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Bacterial Community Composition of Activated Sludge Relative to Type and Efficiency of Municipal Wastewater Treatment Plants

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Abstract Two microbial communities of activated sludge in the same municipal wastewater, but treated with different systems, were studied and compared using molecular microbiological approaches. The bacterial 16S rDNA sequences from 124 clones were analyzed, however, the majority of them were not closely related to any known species, and found to belong to 8 different phylogenetic groups and 3 different unidentified groups. The relative frequencies of each group were similar between the two microbial communities. Fingerprinting using terminal restriction fragment length polymorphism (T-RFLP) showed that the putative *Nitrospira*-related populations were more diverse and quantitatively higher in the KNR process system than in the other system using a conventional activated sludge process. The relationship between the bacterial community composition and the higher removal efficiency of nitrogen and phosphorus in the KNR process is discussed.

Key words: Activated sludge, bacterial community, T-RFLP, 16S rDNA, phylogeny

The microorganisms present in activated sludge have remained a 'black box,' since wastewater treatment processes were first developed. Engineers and scientists are still trying to identify what kind of microorganisms are involved and which one(s) acts in a principal role in those processes. If such information were revealed, this would enable a better process to design and trouble-shooting. However, in reality, there are too many populations of microorganisms in activated sludge, and no one method can identify all the microorganisms in a particular activated sludge. The recent development of molecular biological techniques has began to shed some light on the dark nature of these complex communities and the most ideal target for the identification of bacteria in activated sludge thus far is rRNA. Most rRNA techniques are culture-independent and so represent the structure of the communities [14]. The use of 16S rRNA to detect and identify natural microorganisms has been applied to many fields, including oligotrophic bacteria [12], hot compost [4], foaming in activated sludge [25], and soil community analysis [16].

Yet there are still certain limitations to be solved, in spite of the significance of 16S rRNA approaches, if they were to be applied to complex ecosystem analysis. Techniques based on 16S rRNA basically require sequence information for the target bacteria. This sequence information is often used to identify existing bacteria or to design a probe to detect a desired group of bacteria by *in situ* hybridization [23]. However, the number of reference in the databases is still very small, therefore, most of the sequences obtained from a complex ecosystem cannot be identified correctly nor directly applied to other different samples, because the microbial community structure may be somewhat different between them. Furthermore, the construction of a huge database, especially for those who desire to determine the microbial nature of a complex system, requires much time and effort.

Many newly designed systems have been developed for the effective treatment of various types of wastewater [7, 18]. As such, the need for good and easy methods that can be used by system designers and developers to compare the systems' microbial populations has also increased. Although there are common limitations, the effectiveness of 16S rRNA means that some molecular microbiological tools can be used as population analysis methods. For example, terminal restriction fragment length polymorphism (T-RFLP) partly fulfills the need for a simple method and an understanding about population dynamics [19]. Without full sequencing or cloning, the fingerprint pattern can provide

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information on different systems, and the distinct T-RF length shows a partial result of bacterial identification [8, 21, 27]. Accordingly, the target system should be confined within narrow differences.

In the current research, microbial communities of activated sludges from two different systems, but with the same microbial origin and influent characteristics, were analyzed and compared by cloning, sequencing the 16S rDNA, and T-RFLP technique. Plus, the effect of treatment system on the treatment efficiency was studied from the prospective of the variation in the bacterial community.

Materials and Methods

System Description and Sampling

The KNR (Kwon's Nutrient Removal) system consists of a UBR (Upflow Bio-Reactor), aeration tank, and clarifier. The influent mixes with recycled sludge from the clarifier and upflows through a concentrated sludge bed in the UBR. The overflowing sludge from the UBR then enters into the aeration tank and settles in the clarifier, while the settled sludge is recycled into the UBR. According to the degree of denitrification, the aerated sludge is also recycled into the UBR. The draining of the sludge takes place in the settled region of the UBR using a drain pump coupled with a sensor for detecting the sludge bed depth in the clarifier. For the current study, a large-scale pilot plant was operated using the same influent and seeding sludge as the municipal wastewater treatment plant (WWTP) in T city. The WWTP was operated using a conventional activated sludge process (influent→clarifier→aeration tank→settling tank→effluent). The treatment efficiency in terms of the BOD_5, TSS (total solid substances), TN (total nitrogen), and TP (total phosphorus) was analyzed by Standard Methods [2]. Sludge samples from the aeration tanks of the KNR plant (MA) and T city WWTP (TMA) were taken and used for analysis.

DNA Extraction and 16S rDNA Library

The sludge samples were centrifuged in a micro-centrifuge tube and washed twice with an autoclaved TE buffer. The activated sludge was lysed by treatment with lysozyme, SDS, and proteinase K, then the DNA was extracted using phenol-chloroform treatment. The bacterial 16S rDNA was amplified with the bacterial universal primers, 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1492R (5'-GGY TAC CTT GTT ACG ACT T-3') [17]. The PCR amplification was carried out using a thermal cycler (Primers Thermal Cyclers; MWG-BIOTECH, Ebersberg, Germany) under the following conditions: 94°C for 3 min; 30 cycles of denaturation at 94°C for 1.5 min, annealing at 50°C for 1.5 min, and an extension at 72°C for 2 min; and 72°C for 10 min. For the reaction mixture, a PCR PreMix (final volume 50 μl) was used (Bioneer Co., Chungbuk, Korea). Approximately 10 ng of the DNA template was added to each PCR tube. The amplification products (~1.5 kb) were visualized by electrophoresis, purified, and concentrated using a Qiaquick PCR cleanup kit (Qiagen, Hilden, Germany). A 16S rDNA library was then generated using the purified 16S rDNA from each sample by each ligation into a pGEM-T Easy vector (Promega Co., Madison, WI, U.S.A.). The plasmid preparations for the DNA sequencing were made with Wizard Mini-Preps (Promega Co.). A total of 124 16S rDNA clones were sequenced by the chain termination method on an ABI Prism 370 automatic sequencer (Applied Biosystems, Foster City, CA, U.S.A.) using T7 primer.

Phylogenetic Analysis and T-RFLP

All sequences with approximately 700 bases were compared with sequences available in the EMBL/GenBank database by using a BLAST search [1]. The sequences were aligned using ClustalX software [26] and phylogenetic trees were constructed using the neighbor-joining method [24]. The PHYLIP software package was used to construct the similarity matrix and phylogenetic trees [11].

For the T-RFLP analysis, the 27F primer was fluorescently labeled with fluorescein at the 5'-end (Bioneer Co., Chungbuk, Korea) to allow detection and quantification of any terminal restriction fragments. The reaction mixtures and PCR conditions were the same as described above. The fluorescently labeled PCR products were purified using a Qiaquick PCR cleanup kit (Qiagen, Hilden, Germany) and digested with the restriction enzyme *Rsa*I (New England Biolabs, Inc., Beverly, U.S.A.) at 37°C for 3 h. The length of the terminal restriction fragments (T-RFs) was determined using an ABI Prism 377 automatic sequencer in the GeneScan mode (Applied Biosystems, Foster City, CA, U.S.A.) with the internal size standard of TAMRA 500 (Applied Biosystems, Foster City, CA, U.S.A.).

Results and Discussion

Analysis of 16S rDNA Sequences and Fingerprinting by T-RFLP

When taking the two clone libraries together, a total of 124 clones (56 clones in MA and 68 clones in TMA) were partially sequenced. From the sequences obtained, 35 sequences in MA and 37 in TMA were confirmed as correctly inserted in the forward sequences. These 72 sequences were then deposited in the GenBank database under accession numbers AF495384-495454, AY093424, and used for the following analysis.

The phylogenetic relationships among the clones were analyzed using a multiple step tree construction. For each step, most related sequences obtained from the Ribosomal Database Project (RDP) and GenBank were aligned together.

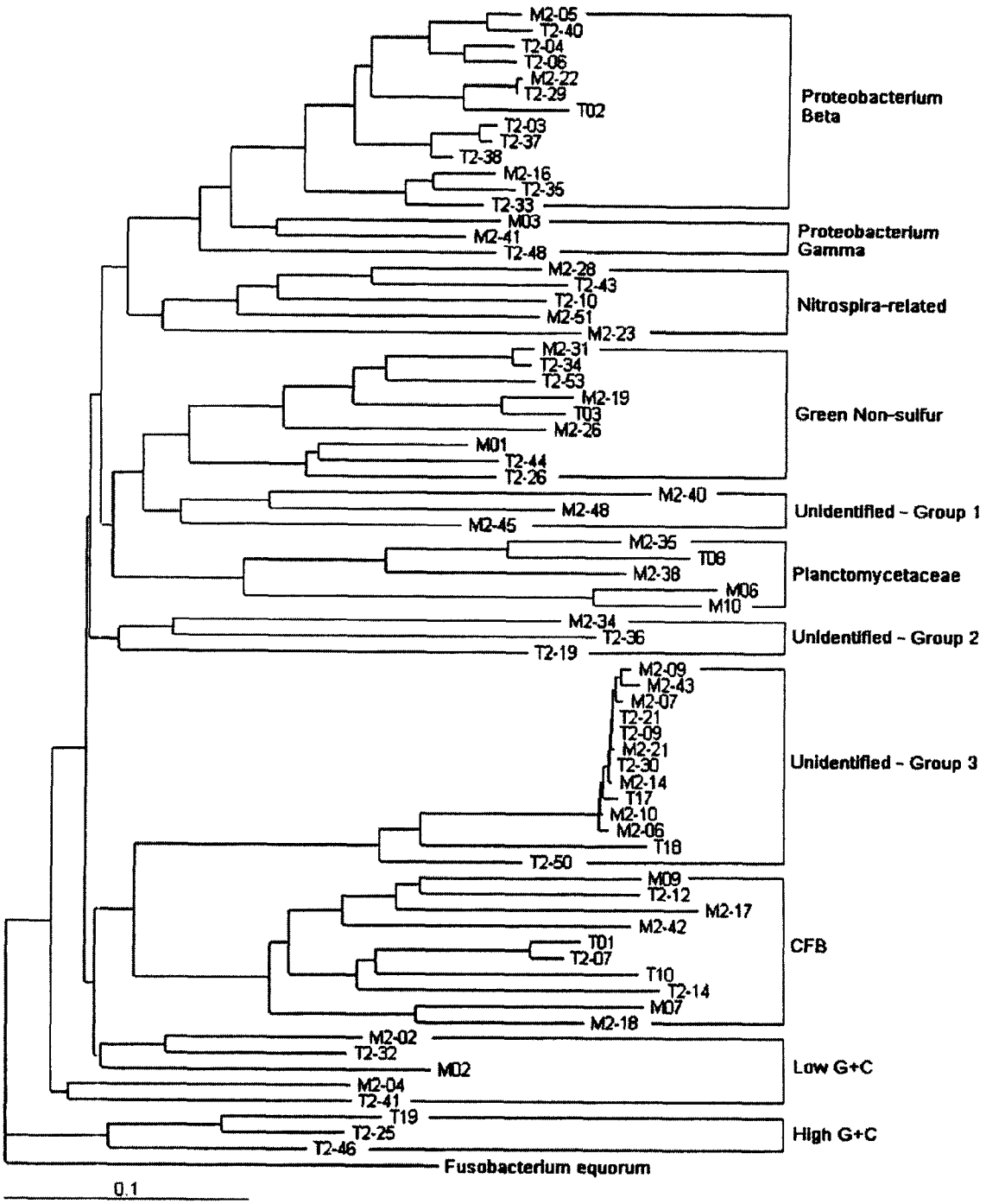

Fig. 1. Dendrogram of clones isolated from aeration tanks of two different types of municipal wastewater treatment plants, MA (abbreviated by M) and TMA (abbreviated by T) with their putative phylogenetic groups.
The DNA distances were calculated using the Jukes-Cantor method, and the tree was generated by neighbor-joining. *Fusobacterium equorum* was used as an outgroup (bar=0.1 distances).

The sequences of the representative strains for each phylogenetic group were also compared. Finally, all the sequences of the clones were categorized into 11 different groups: Eight of them were affiliated with known phylogenetic groups, while the other three groups were arbitrarily referred to as unidentified groups 1, 2, and 3, as the relative relationships with other known groups were distinct (Fig. 1).

Among the 72 sequences of the cloned 16S rDNA, 19 sequences (26.4%) were assigned to the three unidentified groups. RDP suggested that the clones assigned to the

unidentified group 1 were 'clone from nitrogen-removing biofilm from a trickling filter' for clone M2-40, and 'environmental clones' for the others, yet the matching rates were relatively low (88%). The clones grouped into the unidentified group 2 were related with 'uncultured bacteria' (for M2-34 and T2-19; matching rate of 87%) or with *Rhodospirillium photometricum* (for T2-36; matching rate of 92%). *R. photometricum* is a member of the *Proteobacteria* alpha subdivision; however, according to phylogenetic tree mapping, this clone group was not closely related to the *Proteobacteria* alpha subdivision. The unidentified group 3 included 13 clone sequences, two of which (M2-07 and M2-09; matching rate of 86%) were related to the *Cytophaga-Flexibacter-Bacteriodes* (CFB) group. Clones T18 and T2-50 were most closely matched with *Nitrospira* or the *Proteobacteria* beta subdivision, while the remaining clones were matched with 'unidentified eubacteria.' From these RDP matching results and mapping with certain known sequences, it was concluded that the unidentified group 3 could not be allocated to any known group. However, the phylogenetic tree showed that this group could be categorized as CFB, if full sequencing were performed.

The distribution of the clone sequences from MA and TMA were summarized in terms of phylogenetic groups (Table 1). Most of the groups existed in both the MA and TMA samples. Thus, only the high (G+C) Gram-positive bacteria, which were absent in the MA samples, and unidentified group 1, absent in the TMA samples, were characterized. If the number of clones were to be considered, the differences between the MA and TMA sample were relatively low, except for the beta-*Proteobacteria* group. Yet, beta-*Proteobacteria* include some important strains in terms of the nutrient removal process. The *Nitrosomonas* subgroup is related with N-removal processes via nitrification and the *Azoarcus* (especially *Rhodocyclus*) subgroup with the P-removal process [5, 9]. However, there are also other subgroups whose true functions are still unknown. In addition, the distribution data shown in Table 1 only contains qualitative information on each sample, so other quantitative data, such as T-RFLP data, should also be compared with the operation data of the wastewater treatment plants.

The peak data obtained from the T-RFLP was difficult to analyze due to certain basic limitations to the method; 1) the length of the T-RF has an error range of 1 base, 2) the sequence dataset used as the reference in RDP only reflects partial information on the existing bacteria or clones, and 3) different bacteria can have the same T-RF length [10, 20]. However, in spite of these limitations, the T-RFLP data contains useful and quantitative information, if the analyses were carried out along with other data.

Table 1. Relative abundance of clones related to phylogenetic groups in clone libraries from aeration tanks of two different sewage treatment plants.

Phylogenetic group	MA[a]	(%)	TMA[b]	(%)
Cytophaga-Flexibacter-Bacteroides	5	(14.3)	5	(13.5)
Green nonsulfur bacteria	4	(11.4)	5	(13.5)
Nitrospira-related group	3	(8.6)	2	(5.4)
Planctomycetaceae	4	(11.4)	1	(2.7)
High (G+C) Gram-positive bacteria	–	–	3	(8.1)
Low (G+C) Gram-positive bacteria	3	(8.6)	2	(5.4)
Beta-*Proteobacteria*	3	(8.6)	10	(27.0)
Gamma-*Proteobacteria*	2	(5.7)	1	(2.7)
Unidentified - group 1	3	(8.6)	–	–
Unidentified - group 2	1	(2.9)	2	(5.4)
Unidentified - group 3	7	(20.0)	6	(16.2)
	35	(100)	37	(100)

[a]Aeration tank from KNR process.
[b]Aeration tank from TWWP.

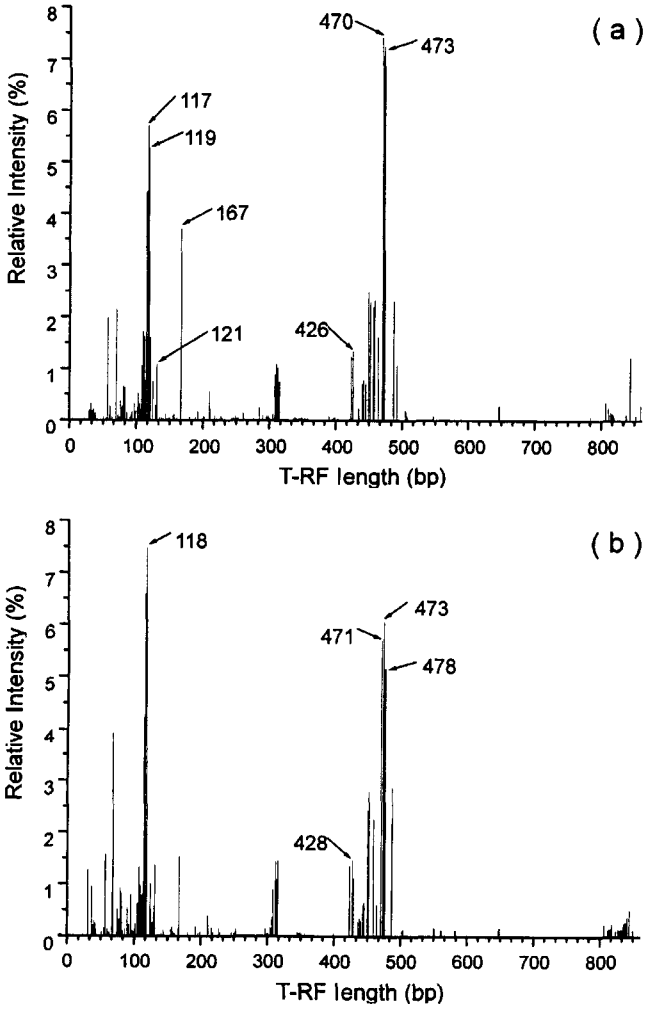

Fig. 2. Chromatogram of *Rsa*I digested terminal restriction fragments (T-RFs) with their relative fluorescent intensity.
The microbial populations from the aeration tanks of two different types of municipal wastewater treatment plants, MA (a) and TMA (b), were analyzed. Values with arrows indicate the peaks exact T-RF length.

Table 2. Putative attribution of each phylogenetic group according to its terminal restriction fragment (T-RF) length on *Rsa*I digested T-RFLP chromatograms.

Phylogenetic groups	T-RF Length from clone (bp)	Relative intensity (%)	
		MA[a]	TMA[b]
Cytophaga-Flexibacter -Bacteroides	110	1.72	-
	116	-	6.59
	118	-	7.47
	311	-	1.44
	314	1.02	-
Green nonsulfur bacteria	310	1.10	-
	448	2.49	-
Nitrospira-related group	118	5.71	-
	121	1.61	-
	167	3.71	-
Beta-*Proteobacteria*	118	5.71	7.47
	427	1.34	1.47
	428	-	1.43
	471	-	5.70
	473	7.24	6.06
Gamma-*Proteobacteria*	457	-	2.27
Unidentified - group 3	469/470/471[c]	7.42	5.70
	478	-	5.15

[a]Aeration tank from KNR process.
[b]Aeration tank from TWWP.
[c]Peaks could not be distinguished according to exact T-RF length, thus total peak intensity was summed.

The sequences of the clone library obtained from the same sample were cut according to the recognition sequence of the restriction enzyme used (GT^AC for *Rsa*I). The length of the artificial T-RF for each clone was determined and matched with the peak data of the T-RFLP (Fig. 2). A total of 42 distinct T-RFs were obtained from the 72 clones and compared with the T-RFLP analysis results, considering an error range of one base. The T-RFs with a relative intensity above 1% were selected, because values under 1% can contain a baseline shift in the GeneScan mode. Finally, 20 distinct T-RFs were selected and their tentative groups determined by the phylogenetic analysis result. The attribution of each phylogenetic group between the MA and TMA samples is summarized in Table 2.

From the results of Table 2 and Fig. 2, the following comparison between the two samples was inferred. The T-RFs showing lengths of 117, 118, 119, and 121 bp in Fig. 2 were likely members of group CFB, *Nitrospira*-related, or beta-*Proteobacteria*. The candidate group of CFB occupied a large portion in the TMA sample, yet disappeared in the MA sample. In contrast, the candidate group of *Nitrospira*-related was abundant in the MA sample, yet absent in the TMA sample. The peaks with T-RF lengths near 470 bp (470, 473, and 478 in Fig. 2) were likely groups of beta-*Proteobacteria* and unidentified group 3. The relative intensity of these two candidate groups in the MA sample was less than that in the TMA sample. Thus, it could be inferred that selective pressure toward the dominance of *Nitrospira*-related group and some of the beta-*Proteobacteria* was facilitated by the introduction of the KNR process in the MA sample.

Efficiency of KNR Process and Bacterial Population Shift

The difference between the reactor types, characteristics of the influent and effluent, and treatment efficiencies for the KNR process and TWWP are summarized at Table 3. Both systems received the same municipal wastewater, plus the HRT (hydraulic retention time) for their aeration tanks was identically maintained. The treatment efficiencies expressed in terms of BOD_5, TSS, TN, and TP were all higher with the KNR process than with the conventional activated sludge process, TWWP. In particular, the increased efficiencies of TN and TP removal showed that the modification of the system by the introduction of a UBR enhanced the system performance through the creation of a small habitat for denitrifier or phosphorus accumulating organisms. It was speculated that the population change created by the habitat was reflected in the aeration tank existing next to the UBR. Thus, the microbial population distribution in the aeration tank, relative to that in the conventional plant, was analyzed

Table 3. Comparison of operation conditions and treatment efficiencies between two different types of municipal wastewater treatment plants, KNR (Kwon's Nutrient Removal) process and TWWP (T-City's Wastewater Treatment Plant).

Operation terms	Units	KNR	TWWP
Flow rate	m^3/day	130	150,000
Retention time			
Settling tank	h	NA[a]	2
UBR[b]	h	3.4	NA[a]
Aeration tank	h	5	5
Clarifier	h	2	3
Influent concentration			
BOD_5	mg/l	265.5	
TSS	mg/l	386.7	
TN	mg/l	33.3	
TP	mg/l	6.3	
Effluent concentration			
BOD_5	mg/l	6.4	32.0
TSS	mg/l	4.8	16.4
TN	mg/l	10.8	16.2
TP	mg/l	0.5	1.5
Removal efficiency			
BOD_5	%	97.6	87.9
TSS	%	98.8	95.8
TN	%	67.6	51.4
TP	%	92.1	76.2

[a]Not Available.
[b]Upflow Biological Reactor.

and compared qualitatively by cloning and quantitatively by T-RFLP.

The highly efficient nutrient removal with the KNR process relative to the conventional process of TWWP was largely due to the bacterial population shift. As already known, nitrogen removal requires both nitrifying- and dentrifying-bacteria, some of which are *Nitrosomonas* in beta-*Proteobacteria* and *Nitrospira* [6, 15]. The identification of the species of phosphorus-removing bacteria has been the focus of several studies, and as a result, some have been found to be beta-*Proteobacteria* [5, 22]. Denitrifying and phosphorus-removing bacteria need anoxic or anaerobic environments. Conventional wastewater treatment processes, which involve only an aeration tank(s), cannot normally create such environments. Yet, this type of bacteria can survive in a clarifier or returning sludge reservoir, although only as a minor population. The UBR in the KNR process helps proliferate these populations so their portion in the aeration tank can be increased. This explanation was supported by the comparison of the two systems, when omitting other interferences, such as the influent characteristics and operation conditions. The above results confirmed the fact that the proportion of *Nitrospira*-related group increased and the community constitution of beta-*Proteobacteria* was changed.

In conclusions, the qualitative data obtained from the cloning in the current study showed that the distribution of each population was similar, yet the quantitative data obtained from the T-RFLP showed that the size of each population relative to the total population varied. Therefore, it was concluded that the UBR in the KNR process acted as a kind of biological selector [3]. In this report, we characterized the total bacterial communities in the KNR and TWWP systems by using 16S rDNA as a target gene. To characterize the bacterial community with respect to nitrogen and phosphorus removal more elucidatively, analyses by using the functional genes, such as *amoA* (ammonia monooxygenase) and *nosZ* (nitrous oxide reductase), should be carried out in the future.

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