*), and with *Methanosarcina,* OTU positively correlated

431 with acetoclastic methanogens, because acetate is a substrate for *Methanosarcina* metabolism. On

432 the other hand, acetate variation was not related to variation in the hydrogenotrophic methanogen

433 *Methanothermobacter thermautotrophicus*, since methane is mainly produced via syntrophic acetate

434 oxidation association followed by hydrogenotrophic methanogenesis. Butyrate, the last VFA

435 considered in this study, resulted significantly in the abundance of *Syntrophomonas*, OTU 203894

436 and *Anaerobaculum* (phylum *Synergistetes*, OTU 533824; P≤0.001, Table 2), a genus able to reduce

437 substrates to butyrate with glucose as an electron donor.

438   The increase in *Anaerobaculum* abundance (P≤0.01; Table 2) was also related to a decrease

439 in the concentration of ammonia. Moreover, ammonia concentration increased in relation to the

440 increase in *Syntrophomonas*, OTU 203894, able to convert atmospheric molecular nitrogen to

441 ammonia (Sieber, 2010). pH variation was only affected by an OTU assigned to *Clostridium* (Table

442 2), whereby an increase in abundance was related to an increase in pH (P≤0.001), and by some

443 other non-abundant *Firmicutes* OTUs (Table S-1). Table S-1 (SI), providing an overview of the less

444 abundant OTUs significantly correlated with AD performance parameters, showed that biogas

445 production process was affected not only by dominant microorganisms, but also by less abundant

446 but crucial microorganisms.

447   Overall, the results of the microbial community analysis show that the composition of

448 feedstock and the process condition affects the diversity of the microbial community. The

449 biochemical correlation also reveals that certain groups of microbes particularly hydrolytic bacteria

450 are significantly correlated with anaerobic digestion process performance parameters. Knowledge of

451 changes in microbial community structures as a response changes in feedstock composition,

452 operational process parameter and reactor performance could help wastewater treatment plants and

453 biogas plant to enhance the methane yield and productivity through bioaugmentation.

454

455 **4. Conclusion**

456   The dominant microbial community, *Proteobacteria*, observed in sludge-based mono-

457 digestion decreased in abundance compared to the anaerobic co-digestion of urban organic waste

458 (UOW). Nevertheless, a new community, *Thermonema*, increased during the co-digestion of UOW.

459 Complex organic polymer degraders *Thermacetogenium*, *Anaerobaculum*,

460 *Ruminococcaceae* and *Clostridium* were significantly abundant in reactor fed with high share of

461 lignocellulosic material (R2) however *S1*, *Thermonema* and *Lactobacillus* were found to be

462 significantly abundant in reactor fed with high share of food waste (R1). The relative abundance of

463 *S1* and *Thermonema* increased, while other taxa such as *Coprothermobacter, Anaerobaculum* and

464 *Dictyoglomus* decreased in line with sequentially decreased HRTs.

465 Syntrophic acetate oxidation, followed by hydrogenotrophic methanogenesis, was

466 established as the main methane formation pathway in both R1 and R2. However, the relative

467 abundance of methanogenic *Euryarchaeota* (*Methanothermobacter*) decreased when the HRT was

468 changed from 15 to 10 days, in which case *Methanosarcina* became dominant. Methane yield was

469 correlated with several *Firmicutes* (*Coprothermobacter, Syntrophomonas, Clostridium*) involved in

470 the hydrolysis stage. The concentration of acetate was correlated with several OTUs, such as

471 *Methanosarcina* and *Acinetobacter iwoffii*, while the concentration of ammonia was associated with

472 *Anaerobaculum* and *Syntrophomonas.*

473 The particular microbial community composition and diversity of the corresponding

474 feedstock composition and operational parameters could support biogas plants to enhance the

475 anaerobic digestion process performance by using bioaugmentation of the respective

476 microorganisms to achieve rapid microbial adaptation and also optimal production of methane yield

477 and productivity.

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List of Figures

Figure 1: Phylogenetic trees of OTUs, describing the entire microbial community observed in both reactors (R1 and R2) during the AD of mixed sludge and co-digestion of urban organic waste at 55°C and HRTs of 30, 20, 15 and 10 days. The letter k_ denotes kingdom, p_ (phylum), c_ (class), o_ (order), f_ (family), g_ (genus) and s_ (species) taxonomical levels. Thick branches indicate bootstrap analysis values higher than 50.

Figure 2: Differences in microbial community diversity shown by principal coordinate analysis ordination (PCoA), considering differences in hydraulic retention time (Phases I to V) and feedstock composition (R1 and R2). The diamond shapes indicate the AD process in R1, while the circles represent R2. The arrows indicate changes in microbial composition.

Figure 3: The relative abundance of microorganisms based on the taxonomical classification of the microbial community in both reactors (R1 and R2) in each operational phase (Phase I to V) (a) identified at phylum, (b) identified at genus level (> 0.5 OTUs of relative abundance) and (c) archaeal community at genus level (> 0.5 OTUs). All other unidentified OTUs were included in "Unclassified". The letter MS denotes sole mixed sludge at HRT of 30 days (Phase I), the numbers 30 (Phase II),20 (Phase III),15 (Phase IV) and 10 (Phase V) denotes the hydraulic retention times at the respective reactors (R1 and R2).

Figure 4: The heat map of the average relative abundance of replicates of dominant microorganisms in the different phases (Phase I to Phase V) within R1 and R2 (on the left panel), and fold changes ($\log_2$(R1/R2)) from R1 to R2 (on the right panel). Colour scales are shown on top of each panel. On the left panel, the most abundant microorganisms are shown in red colour and the less abundant in blue and black. On the right panel, the relative abundance increment in fold change is coloured by red, while the decrease in fold change is coloured in green. The black colour indicates if there was no fold change. The asterisks close to the left and to the right panels indicate the significance of the phase and reactor effects, respectively (* P≤0.05; ** P≤0.01; *** P≤0.001), on the variation in average microbial abundance.

Figure 5: General trends of the most abundant microorganisms classified at genus level with respect to changes in operational phases (Phase I, Phase II, Phase III, Phase IV and Phase V). Abundance was calculated from averaged row data as logarithm of the ratio between each phase (II, III, IV and

V) and the reference phase (Phase I). The obtained results are denoted as Phase II (Phase II versus I), Phase III (Phase III versus I), Phase IV (Phase V versus I) and Phase V (Phase V versus I). Trends are classified in: a) linear; b) quadratic; c) cubic; d) mixed shapes of variation, according to the general linear models analysis (GLM).

Figure 6: The percentage of relative abundance of dominant microorganisms (> 0.5 OTUs) with a change in the operational phase: Phase I (R1 and R2), Phase II (R1 and R2) and Phase V (R1 and R2).

1   **Tables**

2   Table 1. Overview of process conditions and sequencing results. Co-digestion at HRTs of 30, 20, 15

3   and 10 days, with corresponding co-substrate compositions in R1 and R2. Feedstock composition is

4   shown as the ratios of sludge, food waste, grass clippings and garden waste, respectively, for R1

5   and R2 (all VS-based).

Sample	Phase	HRT (days)	Reactor	Feedstock	Reads assigned to taxa	OTUs (>10 reads)	>0.5% of relative abundance				
							Genus (%)	Family (%)	Order (%)	Class (%)	Phylum (%)
R1/MS-I	I	30	1	Sludge*	85069	186	62	87	88	88	90
R1/MS-II	I	30	1	Sludge*	110862	193	73	85	87	87	91
R1/30-I	II	30	1	10:67:16:7	109386	109	60	93	96	96	96
R1/30-II	II	30	1	>>	96073	113	63	92	95	95	95
R1/20-I	III	20	1	>>	67045	108	64	80	96	96	96
R1/20-II	III	20	1	>>	128505	132	60	80	96	96	96
R1/15-I	IV	15	1	>>	101730	135	62	72	94	94	94
R1/15-II	IV	15	1	>>	111771	198	63	74	92	92	93
R1/10-I	V	10	1	>>	123175	79	70	77	95	95	95
R1/10-II	V	10	1	>>	130529	128	63	72	94	94	94
R2/MS-I	I	30	2	Sludge*	136020	360	47	71	73	73	79
R2/MS-II	I	30	2	Sludge*	113342	229	72	86	88	88	90
R2/30-I	II	30	2	10: 44:32:14	108985	161	56	88	91	91	91
R2/30-II	II	30	2	>>	49967	121	54	92	94	94	94
R2/20-I	III	20	2	>>	116453	176	64	75	90	90	90
R2/20-II	III	20	2	>>	129588	133	49	70	90	90	90
R2/15-I	IV	15	2	>>	45503	142	62	75	93	93	93
R2/15-II	IV	15	2	>>	109209	145	68	78	93	93	93
R2/10-I	V	10	2	>>	42890	107	51	57	91	91	91
R2/10-II	V	10	2	>>	58895	134	47	52	91	91	91

6   *Mixture of primary and activated sludge*

7

8

9

Table 2. Significant sources of variation for AD biochemical performance parameters obtained in single-trait linear model analyses considering phases (Phases I-V) and reactors (R1 and R2) as fixed effects and the microbial abundance as a covariate. The P-value of microbial abundance is reported for each model run, and significant results (P≤0.05) are shown in bold and green font. The direction of the variations was indicated by different colours (blue: same variation; red: opposite variation; white: close to zero variation). When phase and reactor effects resulted as significant (P≤0.05) in a model, they were indicated with a P or R superscript close to the P-value of microbial abundance. Only the most interesting OTUs and biochemical parameters were reported (an extended list is provided in Table S-1).

OTUs	Phylum	Genus	CH$_4$ Productivity	CH$_4$ yield	% CH$_4$	VFA	Acetate	Butyrate	Propionate	Ammonia	pH
573124	Proteobacteria	*Acinetobacter*	**0.02** [PR]	**0.02** [PR]	0.28 [P]	0.2 [PR]	**0** [PR]	0.47	0.97	0.28 [P]	0.8 [P]
532569	Proteobacteria	*Acinetobacter*	**0.03** [PR]	**0.03** [PR]	0.32	0.2 [PR]	**0** [PR]	0.42	0.99	0.23 [PR]	0.8
563656	Proteobacteria	*Allochromatium*	**0.01** [PR]	**0.01** [PR]	0.2	0.1 [PR]	**0** [PR]	0.41	0.92	0.26 [P]	0.8
533824	Synergistetes	*Anaerobaculum*	0.84 [P]	0.78	0.77 [P]	0.5 [P]	0.56	**0** [P]	0.69	**0.01** [P]	0.4 [P]
302965	Firmicutes	*Bacillus*	**0.01** [PR]	**0.01** [PR]	0.26	0.2 [PR]	**0** [PR]	0.48	0.98	0.28	0.8
578257	Firmicutes	*Bacillus*	**0.01** [PR]	**0.01** [PR]	0.26 [P]	0.2 [PR]	**0** [PR]	0.46	0.99	0.27 [P]	0.8
210805	Firmicutes	*Caldicoprobacter*	0.37 [P]	0.3 [P]	0.69 [P]	0.7	0.55	0.9	0.15 [P]	1 [P]	0.5 [P]
1108449	Firmicutes	*Caldicoprobacter*	0.2 [P]	0.14 [P]	0.34 [P]	0.5 [P]	0.26	0.35 [P]	0.91	0.38 [P]	0.4 [P]
1047886	Firmicutes	*Clostridium*	0.38 [P]	0.31 [P]	0.58 [P]	0.8	0.6	0.91 [P]	0.27	0.94 [P]	0.4 [P]
220242	Firmicutes	*Clostridium*	0.32 [P]	0.41 [P]	0.08 [P]	0.3 [P]	0.42	0.59	0.47 [P]	0.68 [P]	0.1 [P]
2971192	Firmicutes	*Clostridium*	**0.01** [PR]	**0.01** [PR]	0.21	0.2 [PR]	**0** [PR]	0.46	0.99	0.28 [P]	0.8
1130771	Firmicutes	*Clostridium*	0.65 [P]	0.77 [P]	0.46 [P]	0.6 [P]	0.69	0.36 [P]	0.67	0.53 [P]	**0** [P]
272967	Firmicutes	*Coprothermobacter*	**0.01** [PR]	**0.01** [PR]	0.19 [P]	0.4	**0.05** [PR]	0.93	0.67	0.7 [P]	0.7 [P]
OTU-001	Dictyoglomi	*Dictyoglomus*	**0.01** [PR]	**0.01** [PR]	0.24 [P]	0.2 [PR]	**0** [PR]	0.5	0.97	0.3 [P]	0.8 [P]
189039	Firmicutes	*Exiguobacterium*	**0.05** [PR]	**0.05** [PR]	0.4	0.3 [R]	**0.01** [PR]	0.58	0.84	0.33	0.7
109610	Thermotogae	*Fervidobacterium*	**0.03** [PR]	**0.03** [PR]	0.33	0.2 [R]	**0** [PR]	0.53	0.91	0.3	0.7
559513	Thermotogae	*Fervidobacterium*	**0.02** [PR]	**0.02** [PR]	0.3	0.2 [R]	**0** [PR]	0.48	0.95	0.28	0.8
4415598	Firmicutes	*Lactobacillus*	0.71 [P]	0.65 [P]	0.58 [P]	0.8	0.45	0.19 [P]	0.67	0.08 [P]	0.5 [P]

3851582	Firmicutes	*Lactobacillus*	0.98	P	0.95	P	0.47	P	0.7		0.64		0.15	P	0.99		0.08	P	0.7	P
592689	Euryarchaeota	*Methanosarcina*	0.12	PR	0.13	PR	0.5	P	0.1	P	0.02	PR	0.26	P	0.84		0.11	P	0.8	P
369183	Euryarchaeota	*Methanothermobacter*	0.77	PR	0.8	P	0.65	P	0.8		0.52	R	0.73		0.75		0.45	P	0.7	P
167215	Firmicutes	*Proteiniclasticum*	0.01	PR	0.02	PR	0.27	P	0.2	PR	0	PR	0.48		0.96		0.28	P	0.8	
OTU-002	Thermotogae	S1	0.76	PR	0.82	P	0.18	P	0.3	P	0.85	R	1		0.08	P	0.82	P	0.5	P
777316	Thermotogae	S1	0.05	P	0.05	P	0.21	P	0.6		0.13		0.79		0.57		0.97	P	0.6	P
555945	Firmicutes	SMB53	0.01	PR	0.01	PR	0.22	P	0.1	PR	0	PR	0.44		0.96		0.27	P	0.8	
821325	Firmicutes	*Solibacillus*	0.01	PR	0.02	PR	0.27		0.2	PR	0	PR	0.47		0.97		0.27	P	0.8	
287657	Firmicutes	*Solibacillus*	0.02	PR	0.02	PR	0.31		0.2	PR	0	PR	0.4		0.98		0.22	R	0.8	
1110842	Firmicutes	*Syntrophomonas*	0.57	PR	0.65	P	0.27	P	0	PR	0.47	R	0.59	P	0.03	P	0.63	P	0.3	P
2677385	Firmicutes	*Syntrophomonas*	0.02	PR	0.02	PR	0.3		0.2	R	0	PR	0.45		0.95		0.26	P	0.8	
203894	Firmicutes	*Syntrophomonas*	0.89	P	0.85	P	0.77	P	0.4	P	0.6		0.01	P	0.57		0.02	P	0.5	P
247170	Firmicutes	*Thermacetogenium*	0.34	P	0.27	P	0.8	P	0.8		0.46		0.97	P	0.12		0.82	P	0.5	P
248523	Firmicutes	*Thermacetogenium*	0.35	P	0.27	P	0.87	P	0.9		0.43		0.75	P	0.15	P	0.62	P	0.3	P
242302	Firmicutes	*Thermacetogenium*	0.23	P	0.17	P	0.38	P	0.8		0.36		0.64	P	0.61		0.66	P	0.4	P
566078	Bacteroidetes	*Thermonema*	0	P	0	P	0.11	P	0.1	P	0	P	0.49		0.84		0.36	P	0.8	P

Figure 1

Figure 2

Figure 3

(a) Phylum level

Relative abundance (%)

MS R1/30 R1/20 R1/15 R1/10 MS R2/30 R2/20 R2/15 R2/10

unclassified
Thermotogae
Synergistetes
Proteobacteria
OP9
Firmicutes
Euryarchaeota
EM3
Dictyoglomi
Chloroflexi
Bacteroidetes

(b) Genus level

MS R1/30 R1/20 R1/15 R1/10 MS R2/30 R2/20 R2/15 R2/10

Unclassified
Thermonema
SMB53
Allochromatium
Bacillus
Fervidobacterium
Methanosarcina
Clostridium
Thermacetogenium
Exiguobacterium
Proteiniclasticum
Lactobacillus
Caldicoprobacter
Dictyoglomus
Solibacillus
Acinetobacter
Syntrophomonas
Methanothermobacter
Anaerobaculum
Coprothermobacter
S1

(C) Genus level: archaea communities

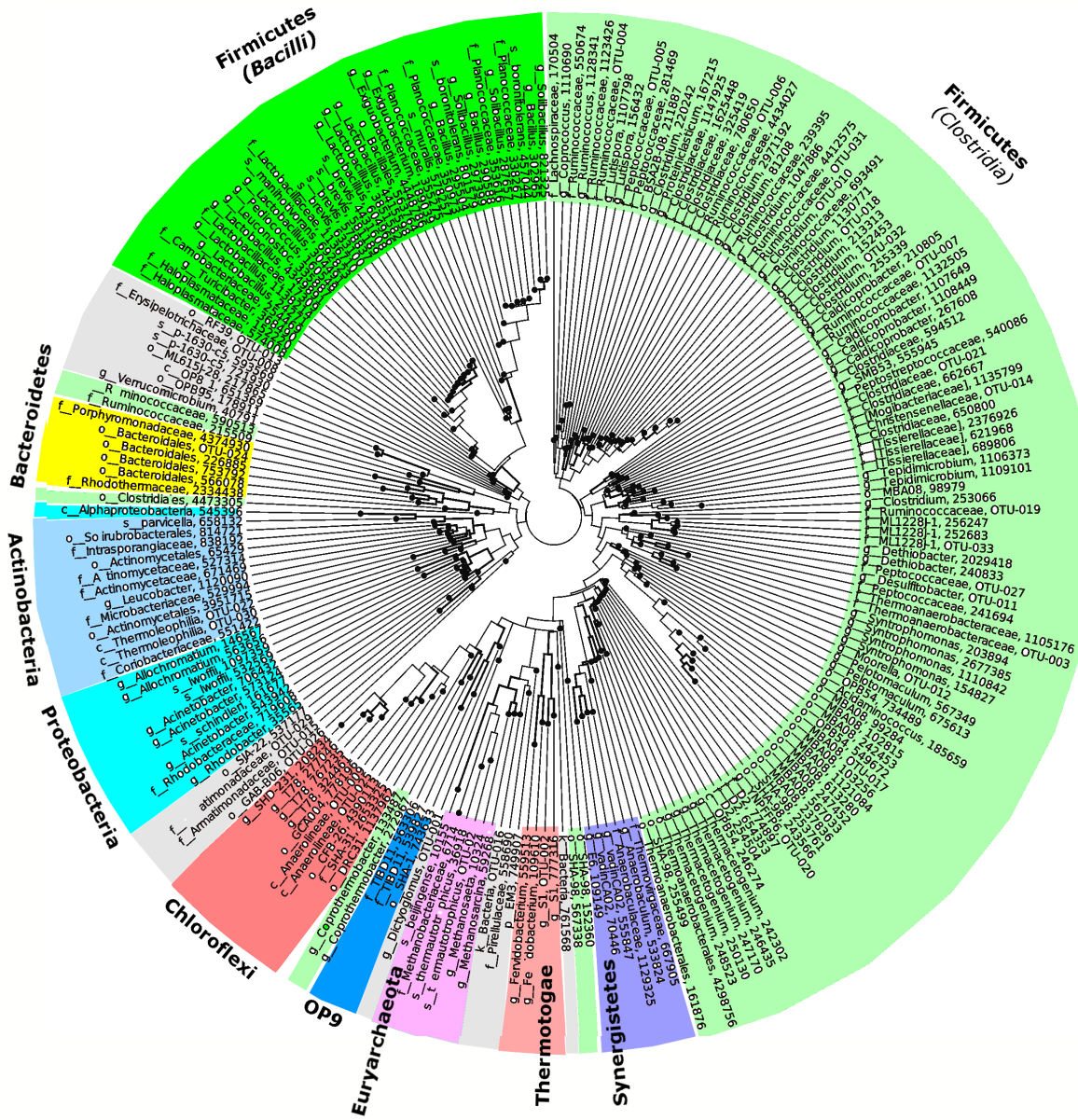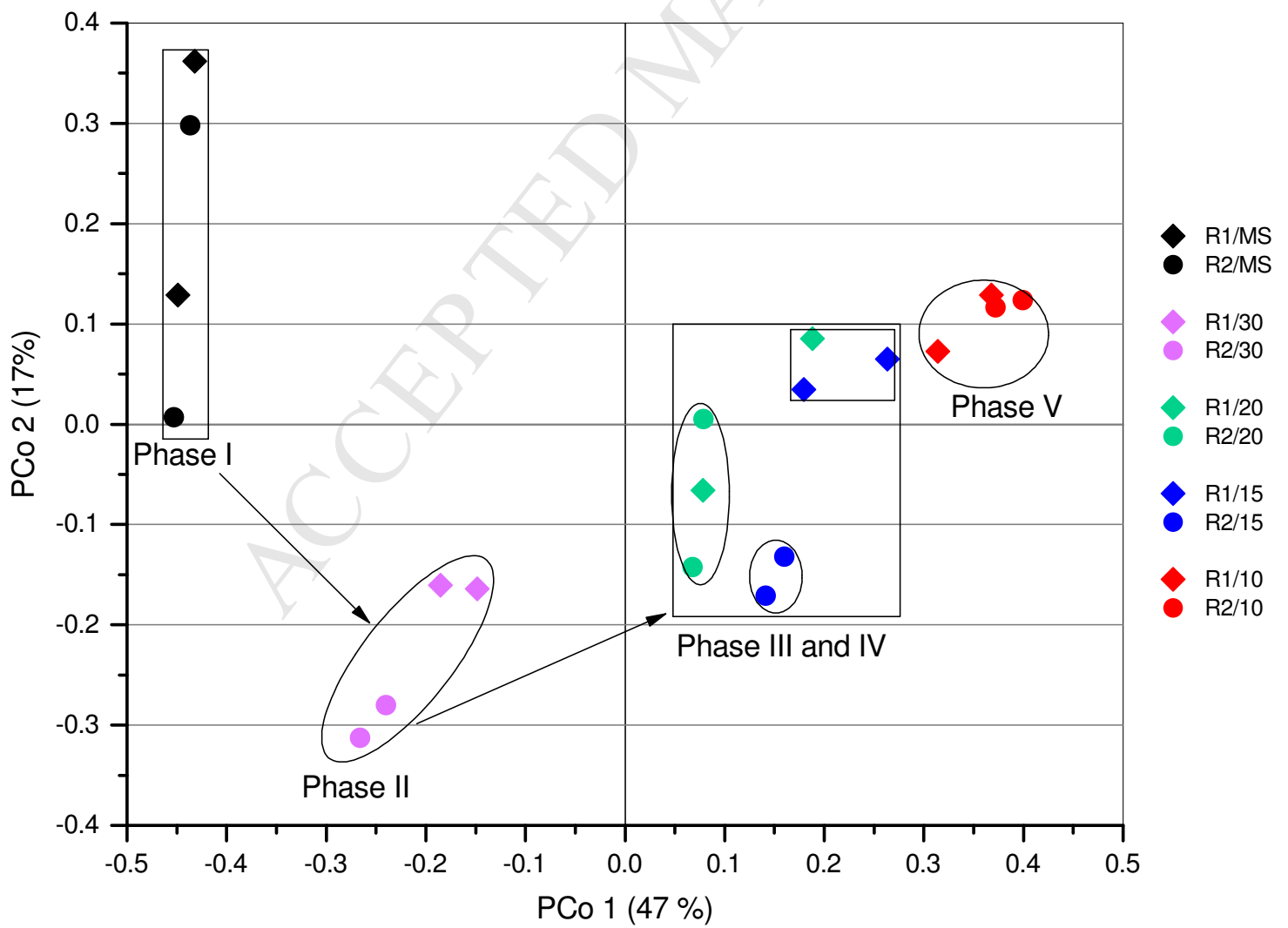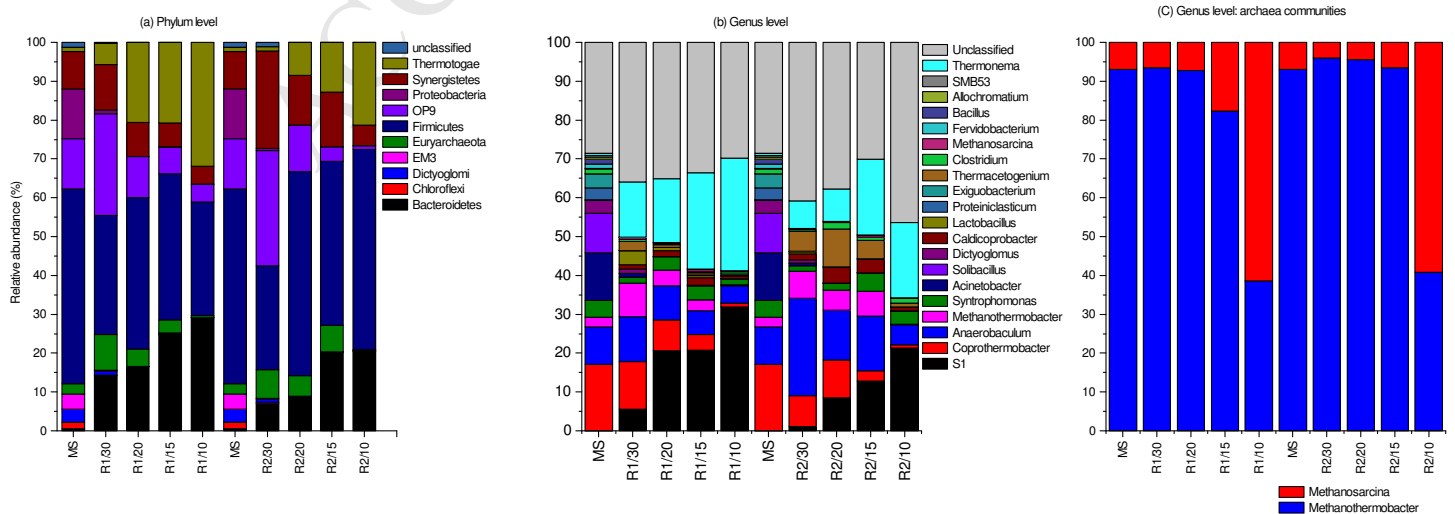MS R1/30 R1/20 R1/15 R1/10 MS R2/30 R2/20 R2/15 R2/10

Methanosarcina
Methanothermobacter

Figure 4
[Click here to download high resolution image](#)
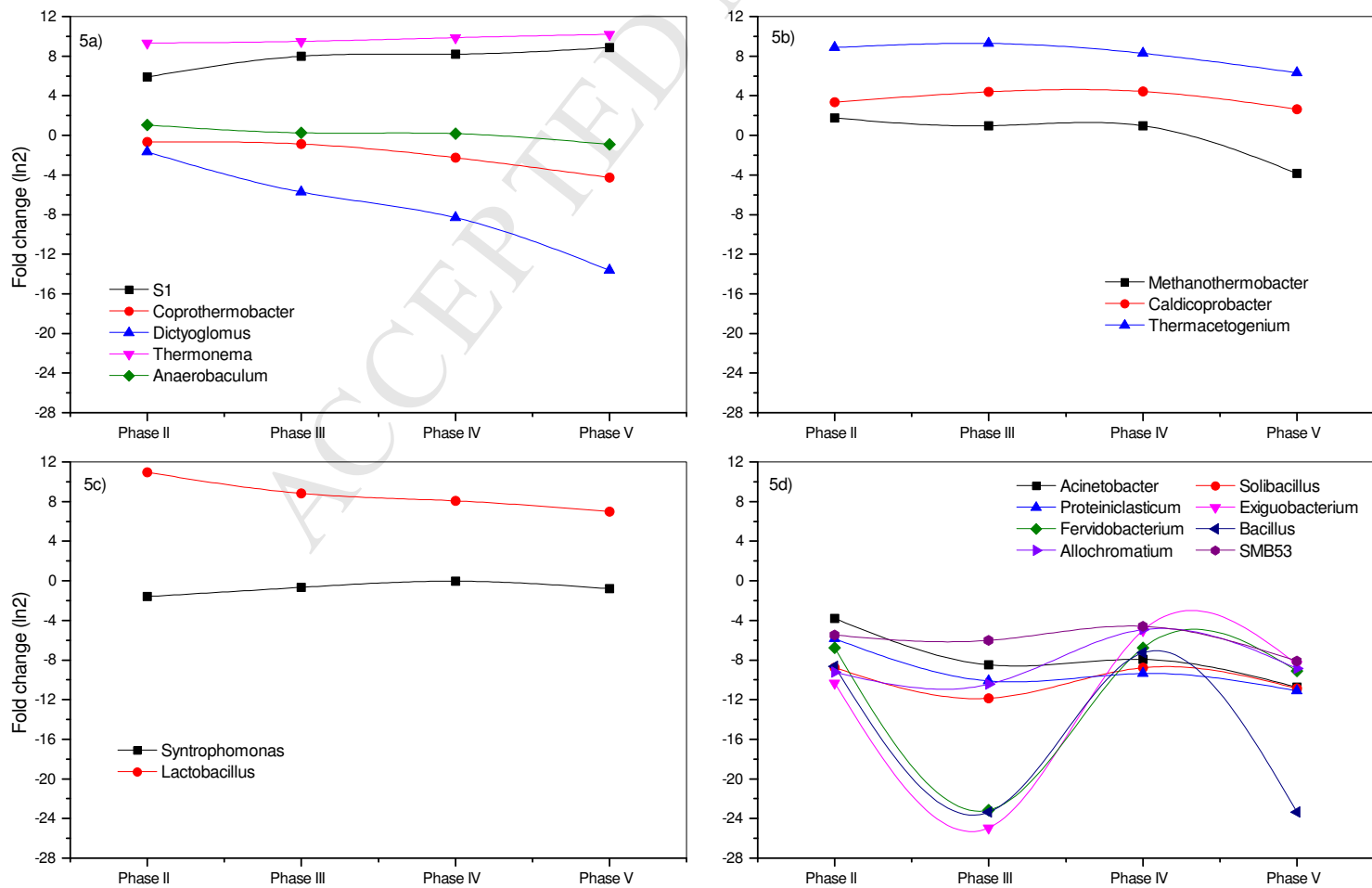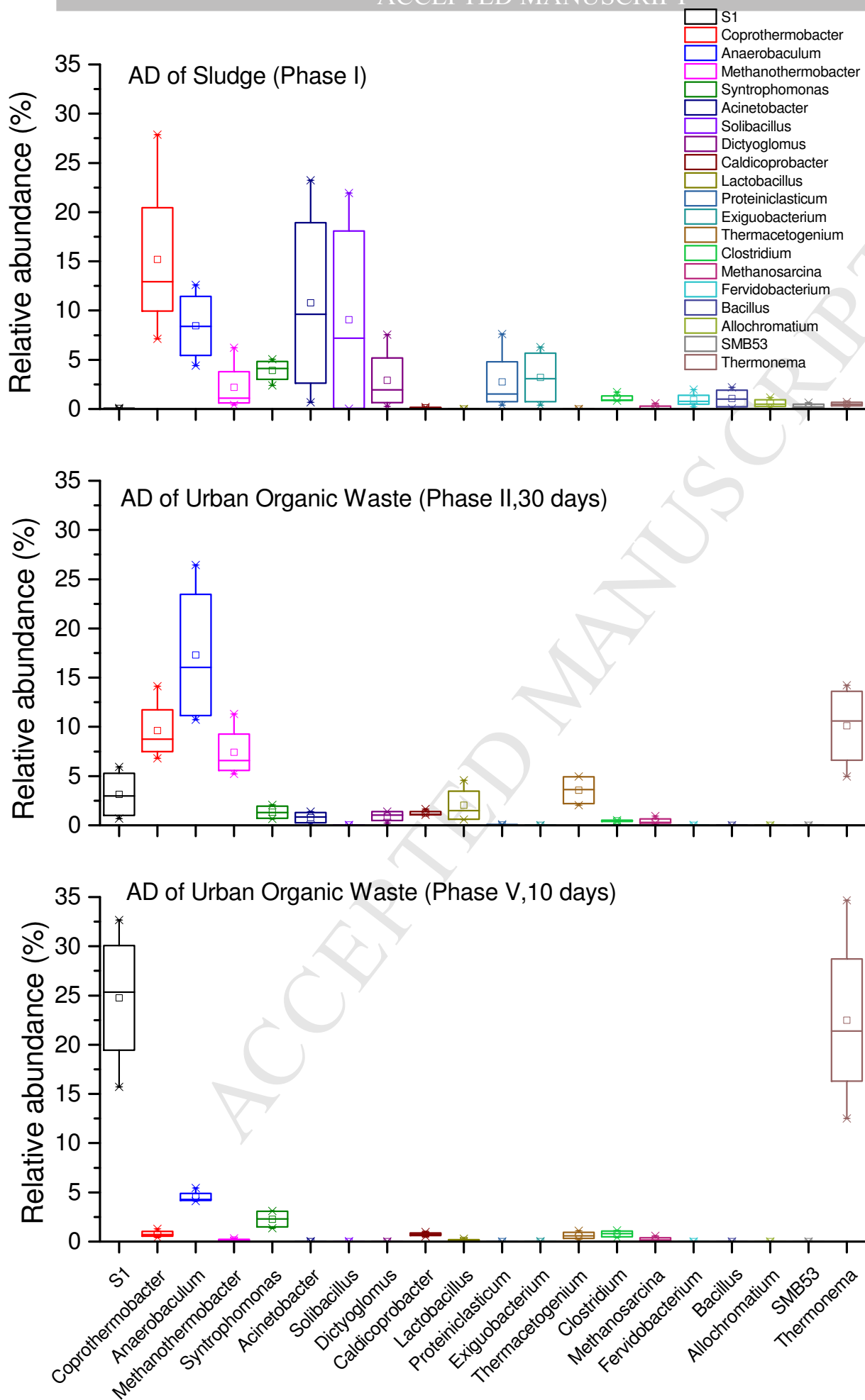
Figure 5

Figure 6

DTU Environment

DTU

To

Water Research

16 March 2017

Highlights: Concerning manuscript for publication in Water Research

➢ *Thermonema* was dominant in co-digestion of sewage sludge and urban organic waste.

➢ Potential pathogenic *Acinetobacter* found in sludge disappeared during co-digestion.

➢ When reducing hydraulic retention time, *Methanothermobacter* decreased in abundance.

➢ Methane and acetate significantly correlated to *Acinetobacter* and *Bacillus* abundance.

➢ Ammonia production significantly increased with the presence of *Syntrophomonas*.

Best regards,

Temesgen Fitamo (PhD student) and Co-authors

DTU Environment

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