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RESEARCH ARTICLE

Methanogenic community structure in simultaneous methanogenesis and denitrification granular sludge

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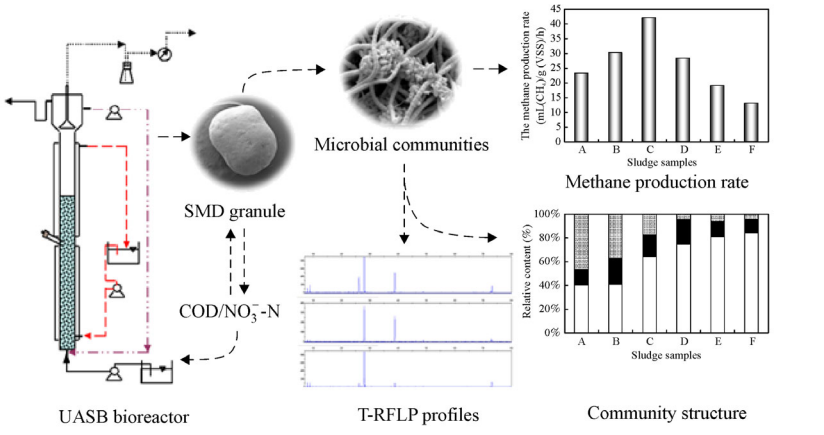
HIGHLIGHTS

- UASB with SMD granules was operated with high removal efficiency of COD and NO_3^-–N.
- *Methanosaetaceae* was absolute predominant methanogen in SMD granules.
- The methanogen quantity and activity decreased as C/N decreased from 20:1 to 5:1.
- Bacterial community succession happened with C/N decreasing.

GRAPHIC ABSTRACT



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ABSTRACT

A laboratory scale up-flow anaerobic sludge bed (UASB) bioreactor fed with synthetic wastewater was operated with simultaneous methanogenesis and denitrification (SMD) granules for 235 days with a gradient decrease of C/N. Molecular cloning, qRT-PCR and T-RFLP were applied to study the methanogenic community structures in SMD granules and their changes in response to changing influent C/N. The results indicate that when C/N was 20:1, the methane production rate was fastest, and *Methanosaetaceae* and *Methanobacteriaceae* were the primary methanogens within the *Archaea*. The richness and evenness of methanogenic bacteria was best with the highest T-RFLP diversity index of 1.627 in the six granular sludge samples. When C/N was reduced from 20:1 to 5:1, the methanogenic activity of SMD granules decreased gradually, and the relative quantities of methanogens decreased from 36.5% to 10.9%. The abundance of *Methanosaetaceae* in *Archaea* increased from 64.5% to 84.2%, while that of *Methanobacteriaceae* decreased from 18.6% to 11.8%, and the richness and evenness of methanogens decreased along with the T-RFLP diversity index to 1.155, suggesting that the community structure reflected the succession to an unstable condition represented by high nitrate concentrations.

1 Introduction

A great amount of wastewater containing high-strength organics and high-strength nitrates is generated in various industries, such as the pharmaceutical factory, dairy, fertilizer, and brewery. If the simultaneous methanogenesis and denitrification (SMD) could be achieved, then organic matter and nitrate could be removed simultaneously, which simplifies the sewage treatment process and reduces overall construction and operational costs. Recently, researchers studying the SMD process (An et al., 2008; Chen et al., 2009; He et al., 2014; Kodera et al., 2017)

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indicated that SMD could be fulfilled by granular sludge in an anaerobic bioreactor. The C/N was thought to be the critical factor affecting the system function and microbial communities (Xie et al., 2012; Akizuki et al., 2013). However, there were two completely different types of microbes involved in the SMD process: methanogenic archaea and denitrifying bacteria, which typically do not coexist. There has been little research reported on how these two different microbes might coexist and exert their function in a bioreactor. It has been presumed that this coexistence can be achieved with the biomembrane or granular sludge, both of which can form clear layers within the bioreactor (Lin and Chen, 1995). More researches are required to further the understanding of the fundamental relationships between *Archaea* and *Eubacteria* in the methanogenic community structure in a SMD granular sludge.

The objective of this study was to investigate the removal efficiency of COD and NO_3^-–N in the up-flow anaerobic sludge bed (UASB) bioreactor running a SMD process at the different influences of C/N. Moreover, the methanogenic community structure and the changes it undergoes in SMD granules and how it is affected by the C/N were investigated. In this research, the UASB bioreactor was incubated with common anaerobic granular sludge and fed with artificial wastewater containing glucose and $NaNO_3$ to domesticate the SMD granular sludge. During the whole process, molecular cloning, qRT-PCR and T-RFLP were used to investigate the methanogenic community structure and the succession process that occurs in response to the changing C/N in the influent from 20:1 to 15:1, 10:1 and 5:1. The relationship between the methanogenic community structure and the C/N were analyzed in-depth, there might be a great potential for applying SMD technology to high strength organics and nitrates in order to accomplish high removal efficiency of COD and NO_3^-–N at low cost.

2 Materials and methods

2.1 UASB operation and sludge samples

The UASB bioreactor was made of poly (methyl methacrylate) with a total height of 1.08 m and a total volume of approximately 3.0 L; the upper settle section of the reactor was 1.1 L, and the lower reactor section was approximately 1.9 L. Artificial influent with suitable proportions of glucose and $NaNO_3$ were provided as input into the reactor from its bottom by a squirm pump and flowed through the reactor section, a three-part separator, and a sedimentation section before flowing out. The gas production was captured and calculated before being emitted. The reflux was adjusted according to influent flux in order to retain the waterpower ascent flow between 0.80 m/h and 1.57 m/h. The temperature in the bioreactor was maintained at 35°C by a bath interlayer, and the pH was adjusted to 7.2–7.8 by adding $NaHCO_3$ into the influent flux. The UASB bioreactor was run for 235 days, and six sludge samples were collected in different phases and labeled as A, B, C, D, E and F. Sample A was incubated sludge, sample B was methanogenic sludge from the start-up phase of the reactor without nitrate in the influent, samples C, D, E and F were SMD sludge when the C/N was 20:1, 15:1, 10:1 and 5:1, respectively. The detailed operational conditions of the UASB bioreactor are provided in Table 1. The six sludge samples were pre-treated, then the morphology of the sludge particles and microbial communities were investigated by Scanning Electron Microscope Quanta 200 (FEI Company, USA).

2.2 Defining the biomethane potential of SMD granular sludge

The methane production rate was measured using a biomethane potential test on granular sludge as described in previous reports (Angelidaki et al., 2009).

2.3 DNA extraction and PCR amplification

The cell wall of the anaerobes in the sludge samples was broken by a cell disruptor instrument Fastprep-24 (MP Biomedicals, USA). DNA extraction and purification were completed by a DP301 kit (Tiangen Biological Company, China) and an A140-2 kit (Dingguo Biological Company, China), respectively. Next, the 16S rDNA segments of *Archaea* and *Eubacteria* were separately amplified by primers 109F-915R (5′ACKGCTCAGTAACACGT3′-

Table 1 Performances of UASB bioreactor under different C/N ratios

Phase	Time (d)	C/N	Loading rate (kg/m^3/d)		Influent strength (mg/L)		Removal efficiency (%)	
			COD	NO_3^-–N	COD	NO_3^-–N	COD	NO_3^-–N
I	0–46	–	10.0	–	3762.6	–	96.5	–
II	47–134	20:1	10.0	0.5	3762.6	188.5	97.7	100.0
III	135–152	15:1	10.1	0.7	3648.1	251.4	98.0	99.6
IV	153–219	10:1	10.7	1.1	3846.2	408.5	98.0	99.6
V	220–234	5:1	10.6	2.0	2216.9	406.4	96.0	100.0

Notes: The data in the table were all average value during each operation phase

5′GTGCTCCCCCGCCAATTCCTT3′) and 8F-1492R (5′ AGAGTTTGATCCTGGCTCAG3′-5′GGTTACCTTGTTACGACTT3′), respectively.

2.4 Clone library construction

A clone library was constructed for sample D (C/N was 15:1). The purified 16S rDNA segments were first ligated into the pGEM-T vector system, transformed into competent cells of *E. coli* DH5α and spread on Luria-Bertani plate with 0.5 mmol/L IPTG, 0.080 mg/mL X-GAL and 0.100 mg/mL amperil. The RsaI and MspI restriction endonuclease (Toyota Biological Company, Japan) were applied to cut the amplified fragments of *Eubacteria*. The PCR products were subjected to electrophoresis and then sequenced by an ABI sequencer according to the manufacturer's instructions.

2.5 QRT-PCR and establishment of standard curve

The primers Arc109f–Arc344r (Grosskopf et al., 1998), P338f–P518r (Ovreås et al., 1997), 331f–518r and 518f–MX825r (Stahl and Amann, 1998) were applied to quantify *Archaea*, *Eubacteria*, *Mehanobacteria* and *Methanosaeta* with Sybr Green florescent dye, respectively. The qRT-PCR optimum parameters were initial denaturation for 3 min at 94°C followed by 32 cycles (for 10 s at 94°C, for 20 s at 57°C and for 30 s at 72°C). The *Archaea* standard sample was made of T plasmids from *Methanobacteriaceae Archaeon*, *Methanosaeta concilli* at a proportion of 1:2 (according to the molecular cloning results). *Eubacteria* was from *Sulfurospirillum sp.*, uncultured *Bacteroidetes*, *Streptococcus suis* with a proportion of 1:1:4, and *Methanobacteria* was from *Methanobacteriaceae Archaeon*. The correlation coefficients of all the standard curves were more than 0.98.

2.6 T-RFLP analysis of community structure

The target fragment of template DNA was amplified by PCR using fluorescence-marked primers (109f–915r-FAM) and purified and processed by restriction endonuclease TaqI (Toyota Biological Company, Japan). The products were further purified to remove salt ions, and a standard sample and formamide were added before denaturation at 95°C for 5 min. T-RFLP analysis was done by a gene analysis instrument (ABI 3730 DNA Analyzer, USA).

3 Results and discussion

3.1 Performance of the UASB bioreactor and SMD granular sludge

The UASB bioreactor was operated for 235 days, which was divided into 5 phases I–V in this paper, and the performance was illustrated in Fig. 1. After the starting period (phase I), the sludge COD loading rate increased quickly to approximately 10 kg/m^3/d, and the COD removal efficiency was stable above 95% in the whole process. Nitrate was introduced into the bioreactor on the 46th day with the loading rate of 0.5 kg/m^3/d, and nitrate concentration in the influent water was increased stepwise in the succeeding days at 0.7, 1.1, to the final concentration of 2.0 kg/m^3/d (as shown in Table 1). The COD removal

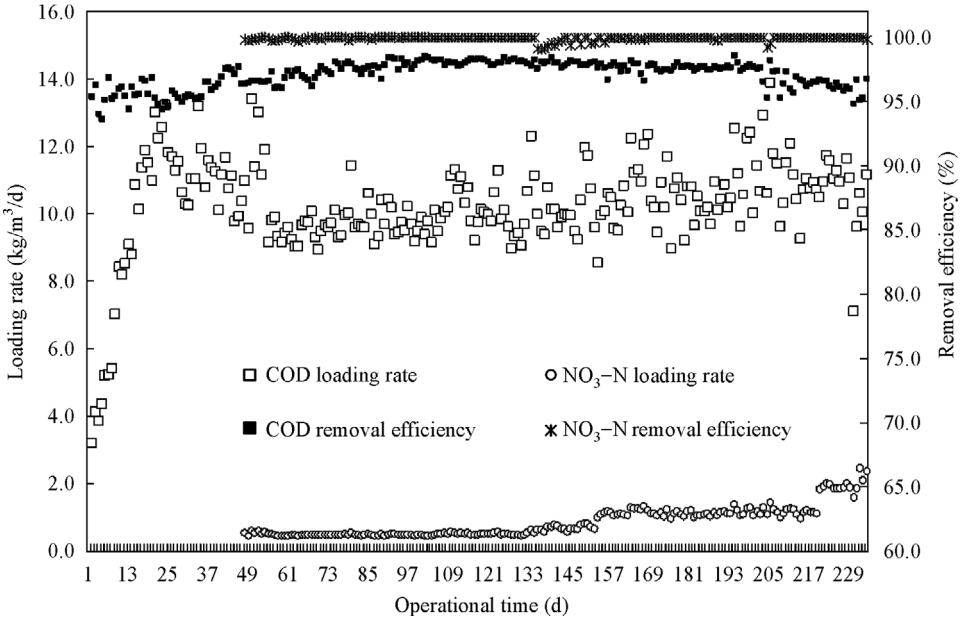

Fig. 1 Changing of loading rate and removal efficiency of COD and NO_3^-–N in the UASB bioreactor for 235 days

efficiency was not affected by nitrate and it was maintained above 97% when C/N was gradually decreased from 20:1 to 15:1 and 10:1 (phases II, III and IV). When the C/N was 5:1 (phase V), the COD removal efficiency declined slightly, but it was mostly above 96%. The NO_3^-–N removal efficiency was excellent, remaining above 99% in phases III–V after nitrate had been added in the influent water.

Upon visual inspection, the incubated sludge was deep-black in color, mostly irregularly spherical. The SEM images of six sludge samples are shown in Fig. 2. Compared with the incubated sludge (sample A), the microbial quantities in granular sludge (sample B) increased greatly although the morphological types consisting mainly of filamentous bacteria, which were predominant outside the granules (Wang et al., 2016), cocci and bacilli did not change considerably. After nitrate was added to bioreactor (samples C, D, E and F), both microorganism quantities and types increased markedly. This significant increase was also reported by other researches (Yi et al., 2017; Yin et al., 2017). In addition to filamentous bacteria, cocci and bacilli, a large number of streptococci were detected on the sludge particle surfaces. Additionally, aside from the large number of cocci and bacilli, a small number of spirilla was found inside the sludge particles. As the ratios of C/N decreased from 20:1 to 15:1, 10:1 and 5:1, the sludge surfaces became rougher, the quantities of streptococci and filamentous bacteria decreased, and the quantities of cocci and bacilli increased. In addition, streptococci and filamentous bacteria were primarily on the surfaces of sludge particles and they were seldom found inside the particles.

3.2 Biomethane potential of SMD granular sludge

The methanogenic activity among sample A–F (shown in Fig. 3) shows that after nitrate was added to the bioreactor, the methanogenic activity of the samples (sample C) was higher than that of the incubated sludge (samples A and B). When C/N was reduced from 20:1 to 15:1, 10:1 and 5:1, the methanogenic activity of the samples also decreased gradually. When the C/N was 10:1 (sample E) and 5:1 (sample F), the methanogenic activity was lower than the activity of the incubated sludge (sample A). These results indicate that nitrate at ambient concentration was effective for the methanogenic activity, while higher nitrate concentrations may inhibit the methanogenic activity of samples. It has been widely reported that nitrate strongly inhibits the methanogenic activity and denitrification occurs before methanogenesis, and the methanogenic process could only occur after denitrification has been completed (Mosquera-Corral et al., 2001; Tugtas and Pavlostathis, 2007;2008). However, the inhibition mechanism of nitrate or denitrification on methanogenesis process has not been thoroughly elucidated to date. It has generally been considered that 1) because the methanogens are strict anaerobe, the presence of NO_3^- and its reduced intermediate products NO_2^-, NO and N_2O leads to higher redox potentials that are not conducive to the growth of methanogens and methanogenic activity (Tugtas and Pavlostathis, 2007; Banihani et al., 2009); 2) denitrifiers would compete against methanogens for hydrogen and acetate (Klüber and Conrad, 1998); 3) NO_2^- and NO_3^- would generate toxic effects on the enzymes involved in the methanogenic process, with NO_2^- being more poisonous than NO_3^- (Chen and Lin, 1993). However, when the carbon source is easily metabolized and the C/N is suitable, these conditions would not inhibit either the methanogenic or denitrification activity (Hendriksen and Ahring, 1996). In the UASB bioreactor system the reduction of NO_3^-–N was very complete; therefore, it could be speculated that NO_3^-–N would not inhibit and

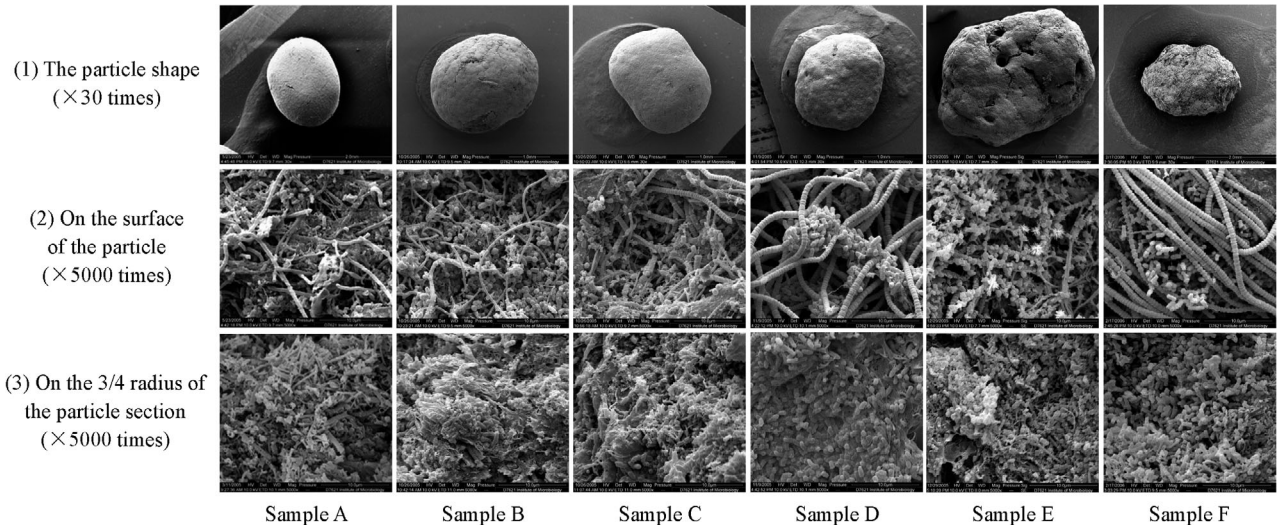

Fig. 2 SEM images of sludge particles and microbial communities

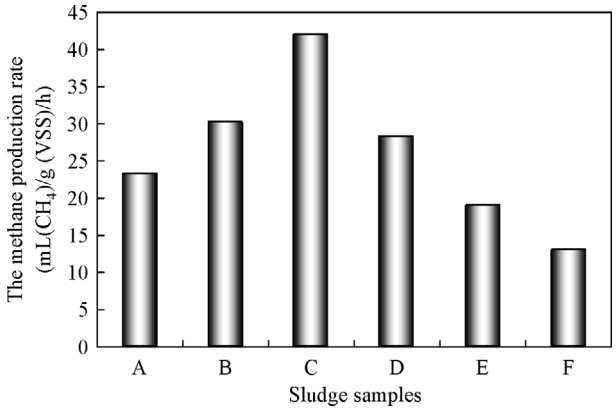

Fig. 3 Methane production rate in biomethane potential test of SMD granular sludge samples

poison methanogens, and the main interaction between denitrifying bacteria and methanogens is the competition for hydrogen and acetate in the system.

3.3 Construction of 16S rDNA clone library

A clone library was constructed for sample D (C/N was 15:1) as the representative sample in order to more accurately assess the microbial community structure in SMD granular sludge and to quantify the proportion of *Methanobacteriaceae Archaeon* and *Methanosaeta concilli* in *Archaea* standard sample for qRT-PCR. After the DNA extraction, the molecular cloning yielded 88 clones that represented 32 OTUs of *Archaea* based on the PCR and ARDRA tests. The 32 OTUs were compared to describe bacterial 16S rDNA in GenBank by the BLAST procedure, and the results are shown in Table 2. In sample D, all the *Archaea* clones were from methanogens and could be classified into 4 main groups, namely, *Methanosaetaceae*, *Methanobacteriaceae*, *Methanospirillaceae* and *Methanomicrobiaceae*, and most of the matches were to uncultured sequences. There were 19 OTUs from 63 clones belonged to *Methanosaetaceae* that accounted for 71.59% of the *Archaea* clones, making *Methanosaetaceae* the most predominant methanogen in the sample; the most abundant clone, A3 (16/88), was most similar to *Methanosaeta concilli.* The abundance of *Methanosaetaceae* could be beneficial for granule formation and maintenance (Song et al., 2010). The second most abundant kind of *Archaea* was represented by 20 clones (22.73%) and 9 OTUs belonging to *Methanobacteriaceae*. There were several clones belonging to *Methanospirillaceae* and *Methanomicrobiaceae*.

Methanosaetaceae is one of the most predominant methanogens in most anaerobic methanogenic granular sludge, and it accounts for 35%–75% of *Archaea* generally (Liu et al., 2002). In the SMD granular sludge, a high concentration of NO_3^-–N would have influenced the growth of methanogens and only four types of methanogens survived (as shown in Table 2). *Methanosarcinaceae*, which often appears in anaerobic acetic-trophic methanogenic granular sludge, was not detected in SMD granular sludge. Both *Methanosarcinaceae* and *Methanosaetaceae* are representatives of acetic-trophic methanogens. *Methanosarcinaceae* have a low affinity for acetate and therefore grow in high acetate concentrations, while the specific surface area of *Methanosaetaceae* is high and its affinity for the matrix is strong, thus, *Methanosaetaceae* grows well in low-acetate conditions. In the UASB bioreactor, more than 95% COD removal efficiency was attained, indicating that the acetate which was produced by fermentative and acetogenic microorganism was utilized by methanogens rapidly and accumulated only slightly. Therefore, the growth of *Methanosarcinaceae* was inhibited and the growth of *Methanosaetaceae* was promoted.

3.4 Analysis of *Archaea* 16S rDNA by qRT-PCR

The relative quantity of *Archaea* and *Bacteria* in SMD granules and *Methanosaetaceae* and *Methanobacteriaceae* within the *Archaea* of samples A–F were analyzed by qRT-PCR. As shown in Fig. 4, *Archaea* was significantly less than *Bacteria* in samples A–F indicating that the content of methanogens was much less than *Bacteria* in SMD granular sludge because all of the *Archaea* clones belonged to methanogens according to the clone library results. In addition, when C/N was reduced from 20:1 to 5:1 (samples C–F), the relative abundance of *Archaea* in SMD granules decreased from 36.5% to 10.9%. As the content of nitrate increased gradually, the quantity of organic matter used for denitrification also increased, leading to a corresponding reduction of organic matter used by methanogens. Although several researchers (Liang and Zuo, 2008) believe that certain *Archaea* have a denitrification function, it is generally considered that denitrifiers are either *Fungi* or *Bacteria*. Therefore, with the increase of nitrate, the methanogens decreased, the denitrifying bacteria increased, and the *Archaea* declined rapidly in samples D, E and F.

Table 2 Clone results of *Archaea* in granules of sample D

Flora classification (*Archaea*)	OTUs quantity	Clone quantity	Content (%)	Representative strain
Methanosaetaceae	19	63	71.59	*Methanosaeta concilli* (X51423)
Methanobacteriaceae	9	20	22.73	*Unculture Methanobacteriaceae Archaeon* (AB236056)
Methanospirillaceae	3	4	4.55	*Unculture Methanoaspirillum* sp. (AY692060)
Methanomicrobiaceae	1	1	1.14	*Unculture Methanomicrobiaceae Archaeon* (AB236985)

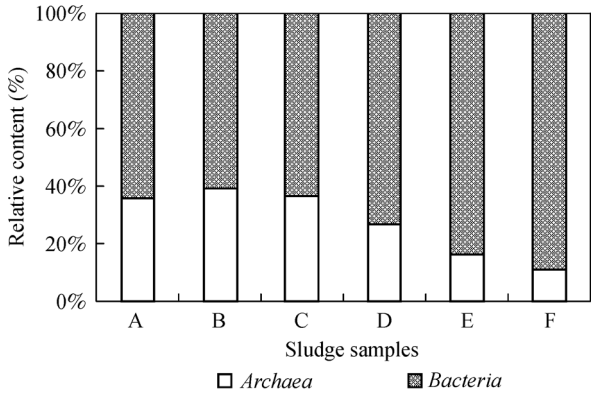

Fig. 4 Relative content of *Archaea* and *Bacteria* in SMD granular sludge samples

In the six sludge samples, the relative contents of *Methanosaetaceae* and *Methanobacteriaceae* are shown in Fig. 5. The content of *Methanosaetaceae* was almost the same in samples A and B (approximately 40%), and the content of *Methanobacteriaceae* in sample A was lower than in sample B, which increased from 13.1% to 21.9%. After adding nitrate into the bioreactor, the abundance levels of *Methanosaetaceae* and *Methanobacteriaceae* changed markedly. With the decline of C/N from 20:1 to 5:1 (samples C–F), *Methanosaetaceae* increased from 64.5% to 84.2%, whereas *Methanobacteriaceae* decreased from 18.6% to 11.8%. According to the previous researches (Song et al., 2010; Tabatabaei et al., 2010), the *Methanosaetaceae* uses acetate as substrate to produce methane, while *Methanobacteriaceae* uses H_2/CO_2 as substrate. The glucose substrate was first degraded by fermentative and acetogenic bacteria into hydrogen and acetate, which were utilized by methanogens to produce methane. In general, 70% of methane is produced from acetate, while the other 30% from H_2/CO_2. Therefore, it is reasonable for the relative content of *Methanosaetaceae* to

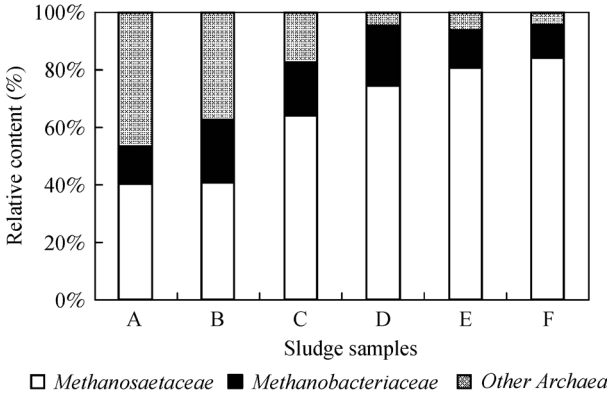

Fig. 5 Relative content of *Methanosaetaceae* and *Methanobacteriaceae* in *Archaea* in SMD granular sludge samples

be approximately 40% of the whole *Archaea*, while *Methanobacteriaceae* was approximately 20%. After the addition of nitrate, the denitrifying bacteria grew quickly, which required abundant electron donors to reduce nitrate into nitrogen gas. Hydrogen and acetate are suitable electron donors, and the denitrifying bacteria are the first to use them because the energy yield in denitrification is higher than that in methanogenesis (as shown in Table 3). This property could affect the growth of methanogens, which is the reason that the content of *Archaea* declined with the decrease of C/N. Compared with acetate, the hydrogen will be prioritized for use by denitrifying bacteria (as shown in Table 3), which mainly resulted in the decrease of hydrogenotrophic *Methanobacteriaceae.* In addition, when the supply of hydrogen had run out, the nitrate ran out mostly because the lowest C/N was 5:1 in this experiment. The remaining acetate would be utilized by methanogenesis, leading to a gradual increase of the relative content of acetic-trophic *Methanosaetaceae* within the *Archaea* with the reduction of the C/N (Fig. 5).

3.5 Analysis of the *Archaea* community with T-RFLP

T-RFLP has been considered a promising way to analyze the microbial community (Osborn et al., 2000; Lueders et al., 2001). The analysis of cloned *Archaea* T-RFLP peak values (as shown in Fig. 6) combined with the molecular cloning results was yielded the followings observations: 1) The peak at 284 bps, which represented *Methanosaetaceae* (compared with the restriction enzyme map of single clones analyzed by T-RFLP), was prominent; 2) The peak at 393–395 bps represented both part *Methanosaetaceae* and *Methanospirillaceae* in the cloned *Archaea*; 3) The peaks at 92 bps and 728–735 bps represented *Methanobacteriaceae* in the cloned *Archaea*; 4) The peak at 82 bps represented *Methanosaetaceae* and *Methanomicrobiaceae*; 5) The peak at 260–265 bps was not observed. In the six sludge samples, it was obvious that the *Archaea* diversity was not high, and *Methanosaetaceae* was the dominant methanogen, while the quantity of *Methanobacteriaceae* was influenced by the C/N, which is consistent with the previous qRT-PCR result.

In addition, peak at 333 in sample A was more abundant than in sample B, while the peak at 284 bps in sample B was much higher than in sample A. After the addition of nitrate, the peak at 393–395 bps which represents both *Methanosaetaceae* and *Methanospirillaceae* in samples C, D, E and F was observed more prominently compared with samples A and B. With the increase of C/N, *Archaea* diversity decreased gradually.

Based on the number of characteristic terminal fragments and the size of the peak area in T-RFLP profiles, Shannon–Wiener index (H') was worked out according to Eq. (1), and the values for the *Archaea* community in six sludge samples are shown in Table 4.

Table 3 Reactions occur in a SMD process

Reactions	$\Delta_r H_m^{\ominus}$ (kJ/mol e donor)	$\Delta_r S_m^{\ominus}$ (J/mol/K e donor)	$\Delta_r G_m^{\ominus}$ (kJ/mol e donor)
Methanogenesis:			
$H_2(g) + 0.25CO_2(ao) \rightarrow 0.25CH_4(g) + 0.5H_2O(l)$	−29	−39	−17
$CH_3COOH(ao) \rightarrow CH_4(g) + CO_2(ao)$	−1	31	−10
Denitrification:			
$H_2(g) + 0.4H^+(ao) + 0.4NO_3^-(ao) \rightarrow 0.2N_2(g) + 1.2H_2O(l)$	−130	−34	−164
$CH_3COO^-(ao) + 2.6H^+(ao) + 1.6NO_3^-(ao) \rightarrow 0.8N_2(g) + 2CO_2(ao) + 2.8H_2O(l)$	−102	33	−155

Notes: $\Delta_r H_m^{\ominus}$, $\Delta_r S_m^{\ominus}$, $\Delta_r G_m^{\ominus}$ were calculated according to the NBS tables of chemical thermodynamic properties (Wagman et al., 1982)

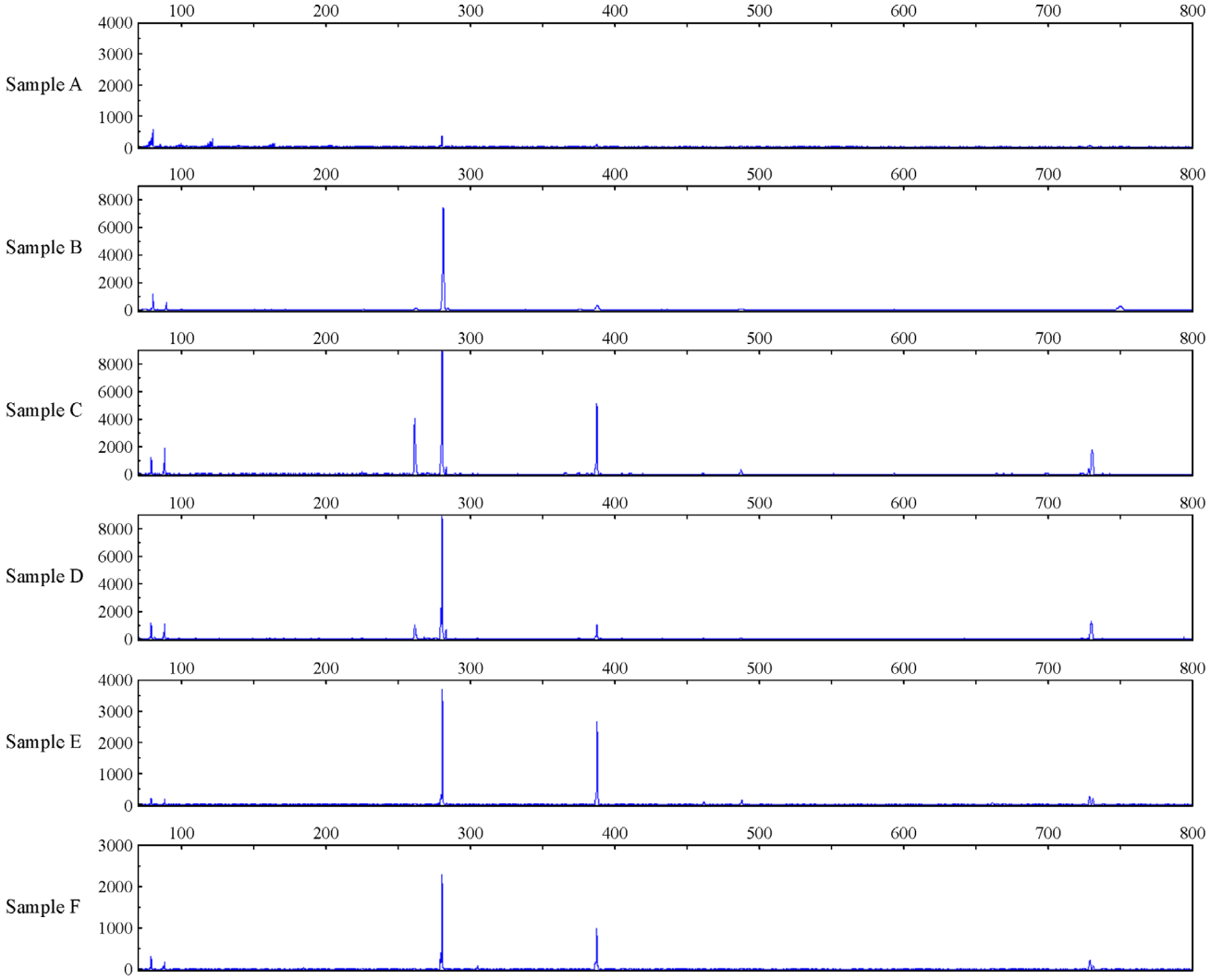

Fig. 6 T-RFLP profiles of *Archaea* 16S rDNA PCR products amplified from SMD sludges at different C/N ratio

$$H' = -\sum_{i=1}^{s} p_i \ln p_i, \quad (1)$$

in this equation, p_i represents the proportion of a certain peak value out of the total peak value.

According to Table 4, the T-RFLP diversity index of *Archaea* in samples A and B without added nitrate were quite low. When the C/N was 20:1 (sample C), the richness and evenness of methanogens provided the highest T-RFLP diversity index value of 1.627, while the removal efficiency was favorable, i.e., the removal efficiency of

Table 4 Diversity index T-RFLP of *Archaea* in six sludge samples

Sample No.	T-RFLP diversity index
A	0.966
B	0.924
C	1.627
D	1.338
E	1.219
F	1.155

COD was above 98% and the removal efficiency of NO_3^-–N was above 99%. However, adding more nitrate (sample D, E and F) led to the decrease of *Archaea* diversity. When the C/N was 5:1, the T-RFLP diversity index decreased to 1.155, which was still higher than that of sample A and B, and the removal efficiency of COD decreased to 96%. Thus, the community structure reflected the succession to an unstable condition represented by high nitrate concentrations.

4 Conclusions

The UASB bioreactor with simultaneous methanogenesis and denitrification (SMD) was operated for 235 days, in which the COD removal efficiency was mostly above 96% and the NO_3^-–N removal efficiency was excellent, remaining above 99%. During the whole process, the methanogenic community structure and the succession process were affected by the changing ratios of C/N in the influent from 20:1 to 15:1, 10:1 and 5:1. When the C/N was 20:1 (sample C), the methane production rate was fastest in the six granular sludge, with *Methanosaetaceae* and *Methanobacteriaceae* being the main methanogens within the *Archaea*; the richness and evenness of the methanogens were best with the highest T-RFLP diversity index of 1.627. With the C/N decrease from 20:1 to 5:1 (sample C to F), the methanogenic activity in the samples decreased gradually, and *Archaea* relative abundance in SMD granules decreased from 36.5% to 10.9%, during which *Methanosaetaceae* increased from 64.5% to 84.2%, while *Methanobacteriaceae* decreased from 18.6% to 11.8%; the T-RFLP diversity index decreased to 1.155. Thus, the community structure reflected the succession to an unstable condition represented by high nitrate concentrations.

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