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Identification of dominant microbial community in aerophilic biofilm reactors by fluorescence *in situ* hybridization and PCR-denaturing gradient gel electrophoresis

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Abstract−This study was conducted by combining fluorescence *in situ* hybridization (FISH) performed on 16S rRNA and polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis (DGGE) with 16S CTOs primers to characterize the nitrifying microbial communities in biofilm processes, which were tested to retrofit the S municipal wastewater treatment plant in Busan, Korea. Four aerophilic biofilm reactors were operated with hydraulic retention times of 2 to 8 h and biofilms were grown on ceramic media. The same low COD/ NH_4^+-N ratio (100 mg/L of COD over 40 mg/L of NH_4^+-N) with the S plant was used. The average relative population ratios of *Nitrosomonas* spp. to ammonia oxidizing bacteria (AOB) as measured by specific FISH probes (%/Nso190) were 75.0%, 80.0%, 73.0% and 73.5%, respectively, while those of *Nitrosospira* spp. to AOB were 21.0%, 14.7%, 24.6% and 24.1% after 180 days of operation. The microbial composition of *Nitrobacter* spp. detected by using a Nit3 probe was below 10% in each reactor. In contrast, *Nitrospira* genus detected with an Ntspa662 probe was around 20%. When CTOs primer was applied in PCR-DGGE analysis to define the nitrifying bacteria, the bands of group B in the R-1 reactor with the highest hydraulic retention time (HRT) had the strongest light intensity compared with two other reactors with lower HRTs after day 64. The bands of the groups were responsible for nitrification with the major dominant population in each reactor depending on the change of ammonia removal rate. These results would directly lead to an understanding of the reactor performance in relation to the ammonia removal, when conventional municipal wastewater treatment plants are retrofitted or upgraded to biological nitrogen removal processes using biofilm.

Key words: Aerophilic Biofilm, Ammonia Oxidizing Bacteria, FISH, Nitrification, PCR-DGGE

INTRODUCTION

Over the past decades, biological nutrient removal (BNR) processes using biofilms have been widely used to treat wastewater containing nutrient chemicals, such as nitrogen and phosphorus, because of their superior stability. In the next decade, the adoption of BNR processes for retrofitting wastewater treatment plants will be a high priority in Korea due to more stringent nitrogen requirements for effluent discharges. Nitrification is considered as an important factor for upgrading the existing wastewater treatment plants. However, the increased volume of influent during rainy season and low water temperature (below 10 °C) in winter can deteriorate the performance of ammonium removal dramatically [1]. Since the researches on biofilm processes for nutrient removal have focused on process development and efficiency promotion rather than on microbial population and activity, a knowledge of the identity and ecology of the microorganisms catalyzing nitrogen removal is still limited [2]. In addition, the characterization of nitrifying bacterial populations in biofilms has been hindered by the limitations in traditional culture-dependent techniques, which often detect a minor portion of the occurring nitrifying bacteria. Recently, various analytical and investigative methods have been developed to analyze the species composition, spatial structure and functional properties of microbial aggregates to overcome the uncertainty associated with culture-dependent enumeration [3].

In this study, an investigation of the nitrifying bacterial communities participating in ammonium conversion in aerophilic biofilms was conducted to treat synthetic wastewater with low C/N ratio. Molecular techniques such as fluorescence in situ hybridization (FISH) using confocal laser scanning microscopy (CLSM) and digital image analysis, and polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis (DGGE) were used to analyze the nitrifying bacterial community of the biofilm complex. A better understanding of the microbiology and ecology of nitrifying bacteria in biofilms will contribute to the improvement of the nitrification process in terms of treatment activity and process stability when a wastewater treatment plant is retrofitted to a biofilm process.

MATERIALS AND METHODS

1. Aerophilic Nitrifying Biofilm Reactors

Inocular for nitrifying biofilms were obtained from an activated sludge tank at S municipal wastewater treatment plant in Busan, Korea, and aerophilic biofilms were cultured on submerged ceramic media in four fixed-bed reactors (Fig. 1). The volume of each reactor was 5.3 L with a medium packing ratio of 20% (v/v). The biofilms were cultured with synthetic nutrient medium containing 95 g/L of glucose as a carbon source and 40 mg/L of NH_4Cl as a nitrogen source. The nutrient medium also contained 5 mg/L yeast extract and minerals including 15.3 mg/L KH_2PO_4, 381.3 mg/L $NaHCO_3$, 17.5 mg/L NaCl, 17.5 mg/L $CaCl_2 \cdot 2H_2O$, and 17.5 mg/L $MgSO_4 \cdot$

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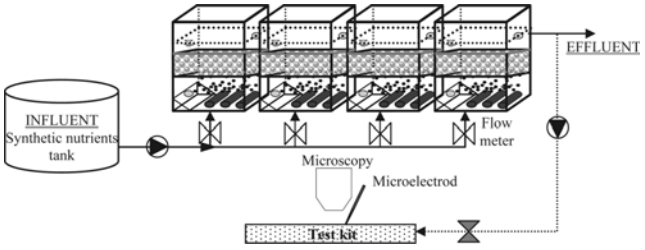

Fig. 1. Schematic diagram of aerophilic nitrifying biofilm reactors.

$7H_2O$ in tap water. The nutrient level in the synthetic medium was designed at the same level with the influent of S wastewater treatment plant. The influent concentrations were adjusted to 100 mg COD/L and 40 mg NH_4^+-N/L. The temperature and dissolved oxygen were maintained at 25±0.5 ℃ and 4±0.2 mg/L, respectively. The reactors were operated under four different conditions. The hydraulic retention time (HRT) of R-1, R-2, and R-3 were 8 h, 2 h, and 4 h, respectively. R-4 was operated at the same HRT but with doubled nutrient medium level of R-1. Analytical procedures were conducted according to the standard methods. The effluent COD, NH_4^+-N, NO_3^--N, and NO_2^--N concentrations were measured by auto analyzer (Bran-Luebbe, Germany). The aerophilic biofilm thickness was directly measured in the test chambers by using microelectrodes.

2. FISH Analysis and Cell Quantification

The ceramic media were taken out carefully from the reactors to avoid detachment of microbial aggregations and the biofilm thickness was measured by using microelectrodes. Immediately after the microelectrode measurements, the biofilms ere sampled and vortexed for 5 min. A cell suspension was centrifuged, washed twice with 1×PBS and then incubated for 1 h at room temperature. For alcohol fixation, the cell pellet was resuspended in 200 μL 2×PBS, and an equal volume of 100% ethanol (50 : 50=PBS : EtOH) was added to eliminate the extracellular polysaccharide materials. The fixed cells were immersed in freshly prepared 4% paraformaldehyde solution. Before pipetting on the slide wells, the samples were dissolved in a solution of 2.8 g/L sodium pyrophosphate in PBS to prevent aggregation.

The following oligonucleotide probes were used: EUB338, Nso 190, Nsm156, Ntspa662, Nsv443, and Nit3. All of the probe sequences, as well as the specificities of the probes and the hybridization conditions, are shown in Table 1. The probes previously developed in other works were used to identify ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) [2,4,5], and labeled with fluorescent dyes fluorescein isothiocyanate (FITC), tetramethylrhodamine isothiocyanate (TRITC), Rhodamin or Cy5. All *in situ* hybridizations followed the procedures described by Manz et al. [6] and Toh et al. [7]. Bacterial populations were detected by CLSM (LSM-510, Zeiss, Germany) and the quantification results were presented as the percentage share of the total bacterial cell area using a digital image analyzer.

3. DNA Extraction and PCR- DGGE Analysis

The biofilms were carefully sampled, and then vortexed for 5 min to extract DNAs. Cell suspensions were centrifuged at 8,000 ×*g* and 4 ℃ for 15 min, and the pellets then stored at –20 ℃ until used. Total community DNAs were extracted from the pellets by using the ultra clean soil DNA isolation kit (Mobio, Inc., Solana beach, CA). The variable V3 region of the 16S rDNA gene in bacterial cells was PCR amplified with primers: 340F (5'-CCT ACG GGA GGC AGC AG-3') with a GC-clamped (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-3') on the 5' end, and 518R (5'-ATT ACC GCG GCT GCT GG-3'). The nested PCR technique previously described by Pynaert et al. [8] was used for 30 cycles with the DGGE to analyze the fragments of the same length for the purpose of increasing the sensitivity.

After the identification of the PCR fragments, PCR for *β*-subdivision AOB was amplified using the primers: CTO189F (5'-GGAGRAAAGCAGGGGATCG-3') with a GC-clamped 5' end, and CTO654R (5'-CTAGCYTTGTAGTTTCAAACGC-3'). These primers were designed to amplify the partial rDNA sequences from *β*-subdivision AOB. DGGE was performed on the Decode Universal Mutation Detection System (BioRad, USA) at 130 V and 60 ℃ on a polyacrylamide gel containing a linear gradient of denaturant ranging from 30% to 55%. After the electrophoresis, the polyacrylamide gel was stained with SYBR Green I (FMC Bioproducts, No. 50513) for 30 min, and then visualized on a UV transilluminator (CSF-35BC, Cosmobio, Japan) coupled with a digital camera (DC120 ZOOM, Kodak, USA).

RESULTS AND DISCUSSION

1. Reactor Performance

Complete nitrification (NH_4^+ oxidation to NO_3^-) was achieved in

Table 1. 16S rRNA targeted oligonucleotide probes used

Probe	Specificity	Probe sequence (5'-3')	Target site[a]	Formamide conc. (%)[b]	NaCl conc. (mM)[c]
EUB 338	Domain bacteria	GCTGCCTCCCGTAGGAGT	338-355	20	215
Nso 190	Ammonia-oxidizing *β*-Proteobacteria	CGATCCCCTGCTTTTCTCC	190-208	20	215
Nsm 156	*Nitrosomonas* spp.	TATTAGCACATCTTTCGAT	156-174	5	630
Ntspa662	*Nitrospira* genus	GGAATTCCGCGCTCCTCT	662-679	20	215
Nsv 443	*Nitrosospira* spp.	CCGTGACCGTTTCGTTCCG	444-462	30	102
Nit 3	*Nitrobacter* spp.	CCTGTGCTCCATGCTCCG	1035-1048	10	46

[a]16S rRNA position according to Escherichia coli numbering

[b]Formamide concentration in the hybridization buffer

[c]Sodium chloride concentration in the washing buffer by Biesterfeld (2003)

R-1 and R-3 within 3 weeks of the operation due to their lower ammonium load. R-2 and R-4 attained steady state approximately 1 month later. The biofilm reactors showed different ammonia removal efficiencies depending on the HRT (R-1 to R-3) and the influent concentration (R-4). The biofilm reactors with HRTs of 8 h (R-1) and 4 h (R-3) showed stable ammonia removal efficiency as high as 90% or greater for 180 days. More than 90% of the removed ammonia was converted to nitrate (data not shown). In contrast, R-2 with HRT of 2 h showed the unstable ammonia removal efficiency of approximately 50%. A strong injection of air bubbles to remove excess biomass of the biofilms at day 50 and day 80 enhanced the ammonia removal efficiency, but the efficiency never recovered to more than 70% due to the increased thickness of biofilms. These results suggested that high loading rate (R-2) and high influent concentration (R-4) induced excess growth of biomass, especially heterotrophs, on the biofilms. This overgrowth of heterotrophs on the biofilms led to a channeling effect and depletion of oxygen, and eventually to the decreased ammonia removal efficiency.

2. Quantification of Nitrifying Bacteria Population Using FISH

Physical and chemical tests about aerophilic biofilms have confirmed that the presence of nitrification reaction in the reactor can explain, but it is not confirmed, what classes and genera of bacteria are present. The problem is that these bacteria cannot be cultured to be tested by the ordinary microbiological tests. At that time, the biofilms were taken from the continuous reactors and analyzed by *in situ* hybridization (FISH) using combinations of group specific probes for AOB (Nso190), *Nitrosomonas* spp. (Nsm156), *Nitrosospira* spp. (Nsv443), *Nitrobacter* spp. (Nit3) and *Nitrospira* genus (Ntspa662). AOB and NOB were quantified by digital image analysis. As shown in Fig. 2, the abundance of nitrifiers in the biofilm reactors was 10% greater than those in the seeding activated sludge. The reactors R-1 and R-3 with 90% ammonia removal efficiency showed higher distribution of AOB. The distributions (%/EUB338) of AOB were 20.1% and 19.6% in R-1 and R-3, respectively, while R-2 and R-4 showed 17.0% and 17.1% of distributions. The averaged relative population ratios of *Nitrosomonas* spp. to AOB as measured by specific FISH probes (%/Nso190) were 75.0%, 80.0%, 73.0% and 73.5%, respectively, while those of *Nitrosospira* spp. to AOB were 21.0%, 14.7%, 24.6% and 24.1% after 180 days of operation. These results show that the differences in the population of AOB among the reactors were too small for definite confirmation, but the AOB population was directly related to the ammonium loading rate or HRT. Especially, *Nitrosospira* spp. was more frequently detected than those in an ammonium poor system [9], but *Nitrosomonas* spp. was the dominant AOB in this system, which had low C/N ratio. The distributions (%/EUB338) of *Nitrospira* genus by Ntspa662 were 13.0%, 14.4%, 10.5% and 9.7% in R-1 to R-4, respectively, while *Nitrobacter* spp. were detected below 10% distribution in all reactors. These suggested that *Nitrospira* genus was often found as a dominant nitrite-oxidizer under the conditions of lower loading rate and low DO in biofilm, while *Nitrobacter* spp. were adjusted with higher substrate loading rate [10].

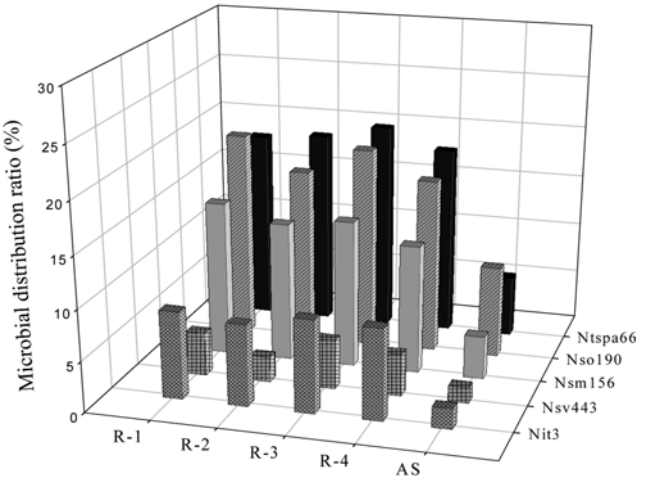

Fig. 2. Quantification of nitrifiers in the biofilm reactors by digital image analysis. Nso190, Nsm156, Ntspa662, Nsv443 and Nit3 probes were used to obtain the distributions of AOB, *Nitrosomonas*, *Nitrospira*, *Nitrosospira* and *Nitrobacter* in each biofilm reactor and the activated seed sludge.

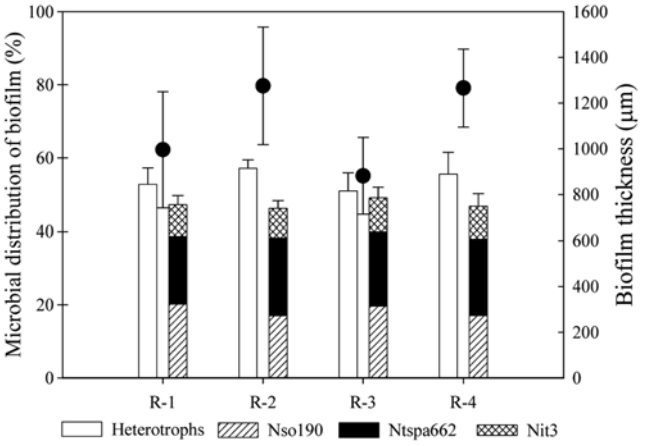

Fig. 3. The distributions of microbial species as against *Eubacteria* and the change of biofilm thickness in each reactor after 180 days of operation.

Fig. 3 presents that the distribution of microbial species and biofilm thickness has changed according to HRT and loading rate in each reactor after 180 days of operation. The distribution ratio of heterotrophs follows the equation (1) by Noqueira et al. [11].

$$\text{Area}_{heterotrophs}=\text{Area}_{EUB338}-(\text{Area}_{Nso190}+\text{Area}_{Nit3}+\text{Area}_{Ntspa662}) \quad (1)$$

The distribution of heterotrophs in R-1 was 58.6%, while that of R-3 and R-4 was 69.4% and 66.0%, respectively. As shown in Fig. 3, the distribution ratio of heterotrophs in R-1 was lower than that in R-2 because heterotrophs in R-1 with enough HRT and DO would have more substrate competition than those in R-3 and R-4 with relatively high organic loading rate. Although R-3 and R-4 were operated with the same organic loading rate, the distribution of heterotrophs was higher in R-4. It appeared that low ammonia removal efficiency was induced because the fast growing heterotrophs were relatively increased due to the increased organic loading ratio. The biofilm thickness was measured approximately as 995 μm (R-1), 882 μm (R-2) and 1,275 μm (R-3) by using microelectrodes. It is explained that the thickness of heterotroph growth related to loading rate changes was affected not by influent concentration but by flow rate; thus the removal efficiency was relatively decreased. The

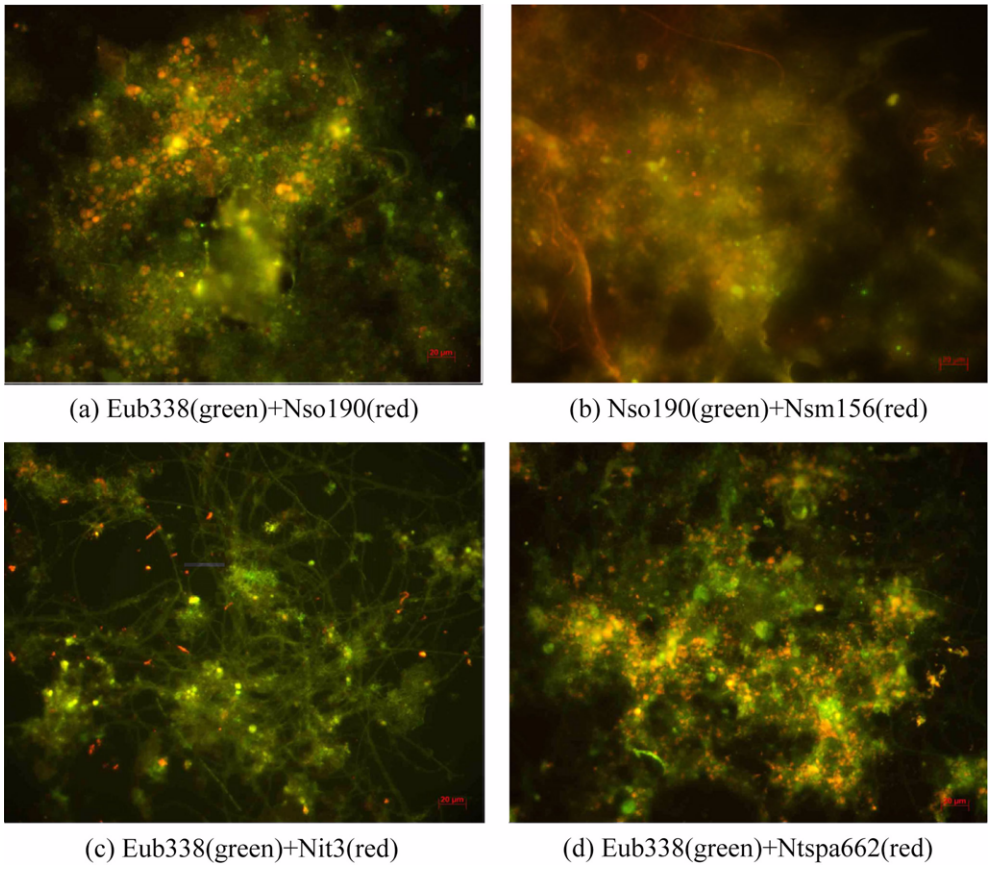

Fig. 4. *In situ* hybridization of nitrifying bacteria in aerophilic biofilms of R-1. Overapping labels are visualized in yellow. Simultaneous *in situ* hybridization is labeled with (a) FITC (green, Eub338) and TRITC (red, Nsol190), (b) FITC (green, Nso190) and Cy5 (red, Nsm156), (c) FITC (green, Eub338) and Rhodamin (red, Nit3), and (d) FITC (green, Eub338) and Cy 5 (red, Ntspa662).

biofilm thickness of R-3, which had less shear stress, was thicker than that of R-1, but the high loading rate influenced the biofilm thickness more than the shear stress in R-2. In conclusion, the factors affecting the biofilm thickness seem to be in the order influent concentration, organic loading rate and finally the influent velocity (shear stress).

Fig. 4 shows the photographs of FISH images in a 180 day biofilm of R-1. Dominant microbial community was identified by FISH images using combination of group specific probes for AOB (%, Nso190/EUB338), *Nitrosomonas* spp. (%, Nsm156/Nso190), *Nitrobacter* spp. (%, Nit3/EUB338) and *Nitrospira* genus (%, Ntspa662/EUB338). As one can see, *Nitrosomonas* spp. made groups and clusters in biofilms, but *Nitrospira* spp. tended to form monospecies. The average relative population ratios of AOB (red and yellow) to *Eubacteria* and *Nitrosomonas* spp. (red and yellow color) to AOB, as measured by digital image analyzer were 18% and 75%. In contrast, the microbial compositions of *Nitrobacter* spp. detected with Nit3 probe in biofilms of all the reactors were below 10%, and those of *Nitrospira* genus detected by the Ntspa662 probe were around 10%. It shows that those species have substrate competition and different distribution clusters depending on the operation conditions [9], as shown in Fig. 4 ((c) and (d)). These results suggested that the distribution of each nitrifier community decreased according to the variation of HRT (Fig. 2), but the dominant AOB and NOB were *Nitrosomonas* spp. and *Nitrospira* genus as evidenced by Kindaichi *et al*. [12] using a relatively ammonium rich system.

3. PCR-DGGE Analysis of Microbial Community in Biofilms

DGGE provides potential for the analysis of bacterial community diversity in a complex natural environment. Based upon the mobility of PCR-amplified 16S rDNA fragments, DGGE was evaluated for the identification of *Eubacteria* and β-subdivision AOB. Fig. 5 shows the DGGE band profiles of the 16S rDNA fragments amplified with the EUB primer set for targeting *Eubacteria* from the biofilms of R-1, R-2 and R-3. The DGGE bands for each reactor varied with the number of operating days and the dominant bacteria populations also sequentially varied in the different biofilms. Although reactors R-1 and R-3 showed similar ammonia removal efficiency of 90% or greater, their DGGE profiles had different light intensities. Reactors R-2 and R-3 with higher loading rates showed a number of different bands compared with R-1. This means that biofilms consisting of various microorganisms, including various heterotrophic bacteria, participated in carbon oxidation or partial denitrification due to the high influent loads and shear stress. Especially, the intensity of the bands between the two red lines matched with the ammonia removal efficiency in each reactor. When ammonia oxidation rate was low, the intensity of the bands was weak. Therefore, these results indicate that the defined DGGE bands were significantly related to AOB.

Based upon the mobility of PCR-amplified 16S rDNA fragments for obtaining more detailed information about the AOB in the biofilm reactors, a DGGE examination of PCR products was con-

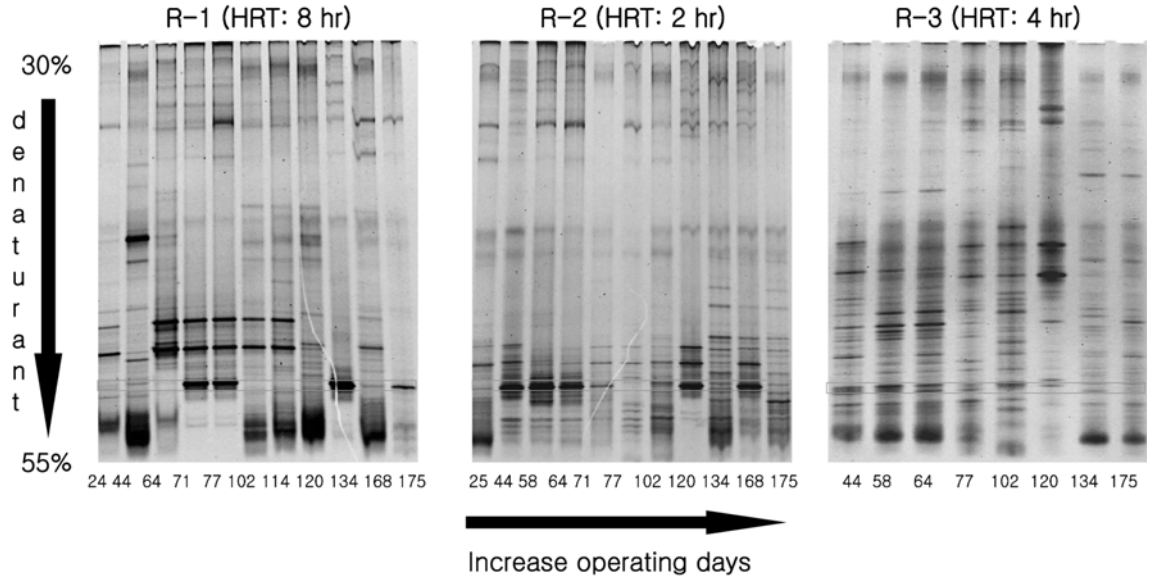

Fig. 5. PCR-DGGE analysis based on the 16S rDNA fragment amplified from the total DNA obtained from biofilms in R-1, R-2 and R-3 according to the number of operating days.

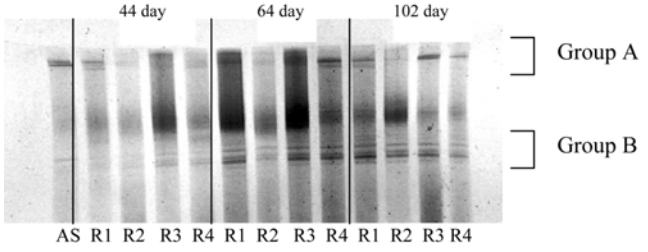

Fig. 6. DGGE analysis of the DNA sample following PCR with the CTOs primers set for AOB.

ducted by using CTO189F and CTO654R primers to amplify the AOB (Fig. 6). The previously designed primers were developed to amplify partial rDNA sequences from AOB. The intensities of the PCR products were believed to be proportional to the abundance of each microbial population [13]. Compared to these of R-1 to R-4, the pattern of the bands of the activated sludge of S treatment plant was simplistic as the number of operating days increased (data not shown). However, the bands of group A, which were vivid in the activated seeding sludge, seemed to participate in the ammonia oxidation of *Nitrosomonas* spp. In case of groups A and B, total band brightness of R-1 and R-3 was higher than that of R-2. These results demonstrated that the ammonia removal of R-1 and R-3 was more stable than that of R-2. In fact, the intensity of the bands of group B for reactor R-1 became stronger as the operating days increased from 44 (75% ammonia removal efficiency) to 64 (95% ammonia removal efficiency). Consequently, the bands of groups A and B seemed to be responsible for nitrification with the major dominant population in each reactor being responsible for the change in the ammonia removal rate.

CONCLUSIONS

The microbial population in biofilm processes as measured by molecular techniques such as FISH and PCR-DGGE varied with the HRT and influent loading rate, which were also directly related to the reactor performance for the ammonia removal. The distributions (%/EUB338) of AOB were 20.1% and 19.6% in R-1 and R-3, respectively, while R-2 and R-4 showed 17.0% and 17.1% of distributions. These results show that the difference in the population of AOB among the reactors was too small for definite confirmation, but the AOB population was directly related to the ammonium loading rate or HRT. Each visualized dominant cell (red color) might be responsible for nitrogen removal. FISH analysis confirmed what was shown in the physiological and microbial characterization. These results suggested that the dominant AOB and NOB were *Nitrosomonas* spp. and *Nitrospira* genus as evidenced by a relatively ammonium rich system. Especially, the intensity of the group B bands detected by PCR-DGGE matched the ammonia removal efficiency in each reactor. These results indicate that the defined DGGE bands were not only significantly related to the dominant population in AOB, but also responsible for nitrification in each reactor.

Finally, microbial community analysis techniques using FISH and PCR-DGGE suggested that the mechanism of treatment activities elucidated by these molecular techniques can be applied to the development of a new wastewater treatment system or the improvement of process performance when conventional wastewater treatment plants are retrofitted to a biofilm BNR process.

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