**Influence of temperature, pH and dissolved oxygen concentration on enhanced
biological phosphorus removal under strictly aerobic conditions**

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Abstract

Previous research has suggested that enhanced biological phosphorus removal (EBPR) from wastewater can be achieved under continuous aerobic conditions over the short term. However, little is known how environmental conditions might affect aerobic EBPR performance. Consequently we have investigated the impact of temperature, pH and dissolved oxygen (DO) concentrations on EBPR performance under strictly aerobic conditions. A sequencing batch reactor (SBR) was operated for 108 days on a 6 hour cycle (4 cycles a day). The SBR ran under alternating anaerobic-aerobic conditions as standard and then operated under strictly aerobic conditions for one cycle every three or four days. SBR operational temperature (10, 15, 20, 25 and 30°C), pH (6, 7, 8 and 9) and DO concentration (0.5, 2.0 and 3.5 mg/L) were changed consecutively during the aerobic cycle. Recorded increases in mixed liquor phosphorus (P) concentrations during aerobic carbon source uptake (P release) were affected by the biomass P content rather than the imposed changes in the operational conditions. Thus, P release levels increased with biomass P content. On the other hand, subsequent aerobic P assimilation (P uptake) levels were both affected by changes in operational temperature and pH, and peaked at 20-25 °C and pH 7-8. Highest P uptake detected under these SBR operating conditions was 15.4 mgP g-MLSS⁻¹ (at 25 °C, pH 7 and DO 2.0 mg/L). The ability of the community for linked aerobic P release and P uptake required the presence of acetate in

the medium, a finding which differs from previous data, where these are reported to occur in the absence of any exogenous carbon source. Fluorescence *in situ* hybridization was performed on samples collected from the SBR, and *Candidatus* ‘*Accumulibacter phosphatis*’ cells were detected with PAOmix probes through the operational periods. Thus *Candidatus* ‘*Accumulibacter phosphatis*’ seemed to perform P removal in the SBR as shown in previous studies on P removal under strictly aerobic conditions.

Keywords

Activated sludge, Enhanced biological phosphorus removal (EBPR), Fluorescence *in situ* hybridization (FISH), Phosphorus accumulating organisms (PAO), *Candidatus* ‘*Accumulibacter phosphatis*’, Wastewater treatment

Introduction

Enhanced biological phosphorus removal (EBPR) is an economically and environmentally attractive process to remove phosphorus (P) from wastewater, and thus it has been used to treat wastewater around the world. This process is based on an enrichment of the activated sludge community with polyphosphate accumulating organisms (PAO) [1–3], and by encouraging the accumulation of phosphorus in PAO cells in the form of polyphosphate (polyP) granules in excess of the levels normally required to satisfy the metabolic demand for growth. When the cells are removed from the process during sludge wasting then so is this immobilized polyP [1–3].

EBPR is performed conventionally by an anaerobic-aerobic process. PAO take up organic substrates (preferably volatile fatty acids-VFAs) from wastewater and store them as poly-hydroxyalkanoates (PHA) under anaerobic conditions. The reducing equivalents and energy to store PHA are provided mainly by glycolysis of internally stored glycogen reserves and hydrolysis of intracellular polyP [4]. Then in the subsequent aerobic zone, PAO assimilate orthophosphate from the bulk water to synthesize the intracellular polyP in excess of that hydrolyzed under anaerobic condition by degrading the stored PHA for energy. They also grow and replenish their glycogen stores using the PHA as carbon and energy sources [5, 6]. Thus, PAO achieve dominance in this anaerobic-aerobic process because they can grow aerobically in the

absence of any exogenous carbon and energy source by using the PHA accumulated anaerobically [3]. Glycogen accumulating organisms (GAO) can also store organic substrates as PHA under anaerobic conditions. The reducing equivalents and energy to synthesise PHA are provided mainly by glycolysis of their internally stored glycogen reserves, and not from hydrolysis of intracellular polyP as in the PAO [7]. Therefore, GAO may compete for organic substrates with the PAO under anaerobic conditions [7]. Consequently GAO are often more relatively abundant than PAO in deteriorated EBPR systems.

Although EBPR is achieved conventionally by anaerobic-aerobic processes as described above, some studies have reported successful EBPR under strictly aerobic conditions in lab-scale reactors [8–10]. P release and uptake seems to switch with or without any available external carbon source in their studies. As with conventional EBPR, the two aerobic phases (with or without carbon source) can also be described as feast and famine respectively [11,12], and are analogous to the anaerobic-aerobic conditions of the anaerobic-aerobic EBPR process [9]. High aerobic EBPR capacity was only maintained during the first four days of operation with acetate as sole carbon source [13], while Vergas *et al.* [14] showed that EBPR performance persisted for 46 days when propionate was used. Nevertheless, aerobic EBPR performance was not

84 observed in full scale WWTP in the absence of an anaerobic phase [13]. It is possible
85 that the inability of a strictly aerobic feast phase to support EBPR in full scale plants
86 may reflect the impact of some operational factors, whereas their influence has not been
87 examined under strictly aerobic condition. The better our understanding of the basic
88 microbiology of EBPR communities, then the better eventually will be our ability to
89 control EBPR processes better.

90 This study was designed to more fully understand the basic microbiology and
91 process chemistry of EBPR processes by looking at a system with quite different
92 operational parameters, where both the feed and famine stages were carried out under
93 aerobic conditions like those encountered occur in heavy rain periods [13]. One aim was
94 to see how the microbial community might adapt to such marked operational
95 modifications. Long term stability of such aerobic EBPR processes has been
96 demonstrated [13–15], but this study focused on the immediate impact of an aerobic
97 feed. To see how an established conventional EBPR process responded immediately
98 after introducing an aerobic feed stage, this paper examines how temperature, pH and
99 DO concentration might affect the stability of aerobic EBPR performance. A lab-scale
100 reactor was first operated under the usual anaerobic-aerobic conditions and then the
101 influence of imposing strictly aerobic condition on the microbial community was

examined at frequent intervals.

Materials and Methods

Anaerobic/aerobic operation of sequencing batch reactor

A laboratory-scale reactor was operated as a sequencing batch reactor (SBR) with a working volume of 4 liters. The SBR was operated for 108 days in a water bath at 20°C on a 6 h cycle consisting of feed (10 min), anaerobic feast phase (120 min), aerobic famine phase (190 min), settling (30 min), and discharge of supernatant (10 min). To maintain anaerobic conditions, nitrogen gas was supplied for 10 min at the beginning of the feed period. SRT and HRT were controlled at 8 days and 12 h, respectively. Reactor volumes were maintained by discarding 250 ml of mixed liquor during the aerobic reaction period each cycle before settling, and 1.75 l of supernatant was removed at the discharge period of each cycle. Bulk liquid dissolved oxygen (DO) concentrations were measured with a galvanic DO probe (DO-24P, DKK-TOA, Tokyo, Japan), and controlled at >3.5 mg/L during the aerobic phase by turning the air from the diffuser on and off. The pH was controlled at 7.0 ± 0.1 with 0.5 M HCl and 0.5 M NaOH using a pH controller (TPX-98 with FX-300H probe, TOKO, Tokyo, Japan).

Inoculum was obtained from an anaerobic-anoxic-aerobic (A₂O) biological

nutrient removal plant (Yokohama, Japan) treating municipal wastewater. Plant influent contained 1.5 mg-P/l of total P (TP). Treated effluent contained 0.28 mg-P/l of TP. Sludge retention time (SRT) and hydraulic retention time (HRT) were controlled at 17 days and 6.6 h respectively.

Aerobic operation of sequencing batch reactor

The SBR was run under alternating anaerobic-aerobic conditions for 108 days as above. It was operated under strictly aerobic conditions for one cycle every three or four days after day 52. The aerobic cycle consisted of feed (10 min), aerobic phase (310 min), settling (30 min), and discharge of supernatant (10 min). The environmental conditions examined were temperature (10, 15, 20, 25 and 30°C), pH (6, 7, 8 and 9) and DO concentration (0.5, 2.0 and 3.5 mg/L), which were changed during each aerobic cycle consecutively.

Synthetic wastewater

The synthetic wastewater for anaerobic/aerobic operation and aerobic operation had the same composition similar to that used by Pijuan *et al.* [10]. Two separate concentrated solutions “C-water” and “P-water” formed the synthetic wastewater. The

C-water contained the following ingredients (g/L milli-Q water): CH_3COONa , 10.9; peptone, 0.5; NH_4Cl , 0.84; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.8; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 3.2; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.84; Bacto yeast extract, 0.4; allylthiourea, 0.01; and a nutrient solution (12 ml) that consisted of (g/L milli-Q water): EDTA, 10; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.12; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.12; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.15; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.03; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 1.5; H_3BO_4 , 0.15; KI, 0.18; $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$, 0.06. The P-water contained only KH_2PO_4 , 2.46 g/L in milli-Q water. The C-water and P-water was sterilized separately by autoclaving. At the beginning of the feed period, 100 ml of C-water and 100 ml of P-water were added together to the SBR with 1.8 L of tap water (1:20 dilution). Thus, the initial acetate and P concentration in each cycle were set to 79.8 mg-C/L and 14.0 mg-P/L respectively.

Chemical analysis

CH_3COO^- in filtered activated sludge samples was measured using a LC-10A ion chromatograph with a Shim-pack IC-A3 column (Shimadzu, Kyoto, Japan). Analysis of P as phosphate in filtered samples by the ascorbic acid method, TP concentration in the mixed liquor by the ascorbic acid method after persulfate digestion, and the determination of MLSS were all performed according to standard methods [16]. Phosphorus content of activated sludge biomass during each aerobic cycle was also

calculated as follows: (TP at the beginning of aerobic phase – phosphate at the beginning of aerobic phase) /MLSS.

Fluorescence in situ Hybridisation (FISH) analyses

FISH was performed as detailed by Daims *et al.* [17] with FITC-labelled EUB-338-I, EUB-338-II and EUB-338-III [18,19] probes applied as a mix to cover most *Bacteria* and Cy-3-labeled PAO462, PAO651 and PAO846 probes [20], applied as a mix to cover most *Candidatus* 'Accumulibacter phosphatis'. EUB probes and PAO probes were purchased from Rikaken (Nagoya, Japan) and Nippon EGT (Toyama, Japan) respectively. Slides were air dried, mounted in Vectashield (Vector Laboratories, CA, US) and then viewed on a BX51 epifluorescence microscope (Olympus, Tokyo, Japan).

Results and Discussion

EBPR performance in an anaerobic-aerobic cycling SBR

Anaerobic-aerobic cycle was carried out for 108 days using acetate as the only carbon source. Fig. 1 shows an experimental profile of P levels in the SBR on day 31. P was released into the bulk liquid under anaerobic condition and then taken up from the

bulk liquid under aerobic conditions as expected. MLSS and phosphate concentrations at the beginning of anaerobic phase, end of anaerobic phase and end of aerobic phase are shown in Fig. 2, and show that net P removal was achieved through the experimental period except for day 1. MLSS remained constant at around 2.5-3.0 g/L after day 31. FISH was performed on samples collected from the SBR, and *Candidatus* 'Accumulibacter phosphatis' cells were detected with PAOmix probes through the all operational periods. Fig. 3 shows FISH micrographs on days 1 (Fig. 3a) and 108 (Fig. 3b). Green and red fluorescence show EUBmix probe and PAOmix probe respectively. Thus *Candidatus* 'Accumulibacter phosphatis' seemed to perform P removal in the SBR as shown in previous studies on P removal under strictly aerobic conditions [13–15].

Fig. 2 also shows the time course of P release per acetate uptake (P-mol/C-mol) under anaerobic conditions. As mentioned above, GAO use glycogen as the sole or primary source of energy for anaerobic organic substrate uptake and thus can survive and grow in EBPR systems without P release and uptake [7]. Therefore, the P release per acetate uptake levels under anaerobic conditions may be changed by the relative abundance of GAO. Fig. 2 shows that P release per acetate uptake kept high levels after day 9, but decreased rapidly between day 62 and 84, which may imply an increase in the relative abundance of GAO in community after day 62.

EBPR performance on permanent aerobic cycles under different environmental conditions

The activated sludge acclimated under anaerobic-aerobic condition was examined during fully aerobic SBR operation after day 52, and its EBPR performance was monitored in response to changes in several operational parameters. The aerobic cycles were performed changing one of the following three factors (20°C, pH 7, and DO 2 mg/L) consecutively. Influence of temperature was examined on days 66 (30°C), 70 (10°C), 73 (25°C), 77 (15°C) and 93 (20°C); pH changes were monitored on days 59 (pH 6), 63 (pH 8), 87 (pH 9) and 93 (pH 7); and DO concentrations were varied on days 52 (0.5 mg/L), 93 (2.0 mg/L) and 105 (3.5 mg/L). The chemical profiles generated in response to these changes are given in Fig. 4. They show that P release and uptake were achieved during every aerobic cycle except at pH 6 and pH 9, and net P removal was achieved except for these. As shown in Fig. 4f (pH 6) and 4h (pH 9), P uptake was not completely achieved at pH 6 and pH 9 under these SBR conditions. More importantly, these results imply that following exposure to fully aerobic conditions at around neutral pH the SBR community showed no deterioration in its EBPR capacity.

Fig. 4 also shows that phosphate release and subsequent uptake did not depend

on the presence of an available external carbon source like acetate. Fig. 4a, 4b, 4e, 4f, and 4i clearly show that P uptake occurred even when acetate was still present, which differs from data from other studies looking at EBPR performance under fully aerobic conditions [13,14]. Moreover, acetate uptake following P uptake was clearly slower than that before P uptake. Such a slower uptake of acetate seems to result from a shift in PAO metabolism in response to limitations in the level of intracellular polyP. If PAO can no longer release P, then synthesis of PHA from acetate is not possible.

Impact of environmental factors on EBPR

Table 1 shows the P release and uptake levels at different temperature, pH and DO concentrations. The data suggest that P uptake levels changed in response to both temperature and pH, and were optimal at 25°C and pH 8, while optimal P release occurred at 20°C. However, pH seems not to affect P release levels. Table 1 also shows that the maximum P release and uptake levels at different DO concentrations were observed at 0.5 mg/L and 2.0 mg/L oxygen, and the minimum levels at 3.5 mg/L. The differences in P uptake levels at the two extreme values were 5.85 mg-P/MLSS, 10.2 mg-P/MLSS and 3.29 mg-P/MLSS in response to temperature, pH, and DO changes respectively, while those for P release were 2.14 mg-P/MLSS, 1.83 mg-P/MLSS and

2.45 mg-P/MLSS respectively. The maximum levels of P uptake were 1.62, 6.21 and 1.40 times the minimum levels in response to temperature, pH, and DO concentration changes respectively, while those for P release were 1.40, 1.38 and 1.52 times respectively. Aerobic P release levels seemed to be less sensitive to these environmental changes than P uptake levels.

The influence of environmental changes on P release level in the anaerobic-aerobic EBPR cycle has been investigated previously. For example, Bradjanovic *et al.* [21, 22] operated an SBR under anaerobic-aerobic conditions and then investigated the influence of temperature on P release during the first cycle after changing temperature. The specific P release rate (g-P/g-active biomass/hour) increased with an increase in temperature until 20°C, and the maximum levels at 20°C were 0.076 [21] and 0.077 [22], which were 1.43 and 1.79 times the minimum levels at 10°C (0.053 and 0.043) respectively. P release data under anaerobic condition from their study suggested less influence of temperature than shown in this present study. Meanwhile, Smolders *et al.* [23] studied the influence of pH (5.5-8.5) on P release under anaerobic conditions over short time period. They concluded that the P release was strongly influenced by pH and P release per acetate uptake ratio varied between 0.25-0.75 P-mol/C-mol in a range of pH 5.5-8.5. Data from this present study on P release levels

per acetate uptake under aerobic condition (Table 1) showed no increase with pH change and thus the same strong influence from Smolders's study under anaerobic condition [23] was not seen here.

Fig. 5 shows the relationship between biomass P content and levels of P release (also listed in Table 1), suggesting a correlation between them. Thus the amount of P release is determined mainly by biomass P content. Furthermore, this relationship explains the reason why P release and uptake levels were not dependent on whether acetate was available or not (Fig. 4). Chuang *et al.* [24] have reported that the sludge with a high P content had a higher anaerobic P release rate in EPBR batch experiments. Thus, it seems to be possible that P release under aerobic condition also increase with P content in biomass.

Conclusions

In this work, influence of temperature, pH and dissolved oxygen concentration on EBPR performance under fully aerobic conditions was studied in short term experiments. The main conclusions are as follows:

- P release and uptake occurred during every aerobic cycle where environmental conditions were varied from the standard parameters except when the pH was

264 changed. Therefore, aerobic condition by themselves do not explain why net P

265 removal deteriorates over a short time period at pH around neutral.

- 266 • Aerobic P uptake levels seem to be more sensitive to environmental change than P
267 release levels.

- 268 • Aerobic P release levels under aerobic conditions correlated with the biomass P
269 content.

- 270 • Aerobic P uptake occurred in the presence of acetate.

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351

352 Table 1 Chemical analytical data obtained from samples taken during the aerobic EBPR
353 cycles exposed to different operational parameters.

354 Fig. 1 Chemical profiles during an anaerobic-aerobic cycle in the SBR.

355

356 Fig. 2 Changes in phosphate (at the beginning of anaerobic, end of anaerobic, and end
357 of aerobic), MLSS, and P release per acetate uptake (under anaerobic condition) levels
358 during anaerobic-aerobic SBR operation.

359

360 Fig. 3 FISH micrographs of Accumulibacter PAOs in the SBR sludge on day 1 (a), and
361 day 108 (b). Green and red fluorescence show EUBmix probe and PAOmix probe
362 respectively.

363

364 Fig. 4 Chemical profiles on the aerobic EBPR SBR cycles in response to changes in
365 operational parameters, each of which is described at the head of the corresponding
366 figure (Fig. 4a-4j).

367

368 Fig. 5 Relationship between biomass P content and aerobic P release levels. These data

369 are provided in Table 1.

370

Table 1

Table 1

day	70	77	93	73	66	59	63	87	52	105
Temperature (°C)	10	15	20	25	30	20	20	20	20	20
pH	7	7	7	7	7	6	8	9	7	7
DO concentration (mg/L)	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	0.5	3.5
Acetate uptake (mg-C/g-MLSS) ¹⁾	15.1	18.7	21.0	28.6	18.8	16.2	20.6	17.1	13.6	27.5
P release (mg-P/g-MLSS)	4.71	5.58	6.58	6.47	4.33	5.55	4.85	6.68	7.18	4.73
P uptake (mg-P/g-MLSS)	9.52	11.7	11.4	15.4	9.07	2.85	12.1	1.95	11.1	8.13
Phosphorus content (mg-P/g-MLSS) ²⁾	89.3	80.2	87.8	108	69.8	91.9	91.3	98.6	123	83.2
P release/Acetate uptake (P-mol/C-mol)	0.121	0.116	0.121	0.0876	0.0891	0.133	0.0911	0.152	0.205	0.0667

¹⁾ Uptake mass during P release.

²⁾ Content at the beginning of cycle.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5