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Denitrifying Dephosphatation Performance Link to Microbial Community Structure

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ABSTRACT

In order to investigate the dynamic relationship of microbial community structure and denitrifying dephosphatation performance of the process, fingerprint profile technique of polymerase chain reaction (PCR) amplified 16S rRNA and denaturing gradient gel electrophoresis (DGGE) were applied to analyze microbial communities in anaerobic/anoxic/oxic enhanced biological phosphorus removal (EBPR) process. The chemical analysis showed that average removal efficiency of COD, NH_4^+–N and TP was 90%, 90% and 97% respectively during the steady-state phase. Herein, about 40% of total phosphorus (TP) was removed by denitrifying dephosphatation. Phylogenetic affiliation of predominant members was assessed by the determination of 16S rRNA sequence of correspondent bands. The comparison of these gradual shifts and removal performance of the process suggested that *Bacteroidetes* was positive for EBPR, while *α-proteobacteria* was related to EBPR deterioration. Moreover, *Uncultured Sphingobacteriaceae* was likely to be one of the denitrifying phosphate accumulating organisms (DPAOs), which could contribute to denitrifying dephosphatation.

Keywords: Denitrifying dephosphatation; Removal performance; DGGE; Microbial community structure; EBPR

1. INTRODUCTION

EBPR has become a widespread technology in wastewater treatment plants (WWTPs) due to its characteristic of high efficiency and low cost (van Loosdrecht et al., 1998; Tian et al., 2011). Denitrifying dephosphatation is one typical EBPR by enrichment of activated sludge with DPAOs, using nitrate as an electron acceptor to achieve anoxic phosphorus uptake and simultaneous denitrification (Zeng et al., 2004; Kishida et al., 2006; Zhang et al., 2006), which could save carbon sources and aeration, reduce sludge production (Kuba et al., 1996; 1997; Shoji et al., 2003; Carvalho et al., 2007). Denitrifying dephosphatation is based on sufficient nitrite or nitrate as electron acceptors for DPAOs and special process design (Zhang et al., 2006). Most of the related studies have mainly focused on the metabolism of DPAOs (Ahn et al., 2001; Oehmen et al., 2007) and electron acceptors (Hu et al., 2003; Zhou et al., 2010).

It has recently been reported that molecular biology approaches were applied to investigate microbial community and diversity, to insight into from the microbiological point of view. Carvalho et al. (2007) investigated the link between the process performance of two denitrifying phosphorus removal bacterium with synthetic wastewater and microbial

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community structure by fluorescence in situ hybridization (FISH). Miura et al. (2007) found that the bacterial community structure is important for the stable performance in MBRs treating municipal wastewater by PCR-DGGE and FISH techniques. Nevertheless, the literatures on the bacterial community structure link to stable removal performance in anaerobic/anoxic/oxic EBPR process treating domestic wastewater are not yet well documented.

The objective of this study was, therefore, to investigate the dynamic relationship of microbial community structure and the removal performance in anaerobic/anoxic/oxic EBPR process by microbial techniques (PCR-DGGE) combined with chemical analysis.

2. MATERIALS AND METHODS

2.1 Experimental Apparatus and Sample Collection

The activated sludge samples were collected from a lab-scale EBPR system, as shown in Figure 1. The reactor was made of plexiglas with a total working volume of 27L, which was separated four functional compartments by removable flashboards. The working volume of anaerobic selector, anoxic selector, anoxic, aerobic 1 and aerobic 2 compartments are 5.4, 1.2, 6.6, 7.8 and 6 L, respectively. The system was fed with raw domestic sewage which the characteristic was shown in Table 1. The operational parameters were as follows: temperature 22±3˚C, DO of 1.5-2.0 mg/L, solid retention time (SRT) of 12 days. The recycling rate of sludge, nitrifying liquor and mixture liquor was set at 1.0, 2.0 and 1.5 time of total influent flow rate, respectively.

The activated sludge sample was taken from anoxic compartment once per 10 days within the start-up phase of the system (30days) and once per 15days within steady-state phase. The sludge was concentrated and stored at -20˚C for DNA extraction.

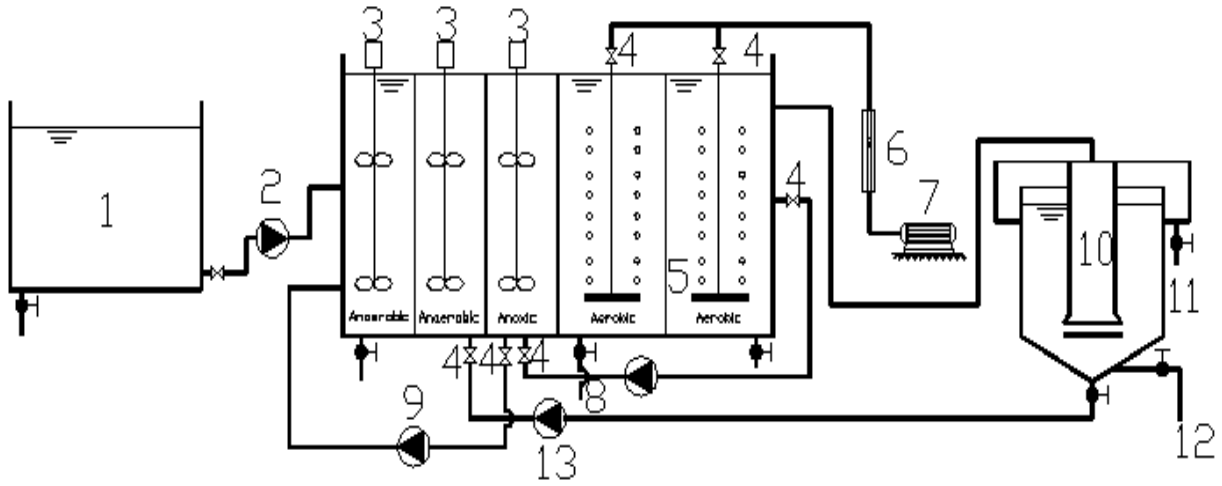

Figure 1 Schematic diagram of lab-scale anaerobic/anoxic/oxic EBPR reactor. 1. Influent tank; 2. feed pump; 3.mechanical mixer; 4.check valve; 5.diffuser; 6.airflow meter;7.air compressor; 8.return nitrifying liquor pump; 9. recycling mixture liquor pump; 10.secondary clarifier; 11. effluent; 12.waste sludge; and 13.return sludge pump

Table 1 Wastewater characteristic

Items	COD(mg/L)	BOD_5	NH_4^+–N(mg/L)	TN(mg/L)	TP(mg/L)	pH
Influent	209.45-281.4	109.65-161.75	45.94-57.85	48.6-64.5	7.44-8.47	7.0-7.7
Effluent	17.83-33.79	9.10-21.12	3.14-12.25	5.78-13.3	0.13-3.31	6.8-7.4

2.2 Chemical Analysis

Ammonia nitrogen (NH_4^+–N), nitrate nitrogen (NO_3^-–N), nitrite nitrogen (NO_2^-–N), total phosphorus (TP), chemical oxygen demand (COD), mixed liquor suspended solids (MLSS), were measured in accordance with standard methods (APHA, 1998). The total nitrogen (TN) concentration was determined with a total organic carbon analyzer (Liqui-TOCII, Elementar, Germany). DO was measured by SG6-ELK (Mettler Toledo, Switzerland), pH and temperature were measured with HI-8424 (HANNA, Italy).

2.3 Microbial Analysis

To characterize the microbial community, total DNA of sludge samples was extracted using the E.Z.N.A Soil DNA Kit, which is a highly efficient mean to extract DNA from environmental samples. The V3 region was selected as the PCR amplified region. The forward primer BSF338 and the reverse primer BSR534 were 5′- ACTCCTACGGG AGGCAGCAG -3′ and 5′- ATTACCGCGG CTGCTGG -3′. PCR amplification was carried out in an automated thermal cycler (Veriti, ABI). Initial denaturation was at 94˚C for 4min which was followed by 30 cycles of denaturation at 94˚C and annealing at 55˚C for 45 s respectively. Each annealing step was followed by extension at 72˚C for 1min except 8min of final extension step. The PCR products were loaded onto 1% agarose gel and stained with ethidium bromide to determine size, purity, and concentration of DNA. Afterward, DGGE was performed with DCode Universal Mutation Detection system (Bio-Rad, USA).The PCR amplicons were applied directly to 8% (w/v) polyacrylamide gels in 1×TAE buffer (20 mM Tris-acetate (pH 7.4), 10mM acetate, 0.5 mM Na_2EDTA) with a denaturing gradient from 40% to 60%. The gradient gel was casted with gradient delivery system (Model 475, BioRad). Electrophoresis was carried out at 60˚C and constant voltage of 180V for 4h. Finally, the gel was fixed with 10% acetic, then was stained by 0.1% silver nitrate solution, developed with 10% sodium hydroxide, terminated with 10% acetic, and photographed by PowerLook 1000 (UMAX Technologies, Taiwan) to obtain DGGE profile (Miura et al., 2007; Tan and Ji, 2010).

The target bands were excised from the gel and were immersed in Tris-EDTA buffer (pH 8) for 24 h. The eluted DNA was reamplified by PCR with the forward primer BSF338 and the reverse primer BSR534 without GC clamp. The PCR products were extracted and cloned with PMD19-T Vector (Takara). Then the formatted plasmid was transformed into competent cells (E. coli Competent Cells DH5α, Takara Inc.). Afterward, the E. coli was added by 1ml SOC medium, then was cultured at 37˚C for 1h, thereafter was sent for sequencing. A search for the closest reference microorganisms in the GenBank database using partial 16S rRNA sequences was carried out with the BLAST program (http://www.ncbi.nlm.nih.gov/blast). Phylogenetic trees were finally constructed by MEGA software (Version 3.1) via the Neighbor-Joining (NJ) algorithm.

3. RESULTS AND DISCUSSION

3.1 TP Removal Performance

The five activated sludge samples were collected on the 10th, 20th, 30th, 45th and 60th day respectively from the start-up phase to the steady state phase. The variation of TP along the flow path and P-uptake rate was shown as Figure 2, in which TP concentration of each stage represented an average value of 10 days. From the bar chart, it was clear to see the percentage of phosphorus release increased gradually under the condition of average influent TP concentration was below 7.9 mg/L, from triple phosphorus release of start-up phase to more than fourfold of steady-state phase, which could enhance phosphorus uptake power in anoxic and aerobic phase. TP concentration in anoxic selector was gradually decreased since the dilution of return sludge and nitrifying liquor, microbial assimilation and denitrifying dephosphatation. Figure 2 also showed that anoxic P-uptake from 1.7 mg/L of the start-up phase up to 5.4 mg/L of steady-state phase, P-uptake rate climbed from 21% to 40%, then keep stable, which indicated that approximate 40% of TP was removed by anoxic uptake at steady-state phase. Moreover, average removal efficiency of TP was 97% and effluent phosphorus was below 0.5 mg/L, which could meet A standard of China Municipal Wastewater Treatment Plant Emission.

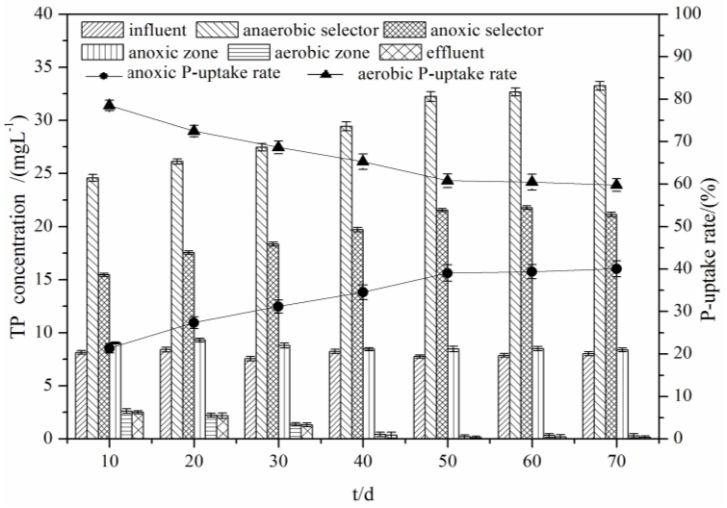

Figure 2 Variation of TP along the flow path and P-uptake rate

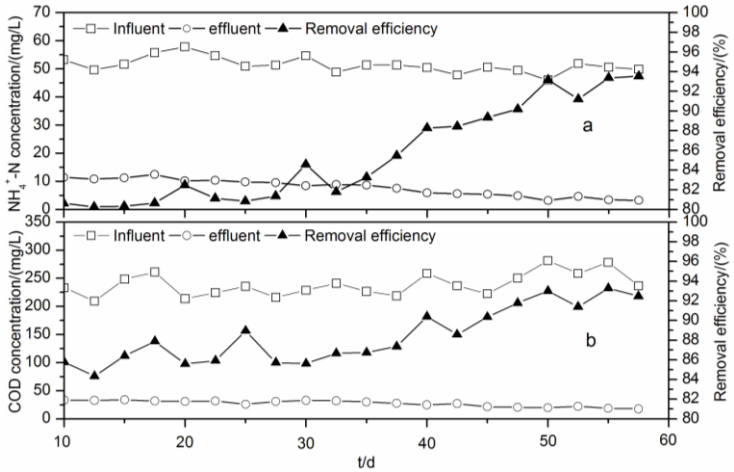

Figure 3 Removal performances of COD and nitrogen in EBPR process

3.2 COD and Ammonia Nitrogen Removal Performance

Generally speaking, heterotrophic denitrifying bacteria compete carbon source with denitrifying phosphorus removal bacteria to take precedence for denitrification. If COD concentration of anoxic compartment was too high, denitrifying phosphorus removal bacteria will be inhibited since electron acceptor of denitrifying dephosphatation (nitrate) is exhausted; reversely, lower COD concentration is beneficial for denitrifying phosphorus removal bacteria to use nitrate as electron acceptor and inner carbon source (PHB) for denitrifying dephosphatation and nitrogen removal, which could save part of aerating consumption.

In this study, COD removal efficiency was higher when phosphorus removal was good, as illustrated in Figure 3. From Figure 3, it was easily found COD removal efficiency keep stable. When the average influent concentration was 239.06 mg/L, average effluent concentration was about 27.3 mg/L, and COD removal efficiency maintained at 90% in steady-state phase. In addition, COD concentration of anoxic compartment was around 50 mg/L, which is consistent with the results of Luo et al. (2005). This result could guarantee denitrifying dephosphatation as well as maintain a better environment for nitrifying bacteria in the subsequent aerobic compartment.

Ammonia nitrogen removal performance was shown in Figure 3. The average influent concentration was 51.37 mg/L, and the removal efficiency was about 78% in start-up phase. The removal efficiency remarkably improved after start-up, and then remained at 90%. Nitrate concentration of anoxic compartments was below 2.0 mg/L with DO concentration of 1.5~2.0 mg/L, which could ensure recycling mixture liquor don't influence anaerobic phosphorus release. This result was according with Wang et al. (2005) and Yuan et al. (2002).

Table 2 The summary of DGGE bands distribution

Band Number \ Day	10	20	30	45	60
Band 1	++	++	++	+	+
Band 2	-	+	++	+	-
Band 3	+	++	++	-	-
Band 4	+	++	++	-	-
Band 5	-	-	-	-	+
Band 6	-	+	+	+	-
Band 7	-	+	-	+	-
Band 8	+	+	-	+	-
Band 9	+	+	+	+	-
Band 10	+	++	+	+	+
Band 11	++	++	+	+	+
Band 12	+	+	+	+	++
Band 13	+	++	+	++	++
Band 14	+	+	+	++	++
Band 15	+	+	+	+	+

3.3 The Variation of Microbial Community Structure and Diversity Analysis

The specific PCR amplification and subsequent DGGE of five sample gained from different period of time was conducted, cloned and sequenced to reveal the microbial structure and diversity. The summary of DGGE bands distribution for five samples were shown in Table 2, it was found that band 10, 11, 12, 13, 14 and 15 were present all long, although sometimes a little bit weak. However, band 1, 3, 4, 8 and 9 were gradually weakened as the non-dominant population. Besides, band 2, 6 and 7 were irregularly changed, which probably had little effect on the performance of phosphorus removal. In addition, band 5 was only present in steady-state period, which was likely to be a sort of denitrifying phosphorus removal bacterium.

Ten predominant different bands (1-15) were carefully excised from the DGGE gel and reamplified, cloned and sequenced. The nucleotide sequences were compared to similar bacteria available in the GenBank database using BLASTN. All sequences were found to be 94-100% homologous with previously identified 16S rRNA gene sequences. A comprehensive phylogenetic 16S rRNA tree reflecting the relationships of nucleotide sequences and similar sequences available on NCBI was shown in Figure 4. Phylogenetic 16S rRNA tree shows that the predominant populations of the system were α-proteobacteria, Bacteroidetes, Firmicutes and TM7. Of all the bands, band 1,3,4,9,11,13 and 14 belonged to α-proteobacteria, band 2,5,7,12 belonged to Bacteroidetes, band 6, 13 belonged to Firmicutes, and band 8,10 belonged to TM7.

From Figures 2 and 4, it was found that most of α-proteobacteria emerged in start-up phase by comparing the corresponding band, which matched poor phosphorus removal performance. However, with the succession of bacteria, some species were no longer the predominant genus in steady-state phase. This result demonstrated that the existence of a large number of α-proteobacteria lead collapse of EBPR system, which was consistent with the results of Wagner and Loy (2002) and Oehmen et al. (2007). The Acinetobacter belonged to Pseudomonadales existed in large numbers from start-up phase to steady-state phase but didn't lead the disruption of EBPR system. Previous researcher found that the Acinetobacter gradually disappeared accompanying by the enhancement of denitrifying dephosphatation in A^2O process (Wu et al., 2009). However, most of researches suggested that Acinetobacter played a role in denitrifying phosphorus and nitrogen removal. With the stable operation to the steady-state phase, Bacteroidetes become the dominant genus. Phosphorus removal and denitrifying dephosphatation were very good and stable accompanied by the emergence of Uncultured Sphingobacteriaceae, which accordingly suggested that it's likely to be one kind of DPAOs. Although its role in phosphorus removal is still not well explored, however, it was practically present in biological phosphorus removal system and has been isolated from EBPR plants. The variation of Firmicutes had no distinct influence on phosphorus removal performance. However, Zhao et al. (2011) found Firmicutes had the relationship with CODcr degradation. Furthermore, it was reported that Uncultured Trichococcus could cause glucose fermentation into acetic acid and propionic acid under anaerobic conditions, which was conducive to EBPR process (Wu et al., 2009). As shown in Figure 4, Uncultured Trichococcus (Band 10) had been always present, which maybe contribute to phosphorus removal.

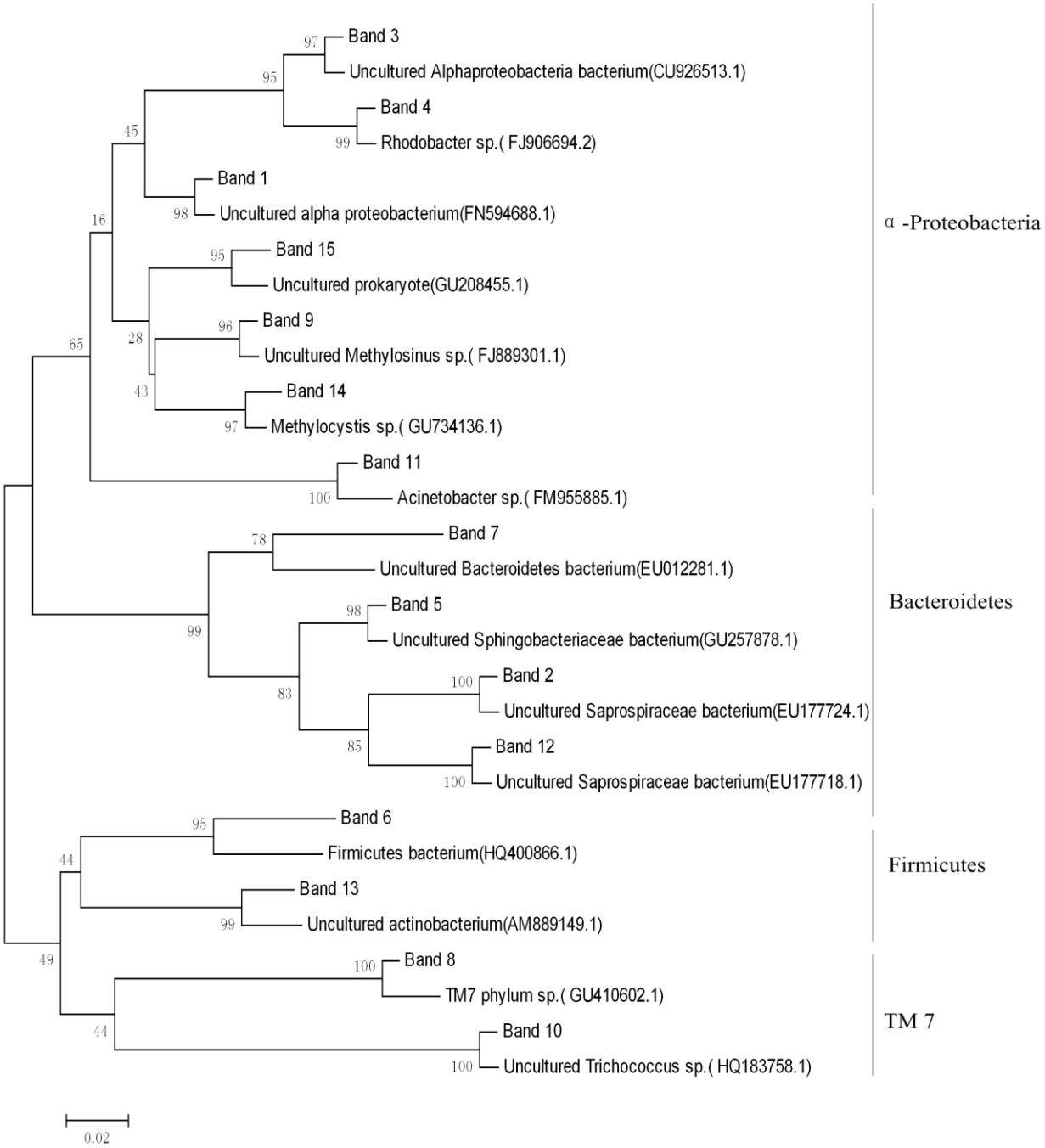

Figure 4 Phylogenetic tree of 16S rRNA sequences from DGGE profiles. Bar represents 2 nucleotides substitution in 100 nucleotides, and Bootstrap probabilities are indicated at the branch nodes

Although a mass of bacterium were well-known present in EBPR process, the corresponding metabolic mechanism and the quantitative function still need to explore by advanced molecular microbiology methods. The operating performance reflected by the microbiological point of view will certainly be a powerful tool to optimize the performance of EBPR.

CONCLUSIONS

In this study, denitrifying dephosphatation performance link to microbial community structure was investigated. It was found that the variation of microbial community had a relative dynamic relationship with denitrifying dephosphatation performance. The sequence results of DGGE bands and removal performance illustrated that TP removal efficiency was lower when *α-proteobacteria* was the dominant species, which mean *α-proteobacteria* was not favorable for EBPR; however, TP removal efficiency and denitrifying dephosphatation were good and stable when *Bacteroidetes* was the dominant species, which should partly attribute to *Uncultured Sphingobacteriaceae*. Accordingly, *Uncultured Sphingobacteriaceae* was likely to one of the DPAOs in this study. Moreover, *Acinetobacter*, *TM7* and *Uncultured Trichococcus* also contributed to phosphorus and nitrogen removal.

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