

# Structure and Succession of Bacterial Communities of the Granular Sludge during the Initial Stage of the Simultaneous Denitrification and Methanogenesis Process

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**Abstract** Batch experiment at COD/NO<sub>3</sub><sup>-</sup>-N ratio of 8.0 was conducted to investigate the initial performance of the simultaneous denitrification and methanogenesis (SDM) process and corresponding granular sludge (SDMGS). The results showed a high level of inhibition of methanogenesis activity with nitrate addition, and the particle size, settling performance, and morphologies of the SDMGS were also different from conventional methanogenesis granular sludge. The structure and succession of bacterial communities of the granular sludge during the initial stage of the SDM process were determined using the high-throughput sequencing method. Sequence analysis indicated that diversity of bacterial communities was significantly decreased due to nitrate

addition. Proteobacteria, Bacteroidetes, Firmicutes, and Spirochaetes were identified to be the dominant bacterial communities (96.06%) of the SDMGS samples, and microbes associated with anaerobic fermentation were reorganized. Alpha-, Beta- and Gamma-proteobacteria, and Bacteroides might be the sources of denitrifiers. Lastly, species associated with animal and human infections, such as Enterobacteriaceae, Bacteroides, and other common human enteric pathogens, were found to be recovered during the initial stage. Short-term assessment of bacterial communities of the SDMGS would strengthen understandings of the effects of nitrate contamination in water bodies and provide vital guidance for operation of nitrate-containing wastewater treatment.

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**Keywords** Anaerobic digestion · Denitrification · Gamma-proteobacteria · Next-generation sequencing · Nitrate

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## 1 Introduction

Increasing nitrate concentration in soil, lakes, surface water bodies, and groundwater due to intensive agricultural and industrial activities has been reported in many parts of the world (Chen et al. 2007; Qu and Fan 2010; Liu et al. 2013; Wolinska et al. 2016). Huge amounts of nitrate in water bodies without proper treatment would cause serious environmental problems and further threaten human and animal health (Su et al. 2013). Simultaneous denitrification and methanogenesis (SDM) process, which coupled the nitrate reduction

and anaerobic digestion together into a bioreactor, has received considerable attention recently and been shown to be a promising technology for the treatment of high-strength carbon- and nitrate-containing wastewater (Chen et al. 2009; Sun et al. 2015) due to advantages of simplifying the operating procedures, supplementing the substrates (organic carbon), reducing cost and space, and partly recovering energy in the form of methane (Andalib et al. 2011; Show et al. 2013).

Integration of denitrification into the conventional anaerobic methanogenesis process makes significant influence on the whole microbial communities and further affects the characteristics of the sludge and performance of the reactor (Hendriksen and Ahring 1996b; Tugtas and Pavlostathis 2007). The distribution and location of the denitrifiers and methanogens on the sludge were reported through microscopic observation in an immobilized mixed culture (Lin and Chen 1995), and the growth of the denitrifiers and facultative anaerobes on the peripheral surface of the sludge was thought to account for the reorganization of microbial communities during the SDM process (Karim and Gupta 2003; Wang et al. 2014). Despite that the bacterial communities of the SDM process within a long-term period were examined using the 16S rDNA clone libraries method (Sun et al. 2008), detailed investigations into the succession and structure of bacterial communities during the initial stage of the SDM process, being connected with the evolution of bacterial communities and the corresponding granular sludge (SDMGS) due to exposure to high levels of nitrate, have not generally been reported. Moreover, there is lack of sufficient gene sequence information to reveal the comprehensive bacterial communities and functional microorganisms in the SDM process.

High-throughput sequencing (HTS) analysis, based on different hyper-variable regions of 16S rDNA/rRNA gene, brings comprehensive insights into the whole microbial communities in terms of taxonomy and diversity, especially for those rare but functionally important species from environmental samples (Logares et al. 2012). Nowadays, the HTS method has been widely applied to evaluate microbial communities of wastewater treatment plants and has become a powerful tool for environmental microbiology research (Zhang et al. 2012; Liao et al. 2013). Owing to much greater coverage and far more informative DNA sequences generated from HTS analysis, information regarding species, abundance, and physiology involved in the SDM

process can be elucidated more accurately and comprehensively.

Influent chemical oxygen demand (COD)/NO<sub>x</sub><sup>-</sup>-N ratio is one of the most critical parameters for the realization of the SDM process and succession of the functional microbes (Akunna et al. 1993; Chen et al. 2009). Suitable COD/NO<sub>x</sub><sup>-</sup>-N ratios with different carbon sources for the SDM process have been well discussed in literatures (Akunna et al. 1992; Del Pozo and Diez 2003; Ruiz et al. 2006), and with glucose as the carbon source, a COD/NO<sub>3</sub><sup>-</sup>-N ratio around 8.0 could be treated as the minimum threshold for the SDM process from the aforementioned reports and used in the experiment.

Therefore, the main objectives of the present work were to: (1) assess the reactor's performance of the SDM process at a COD/NO<sub>3</sub><sup>-</sup>-N ratio of 8.0 and the corresponding granular sludge (SDMGS); (2) investigate the structure and succession of bacterial communities of the SDMGS at the initial stage using the HTS method and conduct a comparative analysis of the different bacterial communities between the SDMGS and methanogenesis granular sludge (MGS); and (3) identify sources of possible denitrifiers and other functional bacterial communities. The results will provide a better understanding of the succession of different bacterial communities with nitrate addition and the effects of nitrate contamination on the water bodies, as well as being helpful for the better treatment of high level of nitrate-containing wastewater.

## 2 Materials and Methods

### 2.1 Batch reactors, Culture Medium, and Seed Sludge

Series of identical serum bottles with a volume of 250 mL were used as the batch bioreactors. The culture medium contained the following constituents: C<sub>6</sub>H<sub>12</sub>O<sub>6</sub>, 4.0 g/L; NH<sub>4</sub>Cl, 0.42 g/L; KH<sub>2</sub>PO<sub>4</sub>, 0.2 g/L; MgSO<sub>4</sub>·6H<sub>2</sub>O, 0.4 g/L; CaCl<sub>2</sub>, 0.15 g/L; and L-cysteine, 0.1 g/L. Trace element solution was prepared and the culture medium was stored at 4 °C before use. Sludge was collected from a local full-scale, mesophilic, anaerobic internal circulation bioreactor and then cultured with synthetic wastewater at a COD/N/P ratio of 200–300:5:1 for months before being used as the inoculum. After removing the large debris from sludge with a 3.0-mm sieve, the volatile suspended solids (VSS) and the volatile-to-total suspended solids (VSS/TSS) ratio

of the granular sludge used as the inoculum for batch experiments were  $50.5 \pm 3.2$  g/L and 0.81, respectively.

To prepare the batch experiments, 20.0 g (measured on a wet weight) of inoculum sludge, 150.0 mL culture medium, and 1.0 mL trace elements solution were respectively added into serum bottles and numbered. The COD/NO<sub>3</sub><sup>-</sup>-N ratio of mixtures was adjusted to 8.0 with sodium nitrate (NaNO<sub>3</sub>); the control group was prepared without nitrate. The initial pH value of mixtures was about  $7.4 \pm 0.5$ , with adjustment of sodium bicarbonate (NaHCO<sub>3</sub>). Mixtures and bioreactors were then flushed with 99.995% argon gas for 10 min, closed with butyl rubber stoppers, and sealed with aluminum caps immediately. An anaerobic glove box filled with argon gas was utilized to ensure the absence of oxygen during the entire preparation period. The cultivation was performed in a reciprocating shaker water bath (SW22, Julabo, Germany) at a constant temperature of  $35 \pm 0.2$  °C. Continuous experiments lasting 15 days were carried out to obtain the sludge samples for further analysis, and the culture medium was replaced every 2 days with the help of an anaerobic glove box filled with argon gas (>99.995%). All the batch experiments were performed in triplicate.

## 2.2 Sampling and Analytical Methods

Liquid samples were collected with sampling syringes and centrifuged at 10,000×g for 10 min at 4 °C (3–18k, Sigma, Germany), then used for chemical analysis. COD, NH<sub>4</sub><sup>+</sup>-N, VSS, and TSS were determined according to standard methods (APHA 1998). The concentrations of NO<sub>3</sub><sup>-</sup>-N and NO<sub>2</sub><sup>-</sup>-N were measured using an ion chromatography (ICS-90, Dionex, USA) equipped with an IonPac AS14 column (4 × 250 mm) with a carbonate/bicarbonate eluent (3.5 mmol/L Na<sub>2</sub>CO<sub>3</sub> and 1.0 mmol/L NaHCO<sub>3</sub> at 1.0 mL/min). The pH and oxidation reduction potential (ORP) values of the liquid samples were examined using a pH meter (PHS-25, LeiCi, China).

The composition of volatile fatty acids (VFAs) in the experiment was examined with a gas chromatograph (A90, Echrom Inc., China) equipped with a capillary column (DB-FFAP, 30 m × 0.32 mm × 0.5 μm) and a flame ionization detector. The nitrogen carrier gas flow rate was 5.0 mL/min. The total volume of the biogas produced was recorded with a gas flow meter, and the composition was determined using the same gas chromatograph equipped with a thermal conductivity detector and a stainless steel column (3 mm, 2-m length)

packed with TDX-01 (60–80 mesh). The injector, oven, and detector temperatures were 150, 100, and 250 °C, respectively. High-purity argon was used as the carrier gas at a flow rate of 30.0 mL/min.

## 2.3 Granular Sludge Characterization

SDMGS and MGS (control group) samples were collected at the end of continuous experiments. Main appearance features of SDMGS and MGS were gained through visual observation and photographed with a digital camera. The particle size of the granular sludge was then determined in triplicate from digital photos using the Image-Pro Plus software (version 6.0, Media Cybernetics, USA). The settling velocity of the sludge was evaluated through recording the average time taken for an individual granule to fall from a certain height in a measuring cylinder filled with tap water. Morphology details of the sludge granules were recognized with a scanning electron microscope (EVO LS10, Carl Zeiss, Germany) after fixation, dehydration of gradient ethanol series, and gold coating.

## 2.4 DNA Extraction and High-Throughput Sequencing

Total DNA was extracted from sludge with a FX Soil DNA MiniPuri Kit (Felix Biotechnology, USA), referring to the manufacturer's instructions. The quality of extracted DNA was checked with 1.0% (w/v) agarose gel electrophoresis and visualized under ultraviolet light after staining with Goldview I<sup>®</sup> (nucleotide dye). The extracted DNA was then stored at -20 °C and used for HTS analysis.

HTS analysis was performed on an Illumina MiSeq platform. Primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') were used to amplify the V4 region of the bacterial 16S rDNA gene. Unique barcodes were utilized to assign sequences to different samples. PCR amplification was performed in a final volume of 50 μL reaction mixtures containing 0.4 μM forward and reverse primers, 1 μL template DNA, 250 nM dNTP, and 1× FastPfu Buffer using a GeneAmp 9700 (Applied Biosystems, USA). The PCR program consisted of an initial denaturation step at 95 °C for 3 min, 25 cycles of denaturation at 95 °C for 30 s, then annealing at 55 °C for 30 s and extension at 72 °C for 30 s, finished with a final extension at 72 °C for 5 min. Sequencing was performed at BGI Tech Co. Ltd., China.

## 2.5 Data Processing and Bioinformatics Analysis

For the bioinformatics analysis, the raw sequence reads were firstly assigned to different samples based on the nucleotide barcodes. After denoising, filtering out the chimeras, and removing archaeal sequences, effective tags were clustered into different operational taxonomic units (OTUs) at a 0.03 limit using the MOTHUR software (<http://www.mothur.org/>). Rarefaction curves, Chao1 richness estimator, and Shannon diversity index were also obtained based on cluster information. Sequences were compared to entries in the SILVA database (version SSU111; <http://www.arb-silva.de>), with a set confidence threshold of 80%, and phylogenetically assigned to taxonomic classification from phylum to the genus level using an RDP classifier at a 0.03 distance. Comparative visualization of the differences in bacterial communities between the SDMGS and MGS samples was performed using the MEGAN (version 5.9.0) software (<http://ab.inf.uni-tuebingen.de/>), and a treemap based on the output from the RDP classifier was constructed for the SDMGS samples using the Treemap program (version 4.1.1; <http://www.cs.umd.edu/hcil/treemap/>).

## 3 Results and Discussion

### 3.1 Performances of the Batch Experiments

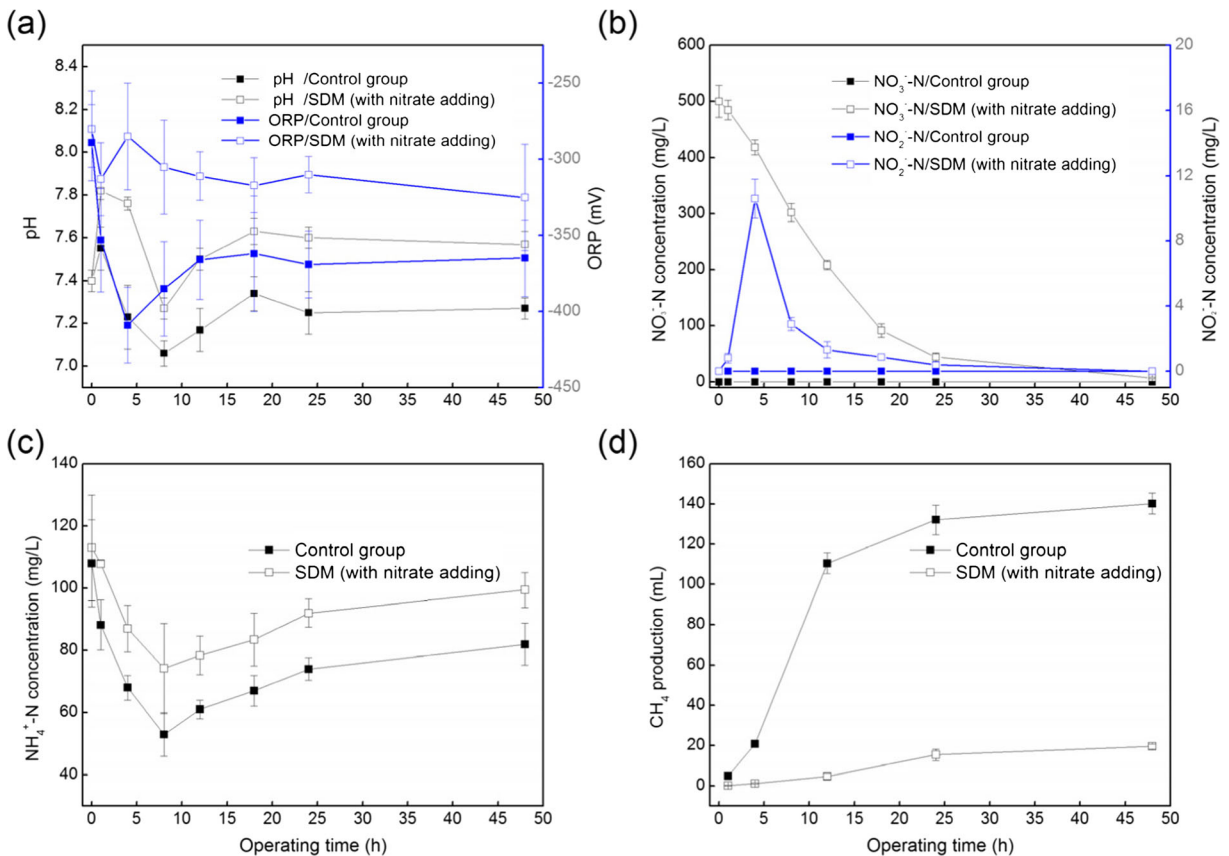
Performances of batch experiments with nitrate addition at a COD/NO<sub>3</sub><sup>-</sup>-N ratio of 8.0 were investigated; the control group was performed without additional nitrate. Variations of pH and ORP values are presented in Fig. 1a. Both the pH and ORP values were increased and showed to be higher than the control group at the same time due to denitrification occurring simultaneously with nitrate addition. At the first 10 h, the pH values of the two groups were decreased to the lowest, 7.26 and 7.06, respectively, which could be owing to the acidic fermentation of glucose. The ORP values were kept around -300 mV during the entire experimental period with nitrate addition and rose to the highest of about -285 mV at 4 h, which could have inhibited the activities of the methanogenesis process since the growth of methanogens was reported to be inhibited at ORP values above -330 mV. About 91.2% of nitrate was removed in the first 24 h, and nitrite was accumulated and rose to about 10.6 mg/L (NO<sub>2</sub><sup>-</sup>-N) at the beginning (Fig. 1b),

and no nitrite could be detected at the end of the experiment. During the experimental period, ammonia was not found to be accumulated, and a lower concentration of ammonia was detected at the end, which might be due to utilization for the synthesis of microbes as a nitrogen source (Fig. 1c). The methane production of the two groups is presented in Fig. 1d; a total of 19.5 mL methane was produced under the COD/NO<sub>3</sub><sup>-</sup>-N ratio of 8.0, which was significantly lower than that of the control group (with a production of 140.3 mL in total). The results showed a high level of inhibition effect of methanogenesis activity with nitrate addition at the COD/NO<sub>3</sub><sup>-</sup>-N ratio of 8.0. Combined with the removal performance of nitrate, the SDM process seems to be observed at the COD/NO<sub>3</sub><sup>-</sup>-N ratio of 8.0.

Continuous experiments lasting 15 days were carried out as aforementioned to obtain the SDMGS and MGS (control group) samples for analysis. The culture medium was replaced every 2 days with the help of an anaerobic glove box. Variations of individual VFA concentration, in terms of acetate and propionate, are shown in Fig. 2a. Acetate was found to be the main component of the total VFAs, and only a little propionate (less than the control group) was detected in the culture of the SDM process, which could be owing to the metabolism of denitrifiers since propionate and butyrate were likely to be preferably metabolized by denitrifiers (Hendriksen and Ahring 1996a). The consumption rate of acetate was increased under the SDM process from 12 to 24 h, which could be associated with the recovery of methanogenesis activities. The composition of the biogas produced is shown in Fig. 2b, and the contents of nitrogen and carbon dioxide were increased from 2.01 to 12.22% and from 30.39 to 43.41%, respectively, due to the metabolism of denitrifiers and facultative anaerobes. The percentage of methane in the total biogas under the SDM reduced to almost 34.8% in comparison to the control group, which might be resulted from inhibition and competition of the carbon source. A small amount of hydrogen (0.29%) was found under the SDM process, which could be due to shift of population or activities of the hydrogenotrophic microbes with nitrate addition

### 3.2 Characteristics of the SDMGS and MGS Samples

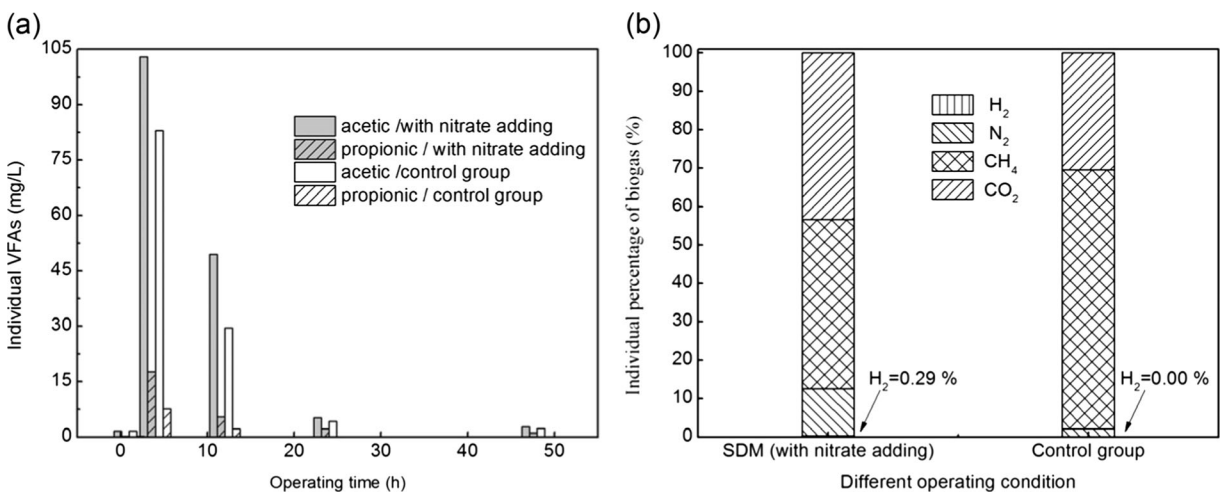
The characteristics of the SDMGS and MGS samples collected from the experiment are presented in Table 1. The MGS samples from the control group presented



**Fig. 1** Variations of pH and ORP values and NO<sub>3</sub><sup>-</sup>-N, NO<sub>2</sub><sup>-</sup>-N, NH<sub>4</sub><sup>+</sup>-N, and CH<sub>4</sub> production under the SDM process at the COD/NO<sub>3</sub><sup>-</sup>-N ratio of 8.0 and the control group. **a** pH and ORP. **b** NO<sub>3</sub><sup>-</sup>-N and NO<sub>2</sub><sup>-</sup>-N. **c** NH<sub>4</sub><sup>+</sup>-N. **d** CH<sub>4</sub> production

almost the same appearance, color, and settling performance as in the initial state, while the SDMGS became

fluffier and less dense and lighter in color (changed from dark black to light white-brown; shown in Fig. 3a, d),



**Fig. 2** Variations of individual VFAs (acetic and propionic acids) and composition of the biogas produced under the SDM process at the COD/NO<sub>3</sub><sup>-</sup>-N ratio of 8.0 and the control group. **a** Variations

of the individual VFAs including acetic and propionic acids. **b** Variations of the composition of the biogas produced

**Table 1** Characteristics and particle size distribution of the SDMGS and MGS samples

	SDMGS	MGS (control group)
Characteristics		
Appearance	Fluffy, buoyant, flocculent	Dense, settleable, flocculent
Color	White-brown	Dark black
Size (mm)	1.4 ± 0.5	1.8 ± 0.25
Settling velocity (m/h)	52.7 ± 3.2	84.2 ± 6.1
VSS/TSS	0.85 ± 0.02	0.81 ± 0.03
Size distribution		
<0.3 mm	4.0 ± 1.1%	3.0 ± 0.9%
0.3–0.5 mm	22.0 ± 1.2%	17 ± 2.3%
0.5–1.0 mm	37.2 ± 3.0%	28 ± 3.1%
1.0–2.0 mm	31.0 ± 2.7%	47.0 ± 5.2%
2.0–3.0 mm	5.3 ± 0.2%	4.7 ± 0.8%
>3.0 mm	0.5 ± 0.1%	0.3 ± 0.1%

and the settling velocity of the SMDGS was decreased to 52.7 ± 3.2 m/h. The average particle sizes of the SDMGS and MGS samples were calculated using the Image-Pro Plus software. Most of the SDMGS samples were found with small sizes of less than 1.0 mm. Meanwhile, floating and flocculent sludge granules were also observed. Large numbers of rod-shaped and spherical bacteria were observed on the surface of SDMGS samples (Fig. 3e, f), and it was found to be rougher than the surface of the MGS samples (Fig. 3b, c), which could be resulted from the excessive growth of denitrificans and fermentative bacteria on the peripheral surface of the sludge granules under the SDM process at the COD/NO<sub>3</sub><sup>-</sup>-N ratio of 8.0.

### 3.3 Biodiversity of Bacterial Communities of the Granular Sludge

With the help of the HTS method, large quantities of DNA sequences were generated from two granular sludges. After denoising, filtering out the chimeras, and removing archaeal sequences, a total of 106,875 effective bacterial sequences were obtained from the SDMGS and MGS samples. Rarefaction analysis was performed, and curves at different cutoff levels of 0.01, 0.03, 0.05, and 0.07 are shown in Fig. 4. The curves at the cutoff level of 0.03 of two sludge samples were found to gradually tend to be a plateau to the right,

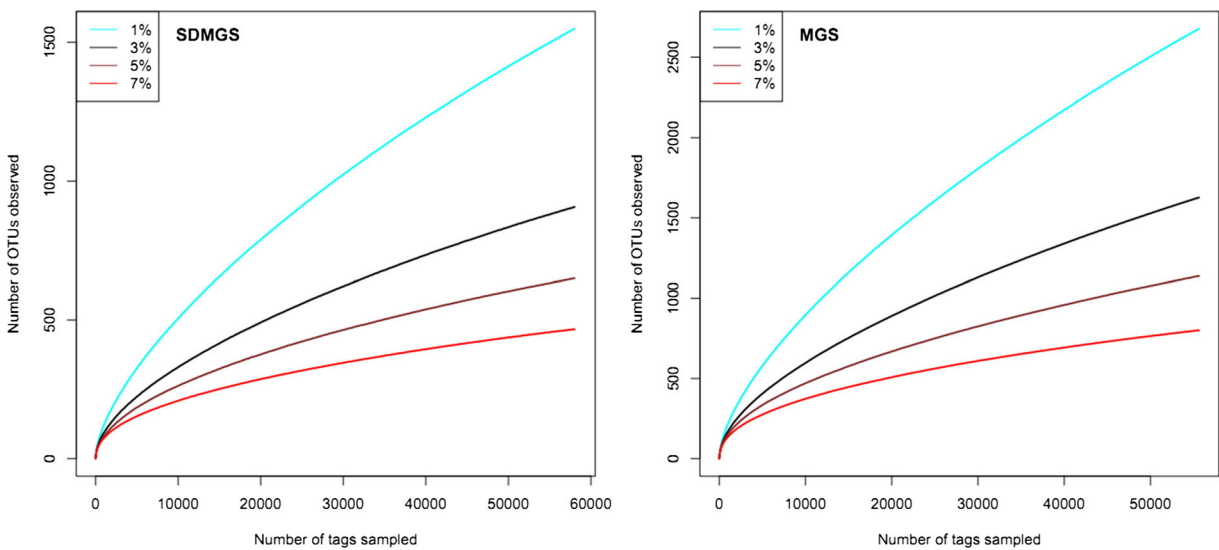
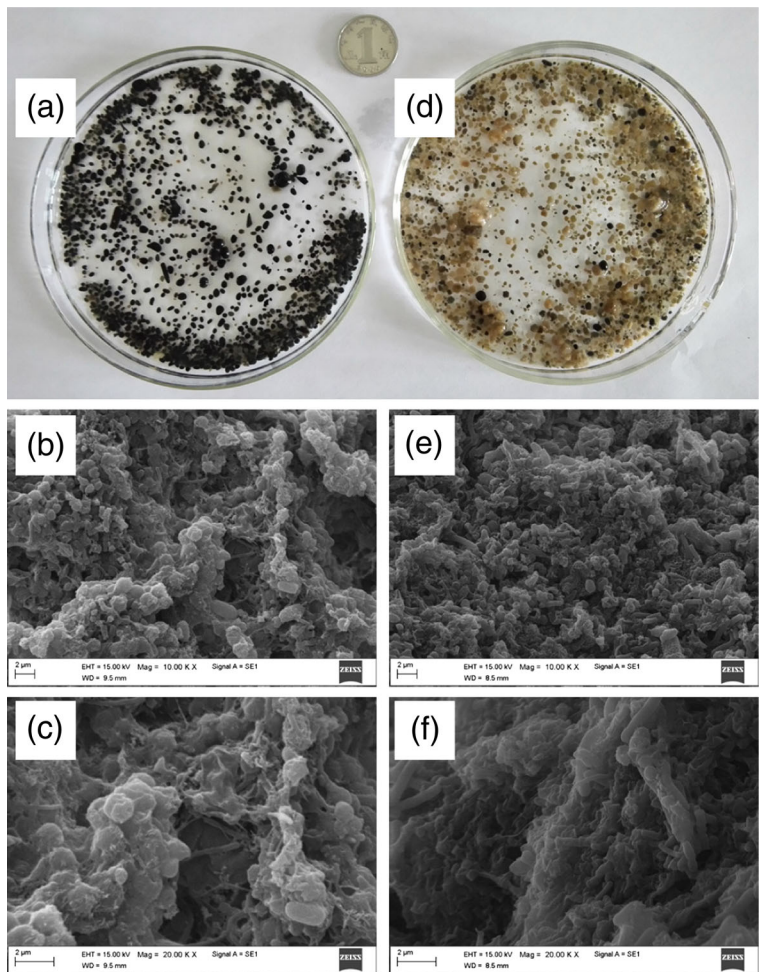
hinting that enough sequencing datasets were obtained to provide useful information about the structure, diversity, and succession of the bacterial communities of two sludge samples. Effective bacterial sequences were assigned to different OTUs at 97% similarity for subsequent analysis. A total of 878 and 1555 OTUs were identified at the 3% cutoff level from the SDMGS and MGS samples, respectively. Based on the OTU information, Chao1, Simpson, and Shannon diversity indexes of the bacterial communities of two sludge samples were calculated and provided in Table 2. Lower Shannon diversity index values (SDMGS = 2.616, MGS (control) = 4.131) were observed, and together with the lower OTU number and Chao1 richness estimator of the SDMGS samples, the results indicated that denitrification happened simultaneously under the anaerobic methanogenesis condition, which made a significant inhibitory effect on the structure and diversity of the bacterial communities of sludge during the initial stage.

### 3.4 Structure and Succession of Bacterial Communities of the Granular Sludge

In total, 56,087 effective sequences were detected from the SDMGS samples, which were 10.43% higher than the MGS samples (50,788 sequences, control group). All the effective bacterial sequences from the two sludges were assigned to different taxon levels (from genus to phylum) using the RDP classifier at 51% threshold, and taxonomic analysis was conducted and visualized using the MEGAN software (Fig. S1).

Except for the unclassified bacterial sequences (852 sequences), Proteobacteria, Bacteroidetes, Firmicutes, Spirochaetes, Thermotogae, and Chloroflexi were identified to be the dominant known phyla in the SDMGS samples, accounting for about 63.00, 21.79, 9.73, 1.54, 1.39, and 0.59%, respectively (shown in Fig. 5). A shift in the bacterial structure (from Bacteroidetes-, Firmicutes-, and Proteobacteria-dominated (MGS, control group) to Proteobacteria- and Bacteroidetes-dominated) was observed over the short-term experimental period (Fig. 5). To investigate the structure of the different taxon levels and the functions of different bacterial communities, the identified phyla of the SDMGS samples were studied as follows. Three dominant phyla (Proteobacteria, Bacteroidetes, and Firmicutes), accounting for almost 94.5% of the total bacterial sequences of the SDMGS, were discussed separately.

**Fig. 3** Morphology and surface details of the SDMGS and MGS samples. **a–c** Images of the MGS samples collected from the control group. **d–f** Images of the SDMGS samples with nitrate addition. Sizes of the actual sludge granule samples were compared to a 1 dime coin (RMB, a diameter of 19.00 mm) in (a) and (d)



**Fig. 4** Rarefaction curves of the SDMGS and MGS (control group) samples at cutoff levels of 1, 3, 5, and 7%, respectively

**Table 2** Number of effective tags, OTUs, and Chao1, Simpson and Shannon diversity indexes of bacterial communities of the SDMGS and MGS samples at the 97% level

	SDMGS	MGS (control group)
Effective tag number	56,087	50,788
OTUs <sup>a</sup>	878	1555
Chao1	1743	3442
Simpson	0.221	0.043
Shannon diversity index	2.616	4.131

<sup>a</sup>Operational taxonomic units calculated using the MOTHUR software at the 3% distance limit

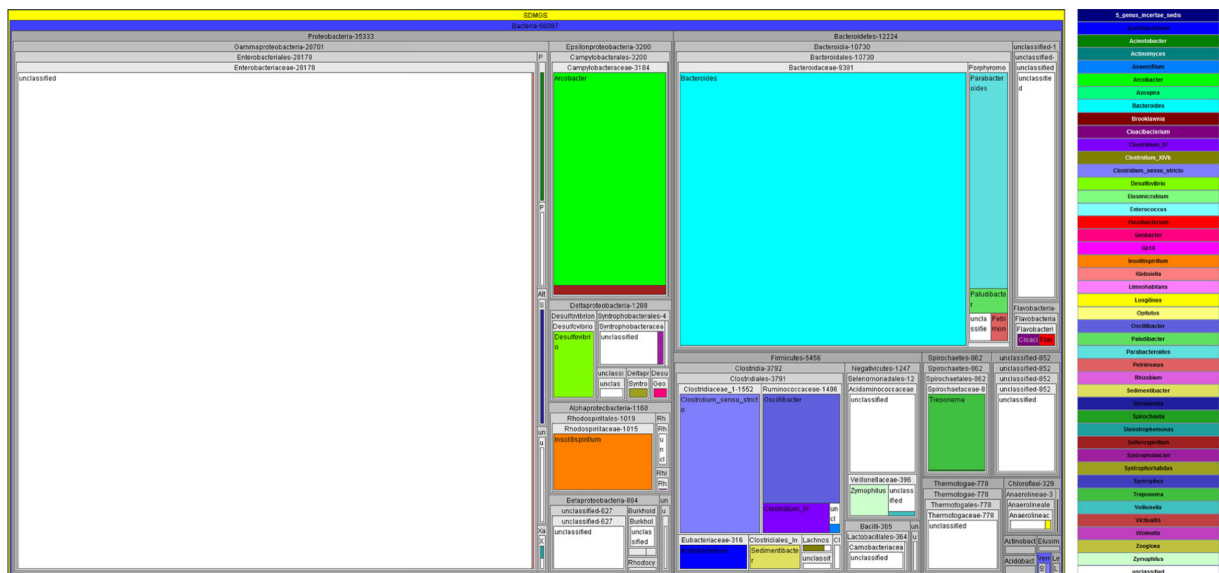
Others, including Spirochaetes, Thermotogae, and other minor groups, were studied as a bacterial group.

### 3.4.1 Proteobacteria

Proteobacteria was the largest bacterial phylum detected from the SDMGS samples, with a content of 63.00% of the total effective bacterial sequences. All the five classes (Gamma, Epsilon, Delta, Alpha, and Beta) within the phylum Proteobacteria were identified from the SDMGS samples (shown in Fig. S2) and studied as follows.

Gamma-proteobacteria class separately represented roughly 81.23% of the total proteobacterial sequences. Enterobacteriaceae (28,178 sequences) was detected to be the most dominant taxonomic family of Gamma-proteobacteria from the SDMGS, accounting for about 50.24% of the total effective bacterial sequences (Fig. S2), but those sequences failed to be classified to any known genus. Most microbes from the family Enterobacteriaceae are reported to be rod-shaped and facultative anaerobes and participants of the biological denitrification process (partly, reduction of nitrate to nitrite). Moreover, the abundant growth of the family Enterobacteriaceae during the short-term period indicated a high recovery of the enteric bacteria, including common human enteric pathogens due to nitrate addition under the anaerobic methanogenesis condition. *Acinetobacter*, a part of the acid-producing communities of anaerobic digesters, was identified as the dominant genus within the Pseudomonadales order (239 sequences) of the SDMGS. Pseudomonadaceae (93 sequences), including type genus *Pseudomonas*, which is capable of high denitrification potential, was also observed from the SDMGS samples as a small bacterial group in the initial stage.

The numbers of Delta- and Epsilon-proteobacteria classes (4488 sequences, 12.70%) of the SDMGS were found to be decreased in comparison with the MGS



**Fig. 5** Treemap visualization of the identified taxa (genus) of all the effective bacterial sequences from the SDMGS samples shown in their hierarchical order with the squarified method using the Treemap software (version 4.1.1). All taxa with sequence numbers

greater than 10 were shown in the treemap. The size of each block is proportional to the number of sequences assigned to that taxon, and each block is labeled referring to its RDP classification and sequence number



samples (7833 sequences, 77.09%). Most of the Delta-proteobacteria class was reported to be detected from the sediment and anaerobic digesters and contained most of the known sulfate- and sulfur-reducing bacteria. Low sulfate concentration and the single carbon source of the culture medium during the entire experimental period, which led to shifting of the metabolic types and competitions among different bacterial communities, could be responsible for the decrease of the Delta-proteobacteria class. *Arcobacter*, the dominant genus within the Epsilon-proteobacteria class, was also found from the SDMGS and MGS. Members of the *Arcobacter* genus are mainly isolated from kinds of environmental waters and associated with animal and human infections. Emergence of the *Arcobacter* could be correlated to the carbon source, scale, and period of the experiment since it can be effectively removed through anaerobic treatment (Collado et al. 2010).

The numbers of Alpha- and Beta-proteobacteria classes were increased a little in the SDMGS samples since their populations are associated with the level of nutrient contamination of the surroundings as they thrive in the soil, wastewater, and lakes with abundant organic matter and nitrogen levels. Denitrifiers were composed of several facultative or strict anaerobes, and most are from the Alpha-, Beta-, and Gamma-proteobacteria classes, such as the genera *Paracoccus* (Alpha-proteobacteria), *Brachymonas* (Beta-proteobacteria), and *Pseudomonas* (Gamma-proteobacteria). The rapid growth of the Proteobacteria phylum from the SDMGS samples during the initial stage with nitrate addition might be associated with the degradation of nitrate and the SDM process.

### 3.4.2 Bacteroidetes

Bacteroidetes was the second largest identified phylum of the SDMGS samples, with 12,224 sequences assigned. Most (approximately 89.66%) of the Bacteroidetes sequences were assigned to the classes Bacteroidia (10,730 sequences, 87.78%) and Flavobacteria (230 sequences, 1.88%); the remaining 1259 sequences (10.30%) failed to be classified to any known class (shown in Fig. S3). *Bacteroides* (9381 sequences), *Parabacteroides* (1066 sequences), and *Paludibacter* (122 sequences) were the dominant genera detected from class Bacteroidia of the SDMGS samples.

*Bacteroides*, a genus of obligate anaerobic and rod-shaped bacteria, has been detected as one of the

dominant bacterial communities from anaerobic digesters and reported to contribute to the hydrolysis and fermentation of organic matter. *Bacteroides graminisolvens* (7769 sequences, 82.82%), type species of the genus *Bacteroides*, was identified from the SDMGS samples, which could be related to the fermentation of glucose as the product of acetate and propionate (Nishiyama et al. 2009). The number of the genus *Bacteroides* from the SDMGS samples was much higher than from the MGS samples (3360 sequences), and various species of *Bacteroides* are commonly associated with kinds of human and animal infections. Moreover, Bacteroidia and Flavobacteria were also identified to be the dominant bacterial communities from the long-term period research (Sun et al. 2008), indicating that the growth of *Bacteroides* could be due to the shift of environmental conditions for nitrate addition and connected with the SDM process. *Paludibacter propionigenes* (122 sequences), type species of the genus *Paludibacter*, which is a mesophilic anaerobe that produced propionate and acetate as its fermentation products (Ueki et al. 2006), was also observed and found to be slightly decreased in comparison with the MGS samples.

### 3.4.3 Firmicutes

Firmicutes, accounting for 9.73% of the total effective bacterial sequences with a content of 5456 sequences, was the third largest identified phylum of the SDMGS samples. Approximately 69.50% of the sequences within the phylum Firmicutes were classified to the class Clostridia (shown in Fig. S4). *Clostridium sensu stricto* (1552 sequences) was the main taxonomic genus within the class Clostridia. The *Oscillibacter* genus (1173 sequences), within the family Ruminococcaceae (1496 sequences) of the SDMGS samples, was found to be much higher than that of the MGS samples (60 sequences). Previous studies suggested that Ruminococcaceae might be related to the biological hydrogen production process (Huws et al. 2011), and the growth of the family Ruminococcaceae could be accounted for the hydrogen produced during the SDM process. The *Acetobacterium* genus (313 sequences), within the family Eubacteriaceae (316 sequences), was also found from the SDMGS samples. Most of the *Acetobacterium* are homo-acetogens, which solely produced the acetic acid as a by-product of the anaerobic metabolism of glucose.

Negativicutes (1247 sequences) was identified to be the second taxonomic class of the phylum Firmicutes in the SDMGS samples. Acidaminococcaceae (849 sequences) and Veillonellaceae (396 sequences) were two principal taxonomic families within the order Selenomonadales (the only order of Negativicutes). The population of the class Negativicutes was sharply reduced in comparison with the MGS samples, especially the Acidaminococcaceae family, which might be due to the competition and succession of bacterial communities with nitrate addition.

A total of 365 sequences were classified to the class Bacilli (365 sequences) from the SDMGS samples, which was higher than that from the MGS samples (16 sequences). Within the class Bacilli, Lactobacillales and Carnobacteriaceae were the main taxonomic order and family of the SDMGS samples, respectively. Members of the Carnobacteriaceae family are facultative anaerobic bacteria, and the metabolic end products of carbohydrate are lactic and acetic acids. The emerging growth of the family Carnobacteriaceae during the SDM process might be due to the change of the fermentation type as variations of environmental factors (pH, ORP, and others) with nitrate addition.

#### 3.4.4 Spirochaetes, Thermotogae, and Other Minor Classified Groups

Spirochaetes, Thermotogae, and other minor phyla, including the Chloroflexi, Actinobacteria, and Acidobacteria phyla, were studied as an entire group (shown in Fig. S5). *Treponema* (842 sequences) and *Spirochaeta* (18 sequences) were the main genera of the Spirochaetes phylum detected from the SDMGS samples. Phylum Spirochaetes consists of lots of pathogenic bacteria. Some species of the *Treponema* and *Spirochaeta* genera are correlated to the degradation of cellulose, with hydrogen, carbon dioxide, acetate, and lactate as the end products of fermentation (Pohlschroeder et al. 1994). During the short-term experimental period, the number of the *Treponema* genus of the SDMGS samples was higher than that of the MGS samples (172 sequences), and the *Spirochaeta* genus was found to be decreased at the same time, indicating that the *Treponema* genus might be more adapted to nitrate or the SDM process than the *Spirochaeta* genus. Anaerolineaceae (302 sequences) was the main family of the phylum Chloroflexi detected from the SDMGS samples; the total sequence number of the

Anaerolineaceae family was lower than that from the MGS samples (2064 sequences). Phylum Chloroflexi has been reported to be mainly distributed in the outermost layer of the granules and correlated to the granulation and bulking of anaerobic granular sludge (Yamada et al. 2005). The notable reduction of the population of the Chloroflexi phylum might be associated with changes in the physical structure of sludge granules under the SDM process and the competition among different bacterial communities since the Chloroflexi phylum mainly contributes to the biodegradation of carbohydrates.

Other minor groups, including the Acidobacteria (65 sequences) and Verrucomicrobia (39 sequences) phyla and the genera *Elusimicrobium* (40 sequences) and *Victivallis* (21 sequences), were also observed from the SDMGS samples. Little content of the emerging *Elusimicrobium* and *Victivallis* genera was identified from the SDMGS samples. *Elusimicrobium*, a mesophilic and obligately anaerobic bacteria, was reported to grow heterotrophically on sugars and convert glucose to acetate, ethanol, hydrogen, and alanine as end products (Herlemann et al. 2009). *Victivallis*, a strictly anaerobic bacterium and first isolated from human feces samples, could convert glucose to acetate, ethanol, and hydrogen. Both genera might have contributed to the fermentation process during the SDM process.

#### 3.5 Possible Sources of Denitrifiers during the SDM Process

Biological denitrification needs the participation of several facultative or strict anaerobes. *Pseudomonas*, *Achromobacter*, and *Bacillus* are known as some of the bacteria with high denitrifying capabilities, and most denitrifiers are mainly from the Alpha-, Beta-, and Gamma-proteobacteria classes of the Proteobacteria phylum. During the short-term experimental period, the numbers of Gamma-, Alpha-, and Beta-proteobacteria classes were sharply increased in the SDMGS samples, which might be associated with denitrification and the SDM process. Known denitrifiers, Pseudomonadaceae, including the type genus *Pseudomonas*, were identified from the SDMGS samples as a small bacterial group. The Enterobacteriaceae family, which accomplished only the first step (reduction of nitrate to nitrite) of denitrification, was also noted as one of the dominant bacteria using the HTS method. *Bacteroides*, a genus of obligate anaerobic and rod-

shaped bacteria, has also been detected as a dominant group from the SDMGs samples, which might participate in the fermentation and denitrification process. Some unknown bacterial groups contributing to the denitrification process within the SDM still need to be further investigated.

#### 4 Conclusion

Performances of the batch experiments at a COD/NO<sub>3</sub><sup>-</sup>-N ratio of 8.0, including the removal of contaminants and production and component of biogas, were shown to be significantly influenced by nitrate addition. Proteobacteria, Bacteroidetes, Firmicutes, and Spirochaetes phyla were detected to be the dominant bacterial communities of the SDMGs during the initial stage of the SDM process. The structure and diversity of the fermentation microbes were reorganized due to the addition of nitrate, and Alpha-, Beta-, and Gamma-proteobacteria and Bacteroides could be the sources of possible denitrifiers. A dramatic flourish of species associated with animal and human infections, such as Enterobacteriaceae, Bacteroides, and Arcobacter, was noted to be recovered, hinting nitrate contamination, which should be properly treated especially in the initial stage under the anaerobic condition of water bodies.

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