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Microbial Structure and Community of RBC Biofilm Removing Nitrate and Phosphorus from Domestic Wastewater

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Using a rotating biological contactor modified with a sequencing bath reactor system (SBRBC) designed and operated to remove phosphate and nitrogen [58], the microbial community structure of the biofilm from the SBRBC system was characterized based on the extracellular polymeric substance (EPS) constituents, electron microscopy, and molecular techniques. Protein and carbohydrate were identified as the major EPS constituents at three different biofilm thicknesses, where the amount of EPS and bacterial cell number were highest in the initial thickness of 0–100 μm. However, the percent of carbohydrate in the total amount of EPS decreased by about 11.23%, whereas the percent of protein increased by about 11.15% as the biofilm grew. Thus, an abundant quantity of EPS and cell mass, as well as a specific quality of EPS were apparently needed to attach to the substratum in the first step of the biofilm growth. A FISH analysis revealed that the dominant phylogenetic group was β- and γ-*Proteobacteria*, where a significant subclass of *Proteobacteria* for removing phosphate and/or nitrate was found within a biofilm thickness of 0–250 μm. In addition, 16S rDNA clone libraries revealed that *Klebsiella* sp. and *Citrobacter* sp. were most dominant within the initial biofilm thickness of 0–250 μm, whereas sulfur-oxidizing bacteria, such as *Beggiatoa* sp. and *Thiothrix* sp., were detected in a biofilm thickness over 250 μm. The results of the bacterial community structure analysis using molecular techniques agreed with the results of the morphological structure based on scanning electron microscopy. Therefore, the overall results indicated that coliform bacteria participated in the nitrate and phosphorus removal when using the SBRBC system. Moreover, the structure of the biofilm was also found to be related to the EPS constituents, as well as the nitrogen and phosphate removal efficiency. Consequently, since this is the first identification of the bacterial community and structure of the biofilm from an RBC simultaneously removing nitrogen and phosphate from domestic wastewater, and it is hoped that the present results may provide a foundation for understanding nitrate and phosphate removal by an RBC system.

Keywords: Biofilm, rotating biological contactor, FISH, 16S rDNA, bacterial community

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A rotating biological contactor (RBC) is used to treat sewage in areas with a population of up to 5,000 [13, 54]. RBCs were first introduced in the early part of the century, and today there are many thousands of units operating worldwide. An RBC consists of a series of segmented corrugated polymer discs on which a biofilm is formed, plus RBC units can be designed for both organic carbon removal and nitrification. Several reports of oxygen and organic carbon-limited autotrophic nitrification-denitrification by lab-scale RBC systems have already been published [10, 42, 52], where the RBC biofilm has been found to be dominated by anaerobic ammonia-oxidizing bacteria (members of the genus *Nitrosomonas* and close relatives of *Kuenenia stuttgartiensis*) and anammox bacteria (members of the order *Planctomycetales*) based on FISH, RT-PCR, and DGGE methods. In these processes, NH_4^+ is directly converted to N_2 by nitrification and denitrification.

To increase efficiency, the existing RBC system has also already been redesigned and retrofitted to a sequencing bath reactor system (SBRBC) [58], which allows wastewater to pass through the basin as the disks slowly rotate at approximately 1 rev/min, thereby exposing the biological growth (biomass) alternately to the wastewater and surrounding air [58]. The SBRBC reactor has a 3-unit RBC configuration, and each RBC unit is operated in the sequencing batch reactor (SBR) mode. Although a thinner biofilm has been found to promote phosphorus removal, a thicker biofilm

enhances denitrification and reduces phosphorus removal [58]. Therefore, this study analyzes the microbial community structure and mechanisms of the SBRBC biofilm formed on the 3rd unit, which has been found to remove phosphate and nitrate simultaneously. Additionally, a comparison is made between the general biofilm from autotrophic nitrogen removal reactors and the SBRBC biofilm. To obtain a global view of the bacterial diversity of the SBRBC biofilm [58], the microbial community structures are investigated using molecular techniques, such as restriction fragment length polymorphism (RFLP) analysis of the 16S rDNA gene, fluorescence *in situ* hybridization (FISH), and electron microscopy. The resulting phylogenetic information is then used to infer the major metabolic abilities of the nitrate- and phosphate-removing bacteria to understand the potential role of these organisms in the biofilm.

Materials and Methods

Experimental Set-Up

The SBRBC reactor had a 3-stage RBC configuration with acrylic circular plates, and each RBC unit was operated in the SBR mode [58]. The effective volume of each reactor was 6 l, and the disk thickness was 1 mm, disk diameter 17 cm, shaft diameter 10 cm, and disk spacing 1 cm. The rotational speed was 3–7 rpm, the peripheral velocity 51 cm/min, and the exposed area of each reactor 0.59 m^3. The operation conditions for the SBRBC are given in Table 1, and Fig. 1 shows a schematic diagram of the SBRBC processes. First, 6 l of influent was fed into the 1st and 2nd units kept under anaerobic conditions to allow for the release of phosphate for 2 h. Thereafter, an aerobic zone was included to extend the solid retention time (SRT) of the nitrifier and avoid conflict between the nitrification and the phosphate removal (Table 1 and Fig. 1). Meanwhile, the 3rd unit was operated to remove phosphate, along with the nitrate and nitrite produced by the 1st and 2nd units as a result of the nitrification under oxic conditions. Following the anaerobic and aerobic steps, the supernatant from the oxic 1st and 2nd units was repeatedly mixed with the supernatant from the anoxic 3rd unit during anoxic steps.

Table 1. Influent characteristics and operating conditions of the SBRBC.

Parameter	SBRBC
TCOD	128–355 mg/l
SCOD	44–166 mg/l
TKN	21–53 mg/l
TP	5.6–20.2 mg/l
SP	4.9–16 mg/l
COD/TN	6–7
COD/TP	11–44
NH_4-N	17–45 mg/l
NO_3-N	0–0.2 mg/l
Sulfate (SO_4^{2-})	1–3 mg/l
Sulfide (S^{2-})	5–11 mg/l
SS and VSS	47–102/45–97 mg/l
VSS as HAc	27–82 mg/l
Alkalinity as $CaCO_3$	98–203 mg/l
pH	7.0
Temp.	18–20°C
Total HRT (h)	11.5
An/Ax/Ox (h)	2/3.5/6
Organic loading rate (g COD/m^2/d)	6.9
Hydraulic loading rate (l/m^2/d)	30.6
Submerged ratio	An (100%), Ox (54%), Ax (100%)
Type	Partially and fully submerged type

TCOD, total chemical oxygen demand; SCOD, soluble chemical oxygen demand; SP, soluble phosphate; VSS, volatile suspended solid; SS, suspended solid; TN, total nitrogen; TP, total phosphate; TKN, total Kjeldahl nitrogen; HAc, acetic acid; HRT, hydraulic retention time; VFA, volatile fatty acid.

Wastewater Quality Parameters

A total chemical oxygen demand (soluble COD) concentration range of 128 to 355 (44–166) mg/l was used throughout the experiments. The other characteristics of the influent wastewater are shown in Table 1. All the analytical procedures followed Standard Methods [3], except for the extracellular polymeric substance (EPS) measurements, where the EPS contents in the biofilm samples were determined using the method reported by Zhang *et al.* [60]. The nucleic acid content was determined to check for contamination by intracellular polymers, the carbohydrate concentration measured according to the phenol-sulfuric acid method [12], and the protein content determined using the method suggested by Lowry *et al.* [24].

Biofilm Slices and Scanning Electron Microscopy

Biofilm samples (Fig. 2) were taken from the 3rd unit of the SBRBC [58] in which wastewater was periodically loaded. The feed wastewater was obtained from the Joograng sewage treatment plant in Korea. The biofilm, including the support material, was removed from the outer layer of the SBRBC, and care was taken to maintain the structure of the biofilm. After fixation with paraformaldehyde, various parts of the biofilm were embedded in an O.C.T. embedding compound on a cryostat chuck for low and medium temperatures (Microm. Labor AG, Walldorf, Germany) and frozen at −18°C. Small pieces were then serially selected and cut into slices with a thickness of approximately 10 mm using a Microm HM 505 E microtome (Carl Zeiss AG, Oberkochen, Germany), while being maintained at −18°C. This step was performed to provide samples from three different biofilm thicknesses (0–100 μm, 100–250 μm, and over 250 μm). The biofilms were then fixed with 2.5% glutaraldehyde, postfixed with 2% osmium tetroxide, and dehydrated using acetone. After critical-point drying, the samples were sputter-coated with gold and observed using a model JSM-840A scanning electron microscope (Jeol, Ltd., Tokyo, Japan).

Oligonucleotide Probes and FISH

The 16S rRNA-targeted oligonucleotide probes and hybridization conditions used in this study are shown in Table 3. The probes were labeled with tetramethylrhodamine 5-isothiocyanate (TRITC). The FISH and dehydration and were performed using the procedure described by Amann *et al.* [2]. Staining with 4',6'-diamidino-2-phenylindole (DAPI) (1 μg/ml) was used to determine the total number of cells for 10 min in the dark [20]. The slides were then rinsed briefly with

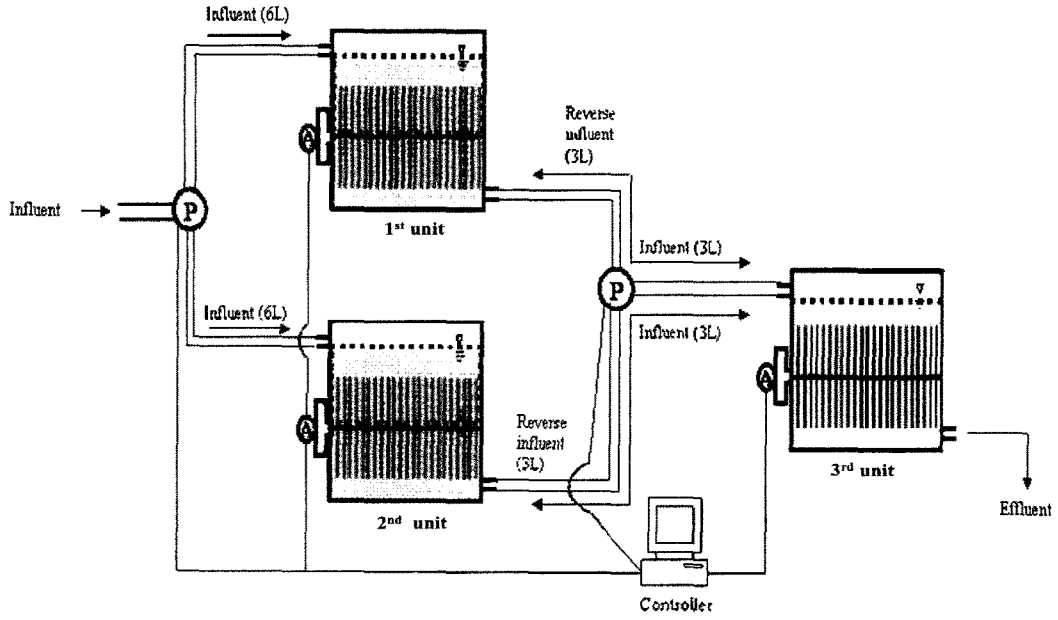

Fig. 1. Schematic diagram of the SBRBC process.
A, rotating axis; P, pump.

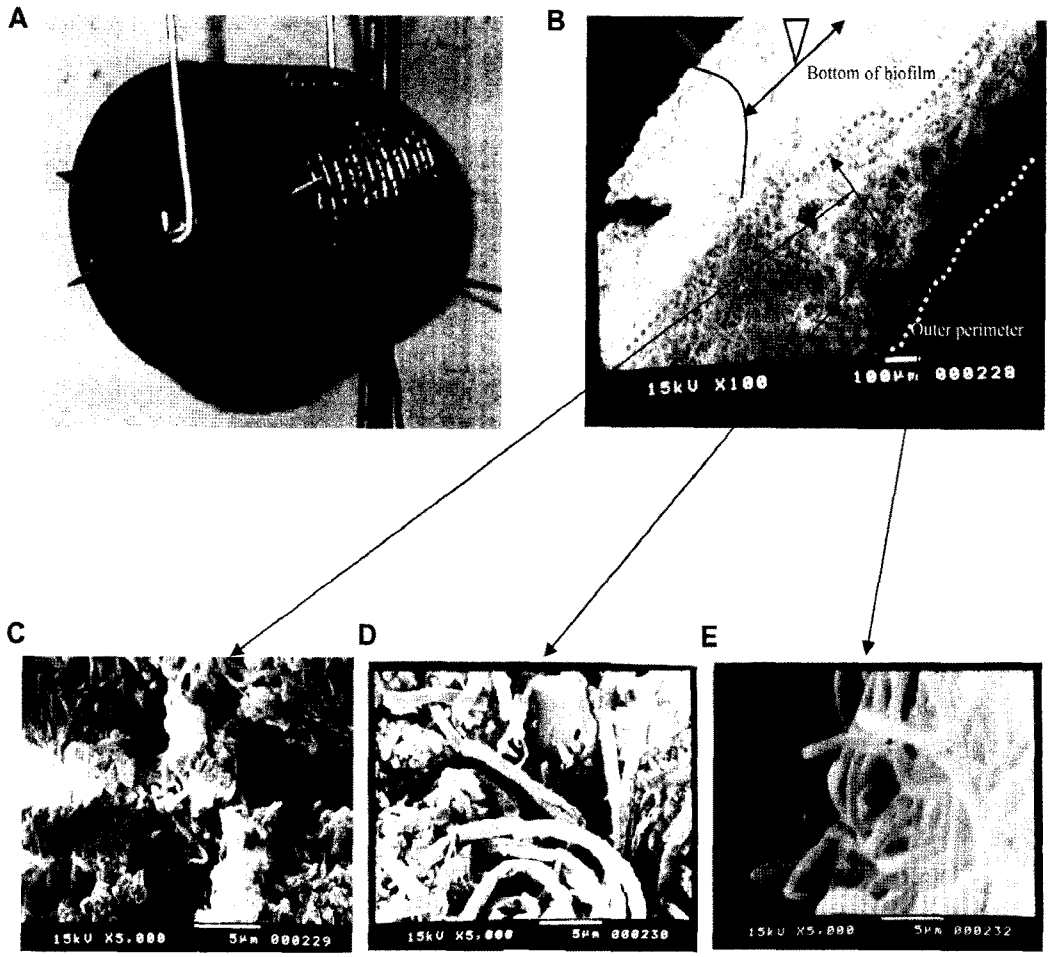

Fig. 2. Electron micrographs showing bacterial structures of the SBRBC biofilm.
A. View of biofilm disk. **B.** Bacterial structure of whole biofilm attached to matrix. **C.** Part A, bacterial structure for biofilm thickness of 0–100 μm. **D.** Part B, bacterial structure for biofilm thickness of 100–250 μm. **E.** Part C, bacterial structure for biofilm thickness of over 250 μm. ⇔, solid (disc)-biofilm interface; dotted line, biofilm-water interface; double arrow, thickness of biofilm; single arrow, each part of biofilm.

highly distilled H_2O, allowed to air dry, and mounted in an anti-fading solution. The hybridized and DAPI-stained samples were examined under an epifluorescence microscope using an Axiophot 2 microscope (Zeiss, Thornwood, NY, U.S.A) with filter sets 01 (DAPI staining) and 15 (TRITC-labeled probe). To count each probe, each part of the biofilm was sonicated (40 to 50 Hz, 117 V, 1.0 A; Branson Ultrasonics, Danbury, CT, U.S.A.) for 30 s in 10 ml of distilled water to separate the bacterial cells and flocs. About 1,000 cells stained with DAPI were enumerated. Two replicate samples were counted for each hybridization [21].

DNA Extraction

Genomic DNA extraction from the various biofilm samples (0.5 g [wet weight]) was performed using a modification of the method of Lee *et al.* [23], where the modified step involved extraction of the lysate with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and chloroform-isoamyl alcohol (24:1). The DNA concentrations were measured based on the absorbance at 260 nm.

DNA Preparation, Sequencing, and Analysis of 16S rDNA Sequencing Data

The amplification of the 16S rDNA from the chromosomal DNA was carried out in a DNA thermal cycler model 480 (Perkin-Elmer, Norwalk, CT, U.S.A.) using universal bacterial primers, 27F and 1492R [5, 18]. The PCR amplification program consisted of an initial 5-min denaturation step at 94°C, followed by 30 cycles at 94°C for 1 min, 57°C for 1 min, and 72°C for 1 min, plus a final 10 min extension step at 72°C. The PCR products were purified using a QIAquick PCR purification kit (Qiagen, Germany) [39] and cloned into a pGEM-T Easy vector system I according to the manufacturer's instructions (Promega, Madison, WI, U.S.A.) [17]. The clones containing the appropriate-sized inserts were identified by agarose gel electrophoresis of the PCR products obtained from the host lysates using primers complementary to the vector's flanking insertion sites. Approximately 100 ng of the purified PCR product from the re-amplified insertion site from each clone was digested overnight at 36°C with 5 U of RSAI and HaeIII (Promega, Madison, WI, U.S.A.). Unique clones were grouped using an RFLP analysis for operational taxonomic units (OUT).

The nucleotide sequences of the cloned products were determined from plasmid DNA preparations using an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer) and ABI310 Sequencer (Applied Biosystems, Foster City, CA, U.S.A.) according to the manufacturer's instructions. The vector primers T7 and SP6 were used for the sequencing reactions [21]. All the sequences were assembled using the BioEdit sequence alignment editor freeware (version 7.0.8; http://www.mbio.ncsu.edu/BioEdit/bioedit.html) [15]. The assembled sequences were then examined for chimeric artifacts using CHIMERA_CHECK from the Ribosomal Database Project II (RDP) [26], and potentially chimeric sequences were not considered further. The nonchimeric sequences were aligned to 16S rDNA sequences from the GenBank and RDP databases using the ClustalW alignment tool in BioEdit. A distance matrix and neighbor-joining tree were constructed using 1,000 bootstrap replicates and the PHYLIP v. 3.6 software package (J. Felsenstein, University of Washington, Seattle, 2005). Phylogenetic trees were also drawn from the neighbor output using the TreeView program. The robustness of the inferred tree topologies was evaluated after 1,000 bootstrap resamplings of the neighbor-joining data, and only values >70% are shown on the trees.

Nucleotide Sequence Accession Numbers

The 16S rDNA partial sequences obtained in this study are available from the GenBank nucleotide sequence database under accession numbers AY444157 to AY444165.

Results and Discussion

Performance and Nutrient-Removal Characteristics of SBRBC

As described above, the SBRBC was designed and operated to remove phosphate and nitrogen from domestic wastewater [58]. Nitrate and nitrite were generated from the 1st and 2nd units by nitrification under aerobic conditions (Fig. 1). Nitrate and phosphate were then removed for 6 h under anoxic conditions in the 3rd unit. COD was removed in all the steps. The COD removal efficiency of the SBRBC was 85% of the total COD. The NH_4-N (17–45 mg/l) in the domestic wastewater was oxidized to NO_3-N by nitrification during the oxic stage in the 1st and 2nd units (90%), and then the generated NO_3-N was removed by denitrification under the anoxic conditions of the 3rd unit. The specific denitrification rate (SDNR) measured from the 3rd unit was 2.44–2.41 mg NO_3N/gMLVSS/h, the estimated SRTs of the main units were about 19–23 days, and the T-N removal efficiency was 55% in the SBRBC during these steps [58].

In general, RBC systems are intended to reduce nutrients, such as ammonia and COD, by nitrification and denitrification processes [10, 42, 52] or remove coliform bacteria, such as *Escherichia*, *Klebsiella*, *Citrobacter*, and *Enterobacter* spp. [10, 14, 53, 57]. However, the system reported in this paper was designed to remove phosphate, as well as nitrate, under anoxic conditions in the 3rd unit. Although the T-N and T-P removal efficiencies were not higher than those previously reported for SBR or EBPR systems [4, 21, 22, 32], the results indicated that the present system simultaneously removed COD, phosphate, and nitrogen during 1 cycle [58]. A previous study [6, 58] also clearly reported that nitrogen and phosphate removal was possible by an RBC system with a modified SRC system.

Relationship Between Biofilm Growth and Nutrient-Removal Characteristics

The biofilm formed on the disk was spontaneously sloughed after its thickness reached 500 μm. When the biofilm was spontaneously removed, the thickness was reduced by 400 μm, leaving an average thickness of approximately 100 μm on the disk. After 17 days, the biofilm grew to a thickness of approximately 200 μm on the disk of the 3rd unit, making the biofilm growth rate 0.103 mm/day [58]. Although the relationship between biofilm growth and T-P

Table 2. Composition of EPS and cell counts in biofilm samples from the SBRBC system.

	Biofilm		
	Part A	Part B	Part C
Carbohydrate[a]	121.6±5.4 (71%)	74.5±4.6 (66%)	64.2±5.7 (59.7%)
Protein[a]	49.6±6.3 (28.9%)	37.6±6.6 (33%)	43.1±5.5 (40.1%)
Nucleic acid[a]	0.06±0.02 (0.03%)	0.07±0.02 (0.06%)	0.14±0.02 (0.13%)
Total[b]	171.3	112.2	107.4
Cell count[c]	2.35×10^{12}	9.23×10^{10}	3.93×10^{10}

[a]mg/g.
[b]Sum of proteins, carbohydrates, and nucleic acid.
[c]Cell counts/g using DAPI stain. The numbers in parentheses denote the percentage of carbohydrates, proteins, and nucleic acid in the total EPS content. Part A, thickness of 0–100 μm; part B, thickness of 100–250 μm; part C, thickness over 250 μm. Values represent the average from triplicate experiments.

removal efficiency has already been reported in previous studies [58], in the present study, the T-P removal efficiency decreased with the growth of the biofilm in the 3rd unit. At a thickness of approximately 150 μm, the T-P removal efficiency ranged from 70% to 80%, whereas the T-P removal efficiency was approximately 40% at a thickness of 250 μm. Phosphate was not removed with a biofilm thickness of over 300 μm (data not shown). Therefore, these results indicated that maintaining the biofilm at a constant thickness (approximately 300 μm) was necessary to increase the T-P removal efficiency in this process. The best COD/phosphate ratios were 11–15 and 13–18 with a 250 μm biofilm thickness [58], which also suggested that the thickness of the biofilm inhibited the T-P removal efficiency. Consequently, it can be inferred from a previous report [58] that very different bacterial groups inhabit a thickness of over 250 μm in comparison with the bacterial groups inhabiting a thinner biofilm layer (a biofilm thickness of approximately 250 μm and below). Moreover, the bacterial types in the 250-μm-thick biofilm were not apparently related to phosphate-removal bacteria.

Extracellular Polymeric Substances (EPS) of Biofilm Structure

The biofilm from the SBRBC was scrutinized based on dividing it into three parts for detailed observation (part A, from 0 to 100 μm; part B; 100–250 μm; part C, 250–500 μm), and the EPS extracted from each part. The total EPS content and composition in each part are shown in Table 2, where the total EPS content in parts B and C were 1.52- and 1.59-fold lower, respectively, than the total EPS content in part A. Protein and carbohydrate were also the major EPS constituents. Notably, carbohydrate was the dominant organic compound, representing about 71%, 66%, and 59.7% of the total amount of polymeric material in parts A, B, and C, respectively. The carbohydrate to protein (C/P) ratio decreased from 2.5 in part A to 1.28 in part C, and DNA only accounted for 0.04–0.14% of the EPS extracted from all parts of the biofilm.

In part A of the biofilm, the amount of EPS and total bacterial cell number were higher than those measured in the other two parts (Table 2). Thus, it appeared as if an abundant quantity of EPS and cell mass were needed to attach to the substratum in the first step of the biofilm growth.

Table 3. 16S and 23S rRNA-targeted oligonucleotide probes.

Probes	Sequence (5' to 3')	Specificity	Reference
EUB338	GCTGCCTCCCGTAGGAGT	Most bacteria	[2]
ALF968	GGTAAGGTTCTGCGCGTT	α-*Proteobacteria*, except *Rickettsiales*	[34]
BET42a[a]	GCCTTCCCACTTCGTTT	β-*Proteobacteria*	[28]
GAM42a[b]	GCCTTCCCACATCGTTT	γ-*Proteobacteria*	[28]
CF319a/b	TGGTCCGTRTCTCAGTAC	CF cluster of the *Bacteroidetes*	[27]
Amx820	AAAACCCCTCTACTTAGTA	Anammox-specific	[48]
NSV443	CCGTGACCGTTTCGTTCCG	*Nitrosospira* spp.	[33]
NSR1156	CCCGTTCTCCTGGGCAGT	*Nitrospira* spp.	[49]
NSO190	CGATCCCCTGCTTTTCTCC	Ammonia-oxidizing β-*Proteobacteria*	[33]
HGC69a	TATAGTTACCACCGCCGT	Actinobacteria	[46]
Rc988	AGGATTCCTGACATGTCAAGGG	*Rhodocyclus* group	[7]
PAO846	GTTAGCTACGGCACTAAAAGG	PAO cluster	[7]

[a]The unlabeled probe GAM42a was used as a competitor to enhance the specificity.
[b]The unlabeled probe BET42a was used as a competitor to enhance the specificity.

For years, carbohydrate and protein have been considered the main constituents of EPS in pure cultures and biofilm samples [30, 50, 51, 56]. Recent studies of mixed cultures in wastewater treatment systems have found that protein is an important constituent in EPS, possibly due to the large quantities of exoenzymes entrapped in the EPS [9]. Yet, this study found that the carbohydrate content was the major component in all three parts, which may have been due to the conversion of the substrate into organic acids by fermenting organisms that gain their carbon and energy from various substrates [8]. Nonetheless, the carbohydrate content in the total EPS decreased by about 11.23%, whereas the protein content increased by about 11.15% as the biofilm grew (Table 2), suggesting that the quantity of EPS determined the special role played by the polymeric material that formed the "glue" holding the floc together, as the SBRBC biofilm was spontaneously sloughed off at a thickness of approximately 100 μm (part A), which contained a higher quantity of EPS than parts B and C. This result was expected, as when EPS covers a cell surface, it alters the physicochemical characteristics of the surface, including the charge, hydrophobicity, and polymeric property [8, 31, 35, 44].

EPS is an important bonding agent of the biofilm structure [8, 31, 35], and since different bacterial species are involved within the spatial biofilm structure, the role of EPS may require further biochemical characterization in biofilm formation. For instance, fewer or no filamentous organisms were observed in part A of the biofilm, indicating that filamentous organisms could not compete with rod- and coccoid-shaped organisms, although a high volume-to-surface ratio of filamentous organisms can endure a low substrate and dissolved oxygen environment. Thus, because of the diverse microbial species involved in the biofilm formation, the strong extraction method used for the EPS measurement may have disrupted the intracellular polymeric substances (IPS) for certain microbial species. Therefore, as indicated in a recent study by Yun *et al.* [59] and earlier studies [11, 37], EPS measurements require a careful check procedure for such contamination.

Preliminary FISH Analysis of Biofilm for Bacterial Composition

As mentioned earlier, the bacteria were quantified by FISH using *Proteobacteria*-specific probes. Up to 72.6%±5.8% (part A), 73.2%±6.2% (part B), and 64.5%±7.2% (part C) of the total DAPI cell counts were hybridized to the eubacterial probe EUB338. The percentages of the subclass probe ALF968, BET42a, GAM42a, CF319a, and HGC 94a counts are given in Fig. 3. The percentage of GAM42a was up to 47.1% for all the sample thicknesses. Remarkably, the percentage of GAM42a was 60.7% of the EUB probe for the samples representing a thickness of over 250 μm (Part C). The unknown groups in the parts A, B, and C

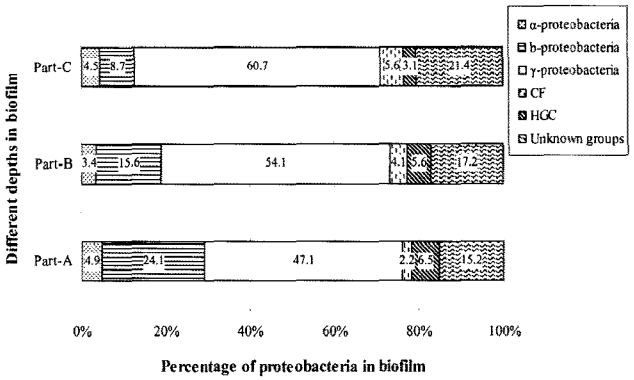

Fig. 3. Microbial community composition of different SBRBC biofilm thicknesses quantitatively determined by FISH.
Part A, thickness of 0–100 μm; part B, thickness of 100–250 μm; part C, thickness over 250 μm. The values are the average of duplicate samples.

samples accounted for 15.2%, 17.2%, and 21.4% of the EUB probe, respectively. The quantity of the unknown groups was obtained by calculating the difference between the total quantity for all the groups and the total quantity for the known groups. Therefore, the results from this system showed that the percentage of GAM42a increased with the growth of the biofilm, whereas the percentage of BET42a decreased with the growth of the biofilm (Fig. 3). The FISH analysis revealed that the γ- and β-*Proteobacteria* groups were the dominant bacteria groups in the part A samples (thickness of 0–100 μm), whereas the γ-*Proteobacteria* group was an important subclass of *Proteobacteria* in the biofilm for the part C samples (thickness over 250 μm).

To quantify the polyphosphate-accumulating and nitrogen-removing bacteria genera and species in the biofilm, the following probes were used. A *Rhodocyclus* relative probe and PAO846 probe [7] were used to assess the proportion of polyphosphate-accumulating organisms (PAOs). These fluorescently labeled probes were not counted in the various parts of the biofilm. Meanwhile, an NSO190 probe [33], NSV443 probe [33], and NSR1156-probe [49] were used to quantify the ammonia-oxidizing bacteria and nitrite-oxidizing bacteria in the biofilm. These probes were not investigated in the biofilm samples. Finally, an Amx820 probe was used to quantify the anaerobic ammonium-oxidizing (anammox) bacteria [48, 52]. The FISH method using the anammox-specific probe confirmed the absence of the anammox *Planctomycetales* from any of the biofilm samples exposed to the surrounding environment and anoxic wastewater during the operation. As a result, it was found that neither polyphosphate-accumulating bacteria, such as *Rhodocyclus* relatives and *Actinobacteria*, nor nitrogen-removing bacteria, such as the anammox *Planctomycetales*, *Nitrosospira* spp., *Nitrospira* spp., and ammonia-oxidizing β-*Proteobacteria*, were related to the nitrogen and phosphate removal in this system.

Microbial Community and Structure of Biofilm

The typical microbial structure of the SBRBC biofilm is shown in Fig. 2. The morphology of this biofilm was observed according to the thickness. As shown in Fig. 2C, rod- and coccoid-shaped bacteria coexisted in part A of the biofilm, part B was colonized by complex bacterial shapes, such as rod-, coccoid-shaped, and filamentous organisms, that formed a dense mat structure (Fig. 2D), and part C revealed no rod- or coccoid-shaped bacteria, as filamentous organisms were the dominant morphology on the surface of the biofilm (Fig. 2E).

For a full characterization of the bacterial community and composition of the three parts of the biofilm, 16S rDNA amplified from the total DNA extracted from each of the three parts of the biofilm was cloned and analyzed using an RFLP analysis. In part A of the biofilm (Fig. 2C), a total of 46 clones were grouped by the RFLP analysis, where three operational taxonomic units (OTUs) were identified that contained at least three clones (SBRBC1, 2, and 3). SBRBC1 (47.8% of the total clones) and SBRBC 2 (37% of total clones) were the dominant clones, where SBRBC1 and SBRBC2 were most closely matched to *Citrobacter* sp. (EF491832) and *Enterobacter* sp. (AB098582), respectively. SBRBC 3 was most closely matched to β-*Proteobacteria* (Z93987) previously detected in biological wastewater treatment reactors designed for bioregenerative life support [47]. In part B of the biofilm (Fig. 2D), a total of 54 clones were classified by RFLP, resulting in the selection and partial sequencing of three dominant groups (SBRBC4, 5, and 6) (Table 4). The closest relative of SBRBC4 (24.1% of the total clones) was *Thiothrix fructosivorans* (L79962), the closest relative of SBRBC5 (33.3% of the total clones) was *Citrobacter* sp. (EF422184), and the closest relative of SBRBC6 (31.5% of the total clones) was *Klebsiella* sp. (U31076). Finally, in part C of the biofilm, a total of 57 clones were analyzed by an RFLP analysis, and the closest relative of SBRBC7 (33.3% of the total clones) was *Citrobacter* sp. (EF491832), the closest

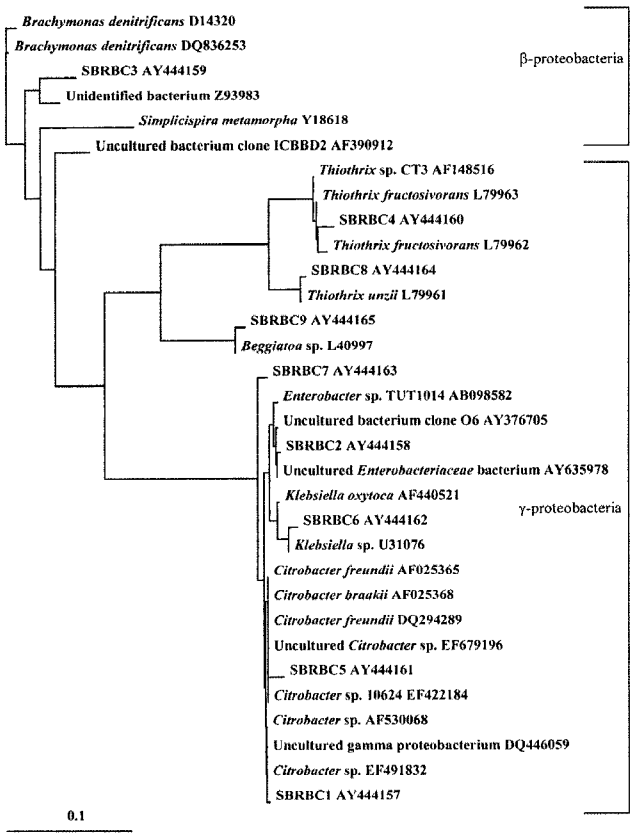

Fig. 4. Phylogenetic tree representing taxonomic positions of members of beta- and gamma-proteobacterial OTUs isolated from biofilms.
The phylogenetic tree was constructed using a neighbor-joining analysis with 1,000 bootstrap replicates. The scale bar indicates 0.1 nucleotide substitutions per site.

Table 4. Phyla from domain bacteria represented in the SBRBC clone library of biofilm determined by BLAST comparisons of partial clone insert sequences.

Clones of SBRBC	16S rRNA gene sequencing			Subclass of *Proteobacteria*
	Closest match (Accession number)	No. of nucleotides compared	% Similarity with closest match	
SBRBC1 (22/46)[a]	*Citrobacter* sp. (EF491832)	400	98	γ
SBRBC2 (17/46)	*Enterobacter* sp. (AB098582)	400	98	γ
SBRBC3 (5/46)	Unidentified bacterium (Z93987)	455	98	β
SBRBC4 (13/54)	*Thiothrix fructosivorans* (L79962)	400	98	γ
SBRBC5 (18/54)	*Citrobacter* sp. (EF422184)	426	98	γ
SBRBC6 (17/54)	*Klebsiella* sp. (U31076)	400	98	γ
SBRBC7 (19/57)	*Citrobacter* sp. (EF491832)	400	99	γ
SBRBC8 (16/57)	*Thiothrix unzii* (L79961)	426	99	γ
SBRBC9 (15/57)	*Beggiatoa* sp. (L40997)	403	97	γ

[a]No. of clone inserted 16S rDNA/ total clones.
[b]SBRBC1, SBRBC2, and SBRBC3 were dominant groups in initial biofilm thickness of 0–100 μm (part A), SBRBC4, 5, and 6 were the dominant groups in the biofilm thickness of 100–250 μm (part B), and SBRBC7, 8, and 9 were the dominant groups in the biofilm thickness over 250 μm (part C).

relative of SBRBC8 (28.1% of the total clones) was *Thiothrix unzii* sp. (L79961), and the closest relative of SBRBC9 (26.3% of the total clones) was *Beggiatoa* sp. (L40997). The 9 sequences of the dominant bacteria were then used to construct a phylogenetic tree using a neighbor-joining algorithm (Fig. 4). Furthermore, examination of Fig. 2 and Table 4 confirms that the morphological analysis results by scanning electron microscopy supported the molecular analysis results. The clones associated with part A (SBRBC1-SBRBC3) (Table 4) were rod-shaped bacteria, those associated with part B (SBRBC4-SBRBC6) (Table 4) were a mixture of rod-shaped and filamentous bacteria, and those associated with part C (SBRBC7-SBRBC9) (Table 4) were filamentous bacteria, as also shown in Fig. 2.

Members of *Citrobacter* have characteristics of facultative anaerobic organisms, including both a respiratory and fermentative type of metabolism. Thus, nitrate is reduced to nitrite, while glucose is fermented with the production of acid and gas. Notably, the majority of *Citrobacter* sp., such as SBRBC5, produces abundant H_2S in an anoxic environmental [16]. Members of *Klebsiella* have characteristics that make them practically immobile, facultative anaerobic, including both a respiratory and fermentative type of metabolism. The outermost layer of *Klebsiella* bacteria consists of a large polysaccharide capsule, plus *Klebsiella* strains possess fimbriae [16]. All members of *Klebsiella*, such as *Klebsiella oxitoca* CECT 4460, can remove a high concentration of nitrate from industrial wastewater without the accumulation of nitrite and ammonium under optimal growth conditions [38, 40, 41].

Thiothrix sp. was the third dominant bacterial group in part B of the biofilm, and *Thiothrix unzii* and *Beggiatoa* sp. were the second and third dominant bacterial groups, respectively, in part C of the biofilm. The members of *Thiothrix* are filamentous sulfur-oxidizing bacteria and a mixotroph [25, 29, 55]. Some *Thiothrix* strains are able to form nitrite from nitrate in the absence of oxygen [55], plus *Thiothrix* has been found to be physiologically very active with nitrate as an electron acceptor, being able to form S globules from thiosulfate and take up acetate in the presence of thiosulfate or intracellular S globules [36]. Organisms of the genus *Beggiatoa* are filamentous, gliding sulfur-oxidizing bacteria. When sulfide is present in the influent wastewater, *Beggiatoa* grow on the biofilm surface. Enzyme preparations from partially purified *Beggiatoa* samples have been found to exhibit a high nitrate reductase and ribulose-1,5-bisphosphate carboxylase-oxygenase activity, providing the first biochemical evidence for the use of nitrate as an electron acceptor for sulfide oxidation and chemoautotrophic growth [27, 61]. Moreover, a few *Beggiatoa* sp. filaments have the capacity to internally concentrate nitrate at levels ranging from 150 to 500 mM in an anoxic sediment [1]. The most common sulfur compounds used as energy sources are H_2S, S^0, and $S_2O_3^{2-}$. The final product of sulfur oxidation in most cases is sulfate [25].

Various authors have already proposed that *Rhodocyclus*-related bacteria are important PAOs in lab-scale P-removing systems when using molecular techniques, whereas Gram-positive bacteria, such as *Microlunatus phosphovorus*, the gram-negative *Lampropedia*, plus *Actinobacteria* and α-*Proteobacteria*, are important PAOs in P-removing systems when using culture-dependent techniques [7, 32, 45]. However, in the present system, no polyphosphate-accumulating bacteria were detected based on analyses using FISH and RFLP techniques. Anaerobic ammonia-oxidizing bacteria, such as *Nitrosomonas*-like species, and the anaerobic ammonia-oxidizing bacterium (AnAOB) *Kuenenia stuttgartiensis* dominate the biofilm of an RBC for nitrogen removal [10, 43], whereas the dominant groups of microorganisms constituting an RBC biofilm have been found to be ammonium-oxidizing bacteria from the *Nitrosomonas europaea/eutropha* group, anaerobic ammonium-oxidizing bacteria of the "*Candidatus Kuenenia stuttgartiensis*" type, filamentous bacteria from the phylum *Bacteroidetes*, and nitrite-oxidizing bacteria from the genus *Nitrospira* [10]. The genera *Nitrosospira* and *Nitrospira* have also been found to be present in all biofilm samples from a combined activated sludge-rotating biological contactor process and A2O systems [57]. Yet, with the present system, the FISH and RFLP methods detected no anaerobic ammonia oxidizers, aerobic ammonia-oxidizing bacteria, or nitrite-oxidizing bacteria in the biofilm. Thus, when compared with other systems or biofilms, the present results identified very different bacterial groups related to the removal of nitrogen and phosphate from wastewater.

Based on the pollutant-removal characteristics and overall structural morphology of the bacterial community composition of the biofilm, the SBRBC produced simultaneous P and N removal, where the COD removal efficiency was 85% of the total COD, the T-N removal efficiency was 55%, and the average T-P removal efficiency was 65% [58]. Although an abundant quantity of EPS and cell mass was needed to attach to the substratum in the first step of biofilm growth, the biofilm was subsequently constructed based on a co-relationship with other bacteria. Specifically, *Citrobacter* sp. and *Klebsiella* sp. appeared to play a role in the nitrate and phosphate removal from the wastewater, as they were the main bacterial groups in the initial biofilm thickness of 0–150 μm in the anaerobic stage (3rd unit) of the operation, during which the P release and uptake rates with a biofilm of less than 1.8 mm showed a tendency to increase with the % P content in the biofilm [58]. The P uptake rate was about 25–35 mg/l when the P release rate was about 17–27 mg/l. Yun *et al.* [58] previously reported on the influent and effluent NH_4-N concentration profiles during the operating period, and complete nitrification was generally achieved by the modified RBC system with

effluent NH_4-N concentrations of 0.3–1.0 mg/l, except during the period of 90–120 days when the RBC was overloaded with high NH_4-N and organic sewage in the 1st and 2nd units. However, the overall nitrogen removal efficiencies were generally lower than the normal efficiencies expected in the case of suspended growth systems with domestic sewage. Yet, this lower N removal efficiency was due to the system design to secure phosphorus removal when using low-strength domestic sewage [58]. The present results of the bacterial community structure inferred that coliform bacteria, such as *Citrobacter* sp. and *Klebsiella* sp., took part in the denitrification and phosphate removal in the 3rd unit during the operation. These organisms can also use organic acids in wastewater, and then produce H_2 or H_2S under anaerobic conditions. In this study, the initial concentration of sulfide averaged 9 mg/l in the 3rd unit (Table 1). For a biofilm thickness of 250 μm surface, sulfide-oxidizing bacteria, such as *Thiothrix* and *Beggiatoa*, were identified as the main bacterial groups growing on the biofilm surface using the H_2S produced by the *Citrobacter* sp. and *Klebsiella* sp. at the base of the biofilm. No sulfur compounds, such as S^{2-} or S^0, were detected in the effluent wastewater from the 3rd unit, and the sulfate concentration was kept around 1.5 mg/l after operation. Thus, it would appear that any sulfur compounds were oxidized by *Thiothrix* and *Beggiatoa*, resulting in sulfate as the final compound. The sulfate derived from the sulfide-oxidizing bacteria may have been used as organic sulfur compounds based on assimilative sulfate reduction, as sulfate was not accumulated in the effluent wastewater and no sulfate reducers were found in the biofilms.

In conclusion, given the feasibility of nitrate and phosphate removal when using an RBC system with a modified SBR-type operation [58], the results from this study revealed that a biofilm formed by coliform bacteria, such as *Enterobacter* sp., *Citrobacter* sp., and *Klebsiella* sp., contributed to the nitrate and phosphate removal, whereas sulfide-oxidizing bacteria, such as *Thiothrix* and *Beggiatoa*, participated in the nitrate removal and sulfur cycle. In addition, the structure of the biofilm was found to correlate with the EPS constituents, as well as the SBRBC processes. Nonetheless, the critical operational factors were of controlling the biofilm thickness, while minimizing the nitrate effect on the PAOs in the case of weak sewage, which generally has a limited usable carbon content. Therefore, it is hoped that these findings may provide a foundation and bacteria community for understanding the nitrogen and phosphate removal in an RBC system.

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