Denitrification kinetics indicates nitrous oxide uptake is unaffected by electron competition in Accumulibacter

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ABSTRACT

Denitrifying phosphorus removal is a cost and energy efficient treatment technology that relies on polyphosphate accumulating organisms (DPAOs) utilizing nitrate or nitrite as terminal electron acceptor. Denitrification is a multistep process and many organisms do not possess the complete pathway, leading to the accumulation of intermediates such as nitrous oxide (N_2O), a potent greenhouse gas and ozone depleting substance. *Candidatus* Accumulibacter organisms are prevalent in denitrifying phosphorus removal processes and, according to genomic analyses, appear to vary in their denitrification abilities based on their lineage. Yet, denitrification kinetics and nitrous oxide accumulation by Accumulibacter after long-term exposure to either nitrate or nitrite as electron acceptor have never been compared. We investigated the preferential use of the nitrogen oxides involved in denitrification and nitrous oxide accumulation in two enrichments of Accumulibacter and a competitor – the glycogen accumulating organism *Candidatus* Competibacter. A metabolic model was modified to predict phosphorus removal and denitrification rates when nitrate, nitrite or N_2O were added as electron acceptors in different combinations. Unlike previous studies, no N_2O accumulation was observed for Accumulibacter in the presence of multiple electron acceptors. Electron competition did not affect denitrification kinetics or N_2O accumulation in Accumulibacter or Competibacter. Despite the presence of sufficient internal storage polymers (polyhydroxyalkanoates, or PHA) as energy source for each denitrification step, the extent of denitrification observed was dependent on the dominant organism in the enrichment. Accumulibacter showed complete denitrification and N_2O utilization, whereas for Competibacter denitrification was limited to reduction of nitrate to nitrite.

These findings indicate that DPAOs can contribute to lowering N_2O emissions in the presence of multiple electron acceptors under partial nitritation conditions.

Keywords

Nitrous oxide (N_2O) emission, Accumulibacter, electron competition, free nitrous acid, polyhydroxyalkanoates

1. INTRODUCTION

Biological nutrient removal processes are regarded as cost-effective, sustainable solutions to tackle excess carbon, phosphorus (P) and nitrogen (N) in wastewater. However, complete P and N removal usually requires high aeration, accounting for more than 60% of the overall power consumption at a wastewater treatment plant (WWTP) [1]. To meet rigorous effluent discharge limits, external carbon dosage is often required to achieve complete denitrification resulting in increased operational costs [2]. In this regard, denitrifying phosphorus removal (DPR) has been identified as an optimal treatment solution due to its reduced energy consumption, sludge wastage and carbon requirement for nutrient removal.

In the DPR process, sludge is cycled through anaerobic-anoxic conditions allowing denitrifying phosphorus accumulating organisms (DPAOs) such as *Candidatus* Accumulibacter phosphatis (hereafter referred to as Accumulibacter) to take up organic carbon under anaerobic conditions by utilizing stored polyphosphate as a source of energy. In the subsequent anoxic phase, electron acceptors such as nitrate or nitrite are reduced to meet growth and metabolic requirements [3-5]. These conditions also lead to the growth of denitrifying glycogen accumulating organisms (DGAOs), which compete with DPAOs for organic carbon under anaerobic conditions, but do not aid in phosphorus removal in the subsequent phase; hence recognized as competitors to DPAOs. *Candidatus* Competibacter phosphatis (hereafter referred to as Competibacter) is one such DGAO commonly found in laboratory- and full-scale studies [6-9].

In terrestrial and aquatic ecosystems, the conversion of nitrous oxide (N_2O) to nitrogen gas ($E^{o'}$ = +1.35 V at pH 7) by nitrous oxide reductase (Nos) is the only known biological N_2O attenuation process in the biosphere [10, 11]. Genomic

evidence revealed that many organisms possess only a subset of this denitrification pathway, sometimes lacking the nitrous oxide reductase gene (*nos*), resulting in the accumulation of N_2O [11, 12]. This is particularly important in engineered systems such as WWTPs where denitrification is implemented to remove nitrogen from influent wastewater [13, 14]. To this end, a genomic comparison of the denitrification pathway in Accumulibacter clades IA, IIA, IIB, and IIC showed significant differences and suggested that some clades were incapable of reducing N_2O [15-17]. Likewise, there is evidence that phylogenetically similar members of Competibacter have distinct denitrification capabilities [7, 18-20]. However, most metabolic models ignore the differences in denitrification capabilities of PAOs and GAOs and split the total nitrogen reduced between anoxic and aerobic phases [21-24]. Such an approach overlooks differences in phosphorus removal rates by different Accumulibacter clades that arise due to affinity towards certain nitrogen oxides. In addition to differences in denitrification abilities, environmental factors have been associated with increased N_2O release from WWTPs [24-27]. For heterotrophic organisms, studies demonstrated that the presence of nitrogen oxides (i.e., nitrate or nitrite) coupled with free nitrous acid (FNA) accumulation had a negative effect on the nitrous oxide reduction potential [28]. Nitrous oxide accumulation is also affected by electron competition, which results in a higher flow of electrons to one step of the denitrification pathway, rather than electrons being distributed to all denitrification enzymes [25, 28, 29]. In the context of Accumulibacter, slow polyhydroxyalkanoates (PHA) degradation has often been reported as a reason for N_2O release from DPR systems, although others have been unable to establish a link between the two [30, 31]. Thus, it is unclear whether N_2O emission from the DPR process is due to

104 environmental factors or an incomplete denitrification pathway in dominant
105 denitrifying organisms.

106 Metabolic models have been widely used to predict nutrient removal in
107 wastewater treatment [21-23, 32]. So far, models predicting N_2O accumulation have
108 been developed solely based on external nitrate dosage as terminal electron acceptor
109 [18, 33-36]. However, various additional nitrogen oxides (NO_x) occur simultaneously
110 in biological wastewater treatment, and each can serve as terminal electron acceptor.
111 This necessitates validation of metabolic models to evaluate electron competition and
112 denitrification kinetics in organisms utilizing PHA. The preference for different NO_x
113 has also been shown to affect oxidative phosphorylation and phosphorus transport
114 across cell membranes [26, 36, 37]. Consequently, it is necessary to consider
115 differences in the denitrification pathway and the effect of specific long-term
116 enrichment conditions (nitrate or nitrite as electron acceptor) in modelling
117 approaches.

118 In this study, we compared the denitrification kinetics of two Accumulibacter
119 enrichments acclimated to different anoxic conditions. We tested the effect of electron
120 competition on nitrous oxide accumulation in the presence of multiple electron
121 acceptors. These results for Accumulibacter were contrasted with denitrification
122 characteristics of a Competibacter enrichment. The objectives were to (i) identify any
123 accumulation of intermediates in the denitrification process that would signify a
124 preference for certain terminal electron acceptors, (ii) compare the role of electron
125 competition in nitrous oxide accumulation, and (iii) validate an existing metabolic
126 model to predict the mechanism of phosphorus uptake and nitrogen oxide
127 accumulation in the presence of multiple electron acceptors. We investigated three
128 electron acceptors (nitrate, nitrite and nitrous oxide) dosed in seven different

129 combinations under similar test conditions for all enrichments. We hypothesized that
130 the preference for certain NO_x compounds would be driven either by electron
131 competition or the extent of denitrification performed by the dominant organism in
132 each enrichment culture.
133
134 **2. MATERIALS AND METHODS**
135 *2. 1. Reactor operation for enrichment cultures*
136 We operated three lab-scale aerobic granular sludge sequencing batch reactors
137 (SBRs), each with a 2-L working volume and 200 mL headspace, to obtain
138 Accumulibacter and Competibacter enrichments. Accumulibacter was adapted to
139 nitrate or nitrite as terminal electron acceptor under anoxic conditions leading to two
140 different enrichments, $Accumulibacter_{nitrate}$ and $Accumulibacter_{nitrite}$, respectively. The
141 third reactor enriched Competibacter fed with nitrate under anoxic conditions. The
142 reactors were maintained at a constant temperature of 30°C using a heating jacket,
143 and a cycle length of 6 h consisting of 30 min feeding, 60 min anaerobic react, 120
144 min anoxic react, 120 min aerobic react, 10 min settling and 20 min effluent discharge
145 phases. During the anaerobic phase, 1 L of synthetic feed containing acetate and
146 propionate (3:1 ratio on the basis of total influent chemical oxygen demand, or COD)
147 as carbon source was pumped into each reactor at a rate of 33.33 mL/min.
148 Nitrogen gas was sparged from the bottom of the reactor at 2.0 L/min to
149 provide sufficient mixing during the reaction phases and maintain anaerobic
150 conditions. Subsequently, in the anoxic phase nitrite or nitrate were fed in pulses.
151 Nitrite and nitrate dosages were carefully adjusted to avoid their accumulation in the
152 anoxic phase because this could affect microbial activity in the reactors. During the
153 effluent discharge phase, 1 L of effluent was pumped out of the reactors, leading to a

hydraulic retention time of 12 h and a volumetric exchange ratio of 50%. For each cycle, the pH was controlled at 7.5 to 8.0 with 0.5 M NaOH or 0.5 M HCl solution, and the dissolved oxygen (DO) concentration in the aerobic phase was varied from 1 to 1.5 mg O_2/L. A programmable logic controller was linked to a SCADA interface for data visualization and storage. The bioreactors were assumed to be at pseudo-steady state at the start of the batch tests as no significant changes in mixed liquor volatile suspended solids (MLSS), volatile suspended solids (VSS) and phosphorus concentrations were observed for three consecutive solids retention times (SRTs). Cycle studies were performed regularly and samples were taken during each phase to monitor reactor performance. The operating conditions for the Accumulibacter$_{nitrite}$, Accumulibacter$_{nitrate}$ and Competibacter enrichment reactors were as follows:

Case 1: Accumulibacter$_{nitrite}$. A lab-scale aerobic granular sludge SBR operated at 30℃ and fed with synthetic wastewater in which the influent carbon source was a mixture of acetate and propionate (3:1 ratio for acetate and propionate on the basis of total influent COD) was used to enrich Accumulibacter. The synthetic wastewater consisted of 0.15 L solution A and 0.85 L solution B. Solution A (per litre) contained 3.5 g NaAc.3H_2O and 0.7 mL of 99.5% propionic acid as the carbon source, 1.20 g $MgSO_4$.7H_2O, 0.19 g $CaCl_2$.2H_2O, 1.02 g NH_4Cl, 0.01 g peptone, 0.01 g yeast and 4 mL of trace metals solution. The trace metals solution was prepared as described in Smolders et al. (1994) and consisted (per litre) of 0.15 g H_3BO_3, 1.5 g $FeCl_3$.6H_2O, 0.18 g KI, 0.12 g $MnCl_2$.4H_2O, 0.15 g $CoCl_2$.6H_2O, 0.06 g Na_2MoO_4.2H_2O, 0.03 g $CuSO_4$.5H_2O, 0.12 g $ZnSO_4$.7H_2O and 10 g Ethylenediamine tetra-acetic acid (EDTA). Solution B contained 132 mg K_2HPO_4/L and 103 mg KH_2PO_4/L. Nitrification was inhibited by the addition of 2.38 mg allyl-N thiourea (ATU) per litre feed. After feeding, the mixed liquor contained 12 mg NH_4-N/L, 300 mg COD/L and

15 mg PO_4-P/L resulting in a COD to P ratio of 20:1. The anoxic phase was initiated by adding $NaNO_2$ solution in pulses such that the total concentration in the reactor after each pulse was 10 mg NO_2-N/L. The SRT varied from 12 to 15 d to maintain an MLSS between 2 and 2.5 g/L.

Case 2: Accumulibacter$_{nitrate}$. Accumulibacter was enriched at 30℃ in a lab-scale aerobic granular sludge SBR using the same enrichment protocol and synthetic wastewater as mentioned in Case I, Accumulibacter$_{nitrite}$. The only difference in operation of this reactor was that the anoxic phase was initiated by adding KNO_3 solution in pulses such that the final concentration in the reactor after each pulse was 15 mg NO_3-N/L.

Case 3: Competibacter. Enrichment of Competibacter was achieved in a lab-scale aerobic granular sludge SBR operated at 30℃ with synthetic feed containing acetate as sole carbon source. The synthetic wastewater was similar to that outlined for Case I (Accumulibacter$_{nitrite}$) with the difference that solution A contained 8.5 g NaAc.3H_2O and solution B contained trace amounts of K_2HPO_4 and KH_2PO_4, such that the resulting phosphorus concentration in the reactor after feeding was 2 mg P/L. After feeding, the mixed liquor contained 300 mg COD/L, 12 mg NH_4-N/L and 2 mg PO_4-P/L resulting in a COD:P of 150:1. The reactor operation phases were similar to those of the other reactors and KNO_3 solution was introduced as a pulse into the reactor during the anoxic phase, resulting in a final concentration in the reactor after each pulse of 15 mg NO_3-N/L.

2. 2. Batch tests to measure denitrification kinetics and N_2O accumulation

A total of seven batch tests were performed under different terminal electron acceptor conditions for all enrichments. At the start of each batch test, mixed liquor was

withdrawn from the reactor after completion of the anaerobic phase and immediately transferred to batch reactors with nitrogen gas sparging for 5 min to remove trace oxygen. The batch reactors had a total volume of 1.1 L and a working volume of 1 L. The reactor headspace was minimized in order to reduce stripping of nitrous oxide. For all tests, pH was controlled at 8.0 ± 0.1 using 0.5M HCl or 0.5M NaOH for Accumulibacter enrichments and 0.2M HCl or 0.2M NaOH for the Competibacter enrichment. Nitrous oxide reduction can be severely affected by FNA concentrations as low as 0.7 µg HNO_2-N/L [37]. The aforementioned pH set-point was maintained during the batch tests to reduce susceptibility of sludge to the inhibitory effects of FNA. Once the tests began, no nitrogen was sparged in the batch reactor to minimize N_2O stripping, and the mixed liquor was stirred slowly at a rate of 30 rpm to avoid oxygen intrusion. Dissolved N_2O concentrations were monitored with an online N_2O microsensor (N_2O -R, Unisense A/S, Denmark). Fresh stock of N_2O solution prepared by sparging Milli-Q water with 100% N_2O gas for 5 min at room temperature (24-25°C) was used to calibrate the N_2O probe as per the product instruction manual. N_2O reduced at the cathode of the microsensor produced a current that was converted into a signal by the Picoammeter, and these readings were collected every second.

Different combinations of terminal electron acceptors were added for batch tests with the enrichment cultures (Table 1). In each test, nitrogen oxides were added as a pulse such that their concentrations in each batch test reactor ranged from 12 to 15 mg NO_x-N/L. Four control tests were carried out in duplicate to determine the loss of N_2O due to stripping or other abiotic phenomena. The losses were averaged and deducted from all Picoammeter readings to obtain the true biological N_2O uptake as shown in the supplementary information (Appendix 2). The tests were conducted for 30 min and mixed liquor samples for nutrient analysis were collected every 5 min.

The MLVSS concentration was measured in triplicate before the start of each batch test and all batch tests were carried out in duplicate. Apparent biomass-specific reduction rates for all nitrogen oxides, electron consumption and distribution rates were calculated in duplicate as outlined in previous studies (SI, Appendix 2). Detailed analytical methods for measuring phosphate, ammonium, nitrite, nitrate, VFA, PHA and glycogen have been outlined in the Supplementary Information (Appendix 1). GraphPad Prism (v6) was used for all statistical analyses.

2. 3. *DNA extraction, sequencing library preparation, 16S amplicon sequencing and analysis*

For microbial community analysis, 2 mL of mixed liquor from the three lab-scale reactors was collected on day 0 and day 28, coinciding with the first and last day of the batch tests. The genomic DNA extraction protocol can be found in the SI, Appendix 1. The library pool was sequenced at the Singapore Centre for Environmental Life Sciences Engineering (SCELSE) sequencing facility on a MiSeq (Illumina, US) using MiSeq Reagent kit V3 (2×300 paired end). Sequenced sample libraries were processed according to published DADA2 pipelines using the *dada2* R package (v 1.14). Details of the raw reads processing and community analysis are also explained in details in the SI, Appendix 1.

2.4 *Model details*

It is well established that during the anoxic phase in DPR systems, nitrate, nitrite or nitrous oxide are utilized as terminal electron acceptor by Accumulibacter for replenishment of its polyphosphate base, growth and other metabolic activities. The utilization rate of these terminal electron acceptors in Accumulibacter is associated

with the PHA consumption rate for anoxic growth and subsequent phosphorus uptake. Thus, each step of the denitrification pathway (from NO_3^- to N_2 via NO_2^-, NO and N_2O) can be associated with anoxic growth and phosphorus uptake rates. The model developed by Liu et al. (2015) incorporates these features to evaluate electron competition and nitrous oxide accumulation during denitrifying phosphorus removal [35]. In this study, we extended the application of the model by considering the simultaneous availability of multiple electron acceptors.

Each sequentially occurring denitrification step was associated with individual reaction-specific kinetics, i.e., anoxic growth rate on nitrate (μ_{DPAO1}), nitrite (μ_{DPAO2}), and nitrous oxide (μ_{DPAO4}) and their associated phosphorus uptake rates. Experimental data from the batch tests were used to calibrate the relevant reactions and relationships for Accumulibacter enrichments, polyphosphate (X_{PP}), PHAs (X_{PHA}), DPAOs (X_{DPAO}), residual inert biomass (X_I) and seven soluble compounds - phosphate (S_{PO4}), nitrate (S_{NO3}), nitrite (S_{NO2}), nitric oxide (S_{NO}), nitrous oxide (S_{N2O}), nitrogen gas (S_{N2}) and readily degradable substrate (S_s). The saturation constants for polyphosphate storage ($K_{PP,DPAO}$, $K_{max,DPAO}$) and PHA storage (K_{PHA}) incorporated the relative abundance of DPAOs as assessed by 16S rRNA amplicon sequencing. The stoichiometry, component definition, model fitting parameters for $Accumulibacter_{nitrite}$ and $Accumulibacter_{nitrate}$, composition matrix and kinetic rate expression matrix and literature references are presented in the SI, Appendix 3.

3. RESULTS AND DISCUSSION

3.1 Reactor performance and microbial community characterization

Nutrient concentration measurement and microbial community analysis of the enrichments were conducted regularly. 16S rRNA gene amplicon sequencing was

279 followed by classification of the most abundant ASVs at the highest level of
280 resolution using the SILVA database to determine the degree of enrichment of target
281 organisms and assess whether the community had undergone major shifts during the
282 batch tests (Figure 1). The community structure was complex in all enrichments,
283 although a significant proportion of the microbial community consisted of target
284 organisms along with a low abundance of known competitor organisms.
285 $Accumulibacter_{nitrate}$ and $Accumulibacter_{nitrite}$ had 48 ± 4% and 39 ± 3% relative
286 abundance of target organisms, respectively, and a comparatively lower abundance of
287 Competibacter (< 4% relative abundance). The Competibacter enrichment during the
288 batch tests had a relative abundance of 44 ± 4% target organisms and 6 ± 3% of
289 Accumulibacter. Organisms not known to display PAO or GAO phenotypes detected
290 in the reactors were related to *Thiothrix*, *Thiobacillus*, *Hyphomicrobium*, *SJA-28*,
291 *Denitratisoma*, *Cyanobacteria* and *Terrimonas*; they were approximately fourfold less
292 abundant than target organisms.

293 After an acclimation period lasting one month, pseudo-steady state activity
294 and nitrogen removal was observed for $Accumulibacter_{nitrite}$, $Accumulibacter_{nitrate}$ and
295 Competibacter-enriched sludge. During the anaerobic feeding phase, VFA was
296 completely consumed followed by an increase in intracellular PHA content in all
297 enrichments (Figure 2). Nitrogen oxides were utilized as terminal electron acceptors
298 with concomitant phosphorus uptake in $Accumulibacter_{nitrite}$ and $Accumulibacter_{nitrate}$
299 enrichments. In the anoxic phase, $Accumulibacter_{nitrite}$ showed 99% nitrite removal
300 efficiency along with an anoxic phosphorus removal efficiency of 20%, whereas
301 $Accumulibacter_{nitrate}$ showed 72% nitrate removal efficiency with an anoxic
302 phosphorus removal efficiency of 10%. The phosphorus remaining at the end of the
303 anoxic phase in both enrichment reactors was utilized rapidly in the subsequent

aerobic phase (Figure 2a, b). For Competibacter, the reducing power for PHA formation was obtained through glycolysis; therefore, glycogen reduction was observed with a concomitant increase in intracellular PHA in the anaerobic phase (Figure 2c). In the subsequent anoxic phase, nitrate added to the reactors was utilized to replenish the intracellular glycogen levels. The phosphorus concentration did not change during the entire cycle and remained below 1.5 mg/L.

The microbial community of Accumulibacter enrichments utilized in previous studies included a significant fraction of GAOs, which could have led to confounding factors in understanding the denitrification kinetics of the target organism [26, 35-38]. In this study, we performed batch studies with Accumulibacter and Competibacter enrichments dominated by the target organisms to improve our understanding of their true denitrifying abilities.

3.2 Denitrification kinetics with single terminal electron acceptors

To test the denitrification kinetics and propensity for N_2O accumulation in $Accumulibacter_{nitrate}$, $Accumulibacter_{nitrite}$ and Competibacter enrichments, true nitrous oxide, nitrate and nitrite reduction rates were measured in batch tests A, B and C, respectively (Figure 3a, b, c). These rates were calculated from the observed nitrogen oxide (NO_x) concentration during each test (SI, Appendix 2). A linear decrease in NO_x concentrations was observed in tests with a single terminal electron acceptor, indicating denitrification activity (Fig S1).

In batch test A, nitrous oxide reduction rates for $Accumulibacter_{nitrite}$ and $Accumulibacter_{nitrate}$ enrichments were 7.3 ± 0.43 and 10.6 ± 0.007 mg N $gVSS^{-1}$ h^{-1}, respectively. These rates were significantly higher than that of Competibacter ($p < 0.05$). Previously, it had been hypothesized that denitrification using PHA leads to

329 N_2O emissions [25, 39]. Yet, neither experimental nor modelling studies found any
330 evidence to support this claim, suggesting other factors such as pH, free nitrous acid
331 concentration and excess aeration as causes for increased N_2O emissions [26, 34, 40,
332 41]. It is interesting that we observed higher nitrous oxide (N_2O) utilization rates by
333 Accumulibacter than by Competibacter, despite similar PHA storage and anoxic PHA
334 utilization rates (Table 4). This rules out the possibility of incomplete denitrification
335 and nitrous oxide accumulation solely due to PHA serving as carbon source [13].

336 For batch test B, nitrate reduction rates for $Accumulibacter_{nitrate}$ and
337 Competibacter were 10.9 ± 0.3 and 5.03 ± 0.39 mg N $gVSS^{-1}$ h^{-1}, respectively. These
338 reduction rates were significantly higher ($p < 0.05$) than that of $Accumulibacter_{nitrite}$,
339 indicating that Accumulibacter acclimated to nitrite was less efficient in reducing
340 nitrate. Also, as nitrate was reduced, nitrite accumulation was observed only for the
341 Competibacter enrichment. In batch test C, the nitrite reduction rates for
342 $Accumulibacter_{nitrite}$ and $Accumulibacter_{nitrate}$ were 9.1 ± 0.8 and 10.7 ± 1.2 mg N
343 $gVSS^{-1}$ h^{-1}, respectively, which was almost six times higher than that observed for
344 Competibacter. The preferential utilization of nitrate by Competibacter with
345 concomitant nitrite accumulation was also observed in cycle studies, where the anoxic
346 reaction phase was longer than in the batch test (Figure 1c). These differences in
347 nitrite reduction between the enrichments support the conclusion that Competibacter
348 had a lower preference for nitrite than Accumulibacter ($p < 0.05$).

349 Previously, McIlroy et al. (2015) reported genomes of two Competibacter
350 GAOs (*Ca.* Competibacter denitrificans and *Ca.* Contendobacter odensis) encoding
351 different denitrification pathways [7]. *Ca.* denitrificans encodes the complete
352 denitrification pathway, while *Ca.* odensis only encodes genes for nitrate to nitrite
353 reduction. Another study provided evidence of *nos* gene expression in denitrifying

communities dominated by *Ca.* Competibacter denitrificans, signifying its active role in N_2O reduction [27]. Consistent with our physiological data, the Competibacter enrichment was dominated by members of a Competibacter-lineage that could predominantly reduce nitrate to nitrite, and showed limited N_2O uptake despite adequate PHA storage. Genomic analyses of various Accumulibacter clades have revealed that most members encode the *nos* gene, and have the potential to utilize N_2O as an electron acceptor [16]. This was reflected in batch tests where adequate N_2O reduction was observed in Accumulibacter enrichments. Thus, in addition to adequate environmental conditions required to drive N_2O reduction, enzymatic regulation of the *nos* gene is important in PHA-driven denitrification.

3.3 Denitrification kinetics with multiple terminal electron acceptors

Multiple electron acceptors were simultaneously added in batch tests D to G, and the observed NO_x reduction rates were compared with those from the single electron acceptor batch experiments (tests A, B and C). The tests allowed us to investigate the potential for electron competition limiting N_2O utilization with different combinations of terminal electron acceptors for Accumulibacter and Competibacter. The overall NO_x reduction rates for the Accumulibacter and Competibacter enrichments increased in the presence of multiple electron acceptors, and were not affected by the simultaneous addition of nitrogen oxides (Figure 3). In a previous study, another phosphorus accumulating organism, *Tetrasphaera*, had shown reduced denitrification kinetics when multiple electron acceptors were added simultaneously, indicating a limited electron supply to different denitrification enzymes [42]. In this study, we observed the opposite with an increase in overall NO_x reduction rates in the presence of multiple electron acceptors, suggesting the absence of electron competition.

In batch test D, when nitrite and nitrate were added together as terminal electron acceptors, the nitrate reduction rate was significantly higher for $Accumulibacter_{nitrate}$ compared to $Accumulibacter_{nitrite}$ ($p < 0.0005$). For both Accumulibacter enrichments, the net nitrite reduction rate was observed to be two to five times higher than the net nitrate reduction rate, with no observed N_2O accumulation, suggesting that the Accumulibacter taxa enriched in both reactors were able to take up nitrite provided externally as an electron acceptor, despite the simultaneous presence of nitrate. By comparison, for Competibacter, the true nitrate reduction rate was twice the true nitrite reduction rate, leading to accumulation of nitrite (Figure 3). The Competibacter enrichment displayed preferential utilization of nitrate despite the simultaneous presence of equimolar amounts of nitrate and nitrite, reinforcing our observations from the batch tests of its limited nitrite utilization ability.

In batch test E, when nitrate and N_2O were utilized as terminal electron acceptors, the N_2O utilization rate for $Accumulibacter_{nitrite}$ was twice its nitrate utilization rate. In contrast, for $Accumulibacter_{nitrate}$, the nitrate, nitrite and N_2O reduction rates were similar, and complete denitrification was observed. However, unlike $Accumulibacter_{nitrite}$ the externally provided N_2O was not utilized despite similar PHA levels. This is also reflected in the electron consumption profiles for batch test E (Figure 4), where $Accumulibacter_{nitrate}$ showed an equal fraction of electrons consumed during each denitrification step, while in $Accumulibacter_{nitrite}$ most electrons were consumed for nitrate and N_2O reduction. When nitrite and N_2O were added together (batch test F, Figure 4), $Accumulibacter_{nitrite}$ showed N_2O uptake in excess of that expected by denitrification of nitrite; by comparison, $Accumulibacter_{nitrate}$ exhibited similar nitrite and N_2O reduction rates with no excess

404 N_2O uptake. Finally, in batch test G where nitrate, nitrite and N_2O were added
405 together, the total denitrification rate for both Accumulibacter enrichments was 1.5 to
406 2 times higher than that of Competibacter.

407 In comparison to previous studies measuring denitrification kinetics in DPAOs
408 and DGAOs, nitrous oxide accumulation was not observed in this study when
409 multiple electron acceptors were added simultaneously (Table 2) [36, 43]. FNA
410 concentrations in previous studies ranged from 0.001 to 0.015 mg HNO_2-N L^{-1}, which
411 is much higher than the suggested inhibitory concentrations of 0.0007 – 0.001 mg
412 HNO_2-N L^{-1} [26]. FNA in excess of inhibitory concentrations has been shown to
413 affect the *nos* gene transcriptional process, and directly react with the copper-
414 containing active sites of N_2O reductase (Nos) [44, 45]. Therefore, in this study, the
415 pH and NOx dosage study were adjusted such that the FNA formed was below the
416 inhibitory levels (0.1 – 0.2 µg HNO_2-N L^{-1}). It has also been proposed that the limited
417 bioenergetic advantage of N_2O reduction for a cell reducing nitrate to nitrogen gas
418 (i.e., ~20% of the total energy generated) allows for release of N_2O as the final
419 denitrification product instead of N_2 [29]. Further, utilizing N_2O produced within a
420 cell generates more energy than transporting N_2O into the cell and subsequently
421 reducing it to nitrogen gas [42]. However, in this study, we observed that
422 Accumulibacter was able to utilize N_2O that was externally provided in addition to
423 N_2O produced as a result of denitrification of NO_x. The electron distribution patterns
424 for Accumulibacter, too, indicated a significant portion of electrons was consumed by
425 Nos, which was not limited by the simultaneous presence of other NO_x (SI, Fig S3).
426 Although the gene expression levels of various denitrification enzymes involved were
427 not measured in this study, previous studies have identified a strong correlation

between the transcription of *nosZ* genes (clade I and II) and the N_2O reduction rate [10, 27].

It is likely that a combination of factors such as adequate intracellular PHA storage, higher availability of N_2O and low FNA inhibition in this study allowed for adequate *nos* transcription. This made it bioenergetically feasible for Accumulibacter to transport and reduce externally added N_2O, while simultaneously utilizing N_2O produced by nitrate or nitrite reduction.

3.4 Electron consumption and distribution during denitrification

For the batch tests conducted, we compared electron consumption and distribution trends as they have been previously shown to be affected by electron competition [28]. Here, Accumulibacter enrichments were observed to have a higher electron consumption rate than Competibacter, which can be attributed to higher denitrification rates observed in the former. The total average electron consumptions in multiple electron addition tests for $Accumulibacter_{nitrite}$ and $Accumulibacter_{nitrate}$ were 2.1 ± 0.63 and 2.47 ± 0.58 mmol e^- g VSS^{-1} h^{-1}, respectively (Figure 4). These values were higher than the total electron consumptions calculated for $Accumulibacter_{nitrite}$ with nitrite addition (test C, 1.44 mmol e^- $gVSS^{-1}$ h^{-1}) and for $Accumulibacter_{nitrate}$ with nitrate addition (test B, 2.36 mmol e^- $gVSS^{-1}$ h^{-1}). Thus, despite the simultaneous presence of multiple electron acceptors, the PHA-derived electron supply to denitrification enzymes increased as a function of the total NO_x concentration, demonstrating that intracellular PHA storage was sufficient to support denitrification in Accumulibacter.

Previously, it has been surmised that enzyme-specific affinity to electron carriers and regulation of electron transfer are important factors that determine

electron distribution to enzymes involved in denitrification [28]. It is usually observed that denitrification enzymes upstream (such as Nar) have a greater ability to receive electron carriers than downstream enzymes (e.g. Nir and Nos) [46]. This is because the ubiquinone/ubiquinol pool supplies electrons to the Nar enzymes and the cytochrome c550/pseudoazurin pool, and the latter subsequently supplies electrons to the Nir and Nos enzymes. In this study, Accumulibacter$_{nitrate}$ and Competibacter enrichments showed significantly higher electron consumption rates in batch test E (NO_3^- and N_2O added) compared to batch test F (NO_2^- and N_2O added) (p-value = 0.007 and 0.044 for Accumulibacter$_{nitrate}$ and Competibacter, respectively). However, the trend for electron consumption was reversed for Accumulibacter$_{nitrite}$ with electron consumption in batch test F approximately 1.5 times that in test E (Figure 4) (p-value = 0.028). Thus electron competition was not necessarily the limiting factor for denitrification, enrichment-specific affinity for different electron acceptors also affected the overall NO_x reduction. Further, in Accumulibacter$_{nitrite}$ during nitrite reduction, 37% of total electrons were distributed to nitrite reductase whereas in the presence of other terminal electron acceptors, i.e., tests D, F and G, the fractions of total electrons distributed to nitrite reductase were reduced to 30%, 27%, and 22%, respectively. This proves that nitrite reductase did not display a higher ability to compete for electrons compared to nitrate or nitrous oxide reductase, as has been reported for heterotrophic organisms and some *Tetrasphaera*-related species [28, 42].

We also compared the phosphorus-uptake-to-electron-consumption ratio in all batch tests of Accumulibacter$_{nitrite}$ and Accumulibacter$_{nitrate}$, because the total electron consumption is indicative of the energy utilized for anoxic phosphorus uptake (Figure 5). This ratio, just as the true denitrification rate, did not seem to be affected by increasing concentrations of electron acceptors, but rather by the type of electron

acceptor present. Phosphorus uptake associated with nitrate in Accumulibacter$_{nitrate}$ enrichments was twice that observed for Accumulibacter$_{nitrite}$. Higher phosphorus uptake was associated with nitrite and nitrous oxide compared to nitrate in Accumulibacter$_{nitrite}$ enrichments, emphasizing the impact of long-term adaptation to certain terminal electron acceptors on observed denitrification kinetics and associated phosphorus uptake.

In summary, the analysis of electron consumption and distribution in this study leads to the conclusion that electron supply increased as a function of nitrogen oxide concentration, indicating that adequate PHA levels can drive denitrification in Accumulibacter. Upstream denitrification enzymes did not limit the flow of electrons to Nos, and hence there was no electron competition for nitrous oxide reduction in Accumulibacter. A comparison of the electron distribution in the two Accumulibacter enrichments suggests an enrichment-specific affinity for terminal electron acceptors.

3.5 Model evaluation of denitrification kinetics and electron competition

The nitrate, nitrite, and nitrous oxide reduction rates for Accumulibacter measured during the batch tests with low FNA inhibition were employed in a metabolic model to predict anoxic growth at each denitrification step and the associated phosphorus uptake for DPAOs [35]. The model used kinetic parameters specific for each step in the denitrification process, and calculated a carbon oxidation rate to predict the accumulation of denitrification products. As the flanking community members were much less abundant than the Accumulibacter cells present in the enrichments, their contribution to NO_x utilization was considered negligible. The model was optimized by recalibrating four key kinetic parameters: rate constant for storage of polyphosphate (q_{PP}), anoxic growth rate on nitrate (μ_{DPAO1}), anoxic growth rate on

nitrite (μ_{DPAO2}) and anoxic growth rate on N_2O (μ_{DPAO4}). In addition to these parameters, another 17 kinetic and 6 stoichiometric parameters were obtained from the literature (Table S1).

The optimized model captured the trends well for the reduction of various nitrogen oxides (NO_x) and phosphorus uptake in the batch tests. The R^2 values obtained for both Accumulibacter enrichments were 0.97 ± 0.01, 0.96 ± 0.09, 0.97 ± 0.05 and 0.96 ± 0.10 for nitrate, nitrite, N_2O and phosphorus, respectively, confirming the robustness of the model and parameter values to accurately predict N_2O accumulation in Accumulibacter enrichments (Figure 6). A comparison of the anoxic growth rates optimized with the model revealed that the growth on nitrite, μ_{DPAO2}, was lower for $Accumulibacter_{nitrate}$ compared to $Accumulibacter_{nitrite}$, indicating differences in nitrite reducing abilities (p-value = 0.04). These observed differences also highlight the effect of long-term adaptation to certain terminal electron acceptors.

In batch tests where N_2O was added in addition to nitrite or nitrate, Accumulibacter effectively utilized N_2O that was produced from denitrification and, more importantly, did not lead to any N_2O accumulation. A previous study reported a similar observation for *Tetrasphaera*-related organisms, suggesting that high availability of N_2O and energy from nitrate/nitrite reduction in these tests led to an increased synthesis of nitrous oxide reductase (Nos) [42]. Here, we also used the model to evaluate energy supplied for Nos synthesis (SI, Appendix 3, Table S6). For Accumulibacter, the anoxic growth rate on N_2O increased approximately 1.5 to 2 times when multiple electron acceptors were added simultaneously (tests D to G) as compared to tests with single terminal electron acceptors (tests A to C) (p-value = 0.03 and 0.0048 for $Accumulibacter_{nitrite}$ and $Accumulibacter_{nitrate}$, respectively). This further confirms our hypothesis that the increased availability of N_2O and energy

528 derived from the reduction of multiple electron acceptors allowed for the increase in
529 anoxic growth rate.

530 Other mathematical models have been used to predict phosphorus removal,
531 but few compared the nitrous oxide (N_2O) accumulation from denitrifying phosphorus
532 removal processes. A limitation is that the denitrification process is usually
533 represented as a single- or two-step process. One study utilized a similar four-step
534 denitrification model as our approach to predict nitrous oxide (N_2O) accumulation
535 during biological nitrogen removal in anaerobic/anoxic/oxic sequencing batch
536 reactors (A^2O-SBR), but abstained from accounting for the abundant Accumulibacter
537 and Competibacter organisms usually found in these systems [47]. It can be surmised
538 that lack of organism-specific parameters in the model can lead to a lower precision
539 of N_2O accumulation prediction. In contrast, the model applied in this study lacks the
540 aforementioned shortcomings and adequately describes N_2O accumulation during
541 denitrification [35].

542 The model heuristic can also be integrated with other N_2O models for
543 nitrification and denitrification to gain more insight into N_2O dynamics during various
544 stages of biological wastewater treatment. FNA concentrations kept below inhibitory
545 levels in this study allowed for modelling of the true denitrification kinetics in
546 Accumulibacter. FNA inhibition leading to N_2O accumulation is not limited to
547 polyphosphate accumulating organisms, but has also been documented for
548 heterotrophic, hydrogenotrophic, ammonia-oxidising and Anammox organisms [48-
549 51]. A lower tendency for N_2O accumulation and higher anoxic growth rate on N_2O
550 was observed when FNA concentrations were low. This identifies FNA as an
551 important parameter that must be included in models developed to predict N_2O
552 accumulation based on nitrite concentration and pH. To supplement these modelling

553 predictions, future studies can also include metatranscriptomic analysis of the
554 denitrification enzymes in Accumulibacter, and compare levels of transcription at
555 different FNA concentrations.
556

557 **4. CONCLUSIONS**

558 Denitrifying phosphorus removal (DPR) is a promising technology. In this work we
559 have focussed on the potential for nitrous oxide accumulation by dominant microbial
560 community members - Accumulibacter and Competibacter - in DPR systems. Our
561 batch test results and validation with a metabolic model suggest that denitrification by
562 Accumulibacter and Competibacter is not limited by electron competition. In fact in
563 Accumulibacter, anoxic growth on nitrous oxide (N_2O) in the presence of other
564 nitrogen oxides was significantly higher compared to when nitrous oxide added as
565 sole electron acceptor. Our observations indicate that sufficiently high PHA levels
566 and low free nitrous acid (FNA) inhibition resulted in increased transcription of the
567 *nos* gene, allowing for higher N_2O reduction than previously observed. The
568 denitrification kinetics for Competibacter revealed poor nitrite or nitrous oxide
569 utilization despite sufficient PHA storage and low FNA inhibition, implying a
570 truncated denitrification pathway. To conclude, the reduction of nitrogen oxides by
571 Accumulibacter and Competibacter is largely governed by their denitrification
572 enzyme-specific affinity to electron carriers, the availability of sufficient internal
573 storage polymers to provide energy for electron transfer and low concentrations of
574 FNA. Understanding the nitrous oxide reduction potential of the dominant microbial
575 community members in DPR can be leveraged to design energy efficient treatment
576 processes for biological phosphorus removal and subsequently reduce unwanted
577 emissions.

AUTHOR CONTRIBUTIONS

The study was designed by SR, SW and HYN. SR conducted the batch test experiments and analysed the microbial community. SR, NP and SW analysed and interpreted the data obtained over the course of this study and wrote the manuscript with contributions from HYN.

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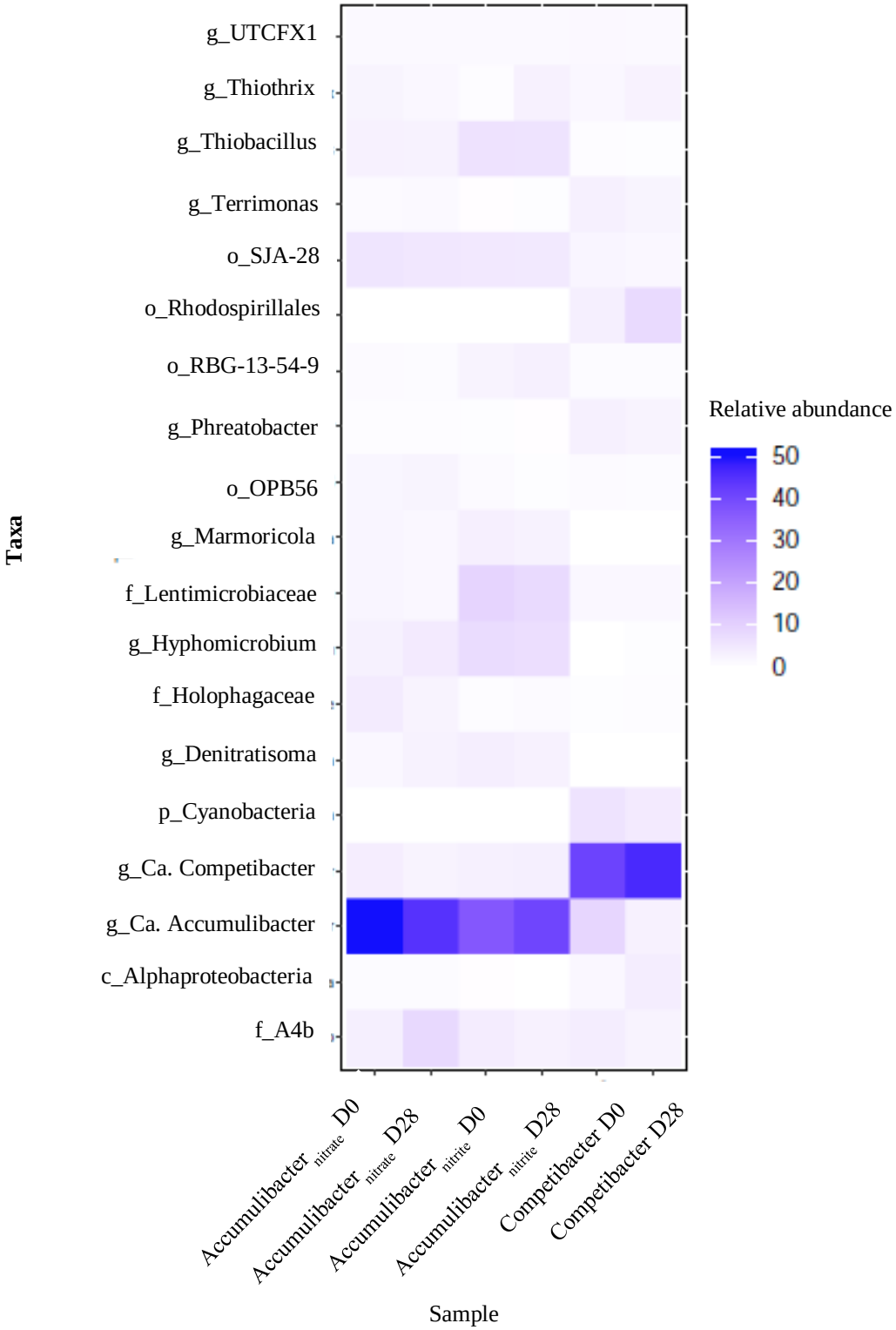
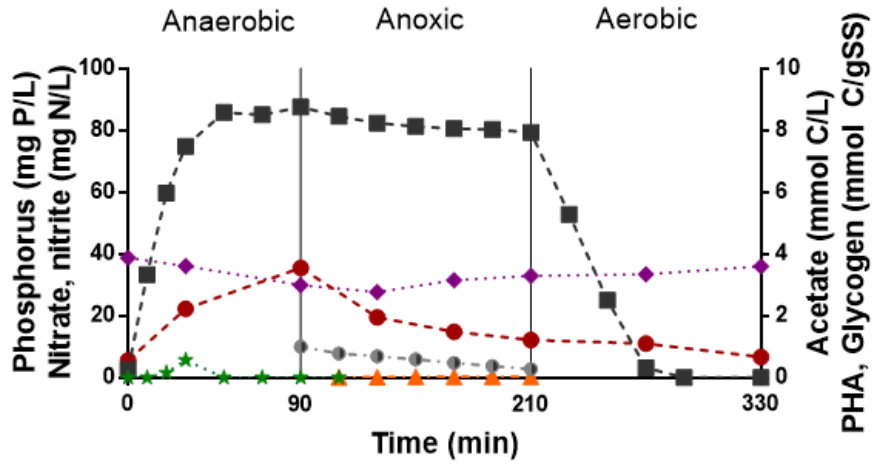


Figure 1. Microbial community analysis for samples collected at the start (D0) and end (D28) of batch tests from Accumulibacter$_{nitrite}$, Accumulibacter$_{nitrate}$, and Competibacter enrichment. Shown are the 15 most abundant organisms based on 16S rRNA gene amplicon sequencing and classified by the highest level of resolution obtained for each V1-V3 ASV annotated using the SILVA database. The microbial

community was dominated by the respective target organism for each reactor and the abundance remained stable during the course of the experiment.

(a)



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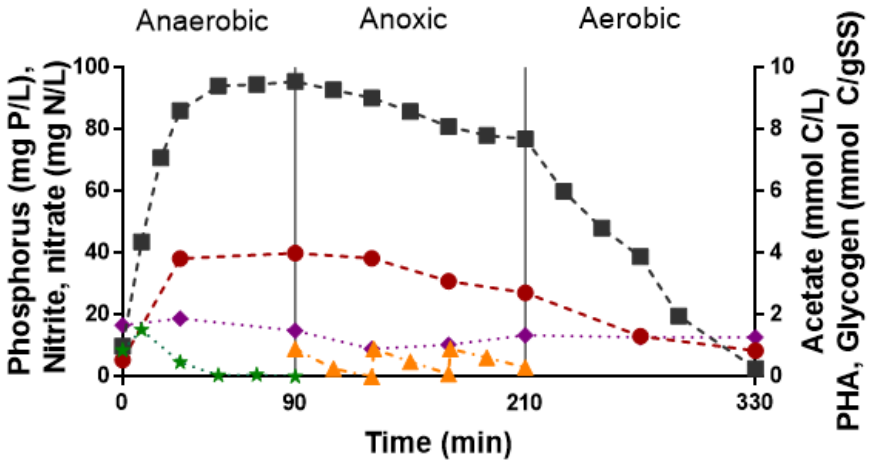

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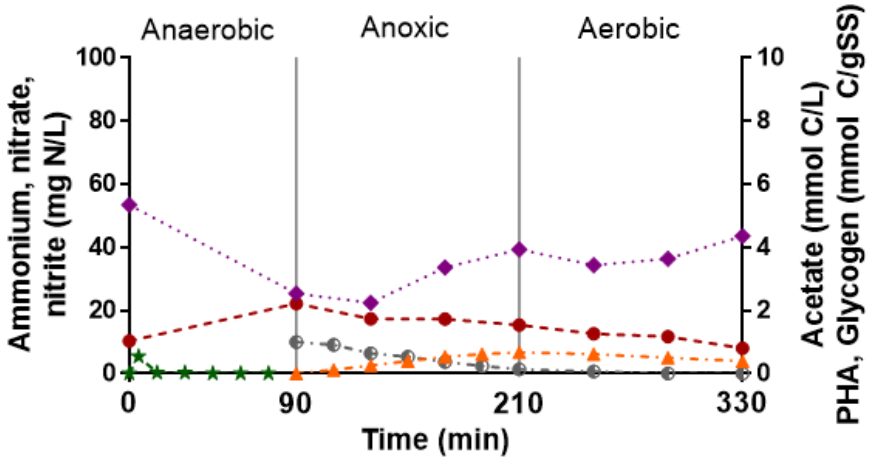
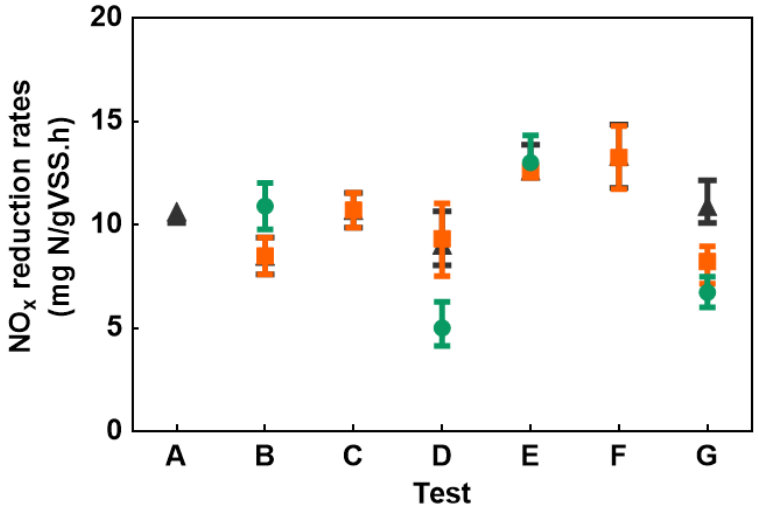
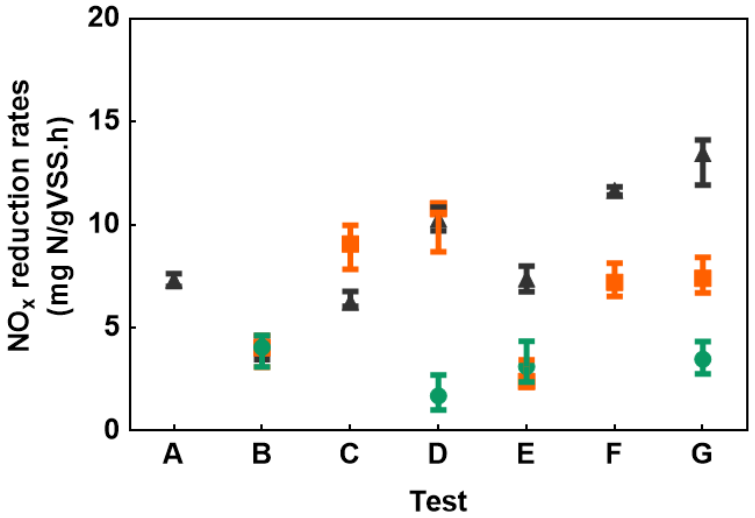
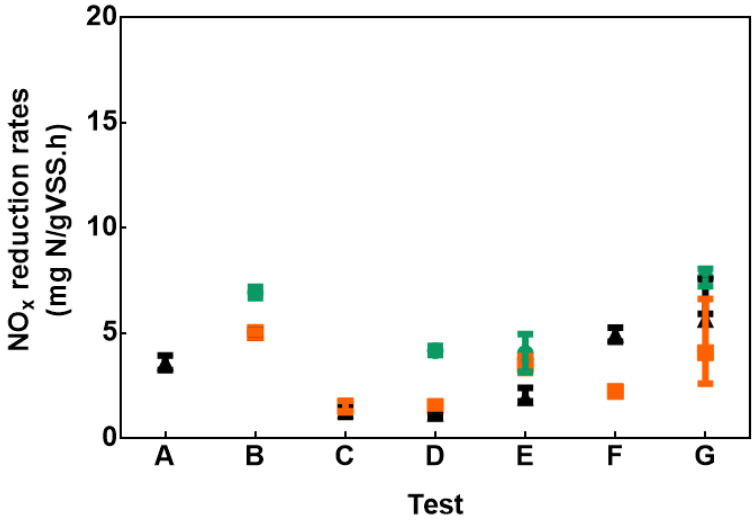


Figure 2. Transformation of nutrients and intracellular storage compounds during a typical SBR cycle of (a) Accumulibacter$_{nitrate}$, (b) Accumulibacter$_{nitrite,}$ and (c) Competibacter.



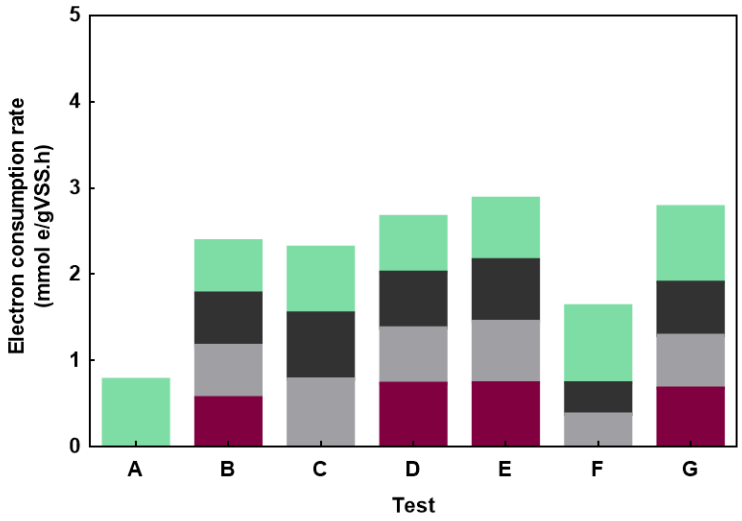


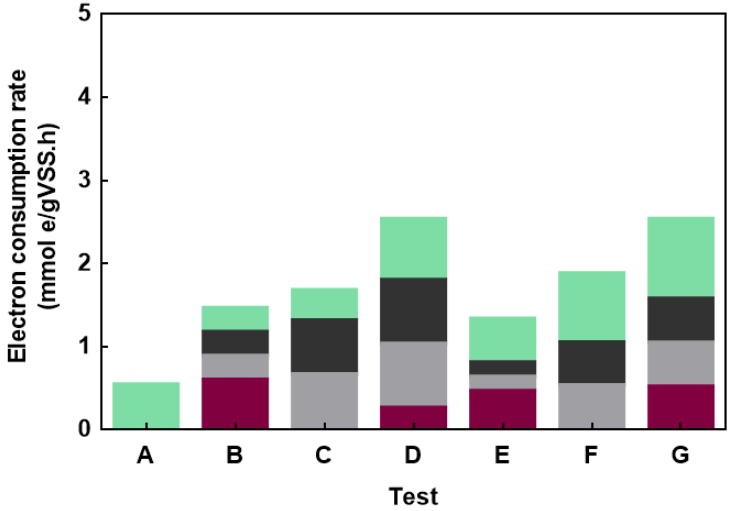


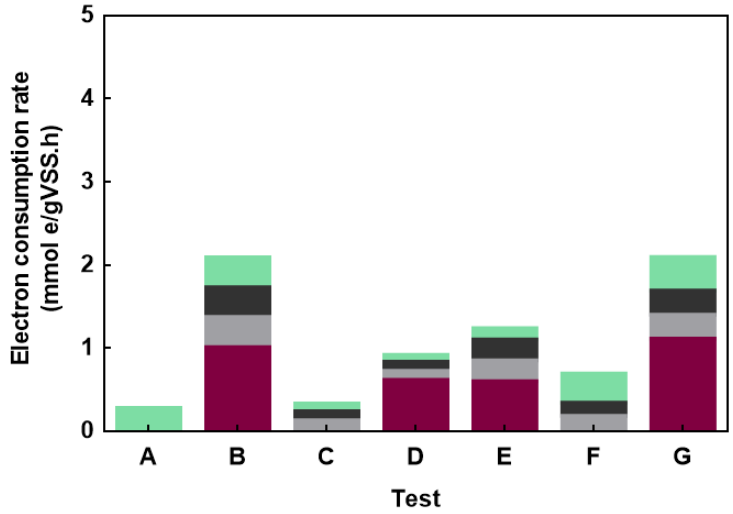
NO_3
NO_2
N_2O

Batch test	Electron acceptor added
A	N_2O
B	NO_3^-
C	NO_2^-
D	NO_3^-, NO_2^-
E	NO_3^-, N_2O
F	NO_2^-, N_2O
G	NO_3^-, NO_2^-, N_2O

Figure 3. True reduction rate of nitrate, nitrite and N_2O in batch test conditions A to G for (a) $Accumulibacter_{nitrate}$ (b) $Accumulibacter_{nitrite}$, and (c) Competibacter enrichments. The mean and range shown here were obtained from two replicates.



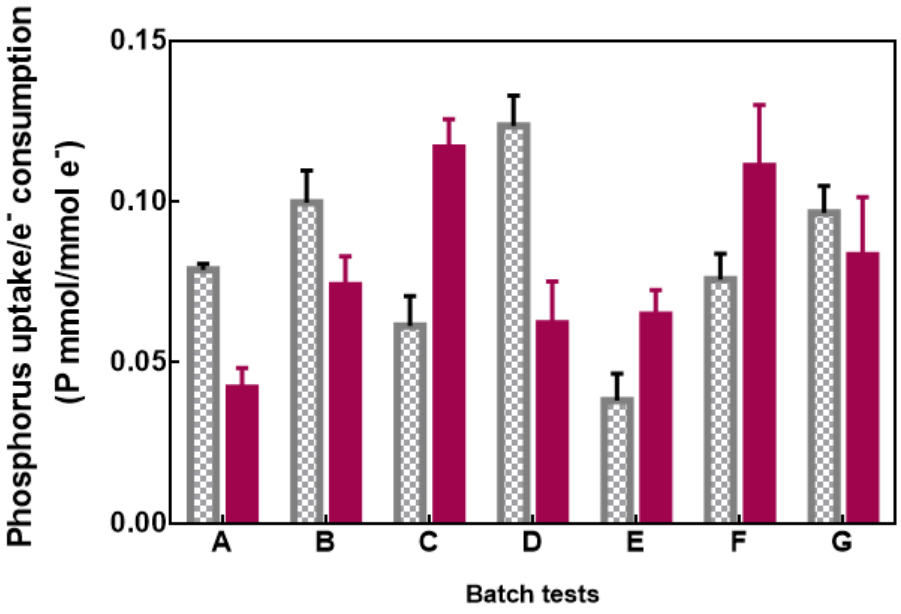




Nos
Nor
Nir
Nar

Batch test	Electron acceptor added
A	N_2O
B	NO_3^-
C	NO_2^-
D	NO_3^-, NO_2^-
E	NO_3^-, N_2O
F	NO_2^-, N_2O
G	NO_3^-, NO_2^-, N_2O

Figure 4. Electron consumption rates by specific denitrification enzymes for batch test conditions A to G for (a) Accumulibacter$_{nitrate}$, (b) Accumulibacter$_{nitrite}$, and (c) Competibacter. Denitrification enzymes are nitrate reductase (Nar, shown in maroon), nitrite reductase (Nir, in grey), nitric oxide reductase (Nor, in black) and nitrous oxide reductase (Nos, in green).



	Batch test	Electron acceptor added
Accumulibacter$_{nitrate}$	A	N_2O
	B	NO_3^-
	C	NO_2^-
Accumulibacter$_{nitrite}$	D	NO_3^-, NO_2^-
	E	NO_3^-, N_2O
	F	NO_2^-, N_2O
	G	NO_3^-, NO_2^-, N_2O

Figure 5. Ratio of P uptake per electron consumption (mmol e^-) obtained for the batch tests performed with various terminal electron acceptors for the two Accumulibacter enrichment cultures – Accumulibacter$_{nitrite}$ and Accumulibacter$_{nitrate}$.

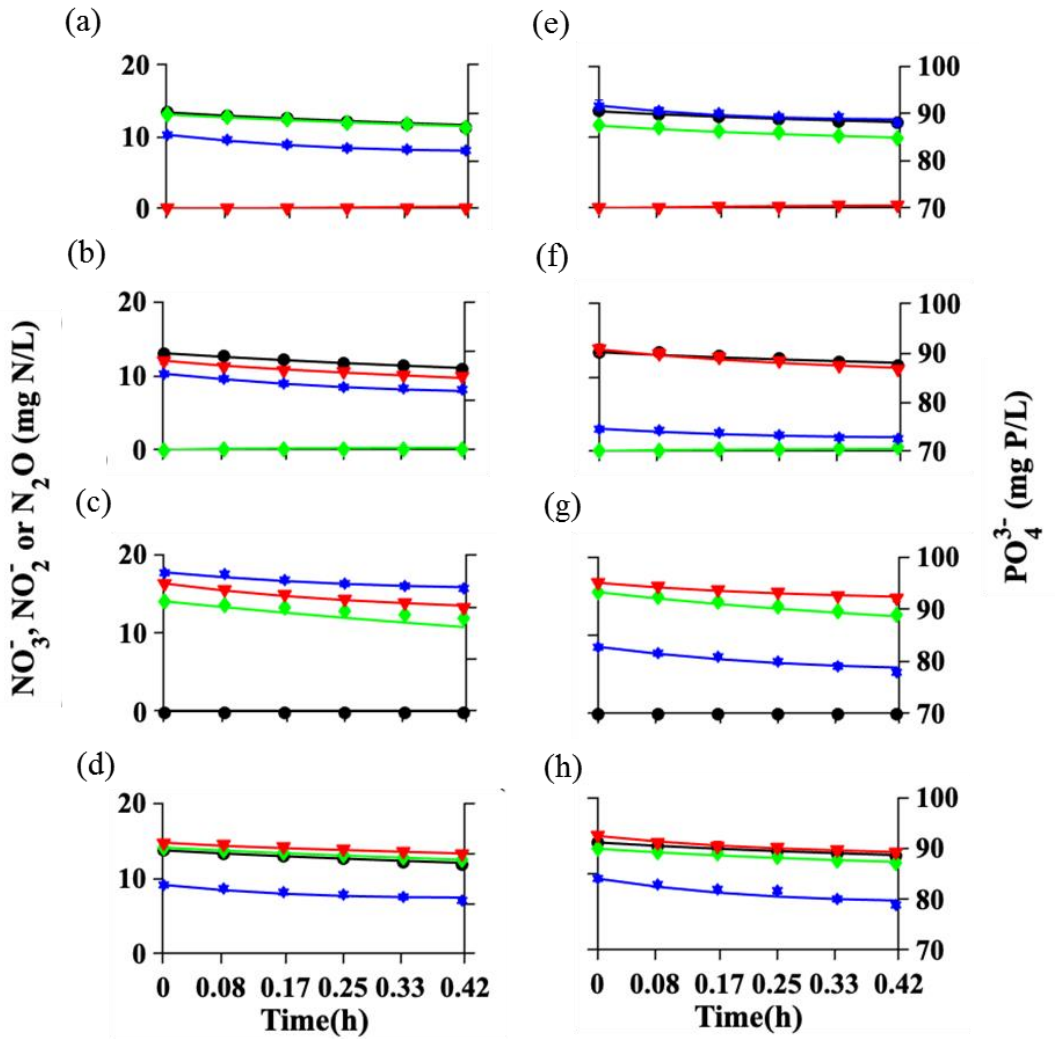


Figure 6. Simulation of batch perturbation studies of nitrate-enriched Accumulibacter with (a) NO_3^- and NO_2^-, (b) NO_3^- and N_2O, (c) NO_2^- and N_2O, and (d)) NO_3^-, NO_2^- and N_2O. Simulation of batch perturbation study of nitrite-enriched Accumulibacter with (e) with) NO_3^- and NO_2^-, (f)) NO_3^- and N_2O, (g) NO_2^- and N_2O, and (h) with) NO_3^-, NO_2^- and N_2O. x_axis limit (0 – 0.42), y_axis_left limit (0 – 20) and y_axis_right limit (70 – 90). Figure legend: predicted PO_4^{3-} (–, solid blue line), predicted NO_3^- (–, solid black line), predicted NO_2^- (–, solid green line), predicted N_2O (–, solid red line), measured PO_4^{3-} (★), measured NO_3^- (●), measured NO_2^- (✦), and measured N_2O (▼).

Table 1. Electron acceptors added in different batch tests

Batch test	Electron acceptor	Intermediate measured
A	N_2O	N_2O
B	NO_3^-	NO_3^-, NO_2^-, N_2O
C	NO_2^-	NO_2^-, N_2O
D	NO_3^-, NO_2^-	NO_3^-, NO_2^-, N_2O
E	NO_3^-, N_2O	NO_3^-, NO_2^-, N_2O
F	NO_2^-, N_2O	NO_2^-, N_2O
G	NO_3^-, NO_2^-, N_2O	NO_3^-, NO_2^-, N_2O

Table 2. Comparison of the amount of N_2O accumulated per N reduced (in mg/L) with values in previous studies

Study	FNA level calculated (HNO_2-N. L^{-1})	Batch test (electron acceptor added)					
		Test B (NO_3^-)	Test C (NO_2^-)	Test D (NO_2^- & NO_3^-)	Test E (NO_3^- & N_2O)	Test F (NO_2^- & N_2O)	Test G (NO_2^-, NO_3^- & N_2O)
Acc[a]	0.1 - 0.2 µg	0.31 ± 0.01	6.93 ± 0.38	3.63 ± 0.18	-	-	-
Acc[b]	0.1 - 0.2 µg	-	30.97 ± 1.06	8.40 ± 0.01	-	-	-
Comp[c]	0.1 - 0.2 µg	-	15.83 ± 2.59	1.07 ± 0.97	1.57 ± 0.26	-	-
Tet[d]	5 µg	16.7 ± 0.8	15.5 ± 0.8	17.7 ± 0.9	-	-	-
DPAO[e]	1.58 µg	8.72 ± 0.2	17.4 ± 5.9	31.2 ± 2.70	-	20.11 ± 1.90	11.30 ± 3.10
DGAO[f]	1.58 µg	7.12 ± 2.16	82.95 ± 4.79	45.45 ± 0.89	13.71 ± 5.81	56.90 ± 4.92	48.45 ± 5.94

[a] *Ca.* Accumulibacter (enriched with nitrate as terminal electron acceptor in this study) (this study)
[b] *Ca.* Accumulibacter (enriched with nitrate as terminal electron acceptor in this study) (this study)
[c] *Ca*. Competibacter (enriched with nitrate as terminal electron acceptor in this study)
[d] *Tetrasphaera* culture (Marques et al. 2018)
[e] DPAO culture (Accumulibacter clade I and II present) (Ribera et al., 2016)
[f] DGAO culture (Competibacter and Defluviicoccus present) (Ribera et al., 2016)

- Below detection limit

Table 3. Mean PHA concentration and standard deviation at the beginning and end of batch tests

Enrichment	Phase	Batch test (electron acceptor added)						
		Test A (N_2O)	**Test B (NO_3^-)**	**Test C (NO_2^-)**	**Test D (NO_2^-& NO_3^-)**	**Test E (NO_3^-& N_2O)**	**Test F (NO_2^-& N_2O)**	**Test G (NO_2^-, NO_3^-& N_2O)**
$Accumulibacter_{nitrate}$	Start (90 min)	10.59 ± 1.58	7.16 ± 1.09	7.37 ± 0.16	8.60 ± 0.80	8.74 ± 1.02	9.14 ± 0.01	6.07 ± 0.39
	End (120 min)	9.54 ± 0.57	5.71 ± 1.13	6.09 ± 0.08	6.13 ± 0.10	4.77 ± 0.82	6.64 ± 0.71	3.80 ± 0.23
$Accumulibacter_{nitrite}$	Start (90 min)	6.57 ± 0.44	10.06 ± 0.33	10.75 ± 0.97	9.97 ± 0.07	10.15 ± 0.31	13.88 ± 0.34	7.64 ± 0.51
	End (120 min)	5.67 ± 0.96	9.43 ± 0.88	9.18 ± 1.60	8.89 ± 0.15	9.07 ± 0.52	12.67 ± 0.60	7.21 ± 0.49
Competibacter	Start (90 min)	5.03 ± 0.91	8.16 ± 1.06	6.23 ± 0.34	9.97 ± 1.14	7.96 ± 0.29	6.16 ± 0.16	8.16 ± 0.07
	End (120 min)	4.04 ± 1.21	7.66 ± 0.32	6.52 ± 0.08	8.12 ± 0.76	6.19 ± 0.15	5.63 ± 0.02	6.36 ± 0.14