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Analysis of Microbial Communities in Biofilms from CSTR-Type Hollow Fiber Membrane Biofilm Reactors for Autotrophic Nitrification and Hydrogenotrophic Denitrification

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Two hollow fiber membrane biofilm reactors (HF-MBfRs) were operated for autotrophic nitrification and hydrogenotrophic denitrification for over 300 days. Oxygen and hydrogen were supplied through the hollow fiber membrane for nitrification and denitrification, respectively. During the period, the nitrogen was removed with the efficiency of 82–97% for ammonium and 87–97% for nitrate and with the nitrogen removal load of 0.09–0.26 kg NH_4^+-N/m^3/d and 0.10–0.21 kg NO_3^--N/m^3/d, depending on hydraulic retention time variation by the two HF-MBfRs for autotrophic nitrification and hydrogenotrophic denitrification, respectively. Biofilms were collected from diverse topological positions in the reactors, each at different nitrogen loading rates, and the microbial communities were analyzed with partial 16S rRNA gene sequences in denaturing gradient gel electrophoresis (DGGE). Detected DGGE band sequences in the reactors were correlated with nitrification or denitrification. The profile of the DGGE bands depended on the NH_4^+ or NO_3^- loading rate, but it was hard to find a major strain affecting the nitrogen removal efficiency. *Nitrospira*-related phylum was detected in all biofilm samples from the nitrification reactors. *Paracoccus* sp. and *Aquaspirillum* sp., which are an autohydrogenotrophic bacterium and an oligotrophic denitrifier, respectively, were observed in the denitrification reactors. The distribution of microbial communities was relatively stable at different nitrogen loading rates, and DGGE analysis based on 16S rRNA (341f /534r) could successfully detect nitrate-oxidizing and hydrogen-oxidizing bacteria but not ammonium-oxidizing bacteria in the HF-MBfRs.

Keywords: CSTR-type hollow fiber membrane biofilm reactor, nitrification, autotrophic denitrification, microbial community, DGGE

Introduction

Nitrogenous compounds in wastewater are removed mainly by a sequential process consisting of nitrification and denitrification. Nitrification is a two-step process in which ammonia or nitrite serves as electron-donor substrates and oxygen serves as the electron acceptor; *Nitrosomonas* and *Nitrosospira* species transform ammonia to nitrite as ammonia-oxidizing bacteria (AOB), whereas *Nitrobacter* and *Nitrospira* species transform nitrite to nitrate as nitrite-oxidizing bacteria (NOB) [1]. Denitrification is a nitrate reduction process to nitrogen and it can be divided according to external electron donors. Heterotrophic denitrifiers need organic carbon and are efficient for nitrate removal in wastewater with high C/N ratios, whereas autotrophic denitrifiers use inorganic energy sources such as hydrogen [26], Fe^{2+} [28], and reduced sulfur compounds [36].

Among the autotrophic denitrifiers, hydrogen-oxidizing bacteria have a number of advantages over heterotrophic bacteria, such as lower biomass yield, low cost, and removal of residual electron donors, and have been widely studied for the removal of nitrate from wastewater and groundwater with low C/N ratios [4, 11]. In particular, potable water treatment using hydrogen-oxidizing denitrification is more promising than other biological processes because dissolved hydrogen is not harmful to human health and does not interfere with subsequent water treatment [22, 31]. However, autotrophic denitrification using H_2 in tank-type wastewater treatment reactors has the risk of explosion in excessive amounts owing to the low solubility of hydrogen in water. These disadvantages of H_2 use were overcome by studies that used bubbleless membrane-diffusion devices, such as hollow fiber membranes (HF-Ms) [10, 37]. A denitrification reactor using HF-Ms could simply consist of a reactor tank and immersed HF-Ms, into which liquid and gas are supplied, respectively. H_2 supplied into the lumen of the HF-Ms is used by denitrifying biofilms that are formed on the outer surface of the HF-Ms [17, 18]. We tested for the presence of hydrogen-oxidizing bacteria participating in the denitrification process.

In our previous study, two HF-MBfRs for nitrification and denitrification were supplied with O_2 and H_2 in their respective lumens and were successfully operated for the removal of NH_4^+ and NO_3^- in the influent [24]. Specifically, hydrogenotrophic denitrification was observed in the HF-MBfR for denitrification. There has been little study on the analysis of microbial communities involved in autotrophic nitrification and hydrogenotrophic denitrification over a long period of operation. In our study, the diversity of the bacterial communities in the biofilms of the two HF-MBfRs during long-term operation for over 300 days was investigated by the analysis of 16S rRNA gene partial sequences after denaturing gradient gel electrophoresis (DGGE) of the PCR-amplified 16S rRNA gene sequences, and the major microbes involved in nitrification and denitrification were identified.

The DGGE analysis for the nitrification/denitrification reactor used nitrifying bacteria-specific primers or 16S rRNA gene-based primers. Microbial community analysis using DGGE (AOB-specific primers) in membrane-aerated biofilm reactors (MABR) showed a *Nitrosomonas*-dominant profile for the AOB community with Anammox bacteria [6]. DGGE analysis (AOB-specific primers) with nitrifying bacteria (granule formation) in an aerobic upflow fluidized bed (AUFB) reactor indicated *Nitrosomonas*-like bacteria were dominant [33]. DGGE analysis based on the 16S rRNA gene showed an aerobic nitrification reactor was enriched with *Nitrospira* genus (NOB) [35] and an anaerobic denitrification reactor with *Firmicutes*, *Proteobacteria*, *Chloroflexi*, and *Bacteroidetes* [20]. We used eubacterial primers based on the 16S rRNA gene [15] and performed DGGE analysis to analyze the whole microbial community structure.

Materials and Methods

Experimental System for the CSTR-Type HF-MBfRs

Two CSTR-type HF-MBfRs, designed by Shin *et al*. [25], were used for the removal of NH_4^+ and NO_3^- from synthetic wastewater. O_2 was supplied to the nitrification reactor as an electron acceptor for the ammonium oxidation and H_2 to the denitrification reactor as an electron donor for the reduction of nitrate (Fig. 1). The

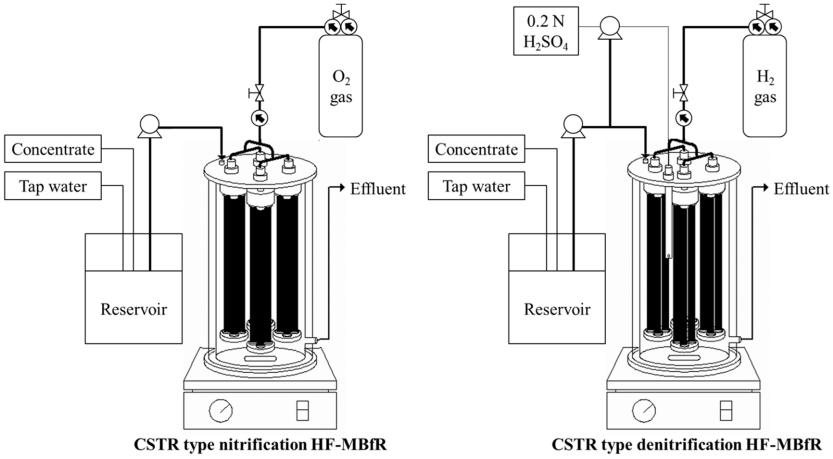

Fig. 1. Schematic diagram of the CSTR-type HF-MBfRs.

CSTR-type HF-MBfRs consisted of a transparent acrylic cylinder tank and four HF-M modules. The HF-M modules were directly submerged in each tank. The reactors had a height of 400 mm and a diameter of 160 mm. A completely mixed state on the membrane surface was maintained with a magnetic stirring device at the bottom of each reactor.

The HF-M modules were composed of gas permeable hollow fibers (Model MHF 200TL; Mitsubishi Rayon, Japan). The hollow fibers had an I.D. and O.D. of 0.135 mm and 0.27 mm, respectively. Each module consisted of three separately potted bundles of HF-Ms, each of which contained 320 HF-Ms. Each bundle was potted into a cylindrical plug of polyurethane (10 mm in diameter and 20 mm in height), and was 250 mm in length to yield a specific area of 148 m^2/m^3, providing 67,900 mm^2 of membrane surface area. The four HF-M modules provided 8,143 cm^2 of membrane surface area, with 3,840 hollow fiber membranes. The void ratio of the working reactor volume (volume of the reactor, 6,554 ml; volume of fibers, 54 ml) was 99.2% when the HF-Ms were free of biofilm. Pure O_2 and H_2 were supplied to the lumen side of the fibers from a pressurized gas tank through a metering valve (Parker, Cleveland, OH, USA).

Table 1. The operation condition dependent on hydraulic retention time (HRT) at different operating steps.

Operating step	Time (d)	HRT (h)
CSTR-type nitrification HF-MBfR		
Run 1	0–67	12
Run 2	68–126	10
Run 3	127–175	8
Run 4	176–256	7
Run 5	257–361	6
Run 6	362–403	5
CSTR-type denitrification HF-MBfR		
Run 1	0–39	12
Run 2	40–98	10
Run 3	99–171	8
Run 4	172–217	7
Run 5	218–298	6

Each run was distinguished by the same HRT operation.

Operation of the CSTR-Type Nitrification and Denitrification HF-MBfRs

The nitrification and denitrification reactors were operated separately with synthetic wastewater, following the procedures described by Shin *et al*. [25]. For nitrification, $NaHCO_3$ was used as the sole inorganic carbon source to grow autotrophic microorganisms and to prevent pH drops, and $(NH_4)_2SO_4$ was also used as a nitrogen source. For denitrification, $NaNO_3$ was used as the nitrogen source and a phosphate buffer (K_2HPO_4 + KH_2PO_4) and H_2SO_4 (0.2 N) were used to prevent pH increase. The phosphate buffer was added to the raw synthetic wastewater from days 65 to 205. Apart from days 65 to 205, the pH was controlled by an auto controller, and 0.2 N H_2SO_4 was added automatically to the denitrification reactor when the pH was over 8. $NaHCO_3$, phosphate buffer, and H_2SO_4 were intermittently added during the monitoring of pH. The synthetic wastewaters were supplied to the reactors by a peristaltic pump (Masterflex, Cole-Parmer, Vernon Hills, IL, USA).

The concentrations of NH_4^+-N and NO_3^--N in the influent were both 50 mg N/l, and the hydraulic retention time (HRT) was reduced to increase the NH_4^+ and NO_3^- loading rates (kg $N/m^3/d$) (Table 1). The operation times at different HRTs are shown in Table 1. Activated sludge from a well-operated municipal wastewater treatment plant was inoculated into the nitrification reactor, and the reactor was continuously operated for 403 days. In addition, the anaerobic sludge was taken from a sludge digester at the same plant and transferred into the denitrification reactor, continuously operated for 298 days.

Analytical Methods

Samples to measure the concentrations of NH_4^+, NO_2^-, and NO_3^- were stored at 4°C and analyzed within 2 days. Both NO_2^- and NO_3^- concentrations were determined by ion chromatography (DX-120, Dionex, Sunnyvale, CA, USA), and NH_4^+ was analyzed with a Kjeldahl analyzer (KJELTEC 1035 analyzers, Cheshire, UK). Alkalinity was measured according to standard methods [21].

Sampling and 16S rRNA Gene Amplification

Biomass from the biofilm was collected from different topological positions at diverse N loading rate conditions (Table 2). From the nitrification reactor, samples were retrieved on days 170, 245, 355, and 380 from the upper and lower parts of the HF-M. For the denitrification reactor, samples were retrieved on days 35, 93, 148, 215, and 275. Biofilm samples were collected from the outer surfaces of the HF-M and the inner surface of the denitrification reactor tank. A thick biofilm was formed on the outer surfaces of the HF-M in the reactor, and this was divided into inner and outer layers for biomass collection.

The genomic DNAs of the seed sludge and the biomass from the two reactors were extracted with UltraClean Soil DNA kits (MoBio Laboratories, Solana Beach, CA, USA) according to the manufacturer's instructions. The partial 16S rRNA genes from the biomass were amplified by PCR using universal eubacterial primers 27F and 1492R [27]. For the DGGE analysis, nested PCR was performed on the initial PCR products using eubacterial primers 341f (5′- CCT ACG GGA GGC AGC AG-3′) with a GC clamp, and 534r (5′-ATT ACC GCG GCT GCT GG-3′) [15]. For DGGE PCR, the touch-down PCR method was used to effectively eliminate the nonspecific annealing of primers to non-target DNA. Secondary PCR was carried out with the following program: 94°C for 5 min; 30 cycles of denaturation at 94°C for 30 sec, annealing at

Table 2. Practical value measurement for evaluation of nitrification/denitrification operation.

Ammonium-N removal in nitrification reactor				
Run	Influent NH_4^+-N (mg N/l)	Effluent NH_4^+-N (mg/l)	NH_4^+-N loading rate (kg N/m^3/d)	Removal efficiency (%)[a]
1	45.6 ± 5.8	10.4 ± 13.2	0.09 ± 0.01	83.5 ± 22.4
2	50.0 ± 1.9	8.9 ± 7.0	0.12 ± 0.01	82.3 ± 13.9
3	50.7 ± 2.0	1.9 ± 1.4	0.15 ± 0.01	96.2 ± 2.9
4	51.8 ± 2.2	1.6 ± 0.8	0.16 ± 0.01	96.8 ± 1.5
5	51.5 ± 3.0	4.8 ± 6.6	0.19 ± 0.02	90.6 ± 13.1
6	53.1 ± 4.0	3.8 ± 2.1	0.26 ± 0.01	92.6 ± 4.3
Nitrate-N removal in denitrification reactor				
Run	Influent NO_3^--N (mg/l)	Effluent NO_3^--N (mg/l)	NO_3^--N loading rate (kg N/m^3/d)	Removal efficiency (%)
1	55.4 ± 2.6	2.2 ± 1.6	0.10 ± 0	96.0 ± 2.7
2	55.0 ± 2.4	4.5 ± 5.5	0.13 ± 0.02	91.9 ± 10.0
3	54.5 ± 3.2	1.9 ± 3.0	0.16 ± 0.01	96.6 ± 5.6
4	54.7 ± 3.4	2.4 ± 4.1	0.18 ± 0.01	95.6 ± 7.8
5	55.1 ± 4.1	7.2 ± 4.5	0.21 ± 0.02	86.8 ± 8.1

[a] $\text{Removal efficiency (\%)} = \frac{\text{influent}_{NH_4 \text{ or } NO_3^-} - \text{effluent}_{NH_4 \text{ or } NO_3^-}}{\text{influent}_{NH_4 \text{ or } NO_3^-}} \times 100$

63°C for 30 sec, and extension at 72°C for 30 sec; 20 cycles of denaturation at 94°C for 30 sec, annealing at 54°C for 30 sec, and extension at 72°C for 30 sec; and a single final extension at 72°C for 10 min. The secondary PCR products were purified with the Power Gel Extraction Kit (TaKaRa, Otsu, Japan), following the manufacturer's instructions.

DGGE and 16S rRNA Gene Sequence Analysis

DGGE was performed with a D-Code universal mutation detection system (Bio-Rad Laboratories, Hercules, CA, USA). Amplified 16S rRNA gene sequence fragments were loaded onto 10% (w/v) polyacrylamide gels. The polyacrylamide gels were made with denaturing gradients ranging from 30% to 60% according to the protocol of Muyzer *et al*. [15]. The DNA in the polyacrylamide gel was run at a constant 60°C for 16 h at 60 V. After staining the DNA in the polyacrylamide gel, distinct bands were manually excised from the gel. The DNA from the excised gel was extracted into a 0.1× Tris-EDTA buffer and used as the template for subsequent PCR, with 341f and 534r primers. PCR products were purified with a Power Gel Extraction Kit (TaKaRa) and then were ligated into the T&A cloning vector (Real Biotech, Taipei, Taiwan) following the manufacturer's instructions. The 16S rRNA gene of the DGGE band in each clone was sequenced with the universal vector primers M13f or M13r (Macrogene Co., Seoul, Korea). The 16S rRNA gene partial sequences obtained from the DGGE band were compared with the reference sequences available in the GenBank database using the Basic Local Alignment Search Tool (BLAST). The 16S rRNA gene partial sequences were aligned with Clustal_X [32], and a neighbor-joining [23] phylogenetic tree was constructed with the MEGA5 software [30].

Results and Discussion

Removal of NH_4^+ and NO_3^-

The nitrification and denitrification reactors were separately operated over 403 and 298 days, respectively. The loading rate of the ammonium and nitrate is shown in Table 2. The practical NH_4^+ loading rate for nitrification was 0.09–0.26 kg N/m^3/d, and the NO_3^- loading rate for denitrification was 0.10–0.21 kg N/m^3/d. The nitrogen removal rate was different for the loading rates. Maximum removal was achieved on Run 4 (HRT = 7 d) at 96.8 ± 1.5% for ammonium and on Run 3 (HRT = 8 d) at 96.6 ± 5.6% for nitrate (Table 2). The concentrations of NH_4^+, NO_2^-, and NO_3^- in the influent and effluent are described for a time series with the loading rate in Fig. 2A for nitrification and in Fig. 2B for denitrification.

Sequencing of DGGE Fragments and Phylogenetic Analysis of the CSTR-Type of Nitrification HF-MBfR

DGGE band profiles of the amplified 16S rRNA gene sequences obtained from the inoculated activated sludge and biofilms in the nitrification HF-MBfR are shown in Fig. 3A. Biomass samples were collected from the upper (UP; DGGE bands 1, 4, and 7) and lower (LP; DGGE bands

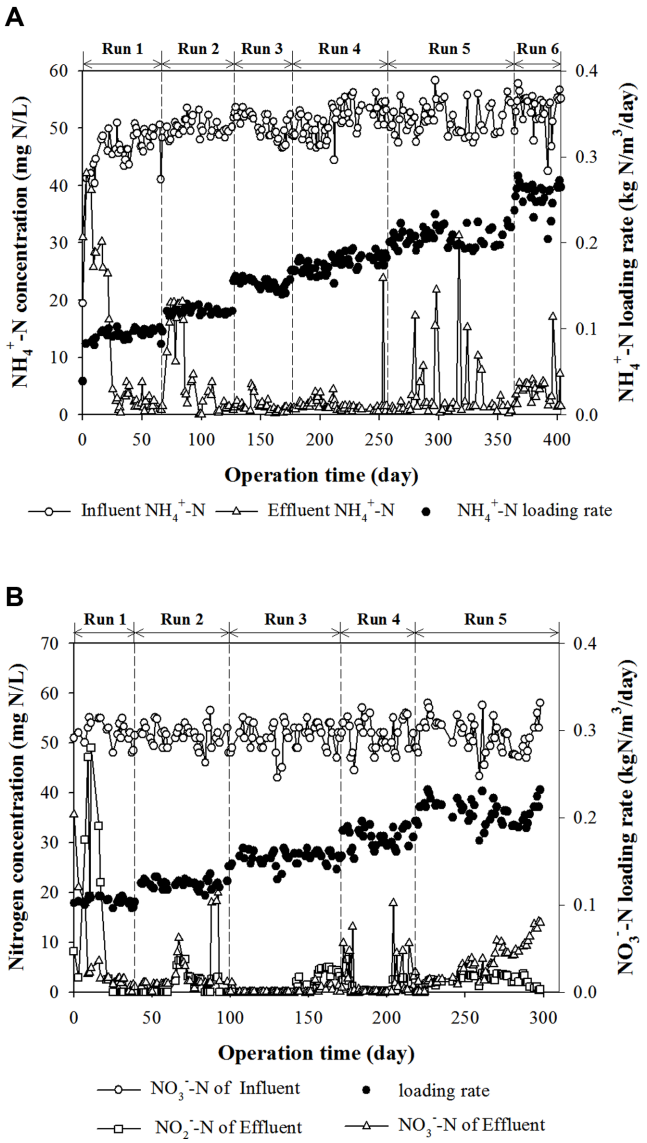

Fig. 2. Nitrification (**A**) and denitrification (**B**) profiles in CSTR- HF-MBfR.

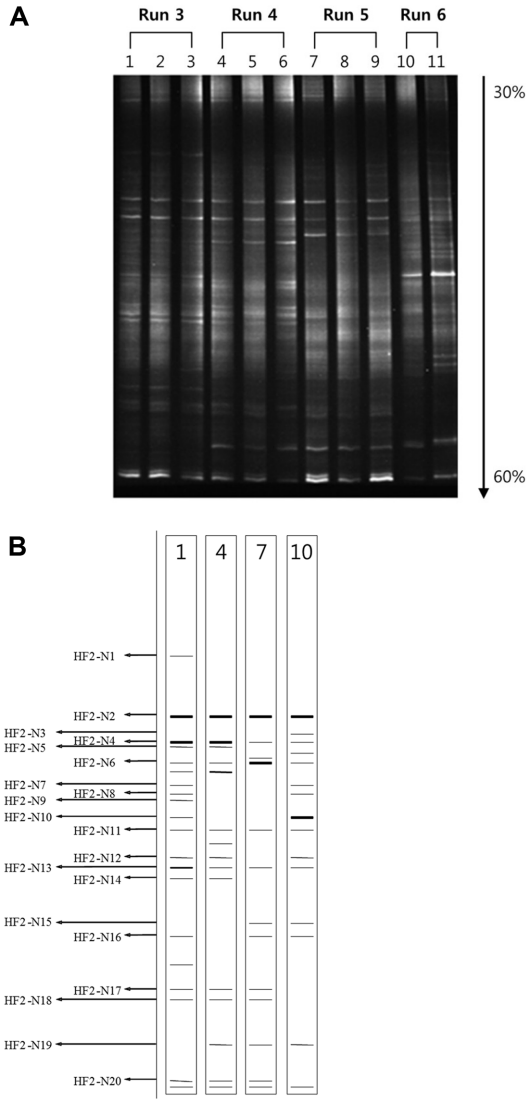

Fig. 3. DGGE band profile of the amplified 16S rRNA gene sequences (**A**), schematic of the bands for comparison of microbial community (**B**) in biofilms from the CSTR-type nitrification HF-MBfR; and neighbor-joining phylogenetic tree based upon 16S rRNA sequences derived from DGGE bands recovered from biofilm in the CSTR-type nitrification (**C**).
The scale bar indicates 0.02 estimated substitution per nucleotide. Biomass was collected at diverse topological positions in the CSTR-type nitrification HF-MBfR at different NH_4^+ loading rates. NH_4^+ loading rates: Lane 1 for Run 3 (0.15 kg N/m^3/d); Lane 4 for Run 4 (0.16 kg N/m^3/d); Lane 7 for Run 5 (0.19 kg N/m^3/d); and Lane 10 for Run 6 (0.26 kg N/m^3/d). Topological positions: Lanes 1, 4, and 7 for biomass from the upper part of the HF-M; Lanes 2, 5, 8, and 10 for biomass from the lower part of the HF-M; Lanes 3, 6, 9, and 11 for biomass formed on the inner surface of the reactor tank; and Lane 12 for the inoculated activated sludge. Sampling dates: Lanes 1, 2, and 3 at 170 d; Lanes 4, 5, and 6 at 245 d; Lanes 7, 8, and 9 at 355 d; and Lanes 10 and 11 at 380 d.

2, 5, 8, and 10) parts of the HF-M modules, and the rest of the samples (DGGE bands 3, 6, 9, and 11) were collected from biofilms formed on the inner surface (IS) of the reactor tank. The lanes for the samples from each run did not show any distinguishable local variation in the microbial community of the CSTR HF-MBfR shown in Fig. 3A. For example, Lanes 1, 2, and 3 for Run 3 (D-170) look similar. This result suggested that the gas (oxygen) was well distributed, and the liquid component (ammonium and nutrient) also did not limit nitrification. On the other hand, the samples between runs showed slight variations in the microbial community; DGGE bands HF2-N6 and HF2-N10

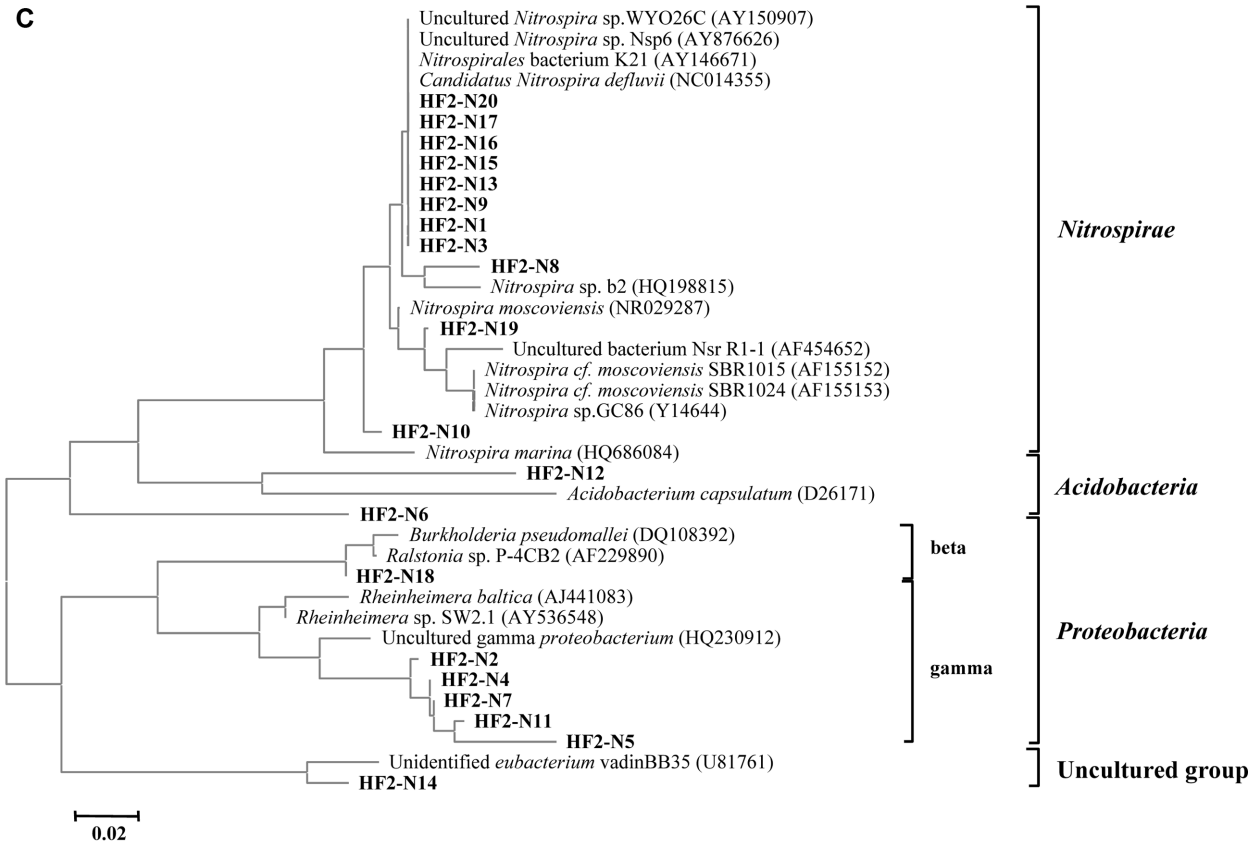

Fig. 3. Continued.

were clearly observed for Runs 5 (D-355) and 6 (D-380), respectively, shown in Fig. 3B. The temporal variations in the microbial community observed by DGGE analysis were attributed to the variations in the NH_4^+ loading rate. The results suggest that the majority of the nitrifying bacteria were altered in the nitrification reactor at varying NH_4^+ loading rates. However, it was hard to distinguish the structure of microbial community, or the major strain affecting the nitrification efficiency (Table 2).

A neighbor-joining phylogenetic tree was constructed with the sequences obtained from the DGGE bands established for the biofilm of the nitrification reactor and the sequences of phylogenetically related bacteria, shown in Fig. 3C. The phylum *Nitrospirae* was detected in all samples as NOB. Among the 20 analyzed DGGE bands, 11 bands were clustered in *Nitrospira*. *Nitrospira* was a dominant NOB in the nitrifying reactors. It was reported that *Nitrospira* is present in the micro-aerobic zone, whereas *Nitrobacter* was shown to dominate the high oxygen zone near the biofilm interface [34]. In addition, *Nitrospira* is more likely to dominate nitrite oxidation under conditions with low ammonium and nitrite concentrations [2]. When increasing the nitrite loading rate, *Nitrospira* was highly dominant over *Nitrobacter* [9].

However, AOB were not observed in the current DGGE analysis. AOB have been found among beta and gamma proteobacteria. Several molecular community analysis studies have shown no detectable results on the DGGE bands for AOB [3, 8, 14]. *Nitrosomonas europaea*-like AOB could not be successfully visualized under the gel conditions for this DGGE [3]. AOB were detected with FISH but not detected with DGGE, when 16S rRNA gene fragments with primers specific for the bacteria were used [14]. In addition, we believe there is a possibility that the presence of Ammonium-oxidizing archaea (AOA) plays a role in ammonium oxidation. AOA are present in soil [7, 16] and ocean [5] samples. Recently, AOA were also found in activated sludge samples [19]. Therefore, these molecular data within the limitations of the techniques used showed that the DGGE analysis based on the 16S rRNA region (341f/534r) could not completely detect the microorganisms responsible for nitrification, or AOB was easily grown as planktonic cell and it could be missed from DNA extraction samples as sludge forms [35].

Sequencing of DGGE Fragments and Phylogenetic Analysis of the CSTR-Type Denitrification HF-MBfR

Fig. 4A shows the DGGE band profiles of the PCR amplification products obtained from the inoculated anaerobic sludge and biofilm in the denitrification reactor. Samples were collected from the inner layer of the biofilm (IL; DGGE bands 1, 4, 6, 8, and 11) and the outer layer of the biofilm (OL; DGGE bands 2 and 9) formed on the HF-Ms. The rest of samples (DGGE bands 3, 5, 7, 10, and 12) were collected from biofilms formed on the IS of the reactor tank. As shown in Fig. 4A, most samples collected for each run did not show local variations in the microbial community, except for Run 4 (D-215). The band patterns were similar in lanes 8, 9, and 10, but the intensity of each band was different. This result might be due to the presence of a concentration gradient of electron donors (H_2) and acceptors (NO_3^-) across the biofilm. Fig. 4B shows a comparison of the DGGE band patterns of the samples collected from the inner biofilm samples. The major bands in each sample differed, and the DGGE band pattern became simpler over the experimental period. The temporal variations in the microbial community were attributed to the NO_3^- loading rate applied to the denitrification HF-MBfR.

A neighbor-joining phylogenetic tree was constructed with the sequences obtained from the DGGE bands in the denitrification reactor and the sequences of the phylogenetically related bacteria (Fig. 4C). Because hydrogen was the sole electron donor for the denitrification process, autotrophic denitrifiers could be found in the denitrification reactor. One of the major groups showed high similarity to the alpha class of the phylum *Proteobacteria*. Another major group showed high similarity to the phylum *Bacteroidetes*. DGGE bands HF2-DN 2 and 4 showed low similarity to identified bacteria in the database, and the sequences of these bands most closely matched with uncultured anaerobic bacteria. HF2-DN 16, which was one of the major species identified in Run 2 (D-93), was also observed in Runs 1 (D-35), 3 (D-148), and 4 (D-215). HF2-DN 16 showed high similarity to *Paracoccus* sp., which was reported as an autohydrogenotrophic bacterium [13]. HF2-DN 18 observed in Runs 2 (D-93), 3 (D-148), 4 (D-215), and 5 (D-275) showed high similarity to *Ochrobactrum anthropi*, which was also reported as an autohydrogenotrophic bacterium [29]. HF2-DN 7 identified in Runs 2 (D-93), 3 (D-148), and 4 (D-215) showed high similarity to *Aquaspirillum* sp., which is capable of oligotrophic denitrification [12]. HF2-DN 3 and 15 are most closely related to the heterotrophic denitrifying bacteria that were identified from a diversity analysis of

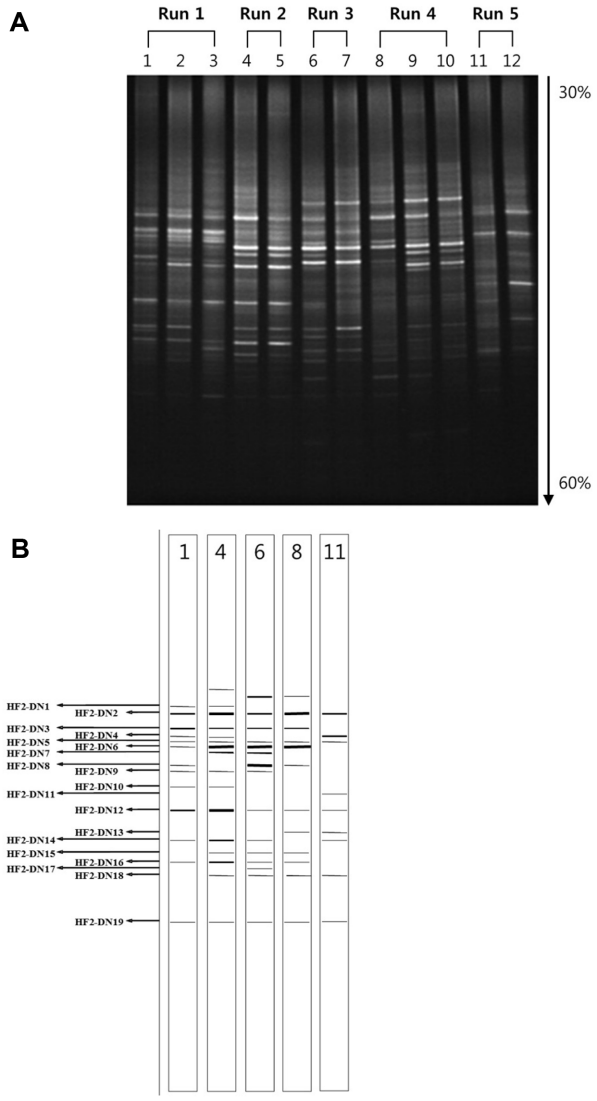

Fig. 4. DGGE band profile of the amplified 16S rRNA gene sequences (**A**), schematic of the bands for comparison of microbial community (**B**) in biofilms from the CSTR-type denitrification HF-MBfR, and neighbor-joining phylogenetic tree based upon 16S rRNA sequences derived from DGGE bands recovered from biofilm in the CSTR-type denitrification (**C**).
The scale bar indicates 0.02 estimated substitution per nucleotide. Biomass was collected at diverse topological positions in the CSTR-type HF-MBfR at different NO_3^- loading rates. NO_3^- loading rates: Lane 1 for Run 1 (0.10 kg N/m^3/d); Lane 4 for Run 2 (0.13 kg N/m^3/d); Lane 6 for Run 3 (0.16 kg N/m^3/d); Lane 8 for Run 4 (0.18 kg N/m^3/d); and Lane 11 for Run 5 (0.21 kg N/m^3/d). Topological positions: Lanes 1, 4, 6, 8, and 11 for biomass from the inner layer of the biofilm; Lanes 2 and 9 for biomass from the outer layer of the biofilm; Lanes 3, 5, 7, 10, and 12 for biomass of biofilm formed on the inner surface of the reactor tank; and Lane 13 for the inoculated anaerobic sludge. Sampling dates: Lanes 1, 2, and 3 at 35 d; Lanes 4 and 5 at 93 d; Lanes 6 and 7 at 148 d; Lanes 8, 9, and 10 at 215 d; and Lanes 11 and 12 at 275 d.

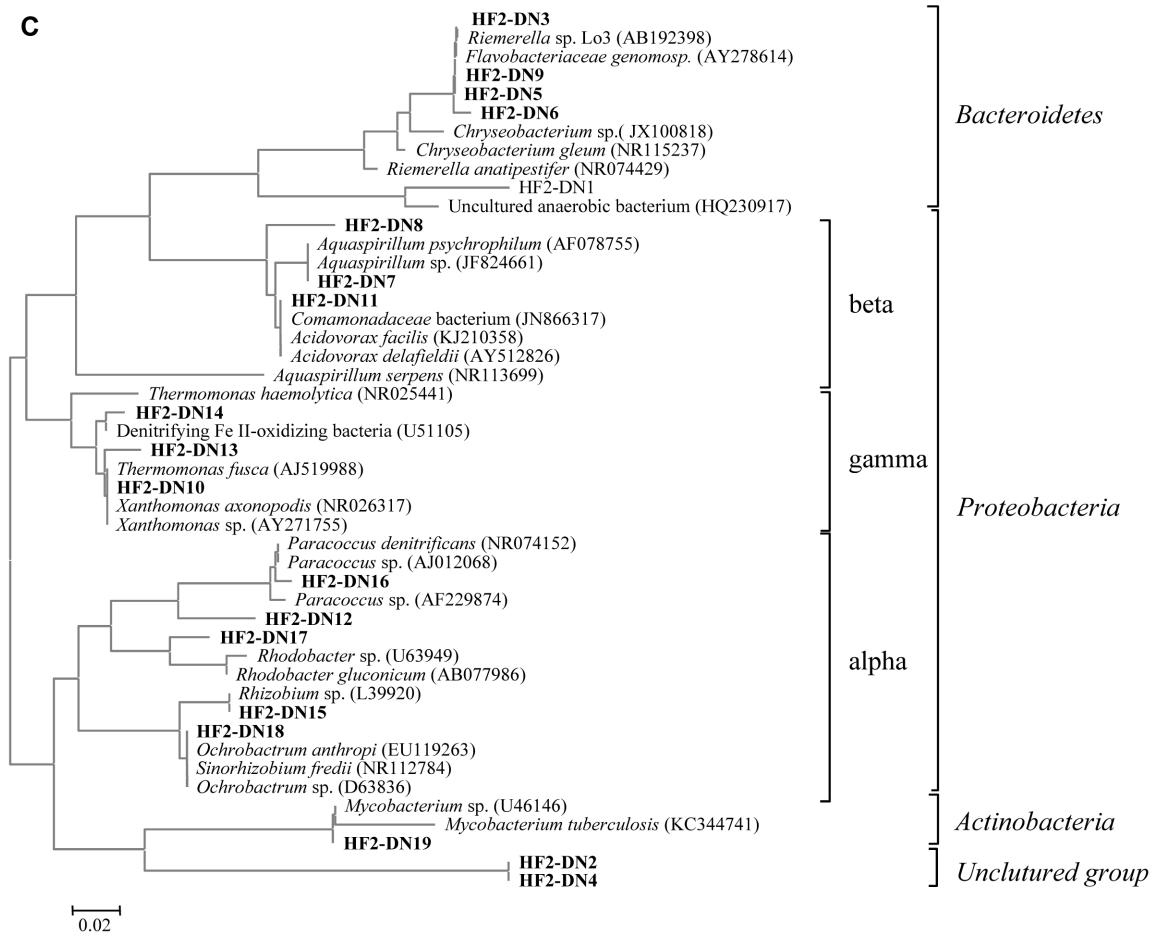

Fig. 4. Continued.

denitrifiers in activated sludge systems, indicating the presence of organic electron donors. As a result, the analysis of the microbial community by PCR-DGGE confirmed that the microbial community in the CSTR-type denitrification HF-MBfR consisted mainly of the alpha class of the phylum *Proteobacteria*. The results suggest that autohydrogenotrophic denitrifying and heterotrophic denitrifying bacteria coexisted in the CSTR-type denitrification HF-MBfR. Additionally, the fact that there was no big difference in the microbial community according to topological positions showed a uniformed distribution of H_2 (electron donor) and NO_3^- (electron acceptor) in the denitrification HF-MBfR.

Hollow fiber membrane biofilm reactors were operated for autotrophic nitrification and denitrification. Through a hollow fiber membrane, O_2 and H_2 were successfully supplied for nitrification and denitrification, respectively. The nitrogen loading rate affected the structure of microbial community. Different intensities of DGGE bands were observed for the denitrification biofilm, and the spatial difference suggests that within biofilms, there was a concentration gradient of bioavailable H_2, nitrate, or nutrients due to thick biofilms. The types of band distribution on DGGE did not show any big differences, but the intensity of the bands did change according to the loading rate. This was more clearly observed from the denitrification reactor (Fig. 4A). DGGE analysis based on 16S rRNA (341f/534r) successfully detected nitrate-oxidizing bacteria and autohydrogenotrophic denitrifying bacteria but not ammonium-oxidizing bacteria in the HF-MBfRs. Nitrogen removal in the HF-MBfRs was autotrophically operated by supplementing O_2 and H_2, with bacteria playing a role in autotrophic nitrification and denitrification.

In conclusion, the hollow fiber membrane biofilm reactors were operated for nitrification and denitrification. The nitrogen removal was dependent on its loading rate. The DGGE analysis showed there was no local variation of microbial community in nitrification but a presence of

special variation in denitrification. The *Nitrospira*-related phylum was detected in all samples from the nitrification reactors, whereas *Paracoccus* sp. and *Aquaspirillum* sp., which are an autohydrogenotrophic bacterium and an oligotrophic denitrifier, respectively, were observed in the denitrification reactors.

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References

1. Alawi M, Lipski A, Sanders T, Eva Maria P, Spieck E. 2007. Cultivation of a novel cold-adapted nitrite oxidizing betaproteobacterium from the Siberian Arctic. *ISME J.* **1:** 256-264.
2. Blackburne R, Vadivelu VM, Yuan Z, Keller J. 2007. Kinetic characterisation of an enriched *Nitrospira* culture with comparison to *Nitrobacter*. *Water Res.* **41:** 3033-3042.
3. Burrell PC, Phalen CM, Hovanec TA. 2001. Identification of bacteria responsible for ammonia oxidation in freshwater aquaria. *Appl. Environ. Microbiol.* **67:** 5791-5800.
4. Ergas SJ, Reuss AF. 2001. Hydrogenotrophic denitrification of drinking water using a hollow fibre membrane bioreactor. *J. Water Supply Res. Technol.* **50:** 161-170.
5. Francis CA, Roberts KJ, Beman JM, Santoro AE, Oakley BB. 2005. Ubiquity and diversity of ammonia-oxidizing archaea in water columns and sediments of the ocean. *Proc. Natl. Acad. Sci. USA* **102:** 14683-14688.
6. Gong Z, Liu S, Yang F, Bao H, Furukawa K. 2008. Characterization of functional microbial community in a membrane-aerated biofilm reactor operated for completely autotrophic nitrogen removal. *Bioresour. Technol.* **99:** 2749-2756.
7. He J-Z, Shen J-P, Zhang L-M, Zhu Y-G, Zheng Y-M, Xu M-G, Di H. 2007. Quantitative analyses of the abundance and composition of ammonia-oxidizing bacteria and ammonia-oxidizing archaea of a Chinese upland red soil under long-term fertilization practices. *Appl. Environ. Microbiol.* **9:** 2364-2374.
8. Hollibaugh JT, Bano N, Ducklow HW. 2002. Widespread distribution in polar oceans of a 16S rRNA gene sequence with affinity to *Nitrosospira*-like ammonia-oxidizing bacteria. *Appl. Environ. Microbiol.* **68:** 1478-1484.
9. Kim YM, Cho HU, Lee DS, Park C, Park D, Park JM. 2011. Response of nitrifying bacterial communities to the increased thiocyanate concentration in pre-denitrification process. *Bioresour. Technol.* **102:** 913-922.
10. Lee K-C, Rittmann BE. 2000. A novel hollow-fibre membrane biofilm reactor for autohydrogenotrophic denitrification of drinking water. *Water Sci. Technol.* **41:** 219-226.
11. Lee KC, Rittmann BE. 2002. Applying a novel autohydrogenotrophic hollow-fiber membrane biofilm reactor for denitrification of drinking water. *Water Res.* **36:** 2040-2052.
12. Maier RM, Pepper IL, Gerba CP. 2008. *Environmental Microbiology*, pp. 287-318. Academic Press, San Diego, CA.
13. Matějů V, Čižinská S, Krejčí J, Janoch T. 1992. Biological water denitrification — A review. *Enzyme Microb. Technol.* **14:** 170-183.
14. Milner MG, Curtis TP, Davenport RJ. 2008. Presence and activity of ammonia-oxidising bacteria detected amongst the overall bacterial diversity along a physico-chemical gradient of a nitrifying wastewater treatment plant. *Water Res.* **42:** 2863-2872.
15. Muyzer G, de Waal EC, Uitterlinden AG. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* **59:** 695-700.
16. Nicol GW, Leininger S, Schleper C, Prosser JI. 2008. The influence of soil pH on the diversity, abundance and transcriptional activity of ammonia oxidizing archaea and bacteria. *Appl. Environ. Microbiol.* **10:** 2966-2978.
17. Pabby AK, Rizvi SSH, Requena AMS. 2008. *Handbook of Membrane Separations; Chemical, Pharmaceutical, Food, and Biotechnological Applications*, pp. 7-24. CRC Press Danvers, MA.
18. Pankhania M, Stephenson T, Semmens MJ. 1994. Hollow fibre bioreactor for wastewater treatment using bubbleless membrane aeration. *Water Res.* **28:** 2233-2236.
19. Park H-D, Wells GF, Bae H, Criddle CS, Francis CA. 2006. Occurrence of ammonia-oxidizing archaea in wastewater treatment plant bioreactors. *Appl. Environ. Microbiol.* **72:** 5643-5647.
20. Ren L, Wu Y, Ren N, Zhang K, Xing D. 2010. Microbial community structure in an integrated A/O reactor treating diluted livestock wastewater during start-up period. *J. Environ. Sci. (China)* **22:** 656-662.
21. Rice EW, Bridgewater L, American Public Health A ssociation, American Water Works A ssociation. 2012. *Standard Methods for the Examination of Water and Wastewater*. American Public Health Association, Washington, D.C.
22. Rittmann BE, Huck PM, Bouwer EJ. 1989. Biological treatment of public water supplies. *Crit. Rev. Environ. Sci. Technol.* **19:** 119-184.
23. Saitou N, Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4:** 406-425.
24. Shin J-H, Sang B-I, Chung Y-C, Choung Y-K. 2005. The

removal of nitrogen using an autotrophic hybrid hollow-fiber membrane biofilm reactor. *Desalination* **183:** 447-454.

25. Shin J-H, Sang B-I, Chung Y-C, Choung Y-K. 2008. A novel CSTR-type of hollow fiber membrane biofilm reactor for consecutive nitrification and denitrification. *Desalination* **221:** 526-533.
26. Smith RL, Ceazan ML, Brooks MH. 1994. Autotrophic, hydrogen-oxidizing, denitrifying bacteria in groundwater, potential agents for bioremediation of nitrate contamination. *Appl. Environ. Microbiol.* **60:** 1949-1955.
27. Stackebrandt E, Goodfellow M. 1991. *Nucleic Acid Techniques in Bacterial Systematics*, pp. 115-175. John Wiley & Sons, NY.
28. Straub KL, Benz M, Schink B, Widdel F. 1996. Anaerobic, nitrate-dependent microbial oxidation of ferrous iron. *Appl. Environ. Microbiol.* **62:** 1458-1460.
29. Szekeres S, Kiss I, Kalman M, Soares MI. 2002. Microbial population in a hydrogen-dependent denitrification reactor. *Water Res.* **36:** 4088-4094.
30. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **28:** 2731-2739.
31. Tang Y, Zhou C, Van Ginkel SW, Ontiveros-Valencia A, Shin J, Rittmann BE. 2012. Hydrogen permeability of the hollow fibers used in H_2-based membrane biofilm reactors. *J. Membr. Sci.* **407–408:** 176-183.
32. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The CLUSTAL_X Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **25:** 4876-4882.
33. Tsuneda S, Nagano T, Hoshino T, Ejiri Y, Noda N, Hirata A. 2003. Characterization of nitrifying granules produced in an aerobic upflow fluidized bed reactor. *Water Res.* **37:** 4965-4973.
34. Vázquez Padín J. 2010. Autotrophic nitrogen removal in granular sequencing batch reactors. PhD thesis. Universidad de Santiago de Compostela.
35. Ye L, Zhang T. 2010. Estimation of nitrifier abundances in a partial nitrification reactor treating ammonium-rich saline wastewater using DGGE, T-RFLP and mathematical modeling. *Appl. Microbiol. Biotechnol.* **88:** 1403-1412.
36. Zhang TC, Lampe DG. 1999. Sulfur:limestone autotrophic denitrification processes for treatment of nitrate-contaminated water: batch experiments. *Water Res.* **33:** 599-608.
37. Zhang Y, Zhong F, Xia S, Wang X, Li J. 2009. Autohydrogenotrophic denitrification of drinking water using a polyvinyl chloride hollow fiber membrane biofilm reactor. *J. Hazard. Mater.* **170:** 203-209.