Impact of anaerobic co-digestion between sewage sludge and carbon-rich organic waste on microbial community resilience

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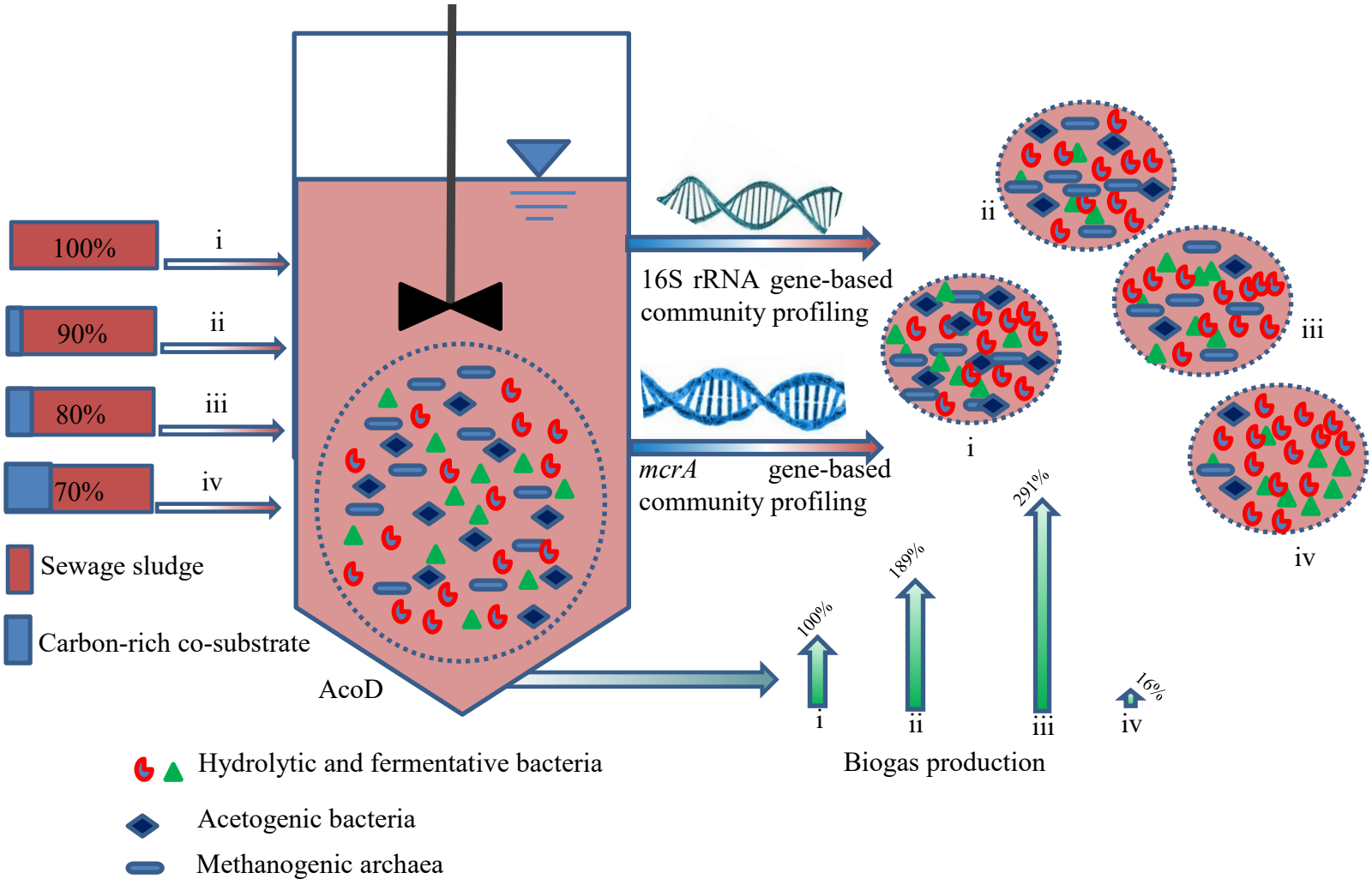
ABSTRACT

This study examines the changes in microbial community diversity and structure in response to anaerobic co-digestion (AcoD) between sewage sludge and a carbon-rich organic waste. Biomass samples were collected at different carbon-rich co-substrate mixing ratios to cover a large range of organic loading rate (OLR) for microbial community analysis by amplicon sequencing of 16S rRNA and *mcrA* marker genes on the Illumina Miseq platform. The results show a reduction in community diversity (i.e. richness and evenness) and a shift in community structure as the OLR increased due to the addition of the carbon-rich co-substrate. Despite the decrease in community diversity, biogas production increased proportionally to the increase in OLR of up to 3.03 kg COD/m^3/d (corresponding to 171% OLR increase compared to anaerobic digestion of only sewage sludge). Further OLR increase led to the collapse of biogas production as well as significant reduction in both the microbial diversity and methanogenic population. The methanogenic community was more sensitive to the increase in OLR compared to hydrolytic and fermentative bacteria. These results show that there is an OLR threshold at which the function and resilience of the anaerobic ecosystem could be maintained. Beyond this threshold, the enrichment of hydrolytic and fermentative bacteria, as well as inhibition of methanogenic community, can cause anaerobic digestion failure.

KEYWORDS. Anaerobic co-digestion, sewage sludge, carbon-rich organic waste, beverage waste, microbial community diversity.


100%
i
90%
ii
80%
iii
70%
iv
Sewage sludge
Carbon-rich co-substrate
AcoD
16S rRNA gene-based community profiling
mcrA gene-based community profiling
ii
iii
i
iv
100%
189%
291%
16%
i
ii
iii
iv
Biogas production
Hydrolytic and fermentative bacteria
Acetogenic bacteria
Methanogenic archaea

35

1. Introduction

Wastewater treatment is essential for the protection of public health and the environment. It is, however, also an energy-intensive exercise. Municipal wastewater treatment accounts for about 3% of global electricity consumption and 5% of global greenhouse gas emission[1]. A promising approach for the water industry to reduce its energy footprint is to co-digest sewage sludge (SS) with organic wastes for the production of biogas, which can then be used to generate electricity. Indeed, a few full-scale trials and successful implementations of anaerobic co-digestion (AcoD) at wastewater treatment plants (WWTPs) have been recently reported[2-4].

Although AcoD has a vast potential[3, 5], the risk of inhibition associated with AcoD at WWTPs is also significant due to organic overloading and the variation in both quality and quantity of organic wastes[6, 7]. Anaerobic digestion is a complex biological process involving four interrelated steps, namely hydrolysis, fermentation (acidogenesis), acetogenesis, and methanogenesis. Each of these steps is accomplished by a consortium of microorganisms with specific functionality and ability to adapt to the environment. For example, while hydrolytic bacteria can thrive under an acidic condition, methanogenic archaea can only grow at near neutral pH. Because these steps occur simultaneously within the digester, the harmonization among them is essential for process stability and efficiency[8, 9].

Previous studies on AcoD have focused mostly on optimizing the abiotic operating conditions such as co-substrate pairing, mixing ratio and organic loading rate (OLR) withvery few efforts have been made to understand key biotic factors. For example, while it is well established in the literature that anaerobic digestion performance can collapse at an excessive OLR value, the underlying changes in microbial community have not been comprehensively studied. Preliminary investigations have revealed the core organisms in AcoD are similar to those in mono-digestion[10, 11], such as *Clostridia* members in hydrolytic and fermentative

bacterial groups[12], *Methanobacteriales*, *Methanomicrobiales*, and *Methanosarcinales* members in methanogens[12, 13]. Nevertheless, the impact of AcoD on the microbial community is still poorly understood and reported observations vary significantly. Jensen et al [13] observed only minimal changes in microbial diversity when co-digesting SS and glycerol using a bench scale-reactor (1 L). In contrast, Zhang et al [14] observed significant changes in the microbial community structure when co-digesting SS and food waste compared to mono-digestion of only SS using a series of biomethane potential batch experiments. Similarly, Fitamo et al [15] reported significant changes in the microbial community structure as the feedstock changed from SS to a mixture of SS, food waste, grass clipping and garden waste. These early studies also highlight the need to better understand the potential impact of AcoD on microbial diversity and subsequently biogas production[12-17].

The inception of next-generation sequencing technologies has paved the way for in-depth investigation of the microbial community from different environmental matrixes. 16S rRNA gene has been widely used for profiling the microbial community in anaerobic digesters. Methyl-coenzyme M reductase (*mcrA*) gene, encoding the enzyme catalyzing the terminal step in methanogenesis, has been suggested as a useful biomarker for specifically targeting methanogen[18]. The complementary between 16S rRNA and *mcrA* marker genes to characterise the methanogen communities during anaerobic digestion has been recently demonstrated by[19] and can also be useful to evaluate any changes in the microbial community during AcoD.

This study examines the effects of AcoD on the digester microbial community. The high throughput Illumina MiSeq platform was utilised to elucidate the response of the microbial community to AcoD. Diversity and structure of the microbial community were characterised by the complementary use of 16S rRNA and *mcrA* marker genes. Changes in three important ecological parameters – diversity level, community structure and community dynamics over time – were elucidated as these parameters contribute to the system functional stability and

robustness. Finally, the relationship between microbial community dynamics and digester function and stability was revealed and discussed.

2. Materials and methods

2.1. Digester operation

Biomass samples for microbial characterization were collected from an AcoD system as reported in a previous study[20]. It involved three identical anaerobic digesters designated as R1, R2 and R3 operated in parallel. Each digester consisted of a 28 L stainless steel conical shape reactor, a temperature control unit (Neslab RTE 7, Thermo Fisher Scientific, Newington, USA), a peristaltic hose pump (DULCO® Flex from Prominent Fluid Controls, Australia), and a biogas counter (Ritter Company™, MilliGascounter). Primary sludge (PS) from the Wollongong WWTP in New South Wales (NSW) Australia was used as the main substrate. The majority of sewage treatment facilities in Australia is near the coastline and only produces(PS). Where secondary treatment is also involved, the volume of waste activated sludge is usually only half of that of PS. A mixture of carbonated soft drinks collecting from a commercial waste collector in NSW Australia was used as the co-substrate. These soft drinks were beverage waste (BW) since they did not meet market requirements (e.g. out of date, contamination, damaged packaging). Since sugar is the only carbonaceous organics in these soft drinks, they are ideal for representing carbon-rich waste without interference from other constituents such as nutrients and inhibitory substances. pH and COD of the PS were 6.1 ± 0.5 and 22 ± 1.4 (g/kg wet weight, n = 7). pH and COD of the BW were 3.3 ± 0.1 and 204 ± 2.3 (g/kg wet weight, n = 4).

The three digesters were operated for over 3.5 months (108 days) with three stages, at each stage, different organic loading rates (OLR) were applied by alternating the mixing ratio (% v/v) of PS and BW. In Stage 1 (52 days), mono-digestion of PS was carried out in all

reactors to obtain baseline performance data. In subsequent stages, co-digestion was carried out in reactor R1 and R2 while reactor R3 was used as the control system. In Stage 2 (31 days), BW was co-digested with PS at mixing ratio of 20 and 10% (v/v) in reactor R1 and R2, respectively. In Stage 3 (25 days), BW ratio of digester R1 was increased further to 30% (v/v). BW addition of 10, 20 and 30% (v/v) resulted in 86, 171, and 240% increase in OLR, respectively. In total, 13 sets of biomass samples were collected with five, three, three and two from the control, 86, 171 and 240% OLR increase, respectively. All the samples were fixed in ethanol (1:1 v/v) and stored at -20 ºC before DNA extraction.

All digesters were fed every day by withdrawing 1 L of digestate and replacing it with 1 L of feed (either PS or a mixture of PS with BW) resulted in 20 days of hydraulic retention time. The temperature of all three reactors was maintained at 35±1ºC for the whole experimental period.

2.2. Co-digestion performance

Anaerobic performance of these digesters has been previously reported by Wickham et al [20]. Briefly, the specific methane yield of PS in Stage 1 was 300 L/kg COD added. The optimum anaerobic operation was observed at Stage 2, where the increase in OLR to 3.03 and 2.08 kg COD/m^3/d due to BW co-digestion in digester R1 and R2, respectively, resulted in 191 and 89% increase in biogas production compared to the control digester (R3). These values are proportional to the increase in OLR of 171 and 86% in digester R1 and R2, respectively. Parameters including pH, TOA, alkalinity, and soluble COD of the three digesters were consistent for 52 and 31 days in stage 1 and 2, respectively. This suggests that the reactors' operations were stable and organic acids did not accumulate in the digester.

2.3. DNA extraction and quality monitoring

Genomic DNAs were extracted from samples using FastDNA® SPIN Kit for soil (MP Biomedicals, Santa Ana, CA) following manufacturer's instructions. The integrity, purity and concentration of the extracted DNA were evaluated by electrophoresis in a 1% (w/v) agarose gel and the NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). DNA amount in all samples was more than 10 µg and the concentration of all samples was normalized to 10 ng/µl using DNase/Pyrogen-Free Water provided in the extraction kit before sending to the sequencing facility.

2.4. Amplicon sequencing and bioinformatics analysis

The universal primer set Pro341F (*5'-CCTACGGGNBGCASCAG-3'*) and Pro805R (*5'-GACTACNVGGGTATCTAATCC-3'*) was used to target both bacterial and archaeal 16S rRNA V3 – V4 regions for characterisation of the whole microbial community [21]. The *mcrA* gene was PCR-amplified using the primer set ML-F (*5'-GGTGGTGTMGGATTCACACARTAYGCWACAGC-3'*) and ML-R (*5'-TTCATTGCRTAGTTWGGRTAGTT-3'*) specifically for profiling the methanogenic community [22]. Paired-end amplicon sequencing (2 x 300 bp) for both marker genes was carried out on the Illumina MiSeq platform (Australian Genome Research Facility, Queensland, Australia).

Raw reads were imported into Quantitative Insights into Microbial Ecology (QIIME 1.9.1) [23] for computational analysis. Paired-end reads were merged using USEARCH (version 8.1.1861) [24] tool and primers were identified and trimmed with Seqtk tool. Quality filtering, dereplication and operational taxonomic unit (OTU) clustering were performed following UPARSE pipeline [25]. Reads were mapped back to OTUs with a minimum identity of 97% to obtain the number of reads in each OTU. Taxonomy assignment was performed using Greengenes database (version 13_8) [26] for the whole microbial community (cut-off value 90%

156 similarity) and *mcrA* taxonomic database [19] for the methanogenic community (cut-off value
157 80% similarity). The rarefaction curves of all datasets approached the saturation plateau and
158 the Good's coverage of > 98.8 and > 99.7% for the whole microbial and the methanogenic
159 community (Fig. S1) confirming the sufficient sequencing depth in this study. The sequences
160 were rarefied to 55,000 and 30,000 sequences (the lowest number of sequences per sample) for
161 the whole microbial community and the methanogenic community, respectively, to estimate
162 alpha diversity indices (Observed species, Chao 1 value, Shannon and Simpson) using the
163 QIIME package. All sequencing data in this study are available at the Sequence Read Archive
164 (Accession Number: SRP139419) in the National Center for Biotechnology Information.

165 Statistical analyses including principle coordinates analysis (PCoA), Canonical
166 correspondence analysis (CCA) and Pearson's correlation coefficient analysis were performed
167 using QIIME and PAST package. Statistical testing for differential community characteristics
168 (e.g. alpha diversity indices, community composition, and community phenotype) was
169 conducted using the Student's t-test in Excel.

170 **3. Results and discussion**

171 **3.1. Effects of organic loading rate on microbial community diversity**

172 The diversity index measurements based on 16S rRNA and *mcrA* marker genes showed
173 a decrease in alpha diversity of the whole microbial community and the methanogenic
174 community, respectively, in response to the increase of OLR due to BW addition (Fig. 1). The
175 alpha diversity was assessed in terms of microbial community richness (Observed species and
176 Chao1 value) and evenness (Shannon and Simpson indices). Increasing the OLR to 86%
177 decreased Observed species, Chao1 value, Shannon and Simpson indices of the whole
178 microbial community by 6.7, 6.6, 3.4 and 0.6%, respectively. The decrease further extended to
179 29.0, 28.8, 25.8 and 14.3% when the OLR increased further to 171%. At the highest OLR (i.e.

240% increase), the alpha diversity indices were lower ($P < 0.05$ by Student's t-test [Table S1]) than values from the control community, indicating the impact of OLR on the microbial community diversity (Fig. 1). Several previous studies have reported the fluctuations in microbial diversity induced by co-substrate addition and their results could be attributed to co-substrate characteristics[15, 16]. On the other hand, our study appears to be the first to systematically demonstrate a decrease in microbial diversity as the OLR value increases due to the addition of a carbon rich co-substrate.

Of a particular note, the methanogenic community was more susceptible to AcoD compared to the whole microbial community in the digester. Significant decrease in the methanogenic community richness (15.8 and 14.4% decrease of Observed species and Chao 1 value) and evenness (2.4% decrease of Shannon and Simpson indices) were observed at 86% OLR increase (Fig. 1). Further increasing the OLR to 240% caused uneven methanogenic community with 18.4 and 10% decrease in Shannon and Simpson indices, respectively. On the other hand, at 240% OLR increase, the richness and evenness of the whole microbial community were not statistically different compared to those at 171% OLR increase ($P > 0.05$ by Student's t-test, 171% *vs* 240% OLR). Furthermore, the quantitative ratio between bacterial and archaeal abundance increased from 9.0 (control) to 12.7 and 24.6 at OLR increase of 171 and 240%, respectively, indicating the underrepresentation of archaea as OLR increased due to AcoD. Results in Fig. 1 suggest that increase in OLR favored the bacterial population and perturbed the archaeal community at a greater extent in the anaerobic digester. The degree of perturbation caused by OLR increase in the microbial community and methanogenic community diversity could be an indicator of digester performance. The impact of increase OLR on the alpha diversity prompted a detailed investigation into the microbial community structure as presented below.

[FIGURE 1]

205 **3.2. Shifts in microbial community structure**

206 The OTU (n = 5811 for the whole microbial community and n = 762 for the
207 methanogenic community) relative abundance data were used to carry out PCoA using Bray-
208 Curtis dissimilarities metric. The shifts in the community structure under changing operation
209 conditions (i.e. OLR increase) are summarized in Fig. 2. The control (0.27 ± 0.06 by Bray-
210 Curtis dissimilarities metric for the whole microbial community and 0.24 ± 0.07 for the
211 methanogenic community) communities clustered closely, suggesting a stable community
212 structure in the control digester during the experimental period and allowing the better
213 comparison among the control and co-digestion reactors.

214 There was an increasing and more profound variation in the whole microbial
215 community as the OLR increased due to the addition of a carbon rich co-substrate compared to
216 the control digester when examining the PCoA plot and the Bray-Curtis dissimilarities of the
217 different communities based on the analysis of the 16S rRNA marker gene (Fig. 2A and C).
218 Results from Fig. 2A & C conclusively demonstrate that excessive addition of carbon-rich co-
219 substrate could destabilize the microbial community. Indeed, the pairwise distance between the
220 control and AcoD increased as OLR increased (i.e. 0.45 ± 0.05 (control *vs* 86%), 0.64 ± 0.10
221 (control *vs* 171%) and 0.76 ± 0.05 (control *vs* 240%)). Although the intra-community distances
222 were lower than the inter-community distances, a PERMANOVA test revealed only significant
223 difference (Bonferroni-corrected $P < 0.05$) in the community structure of the digester at 171
224 and 240% OLR increase.

225 Fig. 2B & D show the impact of AcoD on the methanogenic community. The first two
226 principal coordinate axes (PC1 and PC2) explained approximately 75% of the variation in the
227 methanogenic community. Increasing OLR appears to destabilize the methanogenic
228 community structure with more profound variations among samples at high OLR. The distance
229 metrics within communities were also lower than that of pairwise communities (Fig. 2D).

[FIGURE 2]

The shift in the community structure upon the addition of co-substrate has been reported in the literature with the degree of shift varied by different co-substrate types and ratios. Yang et al [12] observed clear distinction of the microbial structure of an AcoD digester with SS and fat, oil and grease compared to a mono digester with only SS. In this study, a larger degree of alteration of microbial structure was simulated to observe process failure in the AcoD (e.g. at 240% OLR increase). Overall, results from 16S rRNA and *mcrA* gene-based community analysis suggest that increase OLR (86 – 240%) by addition of BW in AcoD altered the microbial community richness, evenness and structure. Thus, community dynamics were evaluated further by examining different taxonomical levels as discussed in the next section.

3.3. Phylogenetic community structure

16S rRNA gene sequence analysis showed the predominance of *Firmicutes* (20.2 ± 2.0%), followed by *Bacteroidetes* (13.5 ± 2.2%), *Proteobacteria* (7.2 ± 1.5%) in the control digester. In total, 16S rRNA gene sequence analysis reveals 19 major phyla (> 1% of the total) (Fig. S2). Consistent with the results in Fig. 2, OLR increase caused a notable shift in microbial community composition at the phylum level. For example, phyla *Firmicutes* were significantly higher in co-digestion digesters than in the control digester, with its population of 23.1 ± 3.0% and 53.3 ± 19.4% as the OLR increased to 86 and 171%, respectively (Fig. S2).

Changes in the relative abundances of major orders as a function of OLR increase due to AcoD are shown in Fig. 3. The orders belong to different microbial groups in the digester community and the extent of these changes varies from group to group. The *Clostridiales*, *Bacteroidales*, and *Anaerolineales* orders were the most predominant hydrolytic and fermentative bacteria in the three digesters. The sum relative abundance was 41.3% in the control digester. These orders continued maintaining their population under OLR increase with

254 the sum relative abundance of 39.1, 63.1 and 52.2% in communities at OLR increase of 86,
255 171 and 240%, respectively. Of interest was the significant enrichment of *Clostridiales* in the
256 co-digestion reactors at 86 and 171% OLR (21.1 ± 2.5%) and (52.2 ± 24.8%), respectively
257 compared to the control digester (19.8 ± 2.1%). The highest abundance of *Clostridiales* at
258 171% OLR increase correlated with the optimal digester performance (i.e. biogas production
259 increased by 191% and COD removal increased from 75.3 (control) to 86.2% [at 171% OLR
260 increase]). Members of the order *Clostridiales* are known to be associated with diverse
261 hydrolysis and fermentation pathways [27, 28] and they benefit from the syntrophic relationship
262 with hydrogenotrophic methanogens [29, 30]. It is noted that at 240% OLR increase, the sum of
263 relative abundances of six hydrolytic and fermentative bacterial orders was above 74.8% (*vs*
264 48.0% in control digester), indicating the significant enrichment of these bacterial groups. This
265 observation is consistent with the significant decrease in the community diversity and shifts in
266 community structure discussed previously.

267 The acetogenic bacteria order of *Synergistales*[27, 31] was prevalent in the control digester
268 (9.5 ± 2.0%). The abundance of *Synergistales* slightly increased in the OLR 86% community
269 (9.7 ± 1.5%) although the change was not statistically significant. At 240% OLR increase, the
270 *Synergistales* decreased significantly to 1.6 ± 0.2%. *Spirochaetales* and *Syntrophobacterales*
271 relative abundances exhibited some degree of variations among communities; however, the
272 changes were not statistically significant (Fig. 3). Therefore, members of the order
273 *Synergistales* could be more susceptible to OLR increase and their presence appears to play an
274 important role in the digester performance. The underrepresentation of *Synergistales* was in
275 good agreement with the accumulation of TOA in the digester at 240% OLR increase.

276 The three main orders of the methanogenic community were *Methanosarcinales*,
277 *Methanomicrobiales* and *Methanobacteriales*. The relative abundance of *Methanosarcinales*
278 and *Methanomicrobiales* increased significantly as OLR increased to 86%. Their populations

were maintained at a similar level to the control digester as the OLR increased to 171% and decreased further when the OLR increased to 240% ($P < 0.05$, by Student's t-test). The inhibition of the growth of *Methanosarcinales* and *Methanomicrobiales* could be directly attributed to the significant reduction of biogas production when OLR increased to 240%. Likewise, the optimum biogas and COD removal also coincided with their most abundance at 86% OLR increase. On the other hand, the order *Methanobacteriales* remained stable (< 1.5%) regardless of OLR increase.

[FIGURE 3]

3.4. Comparing methanogenic community from 16S rRNA and *mcrA* marker genes

The relative abundance of the methanogens from 16S rRNA analysis was normalized against the total abundance of methanogens for comparison. Fig. 4 presents the profile of methanogenic community in digesters at different OLR increase (0, 86, 171 and 240%). At the class level, two major classes *Methanomicrobia* and *Methanobacteria* were both detected by 16S rRNA (> 95% of the total) and *mcrA* (> 83% of the total) marker genes. However, the relative abundance of each class was significantly different in pairwise comparisons. For example, the relative abundance of the two classes *Methanomicrobia* and *Methanobacteria* revealed by 16S rRNA and *mcrA* marker genes were (87.1% *vs* 52.5% and 11.6% *vs* 36.8%), respectively in the control digester. The distribution of two classes was more even based on the *mcrA* marker gene under all tested conditions (Fig. 4), suggesting the complementary between the two methods. Both results indicated the presence of non-methanogenic *Euryarchaeota* (class *Thermoplasma*) at a very low abundance (< 0.05% of the total), while *Miscellaneous Crenarchaeotal Group* (MCG) was detected based on 16S rRNA gene. Recently, the presence of these groups has been reported in the anaerobic digestion process, but their roles are still unknown. Further analysis of methanogenic community at a more refined level revealed the predominance of three orders *Methanobacteriales, Methanosarcinales* and

304 *Methanomicrobiales*, suggesting the occurrence of both the hydrogenotrophic and acetoclastic
305 methanogenesis in digesters. Another notable observation was the higher abundance of
306 *Methanomicrobiales* at 0, 86 and 171% OLR increase detected by the *mcrA* marker gene. At
307 the highest OLR increase, a new *Methanomicrobia* order namely *YC-E6* was detected at 19.1%
308 by the 16S rRNA marker gene. A high number of unassigned microorganisms was observed in
309 taxonomical identification with the *mcrA* gene, suggesting the phylogenetic diversity of
310 methanogens in Archaea that remains to be discovered[32].

311 [FIGURE 4]

312 **3.5. Community correlations and indications on digester stability**

313 The relative abundances of seven major orders representing the hydrolytic, fermentative,
314 acetogenic and methanogenic microbial groups in all digester communities were selected for
315 community correlation analysis. Negative correlations (Pearson's correlation coefficient < 0)
316 were observed between hydrolytic/fermentative group (i.e. *Clostridiales*) and the orders of
317 other groups (acetogenic and methanogenic) (Fig. 5A). Statistical analysis revealed significant
318 correlation coefficients between *Clostridiales* and *Synergistales*, *Spirochaetales*,
319 *Syntrophobacterales* ($P < 0.05$). No significant correlation coefficient was observed between
320 *Clostridiales* and three orders of methanogens (Table S4). These results indicate that the
321 increase of *Clostridiales* could cause the decrease in abundance of others. This observation is
322 consistent with the data in Fig. 3. On the other hand, acetogenic and methanogenic groups
323 exhibited positive correlation within each group and inter-groups. These correlation
324 coefficients are insignificant, except for the ones between *Synergistales* and
325 *Methanosarcinales*, *Methanomicrobiales* (Table S4).

326 The relationship between environmental variables (i.e. TOA, pH, alkalinity, OLR),
327 performance variables (i.e. VS removal, COD removal and biogas production), and the

328 microbial community was examined by CCA. In this study, seven dominant microbial groups
329 and four environmental variables and three performance variables were screened for CCA plots
330 calculation (Fig. 5B). The CCA1 and CCA2 explained 97.5% of the total variation. The seven
331 variables were divided into four quadrants. OLR%, biogas production, TOA and COD removal
332 were distributed in the same quadrant, while pH and alkalinity were plotted into two quadrants.
333 BW addition (i.e. OLR increase) favored the growth of hydrolytic and fermentative bacteria
334 (e.g. *Clostridiales*). *Clostridiales* showed positive correlations with biogas production, COD
335 removal, and VS removal, indicating the important role of the order in digesters. However,
336 high abundance of this order increased the amount of TOA accumulated in the system which
337 led to a decrease in alkalinity and pH (Fig. 5B).

338 [FIGURE 5]

339 **4. Conclusion**

340 This study demonstrates that the introduction of a carbon-rich co-substrate to AD of SS
341 can lead to a decrease in microbial community diversity. Increasing OLR by further addition
342 of co-substrate extended the reduction of diversity indices (decreased by > 14.3%). In
343 particular, the methanogenic community was more susceptible to OLR increase when
344 comparing to the bacterial community. The shift in the community structure was most profound
345 at high OLR (240% increase) suggesting that there exists an OLR threshold at which the
346 function and resilience of the anaerobic ecosystem could be maintained. Excessive OLR value
347 (240% increase) enriched hydrolytic and fermentative bacteria (> 74.8% of the total
348 abundance) and perturbed acetogenic and methanogenic community. Results reported here also
349 show the complementary application of the *mcrA* and 16S rRNA marker genes to provide a
350 better assessment of the methanogenic community in the anaerobic digestion process.

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CONFLICTS OF INTEREST

There are no conflicts to declare.

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List of Figures:

Figure 1. Alpha diversity indices of AcoD communities under different OLR increase in terms of (A) Observed species, (B) Chao 1 value, (C) Shannon index, and (D) Simpson index. The data presents the mean and one standard deviation at 0% (5 samples), 86% (3 samples), 171% (3 samples) and 240% (2 samples) OLR increase. All indices were calculated at the minimum sequencing depth of all samples (i.e. at 55,000 and 30,000 sequences per sample for 16S rRNA and *mcrA* marker genes, respectively).

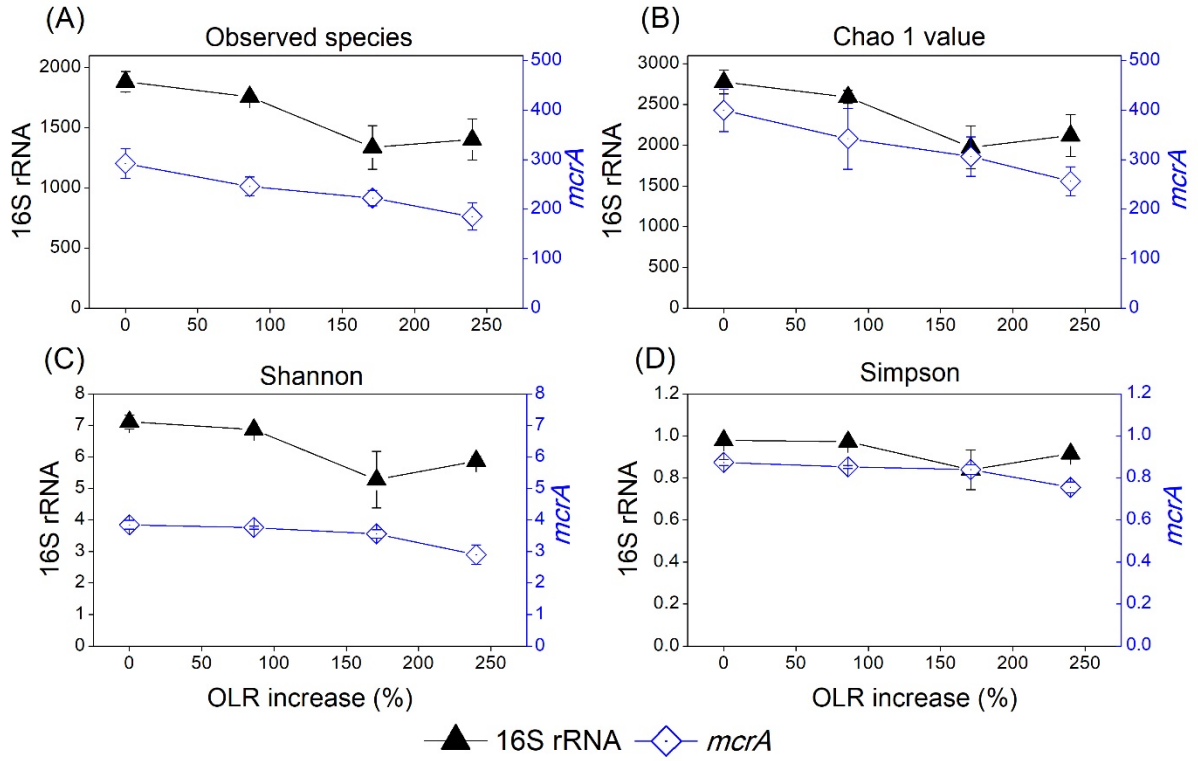
Figure 2. Shifts in the community structure based on (A) 16S rRNA and (B) *mcrA* marker genes principal coordinates analysis (PCoA) using the Bray-Curtis dissimilarities metric as well as the corresponding Bray-Curtis dissimilarities within and between communities from (C) 16S rRNA and (D) *mcrA* marker genes. The whiskers of the box represent the minimum and maximum values. The bottom and top of the box are the first and third quartiles, respectively, and the line inside the box denotes the median.

Figure 3. Relative abundances of the major microbial orders revealed by 16S rRNA marker gene. The error bars represent the mean and one standard deviation from the mean.

Figure 4. Relative abundances within the methanogenic community at (A) class and (B) order level revealed by 16S rRNA and *mcrA* marker genes. The bar presents the mean value at of 0%, 86%, 171% and 240% OLR increase.

Figure 5. Relationships between environmental variables and performance variables and the microbial community. (A) Heat map for the frequency correlation between selected orders of different microbial groups (i.e. hydrolytic, fermentative, acetogenic and methanogenic). The color scale indicates the Pearson's correlation coefficient (between -1 and 1) with red color for positive correlations and blue color for negative correlations. (B) Canonical correspondence

482 analysis (CCA) between selected orders of different microbial groups and environmental
483 variables (i.e. TOA, pH, alkalinity, OLR) and performance variables (i.e. VS removal, COD
484 removal and biogas production). The symbols: blue diamond, red circle, black triangle and
485 green star denotes the communities at 0, 86, 171 and 240% OLR increase. Solid blue circle
486 denotes seven orders with their names placed beside.



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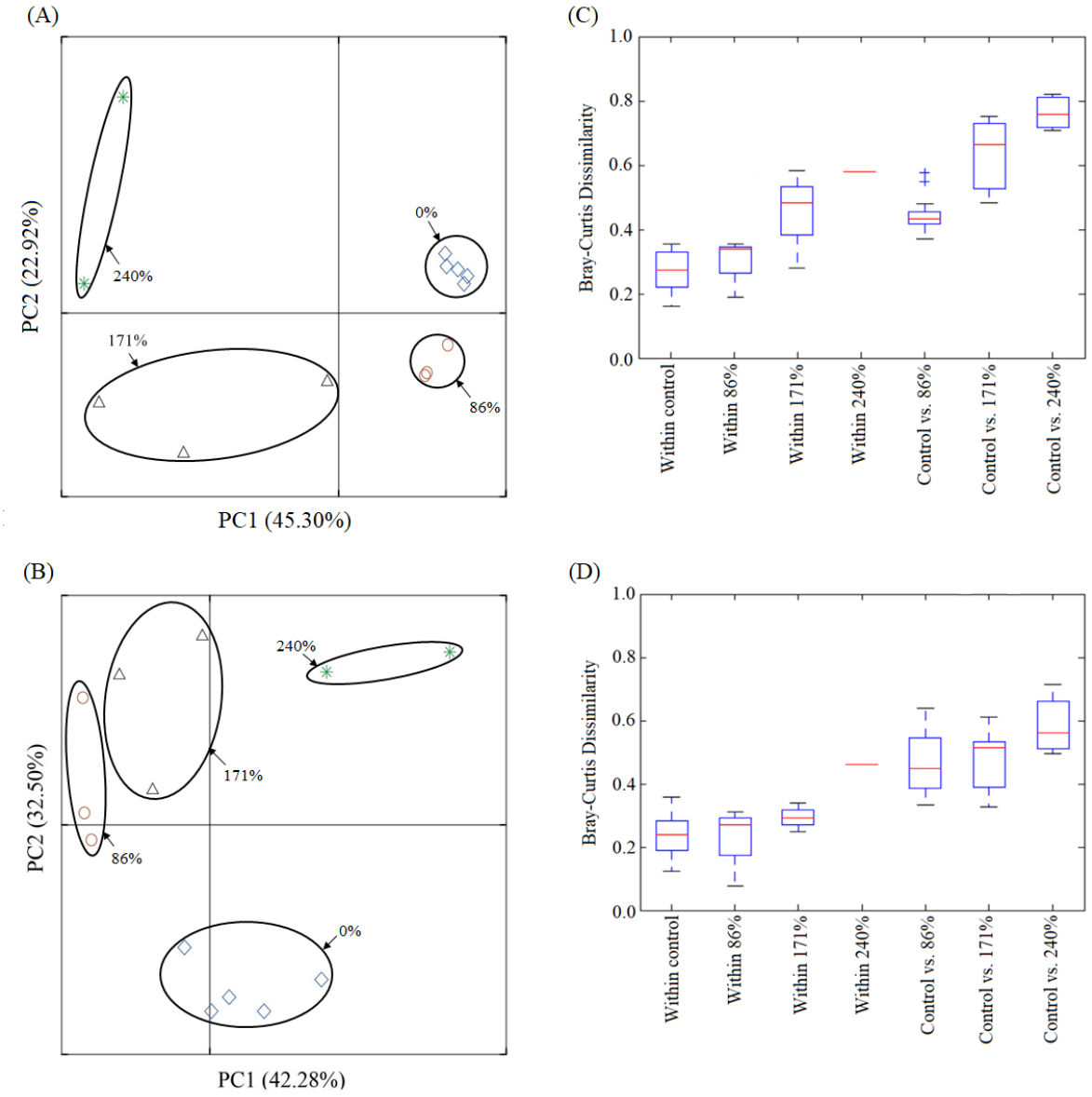
488 **Figure 1**



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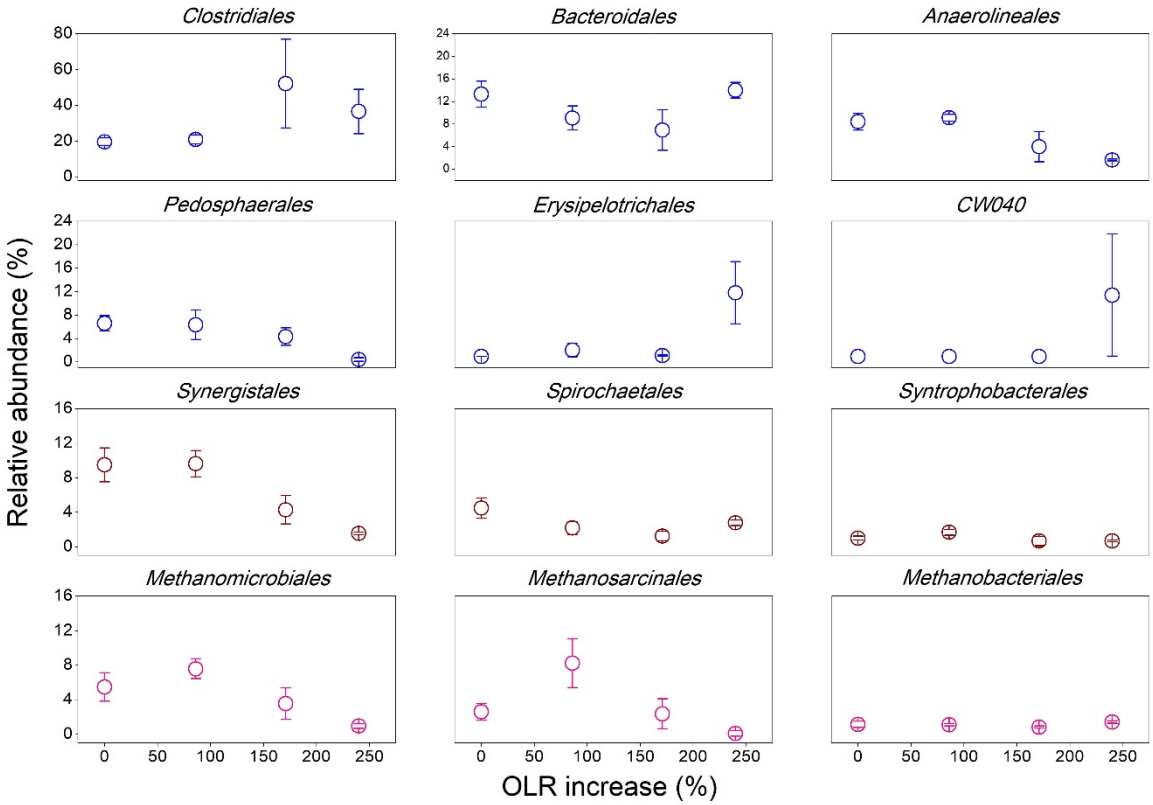

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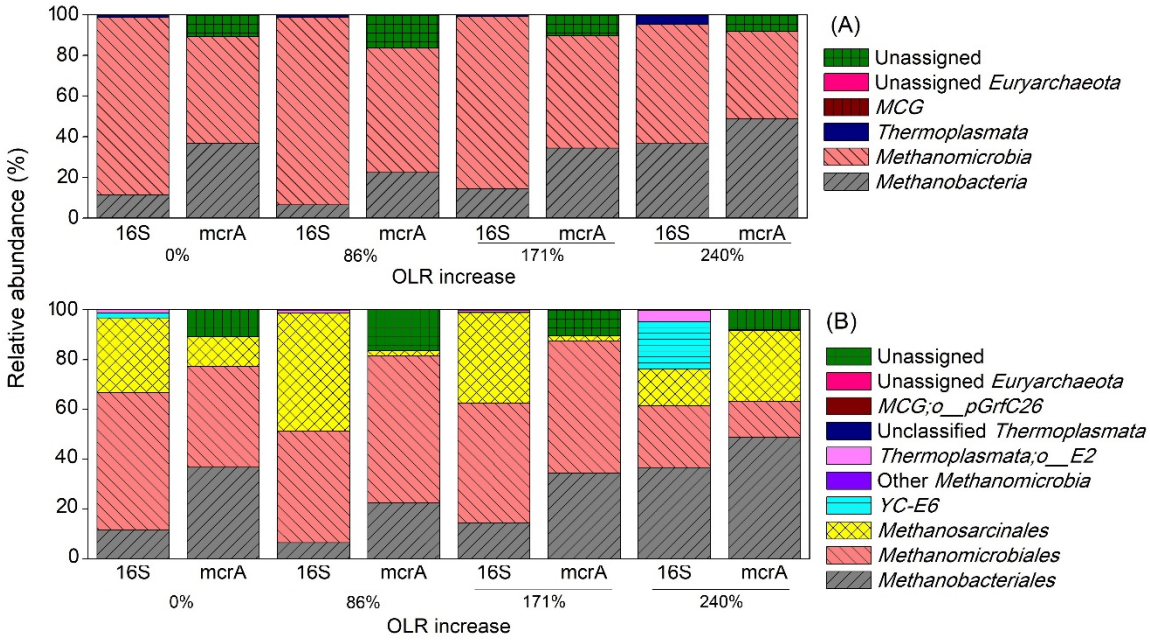

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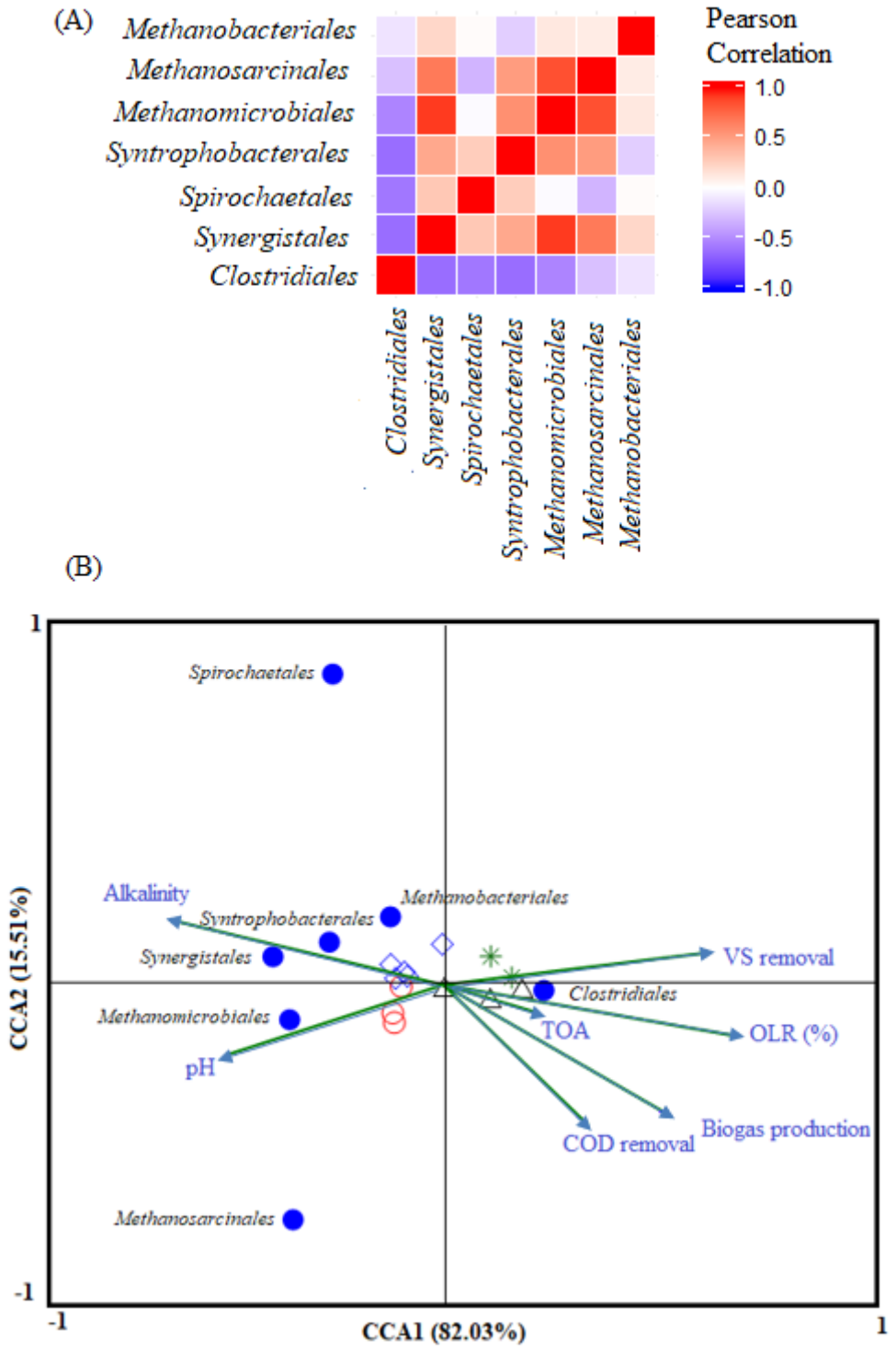
494 **Figure 4**



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496 **Figure 5**

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