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# High variations of methanogenic microorganisms drive full-scale anaerobic digestion process

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ABSTRACT

Anaerobic digestion is one of the most successful waste management strategies worldwide, wherein microorganisms play an essential role in reducing organic pollutants and producing renewable energy. However, variations of microbial community in full-scale anaerobic digesters, particularly functional groups relevant to biogas production, remain elusive. Here, we examined microbial community in a year-long monthly time series of 3 full-scale anaerobic digesters. We observed substantial diversification in community composition, with only a few abundant OTUs (e.g. *Clostridiales*, *Anaerolineaceae* and *Methanosaeta*) persistently present across different samples. Similarly, there were high variations in relative abundance of methanogenic archaea and methanogenic genes, which were positively correlated ($r^2 = 0.530$, $P < 0.001$). Variations of methanogens explained 55.7% of biogas producing rates, much higher than the explanatory percentage of environmental parameters (16.4%). Hydrogenotrophic methanogens, especially abundant *Methanomicrobiales* taxa, were correlated with biogas production performance ($r = 0.665$, $P < 0.001$) and nearly all methanogenic genes ($0.430 < r < 0.735$, $P < 0.012$). Given that methanogenic archaea or genes are well established for methanogenesis, we conclude that high variations in methanogenic traits (e.g. taxa or genes) are responsible for biogas production variations in full-scale anaerobic digesters.

## 1. Introduction

The key target to treat municipal solid wastes and excessive sewage sludge in wastewater treatment facilities lies in the biodegradable organic fraction, which constitutes over 53% of total waste (Kayhanian, 1995). The organic waste can be efficiently removed through the anaerobic digestion process, which recovers methane ($CH_4$) as a sustainable energy source (Werner et al., 2011; Zhang et al., 2008). Methane fermentation is carried out by complex consortia containing a series of bacteria and methanogens of individual degradation steps (Li et al., 2015; Weiland, 2010) including hydrolysis, acidogenesis, acetogenesis and methanogenesis. Understanding the intrinsic variations of different functional groups of microbial communities is essential for deciphering the individual roles of various microorganisms and for investigating the reasons during operational process failure (Bouallagui et al., 2009). Nevertheless, internal linkages between these functional groups and system performance remain obscure, since long-term monitoring studies of full-scale anaerobic digestion systems are scarce (Wang et al., 2018).

Both biotic and abiotic factors are important for engineering management of methanogenesis in anaerobic digestion systems. While abiotic factors such as ammonia and pH have been extensively studied for improving biogas production and system stability (Dini-Andreote et al., 2015; Ju et al., 2017; Pervin et al., 2013), biotic contribution to these processes is poorly understood. Recently, phylogenetic analyses targeting 16S rRNA gene have been applied in bioreactors to determine microbial community composition, which show that substrate types and inoculum sources are influential (De Vrieze et al., 2015; Narihiro et al., 2015; St-Pierre and Wright, 2013; Yang et al., 2011). Those deterministic factors guide microbial community succession, which allows for predicting how microbial community shifts under environmental disturbance (Peces et al., 2018). However, most of these studies are

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carried out in lab-scale or pilot-scale bioreactors.

Insights into sludge community composition mainly focus on important syntrophic consortia that contain metabolically distinct microorganisms tightly associated with methanogens (McInerney et al., 2009; Narihiro et al., 2015). These syntrophs are usually resistant and resilient to disturbances, which are important to maintain robust anaerobic digestion performance over time (Allison and Martiny, 2008; Narihiro et al., 2015; Werner et al., 2011). Each anaerobic metabolic step is performed by a group of functionally redundant but taxonomically distinct populations that can coexist and replace each other to sustain process performance upon environmental disturbances, which is termed as functional redundancy (De Vrieze et al., 2017; Louca et al., 2018; Peces et al., 2018). However, these studies in full-scale digesters only generate taxonomic information without knowledge about functional potentials, which can be complemented by high-throughput genomic technologies such as GeoChip to dissect microbial community functional structure in natural and engineering environments (Liu et al., 2015; Sun et al., 2014; Yue et al., 2015).

Here, we have carried out an integrative study to identify microbial assemblages of anaerobic sludge in different full-scale anaerobic digesters and link them to digester performance. Microbial communities are spatially diversified in various ecosystems (Ruff et al., 2015; Fierer et al., 2012; Werner et al., 2011), but are temporally stable in anaerobic digesters to treat brewery wastewater (Werner et al., 2011). Therefore, we test whether the hypothesis of spatially distinct and temporally stable community composition is applicable for other anaerobic digesters. To this end, we examined microbial communities from 3 full-scale anaerobic digesters on the monthly basis to determine community assembly. Given that dominant methanogens are indispensable in anaerobic digesters and are responsible for key functions in metabolic pathways of methanogenesis, we further hypothesize that linkages can be established between the methanogenic groups and biogas production.

## 2. Material and methods

### 2.1. Sample collection and process performance monitoring

Anaerobic sludge samples were collected from three full-scale anaerobic digesters located in Beijing of northern China, Qingdao of central China, and Ningbo of southern China, whose operational information is shown in Table S1. All anaerobic digesters were under mesophilic conditions operating at 35–40 °C. Beijing and Ningbo digesters treated excess sludge generated in municipal wastewater treatment facilities, while Qingdao digester treated municipal solid wastes. We collected sludge samples on a monthly basis throughout a year and named samples by location of digesters and sampling months. At least 2 l of samples were taken near the outlet of anaerobic digesters, which were immediately transferred to laboratory in air-tight bottles and kept at 4 °C to analyze environmental parameters.

Information of the feed volume, alkalinity and daily biogas production volume was measured online by WWTP facilities. Total solid contents were below 10% in all digesters and methane accounted for 60–70% of generated biogas volume. All analytic methods for environmental parameters were based on the standard methods for the examination of water and wastewater (Apha, 2005). pH was measured by Hach HQ40d meter (Hach, Loveland, CO, USA). Volatile fatty acids (VFAs) concentrations were measured using titration methods and calculated in acetate concentration (Anderson and Yang, 1992). Concentrations of inorganic anions including ammonia nitrogen ($NH_4^+$-N), sulfate ($SO_4^{2-}$) and chloride ($Cl^-$) and cations including Fe, Cu, and Cr were measured by Inductive Coupled Plasma Emission Spectrometer (ICP) (PerkinElmer, Waltham, MA, USA). Chemical oxygen demand (COD), total solid (TS) and volatile solid (VS) were also measured according to the standard methods (Apha, 2005).

### 2.2. DNA extraction, MiSeq sequencing and GeoChip

For DNA extraction, each sample was first centrifugated at 10 krpm for 10 min. The supernatant was decanted and the pellet was air-dried for 30 min before storing at −80 °C. Then, DNA was extracted from 1 g of pellet by a PowerSoil DNA isolation kit (Mo Bio Laboratories, Carlsbad, CA, USA). DNA concentration and purity were measured by a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) to ensure that the A260/A280 ratio was between 1.8 and 2.0 and A260/A230 above 1.5.

Amplicon sequencing was performed on a MiSeq platform (Illumina, San Diego, CA, USA) at the Institute for Environmental Genomics (IEG), University of Oklahoma. The V4 region of 16S rRNA genes was amplified with the primer pair of 515F (5′-GTG CCA GCM GCC GCG GTA A-3′) and 806R (5′-GGA CTA CHV GGG TWT CTA AT-3′). Each sample was run in triplicates for PCR amplification using a Gene Amp PCR-System 9700 (Applied Biosystems, Foster City, CA, USA), which contained template DNA, primers, 10 × PCR buffer II and AccuPrime Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA, USA). PCR amplification was conducted under the thermal cycling condition, which started from DNA melting at 94 °C for 1 min, followed by 35 cycles of 94 °C for 20 s, annealing at 53 °C for 25 s, extension at 68 °C for 45 s, and finally the extension at 68 °C for 10 min used a AccuPrime High Fidelity Taq Polymerase (Invitrogen, Grand Island, NY, USA). Subsequently, PCR products were pooled and purified with the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA) and quantified with PicoGreen. Quality trimming was conducted through the Galaxy pipeline at the Institute for Environmental Genomics, University of Oklahoma (http://zhoulab5.rccc.ou.edu/). Sequences with ambiguous bases and chimera with lengths between 245 bp and 258 bp were removed. Then sequences were resampled at 12,674 reads and operational taxonomic units (OTUs) were clustered at the 97% similarity level, resulting in a total of 36,938 OTUs.

GeoChip 5.0, a microarray-based tool containing 167,044 distinct gene probes, were applied to characterize the diversity of microbial functional genes related to methanogenic pathways (Sun et al., 2014; Wu et al., 2016) (Fig. S1). Genomic DNA from each sample was first labeled with fluorescent dye Cy-3 (GE Healthcare, Little Chalfont, UK) and purified using QIAquick Purification kit (Qiagen, Valencia, CA, USA), and then dried at 45 °C for 45 min in a SpeedVac (Thermo Savant, NY, USA). Subsequently, DNA was resuspended in 27.5 μl DNase/RNase-free distilled water and uniformly mixed with 99.4 μl hybridization solution. The mixture was comprised of 10 pM universal standard DNA, 1 × ACGH blocking buffer, 1 × HI-RPM hybridization buffer, 0.05 μg/μl Cot-1 DNA and 10% formamide, which was denatured at 90 °C for 3 min before incubation was carried out for 30 min under 37 °C. GeoChip hybridization was performed in a G2545A hybridization oven (Agilent, Santa Clara, CA, USA) for 24 h under 67 °C. After hybridization, unbounded DNA was removed by washing the slides at room temperature using Wash Buffers (Agilent, Santa Clara, CA, USA). GeoChip arrays were then scanned at 633 nm by a MS200 Microarray Scanner (Roche NimbleGen, Madison, WI, USA). The acquired image data was extracted and quantified using Feature Extraction software 11.5.1.1 (Agilent, Santa Clara, CA, USA). The pre-processing of raw microarray data was conducted on the GeoChip microarray analysis pipeline (http://ieg.ou.edu/microarray/) as described previously (Ding et al., 2015). Spots with a signal-to-noise ratio (SNR) < 2.0 or signal intensity < 1000 were removed. Signal intensity of each sample was normalized by log-transformation and then divided by the mean intensity of the slide. Probes with signal intensity only detected in single sample within a digester was also removed.

### 2.3. Statistical analyses

Detrended correspondence analysis (DCA) was conducted to display community successions. MRPP (Multi-response permutation

procedure), Anosim (Analysis of similarities) and Adonis (Permutational multivariate analysis of variance) using Bray-Curtis distance were performed to examine the differences in community composition and methanogenic genes diversity. An association network comprised of core genera was constructed using a random matrix theory-based approach (Yang et al., 2009), resulting in 29 nodes and 39 links. Sequences obtained in < 75% of 36 samples at the genus level were removed to ensure a reliable network. Multivariate statistical analyses such as Mantel tests were conducted to access the effect of environmental parameters on microbial community succession. The multiple regression model (MRM) analysis was then carried out to establish linkages between biogas production rate and methanogen community, *mcrA* genes as well as environmental parameters. All the above analysis was performed using functions in the Vegan (v. 2.4-1) and Ecodist (v. 1.2.9) packages in R (v. 3.4.3) (http://www.r-project.org).

## 3. Results

### 3.1. The performance of anaerobic digesters

The performance and environmental parameters in three anaerobic digesters were considerably variable (Table S1). Ammonia concentrations in Beijing and Qingdao samples ranged from 311 to 1200 mg/L while those in Ningbo samples were much higher (1440 to 2172 mg/L). pH values in Ningbo samples fluctuated between 7.37 and 8.58, which were similar to those in Beijing and Qingdao samples (in the range of 7.13 and 8.01). Biogas production rates were highly variable. For example, biogas production rates in Qingdao digester ranged from 7238.42 to 15,376.43 $m^3$/d but those in Ningbo digester ranged from 398.50 to 1265.60 $m^3$/d, which can be attributed to ~10-fold differences in the feed rates (Table S1). Fe concentration varied from 0.020 to 0.490 mg/L in Beijing and Qingdao samples, and 0.087 to 0.740 mg/L in Ningbo samples. Cu and Cr concentrations displayed similar trends, which were below 0.018 mg/L in Beijing and Qingdao samples, but fell into the range of 0.005 and 0.120 mg/L in Ningbo samples. Chloride concentration in Qingdao samples ranged from 945.45 to 1762.04 mg/L, much higher than other samples (below 174.38 mg/L). Sulfate concentrations were below 20 mg/L in most samples but were occasionally over 30 mg/L.

### 3.2. High variations of microbial community composition

The high-throughput sequencing of 16S rRNA gene amplicons detected 36,938 OTUs, much higher than currently reported in full-scale activated sludges (Jiang et al., 2018). Those OTUs can be classified into 33 phyla, 205 families and 683 genera, with only 53% classified at the family level and 41% classified at the genus level. *Coprothermobacter* within *Clostridia*, which ferments proteins rather than carbohydrate (Pervin et al., 2013) and produces hydrogen (Tandishabo et al., 2012), was the most abundant genus in Ningbo digester, accounting for 24.6% of total abundance in June. In Qingdao samples, *Saccharofermentans* (up to 16.8%) and *Ruminococcus* (up to 10.9%) belonging to *Firmicutes*, known to ferment polysaccharide and proteins, as well as *Crocinitomix* (up to 11.3%) belonging to *Bateroidetes*, were the top abundant genera. In Beijing samples, *Thauera*, capable of growth on organic acids, and *Comamonas*, responsible for degradation of organic acids including acetate (Nelson et al., 2011), together accounted for up to 13.8% of total abundance. Both genera were affiliated with *Proteobacteria*.

A total of 30,976 out of 36,938 OTUs were detected only in one of the three digesters, which accounted for 33% of total abundance (Fig. S2). Consequently, community composition was largely clustered by anaerobic digesters (Fig. 1 and Table S2). The successional patterns of microbial communities varied substantially by anaerobic digesters. While microbial community dynamics in Qingdao and Ningbo digesters showed a progression away from the initial time point, communities in Beijing digester converged at the beginning and end of the sampling months, suggestive of a potential annual periodicity. To reveal their temporal succession, the time-decay relationship of microbial community was measured by a linear regression between community dissimilarity and time intervals. There was significant time-decay relationship for Qingdao and Ningbo samples, but not Beijing samples (Fig. S3). The succession rate of Ningbo samples was high ($v = 0.025$, $P < 0.001$), revealing a rapid succession that replacing 30% of microbial communities every year.

Only 68 core genera were found in at least 75% of all samples (Table S3). However, those core genera accounted for 24% of total abundance, implying that they were important for anaerobic digester performance. The top two abundant genera were *Syntrophomonas* of *Firmicutes* (up to 5.1% of total abundance) and *Smithella* of *Proteobacteria* (up to 7.6% of total abundance), both well established for syntrophically degrading short-chain fatty acids to $H_2$, formate and acetate (Muller et al., 2010). Members of *Syntrophomonas* are often functioned as syntrophic butyrate oxidizers, while *Smithella* are syntrophic propionate-oxidizing bacteria to produce acetate and butyrate. Subsequently, an association network was constructed for core genera (Fig. 2), resulting in 29 nodes representing genera and 39 edges representing correlations between genera. The network fit well with power law distribution ($r^2 = 0.85$), suggesting that some nodes in the networks had numerous connections while most nodes had only a few connections (i.e., scale free). Among the 29 nodes in the network, 11 were affiliated with *Proteobacteria*, followed by 4 *Firmicutes* and 4 *Euryarchaeota* methanogens. *Treponema* and *Methanolinea* had both high Z and *P* values and hence were identified as the network hubs (Deng et al., 2012). *Treponema* has the max betweenness. *Syntrophus* and *Desulfomicrobium* had the largest clustering coefficient, indicating that they were best connected with their neighbors. The two nodes were positively associated, and their neighbors were *Methanosaeta*, *Methanolinea*, and *Lutispora* (Fig. 2). The two methanogen genera *Methanosaeta* and *Methanolinea* had the max degree (7), showing critical roles in shaping network structure.

Mantel test was conducted to identify important environmental parameters in shaping community variations (Table 1). Ammonia and alkalinity had significant impact on community succession in all digesters ($r > 0.303$, $P < 0.028$). All parameters except COD, which includes refractory organic pollutants resistant to microbial biodegradation (Chamarro et al., 2001), were significantly correlated with microbial community composition in all samples ($r > 0.142$, $P < 0.004$).

### 3.3. High variations of methanogens

Methanogen sequences constituted over 91% of the archaeal 16S rRNA sequences recovered from each digester. A total of 223 OTUs of methanogens belonged to *Methanobacteriales*, *Methanomicrobiales*, and *Methanosarcinales*, which were well known methanogens (YuChen Liu, 2008). The aceticlastic genus *Methanosaeta* was the most abundant methanogenic genus in Beijing samples, averagely accounting for 33.7% to 67.9% of total methanogen abundance. *Methanolinea*, which is a hydrogenotrophic genus belonging to a newly established family *Methanoregulaceae* (Oren, 2014), accounted for 10.4% to 41.3% of total methanogen abundance in Beijing samples. This genus was the most abundant methanogenic genus in Qingdao samples, accounting for 21.6% to 87.9%. In Ningbo samples, the hydrogenotrophic methanogen *Mathanobacterium* accounted for 34.3% to 79.3% of total methanogen abundance. *Methanosarcina*, a versatile methanogen capable of utilizing both acetate and $H_2/CO_2$, was also abundant (10.9%–51.3%). All of four abundant methanogens were present in the association network (Fig. 2), which showed mostly positive edges (10 out of 14 edges).

A total of 334 probes from 18 genes associated with methanogenesis were detected by GeoChip 5.0. An average of 71 probes of *hdrB*, which encodes heterodisulfide reductase that reduces CoM-S-S-CoB to coenzyme M and coenzyme B during methane production, were detected in

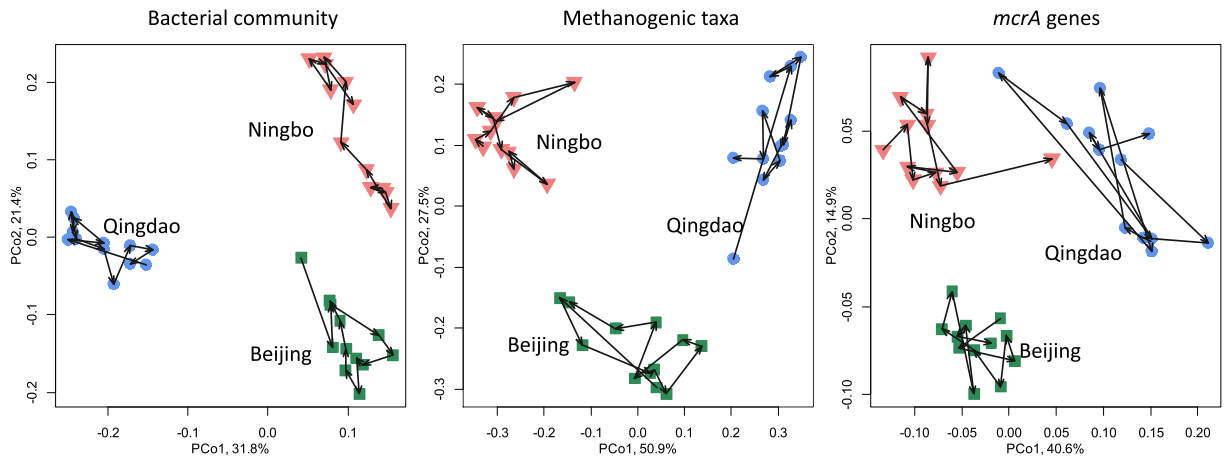


**Fig. 1.** Variations of bacterial communities, methanogenic taxa and *mcrA* genes calculated by Principal Coordinates Analysis (PCoA). Weighted UniFrac distance was used for bacterial and methanogenic communities and Bray-Curtis distance was used for *mcrA* genes. Arrows are drawn in chronological order from October 2012 to September 2013.

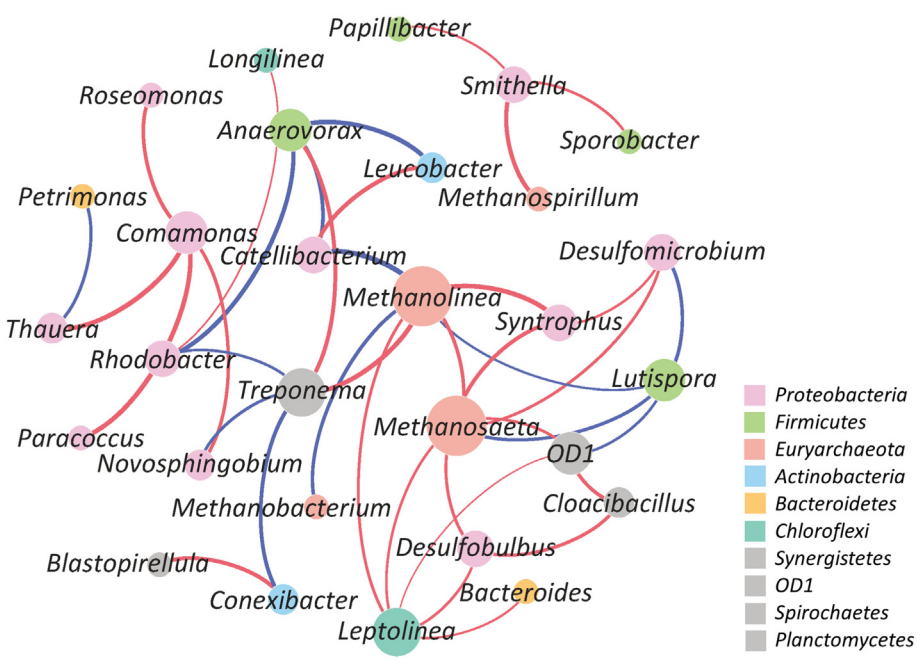


**Fig. 2.** Network interactions among core genera present in > 75% of all samples. Nodes are colored by the phylum that the genus belongs to and the node size is proportional to its degree (i.e., the number of links for the node). Blue edges represent positive associations, while red edges represent negative associations. The thickness of each edge is proportional to strength of correlation between two nodes as calculated by spearman's *r* values. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

**Table 1**
Mantel tests of environmental parameters and microbial communities.

| | Beijing | | Qingdao | | Ningbo | | All | |
|---|---|---|---|---|---|---|---|---|
| | R | p | R | p | R | p | R | p |
| Feed | / | / | 0.089 | 0.243 | **0.628**[a] | **0.001** | **0.650** | **0.001** |
| TS | −0.200 | 0.948 | 0.163 | 0.125 | 0.012 | 0.435 | **0.418** | **0.001** |
| COD | 0.002 | 0.437 | −0.138 | 0.778 | −0.179 | 0.999 | 0.030 | 0.168 |
| pH | **0.297** | **0.038** | −0.060 | 0.658 | 0.204 | 0.075 | **0.142** | **0.001** |
| Alkalinity | / | / | **0.367** | **0.012** | **0.699** | **0.001** | **0.414** | **0.001** |
| Ammonia | **0.369** | **0.017** | **0.360** | **0.011** | **0.303** | **0.028** | **0.175** | **0.004** |
| Sulfate | 0.206 | 0.107 | 0.182 | 0.099 | **0.784** | **0.001** | **0.239** | **0.001** |
| Chloride | 0.004 | 0.441 | 0.020 | 0.488 | −0.116 | 0.905 | **0.495** | **0.001** |
| Fe | −0.008 | 0.447 | 0.092 | 0.298 | **0.552** | **0.004** | **0.260** | **0.001** |
| Cu | 0.022 | 0.291 | 0.033 | 0.462 | 0.037 | 0.306 | **0.183** | **0.001** |
| Cr | 0.102 | 0.179 | **0.367** | **0.013** | **0.384** | **0.005** | **0.387** | **0.001** |
| Air temperature | 0.098 | 0.164 | **0.494** | **0.002** | **0.735** | **0.001** | 0.037 | 0.154 |

[a] Bold values denote significance at the $P < 0.050$ level.

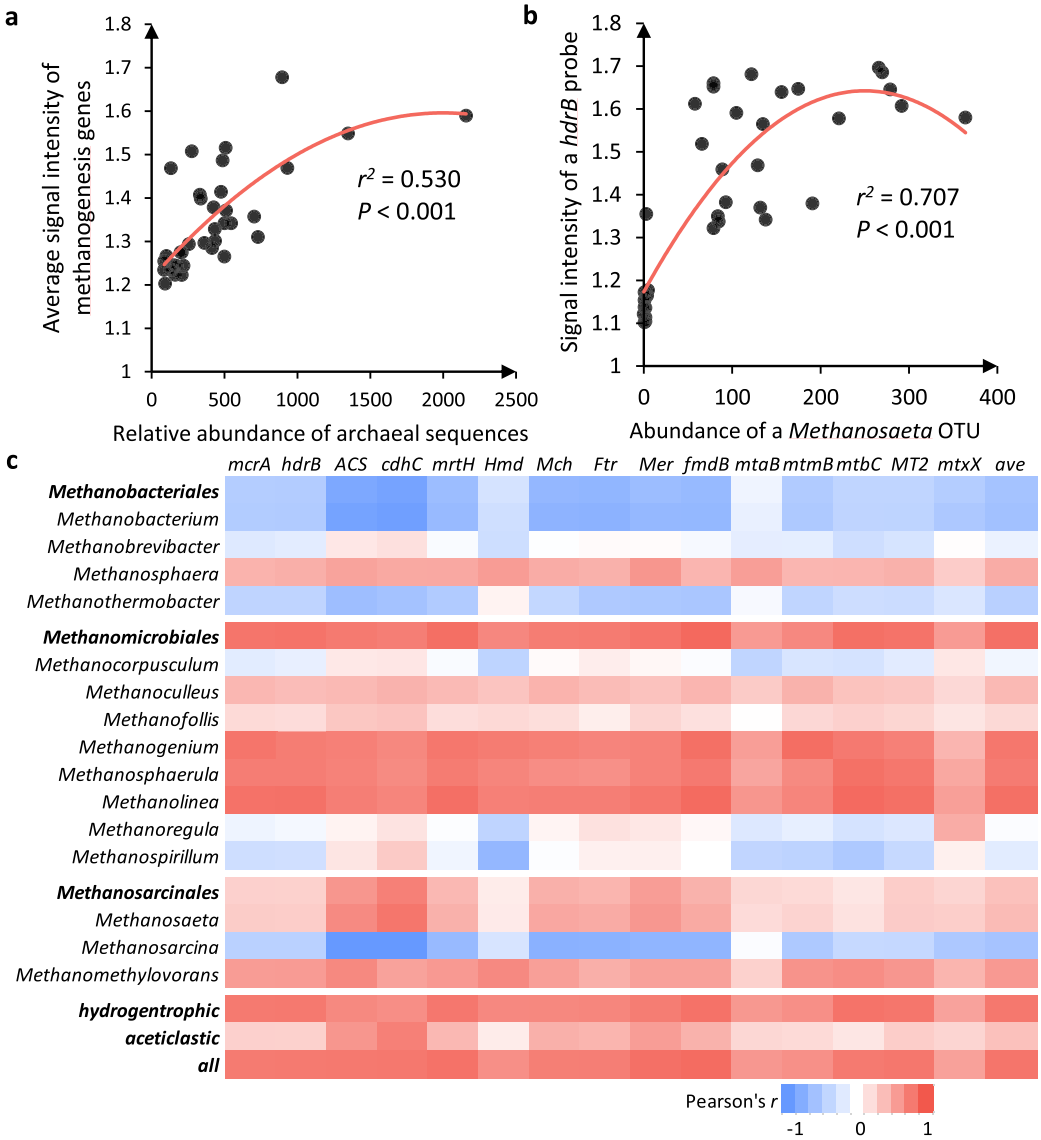


**Fig. 3.** Correlations of methanogenic groups between sequencing and GeoChip data. (a) Correlation between archaea abundance and methanogenic genes fitting parabola curves; (b) correlation between a *Methanosaeta* OTU and a *hdrB* probe fitting parabola curves; and (c) Pearson correlations of linear curves between methanogenic taxa and genes.

every sample. Similarly, 59 *mcrA* probes encoding the α subunit of methyl-coenzyme M reductase, widely regarded as the biomarker gene of methane production, were detected. The other 16 detected genes are associated with different methanogenic pathways utilizing acetate, $H_2$/$CO_2$ or other substrates (Fig. S1).

The methanogenic gene structure was clustered by anaerobic digesters (Fig. 1, Table S2 and Fig. S4b), suggesting that functional potentials of various methanogenic pathways varied in three digesters. The average signal intensities of methanogenesis genes in Ningbo were substantially lower than those in Beijing and Qingdao (Fig. S4b). Aceticlastic genes *ACS* encoding acetyl-CoA synthetase and *cdhC* encoding acetyl-CoA decarbonylase/synthase complex subunit beta were also lower in relative abundance than those in Beijing and Qingdao ($P < 0.001$).

We evaluated the consistency of sequencing and GeoChip data by correlation analysis, resulting in a strong, positive correlation ($r^2 = 0.530$, $P < 0.001$) (Fig. 3a) between relative abundance of methanogenic archaea and average signal intensity of methanogenesis genes. Most methanogenic genes were positively correlated with their corresponding methanogens utilizing methanol, acetate and $CO_2$ (Fig. 3c). For example, *mtaB* encoding methanol-5-hydroxybenzimidazolylcobamide *Co*-methyltransferase, a gene associated with methanol metabolism, was positively correlated with *Methanobacterium* ($r = 0.554$, $P < 0.001$) and *Methanosarcina* ($r = 0.589$, $P < 0.001$), while it was negatively correlated with other methanogens absent in the methanol pathway. Aceticlastic *Methanosaeta* displayed significant correlation with *ACS* ($r = 0.580$, $P < 0.001$) and *cdhC* ($r = 0.418$, $P < 0.001$), the biomarker genes for aceticlastic methanogenesis. The correlations were highly specific, as *Methanosaeta* was not correlated with non-aceticlastic methanogenic genes. The only core archaeal OTU, which is affiliated with *Methanosaeta*, was correlated with many core methanogenic genes. For example, a strong, significant correlation ($r^2 = 0.707$, $P < 0.001$) was detected between the *Methanosaeta* OTU and a core probe of *hdrB* (Fig. 3b).

To explain the high consistency between sequencing and GeoChip data of methanogens, we calculated functional redundancy indices by the ratio of functional diversity to taxonomic diversity, as recently established (Fierer et al., 2013). Methanogens displayed lower functional redundancy indices in all three digesters than fermentative microbes (Table S4), which explained their high consistency between taxonomy

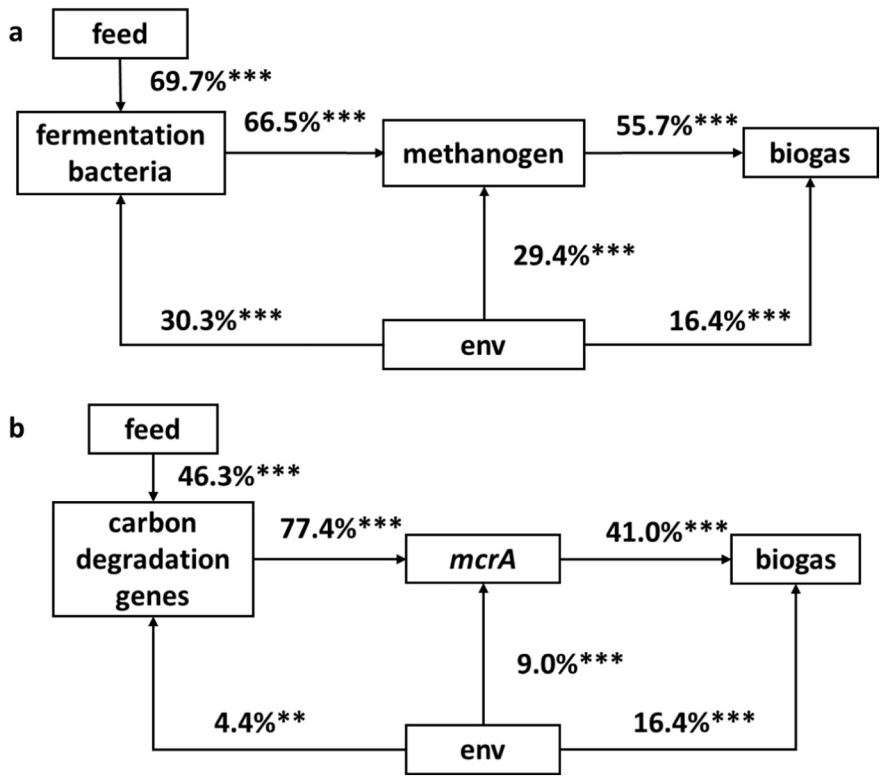


**Fig. 4.** Multiple regression models (MRM) coupling biogas production and selected environmental parameters with (a) taxonomic composition and (b) functional traits of microbial communities. Regression $R^2$ values from the permutation test are presented on the arrows, which are all significant ($P < 0.005$).

and function in methanogens.

### 3.4. Microbial and environmental contributions to the performance of biogas production in anaerobic digesters

The multiple regression model (MRM) analysis was conducted to assess the linkages between methanogens and environmental parameters as well as their contributions to biogas production rates. Using data from sequencing or functional genes, we generated two significant ($P < 0.005$) models (Fig. 4). Variations of feed were closely related to fermentative microbes, explaining 69.7% of fermentative microbial succession and 46.3% of carbon degradation gene variations. High explanatory percentages were also found between fermentative microbes and methanogens, which were 66.5% for sequencing data and 77.4% for functional gene data. For their contributions to biogas production, methanogen variation explained 55.7% of biogas production rate and *mcrA* gene variation explained 41.0% of biogas production rate, much higher than explanatory percentages by environmental parameters including pH, ammonia, TS, COD, Fe, Cu, Cr, Cl and sulfate and metals (16.4%).

## 4. Discussion

### 4.1. High variations of microbial community composition

Microbial communities in natural environments, such as the ocean (Gilbert et al., 2009) and hot springs (Briggs et al., 2014), show clear seasonal dynamics. In contrast, microbial communities in anaerobic digesters, which is a well-controlled engineering system, were shown to only exhibit weak composition variations (Jiang et al., 2018; Werner et al., 2011). However, our in-depth investigation of microbial community revealed high variations. Although community variations were mainly determined by individual digesters (Fig. 1), temporal succession within a digester varied more substantially than previously reported (Jiang et al., 2018; Werner et al., 2011). Deeper sequencing efforts in this study might hold the liability, as weighted UniFrac distances between the same time interval in this study (0.28 on average, ranging from 0.12 to 0.47) (Fig. S3) were apparently higher than those in the recent study (Jiang et al., 2018). However, please bear in mind a caveat that the use of V4 region of 16S-rRNA amplicon-based sequencing approaches can be of low reproducibility due to the artifacts associated with inadequate random sampling, amplification biases, and/or sequencing errors (Zhou et al., 2015).

There were only a few core genera, which were associated with hydrogenesis, acidogenesis and methanogenesis (Table S3). Hydrogen-producing *Syntrophomonas* and *Smithella* were among the core genera, together accounting for 4.6% of microbial communities on average. Both taxa are typical syntrophs with hydrogen-scavenging *Methanomicrobiales* or *Methanobacteriales* (Embree et al., 2015; Ju et al., 2017) in the hydrogenotrophic methanogenesis pathway, which is essential for stable operation of anaerobic digesters (Werner et al., 2011). Similarly, acetate producer *Anaerolineaceae* and aceticlastic methanogen *Methanosaeta*, another two core genera, are syntrophs in the aceticlastic pathway (Liang et al., 2015; McIlroy et al., 2017; Zhao et al., 2016), as verified by our network analysis showing that *Leptolinea* from *Anaerolineaceae* was positively correlated with *Methanosaeta* (Fig. 2). Despite high variations of community composition, high occupancy and abundance of those a few hydrogenotrophic and aceticlastic methanogens suggest that there must be a selection pressure in anaerobic digesters to maintain methane production because fluctuations of key populations can reflect the dynamics of biochemical processes in ecosystems (Ruff et al., 2015).

As microorganisms rely on complex networks of interactions within a community, the network analysis has been powerful to reveal potential microbial interactions (Wu et al., 2016). The two network hubs (*Methanolinea* and *Treponema*) shown in Fig. 2 verified the critical roles of methanogen and acetogen in anaerobic digesters. *Treponema* affiliated with phyla *Spirochaetes* is a homoacetogen using $H_2$ and $CO_2$ to

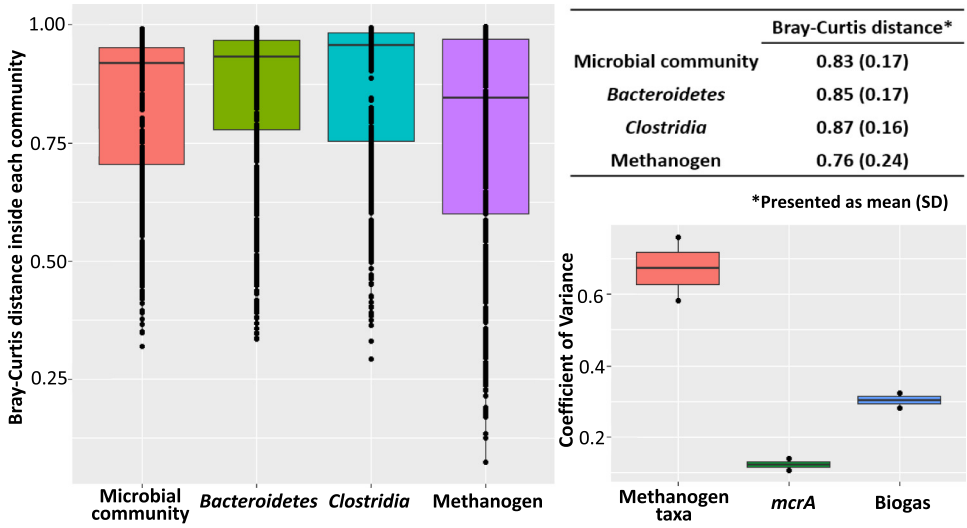


**Fig. 5.** Dynamics and diversity of different microorganisms and biogas production measured by Bray-Curtis distance and coefficient of variance.

produce acetate, which can then be used by aceticlastic methanogens. There was evidence showing its interaction with cellulolytic bacteria (Kudo et al., 1987), serving as a link between hydrolysis and acetogenesis. In contrast, *Desulfomicrobium* and *Syntrophus* had the largest clustering coefficient (Fig. 2). *Desulfomicrobium* is a strictly anaerobic, mesophilic, sulfate-reducing bacterium that oxidizes pyruvate and lactate to acetate, supplying the substrate to aceticlastic methanogens (Copeland et al., 2009). *Syntrophus* can degrade fatty acids and benzoate and well known for syntrophic association with hydrogen-using microbes (Jackson et al., 1999). The positive correlations between *Desulfomicrobium* and aceticlastic *Methanosaeta*, *Syntrophus* and *Methanolinea* were observed in the network, reflecting a syntrophic community efficiently utilizing complex carbon substrates (McInerney et al., 2009). Interestingly, the photosynthetic bacterium *Rhodobacter* was connected to *Treponema*, *Comamonas* and *Anaerovorax* (Fig. 2), possibly owing to the metabolic versatility of *Rhodobacter* in hydrogen production (Koku et al., 2002).

High variations of microbial communities can stem from changes in environmental parameters. Our study is consistent with several previous studies that pH, temperature and ammonia concentration are important in shaping anaerobic digester communities (De Vrieze et al., 2015; Ju et al., 2017; Tonanzi et al., 2018). In our study, environmental parameters explained 30.3% of fermentation bacterial variations and 29.4% of methanogen variations (Fig. 4a), verifying the importance of deterministic processes. The number of deterministic factors significantly affecting community composition was the largest for Ningbo samples (Table 1), which might be attributed to the presence of *Methanosarcina*. It was shown that *Methanosarcina* required a larger concentration of acetate than *Methanosaeta* and was more susceptible to environmental changes (YuChen Liu, 2008). Coincidently, *Methanosaeta* was the dominant aceticlastic methanogen in Ningbo digester, while *Methanosaeta* was the dominant aceticlastic methanogen in Beijing and Qingdao digesters.

Hydrogenotrophic methanogens are more tolerant to high ammonia concentration than aceticlastic methanogens (Westerholm et al., 2016). In the absence of aceticlastic methanogens, acetate can be converted to hydrogen by syntrophic acetate-oxidizing bacteria, thus stimulating hydrogenotrophic pathway (Karakashev et al., 2006). Syntrophic acetate oxidization is often performed by members of *Clostridia* class, which are also more tolerant to high ammonia concentration (De Vrieze et al., 2015; Muller et al., 2016). We observed positive correlations ($r > 0.500$, $P < 0.050$) between hydrogenotrophic methanogens and *Clostridia* populations in anaerobic digesters, which had syntrophic associations (Jaenicke et al., 2011). Similarly, high ammonia concentration and high abundance of *Clostridia* and hydrogenotrophic methanogen were observed in Ningbo digester (Table S1, Figs. S5 & S4a). *Coprothermobacter* may contribute to the high ammonia concentration by fermenting proteins as the most abundant genus in Ningbo digester. In contrast, syntrophic acetate oxidizers are slow growers, rendering them less competitive than aceticlastic methanogens in general (Westerholm et al., 2016) as we have observed in Beijing digester (Fig. S4a).

### 4.2. Low functional redundancy of methanogens

Microbial diversity in natural and engineering environment is typically high. Consequently, a metabolic function can be performed by taxonomically distinct organisms (Louca et al., 2018). High functional redundancy leads to decoupling of taxonomic composition and functional structure because functionally similar but taxonomically distinct species can readily replace each other. As we observed a strong, positive correlation between methanogenic archaea and methanogenic genes (Fig. 3), functional redundancy of the methanogenic group should be low in anaerobic digesters. However, some functional redundancy was notable. In lab-scale bioreactors, *Methanomicrobiales* could replace *Methanobacteriales* without changes in formate consumption rate (Peces et al., 2018). Similarly, *Methanoregula* replaced *Methanosaeta* while maintaining methanogenic community diversity (De Vrieze et al., 2017). In our study, variations of methanogenic archaea were higher than those of biogas production and methanogenic genes (Fig. 5), which also indicated some functional redundancy in methanogenic groups. However, methanogenesis is a narrow function in that it is restricted to a few phylogenic clades, which is believed to be less functional redundant than broad functions such as carbon catabolism and respiration (Louca et al., 2018). In accordance, broad functions are more resistant to changes in taxonomic composition than narrow functions (Langenheder et al., 2006; Peter et al., 2011). We found that the dynamics of broad functional groups of carbon catabolism, such as *Clostridia* and *Bacteroidetes*, were more dynamic and diverse than average populations (Fig. 5) relying more on redundancy to maintain the overall community function. The dynamics of narrow functional group of methanogenesis was markedly different, with core archaea OTUs averagely composing of 76% of archaeal community in each digester (Fig. S2). Consistently, methanogens had the highest node degree in the network (Fig. 2). The relatively low functional redundancy of methanogens was further verified by the ratio of functional to taxonomic diversity, which was lower than fermentative microbes (Table S4).

### 4.3. Linkages between methanogens and biogas production

Relationships between methanogenic functional traits and biogas production are challenging to establish. For example, *mcrA* gene copy numbers, measured by qPCR experiments, failed to show a linkage with biogas production (Hidaka et al., 2018). Based on the variation analysis, we found that biogas production rates were well correlated with variations of methanogens (Fig. 4), verifying that methanogens played a crucial role in anaerobic digesters (Bialek et al., 2011). It was shown that the aceticlastic pathway was the main methanogenic process in a number of anaerobic digesters (Demirel, 2014; YuChen Liu, 2008). Here, high consistency between aceticlastic genes and microbes participated in aceticlastic pathway were observed (Fig. 3). However, a $^{13}$C-labeled acetate study suggested that nonaceticlastic pathway generated 80% of methane (Bialek et al., 2011). We found that aceticlastic methanogen *Methanosaeta* were most abundant methanogens in Beijing digester, but hydrogenotrophic methanogen *Methanomicrobiales* showed strong, positive correlations with nearly all methanogenic genes as well as biogas production (Fig. 3). Similarly, a recent study with 29 full-scale anaerobic digesters also revealed a positive correlation between hydrogenotrophic methanogens and biogas production (De Vrieze et al., 2015).

## 5. Conclusive remarks

Our results demonstrate high variations of microbial community including methanogens, which is substantially driven by deterministic processes. Our finding agrees with the niche-based theory that environmental selection is vital in guiding community composition and dynamics, as has been observed in other anaerobic digesters (De Vrieze et al., 2015; Ju et al., 2017; Vanwonterghem et al., 2014). Interestingly, we showed that methanogenic functional traits were more important than bacterial taxonomic traits and environmental parameters in shaping biogas production variation, which provided valuable insights for developing an efficient, reliable biogas production under fluctuating environmental conditions. As microbial communities in many full-scale anaerobic digesters are continuously facing unstable influents and operation, the number of ecological niches can theoretically increase within anaerobic digesters. Consequently, microbial communities show higher diversity and dynamics than those in stably operated digesters. However, relatively low functional redundancy in methanogens could limit biogas production. As occurrence of perturbations and disturbances are unavoidable in real-world systems of anaerobic digestion, increasing the knowledge base on methanogens by monitoring anaerobic digesters remains an essential step towards the goal of controlling and optimizing process performance of biogas production.

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## Conflict of interest

The authors declare no conflict of interest.

## Data accessibility

MiSeq sequencing data of 16S rRNA gene are available online in the NCBI database (https://trace.ncbi.nlm.nih.gov/Traces/study/?acc=SRP060281&go=go) with the accession number SRP060281. GeoChip data are available online (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE92978) with the accession number GSE92978.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2019.03.005.

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