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Dynamics of microbial community in a mesophilic anaerobic digester treating food waste: Relationship between community structure and process stability

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HIGHLIGHTS

- The linkages between community structure and process stability were investigated.
- OLR disturbances were introduced to induce stable and deteriorative phases.
- Microbial community was investigated by 454 pyrosequencing technique.
- The metabolic function of bacteria and archaea mismatched at deteriorative phase.
- The degradation of intermediate metabolites was inefficient at deteriorative stage.

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ABSTRACT

Organic loading rate (OLR) disturbances were introduced into a mesophilic anaerobic digester treating food waste (FW) to induce stable and deteriorative phases. The microbial community of each phase was investigated using 454-pyrosequencing. Results show that the relative abundance of acid-producing bacteria and syntrophic volatile fatty acid (VFA) oxidizers increased dramatically at deteriorative phase, while the dominant methanogens did not shift from acetoclastic to hydrogenotrophic groups. The mismatching between bacteria and methanogens may partially be responsible for the process deterioration. Moreover, the succession of predominant hydrogenotrophic methanogens reduced the consumption efficiency of hydrogen; meanwhile, the dominant *Methanosaeta* with low acetate degradation rate, and the increase of inhibitors concentrations further decreased its activity, which may be the other causes for the process failure. These results improve the understanding of the microbial mechanisms of process instability, and provide theoretical basis for the efficient and stable operation of anaerobic digester treating FW.

1. Introduction

Food waste (FW) is generated at an ever-increasing rate, causing "waste oil", "garbage pig" and some other problems of food safety; the disposal of FW is attracting wide social attention in China. FW is a high organic waste rich in energy content; among the current approaches (e.g., incineration, landfill, compost and anaerobic digestion (AD)) for FW disposal, AD is considered to be the most effective approach for resource utilization. Accordingly, among the demonstration projects for FW disposal, more than 70% of the projects have chosen AD technology.

However, despite a continuously increasing interest and popularity on AD, large-scale anaerobic digesters are usually operated at low organic loading rate (OLR) to maintain stable operation, and the resulting low biogas production make the process less efficient and economically feasible (Tampio et al., 2014). The increase in operational OLR can achieve higher gas production and thus improve the process efficiency, but the process instability under high OLR is a concern. Most previous researches have focused on process monitoring and control to improve the process stability and efficiency (Feitkenhauer et al., 2002; Li et al., 2014). Microorganisms are the core of the digesters as the AD is a biochemical process mediated by a variety of microbial groups. Thus, the understanding of the microbial community is crucial for improving efficiency and process stability of anaerobic digesters (Zhang et al., 2009; Kim et al., 2013; Lim et al., 2013). The

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investigation of microbial community in anaerobic digesters has attracted increasing attention in recent years.

AD is a multi-stage biochemical process in which the complex organic materials undergo hydrolysis, acidogenesis, acetogenesis and methanogenesis in series. These metabolic stages are functioned by different types of microorganisms, which differ in their nutritional needs, habitat requirements, growth kinetics and ability to tolerate environment stresses (Yi et al., 2014). Many previous studies explored the microbial community in anaerobic digesters, but most of them only paid attention to the community composition under certain operational state of the digester, or the succession of microbial community over time (Supaphol et al., 2011; Cho et al., 2013; Williams et al., 2013). Researchers also tried to link OLR disturbances with microbial communities. However, most of them just focused on the succession of dominant groups or the fluctuation of their relative abundance under stable stage of the digester (Guo et al., 2014; Jang et al., 2014). A few studies considered the microbial community at both stable and deteriorative phase. For example, Rinćon et al. (2008) investigated the effect of OLR on the performance and microbial communities of anaerobic digester. Results showed the predominant bacteria were different at two stages, while archaea were mainly represented by *Methanosaeta* independently of the OLR. Lerm et al. (2012) studied the influence of archaeal community composition on the function of anaerobic co-digesters in response to organic overload. They found that the overload resulted in a decrease of methane production and an accumulation of volatile fatty acid (VFA); besides, hydrogenotrophic methanogens became more dominant, especially in the reactor with a higher OLR. Razaviarani and Buchanan (2014) investigated the linkage between reactor performance and microbial community dynamics at steady and overloading stages during mesophilic anaerobic co-digestion of restaurant grease waste with municipal wastewater sludge. Results indicate that the dominant methanogens differ in two process states, and under overload condition, the pH, alkalinity and methane production decreased while VFA concentrations increased dramatically. Overall, the available literature is mainly about process parameters and the corresponding structure and dynamic of microbial community in anaerobic digester operated under different process stages, or only simply about microbial community succession, the microbial mechanisms of process instability has not yet been fully explored.

This study introduced OLR disturbances into a mesophilic anaerobic digester treating FW to induce stable and deteriorative phase, during which physico-chemical analysis along with the pyrosequencing microbial technique were performed to monitor state parameters and microbial population of each phase, respectively. The objectives of this study were to investigate the linkages between process stability and community structure, and to improve the understanding of the microbial mechanisms of system instability.

2. Methods

2.1. Feedstock and seeding sludge

FW was collected from a school dining facility, and was shredded into particles with an average size of 5.0 mm by a Robot-Coupe Shredder after the removal of coarse impurities such as bones and plastics. The prepared materials were then packed into 4-L plastic storage bags, and cryopreserved at −18 °C. The frozen feedstock was thawed and stored at 4 °C a week prior to use. The seed sludge was obtained from a rural household biogas digester operated at the ambient temperature. The characteristics of the FW and sludge are shown in Table 1.

Table 1
Characteristics of substrate and inoculum.

Item	Unit	Food waste	Inoculum
pH	–	6.4 ± 0.2	7.5 ± 0.3
Total solids (TS)	% of wet weight	28.4 ± 0.7	9.1 ± 0.1
Volatile solids (VS)	% of wet weight	26.5 ± 0.7	5.4 ± 0.1
VS/TS	%	93.2 ± 1.6	59.2 ± 0.3
Carbon content	% of TS	53.3 ± 0.1	27.5 ± 0.1
Nitrogen content	% of TS	3.6 ± 0.1	2.8 ± 0.1
Carbon/nitrogen	–	14.7 ± 0.2	9.7 ± 0.4

2.2. Reactor and operation

A completely stirred tank reactor (CSTR) with a working volume of 30-L was operated at 36 ± 1 °C. The constant temperature was maintained by a water jacket heated by a thermostat. Motorized automatic stirring was provided at the top of the digester, and the rotary speed was set at a rate of 60 rpm for 1 h stirring and 2 h break repeatedly. Parameters including pH, oxidation–reduction potential (ORP), gas production and composition (methane and carbon dioxide) can be monitored on-line. However, due to total solid content (TS) of the substrate is too high, automatic feeding system did not work properly, so manual feeding mode was adopted.

The digester was operated in semi-continuous mode after started-up successfully. The semi-continuous operation was achieved by the daily removal of digestate through the sampling opening followed by substrate addition via the feed port. The volume of daily withdrawal was 200 mL, and concentrated discharge was conducted once a week to maintain a working volume of 30-L. The reactor was operated with the OLR of 3 g VS L^{-1} d^{-1} at the first 45 days of the experiment, and this period was considered as the stable stage based on stable process parameters. After Day 45, the OLR was increased from 3 to 6 g VS L^{-1} d^{-1} with an interval of 1 g VS L^{-1} d^{-1} every 15 days. Process parameters indicated the process failure by Day 90 when the experiment was terminated.

2.3. DNA extraction, PCR and pyrosequencing

The digestate samples were respectively collected on Days 45 and 90 to determine the microbial communities at both stable and deteriorative phases of the digester. The samples were frozen at −80 °C immediately. Genomic DNA was extracted from each sample with the E.Z.N.A Soil DNA kit (OMEGA, USA) following the manufacturer's instructions. 16S rRNA genes segments were amplified using bar-coded primer pairs of 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 533R (5′-TTACCGCGGCTGCTGGCAC-3′) for bacteria and 344F (5′-ACGGGGYGCAGCAGGCGCGA-3′) and 915R (5′-GTGCTCC CCCGCCAATTCCT-3′) for archaea. To achieve the sample multiplexing during pyrosequencing, barcodes were incorporated in the 5′ end of reverse primers 553R and 915R. The PCR amplification program for bacteria contained an initial denature at 95 °C for 2 min, followed by 25 cycles of denaturing at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. The thermal cycling for archaea was similar to that for bacteria except that the cycles were 27 rather than 25. After amplification, the PCR products were purified and quantified and then pooled at equal concentrations. Finally the PCR products were sent to Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China).

2.4. Analysis of pyrosequencing-derived data

The raw sequences were trimmed, qualified and then clustered to operational taxonomic units (OTUs) following the procedures

described by Yi et al. (2014) except that the possible chimeras were checked and removed from the data using UCHIME described by Edgar et al. (2011). Mothur project (Mothur v.1.30.1) (http://www.mothur.org) was used to conduct rarefaction curve, abundance base coverage estimator (ACE), richness (Chao), Shannon diversity, Simpson diversity indices and Good's coverage analysis, assign sequences to operational taxonomic units (OTUs, 97% similarity) using furthest neighbor approach. For taxonomy-based analysis, the SILVA database project (http://www.arb-silva.de) was used as a repository for aligned rRNA sequences.

2.5. Analytical methods

TS and volatile solids (VS) were measured according to standard methods (APHA, 1998). pH was measured using a pH meter (Horiba, B-212). The C and N elemental contents were quantified using an Element Analyzer (Elementar Vario ELIII, Germany). The methods for analyzing VFAs, total ammonia–nitrogen (TAN), total VFA, total alkalinity (TA) and bicarbonate alkalinity (BA) were described in a previous report (Li et al., 2014). For sodium (Na^+) determination, the samples were digested with HNO_3, followed by elemental analysis using inductively coupled plasma optical emission spectrometry (Optima 2100 DV, PerkinElmer, US). VS removal rate (VS_r) was calculated using the equation reported by Koch et al. (2009). Free ammonia (FAN) concentration was calculated based on the method described by Körner et al. (2001).

3. Results and discussion

3.1. Reactor performance and process stability

The performance of AD reactors is mainly evaluated based on the OLR, methane yield and VS_r (Nagao et al., 2012), and Fig. 1a shows their evolutions during the experiment. As shown in Fig. 1a, with the OLR increasing from 3 to 5 g VS L^{-1} d^{-1}, methane yield and VS_r kept almost constant at 0.50 ± 0.03 L CH_4 g VS^{-1} and 89.42 ± 0.64%, respectively. These results were similar to those reported in previous studies (Nagao et al., 2012; Li et al., 2014), and indicated the stability of the system. With the OLR increased to 6 g VS L^{-1} d^{-1}, a temporary increase in methane yield was observed; however, a significant decrease in methane yield occurred from Day 86 and more than 50% reductions appeared at Day 90, which indicated the process failure of the digester. A wide range of OLR thresholds have been reported in literature, ranging from 1 to 10 g VS L^{-1} d^{-1}, that were used to ensure the stable operation of FW digesters, depending on inocula, substrates, reactor configurations and operation conditions (Nagao et al., 2012; Tampio et al., 2014). The process failure of studied digester occurred at OLR of 6 g VS L^{-1} d^{-1}, which was comparable with the range of previous study.

Fig. 1b shows the responses of pH, methane content and gas production during the experiment. These three parameters were considered lagging indicators when used as early warning for process failure, as significant change only occurred after severe inhibition developed (Li et al., 2014). Nonetheless, their trends presented in the Fig. 1b further confirmed that the digester operated well when the OLR fluctuated form 3–5 g VS L^{-1} d^{-1}, while completely process deterioration developed at the end of the experiment. The ratio of VFA to TA (VFA/TA) and the ratio of BA to TA (BA/TA) are commonly used as early warning indicators, and they were reported to indicate the stable operation of the digester when the ratios are less than 0.35 and more than 0.8, respectively (Li et al., 2014). As can be seen from Fig. 1d, VFA/TA and BA/TA beyond their thresholds from Day 83, then continued deteriorations were observed, and their ratios were 1.27 and 0.42, respectively at the end of the experiment. The results further illustrate that the system operated well during the initial 82 days, and then process imbalance occurred from Day 83, finally the process completely failed on Day 90.

The process deterioration of organic overload was usually caused by two reasons. First, with an extremely high OLR, the rate of hydrolysis/acidogenesis could be higher than methanogenesis, and the accumulated VFA can eventually lead to an irreversible acidification; secondly, inhibitors may exceed their thresholds under high OLR, then gas production would be inhibited and even ceased. As Fig. 1c shows, with the OLR increased from 3 to 5 g VS L^{-1} d^{-1}, the mean concentration of total VFA was 2214 ± 185 mg L^{-1}. The dramatic rise appeared on Day 83, and reached a final concentration of 9443 mg L^{-1} at Day 90. Thus, the acid accumulation may be one cause of process deterioration. Acetate and propionate were the major VFA produced during the AD process. Among them, acetate increased more than 3 times during the instability stage, to a final concentration of 5692 mg L^{-1}; propionate rose about 7 folds and reached the final concentration of 2761 mg L^{-1}. Except for the high yield under high OLR, the dramatic increase in propionate may be also caused by the elevated H_2, because the bioconversion of propionate is not thermodynamically spontaneous under standard conditions and the degradation stops when the H_2 concentration is above 10^{-4} atm (Tale et al., 2011; Shigematsu et al., 2006). Unfortunately, the current study did not measure the concentration of H_2 to verify this inference.

FAN, TAN and Na^+ are known the potential inhibitory chemicals for AD. It has been reported that the FAN concentrations above 600 mg L^{-1} will significantly affect the activity of methanogens (Dai et al., 2013). During the reactor operation, FAN concentrations in the digester were generally below 150 mg L^{-1}, therefore the inhibition effect of FAN is likely negligible. A wide range of TAN inhibition thresholds have been reported in literature, ranging from 1.7 to 14 g L^{-1}, which cause a 50% reduction in methane production, depending on operation conditions (Lü et al., 2013). Similarly, some previous studies suggested that Na^+ concentrations ranging from 3.5 to 5.5 g L^{-1} to be moderately and 8.0 g L^{-1} to be strongly inhibitory to methanogens at mesophilic temperatures (Dai et al., 2013). Fig. 1d shows the change of these two parameters during the reactor operation. Both of them seem to accumulate at a constant rate regardless of the process stage. The concentrations of TAN and Na^+ at initial, middle and terminal stage of the experiment were 1035 and 693 mg L^{-1} (Day 1), 1767 and 2078 mg L^{-1} (Day 43) and 2695 and 4530 mg L^{-1} (Day 90), respectively. At the initial stage their concentrations were lower than the inhibition thresholds, while the inhibition on the process was likely to occur during the later period of the experiment. Therefore, the accumulation of inhibitors may be another reason of process deterioration.

3.2. Overall analysis of pyrosequencing

Based on the above analysis, the process maintained stable and no any inhibition occurred by Day 45, while completely deteriorated was developed until Day 90. To analyze the responses of microbial community structure in both stable and deteriorative phases, sludge samples retrieved on Days 45 and 90 were used to obtain DNA for the amplification and subsequent pyrosequencing of a region of archaebacterial 16S rRNA genes. The sequencing depth of bacteria and archaea were 10,000 and 5000, respectively, and the rarefaction curves of two samples generated at 3% cut-off for bacterial and archaeal communities are shown in Fig. 2. The curves of archea have completely reached a plateau, the curves of bacteria also approached a plateau, suggesting that this sequencing depth was enough to cover the whole microbial diversity for each sample. The Good's coverage of sequencing for each sample (Table 2) further confirmed this hypothesis. Besides,

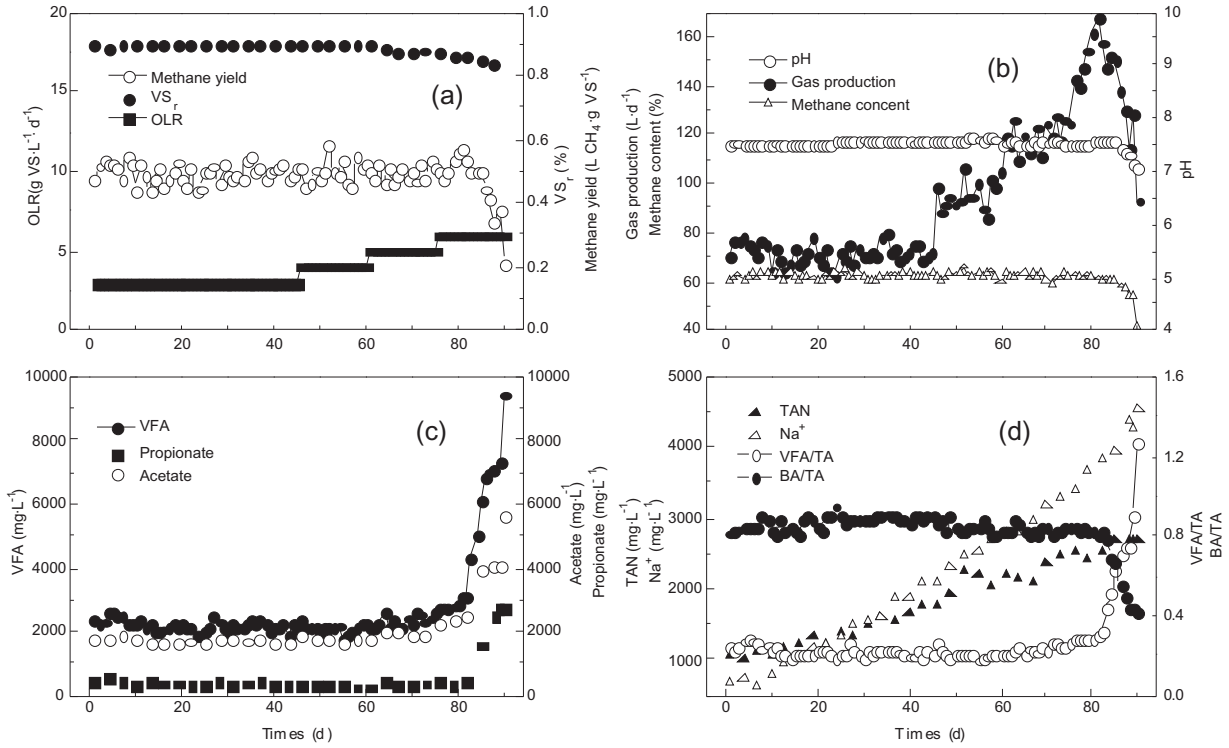

Fig. 1. Evolution of OLR, VS_r, methane yield (a), pH, gas production, methane content (b), total VFA, acetate, propionate (c) and TAN, Na^+, VFA/TA, BA/TA (d) in digester during the experiment.

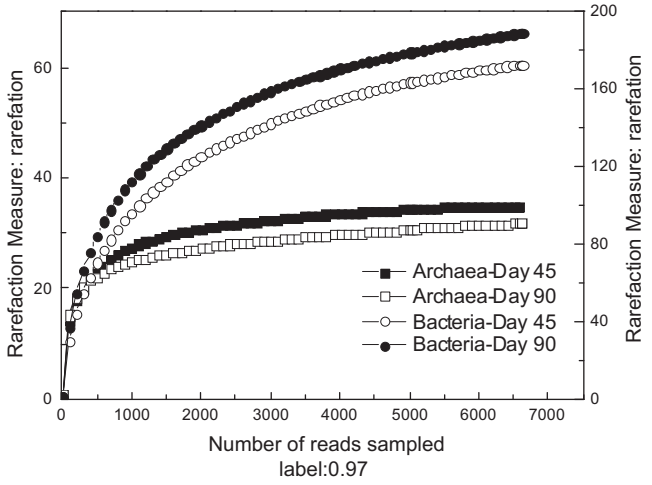

Fig. 2. The rarefaction curves of samples generated at 3% cutoff for bacterial and archaeal sequences.

Table 2 shows more information about microbial community diversity. As can be seen, a total of 6625 and 6614 trimmed bacterial 16S rRNA gene sequences and 6221 and 6552 trimmed archaeal 16S rRNA gene sequences were recovered from samples on Days 45 and 90, respectively. The sequences were grouped into OTUs at a distance level of 3% to estimate the phylogenetic diversities of microbial communities. The larger OTUs number in bacterial sequences along with the diversity estimators including ACE, Chao1, Shannon index and Simpon index jointly implied the higher diversity of bacterial communities than archaeal, which was also consistent with previous studies (Kim et al., 2014). Further compare the sample information on Day 45 and Day 90, we can see that the diversity indexes of both bacteria and archaea had no significant differences between stable and deteriorative phases. Therefore, the diversity indexes are not good indicators of process state. This may be because the diversity indexes are statistical data used to describe the community diversity independently of community composition, thus different community structure may lead to the same diversity indexes. Dearman et al. (2006) had a similar conclusion that microbial diversity was not important in developing a functionally successful anaerobic microbial community, rather the structure of the community is of greater importance. So further comparing the microbial community structure on different process phases was required to improve the understanding of the microbial mechanisms of process instability.

3.3. Dynamics of bacterial communities under different process state

Bacteria directly involved in the degradation of the FW to generate the intermediate metabolites that can be later utilized by

Table 2
Sample information and statistics analysis of the microbial 16S rRNA gene libraries obtained from the pyrosequencing. All values were calculated at 0.03 distance limit.

	Sample	Reads	OTU	ACE	Chao	Shannon	Simpon	Coverage
Bacteria	Day 45	6625	173	192	188	3.14	0.0991	0.9955
	Day 90	6614	189	212	211	3.66	0.0512	0.9950
Archaea	Day 45	6221	35	36	36	2.01	0.2140	0.9995
	Day 90	6552	32	36	37	2.05	0.2333	0.9992

methanogens, therefore bacterial community structures will be firstly affected by the OLR fluctuation (Rinćon et al., 2008). Distribution of sequences at the phyla level in each sample was shown in Fig. 3. *Bacteroidetes*, *Firmicutes*, *Chloroflexi*, *Spirochaetae* and *Synergistete* were the common major phyla at stable stage of the digester, and formed a considerable proportion of 97.43%, while at deteriorative phase, the relative abundance of the above phyla all decreased. In contrast, the relative abundance of *Actinobacteria* increased from 0.03% on Day 45 to 1.41% on Day 90, and amounts of *Tenericutes* sharply increased from 0.08% to 13.30%. Members of the *Actinobacteria* are commonly found in soils and natural waters (Wirth et al., 2012; Supaphol et al., 2011), and may be responsible for hydrolyzing and degrading FW into VFAs in the digester (Ike et al., 2010). Besides, some bacteria in *Actinobacteria* produce propionate (Jang et al., 2014), which may be one of the reasons of propionate accumulation during deteriorative stage. The significant increase of *Tenericutes* at deteriorative stage could explain the decrease of other bacteria phyla. In addition, there may have some correlation between the increase of *Tenericutes* and the failure of process, since Guo et al. (2014) also observed similar phenomenon in thermophilic anaerobic digester treating FW.

To further compare the difference of bacterial communities under different process state and explain the microbial mechanism of process instability, it is preferable to deconstruct the sequencing data at the subdivision level. Therefore, the relative abundance of each class and genus in two samples was calculated, and the sequence distributions were shown in Table 3. The members of *Tenericutes* were all affiliated with class *Mollicutes*, which can further be categorized into genus *Acholeplasma*. The members of the *Mollicutes* are facultative anaerobes, and they produce organic acids under anaerobic condition (Wirth et al., 2012). In addition, *Clostridia* and *Erysipelotrichia* also changed dramatically during the experiment. *Clostridia* and *Erysipelotrichia* were the major class in *Firmicutes*. *Erysipelotrichia* is a constituent of the intestinal flora, and dominant at stable stage, while its relative abundance decreased from 21.46% to 1.09% at deteriorative phase; meanwhile the relative abundance of *Clostridia* increased from 8.18% to 26.82%. It is known that the *Clostridia* are efficient hydrogen producers, various *Clostridia* strains degrade organic acids in a syntrophic association with hydrogenotrophic methanogens (Kim et al., 2014). The dramatic proliferation of *Clostridia* suggested that excessive hydrogen was generated and supplied to hydrogenotrophs. Corresponding, the amounts of genus *Treponema* increased from 0.50% (Day 45) to 3.28% (Day 90). *Treponema*-affiliated bacteria are likely homo-acetogens (one kinds of hydrogenotrophic microbes), consuming H_2 and CO_2 to make acetate, which in turn can be used by acetoclastic methanogens (Zhang et al., 2009).

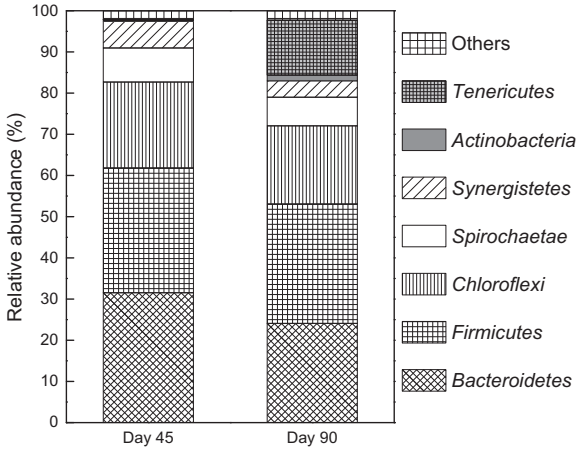

Fig. 3. Taxonomic compositions of bacterial communities at phyla level in each sample retrieved from pyrosequencing. The number in the sample names represented the day when sampling occurred.

Table 3
Taxonomic compositions of bacterial communities at the class and genus level for the sequences retrieved from each sample (only genus with relative abundances higher than 0.5% at least in one sample were listed).

%, relative abundance					
Class	Day 45	Day 90	Genus	Day 45	Day 90
Bacteroidia	27.58	23.16	*Bacteroides*	2.84	1.30
			Petrimonas	18.85	17.54
			Proteiniphilum	0.78	2.59
			vadinBC27_wastewater-sludge_group	4.33	0.17
			Alkaliflexus	0.14	1.24
Sphingobacteriia	2.49	0.62	*ST-12K33_norank*	2.49	0.62
SB-1	0.56	0.11			
vadinHA17	0.91	0.20	*vadinHA17_norank*	0.91	0.20
Erysipelotrichia	21.46	1.09			
Clostridia	8.18	26.82	*Fastidiosipila*	1.69	3.69
			Gelria	0.53	0.30
			Syntrophomonas	0.50	1.78
			Sedimentibacter	0.24	1.22
			Caldicoprobacter	0.45	0.50
Anaerolineae	20.88	18.99			
Spirochaetes	8.27	6.95	*Treponema*	0.50	3.28
Synergistia	6.48	3.98	*Thermovirga*	4.82	2.24
Actinobacteria	0.33	1.41	*Actinomyces*	0.29	1.36
Mollicutes	0.08	13.31	*Acholeplasma*	0.08	13.27
Others	2.79	3.36	Others	61.39	51.66

The highly dynamic of genus *Proteiniphilum*, *Sedimentibacter* and *vadinBC27_wastewater-sludge_group* under different process state were also observed. Among them, relative abundance of genus *Proteiniphilum* and *Sedimentibacter* increased at deteriorative phase, while the remaining one decreased. They are all capable of anaerobic amino acids degradation (Ziganshin et al., 2011; Guo et al., 2014; Tang et al., 2005). However, The first two genus were found to degrade certain amino acids via the Stickland reaction, while the later one degrade amino acids in syntrophic association with hydrogenotrophic methanogens. VFA, $NH4^+$ and CO_2, $NH4^+$ were the metabolites of these two degradation pathways, respectively (Tang et al., 2005). As can be seen, the transfer of amino acids degradation pathway may be associated with the accumulation of VFA at deteriorative phase. In addition, the relative abundance of *Syntrophomonas* was also significantly different in two stages. *Syntrophomonas* are capable of butyrate and propionate degradation (Nelson et al., 2011), their proliferation at deteriorative phase was consistent with the propionate accumulation in digester. *Thermovirga*-affiliated bacteria are thermophilic organisms, capable of utilizing carbohydrates, proteinous compounds, amino acids and organic acids. Their abundance decline at deteriorative stage may be caused by undesirable temperature or transition of degradation pathway.

3.4. Dynamics of methanogens communities under different process state

As the inherent phylogenetic low diversity of methanogens, the common detected methanogens in anaerobic digesters include the order of *Methanomicrobiales*, *Methanobacteriales*, *Methanosarcinales* and *Methanococcales*. The first three orders were detected in the studied digester, among which *Methanomicrobiales* and *Methanosarcinales* were the dominant orders during the overall periods, while *Methanobacteriales* formed a relatively small

population abundance of less than 0.5%. Despite this, the pyrosequencing data showed that community structures of methanogens responded to process state.

The sequences distribution at genus level was shown in Fig. 4. As Fig. 4 shows, there was no notable change between stable and deteriorative phases in terms of methanogens compositions, but distinct discrimination was observed in the relative abundance of each genus. *Methanosaeta* were the most abundant genus independently of the process state. Their amounts even increased form 46.97% to 58.47% during the deteriorative phase. In contrast, another acetoclastic methanogenesis *Methanosarcina* with lower abundance, and decreased form 4.90% to 3.34%. *Methanosarcina* and *Methanosaeta* were the only two methanogens who can use acetate as a substrate for methane production (Ike et al., 2010). As they compete for acetate, usually only one of them dominates in digester (Lin et al., 2012). *Methanosarcina*, a generalist known to have a high metabolic versatility and growth rate, always became dominant at acetate concentration above 250–500 mg L^{-1} (Franke-Whittle et al., 2014; Lim et al., 2013). While members of *Methanosaeta* use only acetate to produce methane, and they are known for competing well against *Methanosarcina* at acetate concentrations not exceeding 100–150 mg L^{-1}, due to their higher acetate affinity (Kim et al., 2014; Lim et al., 2013). In addition, ammonia is another factor that affect their competition. TAN concentrations of 1700 mg L^{-1} or higher are known to inhibit *Methanosaeta* (Franke-Whittle et al., 2014); while *Methanosarcina* were reported to be tolerant of TAN concentrations up to 7000 mg L^{-1} (Cho et al., 2013). Corresponding, many studies have reported the dominance of *Methanosarcina* instead of *Methanosaeta* at high VFA and TAN concentrations (Guo et al., 2014; Lerm et al., 2012; Kim et al., 2014). However, our results contrasted with those of previous studies. The reason for this phenomenon may be due to that competition between *Methanosarcina* and *Methanosaeta* was also affected by other factors, such as inoculum source, operation conditions (stirring intensity, hydraulic retention time (HRT)) and cell morphology, etc (Lerm et al., 2012; Lin et al., 2012). Study conducted by Tale et al. (2011) concluded that the origin of the inoculum have exerted a strong influence on the enrichment of specific methanogenic populations. The relative abundance of *Methanosaeta* in inoculum was 85.01%, while *Methanosarcina* only account for 0.39% (data not shown), which greatly facilitated the dominant position of *Methanosaeta*. Hoffmann et al. (2008) have studied the effect of shear on their competition, and concluded that *Methanosaeta* was the dominant Methanogens with low mixing intensity (50 r min^{-1}), because which protected the filaments of *Methanosaeta* from shear and turbulence. The mixing intensity in this study was 60 r min^{-1}, which may also one cause of the enrichment of *Methanosaeta*. Moreover, the studied digester operated at long HRT (22–44 d), which facilitated the growth of *Methanosaeta* with a lower μ_{max} of 0.24–0.26 d^{-1} (Shigematsu et al., 2006). Some other researchers also attributed this anomaly to cell morphology. For example, Lin et al. (2012) have pointed that *Methanosaeta* may form multicellular aggregates to resist the inhibition caused by VFA and TAN, because the slow diffusion rate of acids and ammonia limits their concentration in the aggregates. Besides, other researches such as Franke-Whittle et al. (2014), Lerm et al. (2012) and Kim et al. (2013) have also observed the dominance of *Methanosaeta* at high VFA or TAN concentrations. However, they have not explained the reasons for this anomaly. Further studies may be needed to reveal the mechanisms of competition between *Methanosaeta* and *Methanosarcina*.

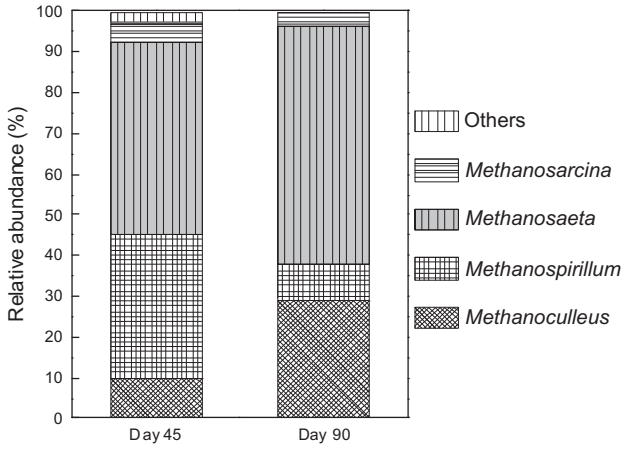

Fig. 4. Taxonomic compositions of methanogens at the genus level in each sample retrieved from pyrosequencing. The number in the sample names represented the day when sampling occurred.

The other two genuses with relative high abundance were *Methanospirillum* and *Methanoculleus*. They were hydrogenotrophic methanogens. Overall, the abundance of hydrogenotrophic methanogens decreased from 45.24% (Day 45) to 37.68% (Day 90). This decline will reduce the consumption efficiency of hydrogen, resulting in increased H_2 partial pressure, which may be the other cause of propionate accumulation. More specifically, *Methanospirillum* was the dominant genus in hydrogenotrophic methanogens at stable phase, while their dominance was replaced by *Methanoculleus* at deteriorative stage. It is reported that once a state of equilibrium has been reached, the community structures of methanogens are likely to keep "their leading position" even under sub-favorable digester conditions (Wagner et al., 2011). Therefore, the shift from *Methanospirillum* to *Methanoculleus* was worth considering. The reported μ_{max} of *Methanospirillum* and *Methanoculleus* were 0.98 and 0.92–1.53 d^{-1}, respectively (Shigematsu et al., 2006). The similar growth rate excluded it as the reason of the shift. Franke-Whittle et al. (2014) suggested that genus *Methanoculleus* dominance over other hydrogenotrophic methanogens because its tolerance of high salt concentrations, while the optimal Na^+ concentration for *Methanospirillum* was below 2300 mg L^{-1}. As mentioned in the previous section, the Na^+ concentration at deteriorative stage exceeded the optimal range of *Methanospirillum*, which could explain the succession properly. However, the succession had negative impact on process stability. As reported, the H_2 utilization rate for *Methanospirillum* is greater than *Methanoculleus*. The minimal hydrogen partial pressure is 3.0 Pa for *Methanospirillum*, while *Methanoculleus* with higher H_2 affinity and its H_2 threshold is approximately 0.1 Pa (Shigematsu et al., 2006). The succession from *Methanospirillum* to *Methanoculleus* will further reduce the efficiency of hydrogen consumption, and lead to the accumulation of H_2, then further impede the degradation of VFA in positive direction, finally result in the accumulation of VFA and process deterioration.

3.5. Microbial mechanisms of process instability

Acid-producing bacteria such as genus *Acholeplasma* and *Actinomyces* increased dramatically at deteriorative stage compared with stable phase. The significant growth of acid-producing bacteria resulted in the accumulation of VFA in digester, which is an important factor that may explain the process deterioration. The accumulation of VFA induced the proliferation of *Clostridia*; *Clostridia* oxidize fatty acids with 4–11 carbon atoms in association with several different hydrogenotrophic methanogens. The delicate balance between the *Clostridia* and hydrogenotrophic methanogens is a determining factor of process stability (Wirth et al., 2012). However, with the increase of *Clostridia* abundance, the

dominant methanogens did not shift from acetoclastic to hydrogenotrophic groups. In contrast, the total number of hydrogenotrophic methanogens declined 7.56%. The decline in hydrogenotrophic methanogens not only impeded the decomposition of VFA, but also promoted the transfer of amino acids degradation pathway, which further exacerbated the accumulation of VFA. Therefore, the mismatch of metabolism between bacteria and methanogens is the second reason of process deterioration. Besides, the dominant hydrogenotrophic methanogens shifted from genus *Methanospirillum* to *Methanoculleus* in response to Na^+ inhibition could be another possibility. The dominance of *Methanospirillum* at stable stage indicated that the hydrogen partial pressure in digester was in their optimum range. At deteriorative stage, the system with higher OLR and have accumulated more VFA, which was bound to bring higher hydrogen partial pressure. While the corresponding hydrogenotrophs with slower H_2 consumption rate, which would further exacerbate the accumulation of H_2. Homo-acetogens may be a product of this stress. Members of *Treponema* consumed part of H_2, and provided the generated acetate to acetoclastic methanogens. However, the activity of acetoclastic methanogens was also a concern. Despite the variety of operating conditions caused the dominance of genus *Methanosaeta*, it is an indisputable fact that this genus with lower acetate utilization rate and was more susceptible than *Methanosarcina* (Cho et al., 2013; Kim et al., 2014). The dominant *Methanosaeta* was inefficient in degradation the increasing acetate produced under high OLR, and resulted in its accumulation, which may be another reason of process deterioration.

4. Conclusion

This study showed significant correlations between microbial community structures and process stability. Acid-producing bacteria and syntrophic VFA oxidizers increased dramatically at deteriorative phase, while the dominant methanogens did not shift from acetoclastic to hydrogenotrophic groups, the mismatch of metabolism between bacteria and methanogens may be one reason of process deterioration. In addition, the dominant methanogens was inefficient in degradation of intermediate metabolites, and resulted in their accumulation, which may the other cause of process deterioration. Optimization of operating conditions to breed predominant methanogens selectively may be helpful to the efficient and stable operation of anaerobic digester.

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