STABILITY OF PHOSPHORUS REMOVAL AND POPULATION OF BIO-P-BACTERIA UNDER SHORT TERM DISTURBANCES IN SEQUENCING BATCH REACTOR ACTIVATED SLUDGE PROCESS

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ABSTRACT

Laboratory-scale sequencing batch reactor (SBR) activated sludge processes were operated to investigate the stability of phosphorus removal capacity and population of bio-P-bacteria under short term disturbances (2 to 5 days) and to characterize the structure and dynamics of bacterial population of activated sludge for phosphorus removal. The performance on phosphorus removal deteriorated in 3 days, whereas it took more than 1 week for the recovery and the time for the recovery prolonged with the length of disturbances. The responses of phosphorus removal activity and quinone profiles suggested that the deterioration and the slow recovery were dependent not on the decrease in the activity of each bio-P-bacteria but on the decrease in their population, i.e., species succession of bacteria. The isolated strains of Acinetobacter and Pseudomonas were seen to be predominant species in the total bacterial population in the activated sludge. These strains showed high activity of phosphorus removal and low specific growth rate indicating also the slow recovery.

KEYWORDS

Activated sludge population dynamics; phosphorus removal; rainwater intrusion; quinone profile; Acinetobacter; Pseudomonas.

INTRODUCTION

Biological phosphorus removal processes are studied widely to prevent water pollution caused by artificial eutrophication. Although most studies have been carried out by continuous flow reactors (CFS), sequencing batch reactor, SBR, activated sludge processes with anaerobic and aerobic operations have also been accepted to be one of the promising processes for phosphorus removal (Okada et al., 1991).

Both in SBR and CFS systems for phosphorus removal, it is widely known that activated sludge must be subjected cyclically to anaerobic and aerobic conditions. An adequate supply of volatile fatty acids was also shown to be a key factor in successful phosphorus removal (Mostert et al., 1989; Jones et al., 1987; ). In addition to these environmental or operational conditions, it is also essential to have a specific group of bacteria, bio-P-bacteria, that is able to accumulate polyphosphate and, hence, to remove phosphate in wastewater (Okada et al., 1987; Wentzel et al., 1986). Selection, accumulation, or enrichment of bio-P-bacteria are regarded to be the most important factors for starting up and stable operation of effective biological phosphorus removal processes (Bowker and Stensel, 1987).

Among numerous bacteria in activated sludge ecosystem, Acinetobacter spp. were claimed to be responsible for phosphorus removal. Actually, many strains of the genus Acinetobacter have been isolated from activated...
sludge processes for biological phosphorus removal and was regarded to be a dominant polyphosphate-accumulating species in the sludge (Fuhs and Chen, 1975; Hao and Chang, 1987; Murphy and Lotter, 1986; Lotter et al., 1986). Numerous studies to elucidate the metabolic control mechanisms involved in the enhanced removal of phosphorus, therefore, have been reported presupposing a strain of Acinetobacter as a model organism (Lotter and Dubery, 1987; Groenestijn and Deinema, 1987; Groenestijn et al., 1989).

Acinetobacter spp., however, were found to form a large fraction (40%) of isolated strains not only in the activated sludge for phosphorus removal but also in conventional activated sludge with fully aerobic condition (Lotter et al., 1986). Those strains isolated from conventional sludge were equivalent in terms of substrate utilization, polyphosphate and polyhydroxybutyrate (PHB) accumulations with those isolated from phosphorus removal sludge.

It was also demonstrated that a strain of Acinetobacter calcoaeticus did not exhibit typical features of activated sludge for phosphorus removal, i.e. anaerobiosis did not accelerate the rate of phosphorus uptake, and no significant uptake of acetate was observed in anaerobic condition (Ohtake et al., 1985). Another strain of Acinetobacter sp. isolated from activated sludge for phosphorus removal did not release phosphate under anaerobic condition fed with acetate (Hao and Chang, 1987). The anaerobiosis also reduced viability of the strain in spite of the reported fact that prolonged anaerobic condition in a Phoredox system had no detrimental effect on sludge characteristics and improved phosphorus removal capacity.

The efforts to identify or isolate other strains than Acinetobacter spp. revealed that such genus as Pseudomonas and Klebsiella could be regarded as bio-P bacteria (Suresh et al., 1985; Gersberg and Allen, 1985). The isolation of new strains, however, does not always assure that total population of bacteria in the activated sludge was examined. It is practically impossible to recover all kinds of bacteria present in activated sludge (Pike and Curds, 1971). In fact, the number of aerobic heterotrophic bacteria detected as "viable colonies" only accounts for small percentages of total population. The isolation and pure culture studies provides only limited information on the "countable and cultivable" bacterial population rather than the total population.

Recent development of methods for in situ identification and enumeration of specific microorganisms presented quantitative information on the role of Acinetobacter. By using electron dispersive micro-analysis of X-rays, Cloete and Steyn (1988) estimated that a maximum of 34% of the observed phosphorus removal could be removed by Acinetobacter as polyphosphate. Also, in situ counts of Acinetobacter by the fluorescent antibody technique indicated that they constituted less than 10% of total bacterial count by the acridine orange staining, whereas the common API-20E identification system based on cultivation indicated that Acinetobacter was the dominant species.

Although the above mentioned methods give us quantitative information on Acinetobacter or a selected species of bacteria, still they do not provide much information on the total population. The microbial chemotaxonomy has been applied to mixed bacterial populations such as activated sludge for their in situ characterization of total population (Hiraishi, 1988). The analyses of isoprenoid quinones in the activated sludge both for conventional and phosphorus removal revealed that there was no significant difference in the structure of bacterial community, nor any evidence on the dominancy of Acinetobacter (Hiraishi et al., 1989).

Practical applications and pilot plant studies in Japan on biological phosphorus removal during the decade pointed out that disturbances such as rapid decrease in temperature and inflow of rainwater, i.e. hydraulic shock loading and/or dilution of wastewater, may cause serious damage to plant performance (JSWA, 1988; Minagawa et al., 1989). It was recommended, in addition to prevent rainwater intrusion, to utilize primary sludge as an agent to increase inflow organic concentration and stabilize effluent quality and activated sludge bacterial population.

The purpose of this study is to investigate the stability of phosphorus removal and population of bio-P-bacteria under short term disturbances such as rainwater intrusion and to characterize the structure and dynamics of bacterial population in activated sludge for phosphorus removal. Laboratory systems of SBR were given disturbances from 2 to 10 days by introducing full-time aeriation or diluted wastewater and the responses of bacterial population in activated sludge was monitored by phosphorus removal activity, quinone profiles, and species identification in addition to plant performance.
MATERIALS AND METHODS

Configuration and operation of SBR activated sludge processes

Two laboratory-scale SBR activated sludge processes were operated in an air-conditioned room of 20±2 °C. Each reactor consisted of a 5 l acrylic vessel with facilities for wastewater inflow, mixing, aeration, effluent discharge and excess sludge removal (Okada, et al., 1991). Acetate was used as a carbon source (TOC = 320 mg l⁻¹) and inorganic nutrients were added to have T-N and T-P concentrations of 106 mg l⁻¹ and 15 mg l⁻¹, respectively. Actually, concentrated wastewater was prepared and fed into reactors with tap water for dilution after autoclaving it for 15 min at 120 °C. BOD loading was 0.36 kg BOD m⁻³ d⁻¹. BOD concentration of the wastewater was 720 mg l⁻¹ and the ratio of BOD : N : P was 100 : 14.7 : 2.1. Hydraulic retention time (HRT) was kept at 2 days. MLSS was maintained at around 4,000 mg l⁻¹.

![Fig. 1. Operational schedules for normal operation and disturbances.](image)

The SBR systems were operated in a cycle of 24 h. Fig. 1 shows operational schedules of normal operation and two types of disturbances, i.e. OX and 1/4. The SBR was operated on the schedule of normal operation for more than one month to confirm steady-state operation both in effluent quality and activated sludge microbial ecosystem. Anaerobic/aerobic operations in the normal operation were changed into fully aerobic operation throughout a cycle, i.e. continuous aeration from the beginning to 23.5 h, with the same wastewater inflow for 2 to 10 days and returned to the normal operation in the disturbance OX. In the disturbance 1/4, the wastewater was diluted down to 1/4 of the original concentration without any change in the operational schedule.

The SBR operations were monitored by MLSS, SVI and effluent water quality, i.e. dissolved organic carbon (DOC), volatile fatty acids (VFA), orthophosphate (PO₄-P), redox potential (ORP) and dissolved oxygen concentration (DO). All the chemical analyses were carried out according to Standard Methods (APHA, 1985).

Activity of phosphorus removal

The activity of phosphorus removal by activated sludge in the reactor was determined by separated batch cultivations. A sample of activated sludge taken from the reactor was washed by the same synthetic wastewater as in the reactor and separated by centrifugation to remove remained organic substrate and phosphorus. The sludge was suspended into the synthetic wastewater. The mixed liquor was kept anaerobic for 8 h after purging DO less than 0.2 mg l⁻¹ by nitrogen gas. The amount of phosphorus release from the activated sludge was determined in 8 h. Based on the fact that the activity of phosphorus removal in anaerobic condition is in proportion to that of phosphorus release, the activity of phosphorus removal was defined as the amount of phosphorus release for 8 hours per unit mass of MLSS, mg phosphorus mg⁻¹ MLSS (Okada et al., 1991).

Isolation of bio-P-bacteria and determination of phosphorus removal activity

Plate culture was used to isolate bacteria in the SBR systems using the medium with the same composition as the synthetic wastewater. Actually the concentration was increased up to 5 times of that of original. After the number of colonies became maximum, typical and representative colonies in shape and color were
purified. The strain was cultivated under aerobic condition at 20 °C in a liquid culture of the same medium. Specific growth rate of the strain was estimated from turbidometric determinations of the increase in the cell mass concentration. When the growth of bacteria levelled off, the culture was turned into anaerobic condition for 8 h and the activity of phosphorus removal by the strain was determined.

Quinone profile analysis in activated sludge

Activated sludge was collected by centrifugation and washed with distilled water and freeze-dried. Quinones were extracted from the dried sludge (0.2 - 0.5 g) by chloroform-methanol (vol:vol = 2:1). The extract was purified by thin-layer chromatography developed with benzene. Quinone components were separated by reverse-phase high performance liquid chromatography (Shimazu LC-6A, column; Zobax ODS) and then identified by comparing their retention times with those of standard quinones (Katayama et al., 1980; Hiraishi et al., 1984; Hiraishi, 1988). Identified quinones were ubiquinones and menaquinones with n isoprene units and expressed as Q-n and MK-n, respectively.

RESULTS AND DISCUSSION

Responses of reactor performance to the disturbance

Profiles of DO in a cycle of operation before and during the disturbance of 5 days are shown in Fig. 2. Changes in DO in a cycle before the disturbance was typical in SBR operation with anaerobic/aerobic condition. As shown in Fig. 2(a), no DO was observed for 8 hours. Although not shown here, ORP dropped from +100 mV down to -100 mV and nitrate nitrogen remaining in the beginning of the cycle was removed by denitrification indicating anoxic and anaerobic reactions. Corresponding profiles of orthophosphate are also shown in Fig. 2(a). Remarkable release of phosphorus was noted during anaerobic period. The released phosphorus was taken up in the following aerobic reaction.

Both the disturbances changed DO and PO₄-P profile as shown in Fig. 2(b). The system was changed into aerobic condition throughout a cycle in the disturbance OX. Although no aeration was carried out in the disturbance 1/4 for 8 hours at the beginning of each cycle, the diluted wastewater could not turn the system into anaerobic condition. The aerobic condition suppressed phosphorus release in the beginning of each cycle and subsequent luxury uptake of phosphorus. Effluent phosphorus concentration, therefore, increased as shown in Fig. 3 (see later on).

![Fig. 2. Profiles of dissolved oxygen (DO: upper) and orthophosphosphate (PO₄-P: lower) concentration in a cycle of operation before (a: left) and during (b: right) the disturbance for 5 days.](https://iwaponline.com/wst/article-pdf/26/3-4/483/24331/483.pdf)
The profiles shown in Fig. 2(b) were observed in day 3, whereas similar profiles were observed from the beginning to the end of the disturbances. It must be noted that it took more than 3 weeks to recover the same profile of phosphorus release and uptake as that shown in Fig. 2(a). For the disturbances of 2, 3, and 10 days, the similar profiles of DO and PO₄-P were noted. In case of 2 days disturbance, however, effluent phosphorus concentration was kept unchanged.

Fig. 3 shows responses of effluent quality and phosphorus release activity in the disturbances of 5 days. Effluent phosphorus concentration was kept low for two days both for the disturbances of OX and 1/4. It increased remarkably after day 3 and showed maximum values at day 5. The disturbances were removed at day 5 and returned to the normal operation after that, whereas it took more than three weeks to recover the same performance as that in the steady-state condition in the normal operation. Significant differences were not noted between disturbances of OX and 1/4.

The disturbance of 3 days showed very similar responses to those of 5 days. Effluent phosphorus concentration, however, returned to the normal level within 1 week (not shown here). Prolonged disturbances such as 10 days deteriorated the system performance more than 4 weeks (also not shown here). Taking the fact that the hydraulic retention time was kept at 2 days throughout the operation, it is most probable that phosphorus removal activity of the activated sludge was damaged during the disturbances. No deterioration in effluent quality was noted in the disturbances for 2 days. The longer disturbances seemed to have resulted in the more serious damage.

**Responses of activated sludge**

As shown in Fig. 3, phosphorus removal activity was kept unchanged for 2 days and decreased after day 3 of disturbance. It decreased less than 50% of the activity in the steady-state in day 5 and took more than 3 weeks to recover the original level. Similar to effluent phosphorus, the activity decreased at day 3 and took 1 week to recover the original level in the disturbance of 3 days. There was no change in the activity, however, for the disturbance of 2 days. This behavior is well coincident with that of effluent phosphorus concentration. Phosphorus removal capacity, therefore, seemed to be dependent on the activity of phosphorus removal by activated sludge.

SVI increased from 40 to 60 by the disturbance OX, whereas it was kept unchanged throughout the disturbance 1/4. Thus MLSS did not washout during the disturbances and MLSS was kept between 3,000 and 4,000 mg l⁻¹. SRT estimated from excess sludge removal was more than 40 days during these operations. Taking the facts that phosphorus removal activity dropped during the disturbance and total biomass, i.e. MLSS, was kept unchanged without significant removal of biomass, the deterioration of plant performance can be referred to degradation of bio-P-bacteria in the activated sludge.
**Responses of bacterial population**

Quinone profiles in the activated sludge was determined before (day -2), during, and after the disturbances. Ubiquinone contents per unit mass of MLSS are shown in Fig. 4 with the corresponding phosphorus removal activity. The major ubiquinone detected in the sludge with high activity of phosphorus removal (day -2; steady-state operation) had 9 isoprene units, i.e. ubiquinone-9 (Q-9). The second was Q-8. Although small amount of Q-10 was detected, other quinone homologs were negligible.

Representative bacteria which contain Q-9 as a major constituent are *Acinetobacter* and *Pseudomonas* (Hiraishi and Morisawa, 1990). It is most probable, therefore, that the dominant bacteria in the sludge studied are one or both of these two genus. This supports the most common understandings based on isolated strains that *Acinetobacter* and/or *Pseudomonas* are predominant in activated sludge for phosphorus removal (Fuhs and Chen, 1975; Hao and Chang, 1987; Murphy and Lotter, 1986; Lotter et al., 1986; Suresh et al., 1985; Gersberg and Allen, 1985).

On the contrary, Hiraishi et al. (1989) reported that Q-8 was present as the predominant ubiquinone, Q-10 was the second most common type, and Q-9 and other homologs appeared as minor components in activated sludge fed with peptone irrespective of anaerobic/aerobic or aerobic operation, i.e. activated sludge for phosphorus removal do not have different microbial population from conventional activated sludge processes based on quinone profile. This discrepancy may partly due to the substrate used. Although not shown here, activated sludge fed with peptone, the substrate Hiraishi et al. used, contained larger amount of Q-8 than those fed with acetate. Major constituent was Q-8 when the sludge was operated under aerobic condition. Cyclic anaerobic and aerobic reactions, however, increased Q-9 to be the predominant.

**Fig. 4.** Ubiquinone contents and phosphorus removal activity in the activated sludge for the disturbance of 5 days from day 0 to day 5.

**Fig. 5.** Menaquinone contents in the activated sludge for the disturbances of 5 days.
Fig. 5 shows menaquinone contents in the activated sludge for the same disturbances of 5 days. Major menaquinones detected were MK-7 and MK-8. The other homologs were not significant. This is in agreement with the previous reports on activated sludge (Hiraishi, 1988; Hiraishi et al., 1989).

The disturbances of 5 days decreased quinone contents in the activated sludge. Although the decrease was not significant on day 1, both ubiquinone and menaquinone decreased remarkably after day 3 and took more than 3 weeks to recover their original contents. It is well known that quinone contents of bacteria are species specific and, in most cases, constant irrespective of environmental conditions. In fact, no significant differences in quinone profiles of isolated strains of *Pseudomonas* from this sludge (strain 2-15-W1 and 2-17-W3, see Table 1) were noted between aerobic and anaerobic conditions.

Thus it is most likely that the decrease in the activity of phosphorus removal was dependent not on the decrease in the activity of each bio-P-bacteria but on the decrease in their population. The facts that it took longer time to recover phosphorus removal activity than to lose it and the longer time was necessary if the duration of disturbances were longer may also support the decrease in bio-P-population.

Fig. 6. Ubiquinone homologs as percentages out of total in the disturbances of 5 days.

Homologs of ubiquinones were shown in Fig. 6 by percentages out of total ubiquinones. Although little change in percentages of menaquinone homologs was noted (not shown here), those for ubiquinone showed significant change, i.e. Q-9 decreased during the disturbances. This may correspond to the decrease in the population of bacterial species with high Q-9 contents.

Previous studies on quinone profiles of activated sludge suggested that the introduction of anaerobic conditions into totally aerobic operation has little influence on its bacterial community structure (Hiraishi et al., 1989; Hiraishi and Morishima, 1990). Also a conventional study on isolation and enumeration of activated sludge bacteria revealed that the cyclic change in anaerobic and aerobic conditions did not stimulate microbial succession to a new species of *Acinetobacter*, but rather to stimulate the ability for the accumulation of polyphosphate and PHB inherent in the strains already present (Lotter et al., 1986).

Fig. 7. Relationships between specific growth rate of isolated strains of bacteria and their phosphorus removal activities.
The present study, however, suggested completely different response of bacterial community. Although further studies would be necessary, not only the quinone profile but also slow responses in plant performance and phosphorus removal activity may support the change in bacterial community rather than the physiological changes in bacterial community.

Fig. 7 shows relationships between specific growth rate of isolated strains from the sludge in steady-state operation and their activity of phosphorus removal. Similar to the previous study (Okada et al., 1991), strains with high activity of phosphorus removal were low in specific growth rate. This result also supports the above mentioned slow succession of bacterial population and recovery of phosphorus removal after the disturbances.

Table 1 shows specific growth rate, phosphorus removal activity and ubiquinone homologs of representative strains. The bacteria with high activity of phosphorus removal and low specific growth rate were identified as genus of *Acinetobacter* or *Pseudomonas*. The fact that major ubiquinone detected was Q-9 suggests that these strains were predominant species in the total bacterial population of activated sludge. Their high activity also suggests that they played major role for phosphorus removal. Their slow rate of growth may explain the slow recovery of bacterial population and activity. The strain with low activity and high specific growth rate, however, had Q-8 indicating not to play major role for phosphorus removal.

**TABLE 1** Specific growth rate, phosphorus removal activity and ubiquinone homologs of isolated strains

<table>
<thead>
<tr>
<th>code</th>
<th>species</th>
<th>specific growth rate (day⁻¹)</th>
<th>activity (mg-P mg⁻¹·MLSS)</th>
<th>ubiquinone (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-12-W1</td>
<td><em>Acinetobacter lwoffi</em></td>
<td>0.66</td>
<td>0.18</td>
<td>Q-9</td>
</tr>
<tr>
<td>2-13-W1</td>
<td><em>Acinetobacter anitratus</em></td>
<td>0.06</td>
<td>0.19</td>
<td>Q-8</td>
</tr>
<tr>
<td>2-15-W1</td>
<td><em>Pseudomonas aeruginosa</em></td>
<td>0.50</td>
<td>0.18</td>
<td>33</td>
</tr>
<tr>
<td>2-17-W3</td>
<td><em>Pseudomonas maltophilia</em></td>
<td>0.80</td>
<td>0.18</td>
<td>91</td>
</tr>
<tr>
<td>4-08-W1</td>
<td><em>Pseudomonas aeruginosa</em></td>
<td>0.50</td>
<td>0.13</td>
<td>14</td>
</tr>
<tr>
<td>4-18-W1</td>
<td><em>Pseudomonas aeruginosa</em></td>
<td>0.43</td>
<td>0.15</td>
<td>7</td>
</tr>
<tr>
<td>2-08-W2</td>
<td><em>Pseudomonas maltophilia</em></td>
<td>0.80</td>
<td>0.07</td>
<td>93</td>
</tr>
<tr>
<td>4-02-W1</td>
<td><em>Enterobacter agglomerans</em></td>
<td>1.94</td>
<td>0.01</td>
<td>92</td>
</tr>
<tr>
<td>4-03-W1</td>
<td><em>Enterobacter agglomerans</em></td>
<td>1.84</td>
<td>0.01</td>
<td>92</td>
</tr>
</tbody>
</table>

**CONCLUSION**

Laboratory-scale sequencing batch reactor (SBR) activated sludge processes were operated to investigate the stability of phosphorus removal capacity and population of bio-P-bacteria under short term disturbances (2 to 10 days) and characterize the structure and dynamics of bacterial population of activated sludge for phosphorus removal. Specific conclusions derived from this study are as follows;

1) The performance on phosphorus removal deteriorated in 3 days, whereas it took more than 1 week for the recovery and the time for the recovery prolonged with the length of disturbances.

2) The responses of phosphorus removal activity and quinone profiles suggested that the deterioration and the slow recovery were dependent not on the decrease in the activity of each bio-P-bacteria but on the decrease in their population, i.e. species succession of bacteria.

3) The isolated strains of *Acinetobacter* and *Pseudomonas* were seemed to be predominant species in the total bacterial population in the activated sludge. These strains showed high activity of phosphorus removal and low specific growth rate indicating also the slow recovery.

**REFERENCES**


