J. Microbiol. Biotechnol. (2007), **17**(2), 253–261

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Molecular and Ecological Analyses of Microbial Community Structures in Biofilms of a Full-Scale Aerated Up-Flow Biobead Process

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Received: August 16, 2006
Accepted: October 19, 2006

Abstract Molecular and cultivation techniques were used to characterize the bacterial communities of biobead reactor biofilms in a sewage treatment plant to which an Aerated Up-Flow Biobead process was applied. With this biobead process, the monthly average values of various chemical parameters in the effluent were generally kept under the regulation limits of the effluent quality of the sewage treatment plant during the operation period. Most probable number (MPN) analysis revealed that the population of denitrifying bacteria was abundant in the biobead #1 reactor, denitrifying and nitrifying bacteria coexisted in the biobead #2 reactor, and nitrifying bacteria prevailed over denitrifying bacteria in the biobead #3 reactor. The results of the MPN test suggested that the biobead #2 reactor was a transition zone leading to acclimated nitrifying biofilms in the biobead #3 reactor. Phylogenetic analysis of 16S rDNA sequences cloned from biofilms showed that the biobead #1 reactor, which received a high organic loading rate, had much diverse microorganisms, whereas the biobead #2 and #3 reactors were dominated by the members of *Proteobacteria*. DGGE analysis with the ammonia monooxygenase (*amoA*) gene supported the observation from the MPN test that the biofilms of September were fully developed and specialized for nitrification in the biobead reactor #3. All of the DNA sequences of the *amoA* DGGE bands were very similar to the sequence of the *amoA* gene of *Nitrosomonas* species, the presence of which is typical in the biological aerated filters. The results of this study showed that organic and inorganic nutrients were efficiently removed by both denitrifying microbial populations in the anaerobic tank and heterotrophic and nitrifying bacterial biofilms well-formed in the three functional biobead reactors in the Aerated Up-Flow Biobead process.

Key words: Biobead, biofilm, 16S rRNA gene, DGGE, *amoA* gene, bacterial community

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Since the introduction of wastewater treatment practices, most treatment plants in Korea have been designed and operated to remove organic matters and suspended solids from wastewater. In 1996, total nitrogen (TN) and total phosphorus (TP) concentration levels were included in water quality regulations of effluents of sewage treatment plants (STPs) by the Ministry of Environment, Republic of Korea [24]. Furthermore, by 2008, the existing plants in Korea, including all kinds of small and medium size STPs (>capacity 50 m^3/day), must satisfy more strict discharge regulations. To satisfy these strict regulations, the existing plants must have been retrofitted and upgraded by introducing tertiary treatment systems or implementing biological nutrient removal (BNR) systems into the existing treatment facilities.

Under the imperative requirements mentioned above, an Aerated Up-Flow Biobead process was developed and has been used as small and middle sized sewage treatment plants in the domestic area since 1997. The main components of the system consisted of three biobead reactors in series [7]. The packed media were granular type and the packing ratio was over 60%. The biofilm had non-homogeneous spatial distribution and the microbial colonies were embedded in the sunken area of the biobead. Biofilm thickness (5.0–29.4 μm) was very thin compared with that of other biological aerated filters (BAFs), at 200–300 μm [39]. Inorganic nutrients

have been observed to be efficiently removed in this Aerated Up-Flow Biobead process, but the removal mechanism is unclear. Specifically, little is known about the characteristics of microbial communities forming biofilm at each biobead reactor.

Various methods have been used to characterize the diversity of microbial populations in environmental samples. When microbial diversity is studied by conventional cultivation techniques, the results are often biased, because a majority of microorganisms are unculturable with standard cultivation techniques [2, 4]. During the last decade, molecular methods based on total microbial community DNA analysis were developed and allowed a more comprehensive analysis of microbial communities in comparison with cultivation-based techniques. The amplified fragments of 16S rRNA genes and especially temperature or denaturing gradient gel electrophoresis analysis of these genes have been frequently used to investigate the microbial diversity and change in environmental samples [9, 10, 17, 26, 31, 37].

In this study, microbial population dynamics and biofilm characteristics of biobead reactors employed in a full-scale sewage treatment plant were investigated by using culture-dependent methods and molecular methods. The treatment efficiency of the Aerated Up-Flow Biobead process was analyzed by examining the levels of various chemicals in wastewater. The population densities of ammonia-oxidizing bacteria (AOB), denitrifying bacteria (DNB), and nitrite-oxidizing bacteria (NOB) were estimated by using the most probable number (MPN) method. In addition, the microbial community structures were analyzed by using 16S rDNA random cloning and denaturing gel gradient electrophoresis (DGGE) analysis of both the 16S rDNA and the *amoA* genes.

Materials and Methods

Description of the Full-Scale Up-Flow Biobead Process

The schematic diagram of the full-scale Aerated Up-Flow Biobead process is shown in Fig. 1A. The designed capacity is 140.0 m^3/day. The process was installed for

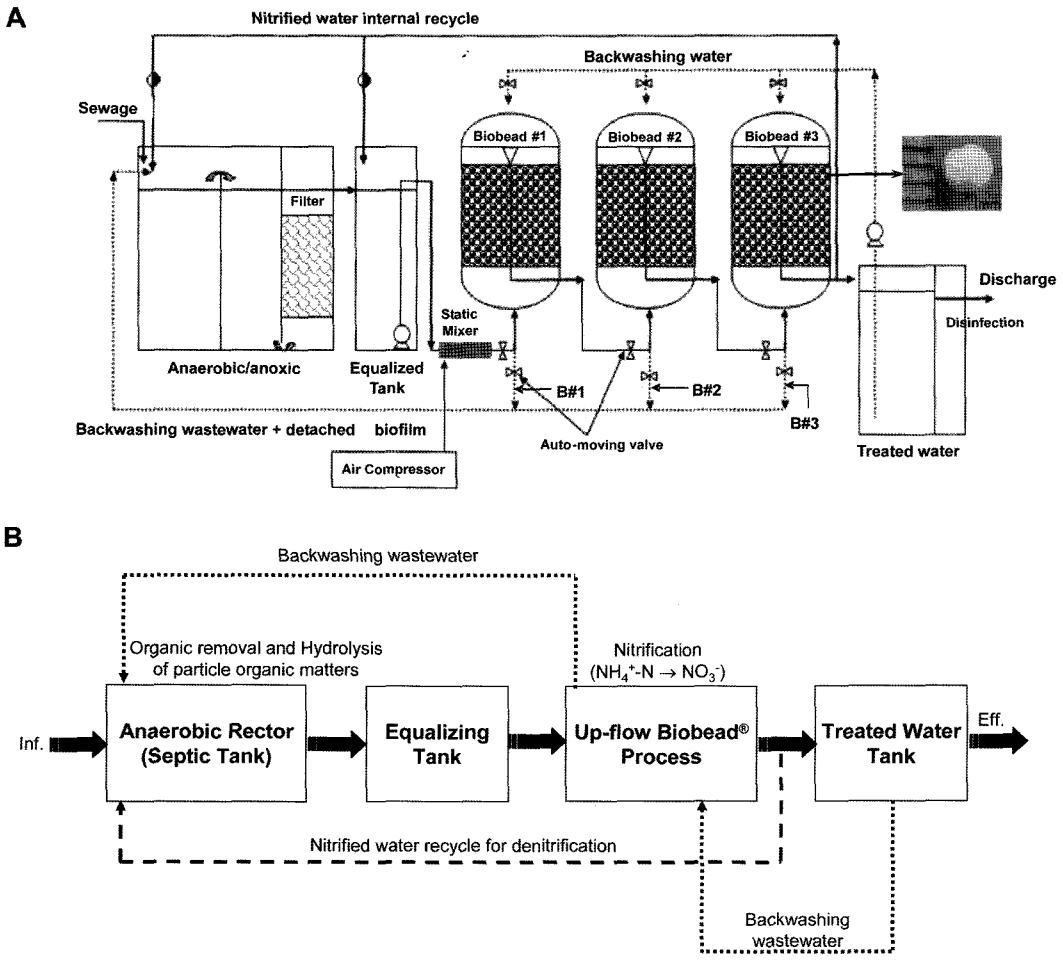

Fig. 1. Schematic diagram (**A**) of the Aerated Up-Flow Biobead process. The sludge samples for microbial analyses were taken from the backwashing wastewater of each biobead reactor (B#1, B#2, and B#3). The diameter of a biobead (photograph) is 2–3 mm. Flow diagram (**B**) of the Aerated Up-Flow Biobead process.

treatment of sewage discharged from army camps at Young-in City, Gyeonggi Province of Korea. The full-scale plant consisted of an anaerobic/anoxic tank, an equalized tank, and three aerated up-flow biobead reactors. The designed hydraulic retention time (HRT) of the biobead process was 2.76 h, and the media packing ratio was over 60% (v/v). Biobead (specific surface area, >2,000 m^2/m^3) was made of polystyrene and the diameter was 2–3 mm. The specific weight and porosity was 0.025 (density= 25 kg/m^3) and 33%, respectively. The flow diagram of the Aerated Up-Flow Biobead process is shown in Fig. 1B. The periodic automatic backwashing (down flow) was carried out using treated water for 2 min. The internal recycle ratio from the biobead #3 reactor to the anaerobic/anoxic reactor was 50%.

Chemical Analysis

Total chemical oxygen demand ($TCOD_{Cr}$), soluble chemical oxygen demand ($SCOD_{Cr}$), suspended solid (SS), and alkalinity were analyzed at 2- or 3-day intervals according to *Standard Methods of Examination of Water and Wastewater* [3]. Total nitrogen (TN), total phosphate (TP), and ionic compounds (NH_4^+-N, NO_3^-, NO_2^-, and PO_4^{3-}) were analyzed by the Hach procedure (Hach Co., Loveland, U.S.A.).

Sampling and DNA Extraction

The biofilm samples for microbial analyses were taken from each biobead reactor (B#1, B#2, and B#3) by backwashing at a sufficient flow rate to get a thorough detachment of biofilms from the biobead. The operation period for this study was from April to October, 2004. For analysis of biofilm microbial communities, biofilms of May were used as the initial phase and those of September as the stabilized phase in this study. From our experiences, the early biofilms become settled in the biobeads about one month after the operation, and the biofilms of September are expected to be fully stabilized after vigorous treatment activity of microorganisms through summer. Total microbial community DNA of the detached biofilms was extracted by using the AccuPrep Genomic DNA Extraction Kit according to the manufacturer's instructions (Bioneer, Daejon, Korea). The quality of extracted DNA was examined by standard agarose gel electrophoresis and stored at −20°C.

MPN Test for Denitrifying Bacteria, Ammonia-Oxidizing Bacteria, and Nitrite-Oxidizing Bacteria

Detached biofilms of each biobead reactor's backwashing wastewater were diluted up to 10^{-8} by using 1.0 mM phosphate buffer. Diluted biofilm samples were inoculated into denitrifying bacteria (DNB) media, ammonia-oxidizing bacteria (AOB) media, and nitrite-oxidizing bacteria (NOB) media [3]. While they were incubated at 28°C for 4–12 weeks, their growth activity was checked every week by using diphenylamine reagent for DNB, and diazotizing reagent and coupling reagent for AOB and NOB (Hach Co., Loveland, U.S.A.). The number of these bacteria in wastewater was determined by an MPN table [38].

Construction of 16S rDNA Clone Library

PCR was carried out with the primers 27mf (5'-AGAGTT-TGATCMTGGCTCAG-3') and 1492r, which target the 16S rRNA genes of a wide range of members of the domain *Bacteria* at positions 28 through 1491 (*E. coli* 16S rRNA gene sequence numbering) [21]. PCR amplification of nearly full-length 16S rRNA genes was performed in 50-μl reaction mixtures as described previously [8]. PCR products were purified by using a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and cloned into the pGEM-T Easy Vector (Promega, Madison, U.S.A.). *E. coli* transformation was performed with ligated DNA according to the procedure of Sambrook *et al.* [33]. The preparation of randomly selected clones (43 clones, 45 clones, and 40 clones from the September biobead #1 biofilm, #2 biofilm, and #3 biofilm, respectively), followed by PCR amplification of cloned inserts, was carried out as described previously [15].

Sequencing of 16S rDNA Clones and Phylogenetic Analysis

Sequencing was performed with an ABI Prism BigDye Terminator Cycle Sequencing Ready Kit (Applied Biosystems, Foster City, U.S.A.) according to the manufacturer's instructions with the sequencing primer 519r [16]. Approximately 400 unambiguous nucleotide positions were used for comparison with the data in GenBank using the Basic Local Alignment Search Tool (BLAST) [1]. Sequences from nearest relatives were identified from BLAST and used as reference sequences for phylogenetic analysis. DNA sequences were aligned with ClustalX version 1.81 [35]. Clustering of the sequences was performed by the neighbor-joining algorithm with Jukes-Cantor distances by using the computer program PAUP version 4.10b [34]. A bootstrap analysis with 1,000 resamplings was made for all datasets to evaluate the tree topology derived from distance matrices [12]. The phylogenetic affiliation of a clone was determined according to the relationship with the reference sequences in the phylogenetic tree. The partial 16S rDNA sequences of 128 clones have been deposited in the GenBank nucleotide sequence databases under accession numbers EF061518 through EF061645.

Denaturing Gradient Gel Electrophoresis (DGGE) Analysis for 16S rDNA

PCR amplification of the 16S rRNA genes was performed with primers 1070f and 1392r (*E. coli* 16S rRNA gene sequence numbering) as described previously [13]. The PCR product contained a GC clamp of 40 bases, added to the reverse primer, and had a total length of 392 bp,

including the highly variable V9 region. PCR reaction mixtures were prepared as described previously [28]. PCR amplification and DGGE separation were performed as described previously [13, 18, 25]. After electrophoresis, the gels were stained with SYBR Green I, rinsed for 15 min, and photographed with UV transillumination (302 nm). During this study, repeated PCR and DGGE analyses produced nearly identical banding patterns.

DGGE Analysis for the *amoA* Gene

PCR amplification of the ammonia monooxygenase subunit A gene (*amoA*) was performed with primers amoA-1F (forward) and amoA-2R-TC (reverse), targeting a stretch corresponding to positions 332 to 349 and 802 to 822 of the open reading frame of *Nitrosomonas europaea*, respectively [27, 32]. This primer pair targets the *amoA* gene from ammonia-oxidizing bacteria (AOB) affiliated with β-*Proteobacteria*. PCR was performed as semi-nested PCR. An initial PCR amplification using primers without the GC-clamp was performed in 50-μl reaction mixtures containing 37.9 μl dH_2O, 5 μl 10× PCR buffer (200 mM Tris/HCl, 500 mM KCl, 25 mM $MgCl_2$, pH 8.4), 1 μl of template DNA, 0.3 μl each of primer (50 pmol/μl), 5 μl dNTP (1.25 mM of each dNTP) (Boehringer Mannheim, Indianapolis, U.S.A.), and 2.5U of *Taq* polymerase (GeneCraft GmbH, Ludinghausen, Germany). The PCR thermocycling regime was 95°C for 15 min, 92°C for 1 min, 35 cycles of 92°C for 30 s, 57°C for 30 s, 72°C for 45 s+1 s/cycle; the last cycle was a 5 min final extension [20]. The product was evaluated by agarose gel electrophoresis. Bands of the expected length from this PCR were cut out and used as templates for a second PCR that was run with 16–18 cycles in a 50-μl setup as described for the initial PCR, with changing into 1 μl primer and 5U *Taq* polymerase [27]. The latter PCR reaction was performed with GC-clamp primers for DGGE analysis. PCR samples were purified from agarose gel slices by using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Extracted samples were applied onto 8% (wt/vol) polyacrylamide gels with a denaturing gradient, ranging from 30% to 70%. PCR products were subjected to DGGE with the Dcode Universal Mutation Detection System (Bio-Rad, Hercules, U.S.A.) according to the procedure described previously [13]. The gels were electrophoresed for 17 h at 60°C and a constant voltage of 40 V. Subsequently, the gels were stained and photographed as described in the previous subsection.

Sequencing of DGGE Bands

For sequencing, selected bands were excised from DGGE gels by using a sterile scalpel and placed in a sterile Eppendorf tube containing 40 μl sterile water and then DNA was eluted through five cycles of freeze-thawing (−70°C/37°C). Two μl of the solution was used as template in PCR with non-GC clamp primers as described previously [23]. The amplified products were purified from agarose gel slices using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). The extracted products were cloned into the pGEM-T Easy Vector as recommended by the manufacturer (Promega, MA, U.S.A.). Two or three white colonies were randomly picked from each cloned sample for plasmid isolation, and the sequencing was done as described above, using 1070f or *amo*A1F primers for the sequence reactions.

RESULTS

Plant Performance

Table 1 summarizes the plant performance of the Aerated Up-Flow Biobead process from April to October, 2004.

Table 1. Chemical characteristics of the effluent of the aerated up-flow biobead process.

	Temp. (°C)	$TCOD_{Cr}$ (mg/l)	$SCOD_{Cr}$ (mg/l)	$TBOD_5$ (mg/l)	TSS (mg/l)	T-N (mg/l)	T-P (mg/l)
April	15.5	356.7/49.8[a] (86.1%)[b]	85.4/13.5 (84.2%)	269.0/59.9 (77.8%)	192.4/29.8 (84.6%)	47.0/19.8 (57.9%)	5.2/3.4 (34.7%)
May	18.3	212.3/33.2 (84.4%)	55.6/15.1 (72.9%)	126.7/23.9 (81.2%)	105.5/12.5 (88.2%)	36.7/20.5 (44.2%)	3.8/2.9 (23.7%)
Jun.	20.6	262.9/25.6 (90.3%)	95.9/14.6 (84.8%)	175.8/20.0 (88.7%)	123.6/8.3 (93.3%)	47.1/21.2 (55.0%)	4.7/3.1 (34.1%)
July	21.9	219.5/23.9 (89.2%)	71.7/11.6 (83.7%)	170.0/29.0 (83.0%)	149.3/10.1 (93.3%)	37.3/16.5 (55.8%)	3.9/2.2 (43.6%)
Aug.	25.3	206.5/36.2 (82.5%)	62.0/12.5 (79.9%)	34.5/7.0 (79.8%)	209.3/18.3 (91.3%)	37.7/20.1 (46.7%)	4.6/2.9 (37.0%)
Sep.	23.5	157.4/36.8 (76.7%)	47.5/19.8 (58.4%)	39.6/6.5 (83.6%)	117.3/20.0 (83.0%)	33.3/20.1 (39.7%)	4.4/2.9 (34.1%)
Oct.	20.8	123.6/39.5 (68.0%)	44.0/22.0 (50.0%)	35.7/6.0 (83.2%)	62.0/13.7 (78.0%)	27.4/19.7 (28.2%)	3.7/2.8 (24.4%)

[a]Influent/effluent (average values from 8–9 samplings)
[b]Removal rate

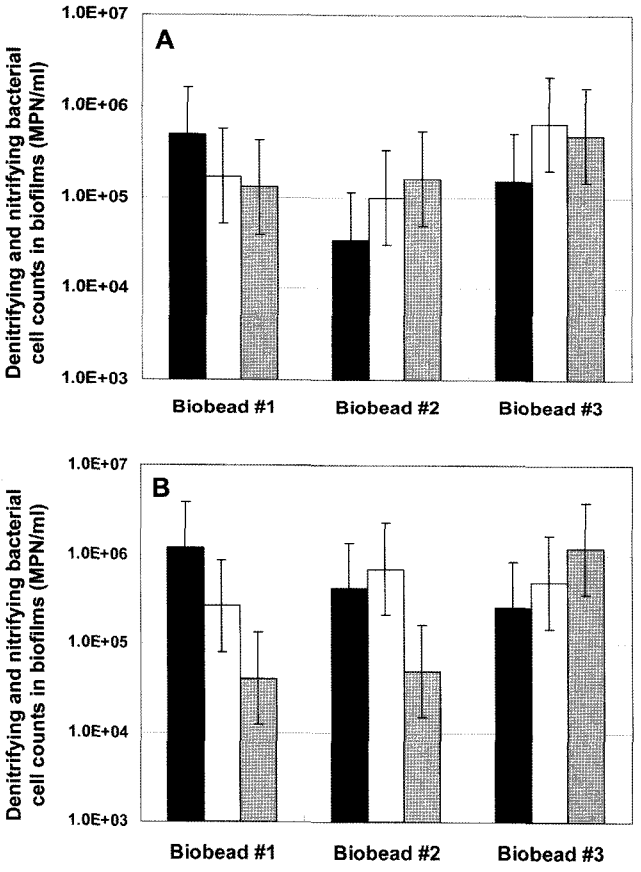

Fig. 2. Comparison of biofilm characteristics of each aerated up-flow biobead reactor using MPN in May (**A**) and September (**B**).
Symbols: ■, denitrifying bacteria (DNB); □, ammonia-oxidizing bacteria (AOB); ▨, nitrite-oxidizing bacteria (NOB). Bars represent the standard deviation from the mean of three replicate measurements.

During the operation period, average monthly influent flow rates fluctuated from 110.0% to 135.5% for the designed flow rate, 140 m^3/day. This variation was known to be due to army training activities. Daily values of COD, SS, and TN during the operation period were affected by the variation of influent flow rate. pH was between 6.3 and 7.0 and remained around 6.68 on average. The average dissolved oxygen (DO) values of the biobead #1 reactor, #2 reactor, and #3 reactor were 6.5 mg/l, 5.2 mg/l, and 3.9 mg/l, respectively, during the operation period. The temperature was from 12.9°C to 26.7°C, on average 18.9°C. The plant was placed in the basement, so the temperature remained below 30°C all the time, in spite of the highest atmospheric temperature (35°C) in midsummer. The alkalinity was between 100.0 mg/l and 240.0 mg/l, on average 165.1 mg/l. This range was adequate to buffer the decrease of pH resulting from the nitrification activity.

Although the plant treatment efficiency fluctuated more or less during the operation period, $TCOD_{Cr}$, $TBOD_5$, and TSS (except for April) were kept below the regulation limits ($TCOD_{Cr}$, 40.0 mg/l; $TBOD_5$, 30.0 mg/l; TSS, 20.0 mg/l) in the effluent. The values of TN and TP in the effluent were kept under 21.2 mg/l and 3.4 mg/l, respectively, which satisfied the regulation limits (TN, 60.0 mg/l; TP, 8.0 mg/l) of the effluent quality of the sewage treatment plant [24]. The initial average values of NH_4^+, NO_2^-, and PO_4^{-3} in the influent, 23.4 mg/l, 0.27 mg/l, and 3.32 mg/l, were decreased to 12.1 mg/l, 0.06 mg/l, and 2.67 mg/l, respectively, in the effluent. By contrast, the average value of NO_3^- in the influent, 0.98 mg/l, was increased to 6.82 mg/l in the effluent owing to microbial nitrification activities of the biobead reactors. The $SCOD_{Cr}$ values were also maintained at low levels throughout the operation period. In the anaerobic/anoxic reactor, approximately 20% of sewage organic materials were removed by microbial degradation and gravity precipitation. The remaining organic material of the sewage was mostly removed at the aerobic up-flow biobead reactors, which are the key components of this treatment system. Hereafter, this study focused on the distribution and diversity of microbial communities in the biofilms that played crucial roles in the removal of nutrients from sewage in the biobead reactors.

Microbial Population Estimation for DNB, AOB, and NOB

Fig. 2 shows the distribution of DNB, AOB, and NOB among the three aerated up-flow biobead reactors in May and September, which were contrasting as the initial phase and the stabilized phase in the treatment process, respectively. Among the three reactors, the biobead #1 reactor received the highest organic loading rate in the treatment system. In May, the population level of DNB, which are heterotrophs, was abundant in the biobead #1 reactor, whereas the populations of AOB and NOB, which are autotrophs, outnumbered DNB in the biobead #2 and #3 reactors (Fig. 2A). The biofilm community of September exhibited a clearer trend than that of May in the distribution of these DNB, AOB, and NOB between the biobead reactors, showing a high density of DNB near the input area and high levels of nitrifying bacteria near the output area (Fig. 2B). Since, in September, the operation condition was optimal and the microbial community in the biofilm was in the stabilized condition, the observed result of the three metabolic groups in the reactors could represent the spatial distribution of the bacterial community performing biological nitrogen removal in the biobead reactors.

Bacterial Diversity Analysis of Biofilm by 16S rDNA Cloning

To investigate the diversity and change of the bacterial community using a molecular method, 16S rDNA sequences were cloned from biofilm DNAs of September, which contained the stabilized microbial community. Phylogenetic analysis of the 16S rDNA sequences of 128 clones revealed

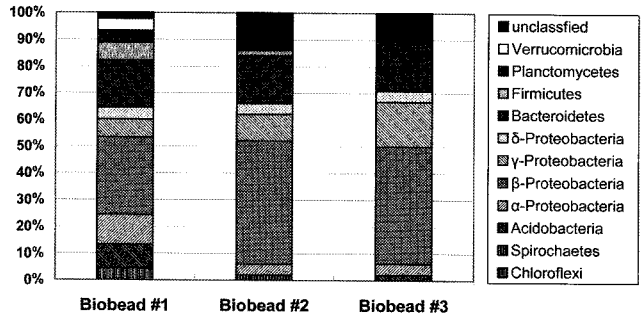

Fig. 3. Comparison of the biofilm microbial community structure of each aerated up-flow biobead reactor using 16S rDNA cloning in September.
Forty-three clones from the biobead #1 reactor, 45 clones from reactor #2, and 40 clones from reactor #3 were sequenced and compared.

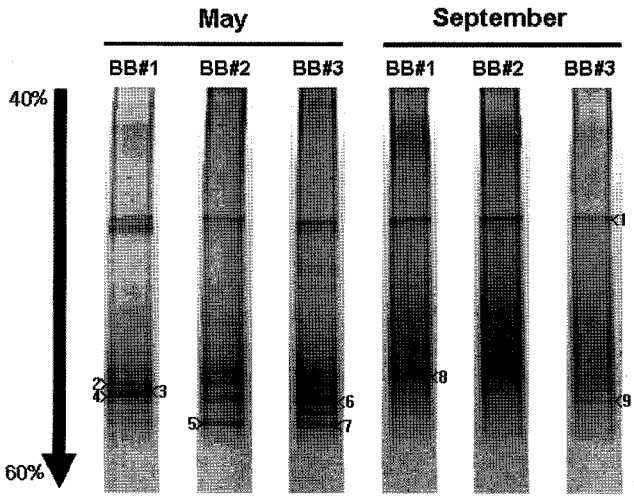

Fig. 4. DGGE of 16S rDNA amplicons obtained from biofilms of each biobead reactor.
BB#1–BB#3 represent biobead #1, #2, and #3 reactors, respectively. DNA bands marked with numbers were excised and sequenced.

that they were closely affiliated with a wide range of bacterial species of various divisions (Fig. 3). In particular, the biofilm community of biobead reactor #1 exhibited eleven phylogenetic groups of bacteria, showing the highest phylogenetic diversity among the three biobead reactors. This could be due to diverse organic materials being undegraded in the biobead #1 reactor compared with the biobead #2 and #3 reactors. The three reactors were commonly dominated by the β-*Proteobacteria* group, mainly composed of *Zoogloea* species (sequence similarities, 94–98%), which are typically found in wastewater [22]. The next most abundant group in the three reactors was the *Bacteroidetes* group, mainly composed of *Flavobacterium* (sequence similarities, 93–95%), *Cytophaga* (90–97%), and *Flexibacter* (96%) species. Overall, cloned sequences affiliated with β- and γ- *Proteobacteria* were gradually increased from the biobead #1 reactor to #3 reactor in concurrence with a decrease of the phylogenetic diversity in that order.

DGGE Analysis of Biofilm Community with PCR-Amplified 16S rDNA Fragments

Although some common bands were detected in the samples of May and September, each of the three biobead reactors exhibited different DGGE profiles in the respective May and September samples (Fig. 4). The DGGE banding patterns of the May sample, which was in the initial biofilm-forming step, were more complex than those of September, in which biofilms were stabilized. It appeared that the stabilized biofilms of September were dominated by some specific microbial populations, as shown by a few intense DGGE DNA bands in September. To investigate the phylogenetic identities of some intense DGGE DNA bands, these DNA bands were sliced from the DGGE gel, and then their DNA sequences were determined. The DNA band no. 1, which was commonly dominant in both May and September, was closely related to *Zoogloea ramigera*. DNA bands no. 2, 3, and 4 were identified as *Flavobacterium* species, and DNA bands no. 5, 6, and 7 were closely affiliated with *Xanthomonas*, *Plantomyces*, and a *δ-Proteobacteria* clone AKYG1852, respectively. DNA bands no. 8 and 9 were closely related to *Comamonadaceae* bacterium and *Sterolibacterium* species, respectively. From the previous clone analyses, 7 out of 128 clones were observed to be closely related to *Zoogloea* species at similarities of 94–98% and two to five clones were related to each of the other species, except for the *δ-Proteobacteria* clone AKYG1852 and *Sterolibacterium* species with which no clones were affiliated, at similarities of 92–98%. The phylogenetic distribution patterns of bacterial populations in the reactors were generally coincident with the observations from the above 16S rDNA cloning experiments.

DGGE Analysis of Biofilm Community with PCR-Amplified *amoA* Gene

To investigate the changes of ammonia-oxidizing bacterial community in the biofilms of the three biobead reactors, total microbial community DNAs extracted from biofilms were analyzed by DGGE after PCR amplification of the ammonia monooxygenase (*amoA*) gene. Each of the three reactors exhibited distinct DGGE profiles with the *amoA* gene in both May and September (Fig. 5). Although the DGGE DNA band patterns of May were getting extensive from the biobead #1 reactor to the #3 reactor, the band intensities were similar to one another among the three reactors. This might be because the microbial biofilms were not fully developed and specialized in the three biobead reactors of May. By contrast, in September, the DGGE DNA band patterns of the *amoA* gene were generally specified and the band intensities were substantially increased from the biobead #1 reactor to the #3 reactor. In

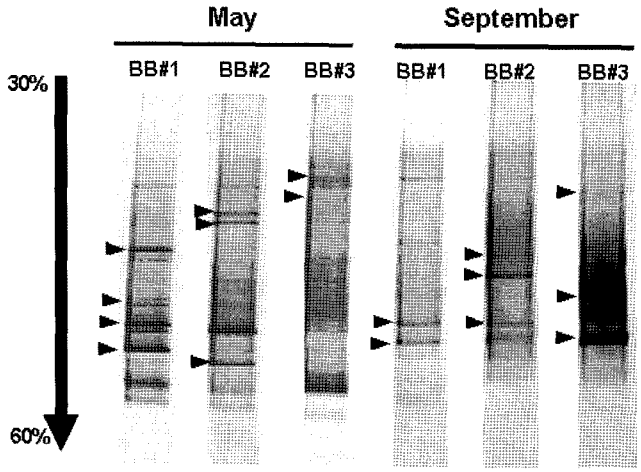

Fig. 5. DGGE of *amoA* gene amplicons obtained from biofilms of each biobead reactor.
BB#1–BB#3 represent biobead #1, #2, and #3 reactors, respectively. DNA bands marked with arrowheads were excised and sequenced.

addition, the #2 and #3 reactors showed more extensive DGGE band patterns than the #1 reactor. The results suggested that the biofilms of the #3 reactor (and possibly the #2 reactor) were fully developed and specialized to function as nitrification reactors in September. The abundance of ammonia-oxidizing bacteria in the biobead # 2 and #3 reactors observed with the *amoA* gene was coincident with the distribution pattern of nitrifying bacteria estimated by the MPN procedure. Some DNA bands (Fig. 5, indicated by arrowheads) were sliced from the DGGE gel, and then the sequences of the *amoA* genes were determined. The sequence data revealed that all the DNA bands examined were closely related to the ammonia monooxygenase A gene of *Nitrosomonas* species at similarities of 90–99% (data not shown), suggesting that the *Nitrosomonas* group predominated the biofilm community of ammonia-oxidizing bacteria in the biobead reactors.

DISCUSSION

In spite of the highly fluctuating conditions of the influent, the removal efficiencies of $TCOD_{Cr}$, $SCOD_{Cr}$, $TBOD_5$, and TSS, which were in the range of 68.0–90.3%, 50.0–84.8%, 77.8–88.7%, and 78.0–93.3%, respectively, were relatively stable during the operation period with the Aerated Up-Flow Biobead process. This achievement is partly due to the equalizing tank, which regulates influent flow rate, and the acclimated biofilms formed in the biobead reactors, which resist loading shock. The TN removal efficiency with our system was generally related to the carbon and nitrogen contents of the influent (Table 1). This is because organic matter is required for removal of nitrogen through microbial denitrification [19, 40]. One of the characteristics of our treatment system is that, compared with other systems [11, 30], it does not use additional carbon sources other than sewage organic carbons for denitrification to remove inorganic nitrogen. Through several recycling experiments, we reached the conclusion that the optimal recycle rate was 50% to the anaerobic reactor for efficient denitrification with the given army sewage.

In wastewater, diverse microorganisms, represented as heterotrophs and autotrophs, grow together in biofilm. Competition for oxygen and space and restriction of substrate transfer inevitably occur between heterotrophs and autotrophs. Thus, through various biotic and abiotic interactions, heterotrophs and autotrophs become dominant in specific habitats of the treatment process that confer the most optimal conditions for each group of microorganisms. In the Aerated Up-Flow Biobead process, according to the results of the MPN test for September, denitrifying bacteria (DNB) outnumbered nitrifying bacteria in the biobead #1 reactor and nitrifying bacteria prevailed over DNB in the biobead #3 reactor, whereas DNB, AOB, and NOB showed the tendency of coexistence in the biobead #2 reactor. The results of this study were congruent with the observations of Ohashi *et al.* [29], who reported that the heterotroph density in the biofilm was high near the input area where high organic loading rates were applied, and the autotroph density was high near the output area where the loading rates were relatively low. It appeared that the biobead #1 reactor was dominated by heterotrophs, the biobead #2 reactor was a transition zone, and the biobead #3 reactor was functionally specialized by the autotrophic nitrifying bacteria in our system.

From the 16S rDNA cloning experiments, the biofilms of the biobead #1 reactor were shown to contain various microorganisms belonging to diverse divisions, such as *Proteobacteria*, *Bacteroidetes*, *Chloroflexi*, *Verrucomicrobia*, *Firmicutes*, *Acidobacteria*, and *Planctomycetes* (Fig. 3). Since the biobead #1 reactor was located near the input area, it received a high organic loading rate, thus leading to proliferation of diverse microorganisms. In the biobead #1 reactor of September, 46.5% of the clones belonged to the *Proteobacteria*. By contrast, the proportions of the *Proteobacteria* in the biobead #2 and #3 reactors were substantially increased to 84.8% and 80.0%, respectively, in concurrence with decrease of phylogenetic diversity. This trend shows how the biofilm microorganisms respond and adapt to changing reactor conditions, eventually forming specialized and functional biofilms under the given conditions. The β-*Proteobacteria* group was observed to be dominant in all of the three biobead reactors. Many clones of the β-*Proteobacteria* group were closely affiliated with slime-forming bacteria, including *Zoogloea ramigera*. These bacteria form an extensive polysaccharide matrix, which is referred to as the glycocalyx [22]. Since the glycocalyx anchors microorganisms and help in the removal of complex

organic and inorganic materials from wastewater [5, 6, 36], these *Zoogloea* species are believed to play a crucial role in the formation of biofilms in the reactors.

In nitrification, ammonia and nitrite are oxidized aerobically by two respective groups of chemoautotrophic microorganisms, *Nitrosomonas* and *Nitrobacter* groups [14]. The product of this process is nitrate, which can be further metabolized to nitrogen gas through denitrification [19, 40]. In the Aerated Up-Flow Biobead process, denitrification mainly occurs in the anaerobic/anoxic tank, which receives recycled wastewater from the biobead #3 reactor, and nitrification occurs in the aerated biobead reactors. Since the biobead #1 reactor receives wastewater from the anaerobic/anoxic tank through the equalizing tank, the wastewater in the biobead #1 reactor contains relatively high amount of organic materials, allowing heterotrophs to outcompete autotrophs. As the organic materials decline through the consumption by heterotrophs, the autotrophs, such as nitrifying bacteria, gradually become prevalent in the subsequent biobead reactors. The increase of nitrifying bacterial population in the biobead #2 and #3 reactors was shown by the comparatively intense and complex DGGE banding patterns with the *amoA* gene of ammonia-oxidizing bacteria in September (Fig. 5). The gradual increase of nitrifying bacterial population from the biobead #1 to #3 reactors was consistent with the MPN result for DNB, AOB, and NOB. The specialization of the three reactors into a heterotrophic zone, a transition zone, and an autotrophic zone would be one of the main characteristics of the Aerated Up-Flow Biobead process. The high efficiency of wastewater treatment and the microbiological information from this work could be useful for the application of the Aerated Up-Flow Biobead process to the existing treatment plants to improve wastewater treatment efficiency.

Acknowledgments

This study was supported by a grant from Biontech Co. and by the RDA Genebank Management Program from the Genetic Resources Division, National Institute of Agricultural Biotechnology, and by the BioGreen 21 Project.

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