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New insights into the key microbial phylotypes of anaerobic sludge digesters under different operational conditions

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ABSTRACT

Analyses on bacterial, archaeal communities at family level and methane-production metabolism were conducted in thirteen full-scale and pilot-scale anaerobic sludge digesters. These digesters were operated at different conditions regarding solids concentration, sludge retention time, organic loading rate and feedstock composition, seeking to optimize digester capacity. Correlations between process parameters and identified microbial phylotypes were evaluated based on relative abundance of these phylotypes determined by Quantitative PCR and 16S rDNA amplicon sequencing. Results showed that, Total Solids concentration (TS), among the evaluated operational parameters, demonstrated the most positive correlation with chemical parameters (including NH_3 and VFAs) and significant impact on the abundance of key microbial phylotypes regardless of other factors. Digesters were grouped into 'Higher-TS' with higher stress (TS > 44 g/L, NH_3 > 90 mg/L, VFAs > 300 mg/L) and 'Lower-TS' under easier status (TS ≤ 44 g/L, NH_3 < 120 mg/L, VFAs < 525 mg/L) in this study. We identified the key microbial phylotypes, i.e. the most abundant and discriminating populations, in 'Higher-TS' digesters with high biogas production rate, which were the class Clostridia, the family Methanosarcinaceae and the order Methanobacteriales. Thermoanaerobacteraceae and Syntrophomonadaceae were identified as key families of Clostridia. Methane was produced both from acetoclastic and hydrogenotrophic methanogenesis. By contrast, in 'Higher-TS' digesters with low biogas production rate, the classes Alpha-, Beta- and Gamma-proteobacteria were detected in higher percentages, of which Rhodobacteraceae, Comamonadaceae and Xanthomonadaceae were the most abundant families respectively, and Methanomicrobiales was the prevailing methanogen order. Consistently, hydrogenotrophic pathway was predominant for methanogenesis, indicating existence of syntrophic acetate oxidation in such 'high-stress', low biogas production rate digesters. These microbial phylotypes were therefore considered to be associated to 'Higher-TS' operation. In 'Lower-TS' digesters, the abundance of the class Delta-proteobacteria, the families Anaerolineaceae, Rikenellaceae, Candidatus Cloacamonas and Methanosaetaceae was obviously higher compared with those in 'Higher-TS' digesters, which were thus considered to be marker phylotypes of easy status. The influence of TS and NH_3 on the microbiome should be considered when a 'TS-increasing' strategy is applied to increase digester capacity.

1. Introduction

The Anaerobic Digestion (AD) process has been used for years in sewage sludge treatment and represents an attractive technique for sludge reduction along with energy production *via* biogas (Rivière et al., 2009). This biological process relies on a very delicate balance between four functional groups of microorganisms through a food web: hydrolytic bacteria decompose insoluble macromolecules to soluble molecules, acidogens and acetogens further

Abbreviations: AD, anaerobic digestion; AM, acetoclastic methanogenesis; HM, hydrogenotrophic methanogenesis; SAO, syntrophic acetate oxidation; OLR, organic loading rate; SRT, sludge retention time; TS, total solids; VS, volatile solids; PSS, primary sewage sludge; BSS, biological sewage sludge; HyBSS, hydrolyzed biological sewage sludge.

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degrade them into smaller intermediates and methanogens convert these smaller substrates into methane and carbon dioxide through Acetoclastic (AM) and Hydrogenotrophic (HM) pathways. This microbiome is highly complex in terms of functionality and community diversity.

Due to insufficient knowledge on the microbial community ecology and function, the AD process has limited performance, below its optimum capacity (Regueiro et al., 2015; Rivière et al., 2009; Couras et al., 2014). Since anaerobic digesters are costly to build and operate, many possible solutions are considered to increase their capacity without affecting biogas yield and solids reduction: for instance, to increase the Organic Loading Rate (OLR) by increasing the flow rate, which then induces a substantial decrease of Sludge Retention Time (SRT) (this approach is named 'hydraulic increase'). Too short SRT can however lead to the washout of microorganisms and impair the digester performance (Regueiro et al., 2015). Thus, an 'organic increase' solution, which consists in raising the concentration of Total (TS) and Volatile Solids (VS) in the feedstock, can be considered in order to circumvent the short SRT problem induced by only hydraulic increase (Regueiro et al., 2015; Couras et al., 2014; Mcleod et al., 2015). Other solutions include pretreating the feeding sludge by processes like thermal hydrolysis to improve its dewaterability and biodegradability (Carrère et al., 2010; Gagliano et al., 2015). During these attempts to optimize the digester capacity, the delicate balance between the four microbial groups can easily suffer inhibition from ammonia, sulphides, metals, pH, fatty acids and other organic compounds (Chen et al., 2008), or even wash out of microbes at low SRT. The sensitivity of anaerobic microorganisms to operational and environmental factors may lead to decreased digestion efficiency under increased capacity, or in extreme cases to complete failure. Therefore, it becomes important to identify the key microbial phylotypes under different operational strategies (De Vrieze et al., 2012) and to get an in-depth understanding of their functionalities, which may help to achieve higher digester capacity (Vanwonterghem et al., 2014).

Operational conditions of the digester, such as OLR, SRT, solids concentration (in the influent and effluent), temperature and reactor configuration have been reported to determine the microbial community composition to a large extent, which in turn influenced the digestion efficiency (De Vrieze et al., 2015). Several attempts have already been carried out to correlate the microbial community composition with the reactor operating conditions and performance in AD (De Vrieze et al., 2015; Abendroth et al., 2015; Regueiro et al., 2015; Karakashev et al., 2005, 2006; Lerm et al., 2012). During these attempts, little information is available yet concerning the relationship between operational strategies, digestion efficiency and bacterial populations of lower taxonomic levels (below class level), especially in sewage sludge AD process. That is however essential to understand the potential functionalities of the microbial communities and their interaction with environmental factors (Narihiro and Sekiguchi, 2007; Ariesyady et al., 2007).

Additionally, the microbial community structure varies a lot depending on the inoculum (Wilkins et al., 2015), feedstock composition and feeding pattern (Sundberg et al., 2013; Abendroth et al., 2015; De Vrieze et al., 2015). As a major feedstock of AD, sewage sludge itself is prone to natural and unavoidable variation caused by numerous aspects such as type and source (Mcleod et al., 2015), which lead to highly diverse and specific structured microbial communities. This further increases the difficulty of investigating the correlations between microbial populations and physical-chemical parameters, and accessing to the common rules that may uncover the mechanisms driving differentiation of microbial communities under different operational conditions.

To better understand the interactions between the key microbial phylotypes and operational factors in anaerobic digestion of sewage sludge, we investigated the archaeal and bacterial communities in 13 digesters working under different operational conditions including TS, SRT, OLR and substrate compositions. Relative abundance of archaeal and bacterial phylotypes was analyzed at the family level and their correlations with operational factors and methane-production efficiency and pathways were evaluated by statistical tools.

2. Materials and methods

2.1. Description of digesters and samples

Two full-scale, five pilot-scale and six laboratory-scale anaerobic digesters were investigated, which have been operated for >150 days after being set up and inoculated with anaerobic sludge originating from five different sources (Table S7). In these digesters, three types of sewage sludge from different sources were used as feedstock, including Primary Sewage Sludge (PSS), Biological Sewage Sludge (BSS) and Hydrolyzed Biological Sewage Sludge (HyBSS), which were produced in wastewater treatment plants located in three sites named "A", "B" and "C". A wide range of operational conditions were set among the different installations, and in some digesters, operational conditions (mainly OLR) changed periodically.

For microbiological analyses, 200 mL of sludge from full- and pilot-scale digesters or 50–100 mL from laboratory-scale digesters were taken from the recirculation loop to obtain representative samples, which were then divided into several aliquots, snap-frozen in dry ice and stored at −80 °C for further analyses. From these 13 digesters, 21 samples were collected for Quantitative PCR (QPCR) analyses, each from one specific operational condition of one or different digesters, during a relatively steady-state period with constant biogas production and stable VFA concentrations. Among them, 10 representative samples from distinct digesters were used for amplicon sequencing.

Samples were named according to the digester's scale and the substrate's characteristics: L-, P-, F- for Lab-, Pilot- and Full-scale respectively; A-, B-, C- for source of substrate; B-, BP-, hyBP- for BSS, mix of BSS and PSS, and mix of hyBSS and PSS. The following first number expressed TS concentration of the substrate. The last part indicated: the operational period with a number, for example, P-C-hyBP45_**2** represented the second period of digester P-C-hyBP45; or distinguished digesters of the same type with a letter, for instance, F-C-BP42-**F** represented the **F**irst digester of type F-C-BP42. Information concerning characteristics of liquid and biogas in the effluent (except stable isotopic composition), OLR, SRT, temperature, reactor type and volume, and composition of influent stream for different digesters was obtained directly from the digester operators.

2.2. DNA extraction and QPCR

Sludge was thawed on ice and centrifuged at 18,000 *g*, 4 °C for 5 min to obtain pellets. DNA was extracted from 0.20 to 0.25 g pellets using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions.

QPCR analysis for methanogenic members of Methanobacteriales, Methanomicrobiales, Methanosarcinaceae and Methanosaetaceae was performed on a BIO-RAD CFX96 Touch™ Real-Time PCR Detection System (Carlifornia, USA) by the Taqman method. The order Methanococcales was neither detected in a first QPCR test (unpublished data) nor in 16S rDNA sequencing results

(data in this work); it was therefore not analyzed in this study. The primer-probe sets described by (Yu et al., 2005) were used. To better amplify the family Methanosarcinaceae, we modifed the original primers Msc380F (5′-GAAA CCGY GATA AGGG **K**A-3′, the underlined letter indicates the modified position) and Msc828R (5′-TAGC GARC AT**M**G TTTA CG-3′). All analyses were performed in duplicates using a 10-fold dilution of the DNA extracts as template. Results were presented as copies per nanogram of DNA (refer to Supplemental Section 2 for details).

2.3. Ion Torrent 16S rDNA amplicon sequencing

PCR amplifications were performed using fusion universal primers targeting the V4 region of the 16S rRNA gene (Baker et al., 2003), prepared according to Ion Torrent guidelines. The forward primer consisted of adapter sequence A, a barcode of 13–15 bases specific for each sample and primer 515F (5′-GTGY-CAGCMGCCGCGGTA-3′). The reverse primer consisted of adapter sequence trP1 and primer 806R (5′-GGACTACHVGGGTWTCTAAT-3′). For PCR amplification, the Platinum *Pfx* DNA Polymerase (Invitrogen, USA) was used and the PCR mix was prepared according to the enzyme manufacturer's instructions. The PCR run consisted of an initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation (94 °C for 15 s), annealing (50 °C for 30 s) and extension (68 °C for 60 s), and by a final extension at 68 °C for 5 min. Obtained amplicons were purified according to the Agencourt AMPure Protocol and quantified using Agilent DNA 1000 Kit (Agilent Technologies). They were subsequently pooled in equimolar amounts (100 pM concentration) and sequencing templates were prepared using Ion PGM™ Template OT2 400 Kit according to the manufacturer's instructions. Sequencing was performed on an Ion Torrent PGM (Life Technologies) on 314V2 chips using Ion PGM™ 400 Sequencing Kit.

2.4. Fluorescence In Situ hybridization (FISH)

Pellets originating from 0.75 mL of raw sludge were immediately fixed by 3% paraformaldehyde in phosphate buffered saline solution (PBS) during 3 h at 4 °C. Samples were then washed twice in PBS (1×), and stored in PBS:Ethanol (1:1) solution at −20 °C. All samples were further dehydrated by immersion in 50%, 80% and 100% ethanol solutions for 3 min each. Hybridization was performed following the protocol described by Qu et al. (2009). The probes applied are listed in Table S3.

2.5. Isotopic composition analyses

Storage of biogas samples and measurement of stable carbon isotopic compositions of CH_4 ($\delta^{13}CH_4$) and CO_2 ($\delta^{13}CO_2$) were performed according to methods used by Qu et al. (2009). The apparent fractionation factor (α_C) was used to characterize the methanogenic pathways, which was determined by $\alpha_c = (\delta^{13}CO_2 + 10^3)/(\delta^{13}CH_4 + 10^3)$ (Whiticar et al., 1986).

2.6. Bioinformatic and statistical analyses

16S rDNA sequences were analyzed by the UPARSE pipeline using USEARCH v8.0.1623 software. Short sequences less than 200 bp were discarded, the remaining ones were truncated to 200 bp and filtered for quality. Those with expected errors below 1 were then selected, dereplicated and sorted by size to discard singletons. The high quality reads obtained were used for Operational Taxonomic Units (OTUs) clustering at 97% identity. Chimeras were removed using UCHIME against the "gold" database (http://drive5.com/uchime/gold.fa) and a taxonomy was attributed for each OTU using the mothur method implemented in QIIME 1.8.0 against Silva database release 119 with a minimum confidence of 0.8. OTU tables and taxonomy summary files were generated with QIIME.

Principal Component Analysis (PCA) was performed using R package FactoMineR based on the parameter datasets of each digester, and the relative abundance of methanogen populations and bacterial phylotypes (Supplemental Section 1). All variables were mean-centered and scaled to unit variance. Figures were drawn by ggplot2. A two-tailed Spearman's Rank Order Correlation test was run to determine the correlations between methanogene groups, bacterial families, stable carbon isotope data, and various parameters (operational, environmental and efficiency) by using the cor () function in RStudio. Figures were drawn by using a script available from http://www.phaget4.org/R/myImagePlot.R.

2.7. Data deposition

Sequence data were deposited in the NCBI Sequence Read Archive under accession number SRP071030.

3. Results

3.1. Operation and performance of anaerobic digesters

As shown in Table 1, a wide range of operational conditions were set among the different installations to seek for higher digester capacity, especially in the pilot- and laboratory-scale digesters. TS of feedstock ranged from 30.5 to 60.3 g/L; SRT and OLR were set at 14–57 days and 0.86–2.30 g-VS/(L·day) respectively. Performance of digesters varied under different operational conditions, as reflected by the wide range of environmental factor values: NH_4^+-N of 1085–3537 mg/L, NH_3 of 28–387 mg/L, VFAs of 193–909 mg/L, pH of 7.20–7.86. Based on these parameters, the investigated digesters were grouped into 'Higher-TS' with higher stress (TS > 44 g/L, NH_3 > 90 mg/L, VFAs > 300 mg/L) and 'Lower-TS' under easier status (TS ≤ 44 g/L, NH_3 < 120 mg/L, VFAs < 525 mg/L). Digestion efficiency was quite different, with biogas and CH_4 production rates of 0.24–0.93 and 0.14–0.61 L/(g-VS·day) respectively, and VS reduction rate of 35%–68%, leading to categorization of 'High-Rate' (CH_4 > 0.25 L/(g-VS·day)) and 'Low-Rate' (CH_4 < 0.25 L/(g-VS·day)) digesters in this study (Table S7). Characterization of different groups of digesters was further described in Section 3.4.

Isotope fractionation effects expressed by $\delta^{13}CH_4$, $\delta^{13}CO_2$ and α_c were used to evaluate methane-production pathway. $\delta^{13}CH_4$ of −61.75 ~ –65.42‰, $\delta^{13}CO_2$ of 18.03–19.11‰, and α_c of 1.085–1.090 in L-B-B50 (Periods 1–2) and L-C-B60 suggested, methane was mainly produced from hydrogenotrophic pathway in both digesters; whereas $\delta^{13}CH_4$ of −39.29 ~ –53.51‰, $\delta^{13}CO_2$ of 1.50–9.23‰, and α_c of 1.055–1.065 in other digesters indicated that methane was produced from both acetoclastic and hydrogenotrophic pathways.

3.2. Archaea community structure

3.2.1. QPCR results

Four methanogen populations were quantified using QPCR by targeting the 16S rRNA genes of members of Methanobacteriales, Methanomicrobiales, Methanosarcinaceae and Methanosaetaceae. As shown in Fig. 1 and Fig. S1, abundance of Methanosarcinaceae members demonstrated the largest variations between various digesters and contributed to much higher percentages of total methanogens in 'Higher-TS' digesters (L-C-B60, P-C-BP55) and the one (P-C-hyBP45) treating HyBSS. Besides, Methanosaetaceae members in these digesters were much less abundant compared to

other digesters (refer to Supplemental Section 5). These variations of Methanosarcinaceae and Methanosaetaceae in different digesters were also confirmed by FISH experiment (Fig. S2).

3.2.2. 16S rDNA sequencing results

Microbial communities were analyzed at lower taxonomic levels by 16S rDNA amplicon sequencing for 10 representative digesters, including L-A-B30, L-A-B45, L-A-B60, L-B-B30_2, L-B-B55_2, L-C-B60, F-C-BP42-F, F-C-BP42-S, P-C-BP55_2 and P-C-hyBP45_2. A total of 199,298 tags were obtained and subsequently classified into 861 bacterial OTUs and 13 archaeal OTUs (OTUs with less than 5 sequences were filtered). For the bacterial community, 15 phyla, 20 classes, 21 orders and 27 families were identified (phylotypes contributing to ≥1% of total sequences in at least one sample), while 5 orders and 6 families were identified for the archaeal community.

As shown in Fig. 2a and Table S4, the orders Methanobacteriales, Methanomicrobiales, Methanosarcinales and the class Thermoplasma were detected. Methanobacteriales only comprised the family Methanobacteriaceae (mostly *Methanobacterium* and low amounts of *Methanobrevibacter* at genus level), which demonstrated high abundance in L-A-B30, L-A-B45, L-A-B60 and P-C-BP55. Methanomicrobiales comprised 2 families: Methanomicrobiaceae (mainly genus *Methanoculleus*) and Methanospirillaceae (mainly *Methanospirillum*). 16S rDNA tags from Methanospirillaceae were however much more abundant than those from Methanomicrobiaceae in most digesters except L-B-B55_2. Methanosaetaceae (genus *Methanosaeta*) and Methanosarcinaceae (genus *Methanosarcina*) illustrated significant variability: the latter was mainly detected in P-C-hyBP45_2 and P-C-BP55_2, while the former was not detected in P-C-hyBP45_2.

3.3. Bacteria community structure

At the phylum level, Proteobacteria, Firmicutes and Bacteroidetes were the most abundant phyla by contributing in total to 52.8–76.6% of bacterial tags in 10 samples (Fig. 2b). The phylum Cloacimonetes was detected in 9 samples with substantially variable proportions (0.1–19.4%). Lentisphaerae, Thermotogae, Chloroflexi, Spirochaetae, Actinobacteria, Candidate_division_OP9 and Synergistetes formed a second group (relative abundance in decrement, ≥ 4% in at least one sample) distributed in all digesters with much lower abundance (0.1–10.4%). These phyla, since shared by most digesters in this study, were considered as the core of the bacterial community. From this core microbiome, 27 bacterial families (≥1% in at least one sample) were identified (refer to Supplemental Section 7).

3.4. Correlations between identified microbial phylotypes and process parameters

3.4.1. PCA based on microbial abundance and process parameters

PCA based on composition of microbial communities showed that, bacterial (at the class level) and methanogenic community structures were firstly related to the substrates' features, leading to 3–4 sample clusters according to the type and source of feedstock (Figs. S4 and S5). However, a further investigation into the correlations between microbial phylotypes and process parameters illustrated some common rules in these diverse and complex microbial communities, which may indicate the underlying mechanisms driving the differentiation of microbial community structure regardless of substrate itself.

PCA based on process parameter datasets revealed that, the analyzed digesters were clearly differentiated by operational, environmental and efficiency parameters (Fig. 3a–b), among which solids concentration (expressed by TS and VS in the influent and effluent), NH_3, SRT, OLR and biogas (CH_4) production rate contributed the most. The digesters fed with mixed sludge formed a group with higher OLR and biogas production ('High-Rate' group), compared with the ones fed with only BSS ('Low-Rate' group). L-A-B45, L-A-B-60, L-B-B50, L-C-B60 and P-C-BP55 as characterized by higher solids concentration, SRT, ammonia and VFAs concentrations formed the 'Higher-TS' group under higher stress, while others were comprised in the 'Lower-TS' group under easier status (Fig. S6). PCA based on relative abundance of methanogen populations (Fig. 3c) and bacteria classes (Fig. 3d, Fig. S7) demonstrated similar influence of operational conditions on clustering pattern of methanogen and bacteria communities, besides the impact from type and source of substrates.

Distribution of microbial phylotypes was closely related with these parameters. Methanobacteriales, Clostridia and Alphaproteobacteria showed strong positive correlation with solids concentration, NH_3, VFAs etc., while the former two also displayed positive relationship with efficiency factors; on the contrary, Methanosaetaceae, Anaerolineae and Candidatus Cloacamonas illustrated negative correlation with these parameters; Methanosarcinaceae and OPB35_soil_group demonstrated positive correlation with OLR and biogas production rate.

3.4.2. Spearman correlations between methanogens, methane-production pathway and process parameters

A Spearman correlation analysis demonstrated that (Fig. 4), SRT and TS, VS in the substrate were strongly positively correlated with most environmental factors including NH_4^+-N, NH_3, pH, VFAs, alkalinity, TS, VS and COD_{total} inside the digesters ($P > 0.5$). It indicates that long SRT and high influent TS lead to accumulation of NH_3, VFAs and other potential inhibitors, thus increased the environmental stress on microbial communities therein. The correlations between efficiency parameters with SRT ($P \approx -0.2$) and TS-inf ($P \approx 0.1$) were however not statistically significant, suggesting that microbial communities were working at similar efficiency under higher stress compared with that under easier status. OLR, which was only controlled by flow rate in this study, was mainly in negative correlation with environmental factors (P of $-0.1 \sim -0.6$) except $COD_{soluble}$, and moderate positive correlation with efficiency parameters ($P \approx 0.5$). Consistently, higher flow rates can help to flush out accumulated intermediate metabolites and inhibitors, but may also wash out slow-growing microorganisms and affect digestion efficiency if SRT is too low (such as < 20 days in pilot-scale digesters, Table 1).

Methanobacteriales and Methanomicrobiales members, as quantified by QPCR, were both positively correlated with SRT, solids concentration and nearly all environmental factors (P of 0.4–0.8 and 0.1–0.7), but showed respectively slight positive ($P \approx 0.2$) and slight negative ($P \approx -0.1$) correlations with efficiency parameters, and no significant relationship with OLR. Methanosarcinaceae members demonstrated moderate positive (P of 0.1–0.4) correlation with solids concentration, OLR, most environmental factors, and relatively stronger correlation with efficiency parameters ($P \approx 0.4$), but no significant relationship with SRT. Nevertheless, Methanosaetaceae members showed negative correlation with all aforementioned parameters. Among all these populations, members of Methanobacteriales, Methanomicrobiales and Methanosarcinaceae demonstrated positive correlation with each other, but showed negative correlation with members of Methanosaetaceae. Consequently, Methanosarcinaceae and Methanobacteriales (mainly Methanobacteriaceae) were recognized as the key methanogen phylotypes for 'Higher-TS' operation at higher SRT and OLR with unaffected efficiency.

Regarding the isotope fractionation effect in methanogenesis,

Table 1
Process parameters of anaerobic digesters when sampled for microbiological analysis. PSS = Primary Sewage Sludge; BSS = Biological Sewage Sludge; HyBSS = Hydrolyzed Biological Sewage Sludge; TS-inf = Total Solid in the influent; VS-inf = Volatile Solid in the influent; OLR = Organic Loading Rate; SRT = Sludge Retention Time; CODt-ef = Total Chemical Oxygen Demand in the effluent; CODs-ef = Soluble Chemical Oxygen Demand in the effluent; α_c = apparent stable carbon isotope fractionation factor in methanogenesis; AM = Acetoclastic methanogenesis; HM = Hydrogenotrophic methanogenesis. Samples with the same reactor name and a different number originate from the same digester at a different period. Average values of each parameter during 21 days before the sampling event are presented.

Reactors	Volume capacity	Substrate (percentage)	Temperature	TS-inf	VS-inf	OLR	SRT	Biogas production rate	CH_4 production rate	VS reduction rate
			°C	g/L	g/L	g-VS/(L·day)	day	L/(g-VS·day)	L/(g-VS·day)	%
L-A-B30[a]	2 L	BSS	37.5	32.4	25.4	1.04	28	0.40	0.28	51
L-A-B45	2 L	BSS	37.5	55.5	43.6	1.06	45	0.41	0.27	46
L-A-B60	2 L	BSS	37.5	68.1	53.8	1.08	57	0.40	0.27	53
P-A-B30	10 L	BSS	38.3	32.5	25.6	1.21	22	0.44	0.31	51
P-A-BP47	10 L	BSS(50) and PSS(50)	38.3	47.0	37.0	1.20	31	0.42	0.33	51
L-B-B30_1	1.6 L	BSS	37.0	30.5	24.5	0.86	28	0.25	0.15	35
L-B-B30_2	1.6 L	BSS	37.0	30.7	24.4	0.86	28	0.24	0.14	46
L-B-B50_1	1.6 L	BSS	37.0	50.8	40.7	0.86	47	0.26	0.16	40
L-B-B55_2	1.6 L	BSS	37.0	54.2	43.3	0.86	50	0.25	0.15	49
L-C-B60	1.6 L	BSS	37.0	60.3	49.9	0.86	57	0.31	0.19	48
F-C-BP42-F	12,600 m^3	BSS(50) and PSS(50)	34.6	41.9	32.8	1.77	19	0.51	0.31	48
F-C-BP42-S	15,000 m^3	BSS(50) and PSS(50)	34.7	41.9	32.8	1.68	20	0.58	0.35	49
P-C-BP55_1	2.6 m^3	BSS(50) and PSS(50)	35.8	54.8	43.6	1.32	34	0.93	0.61	52
P-C-BP55_2	2.6 m^3	BSS(50) and PSS(50)	35.7	52.9	41.9	1.50	28	0.74	0.51	55
P-C-BP55_3	2.6 m^3	BSS(50) and PSS(50)	35.8	53.3	43.1	2.00	24	0.55	0.35	53
P-C-BP40_1	2.6 m^3	BSS(50) and PSS(50)	35.8	37.6	29.7	1.32	23	0.85	0.56	49
P-C-hyBP40_2	2.6 m^3	HyBSS(50) and PSS(50)	35.7	42.0	31.4	1.32	25	0.49	0.36	68
P-C-hyBP40_3	2.6 m^3	HyBSS(50) and PSS(50)	35.8	38.8	28.9	2.00	15	0.27	0.17	61
P-C-hyBP45_1	1.5 m^3	HyBSS(50) and PSS(50)	35.4	44.8	35.8	1.11	32	0.57	0.37	58
P-C-hyBP45_2	1.5 m^3	HyBSS(50) and PSS(50)	35.6	44.0	32.8	1.92	18	0.49	0.35	47
P-C-hyBP45_3	1.5 m^3	HyBSS(50) and PSS(50)	35.7	43.9	32.7	2.30	14	0.38	0.23	53

Reactors	pH	NH_4^+-N	NH_3	TS-ef	VS-ef	VFAs	CODt-ef	CODs-ef	Alkalinity	$\delta^{13}CH_4$	$\delta^{13}CO_2$	α_C	Predominating methanogenic pathway
		mg/L	mg/L	g/L	g/L	mg-acetate/L	mg/L	mg/L	mg-$CaCO_3$/L	‰	‰		
L-A-B30	7.63	1717	117	18.2	11.8	410	20,200	1600	6037	−50.73	1.50	1.055	AM + HM
L-A-B45	7.76	2697	248	28.7	19.1	673	32,360	2500	9300	−49.91	2.48	1.055	AM + HM
L-A-B60	7.86	3340	387	35.4	22.7	909	38,700	3370	11,767	−53.51	6.40	1.063	AM + HM
P-A-B30	7.49	1688	84	18.4	12.2	401	20,630	1520	6076	−51.34	2.10	1.056	AM + HM
P-A-BP47	7.52	1750	93	24.1	15.9	349	27,500	1400	6346	−50.79	4.60	1.058	AM + HM
L-B-B30_1	7.24	1161	28	20.9	14.9	523	24,480	500	4711	−51.97	3.31	1.058	AM + HM
L-B-B30_2	7.20	1626	41	20.4	14.6	499	25,100	622	4541	−50.38	3.58	1.057	AM + HM
L-B-B50_1	7.53	2166	98	32.0	22.1	828	40,170	590	7493	−65.42	19.11	1.090	HM
L-B-B55_2	7.54	2999	166	31.0	21.7	833	37,000	682	7478	−62.72	18.91	1.087	HM
L-C-B60_1	7.70	3537	284	31.4	22.3	661	36,100	770	9537	−61.75	18.03	1.085	HM
F-C-BP42-F	7.37	1582	48	25.5	17.0	316	18,388	636	5473	−49.92	4.80	1.058	AM + HM
F-C-BP42-S	7.37	1521	46	25.3	16.8	381	21,835	775	5506	−49.38	4.68	1.057	AM + HM
P-C-BP55_1	7.79	1754	176	31.9	20.5	738	36,450	2520	7793	−45.80	8.04	1.056	AM + HM
P-C-BP55_2	7.80	2403	243	31.6	20.9	667	36,500	2440	7709	−39.29	6.74	1.058	AM + HM
P-C-BP55_3	7.75	2276	210	31.5	20.3	629	33,930	2450	7251	−46.68	5.90	1.055	AM + HM
P-C-BP40_1	7.59	1329	83	22.7	14.5	291	24,180	1670	5171	−48.19	6.85	1.058	AM + HM
P-C-hyBP40_2	7.62	1476	98	25.8	15.2	234	25,800	898	5138	−43.21	7.50	1.056	AM + HM
P-C-hyBP40_3	7.42	1085	47	25.0	14.1	193	26,370	1860	3673	−47.49	5.40	1.056	AM + HM
P-C-hyBP45_1	7.67	1857	140	24.3	15.3	543	28,720	2440	7193	−52.51	9.23	1.065	HM
P-C-hyBP45_2	7.60	1637	104	25.9	16.1	331	29,980	1390	5118	−39.75	8.79	1.055	AM + HM
P-C-hyBP45_3	7.44	1177	53	25.7	15.8	308	28,580	1810	4016	−45.35	7.21	1.055	AM + HM

[a] Reactors in bold were analyzed by sequencing.

values of $\delta^{13}CH_4$ displayed negative relationship with parameters of SRT, NH_4^+-N, alkalinity and VFAs, but positive correlations with both OLR and efficiency factors. The relationship between α_c and these factors showed opposite trends. It suggested that, contribution of hydrogenotrophic methanogenesis increased under higher environmental pressure resulting from high SRT, but the digestion efficiency was slightly lower. Regarding the hydrogenotrophic methanogens, only Methanomicrobiales members demonstrated positive correlation with higher isotope fractionation effect: *P* of 0.6, 0.2, −0.1 with $\delta^{13}CO_2$, α_c and $\delta^{13}CH_4$, revealing its importance in the HM-dominated systems (like L-B-B50 and L-C-B60).

3.4.3. Spearman correlations between bacteria, methanogens and process parameters

Spearman correlation analysis between the identified bacterial families and other factors (Fig. 5a) revealed that the identified families of Clostridia mostly showed positive correlations with the tested process parameters. Among them, Thermoanaerobacteraceae and Syntrophomonadaceae members demonstrated strong positive correlations with nearly all parameters. They were highly abundant in 10 digesters and displayed higher percentage in the digesters operated at higher SRT, TS or OLR with unaffected digestion efficiency (Fig. 5b, Table S5). They were both associated to higher abundance of Methanosarcinaceae (mainly *Methanosarcina*) and Methanobacteriales (mainly *Methanobacterium*) members.

A second group was formed by Thermotogaceae, Synergistaceae and Porphyromonadaceae, members of which were shared by 10 digesters at lower abundance. They positively correlated with SRT and TS (Fig. 5a), and presented higher amounts in the high-rate digesters fed with both BSS and PSS at higher OLR (Table S5). Their richness was associated with members of Methanosarcinaceae and Methanomicrobiales (mainly

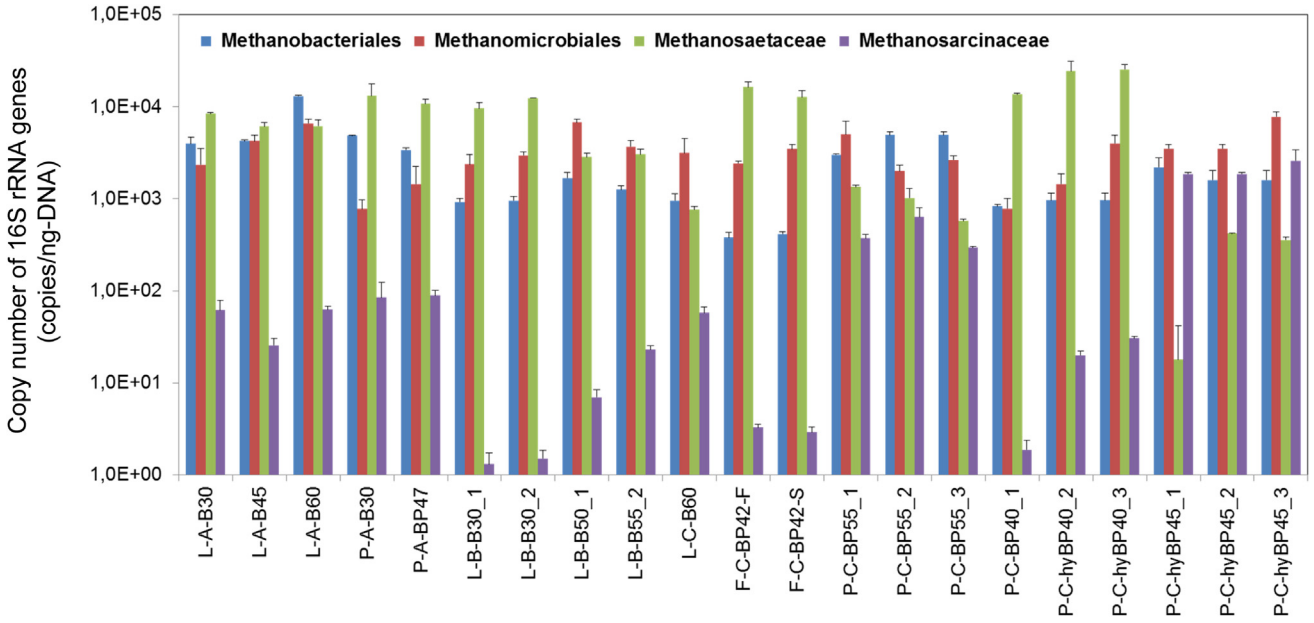

Fig. 1. Relative abundance of targeted methanogens in 21 samples detected by QPCR, expressed as the copy number of 16S rRNA genes per nanogram of DNA. Average values of duplicate analyses are presented with the standard deviations.

Methanospirillaceae), and methane-production pathway involved AM and HM. Another class-level taxon named OPB35_soil_group (belonging to the phylum Verrucomicrobia) was also primarily detected in digesters fed with BSS and PSS.

Microorganisms in these two first groups can be speculated to form the core of the robust community for 'Higher-TS', high rate digesters, in which Thermoanaerobacteraceae, Syntrophomonadaceae and *Methanosarcina*, *Methanobacterium* appear as the crucial phylotypes. Representative digesters included P-C-BP55 and P-C-hyBP45.

The third group revealed strong positive correlation with SRT and TS, but was more abundant in digesters fed with BSS at low OLR (refer to Supplemental Section 11). The representative families were Rhodobacteraceae (Class Alphaproteobacteria), Xanthomonadaceae (Class Gamaproteobacteria) and Comamonadaceae (Class Betaproteobacteria), as existed in 10 digesters at high abundance (Fig. 6c). Members of these families showed strong positive association with Methanobacteriales or Methanomicrobiales (especially *Methanoculleus*), but no significant link with Methanosarcinaceae. HM pathway and low biogas production rate were more related to this group.

These microbial phylotypes were therefore considered to be responsible for a stable but low biogas-production performance of BSS-fed digesters, which were called 'Higher-TS', low-rate digesters at unfavorable conditions (with higher levels of VFAs and ammonia). Strong isotope fractionation effect indicated inhibition of acetotrophic methanogens, while low biogas production suggested other acetate utilizing microorganisms. Representative digesters included L-A-B45, L-A-B60 and L-B-B50, L-C-B60.

The last group of families including Syntrophaceae (Class Deltaproteobacteria), Anaerolineaceae (Phylum Chloroflexi), Rikenellaceae and WCHB1-69 (Phylum Bacteroidetes) demonstrated strong negative correlation with nearly all process parameters (Fig. 6c–d), and were positively correlated with the abundance of Methanosaetaceae members. The class-level taxon Candidatus Cloacamonas had similar features, as well as the taxon vadinHA17 (Phylum Bacteroidetes) which was specific to digesters fed with BBS from A. These phylotypes formed the core of the community for easy-status digesters operated at relatively lower SRT, TS and OLR. Methane was produced from both AM and HM metabolisms by Methanosaetaceae and a small amount of Methanomicrobiales (*Methanospirillum*) or Methanobacteriales (*Methanobacterium*). Representative digesters included full-scale digesters (F-C-BP42-F, F-C-BP42-S), L-A-B30 and L-B-B30.

4. Discussion

13 anaerobic sludge digesters were operated under different conditions by setting different TS, SRT and OLR to seek for higher digester capacity. We hypothesized that different operational strategies lead to particular key microbial phylotypes due to change of the chemical environment. Archaeal and bacterial communities from these digesters were thus studied at the family level. A further correlation analysis between process parameters and relative abundance of identified phylotypes led to identification of several key populations associated to different operational conditions and reactor performances, which emphasized the influence of TS on the microcosms.

4.1. Description of identified phylotypes according to the literature

'Higher-TS', high-rate digesters were characterized by Clostridia, Methanosarcinaceae and Methanobacteriaceae. Syntrophomonadaceae and Thermoanaerobacteraceae, two abundant families of Clostridia, both demonstrated positive correlation with SRT and TS and showed high abundance in hyBSS-fed digesters.

Syntrophomonadaceae-associated bacteria were dominated by the genus *Syntrophomonas* in this study, which have been widely reported as syntrophic bacteria playing an important role in the oxidation of butyrate and long chain fatty acids (Narihiro et al., 2015; Liu et al., 2011; Sousa et al., 2009; Hatamoto et al., 2007). Members of Syntrophomonadaceae are mostly described to be anaerobically oxidizing saturated fatty acids containing 4 to 18 carbon atoms, in syntrophic association with hydrogenotrophic microorganisms such as *Methanospirillum* and *Methanobacterium*, producing acetate, propionate or other fatty acids (Sekiguchi,

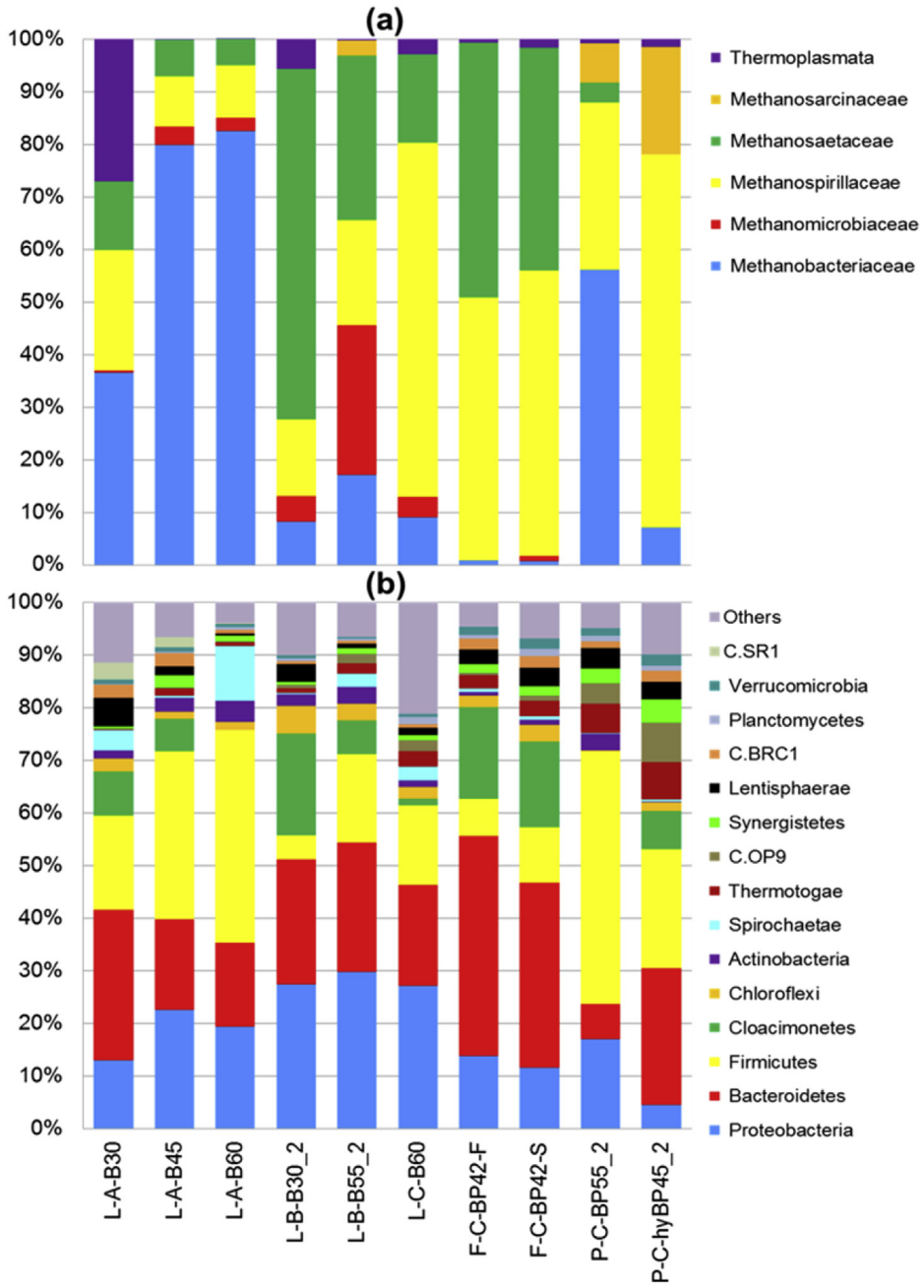

Fig. 2. The relative abundance of (a) Archaea families and (b) Bacteria phyla in 10 anaerobic digesters analyzed by 16S rDNA amplicon sequencing, expressed by percentage of archaeal and bacterial 16S rDNA tags respectively. Others in (b) include unclassified bacteria, and the phyla accounting for less than 1% of the total sequences in all samples, including Candidate_division_OP8, Fusobacteria, Acidobacteria, Deferribacteres. C.OP9 represents Candidate_division_OP9; C.BRC1 represents Candidate_division_BRC1; C.SR1 represents Candidate_division_ SR1.

2009). In a study by Narihiro et al. (2015), *Syntrophomonas* members were recognized as the functional syntrophic butyrate-oxidizing bacteria, in partnership with *Methanosarcina* members and the hydrogenotrophic *Methanoculleus* and *Methanobacterium* methanogens, derived from anaerobic digesters treating sewage sludge and swine manure. The higher abundance of *Syntrophomonas* bacteria under these conditions coupled with large amount of Methanosarcinaceae and *Methanobacterium* may indicate an enhanced butyrate metabolism.

Thermoanaerobacteraceae bacteria were dominated by the genus *Gelria* in our studied systems. *Gelria* bacteria can grow on pyruvate, lactate, glycerol, amino acids and sugars, and convert the substrates to acetate, propionate, HCO_3^-, H_2 and NH_4^+ as described by Wiegel (2009). Although it was reported to be likely to perform Syntrophic Acetate Oxidation (SAO) in rice field or paddy soil at 50 °C with hydrogenotrophic *Methanocella* (Liu and Conrad, 2010), its capability of using acetate in mesophilic anaerobic digesters is however not known (other less abundant phylotypes were described in Supplemental Section 12).

In the **'Higher-TS', low-rate digesters**, the three families Rhodobacteraceae (Alphaproteobacteria), Comamonadaceae (Betaproteobacteria) and Xanthomonadaceae (Gammaproteobacteria) were especially enriched. Rhodobacteraceae members have been described as chemoorganotrophs with strictly aerobic or

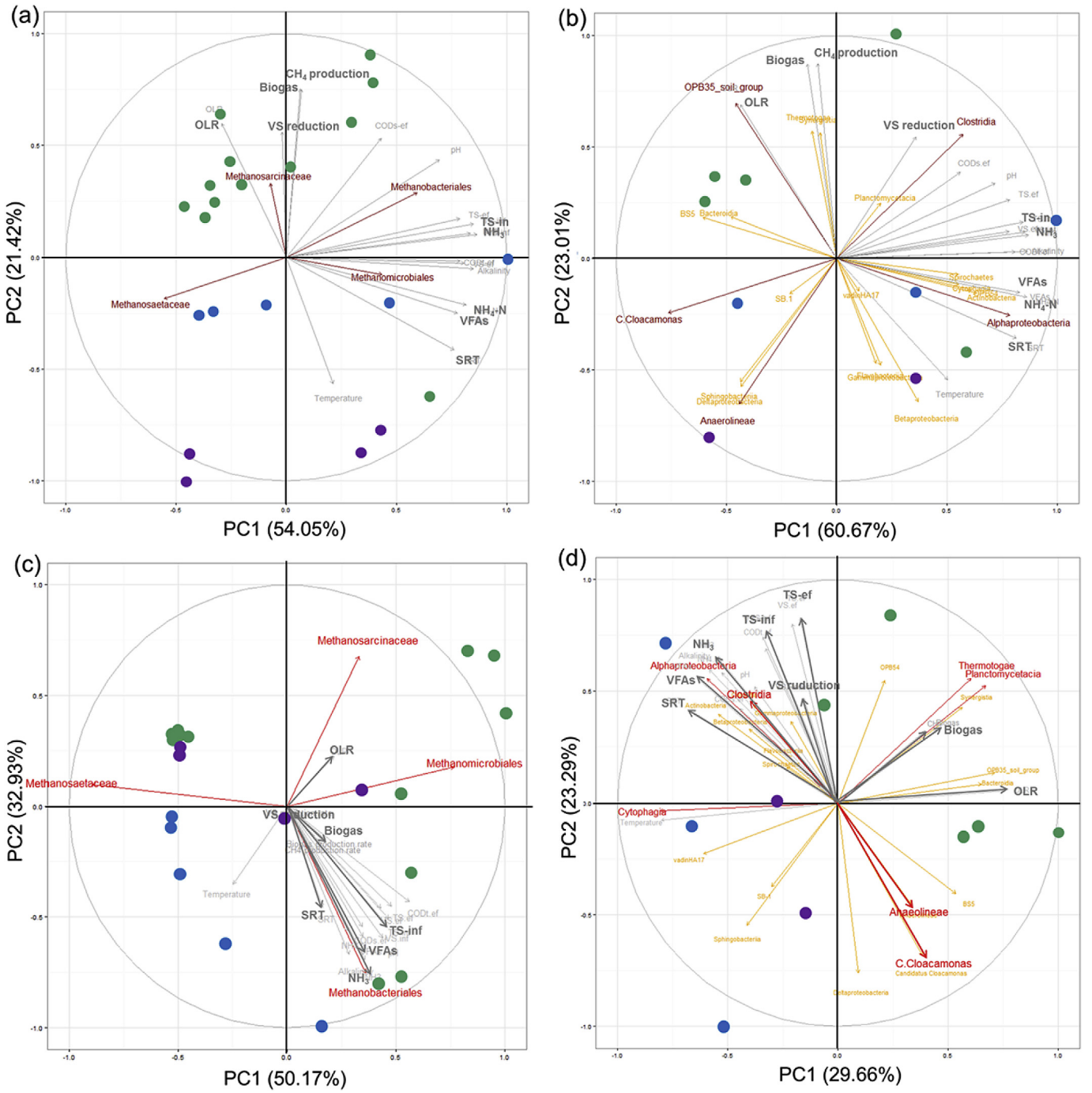

Fig. 3. Principal Component Analysis based on values of process parameters (a, b), relative abundance of methanogen populations (c) and bacteria classes (d) as active variables. 21 samples were included for (a, c) and 10 samples for (b, d). Relative abundance of methanogen populations (derived from QPCR results), bacteria classes (≥1% in at least one sample, derived from 16S rDNA sequencing results) and process parameters as shown in Table 1 were respectively used as supplemental variables in (a), (b), (c), (d). They had therefore no influence on the clustering pattern of samples. Red arrows in (b, d) represent important classes with vector length higher than 0.8, and orange arrows displayed other classes. Grey arrows represent physical-chemical parameters. Purple, blue and green circles represent digesters treating sludges from A, B and C respectively. Detailed location of each digester in PCA is shown in Fig. S8. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

facultatively anaerobic respiratory metabolism (Garrity et al., 2005). *Rhodobacter* and *Gemmobacter* were found here as two minor genera of this family. Both can perform chemotrophic growth by anaerobic/anoxic respiration of denitrification with sugars, ethanol/methanol, pyruvate, acids etc. as carbon or electron sources. Comamonadaceae members are chemoorganotrophic or facultatively chemolithotrophic bacteria with H_2 or CO oxidation. They possess a strictly respiratory type of metabolism with oxygen or nitrate as the terminal electron acceptor and are able to use a wide variety of organic acids, including acetate and amino acids for their growth (Willems and Gillis, 2005). Xanthomonadaceae was described as obligate aerobes, performing strictly respiratory metabolism with oxygen as the terminal electron acceptor (Saddler and Bradbury, 2005). A respiratory type of metabolism with

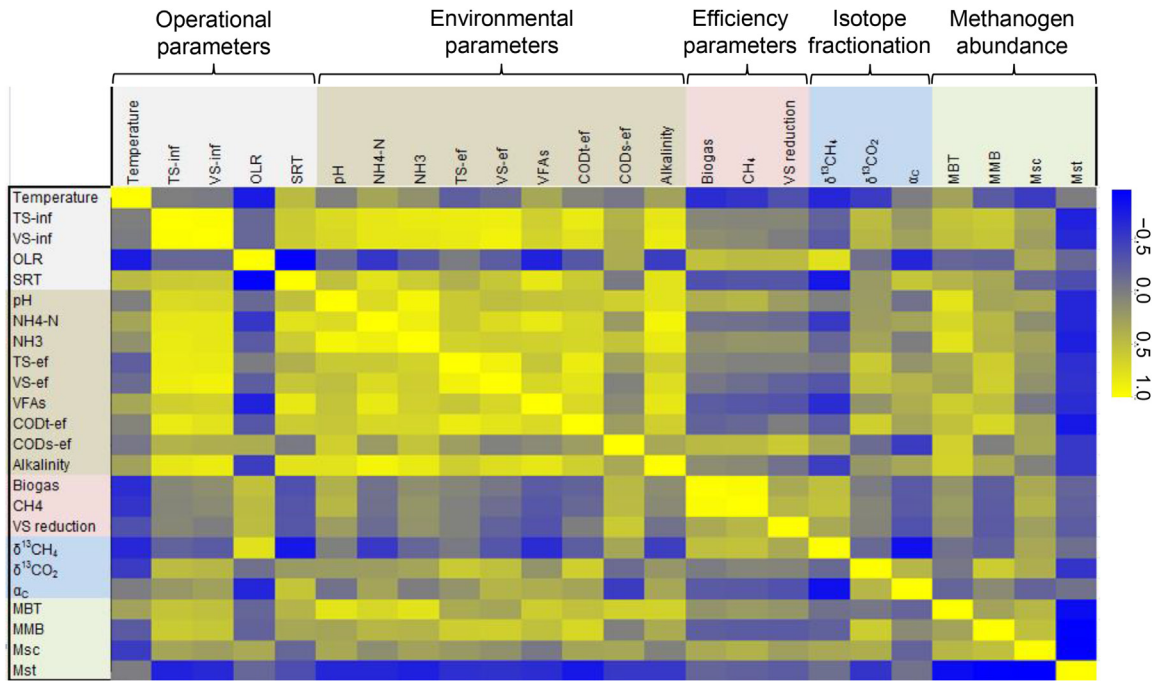

Fig. 4. Correlations between process parameters, isotope fractionation effect and relative abundance of different methanogen groups derived from QPCR results (percentage of copies were used) in 21 samples, calculated by Two-tailed Spearman's Rank Order Correlation statistic. Light yellow represents positive correlations and dark blue represents negative correlations. MBT = Methanobacteriales; MMB = Methanomicrobiales; Msc = Methanosarcinaceae; Mst = Methanosaetaceae. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

electron acceptors (such as nitrate or sulphate) could be suspected in these BSS-fed digesters.

Actually, among all the phylotypes identified in our study, families Comamonadaceae (including *Comamonas* genus) and Rhodocyclaceae (including *Dechloromonas* genus) of the class Betaproteobacteria, and family Rhodobacteraceae (including *Rhodobacter* genus) of the class Alphaproteobacteria, are known as denitrifiers using acetate as carbon source in wastewater treatment systems, while the methanol-utilizing denitrifiers were affiliated to the family Methylophilaceae (Osaka et al., 2006) which was also detected here. All these phylotypes were observed primarily in L-B-B50_2, L-C-B60, followed by L-A-B45, L-A-B60, and P-C-BP55_2; they were also detected but at lower abundance in 'Lower-TS' digesters and the HyBSS-fed digester (P-C-hyBP45_2). Their facultative or aerobic characteristics suggest that, they could be introduced together with the substrate, but could survive in the digesters *via* chemotrophic growth and compete for organic carbon. Such a phenomenon could hypothetically contribute to explain the low biogas production rate of certain BSS-fed digesters under similar VS reduction levels, especially the L-B-B30, L-B-B50, L-C-B60. This however has to be further investigated to confirm the *in situ* function and activity of these populations in AD systems, as their entering and accumulation just depending on the substrate can still not be excluded (other phylotypes were described in Supplemental Section 13).

Regarding **methanogens**, dominance of hydrogenotrophs like Methanobacteriales (*Methanobacterium*) or Methanomicrobiales (*Methanospirillum* and *Methanoculleus*), and lack of acetotrophic methanogens (Methanosaetaceae and Methanosarcinaceae) were the main trends for 'Higher-TS', low-rate digesters, with HM pathway predominating for methanogenesis. Inhibition by accumulated ammonia, VFAs and other potential inhibitors at high SRT and TS was previously described to lead to differentiation of methanogen populations, since they display different resistance levels: Methanosaetaceae is the most vulnerable, Methanosarcinaceae is moderately tolerant, while hydrogenotrophs are the most resistant (Lü et al., 2013; De Vrieze et al., 2012; Karakashev et al., 2006). With higher environmental pressure, hydrogenotrophic methanogens and Methanosarcinaceae all demonstrated positive correlation, however, the amount of Methanosarcinaceae was much lower in these low biogas producing digesters compared to those with high biogas production rate. It can therefore be speculated that, Methanosarcinaceae is closely associated with high biogas production rate under higher environmental stress, as previously indicated by Regueiro et al. (2015). On the other hand, the abundance of Methanobacteriales in high-rate digesters was higher than that of Methanomicrobiales, which was also reported by De Vrieze et al. (2015).

As methane was mainly produced from H_2/CO_2 in such 'Higher-TS', low-rate digesters, SAO is indicated as the metabolism for transformation of acetate to H_2/CO_2. Surprisingly, the well-known bacteria performing SAO were only detected at quite low abundance (Table S6), indicating other acetate-oxidizing or -consuming bacteria which carved up organic carbon therein (refer to Supplemental Section 15).

Digesters at 'Lower-TS' and easy status harbored more Syntrophaceae and Syntrophobacteraceae (of the class Deltaproteobacteria), Anaerolineaceae (phylum Chloroflexi), Rikenellaceae and WCHB01-69 (phylum Bacteroidetes), Candidatus Cloacamonas and high abundance of Methanosaetaceae.

Deltaproteobacteria, hosting several propionate-oxidizing populations (Ariesyady et al., 2007), displayed a negative correlation with SRT and TS; it was dominated by the family Syntrophaceae and the less abundant Syntrophobacteraceae in our studied systems. Both families grow in syntrophic association with H_2- and formate-utilizing microorganisms and some members can use sulfate as an electron acceptor (Kuever et al., 2005). Syntrophaceae mainly comprised by unidentified members, the genera *Syntrophus*,

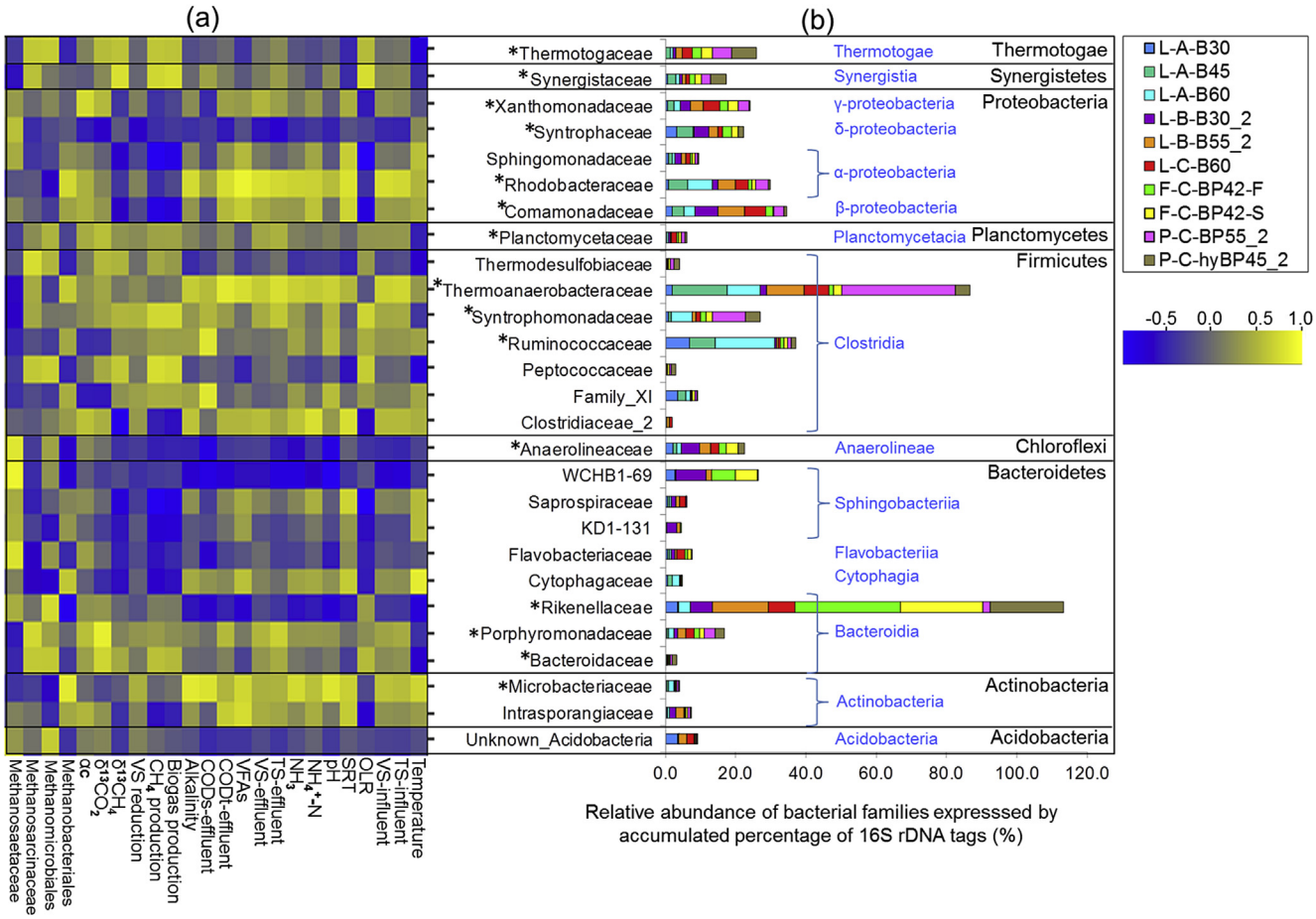

Fig. 5. (a) Correlations between process parameters, isotope fractionation effects and relative abundance of different bacterial families based on 16S rDNA sequencing analysis (percentage values were used) and (b) relative abundance of bacterial families in 10 anaerobic digesters, expressed as percentage in total 16S rDNA tags. Two-tailed Spearman's Rank Order Correlation statistic was used. Light yellow represents positive correlations and dark blue represents negative correlations. Family names with stars indicate the families shared by all 10 digesters. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Smithella and *Syntrophobacter*. *Syntrophus* has been suggested as functional syntrophic benzoate oxidizing bacteria which was coupled with *Methanoculleus* as H_2 consumer and *Methanosaeta* as acetate utilizer in anaerobic digestion (Narihiro et al., 2015). *Smithella* and *Syntrophobacter* of Syntrophobacteraceae family were described as syntrophic propionate-oxidizing bacteria (Kuever et al., 2005; Narihiro et al., 2015; Ariesyady et al., 2007) in partnership with H_2/formate utilizers like *Methanospirillum* or *Methanoculleus*, together with acetate consumers as *Methanosarcina* or *Methanosaeta* (other phylotypes were described in Supplemental Section 14). As several members of these Deltaproteobacteria bacteria could be involved in propionate metabolism (synthesis or degradation), propionate pathway inhibition could occur under higher environmental pressure. That could be due to the high sensitivity of syntrophic propionate oxidation process: it can be easily inhibited by increases in hydrogen, formate or acetate concentrations (Schmidt and Ahring, 1993), which is the case at increased SRT and TS. Regarding the methanogens, higher ammonia and VFAs could inhibit *Methanosaeta*, but promote the growth of *Methanosarcina*.

It can be seen from this study, the fermentative bacteria, acidogens, acetogens and methanogens involved in anaerobic sludge digestion can all be influenced by the operational conditions, especially by TS of the substrate and the SRT. A core of microbiome formed by Clostridia (with Syntrophomonadaceae and Thermoanaerobacteraceae as two most abundant families), Methanosarcinaceae and Methanobacteriaceae, as well as Thermotogaceae and Synergistaceae can better adapt to 'Higher-TS' and high capacity operation with increased environmental pressure but unaffected efficiency, which can represent a robust microbial community and should thus be emphasized. High abundances of Alpha-, Beta- and Gamma-proteobacteria seem to be related with low biogas production, as well as some novel bacterial phylotypes indicated to perform SAO in 'Higher-TS' digesters. Their in-situ function and activity should however be further studied to reveal the influence on biogas production.

Our observations were to some extent consistent with previous studies which targeted higer taxonomic levels in various anaerobic digesters, for instance, the positive correlations between ammonia with Clostridiales (De Vrieze et al., 2015), biogas yield with Firmicutes (Abendroth et al., 2015), OLR and TS with Methanosarcinaceae (De Vrieze et al., 2012); and the negative correlations between biogas yield with Proteobacteria (Abendroth et al., 2015), ammonia, VFAs with Methanosaetaceae (Karakashev et al., 2005, 2006). It thus tend to demonstrate the universility of these common rules for the influence of operational conditions on microcosms, regardless of the sources of inocula and substrates. Description of identified microbial phylotypes at family and genus levels in this study better correlated their ecophysiology with the change of environmental factors, indicating that an 'organic increase' solution to seek for higher digester capacity by increasing TS of substrate may face up to higher concentration of ammonia and

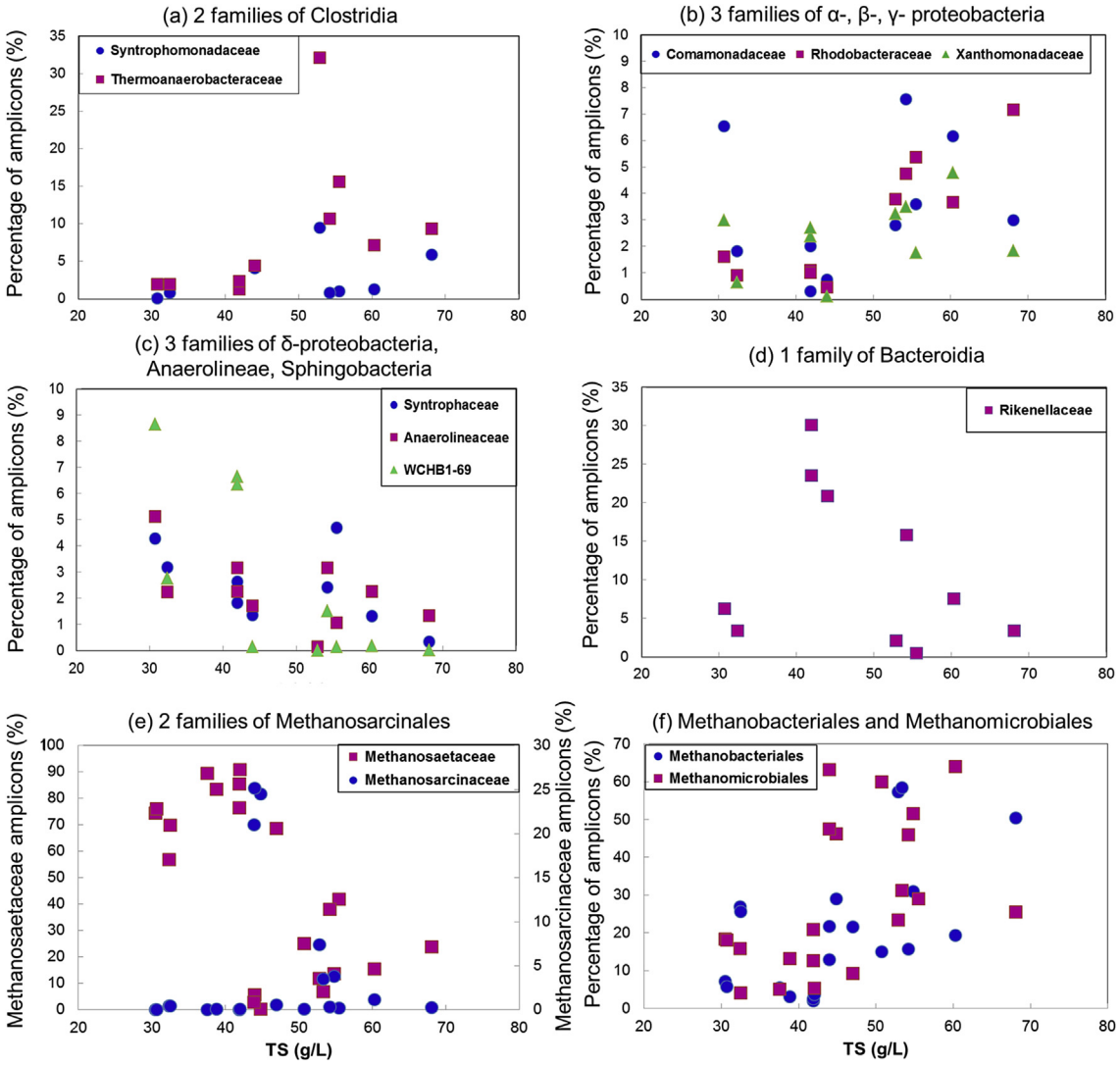

Fig. 6. Dot plot of relative abundance of bacterial families (derived from 16S rDNA sequencing datasets) versus total solids concentration (TS) of feeding sludge (a–d), and relative abundance of methanogen populations (derived from QPCR datasets) versus TS of feeding sludge (e–f). Relative abundance was expressed by percentage of phylotype-relevant amplicons in total number of bacteria or methanogen amplicons. Only representative bacterial families that were abundant and demonstrated significant change with TS are illustrated. Titles indicate the class-, order- or family-level nomenclature of these phylotypes.

VFAs accumulated in the system, which further lead to establishment of a more robust microbial community. A compromise between inlet TS and flow rate should be further studied to facilitate adaptation of microorganisms to higher digester capacity.

5. Conclusions

Results demonstrated that, different operational strategies and the subsequently changed environmental factors (NH_3, VFAs) significantly influenced the microbial communities, with the role of substrate TS emphasized. Marker phylotypes and metabolisms were identified for digesters operated at different status:

1. Clostridia (Thermoanaerobacteraceae and Syntrophomonadaceae as the core families) together with Methanosarcinaceae and Methanobacteriales are identified as key phylotypes of the microbiome working under TS > 44 g/L but with high methane production rate (>0.25 L/(g-VS·day));
2. Microbiome in 'Higher-TS' digesters with low methane production rate (< 0.25 L/(g-VS·day)) were characterized by higher abundance of Rhodobacteraceae, Comamonadaceae, Xanthomonadaceae bacteria, the prevailing Methanomicrobiales methanogens and predominance of hydrogenotrophic methanogenesis; Syntrophic acetate oxidation was also observed which may result from the higher NH_3 concentration under this condition;
3. In digesters with TS $\leq$ 44 g/L at relatively easy status, Deltaproteobacteria, Anaerolineaceae, Rikenellaceae, Candidatus Cloacamonas and Methanosaetaceae were much more abundant;

These influences of TS and NH_3 on the microbiome suggested the importance of a compromise between inlet TS and flow rate for stable digester performance when seeking for higher capacity.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2016.06.014.

References

Abendroth, C., Vilanova, C., Günther, T., Luschnig, O., Porcar, M., 2015. Eubacteria and archaea communities in seven mesophile anaerobic digester plants in Germany. Biotechnol. Biofuels 8 (1), 87.

Ariesyady, H.D., Ito, T., Okabe, S., 2007a. Functional bacterial and archaeal community structures of major trophic groups in a full-scale anaerobic sludge digester. Water Res. 41 (7), 1554–1568.

Ariesyady, H.D., Ito, T., Yoshiguchi, K., Okabe, S., 2007b. Phylogenetic and functional diversity of propionate-oxidizing bacteria in an anaerobic digester sludge. Appl. Microbiol. Biotechnol. 75 (3), 673–683.

Baker, G.C., Smith, J.J., Cowan, D.A., 2003. Review and re-analysis of domain-specific 16S primers. J. Microbiol. Methods 55, 541–555.

Carrère, H., Dumas, C., Battimelli, A., Batstone, D.J., Delgenès, J.P., Steyer, J.P., Ferrer, I., 2010. Pretreatment methods to improve sludge anaerobic degradability: a review. J. Hazard. Mater. 183 (1–3), 1–15.

Chen, Y., Cheng, J.J., Creamer, K.S., 2008. Inhibition of anaerobic digestion process: a review. Bioresour. Technol. 99 (10), 4044–4064.

Couras, C.S., Louros, V.L., Gameiro, T., Alves, N., Silva, A., Capela, M.I., Arroja, L.M., 2014. Effects of operational shocks on key microbial populations for biogas production in UASB (Upflow Anaerobic Sludge Blanket) reactors. Energy 73, 866–874.

Gagliano, M.C., Braguglia, C.M., Petruccioli, M., Rossetti, S., 2015. Thermophilic anaerobic digestion of thermal pretreated sludge: role of microbial community structure and correlation with process performances. Water Res. 68, 498–509.

Garrity, G.M., Belland, J.A., Lillburn, T., 2005. Family I. Rhodobacteraceae fam. nov. In: Brenner, Krieg, Staley, Garrity (Eds.), Bergey's Manual of Systematic Bacteriology, The Proteobacteria, Part C, The Alpha-, Beta-, Delta-, and Epsilonproteobacteria, second ed., vol. 2. Springer, New York. 161–268.

Hatamoto, M., Imachi, H., Fukayo, S., Ohashi, A., Harada, H., 2007. Syntrophomonas palmitatica sp. nov., an anaerobic, syntrophic, long-chain fatty-acid-oxidizing bacterium isolated from methanogenic sludge. Int. J. Syst. Evol. Microbiol. 57 (9), 2137–2142.

Karakashev, D., Batstone, D.J., Trably, E., Angelidaki, I., 2006. Acetate oxidation is the dominant methanogenic pathway from acetate in the absence of Methanosaetaceae. Appl. Environ. Microbiol. 72 (7), 5138–5141.

Karakashev, D., Batstone, D.J., Angelidaki, I., 2005. Influence of environmental conditions on methanogenic compositions in anaerobic biogas reactors. Appl. Environ. Microbiol. 71 (1), 331–338.

Kuever, J., Rainey, F.A., Widdel, F., 2005. Order VI. Syntrophobacterales ord. nov. In: Brenner, Krieg, Staley, Garrity (Eds.), Bergey's Manual of Systematic Bacteriology, The Proteobacteria, Part C, The Alpha-, Beta-, Delta-, and Epsilonproteobacteria, second ed., vol. 2. Springer, New York, pp. 1021–1039.

Lerm, S., Kleyböcker, A., Miethling-Graff, R., Alawi, M., Kasina, M., Liebrich, M., Würdemann, H., 2012. Archaeal community composition affects the function of anaerobic co-digesters in response to organic overload. Waste Manag. 32 (3), 389–399.

Liu, F., Conrad, R., 2010. Thermoanaerobacteriaceae oxidize acetate in methanogenic rice field soil at 50°C. Environ. Microbiol. 12 (8), 2341–2354.

Liu, P., Qiu, Q., Lu, Y., 2011. Syntrophomonadaceae-affiliated species as active butyrate-utilizing syntrophs in paddy field soil. Appl. Environ. Microbiol. 77 (11), 3884–3887.

Lü, F., Hao, L., Guan, D., Qi, Y., Shao, L., He, P., 2013. Synergetic stress of acids and ammonium on the shift in the methanogenic pathways during thermophilic anaerobic digestion of organics. Water Res. 47 (7), 2297–2306.

Mcleod, J., Othman, M., Beale, D., Joshi, D., 2015. The use of laboratory scale reactors to predict sensitivity to changes in operating conditions for full-scale anaerobic digestion treating municipal sewage sludge. Bioresour. Technol. 189, 384–390.

Narihiro, T., Nobu, M.K., Kim, N.K., Kamagata, Y., Liu, W.T., 2015. The nexus of syntrophy-associated microbiota in anaerobic digestion revealed by long-term enrichment and community survey. Environ. Microbiol. 17 (5), 1707–1720.

Narihiro, T., Sekiguchi, Y., 2007. Microbial communities in anaerobic digestion processes for waste and wastewater treatment: a microbiological update. Curr. Opin. Biotechnol. 18 (3), 273–278.

Osaka, T., Yoshie, S., Tsuneda, S., Hirata, A., Iwami, N., Inamori, Y., 2006. Identification of acetate- or methanol-assimilating bacteria under nitrate-reducing conditions by stable-isotope probing. Microb. Ecol. 52 (2), 253–266.

Qu, X., Mazéas, L., Vavilin, V.A., Epissard, J., Lemunier, M., Mouchel, J.M., He, P.J., Bouchez, T., 2009. Combined monitoring of changes in delta13CH4 and archaeal community structure during mesophilic methanization of municipal solid waste. FEMS Microbiol. Ecol. 68 (2), 236–245.

Regueiro, L., Lema, J.M., Carballa, M., 2015. Key microbial communities steering the functioning of anaerobic digesters during hydraulic and organic overloading shocks. Bioresour. Technol. 197, 208–216.

Rivière, D., Desvignes, V., Pelletier, E., Chaussonnerie, S., Guermazi, S., Weissenbach, J., Li, T., Camacho, P., Sghir, A., 2009. Towards the definition of a core of microorganisms involved in anaerobic digestion of sludge. Int. J. Syst. Evol. Microbiol. 3 (6), 700–714.

Saddler, G.S., Bradbury, J.F., 2005. Family I. Xanthomonadaceae fam. nov. In: Brenner, Krieg, Staley, Garrity (Eds.), Bergey's Manual of Systematic Bacteriology, The Proteobacteria, Part B, The Gammaproteobacteria, second ed., vol. 2. Springer, New York, pp. 63–119.

Schmidt, J.E., Ahring, B.K., 1993. Effects of hydrogen and formate on the degradation of propionate and butyrate in thermophilic granules from an upflow anaerobic sludge blanket reactor. Appl. Environ. Microbiol. 59 (8), 2546–2551.

Sekiguchi, Y., 2009. Family IX. Syntrophomonadaceae Zhao, Yang, Woese and Bryant 1993a, 284VP. In: De Vos, P., Garrity, G., Jones, D., Krieg, N.R., Ludwig, W., Rainey, F.A., Schleifer, K.H., Whitman, W.B. (Eds.), Bergey's Manual of Systematic Bacteriology, The Firmicutes, second ed., vol. 3. Springer, New York, pp. 1044–1055.

Sousa, D.Z., Smidt, H., Alves, M.M., Stams, A.J.M., 2009. Ecophysiology of syntrophic communities that degrade saturated and unsaturated long-chain fatty acids. FEMS Microbiol. Ecol. 68 (3), 257–272.

Sundberg, C., Al-Soud, W.A., Larsson, M., Alm, E., Yekta, S.S., Svensson, B.H., Sørensen, S.J., Karlsson, A., 2013. 454 Pyrosequencing analyses of bacterial and archaeal richness in 21 full-scale biogas digesters. FEMS Microbiol. Ecol. 85 (3), 612–626.

Vanwonterghem, I., Jensen, P.D., Ho, D.P., Batstone, D.J., Tyson, G.W., 2014. Linking microbial community structure, interactions and function in anaerobic digesters using new molecular techniques. Curr. Opin. Biotechnol. 27, 55–64.

De Vrieze, J., Saunders, A.M., He, Y., Fang, J., Nielsen, P.H., Verstraete, W., Boon, N., 2015. Ammonia and temperature determine potential clustering in the anaerobic digestion microbiome. Water Res. 75 (0), 312–323.

De Vrieze, J., Hennebel, T., Boon, N., Verstraete, W., 2012. Methanosarcina: the rediscovered methanogen for heavy duty biomethanation. Bioresour. Technol. 112, 1–9.

Wiegel, J., 2009. Family I. Thermoanaerobacteraceae fam. nov. In: De Vos, P., Garrity, G., Jones, D., Krieg, N.R., Ludwig, W., Rainey, F.A., Schleifer, K.H., Whitman, W.B. (Eds.), Bergey's Manual of Systematic Bacteriology, The Firmicutes, second ed., vol. 3. Springer, New York, pp. 1225–1256.

Willems, A., Gillis, M., 2005. Family IV. Comamonadaceae Willems, De Ley, Gillis and Kersters 1991a, 447VP. In: Brenner, Krieg, Staley, Garrity (Eds.), Bergey's Manual of Systematic Bacteriology, The Proteobacteria, Part C, The Alpha-, Beta-, Delta-, and Epsilonproteobacteria, second ed., vol. 2. Springer, New York, pp. 686–759.

Wilkins, D., Rao, S., Lu, X., Lee, P.K.H., 2015. Effects of sludge inoculum and organic feedstock on active microbial communities and methane yield during anaerobic digestion. Front. Microbiol. 6, 1114–1124.

Yu, Y., Lee, C., Kim, J., Hwangs, S., 2005. Group-specific primer and probe sets to detect methanogenic communities using quantitative real-time polymerase chain reaction. Biotechnol. Bioeng. 89 (6), 670–679.