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Influence of anoxic to aerobic volume ratio on sludge settleability and bacterial community structure in a denitrifying–nitrifying activated sludge system

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ABSTRACT

Biological treatment processes under nitrifying and denitrifying conditions are complex systems, in which different kinds of microbes dynamically cultivate and interact. The sludge bulking in these systems can be controlled by understanding the relationship between reactor performance and microbial diversity. In this study, sludge settleability and bacterial community structure were investigated in a modified Ludzack–Ettinger system as anoxic to aerobic volume ratio increased from 1:3 to 1:2 and finally to 2:1. Results indicated that settleability significantly improved as the anoxic to aerobic volume ratio increased ($p < 0.01$), accompanied by the increase in nitrogen removal efficiency and the change in filamentous bacteria. Polymerase chain reaction-denaturing gradient gel electrophoresis analysis revealed that the diversity and richness of bacterial species increased with a small volume ratio but decreased as the volume ratio increased, because of the low energy obtained from anoxic respiration. *Proteobacteria* were the predominant group, followed by *Bacteroidetes*. The influence of the anoxic condition on bacterial community structure was evaluated through correspondence analysis. The changes in bacterial community structure concurred with the reactor performance in terms of nitrogen removal efficiency and settleability. Samples with different settleability values were inclined to cluster into different groups.

Keywords: Anoxic zone; Nitrogen removal; Sludge settleability; Diversity; Bacterial community

1. Introduction

Water eutrophication has emerged as a serious problem in China because of rapid industrialization and urbanization [1]. Activated sludge systems are widely used in wastewater treatment for their low cost and high efficiency. Previous studies have indicated that the initial anoxic zone in the activated sludge system inhibits the growth of filamentous bacteria [2,3]. However, sludge bulking has been regularly reported in several nutrient removal systems with initial anoxic zone [4,5]. Therefore, further study is required to reveal the effect of anoxic condition on sludge settleability.

Several researchers have recently attempted to control sludge bulking in nutrient removal systems.

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Lakay et al. found that sludge settleability was significantly influenced by the anoxic mass fraction of the system [6]. Casey et al. proposed the nitric oxide (NO) hypothesis for the proliferation of low F/M filamentous bacteria [7,8]. Tsai et al. extended the NO hypothesis and suggested an alternative hypothesis based on the requirement of residual ammonia for *Microthrix parvicella* growth [9]. Both hypotheses relied on the biochemical behavior of organisms under alternating anoxic and aerobic conditions, and each hypothesis had its own merits. However, these hypotheses did not address the effect of anoxic condition on the overall bacterial community.

Insight into microbial community diversity and its function is useful in monitoring and controlling biological treatment systems [10]. A comprehensive range of literature has been published on the relationship between reactor performance and community diversity [11–13]. Xia et al. used polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) analysis to demonstrate that the diversity of the ammonia-oxidizing bacteria community decreased with the influent C/N ratio [14]. However, only a few studies have investigated the influence of reactor configuration on bacterial diversity. In addition, several researchers have investigated the phylogeny and ecophysiology of filamentous bacteria in activated sludge systems [15,16]. However, only a few studies discussed the competition between filamentous bacteria and floc-forming bacteria from a bacterial community perspective as reactor configurations changed.

The influence of anoxic to aerobic volume ratio on sludge settleability and bacterial community was investigated in this study. This study aims (1) to address the effect of the anoxic to aerobic volume ratio on sludge settleability, (2) to investigate the changes in the bacterial community structure as the anoxic to aerobic volume ratio increased, and (3) to determine the relationship between sludge settleability and bacterial community structure.

2. Materials and methods

2.1. System setup

A modified Ludzack–Ettinger system (MLE) and a completely aerobic activated sludge system were operated for 230 d. The MLE system included a denitrifying (nitrate reducing) anoxic zone, followed by a nitrifying (ammonia oxidizing) aerobic zone. An internal recycle linked the two zones. The entire experiment process consisted of three periods. The anoxic to aerobic volume ratios were set to 1:3, 1:2, and 2:1 for the first period (day 1 to day 71), the second period (day 72 to day 170), and the third period (day 171 to day 230), respectively. The completely aerobic activated sludge system without anoxic zone [conventional activated sludge system (CAS)] was operated as control. The working volume of both reactors was 6 L. The reactors were inoculated with sludge from the secondary clarifier of a local municipal wastewater treatment plant in Xi'an, China. The seed sludge with sludge volume index (SVI) value of approximately 250 mL/g contained a few filamentous bacteria (Fig. 1(A)). Nocardia-like organisms (*Actinobacteria*) were the dominant filaments in the sludge, which also had few *Sphaerotilus natans* (*Proteobacteria*) and type 0041 (Fig. 1(B)). The characteristics of the synthetic municipal wastewater fed to both systems are shown in Table 1. Air was introduced by two fine bubble aerators. The dissolved oxygen in the aerobic zone was maintained at 4.0 mg/L. The reactors were operated at room temperature (20–25°C). The flow rates of the influent, the return sludge, and the internal cycle were controlled by separate peristaltic pumps. The recycle ratio of the return sludge and the internal cycle were 100 and 200%, respectively. The influent flow rate was 18 L/d, and the hydraulic retention time (HRT) of the reactor was 8 h. The mixed liquor suspended solid (MLSS) concentration was maintained at approximately 3,500 mg/L, whereas the organic loading was maintained at approximately 0.40 kg chemical oxygen demand (COD)/(kgSS·d). The solid retention time was approximately 20 d. The concentrations of total nitrogen (TN) and total phosphorus (TP) were 38 and 4 mg/L, respectively.

Both systems reached steady state at 60 d (three sludge ages). During this time, the COD removal efficiency and settleability in the CAS system were relatively stable, whereas the nitrogen removal and settleability in the MLE system slightly changed. Notably, given that the original shape of the reactor in the CAS system was cuboid, the hydraulic condition of the system was unsatisfactory. To eliminate the dead zone, the reactor shape was changed to a cylinder on day 60. Thereafter, the SVI in the CAS system continuously decreased until day 90. The reactor shape apparently had a significant effect on sludge settleability in the CAS system.

2.2. Analytical methods

COD, ammonia, nitrite, nitrate, TN, TP, MLSS, and SVI were measured twice a week according to standard methods [17]. COD was determined by using the open reflux method. Ammonia, nitrite, and nitrate were measured through phenate, colorimetric, and

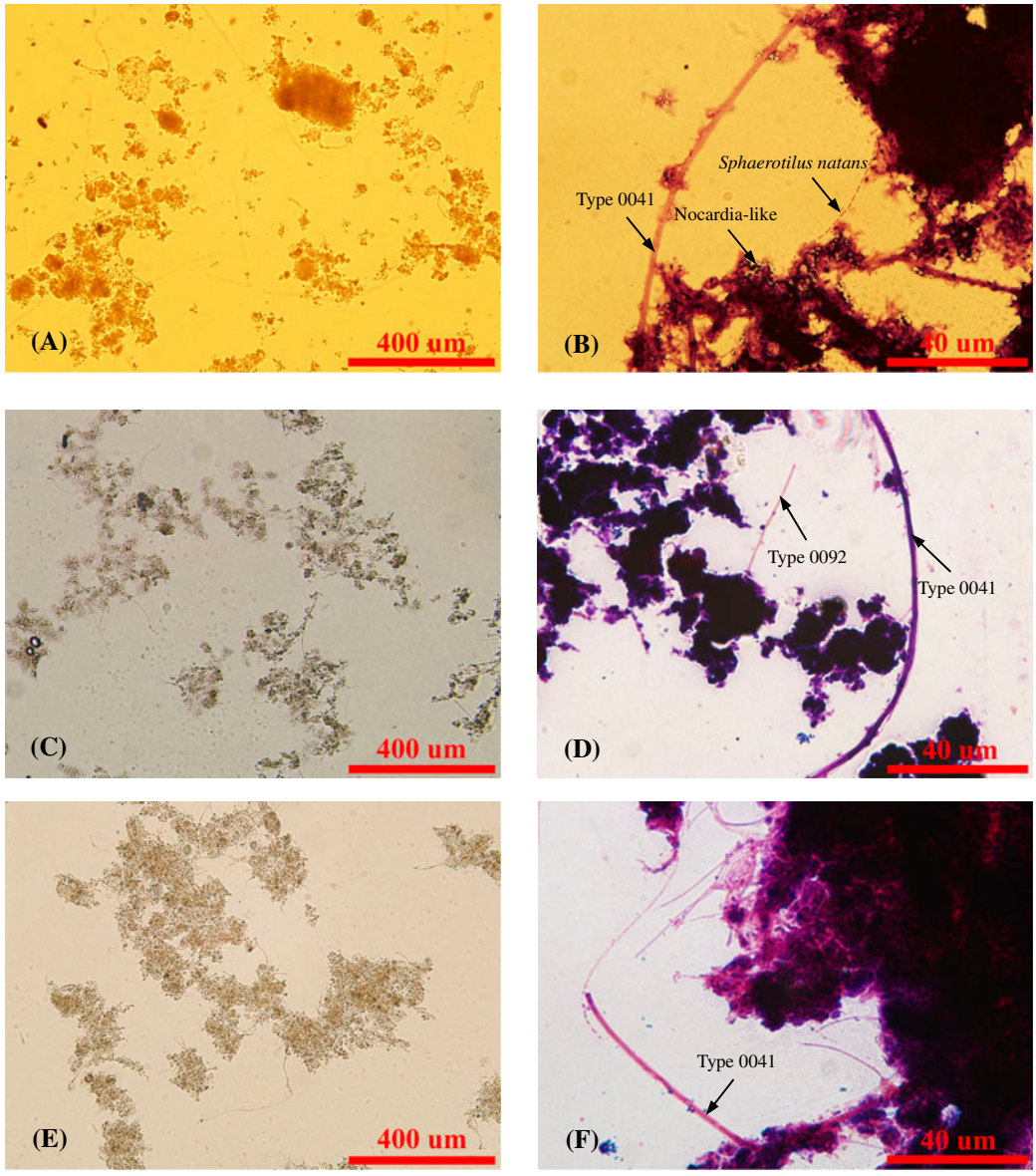

Fig. 1. Filament abundance categories using the subjective scoring system and micrographs of Gram staining of filaments: (A) 1, few; (B) Nocardia-like organisms, *S. natans* and type 0041; (C) 2, some; (D) Types 0092 and 0041; (E) 2, some; and (F) Type 0041.

ultraviolet spectrophotometric screening methods, respectively. TN and TP were measured by using the persulfate method and manual digestion, respectively. The *t*-test of the nitrogen removal efficiency and sludge settleability was performed by using SPSS 16.0 (SPSS Inc, USA) [18]. Sludge samples from both reactors were examined weekly through microscopy. Filaments were identified on the basis of the characteristics determined by Jenkins et al. [19]. First, filamentous species characteristics, including branching (true or false), filament shape, attached bacteria, sheath, filament width, filament length, sulfur deposits (sulfur granule), other granules (polyphosphate and PHAs), and staining reactions (Gram and Neisser), were recorded and summarized in a table. Then, the filamentous species were identified and carefully checked by using the key against the typical filamentous organisms listed in the tables and presented in the descriptions. Photographs of each organism in the book by Jenkins et al. were also used to identify the filamentous species [19]. Three independent microscopic examinations of each sample were performed

Table 1
Composition of synthetic municipal wastewater

Chemical compounds and food ingredient		Concentration (mg/L)	COD (mg/L)
Urea		41.67	
NH_4Cl		20.83	
$K_2HPO_4{\cdot}3H_2O$		7.34	
KH_2PO_4		4.40	
Readily biodegradable substrate (RBCOD)	Na-acetate	79.37	100
Slowly biodegradable substrates (SBCOD)	Potato starch (Particulate matter)	122	150
	Peptone	17.41	200
	Milk powder	96.83	
	Yeast	52.24	
Minerals and trace element[a]			

[a]$MgSO_4{\cdot}7H_2O$, 7.13 mg/L; $CaCl_2$, 5 mg/L; $NaHCO_3$, 25 mg/L; $FeSO_4{\cdot}7H_2O$, 10 mg/L. Trace element: KI, 60 μg/L; H_3BO_3, 300 μg/L; $MnSO_4{\cdot}H_2O$, 100 μg/L; $CoCl_2{\cdot}6H_2O$, 49.45 μg/L; $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 105.34 μg/L; $CuSO_4{\cdot}5H_2O$, 702.99 μg/L; $ZnSO_4{\cdot}7H_2O$, 379.78 μg/L.

to confirm the identifications. Micrographs of Gram staining are used to illustrate the types of filaments. The proliferation of filaments was based on the microscopic observation of the wet mount sludge samples and a subsequent examination of stained samples. On the basis of these observations, we subjectively rated overall filament abundance on a scale from 0 (none) to 6 (excessive) according to the method described by Jenkins et al. [19].

2.3. Microbial community analysis

The bacterial community structure of the sludge was analyzed by using PCR-DGGE as in a previous study [20]. Sludge samples were collected from both reactors on days 1, 71, 83,142, 198, and 228. Day 71 represented the end of period I. In period II, nitrogen removal efficiency in the MLE system began to increase on day 83. Days 142, 198, and 228 represented the time when both reactors were at steady state. The genomic DNA was extracted with the use of a soil DNA kit (Omega Bio-tek Inc, USA). Universal primers (341F with GC clamp and 534R) were used to amplify the conserved V3 region of 16S rRNA for most bacteria and archaea [20]. The nucleotide sequences of the primers were as follows: GC-341F (5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG-3′) and 534R (5′-ATTACCGCGGCTGCTGG-3′). The amplified PCR products were verified with 1.5% agarose gels and then separated through DGGE with polyacrylamide gel (8%, 37.5:1 acrylamide:bisacrylamide) with a linear gradient of 40–60% denaturant. The gel was run for 13 h at 70 V using 1 × TAE buffer maintained at 60˚C (Dcode™, Bio-Rad, USA), stained with silver, and then observed by using a UV transilluminator (GBoxEF, SYNGENE, UK). DNA was recovered by cutting the target band from the gel, after which it was reamplified and cloned into a pEASY-T vector, which was sent to Sangon Biotech Co., Ltd (China) for sequencing.

Quantity One (Bio-Rad, USA) was used to determine the relative intensity of each DGGE band, which was considered as a single species [21]. The relative intensity of the bands in DGGE profiles was confirmed in replicate gels. Shannon–Wiener diversity index (H), Margalef's index (R), and Pielou's Evenness Index (E) were introduced to analyze the diversity, richness, and evenness of the species, respectively [22–24]. The indices were calculated for each sample as follows:

$$H = -\sum(n_i/N)\log(n_i/N) \quad (1)$$

$$R = (S-1)/\ln N \quad (2)$$

$$E = H/\log S \quad (3)$$

where n_i is the relative intensity of each DGGE band, S is the number of DGGE bands (used to indicate the number of species), and N is the sum of the relative intensity of all bands in a given sample [25,26].

A matrix that contained the migration and intensity data of the band was exported from Quantity One to investigate the relationship between reactor performance and bacterial community structure. A correspondence analysis (CA) was then performed on the matrix using SPSS 16.0 (SPSS Inc, USA) [27]. CA is a geometric technique for displaying the rows and columns of a two-way contingency table as points in a low-dimensional space, such that the positions of the row and column points are consistent with their

associations in the table. CA results have two types of point symbols, which are row points for the rows and column points for the columns [28]. In this study, the row points represent the specific bacteria, whereas the column points represent the samples with different properties (sludge settleability and nitrogen removal efficiency). The distance between the samples is a measure of similarity between the row-frequency profiles, whereas the distances between the points representing the bacteria can be interpreted in a similar manner [29].

To determine the phylogenetic affiliation, 16S rRNA sequence similarity analyses were performed using GenBank, National Center for Bio-technology Information (NCBI), and BLAST algorithm (http://www.ncbi.nlm.nih.gov). The phylogenetic tree was constructed using MEGA version 5.0 [30].

The sequence data have been submitted to the GenBank databases under accession numbers KF049565 to KF049605.

3. Results

For both reactors, the ammonia of the effluent was below 0.5 mg/L. COD removal efficiency was always above 94%. The mixed liquor volatile suspended solid was relatively constant and was approximately 82 and 80% of the MLSS in the CAS and MLE systems, respectively. During the entire experiment, sludge settleability, filamentous bacteria, and bacterial community were substantially affected by the anoxic to aerobic volume ratio.

3.1. Influence of the anoxic to aerobic volume ratio on sludge settleability

The changes in MLSS and sludge settleability for both reactors are illustrated in Fig. 2(A). For the CAS system in which the anoxic to aerobic volume ratio was zero, nearly no denitrification occurred. The concentrations of nitrate and nitrite in the effluent were approximately 25 and 0.02 mg/L, respectively. The sludge settleability was relatively good, and the SVI was approximately 122 mL/g (<150 mL/g) at a steady state. Sludge settleability was unaffected by the nitrate and nitrite in the effluent.

By contrast, the concentrations of the ammonia, nitrite, and nitrate in the effluent notably changed as the anoxic to aerobic volume ratio increased in the MLE system (Fig. 2(B)). Sludge settleability was relatively poor (>150 mL/g) and was closely related to the nitrate in the effluent. During the first period (from day 1 to day 71), the anoxic to aerobic volume

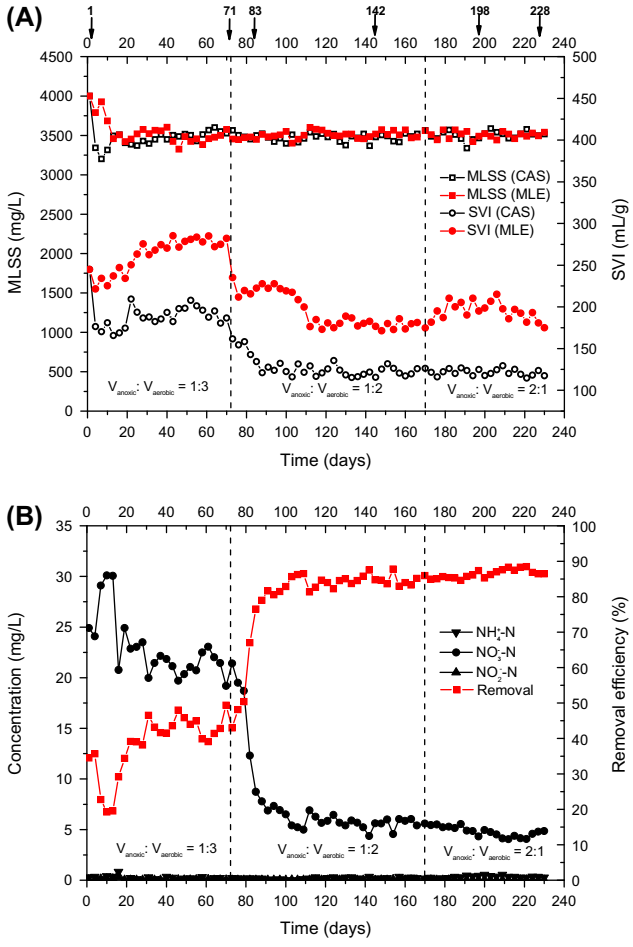

Fig. 2. (A) Variations of MLSS and sludge settleability in both reactors; (B) Variations of the ammonia, nitrite, the nitrate in the effluent, and the TN removal efficiency in the MLE system.

ratio was 1:3. The nitrogen removal efficiency of the system was approximately 38.46%, and the nitrate concentration in the effluent was approximately 23.39 mg/L, indicating the low denitrification potential of the system. Sludge settleability was poor, and the SVI was approximately 258 mL/g. During the second period (from day 72 to day 170), as the anoxic to aerobic volume ratio increased to 1:2, the nitrogen removal efficiency significantly increased to approximately 79.91% ($p < 0.01$). The SVI of the sludge remarkably decreased to approximately 195 mL/g ($p < 0.01$). During the third period (day 171 to day 230), as the anoxic to aerobic volume ratio increased to 2:1, the nitrogen removal efficiency slightly increased to approximately 86.43% ($p < 0.01$). However, sludge settleability was not further improved ($p = 0.985$).

The anoxic zone in an MLE system generally tends to degrade sludge settleability ($p < 0.01$). However, the increase in anoxic to aerobic volume ratio accompanied by the increase in nitrogen removal efficiency significantly alleviates sludge bulking in the MLE system ($p < 0.01$).

3.2. Influence of the anoxic to aerobic volume ratio on filamentous bacteria

During the entire experiment, the filamentous bacteria in the CAS and MLE systems changed in different ways. In the CAS system, the filamentous bacteria of Nocardia-like organisms and *S. natans* in the initial seed sludge were washed out during the first period, accompanied by the growth of type 0092 (Fig. 1(C) and (D)). From day 72 to day 230, the quantity of the filaments remarkably decreased, and the flocs slowly became large and compact. This condition gradually improved sludge settleability (Fig. 1(E) and (F)).

In the MLE system, the filaments in the seed sludge were slowly washed out, and types 0092, 0675, and 0041 proliferated during the first period (Fig. 3(A) and (B)). As the anoxic to aerobic volume ratio increased to 1:2 during the second period, types 0092 and 0675 were quickly covered by thick floc-forming bacteria and embedded in the flocs. Meanwhile, type 1851 rapidly grew and dominated the system (Fig. 3(C) and (D)). As the nitrogen removal efficiency increased to approximately 76%, both type 1851 and floc-forming bacteria thrived well in the system and competed for the influent substrate for the first few days. Then, the floc-forming bacteria overgrew the type 1851 and became the predominant bacteria in the system, thus remarkably improving sludge settleability. Microscopy observation at the end of this period showed that type 1851 was covered by thick floc-forming bacteria, and only some type 0092 grew in the system (Fig. 3(E) and (F)). During the third period, the flocs in the MLE system became round and compact, and some type 0675 extended from the floc surface (Fig. 3(G) and (H)).

3.3. Influence of the anoxic to aerobic volume ratio on the bacterial community structure

The PCR-DGGE patterns of the samples from both reactors are illustrated in Fig. 4. Although the dominant species in both reactors were similar, the quantitative distribution of the bacteria differed. Fig. 5 shows the changes in the evenness, diversity, and richness of the species for both reactors. The evenness in both reactors was initially nearly similar, changed slightly, and then continuously decreased during the entire experiment. By contrast, the diversity and richness of the sludge in the CAS and MLE systems changed in different ways. During the first period (from day 1 to day 71), the diversity and richness were almost unchanged in the CAS system but remarkably increased in the MLE system. At the end of this period, these two parameters were significantly higher in the MLE system than in the CAS system. From day 72 to the end of the experiment, the diversity and richness of the sludge in the CAS system continuously increased. However, these two parameters remarkably decreased in the MLE system.

Bands of interest were excised from the gel, reamplified, cloned, and sequenced. The sequences were compared with the GenBank database using BLAST. All sequences showed 94–100% similarity with previously identified 16S rRNA gene sequences (data not shown). Fig. 6 shows the neighbor-joining phylogenetic tree of all the species in both reactors. A total of 41 separate sequences were obtained from the 11 samples. *Proteobacteria* were found to be the most prevalent (21 of 41 sequences), followed by *Bacteroidetes* (14 sequences), *Chloroflexi* (2 sequences), *Firmicutes* (2 sequences), and TM7 (1 sequence).

CA was performed using SPSS 16.0 (SPSS Inc, USA) to investigate the relationship between reactor performance (nitrogen removal efficiency and sludge settleability) and bacterial community structure (Fig. 7). In the CAS system, the dominant bacteria (bands 12, 13, 14, 31, and 39) affiliated with β-*proteobacteria*, *Bacteroidetes*, or *Chloroflexi* significantly increased. Bacteria (bands 5, 6, 23, 28, and 33) that belonged to either the *Bacteroidetes* or other classes of *Proteobacteria* were slowly washed out. In the MLE system, the bacterial community structure inversely changed. The relative dominant bacteria (bands 5, 6, 23, and 29) affiliated with either *Proteobacteria* (α and β) or *Bacteroidetes* significantly increased. Bacteria (bands 12, 13, and 14) that belonged to β-*proteobacteria*, *Bacteroidetes*, or *Chloroflexi* slowly decreased. Although the microscopic observation showed several filaments in the MLE system, none of the obtained sequences can represent the filaments. The sludge with different settleability values clustered into different groups on the graphical map of CA (Fig. 7). Samples with relatively poor sludge settleability (>150 mL/g) were in the second quadrant, whereas samples with good sludge settleability were always in the third quadrant.

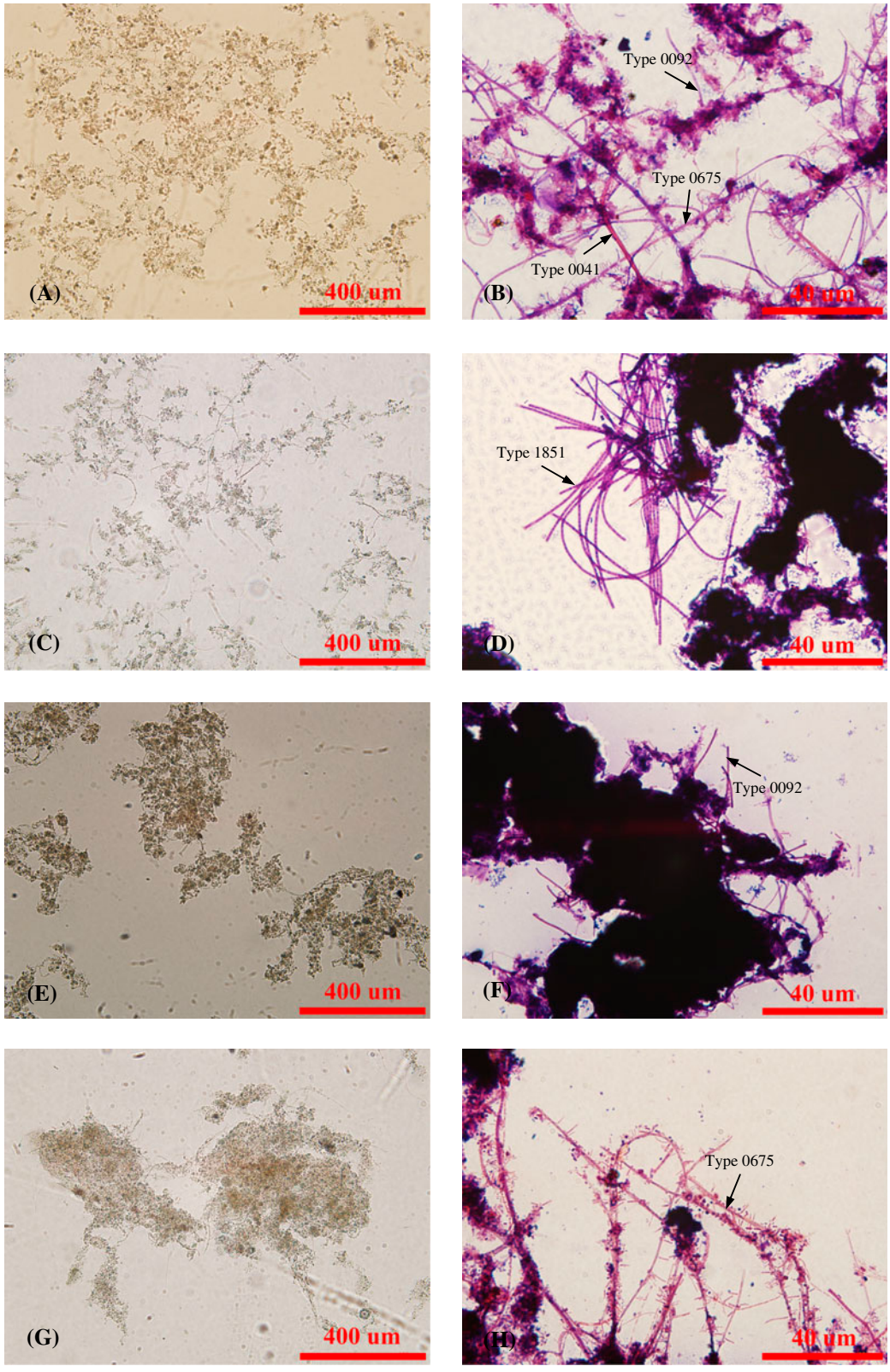

Fig. 3. Filament abundance categories using the subjective scoring system and micrographs of Gram staining of filaments: (A) 4, very common; (B) Types 0092, 0675, and 0041; (C) 3, common; (D) Type 1851; (E) 2, some; (E) Type 0092; (G) 3, common; and (H) Type 0675.

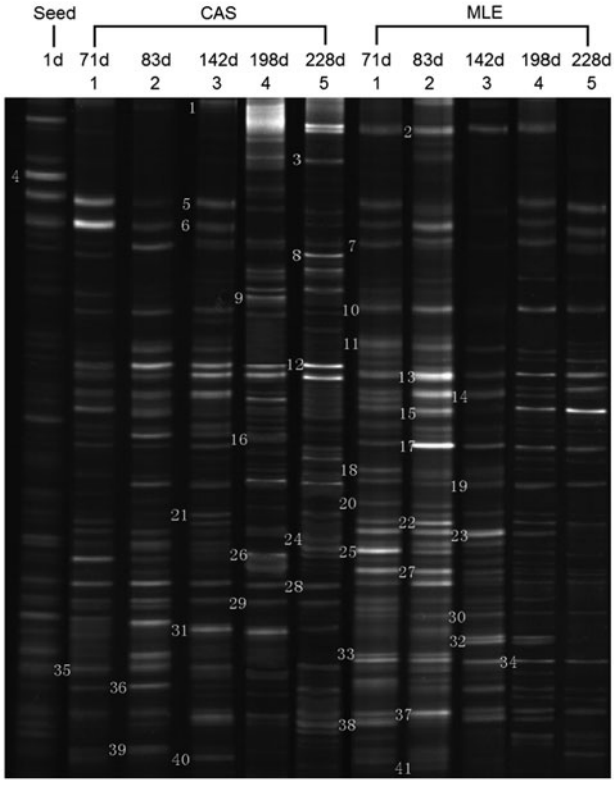

Fig. 4. DGGE pattern of the samples from both reactors.

4. Discussion

4.1. Slowly biodegradable COD and denitrification process

Previous studies indicated that sludge settleability is closely related to both the denitrification process and the influent slowly biodegradable COD (SBCOD), which has to be hydrolyzed before diffusion in the EPS matrix to reach the microbial cells [7,8,31]. During the entire experiment, the quantities of the filamentous bacteria and the SVI of the sludge in the MLE system were always higher than those in the CAS system. The incorporation of an anoxic zone in the MLE system is likely to be beneficial to the low F/M filamentous bacterial growth, which results in relatively poor sludge settleability.

In this study, a large quantity of SBCOD was found in the influent. In the CAS system, given that nearly no denitrification occurred, the influent SBCOD tended to serve a major function in the competition between the floc-forming bacteria and the filamentous bacteria. When the SBCOD enters into the system, most of these substrates will be absorbed in the flocs and then hydrolyzed into readily biodegradable COD (RBCOD). RBCOD can be directly utilized by the bacteria in the flocs. The aerobic hydrolysis rate is slow, and is approximately one-fifth to one-sixth of the rate of utilization of RBCOD. This parameter is the limiting factor in the hydrolysis-absorption-synthesis sequence [32]. The gradient of substrate concentration over the floc radius is quite small. The concentration of monomers at floc level is low and growth-rate-limiting. Therefore, the floc-formers outcompete the filaments for substrate because of higher substrate utilization rates. The filamentous bacteria remain inside the flocs, thus resulting in good sludge settleability in the CAS system [32,33].

However, in the MLE system with anoxic condition for nitrogen removal, the SBCOD is likely to be beneficial for filament growth, which results in poor settleability. This phenomenon agrees well with the nitric oxide (NO) hypothesis proposed by Casey et al. [7,8]. The NO hypothesis assumes that the floc-forming bacteria and filamentous bacteria in the activated sludge compete for mutually growth-limiting substrates. In a nitrogen removal system (i.e. MLE system), the competition between floc-forming bacteria and filamentous bacteria is influenced by the inhibition of substrate utilization by floc-forming bacteria under aerobic conditions. Under anoxic conditions, the floc-forming bacteria denitrify nitrate through each of the denitrification intermediates to dinitrogen. At high SBCOD concentration, the intermediate NO is intracellularly accumulated in the floc-formers because of the low rate of supply of electrons from SBCOD. Under aerobic conditions, the intracellular NO interacts with one of the enzymes specific to aerobic respiration, thus inhibiting the aerobic respiration of floc-forming bacteria. In contrast to floc-forming bacteria, the filamentous bacteria are nitrate reducers and do not intracellularly accumulate NO. The filamentous bacteria are uninhibited under aerobic conditions. Therefore, in systems with anoxic conditions for nitrogen removal, filamentous bacteria have a competitive advantage over floc-forming bacteria at high SBCOD concentrations, which results in poor sludge settleability.

In this study, when the anoxic to aerobic volume ratio was 1:3, the HRT of the anoxic zone was insufficient (2 h) for the hydrolysis of the SBCOD. The amount of electrons provided by the SBCOD for denitrification was small. Considerable NO would accumulate in the floc-forming bacteria, thus inhibiting their growth under aerobic conditions. Abundant SBCOD would likely undergo hydrolysis in the aerobic zone and will then be utilized by types 0092 and 0675 [19]. Consequently, the filaments proliferated and resulted in poor settleability in the MLE system. As the anoxic to aerobic volume ratio increased (1:2, 2:1), the time for the SBCOD to be hydrolyzed in the anoxic zone became adequate because SBCOD

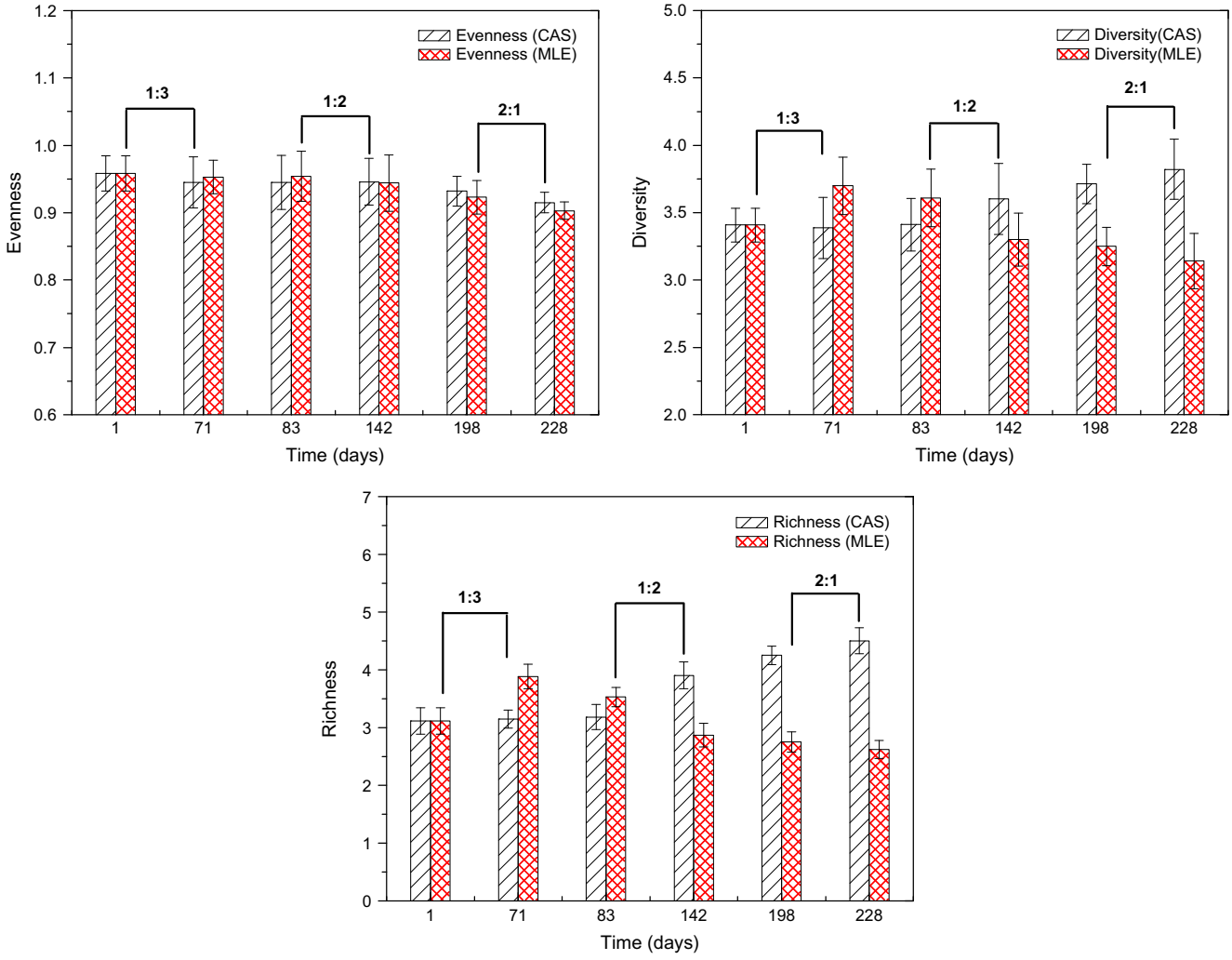

Fig. 5. Changes in the evenness, diversity, and richness of the species in both reactors.

hydrolysis only relied on time [34]. Considerable RBCOD would be available for the denitrifying bacteria in the anoxic zone, which can be verified by the increase in nitrogen removal efficiency and the proliferation of type 1851. Type 1851 is a filament known to prefer simple sugars and soluble starches as substrate. Accompanied with the increase in nitrogen removal efficiency, the increase in RBCOD hydrolyzed from SBCOD relieves the NO accumulation in the floc-forming bacteria and reduces the amount of SBCOD entering the aerobic zone. Therefore, the competitive advantage of filamentous bacteria was weakened, and the sludge bulking was mitigated in the system [35].

4.2. Bacterial community aspects

The initial seed sludge was collected from the system operated under anaerobic–anoxic–aerobic condition; thus, the majority of the bacteria that remained in both reactors were facultative bacteria, similar to those in a previous study [36]. When the anoxic to aerobic volume ratio was 1:3 in the MLE system, the diversity and richness were significantly higher than those in the CAS system. The relatively small anoxic zone tended to increase the environmental complexity, thereby increasing the diversity [37,38]. However, as the anoxic to aerobic volume ratio increased to 1:2 and 2:1, these parameters in the MLE system significantly decreased, contrary to the continuous increase in diversity and richness in the CAS system. During this time, a large quantity of COD in the influent was utilized in a large anoxic zone. The main electron acceptor in the MLE system changed from O_2 to NO_3^-. A previous study indicated that the energy obtained through anoxic respiration is less than the energy obtained through aerobic respiration

Fig. 6. Neighbor-joining phylogenetic tree of all the species in both reactors on the basis of 16S rRNA V3 region sequences derived from DGGE band.

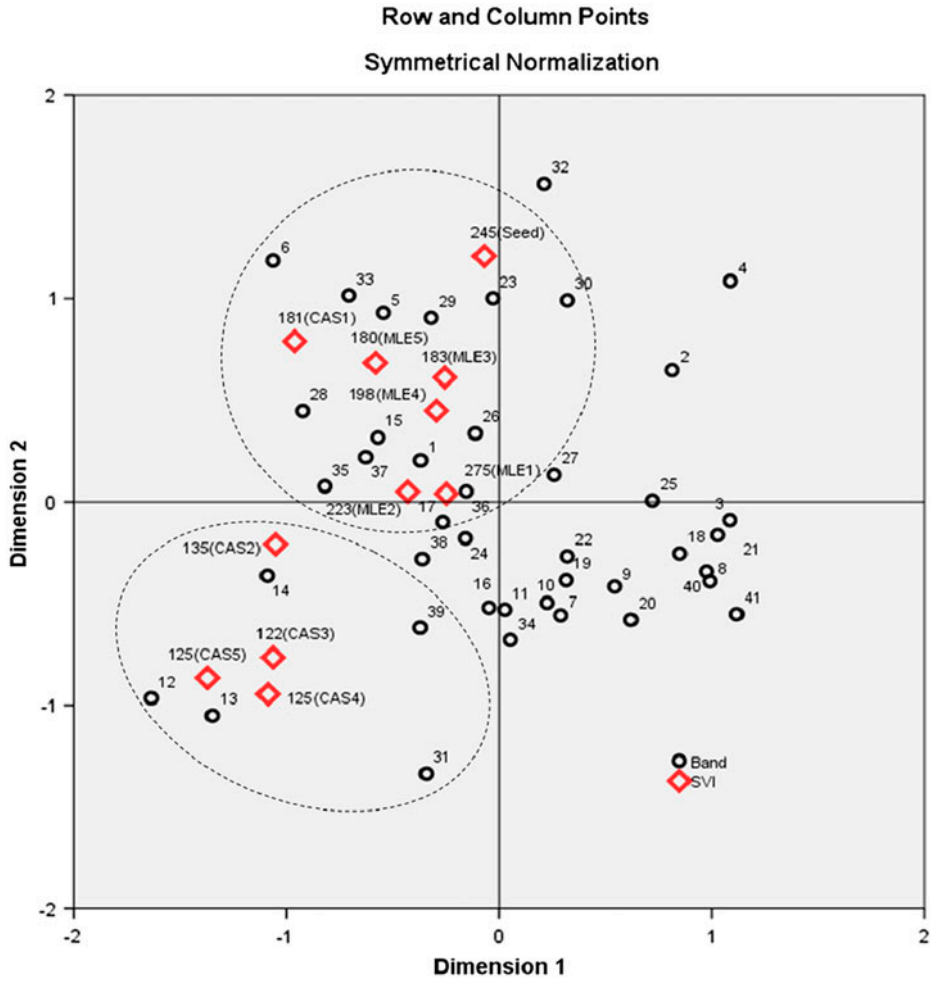

Fig. 7. CA of all the samples and species from both reactors.

[39]. Therefore, according to the species–energy hypothesis, which suggested that the decreased energy causes decreased diversity and richness, the diversity and richness in the MLE system with a large anoxic zone would be considerably lower than that in the CAS system [40].

The β-*proteobacteria* was the most predominant species (11 sequences) in both systems; this finding is consistent with that of recent studies [41–43]. The majority of the β-*proteobacteria* in this study were identified as *Rhodocyclus*, *Comamonadaceae*, or *Burkholderiales*. According to previous studies, these β-*proteobacteria* were potential denitrifiers [44,45]. *Bacteroidetes* were the second predominant group. The most dominant species within this group was *Saprospiraceae*. Xia et al. indicated that *Saprospiraceae* usually attached to the filamentous bacteria and hydrolyzed proteins [46]. *Saprospiraceae*, which provides amino acids to the denitrifiers and other bacteria, has an important function in the denitrification process.

The bacterial community structure in the MLE and CAS systems changed in different manners is illustrated in Figs. 4 and 7. The bacteria associated with bands 17 and 37 proliferated when the anoxic zone was enlarged in the MLE system. Band 17 was the most intense band in sample MLE2 collected when the anoxic zone was enlarged for 11 d. The bacteria represented by band 17 were similar to uncultured *Bacillus sp.* (JF818147.1) affiliated with *Firmicutes*. *Bacillus* sp. is an anaerobe that not only utilizes nitrate as a terminal electron acceptor but also degrades sugars and peptides [47]. Band 37 was closely related to *Saprospiraceae* (EU177677.1), which hydrolyzed the proteins. These two types of bacteria provide the carbon source for the denitrification process, thereby increasing the nitrogen removal efficiency. The bacteria associated with band 23, which predominated in the MLE system, were closely related to *Rhodoferax* sp. (HQ222266.1) affiliated with *Comamonadaceae*. According to the studies by Srinandan et al., bacteria

represented by band 23 are the principal denitrifiers in the MLE system [48]. The bacteria represented by band 12 remarkably increased in the CAS system, but slowly disappeared in the MLE system. These bacteria are closely related to ammonia-oxidizing β-*proteobacteria* (AY631517.1) found in tropical soil [49]. The aerobic environment in the CAS system stimulated the growth of these nitrifiers, whereas exposure to anoxic environment in the MLE system suppressed their growth. The shifts in the bacterial populations strongly coincide with the different configurations in both reactors.

Recent studies have shown that the dominant filamentous bacteria in nutrient removal systems are mainly affiliated with the *Actinobacteria* (*M. parvicella*), β-*proteobacteria* (type 0041/0675), and *Chloroflexi* (types 0092 and 1851) [50]. However, none of the sequences obtained in this study can represent the filamentous bacteria in both systems because only species of more than 1% of the analyzed community can be detected by PCR-DGGE. Therefore, although the filaments in this study may be less than 1%, they significantly affect the settleability of the sludge. Furthermore, the samples with different settleability clustered into different groups on the graphical map of CA. Considering that both the floc-forming bacteria and the filaments coexisting in the flocs were affected by the same operation conditions, the floc-forming bacteria may be useful indicators for sludge settleability.

In conclusion, the anoxic zone in the MLE system tends to degrade sludge settleability ($p < 0.01$). As the anoxic to aerobic volume ratio increases, the large quantity of RBCOD hydrolyzed from SBCOD can significantly mitigate sludge bulking in the MLE system ($p < 0.01$). The diversity and richness of bacterial species in the MLE system with large anoxic zone notably decreased because of the lower energy obtained through anoxic respiration. CA indicates that the bacterial community structure in both reactors was closely related to reactor performance (sludge settleability and nitrogen removal efficiency). Sludge with different settleability values clustered into distinct groups on the graphical map of CA.

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List of symbols

H — shannon–Wiener diversity index
R — margalef's index
E — pielou's Evenness Index
n_i — the relative intensity of each DGGE band
S — the number of DGGE bands
N — the sum of the relative intensity of all bands in a given sample

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