Bioresource Technology 202 (2016) 125–132

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

# Monitoring the microbial community shift throughout the shock changes of hydraulic retention time in an anaerobic moving bed membrane bioreactor

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## HIGHLIGHTS

- The drastic HRT change can effect on microbial and physical reactor performance.
- The optimized PCR–DGGE procedure was used to monitor microbial consortia and their functional behavior.
- Combination of molecular techniques and bioinformatics interpreted data on the taxonomic level.
- The specific microorganisms identified gave information on communities related to methane generation under HRT variables.

## ARTICLE INFO

*Article history:*
Received 8 September 2015
Received in revised form 23 November 2015
Accepted 28 November 2015
Available online 5 December 2015

*Keywords:*
AnMBMBR
PCR–DGGE
Biogas
HRT shock
Microbial community

## ABSTRACT

An anaerobic moving bed membrane bioreactor (AnMBMBR) fed with synthetic domestic wastewater was investigated under hydraulic retention time (HRT) shocks to assess the effects on the microbial (bacteria and archaea) community and reactor performance. 16S rDNA targeted polymerase chain reaction–denaturing gradient gel electrophoresis (PCR–DGGE) approach was optimized to relate the metabolic and community composition with biogas generation, methane content and COD removal efficiency. From the drastic decrease of HRT (from 8 h to 4 h), the methane production was significantly reduced due to the HRT shock, while the COD removal efficiency was not affected. The enhanced growth of homoacetogenic bacteria, *Thermoanaerobacteraceae* competes with methanogens under shock period. When the HRT was recovered to 8 h, the methane generation rate was higher than the initial operation before the shock HRT changes, which would be ascribed to the activity of new emerging hydrogenotrophic archaea, *Methanocella* sp. and *Methanofollis* sp.



## 1. Introduction

Anaerobic processes have traditionally been recognized as an attractive option for wastewater treatment because of their ability to save and/or harvest energy. Recent improvements in anaerobic wastewater treatment are attributed to an efficient uncoupling of solid retention time (SRT) from hydraulic retention time (HRT) through biomass immobilization, usually via the formation of bio film or granules. The immobilized biomass can be efficiently separated from the effluent using membrane technology to harvest polished effluent and to retain the biomass within the anaerobic reactor (Visvanathan and Abeynayaka, 2012). Despite the advances made in anaerobic membrane bioreactors (AnMBRs) that have been successfully applied to wastewater treatment, membrane fouling is still one of the major limitations in a long-term operation. To this end, we recently demonstrated that rotary-disks and biomass immobilization on a moving bed can effectively mitigate the fouling in AnMBRs with relatively small energy requirement (Kim et al., 2014).

A significant body of literature has reviewed for previous research that focused on the influences of parameters such as organic loading rate, HRT, SRT, start-up time, and reactor configuration on the microbiology, biomass yield, and substrate utilization rate of anaerobic bioreactors (Khanal, 2008; Visvanathan and Abeynayaka, 2012). In particular, the HRT and organic loading rate have often been the key factors that primarily determine the size and capital costs of the processes (Salazar-Peláez et al., 2011).

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http://dx.doi.org/10.1016/j.biortech.2015.11.085
0960-8524/

While most of the previous reports performed parametric studies under queasy-stationary condition, evidence has been presented that the significant fluctuation in the HRT or organic loading rate can have a short- or long-term detrimental impact on the performance of anaerobic bioreactors (Dereli et al., 2012). For example, hydraulic shocks result in an increase of the suspended solid concentration in effluent due to the washing out of biomass (Blaszczyk et al., 1994). Microbiological tools, with their metabolic characteristics, used to monitor microbial communities, are useful to understand the behavior of a biological system particularly under dynamic and transitional conditions. Nevertheless, the shift in microbial community in AnMBR caused by the shock of HRT (or organic loading rate) has not been reported much previously.

Numerous culture-independent molecular methods have been reported in the literature to understand the microbial community in various types of biological wastewater treatment processes (Talbot et al., 2008; Theron and Cloete, 2000). Among these techniques, polymerase chain reaction based denaturing gradient gel electrophoresis (PCR–DGGE) has been reported as the most versatile method. Among the many advantages of the PCR–DGGE, it can quickly confirm the genetic diversity of the natural microbial and phylogenetic relationship between population members (Douterelo et al., 2014; Zhou et al., 2011; Theron and Cloete, 2000). As the procedure of DGGE is relatively rapid and multiple samples can be electrophoresed simultaneously, this method is particularly useful for analysis of time series population dynamics.

The objective of this study was to investigate the effects of HRT shock on a microbial community in an anaerobic moving bed membrane bioreactor (AnMBMBR) using a rapid, simple, and efficient molecular fingerprinting method. The variation in the microbial community was quantitatively and qualitatively linked to the performance of the AnMBMBR, such as the conversion of influent organics and its stability. Consequently, the results fully confirmed that the AnMBMBR is a feasible strategy for membrane fouling control and microbial enrichment, even under influent fluctuation.

## 2. Methods

### 2.1. Reactor operation and sampling

As described in our previous study (Kim et al., 2014), the AnMBMBR system consists of two submerged membranes, two rotary disks, and 15% (v/v) of 8 mm diameter polypropylene fabric ball-type media. The inoculum was collected from an anaerobic digestion rector of a municipal wastewater treatment plant in Korea. The system was fed with a given composition of synthetic wastewater reported in an earlier study (Kim et al., 2014) for 90 days under variable HRTs (or organic loading rate). After a 30 day acclimation period under the HRT of 8 h (HRT8-1), the HRT was reduced to 4 h for 15 days (shock period, HRT4) and then adjusted back to 8 h (HRT8-2). The effluent was periodically sampled for further chemical analysis. The moving bed and bulk liquor in the AnMBMBR were also collected before and after the HRT shock for microbial analysis. The specification of the system and the operational parameters are summarized in Table 1.

**Table 1**
Operational parameters and specification of AnMBMBR.

| Parameters | Conditions | | |
|---|---|---|---|
| Reactor volume (working volume) | 5.2 L | | |
| Temperature | 35 ± 1 °C | | |
| Substrate | Glucose + Acetic acid | | |
| Membrane | PTFE (pore size 0.2 μm) | | |
| Organic loading rate | 0.53 kg COD/$m^3$ d | | |
| Intermittent ratio of pump cycle | 8 min (on), 2 min (off) | | |
| Operating time intervals | 1–30 days | 31–45 days | 46–90 days |
| HRT | 8 h | 4 h | 8 h |
| Organic loading rate | 0.53 kg COD/$m^3$ d | 1.06 kg COD/$m^3$ d | 0.53 kg COD/$m^3$ d |

### 2.2. Chemical analysis

Chemical oxygen demand (COD) was measured according to the Standard Methods (LabNavigator, Forston Labs, USA). Volatile fatty acids (VFAs) were analyzed using an HP 6890 series Gas Chromatograph (GC) with a flame ionization detector (FID). The total biogas production rate was monitored volumetrically using a gas collection apparatus employing the water substitution principle. The biogas composition was measured using a gas chromatograph 6890N (Agilent Technologies, USA) equipped with a thermal conductivity detector (TCD) and Restek PC8779 stainless steel packed column (Restek, USA) was used to assess the generation rate of the individual gaseous product (methane, carbon dioxide, and nitrogen).

### 2.3. Microbial community monitoring using PCR based DGGE analysis

Before DNA extraction, media and suspended solid samples were prepared suitable for commercial extraction kit. Media samples (M) were subject to sonication to detach the biomass from the moving bed sponge. Suspended solid samples (S) were prepared from the bulk liquor as described previously (Singka et al., 2012). The genomic DNA was extracted from each sample using an automated DNA/RNA extractor (MagDEA DNA 200 (GC)) with commercial extraction kits (Migration Technology).

The reaction mixture for PCR was prepared according to the manufacturer's instructions by using BioFact™ F-Star taq DNA polymerase and 100–150 ng of extracted genomic DNA. The amplified PCR products were purified using the HIGENE™ Gel purification kit and PCR purification system for proceeding PCR–DGGE analysis. One-step direct PCR–DGGE analysis was performed for bacterial community targeting at the V6–V8 region of the 16S rRNA gene (Heuer et al., 1997). Two-step nested PCR–DGGE analysis was employed for methanogenic archaea community targeting at V3 region (Dar et al., 2005; Heuer et al., 1997). At least 500 ng of purified PCR product was used for each analytical procedure. The gels were stained with SYBR Green I (1:10,000, invitrogen) and visualized under UV by using the gel documentation system (Bio-Rad). The oligonucleotide sequences, optimal PCR program and DGGE conditions used in this study were summarized in Table 2.

The principal and visible DGGE bands in each DGGE profile were excised directly from the original gels and sequenced using the standard sequencing method of SolGent. Instead of cloning, further PCR and DGGE were repeated until a single band from the PCR product appeared. It appeared to be a reliable approach to obtain the specific sequence from each fingerprint pattern. The sequencing results were compared with the reference library in the BLAST program with the National Center for Biotechnology Information (NCBI) database.

### 2.4. Bioinformatics for statistical analysis

DGGE fingerprinting patterns were further analyzed as described in Diez et al. (2001) using Quantity One D software (BioRad). Structural diversities of the bacterial and archaeal community were estimated on the basis of densitometric measurements of apparent bands as described in previous reports (Marzorati et al., 2008).

**Table 2**
Primer information optimized PCR conditions and DGGE conditions for analysis of bacterial and archaeal community.

| Taxonomic linkage | Target variable region | Primer name | Sequence (5′–3′) | Optimized PCR condition | | | | Optimized DGGE condition | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Number of cycles | Denaturation (°C)/time (min/s) | Annealing (°C)/time (s) | Elongation (°C)/time (s) | Voltage (V) | Time (h) | Denaturant (%) |
| Bacteria | V6–V8 | F984[a,b] | GAACGCGAA GAACCTTAC | 35 | 94 (2 min) 94 (30 s) | 55 (15 s) | 68 | 55 | 24 | 50–70 |
| | | R1378[c] | CGGTGTGTACAA GGCCCG GGAACG | | | | | | | |
| Archaea | Universal | Arch f364[d] | CCTACGGGRBG CAGCAGG | 35 | 94 (5 min) 94 (30 s) | 58 (30 s) | 72 (1 min) 72 (10 min) | 130 | 4 | 20–45 |
| | | Arch r1386[d] | GCGGTGTGTG CAAGGAGC | | | | | | | |
| Archaea | V3 | PARCH340f[a,e] | CCCTACGGGCYG CASCAG | 30 | 95 (5 min) | 53 (first) (30 s) | 72 (1 min) | | | |
| | | PARCH519r[e] | TTACCGCGG CKGCTG | | 95 (30 s) | 55.8 (second) (30 s) | 72 (10 min) | | | |

[a] Attached GC-clamp (5′-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG C-3′).
[b] Nubel et al. (1996).
[c] Heuer et al. (1997).
[d] Skillman et al. (2004).
[e] Ovreas et al. (1997).

In order to assess the variation in microbial communities over the HRT shock, we performed a principal components analysis (PCA) by using the Biodiversity R package. The detailed microbial community variation was ascribed by using FactoMine R package (Kindt and Coe, 2005). Sequencing data at the genus level of the bacterial domain and the order level of the archaeal domain were used to determine the principal components from seven variables (described in Table 4).

## 3. Results and discussion

### 3.1. Performance of the AnMBMBR under the shock changes of HRT

The effects of HRT, one of the important operational parameter for the performance of AnMBMBR, were assessed in terms of COD removal efficiency, biogas production, methane content, and VFA accumulation, as shown in Fig. 1. During the acclimation period of 15 days for the growth and immobilization of biomass (HRT8-1), the AnMBMBR reached a quasi-stationary state with 90% of COD removal efficiency, 0.48 L/d of biogas production rate, and 45% of methane content. In spite of the HRT shock during HRT4, deterioration of COD removal efficiency was not observed. Chu et al. (2005) also reported that the COD removal efficiency in an anaerobic bioreactor was independent on HRT at temperatures higher than 15 °C. Theoretically, inorganic matter such as sulfide can be chemically measured as COD (Gao et al., 2011). On the contrary, biogas production rate and methane content significantly decreased to 0.23 L/d and 16.9% respectively, during the HRT4. A decreased sulfate ion concentration of the effluent (not shown data) and an appearance of sulfate reducing bacteria (refer to Table 4) were observed during HRT4. These observations would be further ascribed to an elevated sulfate reduction. COD could be consumed not only for methane production but also for sulfate reduction during the HRT4 period. Ali Shah et al. (2014) also reported that the influent organic substrates in terms of COD could be consumed not only via anaerobic methanogenesis but also via anaerobic respiration using electron acceptors such as sulfate ion. In addition, Yoo et al. (2012) reported that dissolved methane represented 63% of the total methane production in anaerobic treatment of dilute wastewaters. The decreased HRT (from 8 h to 4 h) increased the amount of effluent containing dissolved methane. Thus, the methane lost as dissolved methane in the effluent may decrease gaseous methane production. Furthermore, an increased VFA accumulation in effluent during the HRT4 indicates a strong inhibition of methanogenesis. The finding of this study suggests that a drastic decrease in HRT (or increase in the organic loading rate) would lead to an elevated contribution of sulfate reduction and an increased loss of dissolved methane in

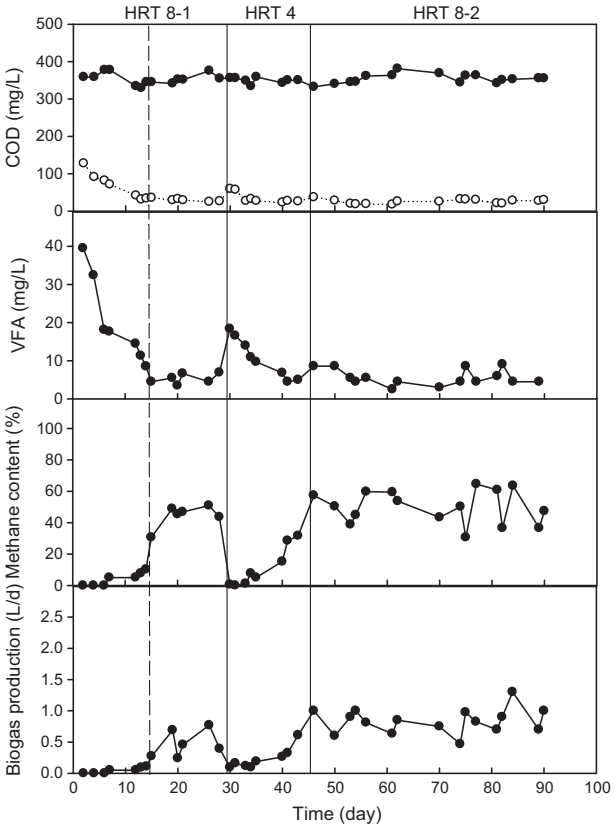


**Fig. 1.** Profiles of the reactor performances at various HRTs.

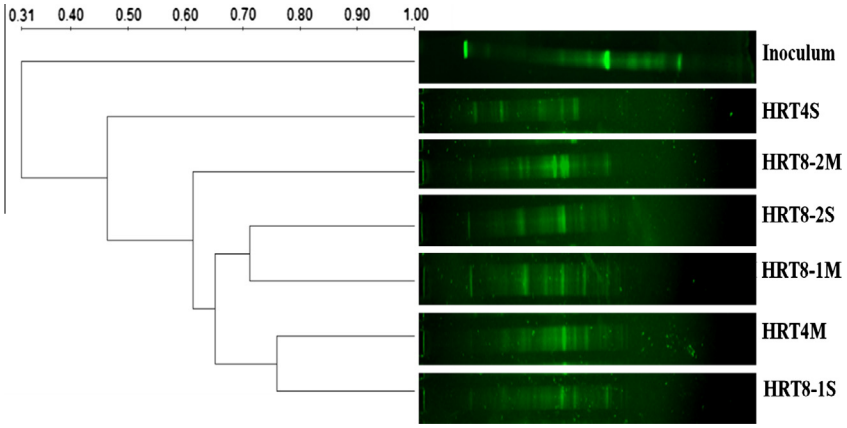


**Fig. 2.** Fingerprinting and corresponding similarity structure of bacterial community.

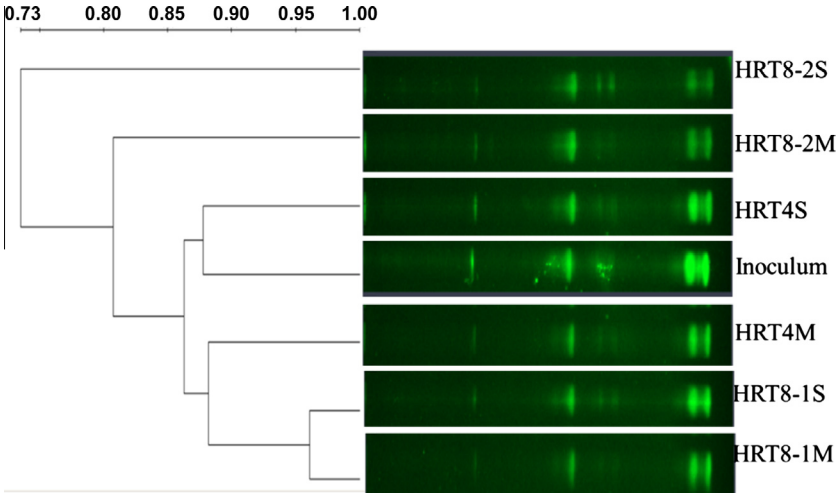


**Fig. 3.** Fingerprinting and corresponding similarity structure of archaeal community.

effluent, which in turn significantly reduces the methane potential (Yoo et al., 2012). Nevertheless, the decreased level of methane production rate appeared to recover when the HRT was again controlled to 8 h (HRT8-2).

### *3.2. Quantitative analysis of microbial community based on PCR–DGGE*

In preliminary experiments of PCR–DGGE (for both bacterial and archaeal domains), the electrophoresis duration, optimal voltage for different PCR product and the amount of sample loading were optimized for an efficient recovery of the DNA from the major band profile. This procedure was necessary for the quantitative fingerprinting of the bacterial and archaeal community pattern and further statistical analysis. The DGGE fingerprinting patterns of bacteria (24 bands) are shown in Fig. 2, where a clear shift in the community was observed during the shock changes of HRT. The dominant bacterial community at HRT8-1 disappeared, while several unforeseen species emerged at HRT4, for both the M and S samples. The clear changes in the bacterial structure between HRT4 and HRT8-1 coincided with the significant decrease in total methane production. Therefore, the shock reduction in HRT presumably resulted in the enrichment of microorganisms adapted to the reduced HRT, which are not favorable for methanogenesis.

On the other hand, a total of 9 bands were observed in the archaeal fingerprint and their similarity matrix is shown in Fig. 3. During HRT4, there was a shift in minor bands, while the dominant band patterns of the archaeal community structure did not change significantly. This observation can be explained by considering that the majority of the archaeal methanogen species are known to be slow-growing organisms (Nauhaus et al., 2007). The archaeal banding patterns are also not as complicated as those in the bacteria fingerprints due to the lower densities of the archaea domain in most microbial complexes (Zhou et al., 2011). A quantitative analysis of the fingerprint (Table 3) showed that the colonized community in the media had a higher diversity index than that of the suspended solids at HRT4. It would indicate that the use of media sponge can effective against membrane fouling control and microbial enrichment under environmental stress.

The moving window analysis (Fig. 4) using the Pearson product-moment correlation coefficient revealed community changes among the sampling points more clearly. This shows that there were 76.1 ± 14.8% dynamics in the bacterial community (Fig. 4a) and 23.06 ± 21.35% in the archaeal community (Fig. 4b) during the HRT shocks. In addition, the bacterial and archaeal consortia were estimated relative functional organization at around a 20% fraction. Therefore, these communities were found to have 'on average' low density functional organisms (Marzorati et al., 2008). However, the microbial community attained a quite stable composition at the recovered HRT (HRT8-2), which led to an enhanced performance in terms of elevated methane yield. According to the quantitative analysis, it can be concluded that HRT stress

**Table 3**
Quantitative indexes of bacterial and archaeal DGGE fingerprints.

| | Bacteria | | | Archaea | | |
|---|---|---|---|---|---|---|
| | Rr[c] | H′[d] | FO (%)[e] | Rr[c] | H′[d] | FO (%)[e] |
| Inoculum[b] | 3.00 | 1.32 ± 0.04 | 20.97 | 0.35 | 1.76 ± 0.03 | 20.29 |
| HRT8-1M[a] | 23.04 | 2.80 ± 0.03 | 18.68 | 3.75 | 1.76 ± 0.03 | 21.80 |
| HRT8-1S[b] | 16.00 | 2.66 ± 0.03 | 21.70 | 3.75 | 1.76 ± 0.03 | 20.80 |
| HRT4-M[a] | 38.25 | 3.22 ± 0.03 | 20.81 | 9.60 | 2.12 ± 0.05 | 20.87 |
| HRT4-S[b] | 28.73 | 3.10 ± 0.03 | 21.76 | 12.15 | 1.99 ± 0.05 | 21.77 |
| HRT8-2M[a] | 27.04 | 3.04 ± 0.04 | 21.68 | 3.75 | 1.52 ± 0.04 | 23.13 |
| HRT8-2S[b] | 40.96 | 3.08 ± 0.03 | 21.17 | 7.35 | 1.89 ± 0.04 | 23.56 |

[a] M: media.
[b] S: suspended.
[c] Rr < 10: low range-weighted richness, Rr < 30 can be correlated with a medium range-weighted richness, Rr > 30: high range-weighted richness (Marzorati et al., 2008).
[d] H′: higher value represents higher diversity (Li et al., 2010).
[e] Functional organization (FO)%: the 20% of (FO) represents a community with high evenness (Marzorati et al., 2008).

seems to affect community stability especially in the bacterial group.

### 3.3. Functionality estimation of microbial community based on sequencing

The optimized PCR–DGGE procedure employed in this study, along with the subsequent qualitative analysis enabled the rapid and reliable monitoring of microbial consortia and their functional behavior. A combination of molecular techniques and bioinformatics was used to interpret data on the taxonomic level. The sequenced affiliations listed in Table 4 were determined in comparison with the NCBI database. The functional possibilities were assessed according to Bergey et al. (1994), Garrity et al. (2004), Whitman and Parte (2009) and Dworkin and Falkow (2006). The inoculum was dominated by *Acinetobacter* sp., *Enterobacter* sp., *Burkholderia* sp., *Clostridium* sp., and *Leclercia* sp. It was apparent that a diversification of the inoculums occurred along with the acclimation in AnMBMBR.

During HRT8-1, the communities of *Acinetobcter* sp., *Sulfurovum* sp., *Enterobacter* sp., *Leclercia* sp., *Acinetobacter* sp., *Stenotrophomonas* sp., *Pseudomonas* sp., *Alteromonas* sp., *Burkholderia* sp., *Pantoea* sp., *Cedecea* sp., *Thermoanaerobacteraceae* sp., *Citrobacter* sp., and *Clostridium* sp. co-existed. *Acinetobcter* sp., *Enterobacter* sp., *Leclercia* sp., *Cedecea* sp. and *Stenotrophomonas* sp. were reported to be fermentative bacteria showing hydrolysis activities. *Burkholderia* sp., *Pantoea* sp., *Citrobacter* sp., *Pseudomonas* sp. and *Alteromonas* sp. were known to play the role of acidogenesis in anaerobic digestion. These acidogens are known to use sugar and amino acid with the end products of hydrogen, $CO_2$, butyrate, and propionate. *Thermoanaerobacteraceae* belongs to firmicutes and homoacetogenic bacteria in the acetogenesis and methanogenesis pathways. Specifically, *Thermoanaerobacteraceae* can reduce thiosulfate to sulfide, and ferment acetate, formate and lactate to hydrogen containing end products (ethanol and carbon dioxide) by producing hydrolytic enzyme. *Clostridium* spp. are syntrophic acetate oxidizing bacteria and are key players in methanogenesis. On the other hand, *Sulfurovum* sp. is mesophilic sulfur- and thiosulfate-oxidizing bacterium, which is very important for biological sulfides removal. The microbial consortium during HRT8-1 appeared to be dominated by gamma-proteobacteria, where mutual interactions between species should be balanced in a quasi-stationary operation. This situation was reported that stable bioreactor performance

**Table 4**
Functionally listed microbial community under the different HRT from sequencing analysis.

| Band no. | Accession no. | Phylogenetic affiliation | Remark | Initial state | HRT8-1 | HRT4 | HRT8-2 |
|---|---|---|---|---|---|---|---|
| 1 | gb\|JQ087085.1\| | *Sulfurovum* sp. | Hydrolysis | – | + | + | + |
| 4 | gb\|KJ638986.1\| | *Cedecea davisae* | | – | + | – | + |
| 5 | gb\|KJ741259.1\| | *Klebsiella pneumoniae* | | – | – | – | + |
| 8 | gb\|KF681003.1\| | *Acinetobacter* sp. | | + | + | + | + |
| 9 | gb\|KJ452162.1\| | *Stenotrophomonas rhizophila* | | – | + | + | + |
| 10 | gb\|KJ632062.1\| | *Enterobacter* sp. | | + | – | + | + |
| 14 | gb\|KJ660958.1\| | *Enterobacter harmaechei* | | + | + | + | + |
| 15 | gb\|KJ732913.1\| | *Alteromonas* sp. | | – | + | + | + |
| 16 | gb\|KJ806423.1\| | *Enterobacter ludwigii* | | – | + | + | + |
| 19 | gb\|KJ748608.1\| | *Stenotrophomonas Pavinini* | | – | – | – | + |
| 21 | gb\|KJ545733.1\| | *Leclercia* sp. | | + | + | + | + |
| 24 | gb\|KC346294.1\| | *Edwardsiella tarda* | $H_2S$ producing bacteria | – | – | + | – |
| 3 | gb\|KC253647.1\| | *Serratia* sp. | | – | – | + | + |
| 6 | gb\|KJ545646.1\| | *Clostridium* sp. | | + | + | + | + |
| 7 | gb\|JN92932.1\| | *Citrobacter* sp. | | – | + | + | + |
| 11 | gb\|KJ194130.1\| | *Pseudomonas putida* | | – | + | + | + |
| 13 | gb\|JX262490.1\| | *Pantoea* sp. | | – | – | + | + |
| 17 | gb\|JQ795139.1\| | *Bukholderia* sp. | | + | + | + | + |
| 18 | gb\|JQ271587.1\| | *Clostridium* sp. | | + | – | + | + |
| 20 | gb\|KJ748612.1\| | *Pseudomonas japonica* | | – | – | – | + |
| 22 | gb\|KC588517.1\| | *Pantoea* sp. | | – | + | – | – |
| 23 | gb\|KJ372438.1\| | *Shewanella irciniae* | Acidogenesis | – | – | + | – |
| 12 | gb\|KF956491.1\| | *Thermoanaerobacteraceae* | Reduce thiosulfate | – | + | + | + |
| 1 | ref\|NC_015574.1\| | *Methanobacterium* sp. | Hydrogenotrophic | + | + | + | + |
| 2 | ref\|NC_015416.1\| | *Methanosaeta concilii* | Aceticlastic | + | + | + | + |
| 3 | ref\|NC_008553.1\| | *Methanosaeta thermophila* | Aceticlastic | + | + | + | + |
| 4 | ref\|NC_013665.1\| | *Methanocella paludicola* | Hydrogennotrophic | – | – | + | + |
| 5 | ref\|NZ_CM001555.1\| | *Methanofollis Liminatans* | Hydrogennotrophic | – | – | + | + |
| 6 | ref\|NC_015562.1\| | *Methanotorris igenus* | Hydrogennotrophic | + | + | + | + |
| 7 | ref\|NC_014408.1\| | *Methanothermus fervidus* | Hydrogennotrophic | + | + | + | + |
| 8 | ref\|NC_015216.1\| | *Methanobacterium* sp. | Hydrogennotrophic | + | + | + | + |
| 9 | ref\|NC_003552.1\| | *Methanosarcina acetivarans* | Aceticlastic | + | + | + | + |

–: Absent.
+: Present.

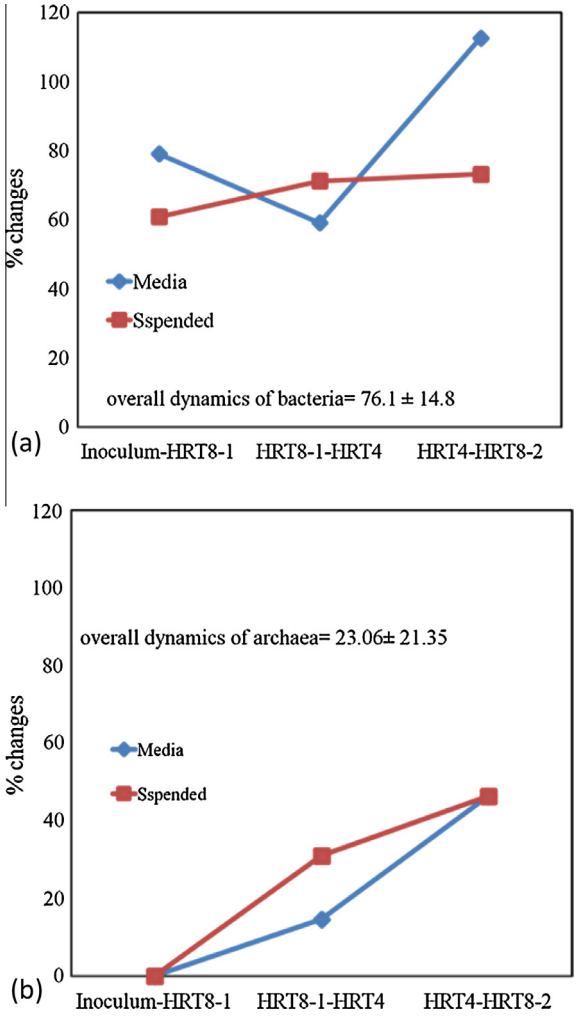


**Fig. 4.** Microbial (a) bacterial and (b) archaeal community dynamics under HRT shock.

requires a certain level of stability in the microbial community (LaPara et al., 2002).

Although some dominant species were adapted to the HRT shock, the fingerprinting pattern and sequencing indicated that some species emerged in HRT4. The emerging new bands during HRT-4 were closely similar to *Shewanella irciniae*, *Serratia* sp., and *Edwardsiella tarda*. *S. irciniae*, a facultative anaerobe is not commonly involved in anaerobic digestion, even though other *Shewanella* species are popular thiosulfates and iron reducers. *Serratia* sp. is chemoautotrophic with a low nutritional requirement and can produce lipase enzyme, possibly allowing hydrolysis activity. *E. tarda* is fermentative bacteria of which the metabolic end product, hydrogen sulfide, can interfere in anaerobic digestion. Relatively weak DGGE bands that represent *Clostridium* sp. and *Thermoanaerobacteraceae* sp. in HRT8-1 became dominant during HRT4. The elevated activities of these sulfate reducing and/or homoacetogenic bacteria enhanced hydrogen utilization activities other than methanogenesis (Lovley, 1985). Since the hydrogen, excreted by fermentative and acidogenic bacteria, is an important intermediate of various metabolic pathways (homoacetogenic or hydrogenotrophic methanogenic pathway), the hydrogen-scavenging reaction might dominate the methanogenesis to render the entire system energetically favorable under the HRT shock (Sekiguchi et al., 2001). Moreover, *Pantoea* sp. and *Cedecea* sp. faded away while the band representing *Sulfurovum* sp. became less dominant under the HRT shock. As the rate limiting steps in anaerobic digestion include hydrolysis (which converts complex organics into simpler derivatives) as well as methanogenesis (Visvanathan and Abeynayaka, 2012), it reduced the density of the hydrolyzing bacteria, which may cause the overall digestion pathway to become unbalanced. Consequently, the volumetric fraction of methane in the biogas was much lower during HRT4, while an accumulation of VFA was observed (Fig. 1). Zinatizadeh et al. (2006) also reported that a decreased HRT in an anaerobic reactor leads to the accumulation of VFAs while reducing methane yield. Visvanathan and Abeynayaka (2012) further explained a consistent observation of an imbalance between VFAs formation and methane generation under an increased organic loading rate.

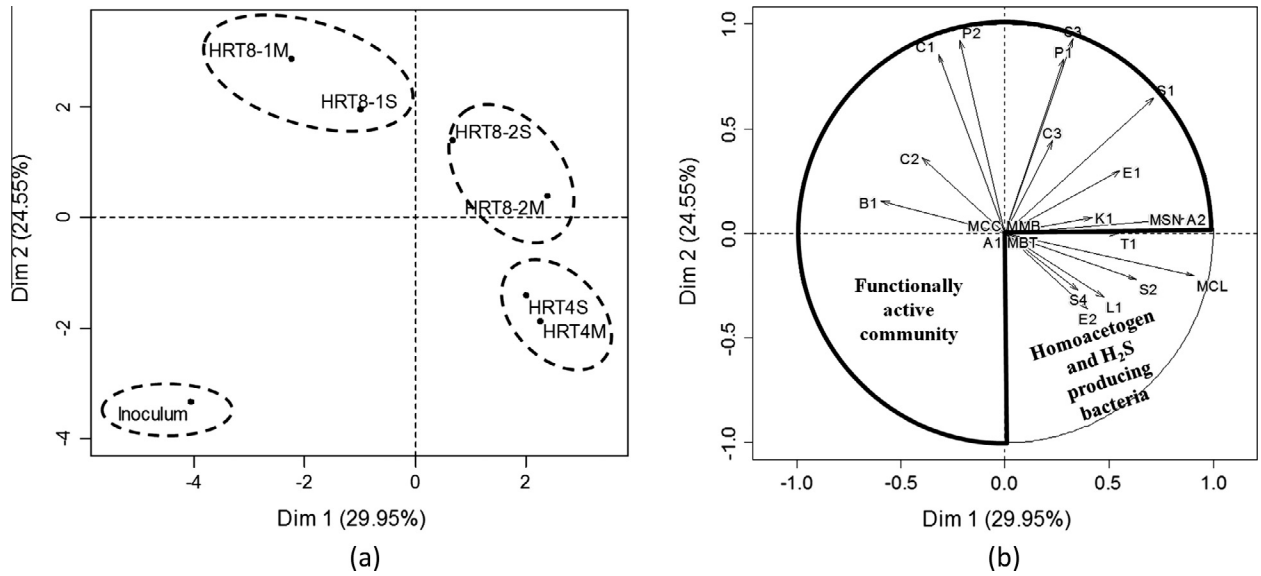


**Fig. 5.** PCA analysis of microbial (bacteria and archaea) community at different HRTs (a) individual factor map and (b) variable factor map. S1 = *Sulfurovum* sp., L1 = *Leclercia* sp., S2 = *Serratia* sp., C1 = *Cedecea* sp., K1 = *Klebsiella* sp., C2 = *Clostridium* sp., C3 = *Citrobacter* sp., A1 = *Acinetobacter* sp., S3 = *Stenotrophomonas* sp., E1 = *Enterobacter* sp., P1 = *Pseudomonas* sp., T1 = *Thermoanaerobacteraceae* sp., P2 = *Pantoea* sp., B1 = *Burkholderia* sp., S4 = *Shewanella* sp., E2 = *Edwardsiella* sp., A2 = *Alteromonas* sp. MBT = *Methanobacterium* sp., *Methanothermus* sp., MSN = *Methanosaeta* sp., *Methanosarcina* sp., MCL = *Methanocella* sp., MCC = *Methanotorris* sp., MMB = *Methanofollis* sp.

During the HRT8-2, *Klebsiella* sp. was observed as a new emerging bacterial member that is a facultative anaerobic, fermentative, and active denitrifier. In contrast, the bands that represent *S. irciniae*, and *E. tarda* were not observed in the fingerprint pattern and hydrogen utilizing activity seemed to become balanced. The minor community shift during HRT8-2 showed that the microbial community in HRT4 could adapt to the increased HRT.

The V3 region of the 16S rDNA gene was targeted to analyze the dominant archaeal community structure and its dynamic variation (Patil et al., 2010). *Methanosaeta* sp., *Methanobacterium* sp., *Methanotorris* sp., *Methanothermus* sp. and *Methanosarcina* sp. were dominant populations regardless of the HRT variation. During HRT4, *Methanocella* sp. and *Methanofollis* sp. emerged as hydrogenotrophic methanogen. *Methanosarcina* sp. and *Methanosaeta* sp. can utilize acetate for methanogenesis (Liu and Whitman, 2008), whereas the others observed methanogens would primarily use hydrogen, carbon dioxide, and formate as the sole energy and carbon sources for methane generation (Liu and Whitman, 2008). Even though the community difference between HRT4 and HRT8-2 was negligible, the methane production rate significantly increased in HRT8-2, even when compared to HRT8-1. This observation could be attributed to the emerged hydrogenotrophic archaea which could utilize the hydrogen for methanogenesis during the HRT8-2.

### 3.4. Statistical analysis

The sequencing affiliation was further analyzed using PCA for an in-depth evaluation of the bacterial/archaeal community shifts over the shock of HRT changes. Analogous methods have been employed to study and predict microbial community-level dynamics under temperature changes, introduction of foreign microorganisms, and exposure to toxic compounds (Wikstrom et al., 1999). In this study, the principal components (PC1 and PC2) covered 54.50% of the total variation; therefore, the resulting two-dimensional PC plot could largely represent the bacterial and archaeal communities' member over the HRT changes. The two-dimensional PC plot in Fig. 5a shows that the microbial communities in the M and S samples are similar to each other, even though DGGE fingerprinting pattern showed some differences. The significant microbial composition shifts from inoculums during acclimation were confirmed by the contrasting factors of inoculums samples compared to the other samples. The coefficients of the HRT4 samples were in inverse relationship to those of the HRT8 samples, confirming that the microbial community accommodated short HRT under hidden strong changes. The microbial structure change during the HRT8-2 was moderate, since the HRT8-2 samples showed positive correlation with HRT4 and HRT8-1 for PC1 and PC2, respectively. This can be explained that the community changes during the HRT shock were restored during HRT8-2. On the other hand, the variable factor map in Fig. 5b clearly showed the evolution of dominant microbial community during the shock changes in HRT. By comparing Fig. 5a and b, it showed different community pattern and predominant species at each operating condition. As discussed in functional estimation of each operation condition, community member at HRT 4 is totally compromised with homoacetogenesis which hinder methanogenesis. Communities' members from both HRT8-1 and HRT8-2 were highly compromised each other's and showed functionality balance for methanogenesis.

## 4. Conclusions

This study confirmed that the drastic HRT change has a strong influence on the microbial community structure as well as the reactor performance. A lower methane generation and VFA accumulation was observed under a reduced HRT (HRT4), where the fingerprinting patterns and its quantitative analysis also revealed a clear shift in the microbial community. In addition, the specific microorganisms identified in this study provided more detailed information on communities with a primary contribution to methane generation under HRT variables. The reactor performance was deteriorated due to the enhanced growth of homoacetotrophic sulfate reducing bacteria during HRT4 might compete with methanogen for hydrogen consumption.

## Acknowledgements

This research was supported by the Institutional Program (2E25312) of the Korea Institute of Science and Technology (KIST), South Korea and partially by the Eco-Innovation Technology Development Project (Grant No. 405-112-037) funded by the Ministry of Environment, South Korea.

## References

Ali Shah, F., Mahmood, Q., Maroof Shah, M., Pervez, A., Ahmad Asad, S., 2014. Microbial ecology of anaerobic digesters: the key players of anaerobiosis. Sci. World J. http://dx.doi.org/10.1155/2014/183752.

Bergey, D., Holt, J., Krieg, P., Sneath, P.H.A., 1994. Bergey's Manual of Determinative Bacteriology. Williams and Wilkins, Baltimore, MD, USA.

Blaszczyk, R., Gardner, D., Kosaric, N., 1994. Response and recovery of anaerobic granules from shock loading. Water Res. 28 (3), 675–680.

Chu, L.-B., Yang, F.-L., Zhang, X.-W., 2005. Anaerobic treatment of domestic wastewater in a membrane-coupled expended granular sludge bed (EGSB) reactor under moderate to low temperature. Process Biochem. 40 (3), 1063–1070.

Dar, S.A., Kuenen, J.G., Muyzer, G., 2005. Nested PCR-denaturing gradient gel electrophoresis approach to determine the diversity of sulfate-reducing bacteria in complex microbial communities. Appl. Environ. Microbiol. 71 (5), 2325–2330.

Dereli, R.K., Ersahin, M.E., Ozgun, H., Ozturk, I., Jeison, D., van der Zee, F., van Lier, J. B., 2012. Potentials of anaerobic membrane bioreactors to overcome treatment limitations induced by industrial wastewaters. Bioresour. Technol. 122, 160–170.

Diez, B., Pedros-Alio, C., Marsh, T.L., Massana, R., 2001. Application of denaturing gradient gel electrophoresis (DGGE) to study the diversity of marine picoeukaryotic assemblages and comparison of DGGE with other molecular techniques. Appl. Environ. Microbiol. 67 (7), 2942–2951.

Douterelo, I., Boxall, J.B., Deines, P., Sekar, R., Fish, K.E., Biggs, C.A., 2014. Methodological approaches for studying the microbial ecology of drinking water distribution systems. Water Res. 65, 134–156.

Dworkin, M., Falkow, S., 2006. The Prokaryotes: Proteobacteria: Gamma Subclass, vol. 6. Springer.

Gao, D.-W., An, R., Tao, Y., Li, J., Li, X.-X., Ren, N.-Q., 2011. Simultaneous methane production and wastewater reuse by a membrane-based process: evaluation with raw domestic wastewater. J. Hazard. Mater. 186 (1), 383–389.

Garrity, G.M., Bell, J.A., Lilburn, T.G., 2004. Taxonomic Outline of the Prokaryotes. Bergey's Manual of Systematic Bacteriology. Springer, New York, Berlin, Heidelberg.

Heuer, H., Smalla, K., van Elsas, J.D., Trevors, J.T., Wellington, E.M., 1997. Application of denaturing gradient gel electrophoresis and temperature gradient gel electrophoresis for studying soil microbial communities. Mod. Soil Microbiol., 353–373.

Khanal, S.K., 2008. Overview of anaerobic biotechnology. Anaerobic Biotechnology for Bioenergy Production: Principles and Applications, vol. 20. Wiley-Blackwell Publishing, New York, pp. 385–406.

Kim, J., Shin, J., Kim, H., Lee, J.-Y., Yoon, M.-H., Won, S., Lee, B.-C., Song, K.G., 2014. Membrane fouling control using a rotary disk in a submerged anaerobic membrane sponge bioreactor. Bioresour. Technol. 172, 321–327.

Kindt, R., Coe, R. 2005. Tree Diversity Analysis: A Manual and Software for Common Statistical Methods for Ecological and Biodiversity Studies. World Agroforestry Centre.

LaPara, T.M., Nakatsu, C.H., Pantea, L.M., Alleman, J.E., 2002. Stability of the bacterial communities supported by a seven-stage biological process treating pharmaceutical wastewater as revealed by PCR–DGGE. Water Res. 36 (3), 638–646.

Li, P., Wang, Y., Liu, K., Tong, L., 2010. Bacterial community structure and diversity during establishment of an anaerobic bioreactor to treat swine wastewater. Water Sci. Technol. 61 (1), 243–252.

Liu, Y., Whitman, W.B., 2008. Metabolic, phylogenetic, and ecological diversity of the methanogenic archaea. Ann. N. Y. Acad. Sci. 1125 (1), 171–189.

Lovley, D.R., 1985. Minimum threshold for hydrogen metabolism in methanogenic bacteria. Appl. Environ. Microbiol. 49 (6), 1530–1531.

Marzorati, M., Wittebolle, L., Boon, N., Daffonchio, D., Verstraete, W., 2008. How to get more out of molecular fingerprints: practical tools for microbial ecology. Environ. Microbiol. 10 (6), 1571–1581.

Nauhaus, K., Albrecht, M., Elvert, M., Boetius, A., Widdel, F., 2007. In vitro cell growth of marine archaeal–bacterial consortia during anaerobic oxidation of methane with sulfate. Environ. Microbiol. 9 (1), 187–196.

Nubel, U., Engelen, B., Felske, A., Snaidr, J., Wieshuber, A., Amann, R.I., Ludwig, W., Backhaus, H., 1996. Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. J. Bacteriol. 178 (19), 5636–5643.

Ovreas, L., Forney, L., Daae, F.L., Torsvik, V., 1997. Distribution of bacterioplankton in meromictic Lake Saelenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. Appl. Environ. Microbiol. 63 (9), 3367–3373.

Patil, S.S., Kumar, M.S., Ball, A.S., 2010. Microbial community dynamics in anaerobic bioreactors and algal tanks treating piggery wastewater. Appl. Microbiol. Biotechnol. 87 (1), 353–363.

Salazar-Peláez, M., Morgan-Sagastume, J., Noyola, A., 2011. Influence of hydraulic retention time on UASB post-treatment with UF membranes. Water Sci. Technol. 64 (11), 2299–2305.

Sekiguchi, Y., Kamagata, Y., Harada, H., 2001. Recent advances in methane fermentation technology. Curr. Opin. Biotechnol. 12 (3), 277–282.

Singka, D., Kumdhitiahutsawakul, L., Rekkriangkrai, P., Pathom-aree, W., 2012. A simple method for DNA extraction from activated sludge. Chiang Mai J. Sci. 39 (1), 111–118.

Skillman, L.C., Evans, P.N., Naylor, G.E., Morvan, B., Jarvis, G.N., Joblin, K.N., 2004. 16S ribosomal DNA-directed PCR primers for ruminal methanogens and identification of methanogens colonising young lambs. Anaerobe 10 (5), 277–285.

Talbot, G., Topp, E., Palin, M., Masse, D., 2008. Evaluation of molecular methods used for establishing the interactions and functions of microorganisms in anaerobic bioreactors. Water Res. 42 (3), 513–537.

Theron, J., Cloete, T., 2000. Molecular techniques for determining microbial diversity and community structure in natural environments. Crit. Rev. Microbiol. 26 (1), 37–57.

Visvanathan, C., Abeynayaka, A., 2012. Developments and future potentials of anaerobic membrane bioreactors (AnMBRs). Membr. Water Treat. 3 (1), 1–23.

Whitman, W.B., Parte, A.C., 2009. Systematic Bacteriology. Springer.

Wikstrom, P., Andersson, A.C., Forsman, M., 1999. Biomonitoring complex microbial communities using random amplified polymorphic DNA and principal component analysis. FEMS Microbiol. Ecol. 28 (2), 131–139.

Yoo, R., Kim, J., McCarty, P.L., Bae, J., 2012. Anaerobic treatment of municipal wastewater with a staged anaerobic fluidized membrane bioreactor (SAF-MBR) system. Bioresour. Technol. 120, 133–139.

Zhou, M., McAllister, T., Guan, L., 2011. Molecular identification of rumen methanogens: technologies, advances and prospects. Anim. Feed Sci. Technol. 166, 76–86.

Zinatizadeh, A., Mohamed, A., Najafpour, G., Hasnain Isa, M., Nasrollahzadeh, H., 2006. Kinetic evaluation of palm oil mill effluent digestion in a high rate up-flow anaerobic sludge fixed film bioreactor. Process Biochem. 41 (5), 1038–1046.