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Quantitative analysis of methanogenic community dynamics in three anaerobic batch digesters treating different wastewaters

Changsoo Lee[a], Jaai Kim[a], Kwanghyun Hwang[b], Vincent O'Flaherty[a], Seokhwan Hwang[b,*]

[a]Microbial Ecology Laboratory, Department of Microbiology and Environmental Change Institute (ECI), National University of Ireland, Galway, Republic of Ireland

[b]School of Environmental Science and Engineering, POSTECH, San 31, Hyoja-dong, Nam-gu, Pohang, Gyungbuk 790-784, Republic of Korea

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ABSTRACT

Quantitative changes in methanogenic community structures, associated with performance data, were investigated in three anaerobic batch digesters treating synthetic glucose medium, whey permeate, and liquefied sewage sludge. All digesters were initially seeded with anaerobic sludge obtained from a local municipal wastewater treatment plant. Dynamics of methanogenic populations were monitored, at order and family levels, using real-time PCR based on the 16S rRNA gene. The molecular monitoring revealed that, in each digester, the quantitative structure of methanogenic community varied continuously over treatment time and the variation corresponded well to the changes in chemical profiles. Biphasic production of methane, associated with successive increases in aceticlastic (mainly *Methanosarcinaceae*) and hydrogenotrophic (mainly *Methanomicrobiales*) methanogenic groups, was observed in each digester. This corresponded to the diauxic utilization of acetate and longer-chain volatile fatty acids (C_3–C_6), mainly propionate. Additionally, the non-metric multidimensional scaling (NMDS) analysis of the quantification results demonstrated that the community shift patterns in three digesters were totally different from each other. Considering that the operating conditions in all trials were identical except substrates, the differences in quantitative shift profiles were suggested to be due to the different substrate compositions. This implied that the composition of wastewater could affect the evolution of quantitative methanogenic community structure in an anaerobic process. Overall, our results suggested that more attention to quantitative as well as qualitative approaches on microbial communities is needed for fundamental understanding of anaerobic processes, particularly under dynamic or transitional conditions.

1. Introduction

Due to the production of methane, a readily usable energy source, and a low generation of sludge, anaerobic digestion has been widely used for treating organic waste (water) (Speece, 1996; Hori et al., 2006). Anaerobic digestion is a series of reactions performed by dozens of interacting microbial populations which can be broadly divided into two groups: acidogens and methanogens. The former is the bacterial group which hydrolyzes and ferments complex organic

* *Corresponding author*. Tel.: +82 54 279 2282; fax: +82 54 279 8299.
E-mail address: shwang@postech.ac.kr (S. Hwang).
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compounds to form hydrogen and organic acids, and the latter is the archaeal group which produces methane using the intermediates. Accordingly, a variety of microorganisms coexist in anaerobic digesters even when a single substrate is utilized (Fernandez et al., 2000), and their concerted activity is necessary for the complete bioconversion of organic materials to methane. Therefore, an understanding of the microbial community structure and dynamics in anaerobic processes is a basic requirement for fundamental improvement of anaerobic digestion technology. Nonetheless, due to the limited knowledge of the microbial ecosystem involved, field-scale anaerobic digesters have been empirically set up and operated to avoid failure, resulting in over-dimensioned digester volumes (Fernandez et al., 2000; Hori et al., 2006).

Application of culture-independent molecular techniques, particularly based on 16S rRNA gene sequences, has provided valuable tools to get insight into microbial communities without cultivation. This enabled us to link microbial community structure and dynamics to process performance. Many molecular approaches have been previously employed to examine the behavior of microbial populations in various anaerobic digestion ecosystems. However, most of them have been carried out using qualitative or semi-quantitative techniques, such as clone library, molecular finger-printing, and nucleic acid hybridization (Zumstein et al., 2000; Ueno et al., 2001; Angenent et al., 2002; Calli et al., 2005), and thus only limited information on the quantitative population dynamics has been available. On the other hand, it is desirable to pay more attention to quantitative as well as qualitative approaches as functional attributes of anaerobic digesters may be related to the relative abundance of microbial populations as well as the composition of the community (Akarsubasi et al., 2005).

Previous studies have mostly focused on the behavior of methanogens because they play a direct role in reducing pollution load and producing biogas in the terminal rate-limiting step, methanogenesis (Yu et al., 2005). Methanogens are also of great interest to researchers also due to their unique physiological characteristics (e.g., low growth rate, high susceptibility to external conditions, and limited substrate utilization range), which make the whole process sensitive to environmental changes (Hori et al., 2006). Although a few studies have recently reported the absolute quantification of methanogens using real-time PCR approaches (Hori et al., 2006; Yu et al., 2006), the majority were performed at steady-state and thus very little information is yet available on how methanogenic communities quantitatively evolve in dynamic or transitional states such as start-up. This community transition is very important, particularly during a start-up period because a successful start-up is critical for long-term stable and efficient digester operation (Leclerc et al., 2001; Show et al., 2004).

In this study, we aimed to quantitatively investigate methanogenic community dynamics in relation to changes in process performance data throughout batch anaerobic digestion. For the purpose, three anaerobic batch digesters treating different wastewaters (i.e., synthetic glucose medium, whey permeate, and liquefied sewage sludge) were operated for 40–60 days. It has been suggested in several steady-state studies that the methanogenic community structure remains stable over time, in terms of population diversity, while the bacterial community is highly variable (Zumstein et al., 2000; Akarsubasi et al., 2005). On the other hand, little has been focused on the quantitative and qualitative shifts in methanogenic community structures in a dynamic state. Temporal changes in methanogenic community composition in each digester were examined using real-time PCR at order or family taxonomic levels, which cover most methanogens relevant to anaerobic digesters. Quantitative community shifts were analyzed employing non-metric multidimensional scaling (NMDS), a multivariate ordination technique, based on the real-time PCR data. This study provides a quantitative insight into the behavior of methanogenic community in anaerobic digestion environments.

2. Materials and methods

2.1. *Bioreactor operation*

Three batch CMTRs (completely mixed tank reactors), denoted as G-, W-, and S-digesters, were anaerobically operated with three different wastewaters, i.e., synthetic glucose medium, whey permeate, and liquefied sewage sludge. Glucose is one of the major energy sources for microbial growth and has been widely used to feed various bioprocesses, including anaerobic digesters, in microbial studies. The glucose medium contained (in mg/L): glucose, 4868; yeast extract, 50; NH_4Cl, 955.5; KH_2PO_4, 63.6; $KH_2PO_4 \cdot 3H_2O$, 123; NaCl, 600; KCl, 185; $MgSO_4 \cdot 7H_2O$, 123.6; NTA, 40; $CaCl_2 \cdot 2H_2O$, 20; $FeCl_3 \cdot 6H_2O$, 0.1; $MnCl_2 \cdot 4H_2O$, 0.9; H_3BO_3, 0.2; $CoCl_2 \cdot 6H_2O$, 1.5; $CuCl_2 \cdot 2H_2O$, 2.2; $NiCl_2 \cdot 6H_2O$, 1.2; 45% Na_2SeO_3, 0.6; $ZnCl_2$, 0.9; citric acid$\cdot H_2O$, 105. The whey permeate wastewater was prepared by dissolving whey permeate powder obtained from Gossner's cheese plant (Logan, UT, USA) in distilled water. Whey permeate, a representative dairy wastewater, is a high organic by-product from the cheese manufacturing process and has been frequently treated in anaerobic systems (Hwang and Hansen, 1992; Kim et al., 2008). No supplementary nutrients were added because whey permeate contains most of the essential nutrients for microbial growth (Hwang and Hansen, 1992; Rajeshwari et al., 2000). While the handling of sewage sludge is one of the most problematical issues in wastewater management (Lundin et al., 2004), anaerobic digestion is now considered as the most common method for sludge stabilization (Baertsch et al., 2007). In this study, to minimize the hydrolysis period of sludge digestion, raw sewage sludge was liquefied by the thermochemical pretreatment with 4 g/L of NaOH at 121 °C for 30 min (Kim et al., 2003) and then filtered through Whatman GF/C filters (1.2 μm pore size). For each reactor, substrate concentration was adjusted to 5 g/L as soluble chemical oxygen demand (SCOD). Physical and chemical characteristics of each substrate are shown in Table 1. The contributions of carbohydrate and protein to substrate SCOD were 99.2% and 0% in G-digester (carbohydrate only), 79.5% and 11.1% in W-digester (carbohydrate rich), and 20.1% and 68.0% in S-digester (protein rich), indicating distinct differences in the wastewater characteristics. The major nutritional compounds composing the substrates were also different. Glucose was the only carbon source in the

Table 1 – Physical and chemical characteristics of the wastewaters studied.

Parameters	Concentrations (mg/L)		
	Synthetic glucose medium	Whey permeate wastewater	Liquefied sewage sludge
Total COD	5038 (28)[a]	5406 (128)	5869 (217)
Soluble COD	4959 (42)	5044 (76)	5021 (27)
Total carbohydrate	4701 (39)	3733 (78)	980 (17)
Soluble carbohydrate	4686 (101)	3757 (27)	945 (24)
Total protein	ND[b]	544 (22)	2169 (10)
Soluble protein	ND	374 (17)	2275 (83)
Total suspended solids	100 (5)	163 (18)	138 (18)
Volatile suspended solids	140 (23)	125 (35)	62 (18)

a Standard deviations are in parentheses.
b ND, not detected.

synthetic glucose medium and whey permeate is mostly composed of lactose and milk protein (Tejayadi and Cheryan, 1995), whereas sewage sludge probably contains much more diverse and complex carbon sources. Each digester of 7 L capacity was inoculated with anaerobic seed sludge with a seeding ratio of 1% (v/v) and the working volume was 6 L. Temperature was held at 35 °C and pH was kept over 7.0 with 3 N NaOH. The sewage- and anaerobic-sludge used were obtained from a local municipal wastewater treatment plant (Pohang, Korea).

2.2. DNA extraction

One milliliter of the sample was centrifuged at 16,000 *g* for 5 min and the supernatant was decanted. The pellet was washed with 1 mL of deionized and distilled water (DDW) and centrifuged again in the same manner to ensure a maximal removal of residual medium. The supernatant was carefully removed, and the pellet was resuspended in 100 μL of DDW. Total DNA in the suspension was immediately extracted using an automated nucleic acid extractor (Magtration System 6GC, PSS, Chiba, Japan). Purified DNA was eluted with 100 μL of Tris–HCl buffer (pH 8.0) and stored at −20 °C for further analyses. DNA extraction was performed in duplicate.

2.3. Real-time PCR analysis

Real-time PCR was performed using a LightCycler 1.2 (Roche, Mannheim, Germany) with five primer and probe sets (Table 2) which cover most methanogens in anaerobic digesters (Yu et al., 2005). Methanogens are classified into five orders within the domain *Archaea* which can be grouped into two guilds, aceticlastic and hydrogenotrophic methanogens. The former includes only *Methanosarcinales* which comprises two families, *Methanosaetaceae* utilizing only acetate and *Methanosarcinaceae* utilizing acetate as well as various methyl compounds and hydrogen (Boone and Castenholz, 2001). The latter comprises the remaining four orders, i.e., *Methanobacteriales*, *Methanococcales*, *Methanomicrobiales*, and *Methanopyrales*, which utilize only $H_2 + CO_2$ or formate to produce methane (Boone and Castenholz, 2001). Because the *Methanopyrales* members are not likely to be present in anaerobic processes due to their extremely high growth temperature (>80 °C) (Boone and Castenholz, 2001), the order was left out of consideration in this study. Additionally, aceticlastic methanogens were monitored at family level, instead of order level, because of their crucial role in overall methanation (i.e., >70% of methane is originated from acetate in most anaerobic systems) and the significant physiological differences between the two aceticlastic families (Hori et al., 2006; Yu et al., 2006). Consequently, five target methanogenic groups, i.e., three hydrogenotrophic orders and two aceticlastic families, were monitored using real-time PCR with the corresponding primer and probe sets listed in Table 2. Each reaction mixture was made using the LightCycler FastStart DNA Master Hybridization Probes kit (Roche): 9.6 μL of PCR-grade pure water, 2.4 μL of $MgCl_2$ stock solution (final concentration 4 mM), 1 μL of each primer (final concentration 500 nM), 2 μL of the TaqMan probe (final concentration 200 nM), 2 μL of reaction mix 10× solution, and 2 μL of template DNA. Amplification was performed in a two-step thermal cycling procedure: predenaturation for 10 min at 94 °C followed by 40 cycles of 10 s at 94 °C and 30 s at 60 °C (except 63 °C for MMB-set). All DNA templates were analyzed in duplicate.

The standard curves for the primer and probe sets used were constructed as previously described (Yu et al., 2006) using the representative strains listed in Table 2. The target rRNA gene sequences were amplified from each strain by PCR with the corresponding primer sets (Table 2) and cloned into pGEM-T Easy vectors (Promega, Mannheim, Germany). For each plasmid, a 10-fold serial dilution series ranging from 10^2 to 10^9 copies/μL was generated and amplified in triplicate using real-time PCR with the corresponding primer and probe sets. The threshold cycle (C_T) values determined were plotted against the logarithm of their initial copy concentrations. The standard curves constructed using different strains for a primer and probe set showed no significant differences in their slopes at a 1% α-level. Thus, the average values of slope and intercept for each set were used to quantify methanogenic rRNA genes.

2.4. Statistical analysis

Non-metric multidimensional scaling (NMDS) was performed based on the rRNA gene quantification results analyzed by real-time PCR. NMDS is a multivariate ordination method

Table 2 – Characteristics of the real-time PCR primer and probe sets used in this study[a].

Set name/target group	Sequence (5′ → 3′)[b]	Representative strains[c]
MBT-set/*Methanobacteriales*	F: CGWAGGGAAGCTGTTAAGT	*Methanobacterium thermoautotrophicum* (DSM1053)
	T: AGCACCACAACGCGTGGA	*Methanobrevibacter arboriphilicus* (DSM 1536)
	R: TACCGTCGTCCACTCCTT	
MCC-set/*Methanococcales*	F: TAAGGGCTGGGCAAGT	*Methanococcus jannaschii* (DSM 2661)
	T: TAGCGGTGRAATGYGTTGATCC	*Methanococcus voltae* (DSM 1537)
	R: CACCTAGTYCGCARAGTTTA	
MMB-set/*Methanomicrobiales*	F: ATCGRTACGGGTTGTGGG	*Methanocorpusculum parvum* (DSM 3823)
	R: TYCGACAGTGAGGRACGAAAGCTG	*Methanomicrobium mobile* (DSM 1539)
	R: CACCTAACGCRCATHGTTTAC	*Methanospirillum hungatei* (DSM 864)
Msc-set/*Methanosaetaceae*	F: GAAACCGYGATAAGGGGA	*Methanosarcina acetivorans* (DSM 2834)
	T: TTAGCAAGGGCCGGGCAA	*Methanosarcina barkeri* (DSM 800)
	R: TAGCGARCATCGTTTACG	*Methanosarcina mazei* (DSM 3647)
Mst-set/*Methanosarcinaceae*	F: TAATCCTYGARGGACCACCA	*Methanosaeta concilii* (DSM 2139)
	T: ACGGCAAGGGACGAAAGCTAGG	*Methanosaeta thermoacetophila* (DSM6194)
	R: CCTACGGCACCRACMAC	

a Yu et al., 2005.
b F, T, and R indicate forward primer, TaqMan probe, and reverse primer, respectively.
c Culture collection numbers are in parentheses.

transforming complex data into a new format by constructing a new set of variables. This procedure condenses a data set to one point in a two-dimensional plane so that highly similar sets are plotted close together (Fromin et al., 2002). Accordingly, a quantitative community profile can be represented as one data point on an NMDS plot, and thus changes in community structure can be visualized by connecting consecutive data points. In this study, the NMDS ordination was carried out based on Sorensen (Bray–Curtis) distance measure in the PC-ORD software (version 5.0, MjM software, Gleneden Beach, OR) (McCune and Grace, 2002).

2.5. *Analytical methods*

COD was measured according to the procedure in Standard Methods (APHA-AWWA-WEF., 2005). Carbohydrate and protein concentrations were determined by the phenol–sulfuric acid method (Dubois et al., 1956) and the Kjeldahl method (Zapsalis and Beck, 1986), respectively. Volatile fatty acids (VFAs) and ethanol were quantified using a gas chromatograph (6890 Plus, Agilent, Palo Alto, CA) equipped with an Innowax capillary column (Agilent) and a flame ionization detector, and biogas composition was analyzed using another identical gas chromatograph equipped with an HP-5 capillary column (Agilent) and a thermal conductivity detector as described previously (Yu et al., 2006). All analyses were performed in duplicate.

3. Results and discussion

3.1. *Anaerobic digester performance*

Figs. 1A, 2A, and 3A represent changes in chemical profiles along with methane production in G-, W-, and S-digesters, respectively. G- and W-digesters showed similar reaction profiles with nearly complete degradation (>96%) of the initial SCOD within 40 d of treatment. The total amounts of methane produced in G- and W-digesters were 6.5 and 6.8 L, respectively. On the other hand, 34% of the initial SCOD remained after 60 d of incubation after the degradation of residual SCOD and the production of methane ceased at 49 d in S-digester. The overall methane production was 2.8 L, less than half of those in the other digesters.

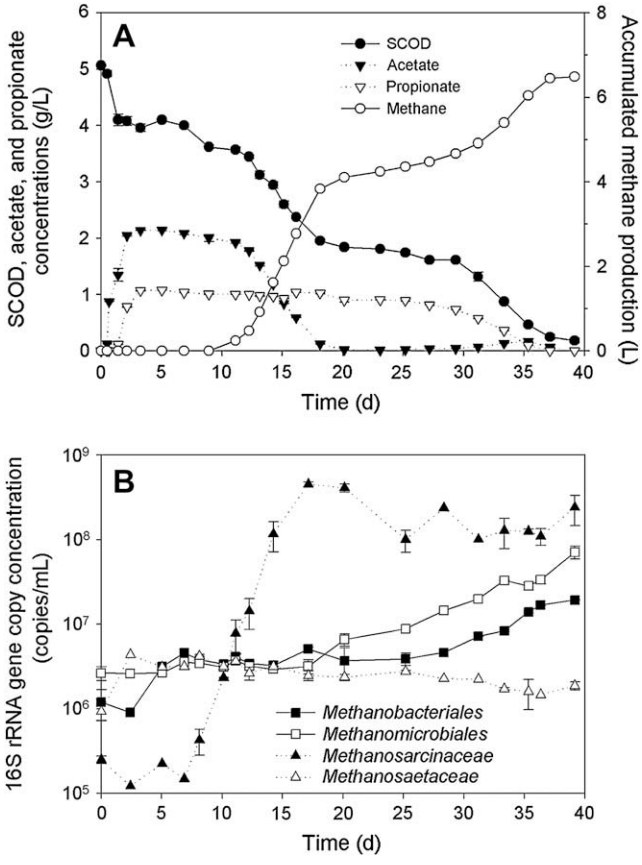

Fig. 1 – Changes in chemical profiles along with methane production (A) and in methanogenic 16S rRNA gene concentrations (B) in G-digester.

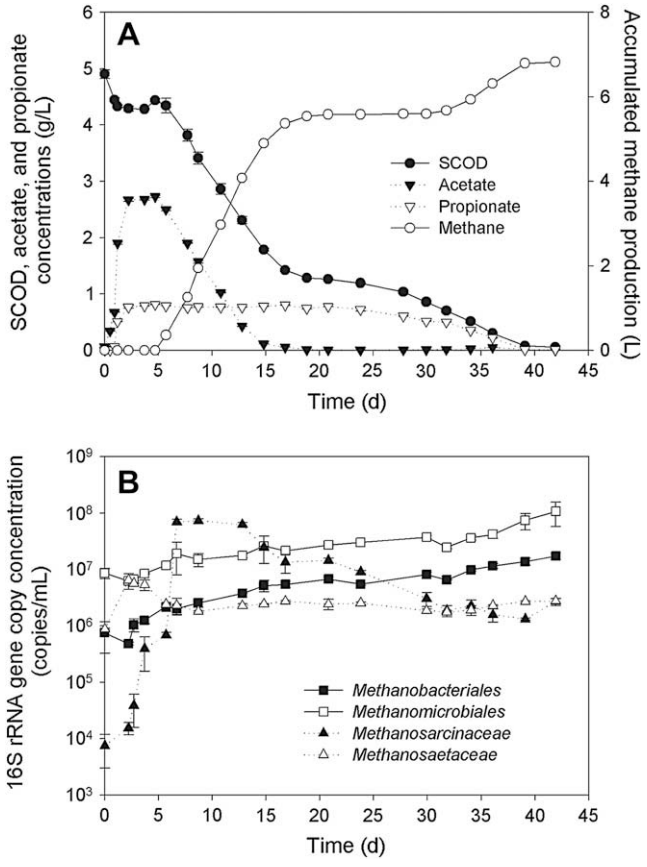

Fig. 2 – Changes in chemical profiles along with methane production (A) and in methanogenic 16S rRNA gene concentrations (B) in W-digester.

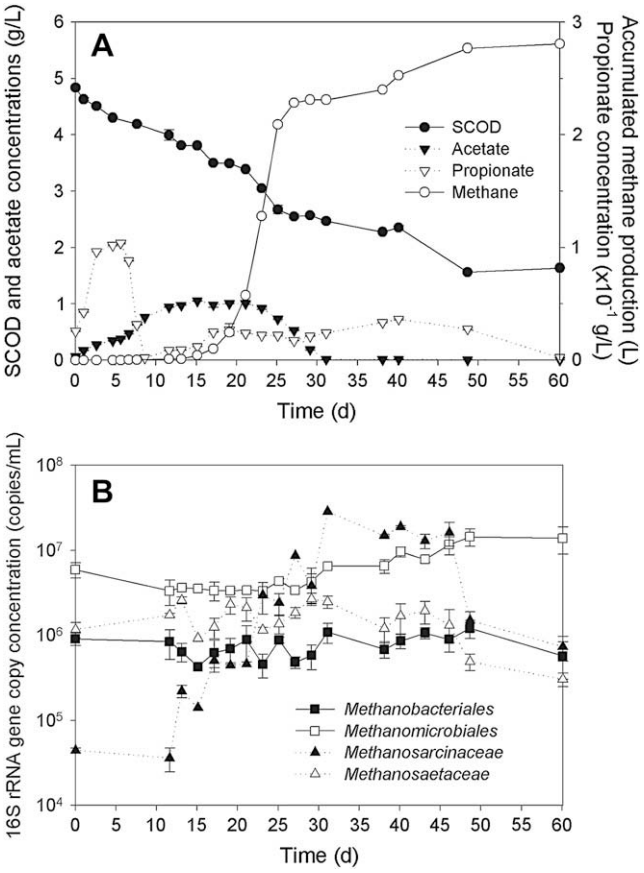

Fig. 3 – Changes in chemical profiles along with methane production (A) and in methanogenic 16S rRNA gene concentrations (B) in S-digester.

In all digesters, acetate and propionate were the first and second most abundant acidogenic products, respectively. A biphasic methane production associated with successive utilization of the two major intermediates was observed in all trials (Figs. 1A, 2A and 3A). Contrary to acetate, propionate cannot be directly utilized by methanogens and should be oxidized first to acetate and hydrogen to be converted to methane. On the other hand, under methanogenic conditions, propionate oxidation is only energetically possible via the syntrophic reactions of acetogenic bacteria and methanogens (Stams et al., 1998). Thus, the complete degradation of propionate in each digester (Figs. 1A, 2A and 3A) indicated the establishment of active propionate-utilizing syntrophic consortia in our experiment (Scholten and Conrad, 2000; Batstone et al., 2002). Acetate and propionate were accumulated up to 2.1 and 1.1 g/L, 2.7 and 0.8 g/L, and 1.1 and 0.1 g/L in G-, W-, and S-digesters, respectively. All VFAs other than acetate, including even propionate, remained at 0.1 g/L or less throughout the operation of S-digester, whereas propionate accounted for more than 75% and 90% of the residual SCOD after the exhaustion of acetate in G- and W-digesters, respectively. Correspondingly, although a biphasic methane production was also shown in S-digesters, the second production was not conspicuous compared to those in the other digesters (Figs. 1A, 2A and 3A).

3.2. *Methanogenic community dynamics*

Changes in the 16S rRNA gene concentration of each target group over operation time in G-, W-, and S-digesters are shown in Figs. 1B, 2B, and 3B, respectively. The concentration profiles showed significant variations in methanogenic populations with respect to performance data. The *Methanosarcinaceae* 16S rRNA gene concentration increased with the degradation of acetate in each digester (Figs. 1, 2 and 3). In G-digester, it increased rapidly following a lag of 7 d and reached its peak of 4.5×10^8 copies/mL (about 1800-fold increase) at 17 d. The concentration increased steeply without a lag up to 6.9×10^7 copies/mL (about 9200-fold increase) after 7 d of operation in W-digester. S-digester showed the longest lag of 12 d and a low increase in the rRNA gene up to 2.8×10^7 copies/mL (about 640-fold increase) at 31 d, which corresponded to the slow and incomplete degradation of SCOD (Fig. 3A). On the other hand, no apparent increase or decrease in the *Methanosaetaceae* 16S rRNA gene was shown in each digester and it was maintained roughly constant at around 10^6 copies/mL level throughout the treatment period. These suggested that *Methanosarcinaceae* was mainly responsible for the methanation of acetate in our trials. This corresponded to the previous findings that *Methanosarcina* species (with higher growth rate) outcompete at a high acetate concentration (>1 mmol), whereas *Methanosaeta* species (with higher substrate affinity)

grow favorably at a low acetate concentration (<1 mmol) (Zinder, 1990; Jetten et al., 1992; Yu et al., 2006). It is noteworthy to mention that the highest increase in *Methanosarcinaceae* 16S rRNA gene was observed in G-digester while its highest increasing ratio was observed in W-digester which showed the most acetate accumulation (Figs. 1 and 2). This suggested that *Methanosarcinaceae* communities in three digesters had different biokinetic characteristics. Each community, as a group of diverse populations, could have a different microbial composition so as to behave in a distinct way. The different initial population size of *Methanosarcinaceae* in G-, W-, and S-digesters (i.e., 33:1:6 in ratio of rRNA gene concentration) might have effects on the different biokinetic characteristics observed (Figs. 1B, 2B and 3B). For more comprehensive insight into this, however, more refined techniques to distinguish and quantify methanogenic populations at lower taxonomic levels, e.g., genus or species, would be required.

Methanococcales was not detected in our trials, probably due to the requirement of high salt concentration for growth (0.3–9.4% (w/v) NaCl) (Boone and Castenholz, 2001). The other two hydrogenotrophic orders, *Methanobacteriales* and *Methanomicrobiales*, were found in all digesters. Both orders increased in abundance after the cessation of aceticlastic methanation and concomitant with the second phase of methane production in G-digester (Fig. 1), whereas they gradually and continuously increased over operation time in W-digester (Fig. 2). The two groups, particularly the dominant *Methanomicrobiales*, more steeply increased within the period of second methane production even in W-digester. In both digesters, the 16S rRNA gene concentrations of *Methanobacteriales* and *Methanomicrobiales* reached their maxima of around 10^7 and 10^8 copies/mL (about 16- and 27-fold increases in G-digester and about 23- and 12-fold increases in W-digester) at the end of treatment (Figs. 1B and 2B). On the other hand, in S-digester, no considerable rise in *Methanobacteriales* was observed while *Methanomicrobiales* slightly increased after the depletion of acetate (Fig. 3). The final concentrations of their 16S rRNA genes were approximately 10 times lower than those in the other digesters, which corresponded to the incomplete removal of SCOD in S-digester (Fig. 3A). In all digesters, longer-chain VFAs (C_3–C_6), particularly propionate, degraded with the increase in hydrogenotrophic populations followed by methane production (Figs. 1 and 2). This suggested that hydrogen derived from the further oxidation of longer-chain VFAs was likely to be utilized by hydrogenotrophic methanogens (Stams et al., 1998; Ahring, 2003).

The 16S rRNA gene quantification results showed that methanogenic community structure continuously changed throughout the treatment period in each digester. Hydrogenotrophic methanogens were dominant in the initial phase but supplanted by aceticlastic methanogens with the rapid increase in *Methanosarcinaceae* (Figs. 1B, 2B and 3B). The rRNA gene concentration ratio of *Methanosarcinaceae* to total measured methanogenic populations (i.e., the sum of all target groups) showed its maximum of 97.7% after 17 d of treatment and remained at greater than 70% thereafter in G-digester (Fig. 1B). On the other hand, W- and S-digesters each showed another transition of dominance from aceticlastic to hydrogenotrophic methanogens, mainly *Methanomicrobiales*, after the exhaustion of acetate (Figs. 2B and 3B). On the basis of 16S rRNA gene concentration, *Methanomicrobiales* constituted 82.3% and 89.6% of total measured methanogenic populations at the end of the operation in W- and S-digesters, respectively, which were even higher than that in G-digester (i.e., 21.4%). It is interesting to note that, contrary to the other two digesters, no significant decrease in *Methanosarcinaceae* was observed in G-digester, even after the complete degradation of acetate. This observation of prolonged maintenance might be contributed by the bias of DNA-based analyses unable to differentiate dead from living cells. However, given that no such a phenomenon was observed in the other digesters and its rRNA gene concentration was maintained at high a concentration ($>10^8$ copies/mL), there were probably other substrates available for *Methanosarcinaceae* than acetate in that period.

3.3. *Quantitative shifts in methanogenic community structures*

Changes in methanogenic community structure were visualized by NMDS analysis because it avoids the assumption of linear relationships among variables and it is reported to be the most generally effective ordination method for ecological community data (McCune and Grace, 2002). The NMDS plot from the analysis of methanogenic 16S rRNA gene concentration profiles demonstrated continuous shifts in methanogenic community structure (Fig. 4). Each point was labeled with the corresponding digester name followed by incubation time in days. For the plot, the cumulative r^2 value represented by the axes was 0.863, the stress value was <10, and instability was $<<10^{-4}$, indicating that our results met the general criteria for good NMDS performance (McCune and Grace, 2002). Because methanogenic community structure was investigated based on the 16S rRNA gene concentrations of target methanogenic groups (Table 2), quantified by real-time PCR assay, the community behavior shown on the NMDS plot would be quantitative as well as qualitative at the corresponding taxonomic levels (Becker et al., 2000; Kuruta et al., 2004).

The early methanogenic communities in three digesters gathered in and around quadrant 2 of the NMDS plot (Fig. 4), but they shifted widely and totally differently with the progress of treatment. The community profiles from G-digester were most widely dispersed and distantly located from the profiles from the other digesters, particularly within the later half period. This seems to be mainly affected by the maintenance of highly dominant *Methanosarcinaceae* populations until the end of incubation, as described above, which was unique in G-digester (Fig. 1B). A big jump, i.e., a relatively more significant change in community structure, between two consecutive time points on the plot was relevant to a remarkable rise or fall in the target microbial populations. For example, the jumps from G12 to G14 and from G14 to G17 were paralleled with the rapid increases in *Methanosarcinaceae* (Fig. 1B) during the corresponding periods. The jumps from W6 to W7 and from S29 to S30 in W- and S-digesters also coincided with the sudden rises of *Methanosarcinaceae* (Figs. 2B and 3B). On the other hand, the one from G20 to G25 coincided with the sudden drop in *Methanosarcinaceae* (Fig. 1B). These suggested that the quantitative shifts in methanogenic community structures were well reflected in the NMDS results. An overlay analysis was also carried out using the

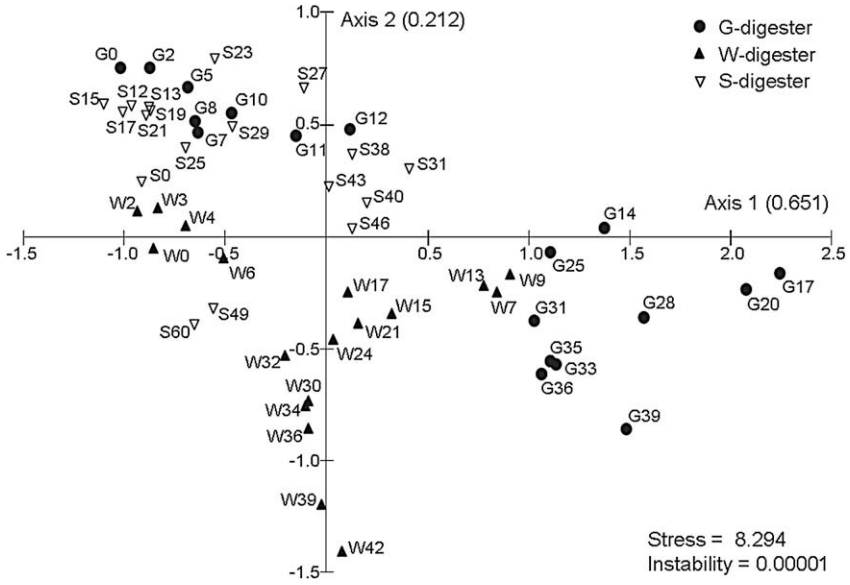

Fig. 4 – NMDS plot showing shifts in the quantitative structure of methanogenic community over treatment time in each digester. Each community profile on the plot is labeled with the corresponding digester name followed by a number indicating the incubation time (days).

residual acetate concentration (RAC) and the methane production rate (MPR) as ordination overlay variables to see if they had significant effects on the community shifts, but both variables showed weak correlations with each axis. The r^2 values against axis 1 and 2 were 0.13 and 0.21 for RAC and 0.29 and 0.04 for MPR, respectively. Furthermore, both variables were ignored by the default cutoff r^2 value of 0.2 in the joint-plot analysis (McCune and Grace, 2002). These results suggested that either of them was not a crucial factor to control the quantitative evolution of methanogenic communities in our systems.

The population diversity (or qualitative community structure) of methanogens is known to be stable and changes little compared to that of bacteria during the steady-state operation of anaerobic digesters (Zumstein et al., 2000; Akarsubasi et al., 2005). This qualitative stability might be because methanogens can utilize only a limited range of simple substrates, primarily converted from complex organics by bacterial populations (Ahring, 2003). On the other hand, as expected, the quantitative structures of methanogenic communities changed over treatment time in all trials. Corresponding to this, the variations in relative abundances of methanogenic populations were observed, by nucleic acid hybridization, during transitional phases, e.g., after a substrate perturbation (i.e., glucose loading shock) in a continuous system (Fernandez et al., 2000) and during the start-up period treating swine waste (Angenent et al., 2002) or a mixture municipal solid waste and sewage sludge (McMahon et al., 2004). There have also been some recent studies which looked at methanogenic community shifts at steady-states with changing operating conditions, such as organic loading, using fluorescence in situ hybridization (FISH) (Boonapatcharoen et al., 2007; Montero et al., 2008). However, most of them provided semi-quantitative results (i.e., relative proportions) which were not absolutely quantitative, and also targeted some specific populations only so as not to be able to cover most methanogens relevant in the systems examined. A notable thing here is that the methanogenic community structures in the digesters tested evolved in totally different ways (Fig. 4). Given that the only difference in the operating conditions was wastewater treated, substrate composition was suggested to affect the shaping of methanogenic community structures. This is supported by the previous study which showed marked and reproducible variations in the relative intensity of archaeal DGGE bands, although not robustly quantitative, in response to gradual transition of substrate from a synthetic glucose wastewater to a pharmaceutical wastewater (Akarsubasi et al., 2005). To our knowledge, this may be the first report on the absolute quantification approach to look at how methanogenic communities evolve differently in anaerobic digesters treating different types of wastewaters, particularly during transitional periods, using real-time PCR associated with a multivariate ordination technique (i.e., NMDS). It should be noted that changes in quantitative microbial composition can impact on microbial diversity analysis, frequently carried out by molecular finger-printing methods based on normal PCR. Because a population which constitutes less than 1% of the total target community is generally not detected by such methods (Forney et al., 2004), a numerically minor but functionally important population may be missed in the diversity analysis results. This, together with the overall results, suggests that more effort on quantitative approaches, particularly at more detailed taxonomic levels, should be made for better understanding of microbial behavior in anaerobic processes.

4. Conclusions

In this study, quantitative shifts in methanogenic community structures, in relation to changes in chemical profiles, were

examined and compared in three anaerobic batch digesters treating different types of wastewaters using a combination of real-time PCR and NMDS techniques.

(1) The quantitative structure of the methanogenic communities varied dynamically and continuously, with dominance shifts between different groups, mainly between *Methanosarcinaceae* and *Methanomicrobiales*, over the treatment time in each digester.
(2) Significantly higher growth, and larger populations, of methanogens were observed in the G- or W-digesters than in the S-digester, corresponding to the incomplete degradation of substrate in the S-digester only.
(3) Biphasic production of methane, associated with diauxic utilization of acetate and longer-chain VFAs (mainly propionate), was observed with successive increases in aceticlastic and hydrogenotrophic methanogens in each trial. *Methanosarcinaceae* and *Methanomicrobiales*, the quantitatively dominant aceticlastic and hydrogenotrophic groups, were suggested to be mainly responsible for the production of methane in each phase.
(4) The methanogenic communities in three digesters tested produced totally different profiles of quantitative evolution during the batch operations (i.e., transitional state). Given that the operating conditions for all trials were identical except wastewater treated, the difference in substrate characteristics was likely to be a factor affecting the direction of community shifts.

Overall, the experimental results emphasize that more attention should be devoted to quantitative as well as qualitative aspects of microbial dynamics for better understanding of their roles and behaviors in anaerobic digestion, particularly under dynamic or transitional conditions such as start-up or loading shock.

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