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Microbial and hydrodynamic properties of aerobic granules in a sequencing batch reactor treating landfill leachate*

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Abstract: A sequencing batch reactor (SBR) seeded with activated sludge was established for landfill leachate treatment. Small bio-aggregates began to appear after 40-d operation, and gradually changed to mature aerobic granules, with a mean size of 0.36–0.60 mm. Their sludge volume index at 5 min ($SVI_{5\ min}$), mixed liquor volatile suspended solids (MLVSS), and wet density were around 35 ml/g, 3.4 g/L, and 1.062 g/cm^3, respectively. The settling velocities of the granules in distilled water ranged from 0.3 to 1.3 cm/s, which were faster than those in landfill leachate with a salt content of 1.4% (w/v), and also slightly faster than those predicted by Stokes' law for porous but impermeable particles. Microbial community evolution during the granulation process and stages under different nitrogen loading rates (NLRs) were monitored and analyzed by polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE), cloning, and sequencing of 16S ribosomal RNA (rRNA) fragments. Results revealed that some primary and dominant communities in inoculating activated sludge died out gradually; while a few common bacteria, inhabiting soils, municipal wastewater, or activated sludge systems, dominated in the SBR system throughout. In addition, some other dominant species, associated with the aerobic granulation process, were thought to play a significant role in the formation and growth of aerobic granular sludge. During the stable operation time under low NLR, a few species were present in abundance, and may have been responsible for the high organic removal efficiency at this time.

Key words: Aerobic granules, Microbial community, Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE), Landfill leachate

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1 Introduction

Aerobic granulation has attracted increased interest in recent years, and extensive research has been done to investigate the basic theoretical issues, such as the mechanism and induced stressors of granulation, physical and hydrodynamic properties of the granular sludge, and organic removal and simultaneous nitrification and denitrification (SND) performances. However, most of these works were based on synthetic wastewater or readily biodegradable wastewater (Wang *et al*., 2007; Bao *et al*., 2009; Ni and Yu, 2010). Information on application of aerobic granules in the treatment of toxic and refractory wastewaters with high-strength ammonium, like landfill leachate, is very limited. Meanwhile, an insight into the microorganisms potentially involved in the aerobic granulation process has not been investigated sufficiently. The correlation between community structures and reactor performance of aerobic granular system has not been clarified. While a better understanding of the ecology and microbiology of microbial population dynamic in wastewater

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treatment systems is necessary to enhance treatment performance and control (Zhang *et al*., 2010).

Because traditional isolation- and cultivation-based techniques typically include less than 15% of the cells observed by direct counts (LaPara *et al*., 2002), novel microbial techniques need to be further developed and applied for understanding the microbial biodiversity and community structure in a biosystem. In recent years, DNA-based molecular techniques, like denaturing gradient gel electrophoresis (DGGE) of polymerase chain reaction (PCR) amplified 16S ribosomal RNA (rRNA) gene fragments, offer a valuable tool for the characterization of the bacterial population diversity in biological wastewater treatment systems (LaPara *et al*., 2002; Li *et al*., 2008). These techniques have been used to characterize the dynamics of microbial evolution in various wastewater treatment reactors (Hirooka *et al*., 2009; Li *et al*., 2010; Zhang *et al*., 2010), and to reveal the effects of feed strength, shock loading, and change of operation strategy on the population structure of the biomass (Liu *et al*., 2007; Li *et al*., 2008). The molecular techniques are also applied in aerobic granular system to study the bacterial community structures. With the PCR-DGGE technique, Zhuang *et al*. (2005) found the existence of a highly stable microbial community with members belonging to the α, β, and δ subdivisions of *Proteobacteria* and the *Cytophaga-Flavobacteria-Bacteroides* (CFB) group in aerobic granules degrading *tert*-butyl alcohol. Adav *et al.* (2007) reported that the *Acinetobacteor* sp. was expected to dominate the pyridine-phenol aerobic granule system. It has also been reported that *Sphingomonas*, *Methylobacterium*, and *Hyphomicrobium vulgare*, associated with methyl *tert*-butyl ether (MTBE) biodegradation, inhabited the aerobic granular system degrading MTBE (Zhang L.L., *et al*., 2008).

In this study, a sequencing batch reactor (SBR) seeded with activated sludge was established for landfill leachate treatment. The granulation process, physical and hydrodynamic properties of the granules, and the performance of granular system were examined. Microbial population dynamics during the aerobic granulation process and under different nitrogen loading rates were explored in detail. The relationship between the community structure and variation of predominant species with the organic removal performances is also discussed.

2 Materials and methods

2.1 Experimental set-up

Fig. 1 shows the schematic diagram of the experimental system. There were two reactors: a biological SBR and a chemical pre-treatment reactor (CPR). Dimensions of the biological SBR unit were: height of 153 cm, inner diameter of 5 cm, and working volume of 3.0 L. It was operated in a sequential mode for a 12-h cycle: 60 min of feeding, 640 min of aeration, 5 min of settling, 5 min of effluent, and 10 min of idling. The reactor was supplied with an airflow rate of 0.10–0.12 m^3/h during the aeration phase, equivalent to a superficial upflow air velocity of 1.41–1.69 cm/s.

Floc-like sludge from an oxidation ditch of a local landfill leachate treatment plant was added into the SBR as inoculums, resulting in an initial mixed liquor suspended solid (MLSS) of 4.5 g/L. Sludge retention time (SRT) was 25–35 d.

CPR, with a working volume of 10 L, was used for ammonia pre-treatment by adding magnesium oxide and phosphoric acid (Di Iaconi *et al.*, 2006). The optimum Mg:N:P ratio of 1.2:1:1 and reaction conditions were determined by preliminary tests.

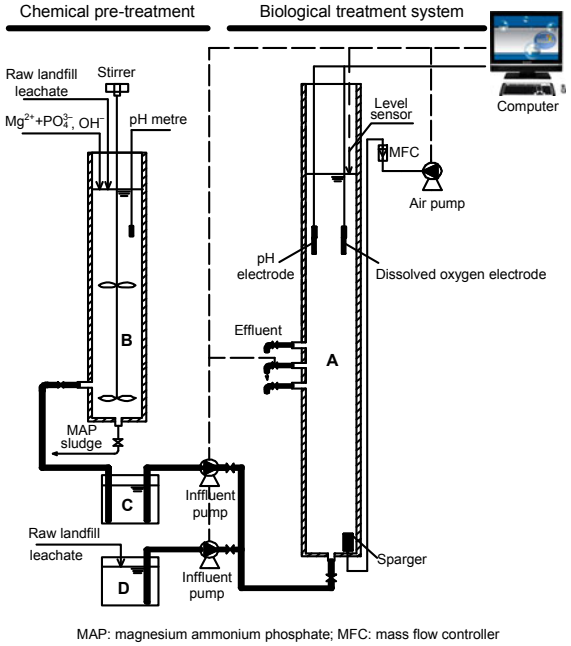

Fig. 1 Schematic diagram of the experimental system
A: biological sequencing batch reactor (SBR); B: chemical pre-treatment reactor (CPR); C: container for leachate after chemical pre-treatment; D: container for raw landfill leachate

2.2 Operational strategy

The experiment lasted for 241 d in total, including the aerobic granulation stage, high NH_4^+-N loading rate (NLR) stage, and low NLR stage (Table 1). During the first two stages, only the biological treatment system was started up. At the low NLR stage, operation of the chemical pre-treatment system and biological treatment system commenced simultaneously, while SBR influent was pre-treated for ammonia removal.

Both systems were operated at room temperature (18–30 °C). Oxygen and pH in the SBR were not controlled and varied between 0–8 mg/L and 7.4–8.9, respectively.

2.3 Analytical methods

Chemical oxygen demand (COD), total nitrogen (TN), NH_4^+-N, NO_2^--N, NO_3^--N, MLSS, mixed liquor volatile suspended solids (MLVSS), and sludge volume index at 5 min ($SVI_{5\,min}$) were measured regularly according to the standard methods (APHA, 1998). Sludge morphology was observed under an optical microscope (HIROX KH-7700, USA). Sludge particle size was determined by a laser particle sizer (Malvern Master Sizer). Settling velocity and wet density of aggregates were measured following the methods proposed by Li and Yuan (2002) and Wang (2006), respectively. Free ammonium (FA) concentration in aqueous phase can be estimated by (Ford *et al*., 1980)

$$\rho_{FA}=\frac{\rho_{NH_4^+\text{-}N}\times 10^{pH}}{\exp[6334/(273+T)]+10^{pH}}, \tag{1}$$

where ρ_{FA} (mg/L) and $\rho_{NH_4^+\text{-}N}$ (mg/L) are the concentrations of FA and ammonium, respectively, and T (°C) is the liquid temperature.

2.4 Stokes' law for impermeable biological aggregates

Stokes' law is usually applied for mathematical analysis of settling performances of particle aggregates in water and wastewater treatment processes (Li and Ganczarczyk, 1989; Li and Yuan, 2002; Zhang *et al.*, 2004; Su and Yu, 2005; Xiao *et al.*, 2008), and the settling velocity (U_s) of microbial aggregates predicted from Stokes' law may be written as (Li and Yuan, 2002):

$$U_s=\left[\frac{8gf}{\pi}\left(\frac{1}{\rho_l}-\frac{1}{\rho_c}\right)\frac{W_d}{C_d d^2}\right]^{1/2}, \tag{2}$$

where ρ_c and ρ_l are the densities of the (wet) aggregate and the liquid, respectively, g is the gravitational constant, d is the size of the aggregate, f is a dimensionless ratio between the wet mass and dry mass of the cells with the aggregate, and W_d is the dry mass of the bacterial aggregate. To avoid a measuring error, aggregates with the same diameter were filtered together to determine the total dry mass, which was divided by the number of granule and the dry mass of a single aerobic granule was given. C_d is the empirical drag coefficient, and can be obtained on the base of Reynolds numbers (Re) (Li and Yuan, 2002):

$$C_d=\frac{24}{Re}+\frac{24}{1+\sqrt{Re}}+0.4. \tag{3}$$

2.5 DNA extraction, polymerase chain reaction (PCR) amplification and denaturing gradient gel electrophoresis (DGGE)

Genomic DNA of the biomass in sludge and granule samples was extracted using an EZNA™ Soil DNA kit (D5625-01, Omega, USA). Subsequently,

Table 1 Parameters at different operational stages

Operational stage	Organic loading rate (kg COD/(m^3·d))	Nitrogen loading rate (kg NH_4^+-N/(m^3·d))	Volumetric exchange ratio of the SBR (%)	Critical settling velocity for flocs (m/h)
Aerobic granulation stage (Day 1–70)	0.67–2.16	0.18–0.38	30–50	5.51–9.16
High NLR stage (Day 71–130)	2.51–4.79	0.94–1.34	50	9.16
Low NLR stage (Day 150–241)	4.30–5.55	0.14–0.37	50	9.16

the 16S rRNA gene was amplified by PCR (Muyzer *et al.*, 1993). The forward primer sequence was F357-GC (5′-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCCCCTACGGGAGGCAGCAG-3′) and the reverse primer was R518 (5′-ATTACCGCGGCTGCTGG-3′). PCR amplification was performed with a PCR authorized thermal cycler (Eppendorf 5331, Hamburg, Germany). The PCR mixture was prepared in a total volume of 50 μl in 0.2-ml Axygen PCR tubes. The reaction solution contained 2.5 U of Taq DNA polymerase (Promega, Shanghai, China), 5 μl of 10× buffer, 2.0 mmol/L $MgCl_2$, 2 μl template DNA (10–100 ng), 0.2 mmol/L dNTPs, and 0.25 μmol/L of each primer. A touchdown thermal profile technique was used for the PCR procedure (Li *et al.*, 2008). Its temperature program began with 5 min of activation of the polymerase at 94 °C. Twenty PCR cycles were then conducted, with the first two cycles consisting of 1 min denaturation at 94 °C, 1 min annealing at 65 °C, and 2 min synthesis at 72 °C for each cycle. While the temperatures for denaturation and synthesis remained the same, the annealing temperature was subsequently decreased by 1 °C after every two cycles, until it reached 55 °C. After the 20 PCR cycles, an 8-min extension step at 72 °C was performed.

The PCR-amplified DNA products were separated by DGGE on polyacrylamide gels (8%, 37.5:1 acrylamide-bisacrylamide) with a linear gradient of 35%–55% denaturant (100% denaturant=7 mol/L urea+40% (w/v) formamide). Gels were run for 7 h at 150 V in 1× TAE buffer (Tris-acetate-EDTA) maintained at 60 °C. Denaturing gradient gels were poured and run by using the DGGE-2001 system (CBS Scientific Co. Inc., USA). Gels were silver-stained, air dried and scanned following the procedures in (Sanguinetti *et al.*, 1994). The gel images were analyzed with the software Quantity One Version 4.26 (Bio-Rad). This was used to calculate the similarity coefficient between lanes, in which the band and pattern in one of the lanes was defined as 100%.

The Shannon-Weaver index H was used as a measurement of the microbial diversity that took into account the richness and the proportion of each species in a population (Li *et al.*, 2008). The index H was calculated using

$$H=-\sum_{i=1}^{s} p_i \log p_i, \tag{4}$$

where p_i is the proportion of band i in the DGGE profile and s is the total number of the bands. DGGE fingerprints were manually scored by the presence of bands with consideration of the band brightness intensity.

2.6 Cloning and sequencing analysis

Selected DGGE bands were excised and dissolved in 50 μl Milli-Q water overnight at 4 °C. DNA was recovered from the gel by freeze-thawing three times. These DNA band samples (3 μl) were reamplified by the same PCR procedure as described previously, using the primer set F357/R518 without GC-clamp. The PCR products were cloned using the PGEM™-T Easy vector system (Promega, Madison, WI). The positive colonies were amplified with F357/R518. PCR amplicons were submitted for sequencing using ABI 3730 capillary sequencers (PE Applied Biosystems, Invitrogen, Beijing, China) (Zhang *et al.*, 2010). Sequence data were analyzed in comparison with the 16S rRNA sequences in the GenBank by BLAST search (National Centre for Biotechnology Information (NCBI)) for species identification.

3 Results and discussion

3.1 Formation and physical characteristics of aerobic granules

The inoculum sludge was typical conventional activated sludge, dominated by filamentous bacteria. The size of the seed sludge was less than 0.15 mm, and its mean floc diameter was 0.04 mm. Raw activated sludge settled relatively well, with the $SVI_{5\ min}$ of 99.6 ml/g (Fig. 2). During the initial operation time, the sludge settling ability deteriorated due to sludge inadaptability to the reactor configuration and operational strategy, causing a significant increase in the sludge $SVI_{5\ min}$. This situation resulted in a severe washout of the suspended biomass and a drop of biomass concentrations in the SBR. After 18 d of acclimation, the settling velocity of sludge began to increase, which led to a corresponding decrease of $SVI_{5\ min}$ and an increase in biomass concentration. Meanwhile, the mean floc size increased to 0.10 mm, with the size distribution being concentrated between 0.05 and 0.19 mm (Fig. 3a). Small granules became

visible after 40 d of operation, and compact mature granules with a clear contour boundary formed on Day 50. Afterwards, the particle size of these granules stabilized at 0.36–0.60 mm (Fig. 3b). A stable granular SBR system was achieved, with the $SVI_{5\ min}$, MLSS, MLVSS, and wet density (ρ_c) of sludge stabilizing at approximately 35 ml/g, 5.0 g/L, 3.4 g/L, and 1.062 g/cm^3, respectively.

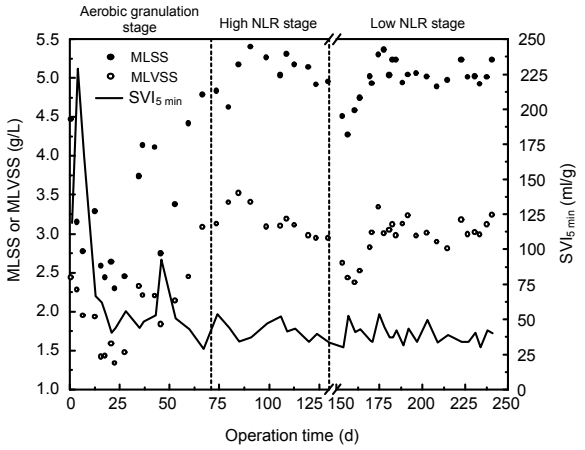

Fig. 2 Variations of MLSS, MLVSS and SVI during the whole operation

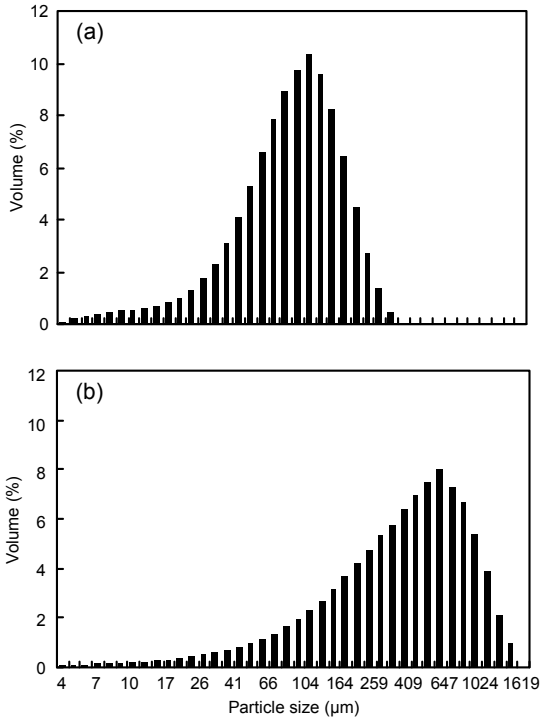

Fig. 3 Comparison of the size distribution between granular sludge on operating Day 18 (a) and Day 113 (b)

3.2 Settling velocities of aerobic granules

A total of 56 aerobic granules with a diameter of 0.3–1.4 mm were recovered and analyzed to determine their dry mass and settling ability. The dry mass W_d was in the range of 0.0006–0.0202 mg (Fig. 4a). Based on the slope of the logarithmic relationship between W_d and d, the fractal dimension D of the aerobic granules was calculated as 2.50, at the range of D>2.1 for flocs produced in bioreactors (Zhang *et al.*, 2004). Settling velocities (U_s) of the granules in distilled water ranged from 0.3 to 1.3 cm/s (Fig. 4b). The corresponding *Re* numbers were ranged from 12.4 to 209.3. Because the wet density of the aggregate in this study was close to the value (1.059 g/cm^3) in Li and Yuan (2002), f=3.45 was applied. Using Eqs. (2) and (3) from *Re*, d, f, and W_d, the settling velocities of the granules predicted by Stokes' law were estimated and are depicted in Fig. 4b.

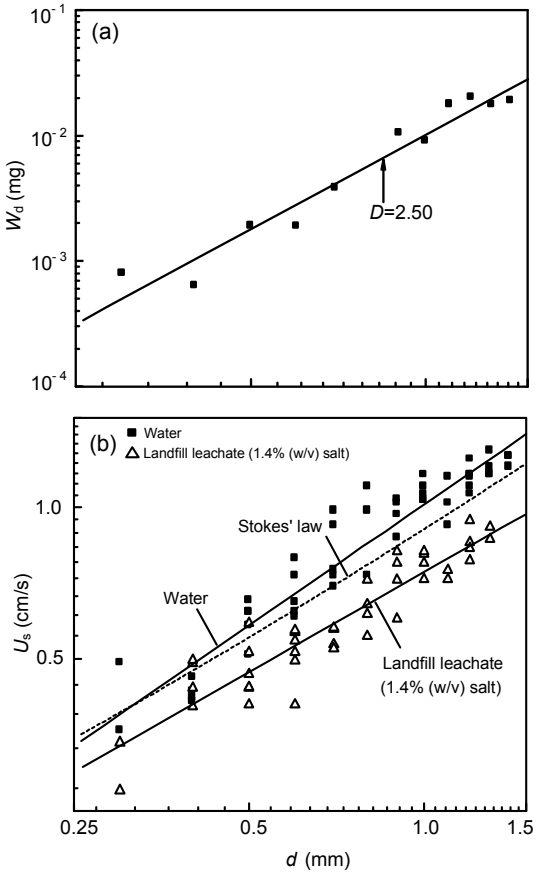

Fig. 4 Fractal characteristics and settling ability of granules: (a) relationship of diameter and dry mass; (b) settling performances

The slope of the observed settling velocity against the size after a log-log transformation was 0.77, larger than the slope of 0.64 predicted by Stokes' law for porous but impermeable particles. Similar results were also observed in previous studies (Table 2). The dimensionless ratios between the observed and predicted settling velocities, Γ, varied from 0.85 to 1.45, with an average of 1.11 (Fig. 5a), suggesting that the internal permeation of granules was not sufficient to affect their settling velocities, and these aggregates could be considered as impermeable in terms of their settling (Li and Yuan, 2002).

When the granules settled from distilled water into landfill leachate with a salt content of 1.4% (w/v), a high reduction in settling velocity was observed. The ratios of the settling velocities in the denser leachate to those in distilled water, Φ, were in the range of 0.56–1.17, with an average of 0.79 (Fig. 5b).

3.3 SBR performances

Fig. 6 shows the changes and removal efficiencies of COD, TN, and NH_4^+-N. For acclimatization of microorganisms to inhibitory compounds present in the leachate, SBR influent was appropriately diluted with tap water in the early phase of the aerobic granulation stage (Days 1–50), with the volume percentage of leachate in the influent stabilizing at 50%. COD and nitrogen removal performances fluctuated sharply during the aerobic granulation stage (Figs. 6a and 6b). From Day 51, the volume percentage of leachate in the influent was raised gradually, and the SBR was fed exclusively with raw landfill leachate from the beginning of the high NLR stage.

In the initial phase of the high NLR stage, a high strength of FA (Fig. 7) had the potential to inhibit the growth rate of microorganisms and microbial activity in the biological process (Li and Zhao, 1999), COD removal efficiency waved violently. Nevertheless, COD removal performance was improved, after 30-d acclimatization of the microorganisms to high NLR. When the influent COD was about 3996 mg/L in the later phase, effluent COD was in the range of 497–911 mg/L, with the average removal efficiency

Table 2 Physical and geometric parameters of aggregates

No.	Aggregate type	Medium source	OLR (kg COD/(m^3·d))	d (mm)	ρ_c (g/cm^3)	U_s (cm/s)	Re	f	D	U_s–d relationship	Obs. slope	Pred. slope	Ref.
1	Granule	AW		1.1–2.1	1.059	0.22–0.64	3.5–14.8	3.45	2.09±0.42	logU_s–log$d$	0.90	0.74	Li and Yuan (2002)
	AS	DW		1.3–2.4		0.17–0.42	2.4–8.8		2.26±0.47		0.94	0.90	
2	Granule	AW	27	1.2		0.3–1.6	1.8–49.6		2.11±0.17	U_s–log$d$			Zhang *et al.* (2004)
			53	3.5					2.20±0.12				
			80	5.8					2.48±0.13				
3	Granule	SPW	6	0.9–2.5	1.017	0.41–1.27			1.87±0.34	U_s–d	0.32	0.30	Su and Yu (2005)
4	F-granule	AW	1.5	1.835–10.745	1.06	0.38–2.67	4.9–274.7	3.45	2.23±0.06	logU_s–log$d$	0.83	<0.83	Xiao *et al.* (2008)
	B-granule			1.835–6.672		0.42–3.21			2.42±0.07		0.68	<0.68	
5	Granule	LL	4.95	0.3–1.4	1.062	0.3–1.3	12.4–209.3	3.45	2.50	logU_s–log$d$	0.77	0.64	Ours

AS: activated sludge; AW: artificial wastewater; DW: domestic wastewater; OLR: organic loading rate; SPW: soybean processing wastewater; LL: landfill leachate; F-granule: fungi-dominated granule; B-granule: bacteria-dominated granule; Obs. slope: observed slope of U_s–d relationship; Pred. slope: predicted slope from Stokes's law

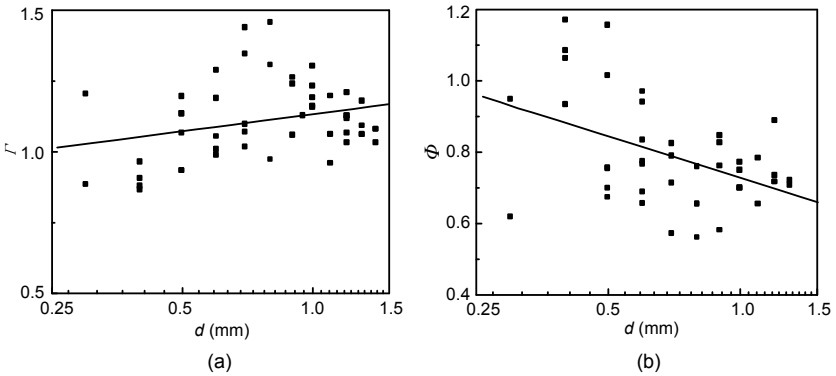

Fig. 5 (a) Ratios of observed and predicted settling velocities, Γ; (b) Ratios of the settling velocities in the landfill leachate with salt content of 1.4% (w/v) to those in distilled water, Φ

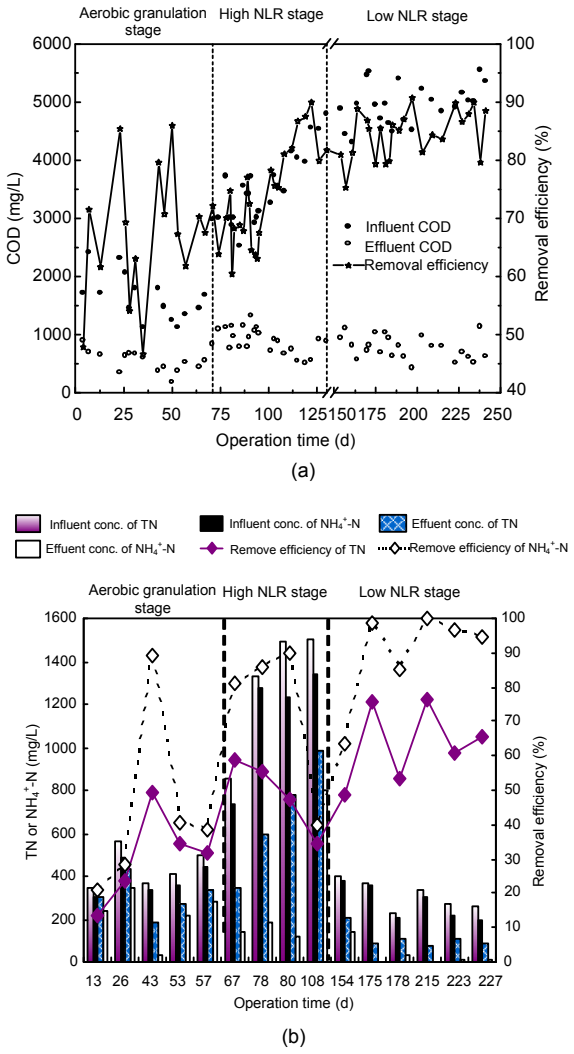

Fig. 6 Organic (a) and nitrogen (b) removal performances at different operational stages

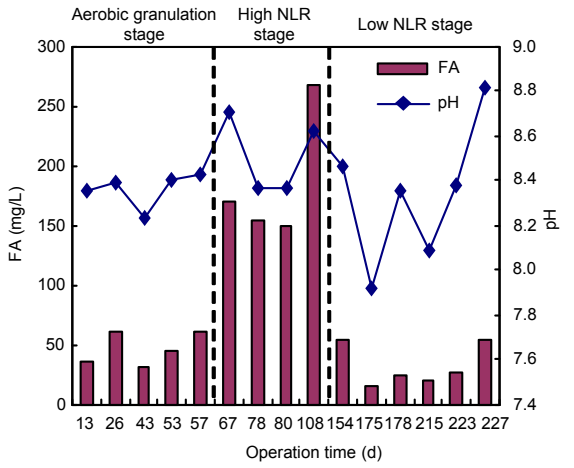

Fig. 7 Influent free ammonium (FA) of the biological treatment system

of 81.8%. Nitrogen removal efficiencies were relatively high in the initial phase of the high NLR stage (Fig. 6b), but decreased gradually in the later phase. On Day 108, when the influent concentrations of TN and NH_4^+-N were 1503 and 1339 mg/L, the effluent values were 982 and 804 mg/L, with the low removal efficiencies of 34.6% and 40.0%, respectively. In addition, due to FA inhibition on microbial activity of nitrite oxidizing bacteria (NOB), nitrite accumulation occurred. Oxidized nitrogen mainly existed in the form of nitrite, with the effluent concentration of 79.8–117.2 mg/L.

During the low NLR stage, FA level of the influent decreased to 17–55 mg/L. Stable effluent of good quality in long-term operations was finally reached, with the average removal efficiencies being 84.4%, 63.4%, and 89.7% for COD, TN, and NH_4^+-N, respectively. Additionally, complete nitrification took place at this stage. The nitrogen oxidation product in the effluent was mainly nitrate, with a concentration of 25 mg/L.

3.4 Microbial population dynamics at different operational stage

Bacterial community DNA was extracted from the sludge, which was sampled once every few days. Using the DNA obtained from DNA kit method as template, 250-bp fragments of the V3 hypervariable region of the 16S rRNA gene were obtained as expected. DGGE was carried out to determine the genetic fingerprint of these PCR amplification products, and the profile is illustrated in Fig. 8a. It is noticeable that greater changes of DGGE bands and patterns were observed at different operational stages. When the DGGE pattern of Day 220 is defined as 100%, the similarity coefficient of the lanes on Day 1 vs. on Day 220 was only 31.5%, as shown in Fig. 8b, indicating a high community shift between the inoculum sludge and the mature aerobic granules.

It can be seen that several dominant bands appeared across all biomass samples, identified by A–C. This indicates that the species represented by these bands were predominant populations throughout the experiment, even under the operational condition of high NLR. There were also some relatively persistent species, like band_D, which were very intense during the later aerobic granulation stage and the low NLR stage, but became depleted or temporarily not

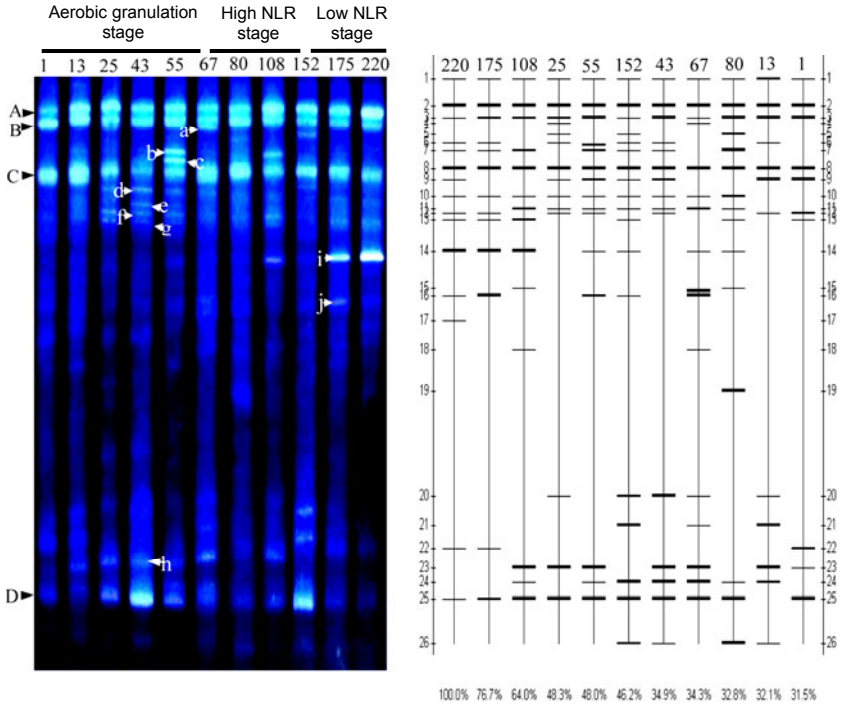

Fig. 8 DGGE profiles of the bacterial communities in the SBR at different operational stages

(a) DGGE profile (the predominant bands are labeled A–D, bands a–j are dynamic species existing in partial samples; (b) Similarity diagram of sample lanes (DGGE pattern of Day 220 is defined as 100%, values along the bottom indicate the similarity coefficient with Day 220). Lane labels along the top show sampling time (days) from startup of the bioreactor

detectable during the high NLR stage. This phenomenon indicates that the population represented by band_D was particularly sensitive to changing of operational conditions.

In addition, some primary and dominant communities in the inoculating activated sludge died out gradually, while secondary microbial species, represented by bands a–h, became predominant with the formation and maturation of the aerobic granules. However, these bands were not observed after the nitrogen shock load. After a few days acclimation, these bands appeared again as shown in lane 108, which means that the corresponding species accommodated the varying reactor conditions. Additionally, band_i and band_j were very intense in the sludge samples of the low NLR stage. Taken together with the results as shown in Fig. 6, it was suggested that the species marked by band_i and band_j may have been responsible for the high pollutants removal efficiency at this time.

The Shannon-Weaver diversity index H is a measurement of DGGE band patterns, which provides a direct indication of the apparent diversity of a microbial community (Li *et al.*, 2008). The profile of H as shown in Fig. 9 indicates a variable microbial diversity during the whole operation time. The initial H of 0.78, decreased to a minimum value of 0.74 on Day 13, signifying microbial populations with fewer species and a disproportionate distribution of individuals at this time. From Day 25 to 67, the H value increased and reached a peak on Day 55, which appeared to coincide with the crucial stage of formation and growth of aerobic granules. The H value decreased when the high NLR was applied, which suggests a larger community shift in response to the nitrogen shock load. In the initial phase of the low NLR stage, the H value increased slightly to 1.04, but decreased to and was maintained at a stable range of 0.87–0.88 in the stationary operational period under low NLR. This phenomenon was consistent with Zhang B. *et al.* (2008), which could be explained by the fact that long-term steady running conditions, including operational strategies and influent wastewater characteristics, brought up the absolute and irreplaceable predominance of a few species of bacteria, and further contributed to a stable, but simple community structure. In addition, the operational period with lower H values (from Day 161 to 241) just corresponded to the running time in which removal efficiencies of COD, NH_4^+-N, and TN were stable and high. This suggests that the small number of predominant species could maintain the optimum stable reactor performance and provide higher effluent quality.

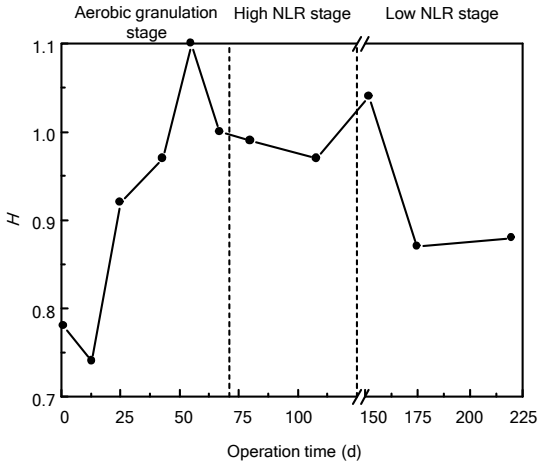

Fig. 9 Bacterial species diversity using the Shannon-Weaver diversity index (*H*)

3.5 Sequencing results and analysis

Six bands were carefully excised, amplified, and sequenced to identify microbial species, and the nucleotide sequences were compared to previously identified 16S rRNA gene sequences in the GenBank database using BLASTN. The results are shown in Table 3. It can be seen that band_A was most closely related to *Arcobacter butzleri* strain *ED-1*, while the *Arcobacter* sp. was found in abundance in municipal wastewater (Moreno *et al.*, 2003). Band_B had relatively low homologies (92%) in nucleotide sequence with uncultured bacterium gene for clone: *13C-A8*, which was thought as one acetate- or methanol-assimilating bacterium (Osaka *et al.*, 2006). Band_C was closely related to uncultured *Burkholderia* sp. clone *GASP-45KB-122-B04*, which was identified in soil microbial community (Tarlera *et al.*, 2008). Band_D was most closely related to *Uruburuella suis* clone *B01*, which may be the common species found in activated sludge system (Parsley *et al.*, 2010).

Among these strains, the presence of band_a and band_d was accompanied the formation and maturation of aerobic granules. Band_a was closely related to uncultured bacterium clone *N1903_90*, which was thought as one of the determinants of activated sludge settling performance. Band_d was closely related to uncultured bacterium clone *JT30*, which was detected in another aerobic granular system (Li *et al.*, 2009). Thus, it was apparent that these bacterial species played a significant role in the formation and growth of aerobic granular sludge.

4 Conclusions

1. Aerobic granules were developed in the SBR treating real landfill leachate, and a stable granular SBR system was achieved with a granule size of 0.36–0.60 mm, $SVI_{5\ min}$, MLSS, and MLVSS of sludge stabilized at around 35 ml/g, 5.0 g/L, and 3.4 g/L, respectively.

2. The granules in the system had a fractal dimension of 2.50, and their settling velocities in distilled water were in the range of 0.3–1.3 cm/s, slightly faster than those predicted by Stokes' law for porous but impermeable particles. The ratios between the

Table 3 Sequence analysis and species identification of selected DGGE bands for the sludge samples

Band	Sequence (bp)	Closest relatives		
		Accession No.	Description in GenBank	Identity (%)
A	175	FJ968634	*Arcobacter butzleri* strain *ED-1* 16S ribosomal RNA gene, partial sequence	100
B	202	AB205653	Uncultured bacterium gene for 16S rRNA, partial sequence, clone: *13C-A8*	92
C	203	EU044334	Uncultured *Burkholderia* sp. clone *GASP-45KB-122-B04* 16S ribosomal RNA gene, partial sequence	94
D	170	GU003819	*Uruburuella suis* clone *B01* 16S ribosomal RNA gene, partial sequence	99
a	164	EU104344	Uncultured bacterium clone *N1903_90* 16S ribosomal RNA gene, partial sequence	98
d	154	FJ645539	Uncultured bacterium clone *JT30* 16S ribosomal RNA gene, partial sequence	95

observed and predicted settling velocities varied from 0.85 to 1.45, with an average of 1.11. When the granules settled from distilled water into landfill leachate with a salt content of 1.4% (w/v), a high reduction in settling velocity was observed. The ratios of the settling velocities in the denser leachate to those in distilled water were in the range of 0.56–1.17, with an average value of 0.79.

3. Results of PCR-DGGE and species diversity index *H* indicated that the microbial community structure changed dramatically during the whole operation time. Some primary and dominant communities in the inoculating activated sludge died out gradually, while a few microbial species became predominant with the formation and maturation of the aerobic granules, and an increase in the *H* value was noted at this time. Under nitrogen shock load, these species became depleted or temporarily not detectable, and the *H* value turned to decrease. After the high NLR stage, some other microbial species became predominant. Taken together with the results of pollutants removal performance, the species marked by band_i and band_j were thought as functional bacteria responsible for the high pollutant removal efficiency at static operation time.

4. Sequencing analysis demonstrated that the dominant species appeared across all biomass samples were common bacteria inhabiting soils, municipal wastewater, or activated sludge systems. The dominant species, associated with the formation and growth of aerobic granules, were detected in other aerobic granular system, and might allow one to determine the settling performance of bioaggregates.

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