A NOVEL APPROACH FOR BIOLOGICAL RECOVERY OF PHOSPHORUS FROM WASTEWATER

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BSc (Hons)

This thesis is submitted in fulfilment of the requirements for the degree of Doctor of Philosophy at the University of Western Australia

School of Pathology and Laboratory Medicine

June 2016
Abstract

Recovering phosphorus (P) from municipal wastewater is an important concept, but can be challenging because of the low P concentration in wastewater (7–10 mg-P/L). For P recovery to be chemically and economically viable, the P concentration needs to be >50 mg-P/L. This challenge was addressed in this study by developing a novel biofilm-based post-denitrification approach termed enhanced biological phosphorus removal and recovery (EBPR-r). This process was designed to achieve nitrogen (N) removal but also facilitate a multi-fold increase in P concentration, enabling P recovery. Underpinning this process was the innovative use of a group of microorganisms termed phosphorus accumulating organisms (PAOs). However, unlike the conventional enhanced biological phosphorus removal (EBPR) processes, the EBPR-r process was designed to use PAOs as a “shuttle”, to transfer the P from the wastewater stream into a separate P recovery stream. This modified process primarily involved two steps. In the first step the PAOs biofilm were exposed to a carbon-deficient wastewater stream (e.g. secondary effluent), whereby their internal carbon storage (i.e. poly-β-hydroxy-alkanoates; PHAs) were oxidised to provide the energy for phosphate (PO$_4^{3-}$) uptake using nitrate (NO$_3^-$) and dissolved oxygen (O$_2$) as electron acceptors. As PO$_4^{3-}$ was taken up from the wastewater and stored internally as poly-phosphate (Poly-P) inside the biofilm, simultaneous P and N removal from wastewater was achieved. During the second step the Poly-P enriched biofilm was exposed to a smaller recovery stream, where external carbon (acetate) was added to trigger the release of cellular P. Because the volume of the recovery stream was only a small fraction of that of the wastewater stream, P was simultaneously recovered and concentrated into that stream.

The study involved a series of laboratory-scale experiments designed to achieve proof-of-concept, process understanding and optimisation of the EBPR-r process. A laboratory-scale reactor, referred to as the master reactor, was constructed and operated to enrich an EBPR-r biofilm using activated sludge as the microbial inoculum; a synthetic wastewater was used as a secondary effluent (Chapter 2). When the reactor was operated at a 4:1 volumetric ratio (wastewater:recovery stream) the PO$_4^{3-}$ was concentrated 4-fold, from 8 mg-P/L in the wastewater (7.2 L) to 28 mg-P/L in the
recovery stream (1.8 L). During the P uptake phase, simultaneous NO$_3^-$ removal from wastewater was achieved at a P$_{upt}$/N$_{den}$ ratio of 1.31 g-P/g-N, confirming the post-denitrification ability of the process. To generate a P-enriched stream suitable for P recovery, the EBPR-r biofilm was repeatedly exposed to the same recovery stream to facilitate P accumulation (via multiple P release), and a P-enriched liquor was generated (>100 mg-P/L). In addition to P recovery, the process also enabled the recovery of other valuable metal ions including magnesium (Mg$^{2+}$), potassium (K$^+$) and calcium (Ca$^{2+}$), which may facilitate some of the chemical requirements for the downstream P recovery processes. These findings suggest that EBPR-r is a post-denitrification strategy that can also facilitate P recovery during secondary wastewater treatment.

As a consequence of the absence of soluble carbon in the secondary wastewater (i.e. upstream biological treatment removes most soluble carbon in wastewater), a high level of dissolved oxygen (DO >6 mg/L) was observed during the P uptake phase. It was demonstrated that the EBPR-r biofilm could still denitrify and uptake P under such conditions. However, the effect of DO on the EBPR-r process was unclear. Therefore, to investigate the impact of DO on storage-driven denitrification and P uptake by the EBPR-r biofilm, a series of batch experiments was conducted in which a PHA-enriched biofilm (obtained following anaerobic carbon replenishment) was exposed to various DO concentrations for P uptake (DO: 0−8 mg/L; NO$_3^-$: 10 mg-N/L; PO$_4^{3-}$: 8 mg-P/L) (Chapter 3). The results suggested that even at a saturating DO concentration (8 mg/L), the biofilm could take up P (0.043 ± 0.001 mmol-P/g-TS.h; TS: total solid) and denitrify efficiently (0.052 ± 0.007 mmol-N/g-TS.h). However, denitrification declined when the biofilm structure was physically disturbed, suggesting that this phenomenon was a result of an O$_2$ gradient across the biofilm. Hence, for a simultaneous denitrification and P removal using EBPR-r, maintaining the biofilm structure is critical. Moreover, analysis of the data also highlighted some operational boundaries (e.g. specific DO and NO$_3^-$ concentrations in the influent) necessary for the EBPR-r biofilm to achieve acceptable P and N removal. This is valuable information for developing EBPR-r as a post-denitrification strategy, where oxygen intrusion is unavoidable under carbon-deficient conditions.

The effectiveness of the EBPR-r process depends on whether the PAOs can efficiently shuttle soluble PO$_4^{3-}$ from a large volume of wastewater into a smaller recovery stream in a cyclic manner. In practice, whether or not a wastewater plant adopts a single cycle
for P uptake and release will depend largely on the availability of land and infrastructure. When these factors are limiting, an alternative mode of operation for P release (i.e. carbon replenishment) may involve sequential P uptake from the wastewater. Under such condition, the biofilm could be exposed to large quantities of electron acceptors (O$_2$ and NO$_3^-$), exceeding that required for P uptake. The impact of such a highly oxidising environment on storage polymers (and thus on the P uptake activity) of PAOs was unknown. Hence, a further objective of the study was to explore the ability of PAOs to conserve P uptake activity under P-deficient and highly oxidising conditions (Chapter 4). The results showed that even after two days of exposure to highly oxidising conditions, upon the addition of 8 mg/L of P the biofilm could facilitate a similar level of P uptake ($1.20 \pm 0.09$ mg-P/g-TS, between 0–48 h). This suggested that the P uptake activity was conserved throughout the period when no external carbon was replenished. Nonetheless, extending this period beyond 2 days was detrimental, and only 15% of the original P uptake activity remained by day 7. This finding is significant, as it is the first evidence of the ability of PAOs to conserve P uptake activity in the context of P recovery. This unique behaviour of PAOs may enable the development of new operational strategies, such as infrequent carbon replenishment to facilitate multiple P uptake phases before anaerobic carbon replenishment. The opportunities for flexibility in operational strategies could reduce the capital and operational costs of the EBPR-r process, and thus enhance the economic viability of P recovery.

One factor that determines the cost of implementing the EBPR-r strategy is the specific use of carbon for P recovery. However, the P$_\text{rel}$/C$_\text{upt}$ (P-release to carbon-uptake) ratio observed in the master reactor was substantially lower than that typically observed in conventional PAOs sludge (0.08 and 0.50–0.75 mol-P/mol-C, respectively). Hence, a strategy for optimising the P$_\text{rel}$/C$_\text{upt}$ ratio of the EBPR-r biofilm was investigated (Chapter 5). This was achieved using a stepwise increase of P-loading (by increasing the volume) and the P uptake period, while keeping the operational settings constant for recovery (1.8 L with 350 mg/L acetate and a P release duration of 2 h). The results showed that an increase in the wastewater volume from 7.2 L (stage I) to 14.4 L (stage II) and 21.6 L (stage III) increased the P$_\text{rel}$/C$_\text{upt}$ ratio marginally from 0.07 to 0.08 and 0.10, respectively. This small increase was because the biofilm displayed a similar P uptake rate ($0.57 \pm 0.05$ mg-P/g-TS.h) when exposed to the same P concentration in
wastewater. To facilitate a higher $P_{rel}/C_{upt}$ ratio, the P uptake duration was extended from 4 h in stage III to 10 h in stage IV. This increased the $P_{rel}/C_{upt}$ ratio markedly, from 0.10 to 0.25, and the biofilm at stage IV (cycle length of 12 h) was able to concentrate $PO_4^{3-}$ 10-fold, from 8 mg-P/L in the wastewater (21.6 L) to >90 mg-P/L in the recovery stream (1.8 L). Corresponding to the improved P recovery capacity, canonical correspondence analysis (based on sequences obtained using 454 pyrosequencing of the 16S rRNA genes) revealed a decreasing abundance of glycogen accumulating organisms (GAOs) (family Sinobacteraceae) and an increasing abundance of PAOs (Ca. Accumulibacter Clade IIA, unable to use NO$_3^-$) during the optimisation process. Based on the chemical and microbiological data, the strategy to optimise the $P_{rel}/C_{upt}$ ratio of the EBPR-r biofilm was validated. A 3-fold increase in the $P_{rel}/C_{upt}$ ratio was achieved (from stage I to IV), implying a more efficient use of carbon for P recovery (3× carbon saving).

In summary, a novel post-denitrification strategy and process to facilitate P recovery from a low P-containing wastewater was proposed, developed and validated. The EBPR-r approach is expected to offer several advantages over conventional post-denitrification processes: (1) it facilitates P recovery in addition to N removal; (2) it enables more efficient use of external carbon (for both P recovery and N removal, rather than just for N removal); and (3) it is associated with a lower risk of carbon discharge in the effluent (as carbon is not added to the wastewater, but to the recovery stream). Importantly, this study demonstrated a pioneering approach to using PAOs to address the global issue of P scarcity. Further research in this direction should be encouraged.
Declaration

I hereby declare that this submission is my own work and that, to the best of my knowledge, it contains no material previously published or written by another person nor material which to a substantial extend has been accepted for the award of any other degree or diploma of the university or other institute of higher learning, except where due acknowledgment has been made in the text.

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Thesis format and Authorship

In accordance with the University of Western Australia’s regulations regarding Research in Higher Degrees, this thesis is presented as a series of journal papers. The bibliographical details of the work and where it appears in the thesis are outlined below. As the primary author for all these publications, the candidate conducted all experimentation, and completed data analyses and writing of the manuscripts with the contributions from the other co-authors.

Publication arising from this thesis

1. Journal publications included as chapters


2. Conferences


Acknowledgement

I would like to take this opportunity to express my sincere thanks to a number of individuals who have helped me through the course of my PhD studies.

First of all, I would like to thank my supervisors for their selfless guidance and invaluable advice throughout the course of my studies. Assoc/Prof David Sutton, who had constantly offered me his support and guidance. I thank him for all the administrative work that he has taken in relation to my studies, as well as his extremely useful feedback on my academic writing. Dr Maneesha Ginige, who had always been there to lend me a helping hand whenever I encountered difficulties. I admire his knowledge, ability to pursue, confidence and enthusiasm toward his research. Dr Ka Yu Cheng, who had supported me all along with great passion and new ideas. His creativity and patience have often inspired me and led to key insights in my research. I really enjoyed the discussions that we had during coffee breaks, on both scientific and social matters. Dr Anna Kaksonen, who had always been kind and effective in her work. I am thankful for the excellent example she has set as a successful, independent woman scientist. She has shown how being soft yet strong can make a beautiful combination.

Secondly, I would like to thank my past and current CSIRO colleagues, Fahimeh Bimakr, Tharanga Weerasinghe Mohottige, Jason Wylie, Annachiara Codello, Suzy Rea, Kayley Usher, Naomi Mcsweeney, Robert Woodbury and many others those names are too numerous to mention in such a brief acknowledgement. Thank all for providing such a warm and joyful research environment for me. I shall never forget the friendship that we had developed the past four years. Furthermore, I am very grateful to Dr Trevor Bastow and Yasuko Geste for their expert advice and invaluable assistance on the chemical analyses.

I would also like to acknowledge the financial support of the Australian Postgraduate Scholarship from the University of Western Australia and the top-up scholarship from CSIRO Land and Water.

Most importantly, I would like to thank my beloved family in particular Kimmy and Shenton, for their continuous support and unconditional love. They have always been
there for me throughout everything, and for that I am truly blessed to have them in my life.

Last but not least, I would like to dedicate this work to the memory of my father and grandmother whom certainly would have been very proud. They taught me one of the most important things in life, that is success only comes to those who work hard. It was their love and strong belief in me and my successes that had stood me in good stead through all the difficult moments in a foreign country far away from home.
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<thead>
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<th>Symbol</th>
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<tr>
<td>µm</td>
<td>Micrometer</td>
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<td>µmol</td>
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<td>DARB</td>
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<td>h</td>
<td>Hour</td>
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<tr>
<td>L</td>
<td>Liter</td>
</tr>
<tr>
<td>M</td>
<td>Mole</td>
</tr>
<tr>
<td>mA</td>
<td>Milliamperre</td>
</tr>
<tr>
<td>MFCs</td>
<td>Microbial fuel cells</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>mL</td>
<td>Milliliter</td>
</tr>
<tr>
<td>MLSS</td>
<td>Mixed liquor suspended solids</td>
</tr>
<tr>
<td>MLVSS</td>
<td>Mixed liquor volatile suspended solids</td>
</tr>
<tr>
<td>mmol</td>
<td>Millimole</td>
</tr>
</tbody>
</table>
mV  Millivolt
N  Nitrogen
n.a.  Not applicable
N_{den}/P_{upt}  Amount of N removed per P taken up during P uptake phase
OC  Open circuit
OCP  Open circuit potential
OLR  Organic loading rate
ORP  Redox potential
OTUs  Operational Taxonomic Units
OURs  Oxygen uptake rate
P  Phosphorus
PAOs  Polyphosphate accumulating organisms
PCoA  Principle coordinate analyse
PCR  Polymerase chain reaction
PHAs  Poly-β-hydroxyalkanoates
Poly-P  Polyphosphate
P_{rel}/C_{upt}  Amount of P released per carbon taken up under anaerobic condition
P_{rel}/P_{upt}  Amount of P released per P taken up
PRRs  Phosphate release rates
P_{upt}/N_{den}  Amount of P taken up per N removed during P uptake phase
PURs  Phosphate uptake rates
QIIME  Quantitative Insights Into Microbial Ecology
s  Second
SBR  Sequencing batch reactor
SND  Simultaneous nitrification denitrification
SNDPR  Simultaneous nitrification, denitrification and phosphorus removal processes
t  Ton
TN  Total nitrogen
TP  Total phosphorus
TS  Total solid
US$  US dollar
VFAs  Volatile fatty acids
VSS  Volatile suspended solids
WWTPs  Wastewater treatment plants
y  Year

XIX
1. Literature Review

This review highlights the need for the wastewater industry to move from phosphorus removal to phosphorus recovery. Existing chemical and biological strategies used for phosphorus removal are discussed in the context of how these strategies could be retrofitted in existing wastewater treatment plants to facilitate phosphorus recovery. The pros and cons of the existing P recovery strategies, and a promising emerging alternative strategy that warrants further research and development are also discussed in detail.

1.1. Phosphorus removal from wastewater

Municipal wastewater commonly contains phosphorus (P). Domestic sources of P to sewers include human faeces and urine, washing machine and dishwasher detergents, and personal care products (Karunanithi et al., 2015). While the contributions from each of the above sources are unclear, it is estimated that on average 0.9 g-P/person/day is excreted in urine and 0.4 g-P/person/day is excreted in faeces (Jönsson et al., 2005). The total P concentration in municipal wastewater is typically 6–8 mg-P/L, but can be higher depending on the source (Parsons & Smith, 2008). To prevent eutrophication of surface waters, P often needs to be removed from wastewater prior to its discharge to the environment. In wastewater treatment plants (WWTPs), P is typically removed using chemical and/or biological methods. In both cases the soluble P from wastewater is converted to solid form, and then removed by sedimentation and centrifugation. When operated properly, these processes can remove >90% of P from wastewater, achieving effluent concentrations of <1 mg-P/L (Parsons & Smith, 2008).
1.1.1. **Phosphorus removal by chemical precipitation**

Chemical precipitation is a relatively simple but effective method for P removal from wastewater. The chemicals commonly used for P removal are calcium hydroxide (lime), and metal salts including aluminium chloride (or aluminium sulfate) and ferric chloride (Pratt et al., 2012). Although effective, achieving satisfactory P removal from wastewater often requires the addition of the chemicals at levels exceeding the stoichiometric requirements for precipitation. Additionally, it is often difficult to dewater the chemical sludge produced (Seviour & Nielsen, 2010). Resolving both issues comes at a major cost to the industry. Moreover, direct agronomic application of the waste sludge is typically restricted because of heavy metal contamination. Consequently, chemical precipitation is generally considered to be an environmentally undesirable approach to P management in the wastewater industry.

1.1.2. **Enhanced biological phosphorus removal**

P can also be removed from wastewater using a biological process referred to as enhanced biological phosphorus removal (EBPR). An EBPR configuration facilitates alternating sequential exposure of biomass to wastewater under anaerobic and aerobic/anoxic conditions (Figure 1.1). This enables the enrichment of a unique group of bacteria termed polyphosphate accumulating organisms (PAOs) (Seviour & Nielsen, 2010). PAOs are able to store soluble orthophosphate (PO$_4^{3-}$) intracellularly as polyphosphate (Poly-P) in excess of their normal cell requirements, and potentially reaching 15–20% of the dry biomass weight (Tchobanoglous et al., 2003). Regular removal of a proportion of the P-enriched biomass enables the wastewater treatment process to achieve P removal. The EBPR process and the biochemistry of PAOs is summarised in Figure 1.2.
Figure 1.1 Schematic diagram of an enhanced biological phosphorus removal (EBPR) process.

Figure 1.2 Schematic diagrams of the anaerobic and aerobic/anoxic metabolism of PAOs. PHAs: poly-β-hydroxy-alkanoates, Poly-P: polyphosphate, VFAs: volatile fatty acids, ATP: adenosine triphosphate, NADH: nicotinamide adenine dinucleotide.

A. Anaerobic metabolism

In a typical EBPR system treating wastewater, returned activated sludge is initially exposed to anaerobic conditions, where PAOs take up short chain volatile fatty acids (VFAs), including acetate, from wastewater as carbon sources (Tchobanoglous et al., 2003). The carbon (C) is converted into intracellular storage products, including poly-β-
hydroxy-alkanoates (PHAs), using energy derived from the hydrolysis of stored Poly-P and glycogen (Janssen, 2002). This process releases $\text{PO}_4^{3-}$, and results in a temporary increase in the P concentration in wastewater (>50 mg-P/L; Figure 1.3) (Janssen, 2002).

**Figure 1.3** Profiles of extracellular phosphate-P (□), acetate (●), intracellular PHA (○) and glycogen (▲) during the anaerobic and aerobic phases of a typical PAOs sludge in a conventional EBPR reactor. Adapted from Bond et al. (1999).

**B. Aerobic/anoxic metabolism**

Subsequent to the anaerobic phase, exposure of the PAOs to an aerobic and/or anoxic phase in the absence of soluble carbon (electron donor) triggers the oxidation of internal PHA reserves to fulfil energy requirements for cell growth, glycogen replenishment and $\text{PO}_4^{3-}$ uptake (Oehmen et al., 2007). PAOs can utilise oxygen ($\text{O}_2$), nitrate ($\text{NO}_3^-$) or nitrite ($\text{NO}_2^-$) as final electron acceptors for this process. Microorganisms that are able to carry out P uptake and denitrification using $\text{NO}_3^-$ or $\text{NO}_2^-$ as a final electron acceptor are referred to as denitrifying PAOs (DPAOs). Compared with PAOs, DPAOs are more significant as they can contribute to: (1) a reduction of aeration demand (because $\text{NO}_3^-$ is used as the final electron acceptor instead of $\text{O}_2$), and thus lower operational costs; (2) more effective use of carbon source (because carbon is used to achieve both
denitrification and P uptake); and (3) a decrease in sludge production by approximately 20–30% (Oehmen et al., 2007).

As the majority of soluble $\text{PO}_4^{3-}$, including that released during the previous anaerobic phase, is taken up by PAOs and stored as Poly-P, the P concentration in wastewater decreases in this phase (see Figure 1.3). This enables WWTPs to discharge effluent having P concentrations as low as 1–2 mg-P/L. To facilitate net P removal from the WWTP, some of the P-enriched biomass must be periodically removed (Seviour & Nielsen, 2010). Unlike the sludge produced from chemical precipitation, this waste sludge can be applied directly as a soil amendment to supplement P requirements of agricultural lands (Janssen, 2002). Consequently, EBPR is an environmentally friendly approach to P reuse. Additionally, there is opportunity to recover the entrapped P from waste sludge using additional sludge treatments, such as anaerobic digestion and acid extraction (Martí et al., 2010).

1.2. Phosphorus recovery from wastewater

1.2.1. Phosphorus recovery is a necessity

P is a non-substitutable resource that is exclusively used in modern agriculture to maintain high crop yields (Cordell et al., 2009). The main source of P is phosphate rock, which is a non-renewable resource (Rittmann et al., 2011). According to van Enk et al. (2011), 27 million tons of P is used in the global agricultural system annually, of which 3 million tons is accountable for human food consumption, and the rest is dissipated into the environment. As the world’s population increases the future demand for P is expected to intensify. At the current rate of consumption, P reservoirs will be rapidly
depleted in the foreseeable future (Cordell et al., 2009). Hence, there is an urgent need to recycle P to enable sustainable development.

“Wastage” of P is largely a result of agricultural runoff and wastewater discharge (Seviour & Nielsen, 2010). Although agricultural runoff contains a relatively large amount of P, recovery of P from this source is difficult (lower P concentration in nonpoint sources) (Cordell et al., 2011). In contrast, the amount of P present in wastewater is low, and even if recovered could only satisfy 15–20% of the global P demand (Yuan et al., 2012). However, with low effluent P discharge limits being increasingly enforced, incorporating P recovery into existing P removal processes has become an attractive proposition for many municipalities worldwide (Karunanithi et al., 2015). Recent reviews by Sartorius et al. (2012) and Karunanithi et al. (2015) have summarised the status of P recovery technology. The available P recovery strategies are summarised in Figure 1.4, with examples given in Tables 1.1 and 1.2.

![Figure 1.4](image)

**Figure 1.4** An overview of available methods for phosphorus recovery from sludge/ash and wastewater.
Table 1.1 Examples of the biological approaches developed for P recovery from wastewater and sludge.

<table>
<thead>
<tr>
<th>Approach</th>
<th>Feedstock</th>
<th>Description</th>
<th>Product</th>
<th>Recovery Efficacy</th>
<th>Scale</th>
<th>Country</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biological</td>
<td>Wastewater</td>
<td>A biofilm process to uptake P from wastewater (5 mg-P/L) and release it into a separate stream. Multiple P release into the same stream generated P-enriched liquor (Kodera et al., 2013).</td>
<td>P-rich liquid (&gt;100 mg-P/L)</td>
<td>11% (of P in biomass)</td>
<td>Lab</td>
<td>Japan</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anaerobic-anoxic/nitrifying/induced crystallisation (A2N-IC) P-enriched liquid from the settler of EBPR anaerobic reactor was partly fed into a crystallisation process to form CaP (Shi et al., 2012).</td>
<td>CaP</td>
<td>n.a.</td>
<td>Lab</td>
<td>China</td>
</tr>
<tr>
<td>P-enriched liquid</td>
<td>Pearl®</td>
<td>A fluidised bed reactor that facilitates struvite crystallisation from thickener liquor, which generated from the treatment of EBPR sludge (via WASSTRIP® sludge treatment and anaerobic digestion). Developer: Ostara (<a href="http://www.ostara.com/">http://www.ostara.com/</a>)</td>
<td>MAP</td>
<td>80–90% (of P to crystallisation reactor)</td>
<td>Full</td>
<td>Canada</td>
</tr>
<tr>
<td></td>
<td>PhoStrip®</td>
<td>Phosphorus recovery by Institute of Environmental Engineering (PRISA) Acidification of EBPR sludge followed by anaerobic digestion. The P-enriched liquids generated from processes are used for struvite precipitation (Montag et al., 2007).</td>
<td>MAP</td>
<td>40% (of P in wastewater)</td>
<td>Full</td>
<td>Germany</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P is biologically released from PAOs sludge under anaerobic condition. P is precipitated from the generated P-enriched liquid (Levin &amp; Della Sala, 1987).</td>
<td>CaP</td>
<td>60% (of P in wastewater)</td>
<td>Full</td>
<td>USA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Portion of sludge was taken at the end of EBPR aerobic phase. In a batch reactor, carbon was added to release P from sludge, generating a P-rich liquid for recovery (Xia et al., 2014).</td>
<td>P-rich liquid (240 mg-P/L)</td>
<td>79% (of P in wastewater)</td>
<td>Lab</td>
<td>China</td>
</tr>
</tbody>
</table>
Table 1.2 Examples of some physical-chemical approaches developed for P recovery from sludge and incinerated ash.

<table>
<thead>
<tr>
<th>Approach</th>
<th>Feedstock</th>
<th>Description</th>
<th>Product</th>
<th>Recovery Efficacy</th>
<th>Scale</th>
<th>Country</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wet-chemical</td>
<td>Sewage sludge</td>
<td><strong>Seaborne</strong> Hydrolysis of sewage sludge using sulphuric acid, precipitation of heavy metals as sulphides, and finally recovery of P as MAP (Müller et al., 2005).</td>
<td>MAP</td>
<td>n.a.</td>
<td>Full</td>
<td>Germany</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>PHOXNAN</strong> A low pressure wet oxidation to release P, followed by a nanofiltration process to separate P from heavy metal, and finally P is recovered as phosphoric acid. (Blöcher et al., 2012)</td>
<td>Phosphoric acid</td>
<td>54% (of P in sludge)</td>
<td>Lab</td>
<td>Germany</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Quick Wash™</strong> P is first solubilised from human/animal waste via acid extraction. The extracted P is then precipitated as CaP. Developer: Renewable Nutrients LLC (<a href="http://www.renewablenutrients.com/">www.renewablenutrients.com/</a>)</td>
<td>CaP</td>
<td>95% (of P in sludge)</td>
<td>Full</td>
<td>USA</td>
</tr>
<tr>
<td>Sewage sludge ash</td>
<td></td>
<td><strong>RecoPhos</strong> Sewage sludge ash is treated with phosphoric acid to solubilise the P and minerals. This liquid is then used to produce fertilizer (Weigand et al., 2013).</td>
<td>P 38 fertilizer</td>
<td>n.a.</td>
<td>Full</td>
<td>Germany</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P was first solubilised from sludge ash using acidic (HCl) and alkali (NaOH) extraction. After heavy metals were removed using cation exchange resin, P was recovered (Xu et al., 2012).</td>
<td>MAP (58% P₂O₅)</td>
<td>97% (of P sludge)</td>
<td>Lab</td>
<td>China</td>
</tr>
<tr>
<td>Thermal</td>
<td>Sewage sludge and/or ash</td>
<td><strong>MEPHREC®</strong> The briquettes of sludge/ash are thermally treated at 1450 °C. Volatile metals are evaporated and non-volatile heavy metals are separated as liquid metal phase. Developer: Ingitec (<a href="http://www.ingitec.de">www.ingitec.de</a>)</td>
<td>Slag (10–25 % P₂O₅)</td>
<td>81% (of P sludge)</td>
<td>Pilot</td>
<td>Germany</td>
</tr>
<tr>
<td></td>
<td>Sewage sludge ash</td>
<td><strong>Ashdec®</strong> The phosphate phases present in sewage sludge ash are transformed into bio-available form (NaCaPO₄), by reaction with Na₂SO₄ at 900–1000°C in a rotary kiln. Developers: Outotec (<a href="http://www.outotec.com">www.outotec.com</a>) and BAM (<a href="http://www.bam.de">www.bam.de</a>)</td>
<td>Calci- (10–25 % P₂O₅)</td>
<td>98% (of P ash)</td>
<td>Pilot</td>
<td>Finland</td>
</tr>
</tbody>
</table>
1.2.2. P recovery from solids

As stated above, P removal processes remove the majority of the soluble P (~90%) in wastewater as biomass/sludge; in this process the P is largely captured in solid form, which can be separated from the wastewater. As a consequence of the large amount of P potentially present, many strategies have been specifically developed to recover the entrapped P. In some cases, up to 90% of the influent P can be recovered (Sartorius et al., 2012). The most economical route for disposal of biosolids (dewatered biosolids) and recycling of P is through direct land application as a fertilizer or soil conditioner (Petzet & Cornel, 2011). However, because of public health concerns associated with heavy metal and pathogen contamination, agricultural application of biosolids is often restricted (Blöcher et al., 2012). For instance, the use of sewage sludge on agriculture has been prohibited in the Netherlands since 1995 (Roeleveld et al., 2004).

Advanced methods exist for recovering P from sludge and from incinerated ash (Figure 1.4, Tables 1.1 and 1.2). These methods can be generally classified into one of three treatment categories: biological, wet-chemical and thermal (Dichtl et al., 2007; Petzet & Cornel, 2011; Sartorius et al., 2012). In biological treatment, sludge produced from an EBPR is treated in an anaerobic digester to solubilise the P from biomass. The solubilised P can then be precipitated as a fertilizer (e.g. struvite and calcium phosphate) (Sartorius et al., 2012). In the wet chemical treatment, P is solubilised from the biosolids or sewage sludge ash via acid or alkali extraction. After separating heavy metals from the P-enriched liquid (e.g. by nanofiltration and cation exchange), the dissolved P can be precipitated as fertilizer (Xu et al., 2012). In the thermal treatment, the P-containing biosolids or ash residue is treated at 1000–2000 °C. At these temperatures the volatile metals are evaporated, and the non-volatile heavy metals are
separated into a liquid metal phase (Boutoussov, 2009). The resulting slag is enriched with P and can be used for land application.

While successful operations have been reported, all three approaches are energy (e.g. heating) or chemical intensive (e.g. treatment with strong acid/base) (Sartorius et al., 2012). Moreover, these strategies are largely restricted to large-scale WWTPs, where facilities including anaerobic digesters and incinerators are already available. As a result, these conventional methods have not been widely embraced by the wastewater industry.

1.2.3. P recovery from wastewater is challenging because of the low concentration

A recent survey suggests that most (53%) researchers in the field of P recovery believe that recovering P directly from wastewater, without the need for anaerobic digesters or incinerators, is a better option than recovery from P-enriched solids (Sartorius et al., 2012).

Recovering P directly from wastewater is feasible using non-EBPR methods, including anion exchange (Bottini & Rizzo, 2011), forward osmosis (Xie et al., 2014), adsorption (Kuzawa et al., 2006) and microbial fuel cells (Ichihashi & Hirooka, 2012). However, these methods can be costly and are mainly restricted to industrial wastewater, which has P concentrations that are higher than in municipal wastewater (Sartorius et al., 2012). Alternatively, P could be recovered from wastewater using biological approach based on the EBPR process (Table 1.1), such as liquor of dewatered EBPR sludge (returned activated sludge, see Figure 1.1). Compared with P recovery from sludge and ash, the amount of P that can be recovered directly from wastewater (without sludge treatments) is relatively low (approximately 40% of the total P load in WWTPs) (Petzet
& Cornel, 2011), largely because the P concentration in wastewater is too low for chemical precipitation.

The most widely used method for direct recovery of P from wastewater is chemical precipitation. In this process soluble P is converted into marketable fertilizer, such as calcium phosphate (CaP) or struvite (magnesium ammonium phosphate, \( \text{NH}_4\text{MgPO}_4\cdot6\text{H}_2\text{O}, \text{MAP} \)) (Marti et al., 2008). Calcium phosphate is favoured because its chemical properties are similar to rock phosphate. Struvite is also popular because of its multi-nutrient content and its low solubility; this obviates the need for frequent application, which minimises the risk of chemical burning of the crop roots (Parsons & Smith, 2008).

For P recovery via chemical precipitation to be economically viable, the concentration of P in wastewater must reach a certain level, typically 50 mg-P/L (Cornel & Schaum, 2009). Currently, chemical precipitation of P is only applied on certain waste streams, such as on liquor of dewatered EBPR sludge (Figure 1.1) and side stream of an anaerobic digester (Table 1.1), where the P concentrations (in the range of 20–100 mg-P/L) are suitable for chemical precipitation (Nieminen, 2010). As municipal wastewater typically contains only 7–10 mg-P/L of P, using chemical precipitation for P recovery from such waste streams is difficult (Parsons & Smith, 2008). Unless the concentration of P can be effectively increased beyond the threshold level (>50 mg-P/L), direct recovery of P from wastewater is both economically and technically challenging.

1.2.4. Current economic status of P recovery

The wastewater industry is largely considered to be a service industry that is typically a financial burden on society. Although this industry has been modernised in response to pressures including more stringent discharge limits, a desire for such processes to have
smaller footprints, a shortage of landfills and concerns about increasing operational costs, it remains a service industry that rarely generates revenue. Despite this, there are many resources that can potentially be recovered from municipal wastewater, including P, carbon, ammonia, water and energy (Guest et al., 2009). However, recovery of these resources is only practical using technologies that are economically viable, and do not further increase the operational costs of wastewater treatment.

Because of the high costs associated with the existing recovery strategies, P recovery is yet to be widely embraced by the wastewater industry. At the current price of rock phosphate there is also little economic incentive to recover P from wastes (Parsons & Smith, 2008), and therefore the reluctance of the wastewater industry to recover P is understandable. However, the recycling of P is inevitable, and it is only a matter of time before the wastewater industry is compelled to implement P recovery strategies. This highlights the need to explore new P recovery strategies that will not incur additional cost in the treatment of wastewater. One plausible strategy is to recover P concurrently with removal of other nutrients, including nitrogen (N). In this context, post-denitrification could be used as a platform to implement P recovery whereby both N removal and P recovery takes place using the same amount of carbon. This approach, if demonstrated to be feasible, would decrease the operational cost of post-denitrification because of the revenue generated from the recovered P. With increasing triple superphosphate prices (increase of 25% per annum over the past 10 year; http://www.indexmundi.com/) and proper management of recovery costs, P recovery could become a revenue-generating stream for the wastewater industry in the near future.
1.3. Can P recovery be accomplished with post-denitrification? A new concept based on EBPR

This study was premised on the merits of using biological methods to facilitate P recovery from wastewater, and the need to incorporate P recovery concurrently with nutrient removal so as a way to minimise operational costs. As noted above (section 1.1.2), the microorganisms responsible for EBPR (PAOs) have the potential to facilitate both P and N removal from wastewater. By harnessing the metabolism of DPAOs, in this study a unique strategy referred to as Enhanced Biological Phosphorus Removal and Recovery (EBPR-r) was investigated to facilitate simultaneous denitrification and P recovery from wastewater (e.g. secondarily effluent). The concept of this novel EBPR-r process is summarised in Figure 1.5.

**Figure 1.5** A conceptual framework for the proposed post-denitrification EBPR-r process using DPAOs to concentrate P from municipal wastewater. In the first step, DPAOs uptake P from a large P-containing stream using NO₃⁻ as electron acceptor. In the subsequent anaerobic phase the DPAOs uptake acetate and release the captured P into smaller volume recovery stream.
As discussed in section 1.1.2B, DPAOs are able to use NO$_3^-$ as a final electron acceptor and temporarily store PO$_4^{3-}$ as Poly-P. Hence, it was hypothesised that DPAOs could be used to remove both N and P from secondary wastewater to a very low level, meeting the stringent discharge limits that are being increasingly imposed on WWTPs worldwide (Boltz et al., 2012). As DPAOs oxidise internal carbon storage polymers to derive energy requirements for this process, nutrient removal can take place in the complete absence of soluble carbon in wastewater (upstream biological treatment removes most soluble carbon in wastewater).

The conceptual framework for the EBPR-r was developed based on the following hypothesised processes. During anaerobic metabolism the internal carbon storage polymers of DPAOs will be restored via carbon uptake. Hydrolysis of Poly-P reserves will fulfil the energy requirements for this process, and the hydrolysis product (PO$_4^{3-}$) will be released into the bulk water. Unlike in the conventional EBPR configuration, the DPAOs in the EBPR-r process will be exposed to a separate small-volume recovery stream supplied with a carbon source to facilitate this P-release step. The use of a smaller volume recovery stream (relative to the wastewater stream) should facilitate the recovery of P at higher concentration than in the wastewater stream. As the recovery stream will be hydraulically separated from the wastewater stream, accidental discharge of carbon with wastewater will be prevented (Figure 1.6). This would make the EBPR-r approach highly advantageous compared with conventional post-denitrification, where extensive monitoring is needed to prevent carbon discharge to the environment. In addition to the potential benefits noted above, the novel EBPR-r is expected to address several drawbacks associated with conventional post-denitrification (Table 1.3). The following section further elaborates the differences between EBPR-r and conventional...
post-denitrification, and highlights the advantages and disadvantages of this novel process.

Figure 1.6 The application of EBPR-r as a post-denitrification strategy in a WWTP.
Table 1.3 Comparison between the conventional post-denitrification and EBPR-r strategy.

<table>
<thead>
<tr>
<th></th>
<th>Conventional post-denitrification</th>
<th>EBPR-r</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number of stream</strong></td>
<td>Single (wastewater)</td>
<td>Two (wastewater and recovery)</td>
</tr>
<tr>
<td><strong>Attached growth system</strong></td>
<td>Yes (To maintain a higher biomass density)</td>
<td>Yes (To facilitate exchange of two streams and to maintain a higher biomass density)</td>
</tr>
<tr>
<td><strong>Nutrients removal &amp; recovery</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N removal</td>
<td>Yes (Using heterotrophic denitrifiers)</td>
<td>Yes (Using storage-driven denitrifiers, DPAOs and DGAOs)</td>
</tr>
<tr>
<td>P removal</td>
<td>Insignificant (Removal as a result of biomass growth)</td>
<td>Significant (Removal via P uptake by PAOs and/or DPAOs)</td>
</tr>
<tr>
<td>P recovery</td>
<td>No</td>
<td>Yes (Through anaerobic P release into a smaller recovery stream)</td>
</tr>
<tr>
<td>Recovery of other resources</td>
<td>No</td>
<td>Mg²⁺, K⁺ (Through anaerobic released by PAOs and/or DPAOs to neutralise the charge of PO₄³⁻)</td>
</tr>
<tr>
<td>Energy source (electron donor)</td>
<td>Via oxidation of soluble carbon</td>
<td>Via oxidation of internal carbon storage polymers</td>
</tr>
<tr>
<td><strong>External carbon addition</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Most commonly used carbon sources</td>
<td>Methanol, ethanol, acetate, sludge hydrolysate (depends on market price)</td>
<td>Acetate and other (depends on the PAOs’ ability to metabolise the carbon source)</td>
</tr>
<tr>
<td>Carbon addition</td>
<td>To the wastewater stream</td>
<td>To the P recovery stream (Hydraulically separated from the wastewater stream)</td>
</tr>
<tr>
<td>Risk of discharging carbon with wastewater effluent</td>
<td>High (require stringent control of carbon addition)</td>
<td>Low (carbon source is introduced into the recovery stream which is hydraulically separated from the wastewater stream)</td>
</tr>
<tr>
<td>Use of soluble carbon to facilitate anoxic condition for denitrification</td>
<td>Yes (some soluble carbon will be aerobically oxidised)</td>
<td>No (some carbon storage polymers may be aerobically oxidised)</td>
</tr>
</tbody>
</table>
1.4. Characteristics of EBPR-r compared with conventional post-denitrification processes

1.4.1. The use of attached growth system to facilitate liquid exchange in EBPR-r

Similar to most conventional tertiary post-denitrification technologies (Boltz et al., 2012), the use of an attached growth system (biofilms) was proposed for the EBPR-r process. Compared with suspended growth systems, attached growth enables the maintenance of a greater biomass density, and thereby leads to higher volumetric nutrient removal rates. This could reduce the residence times and reactor footprint of the process. Secondly, because majority of biomass is retained in the attached growth system, rapid filling and complete decanting of liquor could be achieved. As such, the attached growth systems could be easily retrofitted to facilitate the unique operational requirement of EBPR-r, namely to switch between two streams (wastewater and recovery stream) that are hydraulically separated. As the biomass will be attached to the carrier media, complete decant of liquid from the reactor is feasible, and the risk of cross contamination during liquid exchange is also minimised.

1.4.2. EBPR-r as a post-denitrification process for P recovery from the wastewater

Although conventional post-denitrification enables N removal from wastewater, P removal in this process is minimal. Only a small portion of the P in influent (0.02 mg-P/mg-VSS; VSS: volatile suspended solids) is removed as a result of biological growth (Henze, 2008), and the majority remains in conventional post-denitrification. As post-denitrification is only implemented when very low nutrient discharge limits must be met (e.g. discharge of effluent into sensitive water bodies), a reduction in the P
concentration is essential. This is typically achieved in a separate process, such as via chemical precipitation of P (Henze, 2008).

In contrast, the EBPR-r process is designed as a tertiary treatment to facilitate simultaneous removal of N and P from wastewater (Figure 1.6). This is possible because some DPAOs are able to uptake P using NO$_3^-$ and/or NO$_2^-$ as final electron acceptors (Jabari et al., 2014). Typically, DPAOs remove P and N at $P_{\text{upt}}/N_{\text{den}}$ (the amount of P taken up per N removed during the anoxic P uptake phase) molar ratios of 0.60–1.0 using NO$_3^-$ (Kerrn-Jespersen et al., 1994; Lanham et al., 2011) and 0.27–0.70 using NO$_2^-$ (Ma et al., 2013). Compared with the use of O$_2$ as the final electron acceptor, the amount of energy gained by PAOs with NO$_3^-$ and NO$_2^-$ as final electron acceptors is approximately 40% less (Murnleitner et al., 1997). Possibly as a result of the lower energy generation, the use of NO$_3^-$ and NO$_2^-$ is always associated with a lower level of P uptake (Lanham et al., 2011). Nevertheless, the ability of DPAOs to simultaneously remove N and P in one step makes their use in the EBPR-r process potentially very advantageous.

1.4.3. Recovery of Mg$^{2+}$ and K$^+$ from wastewater along with P recovery

In addition to P recovery, other nutrients including magnesium (Mg$^{2+}$) and potassium (K$^+$) may also be recovered in the EBPR-r recovery stream (Figure 1.6). This is feasible because these metal ions are required to facilitate the transport of PO$_4^{3-}$ across cell membranes. Each PO$_4^{3-}$ molecule contains three negative charges, and thus the extracellular release of each PO$_4^{3-}$ molecule needs to be accompanied by Mg$^{2+}$ and K$^+$, to ensure charge balance (Mulkerrins et al., 2004). Although the mechanism of this process is still unclear, these metal ions are presumed to be co-factors of enzymes inside the bacterium. Specifically, Mg$^{2+}$ is known to assist enzyme catalysis of Poly-P
biosynthesis, and K\(^+\) plays a role in defining the cell membrane permeability and facilitating the transport of PO\(_4^{3-}\) across the cell membrane (Choi et al., 2011b).

Similarly, when P is taken up from wastewater, these metals ions are also removed from wastewater, typically at a molar ratio of P:\(1/3\):Mg:\(1/3\) (Jonsson et al., 1996). The recovery of these metal ions in the EBPR-r process is beneficial for downstream P recovery steps. For instance, the recovered Mg\(^{2+}\) could reduce some of the chemical requirements for struvite production, which require an equimolar amount of NH\(_4^+\), Mg\(^{2+}\) and PO\(_4^{3-}\) (Münch & Barr, 2001). The recovery of K\(^+\) is also a potential revenue stream for the wastewater industry, although it remains unclear whether K\(^+\) would interfere with struvite precipitation.

1.4.4. The addition of external carbon to the EBPR-r

A. The selection of carbon source is dependent on the PAOs’ metabolism

In conventional post-denitrification, external carbon is directly added into the wastewater stream to facilitate denitrification. Depending on the type of carbon used, a distinct microbial community capable of utilising this carbon is selectively enriched. As the ability to denitrify is ubiquitous in many bacterial classes, N removal can be achieved using a wide range of external carbon sources. The commonly used carbon sources for conventional post-denitrification are methanol, ethanol, acetate and hydrolysed sludge (Osaka et al., 2006). The selection of a carbon source is based on the availability, price and period of adaptation by the microbial community. For instance, methanol is often chosen over other organic compounds due of its relatively low cost and low sludge production (Osaka et al., 2006).

Unlike conventional post-denitrification, the type of carbon that can be used in EBPR-r is expected to be less flexible, as it is largely used by PAOs. Although PAOs are able to
use non-VFAs including glucose (Jeon & Park, 2000) and glycerol (Lv et al., 2014). VFAs are generally believed to be the most suitable carbon source for PAOs. In particular, acetate is the most common used VFA, and the metabolic pathways involved have been well studied in the literature. Propionate has also been used because it gives PAOs a selective advantage for growth over non-PAOs, including glycogen accumulating organisms (GAOs) (Oehmen et al., 2007). For instance, it has been demonstrated that by regularly switching the carbon source between acetate and propionate in an EBPR reactor the PAOs population in the microbial community was substantially enriched (with *Accumulibacter* representing >90% of the bacterial population) (Lu et al., 2006).

**B. Unwanted discharge of residual carbon may be minimised in EBPR-r**

In post-denitrification, the timing of addition and the dosing rate for the external carbon is critical. The dosing rate needs to be carefully controlled to ensure satisfactory N removal, and avoidance of overdosing is essential to minimise the operational costs and the risk of discharging excess carbon to the environment (Regan et al., 1998). Various automatic methods have been developed to control the external carbon dosage, and some have been implemented in full-scale operations (Puznava et al., 1998; Yuan et al., 1997).

Unlike conventional post-denitrification, the risk of accidental discharge of carbon into the environment is expected to be minimal in EBPR-r (Figure 1.6). This is mainly because the external carbon is added directly into the recovery stream instead of into the wastewater stream.
C. Use of carbon storage polymers to facilitate anoxic conditions in the EBPR-r

Typically, secondary effluent is highly deficient in readily biodegradable carbon (Mines, 2014). Because of the absence of adequate carbon for microbes to reduce O₂, the secondary effluent (from upstream N removal) entering the post-denitrification step may contain residual levels of dissolved oxygen (DO) (Yuan & Oleszkiewicz, 2011). In conventional post-denitrification, aerobic heterotrophs reduce DO by oxidising some of the external carbon, which creates the anoxic microenvironment (an O₂ gradient) required for denitrification (Wei et al., 2014). Consequently, not all of the supplied external carbon is fully utilised for N removal.

In the EBPR-r process the creation of anoxic conditions is expected to be derived from the oxidation of carbon storage polymers (electron donor) by storage-driven microorganisms (e.g. PAOs and GAOs). By creating an O₂ gradient in the outer part of the biofilm, PAOs and GAOs, may assist in creating anoxic conditions conducive to denitrification by DPAOs and denitrifying GAOs (DGAOs) in the inner part of the biofilm (Zeng et al., 2003a). However, when PAOs predominantly consume O₂ to create an anoxic zone, simultaneous removal of P from wastewater would be achieved. On the other hand, the growth of GAOs (unable to denitrify) in the EBPR-r biofilm would consume carbon without contributing toward P and N removal (Seviour & Nielsen, 2010), and thus minimising the growth of this group of bacteria is important for the success of EBPR-r. It should be noted that during anaerobic P release in the EBPR-r process, both PAOs and GAOs could consume the added soluble carbon and contribute to the creation of the anaerobic condition required for P recovery.
1.4.5. Denitrification by DPAOs

After upstream biological nitrogen treatment (Figure 1.6), secondary effluent entering post-denitrification typically contains a very low level of NH$_4$ (<0.5 mg-N/L) and 10–15 mg-N/L of NO$_3^-$ (Rittmann et al., 2004). The goal of post-denitrification is to reduce the total nitrogen (TN) concentration to <3 mg-N/L (Boltz et al., 2012).

Denitrification is the reduction of NO$_3^-$ to N$_2$, and four enzymes are generally required to facilitate the complete reduction to N$_2$ (Figure 1.7). Because not all denitrifiers have all four enzymes, partial denitrification with the accumulation of intermediate products (e.g., NO$_2^-$) is a common occurrence.

![Figure 1.7 Complete denitrification from NO$_3^-$ to N$_2$ consists of four reduction steps, each catalysed by a different enzyme.](Image)

**A. Nitrate reductase (Nar):**

Nitrate reductase (Nar) catalyses the first step of dissimilatory NO$_3^-$ reduction (Baker 1998). There are two forms of Nar: (1) membrane Nar, which is found on the cytoplasmic side of the cytoplasmic membrane; and (2) periplasmic Nar, which is found on the periplasmic side of the cytoplasmic membrane.

As the membrane Nar is located on the inner surface of the cytoplasmic membrane, a proton motive force and/or a NO$_3^-$–NO$_2^-$ antiporter is needed to transport NO$_3^-$ across the cell membrane. The NO$_3^-$ transporter is regulated by O$_2$ in the environment. The presence of O$_2$ can change the redox state of ubiquinones, and prevent the movement of
NO$_3^-$ across the membrane (Ferguson, 1994). Most denitrifiers are believed to be dependent on the membrane Nar for denitrification, thus these denitrifiers generally require a DO free environments to carry out denitrification.

Unlike the membrane Nar, periplasmic Nar is located inside the cell and less sensitive to O$_2$. Although the physiological function of periplasmic Nar is still not resolved, it is believed that this enzyme enables some denitrifiers to tolerate residual O$_2$ in their environment (e.g. denitrifiers in simultaneous nitrification–denitrification processes; SND) (Holman & Wareham, 2005). Consequently, denitrification can be achieved in both anoxic and oxic environments.

**B. Nitrite reductase (Nir):**

Nitrite reductase (Nir) catalyses the single electron reduction of NO$_2^-$ to nitric oxide (NO) (Shapleigh, 2006). Not all NO$_3^-$ utilisers have Nir, and these denitrifiers (which comprise approximately 31% of all heterotrophs in activated sludge) are considered to be incomplete denitrifiers (Drysdale et al., 2001). Unlike the membrane Nar, Nir is capable of reducing both O$_2$ and NO$_2^-$, and thus it is not sensitive to O$_2$ (Ferguson, 1994). During denitrification, accumulation of NO$_2^-$ is common; two possible explanations have been proposed for that observation (Betlach & Tiedje, 1981; Dendooven & Anderson, 1994):

- The Nar enzyme has a high affinity for NO$_3^-$, and so requires a relatively low NO$_3^-$ concentration. In comparison, the Nir (NO$_2^-$ reduction) enzyme exhibits a low affinity for NO$_2^-$, and so requires a relatively high NO$_2^-$ concentration to achieve maximum reaction velocities. Therefore, the production of NO$_2^-$ by Nar is faster than its removal by Nir; this could result in the accumulation of NO$_2^-$ during denitrification.
Compared with the Nar enzyme, Nir is less persistent (is less stable) and is subject to a higher de-repression rate; this could also contribute to the accumulation of NO$_2^-$.

C. Nitric oxide reductase (Nor):

Nitric oxide reductase (Nor) is a membrane bound protein that catalyses the reduction of NO to gaseous nitrous oxide (N$_2$O) (Shapleigh, 2006). NO is toxic to denitrifiers, and is rapidly reduced to N$_2$O to ensure it remains at very low concentrations (100 nM) in a cell (Ferguson, 1994). Because of its toxicity, it is unlikely that microorganisms can utilise NO directly as a final electron acceptor for respiration (Zumft, 1997).

D. Nitrous oxide reductase (Nos):

Nitrous oxide reductase (Nos) catalyses the reduction of N$_2$O to N$_2$, and is only present in some denitrifiers. Unlike NO, many denitrifiers are able to use N$_2$O as a sole electron acceptor for oxidation of organic compounds. The Nos enzyme is believed to be sensitive to several environmental factors, including pH, NO$_2^-$ accumulation, the presence of O$_2$, and excessive carbon and low N$_2$O concentrations (Dendooven & Anderson, 1994). When Nos is inhibited, N$_2$O is released from the liquid phase into the atmosphere, indicating incomplete denitrification.

Compared with the conventional denitrification process, the generation of N$_2$O has been reported to be substantially higher for the denitrifying P removal process (EBPR). Specifically, the use of PHAs as a carbon source (compared with soluble carbon) and the accumulation of NO$_2^-$ during denitrification (>1 mg-N/L) could contribute to N$_2$O emission from EBPRs (Zeng et al., 2003a). As N$_2$O is an extremely potent greenhouse that is 300 times more powerful than CO$_2$, minimising N$_2$O emission from DPAOs and DGAOs is essential (Li et al., 2013). It has been demonstrated that continuous NO$_3^-$
addition (instead of bulk addition), and the use of propionate instead of acetate as a carbon source, could reduce the generation of N$_2$O (Li et al., 2013). These findings are based on the conventional EBPR process and may be applicable to the novel EBPR-r process, as EBPR-r also exploits the activities of DPAOs.

1.4.6. The microorganisms that facilitate P and N removal in EBPR-r

In conventional post-denitrification, N removal is carried out largely by heterotrophic denitrifying bacteria in the presence of soluble carbon (via external addition). A wide range of bacteria capable of reducing NO$_3^-$ have been reported, and thus are not discussed here (Heylen et al., 2006; Shapleigh, 2006). Unlike conventional post-denitrification, nutrient removal in EBPR-r is performed in the absence of soluble carbon. Rather than heterotrophic denitrifying bacteria, the enrichment PAOs and GAOs in the EBPR-r process is anticipated. These bacteria, commonly found in conventional EBPR processes, are able to store soluble carbon internally, and perform storage-driven N and P removal in the absence of soluble carbon (Oehmen et al., 2007). The microbiology of PAOs and GAOs are discussed in the following section.

A. Phosphate accumulating organisms

As discussed earlier, PAOs are able to uptake P using O$_2$, NO$_3^-$ and NO$_2^-$ as final electron acceptors. The number of electrons gained through the reduction of each electron acceptor is shown in Reactions 1.1 to 1.4. There is no consensus on whether DPAOs (able to use NO$_3^-$) and aerobic PAOs (not able to use NO$_3^-$) are the same organisms. Some authors suggest that all PAOs are able to fully denitrify (Kong et al.,
2004; Zeng et al., 2003b), while others have suggested the involvement of two distinct PAO groups (Ahn et al., 2002; Freitas et al., 2005).

Reactions 1.1 to 1.4:

\[
O_2 + 4e^- + 4H^+ \rightarrow 2H_2O \quad [1.1]
\]

\[
NO_3^- + 5e^- + 6H^+ \rightarrow \frac{1}{2}N_2 + 3H_2O \quad [1.2]
\]

\[
NO_3^- + 2e^- + 2H^+ \rightarrow NO_2^- + H_2O \quad [1.3]
\]

\[
NO_2^- + 3e^- + 4H^+ \rightarrow \frac{1}{2}N_2 + 2H_2O \quad [1.4]
\]

Many candidates have been proposed as potential PAOs, including *Acinetobacter*, *Tetrasphaera*, *Lampropedia*, *Microlunatus phosphovorus*, *Microthrix parvicella* and *Nostocoida limicola* II (Kim et al., 2010; Seviour et al., 2003). However, the most studied PAO is ‘*Candidatus Accumulibacter phosphatis*’, also known as *Accumulibacter*. It belongs to the class of Proteobacteria and is closely related to the family *Rhodocyclaceae* (Hesselmann et al., 1999). *Accumulibacter* can be classified into two main groups (groups I and II), and each group has been further classified into several clades (e.g. clade 1A–E and IIA–G) (He et al., 2007). It has been demonstrated that *Accumulibacter* clades have different morphologies, and also differ in their ability to use NO\textsubscript{3} for P uptake. For example, all *Accumulibacter* clades are able to use O\textsubscript{2} as electron acceptor, but only clade IA is able to reduce NO\textsubscript{3} for P uptake (Flowers et al., 2009). On the other hand, clade IIA has been showed to use NO\textsubscript{2} but not NO\textsubscript{3} (Kim et al., 2013). Beside *Accumulibacter* (clade IA), PAOs capable of reducing NO\textsubscript{3} have also been identified. These potential DPAOs include the genera *Aquaspirillum*, *Azoarcus*, *Thauera* and *Rhodocyclus* (Thomsen et al., 2007).


B. Glycogen accumulating organisms

Another group of bacteria that is expected to coexist with PAOs in the EBPR-r is GAOs. Like PAOs, they take up organic carbon substrates anaerobically and store them internally as PHAs, which can be oxidised for energy generation and growth during the sequential aerobic or anoxic conditions (Seviour & Nielsen, 2010). Unlike PAOs, GAOs use glycogen instead of Poly-P as their primary energy source for anaerobic carbon uptake. Hence, they do not accumulate P under aerobic or anoxic conditions (Oehmen, 2005). Because GAOs consume carbon without contributing toward P removal and recovery, their growth in the EBPR-r process would need to be minimised. Nevertheless, as some GAOs are capable of utilising NO$_3^-$ (in addition to O$_2$) as an electron acceptor (i.e. DGAOs), their presence in the EBPR-r process may be acceptable as they may contribute to N removal.

The most intensively studied group of GAOs belongs to class Gammaproteobacteria and is referred to as “Candidatus competibacter phosphatis” or Competibacter. This is a diverse group of bacteria that are clustered into eight subgroups (e.g. GB1–8) (Kim et al., 2011). The typical phenotypic properties of GAOs have also been reported among other bacterial lineages, specifically in the orders Sphigomonadales and Rhodospirillales within the class Alphaproteobacteria (Beer et al., 2004; Meyer et al., 2006).
1.5. Foreseeable challenges of EBPR-r and the thesis scope

1.5.1. Can PAOs or DPAOs replenish their Poly-P pool in low-P wastewater, and enable P recovery in a separate stream?

During the P uptake phase in the EBPR-r process, PAOs are exposed to a wastewater stream containing a low P concentration (7–10 mg-P/L). This is very different to a conventional EBPR, in which PAOs initiate P uptake at much higher P concentrations (e.g. >50 mg-P/L; Figure 1.3) (Bond et al., 1999). The high P concentration is largely because of the use of a single stream for both P uptake and P release in EBPR and the fact that prior to the P uptake phase, the intracellular P is released from PAOs into wastewater under the anaerobic condition, and this increases the P concentration considerably (e.g. from 7–10 mg/L to >50 mg/L; Figure 1.3). In contrast, providing a high initial P concentration in the EBPR-r process is not feasible, because anaerobic P release takes place in a separate recovery stream. The lack of a high initial P concentration in wastewater and its implications for the ability of PAOs to create a concentrated stream for P recovery would need to be investigated (Kodera et al., 2013). Therefore, as a first step the applicability of EBPR-r process needed to be validated. This is the subject of Chapter 2 of the thesis.

1.5.2. The lack of soluble carbon may affect whether the EBPR-r biofilm can carry out denitrification when anoxic conditions cannot be strictly maintained

There is much evidence about the need to create strict anoxic conditions for denitrification to occur (Gerardi, 2003). However, it has been shown that denitrification can take place in the presence of residual DO in bulk water, such as in the SND process (Zeng et al., 2003a). In these processes, some external carbon in wastewater is
consumed by the microbial culture to create a favourable micro-environment for denitrification. However, in an EBPR-r soluble carbon is lacking in the wastewater (detailed in section 1.4.4.C). Hence, the creation of anoxic conditions for denitrification is expected to be largely through the oxidation of carbon polymers (i.e. PHAs) by PAOs and/or GAOs (Zeng et al., 2003a). To what extent the PHAs in these microorganisms could be effectively used for denitrification, and the impact of DO on both denitrification and P uptake of EBPR-r biofilm are unclear, and warrant systematic study. This is the subject of Chapter 3 of the thesis.

1.5.3. Justifying the economic viability of the EBPR-r process: the ability of PAOs to conserve carbon for P uptake

The effectiveness of this proposed EBPR-r process depends on whether the PAOs can efficiently shuttle the P from a large volume of wastewater into a smaller recovery stream in a cyclic manner. In practice, whether or not a WWTP adopts a single cycle for P uptake and release will depend largely on the availability of land and infrastructure (the requirement of one relatively large tank for P uptake and one smaller tank for P release). When these factors are limiting, an alternative mode of operation for the P release step (i.e. carbon replenishment) may involve multiple P uptake from wastewater streams (e.g. four sequential P uptake phases per 16 h) rather than uptake in a single pass. However, such strategy of infrequent carbon replenishment is only feasible if storage polymers can be conserved in PAOs over an extended period of time (i.e. over the course of the multiple P uptakes).

It is unclear whether the internal carbon storage polymer (i.e. PHAs) in PAOs can be conserved specifically for P uptake in the proposed unique operational system, whereby intrusion of oxygen may occur during the P uptake phase(s) when soluble carbon is
deficient. In this situation large quantities of electron acceptors (both $\text{NO}_3^-$ and $\text{O}_2$) may be present in the wastewater, creating an oxidising environment whereby the PHAs stored in PAOs may be oxidised rapidly without parallel P uptake (Lopez et al., 2006). In the event that PHAs are not conserved for P uptake, frequent carbon replenishment would be needed, which would increase the operating costs of the process. To facilitate economic P recovery, the external supplemental carbon, in the form of internal PHAs storage, will need to be used exclusively by PAOs for P uptake. Therefore, it is important to investigate whether the EBPR-r biofilm can conserve stored carbon reserves for P uptake during a prolonged period of exposure to an oxidising environment, and if so for how long. This is the subject of Chapter 4 of the thesis.

1.5.4. Optimising the use of external carbon to achieve maximal P recovery

To make the EBPR-r process more economically viable, it will be essential to achieve P recovery using as little external carbon as possible. Specifically, carbon consumption by unwanted microorganisms, such as GAOs, should be minimised. A chemical parameter commonly used to indicate the activity of PAOs in sludge is the anaerobic $\text{P}_{\text{rel}}/\text{C}_{\text{upt}}$ ratio, which is the amount of P released per carbon taken up under anaerobic conditions (Lopez-Vazquez et al., 2007). Typically, a ratio of 0.50–0.75 (mol-P/mol-C) is observed for biomass that is dominated by PAOs (Lopez-Vazquez et al., 2007). A ratio approaching zero, indicating the consumption of carbon without P release, is reported for sludge dominated by GAOs (Bond et al., 1995). Studies of the conventional EBPR process have revealed the impacts of several factors on achieving high $\text{P}_{\text{rel}}/\text{C}_{\text{upt}}$ ratios. These include pH (>7.25), temperature (<25 °C), the organic carbon to P ratio in the wastewater influent (10–25 mg-COD/mg-P), the type of carbon source (propionate), and carbon addition rate (slow) (Oehmen et al., 2007; Tu & Schuler, 2013). These factors can be applied to the EBPR-r process to optimise the $\text{P}_{\text{rel}}/\text{C}_{\text{upt}}$ ratio.
However, as the EBPR-r is proposed to operate using alternating streams, an alternative approach to optimise the $P_{rel}/C_{upt}$ ratio may need to be developed. Theoretically, biofilm in the EBPR-r process acts as a transporter to transfer a similar amount of P between two streams. A higher $P_{rel}/C_{upt}$ ratio could be achieved for P recovery if the biofilm could uptake P from a larger wastewater volume while maintaining the recovery stream volume unchanged (carbon supply constant). The feasibility of this strategy for optimising the $P_{rel}/C_{upt}$ ratio of the EBPR-r biofilm needs to be investigated. This is the subject of Chapter 5 of the thesis.

1.5.5. **Obligatory reliance on external carbon supply: is it possible to apply a bioelectrochemical system (BES) to convert an industrial waste stream into VFAs for use in the EBPR-r?**

The addition to the EBPR-r of an external carbon source such as acetate (VFA) is necessary to facilitate post-denitrification and P recovery. Relative to other carbon substrates commonly used in conventional post-denitrification (e.g. methanol), VFAs are the most expensive. To minimise the operational cost of P recovery, instead of dosing the EBPR-r with pure chemical, VFAs can be provided by fermentation of primary sludge (Kodera et al., 2013). To investigate an alternative source of VFAs, the applicability of producing acetate from industrial wastewater using bioelectrochemical systems (BESs) was explored.

Industrial wastewaters, including the effluent from pulp and paper industries, are rich in carbon but low in N (Pratt et al., 2007). To enable efficient biological treatment, the addition of external N as $\text{NH}_4^+$ or $\text{NO}_3^-$ is required. However, it is possible that the requirement for N could also be filled by diazotrophs ($\text{N}_2$-fixing bacteria), which convert atmosphere $\text{N}_2$ into $\text{NH}_4^+$ (via Reaction 1.5). However, diazotrophs are sensitive
to O\textsubscript{2}. To protect the nitrogenase from O\textsubscript{2}, diazotrophs produce large quantities of slime under oxic condition, but this can lead to poor effluent quality (Nair, 2010). To eliminate the negative impact of O\textsubscript{2}, it may be possible to use N\textsubscript{2}-fixing microorganisms in combination with BESs.

Reaction 1.5:

\[
N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16Pi \quad [1.5]
\]

A BES typically consists of anode and cathode compartments separated by a cation-selective membrane (Logan et al., 2008). Electrochemically active microorganisms generally grow as a biofilm on the surface of the anodic electrode by oxidising electron donor substrates (e.g. glucose) and donating electrons to the anode. Subsequently, the electrons flow from the anode to the counter electrode (cathode) through an external circuit (Logan et al., 2008). Because the insoluble anodic electrode acts as electron acceptor (instead of O\textsubscript{2} in activated sludge), treatment of carbon rich wastewater in the complete absence of O\textsubscript{2} is possible. As a result of anodic reactions, CO\textsubscript{2} (via respiration) and hydrogen (H\textsubscript{2}, via N\textsubscript{2} fixation) may be produced in the anode compartment. These substrates, together with the electron generated from the anode, may be used in the cathode compartment as building blocks for acetate production, via a microbial electro-synthesis process by organisms known as homoacetogenic bacteria (Reaction 1.6) (Ragsdale & Pierce, 2008).

Reaction 1.6:

\[
2CO_2 + 4H_2 \rightarrow CH_3COO^- + H^+ + H_2O \quad [1.6]
\]

If demonstrated to be feasible, the use of BESs would enable the treatment of N-deficient waste and also produce acetate from CO\textsubscript{2}. The generated acetate can be used to
meet the carbon requirement of the EBPR-r, and thereby enhance the economic viability of P recovery. While microbial electro-synthesis of acetate on the cathode of BES is well demonstrated (Gong et al., 2013), oxidation of N-deficient wastewater using N₂-fixing bacteria in the anode compartment is yet to be experimentally demonstrated. These considerations are the subject of Chapter 6 of the thesis.

1.6. **Aim and objectives**

The overall aim of this study was to demonstrate the applicability of EBPR-r, as a post-denitrification strategy for P recovery from low P-containing wastewater. This involved systematically investigating the challenges described above.

Objectives:

1) To demonstrate the applicability of the EBPR-r process to concentrate P from low P-containing wastewater, and achieve a P concentration that is suitable for fertilizer production (>50 mg/L) (Chapter 2).

2) To investigate the impact of bulk water DO (0–8 mg/L) and NO₃⁻ (0–50 mg-N/L) on the storage-driven denitrification and P uptake of the EBPR-r biofilm (Chapter 3).

3) To explore the ability of PAOs to conserve carbon storage reserves for P uptake, after exposing the EBPR-r biofilm to P-deficient and highly oxidising conditions for extended periods (up to 7 days) (Chapter 4).

4) To optimise the anaerobic \(P_{rel}/C_{upt}\) molar ratio of the EBPR-r biofilm, by optimising the amount of P uptake from wastewater while keeping the carbon supply unchanged (Chapter 5).

5) To investigate the applicability of using N₂ fixing bacteria to treat N-deficient wastewater in a BES reactor (Chapter 6).
2. A Novel Post-denitrification Configuration for Phosphorous Recovery using Polyphosphate Accumulating Organisms

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2.1. Abstract

Enhanced biological phosphorus removal (EBPR) has been widely used to remove phosphorus (P) from wastewater. In this study I report a novel modification to the EBPR approach, namely enhanced biological phosphorus removal and recovery (EBPR-r) that facilitates biological recovery of P from wastewater using a post-denitrification configuration. The novel approach consists of two major steps. In the first step, a biofilm of phosphorus accumulating organisms (PAOs) is exposed to a wastewater stream in the absence of active aeration, during which P is taken up by the biofilm using \( \text{NO}_3^- \) and residual dissolved oxygen as electron acceptors. Thus, P and nitrogen (N) removal from wastewater is achieved. During the second step, the P enriched biofilm is exposed to a smaller recovery stream supplemented with an external carbon source to facilitate P release under anaerobic conditions. This allows P to be recovered as a
concentrated liquid. The EBPR-r process was able to generate a P recovery stream four times more concentrated (28 mg-P/L) than the wastewater stream (7 mg-P/L), while removing nitrate via denitrification (P_{up} / N_{den} ratio of 1.31 g-P/g-N) from the wastewater stream. Repeated exposure of the biofilm (10 P-uptake and release cycles) to a recovery stream yielded up to 100 mg-P/L. Overall, EBPR-r is the first post-denitrification strategy that can also facilitate P recovery during secondary wastewater treatment.

2.2. Introduction

Phosphorus (P) is a non-renewable resource that is used in modern agriculture to maintain high crop yields. Many reports have indicated that global P reserves could deplete in the foreseeable future, highlighting the need to recycle P (Cordell et al., 2009; Driver et al., 1999; Jasinski, 2012; Rittmann et al., 2011; Van Vuuren et al., 2010). Sewage treatment plants are a potential point source for P recovery (Seviour & Nielsen, 2010). The main challenge in recovering P from municipal wastewater is its low P concentration (typically <10 mg-P/L) (Parsons & Smith, 2008; Shi & Lee, 2006). Existing P recovery techniques (e.g. struvite crystallisation) are only feasible with high P concentration streams (>50 mg-P/L), such as liquors obtained from anaerobic digestion of P-enriched activated sludge (Cornel & Schaum, 2009; Martí et al., 2010; Münch & Barr, 2001; Rittmann et al., 2011).

Enhanced biological phosphorus removal (EBPR) is an established approach that has been widely used for P removal (Seviour & Nielsen, 2010). EBPR is achieved with the aid of a bacteria known as polyphosphate accumulating organisms (PAOs)/or denitrifying PAOs (DPAOs). Under alternating anaerobic and aerobic (PAOs)/or anoxic (DPAOs) conditions, PAOs and DPAOs are able to facilitate P removal from influent wastewaters. Specifically under anaerobic conditions the PAOs/DPAOs take up short-
chain volatile fatty acids (VFAs) and store them intracellularly as poly-β-hydroxy-alkanoates (PHAs). The energy requirement for this process is met through the hydrolysis of stored polyphosphate (Poly-P). As a result, phosphate ($\text{PO}_4^{3-}$-P) is released from the cell, and the P concentration in the wastewater increases (Seviour & Nielsen, 2010). During the subsequent aerobic or anoxic phase, intracellular PHA (stored during the previous anaerobic phase) is oxidised to generate energy for cell growth and P uptake. With P incorporated into the biomass, removal of a portion of biomass results in a net removal of P from the wastewater. This P-enriched biomass can then be anaerobically digested to obtain a concentrated P stream, facilitating the recovery of P in a form such as struvite (Baur, 2009).

Today, with water recycling emerging as a priority area for the wastewater industry worldwide, improving secondary effluent quality prior to further downstream treatment is becoming a necessity. Similarly if secondary effluent is discharged to sensitive environments, more stringent discharge limits are to be complied by the wastewater treatment plants (Boltz et al., 2012). This would translate into a stronger need for better effluent polishing to achieve acceptable nitrogen (N) and P concentrations. Lower total N limits are often achieved using post-denitrification and this requirement is more so for treatment plants having lower C:N ratios (Wei et al., 2014).

In this study, I propose a novel post-denitrification strategy based on PAOs/DPAOs not only to reduce nitrate but also to recover P, maximising the use of externally supplemented carbon. This strategy is termed enhanced biological phosphorus removal and recovery (EBPR-r) and is a two-step biofilm process (Figure 2.1). In the first step, the PAOs biofilm takes up P (as Poly-P) utilising NO$_3^-$ as a final electron acceptor, removing both P and N from the wastewater. In the second step, the P-enriched biofilm is exposed to a smaller volume stream (recovery stream) where external carbon is added.
to facilitate P release under anaerobic conditions. As such, the biomass acts as a carrier to transfer P from a dilute wastewater stream into a concentrated recovery stream. Considering the need to alternate the biomass between two different liquid streams (i.e. a dilute or a concentrated stream), a strategy to retain biomass in the reactor is essential. A biofilm reactor, not only could facilitate this post-denitrification strategy, but also could allow the maintenance of a high biomass density. Unlike conventional two sludge post-denitrification processes where external carbon is directly introduced into wastewater, in EBPR-r, external carbon is introduced into the recovery stream. This feature substantially improves management and simplifies usage of carbon, reducing the risk of carbon discharge with effluent.

**Figure 2.1** The principle of the EBPR-r process: The biofilm takes up P and respires nitrate of a dilute wastewater stream. A subsequent exposure of the biofilm to a smaller recovery stream and external carbon, triggers a release of accumulated P resulting in a concentrated P recovery stream. PHAs: Poly-β-hydroxy-alkanoates; M⁺: metal e.g. Mg²⁺ and K⁺; Poly-P: Polyphosphate.
The aim of this study was to demonstrate this novel post-denitrification strategy in laboratory-scale. The specific objectives were (i) to validate the applicability of the proposed EBPR-r approach to remove P and N from synthetic wastewater, and consecutively recover P into a recovery stream, and (ii) to demonstrate the potential of the strategy to achieve higher P concentrations (e.g. 100 mg-P/L) with repeated release of P into the recovery stream.

2.3. Materials and Methods

2.3.1. Master reactor

A. Reactor configuration, automated operation and online monitoring

A laboratory-scale biofilm reactor (master reactor) with an internal diameter of 130 mm was packed with 1000 biofilm carrier media (Kaldnes® K1 polyethene carrier, Dimension: 10 mm diameter x 7 mm height, Average specific surface area: 800 m²/m³) to a height of 140 mm (1859 cm³ of Kaldnes® media). The carriers were placed in a stainless steel mesh cage, which was divided into six identical compartments. Each compartment contained approximately 166 ± 3 carriers.

The carriers in the master reactor were alternately exposed to a wastewater (low P) and to a recovery stream (¼ the volume of the wastewater stream) over a 6 h cycle, which consisted of a 4 h P uptake phase and a 2 h P release phase (Figure 2.2). During the first 20 min of the cycle, the concentrated stock solution (0.48 L, described in section 2.3.1B) was diluted with DI water (6.72 L) to obtain a low P-containing wastewater stream (7.2 L). This stream was continuously recirculated (337 mL/min) through the reactor during the P uptake phase. At the end of this phase, the wastewater stream was completely decanted (within 10 min) and the recovery stream (1.8 L) was introduced
and recirculated for 105 min. Finally, the P-enriched recovery stream was harvested by decanting the liquor from the master reactor (10 min).

Figure 2.2 Schematic diagram of the master reactor configuration, the biofilm was alternatively exposed to a dilute wastewater stream (7.2 L) to facilitate P uptake (under anoxic conditions) and to a recovery stream (1.8 L) to facilitate P release (with the exposure to an external carbon source).

National Instruments (USA) data acquisition and control devices and software (LabVIEW) were used to automate the system. Online monitoring of dissolved oxygen (DO), pH and redox potential (ORP) was carried out using a luminescent DO probe (PDO2, Barben Analyzer Technology, USA), an intermediate junction pH probe (Ionode IJ44, Ionode Pty Ltd, Australia) and an intermediate junction redox probe (Ionode IJ64, Ionode Pty Ltd, Australia), respectively. The reactor was operated at 22 ± 2°C for over four months without pH or DO control. Peristaltic pumps (Masterflex®, USA) were used for recirculation and exchange of liquid.
B. **Dilute wastewater stream and concentrated P recovery stream**

Both the wastewater stream and the recovery stream contained a standard growth medium consisting of (per L): 39 mg MgSO$_4$, 20 mg CaCl$_2$·2H$_2$O, 11 mg NH$_4$Cl (3 mg/L NH$_4^+$-N), 200 mg NaHCO$_3$ and 0.3 mL of a nutrient solution. The nutrient solution contained (per L) 1.5 g FeCl$_3$·6H$_2$O, 0.15 g H$_3$BO$_3$, 0.03 g CuSO$_4$·5H$_2$O, 0.18 g KI, 0.12 g MnCl$_2$·4H$_2$O, 0.06 g Na$_2$MoO$_4$·2H$_2$O, 0.12 g ZnSO$_4$·7H$_2$O, 0.15 g CoCl$_2$·6H$_2$O and 10 g EDTA. The dilute wastewater stream further contained 8 mg-P/L of phosphate (as 1 M phosphate buffer: 46 g KH$_2$PO$_4$ and 115 g K$_2$HPO$_4$ per L) and 10 mg-N/L of nitrate (as sodium nitrate). In addition, N-Allylthiourea (11.6 mg/L) was also added to prevent nitrification during the anoxic P uptake phase (Ginestet et al., 1998). The recovery stream contained (per L) 520 mg of sodium acetate (corresponding to 400 mg chemical oxygen demand, COD) to restore intracellular PHA reserves during anaerobic P release. Concentrated stock solutions (15×) of these two streams were prepared and the pH was adjusted to 7.0 ± 0.2 using 2 M HCl. Defined volumes of the stock solution and deionised water were simultaneously pumped into the reactor at the beginning of each phase to achieve the desired concentrations.

C. **Seeding of the master reactor**

The master reactor was seeded with 0.5 L activated sludge obtained from a laboratory-scale sequencing batch reactor (SBR) operated for N and P removal. The inoculum had a mixed liquor volatile suspended solids (MLVSS) concentration of approximately 3.0 g/L. During the 24-day start-up period, the decant port of the master reactor was set 20 mm above the bottom of reactor, such that some suspended solids were retained in the system during decant, to facilitate microbial colonisation of carriers. After 24 days the
decant port was moved to the bottom of the reactor to ensure all suspended solids and liquid removal during decant.

**D. Sampling and sample analysis**

Following inoculation, the master reactor performance was assessed weekly by measuring PO$_4^{3-}$-P and NO$_3^{-}$-N in the reactor influent and effluent. When stable removal and recovery was observed, detailed cyclic studies were performed to evaluate the kinetics of P uptake by, and release from, the established biofilm. Each cyclic study involved withdrawing samples (3 mL) from the master reactor every 15−30 min during the entire 6-h cycle. Each sample was immediately filtered using a 0.22 μm pore size syringe filter (Acrodisc® PF, Pall Corporation, UK). The concentrations of soluble NO$_x$-N (NO$_2^-$-N + NO$_3^-$-N), PO$_4^{3-}$-P and acetate in the filtrates were determined using ion chromatography (ICS-3000, Dionex).

**E. Measurement of total solids**

Total solids (TS) attached to the carriers were measured using a modified procedure as reported by Helness (2007). Briefly, after each cyclic study, 50 biofilm carriers were removed from the master reactor at the end of anaerobic phase. The carriers (with biofilm attached) were dried at 60°C (approximately for 24 h) until a constant weight was achieved. TS was obtained by subtracting this weight (50 carriers + biofilm) from the weight of 50 biofilm-free carriers (estimated using an average weight per carrier, obtained by measuring the weight of 100 clean dried carriers).

**F. Calculation of P uptake and release rate**

The maximum P uptake rate, NO$_x$-N reduction rate, P release rate, and acetate uptake rate were calculated from results of cyclic studies. All rates were normalised in three
forms: (1) volumetric rate (mg-P/L/h) based on volume of streams: 7.2 L for wastewater stream and 1.8 L for recovery stream; (2) volumetric rate (mg-P/L/h) based on volume of the master reactor 2.0 L; and (3) specific rate (mg-P/g-TS.h) based on biomass.

2.3.2. Multiple P release test

A. Increase in P concentration with a repeated release of P into the same recovery stream

After the PAOs biofilm was established, a multiple P release test was conducted to test whether the biofilm could repeatedly release PO$_4^{3-}$-P into the same recovery stream, resulting in a sequential increase of P concentration in the stream. On day 1 of this experiment 166 biofilm carriers were removed from one compartment of the master reactor at the end of an anoxic P uptake phase. The carriers were immediately transferred into a 500 mL glass bottle. P release from the biofilm on these carriers was subsequently triggered by recirculating an acetate containing P recovery stream (250 mL) through the carriers for 2 h (as occurred in the master reactor). Recirculation was achieved using a peristaltic pump (86 mL/min; Masterflex®, USA). The headspace in the bottle was purged with N$_2$ (1000 mL/min) at the beginning for 2 min, to create anaerobic conditions. Following 2 h of incubation a liquor sample (3 mL) was removed for chemical analysis, and the carriers were returned back to the master reactor, where they were exposed to three normal 6-h cycles. On day 2, the carriers were once again transferred back to the glass bottle that contained the previous anaerobic P recovery stream. As before the P recovery stream was once again recirculated for 2 h to facilitate P release. As the recovery stream was depleted of carbon during the first exposure of the carriers to the stream, sodium acetate (1 M) was introduced into the stream at the start of the second exposure (sufficient to achieve a COD of approximately 400 mg/L) to
trigger biological P release. The 2-h incubation was carried out as described for day 1. The release of P into the same recovery stream was repeated 12 times over a period of 16 days. This multiple P release test was performed in duplicate. In one of these duplicate experiments, DO (Hach, HQ30d Portable Dissolved Oxygen meter, LDO101 probe) and pH (Checker HI 98103, Hanna Instruments, United States) were measured and recorded manually.

B. Sampling and analytical methods

For chemical analyses, mixed liquor samples (3 mL) were taken at the end of each anaerobic P release. As described for the master reactor, the samples were immediately filtered and analysed for soluble PO$_4^{3-}$-P. An unfiltered sample (100 mL) was removed at the end of 12 release cycles for measurement of both the total and soluble concentrations of PO$_4^{3-}$-P, Ca$^{2+}$, K$^+$, Mg$^{2+}$ and Na$^+$ (carried out by MPL laboratories, WA, Australia). Total P was measured using a discrete analyser (Konelab Aquakem, Thermo Scientific) following persulphate digestion (based on (Seviour & Blackall, 1999)). The total Ca$^{2+}$, K$^+$, Mg$^{2+}$ and Na$^+$ concentrations were measured by inductively coupled plasma–optical emission spectrometry (ICP-OES) (Vista-Pro, Varian) following digestion with nitric/hydrochloric acid (Seviour & Blackall, 1999). Soluble PO$_4^{3-}$-P, Ca$^{2+}$, K$^+$, Mg$^{2+}$ and Na$^+$ were measured after filtering the samples with 0.22 μm pore size syringe filters (Acrodisc® PSF, Pal Corporation, UK).

2.4. Results and Discussion

2.4.1. Enrichment of biofilm using the EBPR-r configuration

EBPR-r system was used to enrich a biofilm, which achieved stable performance after approximately two months of operation. Weekly monitoring of phosphate (PO$_4^{3-}$-P) and
NO$_3$-N showed a gradual increase in both P uptake and NO$_x$-N removal rates during the first 42 days from 0.85 to 1.84 mg-P/L.h and 0.28 to 1.17 mg-N/L.h, respectively (based on a stream volume of 7.2 L). This indicates an increase in the biofilm activity over this period. Thereafter, the P uptake rate (1.84 ± 0.22 mg-P/L.h) and the NOx-N removal rate (1.17 ± 0.40 mg-N/L.h) remained relatively stable. During stable operation, the NH$_4^+$-N consumption in the reactor was approximately 1–2 mg/L. Hence, NH$_4^+$-N remained in excess at all times ensuring no limitation of nitrogen for biomass growth.

2.4.2. The enriched biofilm had similar P and N removal behavior as EBPR sludge, but enabled concentration of P

To study the P uptake and release kinetics of the enriched biofilm, a cyclic study was conducted (Figure 2.3). In general, the behavior of the biofilm was similar to what is typically observed in conventional EBPR processes. During the P uptake phase, 1.31 mg of P was taken up per mg of NO$_3$-N reduced (Table 2.1), which is similar to reported ratios of 1.33 (Carvalho et al., 2007), 1.42 (Yuan & Oleszkiewicz, 2010) and 2.00 (Kerrn-Jespersen et al., 1994) mg-P/mg-N in conventional EBPR processes operated under anaerobic and anoxic conditions. Overall, the removal efficiency of soluble P and N from the wastewater in this study were 83% and 64%, respectively (Table 2.1).

Since nitrification was inhibited (with the use of N-Allylthiourea in feed) during the P uptake phase, the increase of nitrite and the decrease of NO$_x$-N suggests denitrification during P uptake. The observed denitrification was driven by PAOs and/or glycogen-accumulating microorganisms (GAOs) using intracellular carbon stored during the anaerobic phase (Figure 2.3C). The P$_{upt}$/N$_{den}$ ratio of 1.31, which was similar to what was reported in past literature (Carvalho et al., 2007; Kerrn-Jespersen et al., 1994; Yuan
& Oleszkiewicz, 2010) also suggest that anoxic P uptake took place when exposed to the wastewater stream.

**Figure 2.3** Profiles of (A) soluble phosphorus (PO$_4^{3-}$-P), (B) acetate, (C) NO$_x$-N (NO$_2^-$-N + NO$_3^-$-N) and NO$_2^-$-N (D) dissolved oxygen concentration (DO) and pH, (E) oxidation reduction potential (redox), and (F) total phosphorus (PO$_4^{3-}$-P) during a cyclic study using the master reactor.
Table 2.1 Stoichiometry and kinetics of the enriched PAOs biofilm in the master reactor.

<table>
<thead>
<tr>
<th>Phase</th>
<th>Rate</th>
<th>Unit</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>P uptake</td>
<td>Max P uptake rate</td>
<td>(mg-P/L.h)</td>
<td>2.73a, 9.83b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(mg-P/g-TS.h)</td>
<td>1.74</td>
</tr>
<tr>
<td></td>
<td>Max NOx-N removal rate</td>
<td>(mg-N/L.h)</td>
<td>2.27a, 8.15b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(mg-N/g-TS.h)</td>
<td>1.45</td>
</tr>
<tr>
<td></td>
<td>P_{upt}/N_{den} ratio</td>
<td>(g-P/g-N)</td>
<td>1.31</td>
</tr>
<tr>
<td>P removal efficiency</td>
<td>n.a.</td>
<td></td>
<td>85%</td>
</tr>
<tr>
<td>N removal efficiency</td>
<td>n.a.</td>
<td></td>
<td>62%</td>
</tr>
<tr>
<td>P release</td>
<td>Max P release rate</td>
<td>(mg-P/L.h)</td>
<td>21.0a, 18.9b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(mg-P/g-TS.h)</td>
<td>3.35</td>
</tr>
<tr>
<td></td>
<td>Max acetate uptake rate</td>
<td>(mg-Ac/L.h)</td>
<td>191a, 172b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(mg-Ac/g-TS.h)</td>
<td>30.5</td>
</tr>
<tr>
<td></td>
<td>P_{rel}/C_{upt} ratio</td>
<td>(g-P/g-C)</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(mol-P/mol-C)</td>
<td>0.08</td>
</tr>
</tbody>
</table>

*Rate calculated based on the volume of the wastewater stream 7.2 L and recovery stream 1.8 L, *b*Rate calculated based on reactor volume 2.0 L.

Interestingly, the high DO concentration (8.0 ± 0.6 mg/L) in the wastewater stream did not hinder denitrification during the P uptake phase. The affinities of various strains of PAOs to nitrate and oxygen have been well demonstrated (Carvalho et al., 2007) and it is unclear whether the unique operational strategy facilitated the enrichment of unique PAO strains, which have higher affinities towards nitrate even when DO concentrations are high. The Kaldnes media in the reactor supported thick biofilms. Oxygen gradients in the biofilm and higher affinities of PAOs towards nitrate may have collectively contributed to this phenomenon (Zeng et al., 2003a), but further investigation is warranted.
Unlike conventional EBPR processes, where PAOs repeatedly take up and release P into a single stream, the EBPR-r process was designed to use the biomass as a carrier of P from a dilute wastewater stream into a concentrated recovery stream. As shown in Figure 2.3F, during the P uptake phase the biofilm removed 45 mg-P from the low P wastewater stream (7.2 L) over a period of 4 h, and approximately the same amount was released back into the recovery stream (1.8 L) during a subsequent 2 h anaerobic phase. As the volume of the recovery stream was about one fourth of the wastewater stream, P concentration increased 4-fold from 7 mg-P/L in the wastewater stream to 28 mg-P/L in the recovery stream. This result confirms, for the first time that a biofilm containing PAOs could take up P from a low P concentration stream and subsequently release P into a higher concentration stream.

2.4.3. Repeated release of P into a P recovery stream

To increase the practical attractiveness of the EBPR-r approach for P recovery, the P concentration in the recovery stream needs to be increased ideally to concentrations above 50 mg-P/L (Cornel & Schaum, 2009). Hence, an experiment was conducted to explore the possibilities to stepwise concentrate P by repeated exposure of the biofilm to the same recovery stream (Figure 2.4). The results indicated that the biofilm was able to repeatedly release P into the same stream resulting in a recovery stream of approximately 100 mg-P/L (Figure 2.4). When comparing to the P concentration of municipal wastewaters (which is typically approximately 10 mg-P/L), this is a 10-fold increase of P concentration. At this concentration, P can be efficiently recovered via struvite formation (Rittmann et al., 2011).
Figure 2.4 Increase of soluble $\text{PO}_4^{3-}$-$\text{P}$ during repeated (12 cycles) use of a P recovery stream to release P from PAOs biofilm. Regression line is shown for the first 10 cycles.

Figure 2.5 (A) Soluble and total $\text{PO}_4^{3-}$-$\text{P}$, $\text{Ca}^{2+}$, $\text{K}^+$, $\text{Mg}^{2+}$ and $\text{Na}^+$ concentrations in the concentrated stream relative to concentrations present in the wastewater stream used in this study. (B) Increase in the concentration of soluble nutrients compared with the dilute wastewater stream, according to the equation soluble concentration factor $= \frac{\text{Concentrated recovery stream}}{\text{Dilute wastewater stream}}$. 

y = 8.99x + 4.60
$R^2 = 0.99$

0 20 40 60 80 100
0 2 4 6 8 10 12
Soluble PO$_4^{3-}$-P (mg/L) Cycle

0
2
4
6
8
10
Concentration Factor

A Wastewater stream
Recovery stream (Soluble)
Recovery stream (Total)

Concentration (mg/L)

0
100
200
300
400

B

Concentration Factor

$\text{PO}_4^{3-}$-$\text{P}$ $\text{Ca}^{2+}$ $\text{K}^+$ $\text{Mg}^{2+}$ $\text{Na}^+$
During the first ten P release cycles the soluble P concentration increased linearly (R² = 0.99) in the recovery stream (Figure 2.4), and thereafter, no further increase in soluble P was observed. To test whether the released P became insoluble, the total and soluble P content of the concentrated recovery stream was determined at the end of the experiment (Figure 2.5). The result showed that the concentrated liquor contained 150 mg-P/L of total P, of which only 100 mg-P/L remained soluble. This suggests that precipitation of PO₄³⁻-P might have occurred during the repeated P release test. Throughout the test, the build up of P and other nutrients (Mg²⁺, K⁺ and Ca²⁺) in the recovery stream may provide appropriate conditions (pH 7.00–9.02) for some biologically-induced precipitation (Maurer et al., 1999; Yilmaz et al., 2008). It could also be possible that some soluble P was taken up and stored within the suspended biomass (sloughed off biomass from biofilm carriers). Thus, precipitation of P and incorporation of P into biomass could be the reasons why no further increase of soluble PO₄³⁻-P was obtained after 10 cycles.

2.4.4. Practical implications

A. Stoichiometry and kinetics of the enriched biofilm

Table 2.1 illustrates the stoichiometry and kinetics of the enriched biofilm. An 8-fold difference in volumetric P uptake (2.73 mg-P/L.h, based on the stream volume of 7.2 L) and P release (21.0 mg-P/L.h, based on the stream volume of 1.8 L) rate was observed for the enriched biofilm (Table 2.1). Normalising the rates with a fixed volume (2.0 L reactor volume) and biomass reduced the difference from 8-fold to about 2-fold, which is similar to what is commonly observed in conventional EBPR processes (Kuba et al., 1993). Hence the volume difference between the two streams did not appear to have much influence on the biofilm and process optimisation should be feasible by increasing
the biomass density. If a system with a high biomass density could bring about the desired P concentration in the recovery stream in a single uptake and release cycle, the proposed strategy could then be implemented in a fashion similar to a SBR. Nevertheless, biomass density is a key parameter that should be optimised to eliminate the need for repeated use of the recovery stream.

In conventional EBPR systems, GAOs can compete with PAOs for the uptake of carbon substrates. The PO$_4^{3-}$-P and VFA ($P_{rel}/C_{upt}$) ratio is often used to illustrate the activity of enriched sludge (Oehmen, 2005). A $P_{rel}/C_{upt}$ ratio of 0 mol-P/mol-C was reported when the sludge was dominated by GAOs (Zeng et al., 2003c), whereas $P_{rel}/C_{upt}$ ratios of 0.28 (Kerrn-Jespersen et al., 1994), 0.35 (Zeng et al., 2003b), and 0.84 (Hu et al., 2003) mol-P/mol-C were reported when PAOs were dominating. In this study, a lower ratio of 0.08 mol-P/mol-C was noted possibly due to the presence of GAOs competing with PAOs for carbon and/or due to undesirable oxidation of carbon by heterotrophic microorganisms. At the beginning of the P release phase (recovery stream), an approximate DO concentration of 8 mg/L was observed (Figure 2.3D). The DO concentration however, rapidly decreased during the first few minutes of exposure to recovery stream. The rapid decrease of DO in the recovery stream is likely a result of consumption of DO by heterotrophic bacteria in the biofilm. Accordingly, even in this novel post-denitrification strategy, some of the external carbon was wasted to reduce DO levels in liquid streams. Process modification is required to eliminate both GAOs and oxygen intrusion during anaerobic P release.

**B. NO$_3^-$ as an electron acceptor under aerobic conditions**

PAOs generally prefer O$_2$ as an electron acceptor, as the potential energy gain from O$_2$ is greater than that from NO$_3^-$-N (Dabert et al., 2001). However, in this study the
enriched biofilm was able to denitrify and uptake P even when DO (8.0 ± 0.6 mg/L) was high (Figure 2.3D). A similar finding was previously reported by Ahn et al. (2002). The ability of the biofilm to use NO$_3^-$ for P uptake in the presence of DO is desirable as preventing oxygen intrusion into the reactor is not required. In addition, the use of NO$_3^-$ for P uptake facilitates simultaneous N and P removal, which also has advantages: (1) lower energy consumption due to no aeration requirements for P uptake; (2) optimum use of the external carbon source to achieve both denitrification and P uptake; and (3) reduced sludge production (Kuba et al., 1993; Oehmen et al., 2007; Seviour & Nielsen, 2010).

**C. Recovery of other valuable nutrients**

Apart from P, metal ions such as Mg$^{2+}$, K$^+$ and Ca$^{2+}$ were also concentrated in the recovery stream (Figure 2.5B). All three cations are known to be associated with the movement of PO$_4^{3-}$ across the cell membrane of PAOs, to maintain charge balance (Comeau et al., 1986; Flowers et al., 2009). The enrichment of Mg$^{2+}$ along with P is particularly beneficial if P is to be recovered as struvite (Mg$^{2+}$ is required for struvite formation). Similarly if P were to be recovered as calcium phosphate, the Ca$^{2+}$ in the concentrated stream would be of value. The high concentration of Na$^+$ ions observed in the concentrated recovery stream was a result of the addition of sodium acetate to facilitate P release. This gradual increase in Na$^+$ concentration may eventually suppress the activity of the PAOs biofilm possibly due to increase of ionic stress. Replacing sodium acetate with acetic acid could perhaps resolve this issue.

**D. Potential benefit of two alternating streams**

The unique concept of employing alternate streams in the EBPR-r may offer several advantages: (1) It enables incorporation of P recovery as part of secondary treatment.
(2) The presence of nitrate is detrimental to the anaerobic P release in conventional EBPR (Mulkerrins et al., 2004), but in EPBR-r nitrate is completely excluded from the anaerobic recovery stream. Hence, EBPR-r could deliver stable P removal from municipal wastewaters. (3) As the external carbon source is introduced into a separate recovery stream and not into wastewater, the management and usage of carbon has been simplified, further reducing the risk of carbon discharge with effluent. Additionally since no electron acceptor is present in the recovery stream, there is little or no wastage of carbon due to oxidation.

E. Value of recovered phosphate and cost implications

Compared to a conventional two-sludge post-denitrification process, the proposed novel post-denitrification strategy may incur additional costs specifically for downstream processing of the concentrated P stream (i.e. struvite precipitation). Triple superphosphate (TSP) prices have increased by an average 25% per annum over the past 10 years (IndexMundi, 2013). According to IndexMundi the average cost of TSP during the year of 2012 was $929/ton. If trends continue at a rate of 25% per annum, the value of TSP could exceed as high as $8,000/ton by 2022. Hence a recovery of 1000 ton of TSP today although only has a marketable value of around $929,000, it could become as high as $8 million by 2032. The generated profit could largely offset the costs associated with downstream P recovery and operational costs of the novel process.

2.5. Conclusions

This study demonstrated a novel post-denitrification EBPR-r approach for biological N removal and P recovery. The results suggest that:
• EBPR-r process could recover P from low P wastewater streams (<10 mg-P/L) into a P enriched stream (approximately 100 mg-P/L), while removing NO$_3^-$.

• In addition to P recovery, other valuable nutrients such as Mg$^{2+}$, K$^+$ and Ca$^{2+}$ were also recovered as a concentrated liquid.

• From a wastewater treatment perspective this novel process offers the following advantages: (1) simultaneous P and N removal, and P recovery; (2) greater stability due to the elimination of nitrate in the anaerobic phase; and (3) simplified usage of external carbon for post-denitrification reducing the risk of carbon discharge with effluent.
3. Simultaneous Phosphorus Uptake and Denitrification by EBPR-r biofilm under Aerobic Conditions: Effect of Dissolved Oxygen

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3.1. Abstract

A biofilm process, termed enhanced biological phosphorus removal and recovery (EBPR-r), was recently developed as a post-denitrification approach to facilitate phosphorus (P) recovery from wastewater. Although simultaneous P uptake and denitrification was achieved despite substantial intrusion of dissolved oxygen (DO >6 mg/L), to what extent DO affects the process was unclear. Hence, in this study a series of batch experiments was conducted to assess the activity of the biofilm under various DO concentrations. The biofilm was first allowed to take up acetate (stored it as internal PHA storage) under anaerobic condition, and then was subjected to various conditions for P uptake (DO: 0–8 mg/L; nitrate: 10 mg-N/L; phosphate: 8 mg-P/L). The results suggest that even at a saturating DO concentration (8 mg/L), the biofilm could take up P...
and denitrify efficiently (0.70 mmol e\(^-\)/g-TS.h). However, such aerobic denitrification activity was reduced when the biofilm structure was physically disturbed, suggesting that this phenomenon was a consequence of the presence of oxygen gradients across the biofilm. I conclude that when a biofilm system is used, EBPR-r can be effectively operated as a post-denitrification process, even when oxygen intrusion occurs.

### 3.2. Introduction

Low effluent concentrations for total phosphorus (TP) and total nitrogen (TN) are increasingly being imposed on wastewater treatment plants worldwide. Europe and North America in particular have enforced discharge limits of 0.1 mg/L for TP and 3 mg/L for TN (Boltz et al., 2012). While the strict TP limits are largely achieved through chemical precipitation, biological post-denitrification is applied to meet the TN discharge limit. With much of the readily biodegradable organic carbon in the influent being oxidised during upstream aerobic/anoxic oxidative processes, post-denitrification is heavily reliant on the addition of carbon sources (e.g. methanol) (Wei et al., 2014). The cost of adding external carbon is a significant burden to the wastewater industry, and one way to offset this cost is through resources recovery from wastewater.

Among many resources in wastewater, phosphorus (P) is of interest because it is a non-renewable resource, and its scarcity for agricultural purposes could potentially threaten global food security (Cordell et al., 2011). Wong et al. (2013) proposed and validated a post-denitrification process, termed enhanced biological phosphorus removal and recovery (EBPR-r), that facilitates nitrogen (N) removal but also enables P recovery from wastewater. The EBPR-r process involves two steps, in which a biofilm consisted of phosphate accumulating organisms (PAOs) is alternately exposed to a wastewater stream and a separate recovery stream. As the PAOs can use nitrate (NO\(_3^-\)) as a final
electron acceptor for P uptake, the first step of the process facilitates both denitrification and P removal from the wastewater. In the absence of soluble carbon in wastewater, the internal carbon storage polymers (i.e. such as poly-β-hydroxy-alkanoates; PHAs) act as electron donors and an energy source to facilitate this step. In the subsequent anaerobic step, the biologically-captured P is released into a recovery stream which contains a relatively smaller volume. External carbon (i.e. acetate) is added to facilitate P release, and the biofilm simultaneously replenishes its carbon reserves by intracellular storage of carbon supplied to the recovery stream. The volume difference maintained between the wastewater and the recovery stream (e.g. ratio of 4:1) enables recovery of P as a more concentrated solution.

It has been suggested that dissolved oxygen (DO) concentrations >0.2 mg/L inhibit denitrification (Gerardi, 2003). In conventional post-denitrification processes, anoxic conditions prevail largely because of the rapid consumption of oxygen (O₂) by heterotrophic bacteria during carbon oxidation. Facilitating an anoxic environment in a similar manner is not feasible in the EBPR-r process, as external carbon is supplied only to the recovery stream. Except for carbon stored intracellularly, no soluble carbon is available in the wastewater stream. Under these conditions, Wong et al. (2013) observed elevated dissolved oxygen concentrations (DO >6 mg/L) during N and P removal, but surprisingly the high DO concentrations did not appear to inhibit denitrification and P removal.

Storage-driven denitrification is commonly observed in the simultaneous nitrification, denitrification and phosphorus removal processes (SNDPR) (Lemaire et al., 2008; Zeng et al., 2003a). In which nitrifiers make use of the O₂ to facilitate partial nitrification under low DO concentration (<1 mg/L), and hence are largely responsible for creating the anoxic conditions for denitrification. Findings based on the SNDPR process are not
directly useful for understanding denitrification in the EBPR-r process, which has been observed to take place at much higher levels of DO (>6 mg/L) with little contribution of nitrifiers (Wong et al., 2013). If large-scale EBPR-r is to be implemented, a clear understanding of the impact of DO on post-denitrification and P removal is critical.

Hence, the aim of this study was to explore the impact of DO on simultaneous storage-driven denitrification and P removal by an EBPR-r biofilm. It was hypothesised that the enriched EBPR-r biofilm was able to denitrify at high DO concentration because of the presence of an oxygen gradient across the biofilm. The specific objectives included assessment of the importance of the biofilm structure, and the levels of DO that can be tolerated by the bacteria without impeding denitrification. First, batch experiments were conducted to quantify P uptake, NO$_3^-$ removal and O$_2$ consumption kinetics by an intact biofilm exposed to various concentrations of DO (0–8 mg/L) and NO$_3^-$ (0–50 mg-N/L). Secondly, the EBPR-r biofilm was physically disturbed to investigate the effect of biofilm structure on the P and N removal efficiencies.

### 3.3. Materials and Methods

#### 3.3.1. Reactor configuration and synthetic wastewater

A laboratory-scale sequencing batch biofilm reactor (master reactor) was operated continuously in an EPBR-r configuration for a 2-year period, as described previously (Wong et al., 2013). A schematic diagram of the reactor process is shown in my previous study (Wong et al., 2013). In brief, 1000 biofilm carriers (Kaldnes® K1 polyethene) were equally distributed among eight adjoining stainless steel mesh compartments. Over a 6-h cycle the biofilm carriers were alternately exposed for 4 h to
a wastewater stream (7.2 L) for P uptake, and for 2 h to a separate recovery stream (1.8 L, 25% of the volume of the wastewater stream) for anaerobic P release.

Both the wastewater and recovery streams contained a standard growth medium consisting of (per L of DI water): 39 mg MgSO$_4$, 20 mg CaCl$_2$·2H$_2$O, 11 mg NH$_4$Cl (3 mg/L NH$_4^+$-N), 200 mg NaHCO$_3$ and 0.3 mL of a trace element solution (Wong et al., 2013). The wastewater stream also contained 8 mg-P/L phosphate (supplemented as 1 M phosphate buffer), 10 mg-N/L nitrate (as sodium nitrate) and 11.6 mg/L N-allylthiourea, the latter added to prevent nitrification during the P uptake phase (Ginestet et al., 1998). To restore intracellular PHA reserves during the anaerobic P release, 520 mg/L sodium acetate was added to the recovery stream, which corresponded to 400 mg/L chemical oxygen demand (COD). Concentrated stock solutions (15×) of the media comprising each of the streams were prepared, and the pH was adjusted to 7.0 ± 0.2 using 2 M HCl. Defined volumes of the stock solution and deionised water were simultaneously pumped into the reactor at the beginning of each phase to achieve the desired concentrations.

3.3.2. Kinetic experiments using intact biofilm

To elucidate the use of O$_2$ and NO$_3^-$ by the EBPR-r biofilm when both electron acceptors were present, two sets of experiments were performed in duplication (Figure 3.1). (1) The activity of the biofilm was investigated using an initial NO$_3^-$ concentration of 10 mg-N/L, but the bulk DO concentration was varied (0, 2, 4, 6 and 8 mg/L). (2) Constant influent bulk DO concentration of 8 mg/L was maintained, but the initial NO$_3^-$ concentration was varied (0, 5, 10, 20, 30 and 50 mg-N/L). The DO of the influent in the column reactor (DO$_{in}$) was controlled at a particular set point (0–8 mg L$^{-1}$) by feedback aeration in the recirculation bottle (Figure 3.1).
In each experiment, biofilm carriers (~330) were removed from two compartments of the master reactor at the end of the anaerobic P release phase, at which time the biomass had stored PHAs (Bond et al., 1999). The carriers were immediately transferred into a column reactor (440 mL working volume; diameter 45 cm, height 300 cm; Figure 3.1), where biological P uptake was triggered by recirculating (7.85 L/h; Masterflex®, USA) a P-containing wastewater stream (2.4 L, 8 mg-P/L) for 4 h. Two luminescent DO probes (PDO2; Barben Analyser Technology, USA) were installed in the recirculation line, one before (DO_{in}) and one after (DO_{out}) the column reactor. The influent DO_{in} was controlled at 0–8 mg/L by sparging air or nitrogen into the aeration vessel (2.0 L), whilst NO_{3}^{-} was added (as 4 M NaNO_{3}) into the wastewater stream to give an initial concentration of 0–50 mg-N/L. The monitoring and control of DO were performed using a programmable logical controller and software (LabVIEW, National Instruments, USA).

**Figure 3.1** A schematic diagram of the batch experiment setup designed to assess the ability of the enriched biofilm to denitrify, and to remove P from wastewater using stored PHAs.
Liquid samples were collected from the reactor every 15, 30 or 45 min, and immediately filtered using a 0.22 μm pore size syringe filters (Acrodisc® PF; Pal Corporation, UK). The concentrations of soluble NO₂⁻-N, NO₃⁻-N, and PO₄³⁻-P in the filtrates were determined using ion chromatography (ICS-03000, DIONEX). Changes in the concentrations of PO₄³⁻-P, NOₓ-N (NO₃⁻-N + NO₂⁻-N), and NO₂⁻-N were plotted against time, and the specific P uptake rate (PUR) and the NOₓ removal rate (expressed as mg/L.h) were recorded as the slope of the steepest part of the curves. These rates were normalised using the respective total biomass (TS) concentrations, and expressed in mmol/g-TS.h. TS was obtained by subtracting the weight of 50 biofilm-free carriers from the dry weight (dried at 60 °C) of 50 EBPR-r carriers supporting biofilm (Wong et al., 2013). To compare the reduction (electron-accepting) kinetics of NO₃⁻ and O₂ using the storage reducing power (i.e. PHAs), both the OUR (Appendix 1) and the NOₓ removal rate were transformed into a common unit, termed the electron-accepting rate (mmol e⁻/g-TS.h). The percentage of electrons used for O₂ and NO₃⁻ reduction was calculated by assuming that all the electrons from the storage were captured by either O₂ or NO₃⁻. The details of the calculations are given in the supporting information (Appendix 1).

3.3.3. Kinetic experiments using dislodged biomass

To confirm if the observed denitrification in the presence of O₂ was due to the presence of an oxygen gradient across the biofilm, kinetic experiments were conducted using biomass dislodged from the carriers. To obtain the biomass, biofilm carriers (~330) were removed from the master reactor at the end of a P uptake phase (low in PHAs storage) and placed into growth medium in a 500 mL flask. Attached biofilm was physically removed by shaking the carriers in standard medium for 2 min. To break down the size of the flocs, the suspended biofilm was repeatedly drawn up and expelled
through a needle (gauge 19½) using a 50 mL syringe. An acetate-containing recovery stream (200 mL) was added to the dislodged biomass for PHA replenishment for 2 h. Thereafter, the biomass was concentrated by centrifugation and washed twice with standard medium (without N, P and C) under anaerobic condition to remove any excess acetate. The PHA-rich biomass was then resuspended in 220 mL standard medium under anaerobic conditions for use in batch experiments.

Four batch reactors (250 mL Schott bottles) were operated in parallel to compare the denitrification ability of the biomass in the presence of 10 mg-N/L NO$_3^-$ for 4 h: (1) oxygenated, with 8 mg-P/L phosphate; (2) oxygenated, without phosphate; (3) anoxic, with 8 mg-P/L phosphate; and (4) anoxic, without phosphate. To initiate the experiment, 50 mL of suspended biomass was added to 200 mL of synthetic wastewater. The oxygenated and anoxic condition was achieved by continuously sparging air and nitrogen into the liquid, respectively. Mixing was achieved using a multi-position magnetic stirrer (400 rpm; RT10, IKA). To confirm the observation of denitrification under oxygenated conditions, the batch tests for the aforementioned conditions (1) and (2) were repeated on a different day.

Liquid samples were withdrawn and filtered for analysis of soluble NO$_2^-$-N, NO$_3^-$-N, and PO$_4^{3-}$-P. The mixed liquor suspended solids (MLSS) value for the suspended biomass was measured according to the standard method (American Public Health Association. et al., 1995). The PUR, NO$_x$ removal rate and the NO$_2^-$ accumulation rate were normalised with the solids concentration, and expressed as mmol/g-MLSS.h. The size distribution of the suspended biomass flocs, measured using a laser particle sizer (Malvern Master Sizer), was determined by an external laboratory (CSIRO, Division of Mineral Particle Analysis Service, Waterford, Australia).
3.4. Results and Discussion

3.4.1. Storage-driven denitrification and P uptake at very high DO concentrations

In a previous study I observed that an EBPR-r biofilm could take up phosphate and remove NO$_3^-$ in the presence of saturating DO (Wong et al., 2013). However, as both NO$_3^-$ and O$_2$ were provided to the biofilm, it was unable to distinguish the independent effects of these electron acceptors. Hence, in the present study batch experiments were conducted to assess the influence of each of O$_2$ and NO$_3^-$ as sole electron acceptors on P removal. The EBPR-r biofilm could readily use either O$_2$ or NO$_3^-$ for P uptake, or both (Figure S3.1 of supporting information; Appendix 1). The highest PUR was observed when O$_2$ was provided, either alone (0.038 mmol-P/g-TS.h) or in combination with NO$_3^-$ (0.043 mmol-P/g-TS.h). When NO$_3^-$ was used as the sole electron acceptor, the PUR was markedly reduced by 30% (from 0.043 to 0.030 mmol-P/g-TS.h) and 21% (from 0.038 to 0.030 mmol-P/g-TS.h; Table 3.1). This was a 21% reduction compared to when O$_2$ was provided as a sole electron acceptor. According to Kuba et al. (1996), the energy (adenosine triphosphate, ATP) production during oxidative phosphorylation with NO$_3^-$ as the electron acceptor is approximately 40% less than occurs with O$_2$ as the acceptor. While the reduced energy from oxidative phosphorylation based on NO$_3^-$ could have contributed to the 21% reduction in PUR, the possible role of a low-abundance denitrifying PAOs population in the biofilm should not be overlooked.

As expected, the highest NO$_x$ removal rate (0.076 mmol-N/g-TS.h) was observed when NO$_3^-$ was supplied as the sole electron acceptor. When supplemented with DO (8 mg/L), 70% of the denitrification efficiency of the biofilm was retained (Table 3.1), indicating that the enriched EBPR-r biofilm could denitrify under a very high DO concentrations.
Table 3.1 The result for the intact EBPR-r biofilm under three electron acceptor scenarios: (1) O$_2$ alone (8 mg/L of bulk DO); (2) NO$_3^-$ alone (10 mg-N.L); and (3) O$_2$ and NO$_3^-$ in combination. Results are presented as value ± standard deviation, based on two samples.

<table>
<thead>
<tr>
<th>Rates</th>
<th>Electron acceptors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>O$_2$</td>
</tr>
<tr>
<td>Phosphate uptake rate (mmol-P/g-TS.h)</td>
<td>0.038 ± 0.002</td>
</tr>
<tr>
<td>NO$_x$ removal rate (mmol-N/g-TS.h)</td>
<td>n.a.</td>
</tr>
<tr>
<td>P removal efficiency (%)</td>
<td>72 ± 6</td>
</tr>
<tr>
<td>N removal efficiency (%)</td>
<td>n.a.</td>
</tr>
<tr>
<td>P$<em>{up}$/N$</em>{den}$ ratio (g-P/g-N as NO$_3^-$)</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

3.4.2. The oxygen gradient across the biofilm enabled denitrification in the presence of DO

To elucidate the effect of DO and NO$_3^-$ loading on the P uptake and denitrification behaviour of the EBPR-r biofilm, two sets of experiments were conducted in which the concentration of one electron acceptor was maintained constant while the concentration of the other was varied. The concentration profiles for each experiment set are illustrated in the supplementary data Figure S3.2 (Appendix 1).
**Figure 3.2** Effect of bulk DO (0–8 mg/L) and initial NO$_3^-$ (0–50 mg-N/L) concentration on: (A) & (E) phosphate uptake rate; (B) & (F) oxygen uptake rate (OUR) and NO$_x$-N removal rate; (C) & (G) electron accepting rate for O$_2$ and NO$_3^-$; and (D) & (H) the percentage of electrons used for PHAs oxidation using electrons generated by O$_2$ and NO$_3^-$ reduction.
At a NO$_3^-$ concentration of 10 mg-N/L, increasing the bulk DO concentration from 0 to 8 mg/L increased the PUR by 43% (from 0.030 to 0.043 mmol/g-TS.h) (Figure 3.2A). A linear relationship ($R^2 = 0.999$) was obtained between the OUR and the applied bulk DO concentration (Figure 3.2B). Such a first order kinetic behaviour suggested that the biofilm was limited by O$_2$ (Meyer et al., 2005). Increasing the bulk water DO concentration from 0 to 8 mg/L could result in deeper penetration of O$_2$ into the biofilm, triggering the higher OURs (from 0 to 0.091 mmol/g-TS.h) and the lower denitrification rates (1.5-fold reduction from 0.076 to 0.052 mmol-N/g-TS.h) (Figure 3.2B). This result confirms the presence of an oxygen gradient in the biofilm, with the inner anoxic environment facilitating the observed denitrification despite the bulk water being saturated with oxygen.

In contrast, when the bulk DO concentration was fixed at 8 mg/L, increasing the NO$_3^-$ concentration from 0 to 50 mg-N/L resulted in an increase in the denitrification rate (from 0 to 0.096 mmol-N/g-TS.h) (Figure 3.2F). This result was expected because increased NO$_3^-$ availability in the bulk water could facilitate the penetration of NO$_3^-$ into the deeper anoxic layers of the biofilm, as observed in conventional EBPR processes under anoxic conditions (Ahn et al., 2001; Yuan & Oleszkiewicz, 2010; Zhou et al., 2010). In terms of P uptake, only a slight decrease in the PUR was observed with increasing NO$_3^-$ concentration (16% of overall inhibition, from 0.038 to 0.032 mmol/g-TS.h) (Figure 3.2E). No increase in PUR and a continuous increase in denitrification (evident in Figure 3.2F) could be a result of some NO$_3^-$ being utilised by denitrifying glycogen accumulating organisms (denitrifying GAOs) in the biofilm. The slight decrease in PUR is consistent with the findings of Yuan and Oleszkiewicz (2010), who observed an increased anoxic PUR and a decreased aerobic PUR with increasing NO$_3^-$ concentrations in the bulk water. It is also possible that when the NO$_3^-$ concentration
increased in the bulk water, some PAOs were able to switch from using O\textsubscript{2} as electron acceptor to use of NO\textsubscript{3}\textsuperscript{−}. With more PAOs using NO\textsubscript{3}\textsuperscript{−} as electron acceptor, the OUR and PUR may have decreased. Whether the elevated level of O\textsubscript{2} inhibited the activity of denitrifying PAOs remains unclear, and should be the subject of further research.

3.4.3. More than half of the stored reducing power was used for denitrification at 8 mg/L of DO

In the absence of a soluble carbon source, the observed P uptake and denitrification activities were driven by internal carbon storage (e.g. PHAs) in the EBPR-r biofilm. To compare the reduction kinetics of NO\textsubscript{3}\textsuperscript{−} and O\textsubscript{2} for the biofilm, the OUR and NO\textsubscript{x} removal rates (Figure 3.2B & 3.2F) were transformed into a common unit (electron accepting rate) (Figure 3.2C & 3.2G), and were also expressed as a percentage of the electrons used for internal carbon oxidation (Figure 3.2D & 3.2H).

At a bulk DO concentration of 8 mg/L and a NO\textsubscript{3}\textsuperscript{−} concentration of 0 mg-N/L, the electrons stored in the biofilm were predominately used to reduce O\textsubscript{2} at a maximum electron accepting rate of 0.46 mmol e\textsuperscript{−}/g-TS.h (Figure 3.2G). With increasing bulk water NO\textsubscript{3}\textsuperscript{−} concentration the electron reduction rate for O\textsubscript{2} decreased only slightly, whereas the electron reduction rate for NO\textsubscript{3}\textsuperscript{−} increased dramatically. At a NO\textsubscript{3}\textsuperscript{−} concentration of approximately 8 mg-N/L, the biofilm appeared to be transferring electrons at a similar rate to both O\textsubscript{2} and NO\textsubscript{3}\textsuperscript{−}. At a bulk water NO\textsubscript{3}\textsuperscript{−} concentration exceeding 8 mg-N/L, NO\textsubscript{3}\textsuperscript{−} became the dominant electron acceptor (>50%) (Figure 3.2H). Thus, a NO\textsubscript{3}\textsuperscript{−} concentration of >8 mg-N/L in the wastewater is favorable for efficient NO\textsubscript{3}\textsuperscript{−} reduction.

At a NO\textsubscript{3}\textsuperscript{−} concentration of 10 mg-N/L and a DO concentration of 0 mg/L, the electrons stored in the biofilm were solely used to reduce NO\textsubscript{3}\textsuperscript{−} at a maximum electron accepting
rate of 0.98 mmol e\(^{-}\)/g-TS.h (Figure 3.2C). When the DO concentration increased from 0 to 8 mg/L, the proportion of electrons accepted by O\(_2\) gradually increased from 0 to 35\% (Figure 3.2D). It is noteworthy that even at such a high bulk water DO concentration the biofilm was able to channel approximately 65\% of the electrons to NO\(_3^-\) reduction. This unique ability of the biofilm to reduce NO\(_3^-\) in the presence of DO is critical for the EBPR-r process, and is probably a consequence of the presence of an oxygen gradient across the biofilm, as discussed above.

3.4.4. The biofilm structure is essential for denitrification in the presence of O\(_2\)

To determine whether the cells in the biofilm could continue to denitrify when the DO gradient was disrupted, the biofilm was removed from the carriers and physically disturbed to form suspended aggregates (mean size 185 ± 11 µm). The aerobic and anoxic ratio difference of P uptake (PUR\(_{aer}^\)/PUR\(_{anx}^\)) and denitrification activities of the suspended biomass (MLSS of 1.03 ± 0.05 g/L) were compared with that of the intact biofilm.

Similar to the intact biofilm, the suspended biomass showed the highest PUR (0.53 mmol-P/g-MLSS.h) when both O\(_2\) and NO\(_3^-\) were provided as electron acceptors (Table 3.2). When NO\(_3^-\) was the sole electron acceptor the PUR decreased by 38\%, which is consistent with the observed decline in the previous experiments with the intact biofilm (~30\% in Table 3.1). Thus, the decreased PUR observed with NO\(_3^-\) was not caused by the biofilm structure, but rather appeared to be influenced by the type of electron acceptor.
### Table 3.2 The result for the dispersed biofilm under four conditions tested.

<table>
<thead>
<tr>
<th></th>
<th>Rates</th>
<th>Aerobic</th>
<th>Anoxic</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>With P</strong></td>
<td><strong>NO\textsubscript{x} removal rate (mmol-N/g-MLSS.h):</strong></td>
<td>0.16</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td><strong>NO\textsubscript{3}⁻ removal rate (mmol-N/g-MLSS.h):</strong></td>
<td>n.a.</td>
<td>2.23</td>
</tr>
<tr>
<td></td>
<td><strong>NO\textsubscript{2}⁻ formation rate (mmol-N/g-MLSS.h):</strong></td>
<td>n.a.</td>
<td>2.39</td>
</tr>
<tr>
<td><strong>No P</strong></td>
<td><strong>PUR (mmol-P/g-MLSS.h):</strong></td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td><strong>NO\textsubscript{x} removal rate (mmol-N/g-MLSS.h):</strong></td>
<td>0.00</td>
<td>0.78</td>
</tr>
<tr>
<td></td>
<td><strong>NO\textsubscript{3}⁻ removal rate (mmol-N/g-MLSS.h):</strong></td>
<td>n.a.</td>
<td>1.58</td>
</tr>
<tr>
<td></td>
<td><strong>NO\textsubscript{2}⁻ formation rate (mmol-N/g-MLSS.h):</strong></td>
<td>n.a.</td>
<td>1.89</td>
</tr>
</tbody>
</table>

As with the intact biofilm, the highest level of denitrification by the suspended culture was observed in the absence of DO (Table 3.2). However, when O\textsubscript{2} (DO >6 mg/L) was introduced into the bulk water a 6-fold decrease in the denitrification rate (from 1.00 to 0.16 mmol-N/g-MLSS.h) was observed. This was remarkably different from the intact biofilm, for which the respective decline in the denitrification rate was only 1.5-fold (Table 3.1). One plausible explanation for the decrease in denitrification activity after the disruption of oxygen gradient is the inhibition of nitrate reductase, which is a membrane-bound enzyme that catalyses the reduction of NO\textsubscript{3}⁻ to NO\textsubscript{2}⁻, and is sensitive to O\textsubscript{2} (Ogunseitan, 2005). Under anoxic conditions, the rate of reduction of NO\textsubscript{3}⁻ (2.23 mmol-N/g-MLSS.h) far exceeded that of NO\textsubscript{2}⁻, resulting in the accumulation of the NO\textsubscript{2}⁻ observed in this study (an accumulation rate of 2.39 mmol-N/g-MLSS.h, Figure 3.3C). Only when NO\textsubscript{3}⁻ became limiting was an overall reduction in NO\textsubscript{x} observed. Accumulation of NO\textsubscript{2}⁻ is a common observation during denitrification and has been extensively discussed in the literature (Ahn et al., 2001; Zhou et al., 2010). Under aerobic conditions the nitrate reductase enzyme was exposed to O\textsubscript{2}, resulting in
inhibition of the enzyme and a significant decrease in the NO$_3^-$ reduction rate (Figure 3.3A). These results demonstrate that maintenance of the biofilm structure for bacterial growth is critical for the EBPR-r process to achieve satisfactory rates of denitrification, particularly when strict anoxic conditions cannot be maintained.

3.4.5. The dependency of denitrification on P

The observed denitrification in suspended biomass could be performed by either PAOs or other non-PAOs bacteria, including glycogen accumulating organisms (GAOs). By definition, GAOs do not require storage of P under either aerobic or anoxic conditions (Oehmen et al., 2006). To investigate the denitrification activities of non-PAOs organisms, the suspended biomass experiment was conducted aerobically and anoxically with no P in the bulk water.

**Figure 3.3** Concentrations of soluble PO$_4^{3-}$-P, NO$_x$-N (NO$_3^-$-N + NO$_2^-$-N), NO$_3^-$-N and NO$_2^-$-N over time associated with suspended biomass incubated with 10 mg-N/L of NO$_3^-$ under four conditions: (A) oxygenated, with phosphate; (B) oxygenated, without phosphate; (C) anoxic, with phosphate; and (D) anoxic, without phosphate.
Under anoxic conditions the absence of phosphate decreased the NO₃⁻ removal rate only by 22%, from 1.00 mmol-N/g-MLSS.h in the presence of P to 0.78 mmol-N/g-MLSS.h in the absence of P (Table 3.2). This confirmed the role of non-PAO organisms (e.g. denitrifying GAOs) in biofilms carrying out denitrification or DPAOs performing denitrification without taking up P (the ability of PAOs to conserve their carbon storage in the absence of P is discussed in Chapter 4). However, exposing the suspended biomass to aerobic conditions in the absence of phosphate resulted in a complete inhibition of denitrification (Figure 3.3B), indicating that the non-PAOs denitrifiers in the EBPR-r culture were unable to denitrify when exposed to O₂. It is plausible that they had reduced affinity for NO₃⁻ than did the PAOs, or that they were more sensitive to O₂. Alternatively, it is possible that the denitrifying GAOs predominately occupied the inner parts of the biofilm, where the penetration of O₂ was reduced (Lemaire et al., 2008), as has been reported for the granules enriched in the SNDRP process.

### 3.4.6. Implications of the study

EBPR-r is a novel post-denitrification process that enables P recovery. The success of this strategy depends on whether denitrification can be efficiently driven by the reducing power stored in the biofilm. The lack of soluble carbon and ammonia in the influent of this process could result in an elevated bulk water DO, which might affect the denitrification process. The previous chapter suggested that the EBPR-r process can facilitate denitrification without the need to maintain a strictly anoxic environment in the bulk water (Wong et al., 2013). This was confirmed in the current study, where 60% of the reducing power stored in the biofilm was found to be expended on denitrification, even when the bulk water DO concentration was near saturation (8 mg/L). However, the denitrifying ability of the EBPR-r process was remarkably compromised when the biofilm structure was physically disturbed, implying that maintenance of the biofilm
structure is critical for the success of EBPR-r as a post-denitrification strategy when oxygen intrusion occurs.

3.5. Conclusions

The results of this study suggest that:

- the EPBR-r biofilm facilitated P and N removal in a process that was not sensitive to oxygen intrusion;
- at a NO₃⁻ concentration of 10 mg-N/L, increasing the DO concentration (from 0 to 8 mg/L) increased the PUR by 43% and decreased the denitrification rate by 31%;
- at a DO concentration of 8 mg/L, increasing the NO₃⁻ concentration (from 0 to 50 mg-N/L) increased the denitrification rate (from 0 to 0.096 mmol-N/g-TS.h).

In summary, this study highlights the importance of the EBPR-r biofilm structure in enabling denitrification to take place at the same time as P removal for recovery. The data also suggest some operational boundaries (e.g. specific DO and NO₃⁻ concentrations in the influent) necessary for the EBPR-r biofilm to reduce P and N to acceptable levels in the effluent.
4. The Ability of PAOs to Conserve Their Phosphorus Uptake Activities during Prolonged Aerobic P- and C-starvation Conditions

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4.1. Abstract

A storage-driven post-denitrification process, known as enhanced biological phosphorus removal and recovery (EBPR-r), was recently developed to facilitate phosphorus (P) recovery from municipal wastewater. This process utilises a biofilm containing phosphate-accumulating organisms (PAOs) to capture P from wastewater and then release the captured P in a separate smaller stream for recovery. As a post-denitrification strategy, the EBPR-r biofilm is exposed to carbon-deficient wastewater that contains greater quantities of electron acceptors (O\textsubscript{2} and NO\textsubscript{3}\textsuperscript{−}) than are required for P uptake. The impact of such high concentrations of electron acceptors on the storage-driven P uptake activities of PAOs is unknown. Hence, this study explored the ability of PAOs to conserve their P uptake activities, after exposing the biofilm to an oxidising
and P-deficient condition for extended periods (up to 7 days). Results showed that even after 2 days of exposure to such conditions, upon the addition of 8 mg/L of P the biofilm could facilitate a similar level of P uptake (1.20 ± 0.09 mg-P/g-TS, between 0–48 h). Beyond 2 days of exposure, a decline of P uptake activity was noted, with only 15% activity remained by day 7. Overall, the study reports first line of evidence towards PAOs’ ability to conserve their storage-driven P uptake activities. This unique behaviour of PAOs provides opportunities for new operational strategies such as infrequent carbon replenishment to be implemented (i.e. facilitate multiple P uptake phases before anaerobic carbon replenishment). Such flexibility could reduce the capital and operational costs of the EBPR-r process, and thus enhance the economic viability of P recovery.

4.2. Introduction

The recycling of phosphorous (P) from municipal wastewater is an environmentally sustainable initiative because P is a scarce resource (Rittmann et al., 2011). Sewage treatment plants are potential sites for P recovery, but municipal wastewater typically has a low P concentration (<10 mg-P/L), making P recovery from this source technically and economically challenging (Parsons & Smith, 2008). Generally, a concentration >50 mg-P/L is recommended for P recovery (Cornel & Schaum, 2009; Shi & Lee, 2006).

Recently, a post-denitrification process, referred to as enhanced biological phosphorus removal and recovery (EBPR-r), was developed to facilitate P recovery from municipal wastewater (Wong et al., 2013; Wong et al., 2015). Similar to conventional enhanced biological phosphorus removal (EBPR), the EBPR-r process uses phosphorus accumulating organisms (PAOs) to uptake and release P from wastewater. However,
unlike conventional EBPR, which utilises suspended cells and operates using a single wastewater stream, EBPR-r makes use of biofilms to facilitate P recovery in a two-step process, and is operated using two streams that are hydraulically separated. In the first step, the PAOs uptake phosphate (PO$_4^{3-}$) from low P-containing wastewater using nitrate (NO$_3^-$) and/or oxygen (O$_2$) as final electron acceptors, and P is stored intracellularly as polyphosphate (Poly-P). The internal carbon storage polymers (poly-β-hydroxy-alkanoates; PHAs) of PAOs are used as the energy source to satisfy the carbon requirements for this process. In the subsequent step the P-enriched PAOs biofilm is exposed to a recovery stream of smaller volume under anaerobic conditions. External carbon source (acetate) is introduced into the recovery stream and the PHA reserves of PAOs are replenished via acetate uptake. Energy requirements for this process are fulfilled by the hydrolysis of Poly-P and this releases PO$_4^{3-}$ into the recovery stream.

The capture of P from wastewater in a concentrated recovery stream provides the opportunity for the precipitation of P for use as a fertiliser, which could generate revenue for the wastewater industry.

The effectiveness of this proposed EBPR-r process depends on whether the PAOs can efficiently shuttle the P from a large volume of wastewater into a smaller recovery stream in a cyclic manner. In practice, whether or not a wastewater treatment plant (WWTP) adopts a single cycle for P uptake and release will depend largely on the availability of land and infrastructure (the requirement of one relatively large tank for P uptake and one smaller tank for P release). When these factors are limiting, an alternative mode of operation for the P release step (i.e. carbon replenishment) may involve multiple P uptake (e.g. four sequential P uptake phases per 16 h) from wastewater streams rather than uptake in a single pass. However, such strategy of infrequent carbon replenishment is only feasible if storage polymers (e.g. PHAs) can be
conserved in PAOs over an extended period of time (i.e. over the course of the multiple P uptakes). In EBPR-r process, intrusion of oxygen may occur during the P uptake phase(s) where soluble carbon is deficient. In this situation large quantities of electron acceptors (both NO₃⁻ and O₂) may be present in the wastewater, creating an oxidising environment whereby the PHAs stored in PAOs may be oxidised rapidly without parallel P uptake (Lopez et al., 2006). In the event that storage polymers are not conserved for P uptake, frequent carbon replenishment would be needed, which would increase the operating costs of the EBPR-r process.

To date no studies have investigated the ability of PAOs to conserve their carbon storage for P uptake. However, many studies have investigated the effect of electron donor (carbon) starvation on the activities and endogenous processes of PAOs (Lopez et al., 2006; Lu et al., 2007; Pijuan et al., 2009; Wang et al., 2012; Yilmaz et al., 2007). These findings indicate that PAOs exhibits a higher decay rate when exposed to aerobic conditions compared to anaerobic conditions. However, in these studies the PAOs biomass used was obtained at the end of a P uptake phase (i.e. at the end of an aerobic and/or anaerobic period). It is well known that the internal storage reserves of PAOs are at a minimum at the end of a P uptake phase (Bond et al., 1999). Hence, experiments undertaken with near absence of internal storage reserves in PAOs would not enable the research questions of the present study to be addressed.

The aim of this study was to investigate the impact of excessive electron acceptor concentrations on the storage-driven P uptake activities of PAOs (contain PHAs storage). It was hypothesised that an EBPR-r biofilm could conserve its storage-driven P uptake activities during a prolonged period of exposure to an oxidising and P-deficient environment. This study aimed to investigate how long PAOs could tolerate
such conditions without losing their P uptake ability. The findings of this study are critical to any evaluation of the economic feasibility of the EBPR-r process.

4.3. Materials and Methods

4.3.1. Reactor configuration and synthetic wastewater

A laboratory-scale sequencing batch biofilm reactor (master reactor) was operated continuously in EBPR-r configuration for in excess of 2 years, as described previously (Wong et al., 2013). In brief, 1000 biofilm carriers (Kaldnes® K1 polyethene carrier) were equally distributed among eight adjoining stainless steel mesh compartments. Over a 6-h cycle the biofilm carriers were alternately exposed for 4 h to a wastewater stream (7.2 L) facilitating P uptake and denitrification, and for 2 h to a separate recovery stream (1.8 L, i.e. 25% of the volume of the wastewater stream) to enable anaerobic P release and PHA replenishment.

Both the wastewater and recovery streams contained a mineral salts growth medium consisting (per L) of: 39 mg MgSO$_4$, 20 mg CaCl$_2$·2H$_2$O, 11 mg NH$_4$Cl (3 mg/L NH$_4$+-N), 200 mg NaHCO$_3$ and 0.3 mL of a trace element solution. The trace element solution contained (per L) 1.5 g FeCl$_3$·6H$_2$O, 0.15 g H$_2$BO$_3$, 0.03 g CuSO$_4$·5H$_2$O, 0.18 g KI, 0.12 g MnCl$_2$·4H$_2$O, 0.06 g Na$_2$MoO$_4$·2H$_2$O, 0.12 g ZnSO$_4$·7H$_2$O, 0.15 g CoCl$_2$·6H$_2$O and 10 g ethylenediaminetetraacetic acid (EDTA). The trace element solution pH was adjusted to 7.0. The wastewater stream also contained 8 mg-P/L PO$_4^{3-}$ (as 1 M phosphate buffer: 46 g KH$_2$PO$_4$ and 115 g K$_2$HPO$_4$ per L) and 10 mg-N/L NO$_3^-$ (as NaNO$_3$). To restore intracellular PHA reserves during anaerobic release of P, 375 mg/L acetate (as C$_2$H$_3$NaO$_2$) was added to the recovery stream; this corresponded to 400 mg chemical oxygen demand (COD). Concentrated stock solutions (15×) of the media
comprising each of the streams were prepared, and the pH was adjusted to 7.0 ± 0.2 using 2 M HCl. Defined volumes of the stock solution and deionised water were simultaneously pumped into the reactor at the beginning of each phase to achieve the desired concentrations.

Details of the reactor operation are described in Wong et al. (2013). The experiments described below were carried out during steady state operation of the master reactor, which was indicated by stable total solid (TS) and PO$_4^{3-}$ effluent concentrations.

4.3.2. **Short-term (0–48 h) exposure to P- and C-deficient conditions and a highly oxidising environment**

A series of batch experiments was performed, during which the EBPR-r biofilm was exposed to P- and C-deficient conditions and a highly oxidised environment for various periods (0−48 h). At the end of each starvation period, PO$_4^{3-}$ was introduced to assess the impact of the duration of P- and C-starvation on the storage-driven P uptake activities.

The procedure described below was followed to ensure all batch experiments were initiated using biofilms comprising cells having a high level of internal storage polymers. To achieve this the biofilm carriers (approximately 330; taken from two compartments) were removed from the master reactor at the end of an anaerobic P release phase and briefly washed with deionised water to remove any residual acetate and P. The carriers were immediately transferred into a separate column reactor (440 mL working volume; Figure 4.1), through which a wastewater stream (contained neither soluble C nor P) was recirculated (1.2 L; 130 mL/min). Ion chromatography showed the absence of soluble C and P in the wastewater implying that there was no carryover of residual C and P from the biofilm. To create a highly oxidising environment, NO$_3^-$ was
added to the wastewater at an initial concentration of 10 mg-N/L, and a dissolved oxygen (DO\textsubscript{m}) concentration of 7.8 ± 0.2 mg/L was maintained in the recirculation line of the column reactor. Throughout each batch experiment the inflow (DO\textsubscript{in}) and outflow (DO\textsubscript{out}) oxygen concentrations in the column reactor were monitored online using luminescent DO probes (PDO\textsubscript{2}; Barben Analyser Technology, USA). Monitoring and control of the DO levels were achieved using a programmable logical controller (PLC; National Instruments, USA) and LabVIEW software (National Instruments, USA).

**Figure 4.1** A schematic diagram of the short-term (0–48 h) P- and C-starvation test. The PHA-rich EBPR-r biofilm was removed from the master reactor, immediately transferred to the column reactor, and exposed for various times (0, 3, 12, 24 and 48 h) to oxidising conditions in the absence of soluble PO\textsubscript{4}\textsuperscript{3−}. Thereafter, PO\textsubscript{4}\textsubscript{3−} was added to trigger storage-driven P uptake.

Following exposure of the biofilm to P- and C-deficient wastewater in an oxidising environment for various lengths of time (0, 3, 12, 24, 48 h), PO\textsubscript{4}\textsubscript{3−} (8 mg-P/L) was added to the wastewater to assess the ability of PAOs to uptake P using carbon polymers that had been conserved during starvation. The PO\textsubscript{4}\textsubscript{3−} and O\textsubscript{2} uptake were continuously monitored for a period of 4 h. During this period, liquid samples were
collected every 15–45 min from the column reactor; each sample was immediately filtered using a 0.22 μm pore size syringe filter (Acrodisc® PF; Pall Corporation, UK). The concentration of PO$_4^{3-}$-P in the filtrate was determined using ion chromatography (ICS-03000, Dionex). The specific phosphate uptake rates (PURs) of the biofilm were determined from the steepest part of the PO$_4^{3-}$ concentration profile. The oxygen uptake rates (OURs) were calculated as described previously (Wong et al., 2015). The specific PURs and specific OURs were obtained by normalising the results with the total solid concentration (TS). TS was obtained by subtracting the dry weight of 30 biofilm-free carriers from the dry weight of 30 biofilm-containing carriers (taken at the end of anaerobic phase) following drying at 60°C overnight (Wong et al., 2013). To enable assessment of the effect of the duration of starvation on biofilm activity, the specific PURs and OURs were then plotted against the time of P- and C-starvation.

### 4.3.3. Long-term (7-day) exposure to P- and C-deficient conditions and a highly oxidising environment

To assess the impact of a longer period of P- and C-starvation on storage-driven P uptake, an experiment spanning 7 days was performed in the master reactor. In preparation for this experiment the recovery stream of the master reactor was completely decanted at the end of an anaerobic P release phase, and a newly-added wastewater stream was recirculated (7.2 L, 337 mL/min) in the master reactor for the 7-day experimental period. As in the short-term experiment, this wastewater contained neither soluble P nor C; an initial NO$_3^-$ concentration of 10 mg-N/L and an influent DO concentration of >7 mg/L in the wastewater were supplied to create an oxidising environment.
To quantify the biomass decay during the 7-day period, liquid samples were collected from the master reactor every 1–2 days. Each sample was immediately filtered using a 0.22 μm pore size syringe filter. The concentrations of soluble NO$_3^-$-N, PO$_4^{3-}$-P, and NH$_4^+$-N in each filtrate were determined using ion chromatography. The ammonia (NH$_4^+$) and PO$_4^{3-}$ accumulation rates, indicative of biomass decay, were determined using the slopes of the NH$_4^+$ and PO$_4^{3-}$ profiles, respectively. The NO$_3^-$ concentration was not used as an indication of biomass decay because nitrification of NH$_4^+$ was inhibited by the addition of N-Allylthiourea (11.6 mg/L) to the wastewater in both the short- and long-term experiments (Ginestet et al., 1998).

To assess the change in biomass activity, a cyclic (wastewater stream/recovery stream) study was performed on each of days 0, 2, 4, and 7. For each cyclic study, approximately 160 biofilm carriers were removed from one compartment of the master reactor. The carriers were transferred to the column reactor used in the short-term experiment, where they were exposed to a 1.5 L wastewater stream for P uptake (8 h). A longer P uptake duration was employed to accommodate any lag time for P uptake induced by an extended exposure to a highly oxidised environment. The wastewater stream contained initial PO$_4^{3-}$-P and NO$_3^-$-N concentrations of 9 mg/L, and an influent DO$_m$ concentration of 7.8 ± 0.2 mg/L was maintained throughout this phase. After 8 h the wastewater stream was completely decanted, and a recovery stream (0.375 L) was added to facilitate P release (2 h). The recovery stream contained 325 mg/L of acetate, and was sparged with nitrogen gas for 10 min to purge any DO before recirculating through the column reactor. Both streams were recirculated at a rate of 90 mL/min.

Liquid samples were collected regularly from the column reactor during both the P uptake and P release phases. Each sample was immediately filtered (0.22 μm Acrodisc®). The filtered samples were measured for acetate content using gas
chromatography (GC) with a flame ionisation detector (FID) (Agilent 6890 series), as described previously (Wong et al., 2014), and soluble PO$_4^{3-}$-P was measured using an Aquakem™ 200 photometric analyser (Thermo Scientific, USA). The specific PURs, phosphate release rates (PRRs) and acetate uptake rates (AURs) were determined from the steepest parts of the concentration profiles, and normalised using the TS concentrations. TS were obtained by using the dry weight of 10 biofilm-containing carriers that were taken from the column reactor at the end of 10-h batch tests (day 0, 2, 4, and 7).

A. Decay rate

The biofilm activity decay rates were calculated from the slopes of semi-logarithmic plots of the PUR and PRR as a function of the P- and C-starvation time, using Equation 4.1:

$$b = -ln\frac{R_t}{R_0} \times \frac{1}{t_d} \quad \text{Equation 4.1}$$

Where $b$ is the decay rate (1/day or $d^{-1}$), $R_0$ is the PUR or PRR prior to starvation (mg-P/g-TS.h), $R_t$ is the PUR or PRR following starvation (mg-P/g-TS.h), and $t_d$ is the starvation time (day). This method has been used to calculate decay rates in previous starvation studies (Hao et al., 2010; Lopez et al., 2006; Lu et al., 2007; Vargas et al., 2013).
4.4. Results and Discussion

4.4.1. PAOs are able to conserve their storage-driven P uptake activities for up to 2 days

The EBPR-r biofilm is capable of using O$_2$ and NO$_3^-$ as electron acceptors for P uptake (Wong et al., 2015). To illustrate the activities of biofilm during aerobic starvation (P- and C-deficient conditions), the OURs were measured (Figure 4.2). The OURs in all experiments decreased rapidly (from ~25 to ~6 mg/L.h) during the initial 7 h of starvation, and subsequently a stable rate was observed for up to 48 h (Figure 4.2C–E). The stable OURs suggested a shift in microbial metabolism, including the activities of both PAOs and non-PAOs (e.g. glycogen accumulating organisms; GAOs), from a rapid oxidation state to a slower maintenance state. It has been reported that during electron donor starvation, maintenance energy for PAOs is derived primarily by oxidising PHA (within 4 h); when this is exhausted the oxidation shifts to glycogen, and finally to Poly-P (Lopez et al., 2006). Compared with past studies, the EBPR-r biofilm used in this study was starved of P (i.e. Poly-P) and soluble carbon, but not of internal carbon storage polymers (PHAs). Specifically, the PHAs storage of PAOs was replenished prior to starvation test by exposing the biofilm to anaerobic condition for acetate uptake and release of PO$_4^{3-}$ was noted. Accordingly, the initial higher OURs observed during the initial 7 h of starvation could have been a result of the rapid oxidation of internal storage polymers, possibly PHAs.

To estimate if storage-driven P uptake activities of PAOs were conserved, PO$_4^{3-}$ was introduced into the environment and P uptake was measured (Figure 4.2). Exposed to an oxidised environment and in complete absence of soluble carbon, PAOs are known to uptake P using energy derived from storage polymer oxidation (Bond et al., 1999).
Hence, the approach taken in this study was able to provide an indirect estimate of storage polymer availability for P uptake.

Figure 4.2 Profiles of the oxygen uptake rate (OUR) and soluble PO$_4^{3-}$ concentration following exposure to P- and C-starvation conditions for (A) 0 h, (B) 3 h, (C) 12 h, (D) 24 h and (E) 48 h. The negative time-axis indicates the period of P- and C-starvation in an oxidising environment (presence of O$_2$ and NO$_3^-$). PO$_4^{3-}$ (8 mg-P/L) was added to the bulk water at 0 h (red arrow) to trigger storage-driven P uptake.
Figure 4.3 Effect of short-term P- and C-starvation (0–48 h) on the: (A) specific phosphate uptake rate (PUR; mg-P/g-TS.h) and the oxygen uptake rate (OUR; mg-O_2/g-TS.h) during the P uptake phase; and (B) the respective total oxygen consumption (mg-O_2/g-TS), total P removal (mg-P/g-TS), and O_2/P ratio (g-O_2/g-P).

The results provided clear evidence that even after 48 h of exposure to P- and C-deficient conditions and a highly oxidised environment; PAOs in the biofilm were able to conserve their storage-driven P uptake activities and facilitate a similar level of P uptake (1.20 ± 0.09 mg-P/g-TS in Figure 4.3B) as that in the absence of starvation (0 h treatment). When specific PURs and OURs were examined as a function of the duration of exposure, an initial decrease of both rates was observed. Specially, the PUR decreased from 0.47 to 0.34 mg-P/g-TS.h over a period of 12 h (28% reduction), and thereafter a steady rate was observed for up to 48 h (Figure 4.3A). A higher PUR was accompanied by a higher OUR, and vice versa. The reduction in PURs and OURs during the initial 7 h was most likely a result of enzyme degradation (e.g. polyphosphate
kinase) and storage polymer oxidation. On the other hand, the contribution of biomass decay toward the reduced rates was assumed to be negligible since no soluble \( \text{NH}_4^+ \) and \( \text{PO}_4^{3-} \) (from cell lysis) were detected in the bulk water after 48 h starvation.

As soluble carbon was not present in wastewater, the conservation of P uptake activities implied that PAOs are able to conserve their carbon storage polymers in the presence of electron acceptors exceeding those levels that are stoichiometrically required for P removal. Further study should consider measuring the storage polymers to determine which storage polymers of PAOs was conserved for P uptake. Overall, this study is the first to demonstrate the ability of PAOs to conserve storage-driven P uptake activities. This finding is of significance to the EBPR-r process for two reasons. Firstly, the economic feasibility of the EBPR-r process is greatly enhanced because of the specific use of external carbon by PAOs for P recovery. Secondly, the results suggest the opportunity to develop new operational strategies (e.g. infrequent replenishment of carbon to recover P from a large volume of wastewater) to minimise the footprint of the post-denitrification process, and the capital and operating costs involved.

4.4.2. In the presence of internal carbon storage polymers, the long-term activity and viability of PAOs can be conserved by ensuring complete absence of soluble P

While the previous results demonstrate yet another unique metabolic property of PAOs, it is also of practical importance to explore the limits of this capability, particularly at a time when there is increasing interest in full-scale plant bioaugmentation to enhance the treatment of pollutants. For successful bioaugmentation by bacteria, robust strategies that ensure the long-term bacterial activity and viability are critical. In this study the P-
and C-starvation was extended from 2 to 7 days to assess the long-term effects of exposing PAOs to P- and C-deficient conditions and a highly oxidising environment.

**Figure 4.4** Concentration profiles of dissolved $\text{PO}_4^{3-}$ (mg-P/L) and acetate (mg/L) in the 10 h cyclic tests conducted on each of days 0, 2, 4 and 7 during the long-term P- and C-starvation experiment. The cyclic test consisted of a P uptake phase from a wastewater stream (1.5 L) over 8 h, and P release into a separate recovery stream (0.375 L) over 2 h.

**Figure 4.5** The effect of long-term P- and C-starvation (0–7 days) on the aerobic phosphate uptake rate (PUR; mg-P/g-TS.h), the anaerobic phosphate release rate (PRR; mg-P/g-TS.h), and the anaerobic acetate uptake rate (AUR, mg/g-TS.h). The PUR obtained from the short-term test (Figure 4.3A) is also presented.
To ascertain any loss of bacterial activity, a cyclic study was conducted on each of days 0, 2, 4 and 7; the resulting concentration profiles are shown in Figure 4.4. These data were used to calculate specific PURs, PRRs and AURs, which are shown in Figure 4.5. As in the previous experiment, the biomass retained a significant portion of its P uptake activity (79%; Figure 4.5) for up to 2 days in P-deficient conditions and a highly oxidising environment. Beyond 2 days, a rapid decline in P uptake activity occurred, and only 15% of the activity remained after 7 days (i.e. 0.067 mg-P/g-TS.h compared with an activity of 0.435 mg-P/g-TS.h on day 0). The gradual decrease in the PURs suggests that there was a gradual exhaustion of carbon storage polymers to provide the maintenance energy of PAOs.

During the sequential anaerobic exposure to acetate, the biofilm displayed a reduced P release activity as a result of prolonged starvation (Figure 4.4). The \( \frac{P_{\text{rel}}}{C_{\text{upt}}} \) ratio of the biofilm at day 0 was 0.11 (mol-P/mol-C) (Table 4.1). This ratio continued to decrease with increasing exposure to the starvation conditions (P- and C-deficient), and a 35% reduction had occurred by day 7. This suggests there was increasing use of carbon to recover P. While this could indicate GAO-like activity (i.e. C uptake with little or no P release), a lower \( \frac{P_{\text{rel}}}{C_{\text{upt}}} \) ratio immediately following prolonged exposure to P- and C-deficient conditions could also be explained by an increased reduction of storage polymers (below values normally observed after an aerobic P uptake phase) to fulfil maintenance energy requirements of PAOs.
Table 4.1 The phosphate release/carbon uptake ($P_{rel}/C_{upt}$) and phosphate release/phosphate uptake ($P_{rel}/P_{upt}$) ratios of the EBPR-r biofilm during long-term P- and C-starvation test (0–7 days).

<table>
<thead>
<tr>
<th>Day</th>
<th>$P_{rel}/C_{upt}$ ratio (mol-P/mol-C)</th>
<th>$P_{rel}/P_{upt}$ (g-P/g-P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.11</td>
<td>0.80</td>
</tr>
<tr>
<td>2</td>
<td>0.10</td>
<td>0.86</td>
</tr>
<tr>
<td>4</td>
<td>0.09</td>
<td>1.29</td>
</tr>
<tr>
<td>7</td>
<td>0.07</td>
<td>2.27</td>
</tr>
</tbody>
</table>

4.4.3. Underestimation of PAOs activity because of uptake of P released during biomass decay

The reduced P uptake activities could be a result of the loss of storage polymers and biomass decay (lysis). To measure the biomass decay, the concentration of $PO_4^{3-}$ and $NH_4^+$ were measured in the master reactor during the 7-day experiment (Figure 4.6). From day 2 the $NH_4^+$-N accumulated at a rate of 1.22 mg-N/L.d. The release of $NH_4^+$ is indicative of biomass decay (Lu et al., 2007; Vargas et al., 2013). As the EBPR-r biofilm contained a mixed microbial population, the decay of biofilm biomass could have involved both non-PAOs and PAOs. The latter have been shown to conserve storage polymers in the absence of P (as discussed in above experiment), thus may have a lower rate of decay, assuming decay only occurs following depletion of carbon storage polymers.

According to a published empirical formula ($CH_{2.09}O_{0.54}N_{0.20}P_{0.015}$) proposed by Smolders et al. (1994) and Wang et al. (2012), the decaying biomass (the $NH_4^+$ accumulation observed) would be expected to release $PO_4^{3-}$-P at a rate of 0.01 mg-
P/L. However, there was no increase in PO$_4^{3-}$-P during days 1–4 (Figure 4.6), possibly as a result of PO$_4^{3-}$-P uptake by PAOs. The uptake of this unknown amount of P by PAOs could have decreased their internal storage polymers, which is reflected in the reduced PURs. A measureable quantity of PO$_4^{3-}$-P in the medium was only observed on day 7 (Figure 4.6), suggesting that at this stage the PAOs were no longer able to actively take up P, possibly because of exhaustion of carbon storage polymers. This was substantiated by the cyclic studies (Figure 4.4), in which the biofilm was shown to have limited ability to uptake P. Interestingly, the biofilm was still able to release PO$_4^{3-}$ during anaerobic exposure to acetate, and the P$_{re}/P_{upt}$ ratio was almost 3-fold higher than on day 0 (Table 4.1). This confirms the release of P from biomass decay, and its uptake by PAOs during the P- and C-starvation period.

![Figure 4.6](image)

**Figure 4.6** Concentration profiles for dissolved P-PO$_4^{3-}$, N-NO$_3^-$ and N-NH$_4^+$ in the master reactor during 7 days of P- and C-starvation (pH 6.82–8.30).

The evidence of PAOs taking up P (released from biomass decay) was further supported by the activity decay rates of the biofilm (based on P release and uptake activities) (Table 4.2). A higher activity decay rate (0.283 d$^{-1}$; R$^2 = 0.971$) was found for P uptake than for P release (0.103 d$^{-1}$; R$^2 = 0.971$). The higher activity decay rate for P uptake was probably because of underestimation of P uptake. As noted above, P was released
from decaying biomass, but its uptake by PAOs was not considered in the calculation of decay rates. This might have contributed to the higher activity decay rate estimated for P uptake.

**Table 4.2** The aerobic activity decay rates of EBPR-r biofilm based on changes in the PURs and PRRs during the long-term P- and C-starvation test (0–7 days).

<table>
<thead>
<tr>
<th>EBPR-r stream</th>
<th>Rate</th>
<th>Aerobic activity decay rate (d⁻¹)</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wastewater stream</td>
<td>P uptake</td>
<td>0.283</td>
<td>0.971</td>
</tr>
<tr>
<td>Recovery stream</td>
<td>P release</td>
<td>0.103</td>
<td>0.958</td>
</tr>
</tbody>
</table>

Nevertheless, PAOs in the EBPR-r biofilm were able to retain 50% of their P release activity even after 7 days of exposure to starvation condition. The 50% decline in P release activity could have been a result of biomass decay and the oxidation of carbon storage polymers to fulfil maintenance energy requirements. In summary, it was concluded that when P is in limited supply, PAOs are able to remain active and viable for extended periods of time. This observation is of major significance to the EBPR-r process, and also points to a strategy that could be used for bioaugmentation of PAOs.

### 4.5. Conclusions

- This study is the first to demonstrate the ability of PAOs to conserve their storage-driven P uptake activities when exposed to electron acceptor concentrations greater than those that stoichiometrically required to uptake P;
- Approximately 79% of the P uptake activity of PAOs and 95% of their P release activity was conserved 2 days after exposure to P- and C-deficient conditions and a highly oxidised environment;
• Approximately 15% of the P uptake activity of PAOs and 50% of their P release activity was conserved even after 7 days under P- and C-starvation conditions in a highly oxidising environment.
5. Phosphorus Recovery from Wastewater Using an EBPR-r Approach: Optimising Carbon Usage for P-recovery

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5.1. Abstract

Enhanced biological phosphorus removal and recovery (EBPR-r) is a biofilm process that makes use of polyphosphate accumulating organisms (PAOs) to remove and recover phosphorus (P) from wastewater into a separate recovery stream. The original process was inefficient as indicated by the low P-release to carbon (C)-uptake (P_{rel}/C_{upt}) molar ratio of the biofilm. To enable more efficient use of C for P recovery, this study aimed to optimise the P_{rel}/C_{upt} ratio by developing strategies that are readily implementable in operation of the EBPR-r process. An experimental EBPR-r reactor was operated in four different modes over a period of 450 days. During stages I to III, the wastewater (8 mg-P/L and 10 mg-N/L) hydraulic loading was increased (7.2 >14.4 > 21.6 L); and during stage IV, the P uptake duration was extended (4 h to 10 h). With an
unchanged supply of carbon in the recovery stream (1.8 L with 350 mg/L acetate), a stepwise increase of wastewater volume from 7.2 (stage I) to 14.4 (stage II) and 21.6 L (stage III) resulted in a 43% increase of the $P_{\text{rel}}/C_{\text{upt}}$ molar ratio (0.07, to 0.80 and 0.10, respectively). In contrast, an increase in the duration of the P uptake period from 4 h (stage III) to 10 h during stage IV increased the $P_{\text{rel}}/C_{\text{upt}}$ ratio by 150% (from 0.10 to 0.25). With this $P_{\text{rel}}/C_{\text{upt}}$ ratio, a 10-fold increase in the P concentration (from 8 mg-P/L in wastewater to >90 mg-P/L in recovery stream) could be achieved in a single P-capture and P-release cycle. Bacterial community analysis using 454 pyrosequencing and canonical correspondence analysis revealed an increase in the abundance of PAOs ("Ca. Accumulibacter" Clade IIA), and decreases in the occurrence of glycogen accumulating organisms (GAOs) (family Sinobacteraceae), denitrifiers (family Comamonadaceae) and denitrifying PAOs ("Ca. Accumulibacter" Clade IA). The decrease in denitrifying bacteria was corroborated with detection of a significant decline in the activity of denitrifying activity from stage I to IV (a 5-fold decline in the $N_{\text{den}}/P_{\text{upt}}$ ratio). Overall, a strategy to facilitate more efficient use of carbon in the EBPR-r process was validated (representing a 3-fold carbon saving). However, future studies to develop strategies to improve denitrification in the EBPR-r process are required.

### 5.2. Introduction

Recycling of phosphorus (P) is essential because P is a non-renewable resource (Rittmann et al., 2011). One potential source of P for recovery is municipal wastewater. However, municipal wastewater typically contains only 7–10 mg-P/L, making P recovery from this source challenging (Parsons & Smith, 2008). For P recovery to be chemically and economically viable, a wastewater stream having a P concentration of >50 mg-P/L is generally required (Cornel & Schaum, 2009).
To address this challenge, several approaches based on enhanced biological phosphorus removal (EBPR) have recently been developed (Acevedo et al., 2015; Kodera et al., 2013; Valverde-Pérez et al., 2015; Wong et al., 2013; Xia et al., 2014). Among these, a post-denitrification process termed enhanced biological phosphorus removal and recovery (EBPR-r) was proposed by Wong et al. (2013) to facilitate nitrogen (N) removal and P recovery from wastewater. This two-step process involves the use of a biofilm containing polyphosphate accumulating organisms (PAOs). The first step facilitates storage-driven denitrification and P uptake by PAOs from wastewater. The second step involves exposure of the PAOs biofilm to an anaerobic environment to facilitate replenishment of carbon reserves (via acetate uptake), and release of the stored P into a separate recovery stream. As the volume of the recovery stream is only a small fraction of the volume of the wastewater stream, P is both recovered and concentrated into this separate stream.

Wong et al. (2013) reported that an EBPR-r reactor having a wastewater:recovery stream volumetric ratio of 4:1 was able to achieve a 4-fold concentration of P, from 8 mg-P/L in the wastewater stream (7.2 L) to 28 mg-P/L in the recovery stream (1.8 L). Moreover, by repeated release of P into the same recovery stream, a final P concentration of 100 mg-P/L was achieved in the recovery stream (Wong et al., 2013). However, this mode of operation resulted in a $P_{\text{mol}}/C_{\text{upt}}$ ratio (the amount of P released per carbon substrate taken up by PAOs under anaerobic conditions) of only 0.08 mol-P/mol-C, which was substantially lower than the 0.50–0.75 value typically reported for PAOs biomass in conventional EBPR reactors (Filipe et al., 2001; Lopez-Vazquez et al., 2007). This low ratio implies that a large portion of the consumed carbon could be used for processes not necessarily involving P recovery; for example, uptake by glycogen accumulating organisms (GAOs) (Bond et al., 1995). As carbon addition
represents a substantial operational cost, carbon use in processes not involving P recovery should be minimised. Thus, a strategy to improve the $P_{rel}/C_{upt}$ ratio of EBPR-r biofilm was warranted.

Optimisation of the $P_{rel}/C_{upt}$ ratio has been extensively studied in conventional EBPR processes. Several factors are known to favour the growth of PAOs over GAOs, contributing to an increase in the $P_{rel}/C_{upt}$ ratio. These include pH (>7.25), temperature (<25°C), the organic carbon:P ratio in the wastewater influent (10–25 mg-COD/mg-P), the type of carbon source (propionate), and the mode of carbon feeding (slow feeding rate) (Oehmen et al., 2007; Tu & Schuler, 2013). For instance, Oehmen et al. (2006) reported a higher $P_{rel}/C_{upt}$ ratio (0.30–0.45) in a propionate-fed EBPR reactor, while in an acetate-fed reactor the $P_{rel}/C_{upt}$ ratio decreased from 0.40 to 0.05 after 120 days of operation. In addition, Tu and Schuler (2013) reported an 11-fold increase in the $P_{rel}/C_{upt}$ ratio (from 0.05 to 0.55) when the carbon feeding rate was decreased from 1200 mg/L.h (over 10 min) to 100 mg/L.h (over 120 min).

Increasing the P-loading (by increasing the P concentration) has also been reported to result in an improved $P_{rel}/C_{upt}$ ratio (Choi et al., 2011a; Converti et al., 1993; Liu et al., 1997; Panswad et al., 2007). Choi et al. (2011a) reported an increase in the $P_{rel}/C_{upt}$ ratio from 0.01 to 0.02 with an increase in the P concentration in wastewater from 20 to 80 mg-P/L. Panswad et al. (2007) showed a similar trend of increase in the $P_{rel}/C_{upt}$ ratio (from 0.07 to 0.13) with an increase in the P concentration from 6 to 14.4 mg-P/L. However, increasing the P concentration is impractical for the EBPR-r process because municipal wastewater is used as the process influent. An alternative approach to achieving a higher P-loading is to increase the hydraulic loading. For example, P-loading in the EBPR-r process could be doubled if the volumetric ratio (4:1) of the wastewater to the recovery stream were increased to 8:1. As an increase in the P-loading
does not change the P concentration in the wastewater, it is unclear whether increasing hydraulic loading would enhance the \( \frac{P_{rel}}{C_{upt}} \) ratio of the EBPR-r biofilm. To achieve a higher \( \frac{P_{rel}}{C_{upt}} \) ratio, an increase in P uptake by the biofilm is required at higher hydraulic loadings. In the event that an increase in P uptake rate is not achievable, the duration of P uptake could be increased to facilitate additional uptake of P (hence achieving a higher \( \frac{P_{rel}}{C_{upt}} \) ratio). The impact of an increase of P-loading and the duration of P uptake on the \( \frac{P_{rel}}{C_{upt}} \) ratio is yet to be investigated, but is of particular relevance to optimisation of the EBPR-r process. Accordingly, to enable optimisation of the EBPR-r process the aim of this study was to assess the effect of increasing the hydraulic loading and period of P uptake on the \( \frac{P_{rel}}{C_{upt}} \) ratio. Additionally, bacterial community changes between stages were also examined using 454 pyrosequencing of the 16S rRNA genes.

5.3. Materials and methods

5.3.1. Wastewater stream and P recovery stream

Both the wastewater and recovery streams contained a standard growth medium consisting of (per L): 39 mg MgSO\(_4\), 20 mg CaCl\(_2\cdot2\)H\(_2\)O, 11 mg NH\(_4\)Cl (3 mg/L NH\(_4^+\)-N), 200 mg NaHCO\(_3\) and 0.3 mL of a nutrient solution. The nutrient solution contained (per L) 1.5 g FeCl\(_3\cdot6\)H\(_2\)O, 0.15 g H\(_3\)BO\(_3\), 0.03 g CuSO\(_4\cdot5\)H\(_2\)O, 0.18 g KI, 0.12 g MnCl\(_2\cdot4\)H\(_2\)O, 0.06 g Na\(_2\)MoO\(_4\cdot2\)H\(_2\)O, 0.12 g ZnSO\(_4\cdot7\)H\(_2\)O, 0.15 g CoCl\(_2\cdot6\)H\(_2\)O and 10 g EDTA. The wastewater stream contained 8 mg-P/L of phosphate (supplemented as 1 M phosphate buffer: 46 g KH\(_2\)PO\(_4\) and 115 g K\(_2\)HPO\(_4\) per L) and 10 mg-N/L of nitrate (as sodium nitrate). In contrast the recovery stream contained 350 mg/L of acetate (as sodium acetate). This carbon supply corresponded to 370 mg/L of chemical oxygen demand (COD). Concentrated stock solutions (15×) of these two streams were prepared
and the pH was adjusted to 7.0 ± 0.2 using 2 M HCl. Defined volumes of the stock solution and deionised (DI) water were simultaneously pumped into the reactor at the beginning of each phase to achieve the desired concentrations.

5.3.2. Reactor configuration, automated operation and online monitoring

A laboratory-scale reactor (the master reactor) was operated at 22 ± 2°C to enrich an EBPR-r biofilm, as described previously (Wong et al., 2013). In brief, 1000 biofilm carriers (Kaldnes® K1 polyethene) were equally distributed in the master reactor among eight adjoining stainless steel mesh compartments. Over a 6-h cycle the biofilm carriers were alternately exposed for 4 h to a wastewater stream (7.2 L, containing 8 mg-P/L PO\textsubscript{4}\textsuperscript{3−} and 10 mg-N/L NO\textsubscript{3}−) to enable P uptake, and for 2 h to a separate recovery stream (1.8 L, containing 350 mg/L acetate) to facilitate anaerobic P release. Peristaltic pumps (Masterflex®, USA) were used for recirculation and exchange of liquid. The operation of the reactor was automated using control devices and software (LabVIEW), and dissolved oxygen (DO), pH and redox potential (ORP) of bulk water were recorded online.

The master reactor was seeded using biomass from an another laboratory-scale EBPR-r reactor for which stable P recovery was previously reported (Wong et al., 2013). The master reactor was operated in four stages over 450 days (Table 5.1). The effect of increasing hydraulic loading was examined during the first three stages and the effect of increasing P uptake duration was investigated during the final stage. Different hydraulic loadings were achieved by increasing the volume of wastewater stream from 7.2 L (stage I) to 14.4 L (stage II) and to 21.6 L (stage III). A 4-h P uptake phase was maintained during the initial three stages. During stage IV the P uptake phase was increased from 4 h to 10 h, while maintaining the same volumetric loading used in stage
III. In all stages the amount of carbon supplied to the recovery stream (1.8 L with 350 mg/L acetate) and the duration of P release (2 h) were maintained constant. On achieving a stable operation at each stage, the $P_{rel}/C_{upt}$ ratio and the P and N removal efficiencies of the reactor were determined.

Table 5.1 Experimental settings for the EBPR-r reactor during the four stage operation.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Period</th>
<th>Vol ratio</th>
<th>Cycle length</th>
<th>P uptake phase (wastewater stream)</th>
<th>P release phase (recovery stream)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(d)</td>
<td>(WS:RS)</td>
<td>(h)</td>
<td>Vol (L)  Total $P_{in}$ (mg-P)</td>
<td>Total $N_{in}$ (mg-N)</td>
</tr>
<tr>
<td>I</td>
<td>0–120</td>
<td>4:1</td>
<td>6</td>
<td>7.2 57.6</td>
<td>72</td>
</tr>
<tr>
<td>II</td>
<td>120–316</td>
<td>8:1</td>
<td>6</td>
<td>14.4 115</td>
<td>144</td>
</tr>
<tr>
<td>III</td>
<td>316–360</td>
<td>12:1</td>
<td>6</td>
<td>21.6 173</td>
<td>216</td>
</tr>
<tr>
<td>IV</td>
<td>360–450</td>
<td>12:1</td>
<td>12</td>
<td>21.6 173</td>
<td>216</td>
</tr>
</tbody>
</table>

WS:RS = wastewater stream:recovery stream; $P_{in}$ = P-loading as $PO_4^{3-}$ in the wastewater influent; $N_{in}$ = N-loading as $NO_3^-$ in the wastewater influent.

5.3.3. Chemical analyses to examine the activity of the EBPR-r biofilm

A. Cyclic studies in the master reactor

The performance of the master reactor was assessed weekly by measuring the concentrations of soluble $PO_4^{3-}$-P and $NO_3^-$-N in the wastewater and recovery streams (both influent and effluent). When stable removal and recovery performances were observed, two cyclic studies were performed at least two weeks apart to quantify the steady state activity of the biofilm at each stage.
Each cyclic study involved withdrawing liquid samples from the master reactor every 15–30 min during the entire 6-h or 12-h cycle. Liquid samples were immediately filtered using a 0.22 µm pore size syringe filter (Acrodisc® PF, Pal Corporation, UK). The concentrations of soluble NO$_x$-N (NO$_2^-$-N + NO$_3^-$-N), PO$_4^{3-}$-P and acetate in the filtrates were determined using ion chromatography (ICS-3000, DIONEX). In all cyclic studies, N-Allythiourea (11.6 mg/L) was added to the wastewater stream to prevent aerobic nitrification during the P uptake phase (Ginestet et al., 1998).

**B. Batch test to assess the P and N removal activities**

As all four stages were operated differently (i.e. wastewater volume and P uptake duration), the P and N removal rates determined in the cyclic studies did not facilitate direct comparison of biofilm activity among the stages. To enable direct comparison a series of standardised batch tests was performed, as described previously (Wong et al., 2015).

Biofilm carriers (~330 carriers) removed at the end of the anaerobic phase were used in the batch tests. The carriers were placed in a column reactor (440 mL working volume) and exposed to a P-containing wastewater stream (2.4 L, 8 mg-P/L) for 4 h to facilitate storage-driven P uptake and denitrification. Three batch tests (supplied with different electron acceptors) were performed in duplicate to assess the P and/or N removal activities of the biofilm: (1) oxygen (O$_2$) only (DO of 8 mg/L); (2) nitrate (NO$_3^-$) only with a NO$_3^-$-N concentration of 10 mg/L (with sparging by N$_2$ for 10 min to achieve anoxic conditions); and (3) O$_2$ and NO$_3^-$ (DO of 8 mg/L and a NO$_3^-$ of 10 mg-N/L).

During the 4-h batch tests, liquid samples were collected from the reactor every 15–45 min; each sample was immediately filtered using a 0.22 µm pore size syringe filter (Acrodisc® PF, Pal Corporation, UK). The concentrations of soluble NO$_2^-$-N, NO$_3^-$-N
and \( \text{PO}_4^{3-} \)-P in the filtrates were determined using ion chromatography. Changes in the \( \text{PO}_4^{3-} \)-P and \( \text{NO}_x \)-N (\( \text{NO}_2^- \)-N and \( \text{NO}_3^- \)-N) concentrations were plotted against time. The slopes of the steepest part of the resulting curves were recorded as the specific P uptake rate (PUR) and \( \text{NO}_x \) removal rate, respectively. These rates (expressed as mg/L.h) were normalised using the respective total solids (TS) concentrations, and expressed in mmol/g-TS.h. TS measurements were obtained by subtracting the dry weight of 30 biofilm-free carriers from the dry weight of 30 biofilm-containing carriers, determined following drying at 60°C overnight (Wong et al., 2013).

5.3.4. Bacterial community characterisation

A. DNA extraction, PCR amplification and pyrosequencing

To investigate bacterial community changes among stages, 13 biofilm samples (3 from stage I, 2 from stage II, 4 from stage III and 4 from stage IV) representing different times points during each stage were taken and analysed using 454 pyrosequencing of the 16S rRNA genes. The biofilm was physically removed by gentle sonication of carriers in growth medium for 1 min. The biofilm DNA was subsequently extracted using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc.), as per the manufacturer’s protocols. The extracted DNA was visualised using electrophoresis on a 1% (w/v) agarose gel and quantified using a fluorometer (Qubit ® 2.0, Life Technologies). The samples were then stored at \(-20°C\) until shipment for sequencing. To enable shipment at room temperature, the DNA samples were stabilised using DNAstable Plus (Biometrica, supplied by Diagnostic Technology). The stable DNA samples were then couriered to an external laboratory (MR DNA, Molecular Research LP, Texas, USA) for 454 pyrosequencing of the 16S rRNA genes, using methods described previously by Dowd et al. (2008). In brief, the universal bacterial 16S rRNA
gene primers 27F (5′-AGRGTTTGATCMTGGCTCAG-3′) and 530R (5′-CCGCNGCNGCTGGCAC-3′) were used with the HotStart Taq Plus Master Mix (Qiagen, CA, USA) in a single-step 30 cycle PCR amplification. The thermocycler conditions included an initial denaturing step at 94 °C for 3 min followed by 28 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 40 s, elongation at 72 °C for 1 min, and a final elongation step at 72 °C for 5 min. Amplicon products were diluted to equal concentrations, and purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA, USA). Sequencing was carried out utilising a Roche 454 FLX titanium instrument and reagents.

B. Post-sequence analysis

Following sequencing, post-sequence processing was carried out using the QIIME (Quantitative Insights Into Microbial Ecology) software package (http://www.qiime.org). The split_libraries.py script was used to extract sequences that were relevant to this study. Default arguments were used, with the exception of the maximum sequence length, which was set at 600 bp, because of the use of the 27F and 530R primers for the PCR amplification. Subsequently, the pick_otus.py script (usearch method) was used to group sequences that shared 97% sequence similarity. Groups with a minimum number of 10 sequences were defined as operational taxonomic units (OTUs). Thereafter, a representative sequence from each OTU was selected and aligned (PyNAST method) against the Greengenes imputed core reference alignment using the align_seqs.py script. The script filter_alignment.py was then used to remove gaps, and a taxonomy assignment (using script assign_taxonomy.py) was carried out at a minimum confidence level of 0.8 using a Ribosomal Database Project (RDP) classifier and Greengenes OTUs dataset. The unprocessed DNA sequences have been deposited in the
NCBI (National Centre for Biotechnology) short reads archive database (accession number: SRP061604).

To investigate community changes as a result of process changes, two analyses were performed using PAST (version 2015) (McLellan et al., 2010). (1) An OTU-based cluster analysis (CA) and a principle coordinate analysis (PCoA) were performed to reveal the similarity of bacteria among samples. (2) A canonical correspondence analysis (CCA) was performed to assess the correlations between bacterial communities (abundance of families or OTUs) and the operating parameters (wastewater volumetric loading, P uptake duration, N_{den}/P_{upt} ratio and P_{rel}/C_{upt} ratios).

5.4. Results and Discussion

5.4.1. A 3-fold increase of P-loading resulted in a marginal increase in the

\[ P_{rel}/C_{upt} \text{ ratio} \]

Increasing the volume of the wastewater stream from 7.2 L (stage I) to 14.4 L (stage II) and 21.6 L (stage III) gradually increased the P concentration in the recovery stream from 23 to 29 and 39 mg-P/L, respectively (Figure 5.1C and 5.2A–C). However, only a marginal increase in the \[ P_{rel}/C_{upt} \] molar ratio was observed (from 0.07 to 0.80 and 0.10, respectively) (Table 5.2). This implied that only a marginal improvement in the P uptake activity of the biofilm was achieved with an increase in the P-loading (3× larger wastewater volume). When the PURs of the biofilm were normalised against biomass concentrations, similar specific PURs (0.57 ± 0.05 mg-P/g-TS.h; Figure 5.3B) were observed for the three stages (I to III). The results of the separate batch tests also confirmed that the biofilms of stages I–III had similar P uptake activities (0.492–0.559 mg-P/g-TS.h; Figure 5.3B). As the increased P-loading (via larger volume) did not
increase the specific PURs of the biofilm, a gradual decrease in the volumetric PURs of the biofilm were recorded (1.51, 0.88 and 0.71 mg-P/L.h in stages I–III, respectively; Figure 5.3A). This also led to an increase in the PO$_4^{3-}$ concentration in the wastewater effluent (3.6, 4.8 and 6.5 mg-P/L in stages I–III, respectively; Figure 5.1A), resulting in poor P removal efficiencies (60, 42 and 31% in stages I–III, respectively; Table 5.2).

Figure 5.1 Nutrient concentrations and removal efficiencies in the influent and effluent during EPBR-r operation. (A) Soluble P-PO$_4^{3-}$ and the efficiency of P removal from the wastewater stream. (B) N-NO$_3^-$ and the efficiency of N removal from the wastewater stream. (C) Soluble P-PO$_4^{3-}$ in the recovery stream over the four operational stages.
Figure 5.2 Left: the concentration profiles for soluble P-PO$_4^{3-}$, N-NO$_x$ and acetate in the cyclic studies during (A) stage I, (B) stage II, (C) stage III and (D) stage IV. Right: the profiles of pH, oxidation reduction potential (redox) and dissolved oxygen concentration (DO) in the cyclic studies during (E) stage I, (F) stage II, (G) stage III, and (H) stage IV.

Table 5.2 Summary results for the cyclic studies performed during stages I–IV. Results are presented as value ± standard deviation, based on two cyclic studies.

<table>
<thead>
<tr>
<th>Stage</th>
<th>P removal efficiency (%)</th>
<th>N removal efficiency (%)</th>
<th>P$<em>{upt}$/P$</em>{rel}$ (%)</th>
<th>N$<em>{den}$/P$</em>{upt}$ ratio (mol-N/mol-P)</th>
<th>P$<em>{rel}$/C$</em>{upt}$ ratio (mol-P/mol-C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>60</td>
<td>65</td>
<td>108</td>
<td>2.39</td>
<td>0.07</td>
</tr>
<tr>
<td>II</td>
<td>42 ± 4</td>
<td>31 ± 1</td>
<td>105 ± 0.7</td>
<td>1.76 ± 0.06</td>
<td>0.08 ± 0.002</td>
</tr>
<tr>
<td>III</td>
<td>31 ± 4</td>
<td>14 ± 3</td>
<td>111 ± 22</td>
<td>1.09 ± 0.45</td>
<td>0.10 ± 0.002</td>
</tr>
<tr>
<td>IV</td>
<td>79 ± 6</td>
<td>18 ± 4</td>
<td>104 ± 19</td>
<td>0.47 ± 0.17</td>
<td>0.25 ± 0.02</td>
</tr>
</tbody>
</table>
Figure 5.3 (A) The volumetric PUR and the NO\textsubscript{x} removal rate of the EBPR-r biofilm in stages I–IV during the cyclic studies. (B) The specific PUR and (C) the NO\textsubscript{x} removal rate for the EBPR-r biofilm during stages I–IV, obtained from both the cyclic studies and the batch test. In the batch test the biofilm was supplied with three types of electron acceptor: O\textsubscript{2} alone; O\textsubscript{2} + NO\textsubscript{3}\textsuperscript{−}; and NO\textsubscript{3}\textsuperscript{−} alone.

5.4.2. CA and PCoA reveal a change in the bacterial communities, possibly reflecting decreased denitrification

Analysis of the chemical parameters suggested that an increase in the hydraulic loading (stages I–III) had little impact on the P uptake activity of the biofilm. Accordingly, little change in the structure of the biofilm microbial community was anticipated during these stages. However, the community analysis revealed otherwise.
The CA and PCoA were carried out to quantify statistically the compositional dissimilarities (Bray-Curtis dissimilarity) among the DNA sequences obtained between stages I and III. At the family and OTU levels (Figure 5.4), a gradual shift in the bacterial communities was observed from stage I to stage III. Specifically, samples collected during stages I (Group 1) and III (Group 2) clustered as two separate groups (Figure 5.4). Interestingly, the two samples taken from stage II (II.d192 and II.d284) did not cluster together. Rather, one taken during the acclimation period (II.d192) showed a higher similarity to Group 1, while the other taken during steady state operation (II.d284) clustered into Group 2. These grouping patterns suggested a gradual change of the bacterial community from stage I to III, with stage II resembling a transition period.

One factor that could have contributed to the change in bacterial community structure is a decrease of the abundance of denitrifiers. Cyclic studies revealed a decrease in the volumetric (1.42, 0.66 and 0.31 mg-N/L.h during stages I–III, respectively; Figure 5.3A) and specific (0.52, 0.39 and 0.27 mg-N/g-TS.h during stages I–III, respectively; Figure 5.3C) NOx removal rates by the biofilm. Corresponding to the decrease in the NOx removal rate, the N_{den}/P_{upt} molar ratio decreased by more than 50%, from 2.38 in stage I to only 0.99 in stage III (Table 5.2). Such a significant decrease in the N_{den}/P_{upt} ratio implies that the denitrifying activity of the EBPR-r biofilm was impaired as a result of the increased hydraulic loading. The decrease in denitrification from stage I to III was confirmed by the batch tests (Figure 5.3C). Specifically, in the presence of the electron acceptors O2 and NO3− there was a 34% reduction in denitrification (0.36 to 0.24 mg-N/g-TS.h), and in the presence of NO3− alone there was a 23% reduction (from 0.56 to 0.43 mg-N/g-TS.h) in the NOx removal rate. This decline in denitrification corresponded to the reduction in the abundance of denitrifiers.
Figure 5.4 Cluster analysis (CA) and principal coordinate analysis (PCoA) based on Bray-Curtis distances for all 13 samples at (A and C) the family level, and (B and D) the OTU level. Samples sharing similarities are grouped together (labeled as 1–3 in the square boxes).

5.4.3. Increasing the duration of the P uptake phase facilitated a 3-fold increase in the $P_{rel}/C_{upt}$ ratio

To further reduce the P and N concentrations in the effluent, the duration of the P uptake phase was extended from 4 h in stage III to 10 h in stage IV. This resulted in a slight increase in the N removal efficiency (from 14 ± 3% to 18 ± 4%) and a 2.5-fold increase in the P removal efficiency (from 31 ± 4% to 79 ± 6%, Table 5.2).
Accordingly, the amount of P removed from wastewater increased dramatically, and a P concentration of 90 mg/L in the recovery stream was achieved (Figures 1C and 2D).

As the supply of external carbon (i.e. acetate) was maintained constant throughout the study, the increase in P concentration (90 mg-P/L) in the recovery stream signifies efficient use of the carbon for P recovery. Notably, a 3-fold increase in the \( \frac{P_{rel}}{C_{upt}} \) molar ratio was observed, from 0.07 in stage I to 0.25 in stage IV (Table 5.2). The fact that no additional carbon was required to facilitate a 3-fold increase in P recovery suggests there was a diversion of carbon from GAOS to PAOS (increasing abundance of PAOs), or enlargement of the PHA pools in existing PAOs. The extension of P uptake phase could enable PAOs to efficiently use their internal carbon storage (which could be conserved for P uptake, as shown in Chapter 4) to facilitate a higher amount of P uptake from wastewater. It was also possible that a longer exposure of biofilm to an oxidising condition might have inhibited the growth of denitrifiers (DO inhibits denitrification), and thus favoured the growth of PAOs. In summary, the 3-fold increase in the \( \frac{P_{rel}}{C_{upt}} \) ratio enabled a 3-fold reduction in external carbon demand for P recovery.

5.4.4. **A sufficient contact time was critical to achieve good P recovery when the specific P uptake kinetics of the biofilm remained unchanged**

Although a significant improvement was obtained in terms of the \( \frac{P_{rel}}{C_{upt}} \) ratio, extending the duration of P uptake did not enhance the kinetics of P uptake. Cyclic studies in stages III and IV revealed similar volumetric (0.71 and 0.73 mg-P/L.h; Figure 5.3A) and specific (0.63 and 0.57 mg-P/g-TS.h; Fig 3B) PURs. This was confirmed in separate batch tests (Figure 5.3B), where similar PURs were observed when O\(_2\) was the sole electron acceptor (0.49 and 0.49 mg-P/g-TS.h, for stages III–IV, respectively), and
when O$_2$ and NO$_3^-$ were electron acceptors (0.55 and 0.50 mg-P/g-TS.h, for stages III–IV, respectively). As the strategy adopted in stages I–III (increasing hydraulic loading) did not result in an improvement in the P uptake kinetics (PUR) of the biofilm, the only efficacious way to optimise the P recovery process (P$_{rel}$/C$_{upt}$ ratio) was by extending the time of contact of the EBPR-r biofilm with the wastewater (stage IV). Thereby, the biofilm could capture more P from the wastewater (PUR unchanged, but a longer period for P uptake), and thus achieve a higher P$_{rel}$/C$_{upt}$ ratio. To maximise the overall efficiency of this P recovery process, developing strategies to increase the P uptake kinetics (a higher PUR) of the EBPR-r biofilm is essential.

5.4.5. Canonical correspondence analysis revealed the bacterial communities responded to changes in process parameters

The change in the bacterial community composition (at class level) over the entire period of experiment is shown in Figure 5.5. The most abundant class in all 13 samples was the β-Proteobacteria (15.3–45.5%), followed by SJA-28 (6.2–36.0%), Anaerolineae (12.6–30.0%), α-Proteobacteria (3.1–9.8%), Sphingobacteria (2.3–7.7%) and γ-Proteobacteria (1.8–8.9%).

To assess whether any group of bacteria (the relative abundance at a family level) showed a relationship with operational parameters, a CCA was performed covering stages I–IV (Figure 5.6A). As expected, the CCA biplot revealed a positive correlation (a trend of increase) between the P$_{rel}$/C$_{upt}$ ratio and the operational parameters (wastewater volumetric loading and P uptake duration), and a negative correlation (a trend of decrease) between the N$_{den}$/P$_{upt}$ ratio and the same operational parameters. As more than 97% of the total bacterial community could be explained by the primary and secondary ordination axes of the CCA plot (Razaviarani & Buchanan, 2015), the
variables \( P_{rel} / C_{upt} \) ratio, \( N_{den} / P_{upt} \) ratio, volumetric loading and P uptake duration (Figure 5.6A) appeared to significantly contribute to the changes that occurred in the microbial community in EBPR-r biofilm during the entire experiment.

![Figure 5.5 Abundances of various bacterial classes in the 13 samples collected from the EBPR-r reactor during optimisation. The sample identifiers in the legend comprise the operational stage (I–IV) followed by the day that the biomass was collected (day 0–440).](image-url)

A. **At the family level the CCA revealed a decrease in the abundance of denitrifiers and GAOs**

A strong correlation between the family *Comamonadaceae* and the \( N_{den} / P_{upt} \) ratio (green line in Figure 5.6) was evident in the CCA biplot (Figure 5.6A). Members of this family are known to be capable of performing denitrification (Heylen et al., 2006). Thus, the decline in their abundance was consistent with the chemical data (\( N_{den} / P_{upt} \) ratio), which suggested a decrease in denitrifying activity from stage I to stage IV (\( N_{den} / P_{upt} \) ratio: 2.39, 1.76, 1.09 and 0.437 during stages I–IV, respectively; Table 5.2). The family *Nitrospiraceae* also showed a strong correlation with the \( N_{den} / P_{upt} \) ratio (green line in
Figure 5.6A). One member of this family, the genus *Nitrospira*, is widely known for its ability to oxidise nitrite (Hovanec et al., 1998). During the P uptake phase in the EPBR-r process, accumulation of nitrite was commonly observed (Wong et al., 2015). It is likely some of the nitrite was re-oxidised to NO$_3^-$ by *Nitrospira* in the presence of both O$_2$ and NO$_3^-$; During stages I–IV (when denitrification was suppressed) nitrite accumulation was limited (data not shown), and this could have contributed to a reduction in the abundance of *Nitrospira*.

**Figure 5.6** (A) Canonical correspondence analysis (CCA) of the bacterial abundance and chemical data at the family level. The dots represent bacterial species and the green lines represent quantitative variables (the operating parameters: wastewater volumetric loading, P uptake duration, N$_{\text{den}}$/P$_{\text{upt}}$ ratio and P$_{\text{rel}}$/C$_{\text{upt}}$ ratios). For those sequences where a family name was not available during post-sequence analysis, the class (C1–3) or order (O1–14) names are presented. Species (dots) located near center of the plot indicates their abundance was relatively unaffected by the operating parameters (green lines), while dots located further away from the center and close to the green lines indicated their positive correlation with the operating parameters (i.e. the abundance of *Comamonadaceae* decreased in a relation to the decrease of the N$_{\text{den}}$/P$_{\text{upt}}$ ratio from stage I to IV).
Figure 5.6 (B) Canonical correspondence analysis (CCA) of the bacterial abundance and chemical data at the OTU level (within the family *Rhodocyclaceae*).

Analysis of sequences (1.0–5.2% abundance) classified in the family *Sinobacteraceae* (in class \(\gamma\)-Proteobacteria) using the NCBI Basic Local Alignment Search Tool (BLAST) showed a high level of sequence similarity to the GAO *Competibacter* (accession number: JQ726379; similarity: 89–93%). CCA revealed a negative correlation between the family *Sinobacteraceae* and the \(P_{\text{rel}}/C_{\text{upt}}\) ratio, indicating a decrease in their abundance from stage I to stage IV (Figure 5.6A). This is consistent with previous studies of the conventional EBPR process, where an improvement in the \(P_{\text{rel}}/C_{\text{upt}}\) ratio resulted in a reduction in the population of GAOs (Muszynski et al., 2013; Oehmen et al., 2005). Interestingly, the families *Chitinophagaceae* and *Opitutaceae* showed a strong positive correlation with the \(P_{\text{rel}}/C_{\text{upt}}\) ratio. Although bacteria in these families have not been reported to be PAOs, the increase in their population implies that
member of these families may have played an important role in the EBPR-\(r\) process, facilitating a higher \(P_{rel}/C_{upt}\) ratio.

**B. The CCA revealed a decrease in “Accumulibacter” Clade IA and an increase in Clade IIA**

It is widely known that the one group of PAOs, “*Candidatus Accumulibacter*” (hereafter referred to as “Ca. Accumulibacter”) is a member of the family *Rhodocyclaceae*, in the class \(\beta\)-Proteobacteria (Hesselmann et al., 1999). In this study, *Rhodocyclaceae* was located near the origin of the CCA biplot (Figure 5.6A). While this could imply no correlation (positive or negative) between *Rhodocyclaceae* and the \(P_{rel}/C_{upt}\) ratio, such a result is also feasible if changes were at the genus or species levels, and not at the family level. Hence, another CCA was performed using all *Rhodocyclaceae* OTUs (those having 97% similarity and an abundance of >0.3%) to determine if there was any correlation at the genus or species levels (Figure 5.6B). This showed a clustering of OTUs into four groups (\(a–d\); Figure 5.6B). Interestingly, some groups were correlated positively to the key process variables, and the others negatively, so explaining observations made at the family level. Specifically, groups \(a\) and \(b\) showed a negative correlation with the \(P_{rel}/C_{upt}\) ratio and a positive correlation with the \(N_{den}/P_{upt}\) ratio, suggesting a decreasing abundance of these OTUs between stages I and IV. When these OTUs were analysed using NCBI BLAST (Table 5.3), they were found to be closely related to *Azospira*, *Sulfuritalea*, *Dechloromonas*, *Zoogloea*, “Ca. Accumulibacter” SG 1 (Clade 1A) and *Propionivibrio*. Among these, *Azospira*, *Dechloromonas* and *Zoogloea* are known denitrifiers (Heylen et al., 2006), and “Ca. Accumulibacter” Clade IA (Accession JQ726371) represents a group of denitrifying PAOs (DPAOs) capable of using \(NO_3^{-}\) as a final electron acceptor for P uptake (Flowers et al., 2009). This observation suggests that denitrification was a combined
result of DPAOs and other denitrifiers, and a decline in the abundance of both these groups correspond to a decline in denitrification in the reactor. The decrease in the abundance of DPAOs was consistent with the chemical data (batch test), which also indicated a decrease in denitrification from stage I to stage IV (Figure 5.3C). Specifically, corresponding to a decline in the NO$_x$ removal rate (a 56% reduction from stage I to stage IV), a 50% decline in the specific anoxic PUR was observed. This implies a decline of denitrifiers including DPAOs from stage I to stage IV.

C. The population of aerobic PAOs increased from stage I to stage IV

It has been demonstrated that “Ca. Accumulibacter” Clade IA are able to use NO$_3^-$ and O$_2$ as electron acceptors to facilitate P uptake (Lanham et al., 2011). Hence, when exposed to both O$_2$ and NO$_3^-$, Clade IA (DPAOs) could use either O$_2$ or NO$_3^-$, while other aerobic PAOs (hereafter referred to as aerobic-PAOs) could use O$_2$ as a final electron acceptor for P uptake. Assuming an insignificant change in the abundance of aerobic-PAOs during stages I to IV, a decrease in the DPAOs activity (Clade IA) should be reflected in an overall reduction of the PUR from stage I to stage IV when the biofilm is exposed to both O$_2$ and NO$_3^-$. However, compared to the 50% reduction in PUR observed with NO$_3^-$ as the terminal electron acceptor, an insignificant change in PUR (0.56 ± 0.07 mg-P/g-TS.h; Figure 5.3B) was observed when a mixture of O$_2$ and NO$_3^-$ was supplied as final electron acceptors. This implies that the change of operational condition from stage I to IV resulted in the increase of aerobic-PAOs abundance in the biofilm. The CCA biplot also supports the chemical data, showing increasing abundance of groups $c$ and $d$ from stage I to IV (Figure 5.6B).

Both groups $c$ and $d$ showed positive correlations with the $P_{res}/C_{upt}$ ratio, suggesting an increase of their abundance from stage I to stage IV. Although group $c$ is yet to be
reported among PAOs, group d shares a high level of similarity (95−99%) with “Ca. Accumulibacter” Clade IIA (GeneBank accession HM046425; Table 5.3) (Kim et al., 2010). This group of PAOs (Clade IIA) are able to use O2 and NO−2 (produced by other denitrifiers) but not NO−3 as electron acceptors for P uptake (Kim et al., 2013). Overall, an increase in Clade IIA (aerobic-PAOs) and a decrease in Clade IA (DPAOs) could have resulted in no net change in the aerobic PURs observed in this study.

Table 5.3 Result of the CCA analysis for OTUs within the family Rhodocyclaceae.

<table>
<thead>
<tr>
<th>Group</th>
<th>OTUs (current study)</th>
<th>Total abundance (%)</th>
<th>Bacterial Genus</th>
<th>Accession number (GenBank)</th>
<th>Similarity (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>110, 410</td>
<td>0.8−4.2</td>
<td>Azospira</td>
<td>KJ486371</td>
<td>97</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>0.1−0.9</td>
<td>Sulfuritalea</td>
<td>JQ723633</td>
<td>97</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>42</td>
<td>0.0−1.0</td>
<td>Dechloromonas</td>
<td>EF632559</td>
<td>99</td>
<td>n.a.</td>
</tr>
<tr>
<td>b</td>
<td>22, 66, 105, 205, 359, 617</td>
<td>0.0−12.5</td>
<td>“Ca. Accumulibacter” SG 1 (Clade 1A)</td>
<td>JQ726367</td>
<td>96−99</td>
<td>(Kim et al., 2013)</td>
</tr>
<tr>
<td></td>
<td>12, 133, 178,</td>
<td>0.1−3.9</td>
<td>Zoogloea</td>
<td>KR706006</td>
<td>97−99</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>38, 183</td>
<td>0.0−1.3</td>
<td>Zoogloea</td>
<td>AB736233</td>
<td>97−99</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>349</td>
<td>0.0−0.5</td>
<td>Propionivibrio</td>
<td>NR_025455 (NCBI)</td>
<td>98</td>
<td>(Brune et al., 2002)</td>
</tr>
<tr>
<td>c</td>
<td>5, 58, 68, 275, 497, 610</td>
<td>0.3−12.8</td>
<td>“Ca. Accumulibacter” SG 4 (Clade IIA)</td>
<td>HM046424</td>
<td>94−99</td>
<td>(Kim et al., 2010)</td>
</tr>
<tr>
<td>d</td>
<td>4, 403, 542</td>
<td>0.2−8.6</td>
<td>Unclassified</td>
<td>GU483252 &amp; GU538294</td>
<td>94−99</td>
<td>(Kwon et al., 2010)</td>
</tr>
</tbody>
</table>
Our study showed that increased hydraulic loading and the duration of P uptake impaired the growth of denitrifiers. It has been shown that the length of aerobic/anoxic P uptake phase could exert a selective pressure on “Ca. Accumulibacter” populations (Clade II or I respectively) (Lanham et al., 2011). In addition, Wong et al. (2015) reported that the denitrifying activity of the EBPR-r biofilm was largely dependent on the dissolved oxygen (DO) concentration. Thus, the prolonged exposure of biofilm to a high DO environment could result in increased oxygen penetration into the biofilm thus inhibites the growth of denitrificers. Strategies to better manage the DO concentration in wastewater may enable higher denitrification rates and $P_{rel}/C_{upt}$ ratios to be achieved, but this will require further research.

5.5. Conclusions

In this study, practically implementable strategies to improve the $P_{rel}/C_{upt}$ ratio in the EBPR-r process were investigated. These included: (i) increasing the hydraulic loading to facilitate a non-P limiting environment in the wastewater (larger volume of wastewater loading); and (ii) extending the duration of the P uptake period to enable a larger amount of P to be taken up from the wastewater, because the P uptake rate of the biofilm was constant (a limiting factor).

- The increase in hydraulic loading only increased the $P_{rel}/C_{upt}$ ratio marginally (0.07–0.10).
- Extending the duration of the P uptake period enabled P to be concentrated by 10-fold, from 8 mg-P/L in the wastewater to 90 mg-P/L in the recovery stream.
- As a result, a 3-fold increase in the $P_{rel}/C_{upt}$ ratio was achieved from stage I to stage IV (0.07–0.25), indicating more efficient use of carbon for P recovery (3× carbon saving).
• Long-term operation (>400 days) resulted in a shift in the microbial community, specifically towards bacteria that remove P but not N.

• Corresponding to the improved \( \frac{P_{\text{rel}}}{C_{\text{upt}}} \) ratio, CCA indicated a decline in the abundance of GAOs (family Sinobacteraceae) (negative correlation with \( P_{\text{rel}}/C_{\text{upt}} \) ratio).

• The CCA revealed a positive correlation between the \( P_{\text{rel}}/C_{\text{upt}} \) ratio and an increase in the abundance of known PAOs (“\( Ca. \) Accumulibacter” Clade IIA), and other bacteria whose roles in the EBPR-r process are yet to be defined.

• Transition through the four operational stages corresponded to a significant decline in the denitrifying activity of the biofilm. The CCA indicated that a 5-fold decrease in the \( \frac{N_{\text{den}}}{P_{\text{upt}}} \) ratio corresponded to a decrease in the abundance of denitrifiers (e.g. family Comamonadaceae and the genera Azospira, Dechloromona and Zoogloea) and DPAOs (e.g. “\( Ca. \) Accumulibacter” Clade IA).
6. Enrichment of Anodophilic Nitrogen Fixing Bacteria in a Bioelectrochemical System

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6.1. Abstract

This study demonstrated the ability of a bio-anode to fix dinitrogen (N\textsubscript{2}), and confirmed that diazotrophs can be used to treat N-deficient wastewater in a bioelectrochemical system (BES). A two-compartment BES was fed a N-deficient medium containing glucose for >200 days. The average glucose and COD removal at an anodic potential of +200 mV vs. Ag/AgCl was 100\% and 76\%, respectively. Glucose removal occurred via fermentation under open circuit (OC), with acetate as the key byproduct. Closing circuit remarkably reduced acetate accumulation, suggesting the biofilm could oxidise acetate under N-deficient conditions. Nitrogen fixation required an anode and glucose; removing either reduced N\textsubscript{2} fixation significantly. This suggests that the diazotrophs utilised glucose directly at the anode or indirectly through syntrophic interaction of a N\textsubscript{2}-fixing fermenter and an anodophile. The enriched biofilm was dominated (68\%) by the genus \textit{Clostridium}, members of which are known to be electrochemically active and capable of fixing N\textsubscript{2}.
6.2. Introduction

Industrial wastewaters, including wastewater produced from pulp and paper industries, are carbon (C) rich but nitrogen (N) deficient (Pokhrel & Viraraghavan, 2004; Pratt et al., 2007). To enable efficient biological treatment, a C:N ratio of 100:5 in the raw influent is usually recommended (Peng et al., 2003; Slade et al., 2011). Hence, external supplementation of N (as ammonium or nitrate) is needed to treat N-deficient wastewater (Dennis et al., 2004). N supplementation incurs costs, and intense monitoring is required to prevent discharge of excess N to the environment (Gauthier et al., 2000).

As an alternative to supplementing N, the use of diazotrophic (N₂-fixing) bacteria has been proposed as a method for treating N-deficient wastewater in activated sludge systems (Gauthier et al., 2000; Pratt et al., 2007). N₂-fixing bacteria are capable of converting atmospheric nitrogen (N₂) to ammonia (NH₃) as a means of supplementing N requirements for growth (Nair, 2010). Biological N₂ fixation is catalysed by the nitrogenase enzyme complex, and the reduction of N₂ to NH₃ takes place according to Reaction 6.1 (Nair, 2010):

\[
N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16Pi \quad [6.1]
\]

Although N₂-fixing bacteria could be used in activated sludge processes to oxidise carbon in N-deficient wastewater, the widespread use of this approach has not been possible because nitrogenase is irreversibly inhibited by oxygen (O₂), and conventional activated sludge processes require aeration to facilitate oxidation of organic carbon (Nair, 2010). To prevent O₂ inhibition of nitrogenase, diazotrophs often secrete extracellular polymeric substances (EPS; also known as slime) to limit O₂ diffusion into cells (Nair, 2010). Excessive EPS production can cause sludge bulking, resulting in
poor solid/liquid separation and reduced effluent quality (Peng et al., 2003). Hence, the use of N₂-fixing activated sludge processes to oxidise carbon in N-deficient wastewater is problematic.

One approach to eliminating the negative impact of O₂ on N₂ fixation is to combine N₂-fixing microorganisms with bioelectrochemical systems (BESs). A typical BES consists of an anode and a cathode chamber (Logan et al., 2008). The anodic chamber facilitates the growth of microorganisms (anode respiring bacteria; ARB) under anaerobic conditions, using the electrode (anode) as the sole electron acceptor. If the ARB are diazotrophs, oxidation of N-deficient wastewater in the absence of O₂ becomes feasible because a solid electrode (not O₂) is the final electron acceptor. The anode potential regulates the thermodynamics (free energy change) of bacterial metabolism (Cheng et al., 2008). Therefore, N₂ fixation in diazotrophic ARB is likely to be regulated by the anodic potential. Consequently, inhibitory effects of O₂ on N₂ fixation may be eliminated because of the maintenance of anaerobic conditions in the anode chamber. The electrons donated by the ARB flow to the cathode via an external circuit, where they combine with protons and O₂ to form water (Logan et al., 2008).

Current knowledge of diazotrophic ARB (DARB) is limited. Although potential diazotrophs including *Azoarcus*, *Clostridium* and *Geobacter* have been reported in association with anodes of microbial fuel cells (MFCs) under N supplemented conditions (Kim et al., 2004; Phung et al., 2004), it is unclear whether these bacteria met their N requirements via fixation of atmospheric N₂. Belleville et al. (2011) and Clawaert et al. (2007) operated BESs to treat N-deficient wastewater, but did not provide direct experimental evidence of N₂ fixation, although they assumed that this was how the N requirements of the ARB were met, and did not investigate the bacterial diversity in their systems.
The objectives of this study were to: (1) investigate the efficiency of anodic oxidation of a N-deficient wastewater by an enriched microbial biofilm community; (2) elucidate the possible routes of glucose metabolism by the anodic biofilm; (3) assess the influence of anodic current production on N$_2$ fixation; and (4) characterise the enriched anodic bacterial community using 454 sequencing of the 16S rRNA genes.

6.3. Materials and Methods

6.3.1. Composition of the N-deficient medium

The synthetic N-deficient medium used in this study contained glucose as the sole source of carbon and energy, and represents wastewater characteristic of pulp and paper, and sugar refining industries. The medium contained (per liter of DI water): MgSO$_4$$
\cdot7H_2O$ (25 mg), CaCl$_2$$
\cdot2H_2O$ (25 mg), glucose monohydrate (374–1684 mg), KH$_2$PO$_4$ (2300 mg), K$_2$HPO$_4$ (5750 mg) and 0.40 mL of trace element solution. The trace element solution contained (per litre): nitrilotriacetic acid (5000 mg), H$_3$BO$_3$ (310 mg), FeSO$_4$$
\cdot7H_2O$ (267 mg), CoSO$_4$$
\cdot7H_2O$ (128 mg), CuSO$_4$$
\cdot5H_2O$ (11 mg), MnCl$_2$$
\cdot4H_2O$ (9.6 mg), Na$_2$MoO$_4$$
\cdot2H_2O$ (267 mg) and ZnSO$_4$$
\cdot7H_2O$ (128 mg). The addition of nitrilotriacetic acid (0.15 mg-N/L) resulted in a measurable dissolved organic N level of approximately 0.30 ± 0.14 mg/L in the anodic feed. The medium was continuously supplied to the anodic chamber of the BES at a flow rate of 0.30–1.20 mL/min. The cathodic medium was identical to the anolyte, with the exception of glucose. The catholyte was replaced biweekly to avoid accumulation of ionic species.

6.3.2. Construction and operation of the bioelectrochemical system

A two-chamber BES described by Cheng et al. (2012) was used to enrich an electrochemically active biofilm in the anodic chamber exposed to N-deficient
conditions. Figure 6.1 provides a schematic of the BES reactor setup. The BES reactor had an internal liquid volume of 120 mL (or 316 mL without graphite) (Cheng et al., 2008). The BES was continuously operated for more than 200 days. The anodic and cathodic chambers were filled with granular graphite as the electrode material. The electrode covered with the anodophilic biofilm is referred to as the working electrode, and the cathode is referred to as the counter electrode. An Ag/AgCl reference electrode (MF-2079 Bioanalytical Systems) was embedded among the graphite granules of the working electrode. The reference electrode was intermittently checked against a new reference electrode, and was found to remain functional throughout the experimental period. Anolyte (350 mL) and catholyte (2000 mL) were continuously recirculated through the anodic and cathodic chambers, respectively, at a flow rate of 160 mL/min using a peristaltic pump (Console drive, Cole-Parmer). The anodic chamber of the BES was inoculated with soil microorganisms. The inoculum was prepared by incubating approximately 200 g of soil (obtained from a local garden in Perth, Australia) in 800 mL of anolyte medium at 35°C overnight.

A LabVIEW (National Instruments) program was developed to continuously monitor and control the operation of the process. A constant potential of +200 mV was supplied to the working electrode using a potentiostat (VMP3, BioLogic), and this potential was maintained throughout the study unless noted otherwise. Every 30 min the headspace of the recirculation bottle that contained the anodic electrolyte was sparged with N₂ for 5 min. A gas bubbler (creating a one-way gas valve) was installed into the gas outlet of the recirculation bottle to prevent air intrusion into the BES anode during periods when N₂ was not being sparged. The feed was introduced and waste was withdrawn from the anodic chamber using a peristaltic pump (Masterflex L/S, Cole-Parmer), enabling continuous operation (flow rate of 1.2 mL/h and hydraulic retention time of 4.86 h). The
50 mM phosphate buffer in the influent was unable to maintain a stable pH, and consequently pH control was introduced. The pH was computer controlled at 6.68 ± 0.43 using 4 M NaOH and a peristaltic pump (0.81 mL/min; Masterflex C/L 200 rpm, Cole-Parmer). The anolyte was maintained at 43 ± 3°C throughout the study by recirculating it through a column surrounded by a water jacket. This operational temperature was selected to mimic the wastewater temperatures (30–60°C) used in the pulp and paper industry (Bajpai, 2011). A cleaning regime was also applied on a weekly basis to the BES anode compartment to minimise the retention of dead biomass. The cleaning protocol included gentle disturbance of the anodic biofilm using a 50 mL syringe (gentle liquid agitation by moving the syringe piston 10 times), and subsequent decanting and replacement of the anolyte.

**Figure 6.1** Schematic diagram of the two-chamber BES operated in continuous mode. RE = reference electrode; WE = working electrode; CE = counter electrode.
The anodic and cathodic potentials and the current (I) were continuously measured using a potentiostat (VMP3, BioLogic). All electrode potentials (mV) reported refer to measurements carried out against an Ag/AgCl reference electrode. Online monitoring of pH, temperature, and the oxidation-reduction potential (ORP) of the working electrolyte was carried out using in-line pH and ORP sensors (TPS Ltd. Co., Australia). All measurements were logged every 120 to 360 s using a LabVIEW program.

### 6.3.3. Experimentation

The established anodic biofilm (showing a stable current production) was examined for its (1) electrochemical properties, (2) N\textsubscript{2} fixation activities and (3) community compositions.

**A. Assessment of electrochemical properties through the application of various constant anodic potentials**

Using the potentiostat, an open circuit potential (OCP) and a range of constant potentials (−600 to +200 mV) were systematically applied to the BES anode between days 70 to 80. When a stable current was achieved at an applied potential, three samples of the anolyte were taken at least 9 h apart, and the next constant potential was then applied. The anolyte samples were immediately filtered using 0.22 μm pore size syringe filters (Acrodisc® PF, Pal Corporation, UK) and stored at −20°C until analysed for soluble volatile fatty acids (VFAs), chemical oxygen demand (COD) and glucose concentrations.

VFA measurements were carried out by the Animal Health Laboratories (WA, Australia) using a gas chromatograph (GC) with a flame ionisation detector (FID) (Agilent 6890 series). The GC-FID was equipped with a capillary column (HP-FFAP,
30 m × 0.53 mm × 1.0 m; Agilent). The operational temperatures of the oven, injection port and the detector were 100°C, 260°C and 265°C, respectively. The data were processed using ChemStation software (Agilent) and the following VFAs were quantified: acetic acid, propionic acid, iso-butyric acid, butyric acid, iso-valeric acid, valeric acid and caproic acid.

Soluble COD measurements were performed using HACH reagents (HACH, chemical oxygen demand reagent; cat no. TNT 821; method 8000, LR). Filtered samples were first digested using a thermostat reactor (DRB200, HACH), and the absorbance was measured at 420 nm using a spectrophotometer (GENESYS 20, Thermo Scientific). Analysis for glucose was carried out using a glucose (HK) assay kit (GAHK-50, Sigma), and absorbance measurements were made at 340 nm using the spectrophotometer described above.

**B. Assessment of N₂ fixation using the acetylene reduction assay**

To confirm that N-deficient conditions were maintained, total-N measurements were carried out on unfiltered samples (200 mL) of the influent and effluent of the BES anolyte. Measurements of the concentrations of soluble N, ammonium (NH₄⁺), nitrate (NO₃⁻) and nitrite (NO₂⁻) were carried out on filtered (0.22 μm pore size syringe filter) samples. N measurements were carried out by MPL Laboratories, WA, Australia. Total-N measurements were carried out using a discrete analyser (Konelab Aquakem, Thermo Scientific) using the persulphate method (based on the APHA 4500-N C) as described in the Standard Methods (American Public Health Association. et al., 1995). Soluble-N, NH₄⁺, NO₃⁻ and NO₂⁻ measurements were carried out using the discrete analyser using the persulfate (4500-N C), phenate (APHA 4500-NH₃⁺ F), automated hydrazine
reduction (APHA 4500-NO$_3^-$ H), and colorimetric methods (APHA 4500-NO$_2$ B) of the Standard Methods (American Public Health Association. et al., 1995), respectively.

The acetylene reduction assay (ARA) was used to make in situ measurements of the N$_2$-fixing activity of the anodophilic biofilm under three sets of conditions: (1) with glucose as the sole source of carbon and electrons, under closed circuit conditions; (2) with glucose as the sole source of carbon and electrons, under open circuit conditions; and (3) with acetate as the sole source of carbon and electrons, under closed circuit conditions. The BES was operated at steady state (steady current production) for three days under each set of conditions prior to undertaking the ARA. Glucose or acetate was used in equimolar concentration of COD. The ARAs were carried out in duplicate at least two weeks apart (i.e. during days 165 and 195 of reactor operation). During each experiment the headspace of the anodic chamber was first sparged with argon for 5 min, and then 35 mL of acetylene was introduced into the headspace using a 50 mL syringe. This resulted in an initial acetylene concentration of approximately 20% in the headspace. Changes to the gas composition in the anodic chamber headspace were monitored by withdrawing 5 mL of gas from the headspace every 1–2 h. Gas analyses were carried out using an Agilent 6890 GC fitted with a thermal conductivity detector (TCD), an air actuated heated (75°C) valve box (containing a 0.25 mL gas sampling loop), and a packed column (60/80 Carboxen-1000 SUPELCO®, 15’ × 1/8” SS). Helium was used as the carrier gas at a constant pressure of 40 psi, and the GC oven temperature program was from 35°C (held isothermal for 5 min) at 6°C/min to 225°C (held isothermal for 15 min). The data were processed using ChemStation software (Agilent). Nitrogenase activity (µmol/L.h) was expressed as µmol of ethylene (C$_2$H$_4$) formed per total volume of anodic chamber headspace per hour. The measurements reported are averages of two duplicate measurements.
6.3.4. **Bacterial community analysis of the enriched anodophilic biofilm**

On day 120, biomass from both bulk water and biofilm were recovered by disturbing the anode compartment using a 50 mL syringe (liquid agitation by movement of syringe piston). Genomic DNA was extracted using a PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc.). Extracted DNA was visualised using electrophoresis on a 1% (w/v) agarose gel and was quantified spectrophotometrically using a NanoDrop (ND1000 spectrophotometer; Thermo Scientific). The extracted DNA was stored at −20°C prior to shipment to an external laboratory for 454 pyrosequencing of the 16S rRNA genes (MR DNA, Molecular Research LP, Texas, USA). For shipment, DNA samples were stabilised using DNAstable Plus (Biometrica, supplied by Diagnostic Technology) as per the manufacturer’s instructions, and were transported at room temperature. The method used for the 454 pyrosequencing of the 16S rRNA genes was described by Dowd et al. (2008). In brief, HotStart Taq Plus Master Mix (Qiagen, CA, USA) was used for polymerase chain reaction (PCR) amplification of bacterial 16S rRNA genes using the bacterial primer 27F (5′-AGRGTTTGATCMTGGCTCAG-3′) and the universal primer 530R (5′-CCGCNGCNGCTGAC-3′). The thermocycler conditions used included an initial denaturing step at 94°C for 3 min; 28 cycles of denaturation at 94°C for 30 s; annealing at 53°C for 40 s; extension at 72°C for 1 min; and a final extension step at 72°C for 5 min. The amplified products from different samples were mixed in equal concentrations, and purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA, USA). Samples were then sequenced using a Roche 454 FLX titanium instrument and reagents, following the manufacturer’s instructions.

Post-sequence processing was carried out using the QIIME (Quantitative Insights Into Microbial Ecology; (Caporaso et al., 2010)) software package (http://www.qiime.org). Briefly, fasta, qual and mapping files were used as input for the split_libraries.py script.
with default arguments (except for the maximum sequence length, which was set at 600 due to the use of primers 27F and 530R for PCR amplification) to extract sequences that were relevant to this study. Operational Taxonomic Units (OTUs) were subsequently defined using the pick_otus.py script in QIIME and the usearch method (Edgar, 2010). The sequence similarity threshold was set at 97% and the minimum cluster size was set at 8. The usearch sequence analysis method enables clustering and chimera checking, and also performs quality checks and filtering of de-multiplexed sequences. A representative sequence from each OTU was selected and aligned against the Greengenes imputed core reference alignment using align_seqs.py script (with default alignment method PyNAST (Caporaso et al., 2010)). The script filter_alignment.py was then used to remove gaps in the aligned sequence. A phylogenetic tree was subsequently constructed using the script make_phylogeny.py (with default settings – FastTree). Finally, a taxonomy assignment (using script assign_taxonomy.py) was performed using a Ribosomal Database Project (RDP) classifier and the Greengenes OTUs dataset (minimum confidence level 0.8). The unprocessed DNA sequences of this study were deposited (MG-RAST ID- 4535023.3) in MG-RAST (Meyer et al., 2008).

6.4. Results and Discussion

6.4.1. Establishment of the N₂-fixing anodophilic biofilm

The BES performance during 200 days of operation is summarised in Figure 6.2. Measurable current recorded after approximately 20 days of operation indicated the enrichment of a N₂-fixing anodophilic biofilm. Establishment of the active anodophilic biofilm led to a current increase, and the cathodic potential decreased (Figure 6.2). At an organic loading rate (as COD) of approximately 4 mg/L.h, a current of approximately 100 mA was recorded.
Figure 6.2 (A) Current production, (B) anodic and cathodic potentials, (C) redox,(D) pH and temperature, and (E) organic loading rate (OLR) over time. The arrows indicate the timing of acetylene reduction assays (ARAs).

The removal of glucose from the anodic compartment coincided with a transfer of electrons to the anode, with a 39% coulombic efficiency achieved at an anodic potential of +200 mV. This observation is consistent with previous observations of a N-supplemented glucose-fed MFC anode, where a coulombic efficiency of 45% was reported at a similar anodic potential (approximately 200 mV Ag/AgCl) (Freguia et al., 2008). At an anodic potential of +200 mV, the enriched biofilm showed average glucose
and COD removals of 100% and 76%, respectively. As glucose was the sole source of carbon, the 24% difference between glucose and COD removal implies that the glucose was not completely anodically oxidised to carbon dioxide. This indicates the formation of soluble microbial products and the occurrence of anaerobic processes, such as fermentation and methanogenesis in the BES anode.

Figure 6.3 (A) Current production, carbon removal (soluble chemical oxygen demand (COD) removal, glucose utilisation) rates and carbon accumulation (acetic acid and propionate acid) rates, at anodic potentials ranging from −600 to +200 mV. (B) Electron sinks at open circuit potential (OCP) and anodic potentials ranging from −600 to +200 mV. The percentages of electron recoveries were calculated theoretically from complete glucose oxidation and were categorised as current, volatile fatty acids, soluble-COD (unknown), and insoluble COD unknown.
To better understand the fate of glucose in the anodic compartment, electron balances for the key chemical compounds and current were assessed at various anodic potentials (+200 to −600 mV, and OCP) (Figure 6.3). Complete glucose removal occurred at all anodic potentials tested. While this may not clarify whether anodic glucose oxidation was taking place, the removal of glucose under open circuit conditions suggests the presence of fermentative bacteria in the anode compartment. As the anodic potential was gradually increased from −600 mV (close to OCP) to +200 mV, both the current and the coulombic efficiency increased from 0 to 150 mA and from 0 to 39%, respectively. This indicated that the ability of the anodic biofilm to use the anode as an electron acceptor increased. As with other anodophilic biofilms (Cheng et al., 2008), the established biofilm exhibited a saturation behavior towards the anodic potential (Figure 6.3A), with an increase in potential (in this study beyond −400 mV) not resulting in a further increase of current.

The current production was correlated positively with the COD removal rate and negatively with the accumulation of fermentation products (i.e. VFAs). The main components of the residual COD were acetic acid and propionic acid (Figure 6.3B). The rate of accumulation of acetic acid was dependent on the anode potential, and hence the current. As the anodic potential increased from −600 to +200 mV, the acetic acid content decreased from 46% to 5% (expressed in terms of electrons) in the anodic chamber. Being a final product of fermentation, acetate is not fermentable and thus the acetate removal at higher anodic potentials could only occur through the activity of anodically active bacteria. The evidence of glucose fermentation and anodic oxidation of acetate suggests the possible occurrence of a syntrophic relationship between two groups of microorganisms in the anodic biofilm, with a fermenting community fermenting glucose to acetate, and an anodophilic community oxidising acetate using
the electrode as a final electron acceptor. The observation of a similar current when glucose was substituted with acetate (with an equimolar COD concentration to that of glucose) also provides evidence in support of the proposed syntrophic interaction in the anodic biofilm. A similar syntrophic relationship has been reported in a N-supplemented glucose-fed BES (Freguia et al., 2008; Kiely et al., 2011). The results of this study indicate that anodic glucose removal via a syntrophic pathway is essential under N-deficient conditions.

I was unable to establish the sink for approximately 40% of the electrons derived from glucose (Figure 6.3B), that could be divided into insoluble COD (for example, biomass and gas) and soluble COD products (for example, ethanol and EPS). As reported by Freguia et al. (2008), methane was also detected in the BES (quantitative measurements were not performed), suggesting that some of the insoluble COD electron sink reflects methanogenesis.

### 6.4.2. N₂ fixation as a source of N for the anodophilic biofilm

Although no inorganic N was supplied in the BES influent, a substantial concentration of total-N (12.5 ± 2.1 mg/L) was detected in the effluent (Table S6.1 Appendix 2), and <33% was in the form of soluble N. The concentrations of inorganic N (NH₄⁺-N, NO₂⁻-N and NO₃⁻-N) in the BES were negligible. Hence, the soluble N detected in the effluent is likely to have been organic in nature (for example, soluble microbial products).

Based on the relative absence of inorganic N in the feed, it was concluded that N₂ fixation supplied the N requirements of the BES. To confirm N₂ fixation in the BES, the ARA was performed under closed circuit conditions (Table 6.1). Substantial N₂-fixing activity (237 ± 53 µmol C₂H₄/L.h) was observed, suggesting the presence of N₂-fixing
bacteria in the BES. However, under open circuit conditions, a marked reduction (30-fold) in N$_2$-fixing activity (7 ± 4 µmol C$_2$H$_4$/L.h) was observed, suggesting that electron flow to the anode facilitated higher N$_2$ fixation rates. If glucose utilisation was occurring via a syntrophic interaction, it is possible that N$_2$ fixation was carried out by the fermenting bacterium or the anodophilic bacterium, or both. To assess which bacteria contributed to N$_2$ fixation, glucose in the feed was substituted with acetate (at an equimolar COD concentration to that of glucose) and N$_2$ fixation was assessed under closed circuit conditions. Although current production was similar to that when glucose was the sole source of carbon, considerably lower N$_2$-fixing activity (7 ± 8 µmol C$_2$H$_4$/L.h) was observed. This indicated that N$_2$-fixing activity in this BES reactor was independent of acetate utilising anodophilic bacteria, and suggests that the glucose utilising bacteria (fermentative bacteria or anodophilic glucose oxidising bacteria) were responsible for the observed N$_2$ fixation (Figure 6.4).

Table 6.1 The N$_2$-fixing activities of enriched biofilm under three different scenarios.

<table>
<thead>
<tr>
<th>Scenarios</th>
<th>Substrate</th>
<th>Circuit</th>
<th>N$_2$-fixing activity (µmol C$_2$H$_4$/L.h)</th>
<th>Implications</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose</td>
<td>Acetate</td>
<td>Close</td>
<td>Open</td>
</tr>
<tr>
<td>1</td>
<td>✓</td>
<td></td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>2</td>
<td>✓</td>
<td></td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
</tbody>
</table>
This study provides experimental evidence of a syntrophic interaction to facilitate glucose oxidation in the BES. The inability of the acetate-utilising anodophilic bacteria to fix N\textsubscript{2} suggests that a fermenting bacterium (or bacteria) was (were) responsible for fixing N\textsubscript{2}. However, the low N\textsubscript{2} fixation rate (7 ± 8 µmol C\textsubscript{2}H\textsubscript{4}/L.h) observed under open circuit conditions suggests that the fermenting bacteria could only fix N\textsubscript{2} when both glucose and an effective anode (current production) were provided. It is possible that the low level of N\textsubscript{2} fixation is a result of feedback inhibition of the fermenting bacteria by the accumulated acetate in the reactor (Figure 6.3B). Under closed circuit conditions, any acetate produced was immediately oxidised by the anodophilic bacteria, minimising acetate-induced feedback inhibition of the fermenting bacteria. A syntrophic interaction of this type could make fermentation of glucose more energetically favourable (by continuous removal of its products), increasing N\textsubscript{2}-fixing activity in the reactor.
B. $N_2$ fixation driven by anodophilic glucose oxidising bacteria

As previously noted an anode (close circuit) and a supply of glucose were essential to maintain substantial levels of $N_2$ fixation, which is an energy intensive process (+630 kJ per mol of $N_2$) that requires 16 ATP to fix one mole of $N_2$ (Ibanez, 2007). Anodic respiration of a mole of glucose to $CO_2$ yields 38 ATP (i.e. glucose, closed circuit) (Mara & Horan, 2003). In contrast, fermentation of a mole of glucose to acetate only yields 4 ATP (Hochachka et al., 2002), and anodic respiration of a mole of acetate yields 12 ATP (Atkinson, 1977). Accordingly, anodic respiration of glucose appears more favourable for meeting the ATP demands of $N_2$ fixation. Zhang and Chen (2012) reported a 10-fold reduction in $N_2$-fixing activity when the carbon source was switched from glucose to acetate. Hence, direct anodic oxidation of glucose (route 2 in Figure 6.4) may also have taken place in this BES reactor. However, if route 2 was the dominant glucose oxidation pathway, a decrease in glucose removal efficiency and an increase of the total “unknown COD” fraction (Figure 6.3B) would be expected during OCP. However, when an OCP was applied, there was neither an increase of total “unknown COD” nor an accumulation of glucose. This suggests that route 2 was not the dominated pathway. Figure 6.4 details the two possible pathways of glucose utilisation in the BES reactor.

6.4.3. Analysis of the mixed microbial communities in the anodophilic biofilm

The 454 pyrosequencing of the 16S rRNA genes revealed that 78% of the bacterial community in the anodophilic biofilm (Figure 6.5) comprised the class Clostridia, with the remaining 22% comprising most other previously reported major classes of anodophilic bacteria (which include Bacilli, Bacteroidia, Alphaproteobacteria,
Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria and Clostridia). Figure S6.1 (Appendix 2) shows the distribution of the bacterial communities across all of the above major bacterial classes. Of the 78% of bacteria associated with the class Clostridia, 68% belonged to genus *Clostridium*.

Clostridia are anaerobic, and a number of species of *Clostridium* (*C. acetobutylicum*, *C. beijerinckii* and *C. butyricum*) have been shown to be capable of fixing N\textsubscript{2} (Chen, 2005). The ability of Clostridia to ferment glucose has also been well established (Yang et al., 2013). Clostridia have also been shown to be associated with BESs. Yates *et al.* (2012) operated a BES with acetate as the sole source of carbon, and reported that Clostridia (5% in taxonomic abundance) appeared to be anodically oxidising acetate. Further, Park *et al.* (2001) used a pure culture of *C. butyricum* and glucose as a sole source of carbon to demonstrate that *C. butyricum* was electrochemically active. *C. butyricum* was also able to ferment glucose to lactate, formate, butyrate and acetate.

![Figure 6.5](image.png)

**Figure 6.5** The relative abundance of 16S rRNA genes belonging to identified bacterial classes, and dominant genera in the class Clostridia estimated using 454 sequence data from biofilm material collected from the N\textsubscript{2}-fixing BES.
The dominance of Clostridium in my BES reactor and the ability of this genus to switch between final electron acceptors (i.e. between organic matter, inorganic matter such as Fe$^{3+}$, or an electrode) suggest that Clostridium may be responsible for the observations made under differing experimental conditions. Thus, it is plausible that Clostridia were anodically oxidising glucose in the N$_2$-fixing BES reactor.

Other bacteria such as members of the genus Geobacter (class Deltaproteobacteria) and members of the classes Bacteroidia and Gammaproteobacteria were low in abundance (2%, 3% and 1%, respectively) in the anodophilic biofilm of the glucose fed BES. In contrast, Yates et al. (2012) reported the dominance of communities of the above groups in a BES fed with acetate. Thus, although these bacteria were in low abundance, they could have been associated with anodic oxidation of acetate in the glucose fed BES. The presence of these bacteria in the anodophilic biofilm also suggests that my hypothesis of indirect “anode-driven” oxidation of glucose in the BES via a syntrophic interaction between glucose fermenting bacteria (e.g. Clostridia) and acetate oxidising bacteria (e.g. Geobacter) is feasible.

Reactor operating conditions including temperature, pH, applied working electrode potential, the presence/absence of a N source, and the type of carbon source, the source of inoculum will determine the microbial community composition of an anodophilic biofilm (Chae et al., 2009; Dunaj et al., 2012; Logan & Regan, 2006). Hence, a single study of this nature can not comprehensively describe the influence of glucose or the absence of a N source on the community composition of an anodophilic biofilm. Future studies specifically focused on different operational conditions are required to reveal the role and importance of the bacterial communities observed in this study in treating N-deficient wastewater that is rich in glucose.
Benefits of using N$_2$-fixing BES technology to treat N-deficient waste

This study is the first to have demonstrated that a BES anode can be successfully used to anaerobically oxidise N-deficient substrates, including glucose. The anode-dependent N$_2$ fixation observed in this study also indicates that N$_2$ fixation can be electrochemically regulated. This may create opportunities to augment N$_2$ fixation rates in certain N limited environments (e.g. soils), by introducing electrodes into these environments. Future studies are recommended to investigate the effect of different electrode potentials on N$_2$ fixation. Such a study would also reveal whether the anode and N$_2$ would compete for electrons.

The pulp and paper industry uses large quantities of caustic soda (sodium hydroxide) to remove lignin from wood. Through combustion of waste liquor (black liquor), much of the caustic is recovered and reused. The use of BES to treat pulp and paper waste creates an opportunity for this industry to recover/produce this important chemical in the cathode compartment of a BES. Rabaey et al. (2010) demonstrated the potential to couple organic matter oxidation to caustic soda production using brewery wastewater as a BES feedstock. In this process, sodium ions migrate from the anode to the cathode compartment, but other cations including NH$_4^+$ also migrate, and could contaminate the caustic soda produced. The absence of NH$_4^+$ in pulp and paper wastewater and the use of N$_2$-fixing microorganisms to treat this waste offer an approach to essentially eliminate the potential contamination of caustic soda with NH$_4^+$. Overall, the recovery/production of caustic soda using BES should be cost effective (Rabaey et al., 2010), and the dilute nature of the chemical would eliminate the need to purchase and dilute concentrated caustic soda, minimising the health and safety risks (caustic soda burns) to workers in this industry.
With ever increasing energy costs, the cost of waste treatment will inevitably rise. Aerating to facilitate oxidation of organic matter is an energy intensive process, so anaerobic oxidation of organic matter using a BES anode could provide significant savings to industry, including pulp and paper manufacturers. Additionally, under anaerobic conditions the production of biological sludge could be substantially less than that resulting from activated sludge processes, which generate large amounts of sludge that must be disposed of. Hence, a considerably lower cost associated with sludge treatment can be expected from BES processes. Importantly, the use of N₂-fixing microorganisms eliminates the need to supplement waste streams with N, which provides further savings and also reduces the risk of accidental N discharge to environmentally-sensitive water bodies.

6.5. Conclusions

It was confirmed that N-deficient wastewater could be treated using a BES anode, provided the first experimental evidence of nitrogen fixation, and characterised biofilm ARB in a BES treating N-deficient wastewater. The results suggest that:

- Good carbon removal efficiencies were achieved under anaerobic conditions
- The N₂-fixing activity was anode- and glucose-dependent (removing either reduced N₂-fixing activity).
- The presence of N₂-fixing bacterial genera was demonstrated using 454 pyrosequencing of the 16S rRNA genes.
- Chemical and microbial characterisation of the anodophilic biofilm revealed two possible pathways of glucose oxidation: directly using the anode as the final electron acceptor, and indirectly by glucose fermentation to VFAs (e.g. acetate) and subsequent anodic acetate oxidation.
7. Conclusions and Recommendations

In this study the applicability of the EBPR-r as a post-denitrification strategy (Figure 7.1) for P recovery from municipal wastewater was proposed and explored. In this novel process a PAOs biofilm was used as a “shuttle” to carry soluble P from a low P-containing wastewater (8 mg-P/L) to a separate concentrated recovery stream (>90 mg-P/L), while removing N from wastewater. The major findings of this thesis and their implications are summarised in this chapter. In addition, the limitations of the study, and recommendations for future improvements, are also discussed.

Figure 7.1 The application of EBPR-r as a post-denitrification strategy in a WWTP.
7.1. The potential of EBPR-r to achieve P concentrations >100 mg-P/L

Two approaches to concentrating P were investigated in this study (Figure 7.2): (A) multiple P-uptake and P-release cycles to achieve a high P concentration in the recovery stream (Chapter 2); and (B) a single P-uptake from a large volume of wastewater followed by the release of P into a smaller recovery stream, maintaining a large volumetric ratio between the wastewater and recovery streams (e.g. 12:1) (Chapter 5). Both strategies were able to achieve a 10-fold increase in the P concentration and generate a P-enriched stream (~100 mg-P/L) suitable for downstream recovery (e.g. as struvite).

Figure 7.2 Two approaches used to increase the P concentration for P recovery. (A) Multiple P-uptake and P-release cycles to facilitate P accumulation in the recovery stream. (B) A single P-release cycle based on a large volumetric ratio between the wastewater and recovery streams.
The cost of downstream P recovery (for uses including fertilizer production) can be substantially reduced if a higher concentration of P in the recovery stream can be achieved. For instance, an increase in the P concentration from 50 to 800 mg-P/L could result in a 5-fold reduction in the cost of struvite production (Dockhorn, 2009). To facilitate a higher P concentration in the recovery stream (>100 mg-P/L), a strategy involving multiple P-uptake phases and a single P-release phase could be used (Figure 7.3). This strategy facilitates a lower P recovery cost by enabling the use of a smaller plant footprint, and more effective use of carbon for P recovery. However, this strategy is only feasible if the internal storage polymers of PAOs can be conserved throughout the multiple P-uptake phases (e.g. P-uptake phase of 4 h × 3 phases = 12 h). Chapter 4 reports the unique ability of PAOs to reserve their internal carbon storage polymers specifically for P uptake, even after being exposed to highly oxidising conditions for prolonged periods of time (up to 2 days). Whether this unique ability of PAOs could be exploited to facilitate the multiple P-uptake strategy should be the focus of future research.

Figure 7.3 Strategy for (1) a single P-uptake and a single P-release; and (2) multiple P-uptake and a single P-release.
Reducing the relative volume of the recovery stream (i.e. using a larger volumetric ratio between the wastewater and recovery streams, such as 12:1 instead of 4:1) is another method to achieve a higher concentration of P in the recovery stream (Chapter 5). The biofilm carries used in this study were rigid bulky structures, which made a further reduction in the volume of the recovery stream difficult. Future research could be directed at investigating whether a granular biomass could replace biofilm carriers of the EBPR-r process. Granular biomass has similar structure as biofilm and its excellent settling properties would probably facilitate further reduction in the volume of the recovery stream, thus enabling recovery of P at concentrations >100 mg-P/L.

7.2. A broader perspective: how much P could potentially be recovered using the EBPR-r process?

Australia is used as an example to demonstrate the potential for P recovery using the EBPR-r process. The annual domestic P-loading to a WWPT is estimated to be ~1 kg-P/person (Parsons & Smith, 2008). Hence, an Australian population of 23 million would be expected to generate ~23,800 ton (t) of P per year. Of this quantity, approximately 66% (15,700 t-P/y, i.e. from the urban population) enters the major WWTPs of capital cities. Of the P load that enters WWTPs not designed to remove P (Figure 7.1), 39% is removed during primary (11%; primary solids) and secondary (28%; biomass growth) treatment (Cornel & Schaum, 2009). As a result, only 61% (9,580 t-P/y) of the entire P load would be available for recovery using the EBPR-r process. Assuming EBPR-r biofilm has a P removal and recovery efficiency of 79% and 71%, respectively (achieved after optimisation; Chapter 5), the amount of P removed and recovered would be 7,560 and 6,810 t-P/y. In this scenario the EBPR-r process would have an overall P recovery efficiency of 43% (Table 7.1), which is similar to that other P recovery
strategies are currently achieving from wastewater (40–50%; listed in Table 1.1 of Chapter 1) (Cornel & Schaum, 2009).

If P were to be recovered as struvite from the recovery stream, up to 6,130 t-P/y could be recovered (assuming an efficiency of 90%) (Münch & Barr, 2001), potentially reducing Australia’s fertilizer demand by 1.5% (Cordell & White, 2014). A similar estimate was reported by Shu et al. (2006), who calculated that globe phosphate rock mining could be reduced by 1.6% if P were to be recovered as struvite from WWTPs worldwide.

Table 7.1 Estimating the recovery potential of P from wastewater using the EBPR-r process.

<table>
<thead>
<tr>
<th>P load enter WWTPs in Australia</th>
<th>Conventional post-denitrification</th>
<th>EBPR-r</th>
</tr>
</thead>
<tbody>
<tr>
<td>P removal efficiency</td>
<td>Insignificant (via biomass growth)</td>
<td>48.0%</td>
</tr>
<tr>
<td>P recovery efficiency</td>
<td>n.a.</td>
<td>43.0%</td>
</tr>
<tr>
<td>P removal (t-P/y)</td>
<td>n.a.</td>
<td>7,560</td>
</tr>
<tr>
<td>P recovery as liquid (t-P/y)</td>
<td>n.a.</td>
<td>6,810</td>
</tr>
<tr>
<td>P recovery as struvite (t-P/y)</td>
<td>n.a.</td>
<td>6,130</td>
</tr>
<tr>
<td>Contribution toward Australia’s P consumption in fertilizer</td>
<td>n.a.</td>
<td>1.5%</td>
</tr>
<tr>
<td>N removal (t-N/y)</td>
<td>8,170 (assumption)</td>
<td>8,170*; 1,550**</td>
</tr>
<tr>
<td>Carbon consumption (t/y)</td>
<td>26,300 (methanol); 46,950 (acetate) (USEPA, 2013)</td>
<td>93,650*; 25,530**</td>
</tr>
<tr>
<td>Carbon cost (A$ million/y)</td>
<td>21.0 (methanol); 38.5 (acetate)</td>
<td>76.8*; 20.9**</td>
</tr>
</tbody>
</table>

*Estimated values before optimisation; **Estimated value after optimisation described in Chapter 5; Price of acetate and methanol were obtained from www.orbichem.com by converting US$1.00 to A$0.66.
7.3. Optimising the $P_{\text{rel}}/C_{\text{upt}}$ ratio is critical for economic recovery of P

The addition of external carbon is expected to be the main operating cost associated with the EBPR-r process. Based on a $P_{\text{rel}}/C_{\text{upt}}$ ratio of 0.25 mol-P/mol-C (obtained after optimisation; Chapter 5), the cost of external carbon (acetate) to recover 6,810 t-P/y from wastewater is estimated to be A$21 million/y (6,130 t-P/y is recovered). This cost is significantly lower than the A$77 million/y estimate derived using a $P_{\text{rel}}/C_{\text{upt}}$ ratio of 0.08 (the $P_{\text{rel}}/C_{\text{upt}}$ ratio prior to optimisation of the process). By increasing the $P_{\text{rel}}/C_{\text{upt}}$ ratio 3-fold (from 0.08 to 0.25; Chapter 5), a significant saving on carbon usage is achievable (almost A$56 million/y). As even higher $P_{\text{rel}}/C_{\text{upt}}$ ratios (0.50–0.70) have been reported for PAOs, future research should aim to further improve the $P_{\text{rel}}/C_{\text{upt}}$ ratio in the EBPR-r process.

In addition, the use of cheaper carbon sources should be investigated. For instance, carbon-rich waste streams from other industries (e.g. pulp and paper), and VFAs produced from primary sludge (via fermentation), could be used as inexpensive carbon sources for the EBPR-r process. If VFAs were to be largely used to supplement the carbon and energy requirements of PAOs, BES technologies may be useful in meeting the VFA requirements for the EBPR-r process, via microbial electro-synthesis of acetate from CO$_2$ (Batlle-Vilanova et al., 2016). The energy requirements of microbial electro-synthesis are high. Therefore, this study examined whether N-deficient wastewater, such as that from the pulp and paper industry, could be oxidised in the anode compartment of a BES reactor to off-set some of the energy demands (Chapter 6). Future research should examine benefits of anodic organic carbon oxidation on the acetate production in the cathode compartment of BES reactor.
According to Lee & Welander (1996), a carbon source used for denitrification should
fulfil five criteria, one of which is in relation to sludge yield. As the effluent from the
EBPR-r process may not be subject to any downstream sedimentation, maintaining a
low sludge yield is critical for minimising discharge of turbid effluent. Hence, the
carbon source added to the EBPR-r should not result in a high sludge yield. Assessing
the sludge yield from the acetate-fed EBPR-r biofilm was beyond the scope of this
study, but future studies should investigate the impact of various carbon sources on
sludge yield (effluent turbidity), based on both biofilm and granular biomass operation.

7.4. Optimising the $N_{\text{den}}/P_{\text{upt}}$ ratio increases the economic feasibility
of EBPR-r as a post-denitrification strategy

The optimisation described in Chapter 5 resulted in a significant reduction in
denitrification activity (a 5-fold decrease in the $N_{\text{den}}/P_{\text{upt}}$ ratio, from 2.39 to 0.47 mol-
N/mol-P). This equates to removal of 1,550 t-N/y during the removal of 7,560 t-P/y
from wastewater (described in section 7.3). If it is assumed that denitrification was
unaffected (i.e. a $N_{\text{den}}/P_{\text{upt}}$ ratio of 2.39), the removal of 8,170 t-N/y would cost A$21
million/y, because of the acetate consumed. This is comparable to the cost to remove a
similar quantity of N through a conventional post-denitrification process (Table 7.1).
Hence, with further optimisation ($P_{rel}/C_{upt}\geq 0.25$ and $N_{\text{den}}/P_{\text{upt}}\geq 2.39$), the EBPR-r
process has the potential to facilitate P recovery at a cost that is comparable to a
conventional post-denitrification process.

During this study the DO content of wastewater was identified as a major factor that
could affect the denitrification activity of the EBPR-r biofilm (Chapter 3). Maintaining
a DO gradient across a biofilm or in granular biomass is important for achieving a better
$N_{\text{den}}/P_{\text{upt}}$ ratio. Hence, investigating the minimum effective biofilm thickness/
size that needs to be maintained to support denitrification should be the subject of further research.

According to Zeng et al. (2003a), storage-driven denitrification contributes to greater emissions of N$_2$O relative to the direct use of soluble carbon for denitrification. As N$_2$O is an extremely potent greenhouse gas, future studies should characterise the gaseous byproducts of the EBPR-r process.

7.5. Reducing the downstream P recovery cost is essential for economical P recovery

The economic feasibility of P recovery is illustrated by considering the costs associated with downstream struvite precipitation (Table 7.2). For example, ignoring the capital costs (e.g. plant construction costs) and other operational expenses, the processing of 6,810 t-P/y to form struvite would cost A$7.0 million/y for chemicals alone, specifically for ammonia (NH$_3$) and magnesium hydroxide (Mg(OH)$_2$). On the other hand, the estimated market value of struvite is A$300–1150/t (€198–763) (Dockhorn, 2009; Münch & Barr, 2001). Hence, the sale of P recovered as struvite (based on 90% recovery efficiency; 6,130 t-P/y) could at the most generate only sufficient revenue to offset production costs (A$7.0 million/y) (Table 7.2). Therefore, downstream processing of recovered P to form struvite using the EBPR-r process would not be profitable.

In contrast, conventional P recovery processes that utilise anaerobic digesters have been able to generate revenue from sale of the struvite produced because the digester effluent is rich in both P and NH$_4^+$ (700–800 mg-N/L) (Münch & Barr, 2001). In the EBPR-r process, NH$_4^+$ is not recovered and must be supplemented externally (a molar N:P ratio
This would increase the production cost if struvite is the desired end product of P recovery. To enable the EBPR-r process to be cost competitive, the production of other forms of P fertilizer (e.g. concentrated liquid P sprays) having lower production cost will need to be investigated.

**Table 7.2** Estimating the cost of P recovery from the recovery stream.

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Annual amount</th>
<th>Assume price (A$/t)</th>
<th>Annual cost (A$ million/t)</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-PO₄³⁻</td>
<td>6,810 t</td>
<td>0</td>
<td>0</td>
<td>Recovery stream</td>
</tr>
<tr>
<td>NH₃ (100%)</td>
<td>3,740 t</td>
<td>960</td>
<td>3.60</td>
<td>Assume a molar N:P ratio of 1:1.</td>
</tr>
<tr>
<td>Mg(OH)₂ MHS-60</td>
<td>10.4 ML</td>
<td>500</td>
<td>3.45</td>
<td>Assume a molar Mg²⁺:P ratio of 1:1 and 33% of Mg²⁺ was supplied via EBPR-r.</td>
</tr>
<tr>
<td>Struvite production cost</td>
<td></td>
<td></td>
<td>-7.00</td>
<td>A$1.15/kg-P</td>
</tr>
<tr>
<td>Profit from struvite</td>
<td>6,130 t</td>
<td>1150</td>
<td>+7.00</td>
<td>Struvite price from (Dockhorn, 2009)</td>
</tr>
<tr>
<td>Profit</td>
<td></td>
<td></td>
<td>0.00</td>
<td></td>
</tr>
</tbody>
</table>

Currency conversations: €1 equal to A$0.66 and US$1 equal to A$1.37. Magnesium hydroxide slurry (brand name MHS-60 contain, Orica, Australia) contains 55% w/w Mg(OH)₂ and a bulk density of 1.5 t/m³ (Münch & Barr, 2001).
8. References

Acevedo, B., Camiña, C., Corona, J.E., Borrás, L., Barat, R. 2015. The metabolic versatility of PAOs as an opportunity to obtain a highly P-enriched stream for further P-recovery. Chemical Engineering Journal, 270(0), 459-467.


conditions in post-denitrification biofilm reactors within systems designed for simultaneous low-level effluent nitrogen and phosphorus concentrations. Water Research, 46(19), 6228-6238.


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Muszynski, A., Lebkowska, M., Tabernacka, A., Milobedzka, A. 2013. From macro to lab-scale: Changes in bacterial community led to deterioration of EBPR in lab reactor. Central European Journal of Biology, 8(2), 130-142.


Appendix 1

Supporting documents to Chapter 3

Figure S3.1 Concentrations of soluble (A) $\text{PO}_4^{3-}$-P, (B) $\text{NO}_x$-N ($\text{NO}_3^-$-N + $\text{NO}_2^-$-N), and (C) $\text{NO}_2^-$-N associated with the enriched biofilm over time under three electron acceptor scenarios: (1) $\text{O}_2$ alone (8 mg/L of bulk DO); (2) $\text{NO}_3^-$ alone (10 mg-N/L); and (3) $\text{O}_2$ and $\text{NO}_3^-$ in combination.
Figure S3.2 The concentrations of soluble (A) & (E) PO$_4^{3-}$-P, (B) & (F) NO$_x$-N (NO$_3^-$-N + NO$_2^-$-N), (C) & (G) NO$_2^-$-N, and (D) & (H) the oxygen uptake rate (OUR) for the EBPR-r biofilm in two sets of batch experiments: (1) varying bulk DO concentrations (0–8 mg/L) at an initial NO$_3^-$ concentration of 10 mg-N/L; and (2) varying initial NO$_3^-$ concentrations (0–50 mg-N/L) at a constant bulk DO concentration of 8 mg/L.
Rate calculations – Chapter 3

Table 3.1, Table 3.2, Figure 3.2A, 3.2B, 3.2E and 3.2F:

P uptake rate (PUR), NOx removal rates and oxygen uptake rate (OUR) were calculated from the steepest part of the kinetic profile of Figure S3.2, and are expressed in either mg/L.h or mmol/g-TS.h:

\[
\frac{PUR}{NO_x \text{removal rate (mmol/gTS.h)}} = \frac{\text{Maximum rate of kinetic profile (mmol/L.h)} \times \text{Volume of solution (2.4 L)}}{\text{Total biomass (g)}}
\]

\[\text{OUR (mmol/gTS.h) in the column reactor} = \frac{(\text{Influent DO} - \text{Effluent DO})(\text{mmol/L}) \times \text{Volume of solution (0.36 L)}}{\text{HRT in column reactor (0.0459 h)} \times \text{Total biomass}(g)}\]

Figure 3.2C and 3.2G:

The electron accepting rate for O2 and NO3− reductions were calculated according to the equation below:

Reduction rate of O2 (mmol e−/gTS.h) = OUR × 4e−

It was assumed that the reduction of a mole of O2 to H2O consumes 4 moles of electrons. The OURs were recorded as the average value of the steepest part of the OUR profile in Figure S3.2 D & H of the supporting information.

Reduction rate of NO3− (mmol e−/gTS.h)

\[= \text{NO}_2^− \text{accumulation rate} \times 2e^− + \text{net NO}_3^− \text{removal rate} \times 5e^−\]

It was assumed that the reduction of a mole NO3− to NO2− consumes 2 moles of electrons, and that the reduction of a mole of NO3− to nitrogen gas consumed 5 moles of electrons. The net NO3− removed (the total NO3− removed minus NO2− formed) was the net NO3− removed in the system to form nitrogen gaseous product.
Figure 3.2D and 3.2H:

The % of electron used for P uptake with O\textsubscript{2} and NO\textsubscript{3}\textsuperscript{−} as electron acceptors was calculated as follows:

\[
\% \text{ electrons for PHAs oxidation with } O_2 \text{ reduction} = \frac{\text{Reduction rate of } O_2}{\text{Reduction rate of } O_2 + \text{Reduction rate of NO}_3\textsuperscript{−}} \times 100
\]

\[
\% \text{ electrons for PHAs oxidation with } NO_3\textsuperscript{−} \text{ reduction} = \frac{\text{Reduction rate of } NO_3\textsuperscript{−}}{\text{Reduction rate of } O_2 + \text{Reduction rate of NO}_3\textsuperscript{−}} \times 100
\]

It was assumed that all electrons consumed in the system through the reduction of O\textsubscript{2} and NO\textsubscript{3}\textsuperscript{−} were used for PHAs oxidation.
Appendix 2

Supporting documents to Chapter 6

Table S6.1 The N content of the anodic influent and effluent.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Anodic influent</th>
<th>Anodic effluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total-N (mg/L)</td>
<td>0.30 ± 0.14</td>
<td>12.50 ± 2.12</td>
</tr>
<tr>
<td>Soluble-N (mg/L)</td>
<td>0.30 ± 0.14</td>
<td>4.10 ± 2.55</td>
</tr>
<tr>
<td>NH₄⁺-N (mg/L)</td>
<td>ND</td>
<td>0.06 ± 0.05</td>
</tr>
<tr>
<td>NO₃⁻-N (mg/L)</td>
<td>ND</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>NO₂⁻-N (mg/L)</td>
<td>ND</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

ND = not detected
Figure S6.1 Evolutionary distance dendrogram of major bacterial classes based on phylogenetic analyses of 16S rRNA gene data. The sequences from this study were compared with anodic biofilm sequences derived from publicly accessible databases. The scale indicates 0.06 nucleotide change per nucleotide position.
Appendix 3

Laboratory photos

Figure S3.1 The Kaldnes® K1 carrier media used for biofilm growth in the EBPR-r process.

Figure S3.2 The master reactor operated in this study using the EBPR-r configuration.
Figure S3.3 The master reactor operated in this study using the EBPR-r configuration.

Figure S3.4 The batch experiment setup designed to assess the ability of the enriched biofilm to denitrify and remove P from wastewater (as described in section 3.3.2 of Chapter 3 and section 4.3.2 of Chapter 4).
Figure S3.5 The batch experiment designed to assess the N and P removal activities of the dislodged biomass from carriers (section 3.3.3 of Chapter 3).