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ORIGINAL ARTICLE

Coexistence of nitrifiers, denitrifiers and Anammox bacteria in a sequencing batch biofilm reactor as revealed by PCR-DGGE

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Abstract

Aims: The bacterial diversity in a sequencing batch biofilm reactor (SBBR) treating landfill leachate was studied to explain the mechanism of nitrogen removal.

Methods and Results: The total microbial DNA was extracted from samples collected from landfill leachate and biofilm of the reactor with the removal efficiencies of NH_4^+-N higher than 97% and that of chemical oxygen demand (determined by $K_2Cr_2O_7$, COD_{Cr}) higher than 86%. Denaturing gradient gel electrophoresis (DGGE) fingerprints based on total community 16S rRNA genes were analyzed with statistical methods, and excised DNA bands were sequenced. The results of phylogenetic analyses revealed high diversity within the SBBR biofilm community, and DGGE banding patterns showed that the community structure in the biofilm remained stable during the running period.

Conclusions: A coexistence of nitrifiers, including ammonia-oxidizing bacteria and nitrite-oxidizing bacteria, denitrifiers, including aerobic or anaerobic denitrifying bacteria and Anammox bacteria were detected, which might be the real matter of high removal efficiencies of NH_4^+-N and COD_{Cr} in the reactor.

Significance and Impact of the Study: The findings in this study indicated that PCR-DGGE analysis could be used for microbial community detection as prior method, and the SBBR technique could provide preferable growing environment for bacteria with N removal function.

Introduction

Containing a wide variety of intermediate organic degradation products and inorganic contaminants, including chemical oxygen demand (determined by $K_2Cr_2O_7$, COD_{Cr}) and nitrogenous contaminants, as a result of microbial activity within a landfill, compression and water flows, landfill leachates pose dangerous environmental and health risks (Kalyuzhnyi *et al.* 2003). Some techniques (Lin and Chang 2000; Wang *et al.* 2000; Kalyuzhnyi and Gladchenko 2004; Zeng *et al.* 2008) have been applied to treat landfill leachates. The technique of sequencing batch biofilm reactor (SBBR), which uses a packed support medium of biofilms, provides periodic changes in conditions to achieve various wastewater treatments (Wilderer *et al.* 2001). The SBBR system is of the advantages of higher biomass concentration in the reactor, higher volumetric load, better treatment stability, higher specific removal rates, less influence by toxic substances, easier accumulation of slow growing organisms and compactness in reactor. So, it has been widely used for treating various wastewater such as acrylnitrile, butadiene and styrene wastewater (Chang *et al.* 2000), cyanide containing wastewater (White *et al.* 2000) and tannery wastewater (Cho *et al.* 2001; Di Iaconi *et al.* 2002, 2003, 2004; Carini *et al.* 2003), and was strongly recommended

to treat landfill leachate (Dollerer and Wilderer 1996). Though so many investigations have been performed well on treating landfill leachate or removing nitrogenous contaminants with SBBR, only a few reports (Daims *et al.* 2001; Gieseke *et al.* 2001, 2003) have been focused on studying the microbial community in reactor.

Recent applications of molecular biology have provided tools to determine microbial presence and diversity in the SBBR system (Daims *et al.* 2001; Gieseke *et al.* 2001, 2003). A number of molecular genetic techniques, such as total DNA isolation and characterization, G + C composition, rRNA sequences, PCR amplification of rDNA or functional genes, and *in situ* hybridization of rRNA oligonucleotide probes, are used to study environmental microbial communities. Fingerprint profiles of rDNA sequences amplified by PCR, such as denaturing gradient gel electrophoresis (DGGE), have been used to study microbial communities (Muyzer *et al.* 1993, 1995; Ferris *et al.* 1996). The method has been used successfully to compare microbial communities in different environments (Nakatsu *et al.* 2000; Müller *et al.* 2004; Green *et al.* 2006), but has not been used to study the microbial community in SBBR yet. The validity of this method for studying SBBR microbial ecology requires further investigation.

The objective of this study was to use PCR-DGGE combined with sequential DNA sequencing and phylogenetic tree construction to detect the variety of bacterial community in the SBBR which worked for nitrite removal and also to study the dynamics of bacterial population during the reactor acclimation period and a single running cycle.

Materials and methods

SBBR system

The experiment was performed in a laboratory-scale SBBR system with an effective volume of 3 l. The biofilm was inoculated with activated sludge from an oxidation ditch municipal wastewater treatment plant (with a capacity of 150 000 m^3 per day) and acclimated with landfill leachate from a landfill site for 3 months at 25–30°C. During the acclimation period, treated wastewater of 600 ml was taken out from the reactor, and a 600-ml mixture of fresh landfill leachate and distilled water was added into the reactor at 10:00 a.m. everyday, with an increase of fresh landfill leachate in the mixture beginning from 100 to 300 ml at the end of the acclimation. The reactor was operated as the following four repeats of a 4-h aeration and then a 2-h anaerobic incubation, which was found to be the optimal operation conditions before the acclimation (data not shown). During the normal running period of 1 month, the SBBR was operated with a constant 300-ml of fresh landfill leachate in the 600 ml mixture during the acclimation period.

Analytical methods

Six water quality parameters, pH and concentrations of COD_{Cr}, DO, NH_4^+-N, NO_2^--N and NO_3^--N, were measured every 2 days during the acclimation period and the normal running period, and were measured every 2 h on the day of sampling as well. pH was determined by a pH meter, DO was determined by a DO analyzer, and the measurements of COD_{Cr}, NH_4^+-N, NO_2^--N and NO_3^--N were performed following standard methods (Eaton *et al.* 2005).

Removal efficiencies (Re) of NH_4^+-N and COD_{Cr} were calculated as the following equation: $Re = (1 - Ci/Ce) \times 100\%$, where Re was the removal efficiency, Ci was the concentration of NH_4^+-N or COD_{Cr} in the added 600-ml inflow, Ce was the concentration of NH_4^+-N or COD_{Cr} in the treated effluent.

Sample collection for DNA extraction

Eight samples, N1–N8, were collected for DNA extraction in this study. Samples N1–N6 were collected from biofilm on the surface of the fillings in the normally running reactor by sampling every 4 h begin at 2:00 p.m. on day 15 to 10:00 a.m. the next day during the 1-month normal running period. Sample N7 was collected from the acclimated biofilm at the end of the acclimation. Sample N8 was collected from the precipitate of 10 ml fresh landfill leachate after centrifuged at 3500 ***g*** for 10 min. All the samples were stored at −20°C.

DNA extraction and PCR amplification

Community total DNA was extracted with protease K and CTAB according to the previous report (Yang *et al.* 2006). Purified DNA was dissolved in 200 μl sterilized Milli-Q water (Millipore, USA), and 5 μl of DNA was used for agarose gel electrophoresis.

Bacterial universal 16S rRNA gene primer pair GC341f (5′-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC GCC TAC GGG AGG CAG CAG-3′) (Muyzer *et al.* 1998) and 907r (5′-CCG TCA ATT CCT TTG AGT TT-3′) (Muyzer *et al.* 1998; Suzuki *et al.* 2004; Hashizume *et al.* 2005) was used to directly amplify partial 16S rRNA gene fragments from total DNA for DGGE analysis. Each 100 μl PCR reaction mixture contained 2 μl of template DNA, 10 μl of 10× buffer (TianGen, China), 5 μl of 10 mmol l^{-1} dNTP mixture (TianGen), 2 μl of 10 μmol ml^{-1} each primer (Sangon, China), 2 μl of 2·5 U μl^{-1} Taq DNA polymerase

(TianGen), 1 μl of 10 mg ml^{-1} bovine serum albumin (Sangon) and 78 μl of sterilized Milli-Q water. Cycle conditions for the amplification were as follows: 5 min at 94°C; 30 cycles with each cycle consisting of 40 s at 94°C, 40 s at 55°C and 45 s at72°C; followed by a final 7-min extension at 72°C. Products were stored at −20°C before analysis. PCR products were visualized on 1% agarose gel run at 10 V cm^{-1} for 30 min before being stained with 0·5 μg ml^{-1} ethidium bromide for 20 min, destained in distilled water for 10 min and visualized under a Gel Doc 2000 System (Bio-Rad, USA).

DGGE analysis

The DGGE was carried out using a DCode^{TM} Universal Detection System instrument and gradient former model 475 according to the manufacturer's instructions (Bio-Rad). The acrylamide concentration in the gel was 6% and the denaturing gradient was 30–60%. The 100% denaturant solution contains 7 mol l^{-1} urea, 40% (v/v) formamide, 6% acrylamide/bis-acrylamide (37·5 : 1) and 0·5× TAE buffer (pH 8) in Milli-Q water. The 0% denaturant solution contains 6% acrylamide/bis-acrylamide (37·5 : 1) and 0·5× TAE buffer (pH 8) in ultrapure water. Twenty μl purified PCR products mixed with 4-μl loading buffer were transferred to the bottom of the sample holes in the gel. Gels were run in 0·5× TAE buffer at 55°C for 15 h at 140 V (Muyzer *et al.* 1998). Gels were stained with SYBR^{TM} Green I and digitized in UV light with the Gel Doc 2000 System.

Statistic analysis

The community abundance of each sample was presented by the index of abundance index (Ai) compared with all the analyzed samples in this report. The index is calculated as the following formula, $Ai_j = L_j/L_T$, where j is the analyzed sample, L_j is the number of bands in lane j in the DGGE profile, and L_T is the number of all bands in the DGGE profile when the bands in different lanes but in one parallel line are thought as only one band. A bigger Ai value indicates that it has more abundant community diversity in the sample analyzed.

The phylotype profiles of the samples from every two samples were compared by Sorenson's index, $Cs_{ij} = 2\text{-}L_{ij}/(L_i + L_j) \times 100$, a pairwise similarity coefficient (Murray *et al.* 1996; Gillian *et al.* 1998), where L_{ij} is the number of bands found common in both lanes i and j, L_i is the number of bands at lane i and L_j is the number of bands at lane j. A Cs value of 0 indicates that the two samples are completely different, and a Cs value of 100 indicates that the two samples have identical microbiological abundance.

Sequence and phylogenetic tree construction

Distinct bands were excised from DGGE gel under UV light, and then DNA was extracted and purified from the excised band with a QIAquick gel extraction kit (QIAquick, Qiagen, Germany). The purified DNA was re-amplified using the primer pair GC341f/907r, and the products were ligated into the pGEM-T easy vector (Promega, USA). Ligation products were electroporated into competent *Escherichia coli* DH5α cells using a Bio-Rad MicroPulser Electroporator (Bio-Rad), and blue/white screening was used to identify transformants containing inserts. Plasmid DNA was obtained from transformants using alkali lysis methods and the cloned inserts were amplified using the primer pair GC341f/907r for re-DGGE to affirm that the inserts was the wanted bands. After the affirmance, 29 clones were incubated and sent to Sangon for sequencing.

The sequences of partial 16S rRNA gene fragments were sent to GenBank to get accession numbers using the program Sequin win32. Phylogenetic identity was determined by comparing the cloned partial 16S rRNA gene fragment sequences to sequences found in GenBank using the database BLASTN (http://www.ncbi.nlm.nih.gov/BLAST/), and the phylogenetic trees were constructed using the program MegAlign in DNAStar 7·0 (CLUSTAL V method).

Results

SBBR performance

No obvious accumulation of NO_2^--N and NO_3^--N was detected in the SBBR system, the removal efficiencies of NH_4^+-N and COD_{Cr} during the normal running period (Fig. 1) were higher than 97% and 86%, respectively. It is also shown in Fig. 1 that pH in treated wastewater was above 8·0 all the time and DO kept at a low concentration of 0·6 mg l^{-1} at the end of each single cycle. It indicates that mature biofilm formed in the SBBR and performed a stable function of removing NH_4^+-N and COD_{Cr} after a 3-month acclimation under optimized conditions.

Figure 2 shows that concentration of NH_4^+-N decreased from about 23·0 to 35·0 mg l^{-1} and concentration of COD_{Cr} decreased from about 2400·0 to 513·4 mg l^{-1} from 0 h to the fourth hour in the single running cycle. The reason why these cases occurred was that the water quality measured at 0 h was related to the 600-ml of untreated mixture of fresh landfill leachate and distilled water, and it was measured at the fourth hour related to the mixture in reactor after 4-h aeration. Figure 2 shows that the concentration of NO_3^--N increased after the periodic aerobic run and decreased after the anaerobic run, while the concentration of NO_2^--N decreased after the periodic aerobic

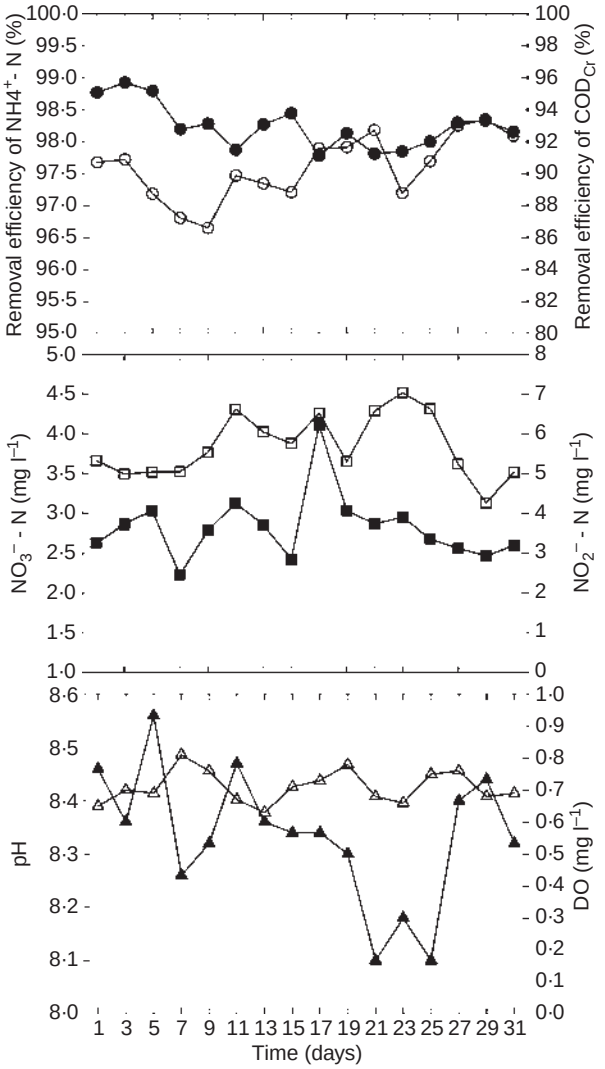

Figure 1 Removal efficiency of NH_4^+–N or COD_{Cr} and water quality of treated wastewater in SBBR during the 1-month running time. (—●—), NH_4^+–N; (—○—), COD_{Cr}; (—■—), NO_3^-–N; (—□—), NO_2^-–N; (—▲—), pH; (—△—), DO.

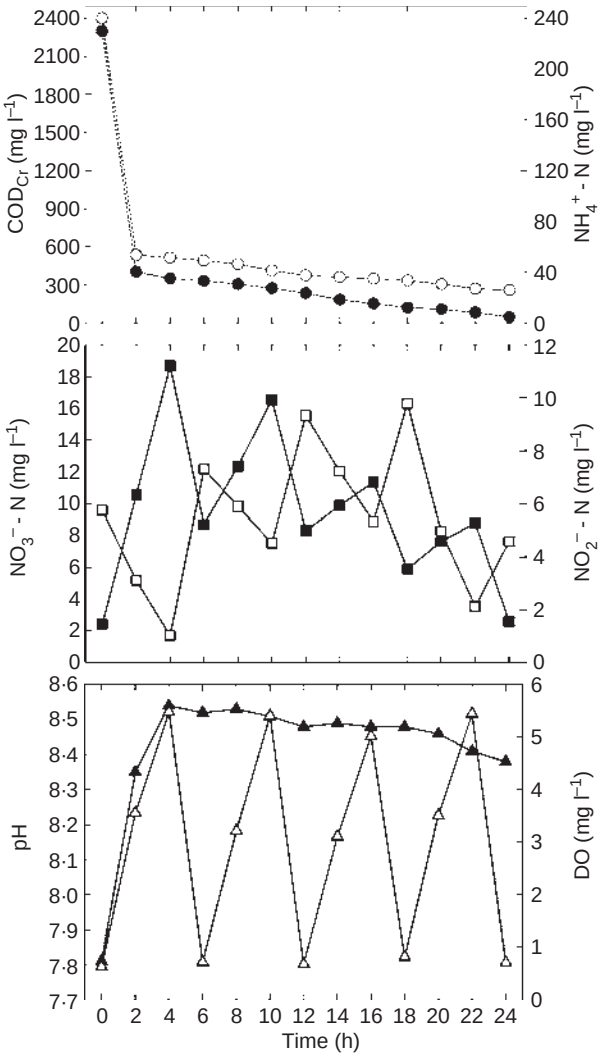

Figure 2 Change curves of water quality of pH and concentration of NH_4^+–N, COD_{Cr}, NO_3^-–N and NO_2^-–N in a single cycle time (24 h) in SBBR. The data of 0 h present the water quality of fresh landfill leachate while the others present the water quality of the mixed water in the SBBR. (—●—), NH_4^+–N; (—○—), COD_{Cr}; (—■—), NO_3^-–N; (—□—), NO_2^-–N; (—▲—), pH; (—△—), DO.

run and increased after the anaerobic run, and the concentration of total inorganic nitrogen kept decreasing within a single cycle. The pH rose sharply from 7·81 to 8·54 and then had a small and continuous fall, but was still higher than 8·0, a suitable pH value for most of nitrogen-removal microbes. The concentration of DO increased clearly during aeration and decreased rapidly during anaerobic period according with the periodic operation.

DGGE and statistic analysis

Total DNA with a length of 23 kb was extracted, and partial 16S rRNA gene fragments of about 630 bp were PCR amplified sequentially, without unexpected products. About 4 μg of PCR products were applied for DGGE analysis. The DNA extraction, PCR and DGGE analysis were performed three times to test its reproducibility. The same DGGE profile was screened by using Gel Doc 2000 System with program Quantity One V4·52 (Bio-Rad). Twenty-nine distinct bands, designated as A–Z and a–c in the DGGE profile (see Fig. 3), were counted out from the SYBR^{TM} Green I stained denaturing gradient gel. That the bands in lane N7, except bands J and V, could be found in N1–N6 indicated that the mature biofilm formed at the end of the acclimation period, and bacteria of bands J

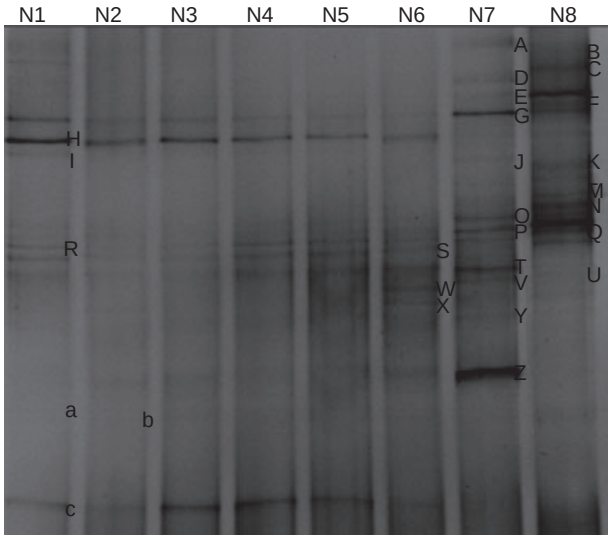

Figure 3 DGGE (30–60%, 140 V, 15 h) profile of PCR amplified 16S rRNA gene products. The letters represented different bands in the figure.

and V might come from the activated sludge as no homologous bands were found in lane N8, which was the sample came from landfill leachate.

The results of abundance index analysis for lanes N1–N8 in the DGGE profile shown in Table 1 indicate that sample N8, the landfill leachate, had the most abundant bacterial diversity as it had an Ai value of 0·828 when N1 had an Ai value of 0·724, N2–N3 had the same Ai values of 0·690 and N4–N7 had the same Ai values of only 0·655. Cs values in Table 2 show that N2 and N3, N4, N5 and N6 had identical bacterial diversities as all their Cs values were 100. High Cs values between N1, N2, N3, N4, N5 and N6 showed that mature biofilm has been acclimated during the acclimation period and the biofilm bacterial community structure changed little during a single cycle. That the Cs value between sample N7 and N8 was 65·1, the minimum value in Table 2, indicates their small similarity to each other. Though the Cs values between N8 and N1–N7 were small, nearly all the bands in lanes N1–N7 could be found in lanes N8 as shown in Fig. 3.

Phylogenetic analysis

Twenty-nine distinct sequences were separated from the excised bands after PCR re-amplification, 16S rRNA gene fragments clone and re-DGGE affirmation. Table 3 shows the corresponding relationship between the bands in DGGE profiles and the accession numbers DQ838671–DQ838699 given by GenBank after the sequences submission with Sequin win32. Twenty-eight sequences, which had the nearest homologous relationships with the bands in DGGE profile and whose accession numbers and species names are also listed in Table 3, were downloaded from GenBank for phylogenetic analysis. Two phylogenetic trees (Fig. 4a,b) were constructed by the program MegAlign in DNAStar 7·0 using the Clustal V method. Figure 4a presents the phylogenetic relationship between most of the nitrifiers including five ammonia-oxidizing bacteria (AOB) of bands A, F, G, R, X and a and four nitrite-oxidizing bacteria (NOB) of bands I, S, T and c, and the denitrifiers including bands D, P, Y and b. And it shows that they had distant phylogenetic relationship especially a strain of *Brocadia anammoxidans*, band G in the DGGE profile. The phylogenetic relationship between other bacteria detected in samples N1–N8 is presented in Fig. 4b.

Table 2 Coefficient similarity (Cs) of bacterial population in SBBR in different periods

Sample	N1	N2	N3	N4	N5	N6	N7	N8
N1	100	97·6	97·6	95·0	95·0	95·0	80·0	81·8
N2	97·6	100	100	97·4	97·4	97·4	87·2	72·7
N3	97·6	100	100	97·4	97·4	97·4	87·2	72·7
N4	95·0	97·4	97·4	100	100	100	89·5	74·4
N5	95·0	97·4	97·4	100	100	100	89·5	74·4
N6	95·0	97·4	97·4	100	100	100	89·5	74·4
N7	80·0	87·2	87·2	89·5	89·5	89·5	100	65·1
N8	81·8	72·7	72·7	74·4	74·4	74·4	65·1	100

Discussion

After 3-month acclimation with landfill leachate, a layer of biofilm with various bacterial species and steady bacterial community structure and function formed on the surface of the filling in the reactor used for nitrogen removal in landfill leachate. Bacteria in the biofilm may mainly come from landfill leachate since that homologous bands of most bands in lane N7 could be found in lane N8 except five bands of D, J, R, V and Z in lane N7. Therefore, more suitable inoculants should be chosen for

Table 1 Abundance index (Ai) of bacterial community in SBBR in different periods

Sample	N1	N2	N3	N4	N5	N6	N7	N8
Number of bands	21	20	20	19	19	19	19	24
Ai	0·724	0·690	0·690	0·655	0·655	0·655	0·655	0·828

Table 3 Sequence size and partial 16S rRNA gene sequence information for the 29 bands in the DGGE profile

			Closest blast match		
Band	Sequence size/bp	Accession number	Accession number	Species name	Sequence similarity/%
A	547	DQ838686	AY123795	*Nitrosomonas eutropha*	97
B	547	DQ838681	U81706	Unidentified eubacterium sp.	64
C	547	DQ838698	DQ196473	*Oxalobacter* sp.	96
D	548	DQ838694	DQ200849	*Hyphomicrobium* sp.	95
E	527	DQ838693	L35517	*Bacteroides cellulosolvens*	97
F	542	DQ838674	AJ298728	*Nitrosococcus mobilis*	95
G	547	DQ838671	AF375994	*Brocadia anammoxidans*	97
H	547	DQ838680	DQ168650	*Sedimentibacter* sp.	77
I	545	DQ838679	AF363288	*Nitrobacter winogradskyi*	97
J	547	DQ838699	X83812	*Terrebacter tumescens*	97
K	547	DQ838692	AJ010960	*Anaerovibrio glycerini*	95
L	528	DQ838687	DQ329895	Uncultured *Chloroflexi* sp.	94
M	547	DQ838688	AY263726	Uncultured *Chloroflexi* sp.	88
N	546	DQ838697	X70954	*Pelobacter propionicus*	97
O	548	DQ838684	AY754832	Uncultured bacterium	95
P	543	DQ838695	AY512826	*Acidovorax delafieldii*	97
Q	547	DQ838690	DQ146765	*Megasphaera elsdenii*	99
R	547	DQ838672	AY123794	*Nitrosomonas* sp.	95
S	547	DQ838678	AF069957	*Nitrobacter alkalicus*	97
T	547	DQ838675	L35504	*Nitrospina gracilis*	93
U	547	DQ838683	DQ329882	Uncultured *Chloroflexi* sp.	90
V	549	DQ838691	DQ394709	*Veillonella parvula*	97
W	547	DQ838682	DQ232379	Bacterium sp.	99
X	547	DQ838677	M96398	*Nitrosococcus oceanus*	95
Y	542	DQ838689	DQ371241	*Bacillus clausii*	95
Z	529	DQ838685	DQ168655	*Clostridiales* sp.	95
a	547	DQ838673	AY123794	*Nitrosomonas* sp.	97
b	532	DQ838696	AY730711	*Corynebacterium variabile*	97
c	539	DQ838676	DQ414438	Uncultured *Nitrospira* sp.	93

the inoculation to a microbial reactor. The bacterial community of mature biofilm in SBBR changed little both in different cycles and different hours of a single cycle so that it could remove NH_4^+-N and COD_{Cr} with high efficiencies during the running period.

According to the phylogenetic analysis, about half of the detected bacteria in biofilm or landfill leachate, 14 of 29, may contribute to nitrogen removal. The previous studies have reported some kinds of aerobic AOB, such as *Nitrosomonas* sp. (Dionisi *et al.* 2002; Bernhard *et al.* 2005) and *Nitrosococcus* sp. (Casciotti and Ward 2005; Coskuner *et al.* 2005). *Brocadia anammoxidans* was affirmed as one of the two anaerobic AOB (Strous *et al.* 2006; Tal *et al.* 2006). In this study, we found that five kinds of aerobic AOB (*Nitrosomonas* sp. of bands A, R and a, and *Nitrosococcus* sp. of bands F and X) and one kind of anaerobic AOB (*B. anammoxidans* of band G) coexisted in the reactor, and we deduced that the aerobic AOB offered anaerobic AOB enough nitrite for the anaerobic ammonia oxidization in the anaerobic circumstance. Thus, the NH_4^+-N should have been partially removed by anaerobic ammonia oxidation (Anammox) techniques. The aerobic AOB also offered the detected four NOB, *Nitrobacter winogradskyi* of band I (Freitag *et al.* 2005; Starkenburg *et al.* 2006), *Nitrobacter alkalicus* of band S (Freitag *et al.* 2005), *Nitrospina gracilis* of band T (Freitag *et al.* 2005) and *Nitrospira* sp. of band c (Freitag *et al.* 2005), nitrite for the aerobic nitrite oxidization. We deduced that because the aerobic AOB offered most of the nitrite needed in the reactor, they had the most abundant species diversity.

Acidovorax delafieldii was considered to be a nutritionally versatile species frequently present in wastewater treatment plants and an anaerobic denitrifying bacterium (Schramm *et al.* 2003; Nicholson and Fathepure 2005) as well as *Bacillus clausii* (Ntougias and Russell 2000) and *Corynebacterium variabile* (Bonin *et al.* 2002). The previous studies indicated that *Hyphomicrobium* sp. have the function of denitrification under aerobic conditions (Meiberg and Harder 1978; Timmermans and Van Haute

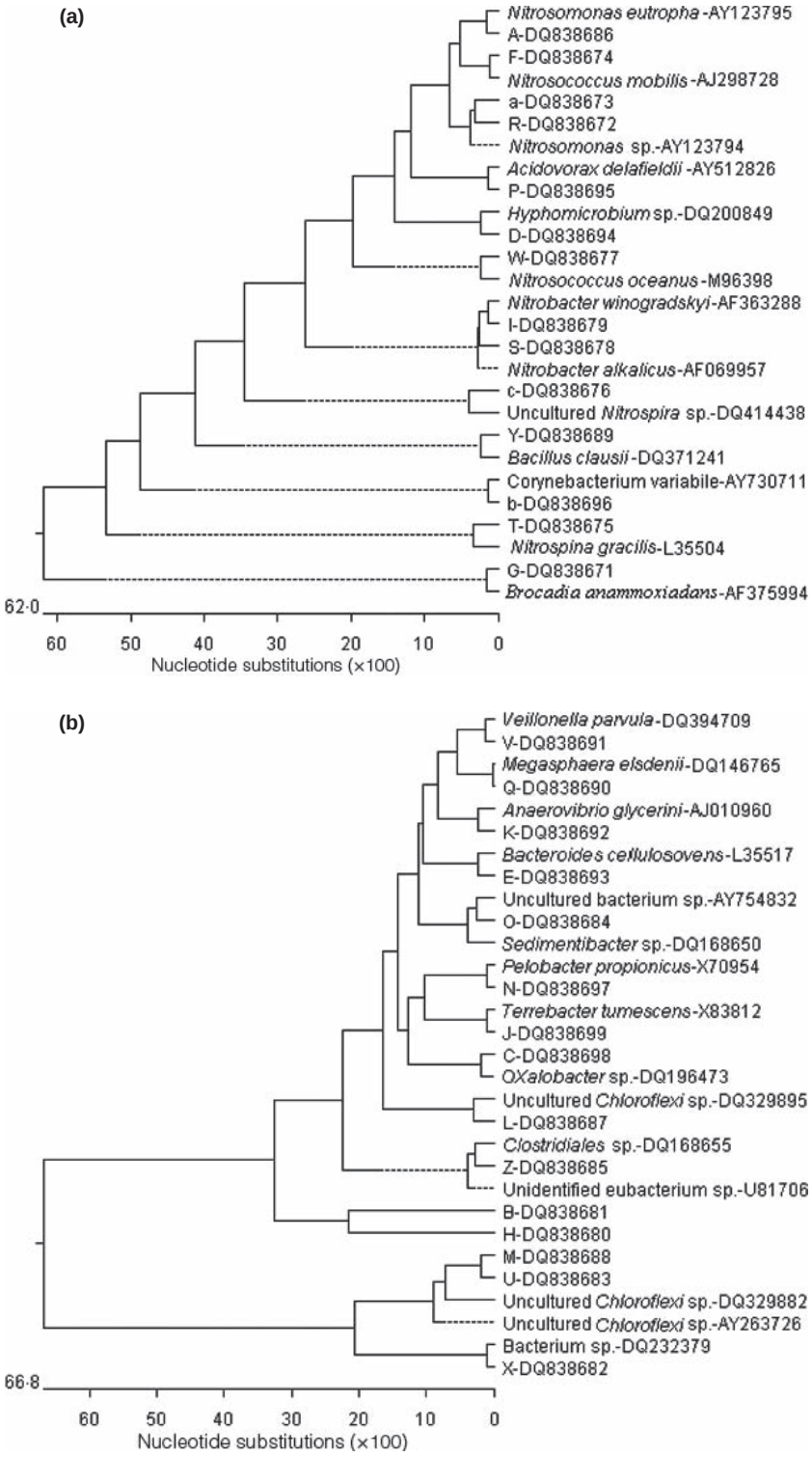

Figure 4 Phylogenetic trees, (a) and (b), of bacteria in SBBR constructed by MegAlign in DNAStar 7·0 using the CLUSTAL V method on basis of the BLAST results of 29 sequences. (a) Phylogenetic relationship between the denitrifying bacteria; (b) phylogenetic tree of other bacteria detected by DGGE in the SBBR biofilm.

1983). In this study, three different anaerobic denitrifying bacteria, *A. delafieldii* of band P, *B. clausii* of band Y and *C. variabile* of band b, and an aerobic denitrifying bacterium, *Hyphomicrobium* sp. of band D which transformed nitrate to nitrite, were detected from the biofilm, and the presence of so many denitrifying bacteria indicated that conventional denitrification may have played an important role in NH_4^+-N removal. What's more, simultaneous

nitrification and denitrification (SND) techniques might have removed some NH_4^+-N with the presence of aerobic denitrifying bacteria (Lee *et al.* 2001).

According to above the discussion, we concluded that NH_4^+-N was removed by a combined techniques of three different nitrogen removal modes, Anammox, conventional denitrification and SND, in the SBBR which had never been reported before. It should be the biofilm in the SBBR, which provided aerobic bacteria such as aerobic AOB, NOB and aerobic denitrifying bacteria an aerobic circumstance on the surface and anaerobic bacteria such as anaerobic AOB and anaerobic denitrifying bacteria an anaerobic circumstance inside the biofilm, making the three denitrifying modes coexisting in a single reactor, and so that SBBR could achieve steady NH_4^+-N removal efficiencies.

The concentration of COD_{Cr} in landfill leachate used in this study was from 3700 to 4900 mg l^{-1}. Though the previous literature reported that the high concentration of COD_{Cr} made against denitrification, it was not detected in this study, and high NH_4^+-N removal efficiency was achieved with the presence of the high concentration of COD_{Cr}. What's more, COD_{Cr} removal efficiencies higher than 86% were achieved. Though many of the denitrifying bacteria in the SBBR could grow autotrophically, they could also grow heterotrophically so that denitrifying bacteria could remove COD_{Cr} and nitrogen at the same time. Because of slow growth of denitrifying bacteria, COD_{Cr} in the reactor may have been removed by other bacteria, such as *Bacteroides cellulosolvens* (Murray 1986; Xu *et al.* 2004) of band E which could decompose cellulose according to the previous reports.

As shown in Fig. 4a, we found that most of nitrogen removal related bacteria had distant phylogenetic relationship at the level of genus, according with the literature reported (Focht and Verstraete 1977; Mobarry *et al.* 1996; Purkhold *et al.* 2000). *Nitrosomonas* sp. and *Nitrosococcus* sp. was far away from *B. anammoxiadans* on the phylogenetic tree, although they all could oxidize NH_4^+ (or NH_3) into NO_2^-. Similarly, the four denitrifying bacteria belonging to four different genera had relative distant genetic relationship despite of their same function of denitrification. However, bacteria that not only grew in the similar circumstance but had the same function, except the denitrifying bacteria, had homologous phylogenetic relationship. Because of demanding different growing conditions and acting different effects in the biofilm or leachate, bacteria shown in Fig. 4b composed some branches on the phylogenetic tree, indicating their distant phylogenetic relationship.

We have got some interesting findings that some different bacteria contributed to different nitrogen removal modes were detected in this reactor using PCR-DGGE technology. So we deduced that these bacteria contributed a lot to the high efficiencies of synchronous nitrogen and COD_{Cr} removal. However, we were still not so sure of that because some reports (MacNaughton *et al.* 1999; Kirka *et al.* 2004) claimed that DGGE was estimated to only detect 1–2% of the microbial population representing dominant species present in an environmental sample. More tools, such as PLFA method (Yu *et al.* 2007; Huang *et al.* 2008) and biosensor (Tang *et al.* 2006, 2008) which have been used to monitor microbial community in our group, should be used to prove what we have deduced or to summarize much more comprehensive conclusions in future research.

Acknowledgements

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