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INCREASING ROLE OF MICROORGANISM WITH UBIQUINONE-9 IN DENITRIFYING PAO SLUDGE

Hansaem Lee[1], Zuwhan Yun[2*]

[1]R&D Division, Hyundai Engineering & Construction Co., Yongin, 16891, Korea
[2]Department of Environmental Engineering, Korea University, Seoul, 136-713, Korea

Abstract

An anaerobic-aerobic (An-Ox) sequencing batch reactor (SBR) was operated for the growth of phosphate accumulating organisms (PAOs), while an anaerobic-anoxic (An-Ax) SBR was used for the growth of denitrifying PAOs (dPAOs). Both reactors exhibited successful enhanced biological phosphorous removal (EBPR) performance. The sludge P contents in the two reactors increased from approximately 2% at the beginning of the operation to 9.1% (An-Ox SBR) and 7.3% (An-Ax SBR) after 140 d. The results of the quinone profile analysis demonstrated that the proportions of UQ-8, UQ-10, and MK-8 (H_4) in the An-Ox sludge increased compared to the beginning of the operation, whereas in the An-Ax sludge, the proportion of UQ-8 was the highest and exhibited the greatest increase, and the mole fraction of UQ-9 more than doubled from 8.3% to 16.9%. Thus, the role of microorganisms with UQ-8 and UQ-9 was found to have significantly increased in the An-Ax sludge. As a result of microbial identification through the fatty acid methyl ester (FAME) analysis, *Comamonas testosteroni* and *Pseudomonas alcaligenes*, which produce the UQ-9 in the An-Ax sludge, as well as *Psychrobacter immobilis*, which produces UQ-8.

Keywords: denitrifying phosphorus accumulating organism, fatty acid methyl ester, phosphorus accumulating organism, quinone profile

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1. Introduction

Unlike the alternating anaerobic-aerobic (An-Ox) conditions for the growth of phosphate accumulating organisms (PAOs), denitrifying PAOs (dPAOs) grow under anaerobic-anoxic (An-Ax) conditions. PAOs use only O_2 as the electron acceptor for P uptake, while dPAOs use chemically bonded oxygen, such as NO_3^- or NO_2^- (Kuba et al., 1996), in which NO_2^- and/or NO_3^- reduction occurs by using the accumulated polyhydroxyalkanoate (PHA) as the internal carbon source during the anoxic period.

In enhanced biological phosphorous removal (EBPR) sludge, many recent studies (He et al., 2007; He et al., 2010; Kim et al., 2010; Peterson et al., 2008; Wang et al., 2016) have given various interpretations of the microbial community structure. In addition to the use of chemical taxonomy, which uses microbial cell components (e.g., cell membranes, cell wall lipids, quinones, polysaccharides, amino acids) as indicators, analyses of the microbial ecosystem using the various techniques of molecular biology (e.g. DNA probes, DNA hybridization, PCR, 16s rDNA profiles) have been performed.

Cellular quinones are utilized as electron transporters through aerobic and anaerobic respiration or photosynthesis because they present in the cell membrane of microorganisms and are essential components for the respiration chain as well as the photosynthetic electron transfer system (Levandowsky and Hutner, 1980). Obligate anaerobic bacteria use menaquinone (MK), whereas ubiquinone (UQ) is synthesized under aerobic or anoxic conditions (Collins and Jones, 1981). A

* Author to whom all correspondence should be addressed: e-mail: envzyun@korea.ac.kr; Phone: +82 2 32903979; Fax: +82 2 9295391

wealth of information has been collected regarding the main components of the quinone species in microorganisms, and this information can be used as a database.

In addition, the quinone profile has been often applied as a useful and simple tool for the dynamic analysis of microorganisms in a mixed culture system because not only can the quinone profile be expressed as a molar ratio for each of the quinone forms for characterization but also can be used to quantify changes in the microbial community. Nevertheless, most analyses of the quinone profile in the EBPR system have been focused on the PAO that are grown within alternating An-Ox conditions (Ahn et al., 2006; Hiraishi, 1998; Okada et al., 1992), whereas the quinone profile analysis for dPAO has been nonexistent. Therefore, the purpose of this study was to analyze the microbial characteristics of the EBPR system using the quinone profile after the PAOs and dPAOs were grown in each SBR system and to investigate the relationship with the quinone profile through microbial identification using a fatty acid methyl ester (FAME) analysis.

2. Experimental

2.1. Reactor setup and operation

The An-Ax SBR (for the growth of dPAO) and the An-Ox SBR (for the growth of PAO) were operated during about 160 days. Using a programmable logic controller (PLC), the cycle for operation of the An-Ax SBR was set as follows: fill (0.5 h); anaerobic stage (2.5 h); anoxic stage (4 h); settling (0.5 h); and decanting (0.5 h). The operation cycle of the An-Ox SBR was controlled similarly: fill (0.5 h); anaerobic stage (2.5 h); aerobic stage (4 h); settling (0.5 h); and decanting (0.5 h). The working volume of both SBRs was 5.9 L. The influent flow was fed to 3.4 L per cycle at every 8-h interval. The daily total inflow was 10.2 L. In the An-Ax SBR, nitrate was supplied externally during 15 min of the initial anoxic period as an electron acceptor for dPAO. For the aerobic condition in the An-Ox SBR, the dissolved oxygen (DO) was maintained at 2.2 ± 0.4 mg/L using an air pump.

2.2. Characteristics of the synthetic wastewater

Table 1 shows the influent characteristics of synthetic wastewater. Propionic acid (HPr) was used as a sole carbon source in the synthetic wastewater because HPr as sodium propionate can minimize the growth of glycogen accumulating organisms (GAOs) known to impede the EBPR when using acetic acid (HAc) due to the difference of substrate affinity (Lopez-Vazquez et al., 2009; Oehmen et al., 2006). NH_4Cl as a nitrogen source and KH_2PO_4 for a phosphate source were used to construct the influent. Trace metal solution which modified the trace salt solution of Weng and Molof (1974) was added to the influent. The influent with the same C/N/P ratio was supplied into both SBRs. For the nitrogen supply, in the An-Ax SBR, 9.0 ± 2.1 mg/L of NH_4^+-N was fed into the influent for microbial synthesis. NO_3^--N was externally supplied at the initial 15 min of the anoxic period as an electron acceptor for dPAO. In the An-Ox SBR, 23.9 ± 1.4 mg/L of NH_4^+-N was fed to the influent.

The actual sludge retention time (SRT_a) in the An-Ax SBR and the An-Ox SBR was calculated to 27 ± 2.8 and 25 ± 3.5 days, respectively. Mixed liquor suspended solids (MLSS) of the An-Ax SBR was 2,180 ± 250 mg/L, while that of the An-Ox SBR was 2,580 ± 210 mg/L.

2.3. Chemical analysis

The analyses including COD, NH_4^+-N, TP, PO_4^{3-}-P, TSS, and VSS were conducted on the basis of Standard Methods (APHA et al., 2005). Ion chromatography (IC-80, Dionex) was used for the measurement of NO_2^--N and NO_3^--N. TN was also measured using a DR4000 (Hach Co.).

Table 1. Influent characteristics of synthetic wastewater

Parameters		*Influent concentration*	*Remarks*
An-Ox SBR	SCOD (mg/L)	147.1 ± 20.0	Sodium propionate as a sole carbon source
	NH_4^+-N (mg/L)	23.9 ± 1.4	
	NO_3^--N (mg/L)	0.2 ± 0.2	
	PO_4^{3-}-P (mg/L)	5.5 ± 0.2	
An-Ax SBR	SCOD (mg/L)	144.1 ± 21.8	Sodium propionate as a sole carbon source
	NH_4^+-N (mg/L)	9.0 ± 2.1	
	NO_3^--N (mg/L)	0.2 ± 0.1	
	External NO_3^--N feed (mg/0.1 L)	95 ± 3.8	After the injection, the average NO_3^--N concentration in the reactor was 15.8 ± 3.2 mg/L
	PO_4^{3-}-P (mg/L)	5.8 ± 0.2	
Trace metal solution	* (mL/L)	0.1	

** $MgSO_4 \cdot 7H_2O$ 0.2 g/L, $FeCl_3 \cdot 6H_2O$ 1.5 g/L, $MnSO_4 \cdot 5H_2O$ 1.5 g/L, $ZnSO_4 \cdot 5H_2O$ 0.1 g/L, $CaCl_2 \cdot 2H_2O$ 0.1 g/L, and $(NH_4)_6Mn_7O_{24} \cdot 5H_2O$ 0.1 g/L*

Samples were taken regularly at the influent, the end of anaerobic period, the end of anoxic (or aerobic) period, and effluent in both SBRs. The biomass P contents were measured as %. PHA, which was calculated from the sum of polyhydroxybutyrate (PHB) and polyhydroxyvalerate (PHV), was measured according to the method of Comeau et al. (1998).

Gas chromatography (GC) equipped with a DB-WAX column (length of 15 m, diameter of 0.52 mm) (Mega bore J&W Scientific Co. capillary column) was used for the PHA measurement. To analyze the glycogen content in biomass, a liquid chromatography with a Bio-Rad Aminex ion-exclusion column was used by the method proposed by Smolders et al. (1994).

2.4. Quinone analysis

The quinone profile analysis was conducted to investigate the microbial community characteristics of the An-Ax SBR and the An-Ox SBR. The quinone analysis was performed by the method suggested by Hirashi et al. (1996). The activated sludge, which was taken from the Joongnang Wastewater Treatment Plant (WWTP) in Korea as seed sludge, the An-Ax sludge and the An-Ox sludge were used for the quinone analysis. Activated sludge process was adopted in Joongnang WWTP, in which biomass P content was 1.8% on average. Quinone was extracted for 3 h by injecting the mixture of chloroform and methanol (2:1, v/v) into the sample. After filtering through filter paper, the solution was concentrated and underwent a re-extraction process by adding 100 μL of another chloroform-methanol mixture (8.5:1.5, v/v). HPLC (Younglin SP930D) was used for the measurement, and a Waters Spherisorb 5 μm ODS2 4.6 mm x 250 mm column (40°C) was used. The solvent for the analysis was methanol-isopropyl ether (4:1, v/v), which was injected at 1.0 mL/min. The detection wavelength was 254 mm, and a Younglin UV730D was used as the detector.

2.5. Microbial identification through fatty acid methyl esters (FAME) analysis

The FAME analysis was performed to identify the microbes in the An-Ax SBR and An-Ox SBRs. The seed activated sludge was used as a control. A Trypticase soy agar (BBL) medium (17.0 g of pancreatic digest of casein, 3.0 g of pancreatic digest of soybean meal, 5.0 g of sodium chloride, 2.5 g of potassium hydrogen phosphate, 2.5 g of glucose, 15.0 g of agar, and 1.0 L of distilled water) was used for cultivating the strain. After approximately 48 h of cultivation in the culture medium, the cellular fatty acid composition of the isolated strains in each sample was determined according to the method suggested by Miller (1982). Approximately 40 mg of the cultivated cell was transferred to a Teflon-lined screw cap tube, after which 1 ml of solution with 15% NaOH was added to 50% methanol, heated at 100°C for 30 min, and air-cooled at room temperature. Then, 2 mL of methanolic HCl (a mixed solution with 325 ml of 6.0 N HCl and 275 mL of methanol) was added and heated for 10 min. at 80°C. After quenching, 1.25 mL of hexane-methyl tertiary butyl ether (1:1: v/v) was added, mixed well for 10 min, and allowed to stand at room temperature. Once the reaction mixture separated into two layers, the supernatant was separated, and 3 ml of dilute NaOH (10.8 g NaOH per 900 mL dry weight) was added, mixed well for 10 min, and then left to stand at room temperature again. Approximately 2/3 of the supernatant was then transferred to the screw-capped vial (12 × 32 mm) and used as the samples for the analysis. A GC (Agilent Technologies, model 6890) equipped with a cross-linked methyl siloxane column (HP-1) was used for the analysis. Sherlock MIS Software was used for identifying the microbes.

3. Results and discussion

3.1. EBPR performance

The profile of PO_4^{3-}-P concentration in the An-Ax SBR and the An-Ox SBR was shown in Fig. 1 for each reaction stage.

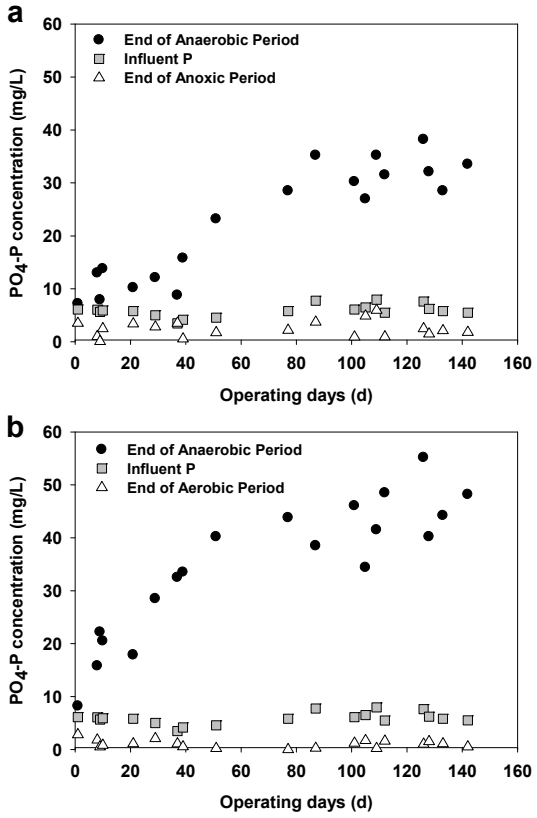

Fig. 1. The profile of PO_4^{3-}-P concentration in (a) the An-Ax SBR and (b) the An-Ox SBR

The samples were collected and analyzed for EBPR performance. In the An-Ax SBR and the An-

Ox SBR, the PO_4^{3-}-P concentration increased as the operation started. Influent concentration of PO_4^{3-}-P was 5.8 ± 0.2 mg/L, and the PO_4^{3-}-P stabilized when it increased to more than 30 mg/L at approximately 100 d after the operation. PO_4^{3-}-P at the end of the anoxic condition tended to remain relatively constant at the concentration of 1.8 ± 0.5 mg/L (Fig. 1a).

In the An-Ox SBR (Fig. 1b), which simulates a typical EBPR by PAOs, the EBPR performance appeared to be relatively better than the operation results from the An-Ax SBR. For the entire period of the operation, an average 5.5 mg/L of PO_4^{3-}-P was fed to the influent. Subsequent to the anaerobic condition for P release, PO_4^{3-}-P was accumulated to 0.8 ± 0.6 mg/L at the end of the aerobic condition. The P release rapidly increased at the initial operation period and, after about 80 d, the concentration has stabilized to more than 40 mg/L.

Fig. 2 exhibits the SCOD, PO_4^{3-}-P, and NO_3^--N concentrations including the changes of PHA and glycogen in both SBRs after the stabilization. For SCOD, both SBRs had a similar tendency at the influent-anaerobic-anoxic (aerobic) conditions. Also, most of SCOD was consumed in the anaerobic period. In the An-Ax SBR (Fig. 2a), PO_4^{3-}-P concentration (5.8 ± 0.2) in the influent was released to 31.2 ± 4.3 mg/L at the end of the anaerobic condition, and then accumulated to 1.8 ± 0.5 mg/L at the anoxic condition. At the same time, approximately 97% of nitrate was denitrified at the anoxic condition. These indicate that simultaneous N and P removal was successfully achieved in the An-Ax SBR although the efficiency of P removal was not higher than expected.

In the An-Ox SBR (Fig. 2b), higher P release (42.9 ± 6.2 mg/L) and uptake (0.8 ± 0.6 mg/L) were observed as compared with that of the An-Ax SBR. NO_3^--N nitrified at the end of the aerobic condition (10.4 ± 1.8 mg/L) had little influence on P release during the subsequent anaerobic period because about 60% of NO_3^--N was discharged to the effluent. On the mass basis, only 26.0 mg NO_3^--N/cycle was returned into the subsequent anaerobic condition, in which 500.1 mg SCOD/cycle was fed into the reactor. About 20% of total SCOD was utilized for denitrification, and then the remained SCOD was mainly utilized for P release.

PHA and glycogen were analysed for verifying the EBPR performance of the An-Ax SBR and the An-Ox SBR. PHA is utilized as the internal carbon source under anoxic and/or aerobic conditions after the PAO or dPAO converts volatile fatty acids (VFA) to PHA at anaerobic condition (Mino et al., 1994; Smolders et al., 1994). In addition, glycogen also measured along with PHA because glycogen provides reducing power when the PAO or dPAO transforms VFA into PHA under anaerobic condition (Smolders et al., 1994).

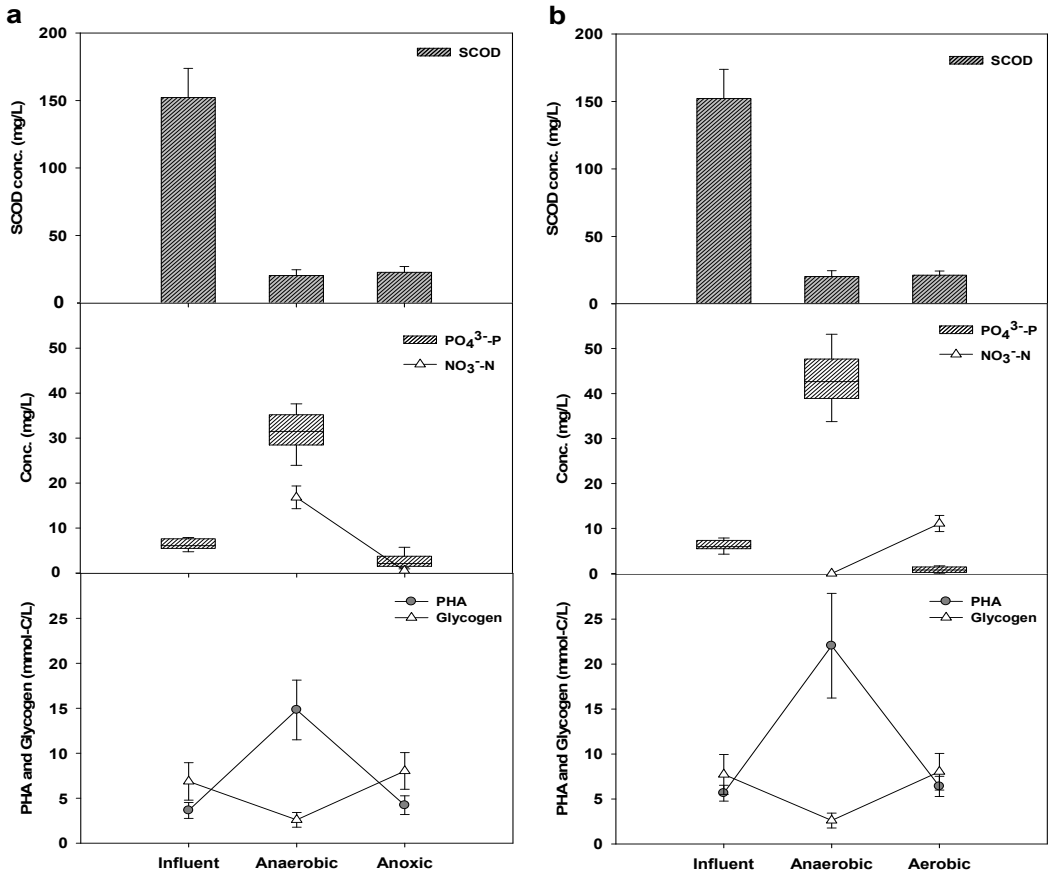

Fig. 2. The EBPR behavior in (a) the influent-anaerobic-anoxic conditions of the An-Ax SBR and (b) the influent-anaerobic-aerobic conditions of the An-Ox SBR

In both SBRs, PHA increased with P release at the anaerobic period. Subsequent to the anaerobic condition, PHA was utilized at the anoxic condition of the An-Ax SBR and the aerobic condition of the An-Ox SBR, in which P uptake occurred in both reactors. On the other hand, glycogen had a tendency to reduce at the anaerobic condition because it was used as a reducing agent to transform VFA to PHA, and then recover at the anoxic period of the An-Ax SBR and the aerobic period of the An-Ox SBR.

3.2. Sludge P contents

The P contents in the sludge can be considered as the easy and simple indicator to correlate the extent of the EBPR activities. The variations of the P contents in the An-Ax sludge and the An-Ox sludge during the operation period are shown in Fig. 3. In general, the P contents in an activated sludge without EBPR is known to be approximately 1.5-2.5% (van Haandel and van der Lubbe, 2007), whereas the P contents in the EBPR sludge is varied with inflow C/P ratio but has been reported to increase by as much as 12.3% (Liu et al., 2000).

Sludges in the An-Ox SBR and the An-Ax SBR had slightly more than 2% of P content at the beginning of the operation. However, the P content increased gradually, and after 140 d, the P content in the An-Ox sludge was 9.1%, and the P content in the An-Ax sludge was found to be 7.3%. P contents in the An-Ax sludge were about 20% less than that in the An-Ox sludge. Although the P contents in the An-Ax sludge and the An-Ox sludge were slightly different, both sludges had high P contents (more than 7%), which means the two SBR systems achieved successful EBPR activities. Thus, the sludges after 140 d of operation, which had the greatest P contents, were used for quinone analysis.

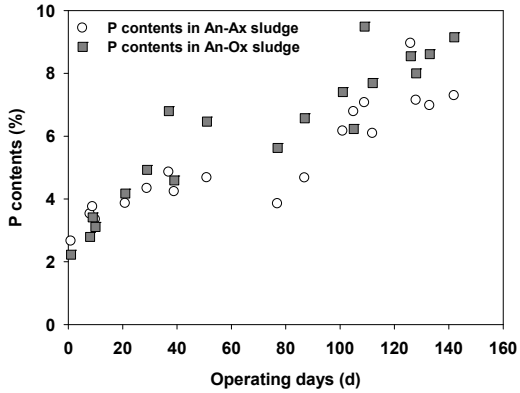

Fig. 3. Daily variations of P contents in the An-Ax sludge and the An-Ox sludge

3.3. Microbial quinone compositions

The respiratory quinones were analyzed to identify the microbial community composition of the initial activated sludge (as a control parameter), the An-Ax sludge and the An-Ox sludge as shown in Fig. 4. Three types of UQ and 10 types of MK were measured. The samples of the An-Ax sludge and the An-Ox sludge were taken at the end of the anoxic and aerobic period in each reactor after 130 d of operation.

At the beginning of the operation, UQ-8 was the predominant quinone. UQ-9, UQ-10, MK-7, MK-8, MK-8(H_4), and MK-9(H_4) were also found to be a large proportion of the total quinones. As the EBPR capacity was increased, the mole fraction of UQ-8 and UQ-9 exhibited the largest increase in the An-Ax sludge. UQ-8 was predominant, and UQ-9 was the second-most common type of quinone, followed by MK-7, MK-8, and MK-8(H_4). UQ-10 and other homologs were minor quinones in the An-Ax sludge.

In the An-Ox sludge, UQ-8 was the most dominant quinone, and UQ-10, MK-8(H_4), and MK-7 were also observed in significant proportions. The mole fraction of UQ-9 was increased from 8.3% (seed sludge) to 16.9% in the An-Ax operation, while UQ-9 in the An-Ox sludge was decreased to 2%. In addition, UQ-10 was decreased from 10.5% to 2.2% in the An-Ax sludge, but it was slightly increased to 12.5% in the An-Ox sludge. In a typical EBPR sludge, which was operated in An-Ox conditions, UQ-8, UQ-10, and MK-8(H_4) were the most abundant quinones in both the lab- and pilot-scale plants (Hiraishi, 1998; Hiraishi and Morishima, 1990; Liu, 1995). UQ-8 was the minor quinone in the PAO-enriched sludge (Hiraishi, 1998; Lee et al., 2002). Their results are consistent with our result of the An-Ox sludge, which was cultivated with the PAO-enriched population.

The increase in UQ-9 in the An-Ax sludge, which demonstrated anoxic polyphosphate accumulation with denitrification, was a remarkable phenomenon, and that could suggest an association between the increase in the cell numbers of bacteria containing UQ-9 and the denitrifying EBPR. Although Okada et al. (1992) reported that UQ-9 was the predominant quinone in the An-Ox SBR with the acetate-fed synthetic wastewater, the increase in UQ-9 has not been reported in An-Ax SBR operation. This result means that the microorganisms containing UQ-9 could be significantly related to the denitrifying EBPR performance.

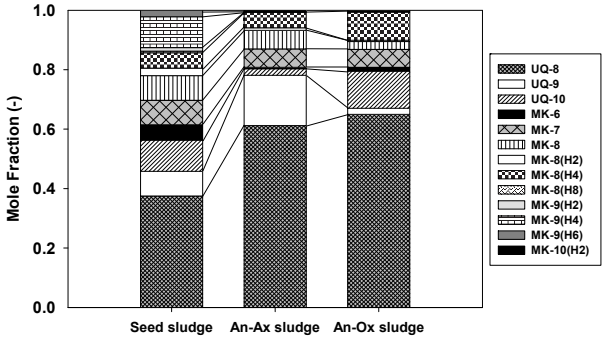

Fig. 4. Quinone compositions of the seed activated sludge, the An-Ax sludge, and the An-Ox sludge

Table 2 shows the results of dissimilarity (D) and diversity of quinone (DQ) based on the quinone profiling. The calculation for D and DQ referred to the methods suggested by Hiraishi et al. (1998) and Hu et al. (1999). The D values between the seed sludge and the An-Ax sludge (0.371) as well as between the seed sludge and the An-Ox sludge (0.365) were very similar each other. However, the dissimilarity between the An-Ax sludge and the An-Ox sludge was even lower (0.212). It demonstrated that there was a change in the microbial community and the initial activated sludge changed into EBPR sludge. It also indicated that there is little difference in the microbial community structures between the An-Ax sludge and the An-Ox sludge

The diversity index of the seed sludge (9.56) at the beginning of the operation was greater than the An-Ax sludge (5.33) and the An-Ox sludge (5.51). This result means that the diversity of the microbial species in the seed sludge was greater than the sludges in the two SBRs. Furthermore, the equitability index (EQ) in both An-Ax sludge and An-Ox sludge, in which EQ can be calculated to DQ/n, was relatively low. It indicates that they exhibited microbial distribution toward the predominant species such as PAOs or dPAOs.

3.4. Identification of the microorganisms by FAME analysis

The principle of microbial identification using the FAME analysis basically uses a GC to determine the FAME profile, which is produced through the methylation of a cellular fatty acid that has very stable dielectric properties within the microbial taxonomic group, and compare this profile with the reference microbial library. The identified result can be determined at the genus or species level.

The results of the microbial identification through the FAME analysis are presented in Table 3. In the seed sludge, *Staphylococcus lentus*, which contains the MK-9(H_4) quinone, and *Acinetobacter*, which contains the Q-9 quinone, were identified. *Staphylococcus lentus* are gram-positive cocci that are known to be a denitrifying microorganism (Harbi et al., 2010; Zumft, 1992). According to the quinone profile in Fig. 4, the proportion of the molecular species of MK-9(H_4) was relatively large, but the proportion of the MK-9(H_4) quinone has been dramatically reduced during the long-term operation of the An-Ax and An-Ox alternating conditions. This result indicates that one or more denitrifying microorganisms could have been reduced because the reactor system was not based on denitrification.

In the An-Ax sludge, the microbial species of *Comamonas testosteroni* and *Pseudomonas alcaligenes*, which produce the quinone Q-9, have been identified, and *Psychrobacter immobilis* (which produces Q-8) has also been detected. The identification of the microorganisms with Q-8 and Q-9, which account for the greatest proportion of the quinone profile in the An-Ax sludge, is very significant. What is distinctive is that *Comamonas testosteroni* and *Pseudomonas alcaligenes* have EBPR functions (Fang et al., 2002; Hiraishi and Morishima, 1990; Streichan et al., 1990) and *Pseudomonas* as well as *Alcaligenes* had a function on denitrifying EBPR (Bao et al., 2007). Accordingly, the *Comamonas testosteroni* and *Pseudomonas alcaligenes* that were identified in this study could be included the dPAO candidates.

In the An-Ox sludge, we were able to identify the microorganisms, *Sphingomonas paucimobilis* and *Psychrobacter immobilis*, which account for the greatest proportion in the quinone profile. According to recent studies, *Psychrobacter* is phylogenetically in the same category as the *Acinetobacter* and *Moraxella* genera (Di Gioia et al., 2008; Ding et al., 2011), and thus, it could be considered a PAO candidate.

Table 2. Quinone compositions and quinone profile-based D and DQ values

Quinone homolog (n)	***mol % in***			***Dissimilarity (D)***			***Diversity of quinone (DQ)***		
	Seed sludge (A)	***An-Ax sludge (B)***	***An-Ox sludge (C)***	***Seed sludge and An-Ax sludge ∑(|B-A|/2)***	***Seed sludge and An-Ox sludge ∑(|C-A|/2)***	***An-Ax sludge and An-Ox sludge ∑(|C-B|/2)***	***Seed sludge $(\sum\sqrt{A})^2$***	***An-Ax sludge $(\sum\sqrt{B})^2$***	***An-Ox sludge $(\sum\sqrt{C})^2$***
UQ-8	0.3750	0.6120	0.6500	0.1185	0.1375	0.0190	0.6124	0.7823	0.8062
UQ-9	0.0830	0.1890	0.0200	0.0530	0.0315	0.0845	0.2881	0.4347	0.1414
UQ-10	0.1050	0.0020	0.1250	0.0515	0.0100	0.0615	0.3240	0.0447	0.3536
MK-6	0.0520	0.0050	0.0130	0.0235	0.0195	0.0040	0.2280	0.0707	0.1140
MK-7	0.0820	0.0620	0.0600	0.0100	0.0110	0.0010	0.2864	0.2490	0.2449
MK-8	0.0830	0.0620	0.0270	0.0105	0.0280	0.0175	0.2881	0.2490	0.1643
MK-8(H_2)	0.0520	0.0090	0.0040	0.0215	0.0240	0.0025	0.2280	0.0949	0.0632
MK-8(H_4)	0.0250	0.0530	0.0950	0.0140	0.0350	0.0210	0.1581	0.2302	0.3082
MK-8(H_8)	0.0050	0.0010	0.0020	0.0020	0.0015	0.0005	0.0707	0.0316	0.0447
MK-9(H_2)	0.0120	0.0020	0.0020	0.0050	0.0050	0.0000	0.1095	0.0447	0.0447
MK-9(H_4)	0.1040	0.0010	0.0010	0.0515	0.0515	0.0000	0.3225	0.0316	0.0316
MK-9(H_6)	0.0210	0.0020	0.0010	0.0095	0.0100	0.0005	0.1449	0.0447	0.0316
MK-10(H_2)	0.0010	0.0000	0.0000	0.0005	0.0005	0.0000	0.0316	0.0000	0.0000
				0.371	**0.365**	**0.212**	**9.563**	**5.328**	**5.516**

Table 3. The microorganisms identified by the culture-dependent FAME analysis

Sample	***Microorganism***	***Dominant quinone***	***Remarks***
Seed activated sludge	*Staphylococcus lentus*	MK-9(H_4)	Gram-positive cocci, Aerobe, known as a denitrifier (Harbi et al., 2010; Zumft, 1992)
	Acinetobacter	Q-9	Gram-negative, γ-proteobacteria
An-Ax SBR	*Comamonas testosteroni* / *Pseudomonas alcaligenes*	Q-9	They can accumulate polyphosphate at the anoxic condition (Fang et al., 2002; Hiraishi and Morishima, 1990; Streichan et al., 1990) ※ *Pseudomonas* and *Alcaligenes* Bao et al. (2007) reported that *Pseudomonas* and *Alcaligenes* could conduct anoxic phosphorus accumulation with denitrification.
	Psychrobacter immobilis	Q-8	Gram-negative, γ-proteobacteria, nitrate reductase (Bozal et al., 2003; Vaneechoutte et al., 2007)
An-Ox SBR	*Sphingomonas paucimobilis*	Q-10	α-proteobacteria, gram-negative, aerobe (David et al., 1996)
	Psychrobacter immobilis	Q-8	Gram-negative, γ-proteobacteria, nitrate reductase (Bozal et al., 2003; Vaneechoutte et al., 2007)

Furthermore, because *Psychrobacter immobilis* was also identified in the An-Ax sludge, *Psychrobacter immobilis* is thought to have not only the function of PAOs but also the denitrification.

4. Conclusions

For approximately 160 d, the An-Ax SBR and the An-Ox SBR exhibited successful EBPR performance, in which the sludge P contents in the two reactors increased up to 7.3% and 9.1%, respectively. The mole fraction of UQ-9 was more than double in the An-Ax SBR and UQ-8 was the predominant quinone in both SBRs.

Through the FAME analysis, *Comamonas testosteroni* and *Pseudomonas alcaligenes*, which produce UQ-9, were identified in the An-Ax sludge, and *Psychrobacter*, which produces UQ-8, was identified in both systems. *Comamonas testosteroni*, *Pseudomonas alcaligenes*, and *Psychrobacter* seemed to play important roles in the denitrifying EBPR system.

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