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Effects of operational disturbance and subsequent recovery process on microbial community during a pilot-scale anaerobic co-digestion

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ABSTRACT

This study investigated changes in microbial community structure and composition in response to operational disturbance and subsequent process recovery by inoculum addition. Amplicon sequencing of 16S rRNA and *mcrA* marker genes on the Illumina Miseq platform was used for microbial community analysis. The results show that imbalance among core microbial groups caused volatile fatty acid accumulation and subsequent deteriorated biogas production (decreased by 45% of daily volume) and methane content (<49%). Operational disturbance led to the enrichment of hydrolytic and fermentative bacteria (accounted for >57% of the total abundance) and reduction of acetogenic and methanogenic microbes (they accounted for <9% and <3% of the total abundance, respectively), suggesting their resilience. Acetogens and methanogens were replenished by inoculum addition to recover digester performance. Although digester performances were similar in stable (prior to disturbance) and post recovery phase, the microbial community did not return to the original state, suggesting the existence of functional redundancy in the community.

KEYWORDS: anaerobic co-digestion, operational disturbance, digester recovery, sewage sludge, microbial community, beverage waste.

1. Introduction

In recent years, water utilities have started to implement anaerobic co-digestion (AcoD) of sewage sludge and organic-rich wastes using existing anaerobic digestion (AD) facility in municipal wastewater treatment plants (Nghiem et al., 2017; Shen et al., 2015; Xie et al., 2018). During conventional AD (mono-digestion), the synchronisation amongst the four key stages namely hydrolysis, acidogenesis, acetogenesis, and methanogenesis results in the production of methane-rich biogas from sewage sludge with no accumulation of intermediate products that can inhibit the process (Sawatdeenarunat et al., 2016). A different picture emerges when AcoD is implemented with organic-rich co-substrate as some intermediate products such as volatile fatty acids (VFAs) can accumulate in the system (Xie et al., 2017). High VFA concentration could inhibit one or more stages of the anaerobic digestion process and result in digester failure (Ratanatamskul and Manpetch, 2016). In addition, operational disturbance such as temperature variation may also occur during full-scale operation. Thus, it is essential to understand how the anaerobic microbial community respond to disturbance during AcoD operation.

Many dedicated efforts have been devoted toward a better understanding of the anaerobic microbiome, especially after the emergence of next-generation sequencing technology (Buettner and Noll, 2018; Centurion et al., 2018; Ju et al., 2017). The 16S rRNA marker gene is frequently used to target both bacterial and archaeal domains, while the *mcrA* marker gene specifically targets methanogens (Friedrich, 2005). The combined use of these two marker genes could offer better methanogenic community characterization (Wilkins et al., 2015). Overall, previous studies have identified key players in the process and their functions. It appears that some microorganisms have one specific function while some can perform multiple functions. For example, *Methanomicrobiales* and *Methanosarcinales* are only involved in methanogenesis while *Firmicutes* and *Bacteroidetes* members are reported to participate in both hydrolysis and fermentation (Venkiteshwaran et al., 2015). Core microbial groups also have different growth conditions, physiology and stress tolerance.

Successful implementation of AcoD at existing WWTPs requires careful management of the risk associated with operational and environmental disturbances (e.g. organic overloading, high ammonia concentration and temperature fluctuation). Disturbances can result in alteration of microbial community structure and composition. An increase of *Syntrophomonadaceae* family followed by digester failure was triggered by overloading of

glycerol in a digester treating cow manure and crude glycerol (Regueiro et al., 2015). Dominant methanogens shifted from acetoclastic *Methanosaeta* to hydrogenotrophic *Methanocorpusculum* and *Methanobrevibacter* during high salinity exposure in digesters treating molasses (De Vrieze et al., 2017). In anaerobic digesters treating organic household waste, *Bacteroidetes* and *Chloroflexi* were dominant at mesophilic temperatures (34 and 27% of total clones), whilst the phylum *Thermotogae* was the major phyla in the thermophilic conditions (61%) (Levén et al., 2007). The shift in dominant taxa from *Firmicutes* (67 – 75%) to *Bacteroidetes* and from *Methanosaeta* to *Methanosarcina* (60%) due to temperature drop has been reported during AcoD of pig manure, fish processing waste, beet molasses residues (Regueiro et al., 2014). It is evidenced that altering the microbial community can cause an imbalance among core microbial groups which eventually lead to VFAs accumulation, microbial community inhibition and digester failure. However, there have not yet been any studies to elucidate the effect of common operational disturbance (i.e. temperature and substrate stratification due to mixing failure) in both mono- and co-digestion.

Despite the significant economic loss associated with digester failure, only a few studies have investigated different recovery strategies (i.e. water dilution, bentonite addition, feeding cessation, inoculum addition, pH adjustment, trace elements supplement). Wu et al. (2015) applied inoculum addition (mixing ratio of 80%) to recover the performance of an AD system fed with grease trap waste. Inoculum addition (mixing ratio 80%) was reported to fully restore performance within 20 days compared to other methods such as bentonite addition (73 days) or water dilution (64 days) (Wu et al., 2015). Aboudi et al. (2016) recovered a failed digester due to overloading of sugar beet by-product and cow manure after 40 days by inoculum addition and feeding cessation. Zhang et al. (2018) reported that VFAs accumulation during anaerobic digestion of food waste could result in system failure and inoculum addition has shown the effectiveness in recovering the failed digester after 3 days.

Although the feedstock and cause of disturbance varied from study to study, previous studies have confirmed the potential of inoculum addition in facilitating digester recovery in a short time. However, these studies have only focused on process performance. The dynamics of the microbial community during the digester recovery period has not been elucidated. Understanding the microbial community dynamics and the subsequent impact on digester performance during stable, disturbance and recovery periods are necessary in order to identify populations that better respond to disturbance and to set microbial indicators of

process performance (Carballa et al., 2015). This new knowledge could be used to develop effective strategies to prevent failure, mitigate disturbance consequences and shorten the recovery time (Regueiro et al., 2014).

This study aims to investigate the microbial community of pilot-scale AD plant during operational disturbance and the subsequent process recovery. Simulation of operational disturbance is carried out by ceasing digester mixing. New inoculum is then added to restore the digester performance. Illumina Miseq sequencing for the total DNA of the digestate is performed with 16S rRNA and *mcrA* marker genes. The digester performance is also monitored along with the experiment period. By comprehensive investigation of digester microbial community and performance, our results provide new insights for the improvement of digester operation and performance.

2. Materials and methods

2.1. Pilot-scale anaerobic digesters setup

A pilot AD plant was constructed and installed at a WWTP (Shellharbour, NSW, Australia). The pilot plant consisted of two parallel and identical anaerobic digester systems. Each system had a 1000 L conical stainless steel reactor, a heating system, a feed pump, a circulation pump for mixing, a propeller mixer, an online biogas meter (Brooks Instrument, Hatfield, PA, USA), and external gas holder (Supplementary Data Fig. S1). Heating was achieved by circulating hot water through a water jacket at the bottom of the conical stainless steel reactor. The reactor and all pipelines were insulated with polystyrene-aluminium foil to prevent heat loss. The recirculation pump was continuously operated at 25 L/min to provide mixing in the reactor. The propeller mixer was also operated for 1 min on and 30 min off cycle to provide additional mixing. The pilot AD plant was equipped with supervisory control and data acquisition (SCADA) and could be remotely operated.

2.2. Feedstock and inoculum

Beverage waste (BW) was collected from a commercial waste collector centre (SUEZ Camellia Resource Recovery Centre, NSW, Australia), stored in a cool room (- 4 °C) on site, and used as the co-substrate. Anaerobic digestate and primary sludge (PS) were collected directly from the Shellharbour WWTP (where this pilot AD plant was located) and used as inoculum and feed sludge, respectively. Characteristics of the feedstocks and inoculum

including total chemical oxygen demand (tCOD), alkalinity, total organic acids (TOA), pH, total solids (TS) and volatile solids (VS) are available in Table S1.

2.3. Digester operation

Apart from the difference in substrate feeding as specified below, both systems of the pilot AD plant were operated under the same conditions. They were operated at a sludge retention time of 20 days by maintaining an active sludge volume of 600 L and a daily feed of 30 L. The systems were fed automatically in four cycles per day in a similar protocol to a full-scale plant. Each feeding cycle was initiated by first discharging 7.5 L of digested sludge, then, 7.5 L of feed (either PS or a combination of PS and BW) was fed into the reactor.

The systems were operated for a total period of 148 days with different operating phases defined as follows: start-up (day 0 – 73), phase I - quasi-stable (day 74 – 84), phase II - operational disturbance (day 85 –99), phase III - inoculum addition (day 100 – 109) and phase IV - new quasi-stable phase (110 – 148).

In the start-up phase, the two systems were fed with PS (organic loading rate (OLR) of 1.81 kg COD/m^3d) and operated under the same operating conditions (Table S2). Once similar performance (shown through biogas production and methane content) has been achieved (after 73 days), phase I commenced. In phase I, one digester was used for the mono-digestion experiment while the other digester was used for the co-digestion experiment. Only PS was fed into the mono-digestion system throughout the experimental period. In the co-digestion system, BW was added with PS at a BW:PS mixing ratio of 10:90 (% v/v), corresponding to an increase of OLR to 2.39 kg COD/m^3d.

In phase II, circulation pumps and mixers of both systems were turned off while hot water circulation to the water jacket at the bottom of the reactor was operated as normal. In the absence of mixing, operational disturbance (i.e. temperature and substrate stratification) was induced in the digester. This disturbance resulted in a deteriorated performance of both systems.

In phase III, new inoculum was added to both systems to facilitate digester recovery. This was achieved by emptying 100 L of sludge from each reactor then adding the same volume of digestate from the full-scale plant on day 100, 102, 107 and 109.

Throughout this study, biomass samples for microbial community characterization were collected weekly to profile the changes in the microbial community from each phase. The feedstock composition of the digesters during different operating phases is available in Table S2. The digester performance and operational conditions were monitored with a set of parameters including biogas production, methane production, pH, TOA and alkalinity.

2.4. Analytical methods

Biogas production was continuously recorded via the SCADA system. Biogas composition was analysed using a portable GA5000 gas analyser (Geotechnical Instruments, UK) every second day (Nghiem et al., 2014). tCOD was measured following the US-EPA Standard Method 8000 by using high range COD vials (Hach, Australia) and Hach DR3900 spectrophotometer. Alkalinity and TOA were measured weekly following the standard method 2320B and 5560C, respectively. Digestate pH was measured every second day using a portable pH meter (Thermo Fisher Scientific, Australia).

2.5. Microbial community analysis

Collected biomass samples were immediately fixed in ethanol (1:1 v/v) and stored at - 20 ºC before DNA extraction. Genomic DNAs were extracted from samples using FastDNA® SPIN Kit for soil (MP Biomedicals, Santa Ana, CA) following the manufacturer's instructions. The integrity, purity and concentration of the extracted DNA were determined by means of 1% (w/v) agarose gel electrophoresis 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. Forward and reverse primers Pro341F (*5'-CCTACGGGNBGCASCAG-3'*) and Pro805R (*5'-GACTACNVGGGTATCTAATCC-3'*) were used to amplify the V3 – V4 region of the *Bacterial* and *Archaeal* 16S rRNA genes for characterisation of the whole microbial community (Takahashi et al., 2014). For characterisation of the methanogenic community, the primer set ML-F (*5'-GGTGGTGTMGGATTCACACARTAYGCWACAGC-3'*) and ML-R (*5'-TTCATTGCRTAGTTWGGRTAGTT-3'*) was used to target the *mcrA* marker gene (Luton et al., 2002). 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).

Computational analysis of the sequencing reads was performed using the Quantitative Insights into Microbial Ecology (QIIME 1.9.1) platform (Caporaso et al., 2010). Forward and reverse reads were assembled using USEARCH (version 8.1.1861) tool (Edgar, 2010) and primers were identified and trimmed with Seqtk tool. UPARSE pipeline (Edgar, 2013) was used to perform quality filtering, dereplication and operational taxonomic unit (OTU) clustering within QIIME environment. OTU abundances were calculated by mapping reads back to OTUs with a minimum identity of 97%. Taxonomical classification was done against the Greengenes database (version 13_8) (McDonald et al., 2012) for the whole microbial community (cut-off value 90% similarity) and *mcrA* taxonomic database (Wilkins et al., 2015) for the methanogenic community (cut-off value 80% similarity). All sequencing data in this study are available at the Sequence Read Archive (Accession Number: SRP150500) in the National Center for Biotechnology Information.

The statistical analysis non-metric multidimensional scaling (NMDS) was performed using PAST package. Statistical testing for differential community characteristics and performance parameters were conducted using Student's t-test in Excel $p < 0.05$ (i.e. statistically significant).

3. Results and discussions

3.1. Overall digester performance

The two parallel AD systems showed similar performance during the start-up phase (i.e. prior to co-digestion). The biogas production and methane content in both systems were comparable at 276 ± 82 L/d and 63.0%, respectively. The theoretical methane potential (L CH_4/ g COD) is widely used to indicate the maximum methane yield from a specific waste. In this period, AD of PS generated a specific methane yield of 0.15 L CH_4 / g COD that was comparable to the previous data from lab-scale digesters (Yang et al., 2017). Other parameters (pH, TOA, and alkalinity) were stable and in the normal range (Table 1) suggesting that no VFA or ammonia accumulation occurred. Two identical systems with similar performance were then set as the baseline for comparison in subsequent experimental phases (i.e. one mono- and one co-digestion).

Co-digestion of PS and BW in phase I (at mixing ratio of 90:10 % v/v) increased in 62% more biogas production (Table 1). Higher biogas production in AcoD coincided with a 32% increase in OLR due to the addition of carbon-rich substrate (i.e. 1.81 *vs* 2.39 kg COD/m^3d).

Although more biogas production was achieved in the co-digestion system, the specific methane yield was similar in both systems (0.15 *vs* 0.16 L CH_4/g COD). Thus, there was probably no synergistic effect between PS and BW substrates. BW substrate increased biogas production mainly due to the addition of carbon source in this study. The synergistic effect may require a complementary of alkalinity, trace elements, nutrients and pH, which increase substrate biodegradability and methane yields. BW is carbon-rich substrate (i.e. 150.8 ± 0.6 COD g/L), but deficient in nutrients. On the other hand, PS is rich in nutrients. The nutrient-rich PS can complement the carbon-rich substrate to a level that allows sufficient digestion and process stability. The addition of BW was quantitatively based on the COD content of PS and BW to achieve 2.39 kg COD/m^3d and followed the suggestion from a previous laboratory scale experiment to avoid overloading the digester (Wickham et al., 2018). A future investigation to determine the synergistic effect and nutrient compositions for optimization of BW addition is suggested.

In phase II, operational disturbance induced significant impacts on the digester performance and conditions in both mono- and co-digestion systems. Biogas production and methane content decreased significantly to 115 ± 42 L/d (decreased by 45%) and 49.0 ± 4.6% in the mono-digestion system and to 129 ± 61 L/d (decreased by 62%) and 46.6. ± 2.8% in the co-digestion system. TOA levels increased by 5.6 and 4.7 times in mono- and co-digestion systems, respectively. Consequently, digestate pH dropped to 6.4 ± 0.4 and 5.7 ± 0.7 in mono- and co-digestion systems, respectively. These results indicate that different microbial groups in anaerobic digesters might be influenced differently by operational disturbance. Low pH is unfavourable for methanogens, however other microbial groups can continue to function. The data was in agreement with previous studies that temperature is a deterministic factor influencing the digester performance by altering the complex microbial community in anaerobic digesters and thus could lead to process failure (Luo et al., 2015; Regueiro et al., 2014). The co-digestion system was more susceptible to operational disturbance than the mono-digestion system. This could be attributed to the addition of a carbon-rich substrate (BW) in the co-digestion system that led to faster hydrolysis and acidogenesis. When the system experience disturbed conditions, faster hydrolysis and acidogenesis decrease pH of the digester. Low pH condition inhibits methanogenic microbes that are characterized as slow-growing microorganisms and sensitive to low pH. In other words, AcoD can negatively affect the resilience of the digester under disturbance (Xie et al., 2016). Further investigation into the microbial community structure could support this finding (Section 3.3 &3.4).

[TABLE 1]

A total of 17% of digester volume was replaced with the inoculum (i.e. digested sludge) from a full-scale AD to initiate the recovery process of digester after phase II. A slight increase in biogas production was observed in both digesters. However, methane content continued to drop to 38% and 32% while pH dropped to 5.7 and 5.4 in mono- and co-digestion system, respectively. The increase in biogas production and a decrease of methane content are consistent with rapid hydrolysis and acidogenesis and the inhibition of the methanogenic process. Another inoculum addition event was carried out on day 102. After this second addition, the digesters showed a gradual increase in pH and methane content. Specifically, pH returned to 6.7 and 5.9 in the mono- and co-digestion system, respectively, and an increase in the methane content of the mono-digestion system from 38 to 41% was observed. Nevertheless, the digesters have not reached the same performance levels as prior to the disturbance (phase I). The third inoculum addition was implemented on day 107 resulted in notable improvements. Methane content in the mono- and co-digestion system increased to 50 and 34%, respectively. Digestate pH in the mono-digestion system also increased to 6.9, however, digestate pH in the co-digestion system was below 6.0. Thus, another inoculum event was carried out on day 109. Notably, digester performance was successfully boosted with biogas production increased from 272 and 148 (day 108) to 373 and 281 L/d (day 111) for the mono- and co-digestion system, respectively. At this phase, both digesters were fed with PS and operational parameters (i.e. temperature and mixing) were maintained. Our results suggest that inoculum addition could shorten the recovery period of a failed digester (i.e. ten days). At the last phase (38 days), the mono and co-digestion system were operated in the same fashion as in phase I. The obtained results from this period confirmed the recovery process (i.e. comparable performance to prior disturbance) (Table 1). Further investigations on the changes in the microbial community in both mono- and co-digestion systems will provide insights into the effects of co-digestion, operational disturbance and inoculum addition.

3.2. Microbial community during stable operation

Analysis of the phylogenetic structure of the microbial community indicated that the overall profile is the same in the two digesters. This is consistent with the identical performance in the start-up phase (Fig. S2). Co-digestion of BW in phase I did not change the overall microbial community structure. Hydrolytic and fermentative bacteria in the two systems

included *Anaerolineales, Thermotogales, Bacteroidales,* and *Clostridiales*. Their relative abundances were 20.1, 9.8, 9.3 and 2.4% in the mono-digestion system and 32.4, 9.0, 6.5 and 2.8% in the co-digestion system, respectively. These bacteria were dominant in anaerobic digestion. *Bacteroidales* was dominant in a digester treating PS with population up to 45.6% (Ju et al., 2017). Members of *Thermotogae* and *Anaerolineae* were also detected in all samples at high abundances of 8.7 ± 7.0% and 2.7 ± 1.2% under different operating conditions (i.e. sludge retention time) (Ju et al., 2017). *Clostridiales* presented at 20 - 30% of the total abundance in thermophilic AD of waste activated sludge (Ho et al., 2013).

The abundance of core acetogenic microorganisms in both systems were also similar. The relative abundance of *Synergistetes*, *Spirochaetes*, *Syntrophobacterales* and *Syntrophomonas* were 5.9, 3.4, 5.4 and 0.5 in the mono-digestion system, and 9.5, 2.4, 2.8 and 0.4% in the co-digestion system, respectively. *Synergistetes* and *Spirochaetes* are able to convert lactate to substrates for methanogens (Detman et al., 2018). The order *Spirochaetales* (phylum *Spirochaetes*) is potential syntrophic acetate-oxidizing bacteria (SAOB) coupled with hydrogenotrophic methanogens in syntrophic acetate-oxidation (SAO) (Deng et al., 2017). While *Syntrophobacterales* is responsible for propionate degradation, *Syntrophomonas* takes part in butyrate and other organic acids oxidation (Venkiteshwaran et al., 2015). *Syntrophomonas* has been detected at high abundance in good performing full-scale anaerobic digesters and also correlated to methane production in digesters treating synthetic wastewater (Regueiro et al., 2012; Venkiteshwaran et al., 2017).

The major methanogens included *Methanomicrobiales, Methanosarcinales* and *Methanobacteriales*. Their relative abundances were 2.5, 1.2 and 0.1% in the mono-digestion system, and 2.1, 4.9 and 0.2% in the co-digestion system, respectively. The methanogenic community was also targeted using *mcrA* marker gene and the results from both marker genes were congruent with each other in terms of presented methanogens and their populations (*Methanomicrobiales* was the most dominant order and accounted for 63.7 and 72.6% of the total methanogens abundance in the mono- and co-digestion system). *Methanosarcinales* are strict acetoclastic methanogens that produce methane directly from acetate, while members of *Methanomicrobiales* and *Methanobacteriales* using hydrogen from SAOB to produce methane (hydrogenotrophic methanogens).

3.3. Transition of microbial community induced by operational disturbance

Significant changes in the abundances of core microbial groups were observed under operational disturbance (Fig. 1). The results suggested an imbalance distribution between microbial groups and the degree of alternations were different in each group. During operational disturbance, the total abundance of hydrolytic and fermentative bacteria increased from 41.6 and 50.6% to 57.2 and 66.6% in mono- and co-digestion system, respectively. Indeed, the relative abundance of *Thermotogales, Bacteroidales and Clostridiales* increased to 12.1 ± 1.5, 12.7 ± 1.6 and 10.9 ± 5.0% in the mono-digestion system, and to 14.1 ± 7.2, 10.3 ± 1.1 and 25.4 ± 16% in the co-digestion system, respectively (Fig. 1). The increase in abundance of *Thermotogales* during operational disturbance could be attributed to the fact that it is a thermophilic bacteria order. The order *Anaerolineales* was still dominant in the digesters in phase II (21.5 ± 6.8% in the mono-digestion system), however, its abundance decreased from 32.3 to 16.8 ± 8.3% in the co-digestion system. Hydrolytic and fermentative bacteria are phylogenetically diverse and are resilient to environmental changes (Venkiteshwaran et al., 2015). The predominance of hydrolytic and fermentative bacteria in the microbial communities is attributable to rapid acid production and lower pH in the digester (Section 3.1).

[FIGURE 1]

A decline was observed in the abundance of syntrophic acetogens in the two digesters communities during operational disturbance (Fig. 1). *Synergistetes* and *Syntrophobacterales* abundance decreased by 2.0 and 4.3% in the mono-digestion system, and by 6.8 and 2.4% in the co-digestion system, respectively. As a result, TOA accumulation occurred (Table 1) and led to dramatic pH drop to 6.4 ± 0.4 and 5.7 ± 0.7 in mono- and co-digestion systems, respectively. This pH level was under optimum pH range for methanogens growth, thus methanogens were inhibited during the disturbance. Specifically, the abundances of orders *Methanomicrobiales* and *Methanosarcinales* decreased by 0.7 and 1.1% in the mono-digestion system while their abundances dropped by 0.9 and 4.8% in the co-digestion system. This explains the decline in biogas production and methane content observed in both systems (Table 1). Our results also suggested that acetogens and methanogens were more susceptible to operational disturbance than hydrolytic and fermentative bacteria.

The increased temperature in the bottom part of the reactor during operational disturbance (Section 3.1) probably provoked the emergence of another thermophilic bacteria order

346 *Thermoanaerobacterales*, whose members can thrive under elevated temperature (50 – 70
347 ºC). Species in the order of *Thermoanaerobacterales* (i.e. *Coprothermobacter sp)* are
348 syntrophic acetate-oxidizing bacteria (SAOB) the convert acetate to H_2 and CO_2 to promote
349 hydrogenotrophic methanogenesis under disturbance conditions (Gagliano et al., 2015b). The
350 abundance of genus *Coprothermobacter* rose from 0.5 (phase I) to 3.4 ± 1.7% (phase II) in
351 the mono-digestion system and from 0.8 (phase I) to 1.4 ± 0.7% (phase II) in the co-digestion
352 system. Phylum *Spirochaetes* has also been suggested to be involved in this SAO pathway
353 (Deng et al., 2017). Its abundance increased to 3.7 ± 0.3% in mono- and maintained at 2.0%
354 in the co-digestion system. In line with the emergence of SAOB, the relative abundance of
355 *Methanobacterales* increased to 0.2 ± 0.1 and 0.5 ± 0.2% in the mono- and co-digestion
356 system, respectively. These observations coupled with the absence of acetoclastic
357 methanogens (*Methanosarcinales*) may indicate the shift from co-occurrence of both
358 methanogenesis pathways to the exclusive occurrence of SAO pathway during disturbance.

359 **3.4. Insights into the microbial community during process recovery**

360 Core microbial groups detected in the inoculum were similar to those in the pilot digester
361 since the same substrate (i.e. PS) was used by the pilot and full-scale AD plant. Dominant
362 hydrolytic and fermentative bacteria included *Anaerolineales* (10.9 ± 1.7%) and
363 *Bacteroidales* (8.8 ± 1.8%) while acetogens representatives were *Synergistetes* (7.2 ± 0.9%),
364 *Spirochaetes* (13.4 ± 3.9%) and *Syntrophobacterales* (4.6 ± 0.4%). Methanogenic archaea
365 order *Methanosarcinales* and *Methanomicrobiales* were also presented in the inoculum at 1.1
366 ± 0.3 and 1.1 ± 0.4%. It is believed that adding inoculum help to supply active microbial
367 groups to the digesters and also reduce the concentration of growth inhibitors (e.g. VFAs) in
368 the digester due to dilution factor, thus accelerate the recovery process (Aboudi et al., 2016).
369 The source and quality of the inoculum used in anaerobic digesters are also of major
370 importance (De Vrieze et al., 2015).

371 Addition of inoculum during phase III resulted in notable changes in the microbial
372 community in both digesters. The total abundance of hydrolytic and fermentative bacteria
373 decreased from 57.2 to 50.2% and from 66.6 to 54.4% in mono- and co-digestion system,
374 respectively. During inoculum addition, only *Bacteroidales* abundance increased from 12.7 ±
375 1.5 to 16.7 ± 3.5% and from 10.3 ± 1.1 to 20.6 ± 5.8% in mono- and co-digestion system,
376 respectively (Table 2). In contrast, other hydrolytic and fermentative orders showed a
377 decrease in their abundances. This is probably due to the high abundance of *Bacteroidales* in

the inoculum and the potential of this order to be a stronger competitor for substrate than other bacteria (Ju et al., 2017).

[TABLE 2]

Meanwhile, acetogens population was restored during inoculum addition, with *Synergistetes* abundance increased to 4.5 ± 1.2% in the mono-digestion system, *Syntrophobacterales* abundance increased to 0.5 ± 0.1% in the co-digestion system. Acetogenic bacteria and potential SAOB phylum *Spirochaetes* abundance increased to 9.2 ± 2.4 and 5.4% in the mono- and co-digestion system, respectively. The increase in acetogens and SAOB abundance promote VFAs degradation and explained for the gradual return of pH to normal range.

A slight increase in the abundance of methanogens was observed during inoculum addition, probably due to the slow-growing characteristics of methanogens compared to other groups and thus, they may take a longer time to recover from disturbance. *Methanosarcinales* abundance in the mono- and co-digestion systems increased from 0.1 ± 0.1 to 0.6 ± 0.4% and 0.1 ± 0.1 to 0.2 ± 0.1%, respectively (this order presented in the inoculum at 1.1 ± 0.4% of the total abundance). *Methanomicrobiales* also restored its population but at a slower pace, its abundance in the mono- and co-digestion system only increased in phase IV from 1.0 ± 0.1 to 4.2 ± 0.4% and 0.5 to 2.5% (this order presented in the inoculum at 1.1 ± 0.4% of total abundance).

Overall, inoculum addition led to the re-establishment of a balance between core microbial groups and promoted recovery process by supplying VFAs-degrading bacteria to reduce the accumulated VFAs concentration. In this study, the inoculum used for digester recovery was taken from a full-scale anaerobic digester treating the same main substrate with the pilot-scale digesters. The fact that the inoculum contained a well-adapted biomass to the substrate and inoculum addition was carried out four times might contribute to the short recovery time (ten days). It is also noticed that the inoculum used at different times showed no difference in the microbial community composition due to stable operation of the full-scale AD (i.e. inoculum source).

3.5. Microbial community prior to disturbance and post inoculum addition

At the end of the experiment (phase IV), the microbial communities in both digesters were very different from the initial stable phase (phase I) despite the retrieval of similar operating conditions and performances. Taxonomical profiling confirmed the distinguished microbial community composition from the initial state. Compared to phase I, hydrolytic and fermentative bacteria in both digesters were enriched to 55.2 and 68.0% of the total abundance. Nevertheless, each hydrolytic and fermentative bacteria order abundance changed differently. *Bacteroidales* and *Clostridiales* abundances increased by 9.6 and 9.1% in the mono-digestion system and 6.7 and 1.0% in the co-digestion system. The order *Anaerolineales* abundance decreased by 5.7 and 3.8% in the mono- and co-digestion system, respectively. Meanwhile, the order *Thermotogales* slightly decreased by 1.6% in the mono-digestion system but increased significantly by 13.6% in the co-digestion system. In the mono-digestion system, acetogens total abundance remained unchanged (13.7% in phase I vs 13.2% in phase IV), however, one dominant member of this group shifted from *Syntrophobacterales* to *Spirochaetes*. Meanwhile, methanogens total abundance was almost doubled (3.8% in phase I vs 6.1% in phase IV). The increase in methanogens abundance was due to the emergence of a new methanogenic order namely *YC-E6* (from 0.0 to 1.6 ± 1.1). This order belongs to the class *Methanomicrobia* and has been detected during mesophilic AD of pig waste (Pampillon-Gonzalez et al., 2017) and thermophilic AD of lignocellulosic biomass (Lin et al., 2017), however, at a very low abundance. On the other hand, acetogens and methanogens populations in the co-digestion system decreased from 13.8 and 7.2% (phase I) to 8.0 and 2.6% (phase IV).

The thermophilic proteolytic bacteria genus *Coprothermobacter* (order *Thermoanaerobacterales*) was still presented in both digesters at 4.3 ± 1.3 and 7.5% in the mono- and co-digestion system, respectively in phase IV. The increase in *Coprothermobacter* abundance is unlikely resulted from migration from the inoculum as its abundance in the inoculum is only 0.2 ± 0.1% (data not shown). This could be due to the fact that *Coprothermobacter* growth is related to proteinaceous substrate availability (PS was the main substrate). In a previous study, this genus dominance was correlated with a high protein content of the feed (Kobayashi et al., 2008). Increase abundance of *Coprothermobacter* was also observed when soluble COD in the feed increased (Gagliano et al., 2015a), explaining

437 the higher abundance of this genus in the co-digestion system compared to the mono-
438 digestion system.

439 [FIGURE 2]

440 The shift in the microbial community structure during the operational period was further
441 examined through NMDS with the distance between samples indicates how similar their
442 communities are to each other (Fig. 2). The whole microbial community and methanogenic
443 community in two digesters formed different groups corresponding to different phases of
444 operation. During phase I (day 80), the community structure remained similar to the start-up
445 phase (Fig. 2) with the methanogenic community structure in the co-digestion system
446 unchanged. Methanogens have been claimed to be less affected by co-substrate addition than
447 by VFAs and ammonia concentration (Xia et al., 2012). Operational disturbance (phase II)
448 induced profound changes in microbial communities shown by their notable progression to
449 specific directions. While the digesters constantly receiving the inoculum in phase III (day
450 103, 106, 108), the microbial structure remained stable and appeared to have higher similarity
451 to the inoculum community. Once the inoculum addition ceased (after day 108), both
452 digesters communities diverged from the inoculum microbiome and neither of them returned
453 to the original structure (Fig. 2). Both microbial structure and composition analyses suggest
454 that the whole microbial community and methanogenic community in both digesters did not
455 return to their original states after operational disturbance and recovery. The new quasi-stable
456 phase achieved by the two digesters with comparable performance to the initial stable phase
457 (prior to disturbance) indicates that there is functional redundancy within the microbial
458 community and similar level of performance can be achieved by different microbial
459 community structure.

460 **4. Conclusion**

461 Operational disturbance resulted in an imbalance between different microbial groups which
462 subsequently led to process failure. The increased organic load in the co-digestion system
463 (from carbon-rich waste addition) led to enhanced biogas production during the quasi-stable
464 phase, but also negatively affected the system resilience under disturbed condition. Inoculum
465 addition effectively facilitated digester recovery through the supplement of acetogens and
466 methanogens to the digester. Although digester performance after process recovery by
467 inoculum addition was comparable to that prior to disturbance, the microbial community did

not return to its original structure and composition, suggest the presence of functional redundancy within the microbial community.

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