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Organic loading rate and hydraulic retention time shape distinct ecological networks of anaerobic digestion related microbiome

Rui Xu[a,b], Zhao-Hui Yang[a,b,*], Yue Zheng[c], Jian-Bo Liu[a,b], Wei-Ping Xiong[a,b], Yan-Ru Zhang[a,b], Yue Lu[a,b], Wen-Jing Xue[a,b], Chang-Zheng Fan[a,b]

[a] College of Environmental Science and Engineering, Hunan University, Changsha 410082, PR China
[b] Key Laboratory of Environmental Biology and Pollution Control (Hunan University), Ministry of Education, Changsha 410082, PR China
[c] Department of Chemical Engineering, University of Washington, Seattle, WA 98195, United States

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ABSTRACT

Understanding of how anaerobic digestion (AD)-related microbiomes are constructed by operational parameters or their interactions within the biochemical process is limited. Using high-throughput sequencing and molecular ecological network analysis, this study shows the succession of AD-related microbiome hosting diverse members of the phylum *Actinobacteria*, *Bacteroidetes*, *Euryarchaeota*, and *Firmicutes*, which were affected by organic loading rate (OLR) and hydraulic retention time (HRT). OLR formed finer microbial network modules than HRT (12 *vs.* 6), suggesting the further subdivision of functional components. Biomarkers were also identified in OLR or HRT groups (e.g. the family *Actinomycetaceae*, *Methanosaetaceae* and *Aminiphilaceae*). The most pair-wise link between *Firmicutes* and biogas production indicates the keystone members based on network features can be considered as markers in the regulation of AD. A set of 40% species ("core microbiome") were similar across different digesters. Such noteworthy overlap of microbiomes indicates they are generalists in maintaining the ecological stability of digesters.

1. Introduction

In the past years the increased significance of the renewable energy (mainly methane) recovered from anaerobic digestion (AD) has attracted considerable interest in the application of this promising technology to wastewater, municipal waste sludge, urban organic waste or new co-digestion feedstocks (Dareioti and Kornaros, 2014; Fitamo et al., 2017; He et al., 2018; Wu et al., 2016; Xu et al., 2015). AD technology supports the energy balance in wastewater treatment plants which are energy consuming (Kundu et al., 2017). Previous studies reported the methane generation strongly correlate with many AD parameters. For example, organic loading rate (OLR), hydraulic retention time (HRT), pretreatment, temperature, pH, etc., have been confirmed to be associated directly with AD process (Gou et al., 2014; Kumar et al., 2016; Xu et al., 2018; Ziganshin et al., 2016). Our previous study also showed that reactor's stability and microbial metabolic activity is strongly affected by OLR and HRT (Xu et al., 2015). Despite the "black box" of AD is partially unraveled, however, as an important microbial process, there is still more to be understood the crucial correlations between microbial community structure and function for more efficient and predictable AD performance.

During the biochemical pathways of AD, critical intermediates are converted to methane *via* different microbial groups, including *Archaea* and *Bacteria* (Dareioti and Kornaros, 2014; Fitamo et al., 2017; Xu et al., 2018). Recent studies have used multiple advanced "-omics" technologies to profile the composition and variation of microbial community in AD process (Anantharaman et al., 2016; De Vrieze et al., 2018; Kundu et al., 2017; Qin et al., 2016; Xu et al., 2017). Former researchers found that the variations of function microbes largely depend on reactor design as well as many operational variables, such as temperature, OLR or HRT (Gou et al., 2014; Razaviarani and Buchanan, 2014; Xu et al., 2018; Ziganshin et al., 2016). In a previous survey across seven full-scale anaerobic digesters located in Europe, Riviere et al., identified the phylum *Chloroflexi*, *Betaproteobacteria*, *Bacteroidetes* and *Synergistetes* as the core members involve in AD of sludge (Rivière et al., 2009). This study also shows current knowledge on the dynamics between microbiomes and AD operation is still limited. Because the microbial communities across AD steps (including hydrolysis, acidogenesis, acetogenesis and methanogenesis) have been characterized to host a high abundance of microbial diversity. Most of them are detected at the low abundance (< 0.1%) of "rare biosphere" (Anantharaman et al., 2016; Lynch, 2015), little reliable information is known about

* Corresponding author at: College of Environmental Science and Engineering, Hunan University, Changsha 410082, PR China.
E-mail address: yzh@hnu.edu.cn (Z.-H. Yang).

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how such complex microbial communities in AD system are structured, and how reactor parameters shape the inter-organism interactions (Kundu et al., 2017; Razaviarani and Buchanan, 2014). This restricts the understanding of microbial population and evolution, or which keystone species affect AD process. This study proposes an assumption about the "core microbiomes" that the species common to all or nearly all AD conditions, which is essential component for methane production or digesters stability. The minimal variation of "core microbiomes" should be detectable from different dataset. These fundamental populations can be considered as marker species that reflecting the conditions in AD digesters.

Furthermore, the previously operation taxonomic units (OTUs)-based investigation of AD-related microbiomes mainly focused on how individual member within each reactor is affected by different operational conditions (Wu et al., 2016). However, this approach can not reveal the complex interactions that occur in microbial communities. Because microbes cooperate within close metabolic interactions, providing each other with critical nutrients for their growth (De Vrieze et al., 2016; Deng et al., 2012; Edwards et al., 2015). For instance, the acetate that utilized by methanogenic *Archaea* for methane generation mainly come from the fermentative *Bacteria* (Wu et al., 2016); while an increase of ammonia concentration in AD digesters often caused a transition of methanogenic pathway from acetoclastic to hydrogenotrophic methanogenesis (De Vrieze et al., 2016). Thus, further studies are required to focus on the microbial cooperation at the overall community level, because it is expected to affect more to ecological functions than individual members (Ma et al., 2016; Wu et al., 2016). Nevertheless, it is a great challenge to identify the interactions within microbial community because their vast diversity and uncultivated status (Deng et al., 2012). Molecular ecological network analysis (MENA) provides a new approach towards deducing microbial interactions within the complicated communities, which has been successfully performed in various habitats, including soils, human gut and oceans (Faust and Raes, 2012; Wu et al., 2016). This analysis can identify the keystone species and their interactions with other taxa. It helps to understand how synergistic biochemical reactions of AD-related microbiomes are affected by the different operational parameters.

To address these questions, this study presents a detailed characterization of the AD-related microbiomes by high throughput sequencing (HTS) approach and network analysis by running three AD reactors under controlled conditions of OLR and HRT. The purpose is the extensive recognition of ecological roles of AD parameters to shape microbial majority. This was achieved by: (1) using HTS targeting microbial communities to cover different operational periods from three AD reactors. (2) comparing microbial distribution and dynamics under different OLR and HRT conditions. (3) correlating the variation of individual microbe within ecological network.

2. Materials and methods

2.1. Preparation of substrates and feedstock materials

The seed sludge (SS) and feedstock materials including municipal waste sludge (MWS), raw food waste (FW) with high a concentration of fat, oil and grease (FOG) were collected from several locations in China, as described in (Xu et al., 2015). The most commonly used substrates of MWS and FW for AD has been widely documented to enhance biogas production or nutrient balance (Kumar et al., 2016; Xu et al., 2015). FOG was separated from raw FW using a cement compressor and Soxhlet extraction method. The MWS and post-treated FW (considered as no FOG) were smashed into small particles using an electric food grinder (XTL-767, IFAVORITE) and mixed with a TS ratio of 1: 1. The mixture of MWS and post-treated FW was identified as "substrates" in the following parts. Materials used in this study was characterized in terms of common AD physico-chemical properties (see Supplementary data). The detail analytical methods and values are described in (Xu et al., 2015).

Table 1
Summary of experiment setups and OTU numbers in R1, R2 and R3.

Sample	Period	HRT (d)	Phylum (59)	OTUs shared ratio (detected OTUs' number)			
				Class (155)	Order (259)	Family (318)	Genus (645)
R1-1	I	20	76%	65%	60%	74%	44%
R1-18	I	20	75%	59%	51%	64%	33%
R1-30	I	20	75%	57%	49%	58%	30%
R1-40	II	20	76%	62%	53%	61%	35%
R1-57	II	20	71%	41%	35%	52%	25%
R1-72	III	20	64%	49%	43%	52%	29%
R1-85	III	20	69%	59%	52%	64%	40%
R1-91	IV	20	64%	52%	46%	57%	32%
R1-109	IV	20	69%	64%	54%	66%	40%
R1-120	IV	20	73%	57%	50%	64%	38%
R2-1	I	20	81%	70%	64%	75%	45%
R2-18	I	20	81%	63%	56%	69%	45%
R2-30	I	20	66%	53%	45%	58%	34%
R2-40	II	20	46%	43%	38%	56%	29%
R2-57	II	20	61%	48%	43%	62%	30%
R2-72	III	20	61%	55%	46%	63%	37%
R2-85	III	20	59%	46%	37%	56%	27%
R2-91	IV	20	64%	53%	44%	58%	31%
R2-109	IV	20	64%	55%	44%	59%	31%
R2-120	IV	20	63%	49%	41%	56%	28%
R3-1	I	15	80%	66%	58%	67%	39%
R3-18	I	15	85%	58%	51%	60%	31%
R3-30	I	15	68%	56%	47%	62%	36%
R3-40	II	15	61%	41%	36%	49%	27%
R3-57	II	15	59%	49%	47%	60%	37%
R3-72	III	15	58%	48%	41%	57%	34%
R3-85	III	15	63%	55%	47%	65%	39%
R3-91	IV	15	61%	54%	46%	58%	32%
R3-109	IV	15	64%	58%	51%	63%	35%
R3-120	IV	15	66%	55%	49%	62%	36%

2.2. AD experiment procedure

AD experiment was conducted in nine (R1, R2, R3 in triplicates) continuously stirred reactors (CSTR) with 2.0 L working volume over 120 days at a mesophilic condition. Each reactor was operated under different OLR and HRT conditions across 4 periods (Table 1). R1 was operated under invariable OLR ($3\,g\,VS\,L^{-1}\,d^{-1}$, only the substrates) and HRT (20 day) as the control. R2 received a gradient increasing OLR from 4.5 to $6.7\,g\,VS\,L^{-1}\,d^{-1}$ (performed by adding different FOG contents in co-digestion with the substrates) in 4 periods with a constant HRT = 20 day. R3 received the OLR as R2 but HRT = 15 day. Samples from each reactor were periodically collected for the routine chemical analysis, including biogas production, pH, chemical oxygen demand (COD), total solids (TS), volatile solids (VS), volatile fatty acids (VFA), alkalinity (ALK), total carbon (TC) and total nitrogen (TN), etc. Detailed information of the set-up and start-up of each reactors can be found in the previous work (Xu et al., 2015). Performance data of typical processes (such as the begin, mid-term and end of each period) used in this study are summarized in Supplementary data.

2.3. DNA extraction and high-throughput sequencing

For the HTS analysis, 90 samples from R1, R2 and R3 were collected on day 1, 18, 30, 40, 57, 72, 85, 91, 109, 120 to cover the whole digestion process. All samples were: (1) stabilized using 50% (v/v) alcohol, (2) flushed three times with 0.1 M Na_3PO_4 (pH = 8), (3) vortexed at maximum speed for 5 min in the sodium dodecyl sulfonate reagent to thoroughly lyse, (4) genomic DNA was extracted from ~1.0 g of each in triplicates according to the instructions of FastDNA