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# Advancing post-anoxic denitrification for biological nutrient removal

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ABSTRACT

The objective of this research was to advance a fundamental understanding of a unique post-anoxic denitrification process for achieving biological nutrient removal (BNR), with an emphasis on elucidating the impacts of surface oxygen transfer (SOT), variable process loadings, and bioreactor operational conditions on nitrogen and phosphorus removal. Two sequencing batch reactors (SBRs) were operated in an anaerobic/aerobic/anoxic mode for over 250 days and fed real municipal wastewater. One SBR was operated with a headspace open to the atmosphere, while the other had a covered liquid surface to prevent surface oxygen transfer. Process performance was assessed for mixed volatile fatty acid (VFA) and acetate-dominated substrate, as well as daily/seasonal variance in influent phosphorus and ammonia loadings. Results demonstrated that post-anoxic BNR can achieve near-complete (>99%) inorganic nitrogen and phosphorus removal, with soluble effluent concentrations less than 1.0 mgN $L^{-1}$ and 0.14 mgP $L^{-1}$. Observed specific denitrification rates were in excess of typical endogenous values and exhibited a linear dependence on the glycogen concentration in the biomass. Preventing SOT improved nitrogen removal but had little impact on phosphorus removal under normal loading conditions. However, during periods of low influent ammonia, the covered reactor maintained phosphorus removal performance and showed a greater relative abundance of polyphosphate accumulating organisms (PAOs) as evidenced by quantitative real-time PCR (qPCR). While GAOs were detected in both reactors under all operational conditions, BNR performance was not adversely impacted. Finally, secondary phosphorus release during the post-anoxic period was minimal and only occurred if nitrate/nitrite were depleted post-anoxically.



## 1. Introduction

Phosphorus (P) and nitrogen (N) are nutrients of primary concern in regard to accelerated surface water eutrophication, and many wastewater treatment plants (WWTPs) are facing increasingly stringent effluent limitations for both nutrients. P and N can be readily removed biologically, with P removal achieved using an engineered process known as enhanced biological P removal (EBPR). EBPR is accomplished by exposing microbes to cyclical anaerobic/aerobic and/or anoxic conditions, with influent wastewater first directed to the anaerobic zone. The prescriptive EBPR configuration provides a selective advantage to organisms capable of storing volatile fatty acids (VFAs) anaerobically as polyhydroxyalkanoates (PHAs), such as polyphosphate accumulating organisms (PAOs), which remove and store excess P as intracellular polyphosphate (poly-P) and are the putative organisms responsible for EBPR. EBPR can also enrich for glycogen accumulating organisms

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(GAOs), which do not appear to contribute to EBPR and are therefore considered undesirable. PAOs generate energy for VFA uptake through hydrolysis of intracellular poly-P and glycogen degradation through glycolysis (Smolders et al., 1994), with glycogen degradation considered the main source of reducing power ($NADH_2$) for PHA storage (Zhou et al., 2010). Under aerobic and/or anoxic conditions, PAOs oxidize PHA via the TCA cycle to provide energy for growth, glycogen replenishment, P uptake, and poly-P storage (Smolders et al., 1995). As will be discussed later, *Candidatus* "Accumulibacter phosphatis" (henceforth referred to as Accumulibacter) has been suggested to be a dominant PAO, based on lab-scale and full-scale studies (He et al., 2007). GAOs exhibit similar metabolisms, with the exclusion of P cycling. Extensive research on factors affecting the PAO-GAO competition can be found elsewhere (Lopez-Vazquez et al., 2009; Oehmen et al., 2010a, 2007).

The combination of P and N removal is referred to as biological nutrient removal (BNR). Most BNR WWTPs accomplish denitrification using a pre-anoxic configuration, where the anoxic zone is located upstream of the aerobic zone. Since denitrification relies on ammonia oxidation in an aerobic zone, high mixed liquor recycle (MLR) rates are needed to provide a nitrate source in the anoxic zone. Although high specific denitrification rates (SDNRs) can be obtained with this configuration, there are several disadvantages associated with MLR pumping: higher energy costs, dissolved oxygen (DO) return from the aerobic, and dilution of influent carbon. Most importantly, the removal of oxidized nitrogen ($NO_x$; nitrate + nitrite) is ultimately limited by the MLR rate, and complete $NO_x$ removable is unattainable (estimated 3–5 mg $L^{-1}$ effluent total N) (Tchobanoglous et al., 2003).

Post-anoxic denitrification eliminates the need for MLR pumping, since the anoxic tank is located downstream of the aerobic nitrifying tank, and can produce effluent less than 3 mg $L^{-1}$ total N (Tchobanoglous et al., 2003). In a non-EBPR system, an exogenous carbon source is typically supplied to drive denitrification. However, this approach cannot be applied to an EBPR system because the addition of carbon could promote phosphorus release (Kuba et al., 1994) and/or lead to proliferation of ordinary heterotrophic organisms which are incapable of excess P removal. Instead, a dual-sludge system is employed to separate the PAO and nitrifying sludges (Bortone et al., 1996; Kuba et al., 1996b). The PAO sludge bypasses nitrification, and intracellular PHA is thus conserved for post-anoxic denitrification. While the dual-sludge configuration eliminates the need for MLR pumping, it requires more underflow pumping and a larger footprint due to additional settlers.

An alternative post-anoxic EBPR-based configuration would leverage residual PAO carbon reserves (PHA and/or glycogen) to drive denitrification. In this operating scenario, use of the influent organic carbon and associated electrons is maximized for efficient nutrient removal. Further, this process configuration could produce lower effluent N and P loads as compared to traditional BNR configurations. Promising results have been obtained with lab- and pilot-scale continuous flow membrane bioreactors (Bracklow et al., 2010; Vocks et al., 2005) and lab-scale sequencing batch reactors (SBRs) (Coats et al., 2011b). These systems achieved SDNRs in excess of endogenous rates, and exhibited high N and P removal efficiencies. Recognizing the potential of this novel post-anoxic BNR process, and considering prior work, the research presented and discussed herein focused on understanding the effects of process operation and wastewater loading on N and P removal. New insight on relevant post-anoxic maintenance metabolisms is provided, and the issue of secondary P release is examined. This research also considered the long-term effects of surface oxygen transfer (SOT) on the anaerobic and anoxic aspects of the process, which has not been studied in relation to EBPR. Others have observed impaired SDNRs as result of SOT/microaerophilic conditions in open anoxic basins (Martins et al., 2004; Oh and Silverstein, 1999; Plósz et al., 2003), and therefore the issue could be especially relevant for a carbon-limited post-anoxic environment. The research also interrogated respective microbial consortia on PAO and GAO fraction. The research presents results based on the use of real municipal wastewater rather than the much more common approach of using synthetic wastewater.

## 2. Materials and methods

### 2.1. Experimental setup

Two independent SBRs (reactors O and C) were operated for over 250 days, with monitoring events as shown (Fig. 1). The reactors were operated identically except for the headspace condition; reactor O had a headspace open to the atmosphere, while reactor C had zero headspace due to a liquid surface covered with a polyethylene disk. Thus, SOT could occur during the anaerobic and anoxic periods in reactor O but not in reactor C. Note that for all of the figures in this manuscript, reactor O is represented by open symbols, while reactor C is denoted by filled symbols. Each SBR (0.9 L operating volume) was inoculated with activated sludge obtained from the Moscow, ID WWTP, which operates a hybrid $A^2$/O-oxidation

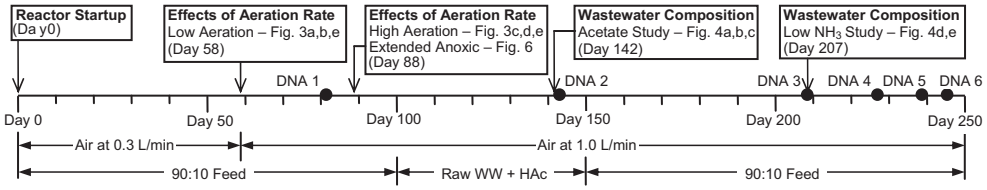


**Fig. 1 – Research timeline showing time points for all sampling investigations in this study (aeration rate and substrate are provided below the timeline).**

ditch process (Tchobanoglous et al., 2003). Each 6 h operational cycle consisted of 1 h anaerobic, 2 h aerobic, 2.5 h anoxic, and 0.5 h for settling/decanting. At the beginning of each cycle, 0.3 L of wastewater was fed to each reactor, resulting in a hydraulic retention time (HRT) of 18 h. The solids retention time (SRT) was maintained at 20 days by wasting 45 mL of mixed liquor at the end of one anoxic period each day. pH was not controlled, and varied between 7.0 and 7.3, 7.3–7.6, and 7.3–7.6 during the anaerobic, aerobic, and anoxic periods, respectively. The air flow rate was controlled at either 0.3 or 1.0 L $min^{-1}$ during the aerobic period (Fig. 1; referred to as low and high aeration studies). Mixing was accomplished using magnetic stirrers and feeding/decanting was performed with peristaltic pumps. All operations were controlled by a bench-top programmable logic controller (PLC). The reactors were operated in a temperature controlled room which resulted in average reactor temperatures of 23 ± 3 °C.

### 2.2. Wastewater source and composition

Screened and de-gritted raw wastewater was obtained weekly from the Moscow, ID WWTP and stored at 4 °C in polyethylene jugs until use. Wastewater was filtered through cheesecloth prior to daily batching of feed. To supply additional VFAs, the raw wastewater was mixed with either VFA-rich fermenter liquor (90:10; 90% raw wastewater and 10% fermenter liquor by volume) or 10 mL per L of a concentrated sodium acetate solution which supplied an additional 200 mgCOD $L^{-1}$ to the feed (raw WW + HAc), as illustrated in Fig. 1. VFA-rich fermenter liquor was recovered from a laboratory fermenter fed with primary solids from the Pullman, WA WWTP (Coats et al., 2011b). The VFA distribution in the 90:10 feed averaged 52 ± 5% acetate (HAc), 34 ± 6% propionate (HPr), 12 ± 2% butyrate (HBu), and 5 ± 1% valerate (HVa) on a Cmol basis; nominal amounts of iso-HBu and iso-HVa were observed as well. Influent P varied from 3.5 to 6.0 mgP $L^{-1}$, and influent ammonia concentrations ranged from 14 to 50 mgN $L^{-1}$. The reactors received the 90:10 substrate for a majority of the study (Fig. 1), and an example of the daily variations in the 90:10 characteristics are shown in Fig. 2 for about 30 days prior to the first sampling run. During this time period, total VFAs ranged from 200 to 275 mgCOD $L^{-1}$ which yielded an influent VFA:P ratio of 35–50 mgCOD $mgP^{-1}$, which was theoretically sufficient to be favorable for PAOs (Oehmen et al., 2007). Reactors were allowed to stabilize for about 3 SRTs between operational changes before performance was assessed. Table 1 provides additional information about the influent wastewater characteristics for each sampling event.

### 2.3. Stoichiometric calculations

Due to the SBR configuration and post-anoxic operational mode, residual $NO_x$ carryover from the anoxic period to the subsequent anaerobic cycle occurred. While the amount of carryover was insufficient to upset EBPR performance, some of the influent VFAs would have been consumed to reduce the residual $NO_x$ (mainly nitrate). Much debate exists regarding how much influent COD would be consumed for nitrate reduction, and others have commonly assumed a ratio of 8.6 mgCOD $mgNO_3$–$N^{-1}$ (Henze et al., 2008). However, this

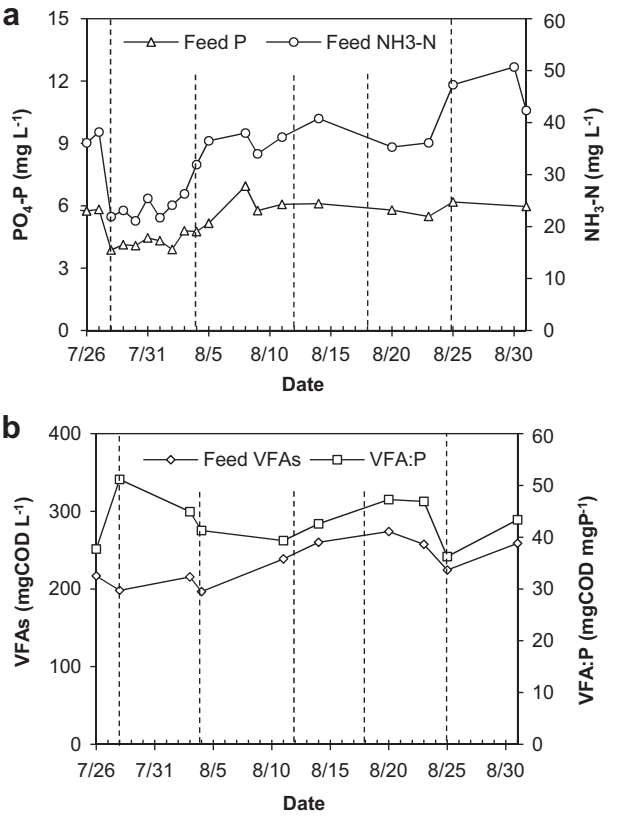


**Fig. 2 – Typical influent characteristics for (a) P and ammonia, and (b) VFAs and VFA:P ratio at the beginning of this study. The dashed lines show each time that a new batch of wastewater was collected.**

ratio likely overestimates COD utilization because it assumes an anoxic yield equal to the typical aerobic heterotrophic yield value of 0.666 mgBiomassCOD $(mgCODutilized)^{-1}$. Others have suggested that the anoxic yield is 60–70% of the aerobic yield and also dependent on the substrate provided (Copp and Dold, 1998). In this study, residual nitrate at the beginning of the anaerobic period was low and available for less than 5 min, which would also limit the anoxic yield. Assuming that acetate was the preferred VFA for denitrification (Elefsiniotis and Wareham, 2007) and that the anoxic yield on acetate was 0.192 mgBiomassCOD $(mgCODutilized)^{-1}$ (Copp and Dold, 1998), a ratio of 3.54 mgCOD $mgNO_3$–$N^{-1}$ was obtained. Accordingly, all EBPR stoichiometric VFA ratios were calculated assuming that 3.54 mgCOD $mgNO_3$–$N^{-1}$ and 1.71 mgCOD $mgNO_2$–$N^{-1}$ were utilized for nitrate and nitrite reduction, respectively (Tchobanoglous et al., 2003). Given that the $NO_x$ residual was small in comparison to the influent VFAs, this assumption caused minimal variation in the ratios.

### 2.4. Analysis

#### 2.4.1. Chemical analyses

All soluble constituents were filtered through a 0.22 μm Millex GP syringe-driven filter (Millipore, MA, USA). Phosphate ($PO_4$–P) and nitrate ($NO_3$–N) were determined colorimetrically

**Table 1 – Influent characteristics for all sampling investigations in this study.**

| Study type | Influent characteristics[a] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | $PO_4$–P (mg $L^{-1}$) | $NH_3$–N (mg $L^{-1}$) | HAc | HPr | HBu | HVa | Total VFAs | Influent VFA:P | Influent VFA:$NH_3$–N |
| Low Aeration | 5.97 | 42.4 | 3.27 | 2.62 | 0.91 | 0.39 | 7.28 | 43 | 6.1 |
| High Aeration | 5.48 | 34.2 | 3.72 | 3.14 | 0.72 | 0.41 | 8.24 | 54 | 8.6 |
| Acetate | 3.87 | 35.9 | 5.62 | 0.57 | 0.00 | 0.00 | 6.19 | 52 | 5.6 |
| Low $NH_3$ - Cycle 1 | 4.42 | 14.6 | 2.95 | 1.53 | 0.55 | 0.21 | 5.24 | 41 | 12.5 |
| Low $NH_3$ - Cycle 2 | 4.99 | 16.0 | 3.45 | 1.79 | 0.00 | 0.00 | 5.24 | 36 | 11.1 |

a VFA units reported as Cmmol $L^{-1}$; VFA:P and VFA:N are mgCOD $mgP^{-1}$ and mgCOD $mgN^{-1}$, respectively.

(Coats et al., 2011b). Ammonia ($NH_3$–N) and nitrite ($NO_2$–N) were measured in accordance with Hach (Loveland, CO, USA) methods 10031 and 10019, respectively. MLSS and MLVSS were analyzed according to the standard methods (APHA, 1998). pH was monitored using American Marine (Ridgefield, CT, USA) Pinpoint® pH controllers. VFAs were measured using a gas chromatograph (GC) equipped with a flame ionization detector (Coats et al., 2011a). Glycogen was determined with dried biomass samples as described by Parrou and Francois (1997); biomass samples were washed with a 1% NaCl solution prior to analysis to minimize potential interference with exopolysaccharide (EPS), the latter being a source of slowly biodegradable carbon for bacteria. Intracellular PHA was quantified using a GC equipped with a mass spectrometer (Coats et al., 2011b). Surface oxygen mass transfer coefficients ($K_La_{SUR}$) were determined using room temperature tap water according to the dynamic gassing out method of Van't Riet (1979), where saturation DO concentrations were estimated using temperature and pressure correction equations (APHA, 1998). $K_La_{SUR}$ values were calculated by minimizing the total sum of square errors between the predicted DO from the mass transfer model and the actual DO measurements. Both DO and temperature were recorded simultaneously using a Hach HQ30d Meter and LDO101 DO Probe. The volumetric oxygen mass transfer coefficient, $K_La_{SUR}$, in reactor O was measured at 0.54 $h^{-1}$.

### 2.4.2. *Microbial population analyses*

Genomic DNA was extracted from each reactor on the dates shown (Fig. 1) according to the procedure outlined in Coats et al. (2011c). Quantitative real-time PCR (qPCR) was used to quantify 16S rDNA genes from total bacteria, Accumulibacter (the model PAO), and GAOs to provide an estimation of relative abundance. qPCR was conducted on a StepOne Plus™ Real-Time PCR system (Applied Biosystems, Foster City, CA) using iTaq™ SYBR® Green Supermix w/ROX (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with a total reaction volume of 25 μl. Total bacterial and total Accumulibacter 16S rDNA genes were quantified with primer sets 341f/534r and 518f/846r, respectively (He et al., 2007). GAOs were quantified using primer set GAOQ431f/GAOQ989r (specifically designed to target *Candidatus* Competibacter phosphatis, which is a putative model GAO (Crocetti et al., 2002)) and the total bacteria primer set. In addition, a primer set targeting the GB lineage (specifically GB612f/GAOQ989r (Kong et al., 2002), coupled with the total bacteria primer set) was employed to quantify *Gammaproteobacteria*. The GB lineage, also referred to as the *Competibacter* lineage, is proposed to capture the predominant GAOs within the class *Gammaproteobacteria* that would be present in EBPR WWTPs (Kong et al., 2006; Oehmen et al., 2007). qPCR conditions were as follows: 3 min at 95 °C, 45 cycles of 30 s at 95 °C, 45 s at 60 °C, and 30 s at 72 °C. All unknown samples were assessed in triplicate with 5 ng of total genomic DNA per reaction. Amplification efficiency was estimated for each primer set using baseline-corrected fluorescence data (from StepOne Software v2.0) with LinRegPCR (Ramakers et al., 2003). For PAO quantification, mean amplification efficiencies for the total bacterial and PAO primer sets were 96.8 ± 1.8% ($n = 37$) and 93.9 ± 1.5% ($n = 36$), respectively. For GAO and GB lineage quantification, mean amplification efficiencies for the respective primer sets were 85.2 ± 3.1% (GAOs; $n = 76$), 93.6 ± 3.4% (GB lineage; $n = 76$), and 93.9 ± 3.0% ($n = 76$). The cycle threshold was set at a constant value across all samples based on location within the log-linear region for

**Table 2 – Anaerobic biochemical transformations and P removal performance.**

| Study type | Feed | Reactor name | MLVSS (mg $L^{-1}$) | EBPR anaerobic stoichiometry (normalized to VFAs)[a] | | | | | | P uptake rate[b] | Effluent $PO_4$–P[b] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | $P_{rel}$ | Gly | PHB | PHV | PH2MV | PHA | | |
| Low Aeration | 90:10 | O | 3560 | 0.29 | 0.18 | 0.36 | 0.53 | 0.12 | 1.02 | 13.6 | 0.08 |
| | | C | 3770 | 0.29 | 0.40 | 0.36 | 0.46 | 0.12 | 0.94 | 8.8 | 0.10 |
| High Aeration | 90:10 | O | 5060 | 0.20 | 0.68 | 0.28 | 0.73 | 0.16 | 1.17 | 25.7 | 0.09 |
| | | C | 5470 | 0.21 | 0.50 | 0.30 | 0.81 | 0.14 | 1.24 | 22.0 | 0.14 |
| | HAc | O | 1520 | 0.15 | 0.71 | 0.84 | 0.27 | 0.00 | 1.11 | 7.4 | 0.02 |
| | | C | 1996 | 0.16 | 0.61 | 0.93 | 0.33 | 0.00 | 1.18 | 9.1 | 0.03 |

a All ratios reported as Cmol $Cmol^{-1}$ except for $P_{rel}$/VFA (Pmol $Cmol^{-1}$).
b Aerobic P uptake rate reported as mgP $gVSS^{-1}$ $h^{-1}$; Effluent $PO_4$–P reported as mg $L^{-1}$.

determination of Cq values (cycle number at which the measured fluorescence exceeds the cycle threshold). Relative PAO abundance (Table 3) was estimated using the mean efficiencies for each primer set and the Cq values for the individual samples, assuming that the average 16S rDNA gene copy number is 4.1 per bacterial cell and 2 per Accumulibacter cell (He et al., 2007). For relative and comparative GAO and GB lineage abundance between sampling events, lacking a mapped genome, an average 16S rDNA gene copy number of 4.1 per bacterial cell was assumed for the targeted populations. Gel electrophoresis of qPCR products confirmed the presence of a single band for all GAO and PAO samples.

## 3. Results and discussion

Two SBRs were operated continuously for over 250 days (Fig. 1). Numerous investigations were conducted on each reactor over the course of this time period in order to understand core operating fundamentals associated with this BNR process. Results from these investigations are presented and discussed below.

### 3.1. *Effects of aeration rate on process performance*

Considering that aeration accounts for upwards of 70% of the total energy cost at biological WWTPs (Cornel et al., 2003), minimizing air requirements represents a significant cost savings opportunity. Thus, performance was assessed at both low and high aeration rates during the normal reactor cycle on the dates shown (Fig. 1). Our process configuration demands oxygen principally for nitrification, because incomplete ammonia oxidation would potentially impair the post-anoxic based process configuration. For the low aeration study, the DO remained below 1 mg $L^{-1}$ for the first 1.5 h before increasing to 6.25 mg $L^{-1}$ near the end of the aerobic period (Fig. 3a). In contrast, the high aeration study showed a rapid increase in the DO profile, surpassing 2 mg $L^{-1}$ within the first 7 min of aeration (Fig. 3c). DO profiles were only measured for the open reactor. The aeration, influent, and effluent piping were hard-connected through the closed reactor cover, and we elected not to remove the cover to measure DO in order to maintain the integrity of our experimental design. Although the closed reactor exhibited higher MLVSS (Table 2), which would have imposed a higher oxygen demand, considering that the applied aeration rate was the same for both reactors the DO profiles in the closed reactor were assumed to be comparable to that measured in the open reactor. The observed removal of ammonia, VFAs, and phosphorus during the aerobic period (Fig. 3) supports this assumption.

#### 3.1.1. *Phosphorus removal*

Excellent P removal was achieved in both reactors regardless of the aeration rate (Fig. 3a, c; Table 2), and P cycling was consistent with current EBPR theory (Smolders et al., 1994). At each aeration rate, the anaerobic stoichiometric P release ($P_{rel}$/VFA; Pmol $Cmol^{-1}$) was identical between reactors (Table 2), suggesting negligible impact of SOT on anaerobic EBPR metabolisms. However, comparatively higher $P_{rel}$/VFA ratios were observed at the low aeration rate. Zhang et al. (2008) reported $P_{rel}$/VFA ratios in the range of 0.43–0.51 for their PAO-enriched SBRs fed a mixture of acetic and propionic acids; similarly, PAO-enriched cultures exhibited $P_{rel}$/VFA ratios of 0.48 and 0.42 for acetate (Smolders et al., 1995) or propionate (Oehmen et al., 2005), respectively. Comparatively, the ratios observed in this study were markedly lower. Current theory would suggest that the decrease in $P_{rel}$/VFA ratios and the corresponding increase in anaerobic glycogen degradation between aeration rates (Table 2) indicates a population shift toward GAOs at the higher aeration rate (Schuler and Jenkins, 2003). However, in both reactors under high aeration conditions Accumulibacter abundance was estimated to be relatively high at 24–25%, while the GAO fraction was lower (2.8–5.4%; DNA1, Table 3); DNA was not available to comparatively characterize the population at the low aeration rate. Since both configurations achieved excellent P removal, clearly the respective consortia were sufficiently enriched with PAOs. The lower $P_{rel}$/VFA ratios, and the associated decrease in this ratio, would appear to have been influenced more by the use of real wastewater, which is inherently more complex than synthetic wastewater used in most EBPR studies.

At the low aeration rate, biomass in both reactors accumulated comparable amounts of PHA anaerobically, producing PHB, PHV, and PH2MV (Table 2). A similar PHA composition was observed by Zhang et al. (2008) for their PAO-enriched SBR fed an equal concentration of acetate and propionate. At the high aeration rate, PHV synthesis increased moderately despite nearly identical influent VFA fractions

**Table 3 – Relative fraction of PAOs ($n = 3$) and GAOs ($n = 6$; except DNA5 $n = 8$) within the bacterial community as estimated by qPCR for the DNA extraction dates shown in the timeline (Fig. 1).**

| Sample ID | PAOs | | GAOs | | | |
|---|---|---|---|---|---|---|
| | | | GAO primer set | | GB lineage | |
| | Reactor O | Reactor C | Reactor O | Reactor C | Reactor O | Reactor C |
| DNA1 | 24.5 ± 1.4 | 24.1 ± 1.8 | 2.8 ± 0.7 | 3.5 ± 0.2 | 3.6 ± 0.2 | 5.4 ± 0.8 |
| DNA2 | 1.0 ± 0.06 | 1.5 ± 0.11 | 5.3 ± 1.3 | 6.0 ± 0.5 | 10.4 ± 0.8 | 11.4 ± 1.7 |
| DNA3 | 0.9 ± 0.09 | 1.5 ± 0.12 | 2.4 ± 0.3 | 11.5 ± 1.0 | 12.9 ± 1.0 | 46.6 ± 3.6 |
| DNA4 | 4.1 ± 0.11 | 12.4 ± 0.11 | 0.3 ± 0.04 | 3.5 ± 0.3 | 3.4 ± 0.6 | 8.9 ± 0.4 |
| DNA5 | 8.7 ± 0.51 | 12.5 ± 0.54 | 2.6 ± 0.6 | 3.6 ± 0.5 | 14.9 ± 1.8 | 15.2 ± 2.4 |
| DNA6 | 4.7 ± 0.24 | 17.0 ± 1.53 | 0.2 ± 0.1 | 3.4 ± 0.2 | 3.5 ± 0.9 | 10.7 ± 0.7 |

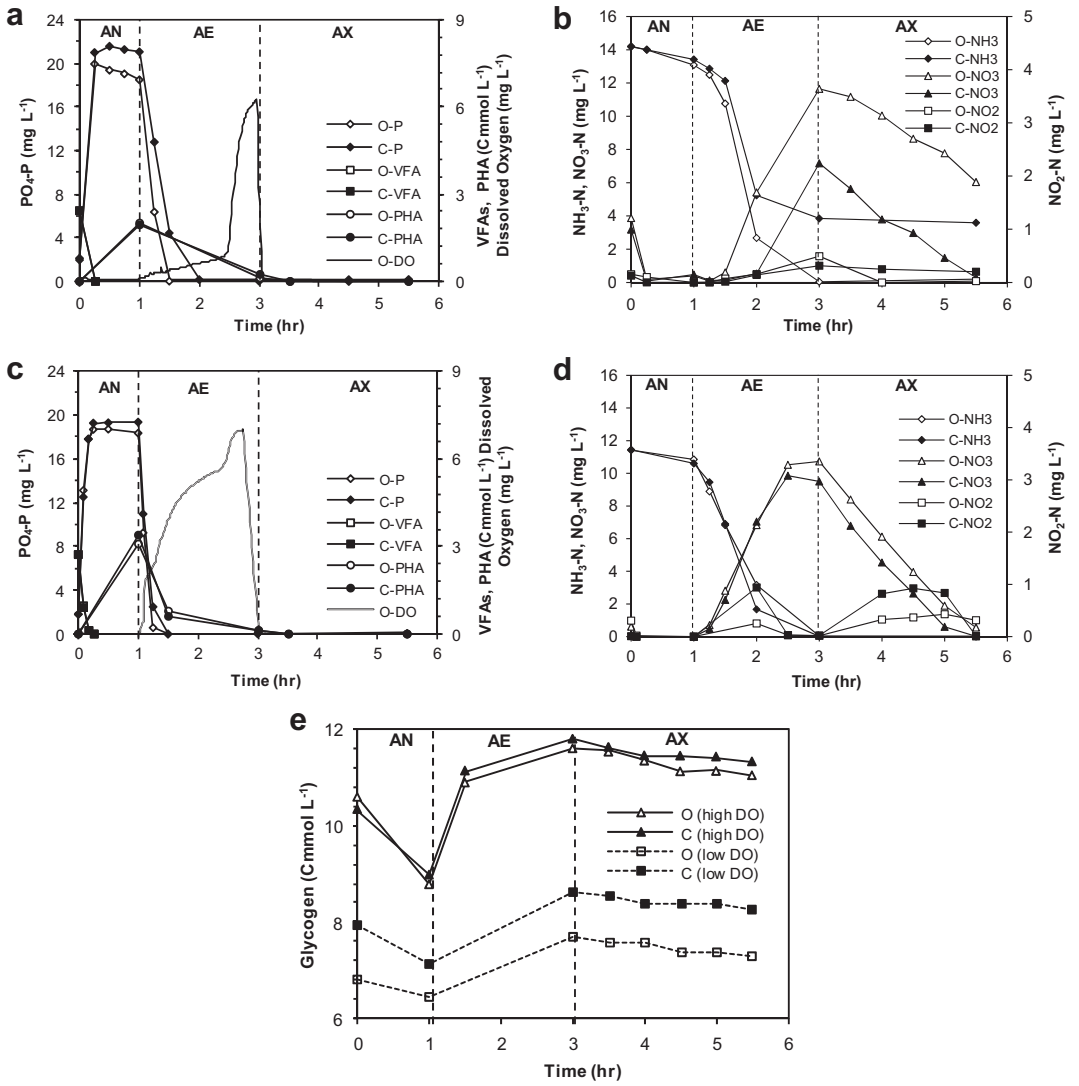


**Fig. 3 – Effects of aeration rate on process performance. Low aeration rate cycle profiles for (a) P, PHA, and DO, and (b) ammonia, nitrate, and nitrite. High aeration rate cycle profiles for (c) P, PHA, and DO, and (d) ammonia, nitrate, and nitrite. Glycogen profiles (e) for both low and high aeration studies.**

(Table 1); as noted, consortia in both reactors also exhibited increased Gly/VFA ratios, which likely contributed to the greater PHV fraction (Table 2). While both PAOs (Lopez et al., 2006; Lu et al., 2007) and GAOs (Liu et al., 1994) have been suggested to produce PHV from glycolysis end products and the propionate-succinate pathway, this is more commonly considered a GAO metabolism. As shown (Table 3), GAOs were present in both respective consortia.

### 3.1.2. *Nitrogen removal*

Total nitrogen removal at the low aeration rate (ammonia, nitrate, and nitrite) was 85% for reactor O and 90% for reactor C, while at the high aeration rate reactors O and C achieved 97% removal and 100% removal, respectively (Table 4; relative to influent ammonia, Table 1). Although reactor C did not achieve complete nitrification during the low aeration rate study (Fig. 3b; Table 4), preventing SOT moderately improved overall N removal at both aeration states. Despite the theoretical transfer of approximately 10.8 $mgO_2\ L^{-1}$ during the denitrification reaction period, reactor O maintained comparable SDNRs to reactor C. The most likely explanation for higher effluent $NO_x$ in reactor O (low aeration) was reduced intracellular glycogen (Fig. 3e).

Recognizing that PHA is the preferred aerobic carbon source of PAOs, glycogen would most commonly be the only intracellular carbon source remaining post-anoxically that could potentially drive denitrification. This, in fact, was observed for both the low and high aeration studies (Fig. 3e) and is consistent with similar studies (Coats et al., 2011b; Vocks et al., 2005). All SDNRs (Table 4) were in excess of typical values for endogenous decay, which have been shown to range from 0.2 to 0.6 $mgNO_3$–N $gVSS^{-1}\ h^{-1}$ (Kujawa and Klapwijk, 1999), and the results suggest that PAO maintenance metabolisms were driving denitrification (see sections 3.2.2 and 3.3). Similar observations supporting PAO maintenance energy production via glycogen oxidation with nitrate

**Table 4 – N removal performance and denitrification rates during post-anoxic period.**

| Study type | Feed | Reactor name | Effluent nitrogen (mgN $L^{-1}$) | | | | Post-AX characteristics[a] | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | $NH_3$–N | $NO_3$–N | $NO_2$–N | $NO_x$ | T (°C) | SDNR | $SDNR_{20}$ |
| Low Aeration | 90:10 | O | 0.20 | 6.04 | 0.23 | 6.27 | 24.4 | 0.71 | 0.63 |
| | | C | 3.59 | 0.29 | 0.20 | 0.49 | 24.4 | 0.90 | 0.80 |
| High Aeration | 90:10 | O | 0.03 | 0.56 | 0.31 | 0.87 | 25.5 | 1.09 | 0.95 |
| | | C | 0.01 | 0.02 | 0.01 | 0.03 | 25.5 | 1.09 | 0.95 |
| | HAc | O | 0.07 | 8.08 | 0.00 | 8.09 | 22.7 | 0.33 | 0.31 |
| | | C | 0.05 | 5.73 | 0.43 | 6.16 | 22.7 | 0.62 | 0.58 |

a SDNR values reported as mg$NO_3$–N $gVSS^{-1}$ $h^{-1}$.

have been reported by others (Coats et al., 2011b; Lu et al., 2007; Vocks et al., 2005).

### 3.2. Wastewater composition and process performance

#### 3.2.1. Acetate effects

Propionate has been postulated to be a more favorable EBPR carbon source than acetate (Oehmen et al., 2007). In this regard, the wastewater used in our study contained significant propionate, as indicated by the HPr-to-total VFA ratio of approximately 0.36–0.38 Cmol $Cmol^{-1}$, and excellent P removal was observed. However, since the majority of EBPR studies use acetate as the sole carbon source, it was of interest to determine if process performance could be maintained with acetate-dominated feed and how a VFA switch might affect the consortia. The reactors were switched from the 90:10 substrate to the "raw WW + HAc" feed (Fig. 1), where acetate generally comprised greater than 90% of the influent VFA fraction (Table 1; Cmol basis). Although $P_{rel}$/VFA ratios decreased moderately, both reactors achieved excellent EBPR, with the lowest effluent P observed in this study (Fig. 4a, b; Table 2). However, the approximately 71% reduction for reactor O and 59% reduction for reactor C in aerobic P uptake rate (Table 2) does suggest a potential population shift associated with the dominant acetate substrate. *Competibacter*, for example, has been shown to prefer acetate as a carbon source and could compete with PAOs at the anaerobic pH range realized in this study (Lopez-Vazquez et al., 2009). Interestingly, as shown (Table 3; DNA2) the Accumulibacter abundance did decrease more than 10-fold in both reactors after switching to the acetate feed. Further, note that the percentages remained relatively low even 3 SRTs after transitioning back to the 90:10 feed (i.e., DNA3). Conversely, the relative GAO abundance, as measured by both the GAO primer set and the GB lineage, increased substantially in both reactors (Table 3; DNA2). These observations generally align with current EBPR theory, which would suggest that the decrease in $P_{rel}$/VFA ratios and corresponding increase in Gly/VFA ratios associated with the shift in substrate (Table 2) is indicative of a shift toward GAOs (Schuler and Jenkins, 2003). Nevertheless, recognizing that P removal remained consistently high in both reactors, it would appear that the Accumulibacter population was sufficient for EBPR success. Alternately, other PAOs not targeted by the Accumulibacter primers were present, or perhaps some of the GAOs performed EBPR.

Nitrogen cycling in the acetate-augmented reactors was generally similar to that observed in the 90:10 wastewater reactors (Fig. 3d vs. Fig. 4b; Table 4), although total inorganic-N removal was 77% for reactor O and 83% for reactor C, the lowest values observed in this study. MLVSS concentrations also decreased from over 5000 mg $L^{-1}$ to less than 2000 mg $L^{-1}$. As contrasted with the 90:10 wastewater-fed reactors (Fig. 3), the synthetic acetate-augmented substrate generated lower intracellular glycogen reserves, and less glycogen was used anoxically (Fig. 4c). Notably, the SDNR in reactor O approached endogenous levels, suggesting that SOT may have been detrimental to process performance (Fig. 4b). It is also possible that the combined effects of SOT and an acetate-rich substrate enriched for a consortium less efficient at, or less capable of, denitrification. Regarding PAOs, as noted the population did decrease quite substantially (Table 3). In this regard, Carvalho et al. (2007) suggested that acetate fed SBRs are more likely to select for an Accumulibacter strain that can use nitrite but not nitrate as electron acceptor (nitrite-DPAOs). Further, Martin et al. (2006) determined that Accumulibacter may not harbor the metabolic capability to reduce nitrate (but could reduce nitrite), and suggested that other members of the microbial consortium provided nitrite. Finally, Flowers et al. (2009) determined that certain clades within the model PAO quantified herein could not readily use nitrate. In addition to potential metabolic limitations with PAOs, it could alternately be suggested that the increase in GAOs could have contributed to reduced denitrification capacity, since not all of the subgroups in the GB lineage can denitrify (Kong et al., 2006; Oehmen et al., 2007).

#### 3.2.2. Post-anoxic denitrification using slowly biodegradable carbon

Real wastewater contains a complex mixture of slowly- and readily-biodegradable carbon sources, whereas synthetic wastewater commonly used in most EBPR and BNR research contains 100% readily biodegradable carbon (i.e., pure VFAs). In this regard, the presence of residual slowly-biodegradable carbon could have been partially responsible for increasing post-anoxic denitrification rates beyond typical endogenous values (although the aerobic period could theoretically induce measurable oxidation of this substrate). To understand if the raw wastewater or the fermenter liquor was providing slowly-biodegradable carbon (in bulk solution; not as EPS) for post-anoxic denitrification, reactor performance was observed over two consecutive cycles, where the feed for

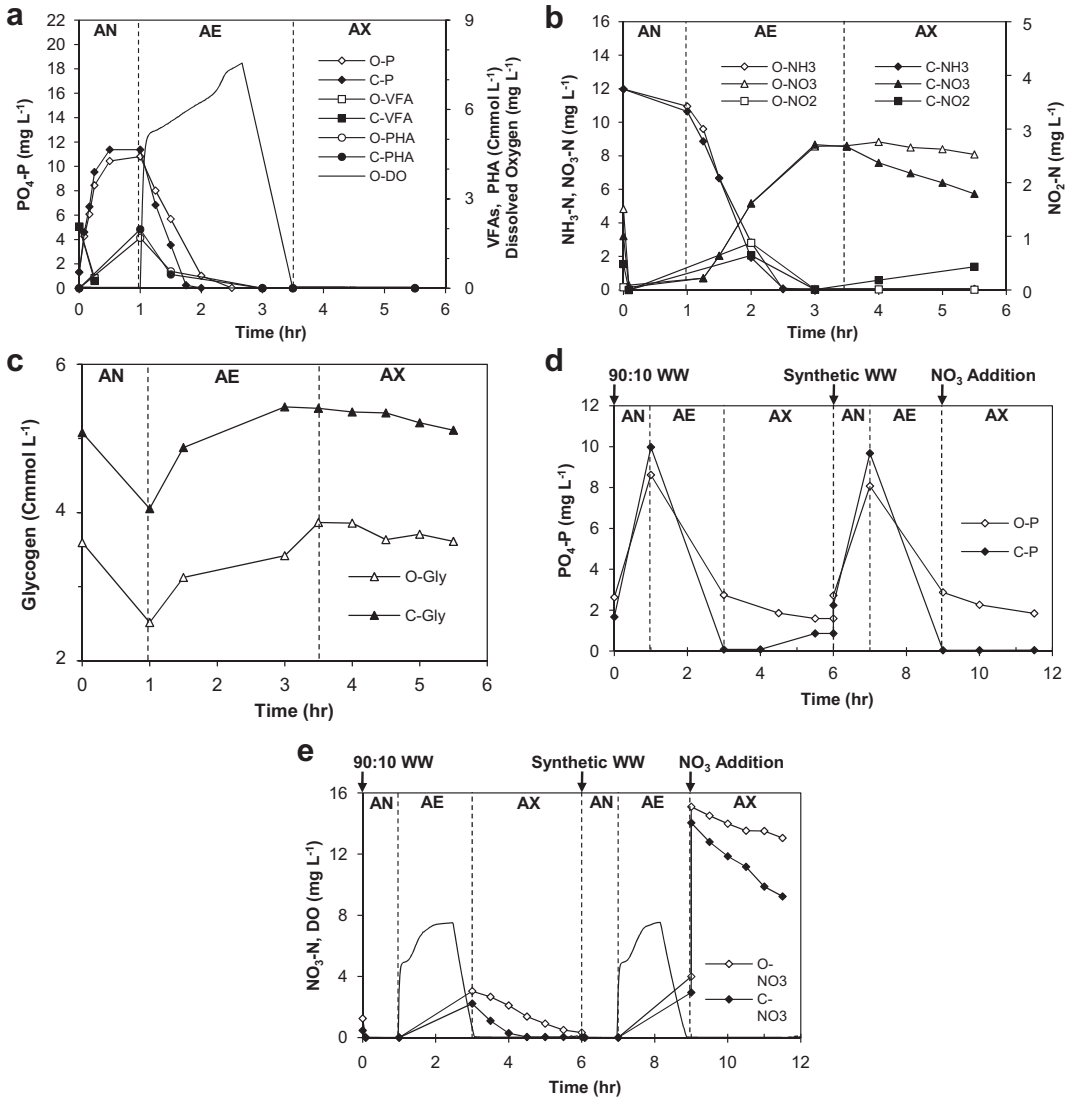


**Fig. 4 – Effects of wastewater composition on process performance. Acetate study cycle profiles for (a) P, PHA, and DO, and (b) ammonia, nitrate, and nitrite, and (c) glycogen. Low ammonia study cycle profiles for (d) P, and (e) nitrate and DO over two consecutive cycles, where the feed for the first cycle (0–6 h) was 90:10 and the feed for the second cycle (6–12 h) was a synthetic WW with similar composition.**

the first cycle was the 90:10 wastewater mixture (5.24 Cmmol $L^{-1}$ VFAs) and the feed for the second cycle was entirely a synthetic wastewater (i.e., no bulk solution slowly biodegradable carbon provided). The synthetic wastewater was designed to contain the same influent P, N, and VFAs (3.45 Cmmol $L^{-1}$ HAc and 1.79 Cmmol $L^{-1}$ HPr) as the 90:10 substrate, with other nutrient concentrations provided as described by Kuba et al. (1996a). Of note, this experiment coincided with a period of low ammonia in the real wastewater (further discussed in section 3.2.3). Due to the low influent ammonia (14.6 mgN $L^{-1}$), minimal nitrate was produced during the first aerobic cycle. To ensure a sufficient anoxic period in the second cycle, each reactor was spiked with an additional 11.1 mg$NO_3$–N $L^{-1}$ at the end of the second aerobic period.

As shown (Fig. 4d, e), there was no apparent difference in performance when the consortia were supplied real vs. synthetic wastewater. The SDNRs for the first cycle were 0.47 and 0.80 mg$NO_3$-N $gVSS^{-1}$ $h^{-1}$ for reactors O and C, respectively, and 0.39 and 0.82 mg$NO_3$–N $gVSS^{-1}$ $h^{-1}$ for reactors O and C in the second cycle, respectively. These comparable results suggest that residual slowly-biodegradable carbon present in the substrate was not a significant source of electrons in post-anoxic denitrification. However, we cannot rule out the contributions of EPS and/or soluble microbial products to the increased SDNRs.

### 3.2.3. *Effects of influent ammonia on post-anoxic BNR*

Nutrient concentrations in real wastewater will vary over time, particularly with excess precipitation. The influent ammonia concentration is particularly important to this post-anoxic configuration because it determines anoxic nitrate availability and associated process metabolisms and stability. As evidenced by the range of ammonia concentrations

observed in this study (Fig. 2a), influent ammonia loads to a WWTP will vary. Coats et al. (2011b) suggested that the influent VFA:$NH_3$–N ratio (mgCOD $mgN^{-1}$) could be an important predictor of process success, where a VFA:$NH_3$–N ratio greater than 4.1 was desirable. A higher VFA:$NH_3$–N ratio typically indicates that sufficient VFAs are available to drive EBPR and that the influent ammonia is not so high as to create excessive nitrate carryover. However, the guideline does not address cases where the ratio is inflated not by high VFAs but by low influent ammonia concentrations (<20 mgN $L^{-1}$), which can lead to nitrate depletion before the end of the anoxic period and potentially induce secondary P release (Barnard and Fothergill, 1998).

As discussed in section 3.2.2, reactor performance was observed over consecutive cycles during a period of low ammonia loading; the VFA:$NH_3$–N ratio was 12.5. While both systems maintained excellent N removal, EBPR efficiency was impaired in the open air reactor (Fig. 4d, e). $P_{rel}$/VFA ratios for reactors O and C were 0.12 and 0.17 for the first cycle and 0.10 and 0.14 for the second cycle, comparable to those observed when the reactors were receiving acetate-augmented substrate. However, incomplete aerobic P uptake was observed in reactor O (Fig. 4d) and the associated aerobic P uptake rate was only 1.4 mgP $gVSS^{-1}$ $h^{-1}$, markedly less than observed with the acetate and 90:10 substrates (Table 2). In contrast, the consortium in reactor C removed all P from solution aerobically for both cycles (Fig. 4d), although the consortium exhibited a slow secondary release (approximately 0.22 mgP $gVSS^{-1}$ $h^{-1}$) if nitrate was depleted anoxically. Similar trends were observed over the remainder of this study. Accumulibacter and GAO abundance was evaluated on four dates while the influent ammonia remained below 20 mgN $L^{-1}$ (Fig. 1; DNA 3, 4, 5, and 6). The end-aerobic P concentrations on these dates averaged 2.4 and 0.20 mgP $L^{-1}$ for reactors O and C, respectively. The fractional PAO population in both reactors increased substantially from DNA3 to DNA4, and then remained relatively steady for the rest of this time period (Table 3). Of note, the qPCR and effluent data indicated that reactor C was consistently enriched for more PAOs than in reactor O. Further, the GAO population also remained relatively high in the closed reactor (although decreasing substantially from the estimated peak when DNA3 was collected), as compared with the open reactor. Overall, these results suggest that limiting the extent of SOT could improve process stability, and also that the relative significant presence of GAOs will not necessarily impair phosphorus removal.

### 3.3. Glycogen and post-anoxic denitrification

Glycogen, and to a lesser extent PHA, have been hypothesized by others to be important carbon sources driving post-anoxic denitrification (Vocks et al., 2005), as contrasted with endogenous decay. As discussed herein, further evidence supporting the use of glycogen for denitrification has been observations of glycogen utilization during the anoxic period and SDNRs in excess of typical endogenous rates. However, to better quantify the potential involvement of glycogen, minimum denitrification carbon requirements were estimated (assuming 2.86 mgCOD $mgNO_3$–$N^{-1}$ and no growth) and compared with the measured carbon utilization (Table 5). For the quantity of nitrate reduced, measured glycogen utilization accounted for 62–76% and 55–62% of the minimum carbon requirements for the low and high aeration studies with the 90:10 wastewater, respectively. Conversely, glycogen utilization in excess of the carbon requirements was measured for both reactors during the acetate study. The excess glycogen utilization observed in these latter configurations (in particular for reactor O, which also reduced very little nitrate) could have been associated with the shift in population toward GAOs (Table 3). As noted, not all subgroups of the GB lineage can denitrify (Kong et al., 2006). Thus, when anoxic conditions were imposed, certain GAOs would have used their glycogen reserves not for nitrate reduction but for maintenance and survival, resulting in excess glycogen consumption.

As further support for the involvement of glycogen in the proposed post-anoxic process, we compared SDNRs vs. glycogen for data from this study and values from Coats et al. (2011b) (Fig. 5). The SDNRs from this study were corrected to a temperature of 20 °C ($SDNR_{20}$) using the average anoxic temperature and an Arrhenius temperature correction coefficient, $\theta = 1.026$ (Tchobanoglous et al., 2003). The SDNR values from Coats et al. (2011b) were not corrected for temperature because no temperature values were reported. As shown, the data shows that SDNRs increase with glycogen content, especially when considering each of the studies separately.

### 3.4. Secondary P release considerations

One concern with our process configuration is the potential for secondary P release during the anoxic period, which could lead to elevated effluent P concentrations (Barnard and Fothergill, 1998). Specifically, process success hinges on

**Table 5 – Theoretical carbon source requirements for post-anoxic denitrification.**

| Study type | Feed | Reactor name | $NO_3$–N reduced | COD required | Internal carbon sources used | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Gly | PHB | PHV | PH2MV | Total |
| Low Aeration | 90:10 | O | 5.61 | 16.0 | 12.2 | 0.4 | 0.4 | 0.0 | 12.9 |
| | | C | 6.88 | 19.7 | 12.3 | 0.6 | 1.0 | 0.0 | 13.9 |
| High Aeration | 90:10 | O | 10.18 | 29.1 | 17.9 | 0.3 | 0.6 | 0.0 | 18.8 |
| | | C | 9.49 | 27.1 | 15.0 | 0.4 | 0.7 | 0.0 | 16.1 |
| | HAc | O | 0.48 | 1.4 | 8.2 | 0.0 | 0.0 | 0.0 | 8.2 |
| | | C | 2.94 | 8.4 | 10.1 | 0.0 | 0.0 | 0.0 | 10.1 |

All units are mgCOD $L^{-1}$ except for $NO_3$–N (mgN $L^{-1}$).

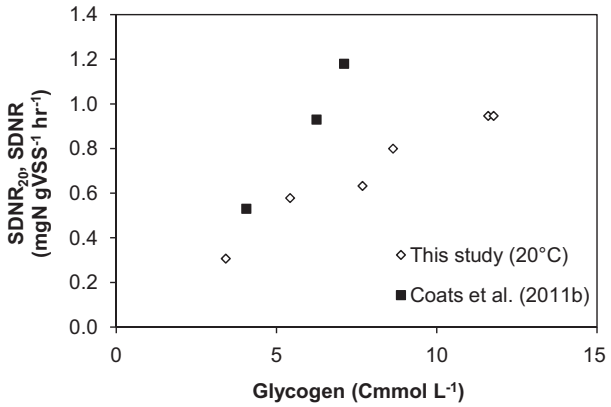


**Fig. 5 – Dependence of SDNR on glycogen concentration at start of anoxic period. Values for this study are corrected to a temperature of 20 °C.**

PAOs deriving maintenance energy during the anoxic period via oxidative and substrate-level phosphorylation rather than poly-P hydrolysis. The results presented herein indicated that secondary P release did not occur during the anoxic period as long as nitrate/nitrite remained available (Fig. 3a, c and Fig. 4a, c). However, to better understand the maintenance energy dynamics in the absence of $NO_x$ (principal electron acceptors driving post-anoxic oxidative phosphorylation), the reactors were monitored for 3 h following the end of the high aeration study (Fig. 3d shows that $NO_x$ was mostly depleted at the start of this test). As shown (Fig. 6), glycogen utilization continued in both reactors, suggesting that maintenance energy was mainly supplied via glycolysis. While secondary P release was not observed in reactor O, a nominal release occurred in reactor C (~0.15 mgP $L^{-1}$).

Notably, the rate of P release in reactor C was markedly lower than reported for PAO anaerobic maintenance. Secondary P release rates in the range of 2–5 mgP $gVSS^{-1}$ $h^{-1}$ have been reported for PAOs (Oehmen et al., 2005; Smolders et al., 1995; Wentzel et al., 1989), but the release rate observed during this extended test (reactor C) was just

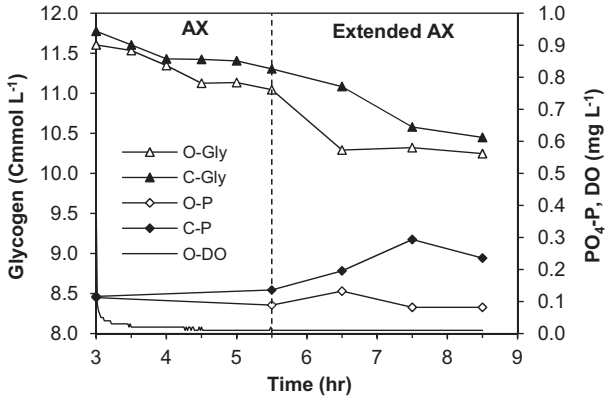


**Fig. 6 – Glycogen and P transformations during the normal post-anoxic period and an extended post-anoxic period after nitrate/nitrite depletion. The DO concentration is plotted for reactor O.**

0.016 mgP $gVSS^{-1}$ $h^{-1}$, more than 100 times slower. This difference cannot be attributed solely to the relative differences in Accumulibacter abundance between this study and the aforementioned studies. Rather, the microbial consortia enriched for in this study appears to have preferentially metabolized glycogen for maintenance energy before poly-P, which is also consistent with the findings of Lu et al. (2007) for their PAO anaerobic starvation experiment. It is also possible that the lower poly-P reserves of the PAOs in this study (VSS/TSS ~0.78–0.84) compared to synthetic-fed EBPR cultures (VSS/TSS ~0.5–0.7) made glycolysis the more favorable pathway for maintenance ATP production. Erdal et al. (2008) similarly concluded that PAOs are capable of a metabolic shift from poly-P to glycogen when poly-P pools are lowered. They postulated that the preference for the glycolytic pathway could be regulated by temperature, where glycolysis is favorable at temperatures above 20 °C. Considering that the temperatures in this study were 23–25 °C, the PAOs may have been relying more on glycogen to meet their ATP demands. Regardless, it would appear that secondary P release is not a significant concern in this post-anoxic BNR process.

### 3.5. *Potential organisms involved in post-anoxic denitrification*

Two types of PAOs have been indentified in EBPR systems, referred to as *Accumulibacter* Type I (PAOI) and *Accumulibacter* Type II (PAOII). PAOI is postulated to be capable of full denitrification, whereas PAOII is only able to denitrify from nitrite onwards (Oehmen et al., 2010b). Both PAO types would be amplified with the primer set employed in this study. Regarding GAOs, *Competibacter* subgroup 6 (amplified within the GAO primer set and GB lineage) is capable of full denitrification (Kong et al., 2006), while *Competibacter* subgroups 1, 4, and 5 (all collectively amplified within the GAO primer set and GB lineage (Table 2)) and *Defluvicoccus* Cluster I (not quantified in this study) are able to reduce nitrate only (Oehmen et al., 2010b). Based on the $P_{rel}$/VFA and Gly/VFA ratios, fractional PAO and GAO abundances, and overall reported N and P removal reported herein, it would appear that the imposed environmental pressures in both reactor configurations selected for a sufficiently enriched mixture of PAOs and GAOs capable of both phosphorus removal and denitrification. Since significant nitrite accumulation was never observed in any of the sampling runs, it is probable that the dominant denitrifying organisms were capable of using both nitrate and nitrite as electron acceptors (Carvalho et al., 2007; Wang et al., 2008). This suggests that most of the denitrification took place within the same microorganism rather than by multiple flanking species (with the exception of reactor O when supplied synthetic acetate, as discussed previously). Further, the PAOs in the system only hydrolyzed poly-P (i.e., secondary P release) in the absence of nitrate/nitrite, and therefore likely were capable of full denitrification (Barnard and Fothergill, 1998). Although some nitrite accumulation was observed in reactor C (Fig. 3d), nitrite was rapidly removed from solution after the depletion of nitrate. The organisms that reduced the nitrite at the end of the anoxic period seemingly relied on nitrate until it was no longer available, at which point they switched to nitrite (a less favorable electron acceptor).

### 3.6. *Considerations for full-scale application*

To the best of our knowledge, a post-anoxic denitrification EBPR process that is designed and/or operated to significantly rely on intracellular glycogen as a carbon source has yet to be applied at full-scale. A membrane-based process was investigated and patented (WO03057632) by a research group out of Berlin in 2004 (Vocks et al., 2005). The same group has operated continuous flow laboratory- and pilot-scale membrane bioreactors to demonstrate the potential viability of this process at full-scale (Bracklow et al., 2010). They observed total N and total P removal efficiencies of 86–94% and 92–99%, with SDNRs ranging from 0.5 to 1.5 mg$NO_3$–N $gVSS^{-1}$ $h^{-1}$. However, Bracklow et al., (2010) reported a limited understanding of the microbes and carbon source driving post-anoxic denitrification.

Complementing our prior work (Coats et al., 2011b), the results of this study suggest that the proposed post-anoxic BNR process is also suitable for SBR configurations, and further that the process may be appropriate for continuous flow WWTPs. Specifically, our results demonstrated that the process is capable of achieving >99% soluble P and inorganic N removal, and that limiting SOT in the anaerobic and anoxic basins can improve EBPR stability under low ammonia loading. At full-scale, variable speed mixers could be installed in the anaerobic and anoxic basins to minimize mixing and reduce SOT. Further, the design of a full-scale facility would have to consider the implications of variable ammonia loadings and secondary P release, both of which were investigated in this study.

## 4. Conclusions

The research presented and discussed herein focused on advancing the understanding of a relatively new post-anoxic BNR process. The major findings from this study can be summarized as follows.

- Post-anoxic denitrification can accomplish near-complete soluble inorganic N and P removal (>99%). Process success is enhanced at elevated aeration rates, but significant removal can be achieved at reduced aeration.
- Intracellular glycogen, synthesized associated with EBPR, is an important carbon source used by the mixed microbial consortium to achieve denitrification. A positive correlation between the SDNR and intracellular glycogen concentration was observed. Furthermore, glycogen oxidization for denitrification does not compromise subsequent anaerobic VFA uptake and PHA storage, which is critical to EBPR.
- A mixed VFA substrate (HAc, HPr, HBu, and HVa) appears to be more beneficial to process performance and supports a higher percentage of PAOs than an acetate-dominated substrate.
- Post-anoxic secondary P release can occur with $NO_x$ depletion. However, P release was only observed when SOT was prevented, and the rate of release was such that effluent P was only moderately increased.
- The proposed process configuration is potentially sensitive to low influent ammonia (<20 mgN/L), but stable performance can be maintained by minimizing SOT.
- All tested reactor configurations achieved significant P removal despite variability over time in the relative PAO fraction, and also considering a relatively significant GAO population.

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