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Review

Advances in enhanced biological phosphorus removal: From micro to macro scale

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ABSTRACT

The enhanced biological phosphorus removal (EBPR) process has been implemented in many wastewater treatment plants worldwide. While the EBPR process is indeed capable of efficient phosphorus (P) removal performance, disturbances and prolonged periods of insufficient P removal have been observed at full-scale plants on numerous occasions under conditions that are seemingly favourable for EBPR. Recent studies in this field have utilised a wide range of approaches to address this problem, from studying the microorganisms that are primarily responsible for or detrimental to this process, to determining their biochemical pathways and developing mathematical models that facilitate better prediction of process performance. The overall goal of each of these studies is to obtain a more detailed insight into how the EBPR process works, where the best way of achieving this objective is through linking together the information obtained using these different approaches. This review paper critically assesses the recent advances that have been achieved in this field, particularly relating to the areas of EBPR microbiology, biochemistry, process operation and process modelling. Potential areas for future research are also proposed. Although previous research in this field has undoubtedly improved our level of understanding, it is clear that much remains to be learned about the process, as many unanswered questions still remain. One of the challenges appears to be the integration of the existing and growing scientific knowledge base with the observations and applications in practice, which this paper hopes to partially achieve.

Contents

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1. Introduction

Phosphorus (P) is a key nutrient that stimulates the growth of algae and other photosynthetic microorganisms such as toxic cyanobacteria (blue-green algae), and must be removed from wastewater to avoid eutrophication in aquatic water systems. The risk of adverse effects to the plant and animal communities in waterways declines as P concentrations approach background levels (Mainstone and Parr, 2002). Around the world, a growing awareness of the need to control P emissions, which is reflected in increasingly stringent regulations, has made P removal more widely employed in wastewater treatment. Enhanced biological phosphorus removal (EBPR) promotes the removal of P from wastewater without the need for chemical precipitants. EBPR can be achieved through the activated sludge process by recirculating sludge through anaerobic and aerobic conditions (Barnard, 1975). Usually, biological nutrient removal (BNR) refers to the combination of biological nitrogen removal and the EBPR process.

The group of microorganisms that are largely responsible for P removal are known as the polyphosphate accumulating organisms (PAOs). These organisms are able to store phosphate as intracellular polyphosphate, leading to P removal from the bulk liquid phase via PAO cell removal in the waste activated sludge. Unlike most other microorganisms, PAOs can take up carbon sources such as volatile fatty acids (VFAs) under anaerobic conditions, and store them intracellularly as carbon polymers, namely poly-β-hydroxyalkanoates (PHAs). The energy for these biotransformations is mainly generated by the cleavage of polyphosphate and release of phosphate from the cell. Reducing power is also required for PHA

formation, which is produced largely through the glycolysis of internally stored glycogen (Mino et al., 1998).

Aerobically, PAOs are able to use their stored PHA as the energy source for biomass growth, glycogen replenishment, P uptake and polyphosphate storage. Net P removal from the wastewater is achieved through the removal of waste activated sludge containing a high polyphosphate content. While the majority of P removal from the EBPR process is often achieved through anaerobic–aerobic cycling, anaerobic–anoxic operation also allows P removal to occur, due to the ability of at least some PAOs (i.e. denitrifying PAOs or DPAOs) to use nitrate or nitrite instead of oxygen as electron acceptors and, therefore, perform P uptake and denitrification simultaneously. Maximising the fraction of P removal achieved anoxically can reduce process operational costs, due to savings in aeration as well as in the amount of carbon sources needed for denitrification. Currently, many different process configurations exist where both P and nitrogen removal are combined (Henze et al., 1997; Tchobanoglous et al., 2002).

When operated successfully, the EBPR process is a relatively inexpensive and environmentally sustainable option for P removal; however, the stability and reliability of EBPR can be a problem. It is widely known that EBPR plants may experience process upsets, deterioration in performance and even failures, causing violations to discharge regulations (Hartley and Sickerdick, 1994; Blackall et al., 2002; Thomas et al., 2003; Stephens et al., 2004). In some cases, external disturbances such as high rainfall, excessive nitrate loading to the anaerobic reactor, or nutrient limitation explains these process upsets. In other cases, microbial competition between PAOs and another group of organisms, known as the glycogen (non-polyphosphate) accumulating organisms (GAOs), has been hypothesised to be the cause of the degradation in P removal. Like PAOs, GAOs are able to proliferate under alternating anaerobic and aerobic conditions without performing anaerobic P release or aerobic P uptake, thus they do not contribute to P removal from EBPR systems. GAOs are believed to use glycogen as their primary energy source for anaerobic VFA uptake and PHA formation, while PHA is oxidised aerobically, leading to biomass growth and glycogen replenishment (Liu et al., 1994; Satoh et al., 1994; Mino et al., 1995). Since GAOs consume VFAs without contributing to P removal, they are highly undesirable organisms in EBPR systems. GAOs have indeed been found in numerous full-scale EBPR plants (Crocetti et al., 2002; Kong et al., 2002b; Saunders et al., 2003; Thomas et al., 2003; Gu et al., 2005; Wong et al., 2005; Kong et al., 2006; Burow et al., 2007), and studies have suggested that they increase the anaerobic VFA requirements of these plants (Saunders et al., 2003; Thomas et al., 2003; Gu et al., 2005). Minimising the growth of GAOs in EBPR systems has been a widely researched topic recently, due to the opportunities that exist for increasing the cost-effectiveness of this process.

The aim of this review is to discuss recent advances in EBPR research, related to the microbiology and biochemistry of the microorganisms involved as well as process modelling and optimisation. The main focus of the review is to summarise the many new findings that have been made since previous reviews of this subject (e.g. van Loosdrecht et al., 1997; Mino et al., 1998; Kortstee et al., 2000; Blackall et al., 2002; Seviour et al., 2003). Future developments for this process are discussed as well.

2. Microbiology

2.1. *Identification of PAOs*

2.1.1. *Isolated organisms proposed as PAOs*

The first attempts to identify the microorganisms involved in EBPR, over 30 years ago, were based on culture-dependent techniques. Postulation of which bacteria were thought to be responsible for P removal were made based on the number of viable bacterial colonies that grew on defined media (Barker and Dold, 1996). Through these techniques, *Acinetobacter* was first proposed to be the primary organism responsible for P removal in EBPR (Fuhs and Chen, 1975), and was long believed to be the sole PAO present in EBPR plants. However, the use of techniques such as fluorescence *in situ* hybridisation (FISH), 16S rRNA-based clone libraries or denaturing gradient gel electrophoresis (DGGE), showed that a high diversity of phylogenetic groups are present in lab- and full-scale EBPR sludges (Seviour et al., 2003). It was with the use of specific FISH probes that *Acinetobacter* was shown to have little significance in full-scale plants when compared to members of other phylogenetic groups, such as the *Betaproteobacteria* and *Actinobacteria* (Wagner et al., 1994).

Microlunatus phosphovorus (Nakamura et al., 1995) was another isolated microorganism that was also hypothesised to play an important role in P removal, but later studies carried out with ^{31}P- and ^{13}C-NMR (Santos et al., 1999) demonstrated that neither glycogen nor PHA were cycled within their cells, unlike the characteristic PAO phenotype. Furthermore, this organism is not generally present in EBPR systems (Seviour et al., 2003) suggesting that it was not a major PAO. Other organisms isolated from EBPR systems, such as *Lampropedia* spp. (Stante et al., 1997) and *Tetrasphaera* spp. (Maszenan et al., 2000) also did not demonstrate the characteristic PAO phenotype (Seviour et al., 2003). In fact, despite multiple efforts, the successful isolation of a PAO has yet to be conclusively achieved.

Potential methods to successfully isolate PAOs have been proposed through the use of e.g. micro-manipulation techniques from enriched cultures of these organisms (Seviour et al., 2003; Saunders, 2005). In order to be able to capture isolates using these techniques, and avoid contamination by other organisms, highly enriched PAO cultures are necessary (Saunders, 2005). An operational strategy has been proposed by Lu et al. (2006) in order to obtain very high PAO enrichments (>90% of all *Bacteria* as assessed by FISH quantification), and eliminate the growth of GAOs (see PAO–GAO competition section for more detail), which could be useful in future attempts to isolate PAOs.

Considerable study has also been devoted to determining the identity of DPAOs, and establishing if they are different organisms than non-DPAOs. Both chemically and microbiologically based methods have been employed for this purpose (see Section 5.1 for more detail).

2.1.2. *Candidatus Accumulibacter phosphatis or Rhodocyclus-related bacteria*

In spite of the difficulty in the isolation of PAOs, the identification of this group of organisms has proceeded in the last decade through the use of molecular techniques. Bond et al. (1995) observed differences in community structure between phosphate and non-phosphate-removing sludges, as determined by phylogenetic analysis of 16S rRNA clone libraries; in particular, the *Rhodocyclus* group from subclass 2 of the *Betaproteobacteria* was represented to a greater extent in the phosphate-removing community. Bond et al. (1999a) later supported these conclusions through observing an abundance of *Rhodocyclus*-related bacteria by FISH in other sludges exhibiting good EBPR performance. Hesselmann et al. (1999) named the subclass 2 *Betaproteobacteria* closely related to *Rhodocyclus* as "Candidatus *Accumulibacter phosphatis*". They are often abbreviated to *Accumulibacter*, as in this paper, but are also referred to as *Rhodocyclus*-related bacteria. Hesselmann et al. (1999) and Crocetti et al. (2000) reported several FISH probes for *Accumulibacter* that target the organism at different areas of the 16S rRNA. These studies each demonstrated that *Accumulibacter* corresponded to the characteristic PAO phenotype of anaerobic/aerobic cycling of poly-P and PHA through chemical staining. Zilles et al. (2002a) proposed modifications of the FISH probes for *Accumulibacter* in order to cover two additional clones obtained from a full-scale plant. An analysis of the specificity and sensitivity of the sets of probes for *Accumulibacter* (Table 1) has been reported by Saunders (2005). It was concluded that a combination of the Crocetti et al. (2000) probes (PAO462, PAO651 and PAO846) is the most sensitive and specific for *Accumulibacter* from the presently available probes for these bacteria.

Since *Accumulibacter* was shown through FISH analysis to be a highly abundant PAO in many lab-scale systems

Table 1 – Most common 16S rRNA-targeted probes used for FISH detection of organisms of relevance in EBPR systems

Probe	Sequence 5′-3′	Specificity	Reference
Probes designed for (potential) PAOs			
PAO462	CCGTCATCTACWCAGGGTATTAAC	Most *Accumulibacter*	Crocetti et al. (2000)
PAO651	CCCTCTGCCAAACTCCAG	Most *Accumulibacter*	Crocetti et al. (2000)
PAO846	GTTAGCTACGGCACTAAAAGG	Most *Accumulibacter*	Crocetti et al. (2000)
RHC439	CNATTTCTTCCCCGCCGA	*Rhodocyclus/Accumulibacter*	Hesselmann et al. (1999)
RHC175	TGCTCACAGAATATGCGG	Most *Rhodocyclaceae*	Hesselmann et al. (1999)
PAO462b	CCGTCATCTRCWCAGGGTATTAAC	Most *Accumulibacter*	Zilles et al. (2002a)
PAO846b	GTTAGCTACGGYACTAAAAGG	Most *Accumulibacter*	Zilles et al. (2002a)
Actino-221a	CGCAGGTCCATCCCAGAC	*Actinobacteria*—potential PAOs	Kong et al. (2005)
Actino-658a	TCCGGTCTCCCCTACCAT	*Actinobacteria*—potential PAOs	Kong et al. (2005)
Probes designed for (potential) GAOs			
Gam1019	GGTTCCTTGCGGCACCTC	Some *Gammaproteobacteria*	Nielsen et al. (1999)
Gam1278	ACGAGCGGCTTTTTGGGA	Some *Gammaproteobacteria*	Nielsen et al. (1999)
GAOQ431	TCCCCGCCTAAAGGGCTT	Some *Competibacter*	Crocetti et al. (2002)
GAOQ989	TTCCCCGGATGTCAAGGC	Some *Competibacter*	Crocetti et al. (2002)
GB	CGATCCTCTAGCCCACT	*Competibacter* (GB group)	Kong et al. (2002b)
GB_G1[a] (GAOQ989)	TTCCCCGGATGTCAAGGC	Some *Competibacter*	Kong et al. (2002b)
GB_G2[a]	TTCCCCAGATGTCAAGGC	Some *Competibacter*	Kong et al. (2002b)
GB_1 and 2	GGCTGACTGACCCATCC	Some *Competibacter*	Kong et al. (2002b)
GB_2	GGCATCGCTGCCCTCGTT	Some *Competibacter*	Kong et al. (2002b)
GB_3	CCACTCAAGTCCAGCCGT	Some *Competibacter*	Kong et al. (2002b)
GB_4[a]	GGCTCCTTGCGGCACCGT	Some *Competibacter*	Kong et al. (2002b)
GB_5	CTAGGCGCCGAAGCGCCC	Some *Competibacter*	Kong et al. (2002b)
GB_6 (Gam1019)	GGTTCCTTGCGGCACCTC	Some *Competibacter*	Kong et al. (2002b)
GB_7[a]	CATCTCTGGACATTCCCC	Some *Competibacter*	Kong et al. (2002b)
SBR9-1a	AAGCGCAAGTTCCCAGGTTG	*Sphingomonas*-related organisms—potential GAOs	Beer et al. (2004)
SBR9-1b	TGTTAGGGGCTTAGACCT	*Sphingomonas*-related organisms—potential GAOs	Beer et al. (2004)
SBR8-4	CACCGAAGCACTAAGTGCCC	*Sphingomonas*-related organisms—potential GAOs	Beer et al. (2004)
TFO_DF218	GAAGCCTTTGCCCCTCAG	*Defluviicoccus*-related organisms (cluster 1)	Wong et al. (2004)
TFO_DF618	GCCTCACTTGTCTAACCG	*Defluviicoccus*-related organisms (cluster 1)	Wong et al. (2004)
DF988[a]	GATACGACGCCCATGTCAAGGG	*Defluviicoccus*-related organisms (cluster 2)	Meyer et al. (2006)
DF1020[a]	CCGGCCGAACCGACTCCC	*Defluviicoccus*-related organisms (cluster 2)	Meyer et al. (2006)

[a] Requires competitor or helper probes.

(Hesselmann et al., 1999; Crocetti et al., 2000; Liu et al., 2001), a number of surveys were carried out to assess their presence in full-scale plants. Wastewater treatment plants with various process configurations and from different countries across four continents were evaluated (Zilles et al., 2002a; Saunders et al., 2003; Kong et al., 2004; Gu et al., 2005; He et al., 2005; Wong et al., 2005). In all of these studies, *Accumulibacter* was present in relative abundance (4–22% of all *Bacteria*); considering the large diversity of organisms that is present in full-scale sludge, and was concluded to be an important organism contributing to P removal in EBPR plants. A survey carried out in six EBPR plants in Australia (Saunders et al., 2003) found that a good correlation was observed between the EBPR performance and the percentage of *Accumulibacter* present in the sludge. Methylene blue and Sudan Black B chemical staining were performed in this study to detect polyphosphate and PHA cycling, respectively, but cells displaying the PAO phenotype other than *Accumulibacter* were not observed. Through combining FISH with microautoradiography (FISH–MAR), Kong et al. (2004) found that the metabolism of *Accumulibacter* in three Danish EBPR plants correlated very well with the PAO phenotype as defined by the biochemical models proposed for PAOs. Some studies have noted that not all cells binding the FISH probes for *Accumulibacter* were observed to store polyphosphate (Zilles et al., 2002a; Wong et al., 2005), while poly-P granules have also been observed in other cells that are not *Accumulibacter* (Zilles et al., 2002a; He et al., 2005; Wong et al., 2005). This suggests that other PAOs may also have been present in these sludges. Wong et al. (2005) combined FISH with chemical staining in sludges displaying this effect, and with group-specific probes showed that most of the bacteria still belonged to the *Betaproteobacteria*, while lower, but still substantial percentages were *Actinobacteria*, *Alphaproteobacteria* and *Gammaproteobacteria*. Further studies focussed on the identification of other PAOs, the development of FISH probes to target these organisms, and their assessment as relevant organisms in full-scale EBPR plants is recommended.

2.1.3. *Are a group of Actinobacteria a new type of PAO?*

Many studies have previously reported the presence of *Actinobacteria* in EBPR systems, often in substantial numbers (Bond et al., 1999a; Kawaharasaki et al., 1999; Crocetti et al., 2000; Liu et al., 2001; Lee et al., 2002), but their relevance as P-removing bacteria had not been conclusively shown. A recent study by Kong et al. (2005) showed that two morphotypes of *Actinobacteria* (closely related to *Tetrasphaera*) were found to take up phosphate aerobically (but not anoxically), only after taking up organic matter such as amino acids anaerobically. However, these organisms could not take up short-chain fatty acids and their intracellular storage polymer (shown not to be PHA) was not identified. If these *Actinobacteria* are PAOs, they do not behave according to most biochemical models that have been proposed for PAOs, and thrive on different carbon sources that may be available in wastewater plants. FISH probes were developed for these particular *Actinobacteria*, and they were detected in high numbers (as high as 35% of *Bacteria*) in many of the 10 full-scale EBPR plants studied in Denmark (Kong et al., 2005). These organisms were particularly abundant in industrial wastewater plants, however, they have also been observed in domestic plants (Kong et al., 2005; Beer et al., 2006). Although the role of these bacteria in EBPR cannot be clarified at present, due to the lack of process performance data and information concerning its biochemical mechanism, further investigation is highly recommended.

2.2. *Identification of GAOs*

Failure of EBPR systems operated under seemingly favourable operational conditions has been widely reported in literature (Cech and Hartman, 1990, 1993; Liu et al., 1994; Satoh et al., 1994; Nielsen et al., 1999; Crocetti et al., 2002; Wong et al., 2004). Fukase et al. (1985) was the first to report the presence of organisms that can potentially compete with PAOs, by taking up VFA under anaerobic conditions and not accumulating polyphosphate under aerobic conditions. Cech and Hartman (1990; 1993) observed that cocci-shaped cells arranged in tetrads were abundant in systems fed with glucose and/or acetate, where carbon uptake was observed without phosphate release under anaerobic conditions. The organisms were initially called “G bacteria”. Subsequently, they have often become known as tetrad-forming organisms or TFOs (Tsai and Liu, 2002). The term GAO was proposed by Mino et al. (1995), and is defined as the phenotype of organisms that store glycogen aerobically and consume it anaerobically as their primary source of energy for taking up carbon sources and storing them as PHAs. These organisms do not always form a tetrad-type morphology, and thus the present definition of GAOs is based on their phenotype rather than their morphotype.

2.2.1. *Isolated organisms proposed as GAOs*

Culture-dependent methods have generated isolates with TFO morphology, similar to that observed in some EBPR systems. The isolate of the original ‘G-bacteria’ candidate of Cech and Hartman (1993) was identified as *Amaricoccus kaplicensis* (Maszenan et al., 1997), but was also reported as *Tetracoccus cechii* (Blackall et al., 1997). However, this organism has not been observed in other anaerobic/aerobic systems fed with acetate and glucose that have exhibited poor EBPR performance (Kong et al., 2001), and furthermore, the organism is not able to take up these substrates anaerobically (Falvo et al., 2001). Other isolated bacteria are phylogenetically diverse, and belong to the *Alphaproteobacteria* (*Amaricoccus* spp., and *Defluviicoccus vanus*), *Betaproteobacteria* (*Quadracoccus* sp.), *Gammaproteobacteria*, and *Actinobacteria* (*Tetrasphaera* spp., *Micropruina glycogenica* and *Kineosphaera limosa*) (Maszenan et al., 1997; Shintani et al., 2000; Kong et al., 2001; Hanada et al., 2002; Liu et al., 2002). Except for *M. glycogenica* (which was isolated from a lab-scale culture fed with glucose), none of the other isolates have been observed in abundance in either full- or lab-scale EBPR processes (Kong et al., 2001; Seviour et al., 2003). It has not currently been shown if *M. glycogenica* or any of the other isolates have the GAO phenotype.

2.2.2. *Gammaproteobacteria GAOs*

The first microbial characterisation of a GAO was carried out by culture-independent methods using DGGE of PCR-amplified

16S rRNA genes. From selected gel bands a novel group of the *Gammaproteobacteria* was identified (Nielsen et al., 1999). Two FISH probes for this novel group (Gam 1019, Gam 1278) were designed and FISH quantification showed that these coccoid cells represented 35% of the total bacterial population of this deteriorated biological P removal reactor.

Using a deteriorated EBPR sludge, Crocetti et al. (2002) generated a 16S rRNA gene clone library from which two new FISH probes were designed (GAOQ431, GAOQ989), which targeted bacteria belonging to the *Gammaproteobacteria*. These cloned sequences were in the same cluster reported by Nielsen et al. (1999). The GAO phenotype was clearly identified and the organisms were named "*Candidatus* Competibacter phosphatis" (henceforth referred to as *Competibacter* (Crocetti et al., 2002)). Kong et al. (2002b) carried out a phylogenetic analysis of 14 pre-existing 16S rRNA sequences (some obtained by Nielsen et al. (1999)) and 18 new sequences and observed a novel cluster with seven subgroups in the *Gammaproteobacteria*. They designed ten FISH probes specifically targeting the novel group at different hierarchical levels, GB, the most general, was divided into GB_G1 (identical to GAOQ989) and GB_G2, and finally GB_1–GB_7, for the seven subgroups. These bacteria exhibited cocci and rod morphologies and were widely distributed in sludge samples taken from nine lab- and full-scale EBPR systems (10–50% of total cells) and four conventional activated sludge systems (1–10%). Thus, *Competibacter* (also known as the GB lineage) can be targeted either through probe GB or a mixture of probes GAOQ989 and GB_G2. Either of these sets of probes will target the known diversity of *Competibacter*, which have frequently dominated lab-scale cultures fed with acetate (Crocetti et al., 2002; Kong et al., 2002b; Zeng et al., 2003d; Oehmen et al., 2004), and are often present in abundance in the biomass from full-scale plants (Crocetti et al., 2002; Kong et al., 2002b; Saunders et al., 2003; Gu et al., 2005; Wong et al., 2005; Kong et al., 2006). A high abundance of *Competibacter* in the sludge has also been observed to correlate well with a low P release to VFA uptake ratio (Saunders et al., 2003; Gu et al., 2005), suggesting that *Competibacter* actively compete with PAOs for anaerobic VFA uptake in full-scale plants.

2.2.3. *Alphaproteobacteria* GAOs

Recently, other groups of TFO GAOs belonging to the *Alphaproteobacteria* have been observed in lab-scale reactors. One such group related to the *Sphingomonadales* has been identified in lab-scale systems with poor P removal capacity (Beer et al., 2004). This study used DGGE and primers specifically designed to amplify DNA from *Alphaproteobacteria*. Retrieved sequences were used to design new FISH probes to target the abundant organism (SBR9-1a, SBR9-1b and SBR4). Two other distinct subgroups of *Alphaproteobacteria* hypothesised to be GAOs have since been identified, both related to *D. vanus* (Wong et al., 2004; Meyer et al., 2006) and displaying the TFO morphotype. Wong et al. (2004) found the organism in an acetate-fed anaerobic–aerobic membrane bioreactor with a deteriorated P removal performance, where the community was dominated by TFOs corresponding to the *Alphaproteobacteria* (85.0±7.0% of total cells). A 16S rRNA gene clone library specific for *Alphaproteobacteria* was prepared, and from four identical clones, two new probes (TFO_DF218, TFO_DF618) were designed. Most of the 16S rRNA gene clones were closely affiliated with *D. vanus*. Wong et al. (2004) found that these *Defluviicoccus*-related TFOs were not abundant in the different full-scale EBPR and non-EBPR plants that were tested.

Meyer et al. (2006) used rRNA-based stable isotope probing followed by full-cycle rRNA analysis to characterise an unidentified GAO highly enriched in a propionate fed lab-scale bioreactor. A new GAO belonging to the *Alphaproteobacteria* was found, which was also closely related to *D. vanus*. Phylogenetic analysis showed that the sequences related to *D. vanus* formed a monophyletic group with two distinct clusters. Cluster 1 included the sequences from Wong et al. (2004), while two new FISH probes (DF988, DF1020) were designed to target the new cluster 2. These microorganisms comprised 33% of all *Bacteria* in the original lab-scale reactor and were also detected in two other deteriorated EBPR lab-scale reactors (51 and 55% of *Bacteria*). The new group identified was also found in two full-scale treatment plants in Australia performing EBPR, although the abundance was low (<5%). In a study of *Defluviicoccus*-related organisms in 10 full-scale EBPR plants in Denmark, the organisms were generally detected in low abundance, although in some plants their population was similar or larger than *Accumulibacter* and *Competibacter*, comprising up to 9% of the total bacterial population (Burow et al., 2007). *Defluviicoccus*-related organisms were shown through FISH–MAR to take up carbon sources such as acetate and propionate anaerobically, suggesting that this group of organisms have the capacity to actively compete with PAOs for anaerobic carbon uptake in full-scale systems. *Sphingomonas*-related organisms were not observed in any of the plants that were tested in this study.

While *Alphaproteobacteria* GAOs have been observed now in numerous lab-scale systems, they have been found in only a small number of full-scale EBPR plants. Further investigation is necessary in order to determine their abundance and role in EBPR systems, as well as to clarify the presence of other potential GAO candidates. Other TFO belonging to the *Alphaproteobacteria* have been shown to exhibit the GAO phenotype, yet do not bind the currently available FISH probes for GAOs from the *Alphaproteobacteria* (Oehmen et al., 2006b). Additional study seems to be necessary in order to design other FISH probes to target GAOs within the *Alphaproteobacteria*, which could potentially affect the frequency that these organisms are detected in full-scale plants.

2.3. *New tools and future trends in PAO microbiology*

In recent years, it is generally recognised that this field requires advances concerning not only the identity and biodiversity of the microorganisms involved in EBPR systems, but also their biochemistry and metabolism (Mino et al., 1998; Blackall et al., 2002; Seviour et al., 2003). Beyond the taxonomy of PAOs, which is now routinely studied using rDNA- and rRNA-based methods, the next challenge is to obtain more information about the genetics and gene expression of the EBPR-related enzymes.

To this end, a near complete genome of *Accumulibacter* was recently obtained from two enriched EBPR sludges, one from the USA and the other from Australia (Martin et al., 2006).

Based on this metagenomic data, it was possible to analyse the presence or absence of genes encoding enzymes involved in important metabolic processes such as: the transport of polyphosphate through the cell membrane and its degradation/generation under anaerobic/aerobic conditions; the metabolic pathways for glycogen degradation and for the generation of additional reducing power necessary for anaerobic PHA production (see Section 3 for more detail); extracellular polymeric substances (EPS) production; denitrification and nitrogen fixation ability. This work opens up new research possibilities. Beyond the knowledge of the metabolic capabilities of *Accumulibacter*, it was also possible to analyse the flanking EBPR species and distinguish which functions could be attributed to *Accumulibacter* itself or to other members of the community. Furthermore, new isolation strategies may be derived from this new knowledge.

Genomics give information regarding the potential metabolism that can occur in a cell or population, as well as its phylogeny. However, the actual metabolic activity in a particular set of conditions is better assessed by studying the gene products, i.e., the transcribed mRNA or the proteins themselves. The application of this research approach in EBPR was first done by McMahon et al. (2002). This study identified the functional gene sequence (*ppk*) of the enzyme polyphosphate kinase (PPK), which is thought to be primarily responsible for poly-P synthesis in numerous bacteria. Four sequence types were obtained from an EBPR sludge highly enriched in *Accumulibacter*. The two dominant sequence types were found to have a high degree of similarity with the *ppk* amplified from *Rhodocyclus tenuis*, which is phylogenetically closely related to *Accumulibacter*, suggesting that they were from *Rhodocyclus*-related PAO. The expression of these *ppk* gene sequences was demonstrated by dot blot quantification of the mRNA transcribed from this gene in the sludge. These results showed the involvement of PPK in poly-P synthesis in EBPR. The expression of the *ppk* gene was further studied by Saunders (2005), who investigated the effect of extracellular phosphate concentration using quantitative reverse transcriptase PCR. It was concluded that the expression of *ppk* by *Accumulibacter* is inhibited at high concentrations of phosphate in the medium (>2 mM).

The global analysis of the products of gene expression (i.e. proteins) in an EBPR system was performed by Wilmes and Bond (2004) through a metaproteomic study of an *Accumulibacter*-enriched sludge. In this study, the entire proteome was extracted, purified and separated by 2D-polyacrylamide gel electrophoresis (PAGE), obtaining a protein map that characterises the microbial activity at a certain time and in a given environment. The most highly expressed proteins can then be excised and identified through mass spectrometry. Results obtained with EBPR sludge (enriched in *Accumulibacter*) and non-EBPR sludge gave clearly different proteomic maps, and some of the proteins involved in the PAO metabolism could be identified with this method (Wilmes and Bond, 2004, 2006).

The presently available 16S rRNA-targeted probes for *Rhodocyclus*-related PAOs were included in a microarray for the *Rhodocyclales* order (Loy et al., 2005). With a higher knowledge of the metagenomics of dominant populations of EBPR systems there may be new and more specific probes that can be designed, and thus new microarrays for PAOs can be developed, potentially including PAOs that belong to other bacterial groups. This would provide full-scale wastewater plants with a rapid tool for evaluating the EBPR potential of a sludge population.

3. Biochemistry

3.1. *Limitations of biochemical studies*

One limitation with many of the biochemical studies discussed below is the lack of characterization of the microbial population present in the culture that was used. In particular, it is likely that GAOs were present and active in a number of previous studies that have focussed on the biochemistry of PAOs (as suggested by low anaerobic P release to VFA uptake ratios), making the results more difficult to interpret. In studies focussed on determining the metabolic pathways of GAOs, the microbial competition between PAOs and GAOs is less problematic, since the microbial selection of GAOs can be ensured through the limitation of P in the feed.

In recent years, molecular methods have been implemented to detect and quantify PAO and GAO organisms through FISH, facilitating the correlation between the microbiology and the biochemistry of EBPR systems. Clearly, PAO and GAO metabolism is highly dependent on which microorganisms are present, thus no 'universal' biochemical model for either group of organisms is available. The determination of PAO biochemical pathways using highly enriched cultures of PAOs that contain few GAOs is highly recommended. Methods of achieving such highly enriched PAO cultures have been proposed (see Lu et al. (2006) and Section 4 for more details).

3.2. *Biochemical pathways of PAOs*

The vast majority of studies on EBPR metabolism employ acetate as the model carbon substrate, likely since it is generally the largest VFA species present in the influent of wastewater treatment plants (Cuevas-Rodriguez and Tejero-Monzon, 2003; Thomas et al., 2003; Ahn and Speece, 2006; Zeng et al., 2006). Most of the biochemical models agree on the fate of acetate and P conversions during the anaerobic phase. Once acetate is taken up by PAO cells, it is activated to acetyl-CoA, which is coupled with the consumption of ATP. ATP is largely generated from the transfer of an energy-rich phosphoric group from polyphosphate to ADP. The hydrolysis of polyphosphate produces cations, like K^+ and Mg^{2+}, and the $H_2PO_4^-$ anion that are released to the external medium. Two molecules of acetyl-CoA condense to form acetoacetyl-CoA, which is reduced by NADH to form 3-hydroxybutyryl-CoA. This last compound is polymerised forming poly-β-hydroxybutyrate (PHB). The major differences in the proposed EBPR metabolic pathways are related to the origin of reducing equivalents for PHA synthesis, the pathway for glycogen degradation (in the models where its utilisation is proposed) and on the transport of acetate across the cell membrane.

3.2.1. *Origin of reducing equivalents*

Initially, two biochemical models were proposed to describe the metabolic transformations of PAOs. The Comeau/Wentzel

model (Comeau et al., 1986; Wentzel et al., 1986) assumed that the reducing equivalents needed for the process were produced under anaerobic conditions by the tricarboxylic acid (TCA) cycle. Mino et al. (1987) proposed a new model in order to account for the experimental observation of variations in intracellular carbohydrates, decreasing under anaerobic conditions and increasing under aerobiosis. According to Mino et al. (1987), the TCA cycle was not operational under anaerobic conditions due to the accumulation of $FADH_2$, which is not used in PHB synthesis and can only be regenerated with an external electron acceptor. The reducing equivalents were believed to be produced from the consumption of internal carbohydrates (i.e. glycogen). Under anaerobic conditions, glycogen was assumed to be converted to pyruvate via the Embden–Meyerhoff–Parnas (EMP) pathway, producing NADH. Pyruvate was further converted to acetyl-CoA and CO_2. Smolders et al. (1994a) later developed a metabolic model based on the Mino model (see Section 6.1 for more detail), which supported the theory that reducing equivalents are generated through glycogen degradation.

Some new insights on the biochemistry of EBPR, concerning TCA and glycogen utilisation, came from the use of *in vivo* ^{13}C and ^{31}P nuclear magnetic resonance (NMR) by Pereira et al. (1996). Using this technique with 2-^{13}C acetate, several cyclic experiments (anaerobic/aerobic/anaerobic) were performed. It was confirmed that under anaerobic conditions, external acetate was mainly used for PHB production, and to a small extent for poly-β-hydroxyvalerate (PHV) production. Under aerobic conditions PHB/V were oxidised, and the labelled carbon was transferred to newly-formed glycogen. In the subsequent anaerobic phase, where unlabelled acetate was provided, the labelled glycogen decreased and was transferred mostly to the propionyl moiety of the new 3-hydroxyvalerate (3HV) formed. The propionyl-CoA was likely formed by way of the left branch of the TCA cycle operating backwards from oxaloacetate to succinyl-CoA and then to propionyl-CoA through the methylmalonyl-CoA pathway (also known as the succinate–propionate pathway), as proposed by Satoh et al. (1992). This study conclusively showed the involvement of glycogen in the EBPR process, and thus strongly supported the Mino model. However, the authors suggested that the full TCA cycle was also functional, due to the observation of a small fraction of dissolved CO_2 (i.e. bicarbonate) generated from the labelled acetate in the first anaerobic period, and also to the need of another source of reducing equivalents to balance the biochemical model. These authors proposed that both glycogen and the TCA cycle were used for the production of reducing equivalents, merging the two initial model schemes (see Fig. 1A).

Further investigations have been devoted to attempt to clarify the role of the TCA cycle in EBPR systems. Louie et al. (2000) used metabolic inhibitors to elucidate the biochemical pathways from an EBPR reactor, and proposed that PAOs employed a partially operating TCA cycle that proceeded through the glyoxylate shunt (Fig. 1B). The isocitrate formed in the TCA cycle would be converted to succinate and malate, via glyoxylate, bypassing the production of CO_2 and lowering the production of reducing equivalents. A problem that was not solved by this proposal was the regeneration of $FADH_2$ formed from the activity of the succinate dehydrogenase enzymes. The possibility that PAOs employ the split TCA cycle (Fig. 1C) anaerobically was also proposed (Pramanik et al., 1999; Hesselmann et al., 2000; Kortstee et al., 2000). In this case, the TCA could operate forwards from citrate to succinyl-CoA (right branch, oxidative) or backwards from oxaloacetate to succinyl-CoA (left branch, reductive). This split TCA cycle would serve to regulate the availability of reducing equivalents, since they are produced by the right branch and consumed by the left branch. The sink for succinyl-CoA would be its conversion to propionyl-CoA via the methylmalonyl-CoA pathway, producing CO_2 that can be further used for the conversion of pyruvate to oxaloacetate, and resulting in 3HV formation.

Clearly, numerous pathways have been proposed to generate the reducing equivalents necessary for anaerobic PAO metabolism. While one possibility is that some of these aforementioned pathways exist only in specific microbial groups of PAOs, another possibility is that these organisms could contain multiple or all pathways. Martin et al. (2006) determined that the genes necessary for glycolysis, and the operation of either the full TCA cycle (Fig. 1A) or the split TCA cycle (Fig. 1C) were present in *Accumulibacter*. If multiple pathways are in fact present in PAOs, it is possible that different metabolic pathways are employed depending on their internal or surrounding environmental conditions. For example, it has been observed in some lab- and full-scale EBPR sludges that glycogen was utilised to a lower extent than was predicted by the metabolic models, suggesting that PAOs might have an increased dependence on the TCA cycle for balancing their internal redox balance (Schuler and Jenkins, 2003b; Pijuan, 2004). Erdal et al. (2002a, b, 2005) have observed different biochemical pathways employed in EBPR reactors operated at different temperatures, where the glyoxalate cycle (Fig. 1B) was active at 5 °C and the split TCA cycle (Fig. 1C) was functional at 20 °C. Further investigation is necessary to clarify if PAOs and/or GAOs are able to change their metabolic pathways based on their internal or external conditions.

3.2.2. *Glycogen utilisation*

Contrasting experimental evidence supporting either the Entner–Doudoroff (ED) or EMP pathways for glycogen degradation has been observed. According to Mino et al. (1987), glycogen was converted under anaerobic conditions to pyruvate via the EMP pathway, producing reducing equivalents. A modification to the Mino model was introduced by Wentzel et al. (1991), where glycogen hydrolysis was believed to occur via the ED pathway, assuming at the time that the organisms responsible for P removal was *Acinetobacter* sp., which did not possess the EMP pathway. The ED pathway generates less energy (i.e. ATP) from glycogen hydrolysis, which would require a higher degradation of polyphosphate.

Using solid state ^{13}C NMR, Maurer et al. (1997) observed in experiments with 1-^{13}C acetate that the labelling of glycogen (carbons 3 and 4) gave rise to a label on carbon 4 of 3-hydroxybutyrate (3HB) under anaerobic conditions. This labelling is a validation that the ED pathway was occurring in the sludge, since via the EMP pathway, carbons 3 and 4 remain oxidised and are converted to CO_2, thus they do not appear in the newly formed PHA polymer. The ED pathway

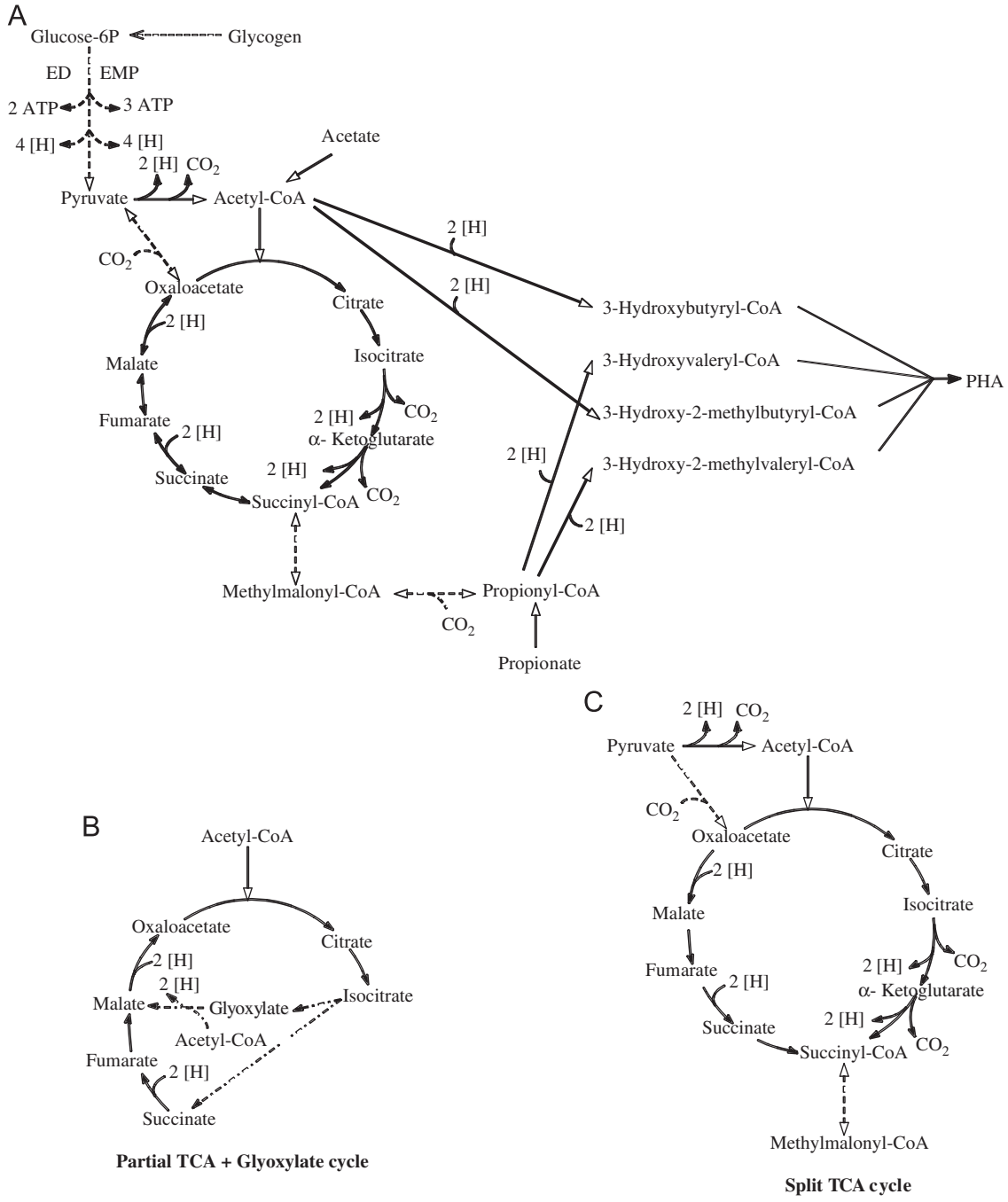

Fig. 1 – The proposed anaerobic metabolic pathways of PAOs for the production of reducing equivalents and precursors for PHA synthesis. Glycolysis has been proposed to be combined with either the full TCA cycle (A), the glyoxylate shunt (B) or the split TCA cycle (C) to regulate the redox balance in PAOs. [H] represents NADH.

was later observed by Hesselmann et al. (2000), where ^{13}C NMR studies with 1-^{13}C acetate were performed in conjunction with enzymatic studies for a culture primarily dominated by a single Gram-negative bacterium (>50% DAPI count) belonging to the *Betaproteobacteria* (Hesselmann et al., 2000). Pramanik et al. (1999) presented a model where the metabolic flux employed by PAOs through theoretical biochemical pathways (obtained from a database of known pathways) was calculated using linear optimisation. The results of this flux-based model suggested that glycogen degradation proceeded through a combination of the ED pathway and the pentose phosphate pathway.

In some studies, however, there are evidences that glycogen degradation proceeds through the EMP pathway. Erdal (2002b) tested the activity of phosphofructokinase, a key enzyme of the EMP pathway, in two EBPR systems operated at different temperatures (5 and 20 °C). They observed the activity of this enzyme, while the activity of glucose-6-P-dehydrogenase, an enzyme for both the ED and pentose phosphate pathways, was not detected. Furthermore, a metagenomic study of two

enriched cultures of *Accumulibacter* (Martin et al., 2006) showed that *Accumulibacter* contained all EMP genes, while the key genes of the ED pathway were not present. One possible explanation for these contrasting results concerning the pathway that PAOs employ for glycogen degradation is the presence of different bacteria in the microbial communities of these different studies. However, it is surprising that differing results were obtained in the studies of Hesselmann et al. (2000) and Martin et al. (2006), where in both cases the sludges were likely dominated by *Accumulibacter*. Another possible explanation is that there are multiple strains of *Accumulibacter* that display different metabolic behaviours. Further research is needed in order to clarify this issue.

3.2.3. *Transport mechanism for VFA uptake*

The mechanism for acetate transport into PAO cells and the energy required for this process has also been studied. The Comeau/Wentzel model (Comeau et al., 1986; Wentzel et al., 1986) assumed that the model substrate, acetate, was transferred into the cell by passive diffusion under anaerobic conditions. Conversely, Mino et al. (1987), proposed an active transport mechanism for acetate by PAOs, which was also suggested by Smolders et al. (1994a). The energy requirement for active transport was assumed to be obtained through the hydrolysis of ATP from polyphosphate. Smolders et al. (1994a) demonstrated that the acetate uptake rate was independent of the external pH value, while the P release rate increased with pH. It was hypothesised that the increased energy consumption at a higher pH was due to the increased energy requirement for acetate transport through the cell membrane, supporting the mechanism of active transport.

Saunders (2005) studied in detail the anaerobic VFA uptake mechanism by PAOs at the membrane transport level from a highly enriched culture of *Accumulibacter* (>90%). Using specific chemicals, processes that may be involved in the transport of acetate under anaerobic conditions were selectively inhibited. It was found that uncoupling of the proton motive force (PMF) by *m*-chlorophenylhydrazone (CCCP) substantially inhibited the rate of acetate uptake by PAOs (this rate decreased by 64%), and concluded that acetate transport into PAO cells was primarily driven by energy contained in the PMF, i.e. by secondary transport. Although one potential method of PMF generation is by the export of protons through the ATPase enzyme, no significant decrease was found in the acetate uptake rate when the ATPase was inhibited by *N*-*N*′-dicyclohexylcarboiimide (DCCD). Another known mechanism for PMF generation is through the efflux of phosphate and a proton through the Pit transporter. This mechanism was proposed to be the primary method that PAOs transport acetate through the cell membrane.

3.2.4. *Aerobic metabolism*

The aerobic biotransformations of PAOs are similar in all of the proposed models, where catabolism proceeds through the TCA cycle. The degradation of PHB and PHV would lead to acetyl-CoA and propionyl-CoA, the former entering the TCA at the oxaloacetate level and the latter entering at the succinyl-CoA level. Both are used as the carbon and energy source for biomass growth, while a portion of the ATP is used for external phosphate uptake and regeneration of polyphosphate. According to the Mino model, and later models based on it, some carbon and energy are also used for the replenishment of glycogen.

3.2.5. *Propionate metabolism*

Wastewater contains a much more diverse mixture of substrates other than acetate and investigations were conducted with other compounds, alone or in mixtures, including: propionate, butyrate, valerate, isovalerate, formate, lactate, malate, pyruvate, glucose, citrate, succinate, glutamate, and aspartate (Comeau et al., 1987; Satoh et al., 1992; Carucci et al., 1994; Satoh et al., 1996, 1998; Lemos et al., 1998; Louie et al., 2000; Oehmen et al., 2004; Pijuan et al., 2004a). In all cases, with the exception of glutamate, the consumption of these substrates gives rise to the production of PHA with different compositions. According to Comeau et al. (1987), PHV production was significantly higher than that of PHB when a substrate with an odd number of carbon atoms was fed to the activated sludge.

Recent research has increasingly focussed on the use of propionate as carbon source, due to its abundance in many plants and its impact on the PAO–GAO competition (see Section 4 for more detail). The metabolism of propionate by an enriched PAO culture was studied by Lemos et al. (2003) using *in vivo* NMR. The fate of labelled 3-^{13}C-propionate was monitored during anaerobic/aerobic/anaerobic cycles from a PAO culture that was enriched with a mixture of VFAs (acetate, propionate and butyrate). The PHA composition produced from propionate was comprised of a high 3HV fraction, as well as 3-hydroxy-2-methylvalerate (3H2MV), 3-hydroxy-2-methylbutyrate (3H2MB) and a very low amount of 3HB. The labelling observed on some 3HV carbons showed that a small amount of propionyl-CoA was converted to acetyl-CoA, via the methylmalonyl-CoA pathway to succinyl-CoA, which was followed by the left branch of the TCA, further converted to pyruvate and finally to acetyl-CoA. The labelling pattern observed in acetyl-CoA demonstrated the operation of succinate dehydrogenase under anaerobic conditions, which allowed for the conversion of succinate to fumarate. However, the regeneration of $FADH_2$ produced through this pathway was not definable, since it is not directly utilised in PHA synthesis. Nevertheless, other authors have also proposed this metabolic reaction under anaerobic conditions in EBPR systems, where an inhibitor of succinate dehydrogenase, malonate, was shown to correlate well with a decreased PHA production, supporting the activity of this enzyme (Louie et al., 2000). The involvement of glycogen in the metabolism of propionate was clearly demonstrated, where acetyl-CoA was preferentially derived from glycogen, generating the reducing equivalents needed for PHA synthesis. The potential contribution of the right branch of the TCA in the production of NADH was unclear.

Since acetyl-CoA and propionyl-CoA are generated through propionate metabolism by PAOs, the resulting PHA polymer (see Fig. 1) can be comprised of 3HB (from 2 acetyl-CoA molecules), 3HV (from one acetyl-CoA and one propionyl-CoA), 3H2MB (an isomer of 3HV), and 3H2MV (from 2 propionyl-CoA). The PHA composition depends on whether PAOs selectively or randomly condense acetyl-CoA and propionyl-CoA to form PHA. In selective condensation,

acetyl-CoA preferentially binds with propionyl-CoA, forming PHV. Since propionyl-CoA is generated in higher abundance than acetyl-CoA by PAOs through propionate uptake, the remaining propionyl-CoA condenses to form PH2MV, while little PHB is produced. In random condensation, the PHA composition would consist of a lower PHV fraction and a higher PH2MV and PHB fraction as compared with selective condensation. While literature studies have shown that all four PHA fractions may be produced by PAOs from propionate uptake (Satoh et al., 1992; Lemos et al., 2003; Oehmen et al., 2005c), these studies have also observed that PHB is only generated in very low abundance. Furthermore, using metabolic modelling techniques (see Section 6.1 for more detail), Oehmen et al. (2005c) found that the PHA composition produced from propionate uptake by an enriched PAO culture (containing 63% *Accumulibacter*) correlated very strongly with the theoretical stoichiometry associated with selective PHA condensation, and not with random condensation. This finding has also been supported by Lu et al. (2006) for a very highly enriched *Accumulibacter* culture (>90% of *Bacteria*). These findings support the hypothesis that PAOs tend to selectively condense acetyl-CoA and propionyl-CoA in PHA formation.

3.3. Biochemical pathways of GAOs

3.3.1. Origin of reducing equivalents for GAOs

In EBPR systems, GAO can compete with PAOs for the uptake of carbon substrates. Carbon metabolism by GAOs under anaerobic conditions has been proposed to be similar to that by PAOs, where carbon is taken up and converted to PHA with reducing equivalents provided primarily through glycogen metabolism. The main difference is based on their source of energy, since GAOs do not cycle polyphosphate, unlike PAOs. Glycogen is believed to be the primary source of energy for GAOs, and is utilised to a much larger extent in these microorganisms as compared to PAOs. Due to the higher glycogen demand required to produce ATP, reducing equivalents are produced in excess of those required for reduction of acetyl-CoA to PHB. In order to maintain the redox balance inside the cells, NADH must be consumed, which was proposed via the reduction of pyruvate to propionyl-CoA (Liu et al., 1994; Satoh et al., 1994). Pyruvate, an intermediate of glycolysis, would enter the left branch of the TCA at the oxaloacetate level, be converted to succinyl-CoA and further converted to propionyl-CoA through the methylmalonyl-CoA pathway. Thus, when consuming acetate, GAOs produced both acetyl-CoA and propionyl-CoA. This led to another major difference between PAOs and GAOs; PAOs produce mainly PHB when fed with acetate, with little PHV production (generally <10% (C-mol basis): Satoh et al., 1992; Smolders et al., 1994a; Mino et al., 1998) while GAOs produce approximately 75% PHB and 25% PHV (C-mol basis) when fed with acetate (Filipe et al., 2001b; Zeng et al., 2003c), with only a very small fraction of PH2MV produced (Satoh et al., 1994; Filipe et al., 2001b). While the activity of the left branch of the TCA cycle has been shown to consume NADH in GAOs, it is currently unclear if the full TCA cycle is also responsible for the generation of some reducing power, for the cases of either acetate or propionate as carbon sources. There has clearly been a lesser research focus on GAO metabolism when compared with PAOs.

Structured metabolic models describing the biochemical transformations of GAOs under anaerobic and aerobic conditions have been proposed for the cases of acetate and propionate uptake (Filipe et al., 2001b; Zeng et al., 2003c; Oehmen et al., 2006b: see Section 6.1 for more detail).

3.3.2. Glycogen utilisation by GAOs

Anaerobically, the hypothesised pathway for glycolysis is the EMP (Satoh et al., 1994), which has been supported through the observation that glucose-6-phosphate dehydrogenase, an enzyme of the ED pathway, was not active in a GAO-enriched sludge (Filipe et al., 2001b). However, Lemos et al. (unpublished results) recently utilised *in vivo* NMR with acetate labelled on positions 1 or 2 in order to clarify the proposed metabolism of GAOs. A lab-scale SBR was used in these experiments, comprised of 82% GAOs (consisting of 46% *Candidatus* Competibacter phosphatis and 36% *D. vanus*-related GAO, with <1% *Accumulibacter*) as quantified by FISH. The NMR experiments consisted of an anaerobic/aerobic/anaerobic cycle. From experiments where 2-^{13}C-acetate was fed it was observed that both PHB and PHV were produced in abundance. The 3HV fraction that was formed was mainly from glycogen, but it was also partially produced from externally labelled acetate. A small fraction of 3HB was also made from the stored glycogen. Evidence of the operation of the ED pathway came from comparing the labelling of 3HV between the first and the second anaerobic phase. Glycogen produced during the aerobic phase, from labelled PHA, became labelled at positions 1, 2, 5 and 6. Since glycogen was the only source of labelled carbon in the second anaerobic phase, its degradation through the ED pathway resulted in the newly formed 3HV becoming more highly labelled at carbon positions 3–5 (with the enrichment of 4 and 5 each being three times larger than 3) when compared with the initial anaerobic period. When the cells were fed with 1-^{13}C-acetate, the glycogen formed during the aerobic phase was labelled at carbon positions 1, 3, 4 and 6 (from chloroform extracts). This labelling pattern suggested that aerobic glycogen synthesis proceeded through gluconeogenesis, with partial carbon cycling through the ED pathway.

3.3.3. Transport mechanism for VFA uptake by GAOs

Saunders (2005) also studied the anaerobic VFA uptake by GAOs and the mechanism of transport through the membrane, in a similar way as was performed for PAOs. They observed that GAOs generate PMF by a combination of the efflux of protons through the ATPase, at the expense of ATP, and the reductive TCA cycle enzyme, fumarate reductase. Interestingly, the acetate uptake rate of GAOs was substantially reduced when the ATPase activity was inhibited, but no such reduction in acetate uptake rate could be observed when the ATPase activity was inhibited in the case of PAOs.

3.3.4. Aerobic metabolism of GAOs

The aerobic metabolism of GAOs is similar to that of PAOs, except that there is no poly-P accumulation. The oxidation of both PHB and PHV produces the energy required for biomass growth, glycogen replenishment as well as cell maintenance

(Filipe et al., 2001b; Zeng et al., 2003c). Lemos et al. (unpublished results) suggested that aerobic glycogen synthesis proceeds through gluconeogenesis, with partial carbon cycling through the ED pathway (see Section 3.3.2).

3.3.5. *Propionate metabolism by GAOs*

Similarly to PAOs, the main difference between the metabolism of acetate and propionate by GAOs is the PHA composition that is formed. While GAOs produce mainly PHB and PHV with acetate, PHV and PH2MV are the primary PHA fractions with propionate uptake (Oehmen et al., 2006b). Oehmen et al. (2006b) found that the PHA composition produced by GAOs correlated more strongly with a metabolic model predicting the random condensation of acetyl-CoA and propionyl-CoA. This is in contrast to PAO metabolism, where a higher correlation has been observed with the selective condensation of acetyl-CoA and propionyl-CoA. Notably, the microbial community in the sludge was dominated by *Alphaproteobacteria*, while *Competibacter* were not present. *Competibacter*-dominated cultures have almost exclusively been enriched with acetate as the sole carbon source, and have been observed to take up propionate at very slow rates as compared to acetate (Oehmen et al., 2004: see Section 4 for more detail).

4. The competition between PAOs and GAOs

Successful operation of the EBPR process depends on numerous process operational factors. Process upsets and the deterioration of P removal in EBPR plants can be explained by such disturbances as the presence of nitrate in the anaerobic zone (Kuba et al., 1994), potassium and/or magnesium limitation (Brdjanovic et al., 1996; Pattarkine and Randall, 1999), over-aeration due to e.g. excessive rainfall (Brdjanovic et al., 1998b) and the microbial competition of GAOs with PAOs (Thomas et al., 2003). Lately, factors affecting the PAO–GAO competition have been the focus of many studies, since eliminating unwanted GAOs from full-scale plants can often be a difficult task, and effective control strategies preventing their growth are only beginning to be developed (Filipe et al., 2001c; Oehmen et al., 2005b; Lu et al., 2006). Since the microbial identities of different groups of PAOs and GAOs have only recently been found, and indeed, other unknown PAOs or GAOs may exist, assessing the microbial dynamics in EBPR systems has been a recent development that is still expanding.

It is well known that one factor affecting the PAO–GAO competition is the ratio of organic carbon to P in the influent, or the so-called COD/P ratio. Numerous studies have found that a high COD/P ratio (e.g. >50 mgCOD/mgP) in the wastewater feed tends to favour the growth of GAOs instead of PAOs (for a review see e.g. Mino et al. (1998)). Thus, a low COD/P ratio (e.g. 10–20 mgCOD/mgP) should be more favourable to the growth of PAOs. On the other hand, a sufficient amount of VFAs has to be provided in order to achieve good P removal. Other factors may also influence the microbial competition in EBPR systems, such as the carbon source, pH and temperature.

4.1. *The effect of carbon substrates*

Different carbon sources, VFAs and non-VFAs have been shown to have an impact on the PAO–GAO competition. The most prevalent VFA in EBPR plants is acetate, though in plants where prefermentation is employed, propionate is often present in substantial quantities (25–45 wt%, see: Cuevas-Rodriguez and Tejero-Monzon, 2003; Thomas et al., 2003; Ahn and Speece, 2006; Zeng et al., 2006). Butyrate, valerate and other VFAs may also be present, but typically in small quantities. Non-VFA organic substrates (e.g. amino acids, sugars) are also present in wastewater, and some may be metabolised by PAOs or GAOs. Most previous studies focused on EBPR systems have used acetate as the sole carbon source. Recently, however, there has been an increased interest in the impact of propionate and other substrates on EBPR performance.

While the use of acetate as a carbon source in EBPR systems has been often documented to yield robust and stable P removal performance, there are also many reported occasions where the P removal deteriorated due to what is believed to be microbial competition of GAOs with PAOs. As shown in Table 2, a high abundance of *Accumulibacter* with little or no GAOs (as determined by FISH targeting previously identified GAOs) has been observed in some cases with acetate as the sole carbon source, where good P removal performance was also achieved (Hesselmann et al., 1999; McMahon et al., 2002; He et al., 2006). However, the proliferation of GAOs has also been frequently observed with this carbon source, under otherwise similar operational conditions in many cases (see Table 2). The GAO population quantified by FISH can also be reflected in the chemical data, where a low ratio of P release/VFA uptake is correlated with a low abundance of *Accumulibacter* and a high GAO population, as well as an incomplete level of P removal. Fig. 2 illustrates the correlation between the abundance of *Accumulibacter* in the sludge and the P release/VFA uptake ratio for many of the studies presented in Table 2 (those operated at a nearly neutral pH). While the P release to VFA uptake ratio is influenced by many factors, such as the pH and carbon source, it is clear that the abundance of PAOs and GAOs also has a strong effect on this ratio. It has been suggested that the relative activity of PAOs and GAOs can be estimated through analysis of the P release to VFA uptake ratio (Saunders et al., 2003; Schuler and Jenkins, 2003a).

Recent studies have suggested that propionate may be a more favourable substrate than acetate for successful EBPR performance (Thomas et al., 2003; Chen et al., 2004; Oehmen et al., 2004; Pijuan et al., 2004b; Chen et al., 2005; Oehmen et al., 2006a). Thomas et al. (2003) has shown that the addition of molasses into the prefermenter of a full-scale EBPR plant resulted in a high level of propionate production, which was hypothesised to be responsible for the improved P removal performance observed in the plant as compared with the direct addition of acetic acid. On numerous occasions, superior P removal performance has been reported with propionate as compared with acetate during long-term enrichment studies at the laboratory scale (Hood and Randall, 2001; Chen et al., 2004, 2005; Oehmen et al., 2005a, 2006a). These studies suggest that a propionate feed source may

Table 2 – Experimental conditions and results from some lab-scale studies where the sludge was fed with acetate as sole carbon source, and the microbial population was characterised using FISH

Study	FISH quantification[a]			COD/P in feed (mgCOD/mgP)	Anaerobic Prel/VFAup (Pmol/Cmol)	Effluent P concentration (mgP/L)	PH		Temperature
	Accumulibacter	*Competibacter*	*Alphaproteobacteria*				Anaerobic	Aerobic	
Hesselmann et al. (1999)	81%	<2%	<3%	43	0.33	<0.5	7.35±0.05		20 °C
Nielsen et al. (1999)	Present	35%	Present	13	0.13	60	6.8–7.1		22 °C
Crocetti et al. (2002)	2%	92%	<2%	22	0.03	12	7.0±0.2		22±2 °C
Kong et al. (2002a)	20%	<1%	Present	10	0.32	<0.5	7.1–7.3	Uncontrolled	21±1 °C
McMahon et al. (2002)	80%	N/A	N/A	14	0.66	<0.5	7.0–7.3		23±3 °C
Zeng et al. (2003b)	41%	N/A	N/A	27	0.32	<0.5	6.95±0.1		18–22 °C
Beer et al. (2004)	N/A	N/A	71%	50	0.05	<0.5	7.1–7.3	Uncontrolled	21±1 °C
Oehmen et al. (2004)	65%	24%	N/A	10	0.50	3	7.0±0.1		20–24 °C
Saito et al. (2004)	30–40%	50–60%	N/A	27	0.21	9	7.4±0.1		20–25 °C
Wong et al. (2004)	<5%	<5%	85%	10	~0	90	8.0±0.1		N/A
Oehmen et al. (2005a)	14%	54%	N/A	15	0.10	40	7.0±0.1		20–24 °C
Oehmen et al. (2005a)	15%	23%	N/A	15	0.36	10	7.8±0.1	8.0±0.1	20–24 °C
He et al. (2006)	80%	N/A	N/A	14	N/A	N/A	7.0–7.3		N/A
Jobbagy et al. (2006)	8–48%	1–18%	10–18%	15–20	N/A	0.6–2.1	7.6–7.8	8.3–8.6	20–23 °C
Oehmen et al. (2006a)	3–64%	33–70%	N/A	15	0.06–0.53	<0.5–50	7.0±0.1		20–24 °C
Pijuan et al. (2006)	50%	<1%	N/A	9	0.29	<0.5	7.0±0.1		25 °C
Wong and Liu (2006)	<5%	2–60%	2–85%	10	~0	N/A	8.0±0.1		N/A

N/A = not available (not reported).

[a] Quantification performed with respect to all *Bacteria*. It should be noted that various FISH probes for PAOs and GAOs (listed in Table I) were used during FISH analysis in these studies.

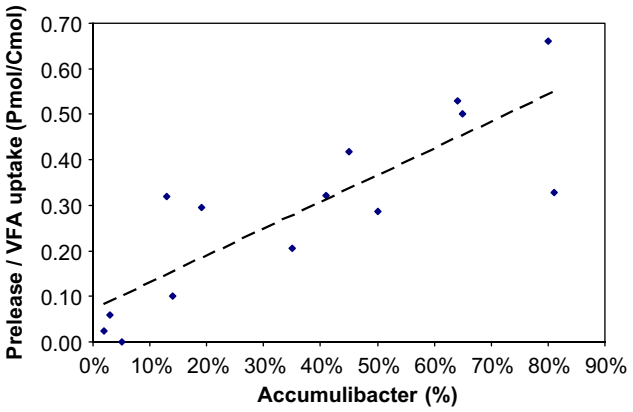

Fig. 2 – Correlation between the P release/VFA uptake ratio and abundance of *Accumulibacter* in the biomass.

provide an advantage to PAOs over GAOs, which has been supported through examining the biomass by FISH. In lab-scale reactors fed with propionate as the sole carbon source, *Accumulibacter* has often been observed to dominate the bacterial community, while *Competibacter* were not present in the biomass (Pijuan et al., 2004a,b; Oehmen et al., 2005c, 2006a). Furthermore, enriched cultures of *Accumulibacter* were able to readily switch between acetate and propionate uptake at similar rates (Oehmen et al., 2004; Pijuan et al., 2004b), while an enriched culture of *Competibacter* fed with acetate as the carbon source consumed propionate at <5% of the acetate uptake rate (Oehmen et al., 2004). It should be noted that using FISH–MAR on full-scale sludge samples, *Competibacter* were found to be capable of taking up propionate (Kong et al., 2006). However, propionate uptake was not observed in many cells, thus Kong et al. (2006) suggested that either not all members of this bacterial community are able to take up propionate, or that the amount of substrate taken up was too low in some cases to be detected by MAR. In contrast, they found that acetate uptake was observed in the vast majority of *Competibacter* cells, thus supporting the hypothesis that these bacteria more easily take up acetate than propionate. Overall, the evidence found thus far strongly suggests that *Competibacter* are less competitive with PAOs for propionate uptake as compared to acetate uptake.

Although a propionate carbon source has been hypothesised to be effective in selecting against *Competibacter*, another group of GAOs, members of the *Alphaproteobacteria*, have been observed to actively take up propionate at substantial rates (Oehmen et al., 2005b, 2006b; Meyer et al., 2006) and have the capacity to compete with PAOs for propionate, leading to the deterioration of P removal performance (Oehmen et al., 2005a; Meyer et al., 2006). *Alphaproteobacteria* GAOs have also been observed to take up acetate (Beer et al., 2004; Wong et al., 2004; Oehmen et al., 2005b), though it has been shown that these bacteria may be more efficient in propionate uptake as compared to acetate uptake (Oehmen et al., 2005b; Dai, 2006). Dai (2006) found that an enriched culture of *Defluviicoccus*-related organisms (95% of all *Bacteria*) that were enriched with acetate as the sole carbon source and displayed the GAO phenotype, took up propionate at a faster rate than acetate when the sole carbon source was switched from acetate to propionate. Furthermore, these GAOs preferentially took up propionate over acetate when both carbon sources were present in the media. Interestingly, these bacteria were shown to anaerobically consume glycogen at similar rates when either acetate or propionate was fed as the sole carbon source. This likely explains their preference for propionate over acetate, as proposed by Dai (2006), since according to the metabolic models proposed for GAOs (Filipe et al., 2001b; Zeng et al., 2002; Oehmen et al., 2006b) a lower amount of glycogen is required for propionate uptake as compared to acetate uptake. Thus, GAOs can maximise their PHA content through maximising propionate uptake, which can be used to maximise their growth in the subsequent aerobic phase (Dai, 2006).

These observations have raised questions concerning the potential effectiveness of a propionate carbon source in providing PAOs with a selective advantage over GAOs. Nevertheless, most studies reported thus far have shown improved P removal performance with a propionate carbon source. While the reason for this remains unclear, one possible explanation is due to the slow rate of propionate uptake by *Competibacter*, and thus one group of GAOs that are commonly observed in EBPR systems are unable to compete with *Accumulibacter* for propionate. On the other hand, both *Competibacter* and *Alphaproteobacteria* GAOs may potentially compete with PAOs for acetate uptake. Therefore, a propionate carbon source may be more advantageous than acetate since a narrower range of GAOs is able to compete with *Accumulibacter* for propionate.

Since *Accumulibacter* have little preference towards acetate or propionate uptake, unlike *Competibacter* and *Alphaproteobacteria* GAOs, the simultaneous presence of acetate and propionate may be more beneficial for PAOs than GAOs. Some studies have incorporated both carbon sources in the feed (Satoh et al., 1992; Lemos et al., 1998; Liu et al., 2001; Levantesi et al., 2002; Onda et al., 2002; Thomas et al., 2003; Chen et al., 2004, 2005), in some cases suggesting beneficial results towards the growth of PAOs and/or a higher level of P removal. Further study concerning the effects of a combined acetate/propionate feed on the PAO–GAO competition is necessary. This approach could be promising for practical application, since both substrates are often produced from prefermenters and the acetate/propionate fraction can be controlled through adjusting the operational conditions of the prefermenter (Thomas et al., 2003; Ahn and Speece, 2006; Zeng et al., 2006). The carbon source preferences observed by PAOs and GAOs has also led to the development of a control strategy whereby the sole carbon source in the feed is periodically alternated between acetate and propionate (Lu et al., 2006). Using this strategy, *Competibacter* and *Alphaproteobacteria* GAOs were almost completely eliminated from the biomass, and very highly enriched *Accumulibacter* cultures were repeatedly obtained, which was correlated with a highly stable level of P removal. Regularly alternating the carbon source appears to be a feasible method of eliminating GAOs for the purposes of lab-scale studies, though suitable modification of this strategy (such as the manipulation of prefermenter operating conditions as described above) would likely be required for practical implementation in full-scale plants.

VFAs other than acetate and propionate (such as butyrate, lactate, valerate and isovalerate) can also be taken up by PAOs and/or GAOs (Liu et al., 1996b; Randall et al., 1997; Lemos et al., 1998; Hood and Randall, 2001; Levantesi et al., 2002). The effect of these substrates on the PAO–GAO competition is currently unclear, though some reports have suggested that isovalerate may be a beneficial carbon source for EBPR systems, leading to a high level of P removal (Randall et al., 1997; Hood and Randall, 2001). It has also been reported in some cases that the uptake rate of butyrate was slower than that of acetate or propionate (Lemos et al., 1998; Levantesi et al., 2002). One complication in these studies is that most of these acids (and other organics, see below) can be quite rapidly converted to acetate and/or propionate in a mixed microbial culture, therefore, raising questions concerning which compounds are actually consumed by the PAOs and/or GAOs. Although clarification of the role of other VFAs on the PAO–GAO competition would be of some interest, it is only of practical relevance in situations where these substrates are present in abundance, which is not generally the case in most EBPR plants.

The most widely studied carbon substrate other than VFAs, has been glucose. Successful EBPR operation has been achieved in some studies fed with glucose as the carbon source (Carucci et al., 1999; Sudiana et al., 1999; Jeon and Park, 2000; Wang et al., 2002). However, it is commonly believed that glucose is fermented to VFAs such as acetate and propionate prior to substrate uptake by PAO (Wentzel et al., 1991; Sudiana et al., 1999; Canizares et al., 2000), which is supported by observations using FISH–MAR for *Accumulibacter* (Kong et al., 2004). The deterioration of EBPR systems has frequently been observed when glucose is fed as the carbon source (Cech and Hartman, 1990, 1993; Tasli et al., 1997; Carucci et al., 1999; Tsai and Liu, 2002), where TFOs (or G-bacteria) have proliferated. It is believed that external glucose can replace internally stored glycogen and serve as the energy source and reducing power for PHA accumulation, reducing the dependency on poly-P and thus selecting for GAOs over PAOs (Mino et al., 1998). However, it has been suggested that *Competibacter*, like *Accumulibacter*, are unable to assimilate glucose directly, and rely on the consumption of fermentation products when fed with this carbon source (Kong et al., 2006). In contrast, glucose has been observed to be directly taken up by *Defluviicoccus*-related organisms (Burow et al., 2007).

Many other non-VFA substrates have been tested (e.g. amino acids, sugars) and have been shown to be able to be taken up anaerobically, by different groups of organisms proposed as PAOs or GAOs (Liu et al., 1996b; Satoh et al., 1996, 1998; Chua et al., 2004; Kong et al., 2004, 2005, 2006). Kong et al. (2005) have found using FISH–MAR that a group of *Actinobacteria* were able to take up amino acids anaerobically and perform aerobic P removal, though it is currently unclear which amino acids were taken up by these bacteria. Interestingly, acetate was not taken up by this group of *Actinobacteria*, and further investigation is necessary regarding the carbon sources that they store and use for P removal. The abundance of these carbon sources in wastewater systems is largely unknown, although they seem to be particularly abundant in industrial wastewater systems.

4.2. *The effect of pH*

Many studies have shown that a higher ambient pH in enriched PAO sludges has resulted in a higher anaerobic P release (Smolders et al., 1994a; Liu et al., 1996a; Bond et al., 1999b; Filipe et al., 2001d). Smolders et al. (1994a) found that the ratio of anaerobic P release to acetate uptake varied linearly from 0.25 to 0.75 P-mol/C-mol when pH rose from 5.5 to 8.5. The reason for this variation was explained as follows: under the assumption that the internal pH of the cell is kept constant, there is an increased pH gradient and a corresponding increase in electrical potential difference across the cell membrane at a high ambient pH. Therefore, more energy is needed for acetate transport through the membrane when external pH is high. This increased energy is generated through an increase in polyphosphate degradation. However, the acetate uptake, glycogen degradation and PHA accumulation rates of PAOs have been shown in batch tests to be independent of pH over the range 6.5–8.0 (Filipe et al., 2001d), indicating that the higher energy requirements to take up acetate does not negatively affect their ability to metabolise VFA. Aerobically, a series of batch tests has shown that P uptake, PHA utilisation and biomass growth were all inhibited by a low pH (6.5), and suggested that a higher aerobic pH (7–7.5) would be more beneficial for PAOs (Filipe et al., 2001a).

To the contrary, the acetate uptake rate of GAOs has been reported in short-term tests to decrease with increasing pH, and is accompanied by an increase in anaerobic glycogen consumption and PHV accumulation (Filipe et al., 2001b). This suggests that a higher pH not only results in a higher energy demand for acetate uptake, but also negatively affects the ability of GAOs to take up acetate. This is likely explained by the fact that PAOs have poly-P as an extra energy source as compared to GAOs, which they expend to meet this higher energy demand. It has been postulated that an anaerobic pH of 7.25 is a critical point, whereby GAOs are able to anaerobically take up VFA faster than PAOs below a pH of 7.25, and PAOs take up acetate faster above this pH value (Filipe et al., 2001c). An improved level of P removal has been observed when the anaerobic pH setpoint was increased from 6.8 to 7.25 (Filipe et al., 2001c). Other studies have also shown higher P removal when the anaerobic and/or aerobic pH level was increased (from $\leqslant$7 to 7.5–8.5) (Bond et al., 1999a; Jeon et al., 2001; Schuler and Jenkins, 2002; Serafim et al., 2002). The reason for the improved performance was hypothesised to be from a shift in the microbial competition from GAOs to PAOs. This hypothesis has been supported through assessing population changes in the microbial community. Zhang et al. (2005) found that the deterioration of P removal accompanied a change in pH from 7.0 to 6.5, while a clear shift in the microbial community structure was simultaneously observed through examination of 16S rRNA clone libraries developed at each pH. The clone library at pH 7.0 consisted mainly of *Betaproteobacteria*, *Alphaproteobacteria* (including *Defluviicoccus*-related clones), *Bacteriodetes/Chlorobi* and *Actinobacteria*. At pH 6.5, the clone library consisted primarily of *Gammaproteobacteria*, *Alphaproteobacteria* (including *Sphingomonas*-related organisms), *Bacteriodetes/Chlorobi* and *Betaproteobacteria*. Furthermore, in a study of two lab-scale reactors, one fed with acetate and the other with propionate, the acetate

reactor was shown to be dominated by *Competibacter* and the propionate reactor by *Alphaproteobacteria* GAOs, when the pH was controlled at 7 (Oehmen et al., 2005a). A clear increase in the ratio of *Accumulibacter* PAOs to GAOs was observed in both systems after the pH was increased to ~8, while the P removal simultaneously increased in both reactors. Interestingly, however, GAOs were not completely eliminated from either bioreactor at high pH, and the propionate reactor exhibited superior P removal performance to the acetate reactor.

Clearly, the results show that pH strongly influences the PAO–GAO competition, and an increase in pH can improve P removal performance by selecting for PAOs over GAOs. This finding may be a very useful control strategy for improving P removal in EBPR systems and confirmation of the above lab-scale results in full-scale plants is recommended. Naturally, excessive pH can be detrimental to EBPR process performance, and there is clearly an upper pH limit where this control strategy will no longer be effective. In some studies, a decrease in the VFA uptake, P release and P uptake rates have been observed at pH values above 8.0 (Liu et al., 1996a; Schuler and Jenkins, 2002; Oehmen et al., 2005a). It should also be noted that the fraction of P removed via biologically induced chemical precipitation increases with increasing pH (Maurer et al., 1999).

4.3. *The effect of temperature*

Temperature also appears to be a factor that has an impact on the PAO–GAO competition. In EBPR systems, a lower temperature has been observed to decrease the rates of biochemical transformations (e.g. P release/uptake, acetate uptake, PHA oxidation, growth), as is the case in most biological reactions (Brdjanovic et al., 1998a). However, successful EBPR operation has been observed at very low temperatures, even 5 °C (Brdjanovic et al., 1998a), though a higher sludge age was necessary at low temperatures due to the decrease in the kinetics of the process at low temperatures. Moreover, low temperatures have been found to improve EBPR performance in some lab-scale studies (Whang and Park, 2002; Erdal et al., 2003; Panswad et al., 2003). This improved performance has been hypothesised to be due to a shift in the microbial community from GAOs to PAOs. Panswad et al. (2003) found that the rate of P release increased with increasing temperature from 20 to 35 °C, while the rate of P uptake decreased. Furthermore, they calculated the mass fraction of PAOs and GAOs through assessing the changes in the ratio of P release to VFA uptake, assuming that PAO stoichiometry is independent of temperature as has been observed previously (Brdjanovic et al., 1997, 1998a). An increase in the fraction of GAOs and decrease in the fraction of PAOs was concluded with an increase in temperature from 20 to 35 °C, which correlated well with a decrease in the P content in the sludge. It is also noteworthy that Panswad et al. (2003) concluded that a substantial portion of GAOs were present in the sludge (28–51% mass fraction) even at 20 °C. Whang and Park (2002) showed that an SBR exhibited good EBPR performance at 20 °C, while another SBR operated at 30 °C exhibited a lower level of P release and uptake with higher anaerobic acetate uptake. A higher level of TFOs was observed in the sludge at 30 °C, suggesting an increased GAO proliferation at higher temperature. Erdal et al. (2003) found a higher P removal capacity and a corresponding decrease in glycogen transformations from biomass acclimatised to 5 °C as compared to 20 °C, and suggested that the low temperature favoured the growth of PAOs over GAOs. They also suggested that PAOs were able to dominate the system at 5 °C through changing their metabolic pathway. While Brdjanovic et al. (1998a) also observed a change in microbial community structure at different temperatures, their finding that PAO stoichiometry was independent of temperature contrasts with those of Erdal et al. (2003, 2005).

The experimental evidence obtained thus far suggests that GAOs tend to become stronger competitors with PAOs at higher temperatures. This implies that competition by GAOs with PAOs in EBPR plants may be more problematic in warm climates, and during the summer months.

4.4. *Other factors affecting the PAO–GAO competition*

The effects of other operational factors on the PAO–GAO competition have been less widely studied. Some studies have suggested that a low sludge age favours PAOs over GAOs (e.g. Rodrigo et al., 1999; Whang and Park, 2006), however, further investigation would be valuable to corroborate these results.

The dissolved oxygen (DO) concentration has been hypothesised to impact the PAO–GAO competition (Griffiths et al., 2002). In this study, the DO concentration was adjusted in numerous full-scale wastewater plants, resulting in changes in the process performance. The abundance of PAOs and TFOs in the sludge (assessed by examining the anaerobic/aerobic cycling of poly-P and PHA through chemical staining) suggested a general, yet indefinitive correlation with DO concentration and plant performance. Poor P removal performance and a high number of TFOs were more frequently observed at very high DO concentrations of 4.5–5.0 mg/L, while DO concentrations of approximately 2.5–3.0 mg/L seemed to correlate with a greater abundance of PAO. Additionally, Lemaire et al. (2006) have observed an increase in the abundance of *Accumulibacter* and a decrease in *Competibacter* during SBR operation at a very low DO level (approximately 0.5 mg/L). Further study is necessary in order to clarify the impact of DO concentration on the PAO–GAO competition. This is particularly relevant as there appears to be a general trend to operate full-scale BNR plants at lower DO concentrations (0.5–1.5 mg/L) than in the past to both reduce aeration costs and improve the nitrogen removal through simultaneous nitrification/denitrification (SND).

It has been observed that aerobic and anoxic P uptake is inhibited by the presence of nitrite (Kuba et al., 1996b; Saito et al., 2004). Furthermore, Saito et al. (2004) have observed that an increase in the *Competibacter* population coincided with the accumulation of nitrite in the anoxic phases, and suggested that it may be a factor that provides GAOs an advantage over PAOs. They also found that the growth rate of PAOs was also inhibited during periods where nitrite was present. Thus, it seems that the presence and accumulation of nitrite inhibits PAOs, thereby favouring the growth of GAOs.

The effects of other factors such as chemical precipitants (de Haas et al., 2001; Tykesson, 2005), and the presence of

inhibitory compounds (Rayne et al., 2005) could potentially have an impact on the PAO–GAO competition, and requires further study.

5. Process operation

Recent advances have been made in relation to the development of novel processes incorporating both P and nitrogen removal. The ability of PAOs to denitrify is a key factor in many EBPR process designs, due mainly to the reduced levels of oxygen and carbon sources that are needed for simultaneous denitrification and P removal. This can lead to savings in plant operational costs.

5.1. *Denitrifying P removal*

Organic carbon sources are necessary to achieve denitrification and EBPR. Often, denitrification proceeds under conditions where both nitrate (i.e. anoxic conditions) and soluble organic carbon sources are present. It is widely accepted that the presence of nitrate in the anaerobic phase of an EBPR system is detrimental to P removal, due to the competition of VFA uptake by PAOs with ordinary denitrifiers (Barker and Dold, 1996). However, when nitrate and PHA-rich sludge are present simultaneously (i.e. when the anoxic zone follows the anaerobic phase), simultaneous denitrification and P removal can be achieved. Denitrifying P removal occurs due to the capacity of at least a fraction of PAOs to use nitrate and/or nitrite as an electron acceptor for P removal instead of oxygen (Kerrn-Jespersen and Henze, 1993; Kuba et al., 1993). The energy production efficiency of PAOs with nitrate seems to be about 40% lower than with oxygen (Kuba et al., 1996a), and, therefore, the rate of P uptake by PAOs under anoxic conditions is generally lower than under aerobic conditions. However, substantially less COD is required for this process as compared to separate P and N removal (Kuba et al., 1996c), which, together with the savings in aeration resulting from the use of nitrate instead of oxygen, make denitrifying P removal economically very attractive. In addition, the lower energy generation efficiency of DPAOs leads to a 20–30% lower cell yield, resulting in lower sludge production (Kuba et al., 1994). It should also be noted that at least a fraction of GAOs have also been found to be able to denitrify (DGAOs), similarly to PAOs (Zeng et al., 2003e).

While nitrite can have a negative effect on the EBPR process by inhibiting the metabolism of PAOs (see the Section 4), it has also been demonstrated that DPAOs can use nitrite as an electron acceptor up to concentrations of 5–10 mgN/L after acclimatisation (Meinhold et al., 1999; Lee et al., 2001).

5.1.1. *Are PAOs and DPAOs the same organisms or not?*

Contrasting results has been found in the literature in relation to the identity of the microorganisms responsible for simultaneous denitrification and P removal. Kerrn-Jespersen and Henze (1993) observed through anaerobic–anoxic–aerobic batch tests that the anoxic P uptake rate gradually diminished until it essentially ceased, but once aeration commenced the P uptake rate increased, to a higher level than was observed under anoxic conditions. These results suggested that the fraction of the PAO population able to utilise either nitrate or oxygen as electron acceptor (DPAOs) took up P until their PHA pools were depleted. In the aerobic phase, another PAO fraction that was able to use only oxygen (non-DPAOs) was able to take up P, since their PHA was not consumed anoxically. Similar assays carried out by Meinhold et al. (1999) supported these results. Further tests carried out by Ahn et al. (2002) showed that sludge acclimatised for 30 days to anaerobic plus aerobic or anoxic conditions had a much lower P uptake capacity when the other electron acceptor (nitrate or oxygen, respectively) was supplied.

The use of molecular tools was employed in the attempt to characterise the microorganisms involved in denitrifying P removal. Based on 16S rRNA-based polymerase chain reaction (PCR)–DGGE, Ahn et al. (2002) demonstrated that three reactors had different population structures when operated with electron acceptors of oxygen, nitrate, and oxygen together with nitrate, respectively. The DGGE band pattern differed among the three reactors, which were seeded from the same inoculum. However, one of the bands that were common to all reactors, which could have come from bacteria that could use either oxygen or nitrogen as electron acceptor, was sequenced and found to be closely related to *Rhodocyclus* spp. This result was confirmed by the high abundance of cells that bound the PAO846 FISH probe, suggesting that *Accumulibacter* represented a considerable fraction of the microbial population regardless of the electron acceptor employed. This result is in agreement with Zeng et al. (2003b), who detected high numbers of *Accumulibacter* with FISH in two SBR operated with either nitrate or oxygen as the electron acceptor. In the latter study, sludge acclimatised to anaerobic–aerobic conditions required a short adaptation period in order to be able to use nitrate as electron acceptor, while sludge acclimatised to anaerobic–anoxic conditions readily took up P in the presence of oxygen. A study performed by Kong et al. (2004) using FISH–MAR demonstrated that *Accumulibacter* were able to take up phosphate with simultaneous denitrification, corroborating these results.

The nearly complete genome of *Accumulibacter* (Martin et al., 2006), did not contain the gene *nar* for respiratory nitrate reductase, although the genes responsible for nitrite reduction to nitrogen were detected. Based on these results, it was hypothesised that nitrate reduction in simultaneous N and P removing systems is not carried out by *Accumulibacter* but by flanking species, which would supply *Accumulibacter* with the necessary nitrite for P removal under anoxic conditions.

However, recent studies on the phylogeny of *Accumulibacter*, based on 16S rRNA gene plus internally transcribed spacer region clone libraries, showed the existence of sub-clades within the *Accumulibacter* group (He et al., 2006). In a recent study, two SBRs were operated under anaerobic–aerobic conditions and gradually acclimatised to anaerobic–anoxic conditions (Carvalho et al., 2006), where the sole carbon source fed to the SBRs was acetate and propionate, respectively. In these systems, similar results to Kerrn-Jespersen and Henze (1993) were obtained in anaerobic–anoxic–aerobic batch tests, suggesting the presence of two different groups of PAOs (DPAOs and non-DPAOs). A high abundance of *Accumulibacter* was found in both reactors, similarly to the results of

Zeng et al. (2003b), although different cell morphotypes were observed. After acclimatisation to denitrifying conditions, *Accumulibacter* with rod-type morphology was abundant in the microbial community of the propionate-fed SBR, which displayed greater denitrifying capacity. In contrast, the cocci morphology was prevalent in the *Accumulibacter* community of the acetate-fed reactor, which ultimately lost its ability to anaerobically take up VFA and perform P cycling during acclimatisation to anaerobic–anoxic conditions. It was hypothesised that DPAOs may be linked to the rod-type morphology and non-DPAOs to the cocci-type morphology, where both organisms are *Accumulibacter*. While this hypothesis has yet to be confirmed, it may explain the seemingly contrasting results that have been observed in previous studies. It is possible that the *Accumulibacter* group contains multiple sub-groups with different phenotypic characteristics (Carvalho et al., 2006; He et al., 2006), such as the ability to denitrify.

5.2. *BNR process configurations*

The processes designed for nitrogen and P removal involve recirculating the sludge through anaerobic, anoxic and aerobic zones. In the anaerobic stage, VFA is taken up and stored as PHA by PAOs, which is accompanied by P release. The biomass is then exposed to anoxic conditions if denitrifying P removal is desired. Finally, the production of the necessary nitrate takes place by oxidation of the ammonia in aerobic conditions, where P uptake also takes place. These three basic steps have been combined in different arrangements, as illustrated in Fig. 3 and described in Table 3. While not all processes involving EBPR are discussed here, many of the processes primarily implemented in full-scale treatment plants are presented, as well as recent trends towards novel process developments.

5.2.1. *EBPR processes with pre-denitrification*

"Classical" processes such as the A_2O, University of Cape Town (UCT) and five-stage Bardenpho processes (Tchobanoglous et al., 2002) are based on pre-denitrification, i.e., the anoxic phase is located prior to the nitrifying aerobic stage. High internal mixed liquor recirculation is, therefore, required from the aerobic into the anoxic section in order to provide the PHA-rich PAO sludge (from the anaerobic stage) with the necessary NO_x for denitrifying P removal. In the A_2O process (Fig. 3A) (Tchobanoglous et al., 2002), the return activated sludge (RAS) is recycled to the beginning of the anaerobic stage. The drawback of recycling all of the sludge to the anaerobic zone is that the NO_x generated in the aerobic stage from nitrification is introduced directly into the anaerobic stage, which can then promote the utilisation of VFA for denitrification, instead of being stored as PHA by PAOs. The ISAH (Institute of Sewage and Waste Management Technology of the University of Hanover: see Henze et al., 1997) process has a similar configuration to the A_2O process, where the main difference is that the recycled sludge is first denitrified in a separate anoxic tank, thus avoiding this problem.

The UCT process (Ekama et al., 1984) differs from the A_2O process in that it has the RAS recycled to the anoxic phase (instead of the anaerobic phase), while an additional recirculation flow from the end of the anoxic to the beginning of the anaerobic phase is provided for increased carbon utilisation and biomass recycling. The modified UCT process (Fig. 3B) has two separate anoxic zones. The first zone only receives the low concentration of nitrate existing in the RAS, which is quickly denitrified. The mixed liquor recycled into the anaerobic zone comes from the end of the first anoxic zone and, therefore, still contains substantial organic carbon but little nitrate. The recycling stream from the aerobic zone goes to the main anoxic zone for denitrification. The Virginia Initiative Plant (VIP: Tchobanoglous et al., 2002) is a process configuration very similar to the UCT, with modifications to the RAS recycle stream and the mixed liquor recycle from the anoxic zone.

The five-stage Bardenpho process (also known as Johannesburg: see Tchobanoglous et al., 2002) offers another means of minimising the undesirable presence of NO_x in the anaerobic zone, through the incorporation of a second anoxic phase for denitrifying the residual NO_x, using stored carbon (Fig. 3C). A final aerobic phase is also included in this process to promote the stripping of N_2 gas from the wastewater and also to optimise P uptake. A similar strategy is employed in the BioDenipho and modified oxidation ditch processes (Henze et al., 1997), which consist of an anaerobic zone followed by alternating anoxic and aerobic zones. A unique feature of these configurations is the interconnected nature of the anoxic and aerobic zones, where the mixed liquor is continuously cycled through both zones, eliminating the need for an internal recycle. Effluent is continuously discharged to the clarifier, typically from the aerobic zone, while the recycled sludge is fed back to the anaerobic zone.

5.2.2. *EBPR processes with post-denitrification*

Other processes have been developed for BNR based on post-denitrification. This reduces the need for mixed-liquor recirculation (as described in most of the processes above) and promotes the use of sequencing batch reactors (SBRs). However, when the same sludge is submitted to a sequence of anaerobic–aerobic–anoxic conditions, the intracellular PHA required for P removal, which was stored during the anaerobic stage, risks being depleted aerobically for P uptake, leaving the anoxic phase with a low level of carbon for denitrification. In order to avoid this waste of electron donor, post-denitrification systems are typically operated with two sludges (Table 3), where the anaerobic–anoxic P removal sludge bypasses the aerobic stage, containing the nitrifying biomass. Examples of this system are the DEPHANOX process (Bortone et al., 1996: see Fig. 3D) and the A_2N process (Kuba et al., 1996c). In the DEPHANOX system, after the initial anaerobic phase, the supernatant is separated from the sludge in an intermediate settling step, and it is then supplied to a separate tank containing nitrifying bacteria grown on fixed-film. An additional aerobic tank (or phase) may also be required at the end of post-denitrification processes if insufficient P removal is achieved by DPAOs. The final aerobic phase can also strip-out residual N_2 gas as well as promote the conversion of PHA into glycogen, which will be necessary in the subsequent anaerobic zone. Two-sludge systems require lower recirculation costs, but involve more settlers

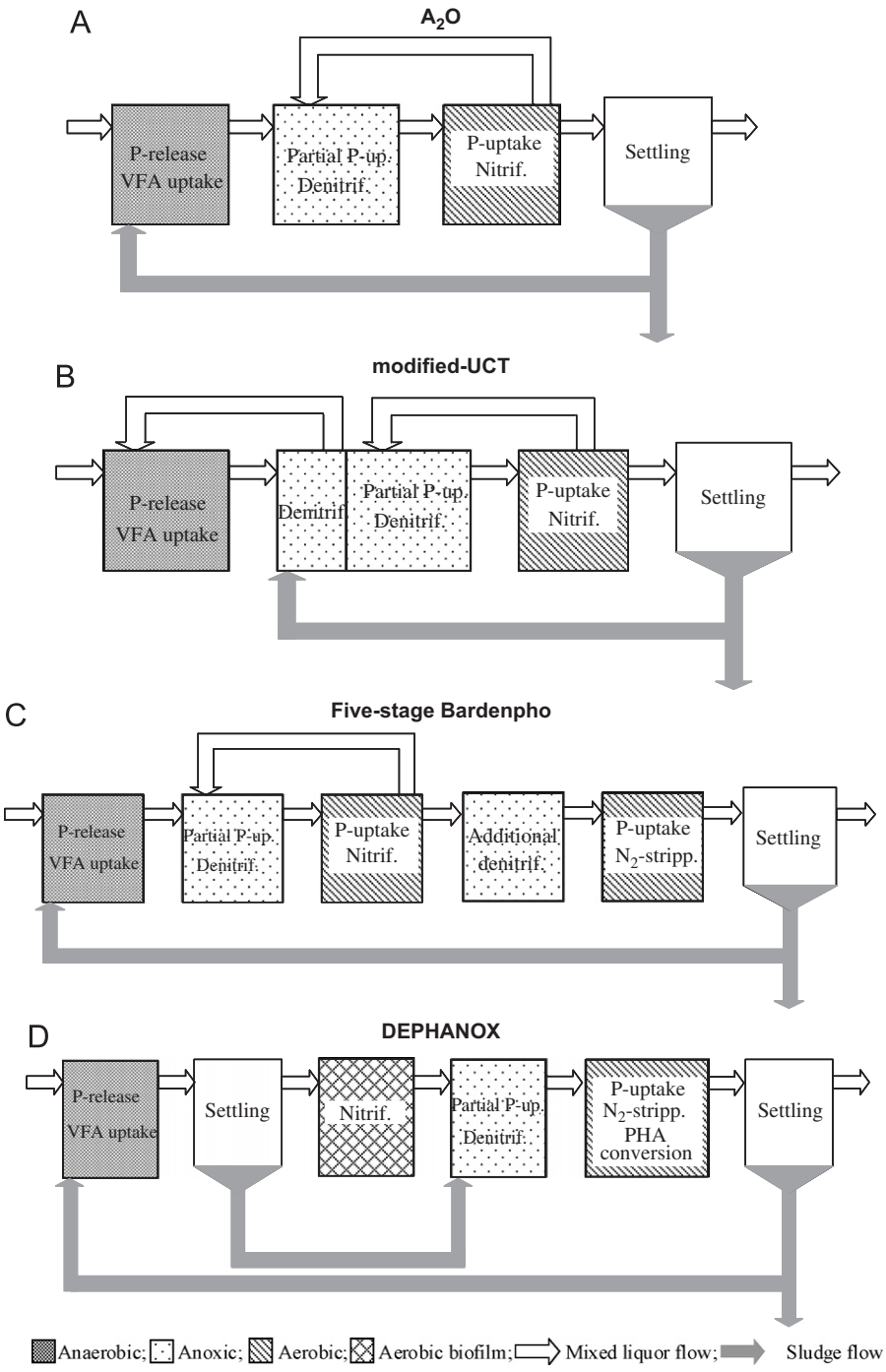

Fig. 3 – Process diagrams for combined biological phosphorus and nitrogen removal. The expected chemical transformations are described in each step.

than the single sludge systems. The number of settlers can be reduced with the use of two SBRs, one anaerobic–anoxic and another aerobic (Kuba et al., 1996c), or one SBR and a biofilm nitrifying system (Bortone et al., 1994).

5.2.3. *EBPR processes combined with chemical precipitation*

In some P removal processes, EBPR is combined with chemical precipitation. In the PhoStrip process (Tchobanoglous et al., 2002), a portion of the RAS is diverted to an anaerobic stripping tank, where P is released. The sludge is then returned to the biological process and P is removed from the supertanant by chemical precipitation. Another process developed and applied in several WWTP in The Netherlands is the BCFS (biological and chemical P and nitrogen removal: van Loosdrecht et al., 1998). This process is similar to the modified-UCT (Fig. 3B) with an integrated "P-stripping" system to deviate part of the effluent of the anaerobic phase into a chemical P removal process when the biological P removal efficiency is not sufficient. An additional area where chemical precipitation is often integrated with the EBPR

Table 3 – Processes for biological phosphorus and nitrogen removal

Process	Pre-denitrification	Post-denitrification	Single sludge	Two sludges	References
A_2O	X		X		Tchobanoglous et al. (2002)
UCT	X		X		Tchobanoglous et al. (2002)
Five stage Bardenpho	X		X		Tchobanoglous et al. (2002)
BioDenipho	X		X		Henze et al. (1997)
BCFS	X		X		van Loosdrecht et al. (1998)
DEPHANOX		X		X	Bortone et al. (1996)
A_2N		X		X	Kuba et al. (1996c)
MBR	X	X	X		Lesjean et al. (2002)
Orbal	Simultaneous nitrif. and denit.		X		Zilles et al. (2002b)
SNDPR	Simultaneous nitrif. and denit.		X		Zeng et al. (2003a)

process is during sludge treatment (often by anaerobic digestion). Under anaerobic conditions, stored polyphosphate can be hydrolysed and released, which when recycled back to the activated sludge process, results in an increase in the P load (Popel and Jardin, 1993). P precipitation as magnesium ammonium phosphate (struvite) can be implemented in this stream, simultaneously reducing the P concentration and recovering it in the form of a potential fertiliser (Booker et al., 1999; Britton et al., 2005; Hao and van Loosdrecht, 2006). This technology is currently being implemented in some full-scale wastewater plants (Ueno and Fujii, 2001; Battistoni et al., 2006).

5.2.4. *Recent trends and developments in EBPR*

The use of membrane bioreactors (MBR) for P and N removal has been tested by Lesjean et al. (2002). Both pre- and post-denitrification processes were adapted to MBRs with similar P removal efficiency, although post-denitrification achieved better N-removal. This process configuration offers advantages in reducing the effluent suspended solids, which is particularly valuable when strict effluent standards are required. EBPR has also been achieved in a biofilm process, however, achieving controlled removal of waste activated sludge (critical for the physical removal of P from the process) is difficult and has thus far prevented wider application (Rogalla et al., 2006).

Other process configurations have been designed to achieve EBPR in conjunction with simultaneous nitrification and denitrification. The Orbal process (Zilles et al., 2002b) and the simultaneous nitrification, denitrification and P removal (SNDPR) process (Zeng et al., 2003a) are among these designs. In the Orbal process, an initial aerated-anoxic phase ($DO < 0.1$ mg/L) provides the conditions for simultaneous nitrification and denitrification, along with P release. P uptake then occurs in the subsequent aerobic phase. The SNDPR process has an initial anaerobic phase, with P release and VFA uptake, followed by an aerobic phase with $DO = 0.5 \pm 0.1$ mg/L, where nitrification and denitrification occur via nitrite, simultaneous to P uptake. This system was successfully operated in bench-scale SBRs, but has not yet been implemented in larger-scale plants. The use of granular sludge has gained increasing interest for achieving simultaneous nitrogen and P removal (Meyer et al., 2005). It seems that SNDPR is favoured by the oxygen gradient observed through the microbial aggregates, which promotes nitrification on the surface of the granules (in the presence of oxygen) while denitrification occurs in the centre of the granules (in the presence of NO_x and absence of oxygen). Lab-scale experiments using granular sludge have demonstrated that stable P and nitrogen removal can be achieved in granules supplied with both synthetic and real wastewater (Dulekgurgen et al., 2003; Cassidy and Belia, 2005; de Kreuk et al., 2005; Lemaire et al., 2006). Moreover, a slow anaerobic substrate feeding rate has been found to improve granule stability by enhancing the growth of slow growing bacteria such as PAOs (de Kreuk et al., 2005). Granular sludge also presents the advantage of improving sludge settleability, thereby requiring smaller settling tanks, resulting in economic advantages.

6. Process modelling

Two different types of models, namely activated sludge models (ASM) and metabolic models, have been used to describe the EBPR process. Both types of models consist of sets of stoichiometric and kinetic expressions that describe the biochemical transformations of the process. One key difference between the two types of models is that the yield coefficients in metabolic models are derived theoretically through substrate, energy and reducing power balances, minimising the need for site-to-site parameter calibration. ASM and metabolic models have also been integrated together to simulate the behaviour of full-scale EBPR plants.

6.1. *Metabolic modelling*

Since neither PAOs nor GAOs have been obtained in pure culture, metabolic models have been proposed through defining the reaction stoichiometry using assumed biochemical pathways (see Section 3). A set of reactions is thus established to describe the metabolism, each with known stoichiometry but unknown reaction rate. The consumption and/or production rate of each compound involved in the reactions either as an initial substrate (e.g. acetate), a final

product (e.g. PHAs) or an intermediate (e.g. ATP and NADH) is then expressed as a function of the rates of the reactions involving this compound with the use of the reaction stoichiometry established. By assuming that the reaction intermediates including ATP and NADH do not accumulate in bacterial cells (i.e. their production rates are equal to their consumption rates), the degree of freedom is reduced so that the substrate consumption and product formation rates are expressed with a minimum number of independent reaction rates. The theoretical stoichiometry is based on the yield coefficients for these independent reaction rates, while the reaction kinetics are also modelled, usually using Monod-type kinetics.

The yield coefficients established above are typically verified by measuring the consumption rates of a set of substrates and the production rates of a set of products. When the number of measured rates is larger than the number of independent reaction rates (see above), equations involving only known stoichiometric coefficients and measured variables are obtained, forming independent checks of the theoretical stoichiometry. At least one such equation is needed. The more equations there are, the more comprehensive the verification. The measured variables for verifying metabolic models of PAOs and GAOs have typically been the rates of VFA consumption, glycogen consumption/regeneration, PHA production/consumption, phosphate release/uptake, oxygen consumption and ammonium uptake (Smolders et al., 1995; Filipe et al., 2001b, d; Yagci et al., 2003). In some cases, the rates of CO_2 and/or proton production have also been used (Smolders et al., 1994a; Zeng et al., 2003c; Oehmen et al., 2005c, 2006b).

A summary of the metabolic models that have been proposed to describe the EBPR process under various conditions is given in Table 4. Smolders et al. (1994a) first developed a metabolic model to describe the anaerobic metabolism of acetate by PAOs, based on the Mino model (see Section 3). The energy required for the active transport of acetate across the cell membrane was described with parameter α, which was illustrated to be dependent on the ambient pH. Metabolic models were later developed to describe the anaerobic metabolism of GAOs with acetate (Filipe et al., 2001b; Zeng et al., 2002), where the primary differences between PAOs were that a portion of the glycogen was degraded through the reductive branch of the TCA cycle and the methylmalonyl-CoA pathway (see Section 3), leading to PHV production, and poly-P was not used as an energy source. Oehmen et al. (2005c, 2006b) adapted the PAO and GAO metabolic models developed for acetate to describe the case of a propionate carbon source.

In each of these aforementioned studies, the proposed metabolic pathways were shown to describe the experimental data very well. Yagci et al. (2003) developed a metabolic model to describe acetate uptake by mixed PAO and GAO cultures. The glyoxalate pathway was assumed for PAOs (see Section 3), and the succinate that is formed was assumed to be converted to propionyl-CoA via the methylmalonyl-CoA pathway, leading to PHV production. Yagci et al. (2003) found that this model correlated very well with experimental results.

The aerobic biochemical processes of PAOs have been described in a metabolic model developed by Smolders et al. (1994b). The yield coefficients for bacterial growth, glycogen generation and phosphate uptake with respect to the

Table 4 – Previously developed metabolic models for describing the activity of PAOs and/or GAOs

Description	PAO	GAO	Anaerobic	Anoxic	Aerobic	Carbon Source	Publication
Model of the anaerobic stoichiometry of PAOs and the influence of pH	x		x			Acetate	Smolders et al. (1994a)
Model of the aerobic stoichiometry of PAOs	x				x	Acetate	Smolders et al. (1994b)
Combined anaerobic/aerobic stoichiometry and kinetics of PAOs	x		x		x	Acetate	Smolders et al. (1995)
Stoichiometric and kinetic model of DPAOs	x			x		Acetate	Kuba et al. (1996a)
Integration of the anaerobic/aerobic andanaerobic/anoxic models	x		x	x	x	Acetate	Murnleitner et al. (1997)
The effect of pH on the stoichiometry and kinetics of PAOs	x		x			Acetate	Filipe et al. (2001d)
Model of the anaerobic stoichiometry and kinetics of GAOs, and the influence of pH		x	x			Acetate	Filipe et al. (2001b), Zeng et al. (2002)
Anaerobic stoichiometric PAO model with glucose	x		x			Glucose	Wang et al. (2002)
Combined anaerobic/aerobic stoichiometry and kinetics of GAOs		x	x		x	Acetate	Zeng et al. (2003c)
Combined PAO and GAO anaerobic model	x	x	x			Acetate	Yagci et al. (2003), Zeng et al. (2003d)
Anaerobic stoichiometric PAO model with propionate	x		x			Propionate	Oehmen et al. (2005c)
Anaerobic/aerobic stoichiometric GAO model with propionate		x	x		x	Propionate	Oehmen et al. (2006b)

consumption of PHB was determined as a function of the respiration efficiency (δ, also known as the P/O ratio), in moles of ATP produced per mole of NADH oxidised. The values of the yields were determined through experimental determination of δ. Zeng et al. (2003c) developed a metabolic model to describe the aerobic metabolism of GAOs. The main differences of the aerobic GAO model are that the reactions of P uptake and polyphosphate accumulation are excluded, while the reactions describing growth and glycogen replenishment required modification. This is due to the fact that the PHA produced by GAOs from acetate uptake consists of both PHB and PHV, as opposed to just PHB as in the case of PAOs. Under aerobic conditions, this leads to the formation of both acetyl-CoA (fraction λ) and propionyl-CoA (fraction β)· In the model, it was assumed that the ratio between acetyl-CoA and propionyl-CoA used for catabolism, glycogen synthesis and biomass growth are all equal to λ:β·

Furthermore, Kuba et al. (1996a) developed a metabolic model to describe denitrification, anoxic P uptake, growth and glycogen replenishment by DPAOs. Combined metabolic models describing the anaerobic/aerobic (Smolders et al., 1995) or anaerobic/anoxic/aerobic (Murnleitner et al., 1997) processes of PAOs are also available.

Metabolic models have often served as a reference basis in the interpretation of data arising from lab- and full-scale studies. The comparison of experimentally determined stoichiometry with the theoretical model predictions has, in many cases, allowed better understanding of the processes under study (Meijer et al., 2001; Schuler and Jenkins, 2003a; Yagci et al., 2003; Zeng et al., 2003d; Pijuan, 2004; Oehmen et al., 2005b, 2006b; Tykesson et al., 2006). Some methods of predicting the relative fraction of PAO/GAO activity have also been proposed based on comparison of experimental data with the PAO and GAO theoretical stoichiometry (Schuler and Jenkins, 2003a; Zeng et al., 2003d). Metabolic models will be a useful tool towards further investigation into the competition between PAOs and GAOs and the optimisation of P removal performance in EBPR systems (Yagci et al., 2003; Zeng et al., 2003d). Some of the model components, particularly the kinetic expressions and the associated parameters, require further experimental verification.

6.2. *ASM models*

The ASM No. 2 (ASM2, (Gujer et al., 1995; Henze et al., 1995)), and later, ASM2d (Henze et al., 1999), have been most widely used to simulate full-scale EBPR processes. ASM2d is an updated model of ASM2, which incorporates the denitrification capability of PAOs. These models, as well as ASM1 (Henze et al., 1987) and ASM3 (Gujer et al., 1999), which describe COD and nitrogen removal processes only, consist of a set of stoichiometric and kinetic expressions describing the bulk biochemical transformations of soluble and particulate compounds in the sludge. In contrast to metabolic models, yield coefficients in these models are not calculated theoretically, but are to be determined experimentally. Default stoichiometric and kinetic parameters are provided as part of these models, and often used in cases when a full-calibration of the model parameters is not possible. Nevertheless, calibration of these parameters is required in most cases, and some tuning procedures involve the identification of the most important model parameters to be calibrated and their interdependencies (Brun et al., 2002). It is common practice that calibration is only performed for the key process parameters, while default values are used for parameters to which the model outputs display a low sensitivity (Melcer et al., 2003). Wastewater characterisation is important for successful model calibration and application.

ASM2d, as well as the extended ASM3 model incorporating the EBPR process (ASM3-BioP, also known as the EAWAG model: Rieger et al., 2001), have been routinely applied to full-scale EBPR plants for process design, optimisation and control (Carrette et al., 2001; Wichern et al., 2001; Ingildsen et al., 2006; Lee et al., 2006). Continuous enhancement of these models is predicted, which will benefit the wastewater industry further.

In all ASM models, acetate is represented as the sole carbon source taken up by PAOs, while the sole carbon storage polymer cycled by PAOs is represented as PHA (more specifically, PHB). Moreover, the existence of GAOs was not considered in these models. Adapted versions of ASM2 have been formulated to incorporate glycogen as a separate storage polymer in addition to PHA, some versions also incorporate the growth and activity of GAOs (Mino et al., 1995; Manga et al., 2001). Others have incorporated the prediction of pH (Serralta et al., 2004), or included components to describe the use of propionate (Pijuan et al., 2004b). These modifications can potentially improve our capability to predict a wider range of EBPR system conditions. For example, Yagci et al. (2006) found that an adapted ASM2d model that accounted for GAOs was able to more accurately describe the process performance in lab-scale SBR systems over a wide range of COD/P ratios. The microbial competition between PAOs and non-polyphosphate accumulating heterotrophs was incorporated into an ASM-based model developed by Schuler (2005). This model accounted for the varying nature of microbial properties in EBPR systems, as opposed to the common assumption of "lumped" or uniform sludge characteristics, and improved prediction of EBPR system performance could potentially be achieved through this approach. Prediction of microbial population dynamics through various techniques, particularly in systems like the EBPR process where the competition between PAOs and GAOs can occur, is likely to be a focus of future modelling applications.

Recent improvements in modelling capabilities do come at an expense, however, which is increased model complexity, making the models more difficult to be used in practice (Gujer, 2006). The combination of metabolic and ASM models discussed below offers an approach to addressing this problem. Another approach could be through hybrid models consisting of a simplified process model and an artificial neural network (Zhao et al., 1997). These hybrid models rely on a preliminary prediction of the desired outputs (e.g. effluent phosphate concentration) by the simplified process model, where the errors are corrected by a 'trained' neural network, resulting in fewer parameters that are necessary for calibration. Although little study has been devoted to the application of this technology for P removal systems thus far, there is the potential that hybrid models may become more widely implemented in the future,

particularly with the increasing availability of reliable on-line measurement tools.

For further information regarding ASM models, readers are referred to reviews by Hu et al. (2003) and Gernaey et al. (2004).

6.2.1. *Combined metabolic/ASM models*

Metabolic models have been combined with ASM models for the purpose of modelling P and nitrogen removal in full-scale WWTPs. The combined metabolic/ASM2d model developed at the Technical University of Delft (known as the TUDP model) has been successfully applied to domestic WWTPs with numerous configurations such as UCT, modified UCT and A_2N (Van Veldhuizen et al., 1999; Brdjanovic et al., 2000; Hao et al., 2001; Meijer et al., 2001). A combined TUDP/ASM3 model called A3DX was developed by Ky et al. (2001) and described well a full-scale SBR plant treating industrial wastewater. The calibration of the TUDP and A3DX models required the adjustment of only 3 or 4 parameters to successfully describe the process. Therefore, as compared to conventional ASM models, combining metabolic and ASM models can enable improved prediction of the process performance, and have the advantage of requiring fewer parameters to be calibrated. Interestingly, there have been cases reported in full-scale plants where the predicted P release/VFA uptake ratio was substantially higher than that observed experimentally, where it was concluded that the discrepancy was likely due to the activity of GAOs (Brdjanovic et al., 2000; Ky et al., 2001) and/or the uptake of other VFAs such as propionate and butyrate (Ky et al., 2001). Thus, incorporation of these metabolisms into future modelling endeavours may further improve the capability of these models to accurately predict the process performance and biochemical transformations in similar cases.

7. Conclusions and future directions

Over the years, our knowledge of the EBPR process has steadily improved, largely due to the high number of valuable studies from various disciplines. However, many unanswered questions about the process still remain in relation to numerous issues. Discussed below are a list of the primary conclusions from this review and some of the main issues that still remain to be resolved.

7.1. *Conclusions*

- PAOs and GAOs of high importance are present in many full-scale EBPR plants, playing substantial roles in terms of anaerobic carbon consumption and/or aerobic/anoxic P uptake.
- While the major source of reducing power for PAOs is generated through glycolysis, the TCA cycle (operating in either a full or partial form as outlined in Fig. 1) is also likely to play a role in the regulation of the redox balance.
- GAOs have been shown to compete with PAOs in EBPR systems for the anaerobic uptake of carbon sources, leading to lower P removal efficiency due to the increased carbon demand. The PAO–GAO competition is influenced by many factors including the COD/P ratio, carbon sources, pH and temperature.
- The denitrification capability of at least some PAOs has been demonstrated. Novel process configurations aimed to achieve anoxic P uptake have been proposed to save oxygen and carbon sources. Recent studies have often focused on the development of simultaneous nitrification, denitrification and P removal processes, sometimes using granular sludge.
- Combining metabolic and ASM models has been shown to be a very useful means of reliably characterising the EBPR process, with fewer parameters to be calibrated.

7.2. *Future directions*

- How diverse are the PAO and GAO populations? Do the putative PAOs or GAOs related to the *Actinobacteria* and *Alphaproteobacteria*, respectively, play a substantial role in EBPR systems, in addition to *Accumulibacter* and *Competibacter*? Further research is needed to clarify the role of these organisms, and characterise them through selective enrichment. In particular, very little is currently known about the metabolism of the novel group of *Actinobacteria* proposed as PAOs, which requires further study. Some studies have suggested that other PAOs or GAOs outside of these groups also exist, thus, research focussed on the determination of other key microorganisms relevant to the process should continue. Furthermore, PAOs and/or GAOs have yet to be obtained in pure culture. The question remaining is whether PAO or GAO isolation can be achieved. There is the possibility that the growth of these organisms is dependent on synergistic interactions with other organisms. This would, however, be quite surprising given the highly enriched PAO and GAO cultures obtained already and the lack of any other specific bacterial group being consistently detected in non-culture-based microbial population studies.
- The detailed metabolic pathways of PAOs and GAOs are yet to be fully revealed. The primary question is: which pathways are employed by the different microbial groups of PAOs and GAOs? A greater correlation is needed between the microbial community present in the sludge and the biochemical pathways that are occurring under anaerobic and aerobic/anoxic conditions. Are PAOs and/or GAOs able to switch between different biochemical pathways as a response to environmental conditions? Further study into the metabolic response of PAOs and GAOs under dynamic conditions may provide clues on their competitive advantage under certain conditions, and result in improved methods of controlling the EBPR process. Recent advances in obtaining the *Accumulibacter* genome should lead to greater knowledge of genomic functions and may yield answers to some of the existing questions in relation to PAO metabolism.
- While some methods of altering the PAO–GAO competition in favour of PAOs have been proposed, testing of these control strategies in full-scale plants has yet to be carried out, and cost-effective methods of implementing these

conditions requires further development. What are the fundamental mechanisms for why key factors such as the carbon source and pH influence the PAO–GAO competition? It is possible that GAOs are more sensitive to environmental changes than PAOs due to their dependence on only one primary energy source (i.e. glycogen), whereas PAOs possess both poly-P and glycogen reserves. What is the most cost-effective way of minimising GAOs in EBPR sludges? Other control strategies that are inexpensive and easy to implement would be highly useful to minimise GAOs and improve P removal performance and reliability.

- Since process performance is likely to be strongly influenced by the microbial composition, it is recommended that further modelling studies be devoted to predicting microbial population dynamics in EBPR systems. Linking the microbial population with the operational factors that influence their proliferation is likely to be the key issue to resolve. Incorporating the metabolism of both PAOs and GAOs and their preferences for different carbon sources may provide improved predictive capabilities of these models.
- The overall goal from each field is to improve the design and operation of EBPR plants in order to maximise the reliability, efficiency and effectiveness of the process. It still remains to be seen how we can implement into practice our improved knowledge of the process, which we hope to assist with through this review and other scientific studies.

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REFERENCES

Ahn, Y.H., Speece, R.E., 2006. Elutriated acid fermentation of municipal primary sludge. Water Res. 40 (11), 2210–2220.

Ahn, J., Daidou, T., Tsuneda, S., Hirata, A., 2002. Characterization of denitrifying phosphate-accumulating organisms cultivated under different electron acceptor conditions using polymerase chain reaction-denaturing gradient gel electrophoresis assay. Water Res. 36 (2), 403–412.

Barker, P.S., Dold, P.L., 1996. Denitrification behaviour in biological excess phosphorus removal activated sludge systems. Water Res. 30 (4), 769–780.

Barnard, J.L., 1975. Biological nutrient removal without the addition of chemicals. Water Res. 9 (5-6), 485–490.

Battistoni, P., Paci, B., Fatone, F., Pavan, P., 2006. Phosphorus removal from anaerobic supernatants: Start-up and steady-state conditions of a fluidized bed reactor full-scale plant. Ind. Eng. Chem. Res. 45 (2), 663–669.

Beer, M., Kong, Y.H., Seviour, R.J., 2004. Are some putative glycogen accumulating organisms (GAO) in anaerobic: aerobic activated sludge systems members of the *α-Proteobacteria*? Microbiology 150, 2267–2275.

Beer, M., Stratton, H.M., Griffiths, P.C., Seviour, R.J., 2006. Which are the polyphosphate accumulating organisms in full-scale activated sludge enhanced biological phosphate removal systems in Australia? J. Appl. Microbiol. 100 (2), 233–243.

Blackall, L.L., Rossetti, S., Christensson, C., Cunningham, M., Hartman, P., Hugenholtz, P., Tandoi, V., 1997. The characterization and description of representatives of 'G' bacteria from activated sludge plants. Lett. Appl. Microbiol. 25 (1), 63–69.

Blackall, L.L., Crocetti, G., Saunders, A.M., Bond, P.L., 2002. A review and update of the microbiology of enhanced biological phosphorus removal in wastewater treatment plants. Anton. Leeuw. 81 (1–4), 681–691.

Bond, P.L., Hugenholtz, P., Keller, J., Blackall, L.L., 1995. Bacterial community structures of phosphate-removing and non-phosphate-removing activated sludges from sequencing batch reactors. Appl. Environ. Microbiol. 61 (5), 1910–1916.

Bond, P.L., Erhart, R., Wagner, M., Keller, J., Blackall, L.L., 1999a. Identification of some of the major groups of bacteria in efficient and nonefficient biological phosphorus removal activated sludge systems. Appl. Environ. Microbiol. 65 (9), 4077–4084.

Bond, P.L., Keller, J., Blackall, L.L., 1999b. Anaerobic phosphate release from activated sludge with enhanced biological phosphorus removal. A possible mechanism of intracellular pH control. Biotechnol. Bioeng. 63 (5), 507–515.

Booker, N.A., Priestley, A.J., Fraser, I.H., 1999. Struvite formation in wastewater treatment plants: opportunities for nutrient recovery. Environ. Technol. 20 (7), 777–782.

Bortone, G., Malaspina, F., Stante, L., Tilche, A., 1994. Biological nitrogen and phosphorus removal in an anerobic/anoxic sequencing batch reactor with separated biofilm nitrification. Water Sci. Technol. 30 (6), 303–313.

Bortone, G., Saltarelli, R., Alonso, V., Sorm, R., Wanner, J., Tilche, A., 1996. Biological anoxic phosphorus removal—the dephanox process. Water Sci. Technol. 34 (1-2), 119–128.

Brdjanovic, D., Hooijmans, C.M., van Loosdrecht, M.C.M., Alaerts, G.J., Heijnen, J.J., 1996. The dynamic effects of potassium limitation on biological phosphorus removal. Water Res. 30 (10), 2323–2328.

Brdjanovic, D., vanLoosdrecht, M.C.M., Hooijmans, C.M., Alaerts, G.J., Heijnen, J.J., 1997. Temperature effects on physiology of biological phosphorus removal. J. Environ. Eng.-ASCE 123 (2), 144–153.

Brdjanovic, D., Logemann, S., Van Loosdrecht, M.C.M., Hooijmans, C.M., Alaerts, G.J., Heijnen, J.J., 1998a. Influence of temperature on biological phosphorus removal: process and molecular ecological studies. Water Res. 32 (4), 1035–1048.

Brdjanovic, D., Slamet, A., van Loosdrecht, M.C.M., Hooijmans, C.M., Alaerts, G.J., Heijnen, J.J., 1998b. Impact of excessive aeration on biological phosphorus removal from wastewater. Water Res. 32 (1), 200–208.

Brdjanovic, D., Van Loosdrecht, M.C.M., Versteeg, P., Hooijmans, C.M., Alaerts, G.J., Heijnen, J.J., 2000. Modeling COD, N and P removal in a full-scale wwtp Haarlem Waarderpolder. Water Res. 34 (3), 846–858.

Britton, A., Koch, F.A., Mavinic, D.S., Adnan, A., Oldham, W.K., Udala, B., 2005. Pilot-scale struvite recovery from anaerobic digester supernatant at an enhanced biological phosphorus removal wastewater treatment plant. J. Environ. Eng. Sci. 4 (4), 265–277.

Brun, R., Kuhni, M., Siegrist, H., Gujer, W., Reichert, P., 2002. Practical identifiability of ASM2d parameters—systematic selection and tuning of parameter subsets. Water Res. 36 (16), 4113–4127.

Burow, L.C., Kong, Y.H., Nielsen, J.L., Blackall, L.L., Nielsen, P.H., 2007. Abundance and ecophysiology of *Defluviicoccus* spp., glycogen accumulating organisms in full-scale wastewater treatment processes. Microbiology 153, 178–185.

Canizares, P., De Lucas, A., Rodriguez, L., Villasenor, J., 2000. Anaerobic uptake of different organic substrates by an enhanced biological phosphorus removal sludge. Environ. Technol. 21 (4), 397–405.

Carrette, R., Bixio, D., Thoeye, C., Ockler, P., 2001. Full-scale application of the IAWQ ASM No. 2d model. Water Sci. Technol. 44 (2–3), 17–24.

Carucci, A., Majone, M., Ramadori, R., Rossetti, S., 1994. Dynamics of phosphorus and organic substrates in anaerobic and aerobic phases of a sequencing batch reactor. Water Sci. Technol. 30 (6), 237–246.

Carucci, A., Lindrea, K., Majone, M., Ramadori, R., 1999. Different mechanisms for the anaerobic storage of organic substrates and their effect on enhanced biological phosphate removal (EBPR). Water Sci. Technol. 39 (6), 21–28.

Carvalho, G., Lemos, P.C., Oehmen, A., Crespo, M.T.B., Reis, M.A.M., 2006. Microbial diversity of the *Candidatus Accumulibacter phosphatis* clade in denitrifying phosphorus removal systems. In: Proceedings of the 11th International Symposium on Microbial Ecology—ISME 11, Vienna, Austria, August 20–25, 2006.

Cassidy, D.P., Belia, E., 2005. Nitrogen and phosphorus removal from an abattoir wastewater in a SBR with aerobic granular sludge. Water Res. 39 (19), 4817–4823.

Cech, J.S., Hartman, P., 1990. Glucose induced break down of enhanced biological phosphate removal. Environ. Technol. 11, 651–656.

Cech, J.S., Hartman, P., 1993. Competition between polyphosphate and polysaccharide accumulating bacteria in enhanced biological Phosphate removal systems. Water Res. 27 (7), 1219–1225.

Chen, Y., Randall, A.A., McCue, T., 2004. The efficiency of enhanced biological phosphorus removal from real wastewater affected by different ratios of acetic to propionic acid. Water Res. 38 (1), 27–36.

Chen, Y.G., Liu, Y., Zhou, Q., Gu, G.W., 2005. Enhanced phosphorus biological removal from wastewater—effect of microorganism acclimatization with different ratios of short-chain fatty acids mixture. Biochem. Eng. J. 27 (1), 24–32.

Chua, A.S.M., Eales, K., Mino, T., Seviour, R., 2004. The large PAO cells in full-scale EBPR biomass samples are not yeast spores but possibly novel members of the beta-Proteobacteria. Water Sci. Technol. 50 (6), 123–130.

Comeau, Y., Hall, K.J., Hancock, R.E.W., Oldham, W.K., 1986. Biochemical-model for enhanced biological phosphorus removal. Water Res. 20 (12), 1511–1521.

Comeau, Y., Rabionwitz, B., Hall, K.J., Oldham, W.K., 1987. Phosphate release and uptake in enhanced biological phosphorus removal from wastewater. J. Water Pollut. Control 59 (7), 707–715.

Crocetti, G.R., Hugenholtz, P., Bond, P.L., Schuler, A., Keller, J., Jenkins, D., Blackall, L.L., 2000. Identification of polyphosphate-accumulating organisms and design of 16S rRNA-directed probes for their detection and quantitation. Appl. Environ. Microbiol. 66 (3), 1175–1182.

Crocetti, G.R., Banfield, J.F., Keller, J., Bond, P.L., Blackall, L.L., 2002. Glycogen-accumulating organisms in laboratory-scale and full-scale wastewater treatment processes. Microbiology 148, 3353–3364.

Cuevas-Rodriguez, G., Tejero-Monzon, I., 2003. Sedimentation and prefermentation of domestic wastewater in a fixed bed biofilm reactor. Water Sci. Technol. 48 (3), 47–55.

Dai, Y., 2006. Polyhydroxyalkanoate Copolymer Production from Synthetic Carbonaceous Wastewater Using Glycogen Accumulating Organisms. University of Queensland, St. Lucia, Australia.

de Haas, D.W., Wentzel, M.C., Ekama, G.A., 2001. The use of simultaneous chemical precipitation in modified activated sludge systems exhibiting biological excess phosphate removal—Part 5: experimental periods using a ferrous-ferric chloride blend. Water Sa 27 (2), 117–134.

de Kreuk, M., Heijnen, J.J., van Loosdrecht, M.C.M., 2005. Simultaneous COD, nitrogen, and phosphate removal by aerobic granular sludge. Biotechnol. Bioeng. 90 (6), 761–769.

Dulekgurgen, E., Ovez, S., Artan, N., Orhon, D., 2003. Enhanced biological phosphate removal by granular sludge in a sequencing batch reactor. Biotechnol. Lett. 25 (9), 687–693.

Ekama, G.A., Marais, G.v.R., Siebritz, I.P., Pitman, A.R., Keay, G.F.P., Buchan, L., Gerber, A., Smollen, M., 1984. Theory, Design and Operation of Nutrient Removal Activated Sludge Processes. University of Cape Town, City Council of Johannesburg and the National Institute for Water Research of the CSIR, Pretoria, South Africa.

Erdal, U.G., 2002a. The effects of temperature on system performance and bacterial community structure in a biological phosphorus removal system. Ph.D. Thesis, Virginia Polytechnic Institute and State University, Blacksburg, Virginia, USA.

Erdal, Z.K., 2002b. The biochemistry of enhanced biological phosphorus removal: role of glycogen in biological phosphorus removal and the impact of the operating conditions on the involvement of glycogen. Ph.D. Thesis, Virginia Polytechnic Institute and State University, Blacksburg, Virginia, USA.

Erdal, U.G., Erdal, Z.K., Randall, C.W., 2003. The competition between PAOs (phosphorus accumulating organisms) and GAOs (glycogen accumulating organisms) in EBPR (enhanced biological phosphorus removal) systems at different temperatures and the effects on system performance. Water Sci. Technol. 47 (11), 1–8.

Erdal, Z.K., Erdal, U.G., Randall, C.W., 2005. Biochemistry of enhanced biological phosphorus removal and anaerobic COD stabilization. Water Sci. Technol. 52 (10–11), 557–567.

Falvo, A., Levantesi, C., Rossetti, S., Seviour, R.J., Tandoi, V., 2001. Synthesis of intracellular storage polymers by *Amaricoccus kaplicensis*, a tetrad forming bacterium present in activated sludge. J. Appl. Microbiol. 91 (2), 299–305.

Filipe, C.D.M., Daigger, G.T., Grady, C.P.L., 2001a. Effects of pH on the rates of aerobic metabolism of phosphate-accumulating and glycogen-accumulating organisms. Water Environ. Res. 73 (2), 213–222.

Filipe, C.D.M., Daigger, G.T., Grady, C.P.L., 2001b. A metabolic model for acetate uptake under anaerobic conditions by glycogen accumulating organisms: stoichiometry, kinetics, and the effect of pH. Biotechnol. Bioeng. 76 (1), 17–31.

Filipe, C.D.M., Daigger, G.T., Grady, C.P.L., 2001c. pH as a key factor in the competition between glycogen-accumulating organisms and phosphorus-accumulating organisms. Water Environ. Res. 73 (2), 223–232.

Filipe, C.D.M., Daigger, G.T., Grady, C.P.L., 2001d. Stoichiometry and kinetics of acetate uptake under anaerobic conditions by an enriched culture of phosphorus-accumulating organisms at different pHs. Biotechnol. Bioeng. 76 (1), 32–43.

Fuhs, G.W., Chen, M., 1975. Microbiological basis of phosphate removal in the activated sludge process for the treatment of wastewater. Microb. Ecol. 2 (2), 119–138.

Fukase, T., Shibata, M., Miyaji, Y., 1985. The role of the anaerobic stage on biological phosphorus removal. Water Sci. Technol. 17, 69–80.

Gernaey, K.V., van Loosdrecht, M.C.M., Henze, M., Lind, M., Jorgensen, S.B., 2004. Activated sludge wastewater treatment plant modelling and simulation: state of the art. Environ. Modelling Software 19 (9), 763–783.

Griffiths, P.C., Stratton, H.M., Seviour, R.J., 2002. Environmental factors contributing to the "G bacteria" population in full-scale EBPR plants. Water Sci. Technol. 46 (4-5), 185–192.

Gu, A.Z., Saunders, A.M., Neethling, J.B., Stensel, H.D., Blackall, L., 2005. In: WEF (Ed.) Investigation of PAOs and GAOs and their effects on EBPR performance at full-scale wastewater treatment plants in US, October 29–November 2, WEFTEC, Washington, DC, USA.

Gujer, W., 2006. Activated sludge modelling: past, present and future. Water Sci. Technol. 53 (3), 111–119.

Gujer, W., Henze, M., Mino, T., Matsuo, T., Wentzel, M.C., Marais, G.v.R., 1995. The activated-sludge model no-2—biological phosphorus removal. Water Sci. Technol. 31 (2), 1–11.

Gujer, W., Henze, M., Mino, T., van Loosdrecht, M., 1999. Activated sludge model no. 3. Water Sci. Technol. 39 (1), 183–193.

Hanada, S., Liu, W.T., Shintani, T., Kamagata, Y., Nakamura, K., 2002. *Tetrasphaera elongata* sp. nov., a polyphosphate-accumulating bacterium isolated from activated sludge. Int. J. Syst. Evol. Microbiol. 52, 883–887.

Hao, X.D., Van Loosdrecht, M.C.M., Meijer, S.C.F., Qian, Y., 2001. Model-based evaluation of two BNR processes—UCT and A(2)N. Water Res. 35 (12), 2851–2860.

Hao, X.D., van Loosdrecht, M.C.M., 2006. Model-based evaluation of struvite recovery from an in-line stripper in a BNR process (BCFS (R)). Water Sci. Technol. 53 (3), 191–198.

Hartley, K.J., Sickerdick, L., 1994. Performance of Australian BNR plants. In: Second Australian Conference on Biological Nutrient Removal, 4–6 October, Albury, Victoria, pp. 65–73.

He, S., Gu, A.Z., McMahon, K.D., 2005. The role of *Rhodocyclus*-like organisms in biological phosphorus removal: factors influencing population structure and activity. In: Water Environment Federation Technical Exhibition and Conference (WEFTEC) 2005, October 29–November 2, Washington, DC, USA.

He, S., Gu, A.Z., McMahon, K.D., 2006. Fine-scale differences between *Accumulibacter*-like bacteria in enhanced biological phosphorus removal activated sludge. Water Sci. Technol. 54 (1), 111–117.

Henze, M., Grady, C.P.L., Gujer, W., Marais, G.v.R., Matsuo, T., 1987. Activated Sludge Model No. 1. IAWPRC Scientific and Technical Report No. 1., London, UK.

Henze, M., Gujer, W., Mino, T., Matsuo, T., Wentzel, M.C., Marais, G.v.R., 1995. Activated Sludge Model. No. 2. IAWQ Scientific and Technical Report No. 3., London, UK.

Henze, M., Harremoes, P., la Cour Jansen, J., Arvin, E., 1997. Wastewater Treatment: Biological and Chemical Processes, second ed. Springer, Berlin, Germany.

Henze, M., Gujer, W., Mino, T., Matsuo, T., Wentzel, M.C., Marais, G.v.R., Van Loosdrecht, M.C.M., 1999. Activated sludge model no.2d, ASM2d. Water Sci. Technol. 39 (1), 165–182.

Hesselmann, R.P.X., Werlen, C., Hahn, D., van der Meer, J.R., Zehnder, A.J.B., 1999. Enrichment, phylogenetic analysis and detection of a bacterium that performs enhanced biological phosphate removal in activated sludge. Syst. Appl. Microbiol. 22 (3), 454–465.

Hesselmann, R.P.X., Von Rummell, R., Resnick, S.M., Hany, R., Zehnder, A.J.B., 2000. Anaerobic metabolism of bacteria performing enhanced biological phosphate removal. Water Res. 34 (14), 3487–3494.

Hood, C.R., Randall, A.A., 2001. A biochemical hypothesis explaining the response of enhanced biological phosphorus removal biomass to organic substrates. Water Res. 35 (11), 2758–2766.

Hu, Z.R., Wentzel, M.C., Ekama, G.A., 2003. Modelling biological nutrient removal activated sludge systems—a review. Water Res. 37 (14), 3430–3444.

Ingildsen, P., Rosen, C., Gernaey, K.V., Nielsen, M.K., Guildal, T., Jacobsen, B.N., 2006. Modelling and control strategy testing of biological and chemical phosphorus removal at Avedore WWTP. Water Sci. Technol. 53 (4–5), 105–113.

Jeon, C.O., Park, J.M., 2000. Enhanced biological phosphorus removal in a sequencing batch reactor supplied with glucose as a sole carbon source. Water Res. 34 (7), 2160–2170.

Jeon, C.O., Lee, D.S., Lee, M.W., Park, J.M., 2001. Enhanced biological phosphorus removal in an anaerobic–aerobic sequencing batch reactor: effect of pH. Water Environ. Res. 73 (3), 301–306.

Jobbagy, A., Literathy, B., Wong, M.T., Tardy, G., Liu, W.T., 2006. Proliferation of glycogen accumulating organisms induced by Fe(III) dosing in a domestic wastewater treatment plant. Water Sci. Technol. 54 (1), 101–109.

Kawaharasaki, M., Tanaka, H., Kanagawa, T., Nakamura, K., 1999. In situ identification of polyphosphate-accumulating bacteria in activated sludge by dual staining with rRNA-targeted oligonucleotide probes and 4′,6-diamidino-2-phenylindol (DAPI) at a polyphosphate-probing concentration. Water Res. 33 (1), 257–265.

Kerrn-Jespersen, J.P., Henze, M., 1993. Biological phosphorus uptake under anoxic and aerobic conditions. Water Res. 27 (4), 617–624.

Kong, Y.H., Beer, M., Seviour, R.J., Lindrea, K.C., Rees, G.N., 2001. Structure and functional analysis of the microbial community in an aerobic: anaerobic sequencing batch reactor (SBR) with no phosphorus removal. Syst. Appl. Microbiol. 24 (4), 597–609.

Kong, Y.H., Beer, M., Rees, G.N., Seviour, R.J., 2002a. Functional analysis of microbial communities in aerobic-anaerobic sequencing batch reactors fed with different phosphorus/carbon (P/C) ratios. Microbiology 148, 2299–2307.

Kong, Y.H., Ong, S.L., Ng, W.J., Liu, W.T., 2002b. Diversity and distribution of a deeply branched novel proteobacterial group found in anaerobic–aerobic activated sludge processes. Environ. Microbiol. 4 (11), 753–757.

Kong, Y.H., Nielsen, J.L., Nielsen, P.H., 2004. Microautoradiographic study of *Rhodocyclus*-Related polyphosphate-accumulating bacteria in full-scale enhanced biological phosphorus removal plants. Appl. Environ. Microbiol. 70 (9), 5383–5390.

Kong, Y.H., Nielsen, J.L., Nielsen, P.H., 2005. Identity and ecophysiology of uncultured actinobacterial polyphosphate-accumulating organisms in full-scale enhanced biological phosphorus removal plants. Appl. Environ. Microbiol. 71 (7), 4076–4085.

Kong, Y.H., Xia, Y., Nielsen, J.L., Nielsen, P.H., 2006. Ecophysiology of a group of uncultured Gammaproteobacterial glycogen-accumulating organisms in full-scale enhanced biological phosphorus removal wastewater treatment plants. Environ. Microbiol. 8 (3), 479–489.

Kortstee, G.J.J., Appeldoorn, K.J., Bonting, C.F.C., van Niel, E.W.J., van Veen, H.W., 2000. Recent developments in the biochemistry and ecology of enhanced biological phosphorus removal. Biochemistry—Moscow 65 (3), 332–340.

Kuba, T., Smolders, G., Vanloosdrecht, M.C.M., Heijnen, J.J., 1993. Biological phosphorus removal from wastewater by anaerobic-anoxic sequencing batch reactor. Water Sci. Technol. 27 (5–6), 241–252.

Kuba, T., Wachtmeister, A., Vanloosdrecht, M.C.M., Heijnen, J.J., 1994. Effect of nitrate on phosphorus release in biological phosphorus removal systems. Water Sci. Technol. 30 (6), 263–269.

Kuba, T., Murnleitner, E., vanLoosdrecht, M.C.M., Heijnen, J.J., 1996a. A metabolic model for biological phosphorus removal by denitrifying organisms. Biotechnol. Bioeng. 52 (6), 685–695.

Kuba, T., vanLoosdrecht, M.C.M., Heijnen, J.J., 1996b. Effect of cyclic oxygen exposure on the activity of denitrifying phosphorus removing bacteria. Water Sci. Technol. 34 (1–2), 33–40.

Kuba, T., VanLoosdrecht, M.C.M., Heijnen, J.J., 1996c. Phosphorus and nitrogen removal with minimal cod requirement by

integration of denitrifying dephosphatation and nitrification in a two-sludge system. Water Res. 30 (7), 1702–1710.
Ky, R.C., Comeau, Y., Perrier, M., Takacs, I., 2001. Modelling biological phosphorus removal from a cheese factory effluent by an SBR. Water Sci. Technol. 43 (3), 257–264.
Lee, D.S., Jeon, C.O., Park, J.M., 2001. Biological nitrogen removal with enhanced phosphate uptake in a sequencing batch reactor using single sludge system. Water Res. 35 (16), 3968–3976.
Lee, N., Jansen, J.L., Aspegren, H., Henze, M., Nielsen, P.H., Wagner, M., 2002. Population dynamics in wastewater treatment plants with enhanced biological phosphorus removal operated with and without nitrogen removal. Water Sci. Technol. 46 (1–2), 163–170.
Lee, S.H., Ko, J.H., Park, J.B., Im, J.H., Kim, J.R., Lee, J.J., Kim, C.W., 2006. Use of activate sludge model No. 3 and Bio-P module for simulating five-stage step-feed enhanced biological phosphorous removal process. Kor. J. Chem. Eng. 23 (2), 203–208.
Lemaire, R., Meyer, R., Taske, A., Crocetti, G.R., Keller, J., Yuan, Z.G., 2006. Identifying causes for N_2O accumulation in a lab-scale sequencing batch reactor performing simultaneous nitrification, denitrification and phosphorus removal. J. Biotechnol. 122 (1), 62–72.
Lemos, P.C., Viana, C., Salgueiro, E.N., Ramos, A.M., Crespo, J., Reis, M.A.M., 1998. Effect of carbon source on the formation of polyhydroxyalkanoates (PHA) by a phosphate-accumulating mixed culture. Enzyme Microb. Technol. 22 (8), 662–671.
Lemos, P.C., Serafim, L.S., Santos, M.M., Reis, M.A.M., Santos, H., 2003. Metabolic pathway for propionate utilization by phosphorus-accumulating organisms in activated sludge: C-13 labeling and in vivo nuclear magnetic resonance. Appl. Environ. Microbiol. 69 (1), 241–251.
Lesjean, B., Gnirss, R., Adam, C., 2002. Process configurations adapted to membrane bioreactors for enhanced biological phosphorous and nitrogen removal. Desalination 149 (1-3), 217–224.
Levantesi, C., Serafim, L.S., Crocetti, G.R., Lemos, P.C., Rossetti, S., Blackall, L.L., Reis, M.A.M., Tandoi, V., 2002. Analysis of the microbial community structure and function of a laboratory scale enhanced biological phosphorus removal reactor. Environ. Microbiol. 4 (10), 559–569.
Liu, W.T., Mino, T., Nakamura, K., Matsuo, T., 1994. Role of glycogen in acetate uptake and polyhydroxyalkanoate synthesis in anaerobic–aerobic activated-sludge with a minimized polyphosphate content. J. Ferment. Bioeng. 77 (5), 535–540.
Liu, W.T., Mino, T., Matsuo, T., Nakamura, K., 1996a. Biological phosphorus removal processes—effect of pH on anaerobic substrate metabolism. Water Sci. Technol. 34 (1–2), 25–32.
Liu, W.T., Mino, T., Nakamura, K., Matsuo, T., 1996b. Glycogen accumulating population and its anaerobic substrate uptake in anaerobic–aerobic activated sludge without biological phosphorus removal. Water Res. 30 (1), 75–82.
Liu, W.T., Nielsen, A.T., Wu, J.H., Tsai, C.S., Matsuo, Y., Molin, S., 2001. In situ identification of polyphosphate- and polyhydroxyalkanoate-accumulating traits for microbial populations in a biological phosphorus removal process. Environ. Microbiol. 3 (2), 110–122.
Liu, W.T., Hanada, S., Marsh, T.L., Kamagata, Y., Nakamura, K., 2002. *Kineosphaera limosa* gen. nov., sp nov., a novel Gram-positive polyhydroxyalkanoate-accumulating coccus isolated from activat sludge. Int. J. Syst. Evol. Microbiol. 52, 1845–1849.
Louie, T.M., Mah, T.J., Oldham, W.K., Ramey, W.D., 2000. Use of metabolic inhibitors and gas chromatography/mass spectrometry to study poly-β-hydroxyalkanoates metabolism involving cryptic nutrients in enhanced biological phosphorus removal systems. Water Res. 34 (5), 1507–1514.
Loy, A., Schulz, C., Lucker, S., Schopfer-Wendels, A., Stoecker, K., Baranyi, C., Lehner, A., Wagner, M., 2005. 16S rRNA gene-based oligonucleotide microarray for environmental monitoring of the betaproteobacterial order "*Rhodocyclales*". Appl. Environ. Microbiol. 71 (3), 1373–1386.
Lu, H., Oehmen, A., Virdis, B., Keller, J., Yuan, Z.G., 2006. Obtaining highly enriched cultures of Candidatus *Accumulibacter phosphatis* through alternating carbon sources. Water Res. 40 (20), 3838–3848.
Mainstone, C.P., Parr, W., 2002. Phosphorus in rivers—ecology and management. Sci. Total Environ. 282, 25–47.
Manga, J., Ferrer, J., Garcia-Usach, F., Seco, A., 2001. A modification to the activated sludge model no. 2 based on the competition between phosphorus-accumulating organisms and glycogen-accumulating organisms. Water Sci. Technol. 43 (11), 161–171.
Martin, H.G., Ivanova, N., Kunin, V., Warnecke, F., Barry, K., McHardy, A.C., Yeates, C., He, S., Salamov, A., Szeto, E., Dalin, E., Putnam, N., Shapiro, H.J., Pangilinan, J.L., Rigoutsos, I., Kyrpides, N.C., Blackall, L.L., McMahon, K.D., Hugenholtz, P., 2006. Metagenomic analysis of two enhanced biological phosphorus removal (EBPR) sludge communities. Nat. Biotechnol. 24 (10), 1263–1269.
Maszenan, A.M., Seviour, R.J., Patel, B.K.C., Rees, G.N., McDougall, B.M., 1997. *Amaricoccus* gen. nov, a gram-negative coccus occurring in regular packages or tetrads, isolated from activated sludge biomass, and descriptions of *Amaricoccus veronensis* sp. nov, *Amaricoccus tamworthensis* sp. nov, *Amaricoccus macauensis* sp. nov, and *Amaricoccus kaplicensis* sp. nov. Int. J. Syst. Bacteriol. 47 (3), 727–734.
Maszenan, A.M., Seviour, R.J., Patel, B.K.C., Schumann, P., Burghardt, J., Tokiwa, Y., Stratton, H.M., 2000. Three isolates of novel polyphosphate-accumulating Gram-positive cocci, obtained from activated sludge, belong to a new genus, *Tetrasphaera* gen. nov., and description of two new species, *Tetrasphaera japonica* sp, nov and *Tetrasphaera australiensis* sp nov. Int. J. Syst. Evol. Microbiol. 50, 593–603.
Maurer, M., Gujer, W., Hany, R., Bachmann, S., 1997. Intracellular carbon flow in phosphorus accumulating organisms from activated sludge systems. Water Res. 31 (4), 907–917.
Maurer, M., Abramovich, D., Siegrist, H., Gujer, W., 1999. Kinetics of biologically induced phosphorus precipitation in wastewater treatment. Water Res. 33 (2), 484–493.
McMahon, K.D., Dojka, M.A., Pace, N.R., Jenkins, D., Keasling, J.D., 2002. Polyphosphate kinase from activated sludge performing enhanced biological phosphorus removal. Appl. Environ. Microbiol. 68 (10), 4971–4978.
Meijer, S.C.F., Van Loosdrecht, M.C.M., Heijnen, J.J., 2001. Metabolic modelling of full-scale biological nitrogen and phosphorus removing WWTP's. Water Res. 35 (11), 2711–2723.
Meinhold, J., Filipe, C.D.M., Daigger, G.T., Isaacs, S., 1999. Characterization of the denitrifying fraction of phosphate accumulating organisms in biological phosphate removal. Water Sci. Technol. 39 (1), 31–42.
Melcer, H., Dold, P.L., Jones, R.M., Bye, C.M., I., T., Stensel, H.D., Wilson, A.W., Sun, P., Bury, S., 2003. Methods for Wastewater Characterisation in Activated Sludge Modelling. Water Environment Research Foundation, IWA Publishing and Water Environment Federation, Alexandria, VA, USA and London, UK.
Meyer, R.L., Zeng, R.J.X., Giugliano, V., Blackall, L.L., 2005. Challenges for simultaneous nitrification, denitrification, and phosphorus removal in microbial aggregates: mass transfer limitation and nitrous oxide production. FEMS Microbiol. Ecol. 52 (3), 329–338.
Meyer, R.L., Saunders, A.M., Blackall, L.L., 2006. Putative glycogen-accumulating organisms belonging to *Alphaproteobacteria* identified through rRNA-based stable isotope probing. Microbiology 152, 419–429.
Mino, T., Arun, V., Tsuzuki, Y., Matsuo, T., 1987. Effect of phosphorus accumulation on acetate metabolism in the

biological phosphorus removal process. In: Ramadori, R. (Ed.), Biological Phosphate Removal from Wastewaters. Pergamon Press, Oxford, pp. 27–38.

Mino, T., Liu, W.T., Kurisu, F., Matsuo, T., 1995. Modeling glycogen-storage and denitrification capability of microorganisms in enhanced biological phosphate removal processes. Water Sci. Technol. 31 (2), 25–34.

Mino, T., Van Loosdrecht, M.C.M., Heijnen, J.J., 1998. Microbiology and biochemistry of the enhanced biological phosphate removal process. Water Res. 32 (11), 3193–3207.

Murnleitner, E., Kuba, T., vanLoosdrecht, M.C.M., Heijnen, J.J., 1997. An integrated metabolic model for the aerobic and denitrifying biological phosphorus removal. Biotechnol. Bioeng. 54 (5), 434–450.

Nakamura, K., Hiraishi, A., Yoshimi, Y., Kawaharasaki, M., Masuda, K., Kamagata, Y., 1995. *Microlunatus phosphovorus* gen-nov, sp-nov, a new gram-positive polyphosphate-accumulating bacterium isolated from activated-sludge. Int. J. Syst. Bacteriol. 45 (1), 17–22.

Nielsen, A.T., Liu, W.T., Filipe, C., Grady, L., Molin, S., Stahl, D.A., 1999. Identification of a novel group of bacteria in sludge from a deteriorated biological phosphorus removal reactor. Appl. Environ. Microbiol. 65 (3), 1251–1258.

Oehmen, A., Yuan, Z., Blackall, L.L., Keller, J., 2004. Short-term effects of carbon source on the competition of polyphosphate accumulating organisms and glycogen accumulating organisms. Water Sci. Technol. 50 (10), 139–144.

Oehmen, A., Vives, M.T., Lu, H., Yuan, Z., Keller, J., 2005a. The effect of pH on the competition between polyphosphate-accumulating organisms and glycogen-accumulating organisms. Water Res. 39 (15), 3727–3737.

Oehmen, A., Yuan, Z.G., Blackall, L.L., Keller, J., 2005b. Comparison of acetate and propionate uptake by polyphosphate accumulating organisms and glycogen accumulating organisms. Biotechnol. Bioeng. 91 (2), 162–168.

Oehmen, A., Zeng, R.J., Yuan, Z.G., Keller, J., 2005c. Anaerobic metabolism of propionate by polyphosphate-accumulating organisms in enhanced biological phosphorus removal systems. Biotechnol. Bioeng. 91 (1), 43–53.

Oehmen, A., Saunders, A.M., Vives, M.T., Yuan, Z., Keller, J., 2006a. Competition between polyphosphate and glycogen accumulating organisms in enhanced biological phosphorus removal systems with acetate and propionate as carbon sources. J. Biotechnol. 123 (1), 22–32.

Oehmen, A., Zeng, R.J., Saunders, A.M., Blackall, L.L., Yuan, Z., Keller, J., 2006b. Anaerobic and aerobic metabolism of glycogen accumulating organisms selected with propionate as the sole carbon source. Microbiology 152 (9), 2767–2778.

Onda, S., Hiraishi, A., Matsuo, Y., Takii, S., 2002. Polyphasic approaches to the identification of predominant polyphosphate-accumulating organisms in a laboratory-scale anaerobic/aerobic activated sludge system. J. Gener. Appl. Microbiol. 48 (1), 43–54.

Panswad, T., Doungchai, A., Anotai, J., 2003. Temperature effect on microbial community of enhanced biological phosphorus removal system. Water Res. 37 (2), 409–415.

Pattarkine, V.M., Randall, C.W., 1999. The requirement of metal cations for enhanced biological phosphorus removal by activated sludge. Water Sci. Technol. 40 (2), 159–165.

Pereira, H., Lemos, P.C., Reis, M.A.M., Crespo, J., Carrondo, M.J.T., Santos, H., 1996. Model for carbon metabolism in biological phosphorus removal processes based on in vivo C-13-NMR labelling experiments. Water Res. 30 (9), 2128–2138.

Pijuan, M., 2004. Effect of different carbon sources and continuous aerobic conditions on the EBPR process. Ph.D. Thesis, Universitat Autonoma de Barcelona, Barcelona, Spain.

Pijuan, M., Baeza, J.A., Casas, C., Lafuente, J., 2004a. Response of an EBPR population developed in an SBR with propionate to different carbon sources. Water Sci. Technol. 50 (10), 131–138.

Pijuan, M., Saunders, A.M., Guisasola, A., Baeza, J.A., Casas, C., Blackall, L.L., 2004b. Enhanced biological phosphorus removal in a sequencing batch reactor using propionate as the sole carbon source. Biotechnol. Bioeng. 85 (1), 56–67.

Pijuan, M., Guisasola, A., Baeza, J.A., Carrera, J., Casas, C., Lafuente, J., 2006. Net P-removal deterioration in enriched PAO sludge subjected to permanent aerobic conditions. J. Biotechnol. 123 (1), 117–126.

Popel, H.J., Jardin, N., 1993. Influence of enhanced biological phosphorus removal on sludge treatment. Water Sci. Technol. 28 (1), 263–271.

Pramanik, J., Trelstad, P.L., Schuler, A.J., Jenkins, D., Keasling, J.D., 1999. Development and validation of a flux-based stoichiometric model for enhanced biological phosphorus removal metabolism. Water Res. 33 (2), 462–476.

Randall, A.A., Benefield, L.D., Hill, W.E., 1997. Induction of phosphorus removal in an enhanced biological phosphorus removal bacterial population. Water Res. 31 (11), 2869–2877.

Rayne, S., Carey, S., Forest, K., 2005. Evidence for tin inhibition of enhanced biological phosphorus removal at a municipal wastewater treatment plant. J. Environ. Sci. Health A-Toxic/Hazard. Subst. Environ. Eng. 40 (3), 535–551.

Rieger, L., Koch, G., Kuhni, M., Gujer, W., Siegrist, H., 2001. The EAWAG bio-P module for activated sludge model no. 3. Water Res. 35 (16), 3887–3903.

Rodrigo, M.A., Seco, A., Ferrer, J., Penya-Roja, J.M., 1999. The effect of sludge age on the deterioration of the enhanced biological phosphorus removal process. Environ. Technol. 20 (10), 1055–1063.

Rogalla, F., Johnson, T.L., McQuarrie, J., 2006. Fixed film phosphorus removal—flexible enough? Water Sci. Technol. 53 (12), 75–81.

Saito, T., Brdjanovic, D., van Loosdrecht, M.C.M., 2004. Effect of nitrite on phosphate uptake by phosphate accumulating organisms. Water Res. 38 (17), 3760–3768.

Santos, M.M., Lemos, P.C., Reis, M.A.M., Santos, H., 1999. Glucose metabolism and kinetics of phosphorus removal by the fermentative bacterium *Microlunatus phosphovorus*. Appl. Environ. Microbiol. 65 (9), 3920–3928.

Satoh, H., Mino, T., Matsuo, T., 1992. Uptake of organic substrates and accumulation of polyhydroxyalkanoates linked with glycolysis of intracellular carbohydrates under anaerobic conditions in the biological excess phosphate removal processes. Water Sci. Technol. 26 (5–6), 933–942.

Satoh, H., Mino, T., Matsuo, T., 1994. Deterioration of enhanced biological phosphorus removal by the domination of microorganisms without polyphosphate accumulation. Water Sci. Technol. 30 (6), 203–211.

Satoh, H., Ramey, W.D., Koch, F.A., Oldham, W.K., Mino, T., Matsuo, T., 1996. Anaerobic substrate uptake by the enhanced biological phosphorus removal activated sludge treating real sewage. Water Sci. Technol. 34 (1–2), 9–16.

Satoh, H., Mino, T., Matsuo, T., 1998. Anaerobic uptake of glutamate and aspartate by enhanced biological phosphorus removal activated sludge. Water Sci. Technol. 37 (4–5), 579–582.

Saunders, A.M., 2005. The physiology of microorganisms in enhanced biological phosphorus removal. Ph.D. Thesis, University of Queensland, St. Lucia, Australia.

Saunders, A.M., Oehmen, A., Blackall, L.L., Yuan, Z., Keller, J., 2003. The effect of GAOs (glycogen accumulating organisms) on anaerobic carbon requirements in full-scale Australian EBPR (enhanced biological phosphorus removal) plants. Water Sci. Technol. 47 (11), 37–43.

Schuler, A.J., 2005. Diversity matters: dynamic simulation of distributed bacterial states in suspended growth biological

wastewater treatment systems. Biotechnol. Bioeng. 91 (1), 62–74.
Schuler, A.J., Jenkins, D., 2002. Effects of pH on enhanced biological phosphorus removal metabolisms. Water Sci. Technol. 46 (4–5), 171–178.
Schuler, A.J., Jenkins, D., 2003a. Enhanced biological phosphorus removal from wastewater by biomass with different phosphorus contents, part I: experimental results and comparison with metabolic models. Water Environ. Res. 75 (6), 485–498.
Schuler, A.J., Jenkins, D., 2003b. Enhanced biological phosphorus removal from wastewater by biomass with different phosphorus contents, part III: anaerobic sources of reducing equivalents. Water Environ. Res. 75 (6), 512–522.
Serafim, L.S., Lemos, P.C., Reis, M.A.M., 2002. Effect of pH control on EBPR stability and efficiency. Water Sci. Technol. 46 (4–5), 179–184.
Serralta, J., Ferrer, J., Borras, L., Seco, A., 2004. An extension of ASM2d including pH calculation. Water Res. 38 (19), 4029–4038.
Seviour, R.J., Mino, T., Onuki, M., 2003. The microbiology of biological phosphorus removal in activated sludge systems. FEMS Microbiol. Rev. 27 (1), 99–127.
Shintani, T., Liu, W.T., Hanada, S., Kamagata, Y., Miyaoka, S., Suzuki, T., Nakamura, K., 2000. *Micropruina glycogenica* gen. nov., sp, nov., a new Gram-positive glycogen-accumulating bacterium isolated from activated sludge. Int. J. Syst. Evol. Microbiol. 50, 201–207.
Smolders, G.J.F., Vandermeij, J., Vanloosdrecht, M.C.M., Heijnen, J.J., 1994a. Model of the anaerobic metabolism of the biological phosphorus removal process—stoichiometry and pH influence. Biotechnol. Bioeng. 43 (6), 461–470.
Smolders, G.J.F., Vandermeij, J., Vanloosdrecht, M.C.M., Heijnen, J.J., 1994b. Stoichiometric model of the aerobic metabolism of the biological phosphorus removal process. Biotechnol. Bioeng. 44 (7), 837–848.
Smolders, G.J.F., Vandermeij, J., Vanloosdrecht, M.C.M., Heijnen, J.J., 1995. A structured metabolic model for anaerobic and aerobic stoichiometry and kinetics of the biological phosphorus removal process. Biotechnol. Bioeng. 47 (3), 277–287.
Stante, L., Cellamare, C.M., Malaspina, F., Bortone, G., Tilche, A., 1997. Biological phosphorus removal by pure culture of *Lampropedia* spp. Water Res. 31 (6), 1317–1324.
Stephens, H.M., Neethling, J.B., Benisch, M., Gu, A.Z., Stensel, H.D., 2004. Comprehensive analysis of full-scale enhanced biological phosphorus removal facilities. In: Water Environment Federation 77th Annual Conference and Exhibition, New Orleans, LA, USA.
Sudiana, I.M., Mino, T., Satoh, H., Nakamura, K., Matsuo, T., 1999. Metabolism of enhanced biological phosphorus removal and non-enhanced biological phosphorus removal sludge with acetate and glucose as carbon source. Water Sci. Technol. 39 (6), 29–35.
Tasli, R., Artan, N., Orhon, D., 1997. The influence of different substrates on enhanced biological phosphorus removal in a sequencing batch reactor. Water Sci. Technol. 35 (1), 75–80.
Tchobanoglous, G., Burton, F.L., Stensel, H.D., 2002. Wastewater Engineering: Treatment and Reuse, fourth ed. Metcalf & Eddy Inc., McGraw-Hill Science Engineering, NY, USA.
Thomas, M., Wright, P., Blackall, L., Urbain, V., Keller, J., 2003. Optimisation of Noosa BNR plant to improve performance and reduce operating costs. Water Sci. Technol. 47 (12), 141–148.
Tsai, C.S., Liu, W.T., 2002. Phylogenetic and physiological diversity of tetrad-forming organisms in deteriorated biological phosphorus removal systems. Water Sci. Technol. 46 (1–2), 179–184.
Tykesson, E., 2005. Enhanced biological phosphorus removal: processes, competing substances and tools for operation of wastewater treatment plants. Lund University, Lund, Sweden.
Tykesson, E., Blackall, L.L., Kong, Y., Nielsen, P.H., Jansen, J.L., 2006. Applicability of experience from laboratory reactors with biological phosphorus removal in full-scale plants. Water Sci. Technol. 54 (1), 267–275.
Ueno, Y., Fujii, M., 2001. Three years experience of operating and selling recovered struvite from full-scale plant. Environ. Technol. 22 (11), 1373–1381.
van Loosdrecht, M.C.M., Hooijmans, C.M., Brdjanovic, D., Heijnen, J.J., 1997. Biological phosphate removal processes. Appl. Microbiol. Biotechnol. 48 (3), 289–296.
van Loosdrecht, M.C.M., Brandse, F.A., de Vries, A.C., 1998. Upgrading of wastewater treatment processes for integrated nutrient removal—the BCFS (R) process. Water Sci. Technol. 37 (9), 209–217.
Van Veldhuizen, H.M., Van Loosdrecht, M.C.M., Heijnen, J.J., 1999. Modelling biological phosphorus and nitrogen removal in a full scale activated sludge process. Water Res. 33 (16), 3459–3468.
Wagner, M., Erhart, R., Manz, W., Amann, R., Lemmer, H., Wedi, D., Schleifer, K.H., 1994. Development of an ribosomal-RNA-targeted oligonucleotide probe specific for the genus *Acinetobacter* and its application for in-situ monitoring in activated-sludge. Appl. Environ. Microbiol. 60 (3), 792–800.
Wang, N.D., Peng, J., Hill, G., 2002. Biochemical model of glucose induced enhanced biological phosphorus removal under anaerobic condition. Water Res. 36 (1), 49–58.
Wentzel, M.C., Lötter, L.H., Loewenthal, R.E., Marais, G.v.R., 1986. Metabolic behaviour of *Acinetobacter* spp. in enhanced biological phosphorus removal—a biochemical model. Water Sa 12, 209–224.
Wentzel, M.C., Lotter, L.H., Ekama, G.A., Loewenthal, R.E., Marais, G.v.R., 1991. Evaluation of biochemical-Models for biological excess phosphorus removal. Water Sci. Technol. 23 (4–6), 567–576.
Whang, L.M., Park, J.K., 2002. Competition between polyphosphate- and glycogen-accumulating organisms in biological phosphorus removal systems—effect of temperature. Water Sci. Technol. 46 (1-2), 191–194.
Whang, L.M., Park, J.K., 2006. Competition between polyphosphate- and glycogen-accumulating organisms in enhanced-biological-phosphorus-removal systems: effect of temperature and sludge age. Water Environ. Res. 78 (1), 4–11.
Wichern, M., Obenaus, F., Wulf, P., Rosenwinkel, K.H., 2001. Modelling of full-scale wastewater treatment plants with different treatment processes using the activated sludge model no. 3. Water Sci. Technol. 44 (1), 49–56.
Wilmes, P., Bond, P.L., 2004. The application of two-dimensional polyacrylamide gel electrophoresis and downstream analyses to a mixed community of prokaryotic microorganisms. Environ. Microbiol. 6 (9), 911–920.
Wilmes, P., Bond, P.L., 2006. Towards exposure of elusive metabolic mixed-culture processes: the application of metaproteomic analyses to activated sludge. Water Sci. Technol. 54 (1), 217–226.
Wong, M.T., Liu, W.T., 2006. Microbial succession of glycogen accumulating organisms in an anaerobic–aerobic membrane bioreactor with no phosphorus removal. Water Sci. Technol. 54 (1), 29–37.
Wong, M.T., Tan, F.M., Ng, W.J., Liu, W.T., 2004. Identification and occurrence of tetrad-forming Alphaproteobacteria in anaerobic–aerobic activated sludge processes. Microbiology 150, 3741–3748.
Wong, M.T., Mino, T., Seviour, R.J., Onuki, M., Liu, W.T., 2005. In situ identification and characterization of the microbial community structure of full-scale enhanced biological phosphorous removal plants in Japan. Water Res. 39 (13), 2901–2914.
Yagci, N., Artan, N., Cokgor, E.U., Randall, C.W., Orhon, D., 2003. Metabolic model for acetate uptake by a mixed culture of

phosphate- and glycogen-accumulating organisms under anaerobic conditions. Biotechnol. Bioeng. 84 (3), 359–373.

Yagci, N., Insel, G., Tasli, R., Artan, N., Randall, C.W., Orhon, D., 2006. A new interpretation of ASM2d for modeling of SBR performance for enhanced biological phosphorus removal under different P/HAc ratios. Biotechnol. Bioeng. 93 (2), 258–270.

Zeng, R., Yuan, Z., van Loosdrecht, M.C.M., Keller, J., 2002. Proposed modifications to metabolic model for glycogen-accumulating organisms under anaerobic conditions. Biotechnol. Bioeng. 80 (3), 277–279.

Zeng, R.J., Lemaire, R., Yuan, Z., Keller, J., 2003a. Simultaneous nitrification, denitrification, and phosphorus removal in a lab-scale sequencing batch reactor. Biotechnol. Bioeng. 84 (2), 170–178.

Zeng, R.J., Saunders, A.M., Yuan, Z., Blackall, L.L., Keller, J., 2003b. Identification and comparison of aerobic and denitrifying polyphosphate-accumulating organisms. Biotechnol. Bioeng. 83 (2), 140–148.

Zeng, R.J., van Loosdrecht, M.C.M., Yuan, Z.G., Keller, J., 2003c. Metabolic model for glycogen-accumulating organisms in anaerobic/aerobic activated sludge systems. Biotechnol. Bioeng. 81 (1), 92–105.

Zeng, R.J., Yuan, Z., Keller, J., 2003d. Model-based analysis of anaerobic acetate uptake by a mixed culture of polyphosphate-accumulating and glycogen-accumulating organisms. Biotechnol. Bioeng. 83 (3), 293–302.

Zeng, R.J., Yuan, Z.G., Keller, J., 2003e. Enrichment of denitrifying glycogen-accumulating organisms in anaerobic/anoxic activated sludge system. Biotechnol. Bioeng. 81 (4), 397–404.

Zeng, R.J., Yuan, Z., Keller, J., 2006. Effects of solids concentration, pH and carbon addition on the production rate and composition of volatile fatty acids in prefermenters using primary sewage sludge. Water Sci. Technol. 53 (8), 263–269.

Zhang, T., Liu, Y., Fang, H.H.P., 2005. Effect of pH change on the performance and microbial community of enhanced biological phosphate removal process. Biotechnol. Bioeng. 92 (2), 173–182.

Zhao, H., Hao, O.J., McAvoy, T.J., Chang, C.H., 1997. Modeling nutrient dynamics in sequencing batch reactor. J. Environ. Eng.-ASCE 123 (4), 311–319.

Zilles, J.L., Peccia, J., Kim, M.W., Hung, C.H., Noguera, D.R., 2002a. Involvement of *Rhodocyclus*-related organisms in phosphorus removal in full-scale wastewater treatment plants. Appl. Environ. Microbiol. 68 (6), 2763–2769.

Zilles, J.L., Peccia, J., Noguera, D.R., 2002b. Microbiology of enhanced biological phosphorus removal in aerated-anoxic orbal processes. Water Environ. Res. 74 (5), 428–436.