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Microbial Community Analysis of 5-Stage Biological Nutrient Removal Process with Step Feed System

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Abstract The 5-stage biological nutrient removal (BNR) process with step feed system showed a very stable organic carbon and nutrient removal efficiency (87% COD, 79% nitrogen, and 87% phosphorus) for an operation period of 2 years. In each stage at the pilot plant, microbial communities, which are important in removing nitrogen and phosphorus, were investigated using fluorescence *in-situ* hybridization (FISH) and 16S rDNA characterization. All tanks of 5-stage sludge had a similar composition of bacterial communities. The total cell numbers of each reactor were found to be around 2.36– 2.83×10^9 cells/ml. About 56.5– 62.0% of total 4,6-diamidino-2-phenylindol (DAPI) cells were hybridized to the bacterial-specific probe EUB388. Members of β-proteobacteria were the most abundant proteobacterial group, accounting for up to 20.6– 26.7%. The high G+C Gram-positive bacterial group and *Cytophaga-Flexibacter* cluster counts were also found to be relatively high. The beta subclass proteobacteria did not accumulate a large amount of polyphosphate. The proportion of phosphorus-accumulating organisms (PAOs) in the total population of the sludge was almost 50% in anoxic-1 tank. The high G+C Gram-positive bacteria and *Cytophaga-Flexibacter* cluster indicate a key role of denitrifying phosphorus-accumulating organisms (dPAOs). Both groups might be correlated with some other subclass of proteobacteria for enhancing nitrogen and phosphorus removal in this process.

Key words: Biological nutrient removal (BNR), community analysis, fluorescence *in-situ* hybridization (FISH), dPAOs, 16S rDNA analysis, PAOs

Denitrifying phosphorus-accumulating organisms (dPAOs) have received a great deal of attention, because they could save carbon through additional denitrification in a system, but the identity of a discrete physiological group capable of removing excess biological phosphate and nitrogen remains unclear [11, 18]. Little is known about the identity of microbial communities involved in biological nutrient removal (BNR) processes. Results from microscopic analysis and visual observation of cultivation are useful only to a certain extent, since diverse microorganisms can have similar morphologies and cultivation limits the detection of organisms that grow under the conditions provided in culture media. Molecular techniques have been applied to samples from laboratory-scale reactors and municipal treatment plants, and suggested the involvement of microorganisms belonging either to the *beta* subclass of the *Proteobacteria*; more precisely, *Rhodocyclus* related bacteria [5] or to the Gram-positive bacteria with high G+C DNA content [24], or to a combination of both. Kim *et al*. [10] tried to remove nitrogen and phosphorus from swine waste using a single strain of *Spirulina platensis*. However, the information on the microorganisms present in reactors still remains unclear, and a better understanding of microorganisms in BNR processes is required for stable operation. This information could assist process retrofitting, design, and operation. The study of enhanced biological phosphorus removal (EBPR) microbiology is important, because the process fails intermittently, the phosphate-accumulating organisms (PAOs) have not unambiguously been identified, and the biochemical pathways for P removal are unknown. Furthermore, design and evaluation procedures for the EBPR system became more complicated by the presence of PAOs that utilize nitrate as an electron acceptor during the P uptake step [11].

In this study, activated sludge samples obtained from the 5-stage BNR process were analyzed by a newly developed molecular biological approach, fluorescence *in-situ* hybridization (FISH) and 16S rDNA clone library technique,

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to directly determine the microbial composition in the complex microbial population. The results obtained are expected to deepen our understanding of the role of microorganisms and help the BNR processes of nitrogen and phosphorus removal in obtaining successful plant performance.

MATERIAL AND METHODS

Process Description of the 5-Stage BNR Process with Step Feed System

The flow diagram of the 5-stage BNR process is shown in Fig. 1. The pilot plant (50 m^3/day) consisted of pre-anoxic, anaerobic, anoxic-1, anoxic-2, and oxic tank operating at S-city sewage treatment plant for 2 years (Kyunggi-do, Korea). Ammonium ion was nitrified in the oxic tank and then denitrified in the anoxic-1 and anoxic-2 tanks. Phosphate to be released in the anaerobic tank was accumulated as the intracellular poly-p level under anoxic/aerobic condition of the anoxic-1 and oxic tanks. Excess accumulated phosphate was designated to be removed through wasting after settling in the clarifier.

The concentration of mixed liquor suspended solid (MLSS) in the tank was maintained by returning activated sludge (RAS) to the pre-anoxic tank. The step feed system that properly distributes raw water containing organics required to remove nitrogen and phosphorus to the pre-anoxic, anaerobic, and anoxic-2 stages, can maximize the phosphorus release and denitrification rates. Operation conditions of the 5-stage BNR process are shown in Table 1. The SRT (sludge retention time), NRCY (nitrified recycle), and RAS ratio of the system were 12–33 d (average 22 d), 200–243% (average 230%), and 36–67% (average 40%, based on influent flow rate), respectively.

Chemical Analysis

The liquid samples were centrifuged at 12,000 rpm (16,000 ×g) at 4°C for 15 min. The supernatants were diluted as required for the relevant analytical methods. Nitrate and phosphate were analyzed by ion chromatography [DIONEX (Sunnyvale, CA, U.S.A.) model DX-500]. All other wastewater analyses were conducted according to *The Standard Methods for Examination of Water and Wastewater* [3]. All analyses were conducted with triplicate samples.

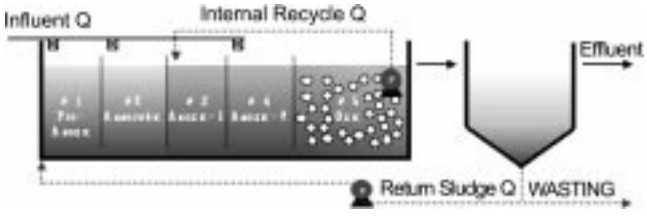

Fig. 1. Schematic diagram of the 5-stage biological nutrient removal process.

DNA Extraction

The genomic DNA was extracted and purified as described by Lee *et al*. [13, 20]. The quality of extracted DNA was checked by standard agarose gel electrophoresis, and DNA concentrations were measured by absorbance at 260 nm.

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

Amplification of 16S rDNA from chromosomal DNA was carried out in a DNA thermal cycler Model480 (Perkin-Elmer, Norwalk, CT, U.S.A.) with universal bacterial primers, 27F (5'-AGAGTTTGATCMTGCTCAG-3') and 1492R (5'-GGTTACCTTTGTTACGACTT-3') [6, 8]. The PCR amplification program consisted of an initial 4-min denaturation step at 94°C, followed by 30 cycles of 94°C for 1 min, 57°C for 1 min, and 72°C for 2 min; and a final 10-min extension step at 72°C [12].

The PCR products were purified with a QIAquick PCR purification kit (Qiagen, Germany), cloned into pGEM-T Easy vector system I according to the manufacturer's instruction (Promega, Madison, WI, U.S.A.). Clones containing inserts of right size were identified by agarose gel electrophoresis of PCR products obtained from host lysates with primers complementary to the vectors flanking insertion sites. Unique clones were identified by restriction fragment length polymorphism (RFLP) analysis of the insert. Thirty-three clones representing the dominant restriction fragment groups were selected from the sludge anoxic-1 tank. The nucleotide sequences of the cloned products were determined from plasmid DNA preparations using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer) and ABI310 Sequencers (Applied Biosystems, Foster City, CA, U.S.A.) according to the

Table 1. Operating conditions of the 5-stage BNR process.

Items		Pre-Ax (#1)	An (#2)	Ax-1 (#3)	Ax-2 (#4)	Ox (#5)	IR (%)	RAS (%)	T (°C)
HRT	h	0.5	1.0	0.5	2.0	3.5	230	40	11–28
MLSS	mg/l	10,840	5,320	4,730	4,430	4,450			
Volume	m^3	1.1	2.1	1.1	4.2	7.7			

*Volatile portion of MLSS was 72–78%, An: anaerobic, Ax: anoxic, Ox: oxic, IR: internal recycle, RAS: return activated sludge, HRT: hydraulic retention time, MLSS: mixed liquor suspended solid.

Table 2. Performance of 5-Stage BNR system process at various temperatures.

	Influent	Effluent (mg/l)			
Temperature	Conc.	11–15	15–20	20–25	25–28
TCODcr	274–348	40.6 (87%)*	35.2 (89%)	24.9 (91%)	22.4 (91%)
SCODcr	102–109	19.8 (81%)	20.2 (81%)	16.0 (84%)	15.6 (84%)
TN	25–31	6.2 (79%)	5.3 (82%)	3.9 (87%)	3.6 (86%)
NH_4+-N	25–31	3.4 (88%)	2.1(93%)	1.0 (97%)	0.7 (97%)
SP	3.8–4.5	0.62 (87%)	0.55 (86%)	0.49 (88%)	0.71 (81%)
SS	108–230	16.5 (90%)	14.0 (90%)	7.3 (93%)	9.4 (91%)

Numbers in the parentheses indicate %. TCODcr: total chemical oxygen demand as chromium, SCODcr: soluble chemical oxygen demand as chromium, TN: total nitrogen, SP: soluble phosphate, SS: suspended solid.

manufacturer's instructions. Vector primers T7 and SP6 were used for the sequencing reactions.

Sequences obtained from 16S rDNA libraries were checked for chimeras with the CHECK-CHIMERA software of Ribosomal Database Project [14] and GenBank database by the BLAST and FASTA programs (Wisconsin Package version 9.1 of the Genetics Computer Group).

Oligonucleotide Probes

The following oligonucleotide probes were used to evaluate the microbial population in sludge: EUB338, a probe designed to detect most bacteria [2]; ALF1b, BET42a, and GAM42a, specific for the α-, β-, and γ-proteobacteria, respectively [16]; HGC, specific for high GC Gram-positive bacteria [16], and CF319a, specific for *Cytophaga-Flexibacter* cluster [15]. All probe sequences, their hybridization conditions, and references are described elsewhere [15, 16, 21, 22]. The probes were labeled with tetramethylrhodamine-5-isothiocyanate (TRITC) at 5'end (Takara Biochemicals Co., Ltd., Japan).

4,6-Diamino-2-Phenylindol (DAPI) Staining and FISH

Define DAPI (4,6-diamidino-2-phenylindole) was used to determine the total number of cells in the samples [16]. FISH of whole cells was performed using the methods described by Snaidr *et al*. [22]. Samples were fixed with paraformaldehyde, and immobilized by air drying on glass slides with a heavy teflon coating forming 9 mm-diameter wells. The samples were hybridized with probes as described by Amann *et al.* [2]. Probes BET42a and GAM42a were used with competitor oligonucleotides [15]. Hybridization was carried out for 90 min at 46°C in a sealed moisture chamber. Washing was conducted under conditions appropriate for each probe [16, 17, 22]. The hybridized and DAPI-stained samples were examined with an epifluorescence microscope (Axioplan; Zeiss, Axiophot 2) with filter sets 01 (DAPI staining) and 15 (TRITC-labeled probe). For counting each probe, more than 10,000 DAPI stained cells were enumerated. Two duplicate samples were counted for each hybridization.

Nucleotide Sequence Accession Numbers

The 16S rDNA partial sequences obtained in this study are available from the EMBL nucleotide sequence database under accession numbers AF485388 to AF485401.

RESULTS AND DISCUSSION

Plant Performance

Table 2 shows the results from average operation for 2 years. The 5-stage BNR system efficiently removed NH_4^+-N (98%) and soluble phosphate (85%) from municipal sewage. The nitrogen and phosphate removal was stable throughout the operation period. In spite of winter period (<15°C), the removal efficiencies of nitrogen and phosphate were maintained over 79% and 87%, respectively. And these

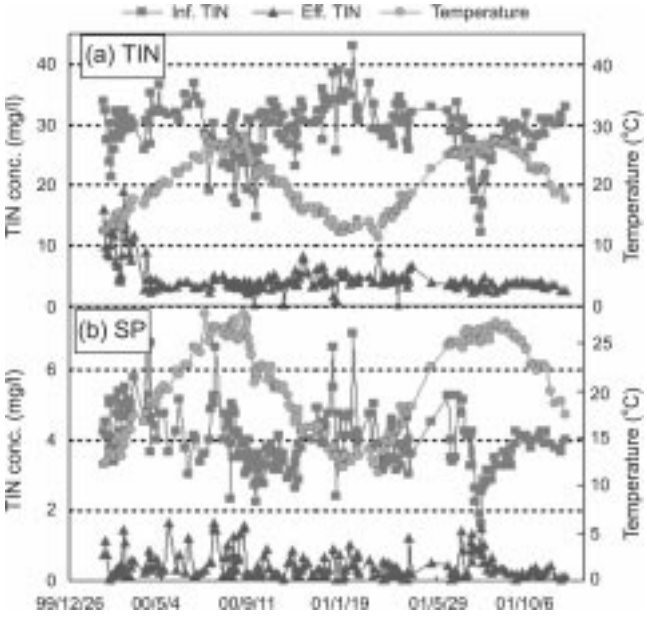

Fig. 2. Variation of TIN (total inorganic nitrogen), SP (soluble phosphate), and temperature during the operating period. Inf.: influent, Eff.: effluent.

Table 3. Proportions of major bacterial divisions in the anaerobic, anoxic-1, anoxic-2, and oxic tanks of the pilot plant sludge by FISH.

Probe	FISH of sludge obtained from each reactor (% binding to probe[a])			
	Anaerobic	Anoxic-1	Anoxic-2	Oxic
ALF1b	4.4±2.1	6.9±2.4	6.2±1.4	8.0±0.9
BET42a	20.6±2.4	26.7±3.1	22.7±3.9	23.1±2.6
GAM42a	3.6±1.3	4.7±1.3	4.0±2.3	3.2±1.4
HGC	8.5±3.6	8.9±3.2	8.8±1.7	9.2±1.0
CF319a	6.8±1.6	6.9±2.3	7.6±1.6	9.6±1.7
Other	18.1	4.4	7.5	7.4
Total	43.9	52.1	49.3	52.7
EUB338	62.0±4.3	56.5±4.7	56.8±3.6	60.1±5.2
Total cell number (cells/ml)	2.36×10^9	2.77×10^9	2.67×10^9	2.83×10^9
PAO[b]	10.0±4.2	50.0±6.1	25.0±3.9	45.0±2.7

a: Percentage of cells in each sludge binding the EUB338 probes for all bacteria, b: as revealed by DAPI staining. EUB388: eubacterial probe, ALF1b: probe for alpha subclass of the class Proteobacteria, BET42a: probe of beta subclass of the class Proteobacteria, GAM42a: probe of gamma subclass of the class Proteobacteria, HGC: probe of high G+C Gram-positive bacteria, CF319a: probe of *Cytophaga-Flexibacter* cluster, Other: probe for other bacteria, PAO: phosphate-accumulating organism.

results are much more excellent than those reported previously [25]. The results of Fig. 2 also show that the nitrogen and phosphate removal was very stable during the operation period.

Total Cell Counts and Domain-Specific Probing

Total cell numbers were determined by DAPI staining. After 2 months of operation during winter, the total cell numbers of each tank were 2.36×10^9, 2.77×10^9, 2.67×10^9, and 2.83×10^9 cells/ml in each activated sludge sample (Table 3). Bacteria were quantified by FISH with domain-specific probes. The bacteria hybridized with the bacterial-specific probe EUB338 were 56.5% to 62.0% of total DAPI cell counts. The microbial community was analyzed by measuring the area of cells that displayed probe conferred fluorescence, and the result was expressed as a percentage of the area that bound DAPI. The label "other" refers to the percentage of bacteria detected by probe EUB338 that could not be explained by the sum of the bacteria detected with the four group-specific probes used in this investigation [1].

Among proteobacteria, the members of the β-subgroup were the most abundant group in each tank, accounting for up to 20.6 to 26.7%, and indicated that these organisms are either directly or synergistically involved in mediating nutrient removal process. The next dominant subclass in each tank was the group of high G+C Gram-positive bacteria (from 8.5 to 9.2%); the *Cytophaga-Flexibacter* cluster, along with b-proteobacteria and high G+C Gram-positive bacteria group, was one of the three most abundant groups in each tank (Table 3). The members of the γ-subgroup were found to be present in relatively low numbers in each tank (from 3.2 to 4.7%). A notable feature of Table 3 is the shift observed for the members of α-subgroup from anaerobic (4.4%), anoxic-1 (4.9%), and anoxic-2 (6.2%) to oxic (8.0%) conditions, indicating that these organisms may not either directly and synergistically play key roles in the denitrification process of this system. The three most abundant groups were maintained in relatively high numbers in all tanks, and these results indicated that the three groups might play important roles in activated sludge, such as degradation of organic materials, removal of nutrients, and formation of flocks. This system maintained a larger number of bacteria in the winter season, and the bacterial population of all tanks by measuring the bacterial specific probes was similar in composition. Therefore, it is proposed that the bacterial population in the 5-stage BNR system was not very much influenced by the external bacteria from wastewater, and we suggest that this system can remove nitrogen and phosphorus in wastewater by stabilized bacterial population.

When used at a high concentration, DAPI also stains polyphosphate granules [23]. Therefore, we can distinguish the bacteria that accumulated polyphosphate *in situ*. With this DAPI staining, 50% of the total bacteria cells fluoresced in bright yellow in anoxic-1 tank (Table 2, anoxic-1). The results indicated that the content of PAOs was almost 50% of the total cell number in anoxic-1 tank and they might play a key role in reducing nitrogen and phosphorus by using PHA/PHB and internal recycled NO_3-N efficiently. From the DAPI staining, we observed that PAOs consisted of five different organisms morphologically in anoxic-1 tank (Fig. 3). Therefore, we suggest that these PAOs are dPAOs, because of simultaneous removed of nitrogen and phosphorus under anoxic condition.

Community Structure Based on 16S rDNA Analysis

More detailed taxonomic information on the dominant bacteria in anoxic-1 tank was obtained by clone libraries of

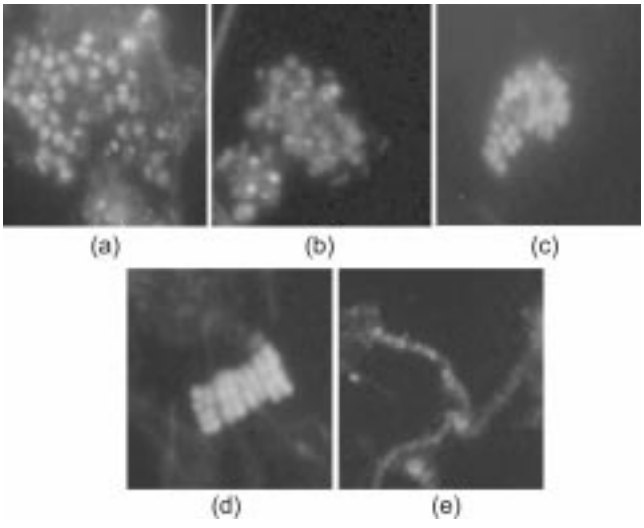

Fig. 3. Epifluorescence micrographs of the dominant dPAO in the anoxic-1 activated sludge.
a: Large coccoid cells, b: Small coccoid cells, c: Large tetrad cells, d: Large sheets cells, e: Filamentous cells.

the 16S rDNA fragment. Genomic DNA directly extracted from activated sludge sample of anoxic-1 tank was cloned and analyzed. Cloned 16S rDNA was analyzed by RFLP analysis. A total of 70 clones were analyzed, and 33 different operational taxonomic units (OUTs) were obtained. RFLP patterns that appeared more than twice were classified into fourteen dominant groups (Table 4; G1-G14). The results of 16S rDNA sequencing are shown in Table 4. Group G1 (14.3 % of the total 70 clones) was the most dominant clone, belonging to the *Cytophaga-Flexibacter* cluster, and it was closely related with uncultured bacterium PHOS-HC35, which were reported as PAOs. The second and the third dominant groups (G2 and G3) contained six and five clones, respectively, and they were more closely related to *Streptococcus suis* (AF009505) and *Microthrix parvicella* (X89560) than high G+C Gram-positive bacteria group and *Acinetobacter* group, respectively. Groups G8 and G9 were related to high G+C Gram-positive bacteria [4, 7]. Groups G4, G5, G6, G7, and G12 were related to α- or β-Proteobacteria, and Groups G8, G10, G11, G13, and G14 were unaffiliated bacterial groups. The overall results by 16S rDNA clone libraries and RFLP analysis indicate that the dominant groups are high G+C% Gram-positive bacteria and *Cytophaga-Flavobacterium* group, except for unaffiliated bacterial groups (G8, G10, G11, G13, and G14).

It is noted that the relative proportions of phylogenetic groups in anoxic-1 tank did not match with those determined by FISH probing (Table 2). Because the unaffiliated bacterial group accounted for 15.7% of total clones, they might have influenced the results on the FISH and clone library. Thus, in order to investigate the proportions of PAOs in the anoxic-1 tank, PAOs were identified by dual staining with group-specific oligonucleotide probes and DAPI. With the DAPI staining, about 90–95% of bacteria hybridized with probe CF319a and probe high G+C fluoresced in bright yellow (Fig. 4). These bacteria were considered to accumulate a large amount of phosphate. Even though the β-subgroup of proteobacteria was the most abundant group on FISH analysis, they did not accumulate a large amount of poly-phosphate (less than 5%; data not shown), and the α- and γ-subgroup of

Table 4. Summary of phylogenetic diversity of domain clones based on 16S rDNA sequences identified by the BLAST database in the 5-stage BNR reactor.

Clones of anoxic-1 tank	16S rRNA gene sequencing			Subclass of Proteobacteria
	Closest match	No. of nucleotide compared	% similarity with closes match	
G 1 (10)[e]	Uncultured bacterium PHOS-HC35(AF314431)	230/243	90	CFB[b]
G 2 (6)	*Streptococcus suis* (AF009505)	320/354	90	Clo[c]
G 3 (5)	*Microthrix parvicella* (X89560)	278/311	89	Actinobacteria
G 4 (4)	*Acidiphilium cryptum* (Y18446)	348/360	96	α[a]
G 5 (4)	*Planctomycetes maris* (AJ231184)	216/237	91	Planctomycetales
G 6 (4)	*Mesorhizobium* sp. N39 (AF282925)	367/382	96	α
G 7 (3)	*Alcaligenes defragrans* (AJ005450)	342/355	96	β[b]
G 8 (3)	Uncultured bacterium GC55 (AJ271048)	324/336	96	UG[f]
G 9 (2)	*Eubacterium formicigenerans* (L34619)	196/213	92	Clo
G10 (2)	Uncultured bacterium (AF125204)	243/260	93	UG
G11 (2)	Uncultured eubacterium SCBP-4D (AJ298275)	98/108	90	UG
G12 (2)	*Hyphomicrobium* sp. (AF 148858)	376/393	95	α
G13 (2)	Uncultured bacterium (AF371539)	353/371	95	UG
G14 (2)	Uncultured bacterium SA6 (AF245321)	371/387	95	UG

a: Alpha subclass of proteobacteria, b: Beta subclass of proteobacteria, c: *Clostridium* group, d: *Cytophaga-Flexibacter* cluster, e: The numbers in parentheses donate the number of colonies involved in the same groups, f: Unaffiliated group.

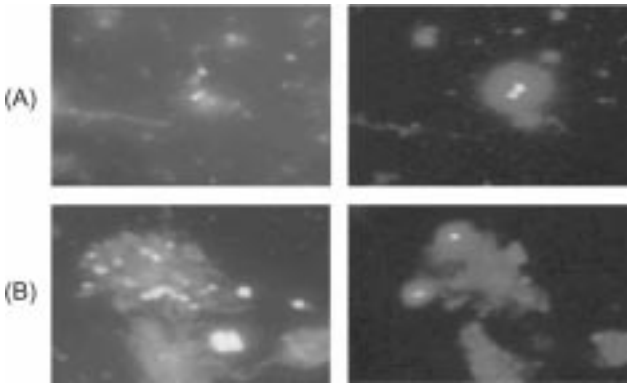

Fig. 4. DAPI stain of activated sludge (left panel) and fluorescent *in situ* hybridization with TRITC labeled probe CF319a and HGC (right panel) for the same microscopic field (×400).
A panel is CF (probe of *Cytophaga-Flexibacter* cluster) and B panel is HGC (probe of high GC Gram-positive bacteria).

proteobacteria did not accumulate polyphosphate at all in this system (data not shown). The overall results indicate that high G+C Gram-positive bacteria and *Cytophaga-Flavobacterium* group might play a key role as dPAOs in anoxic-1 tank and they might cooperate with some other Proteobacteria subclass to enhance nitrogen and phosphorus removal in this process.

Acknowledgments

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