The difference of morphological characteristics and population structure in PAO and DPAO granular sludges

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A B S T R A C T
We examined how long-term operation of anaerobic–oxic and anaerobic–anoxic sequencing batch reactors (SBRs) affects the enhanced biological phosphorus removal (EBPR) performance and sludge characteristics. The microbial characteristics of phosphorus accumulating organism (PAO) and denitrifying PAO (DPAO) sludge were also analyzed through a quantitative analysis of microbial community structure. Compared with the initial stage of operation characterized by unstable EBPR, both PAO and DPAO SBR produced a stable EBPR performance after about 100-day operation. From day 200 days (DPAO SBR) and 250 days (PAO SBR) onward, sludge granulation was observed, and the average granule size of DPAO SBR was approximately 5 times larger than that of PAO SBR. The DPAO granular sludge contained mainly rod-type microbes, whereas the PAO granular sludge contained coccus-type microbes. Fluorescence in situ hybridization analysis revealed that a high ratio of Accumulibacter clade I was found only in DPAO SBR, revealing the important role of this organism in the denitrifying EBPR system. A pyrosequencing analysis showed that Accumulibacter phosphatis was present in PAO sludge at a high proportion of 6%, whereas it rarely observed in DPAO sludge. Dechloromonas was observed in both PAO sludge (3.3%) and DPAO sludge (3.2%), confirming that this organism can use both O₂ and NO₃⁻ as electron acceptors. Further, Thauera spp. was identified to have a new possibility as denitrifier capable of phosphorous uptake under anoxic condition.

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Introduction
The recent rapid developments in molecular biological methods for microbial analysis have accelerated the ability to identify phosphorus accumulating organisms (PAOs). These include analyses by culture-independent methods such as PCR-clone libraries, fluorescence in situ hybridization (FISH), and FISH-microautoradiography (MAR). This type of approach has identified Rhodocyclus-related bacterium, namely, “Candidatus Accumulibacter phosphatis (ca. Accumulibacter),” as perhaps one of

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the most important PAO candidates (Bond et al., 1995; He et al., 2007, 2010; Hesselmann et al., 1999; Kong et al., 2004; Peterson et al., 2008). Furthermore, in addition to potential PAO candidates belonging to the 6-proteobacteria, those belonging to the T-proteobacteria class have often been dominant in pilot-scale and full-scale enhanced biological phosphorus removal (EBPR) communities (Beer et al., 2006; Eschenhagen et al., 2003; Kong et al., 2007). A recent MAR-FISH study of a full-scale EBPR system by Nguyen et al. (2012) also revealed that the genus Halomonas belonging to the T-proteobacteria class is a potential PAO candidate. Menes et al. (2011) found that this genus had good P uptake capacity.

In addition to microbial analysis of PAO, which plays a crucial role in EBPR through the operation of sequencing batch reactors (SBRs) under alternating anaerobic–oxic conditions (He et al., 2010; Jeong et al., 2000; Kim et al., 2010; Wexler et al., 2003; Zhang et al., 2010). The main difference between PAO and DPAO is the use of electron acceptors. Normal PAO uses oxygen under aerobic conditions and DPAO utilizes nitrate under anoxic conditions. As a result, the system utilizing DPAO can not only reduce the oxygen requirement to oxidize poly-β-hydroxyalcanoates (PHA) in the aerobic zone, but also comply with the chemical oxygen demand (COD) utilization (Lee and Yun, 2014). DPAO has an advantage for simultaneous removal of both N and P with a minimized COD utilization. Murleitner et al. (1997) and Kuba et al. (1996) suggested that DPAO produced 20%–30% less sludge than PAO.

There is debate as to whether PAOs and DPAOs are phylogenetically the same microorganisms. In a study by Zeng et al. (2003), FISH analysis performed under anaerobic–oxic and anaerobic–anoxic conditions led to the conclusion that Accumulibacter was dominant in both reactors. Using MAR-FISH analysis, Kong et al. (2004) reported that Accumulibacter could use both O2 and NO3− as electron acceptors, indirectly demonstrating that PAOs and DPAOs can be classified into the same taxonomic group. On the other hand, Carvalho et al. (2007) revealed that Accumulibacter was dominant in both systems, although morphological differences were present. In addition to Accumulibacter, Rhodocyclus-related and Dechloromonas-related groups are also classified as potential PAO candidates (Kong et al., 2007), demonstrating the currently unclear taxonomic differences between PAO and DPAO.

While most studies on PAO and DPAOs have examined EBPR performance under stable operating conditions, there are rarely reports on the duration of reactor operation, or the characteristics that are manifested during operation. In other words, there is a lack of research examining whether it is possible to operate the reactors stably for a prolonged period as the PAO and DPAO continue to grow. Nor is it clear whether there are any time requirements for stabilization of the reaction, whether there are characteristic changes, and whether there are differences between DPAO and PAO microbial community structures. Therefore, the purpose of this study is (1) to examine the changes for a long-term operation in both the EBPR performance and (2) the sludge characteristics including granulation that occurred under alternating anaerobic–oxic and anaerobic–anoxic conditions for the growth of PAO and DPAO. In the case of a long-term stable operation, we also performed a quantitative comparative analysis of their microbial community structures with a pyrosequencing-based analysis as well as their taxonomic characteristics.

1. Materials and methods

1.1. Reactor operation

Two separate laboratory-scale SBRs were operated for about 500 days. One is an anaerobic–oxic SBR (PAO SBR) for the PAO growth and the other is an anaerobic–anoxic SBR (DPAO SBR) for the growth of DPAO. The working volume of both reactors was 5.9 L. The SBRs had a height to diameter ratio of 1.9 (height 320 mm, diameter 170 mm) at the full working volume of 2.5 L, and the decant/feed volume was 60% of the total volume for each cycle. The reactor operated in predefined cycle using a Programmable Logic Controller (PLC) at the room temperature. A mechanical stirrer (60 mm × 130 mm × 2 ea) was used for mixing in the reactor during the operation (about 35% of the apparent volume). The reactor was stirred at 35 ± 10 r/min (diameter one-third of the reactor diameter). As a seed sludge, activated sludge obtained from the Seoul Joongrang Municipal Wastewater Treatment Plant was used. Influent of both reactors was let in 3 times a day at an 8-hr interval at the rate of 3.4 L/cycle. Each cycle was consisted of 0.5 hr feeding, 2.5 hr anaerobic cycle, 4 hr oxic (PAO SBR) or anoxic (DPAO SBR) cycle, 0.5 hr settlement, and 0.5 hr decant. For the aerobic condition in the PAO SBR operation, an air stone with diameter 140 mm and air pump (LP-40A, Yong-nam Co.) were installed for aeration, so that the flow rate was 20 L/min. The aeration was maintained to oxic DO concentration of about 2–3 mg/L. DPAO SBR fed with 100 mL potassium nitrate solution (7.21 g KNO3/L) for 15 min after the anaerobic condition. The solid retention time (SRT) maintained to 20 days during the steady-state operation based on the measured total suspended solids (TSS) and effluent TSS concentrations in both SBRs.

1.2. Synthetic feed

Synthetic wastewater based on propionic acid (HPr) as a sole carbon source, NH4Cl as a nitrogen source and KH2PO4 for a phosphate source was used to construct the influent. Then, 0.1 mL/L trace metal solution to the influent was added (Weng and Molof, 1974). For the single carbon source, sodium propionate (150 mg/L as COD), was used. HPr can be used to minimize the growth of glycogen accumulating organisms (GAOs) known to impede the EBPR when using acetic acid (HAc) as a carbon source, because of the difference in substrate affinity (Lopez-Vazquez et al., 2009; Oehmen et al., 2006). For nitrogen supply, 10 mg/L NH4-N was added to the influent for DPAO SBR, taking account of microbial synthesis, and 15 mg/L NO3-N was supplied from outside in order for DPAO to use it as electron acceptor in the anoxic condition. In the case of PAO SBR, 25 mg/L NH4-N was added.
to the influent for the similar C/N/P ratio with the DPAO SBR. The final pH of influent was 7.2–7.3 (Table 1).

1.3. Chemical analysis and microscopic analysis

Soluble COD, NH$_4^+$-N, PO$_4^{3-}$-P, TSS, and VSS were applied in compliance with the experimental methods according to Standard Methods (APHA, 2005). NO$_3^-$-N and NO$_2^-$-N were measured by ion chromatography (IC-80, Dionex™, USA). Batch tests of two laboratory-scale SBRs in stable operation were conducted for measuring specific phosphorus release rates (SPRRs), specific phosphorus uptake rates (SPURs). The mixture of anoxic and aerobic sludge was used for the batch experiment. 200 mg/L of HPr was added as a sole carbon source. For 2.5 hr, anaerobic condition was maintained for P release. Subsequent to anaerobic condition, air and nitrate were injected for anoxic and aerobic conditions, respectively, to determine the anoxic and aerobic uptake rates. DO was maintained to 2–3 mg/L. 20 mg/L of nitrate was added as electron acceptor for DPAO. Morphological characteristics of sludge were examined by an optical microscope (JSB-133, Green Sci., South Korea) and an environmental scanning electron microscope (ESEM, FEI XL-30 FEG; Philips), after sludge taken from the PAO and DPAO reactor at the anoxic stage, respectively, was harvested by centrifugation (10,000 × g) for 10 min. For ESEM, samples were fixed to the sample holder with silver paste for precise measurements. The granule core was analyzed by X-ray diffraction (XRD) (TTK-450 model, Anton Paar, Austria).

1.4. FISH analysis

FISH was used to observe DPAO and PAO sludge taken form the steady-state operation in both SBRs. Cells were pelleted by centrifugation (12,000 × g, 10 min). The pellet was rinsed twice with 0.01 mol/L phosphate buffered saline (PBS, pH 7.4) and resuspended in 100 μL PBS. Next, 300 μL 4% paraformaldehyde solution was added, and immobilization was allowed to proceed for 2 hr at 4°C. After immobilization, the fixation solution was removed through 3 cycles of centrifugation and rinsing with PBS. Then, the samples were stored at 20°C after the addition of chilled ethanol/PBS (1:1) solution.

Next, 20 μL immobilized cells were put onto a well glass slide and air-dried. To induce dehydration after immobilization, the cells were passed through an ethanol series (50%, 80%, and 99% ethanol) for 3 min each time, after 10 μL hybridization buffer (0.9 mol/L NaCl, 20 mmol/L Tris–HCl, 0.01% sodium dodecyl sulfate [SDS], 1 mmol/L ethylenediaminetetraacetic acid [EDTA], 30% formamide) was added. Using a pre-prepared probe (Table 2), hybridization was performed in a hybridization chamber at 48°C for 120 min (probe concentration, 25 ng/μL). Once the hybridization was completed, the sample was rinsed for 30 min at 46°C with preheated washing buffer (20 mmol/L Tris–HCl, 0.01% SDS, NaCl) and deionized distilled water. Next, the sample was air-dried and mixed with 10 μL mounting medium, and then a cover slide was placed on it. The sample slide transported to a Zeiss Axioshot Epifluorescence microscope equipped with an HBO100 mercury vapor short-arc lamp and Axio-Cam MRm digital camera (Carl Zeiss, Germany) for measurement. Micrographs were analyzed using an Axiovision Version 4.5 software package (Carl Zeiss).

1.5. DNA extraction

Sludge samples were collected for microbial community analysis from the after about 120 days when stable EBPR performance was observed in the DPAO SBR and the PAO SBR. DNA extraction was performed with Blood & Tissue™ DNA Kit (Mo Bio Lab., Inc.). Microbial DNAs were extracted from 200 μL of bacterial sample taken from PAO SBR and DPAO SBR, respectively. Humic acid components released during DNA extraction were removed using the DNA purification kit. The extracted DNA concentration was then measured by Ultraviolet absorbance spectrophotometry at 260 nm. Some of the extracted DNA was freeze-stored at −20°C for further analyses.

1.6. Pyrosequencing and taxonomic analysis with data processing

Pyrosequencing and taxonomic analysis were performed with reference to Kim et al. (2010). PCR (PCR System 9700; Applied Biosystems) was performed using 1 μL DNA as template. PCR was performed beginning with 5 min initial denaturation at 94°C, followed by 30 sec denaturation at 94°C, 45 sec annealing at 55°C, 30 cycles of 90 sec extension at 72°C, and finally, holding at 4°C. In order to verify the successful PCR performance, DGGE was conducted beginning with 50 V for 50 min. After mixing the PCR products obtained from each sample, PCR Purification Kit (QIAAGEN, Cat. No. 28106) was employed for purification. With the Quant-iT™ PicoGreen dsDNA Assay Kit (P-11496; Invitrogen), the PCR product was purified with TBS-380 Mini-Fluorometer (Turner BioSystems) and quantified. The quantified sample was mixed with each DNA in an equal amount in a 1.5-mL Eppendorf tube, and electrophoresis was applied.

Table 1 – Influent characteristics of synthetic wastewater.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Influent concentration</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAO SBR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCOD (mg/L)</td>
<td>147.1 ± 20.0</td>
<td>Sodium propionate as a sole carbon source</td>
</tr>
<tr>
<td>NH$_4^+$-N (mg/L)</td>
<td>23.9 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>NO$_3^-$-N (mg/L)</td>
<td>0.2 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>PO$_4^{3-}$-P (mg/L)</td>
<td>6.2 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>DPAO SBR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCOD (mg/L)</td>
<td>144.1 ± 31.8</td>
<td>Sodium propionate as a sole carbon source</td>
</tr>
<tr>
<td>NH$_4^+$-N (mg/L)</td>
<td>9.0 ± 3.1</td>
<td></td>
</tr>
<tr>
<td>NO$_3^-$-N (mg/L)</td>
<td>0.2 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>External NO$_3^-$-N feed (mg/0.1 L)</td>
<td>95 ± 3.8</td>
<td>After the injection, the average NO$_3^-$-N concentration in the reactor was 15.8 ± 3.2 mg/L</td>
</tr>
<tr>
<td>PO$_4^{3-}$-P (mg/L)</td>
<td>6.0 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Trace metal</td>
<td>* (mL/L)</td>
<td>0.1</td>
</tr>
</tbody>
</table>

*a FeCl$_3$·6H$_2$O 1.5 g/L, MnSO$_4$·H$_2$O 1.5 g/L, ZnSO$_4$·H$_2$O 0.1 g/L, CaCl$_2$·2H$_2$O 0.1 g/L, and [(NH$_4$)$_6$MnO$_2$·5H$_2$O 0.1 g/L.
to 1 μL pooled DNA. Gel extraction (QIAquick Gel Extraction Kit (QIAGEN, Cat. No. 28706)) was performed because short DNA fragments (<300 bp) were found. A final bioanalyzer QC (Agilent) was performed prior to sequencing. Then, the sequencing was performed by capturing beads and primer annealing after emulsion-based clonal amplification (2720 Thermal Cycler; Applied Biosystems) of the DNA library sample.

A raw sff file was generated for each sample through barcode sorting (read lengths with barcoding below 300 were excluded from analysis). Primer and linker sequences within each read were removed. The reads with a length below 300 after the removal of primer and linker sequences were excluded from analysis. Additionally, a pairwise alignment of the primer sequence part of each read and the primer

![Table 2 – Probe and sequence used for FISH analysis.](image)

<table>
<thead>
<tr>
<th>Probe</th>
<th>Sequence (5’-3’)</th>
<th>Target organism</th>
<th>Helper(s) and/or competitor(s)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EUB338I&lt;sup&gt;a&lt;/sup&gt;</td>
<td>GCTGCCCTCCGCTAGGAGCT</td>
<td>Eubacteria</td>
<td></td>
<td>Amann et al. (1995)</td>
</tr>
<tr>
<td>EUB338II&lt;sup&gt;a&lt;/sup&gt;</td>
<td>GCAAGCCACCCTAGGAGTG</td>
<td>Eubacteria</td>
<td></td>
<td>Daims et al. (1999)</td>
</tr>
<tr>
<td>EUB338III&lt;sup&gt;a&lt;/sup&gt;</td>
<td>GCTGCCACCCGCAAGGTTG</td>
<td>Eubacteria</td>
<td></td>
<td>Crocetti et al. (2000)</td>
</tr>
<tr>
<td>PAO651&lt;sup&gt;a&lt;/sup&gt;</td>
<td>CCCCCCCCTTCAAACCCAG</td>
<td>Ca. Accumulibacter</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAO462&lt;sup&gt;a&lt;/sup&gt;</td>
<td>CCCCTCTATCATCWGGGTATTAC</td>
<td>Ca. Accumulibacter</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAO846&lt;sup&gt;a&lt;/sup&gt;</td>
<td>GTTGTATCCGCACTAAAAGG</td>
<td>Ca. Accumulibacter</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acc444</td>
<td>CCGAACAGATTTCTTCCC</td>
<td>Acc-SG1</td>
<td>HAcc462 and HAcc426</td>
<td>Kim et al. (2010)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Used as EUBmix and PAOmix, respectively

Fig. 1 – SCOD, PO<sub>4</sub>3−-P, NH<sub>3</sub>-N, and NO<sub>3</sub>-N profiles in the (a) DPAO SBR and (b) PAO SBR. Phase I indicates the acclimation period, Phase II shows the period of stable EBPR observation, and Phase III represent the period after the sludge granulation within the reactor. DPAO: denitrifying phosphorus accumulating organism; PAO: phosphorus accumulating organism; SCOD: soluble chemical oxygen demand; SBR: sequencing batch reactor; EBPR: enhanced biological phosphorus removal.
sequence used in the experiment was conducted taking into account degeneracy, whereby the reads with mismatch exceeding a specified mark were excluded from analysis. Using the 16S rRNA profile composed of Hidden Markov Model (HMM), non-16S reads were excluded. The data used for generating profiles were those used in setting up the EsTaxon database. Afterward, non-16S reads were removed via BLAST search, thus verifying the HMM-removed non-16S reads. Individual reads were divided into subsets, and the 2 short reads thus divided were subjected to BLAST searches separately; these were removed on the basis of chimera formation. Ultimately, individual reads were identified based on similarity by using the EzTaxon database, and statistical analysis was performed.

2. Results

2.1. EBPR performance

Fig. 1 illustrates the profiles of soluble chemical oxygen demand (SCOD), PO$_4^{3-}$-P, NH$_4^+$-N, and NO$_3^-$-N in DPAO SBR and PAO SBR over the entire duration of operation. In DPAO SBR (Fig. 1a), EBPR was observed at an initial stage of operation, although in insignificant amounts, and then tended to become unstable (Phase I). After about 100 days, however, with the drastic growth of phosphate release under anaerobic conditions, we observed that the reactions of anoxic P uptake and NO$_3^-$-N reduction occurred simultaneously in a stable manner (Phase II). About 200 days after the operation began, the generation of sludge granulation was visualized within the reactor (Phase III). While the denitrifying EBPR continued to be stable at this time, the P release decreased by approximately 15% under anaerobic conditions compared to the level observed prior to the formation of granules. In the case of PAO SBR (Fig. 1b), after unstable EBPR at the initial stage (Phase I), stable EBPR was observed from about 80 days onward (Phase II). After about 250 days of operation, the formation of sludge granulation was also observed with a granule size of about 1/5 compared to DPAO SBR (Phase III). Once the granulation began, as in the case of DPAO SBR, anaerobic P-release tended to be decreased, but the performance of operation remained stable.

2.2. Sludge granulation and the morphological characteristics

Sludge granulation has been observed in both SBR systems. Fig. 2 shows the change of granule size as a function of SBR operating time in PAO SBR and DPAO SBR. In PAO SBR, granules were observed with approximately 0.2 mm in diameter at around 250 days after the operation began, and then they increased up to 0.5 mm at 450 days (Fig. 2a). In DPAO SBR, bigger granules were observed such as up to 2.5 mm in diameter at 490 days but they have been observed at first with approximately 0.5 mm at around 220 days (Fig. 2b). PAO and DPAO granules were ellipsoidal with a brown color, and they had the dense and hard physical characteristics.

Fig. 3 shows images of granules as observed with a 40× electron microscope in DPAO SBR and PAO SBR after 480 days of operation, and ESEM images measured in order to determine morphological characteristics of microbes on the granule surface. The granules in DPAO SBR were approximately 5 times bigger than those in PAO SBR (Fig. 3a and b). In addition, on granule surfaces, a large amount of EPS materials was found to cover the microbes. Rod-shaped microbes were
mainly found in the DPAO granular sludge (Fig. 3c), and coccus-shaped microbes were found in PAO granular sludge (Fig. 3d).

Fig. 4 shows typical images of DPAO granules for more detailed observation on granule surface and inside of the granule. A DPAO granule had the appearance of a round sphere as shown in Fig. 4a. In the surface of the granule, many rod-shaped microorganisms existed and they were covered with extracellular polymeric substance (EPS) (Fig. 4b). A mature granule in DPAO SBR was cut in central slice (Fig. 4c and e), it was found that inorganic precipitates occupied in a significant fraction of total volume of the granule, mainly close to center (Fig. 4c and f). Recent studies (Angela et al., 2011; Wu et al., 2010; Zhang et al., 2011) on EBPR of PAO under alternating anaerobic–oxic conditions have also observed sludge granulation although sludge granulation under alternating anaerobic–anoxic conditions has not been reported yet. Interestingly, of their researches, Manas et al. (2012) found inorganic materials crystallized in a hydroxyapatite form (HAP, Ca_{5}(PO_{4})_{3}OH) in the interior of aerobic granular sludge, whereas in our study, the precipitate was found in the interior of granular sludge only in DPAO SBR. From the X-ray diffraction (XRD) analysis in Fig. 5, the precipitate in the core of granule revealed a major peak coinciding with the HAP spectrum (Entry # 96-901-3628 in Crystallography Open Database). Inorganic precipitates in PAO granule has not been observed yet.

2.3. Abundance of Accumulibacter clade I in the PAO and DPAO sludges

Fig. 6 shows the FISH images of DPAO sludge and PAO sludge. In Fig. 6a and b, Accumulibacter (yellow) are rendered visible against all microbes (green) by using EUBmix and PAOmix probe. In Fig. 6c and d, Accumulibacter clade I (yellow) is highlighted against Accumulibacter (green) by using PAOmix and Acc444 probe. Considering the total population, the portion of Accumulibacter, which was the most common PAO, was higher in both DPAO and PAO sludge. Nevertheless, Accumulibacter clade I was found in a higher proportion only in DPAO sludge, leading us to conclude that in the PAO sludge, a distinct type of Accumulibacter was involved in EBPR. This means that Accumulibacter clade I can use both O_{2} and NO_{3}^{-} as electron acceptors. Moreover, our results are consistent with those of Carvalho et al. (2007) and Oehmen et al. (2010), in which Accumulibacter clade I within EBPR sludge was proved to one of main players in the denitrifying EBPR system.

2.4. Pyrosequencing-based comparative analysis of microbial populations

Fig. 7 reveals the relationship between the total valid reads and operational taxonomic unit (OTU) to ascertain the richness of microbial species in PAO SBR and DPAO SBR.
sludge shows a higher species richness than DPAO sludge, maybe attributing to the fact that PAO SBR, in addition to EBPR, had a relatively variety of microbes with nitrifying and denitrifying functions compared to DPAO SBR.

Fig. 8 reveals the phylum-class comparison between PAO sludge and DPAO sludge. X-axis represents microbial groups by class, and their corresponding phyla are listed on the upper end of the graph. In DPAO sludge under alternating anaerobic–anaerobic conditions (Fig. 8a), with anaerobic microbe Bacteroides (Madigan et al., 2003), class FP245540_C belonging to OP3 phylum group having a function of anaerobic respiration (Glöckner et al., 2010) accounted for up to 55.6% of total bacterial population. \( \beta \)-Proteobacteria involving mostly EBPR-able microbes were the most abundant after FP245540_C and accounted for 23.3% of the population.

In the case of PAO SBR operated under alternating anaerobic–anaerobic conditions (Fig. 8b), Nitrospira having a function of nitrification (Cebon and Garnier, 2005; Park and Noguera, 2008) and the facultative anaerobe Cytophagia (Mergaert et al., 2001) were observed in the proportion of 7.0% and 12.5%, respectively. \( \beta \)-Proteobacteria in the PAO sludge accounted for the majority (70.4%) of the population, which is substantially higher than that in the DPAO sludge (23.3%).

Table 3 shows the comparison of microbes at the genus level within 6-proteobacteria, and Fig. 9 represents the summary of microbial species involved in Rhodocyclaceae and Zoogloeae between PAO and DPAO sludges in terms of % abundance. The genera Accumulibacter and Dechloromonas are within the family of Rhodocyclaceae belonging to the Betaproteobacteria class. It is well known that these two bacteria play a key role in EBPR in

Fig. 4 – ESEM images of (a) DPAO granule (×25), (b) enlarged view of granule surface (×1000), (c) cross sectional view of DPAO granule (×30), (d) enhanced view of the core precipitate inside granule, and microscopic photos of (e) cross sectional view of a DPAO granule and (f) precipitate from the core of a granule.
laboratory-scale systems (Crocetti et al., 2000; Hesselmann et al., 1999; Liu et al., 2001) and full-scale plants (Kong et al., 2004; Saunders et al., 2003; Wong et al., 2005; Zilles et al., 2002). Goel et al. (2005) reported that *Dechloromonas* functions together with *Accumulibacter phosphatis* in the PAO sludge. Consistent with this result, we observed 14.2% *Accumulibacter* in PAO sludge, and *Dechloromonas*-associated microbes were measured at 3.2%.

Fig. 5 – X-ray diffraction (XRD) result from the crystals within the core of the DPAO granular sludge. The experimental pattern is consistent with HAP (Entry # 96-901-3628 in Crystallography Open Database).

Fig. 6 – FISH images of the DPAO and PAO sludges. Fluorescence in situ hybridization (FISH) image showing bacteria hybridized with PAO\textsubscript{mix} (yellow) and EUB\textsubscript{mix} (green) in the (a) DPAO sludge and the (b) PAO sludge PAO\textsubscript{mix} (green) and Acc444 (yellow) in the (c) DPAO sludge and the (d) PAO sludge.

Fig. 7 – Rarefaction curve for estimating species richness.
Interestingly, there were differences in Zoogloea, Thauera sp., and Accumulibacter spp. belonging to 6-proteobacteria in DPAO sludge compared to those in PAO sludge, with the exception of Dechloromonas. For example, both genera Zoogloea and Thauera sp. belonging to the same family of Zoogloea have a denitrifying function (Juretschko et al., 2002; Liu et al., 2006), however, the aspect of their existence in DPAO sludge and PAO sludge are significantly different. A genus of Zoogloea accounted for 0.1% of the total microbial population in the DPAO sludge, while that was 42.2% in the PAO sludge. On the other hand, a genus of Thauera sp. in the DPAO sludge contained at a fraction of 9.6%, whereas none of that was found in PAO sludge. Zilles et al. (2002) reported that the Thauera sp. also possesses an EBPR function, and it is, therefore, highly likely that it performs both denitrification and EBPR. Moreover, Accumulibacter spp. comprised only 1.9% of DPAO sludge while 14.2% of PAO sludge. Accumulibacter phosphatis was found at 9% in the PAO sludge but was observed at only 0.8% in the DPAO sludge, indirectly demonstrating that A. phosphatis had not an important role for DPAO system.

![Fig. 8](image-url) – The comparison of class communities between (a) the DPAO sludge and (b) the PAO sludge.

### Table 3 – Comparison of genus-level population structure within Betaproteobacteria between PAO and DPAO sludges.

<table>
<thead>
<tr>
<th>Class</th>
<th>Order</th>
<th>Family</th>
<th>Genus</th>
<th>% Abundance</th>
<th>Valid Reads</th>
</tr>
</thead>
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In PAO sludge, nitrifying microbes accounted for 9.2% of the population, which is a relatively large proportion; most of those were found to be *Nitrospira* spp. Additionally, *Defluviicoccus* and *Cytophagales* spp., which are GAOs (Burow et al., 2007), were partially observed in the PAO sludge. In the DPAO sludge, EBPR-able microbial genera occupied 6.8%, and 20.8% in the PAO sludge. Considering the role of *Thauera* that has both denitrification and EBPR functions, the proportion of EBPR-able microbial genera in the DPAO sludge increases up to 16.4%, showing little difference from the PAO sludge. Finally, *Thauera* has a high possibility of being a microbe capable of P uptake under anoxic condition by using NO₃⁻. Moreover, we found *Dechloromonas* spp. in both PAO and DPAO sludge; hence, we consider that it can use both O₂ and NO₃⁻ as electron acceptors.

### 3. Discussion

#### 3.1. EBPR behavior and sludge granulation

It has been shown that successful performance of EBPR in both PAO and DPAO SBR systems can be achieved (Ahn et al., 2007; He et al., 2007; Kim et al., 2013; Kong et al., 2004). Those many studies, however, did not provide the detailed information on the duration of reactor operation required for the stabilization of EBPR (or especially denitrifying EBPR) as well as the changes in sludge characteristics for a long-term operation. In this current study, two characteristic changes have been observed during about 500 days of operation (Fig. 1).

Firstly, both SBR systems exhibited not only a stable EBPR in PAO SBR at around 80 days, but also denitrifying EBPR in DPAO SBR after about 100 days of operation. In the DPAO SBR, 5.8 ± 0.2 mg PO₄³⁻-P/L of flowed in, the PO₄³⁻-P exhibited a tendency to stabilize, and it increased to more than 31.3 mg/L in approximately 100 days after the operation. PO₄³⁻-P concentration at the end of the anoxic condition tended to remain relatively consistent throughout the operation period (2.4 ± 1.5 mg PO₄³⁻-P/L) (Fig. 1a). In the PAO SBR, influent concentration averaged to 5.5 mg PO₄³⁻-P/L during the operation period. After the operation of the anaerobic condition, 38.7 mg/L of phosphate was released on average, and phosphate was accumulated to 1.0 mg/L at the end of the aerobic condition (Fig. 1b). The P release began to increase rapidly during the initial operation, and after approximately 80 days, it was confirmed to have stabilized to more than 40 mg/L. Although the phosphate concentration in anaerobic condition of PAO SBR was exhibited a tendency to higher than that of DPAO SBR, SPPR in the DPAO SBR (21.35 ± 2.2 mg P/(g VSS·hr)) and the PAO SBR (21.09 ± 3.9 mg P/(g VSS·hr)) were similar. SPUR under anoxic and aerobic conditions were 25.78 ± 3.8 mg P/(g VSS·hr) in DPAO SBR and 24.89 ± 2.3 mg P/(g VSS·hr) in PAO SBR, respectively, which were also similar. This means that EBPR activity of DPAO is not significantly different from that of PAO. Although Kuba et al. (1996) and Henze et al. (1999) reported that the anoxic P uptake ability is approximately 60% of aerobic P uptake because of electron transport phosphorylation between O₂ and NO₃⁻, the other reported that there was little difference between maximal anoxic and aerobic P uptake rates (Kerrn-Jespersen and Henze, 1993; Lee, 2013). These conflicting results are thought to depend on the operation condition (e.g., influent VFA conc. and recycled NO₃ conc. etc.) and the operating sequence (e.g., anaerobic/anoxic/oxic, anaerobic/anoxic, and anaerobic/oxic).

Secondly, sludge granulation has been visually observed within the reactor after about 200 and 250 days of operation in DPAO and PAO SBRs, respectively. In particular, after the occurrence of granulation, P release at the anaerobic condition was reduced by about 15% in both SBRs. This result was consistent with the comparative study on EBPR performance of flocculent and granular PAO sludge by Li et al. (2012),
which demonstrated that P release rate was decreased in the granular PAO system.

Granular sludge can be considered as a special case of biofilm growth with a three-dimensional and more complex structure, in which microbes are attached to each other and embedded in an extracellular matrix, with different functional microbial populations located in different spaces (Adav et al., 2008). Generally, aerobic heterotrophic microorganisms and some autotrophic microorganisms such as nitrifying bacteria reside in the outer layers of granular sludge, while facultative or anaerobic bacteria such as denitrifiers exist in the inner parts (Winkler et al., 2013). In millimeter sized granules, aerobic and anoxic/anaerobic microenvironments are maintained due to microbial respiration in the outer region of granule along with diffusion limitation (Nancharaiah and Kiran Kumar Reddy, 2018). Presence of different redox conditions in a single granule allow occurrence of simultaneous nitrification and denitrification process even when aeration is on (Nancharaiah et al., 2016; Coma et al., 2012). The parameters that can influence P release include DO in the bulk liquid, size of granules, electron donor availability and microbial activity. Accordingly, the size of flocs or granules, oxygen concentration and COD/P ratio, are able to lead to substrate-rich aggregates with an oxygen-free zone in the interior where the EBPR process can be activated. So, in granular sludge, the P release would be carried on more easily than that can be seen in flocculent activated sludge.

The difference of granular size between PAO and DPAO sludge was also an interesting result. As shown in Figs. 2 and 3, the size of DPAO granular sludge has been increased up to 2.5 mm at 490 days, while PAO granular sludge has been grown slowly and the granule size was 0.5 mm at around 450 days. In ordinary anaerobic and/or aerobic granulation, EPS are known to play a critical role (McSwain et al., 2005). A variety of additional factors also influence sludge granulation, including flow patterns (Liu and Fang, 2002), height-to-diameter ratio of reactor (Beun et al., 1999; Kong et al., 2009), volume exchange ratio (Liu et al., 2005), organic loading rate (Barbusinski and Koscielniak, 1995), shear force (Liu and Tay, 2002), settling time (Gao et al., 2011; Qin et al., 2004), and starvation phase (Liu and Tay, 2008). In our study, there were distinguished characteristics between two SBR systems among the many factors influencing sludge granulation; (1) shear force and (2) starvation phase. According to Dulekgurgen et al. (2008), hydrophobicity and EPS production increased as hydraulic shear force decreased. Hydraulic shear force in both systems was different because of no aeration in DPAO SBR, indicating DPAO SBR had a lower shear force than PAO SBR in which both mechanical mixing and aeration existed. Starvation phase was also different in PAO and DPAO SBRs. Unlike limited NO3 injection at the anoxic condition in DPAO SBR, starvation time under continuous O2 supply as an electron acceptor could be more reduced in PAO SBR than DPAO SBR. Tay et al. (2001) reported that starvation phase makes bacteria more hydrophobic, which promote the granulation from flocs.

Of the recent other studies that observed sludge granulation in anaerobic–oxic SBR (Angela et al., 2011; Wu et al., 2010; Zhang et al., 2011), Angela et al. (2011) reported that the crystal as hydroxyapatite (HAP) was found in the core of the 2 mm-diameter granule. In this study, HAP was also observed in the core of the 2.5 mm-diameter granule in DPAO SBR (Figs. 4 and 5), not in PAO SBR. In activated sludge system, biologically induced phosphate precipitation has been reported (De Kreuk et al., 2005). Some researchers (Saidou et al., 2009; Zhu et al., 2007) also reported that the formation such as calcium phosphate not only can be caused by P release under the anaerobic condition, but also depends on pH. According to other studies (Bellier et al., 2006; Manas et al., 2012), pH plays an significant role and phosphate precipitation is preferred at pH 7.0–8.5. In our case, in DPAO SBR, pH first decreased from 8.5 to 8.0 at the anaerobic condition and then increased to 8.6 at the anoxic condition. However, in PAO SBR where the precipitates have not been observed yet, average pH at the end of the anaerobic and oxic conditions was 7.0 and 7.3, respectively. Difference of pH between DPAO and PAO SBRs was due to the different biological reactions in each system. Moreover, bioreactions such as nitrification, denitrification, and EBPR lead to pH gradients which can be responsible for precipitation in activated sludge. So, it reveals that pH influenced stronger for the HAP formation in DPAO granules than that in PAO granules.

3.2. Characterization of microbial population between PAO and DPAO sludge

It has been well known that rod-shaped Accumulibacter is responsible for denitrifying EBPR (Carvalho et al., 2007; Flowers et al., 2009; Lanham et al., 2011; Oehmen et al., 2010), and coccus-shaped Accumulibacter can use only O2 as an electron acceptor (Carvalho et al., 2007; Guisasola et al., 2009). These were consistent with our results that confirmed the morphological differences between PAO and DPAO sludges from ESEM (Fig. 4c and d) and FISH (Fig. 6c and d) observations. Also, FISH analysis using Acc444 and PAOmix probe showed that the relative abundance of Candidatus Accumulibacter phosphatis (called “Ca. Accumulibacter”) was observed at a high proportion in only DPAO sludge as with the previous studies (Kim et al., 2013; Oehmen et al., 2010). However, morphologies and phylogenies of Ca. Accumulibacter may be different even within the same Ca. Accumulibacter clade (Kim et al., 2013), and in addition to genus-Accumulibacter, Rhodoychys- and Dechloromonas-related microorganisms have been also reported as a potential DPAO candidate (Crocetti et al., 2000; Goel et al., 2005; Kong et al., 2007; Wong et al., 2005). Moreover, Barak and van Rijn (2000) and Zilles et al. (2002) reported the possibility of P removal by denitrifier. These results indicate that the microorganism associated with denitrifying EBPR could vary. Therefore, we hypothesized that (1) various microbes related with denitrifying EBPR could exist besides the genera of Accumulibacter, and (2) DPAO candidates could be distinguished through the quantitative comparison between PAO and DPAO sludge.

After 470 days of operation, the pyrosequencing analysis summarized in Table 4 showed that genera of Accumulibacter in DPAO sludge accounted for only 1.9% of total bacterial population, while PAO sludge contained at a fraction of 14.2%. Especially, A. phosphatis involved in genus-Accumulibacter were observed at a high proportion of 9% in PAO sludge, but measured at only 0.8% in DPAO sludge. Rod-shaped Ca. Accumulibacter at the anoxic condition can use NO3 as electron acceptor (Carvalho et al., 2007; Guisasola et al., 2009), as well...
as Ca. Accumulibacter clade IA have a function with EBPR and nitrate reduction simultaneously (Carvalho et al., 2007; He et al., 2007; Kim et al., 2010, 2013), indicating that A. phosphatis in our observation was seen as the microbial group belonging to Ca. Accumulibacter clade IA. Carvalho et al. (2007) and Kim et al. (2013) reported that A. phosphatis was dominant when they operated the anaerobic–anoxic SBR after the microbial adaptation with anaerobic–oxic condition. However, in this study, the reason for big difference of A. phosphatis property between both sludges was supposed to be hardly predominant under alternating only anaerobic–anoxic condition with the absence of O2, even though A. phosphatis could use both O2 and NO3 as a final electron acceptor.

The genera of Dechloromonas belonging to the family of Rhodocyclaceae are suspected strongly as one of the microbial groups having the role for denitrifying EBPR since they could use NO3 as an alternative electron acceptor (Achenbach et al., 2001; Coates et al., 1999) and accumulate PHA into the cell at the anaerobic condition (Ahn et al., 2007). Also, Kim et al. (2013) reported that the relative abundance of Dechloromonas was increased from 1.2% to 19%, while A. phosphatis were decreased from 55.1% to 29.2% as SBR operation was gradually acclimated from anaerobic–oxic to anaerobic–anoxic–oxic conditions by increases of nitrate concentration and the anoxic time. It means not only increased role of Dechloromonas but also reduced role of Accumulibacter in the process converted from PAO to DPAO. In this study, the genera of Accumulibacter occupied for only 1.9% in DPAO sludge and Dechloromonas observed at relatively higher proportion of above 3% in both sludges, indicating that Dechloromonas could use O2 and NO3 simultaneously as an electron acceptor.

Finally, the possibility of genus-Thauera within the family of Rhodocyclaceae as a DPAO candidate should be mentioned in this research. With a function of nitrate reduction (Liu et al., 2006), the genera of Thauera had a possibility for anoxic P uptake (Zilles et al., 2002) with PHA production (Majone et al., 2006; Yang et al., 2013). Also, Kong et al. (2004) reported that Rhodocyclus-related PAO including Thauera were able to denitrify. In this study, the genera of Thauera were observed in only DPAO sludge at a fraction of 9%, strongly indicating that Thauera could be one of DPAO candidates.

4. Conclusions

For approximately 500 days, we investigated the changes in EBPR performance and microbiological characteristics when PAO SBR and DPAO SBR were operated for prolonged times. In both reactors, unstable EBPR was observed at the initial stage of operation, but after about 3 months, stable EBPR was exhibited successfully. After 200 and 250 days of operation of PAO SBR and DPAO SBR, respectively, the phenomenon of sludge granulation was observed, and the size of DPAO granular sludge was 5 times larger than that of PAO granular
sludge. Furthermore, at the core of granular sludge, crystallized inorganic materials were detected as HAP. FISH analysis revealed that in the DPAO sludge Accumulibacter type I microbe constituted the majority of the total Accumulibacter, but in the PAO sludge, Accumulibacter type I was practically absent. Based on the pyrosequencing analysis, Dechloromonas and Thauera spp. appear to have both denitrification and EBPR functions, and we conclude indirectly that they are microbial groups suitable as DPAO candidates.

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References


