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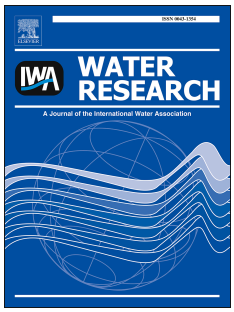

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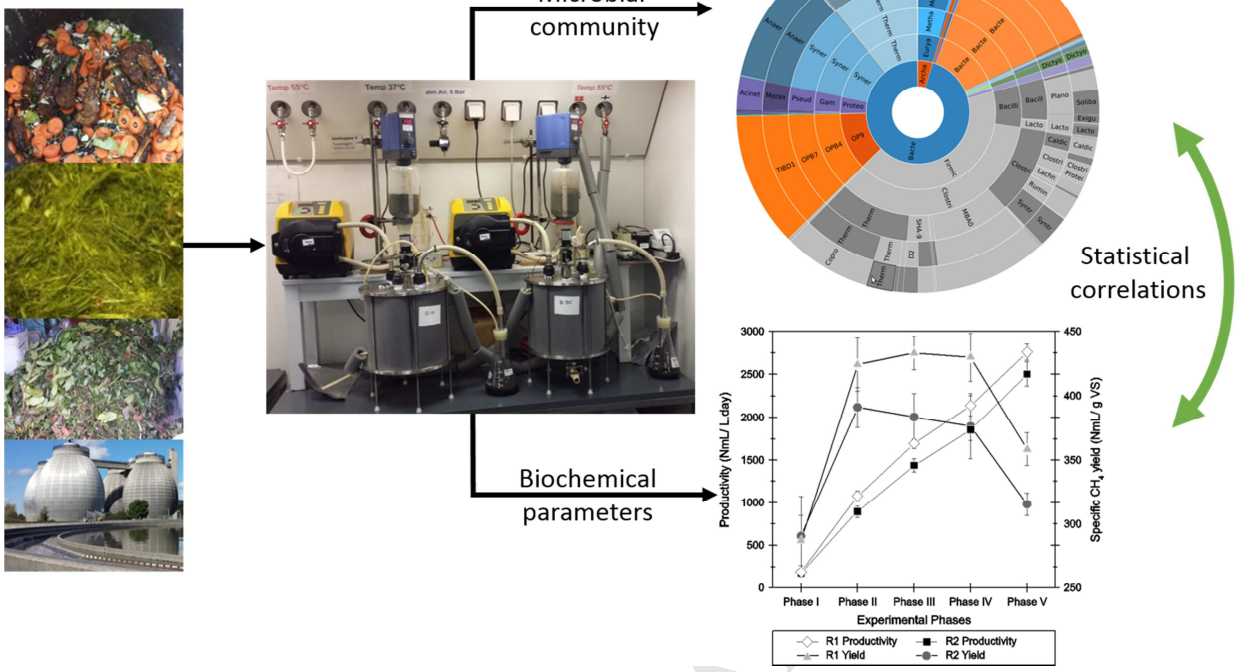

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Microbial population dynamics in urban organic waste anaerobic co-digestion with mixed sludge during a change in feedstock composition and different hydraulic retention times

Temesgen Fitamo[1*], Laura Treu[1,2], Alessio Boldrin[1], Cristina Sartori[2], Irini Angelidaki[1], Charlotte Scheutz[1]

[1]Department of Environmental Engineering, Technical University of Denmark, Miljoevej, Building 115, DK-2800 Kgs. Lyngby, Denmark

[2]Department of Agronomy, Food, Natural resources, Animal and Environment (DAFNAE), viale dell'Università, 16, 35020 Legnaro (Padova), Italy.

[*]Corresponding author

Department of Environmental Engineering,

Technical University of Denmark, Bygningstorvet, Building 115

DK-2800 Kongens Lyngby, Denmark

Phone: (+45) 4525 1413,

Fax: (+45) 4593 2850,

E-mail: tefi@env.dtu.dk

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.

Keywords: anaerobic digestion, methanogenesis, 16S rRNA, microbial diversity, urban organic waste

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).

Biochemical pathways involved in the AD process are based on rather complex and diverse microbial roles. Therefore, it is important to understand the effect of the microbial community's composition and function with regard to the operational parameters required to operate the digester at optimum conditions and maximise energy recovery. Investigations conducted on seven anaerobic digesters fed with sewage sludge have revealed that the core group of bacteria common to all digesters is composed of six operational taxonomical units (OTUs) related to *Chloroflexi*, *Betaproteobacteria*, *Bacteroidetes* and *Synergistetes* (Rivière et al., 2009a). Sludge-based AD digesters – besides strict anaerobes – contain aerobic bacteria originating from the feedstock sludge, which basically consists of aerobic bacteria, and so *Chloroflexi* appear mainly in sludge-based AD processes. Another study regarding sewage sludge digesters has found that the most common archaeal taxa are *Methanomicrobia*, *Methanobacteria* and *Thermoplasmata* (Narihiro and Sekiguchi, 2007). Microbial community variations can influence the AD process and thereby inhibit or enhance the process. For example, it has been shown that the bio-augmentation of hydrogenotrophic methanogen (*Methanoculleus bourgensis* MS2T) in an anaerobic digester can play a significant role in overcoming ammonia inhibition (Fotidis et al., 2014). A shift in methanogenic pathways and methanogenic community composition has been observed when the microbial culture is exposed to increasing concentrations of acetate and ammonia (Fotidis et al., 2013), while specific bacteria such as the filamentous *Microthrix* or *Nocardia* have been shown to be associated with foaming incidents in biogas reactors (Kougias et al., 2014). A common feature of all these studies is that they provide a snapshot of microbial community composition and activity at a given time and in specific conditions. However, the response and development of microbial communities to external changes in process conditions, to date, has not been reported adequately in literature. This information is relevant to ensure smooth transitions when changing process operations or treating specific substrates.

The main objective of this research was to study changes in the microbial population community as a response to variations in the operation of the AD process and co-digestion of urban organic waste (UOW) comprising food waste, grass clippings and garden waste with mixed sludge. This was achieved by: (i) analysing the composition of the microbial community during UOW co-digestion in continuously stirred tank reactors (CSTRs), operated at sequentially reduced hydraulic retention times (HRTs), (ii) comparing two CSTRs fed with different UOW mixing ratios, co-sewage sludge digestion, food waste, grass clippings and garden waste and (iii) analysing changes in the microbial population community in terms of relative abundance and diversity, and correlating these findings with reactor performance and operational process parameters.

2. Materials and methods

2.1 Characterisation of input feedstock materials

The feedstock materials included mixed sewage sludge, food waste, grass clippings and garden waste, which were collected from several locations in Denmark, as described in Fitamo et al. (2016a). The addition of UOW to existing AD operations at wastewater treatment plants (WWTPs) is able to boost biogas production, and current biogas reactor facilities at WWTPs can be used in this regard (Fitamo et al., 2016b). Organic feedstock was shredded into small particles with a shear-shredder (ARP SC 2000) and knife mill (Wiencken 19225 and Fitzmill model D, Daso-6). Individual organic waste materials were then characterised in terms of physicochemical properties (e.g. total solids (TSs), volatile solids (VSs), total Kjeldahl nitrogen (TKN), lipids, VFAs, proteins, total C and total N). The analytical methods are described in Fitamo et al. (2016a).

2.2 Experimental set up and operation

The co-digestion experiment was conducted to maximise biogas production from UOW, by adding food and plant materials (garden waste and grass clippings) to existing sludge digestion at WWTPs. The laboratory experimental work was carried out in two CSTRs, named R1 and R2, each with a

working volume of 7.5 L. The temperature was kept constant in thermophilic conditions (55^{o}C) and with hot water circulation supplied by a circular closed heating system. The co-substrates were fed into the reactor via an automated feeding system, based on the organic loading rate (OLR) of the reactor. The set-up was equipped with an automated stirring system and a water displacement gas-metering counter to measure the amount of biogas produced. The CSTRs were operated in five distinctive operational phases. Phase I aimed at establishing a baseline performance relative to existing sewage sludge AD, and it included the mono-digestion of 100% mixed sludge (primary and secondary sludge mixed at a 1:1 V/V ratio) in both R1 and R2, with a HRT of 30 days (HRT30). After Phase I, UOW was added to the mixed sludge and fed into the reactors in fixed percentage VS mixing ratios throughout Phases II to V. Reactor R1 received 10:67:16:7 and reactor R2 received 10:44:32:14 of sewage sludge, food waste, grass clippings and garden waste, respectively. The ratios were set up in order to have high food waste in R1, while the VS share of food waste was reduced but the lignocellulosic garden and clippings feedstock doubled in R2. An overview of the experimental setup is provided in Table 1, which shows that the HRT was reduced stepwise, from 30 days (HRT30) in Phase II, to 20 days (HRT20) in Phase III, to 15 days (HRT15) in Phase IV 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, 2.8 and 2.5, respectively. Specific methane yield, productivity, concentrations of ammonia and acetate measured during the co-digestion of UOW in R1 and R2 are provided in Figure S-1 in the Supporting Information (SI) (Fitamo et al., 2016a).

<Table 1 here>

2.3 Sampling and DNA extraction

Within each operational phase, duplicate reactor broth samples (10 mL) were taken from both reactors once steady-state conditions were reached – this amounted to 10 samples in total. Residual plant particles present in the samples were removed, using a 100 µm nylon cell strainer

filter. Centrifugation of the filtered samples (10,000 rpm, 10 minutes) was conducted to obtain ~1.5 g of cell pellet. The total microbial DNA extraction (DNA isolation and purification) was performed using the PowerSoil® DNA Isolation Kit protocol (MO BIO Laboratories, Carlsbad, CA) with an additional initial cleaning step by Phenol:Chloroform:Isoamyl Alcohol 25:24:1 pH 8 (Sigma-Aldrich, DK). The quality of the purified DNA was examined with gel electrophoresis, and the DNA concentration was analysed with NanoDrop 2000 (ThermoFisher Scientific, Waltham, MA).

2.4 16S rRNA gene sequences

The samples were sequenced by utilising the Illumina MiSeq platform at Ramaciotti Centre for Gene Function Analysis, University of New South Wales (Sydney, Australia), by amplifying the V4 hypervariable region of the 16S ribosomal gene RNA using 515f-806r primers and following the protocol of the Earth Microbiome Project (Earth Microbiome, 2011). The raw Illumina sequence data obtained in this research work were submitted to the National Centre for Biotechnology Information's (NCBI) sequence read archive database (SRP078424) under the bio-project number (PRJNA328964). The sequences were analysed with CLC Genomic Workbench Software (V.8.0.2), equipped with a microbial genomics module plug-in as previously described (Kougias et al., 2016a). OTUs were aligned using MUSCLE software (Edgar RC, Nucleic Acids Res). The Maximum Likelihood Phylogenetic tree, Alpha diversity index and Beta diversity were computed as described by Kougias et al., (2016). The total number of reads obtained and total OTUs with corresponding taxonomy assignment for the microbial community in both R1 and R2 are reported in Table 1. OTUs with 10 sequences or fewer were considered extremely rare and were discarded from further analysis. Direct comparison of the microbial relative abundance between the samples was performed at genus and phylum level and was calculated as a percentage of the total community for each sample.

The classification used to make the comparison of percentage relative abundance was carried out with highly abundant (> 0.5% relative abundance) and lowly abundant (between 0.01% - 0.5% of relative abundance) OTUs, whereas they were discarded from analysis when lower than 0.01%. Most of the result and discussion section focuses on the most abundant microbes in the community (> 0.5% of relative abundance), while the less abundant microorganisms were considered only when statistically significant. Heat maps showing the relative abundance changes (fold changes), due to comparisons of different retention times and feedstock compositions, were prepared with the Multiexperiment viewer (MeV 4.9.0) (Saeed et al., 2003).

2.5 Statistical analysis

Statistics were performed using a general linear models analysis (GLM Procedure, SAS Institute, 2009). Firstly, differences in microbial abundance in the two reactors (R1 or R2) and in the subsequent phases (Phases I to Phase V) were studied in a series of single-trait analyses, including the reactor and the phase as effects and the abundance of each microorganism as a trait. Each microbial abundance was analysed separately with the GLM. The dataset for the analysis consisted in all the pairs of replicates sampled within each reactor during the different phases (data structure is reported in Table 1). In order to detect the trend of microbial abundance variation with respect to a change in HRT, the phase was alternatively included in the analysis as a linear, quadratic or cubic covariate. The model with the most significant shapes in variation (linear, quadratic or cubic; $P \leq 0.05$) for the phase effect was therefore chosen for each microbial abundance.

Methane yield, methane content of biogas, total VFA, individual VFAs, pH, reactor productivity and ammonia were then used as traits to analyse variations in the operational process parameters. Reactor (R1 or R2) and phase (Phase I to V) were treated as fixed effects (e.g. traits were analysed by considering if they belonged to reactor R1 or R2, or to a specific Phase, I-V), and the abundance

of each microorganism as a linear covariate. In this way, any variation in the operational process parameters was considered as depending on the variation in microbial abundance. Single-trait models were run, all including the same fixed factors (*fixed*) and each one considering different biochemical parameter; *i* (*parameter*) as trait and a different microorganism; *k* (*microorganism*) as covariate, that can be generalized as: $parameter_i = fixed + microorganism_{k:i}$. This approach was used to avoid the over-parameterization of the model (i.e., to have too many parameters for the number of data), and to avoid problems of overlapping variances due to the introduction of microorganisms with similar variations in abundance in the same model.

3. Results and discussion

3.1 General microbial community composition and diversity

The phylogenetic composition of the most abundant bacteria and archaea (OTUs) in the entire microbial community was established, based on the 16S rRNA gene sequence considering all samples from both reactors in all of the considered phases (Figure 1). Between 90 and 96% of the OTUs were classified at the phylum level, showing that the majority of the microorganisms found in the reactors could be identified at the phylum level. In contrast, only 47-73% of the entire community was classified at the genus level (Table 1). This shows strong diversity among the samples. Further research, using advanced sequencing techniques, is needed to classify in detail any unknown microbes and to understand their specific role in the complex anaerobic degradation process.

< Figure 1 here >

In general, the bacterial community consisted of *Firmicutes, OP9, Synergistetes, Proteobacteria, Bacteroidetes, Thermotogae, Dicyoglomi* and *Chrloroflexi* as the main phyla (Figure 1 and Figure S-2). The predominance of phylogenetic groups such as *Firmicutes, 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

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.

Other microbes, with a percentage relative abundance less than 0.5%, were significantly enriched in R2 and belonged to *Porphyromonadaceae* (11 times more abundant in R2) and *Clostridium* (three times more abundant in R2; P≤0.01). Members of the *Clostridium* genus are known to degrade complex cellulose biopolymers (Guo et al., 2015; Nelson et al., 2011) and lignocellulosic material components (Cirne et al., 2007; O'Sullivan et al., 2005).

S1 (Thermotogales) and *Thermonema*, (relative abundance (> 0.5%) decreased significantly in R2 compared to R1 (P≤0.05) by a factor of 2 and 1.3, respectively (Figure 4). *Thermotogales* microorganisms are involved in the fermentation of substrates such as glucose, acetate, methanol and starch as well as reducing elemental sulphur and sulphate (Balk et al., 2002; Feng et al., 2010). R1 was enriched with carbohydrate and fat-degrading microorganisms of *Lactobacillus* (5 times; P≤0.05) (Li et al., 2013). Other less abundant OTUs, such as *Exiguobacterium*, *Bacillus* and *Allochromatium*, decreased in R2 compared to R1 by a factor of 6.4 and 6, respectively (Figure 4). The rest of the microorganisms , apart from *Caldicoprobacter* (P≤0.05), were found in both R1 and R2, irrespective of the feedstock.

3.4.2 The effect of HRT

Differences in microbial relative abundance, due to hydraulic retention times, were detected by considering the effect of the operational phases on the abundance of each microbe. Figure 4 and Figure 5, respectively reports the abundances of microbial communities in the two reactors in the different phases and the changes in the relative abundance of microorganisms between phases. Microbes related to the fermentation of sugars into acetate, lactate, ethanol, CO_2 and H_2, such as *Thermonema*, *S1* and *Caldicoprobacter* (Bouanane-Darenfed et al., 2011), syntrophic acetate oxidiser, such as *Thermacetogenium* (Hattori, 2000), and *Lactobacillus* increased by a factor of at least seven (Figure 5). The GLM analysis (Figure 5) showed a significant trends for these microorganisms, either linear (*Thermonema*; P≤0.01), quadratic (i.e. roughly assumed the shape of a curve: *Caldicoprobacter*, *Thermacetogenium;* P≤0.001), or cubic (i.e. showing an inflection point:

Lactobacillus; P≤0.05). At HRT10 (Phase V) the community populations of *Caldicoprobacter*, *Thermacetogenium* and *Lactobacillus* decreased in abundance (SI: Figure S-5), except those of *Thermonema* and *S1* (Figure 6), which may be due to process inhibition resulting in a yield and methane productivity drop.

< Figure 5 here >

The relative abundance of *Acinetobacter*, *Solibacillus*, *Dictyoglomus*, *Proteiniclasticum*, *Exiguobacterium*, *Fervidobacterium*, *Bacillus*, *Allochromatium* and SMB53 decreased by a factor of at least three in subsequent phases compared to Phase I (Figure 5 and Figure S-5 (SI)). The trend of *Dictyoglomus* was linear (P≤0.01), The shape of variation for *Solibacillus* and *Proteiniclasticum* was mainly linear (P≤0.05), but also a quadratic component was close to significance (P=0.06). The other microorganisms had a mixed pattern of variation, with both linear and quadratic significant components (P≤0.05).

< Figure 6 here >

During the AD process of Phase II to Phase V, OTUs members of *Fervidobacterium*, *Bacillus*, *Allochromatium* and *SMB53* decreased in abundance, as shown in Figure 6 and Figure S-5 (SI). The most dominant genera in Phases II, III and IV at HRT30, HRT20 and HRT15, respectively, were simple and complex sugar-fermenting bacteria (*S1*), proteolitic microorganisms (*Coprothermobacter*), organic acid-degrading bacteria (*Anaerobaculum*), *Methanothermobacter* and *Thermonema* ((Figure 6 and Figure S-5 (SI)). This could be due to increased OLR of UOW in the feedstock. Additionally, Figure 5 shows an increasing trend of the dominant bacterial community, *S1* and *Thermonema*, which could be due to higher specific growth rates surviving washouts at shorter HRTs, and of a taxon belonging to *Firmicutes* (order *MBA08*; Figure S-2), another taxon among the most representative. An almost decreasing trend of *Anaerobaculum* and *Coprothermobacter* with respect to HRT, except for the last phase (HRT10), was also noted. The

methane-producing microorganism, namely *Methanothermobacter*, remained constant at HRT20 and HRT15 but dropped at HRT10.

3.5 Biochemical correlation of the microbial community with AD process parameters

The proper functioning of the AD process is influenced by a number of intertwined microorganisms governing the complex biochemical pathways. Performance parameters measured in the reactors, such as specific methane yield, methane productivity, ammonia concentration and acetate (SI: Figure S-1) (reported in Fitamo et al., 2016a), were correlated with OTUs abundance. The GML analysis (Table S-1, SI) produced a coefficient of linear regression for each biochemical parameter-microbial abundance pair: a positive coefficient indicated that an increase in targeted microbial abundance also caused an increase in the biochemical parameter under consideration, whereas a negative coefficient indicated a decrease in the biochemical parameter, due to an increase in microbial abundance (Table 2 and Table S-1). When comparing microbial community composition with AD performance parameters (Figure S-1,SI), methane yield and productivity significantly increased ($P \leq 0.05$; Table 2) when an increase in abundance variation occurred for the OTUs assigned to Proteobacteria (*Acinetobacter iwoffii*, OTU: 532569; *Allochromatium*), *Thermotogae* (*S1*, *Fervidobacterium*, two OTUs) and Bacteroidetes (*Thermonema*). On the other hand, a significant decrease ($P \leq 0.05$; Table 2) in methane productivity and yield was observed following an increase in the abundance of *Dictyoglomus*, *Fervidobacterium* (two OTUs) and in the OTU 573124 belonging to *Acinetobacter*. Moreover, methane productivity and yield were significantly affected by the abundance of microorganisms belonging to the phylum Firmicutes (*Coprothermobacter*, *Syntrophomonas, Clostridium, Proteiniclasticum, Exiguobacterium, Bacillus*,

two OTUs *including Bacillus muralis, Solibacillus and SMB53*), which is mostly involved in the hydrolysis of complex organic matter. Variation in the methane percentage was instead significantly affected (P≤0.001) only by abundance of the OTU belonging to the phylum *Chloroflexi*, *class Anaerolineae*, even if relatively low in abundance (P<0.05) (Table S-1), members of which may be thermophilic or mesophilic, are generally ubiquitous and play an important role in the environment (Yamada et al., 2006).

Considering the VFAs, a significant decrease in the abundance of these acids (P≤0.001; Table 2), in particular in propionate (P≤0.05; Table 2), was related to an increase in *Syntrophomonas* (OTU: 1110842), known to beta-oxidise saturated fatty acids to acetate or acetate and propionate (Sieber, 2010). Propionate significantly decreased (P≤0.01; Table S-1) following an increase in OTU 254504 belonging to the order *SHA-98* of the class *Clostridia*, phylum *Firmicutes*, known to be involved in syntrophic acetate oxidation activities. The concentration of acetate followed the same trend (P≤0.05) for methane yield and production, apart for *S1* (P=0.013; Table 2). Acetate also significantly increased when *Methanosarcina* increased (P<0.01; Table 2) and seemed also to be significantly associated with the phylum *Firmicutes* (*Coprothermobacter*, *Syntrophomonas*, *Clostridium*, *Solibacillus*), and with *Methanosarcina*, OTU positively correlated with acetoclastic methanogens, because acetate is a substrate for *Methanosarcina* metabolism. On the other hand, acetate variation was not related to variation in the hydrogenotrophic methanogen *Methanothermobacter thermautotrophicus*, since methane is mainly produced via syntrophic acetate oxidation association followed by hydrogenotrophic methanogenesis. Butyrate, the last VFA considered in this study, resulted significantly in the abundance of *Syntrophomonas*, OTU 203894 and *Anaerobaculum* (phylum *Synergistetes*, OTU 533824; P≤0.001, Table 2), a genus able to reduce substrates to butyrate with glucose as an electron donor.

The increase in *Anaerobaculum* abundance (P≤0.01; Table 2) was also related to a decrease in the concentration of ammonia. Moreover, ammonia concentration increased in relation to the

increase in *Syntrophomonas*, OTU 203894, able to convert atmospheric molecular nitrogen to ammonia (Sieber, 2010). pH variation was only affected by an OTU assigned to *Clostridium* (Table 2), whereby an increase in abundance was related to an increase in pH ($P \leq 0.001$), and by some other non-abundant *Firmicutes* OTUs (Table S-1). Table S-1 (SI), providing an overview of the less abundant OTUs significantly correlated with AD performance parameters, showed that biogas production process was affected not only by dominant microorganisms, but also by less abundant but crucial microorganisms.

Overall, the results of the microbial community analysis show that the composition of feedstock and the process condition affects the diversity of the microbial community. The biochemical correlation also reveals that certain groups of microbes particularly hydrolytic bacteria are significantly correlated with anaerobic digestion process performance parameters. Knowledge of changes in microbial community structures as a response changes in feedstock composition, operational process parameter and reactor performance could help wastewater treatment plants and biogas plant to enhance the methane yield and productivity through bioaugmentation.

4. Conclusion

The dominant microbial community, *Proteobacteria*, observed in sludge-based mono-digestion decreased in abundance compared to the anaerobic co-digestion of urban organic waste (UOW). Nevertheless, a new community, *Thermonema*, increased during the co-digestion of UOW.

Complex organic polymer degraders *Thermacetogenium*, *Anaerobaculum*, *Ruminococcaceae* and *Clostridium* were significantly abundant in reactor fed with high share of lignocellulosic material (R2) however *S1*, *Thermonema* and *Lactobacillus* were found to be significantly abundant in reactor fed with high share of food waste (R1). The relative abundance of

S1 and *Thermonema* increased, while other taxa such as *Coprothermobacter, Anaerobaculum* and *Dictyoglomus* decreased in line with sequentially decreased HRTs.

Syntrophic acetate oxidation, followed by hydrogenotrophic methanogenesis, was established as the main methane formation pathway in both R1 and R2. However, the relative abundance of methanogenic *Euryarchaeota* (*Methanothermobacter*) decreased when the HRT was changed from 15 to 10 days, in which case *Methanosarcina* became dominant. Methane yield was correlated with several *Firmicutes* (*Coprothermobacter, Syntrophomonas, Clostridium*) involved in the hydrolysis stage. The concentration of acetate was correlated with several OTUs, such as *Methanosarcina* and *Acinetobacter iwoffii*, while the concentration of ammonia was associated with *Anaerobaculum* and *Syntrophomonas*.

The particular microbial community composition and diversity of the corresponding feedstock composition and operational parameters could support biogas plants to enhance the anaerobic digestion process performance by using bioaugmentation of the respective microorganisms to achieve rapid microbial adaptation and also optimal production of methane yield and productivity.

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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).

Tables

Table 1. Overview of process conditions and sequencing results. Co-digestion at HRTs of 30, 20, 15 and 10 days, with corresponding co-substrate compositions in R1 and R2. Feedstock composition is shown as the ratios of sludge, food waste, grass clippings and garden waste, respectively, for R1 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

**Mixture of primary and activated sludge*

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	P	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

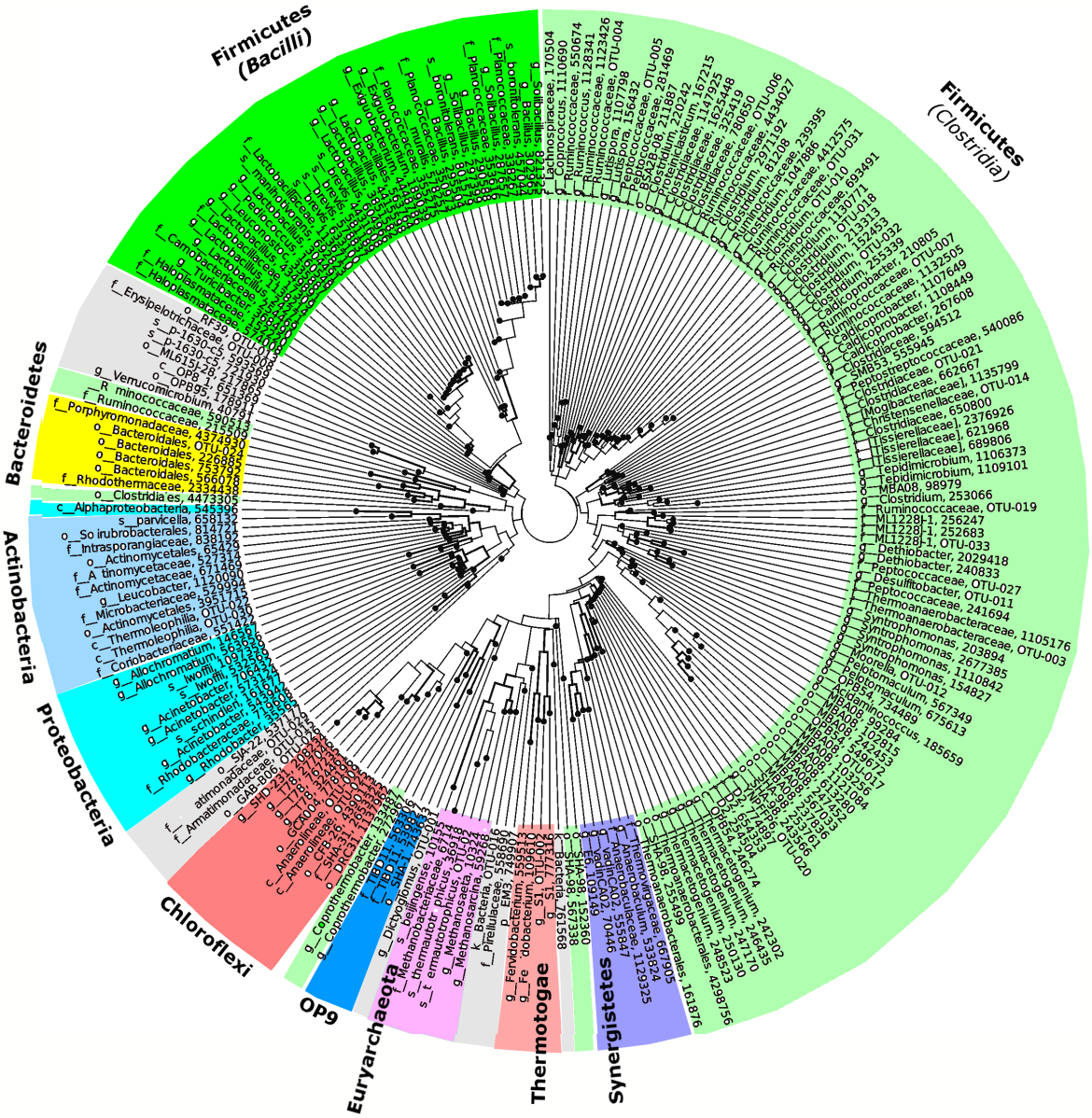
Firmicutes (Bacilli)
Firmicutes (Clostridia)
Bacteroidetes
Actinobacteria
Proteobacteria
Chloroflexi
Op9
Euryarchaeota
Thermotogae
Synergistetes

Figure 1

Figure 2

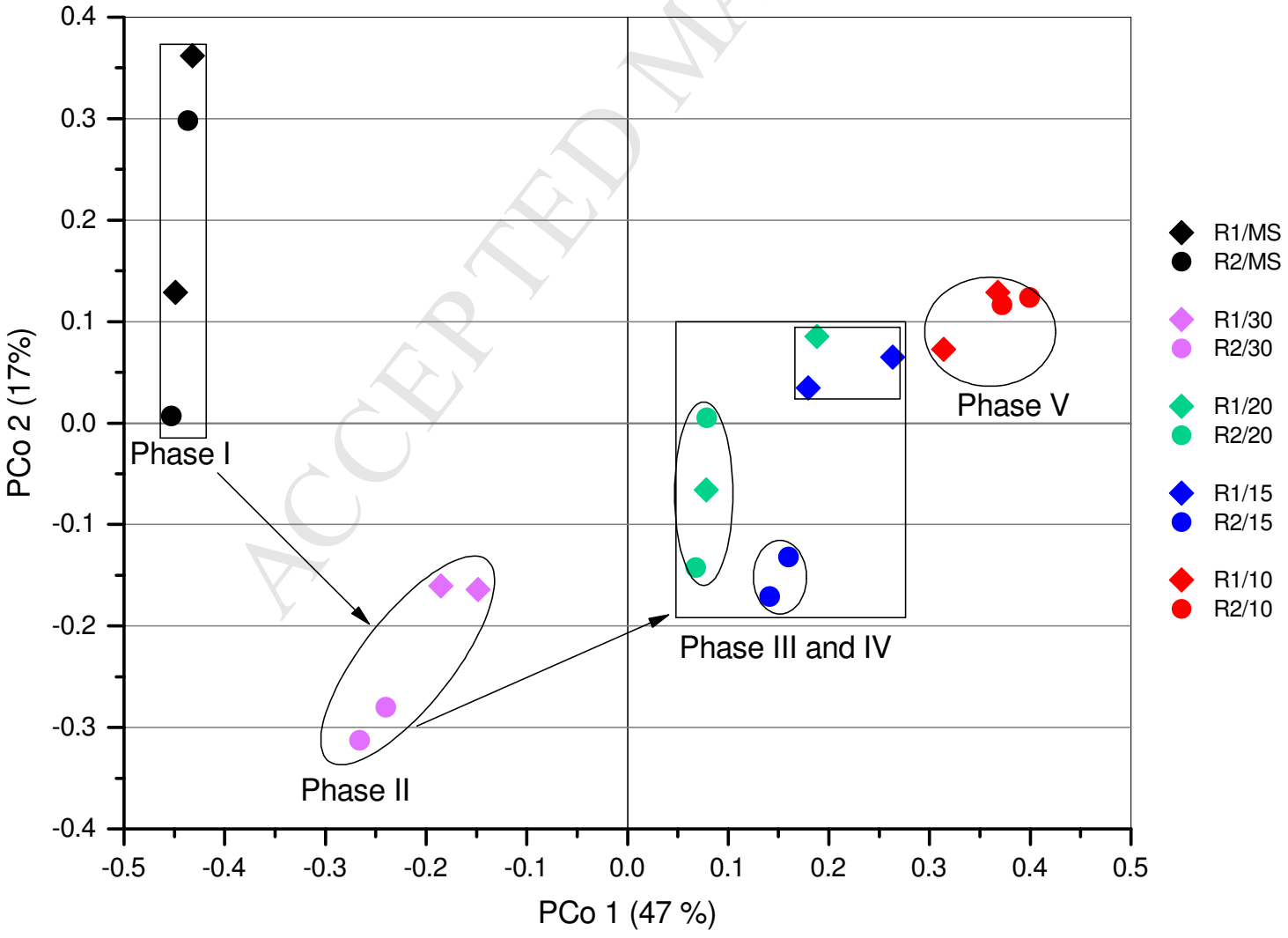

Figure 3

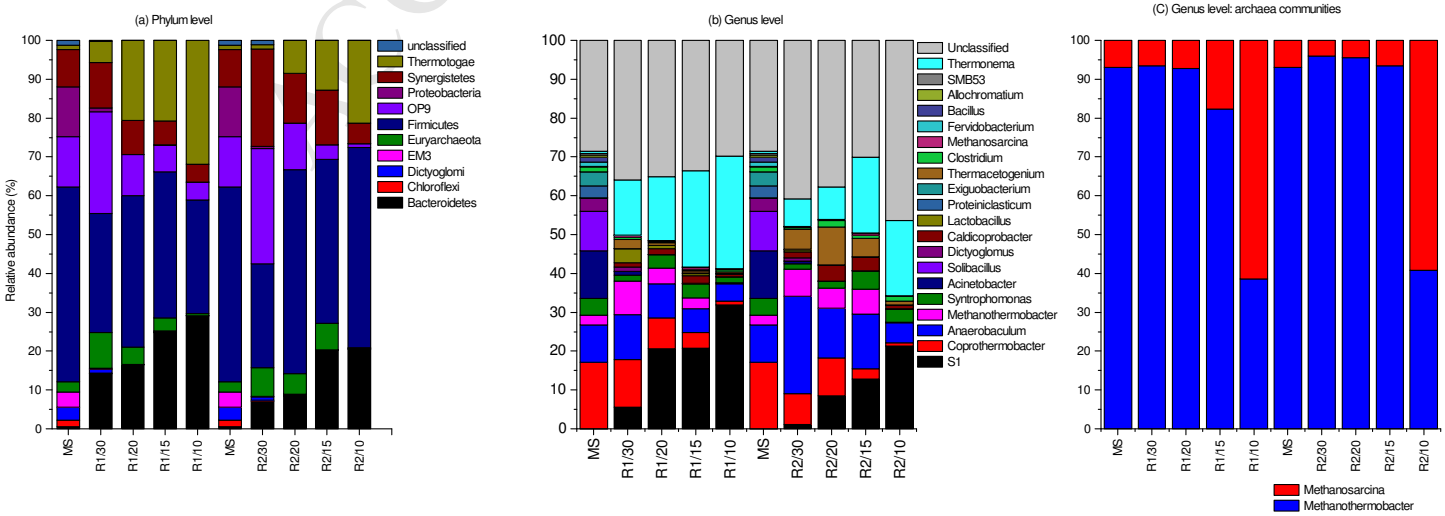
(a) Phylum level
Relative abundance (%)
unclassified
Thermotogae
Synergistetes
Proteobacteria
OP9
Firmicutes
Euryarchaeota
EM3
Dictyoglomi
Chloroflexi
Bacteroidetes
MS
R1/30
R1/20
R1/15
R1/10
MS
R2/30
R2/20
R2/15
R2/10
(b) Genus level
Unclassified
Thermonema
SMB53
Allochromatium
Bacillus
Fervidobacterium
Methanosarcina
Clostridium
Thermacetogenium
Exiguobacterium
Proteiniclasticum
Lactobacillus
Caldicoprobacter
Dictyoglomus
Solibacillus
Acinetobacter
Syntrophomonas
Methanothermobacter
Anaerobaculum
Coprothermobacter
S1
(C) Genus level: archaea communities
Methanosarcina
Methanothermobacter

Figure 4

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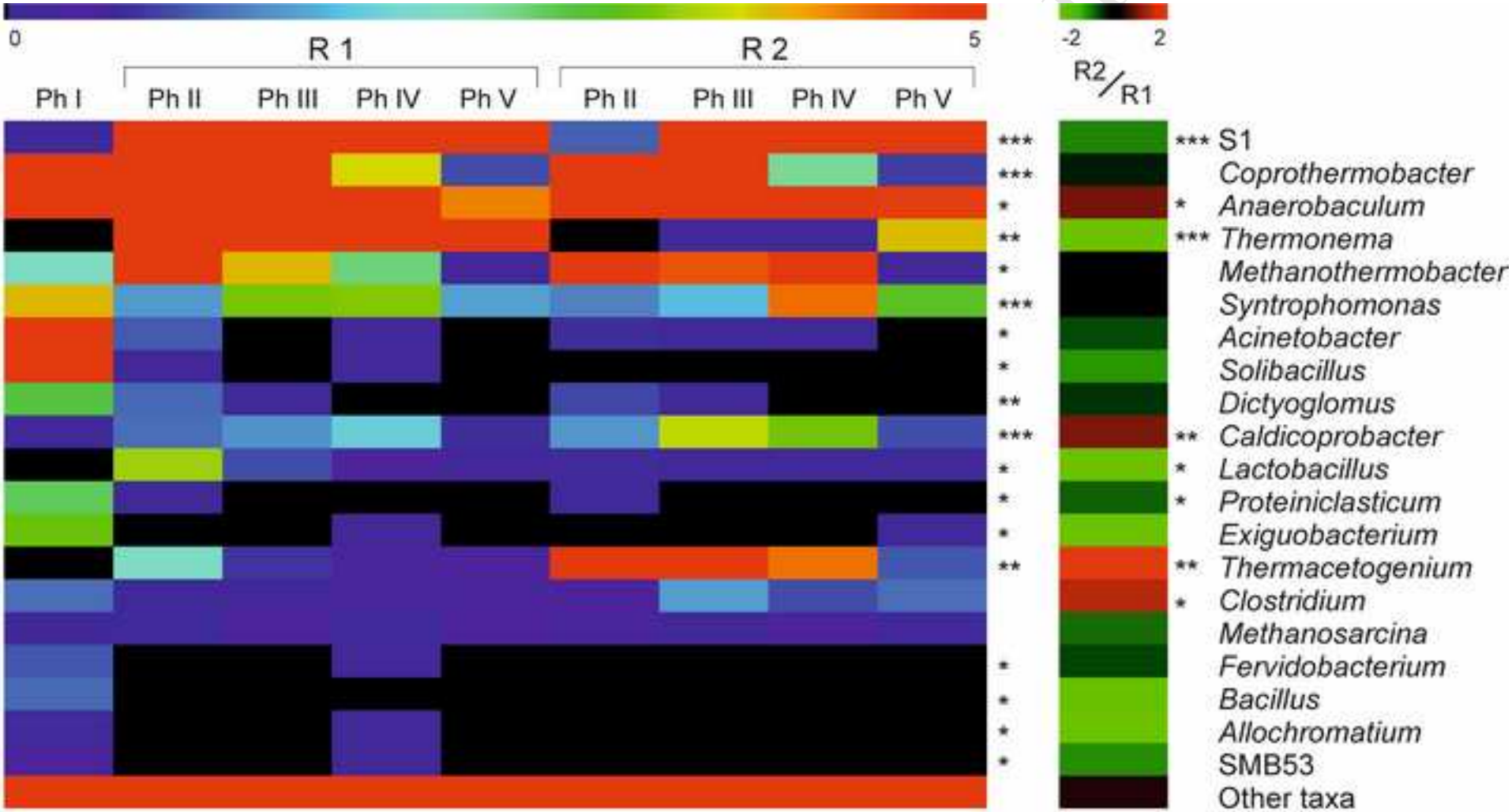


Figure 5

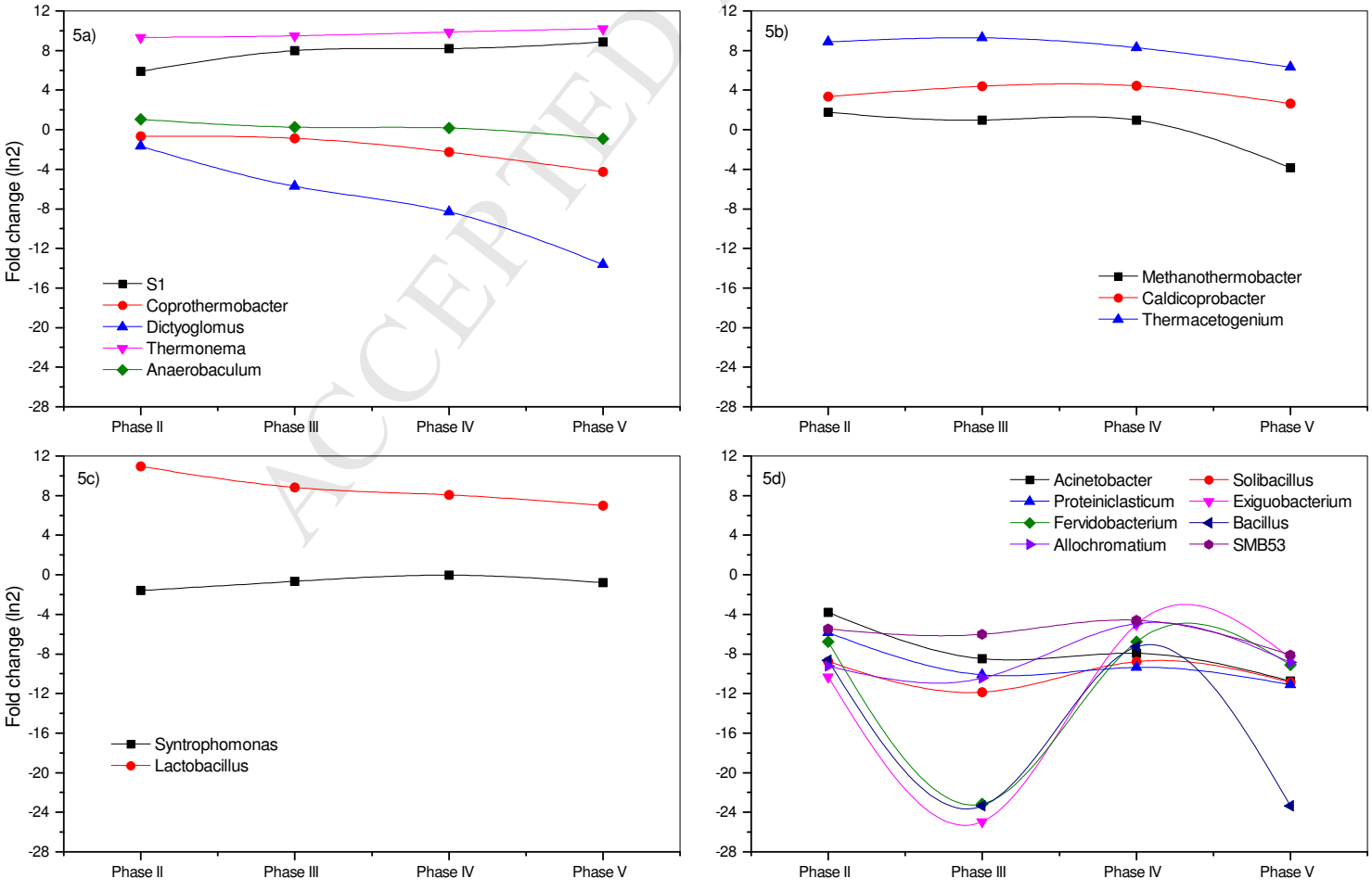
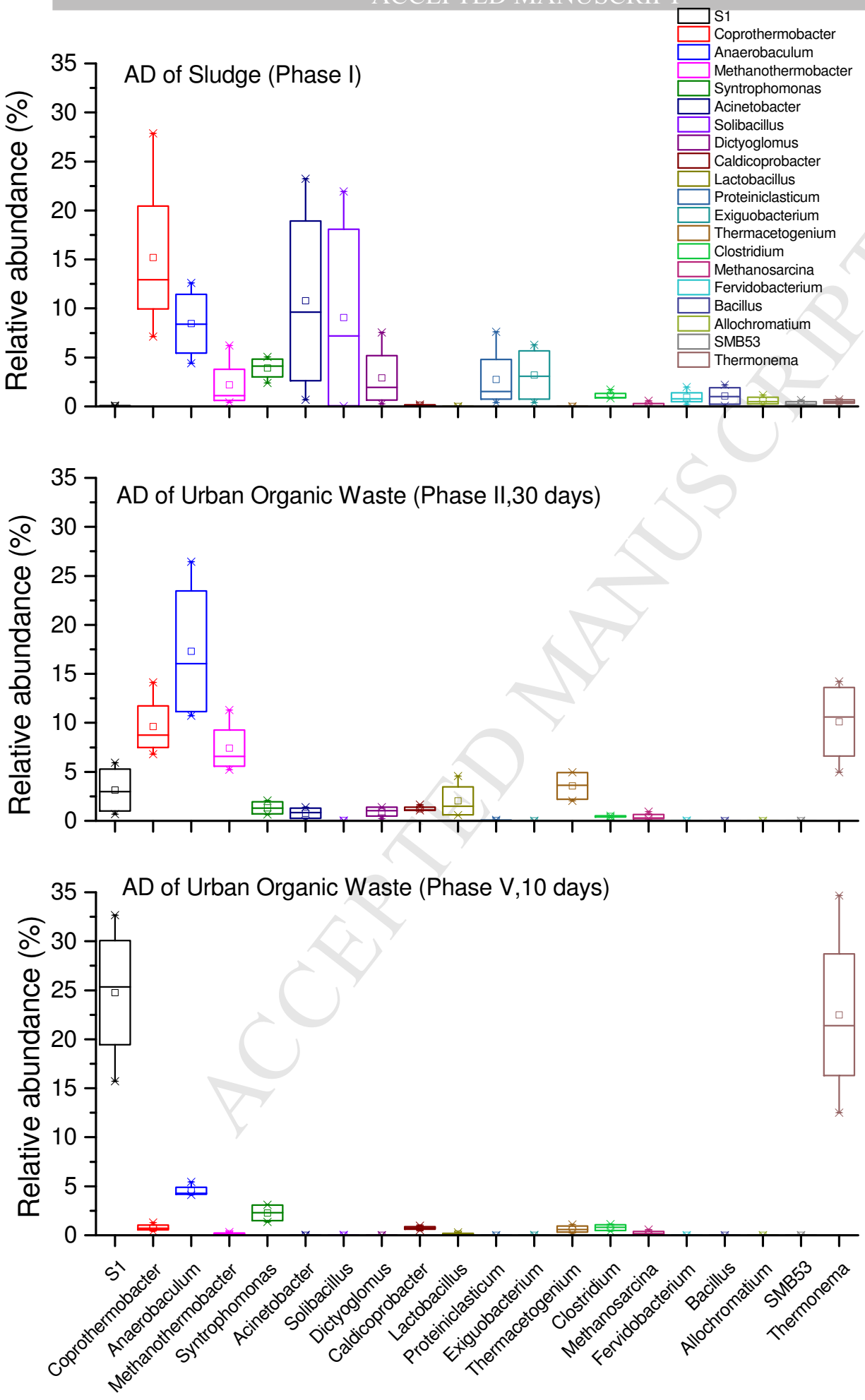


Figure 6



DTU Environment

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

Technical University of Denmark
Department of Environmental Engineering
Miljoevej
Building 113
DK-2800 Kgs. Lyngby
Denmark
Tel +45 45 25 16 00
Dir. +45 45 25 15 85
Fax +45 45 93 28 50
tefi@env.dtu.dk
www.env.dtu.dk