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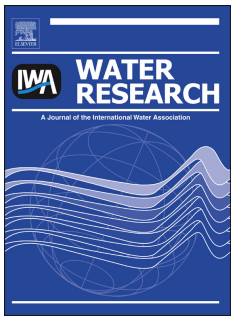

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Quantitative and qualitative analysis of methanogenic communities in mesophilically and psychrophilically cultivated anaerobic granular biofilims

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ABSTRACT

Anaerobic granulation describes the self-immobilisation of methanogenic consortia into dense, particulate biofilms. This procedure underpins the operation of several categories of high-rate anaerobic wastewater treatment system. Full-scale anaerobic granular sludge plants have been generally operated in the mesophilic (20-45°C) or thermophilic (45-65°C) temperature range. On the other hand, recent studies highlighted the economic advantages of treating wastewaters at their discharge temperatures (mostly under 18°C), removing a costly heating process and increasing net biogas yield. However, as yet, relatively little information is available about the microbial behaviour and interactions in anaerobic granular sludge formed under psychrophilic conditions. To this end, and in order to provide a microbial insight into low-temperature anaerobic granulation, we monitored the changes in methanogenic community structure, associated with the changes in process performance. Three, laboratory-scale, expanded granular sludge bed (EGSB) bioreactors treating a synthetic glucose wastewater were tested at two temperatures of 37 ± 1 °C (R1) and 15 ± 1 °C (R2 and 3). Quantitative real-time PCR and specific methanogenic activity assays highlighted a community shift towards hydrogenotrophic methanogens, particularly the order *Methanomicrobiales* in the low-temperature bioreactors. Corresponding to this, denaturing gradient gel electrophoresis (DGGE) analysis identified the emergence and maintenance of a *Methanocorpusculum*-like organism. Our results indicate that hydrogenotrophic methanogens, particularly the *Methanomicrobiales*-related populations, are likely to play important roles in low-temperature anaerobic granular sludge systems. This suggests that the process efficiency could be improved by facilitating the growth and retention of this group.

Keywords: DGGE; Low-temperature anaerobic digestion; Granular biofilm; Methanogenic community, Quantitative real-time PCR

1. Introduction

Anaerobic digestion is an attractive option for treating wastewaters containing biodegradable organic matter due to the ability to reduce pollution load and produce energy simultaneously. Wastewater carbon is converted to biogas, mainly methane, and in effect the treatment process becomes a net producer of energy (Liu et al., 2003; Hulshoff Pol et al., 2004; Collins et al., 2006). Additionally, anaerobic digestion does not require costly aeration and produces less sludge for disposal, indicating that it is generally more economically efficient than aerobic systems (McCarty, 2001). Anaerobic granular biofilms are formed via the self-immobilisation of the microbial populations distributed within the domains *Archaea* and *Bacteria*. The biofilm structure, particularly in granular sludge, has underpinned the development of several categories of high rate anaerobic wastewater treatments conferring a number of advantages over conventional processes (McCarty, 2001).

Most full-scale anaerobic digesters are operated at mesophilic or thermophilic temperatures (Liu et al., 2003; Hulshoff Pol et al., 2004). Ambient or sub-ambient treatments were considered unattractive due to lower microbial activity and increased viscosity of liquids (Rebac et al., 1999; Lettinga et al., 2001). However, the majority of industrial effluents are discharged at ≤ 18°C (Connaughton et al., 2006a), resulting in considerable heating costs for mesophilic or thermophilic operation. Therefore, economic feasibility of anaerobic digestion could be enhanced by treating wastewaters at their discharge temperatures, resulting in a net increase in energy yield (Lettinga et al., 2001). A number of successful low-temperature anaerobic digestion trials, treating various synthetic and real wastewaters, have demonstrated robust and stable operation with comparable performance to mesophilic systems. However, these studies used anaerobic granular sludge which was pre-granulated under mesophilic

conditions, although the microbial populations gradually became psychrotolerent (Collins et al., 2005; Connaughton et al., 2006a; McHugh et al., 2006).

Most studies on the fundamental microbial interactions in anaerobic digestion have focused on bioreactors operating under mesophilic and thermophilic conditions. Previous studies revealed the importance of aceticlastic methanogens, particularly belonging to the family *Methanosaetaceae*, for the stable operation of anaerobic digesters (El-Mamouni et al., 1997; Diaz et al., 2006; Satoh et al., 2007). Efforts at altering digester operating conditions have been made to facilitate the growth and retention of high levels of *Methanosaeta* populations, greatly improving granule formation as well as process efficiency (Liu et al., 2002; Hulshoff Pol et al., 2004). Currently, there is a dearth of basic information on microbial behaviour and interactions in anaerobic granular sludges cultivated at psychrophilic temperatures. Several critical questions have yet to be addressed: "Can granular biofilms be effectively cultivated at low-temperatures?" and "What are the numerically or functionally important populations?" This study focused on these knowledge gaps.

Three lab-scale expanded granular sludge bed (EGSB) bioreactors treating a synthetic glucose wastewater were operated at mesophilic (R1, 37 ± 1 °C) and psychrophilic (R2 and 3, 15 ± 1 °C) temperatures. Formation of granular biofilm was successfully demonstrated in each bioreactor. Methanogenic community structure and population dynamics, assoicated with low-temperature granulation, was assessed by a combination of molecular and statistical tools. The qualitative and quantitative changes in methanogenic community composition were monitored using denaturing gradient gel electrophoresis (DGGE) and real-time PCR techniques.

MATERIALS AND METHODS

Operation of EGSB bioreactors

Anaerobic granular sludge (∅, 0.8-3.5 mm) was obtained from a full-scale internal circulation digester (Carbery Milk Products Ltd, Balineen, Co. Cork, Ireland) treating industrial alcohol production wastewater at 37°C. The granular biomass was crushed and graded by sieving (∅ < 0.4 mm), and subsequently 88 g of volatile solids (VS) were used to seed three identical EGSB bioreactors, with 4-L working volumes, denoted as R1 to 3. All bioreactors were operated under the same conditions except temperature. R1 was operated at 37 ± 1°C, while both R2 and 3 were maintained at 15 ± 1°C. Each system was operated at a 12-h hydraulic retention time (HRT) to treat a synthetic glucose wastewater, which was buffered at pH 7.1 ± 0.2 with $NaHCO_3$ and fortified with trace elements (mg/L): $NiCl_2 \cdot 6H_20$ (0.712), $CoCl_2 \cdot 6H_20$ (0.202), $Na_2Mo_4 \cdot 2H_20$ (0.126), $FeCl_2 \cdot 6H_20$ (0.203) and yeast extract (170). The trial was divided into 5 phases, which were characterised by changes applied to the upflow velocity or organic loading. Glucose was the sole carbon source in the wastewater and its content was adjusted with respect to the organic loading rate (OLR). An OLR of 5.8 kg chemical oxygen demand (COD)/m^3/d was applied during Phases 1 to 4, and upon the commencement of Phase 5, the rate was dropped to 2.8 kg COD/m^3/d. The bioreactor effluent and biogas were routinely sampled for COD, pH, and CH_4 content.

Specific methanogenic activity test

The specific methanogenic activity (SMA) of bioreactor biomass was determined at 30 d and 194 d, using the pressure transducer technique (Colleran et al., 1992)**.** The substrates assayed were acetate (30 mM) and H_2/CO_2 (80:20, v/v) (Colleran et al., 1992; Coates et al., 1996). Each assay was conducted with 3 g VS/L of biomass in triplicate. Each biomass sample was tested at the operating temperature of its corresponding bioreactor.

Granulation rate determination

The granulation rate was defined as the proportion of granules of which diameter is larger than 1.0 mm in the total biomass dry weight. The granulation rate of bioreactor biomass was determined every 30-35 d. Approximately 30 mL of sludge from each bioreactor was graded in a step wise manner using sieves (pore sizes, 1.4, 1.25, 1.0, 0.8, 0.6, 0.4 and 0.2 mm). Each biomass size fraction was dried at 110°C for 24 h. The granulation rate was calculated based on dry weight.

DNA extraction

Total DNA was extracted from bioreactor sludge granules (∅ > 1.0 mm) using an automated nucleic acid extractor (Magtration System 12 GC, PSS Co., Chiba, Japan) (Lee et al., 2008). Prior to extraction, each sample (0.5 g wet weight) was finely crushed using a mortar and pestle, and resuspended in 50 mL of double distilled water. A 100-µL aliquot of the resuspended biomass sample was loaded per extraction. In parallel, the VS concentration of each resuspension was measured to estimate the exact amount of biomass used for DNA extraction. The extracted DNA was eluted in Tris-HCl buffer (pH 8.0) and stored at -20°C. All extractions were carried out in duplicate.

DGGE and phylogenetic analyses

Archaeal community profiles were investigated by DGGE analysis targeting the 16S rRNA gene. Conventional PCR was performed using primers 787F and 1059R (Takai and

Horikoshi, 2000). A 40-bp GC-clamp was attached at the 5'-end of the forward primer for stabilizing the melting behaviour of the PCR products (Muyzer et al., 1993). The PCR amplification was performed as follows: initial denaturation at 95°C for 3 min; a touch-down thermal cycling of denaturation at 95°C for 1.5 min, annealing at 65 to 55°C for 1.5 min (reducing 1°C per cycle), and elongation at 72°C for 1.5 min; additional 20 cycles of 95°C for 1.5 min, 55°C for 1.5 min, 72°C for 1.5 min. A 20 μL aliquot of each PCR product was loaded onto a 10% (w/v) polyacrylamide gel containing a denaturing gradient of 30 to 60% (100% denaturant contained 7 M urea and 40% (v/v) formamide). Electrophoresis was run in a D-Code system (BioRad, Hercules, CA). The DGGE gel was ethidium bromide-stained and photographed under UV transillumination. A binary matrix was constructed from the gel image by scoring the presence or absence of each band with a 1 or 0, and then statistically analyzed based on Sorensen (Bray-Curtis) distance measure using MVSP software (Kovach Computing Services, Pentraeth, UK) to build an unweighted pair group method arithmetic mean (UPGMA) cluster tree.

In parallel, for phylogenetic analysis, bands of interest were excised from the gel using a sterile scalpel blade. For accurate correlation of band homology between two DGGE gels, a selected number of bands (i.e., J1, J5 and J7) were double-checked from both gels (Fig 2). The excised bands were eluted in 25 μL of sterile water, and then reamplified with the same primers (without GC-clamp). The PCR products were gel-purified and cloned into the TOPO TA 2.1 vector (Invitrogen, Carlsbad, CA). The sequence of each PCR fragment was determined by sequencing with M13 primer and compared against the GenBank and RDP II databases. A neighbour-joining tree was constructed using MEGA 4 software (Tamura et al., 2007). All nucleotide sequences reported in this study were deposited in the GenBank database under accession numbers FJ657373-FJ657380.

Real-time PCR analysis

Real-time PCR was performed with five primer/probe sets (Table 1), which cover most methanogens present in anaerobic digesters (Yu et al., 2005; Lee et al., 2008), using a LightCycler 1.2 (Roche, Mannheim, Germany). Each reaction mixture was prepared using the LightCycler TaqMan Master kit (Roche): 8 μL of PCR-grade water, 1 μL of the probe (final concentration 200 nM), 1 μL of each primer (final concentration 500 nM), 4 μL of 5× reaction solution, and 5 μL of DNA template. Amplification was carried out using a two-step thermal cycling protocol consisted of 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).

Quantitative standard curves were constructed as previously described (Yu et al., 2006) using the representative strains corresponding to each primer/probe sets used (Table 1). A total of 12 standard plasmids containing the full-length 16S rRNA gene sequences of the representative strains, were kindly provided by Prof. S. Hwang, POSTECH, Korea (Yu et al., 2005). For each set, an equimolar mixture of its corresponding plasmids was used as the standard solution. The mass concentration of each standard plasmid was measured in duplicate using a Qubit system (Invitrogen) and converted to its copy concentration as previously described (Lee et al., 2006; Yu et al., 2006). For each standard solution, a 10-fold serial dilution series of 10^1 to 10^9 copies/μL was generated and analyzed by real-time PCR in triplicate with its corresponding primer/probe set. The threshold cycle (C_T) values determined were plotted against the logarithm of their input copy concentrations. The 16S rRNA gene copy concentrations of target groups were then estimated against the corresponding standard curves within the linear range ($r^2 > 0.995$). The volume based concentration (copies/μL) was converted to the granule biomass based concentration (copies/g granule VS) using the VS

concentration of each granular sludge sample used for DNA extraction. All DNA samples from each bioreactor were analyzed with each primer/probe set in duplicate.

Analytical methods

Solids and COD were analyzed following the procedure in Standard Methods (APHA-AWWA-WEF, 2005). Methane content was determined using a Philips PYE unicam series 304 gas chromatograph (Cambridge, UK) equipped with a packed capillary column (Propak Q 100-120 mesh). Nitrogen was used as a carrier gas at a flow rate of 60 mL/min. All samples were analysed in duplicate.

RESULTS

Bioreactor performance

Fig. 1 shows the COD removal efficiency profiles of the bioreactors R1 to 3 during the trial (i.e., Operational Phases 1 to 5). The operating parameters and performances are summarized in Table 2. The methane content was maintained at around 59-72% in R1 through out the trial, whereas it increased from 32-34% to 60-65% upon completion of the trial in R2 and 3 (Table 2). During the start-up period (Phase 1), COD removal efficiency remained at around 90% or higher after 20 d of operation in R1, whereas only approximately 60% removal was observed in both R2 and 3. Within the same phase, the granulation ratio was also significantly higher in R1 (63%) than in R2 and 3 (18-22%). This trend in granulation continued during Phase 2, where the upflow velocity was increased from 0.17 to 0.5 m/h. During this phase, the R1 granulation rate increased to 78% but low rates of 19-20% were still maintained in R2 and R3.

During the Phases 2 to 5, the COD removal efficiency in R1 mostly remained above 95%. The upflow velocity was adjusted and maintained at 1 m/h during Phase 3, which was marked by a significant drop in COD removal efficiency (< 50%) in R2 and 3 (Fig. 1). In this phase, the sludge beds in both bioreactors were highly compacted and pistoned severely. This was likely caused by the prevailing liquid viscosity at the low operating temperature, which probably resulted in cumulative compaction of the sludge bed during the previous 90 days of operation. During Phase 4, the upflow velocity was doubled to 2 m/h and the low-temperature bioreactors recovered from performance deterioration and exhibited 60-70% of COD removal efficiencies. Correspondingly, the granulation ratio, which was maintained at approximately 20% during Phase 1 to 3, also increased to between 29 and 33% in this phase. In Phase 5, the OLR was decreased by 50% to 2.9 kg COD/m^3/d and R1 kept a high bioreactor performance of > 95% COD removal. On the other hand, the COD removal efficiencies in R2 and 3 were ≤ 80% (Fig 2). A notable finding was the significant increase in the ratio of hydrogenotrophic to aceticlastic SMA values in the low-temperature bioreactors (Table 2). Throughout the trial, the ratio increased by between 2.1 and 3.3 fold in R2 and 3, whereas no significant change occurred in the ratio of the mesophilic R1 (Table 2). Furthermore, the ratios measured in Phase 1 highlighted that hydrogenotrophic activity was likely to be significantly higher (2.5-3.3 fold) than aceticlastic activity under low-temperature conditions, even in the early stage of operation (Table 2).

Archaeal DGGE profiles

Comparative PCR-DGGE analysis of the methanogenic granular biofilm communities under mesophilic and psychrophillic conditions identified temperature-dependent differences in the community structures (Fig. 2). The R1 mesophilic community displayed no significant

temporal variation and the community profiles analysed at different time points throughout the operation clustered closely together with ≥ 92% similarity. This implies that the qualitative methanogenic community structure (i.e., population diversity) in R1 was reasonably consistent, despite the modification of operating parameters, throughout the trial. The DGGE profiles from two low-temperature bioreactors (i.e., R2 and 3) were highly similar to each other and clustered closely together at each sampling point (Fig 2). A total of 8 bands (J1 to 8) were retrieved from the archaeal DGGE gel and several ribotypes were found (Fig 3). J1, which appeared at 30 d in R1 and at 90 d in R2 and 3, was closely related to *Methanocorpusculum parvum*, with 99.6% similarity (Table 3). This band also corresponded to two other species within the same genus, *M. bavaricum* and *M. labreanum*. J2 to 4 were only detected in R1 from 30 d onwards and showed 95.5 to 100% similarity to *Methanospirillium hungatei*. J8, which appeared only in R2 and 3 from 120 d onwards, was closely related to two *Methanosarcina* species, *M. mazei* and *M. lacustris*, with 100% similarity. The remaining three bands were commonly detected in all DGGE lanes (Fig 2). J5 and 6 were related to *Methanosaeta concilii* (98.5-98.8%) and J7 was related to *Methanobacterium beijingeise* (100%; Table 3). Consequently, 5 out of the 8 deduced sequences were affiliated with hydrogenotrophic methanogens (J1 to 4 and 7) and the remaining three were with aceticlastic methanogens (J5, 6 and 8).

Quantitative analysis of methanogenic community

The real-time PCR results showed clear changes in the quantitative composition of the methanogenic community of each bioreactor over time (Fig. 4). In the seed sludge, in terms of 16S rRNA gene concentration, the aceticlastic family *Methanosaetaceae* was absolutely dominant (98% of the measured methanogenic population, i.e., the sum of all 16S rRNA gene

concentrations quantified with the primer/probe sets used). This corresponds to the previously reported high abundance of *Methanosaetaceae*-related species in stable granular anaerobic digesters (Diaz et al., 2006; Satoh et al., 2007; Fernandez et al., 2008). In R1, the *Methanosaetaceae* 16S rRNA gene concentration decreased by about 100 folds, along with a 100-fold increase in *Methanobacteriales*, after 30 d of operation. The *Methanosaetaceae* concentration increased again up to 8.5×10^{10} copies/g granule VS and remained at around 10^{10} level. On the other hand, *Methanobacteriales* showed no significant change during the trial and remained at a reasonably constant level. *Methanomicrobiales* also showed a considerable increase, particularly after 30 d, and the three methanogenic groups detected in R1 showed similar 16S rRNA gene levels of 2.9 to 4.7×10^{10} copies/g granule VS at trial completion at 194 d. Interestingly, no detectable level of *Methanosarcinaceae* population was found in R1, which maintained a high treatment efficiency throughout the trial (Table 2).

The two low-temperature bioreactors showed similar patterns in quantitative community dynamics. Contrary to R1, *Methanosaetaceae* was missing during the early period and *Methanosarcinaceae* was detected in the later period of operation in R2 and 3. A notable difference between the mesophilic and psychrophilic bioreactors is the dramatic drop and regrowth of the hydrogenotrophic order *Methanomicrobiales*, becoming the most abundant group in the later period under low-temperature conditions. In both R2 and 3, its 16S rRNA gene concentration increased up to about 1.0×10^{11} copies/g granule VS and comprised 66.7-77.2% of the total measured methanogenic population at the end of the trial. In comparison, the other three methanogenic groups were detected at abundances of around 1.0×10^{10} copies/g granule VS.

Consequently, our quantification results clearly demonstrated the significantly higher abundance of hydrogenotrophic populations, particularly *Methanomicrobiales* group, in the low-temperature bioreactors than in the mesophilic one. This indicates that the methanogenic

activities in R2 and 3 were probably significantly more dependent on hydrogenotrophic metabolism. The order *Methanobacteriales* exhibited a relatively even distribution in each bioreactor tested, and the order *Methanococcales* was not detected at any trial point. In this study, the real-time PCR detection limit for each primer/probe set was $< 10^7$ copies/g granule VS level.

DISCUSSION

High COD removal efficiency of around 95% was observed in R1 during the trial, whereas it was considerably lower (ca. 60-70%) in R2 and 3. The granulation rate was also significantly higher (ca. 2.4-4.1 fold) in R1 (Table 2). The replicated low-temperature bioreactors, R2 and 3, showed reproducible performance (Fig. 1), and the methanogenic community compositions of the reactors, qualitatively and quantitatively analyzed by DGGE and real-time PCR analyses, were also highly comparable. These results suggest that the low-temperature bioreactors were reproducible in terms of microbial dynamics as well as system performance.

The clustering analysis of the archaeal DGGE profiles demonstrated that the qualitative archaeal community structures changed significantly during the initial 90 d, and that they remained relatively constant (> 92% similarity) afterwards in R2 and 3. This notable variation during the early period of operation seems to be, at least partly, due to the adaption of methanogens from the mesophilic seed inoculum to low-temperature conditions. In R1, the greatest variation with 30% distance was observed between the seed and 30-d samples, and the community structure varied little (< 6% distance) during the remaining period. This may reflect the faster establishment of a stable granular methanogenic community, developed from the mesophilic seed inoculum, under mesophilic conditions in R1, which would be expectable.

Concerning the real-time PCR quantification, it should be mentioned that the order *Methanococcales* was not detected in any sample assayed. This absence of *Methanococcales*-related organisms was probably due to their growth requirement of high salt conditions (0.3 to 9.4% (w/v) NaCl) (Boone et al., 2001). *Methanobacteriales* was detected in the seed and all bioreactor samples. Its population varied little, except during the initial 30 d period, and was mostly maintained at above 1.0×10^{10} copies/g granule VS in all bioreactors. Among the DGGE band sequences, J7 related to *M. beijingens* was the only one affiliated with the order *Methanobacteriales* (Table 3). This species has previously been isolated from anaerobic digesters treating paper mill wastewater (Ma et al., 2005). Although its optimum growth temperature is known to be 37°C, the growth of the members of the same genus has been reported at temperatures as low as 4°C (Metje and Frenzel, 2007), supporting its potential to adapt to psychrophilic conditions.

The family *Methanosaetaceae* was the most abundant group in the seed biomass, but its quantitative dynamics differed markedly according to the operating temperature (Fig. 4). Although the two *Methanosaeta concilii*-related bands, J5 and 6, were found in all DGGE lanes (Fig. 2), the *Methanosaetaceae* 16S rRNA gene concentration was under the real-time PCR detection limit at 30 and 90 d in R2 and at 30 d in R3. Interestingly, at around 90 d, R2 and 3 exhibited virtually the only disagreement in process performance, that is, the sudden temporary increase in COD removal efficiency from < 60% to > 80% in R3 (Fig. 1). The reasons for which remain unclear, however, it was well correlated with the detection of high abundance (i.e., 1.0×10^{11} copies/g granule VS) of *Methanosaetaceae*, a dominant and vital methanogenic group under stable conditions (Hulshoff Pol et al., 2004; Diaz et al., 2006), at 90 d in R3 only. On the other hand, no apparent relationship between the biomass granulation rate and the abundance of Methanosaetaceae was found in all bioreactors, which is inconsistent with previous reports that the Methanosaeta species play a major role in anaerobic granulation

(Liu et al., 2002; Hulshoff Pol et al., 2004). Additionally, the rapid drop and rise of *Methanosaetaceae* during the early period in the low-temperature bioreactors may indicate that this group was affected by cold-adaptation and is possibly sensitive to temperature change.

The aceticlastic family *Methanosarcinaceae* appeared after 120 d of operation in R2 and 3 only, but it was not detected throughout the operation in R1 (Fig. 4). Interestingly, this group appeared after the deterioration of COD removal (≤ 24%) observed in Phase 3 (Figs. 1 and 4). This corresponds well to the fact that this group favours substrate rich environment (i.e., high organic acids levels) and grows faster than the other aceticlastic family *Methanosaetaceae* (Boone et al., 2001). Previous studies have also suggested that the members of this family are typically associated with high residual acetate concentration accompanied with process deterioration (Wiegant and Deman, 1986; Collins et al., 2003; Hulshoff Pol et al., 2004). Therefore, the appearance of this group was thought to be correlated with the bioreactor deterioration, resulting in the high accumulation of organic acids, in the low-temperature bioreactors. Corresponding to this phase, the acetate concentration in R2 and 3 was c.a. 1.2 g/L, which was almost 1000 times greater than its level in R1. Additionally, R2 and 3 exhibited 0.1-0.3 g/L of propionate and 0.05-0.1 g/L of butyrate, whereas their concentrations were <5 mg/L in R1 (data not shown). Although it has been reported that the anaerobic digesters dominated by *Methanosarcinaceae*-related organisms are also able to perform 'spontaneous granulation', this type of granular biofilm tends to be less dense and to be washed out relatively easily (Hulshoff Pol et al., 2004). The DGGE band J8 affiliated with *Methanosarcinaceae* was assigned to *M. mazei* and *M. lacustris* with 100% similarity (Table 3). *M. mazei* has been frequently observed in anaerobic digestion systems (Diaz et al., 2006) and is able to utilize a variety of substrates, including acetate, H_2/CO_2, methanol, and methylamines (Boone et al., 2001). Additionally, growth of *M. mazei*-like organisms have been reported at temperatures as

low as 5°C (Simankova et al., 2003), indicating its ability to grow under low-temperature conditions.

The most intriguing observation in this study is the quantitative variation of the order *Methanomicrobiales* particularly in the low-temperature bioreactors (Fig. 4). This group was detected in seed biomass and all bioreactor samples, but showed significantly different dynamics under mesophilic and psychrophilic conditions. *Methanomicrobiales* accounted for 1.5 to 44.3% of the total methanogenic 16S rRNA gene concentration in R1, whereas its abundance reached up to 66.7 to 77.2% in R2 and 3. This group showed 1200- to 8400-fold increase, from its minimum to maximum concentrations, in R2 and 3, whereas only a 54-fold increase was noted in R1. Such results indicate that this hydrogenotrophic order would be the key methanogenic group in the low-temperature bioreactors. In this study, it is not evident which factor mainly affected the high dominance of *Methanomicrobiales*, because the upflow velocity or OLR were changed during the 194-d experiment of cold-adaptation. However, given that a remarkable rise or high dominance of *Methanomicrobiales* was not shown in the mesophilic R1, operated with the same strategy as R2 and 3, low temperature seems to be the major factor facilitating the dominance of this group. Supporting this, several previous studies on mesophilic granular biofilms incubated at low temperatures have reported the temporal methanogenic community shifts towards the dominance of hydrogenotrophic methanogens, particularly *Methanomicrobiales* and *Methanobacteriales* (McHugh et al., 2004; Connaughton et al., 2006b). Although four DGGE band sequences (from J1 to 4) were assigned to the order *Methanomicrobiales* only J1 was detected in the low-temperature bioreactors. Interestingly, J1 was solely assigned to the genus *Methanocorpusculum*, whereas J2 to 4 were all related to the genus *Methanospirillum* (Table 3). This suggests that the *Methanomicrobiales* community composition might be affected by the operating temperature. Our observation corresponds to the previous studies reporting increased abundance of *Methanocorpusculum*-like organisms

during the cold adaptation of anaerobic granular sludge (Collins et al., 2003; McHugh et al., 2004). Given the band intensity, although not robustly quantitative, the *Methanocorpusculum*-like organism deduced from J1 was likely to be the major hydrogenotrophic population in the low-temperature bioreactors, particularly from 90 d onwards. This banding pattern is in good agreement with the quantification results of *Methanomicrobiales* (Fig. 4). On the other hand, in mesophilic R1, J1 disappeared over time even with the continuous increase in the 16S rRNA gene concentration of *Methanomicrobiales*, indicating the major population of this group was shifted to the others possibly including the *Methanospirillum*-like organisms deduced from J2 to 4 (Fig. 2).

The physiological profiles generated from the reactor biomass were in good agreement with the quantitative molecular studies. For example, the hydrogenotrophic SMA value was significantly higher than the aceticlastic activity in both R2 and 3, whereas they were evenly distributed in R1. Furthermore, the hydrogenotrophic to aceticlastic SMA value ratio increased significantly (2.1-3.3 times) in R2 and 3 (Table 2) as the trial progressed. This, together with the increase in and dominance of hydrogenotrophic groups, particularly *Methanomicrobiales*, again suggests their numerical and functional importance under low-temperature conditions. Supporting this, the dominance of hydrogenotrophs at low temperatures has been reported in several previous studies (Collins et al., 2005; Connaughton et al., 2006a; McHugh et al., 2006), where very high COD removal efficiencies (>95%) were reported during low-temperature anaerobic digestion. This phenomenon was proposed to be because hydrogen is thermodynamically and metabolically more favourable than acetate and higher level of hydrogen can be retained in the system (i.e., increased gas solubility) at low temperature (Lettinga et al., 2001; Kotsyurbenko, 2005). The fate of acetate in such systems remains a topic of considerable interest, with respect to the possible role of syntrophic actetate oxidation (Schnurer et al., 1994), which would be a subject of further investigation.

CONCLUSIONS

In this study, temporal changes in the archaeal community architecture of granular biofilms were successfully related to physiological methanogenic activity profiles and reactor operating conditions. Low temperature anaerobic digestion was shown to be reproducible, both in terms of operational performance and microbial community structure. Hydrogenotrophic methanogens, from the order *Methanomicrobiales,* emerged as a key functional group during low-temperature anaerobic digestion.

- At applied OLRs of 2.9 to 5.8 kg COD/m3/d, a moderate COD removal efficiency of 46 to 74% with a biomass granulation rate of 18 to 33% was achieved at 15 ± 1 °C, while a high treatment efficiency of 93 to 98% with a biomass granulation rate of 63 to 87% was observed at 37 ± 1 °C.
- The quantitative real-time PCR analysis highlighted the clear dominance shift from aceticlastic towards hydrogenotrophic methanogens, particularly the order *Methanomicrobiales*, in the low-temperature bioreactors.
- The SMA test results also showed the evident increase in the activity of hydrogen-mediated methanogenesis (i.e., 2.1-3.3 folds), while the level of aceticlastic activity showed no change, during the trial period.
- The DGGE banding patterns were in good agreement with the real-time PCR quantification results and suggested that a *Methanocorpusculum*-like organism was likely to be the major hydrogenotrophic population, probably mainly responsible for the dynamic changes and cold adaptation of methanogenic communities.

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Table 1: 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
MBT-set /*Methanobacteriales*	F: CGWAGGGAAGCTGTTAAGT T: AGCACCACAACGCGTGGA R: TACCGTCGTCCACTCCTT	*Methanobacterium thermoautotrophicum* (DSM1053) *Methanobrevibacter arboriphilicus* (DSM 1536)
MCC-set /*Methanococcales*	F: TAAGGGCTGGGCAAGT T: TAGCGGTGRAATGYGTTGATCC R: CACCTAGTYCGCARAGTTTA	*Methanococcus jannaschii* (DSM 2661) *Methanococcus voltae* (DSM 1537)
MMB-set /*Methanomicrobiales*	F: ATCGRTACGGGTTGTGGG T: TYCGACAGTGAGGRACGAAAGCTG R: CACCTAACGCRCATHGTTTAC	*Methanocorpusculum parvum* (DSM 3823) *Methanomicrobium mobile* (DSM 1539) *Methanospirillum hungatei* (DSM 864)
Mst-set /*Methanosaetaceae*	F: GAAACCGYGATAAGGGGA T: TTAGCAAGGGCCGGGCAA R: TAGCGARCATCGTTTACG	*Methanosaeta concilii* (DSM 2139) *Methanosaeta thermoacetophila* (DSM6194)
Msc-set /*Methanosarcinaceae*	F: TAATCCTYGARGGACCACCA T: ACGGCAAGGGACGAAAGCTAGG R: CCTACGGCACCRACMAC	*Methanosarcina acetivorans* (DSM 2834) *Methanosarcina barkeri* (DSM 800) *Methanosarcina mazei* (DSM 3647)

[a] Yu et al., 2005, Lee et al., 2009
[b] F, T, and R indicate forward primer, TaqMan probe, and reverse primer, respectively.
[c] Culture collection numbers are in parentheses.

Table 2: Bioreactor operating parameters and performance data

	Phase 1 Days 0-66			Phase 2 Days 66-90			Phase 3 Days 90-120			Phase 4 Days 120-143			Phase 5 Days 143-194		
	R1	R2	R3	R1	R2	R3	R1	R2	R3	R1	R2	R3	R1	R2	R3
OLR[a]	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8	2.9	2.9	2.9
CRE[b]	93	60	59	98	61	60	97	46	48	96	66	63	95	74	60
	± 6	± 8	± 8	± 1	± 5	± 4	± 2	± 13	± 11	± 2	± 3	± 5	± 4	± 6	± 8
V_u[c]	0.17	0.17	0.17	0.51	0.51	0.51	1.0	1.0	1.0	2.0	2.0	2.0	2.0	2.0	2.0
M%[d]	59	32	34	58	45	45	72	55	55	70	65	64	68	60	61
BGR[e]	63	18	22	78	19	20	80	20	20	80	33	29	87	19	20
HAR[f]	1.0	3.3	2.5										0.9	7.0	8.7

[a] Organic loading rate as measured in chemical oxygen demand per cubic meter per day

[b] Chemical oxygen demand removal efficiencies, determined every 2 or 3 days (average for phase ± standard deviation).

[c] Upflow velocity (m/hr).

[d] Methane composition of biogas produced, determined every 2 or 3 days.

[e] Biomass granulation rate, determined on the final day of each phase, measured as proportion (%) of biomass >1.0 mm

[f] Ratio of hydrogenotrophic to aceticlastic potential SMA values (measured as ml methane g/VSS/d), determined at day 30 and 194.

Table 3. Phylogenetic affiliation of the 16S rRna gene sequences from DGGE bands

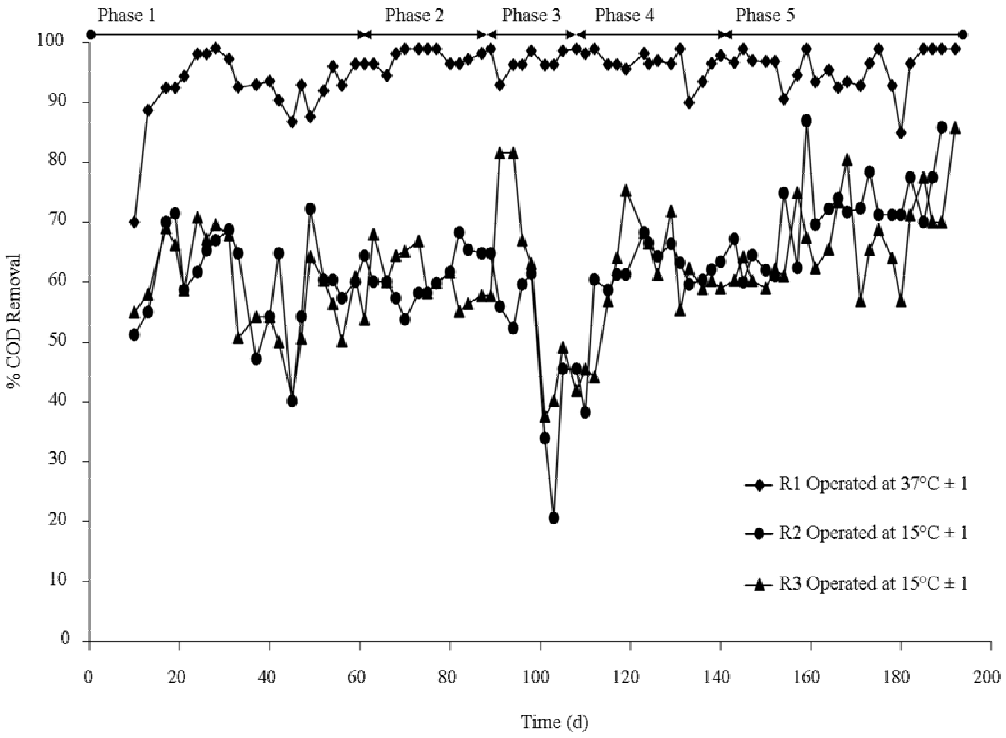
Band	Nearest species and taxon	Accession no.	% Similarity	Phylogenetic group (order / family)
J1	*Methanocorpusculum bavaricum*	AY196676	99.6	*Methanomicrobiales* / *Methanocorpusculaceae*
	Methanocorpusculum parvum	AY260435	99.6	
	Methanocorpusculum labreanum	AF095267	99.6	
J2	*Methanospirilium hungatei*	AY196683	100	*Methanomicrobiales* / *Methanospirillaceae*
J3	*Methanospirilium hungatei*	AY196683	99.6	*Methanomicrobiales* / *Methanospirillaceae*
J4	*Methanospirilium hungatei*	AY196683	95.5	*Methanomicrobiales* / *Methanospirillaceae*
J5	*Methanosaeta concilii*	X16932	98.9	*Methanosarcinales* / *Methanosaetaceae*
J6	*Methanosaeta concilii*	X16932	98.5	*Methanosarcinales* / *Methanosaetaceae*
J7	*Methanobacterium beijingense*	AY350742	100	*Methanobacteriales* / *Methanobacteriaceae*
J8	*Methanosarcina mazei*	AF411467	100	*Methanosarcinales* / *Methanosarcinaceae*
	Methanosarcina lacustris	AY260430	100	

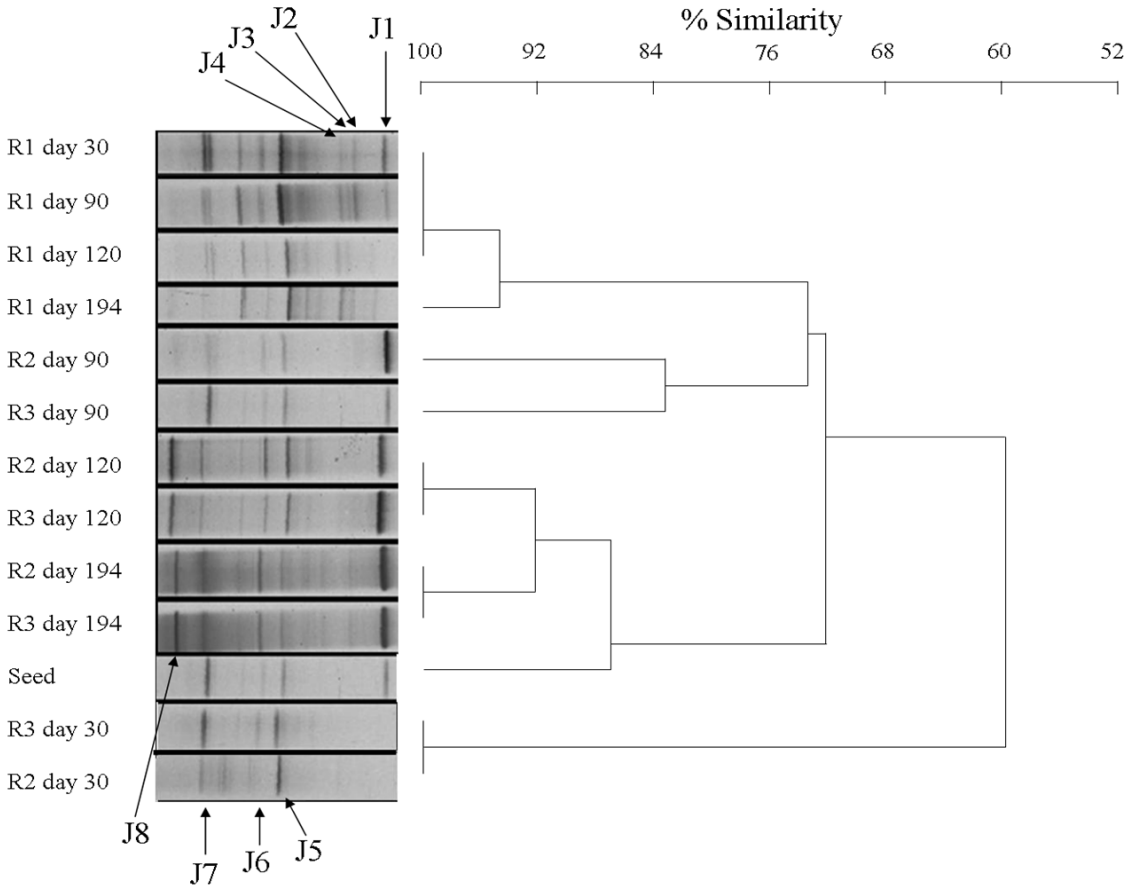
Fig. 1. COD removal efficiency in R1 to 3 during the operational period of 194 d.

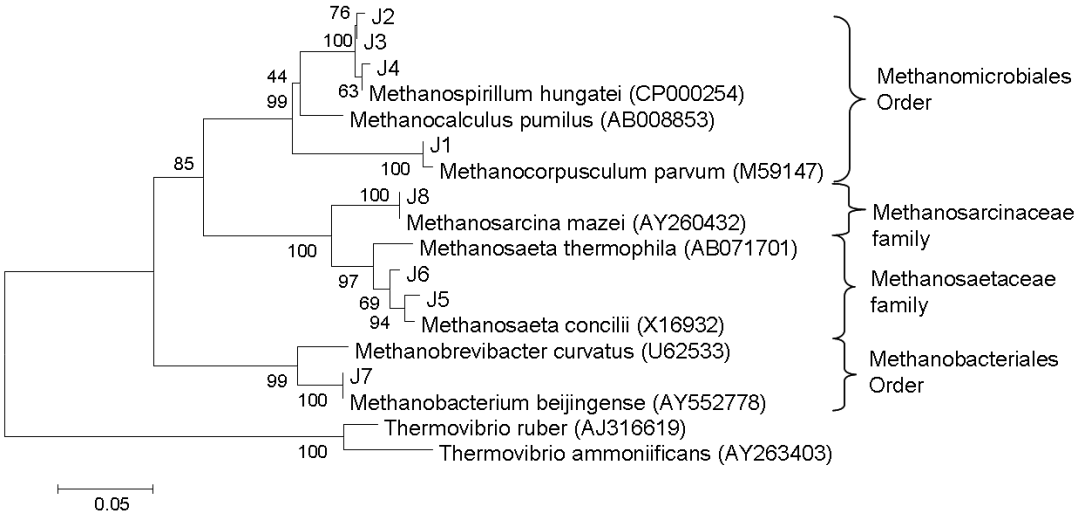
Fig. 2. Archaeal 16S rRNA gene DGGE profiles of the bioreactor granular biofilms accompanied with UPGMA cluster tree.

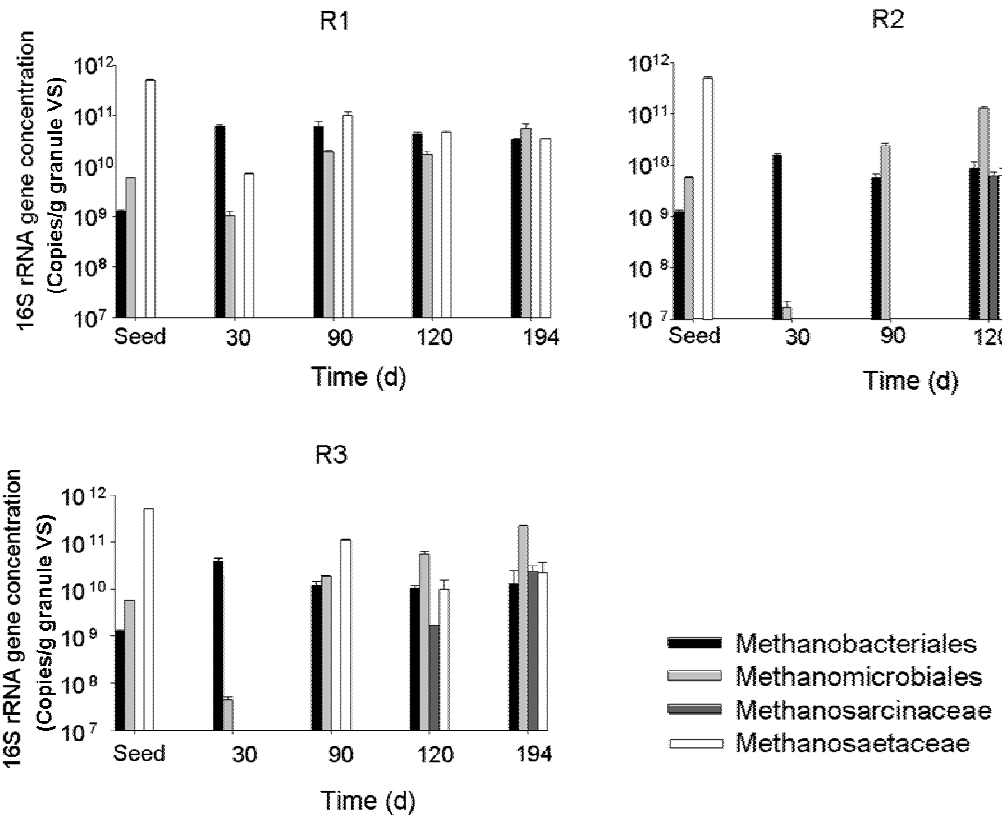
Fig. 3. Neighbor-joining tree illustrating the phylogenetic affiliations of the 16S rRNA gene sequences from DGGE bands.

Fig. 4. Quantitative changes in the 16S rRNA gene concentrations of methanogenic groups. The granular biomass samples assayed were taken from the seed inoculum (i.e., the initial point) and after 30, 90, 120 and 194 d of operation.


Phase 1
Phase 2
Phase 3
Phase 4
Phase 5
% COD Removal
Time (d)
R1 Operated at 37°C ± 1
R2 Operated at 15°C ± 1
R3 Operated at 15°C ± 1


% Similarity
100
92
84
76
68
60
52
J1
J2
J3
J4
R1 day 30
R1 day 90
R1 day 120
R1 day 194
R2 day 90
R3 day 90
R2 day 120
R3 day 120
R2 day 194
R3 day 194
Seed
R3 day 30
R2 day 30
J8
J7
J6
J5




R1
R2
R3
16S rRNA gene concentration (Copies/g granule VS)
Seed
30
90
120
194
Time (d)
Methanobacteriales
Methanomicrobiales
Methanosarcinaceae
Methanosaetaceae