Nutrient removal, microbial community and sludge settlement in anaerobic/aerobic sequencing batch reactors without enhanced biological phosphorus removal
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ABSTRACT
Nutrient removal, microbial community and sludge settlement were examined in two 3-litre laboratory-scale anaerobic/aerobic sequencing batch reactors (SBRs). One SBR was operated at 10°C and the other SBR at 20°C. Different from conventional enhanced biological phosphorus removal, most of the soluble sodium acetate was removed in the aerobic phase and no organic carbon uptake or biological phosphorus release occurred in the anaerobic phase. In this type of anaerobic/aerobic SBR, the phosphorus removal and sludge settlement seemed to be unstable, and the dominant microorganism was Zoogloea sp. Although no excess biological phosphorus removal occurred, extracellular phosphorus precipitation contributed a significant proportion to total phosphorus removed. Sludge volume index decreased with increasing phosphorus contents in the biomass under all conditions. The functions of extracellular polymeric substances in sludge settlement and phosphorus removal depended on the environmental conditions applied.

Key words | enhanced biological phosphorus removal, extracellular polymeric substances, phosphorus precipitation, sludge settlement, Zoogloea sp.

INTRODUCTION
Enhanced biological phosphorus removal (EBPR) has been applied widely in wastewater treatment plants for excess biological phosphorus removal. This process is achieved by enriching polyphosphate-accumulating organisms (PAOs) in alternating anaerobic/aerobic wastewater treatment systems. Under anaerobic conditions, PAOs are able to take up easy-biodegradable organic carbon with energy supplied from polyphosphate degradation. Under the subsequent aerobic conditions, PAOs take up excess phosphorus from wastewater. Wasting the phosphorus-rich biomass following uptake, excess phosphorus removal from wastewater is achieved. However, deterioration or failure of EBPR often happens due to the dominance of glycogen-accumulating organisms (GAOs) or other unknown reasons (Cech & Hartman 1993; Fang et al. 2002; Satoh et al. 2007). In GAO-dominating systems, GAOs are able to take up easy-biodegradable organic carbon under anaerobic conditions but they do not accumulate polyphosphate during subsequent aerobic conditions, and consequently no excess biological phosphorus removal can be achieved (Cech & Hartman 1993). Other phenomena in the deterioration of EBPR are that no organic carbon uptake and biological phosphorus release occur under anaerobic conditions; to date, there is no reasonable explanation for these phenomena (Fang et al. 2002; Satoh et al. 2007). Molecular techniques, such as polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE), are powerful tools in examining the microbial community dynamics and the functioning microorganisms in specific wastewater treatment systems. By combining...
these techniques with chemical and physical examinations, the underlying mechanisms in anaerobic/aerobic wastewater treatment systems may be explored.

The activated sludge separation property is very important for the design and operation of wastewater treatment processes as the effluent total phosphorus concentration can be affected greatly by solid separation treatment processes as the effluent total phosphorus concentration can be affected greatly by solid separation (Ydstebo et al. 2000). Ydstebo et al. (2000) found that low temperature and high organic loading conditions benefited the settlement of activated sludge flocs. On the other hand, Jones & Stephenson (1996) found that low temperature had a harmful effect on sludge settlement. Extracellular polymeric substances (EPS) play important roles in (i) phosphorus removal, (ii) survival of PAOs in EBPR by binding cells in dense flocs, and (iii) settlement of activated sludge (García Martín et al. 2006; Liu et al. 2006).

In this study, characteristics of nutrient removal, microbial community and sludge settlement in anaerobic/aerobic alternating sequencing batch reactors (SBRs)—treating synthetic wastewater with acetate as the main organic carbon—were investigated.

**MATERIALS AND METHODS**

**Anaerobic/aerobic SBRs**

Two SBRs were operated at temperatures of 10°C (SBR10) and 20°C (SBR20). In each reactor, two organic carbon concentrations—influent sodium acetate concentrations of 380 mg/l (Stage 1) and 760 mg/l (Stage 2)—were examined. The reactors had an effective volume of 3 litres. The SBRs had four cycles per day and each cycle comprised the following phases: fill (15 min), anaerobic (105 min), aerobic (180 min), settle (40 min) and draw/idle (20 min). In each cycle, 1.5 litres of synthetic wastewater were exchanged resulting in a hydraulic retention time of 12 hours. The reactors were constantly stirred with magnetic stirrers during the fill, anaerobic and aerobic phases; during the aerobic phase, air was supplied with air diffusers located at the bottom of the reactors. Mixed liquor was withdrawn from the two reactors once a day just before the end of the aerobic phase so as to maintain the solids retention time at 10 days.

For the synthetic wastewater, the sodium acetate concentration was 380 mg/l in Stage 1 and 760 mg/l in Stage 2, and the other components were: 12 mg/l yeast extract, 80 mg/l NH₄Cl, 84.3 mg/l K₂HPO₄ (15 mg P/l), 67 mg/l MgSO₄·7H₂O, 11 mg/l CaCl₂·6H₂O and 1 mg/l allylthiourea (inhibiting nitrification). Tap water was used to supply trace nutrients. The two reactors were seeded with activated sludge taken from a Wastewater Treatment Plant treating slaughterhouse wastewater in Ballyhaunis, Co. Mayo, Ireland.

**DNA extraction, PCR/DGGE and phylogenetic analysis**

The total genomic DNA of the activated sludge was extracted from centrifuged samples using the Mobio Soil DNA Extraction Kit (Cambio Inc., England). Bacterial 16S rRNA genes were amplified in a “touch-down” PCR using primers of E9F (GC-clamp attached) and E533R (Muyzer et al. 1993; McInnery et al. 1995; Watanabe et al. 2001). The PCR program was: an initial denaturation for 10 minutes at 94°C; then 10 cycles of denaturation for 1 minute at 94°C, annealing for 1 minute at 57°C (−1°C/cycle) and extension for 2 minutes at 72°C; followed by 25 cycles of denaturation for 1 minute at 94°C, annealing for 1 minute at 47°C and extension for 2 minutes at 72°C; and finally 7 minutes extension at 72°C. The concentrations of different components in the reaction mix were: 2 μl (10 μM) of each primer, 12.5 μl of AmpliTaqGold PCR Master Mix (Roche, USA) and 8.5 μl of DNase/RNase-free distilled water (Sigma-Aldrich, Ireland). 0.3 μl of extracted DNA was added to the 25 μl PCR reaction mix. PCR products were evaluated by electrophoresis on gels containing 2% agarose in 1 × TAE [40 mM Tris-acetate, 1 mM EDTA, pH 8.3] and then were visualized on a UV transillumination table after staining with SYBR-Green (Sigma-Aldrich, Ireland).

DGGE was performed using the 1001 DGGE system (AGB, Ireland). Polyacrylamide gels (6% acrylamide/bis-acrylamide 37.5:1 in 1 × TAE buffer) were cast with a denaturing gradient ranging from 40% at the top to 60% at the bottom (100% refers to the concentration of 7 M urea and 40% formamide). A total of 20 μl GC-clamped PCR product was loaded in each lane and the gels were run at 60°C for 15 hours at 120 V. Finally, gels were stained with SYBR-Green (Sigma-Aldrich, Ireland) and evaluated on the
UV transillumination table. Individual DGGE bands were excised, re-amplified, and sequenced commercially using the primer E9F (Eurofins MWG Operon, UK). Sequenced DGGE bands were compared with sequences in the Genbank database (Altschul et al. 1997).

Analytical methods

Orthophosphate-P (PO₄-P) was analyzed using a Konelab analyzer (Thermo Clinical Labsystems, Vantaa, Finland). Sodium acetate was measured with high performance liquid chromatography (HPLC, Agilent 1200, Agilent Technology, USA) using a UV index detector and an Aminex HPX-87H column (Bio-Rad, USA). Suspended solids (SS), volatile suspended solids (VSS), and total phosphorus (TP) were determined according to standard methods (APHA 1995). Sludge volume index (SVI) was measured using a 100 ml cylinder. Extracellular phosphorus was measured by the perchloric acid extraction method of De Haas et al. (2000). EPS was extracted from the activated sludge using the formaldehyde–NaOH method given by Liu & Fang (2002). Carbohydrate was measured by means of the sulfureic-phenol method (Dubois et al. 1956). Protein and humic acid were measured using the modified Lowry method (Frohlund et al. 1995).

The fluorescent in situ hybridization (FISH) examination was carried out according to Amann et al. (1990). The probes employed were ZRA23a specific for Zoogloea (Rossello-Mora et al. 1995) and EUB338-mix targeting Bacteria (Amann et al. 1990; Daims et al. 1999).

Nucleotide sequence accession numbers

The phylotypes detected in this study are available from the GenBank nucleotide database, at the NCBI website (http://www.ncbi.nlm.nih.gov), under accession numbers GU176399 to GU176407.

RESULTS

System performance

Different from EBPR, excess biological phosphorus removal was not achieved in all the conditions applied and there were still high PO₄-P concentrations in the effluent (Figure 1). At the influent PO₄-P concentration of 14.8 ± 0.9 mg/l (n = 46), the average PO₄-P removal efficiency was only 22% at Stage 1 (n = 18) and 26% at Stage 2 (n = 32) in SBR10, and 55% at Stage 1 (n = 27) and 46% at Stage 2 (n = 23) in SBR20. In addition, the effluent PO₄-P concentration was unstable.

The profiles of pH, PO₄-P and sodium acetate in typical cycles at Stage 2 from SBR10 and SBR20 on Day 57 are shown in Figure 2. The sodium acetate concentration was not changed much during the anaerobic phase and it was mainly used up during the initial stage of the aerobic phase. This shows that no PAOs or GAOs, which are able to take up organic carbon under anaerobic conditions, were enriched in this type of anaerobic/aerobic SBR. A very small amount of PO₄-P release (0.8 mg/l in SBR10 and 3.3 mg/l in SBR20) in the anaerobic phase could be due to
the decrease in the pH. This shows that it is preferable not to use this type of system for phosphorus removal where a high variation in pH occurs.

In spite of the absence of EBPR, the phosphorus content in the biomass varied with time and even reached 9.8% on Day 32 in SBR20 (Figure 1). In Stage 1, the phosphorus content increased with time in both reactors, ranging from 2.5% to 3.9% in SBR10 and from 2.7 to 9.8% in SBR20. In Stage 2, the phosphorus content in the biomass decreased from 4.4% to 2.2% in SBR10 (from Day 34 on) and from 9.8 to 3.9% in SBR20. The distribution of phosphorus inside and outside the cells was evaluated by the perchloric acid extraction method (Table 1). These results showed that extracellular phosphorus contributed a significant proportion to total phosphorus removed, i.e. 53.3 ± 8.9% in SBR10 and 66.7 ± 4.1% in SBR20.

**Microbial community analysis**

The microbial communities detected by using the PCR/DGGE technique are shown in Figure 3 and the phylogenetic analysis of sequenced bands is given in Table 2. Compared with the seed and the samples on Day 10, the band number decreased in later stages in both reactors, which showed that the microbial diversity was reduced with only acetate as the main organic carbon substrate. In general, most of the sequenced bacteria were in the betaproteobacteria group. The dominant bands (Bands 4 and 5) in both reactors during the study period were affiliated with Zoogloea sp., and this was also confirmed from the FISH analysis (Figure 4).

**Dynamics of activated sludge concentration and settlement**

Figure 5 shows the dynamics of the biomass concentration and SVI. In Stage 1, less biomass grew in SBR10 (10°C) and this caused a rapid decrease in biomass concentrations from 2.5 g SS/l to 1.4 g SS/l within 20 days. To maintain biomass growth, the organic carbon loading rate was doubled in Stage 2 and the biomass concentration increased after 10 days and finally reached 2.8 g SS/l. In SBR20
(20°C), the biomass concentration also decreased from 2.5 g SS/l and was maintained at 1.8 g SS/l during Stage 1; and this rose to 2.5 g SS/l in Stage 2 when the organic carbon loading rate was doubled.

In Stage 1, in SBR10, the SVI was $333 \pm 26 \text{ ml/g}$ ($n = 5$) in the initial 5 days and then decreased to $141 \pm 10 \text{ ml/g}$ ($n = 12$); while in SBR20, the SVI initially increased to 426 ml/g on Day 9 and then decreased thereafter to 73 ml/g on Day 32. In Stage 2, in SBR10, the SVI decreased firstly from 150 ml/g to 60 ml/g, and then it increased to around 200 ml/g; while in SBR20, the SVI increased with time and reached as high as 420 ml/g. In general, the settleability of the activated sludge in these anaerobic/aerobic SBRs was unstable.

**EPS components dynamics and their functions in sludge settlement and phosphorus removal**

The dynamics of protein, carbohydrate and humic acid concentrations in EPS are shown in Figure 6. In both reactors, protein and carbohydrate were the main components; humic acid concentration was high during the initial stage and then decreased to around 14–18 mg/g in the later stage. In SBR10, in Stage 1, protein and carbohydrate concentrations increased, while humic acid concentrations decreased with time; in Stage 2, all components reached steady state, with the concentrations of 82 mg/g for protein, 51 mg/g for carbohydrate and 12 mg/g for humic acid. In SBR20, in Stage 1, all components reached steady state, with the concentrations of 61 mg/g for protein, 32 mg/g for carbohydrate and 14 mg/g for humic acid; in Stage 2, both protein and carbohydrate concentrations increased while humic acid concentrations were reasonably steady.

The contributions of EPS components to sludge settlement and phosphorus removal were analyzed by presenting their linear relationships (Table 3). Different mechanisms were observed for the function of EPS components in sludge settlement. During Stage 1, in SBR10, SVI increased with increasing humic acid or decreasing protein concentrations, while there was a weak relationship with carbohydrate concentrations; in SBR20, the relationships between the SVI and all the EPS components were weak. During Stage 2, SVI was slightly correlated with the protein and humic acid concentrations.

### Table 2  Affiliation of sequenced bands for general bacteria

<table>
<thead>
<tr>
<th>Bands</th>
<th>Closest sequence (GenBank Access No)</th>
<th>Phylogenetic group</th>
<th>Similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Band-1</td>
<td>Acinetobacter sp. clone GI5-006-F04 (FJ192935)</td>
<td>Gamma-proteobacteria</td>
<td>473/482</td>
</tr>
<tr>
<td>Band-2</td>
<td>Burkholderia tropica strain SCu-7265 (FJ436052)</td>
<td>Beta-proteobacteria</td>
<td>243/291</td>
</tr>
<tr>
<td>Band-3</td>
<td>Turmeriella parva strain S-308-81 (AY398688)</td>
<td>Spirochaetales</td>
<td>262/285</td>
</tr>
<tr>
<td>Band-4</td>
<td>Zoogloea sp. EMB 108 (DQ413157)</td>
<td>Beta-proteobacteria</td>
<td>275/280</td>
</tr>
<tr>
<td>Band-5</td>
<td>Zoogloea sp. EMB 108 (DQ413157)</td>
<td>Beta-proteobacteria</td>
<td>477/481</td>
</tr>
<tr>
<td>Band-6</td>
<td>Thauera sp. 'TGOPY13 (T-I)' (EF205255)</td>
<td>Beta-proteobacteria</td>
<td>458/468</td>
</tr>
<tr>
<td>Band-7</td>
<td>Dechloromonas sp. MissR (AF170357)</td>
<td>Beta-proteobacteria</td>
<td>374/379</td>
</tr>
<tr>
<td>Band-8</td>
<td>Fibrobacteres bacterium, clone TH3-98 (AM690985)</td>
<td>Fibrobacteres</td>
<td>399/414</td>
</tr>
<tr>
<td>Band-9</td>
<td>Thauera terpenica strain 58 (AJ005817)</td>
<td>Beta-proteobacteria</td>
<td>469/476</td>
</tr>
</tbody>
</table>
in SBR10, while there were good relationships between SVI and protein or carbohydrate concentrations in SBR20. SVI decreased with increasing phosphorus contents in the biomass and a linear relationship was obtained under all the conditions.

The function of EPS components in phosphorus removal also varied with environmental conditions. At Stage 1, EPS components had a high effect on phosphorus content in biomass at 10°C, while there was not much effect at 20°C. At Stage 2, protein had a positive and humic acid had a negative correlation with the phosphorus content at 10°C; while protein had a negative and carbohydrate had a positive relationship with phosphorus content at 20°C.

DISCUSSION

Deterioration or failure of EBPR caused by dominating GAOs in anaerobic/aerobic SBRs has been intensively investigated. Only a few studies have been focused on the phenomenon that there is no organic carbon uptake or phosphorus release under anaerobic conditions. By microbial community analysis using PCR/DGGE and FISH techniques, proliferation of Zoogloea sp. was responsible for the deterioration of EBPR in this type of anaerobic/aerobic SBR. Zoogloea sp. in the Beta-proteobacteria group has been widely detected in wastewater treatment plants (Rossello-Mora et al. 1995; Juretschko et al. 2002). They are non-filamentous floc-forming bacteria, and they can store polyhydroxyalkanoate and denitrify in wastewater treatment processes (Rossello-Mora et al. 1995; Juretschko et al. 2002). A small amount of Zoogloea sp. in activated sludge flocs can improve the settlement property, while over-proliferation can cause bulking sludge and deterioration of EBPR (Montoya et al. 2008). Poor sludge settlement observed in this study may also support the proliferation of Zoogloea sp. However, bulking sludge was not observed by Okunuki et al. (2004) in anaerobic/aerobic SBR without anaerobic organic carbon uptake and phosphorus release, which shows that there may be other microorganisms contributing to this phenomenon. Deterioration of EBPR by this type of phenomenon may recover: (1) by itself (Okunuki et al. 2004; Satoh et al. 2007), (2) by using nitrogen-limiting wastewater (Harper et al. 2005), or (3) by reducing influent organic carbon and nitrogen concentrations (Montoya et al. 2008).
The function of EPS in phosphorus removal in activated sludge systems has not been studied in detail. By using the scanning electron microscopy-energy dispersive spectroscopy (SEM-EDS), Cloete & Oosthuizen (2001) obtained the phosphorus content in EPS of 27%–30% for the biomass taken from EBPR systems, while Liu et al. (2006) found that the phosphorus contents in EPS were 7.71%, 9.22% and 8.07% for the biomass taken from EBPR systems acclimated with acetate, glucose, and glucose + skim milk, respectively. However, the contribution from EPS and other processes such as precipitation could not be differentiated by the SEM-EDS technique. In a study by Comte et al. (2006), the phosphorus content in the EPS ranged only from 1.8 to 2.8%. The present study showed that the EPS components could be correlated with the phosphorus content in the biomass, which indicates that EPS components may play a role in phosphorus removal. The EPS can contribute to phosphorus removal directly by incorporating phosphorus in the EPS component, which may only remove a very small amount of phosphorus (Comte et al. 2006). However, phosphorus can be removed indirectly through EPS-induced precipitation or trapping. As shown in Table 3, the function of EPS in phosphorus removal depends on both the EPS components and environmental conditions. Further studies on the function of EPS in phosphorus removal are needed.

This study shows that high phosphorus content can enhance settlement properties, where biomass density may be increased with increasing phosphorus contents, while different contributions of EPS components to sludge settlement were observed. A high total EPS concentration can cause a high amount of vicinal water, which may lower the flocculation efficiency of flocs and cause a high SVI. The protein/carbohydrate ratio rather than the individual EPS component was better correlated to the SVI, because protein contributes to the hydrophobic

Table 3 | The relationships between the EPS components and the SVI or the phosphorus content

<table>
<thead>
<tr>
<th>Condition</th>
<th>Data No</th>
<th>Protein (mg/g SS)</th>
<th>Carbohydrate (mg/g SS)</th>
<th>Humic (mg/g SS)</th>
<th>P (mg/g SS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SVI (ml/g)</td>
<td>SBR10/Stage 1</td>
<td>17</td>
<td>$-5.74 (R^2 = 0.67)$</td>
<td>$-8.53 (R^2 = 0.41)$</td>
<td>$12.15 (R^2 = 0.83)$</td>
</tr>
<tr>
<td></td>
<td>SBR10/Stage 2</td>
<td>33</td>
<td>$2.17 (R^2 = 0.34)$</td>
<td>$1.35 (R^2 = 0.04)$</td>
<td>$6.18 (R^2 = 0.46)$</td>
</tr>
<tr>
<td></td>
<td>SBR20/Stage 1</td>
<td>28</td>
<td>$-7.73 (R^2 = 0.36)$</td>
<td>$2.25 (R^2 = 0.01)$</td>
<td>$9.04 (R^2 = 0.18)$</td>
</tr>
<tr>
<td></td>
<td>SBR20/Stage 2</td>
<td>20</td>
<td>$8.03 (R^2 = 0.76)$</td>
<td>$13.29 (R^2 = 0.88)$</td>
<td>$2.15 (R^2 = 0.01)$</td>
</tr>
<tr>
<td>P (mg/g SS)</td>
<td>SBR10/Stage 1</td>
<td>17</td>
<td>$0.26 (R^2 = 0.59)$</td>
<td>$0.31 (R^2 = 0.42)$</td>
<td>$-0.42 (R^2 = 0.47)$</td>
</tr>
<tr>
<td></td>
<td>SBR10/Stage 2</td>
<td>33</td>
<td>$0.16 (R^2 = 0.11)$</td>
<td>$0.05 (R^2 = 0.00)$</td>
<td>$0.02 (R^2 = 0.00)$</td>
</tr>
<tr>
<td></td>
<td>SBR20/Stage 1</td>
<td>28</td>
<td>$1.64 (R^2 = 0.70)$</td>
<td>$-0.04 (R^2 = 0.00)$</td>
<td>$-2.49 (R^2 = 0.53)$</td>
</tr>
<tr>
<td></td>
<td>SBR20/Stage 2</td>
<td>20</td>
<td>$-0.84 (R^2 = 0.58)$</td>
<td>$-1.19 (R^2 = 0.49)$</td>
<td>$1.04 (R^2 = 0.14)$</td>
</tr>
</tbody>
</table>
characteristics and the carbohydrate possesses neutral or hydrophilic characteristics (Liao et al. 2001). However, no good relationship between the SVI and the total EPS amount or the EPS component ratios was found in this study. This study shows that individual EPS components may be well correlated with the SVI and this varied with environmental conditions.

CONCLUSIONS

The nutrient removal, microbial community and sludge settlement were examined in anaerobic/aerobic SBRs at 10°C and 20°C. The following can be obtained.

- In both SBRs, no organic carbon uptake and biological phosphorus release were observed under anaerobic conditions, and the dominating microorganism was Zoogloea sp.
- Phosphorus precipitation contributed a significant proportion to total phosphorus removal, with proportions of 53.3% in SBR10 and 66.7% in SBR20. The functions of EPS in phosphorus removal varied with environmental conditions applied.
- The SVI decreased with increasing phosphorus contents in the biomass, while there was no apparent general relationship between SVI and EPS components at both 10°C and 20°C.

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REFERENCES


