Changes in phosphorus removing performance and bacterial community structure in an enhanced biological phosphorus removal reactor

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Abstract

A lab-scale-enhanced biological phosphorus removal (EBPR) reactor was operated for 204 days to investigate the correlation between phosphorus removing performance and bacterial community structure. The phosphorus removing performance was good from day 1 to 92 and from day 172 to 204. However, the removal activity was in a deteriorated state from day 93 to 171. From day 69 (2 weeks before the beginning of the deterioration) to 118 (2 weeks after the beginning of the deterioration), sludge P content decreased. The amounts of ubiquinone-8 and menaquinone-8 (H$_4$) decreased during this period while the amount of ubiquinone-10 increased. The comparison of these changes and the general attribution of each quinone to the bacterial phylogenetic groups suggested that beta proteobacteria and Actinobacteria contributed to EBPR positively, and that alpha proteobacteria were related to this EBPR deterioration. Glycogen accumulating organisms (GAOs) are considered to detrimentally affect EBPR ability by outcompeting the phosphorus accumulating organisms by using aerobically synthesized glycogen as the energy source to assimilate organic substrates anaerobically to form polyhydroxyalkanoates. However, in this research, there was nearly no substrate uptake during the anaerobic period at the middle of the deteriorated performance period. This suggests that the deterioration observed in this research does not agree with the GAOs inhibition model. In this research, the excess P release at the anaerobic period was concluded to cause the deterioration.

Keywords: EBPR; Bacterial community changes; Quinone; PAOs; GAOs; Deterioration

1. Introduction

Phosphorus (P) contained in domestic, industrial and agricultural wastewater often causes eutrophication in closed water area, such as lakes and inland seas. P in the wastewater can be removed chemically and biologically in wastewater treatment plants. The enhanced biological P removal (EBPR) system with a characteristic array of an anaerobic basin followed by an aerobic has an economical advantage of lower sludge production and less use of chemicals. The EBPR system is therefore widely used all over the world.

In EBPR systems, polyphosphate accumulating organisms (PAOs) are thought to play a significant role in phosphorus removal. The first isolated from an EBPR system were \textit{Acinetobacter} species \cite{1}. In the conventional culture-dependent technique, \textit{Acinetobacter}
species have been found to be dominant among polyphosphate accumulating isolates from EBPR sludge [2]. However, using culture-independent analytical tools for bacterial community study, such as fluorescent antibody, fluorescence oligonucleotide probe, and quinone profiling, it was found that the number of Acinetobacter species in the sludge was much smaller than those reported in literature [3–6] and the nutrient-rich medium used in these studies strongly favored the growth of Acinetobacter species [6]. Subsequently, many culture independent studies were carried out.

Based on a comparison of the bacterial 16S rDNA clone libraries from P-removing and non-P-removing sludge, Bond et al. [7] suggested that bacteria closely related to the genus Rhodocyclus are involved in P removal. Crocetti et al. [8] found the high correlation between the number of Rhodocyclus relatives and the sludge P content using fluorescence probes for Rhodocyclus relatives. Although the presence of bacteria related to the genus Rhodocyclus have been demonstrated in laboratory and plant sludge analysis [9,10], some have reported the simultaneous involvement of bacteria other than Rhodocyclus relatives in EBPR [11,12]. The bacteria that play an important role in EBPR systems are not yet fully understood. It was reported that EBPR system sometimes loses P removing activity, and no way of overcoming this instability has been found owing to lack of knowledge of the microbiological mechanism in EBPR systems [13,14]. More studies are necessary to understand the microbial community in the EBPR systems.

GAOs are usually assumed to cause the deterioration of EBPR by outcompeting PAOs by using aerobically synthesized glycogen as the energy source to assimilate organic substrates anaerobically to form polyhydroxyalkanoates [14,15]. Recently, Fang et al. [16] reported that no substrate uptake was observed while the EBPR performance was in a deteriorated state. However, in his study no EBPR had been established throughout the operation. It has never been reported that sludge that once had a high P removal performance showed almost no total organic carbon (TOC) decrease during the anaerobic period when its P-removal activity deteriorated. This does not agree with the inhibition model by GAOs.

In this study, we operated an EBPR reactor for 204 days to obtain information on the bacterial community structure changes by using quinone analysis to find the correlation between community structure and P removal activity in the EBPR sludge. Consequently, the following results were obtained: (1) the community structure and performance were variable even under stable external conditions, (2) the bacteria belonging to alpha proteobacteria could be responsible for the deterioration of the P removal performance, (3) the GAOs inhibition model could not apply to this EBPR deterioration because there was no substrate uptake under anaerobic periods, and (4) the deterioration was estimated to be caused by the excess P release at the anaerobic period.

2. Materials & methods

2.1. Reactor operation

The reactor operation was almost the same as described previously [17]. A 2.5 l polyacrylamide cylinder with a working volume of 1.8 l was used for the development of EBPR sludge (Fig. 1). The reactor was operated in a cycle with three distinct periods consisting of an anaerobic period (45 min), an aerobic period (90 min) and a period (45 min) for settling sludge and replacing 0.9 l of the supernatant with fresh synthetic wastewater. The synthetic wastewater contained acetate (177 mg), peptone (55 mg), and minerals in 1 l of deionized water [17]. P/C ratio (wt/wt) was 5/100. TOC loading was 0.4 kg C/m$^3$/day and sludge retention time was set at 20 days by removal of suitable amount of sludge every cycle. pH was kept between 6 and 8. Activated sludge in the anaerobic/aerobic/oxic process from domestic wastewater treatment plant was used as the seed sludge.

2.2. Sampling and chemical analyses

The P concentrations in the effluent were measured almost every day. Every 2 weeks, 3 ml of mixed liquor was taken from the reactor and filtered through a 0.45 μm pore size filter (Millex-HV 0.45 μm PVDF 25 mm, Millipore, Bedford, MA) at intervals of 0, 13,
20, 30, 45, 60, 75, 105, and 135 min after the beginning of the anaerobic period for P and TOC measurement. At the end of the aerobic period, sludge P content and mixed liquor suspended solids (MLSS) weight was measured as specified in standard methods [18].

Soluble orthophosphate concentration was measured by ascorbic acid method as specified in standard methods [18], and TOC of the sample was determined with a TOC analyzer (TOC-500, Shimadzu, Kyoto, Japan).

2.3. DAPI staining

DAPI (4', 6'-diamidino-2-phenyl indol dihydrochloride) staining was carried out as described previously [17]. DAPI solution (50 μg/ml in 25 mM Tris-HCl buffered saline pH 7.0) was filtered through a 0.2 μm pore size filter (Millex-GV, Millipore, Bedford, MA). The ultrasonic disruptor (200W, TOMY Digital Biology CO., Ltd., Tokyo, Japan) was used for 10–30 s until the flocks in the sample were almost completely dispersed to the cells. The dispersed cells were then air-dried on a slide and stained with DAPI solution. After 10 s, the slides were washed briefly with distilled water and dried at room temperature. DAPI-DNA fluorescence is blue-white, while the fluorescence of both DAPI—poly-P and DAPI—lipid is yellow. Samples of sludge taken at the end of the aerobic period were used for staining.

2.4. Microscopic observation

An Olympus AX80 fluorescent microscope (Olympus Optical, Tokyo, Japan) equipped with a 100 W high-pressure mercury bulb was used for microscopic observation. For the epifluorescent observation of DAPI stained samples, an Olympus filter set MWU (Olympus Optical, Tokyo, Japan; excitation, 330–385 nm) was used. Yellow cell area and blue cell area were measured using an IPLab-spectrum image analyzing system (Signal Analytical, Virginia) program as follows: split the acquired color image into blue (for all cells) or green (for DAPI stained cells with 525 nm fluorescent maximum) color, segment the split images into the area for all cells (blue and yellow cells) or into the area for PAOs (yellow cells) according to the original DAPI image and finally, quantify the segmented area.

2.5. Quinone profiling

Quinone profiling was carried out basically according to the method described by Hiraishi et al. [19,20].

2.5.1. Quinone extraction

From the reactor, 150 ml of mixed liquor was sampled and sludge was harvested by centrifugation (15,000g, 5 min). After removing the supernatant, the sludge samples were preserved at −80°C. Menaquinone (MK) and Ubiquinone (UQ) fraction were eluted in 20 ml n-hexane–diethyl ether (90:10).

2.5.2. Quinone analysis by HPLC

Quinone components were separated and identified by reverse-phase HPLC: Beckman Coulter System Gold 126 Solvent Module and Beckman Coulter Model AS-100 HPLC Automatic Sampling System (Beckman Coulter Instruments Ltd., Tokyo, Japan), Zorbax ODS column (4.6 i.d. × 250 mm) (Du Pont Co., Wilmington, DE) and photodiode array detection (Beckman Coulter System Gold 166 Detector) with standard quinones. The carrier was methanol:disopropylether (4:1) and its flow rate was 1.0 ml/min. Column temperature was kept at 35°C. The channels for UQ and MK were 270 and 275 nm, respectively. Each quinone was determined by using the standard quinones. The standards, MK-8, MK-8 (H₂) and MK-9 (H₂) were from the quinones extracted from Deinobacter grandis, Rhodococcus equi and Arthrobacter oxydans, respectively. The standards of the other MKs and UQs were the quinones extracted from activated sludge. The ratio of each quinone component was determined from peak areas of HPLC according to Hiraishi et al. [19,21].

2.6. FISH

Sample fixation, hybridization and microscopic observation were carried out according to Crocetti et al. [8]. Sample dispersion and dual staining by FISH probe and DAPI were done as described by Kawaharasaki et al. [17]. The oligonucleotide probes (PAO462, PAO651, PAO846), which are specific for the Rhodocyclus relatives were used to evaluate the microbial population in the sludge [8]. The probes were labeled with Cy-3 at 5’ end.

3. Results

3.1. Reactor performance

The reactor was operated for 204 days, and the P concentration at the end of aerobic period, MLSS and sludge P content (% of dry MLSS) fluctuated (Fig. 2). The EBPR process exhibited excellent P removal from day 1 to 92 (phase I, good EBPR). However, P removal activity deteriorated from day 93 (0.68 mg-P/l P was detected), and more than 7 mg-P/l P was detected at the end of the aerobic period in the EBPR reactor at day 99. This deterioration of P removal activity lasted from day 93 to 171 (phase II, poor EBPR). The excellent P removal activity reappeared between day 172 and 204 (phase III, good EBPR). MLSS stayed in the range of 4000–5000 mg dry-sludge/l from day 25 to 95, but
decreased gradually to 2000 mg dry-sludge/l by day 132. After day 175, it increased to about 4000 mg dry-sludge/l. No sludge bulking was observed throughout the reactor operation period. The sludge P contents were between 5% and 6% from day 14 to 95, but suddenly decreased to 2.1% at day 112 and remained low at around 2% until day 158. It increased to 3.4% on day 190.

On day 14 (in phase I; good EBPR), the reactor exhibited substrate uptake accompanied by phosphate release in the anaerobic period and rapid phosphate uptake in the subsequent aerobic period (Fig. 3a). The P concentration was 40 mg-P/l at the end of the anaerobic period, and the P concentration at the end of the aerobic period was below 0.1 mg-P/l. On day 95 (the beginning of the deterioration) (in phase II; poor EBPR), anaerobic P release increased to 63 mg-P/l, and 2.4 mg-P/l P was detected at the end of the aerobic period (Fig. 3b). On day 118, in the middle of phase II, the anaerobic P release decreased to 10 mg/l and TOC was hardly taken up in the anaerobic period. At the end of the subsequent aerobic period, P concentration of 1.7 mg-P/l remained (Fig. 3c). On day 190 (in phase III; good EBPR), the anaerobic phosphate release increased to 26 mg-P/l at the end of the anaerobic period, and TOC was taken up again at the same level as in phase I. In the subsequent aerobic period, P was taken up rapidly (Fig. 3d).

3.2. DAPI staining

The percentage of yellow cell area in total cell area increased from 7.5% (day 0) to 12.8% (day 55), and after day 55, it was about 14% except on day 95 (Table 1). As shown in Fig. 4, the percentage at day 95 was much lower (4.5%).

Table 1
Percentage of DAPI stained cells. Ratio of yellow to all cells (%) was calculated as: yellow cell area/total (blue and yellow) cell area × 100

<table>
<thead>
<tr>
<th>Day</th>
<th>83</th>
<th>95</th>
<th>132</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLSS (mg/l)</td>
<td>4660</td>
<td>4200</td>
<td>2050</td>
</tr>
<tr>
<td>Sludge P content (%)</td>
<td>5.7</td>
<td>6</td>
<td>2.3</td>
</tr>
<tr>
<td>Yellow cell in total cell (%)</td>
<td>13</td>
<td>4.5</td>
<td>14.8</td>
</tr>
</tbody>
</table>

Fig. 2. P removal of EBPR reactor during 204-day operation.

Fig. 3. Profiles of P and TOC during the anaerobic and aerobic periods. Good P removal was observed at day 14 and 190. Deteriorated P removal was observed at day 95 and 118.
3.3. Quinone profiling

At the beginning of phase I, rapid increases of the amounts of UQ-8 and MK-8 (H4) were observed until day 41 (Fig. 5). UQ-8 was the most dominant quinone throughout. MK-8 (H4) was second most dominant until day 95. Quinone analysis revealed that UQ-10 amount increased from day 69 to 118, concurrently with decreases in the amounts of UQ-8 and MK-8 (H4). The amounts of UQ-8 and MK-8 (H4) increased again when EBPR reappeared after day 174 (phase III).

3.4. FISH

The number of the cells that were positive for PAO probe decreased from day 0 and became small on day 69 when the phosphorus P removal was good (Fig. 6).

4. Discussion

Dynamic bacterial community changes were observed by DAPI staining and quinone analysis in the lab scale EBPR reactor operated for 204 days under stable operational conditions. Concomitantly with the bacterial community changes, the P removal activity of the sludge changed dynamically.

It was found that TOC uptake and P release activity during the anaerobic period were extremely poor in phase II (day 118, Fig. 3c). This showed that bacteria in the sludge hardly took up organic carbon or released P in the anaerobic period. This does not agree with the widely accepted GAOs inhibition model based on the assumption that GAOs inhibit EBPR because they compete with PAOs for substrate during anaerobic periods. Therefore, we need another inhibition model to explain this phenomenon.

The table shows that the sludge P content (%) and MLSS (mg/l) did not change so much, while the percentage of yellow DAPI positive cells (MLSS × yellow cell area/total cell area) was much different from day 83 to 95. This was due to one of these possibilities: (1) P was in the cells but was not stained yellow by DAPI, i.e., P was not poly-P, or (2) P was outside the cells in particulate form, and was trapped on the filter when the sample for P measurement in water was taken at day 95. In either case, we can conclude that polyphosphate in the cells was hydrolyzed in a large amount.

The P concentrations at the end of the anaerobic period were about 40 mg-P/l on day 14 (Fig. 3a), about 50 mg-P/l from day 55 to 83 (data not shown) and 63 mg-P/l at day 95 (Fig. 3b). This shows that the anaerobic phosphate release increased gradually, and this fact also suggests that the hydrolysis of cellular polyphosphate was enhanced.

More than 0.5 mg-P/l P was observed in the effluent after day 93. From day 14 to 95, the concentration of P released at the end of the anaerobic period increased gradually under almost constant sludge concentration. Besides, at day 95, the number of cells stained yellow by
DAPI decreased to one-third of that at day 83 although the sludge P content was about 258 mg/l (Table 1). These facts suggested that the decomposition of cellular polyphosphate had been enhanced by some means. In the cell, ATP was derived from respiration, i.e., oxidation of substrate, and was used for the synthesis of polyphosphate. If the decomposition of polyphosphate was enhanced, ATP and substrates were also wasted. This means a decrease in the biomass yield. It might also cause PAOs death. These dead cells might be lost from sludge due to sludge withdrawal. The rapid decrease of MLSS in our study may be caused by these reasons.

The enhancement of polyphosphate decomposition caused the decrease of sludge P content of PAOs and sludge. The sludge at day 132 with a sludge P content of 2.3% represents this state. PAOs without polyphosphate cannot take up substrate and release phosphate in an anaerobic period (Fig. 3c). If the presence of GAOs had been the cause of this deterioration, the substrate should have been taken up without P release. However, in our observation, there was no substrate uptake in an anaerobic period when the P removal performance deteriorated. Therefore, GAOs were not responsible for the deterioration in our study.

From day 69 (2 weeks before the beginning of the deterioration) to 118 (2 weeks after the beginning of the deterioration), the amounts of UQ-8 and MK-8 (H4) markedly decreased (Fig. 5). The decrease in the amounts of UQ-8 and MK-8 (H4) was accompanied by an increase in the amount of UQ-10 (Fig. 5). The sludge P content, the amount of UQ-8 and MK-8 (H4) simultaneously decreased from day 69 to 118. These similarities suggested that the bacteria containing UQ-8 or MK-8 (H4) played an important role in the EBPR. UQ-8 and MK-8 (H4) are usually attributed to beta proteobacteria and Actinobacteria, respectively [20]. Therefore, it is likely that bacteria belonging to beta proteobacteria and Actinobacteria removed P in phase I in our EBPR reactor. However, FISH with PAO probes [8] showed the number of Rhodocyclus relatives was small (Fig. 6). Therefore, there were many DAPI-positive bacteria other than Rhodocyclus relatives. From day 69 to 118, the rapid increase in the amount of UQ-10 was observed. It should be noted that UQ-10 has usually been attributed to alpha proteobacteria [20]. Therefore, it could be suggested that these alpha proteobacteria could have a negative effect on the bacteria belonging to beta proteobacteria and Actinobacteria during the EBPR deterioration process.

In our reactor, excessive P release seems to have caused the deterioration. The relationships between the excessive P release and the increase of alpha proteobacteria from day 69 to 118 would be an interesting topic for future study. In phase III, the amounts of UQ-8 and MK-8 (H4) increased again. This also suggests the involvement of bacteria containing UQ-8 or MK-8 (H4) in the EBPR.

5. Conclusion

Even when the reactor was operated under the same condition, the bacterial community structure changed dynamically during the reactor operation period. The reactor operation was divided into the three distinct phases. The first EBPR phase was established from day 0 to 92 (phase I), followed by the deteriorated period from day 93 to 171 (phase II), and the second EBPR phase from day 172 to 204 (phase III). Because of the similarity between these changes in the sludge P content and the amounts of UQ-8 or MK-8 (H4), PAOs in phase I were estimated to be beta proteobacteria and Actinobacteria by quinone analysis with general attribution of each quinone to a phylogenetic group. It was also suggested that alpha proteobacteria had a negative effect on the PAOs that were beta proteobacteria and Actinobacteria during the EBPR deterioration process.
References