Investigating the use of anaerobically stored carbon in post-anoxic denitrification

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Abstract
Significant methanol savings are hypothesized to result from anaerobic storage of internal carbon that is used for post-anoxic denitrification. An investigation into this internal carbon-driven denitrification was performed via a series of batch tests using biomass from Hampton Roads Sanitation District’s (HRSD’s) water resource recovery facilities (WRRFs): the Virginia Initiative Plant (VIP), Nansemond Plant (NP), and Army Base (AB) Treatment Plant. Internal carbon specific denitrification rates (SDNRs) increased during winter, by as much as 1 mg N/g MLVSS/h for VIP. Increasing the aeration time by 2–4 h lowered the SDNR by an average of 0.21–0.35 mg N/g MLVSS/h. No internal carbon denitrification was observed for biomass from non-nitrifying/denitrifying, biological phosphorus removal (bio-P) WRRFs. The increase in internal carbon SDNRs when the anaerobic acetate dose increased from 20 to 100 mg COD/L ranged from 0.06 to 0.28 mg N/g MLVSS/h. Higher phosphorus uptake rates were found to correlate to higher internal carbon SDNRs, but no significant post-anoxic P uptake was observed. The first steps are taken towards developing a strategy for full-scale implementation of this relatively novel type of denitrification by evaluating how some factors affect its occurrence.

Practitioner Points
- Significant methanol savings at a full-scale facility may result from use of internally stored carbon for post-anoxic denitrification.
- Short aerobic HRTs and high anaerobic zone VFA loading increase the post-anoxic internal carbon-driven denitrification.
- Non-nitrifying, bio-P biomass is not capable of internal carbon-driven denitrification.
- Internal carbon-driven denitrification is correlated with the activity of polyphosphate accumulating organisms.
INTRODUCTION

In order to meet total nitrogen (TN) concentrations less than approximately 5 mg/L, post-anoxic nitrate (NO$_3$) polishing is needed in addition to the standard pre-anoxic zone with internal nitrate recycle or step-feed BNR. A supplemental carbon source is often added to facilitate post-anoxic denitrification, as any readily biodegradable COD (rbCOD) in the influent has already been utilized. Denitrification via carbon from endogenous decay products will occur, but adding an external carbon source enables faster denitrification rates and smaller tanks, with more control over NO$_3$ removal in meeting strict effluent TN limits. Methanol is a common selection for this purpose, and its use is well documented. Unlike ordinary heterotrophic organisms (OHOs) that can use a variety of carbon sources, only a specialized group of heterotrophs, called methylo trophs, are able to use methanol for denitrification (Tchobanoglous et al., 2014).

Cells are subject to decay from predation and cell lysis, which can be called “external decay” (Van Loosdrecht & Henze, 1999). Alternatively, under famine conditions, cells may undergo “internal decay,” which reduces their size and activity as internal storage compounds are consumed (Kaprelyants & Kell, 1996). Endogenous decay denitrification is the result of NO$_3$/NO$_2$ reduction via substrate generated from decay. When endogenous decay denitrification is discussed in this paper, it is external decay that is being referenced. The use of internal carbon for denitrification as investigated here is considered a distinct process and is suspected to only occur via a select group of microorganisms. Endogenous decay denitrification rates for various processes with post-anoxic stages typically vary between 0.2 and 0.6 mg N/g MLVSS/h (Henze, 1991; Kujawa & Klapwijk, 1999; Shi et al., 2019; Vocks et al., 2005).

Even without an external carbon source, post-anoxic denitrification rates exceeding endogenous decay denitrification rates have been observed (Coats et al., 2011; Vocks et al., 2005; Winkler et al., 2011). It is hypothesized here that this is due to some internal carbon source, likely COD that is taken up and stored in the anaerobic selector, carried through the pre-anoxic and aerobic zones, and used in the post-anoxic zone for denitrification. Such a theory on the role of internal carbon was supported by Vocks et al. (2005) when batch test denitrification rates using washed versus unwashed biomass of the same source were identical and still above expected endogenous decay denitrification rates. A feast/famine regime can be used to select for carbon storing biomass, as Dircks et al. (2001) demonstrated that volatile fatty acids (VFAs) were stored during the feast phase, and this provided a competitive advantage in the famine phase. Few papers mention this internal carbon-driven denitrification phenomenon in peer-reviewed or gray literature. This appears to be a relatively unexplored mechanism for post-anoxic denitrification.

Polyphosphate-accumulating organisms (PAOs) and glycogen-accumulating organisms (GAOs) are heterotrophs that store carbon in the forms of polyhydroxyalkanoate (PHA) and glycogen and could play a role in internal carbon denitrification (Figure S1 shows the modified Mino PAO model, and Figure S2 shows the Filipe-Zeng model for GAOs). Denitrifying PAOs and GAOs (dPAOs and dGAOs) are a subset of these organisms that are able to use NO$_3$/NO$_2$ as an electron acceptor instead of oxygen and, in the case of dPAOs, take up OP anoxically (Meinhold et al., 1999; Zeng et al., 2003).

Multiple studies have measured glycogen in the post-anoxic phase and found its consumption to be almost identical to the theoretical COD requirement for the observed NO$_3$ removal (Shi et al., 2019; Vocks et al., 2005). Vocks et al. (2005) also observed a distinct 36% decrease in a batch test denitrification rate with no external carbon after 6 h, which they attributed to depletion of internal glycogen. It is generally assumed that PHA is no longer available by the post-anoxic phase, as it is consumed in the preceding aerobic phase while glyco gen is replenished (Smolders et al., 1995). Indeed, PHA was depleted by the end of the aerobic phase in the anaerobic/aerobic/anoxic sequencing batch reactors (SBRs) run by Coats et al. (2011), and anoxic glycogen depletion was observed. Augmenting the reactor with VFA led to higher aerobic glycogen replenishment that ultimately increased the subsequent post-anoxic denitrification rate. On the other hand, multiple studies have demonstrated that PHA was degraded for denitrification during the anoxic phase while glycogen was replenished (Chen et al., 2015; Krasnits et al., 2013; Liu et al., 2013). In the study by Liu et al. (2013), shortening the aerobic time increased the PHA in the subsequent post-anoxic stage, increasing the denitrification rate. Regardless of glycogen versus PHA as a carbon source, bio-P biomass has been identified as a prerequisite for internal carbon denitrification (Vocks et al., 2005).

**KEYWORDS**
denitrification, endogenous, internal carbon, methanol, post-anoxic
Three of Hampton Roads Sanitation District’s (HRSD’s) conventional activated sludge WRRFs use pre-anoxic and post-anoxic zones with methanol addition for N removal (five-stage BNR). These include the Virginia Initiative Plant (VIP), Nansemond Plant (NP), and Army Base (AB) Treatment Plant. VIP is located in Norfolk, Virginia, and treats an average flow of 1.02 * 10^5 m^3/day (27 million gallons per day [MGD]). A “VIP + 2” configuration is used here, consisting of the VIP process followed by a post-anoxic and reaeration zone. This general treatment schematic as well as the one for the five-stage Bardenpho process can be found in Figure S3. NP is located in Suffolk, Virginia, and treats an average flow of 6.43 * 10^4 m^3/day (17 MGD). AB is located in Norfolk, Virginia, and treats an average flow of 3.79 * 10^4 m^3/day (10 MGD). A five-stage Bardenpho process is used at both NP and AB.

VIP, NP, and AB all add methanol in the post-anoxic zone to drive the TN concentrations below permitted levels. The comparative methanol doses for each WRRF were represented as the mass ratio of total methanol added to TN removed across the treatment process from the primary clarifier effluent (PCE) to final effluent. The methanol dose averaged over 2017–2020 for VIP is significantly lower than at NP or AB as shown in Table 1, along with other key parameters.

The internal mixed liquor recycle (IMLR) at VIP is restricted due to pumping limitations, yet VIP is still able to meet an average TN of 5.01 ± 0.16 mg/L. Superior post-anoxic denitrification at VIP compensates for the limitation in pre-anoxic denitrification capacity, enabling effluent TN limits to be met while simultaneously achieving significant methanol savings.

Data from nutrient profiles of the full-scale post-anoxic zone at VIP over the past few years suggested denitrification was occurring using an internal carbon source. The theoretical NOx removal attributable to methanol was estimated using the known amount of methanol dosed and assuming 4.8 g COD/g NO3-N removed (Dold et al., 2008). The endogenous decay contribution to NOx removal was determined using the endogenous decay specific denitrification rate (SDNR) measured from batch tests and adjusting for full-scale temperature, mixed liquor suspended solids (MLSS) concentrations, and flow rate. The leftover quantity of NOx removal after subtracting the methanol and decay-related NOx removal from the total was suspected to be due to internal carbon. These contributions are shown in Figures S4 and S5. Of the average 3.8 mg N/L of NOx removed across the zone, approximately 33% (or 1.26 mg N/L) was due to this internal carbon source. The high PCE rbCOD:TKN ratio, PCE rbCOD:TP ratio, and food to mass (F/M) ratio in the anaerobic zone at VIP compared with NP and AB (as shown in Table 1) indicate an excess of influent carbon at VIP. This could potentially be facilitating superior internal carbon storage in the anaerobic zone that is driving post-anoxic denitrification.

A relationship between internal carbon denitrification and bio-P performance was suggested from the correlation of methanol dose and final effluent OP concentrations at VIP, as shown in Figure S6. The deterioration of bio-P at VIP seemed to occur alongside poor denitrification, shown by an increase in methanol demand. This was not necessarily the case at AB or NP (Figures S8 and S10). Figures S7, S9, and S11 compare effluent OP concentration and methanol dose for VIP, AB, and NP; at VIP, the rough correlation of these factors was distinct from the lack of relationship in the graphs for NP and AB.

The full-scale observations at VIP initiated an analysis of internal carbon denitrification, specifically as it was thought to be the driver of VIP’s extremely low post-anoxic methanol demand. The objectives were as follows:

- Determine whether the capacity for internal carbon denitrification can be observed for each plant (AB, NP, and VIP) in a batch test setting, and how this changes over the span of several months.
- Investigate the effect of aeration time on the internal carbon SDNR.
- Determine if non-nitrifying/denitrifying, bio-P biomass can use internal carbon denitrification.
- Verify that the amount of anaerobic VFA affects the internal carbon SDNR.
- Examine how the internal carbon SDNR relates to PAO activity.

### Table 1

| WRRF | Methanol dose (lb COD/lb N removed) | IMLR flow (%) | Effluent TN (mg/L) | PCE rbCOD: TKN (lb/lb) | PCE rbCOD: TP (lb/lb) | Anaerobic F/M (mg rbCOD/mg MLVSS/d) |
|------|-----------------------------------|---------------|-------------------|------------------------|-----------------------|-----------------------------------|
| VIP  | 0.49 (±0.03)                      | 118 (±2)      | 5.01 (±0.16)      | 4.12 (±0.65)           | 31.0 (±4.3)           | 3.57 (±0.57)                      |
| NP   | 1.48 (±0.06)                      | 342 (±4)      | 4.73 (±0.12)      | 2.00 (±0.26)           | 10.4 (±1.2)           | 0.66 (±0.09)                      |
| AB   | 2.11 (±0.15)                      | 201 (±3)      | 4.90 (±0.18)      | 1.63 (±0.32)           | 10.4 (±1.7)           | 0.79 (±0.14)                      |

Note: Values are 2017–2020 averages. (± 95% confidence interval for the average).

Abbreviations: IMLR, internal mixed liquor recycle; PCE, primary clarifier effluent; rbCOD, readily biodegradable COD; TN, total nitrogen; TKN, total Kjeldahl nitrogen; TP, total phosphorus.
With few other papers describing this phenomenon, this study focused on factors affecting internal carbon denitrification and linking it to full-scale observations. This opens a new area of research with the potential to dramatically reduce the cost point for five-stage BNR facilities. Understanding the drivers of internal carbon denitrification is important with the ultimate goal of lowering methanol costs and intensifying treatment processes capable of meeting stringent N and P limits.

**METHODS**

**Biomass sources**

Mixed liquor from six of HRSD’s WRRFs was used for some or all of the tests conducted. Table S1 gives an overview of the relevant treatment process details at each WRRF and sample collection locations. Samples were generally collected at the end of the biological treatment process, prior to secondary clarification.

**Batch test setup**

All bench-scale batch tests were conducted in 4 L or 8 L reactors. Top entry paddle mixers were used. Temperature was controlled to $20^\circ\text{C} \pm 1^\circ\text{C}$ using a water bath. The dissolved oxygen (DO) concentration was maintained at less than 0.1 mg/L for anoxic and anaerobic conditions and greater than 2 mg/L for aerobic conditions via an aquarium pump and aeration stone. DO control was managed via an InsiteIG dual channel controller and sensors. N$_2$ gas sparging was used for removing DO from the reactor to reach anaerobic/anoxic conditions, and a floating Styrofoam cover was applied to minimize surface oxygen transfer. Solutions of 22 g/L of NaHCO$_3$ and 0.6% sulfuric acid were used to maintain pH between 6.9 and 7.2.

**Batch test operation**

After mixed liquor collection, the sample was adjusted from the full-scale WRRF temperature to the standard batch test temperature of $20^\circ\text{C}$. The sample was aerated during this time, which was usually for 1–2 h prior to starting the test. This prevented the mixed liquor from going anaerobic, which could cause phosphorus release.

**Endogenous test**

The reactors were kept under anoxic conditions for at least 24 h prior to measuring the endogenous decay denitrification rate. There was no anaerobic carbon storage phase and no methanol was added, so the only carbon source was endogenous decay products. Additional NO$_3$ was added as necessary to prevent depletion of NO$_x$ and resulting anaerobic conditions. Samples were collected hourly over the course of 4–5 h and analyzed for NO$_3$ and NO$_2$. COD was also measured at the start and end of the sampling period to ensure there were no major changes.

When endogenous SDNR determinations were done for the same mixed liquor as was used for another batch test, the adjustments for determining internal carbon SDNRs were done using that measured endogenous SDNR. For tests when no endogenous decay SDNR was determined for that sample of mixed liquor, factors such as time since a previous endogenous decay SDNR measurement and/or similarity in solids residence times (SRTs) between mixed liquor samples were considered when selecting an endogenous decay SDNR to use for adjustment.

**Standard anaerobic/aerobic/anoxic test**

The standard batch test consisted of anaerobic, aerobic, and anoxic (ANA/AER/ANX) phases at 45 min, 60 min, and 100 min, respectively. Figure S12 shows the test schematic. Sixty milligrams of COD/L of acetate was added at the start of the anaerobic phase. The anoxic phase was split into two parallel reactors: one with methanol added and one without. Approximately 12 mg NO$_3$-N/L of NO$_3$ was added to each reactor at the start of the anoxic phase. This test was conducted for all six of HRSD’s WRRFs listed in Table S1, including the non-nitrifying/denitrifying WRRFs: Atlantic (AT) and Chesapeake-Elizabeth (CE) (although both AT and CE do have bio-P). Tests were repeated over the course of August 2020 to April 2021, primarily for VIP, NP, and AB. There was no reactor with methanol addition for this test when using mixed liquor from CE, AT, and James River (JR) as these WRRFs do not add methanol as a carbon source for denitrification, and thus do not have an established methylo-trophic population capable of using it.

**Long versus short aeration test**

The long versus short aeration test was used to determine the effect of aeration time on post-anoxic SDNR. The same operating sequence as the standard ANA/AER/ANX test was used, with a couple modifications. Figure S13 shows the schematic for the test. After 30 min of the aerobic phase, the reactor was split into two parallel reactors. In one reactor, the anoxic phase was
immediately started by sparging N₂ gas and spiking NO₃ ("short AER" reactor). The aerobic phase was continued for an additional 120 min (or 240 min) in the other reactor ("long AER" reactor) prior to starting the anoxic phase. No methanol was added during the anoxic phase. This was conducted with mixed liquor from VIP, NP, and JR. Initial tests were conducted with a 25 min anaerobic and 150 min long AER phase. Subsequent tests used a 45 min anaerobic and 270 min long AER phase to be closer to full-scale HRTs. Samples were collected and analyzed for bulk PHA and glycogen concentrations during one of these tests with mixed liquor from VIP.

### High versus low acetate addition test

The effect of acetate addition on post-anoxic denitrification was examined by adding either 20 mg COD/L ("low") or 100 mg COD/L ("high") of acetate at the start of the anaerobic phase. Two tests were conducted with the same mixed liquor in parallel: one with the low acetate addition and one with the high acetate addition. Each followed the same procedure as the standard ANA/AER/ANX test (refer to Figure S12). This test was done for VIP, NP, and AB.

A variation of this test was done with NP mixed liquor. The reactor was aerated overnight prior to the test in order to eliminate the effect of residual internal carbon from full-scale. The endogenous decay SDNR was measured after the overnight carbon depletion period, once NO₃ was added and anoxic conditions were met. After the endogenous measurement, methanol was added to remove residual NO₃ and establish anaerobic conditions at the start of the main part of the test. In addition, instead of 20 mg COD/L in the low acetate reactor, no acetate was added.

### Sample analysis and rate determination

Bio-P and denitrification were monitored by taking samples in each phase of the batch tests. Mixed liquor samples were collected with a 20 mL syringe from the 4 or 8 L reactor and passed through 0.45 μm filters. Filtered samples were analyzed for OP and COD in the anaerobic phase for OP release and COD uptake rates. Initial samples were also analyzed for NO₃ and NO₂ until these concentrations were below 1 mg N/L. The COD consumption attributed to bio-P was determined by taking the total COD consumption measured and subtracting the COD required to denitify the initial NO₃ in the reactor (primarily NO₂). This was done by using a consumptive ratio (Cᵣ) of 6.62 g COD/g NO₃-N (Tchobanoglous et al., 2014). Samples were analyzed for OP in the aerobic phase to determine the OP uptake rates. In the anoxic phase, samples were analyzed for NO₃ and NO₂ to determine denitrification rates, COD to determine methanol consumption rates or monitor COD changes when no methanol was present, OP to monitor any potential additional OP uptake (due to dPAOs), and NH₄ to monitor accumulation from decay. Analysis for each parameter was done using Hach TNTPlus vials (HACH Loveland, CO).

MLVSS was measured using a handheld InsiteG Model 3150 suspended solids sensor at the beginning of each test. MLVSS was estimated by assuming an 80% volatile fraction of the MLSS, based on the average volatile fraction reported for the full-scale WRRFs. Specific rates, such as the SDNR, were determined by dividing the measured rates by the MLVSS concentration.

For the standard ANA/AER/ANX test, the methanol-only SDNR was determined by taking the difference between the SDNR in the reactor where methanol was added and subtracting the SDNR in the reactor without methanol. Internal carbon and endogenous decay denitrification would occur in each reactor, so any difference between the two would be attributable to methanol.

Sludge samples from one of the long versus short aeration batch tests were analyzed for PHA and glycogen. For PHA, extraction and analysis were performed according to the method described in Oehmen et al. (2005). The extracted organic phase was analyzed via gas chromatograph with a DB-5MS column and a Shimadzu mass spectrometer GC–MS-QP 5050 (Shimadzu, Japan) with autosampler AOC-1400. The mass spectrometer was run in scan mode at a detector voltage of 1.5 kV, mass range of 40–600 amu, scan speed of 2000 amu/s, and interval of 0.3 s. The automated mass spectral deconvolution and identification system (AMDIS32) was used for deconvolution of GC–MS peaks, with compound identification via the National Institute of Standards and Technology (NIST02) database. The lyophilized samples analyzed for PHA were also analyzed for glycogen using a colorimetric method. Approximately 2 mg of lyophilized cells were added to 2 ml of 0.9 M HCl alongside working standards of glucose ranging from 0 to 200 μg per 2 ml, and all were digested for 3 h at 100°C. Digestion vials were cooled in the dry heat thermoblock for 60 min and then on ice for 30 min. Samples and standards were diluted to concentrations suited for spectrophotometry (<100 ppm). Five milliliters of 0.2% anthrone reagent (0.2 g anthrone powder in 100 ml 95% sulfuric acid) was added to 2 ml of appropriately diluted digested samples and standards and further digested for 10 min at 100°C, then cooled. Colorimetric measurement was taken at 620 nm.
RESULTS AND DISCUSSION

Internal carbon-driven denitrification capacity

The standard ANA/AER/ANX batch tests were performed periodically over the course of 9 months to evaluate the capacity of each of the five-stage WRRFs for internal carbon denitrification, and monitor how that capacity changes over time. The internal carbon denitrification rates were obtained by taking the denitrification rate in the reactor with no methanol feed and subtracting the endogenous decay denitrification rate for that WRRF’s biomass (see Table S2 for batch test determined endogenous decay SDNRs). Figure 1 shows the resulting internal carbon SDNRs for each of the standard batch tests. Mixed liquor from HRSD’s JR WRRF was included due to suspected use of internal carbon full-scale. JR utilizes an anaerobic/anoxic/aerobic (A2O) process with integrated fixed-film activated sludge (IFAS) at low total and aerobic SRT. Following the IFAS tank is a deaeration zone where no methanol is added, but some denitrification still occurs. All of the WRRFs show capacity for internal carbon denitrification in the batch test setting. Therefore, it is not a biomass limitation, but rather an operational factor that affects the initiation of internal carbon denitrification at full scale. These rates are similar to those in literature: Coats et al. (2011) observed SDNRs of 0.69–0.90 mg N/g MLVSS/h against an expected endogenous rate of 0.2–0.6 mg N/g MLVSS/h, thus resulting in a corrected internal carbon SDNR that would fall into the range of 0.09–0.70 mg N/g MLVSS/h.

These SDNRs increased during the winter months, when bio-P performance at the WRRFs is typically better due to repression of GAO competition at lower temperatures. For instance, the internal carbon SDNR was negligible for the initial test with VIP mixed liquor in August 2020, but increased to over 1 mg N/g MLVSS/h for the test in December 2020. This reinforces the idea that internal carbon denitrification depends on bio-P, as initially suggested by the full-scale effluent OP concentrations versus methanol addition rates at VIP (refer to Figure S6).

The internal carbon SDNRs were expected to be highest at VIP or JR. At VIP, the full-scale denitrification from profiles and the rate of methanol addition per N removed is most indicative of this type of denitrification at this WRRF. At JR, no methanol is added in the zone following IFAS, but there is still significant denitrification that is suspected to occur from internal carbon. However, the VIP and JR internal carbon SDNRs were comparable with the SDNRs for the other WRRFs, particularly AB. The maximum rate for NP was significantly lower than the maximum rates observed at the other WRRFs, but these tests were only performed a select number of times during the 9-month period, and so there was no guarantee the maximum SDNR obtainable for each WRRF’s biomass was observed.

One of the distinguishing features of the post-anoxic tanks at VIP is that they are relatively long and narrow compared with those at NP or AB, as shown in Figure 2. This zone at VIP is much closer to plug flow conditions than at the other WRRFs. The batch test setting also offers ideal plug flow conditions. This configuration could encourage internal carbon denitrification, considering this phenomenon is observed for all WRRFs in batch tests but only seems to occur full-scale at VIP. Perhaps the internal carbon denitrifiers have a high NO₃ half-saturation constant and would be more active in the pocket of high substrate concentrations at the start of a plug-flow reactor versus in a continuous stirred-tank reactor (CSTR) where concentrations immediately get diluted. There could also be other aspects of the bench scale batch test operation that favor the use of internal carbon for denitrification regardless of what is happening.
full scale at each WRRF, such as a non-limiting supply of VFA in the anaerobic phase, or a relatively short aerobic phase.

Long versus short aeration tests

The duration of the aerobic phase had an effect on the internal carbon SDNRs observed in the post-anoxic phase during the long versus short aeration batch tests. When the aerobic time was increased from 0.5 to either 2.5 or 4.5 h, the SDNRs decreased. Figure 3 shows the overall SDNRs from each test performed with mixed liquor from VIP, NP, and JR, as well as the estimated endogenous decay SDNRs. The short AER SDNRs ranged from 0.51 mg N/g MLVSS/h to 1.92 mg N/g MLVSS/h. The long AER SDNRs ranged from 0.18 mg N/g MLVSS/h to 1.50 mg N/g MLVSS/h. The difference between the short and long AER SDNRs was generally larger when the long AER time was increased from 2.5 h in the early tests to 4.5 h in the later tests. The average difference between long and short AER SDNRs was 0.21 mg N/g MLVSS/h for the tests with 2.5 h long AER times and 0.35 mg N/g MLVSS/h for the tests with 4.5 h long AER times.

The long AER SDNRs did not exceed the estimated endogenous decay SDNR in the initial tests at VIP and NP (8/26/2020 and 9/8/2020, respectively), and so no internal carbon was assumed to be remaining in the biomass that could be used for denitrification in these reactors. These tests were completed during the summer when temperatures were high at the WRRF and bio-P performance typically deteriorates. The relationship to bio-P will be discussed further. For all of the following tests where rates exceeded endogenous decay SDNRs, it
is possible that a change in carbon source was occurring as aeration time increased. As the aerobic phase progresses, PHA is depleted, and glycogen is replenished. Thus, a short aerobic phase would favor higher PHA concentrations, and a longer aerobic phase would favor glycogen concentrations. Generally denitrification on glycogen has been shown to be lower than that with PHA, where literature values have been in the range of 1.12–10.8 mg N/g MLVSS/h (Carvalho et al., 2007; Qin et al., 2005). For the long AER reactors, either PHA has been partially depleted during the extended aerobic phase and less is available for denitrification in the anoxic phase, or PHA has been fully depleted and glycogen is used for denitrification instead, which could be less favorable. In either case, the drop in SDNR could be explained.

On the other hand, if glycogen is being used as a carbon source in both reactors, the extended aerobic phase may have gotten to the point of glycogen depletion instead of replenishment, as has been shown to occur from over-aeration by Brdjanovic et al. (1998). The point when it switched from replenishment to depletion in the tests by Brdjanovic et al. (1998) occurred sometime between 4 and 10 h aeration. Thus, less would be available for denitrification in the anoxic phase and would explain the lower SDNRs. However, given the long AER time in the tests from this study was similar to full-scale aerobic HRTs (which range from 3.3 h on average for AB to 7.5 h for NP), glycogen depletion seems unlikely.

**PHA and glycogen analyses**

Samples were analyzed for PHA and glycogen analyses during one of the long versus short aeration batch tests with VIP mixed liquor to attempt to address the speculation on the type of carbon source being used for the post-anoxic denitrification. Results are shown in Figure 4. There is no clear trend in post-anoxic PHA nor glycogen consumption for the long-AER nor short-AER tests. This may indicate some other internal carbon source being used for denitrification, or may emphasize the

![Figure 4](image-url)
interchangeability in the form of internal carbon (between PHA and glycogen) that could be used. It has also been suggested that some organisms could actually be producing PHA and/or glycogen while others are consuming it during this post-anoxic phase, and so bulk measurements may not provide clear insight into internal carbon trends for individual groups of denitrifiers (Majed & Gu, 2010). The authors would like to point out that this batch test was only paired with this analysis one time, and would need to be repeated to verify results. This singularity of PHA/glycogen analysis in itself could be a major factor lending to the lack of clear post-anoxic trends for each of these internal carbon stores. In any case, PHA and glycogen should not be ruled out as carbon sources.

Nitrifying versus non-nitrifying biomass

The importance of bio-P biomass for internal carbon denitrification has been demonstrated (Vocks et al., 2005). As discussed in Section 3.1, biomass from the VIP, NP, AB, and JR WRRFs all showed capacity for internal carbon denitrification in the batch test setting, and these WRRFs all nitritify/denitritify as part of their full-scale processes. On the other hand, the standard ANA/-AER/ANX batch test performed with mixed liquor from the bio-P WRRFs that are non-nitrifying/denitrifying (CE and AT) showed no denitrification beyond the endogenous decay SDNRs, as shown in Table S3. The only time when some internal carbon denitrification was observed was when unintentional nitrification was occurring at AT full-scale for the 11/10/2020 test. As indicated by the OP release and uptake rates, each of these WRRFs had biomass performing sufficient bio-P. Thus, it can be concluded that the capability for internal carbon denitrification is dependent on the process of nitrification/denitrification in addition to bio-P. Both CE and AT biomass showed endogenous decay denitrification, so even though no denitrification was occurring full-scale, there was still the capacity for at least some type of denitrification in the biomass. Exposure to NO3/NO2 must then be critical for the establishment of a population that can use internal carbon for denitrification. This exposure is not a problem for WRRFs such as VIP, NP, or AB where nitrification/denitrification is obviously achieved, but it could factor into considerations as for aeration control that would affect NOx concentrations. These results also suggest that internal carbon denitrifiers are a distinct subset of heterotrophs. In other words, this could serve as a differentiator between denitrifying OHs and internal carbon denitrifiers, such as in microbial community analysis. NOx exposure might induce gene expression for the enzymes required for denitrification pathways that use the internal carbon stores in these organisms. Much more advanced analysis would be required to verify this theory.

High versus low acetate test

The average PCE VFA concentration at VIP and NP is just above 45 mg COD/L, and ranges from approximately 12–85 mg COD/L. A “low” value of 20 mg COD/L and “high” value of 100 mg COD/L was loosely based on this range.

Despite the relatively high rbCOD:TNK and rbCOD:TP ratios at VIP, increasing the amount of anaerobic acetate in the batch tests did not lead to a major increase in post-anoxic SDNRs. The resulting SDNRs from the high/low acetate tests (corrected for endogenous decay denitrification) are shown in Figure 5. The increase in internal carbon SDNRs between low and high acetate reactors ranged from 0.06 to 0.28 mg N/g MLVSS/h.

There also seemed to be a slight inhibitory effect on the methylotrophic denitrification for three of the five tests (VIP 9/15, NP 10/8, and VIP 3/3). The methanol-only SDNR dropped by 0.09 mg N/g MLVSS/h on average for these tests when the acetate dose increased, and the overall SDNR did not necessarily improve. This suggests some sort of interference between internal carbon and methylotrophic denitrification. While the NO3 half-saturation constant is 0.5 mg N/L for heterotrophs and 0.05 mg N/L for methylotrophs, the concentrations never got below 3 mg N/L in the batch tests. If internal carbon denitrifiers have a higher half-saturation value, this could have impacted results, but assuming the internal carbon denitrifiers have a half-saturation at least below 3 mg N/L, NO3 was not limiting, and there should not have been competition for substrate. Regardless, it is unlikely that the same organisms are responsible for both types of denitrification, as methylotrophs are not known to be capable of carbon storage.

It is possible that the minor changes in internal carbon SDNRs when acetate was increased was because the internal carbon storage was actually more reflective of residual storage from the full-scale process instead of what was stored during the batch test anaerobic phase. This is despite mixed liquor being collected from the reaeration zone at each WRRF where internal carbon should be at a minimum. To mitigate the potential effect of residual stored carbon from full scale, the second test at NP on 1/25/2021 was altered by initially aerating the reactor overnight. In addition, instead of adding 20 mg COD/L of acetate to the low acetate reactor, no acetate was added. Somehow there was still internal carbon
denitrification observed in this reactor, at a rate of 0.54 mg N/g MLVSS/h. Any contributions from sbCOD would have been included in the endogenous decay SDNR measured after the overnight aerobic period, and thus was already adjusted for when determining the internal carbon SDNR. Methanol was used to deplete residual NOx from the endogenous decay SDNR measurement phase in order to obtain anaerobic conditions at the start of the test, and may have still been present during the anaerobic stage. Again, methylotrophs are not known to be capable of carbon storage, and any residual methanol would have been oxidized in the aerobic phase and not be available in the post-anoxic phase. There must have been some other carbon source besides acetate during the anaerobic phase that facilitated storage for use in the post-anoxic phase. There was also OP release observed to support this, as concentrations reached 13.6 mg P/L at the end of the anaerobic phase. This test should be repeated to verify results and try to identify the source of carbon being stored.

VFA availability is likely only one of many factors relevant to encouraging internal carbon denitrification. As demonstrated with the long versus short aeration test, the aeration time has an effect on the SDNR, likely by changing the amount/form of internal carbon available for post-anoxic denitrification. The low acetate reactors may still have some residual stored carbon from full-scale, or enough stored carbon from the minimal acetate that was added, so there is still some left even after 60 min of aeration. The rate of internal carbon denitrification may take on a Monod-shaped relationship to the amount of stored carbon; if well above the half-saturation, the rate would not change by much for a change in stored carbon concentration. If the aerobic time was extended to a few hours to be closer to actual HRTs, then this internal carbon may finally be depleted in the low acetate reactor, but not in the high acetate reactor. The difference in SDNRs may be much more noticeable. The impact of the quantity of anaerobic VFA on the post-anoxic SDNR needs further examination, particularly in conjunction with the length of the aerobic phase.

**Relationship of internal carbon denitrification to bio-P performance**

Bio-P was monitored during the batch tests: anaerobic OP release and COD uptake rates, OP release: COD uptake ratios, and aerobic OP uptake rates were all compared to post-anoxic internal carbon SDNRs measured during the standard ANA/AER/ANX and the high versus low acetate batch tests. The correlation with OP uptake is shown in Figure 6.

For each WRRF, as the specific OP uptake rate increases, the internal carbon SDNR also increases, and the linear regression for each is included in Figure 6. Unlike for the other two WRRFs, the regression is not statistically significant for VIP. However, it is possible that a linear model is just not a good assumption, and more data would be required to understand the relationship (or lack thereof) between these rates. Furthermore, there could be alternative explanations, such as a major shift in biomass population that caused the SDNRs to increase less drastically with OP uptake, starting a lower branch on the plot for the VIP data. A similar phenomenon could have started to occur with AB, to explain the high OP-uptake and low SDNR outlier point on the plot. These specific tests also may have been outliers for other reasons.

More OP uptake in the aerobic phase would consume more PHA that could be used for either...
denitrification or glycogen replenishment. However, the amount of PHA required for OP uptake is relatively low compared to that for biomass growth or glycogen replenishment (Smolders et al., 1995). In fact, the amount of glycogen replenished was not significantly changed when extra OP was added during aerobic batch tests by Smolders et al. (1994). Therefore, some extra OP uptake would not drastically change PHA consumption or glycogen replenishment, and would not inhibit the post-anoxic SDNR. Aerobic time in general may be more important, as well as the overall quantity of PHA storage. For instance, biomass growth is prioritized when PHA storage is low, so PHA storage would ideally be increased to maximize glycogen replenishment (or PHA leftover for denitrification) and still meet growth requirements (Mino et al., 1995).

Even though OP uptake rates correlated with internal carbon SDNRs, OP concentrations did not tend to drop during the post-anoxic phase in the batch tests. It is therefore unlikely that dPAOs were responsible for the internal carbon denitrification. There is usually not much OP left in the post-anoxic zone full-scale at VIP, as confirmed by nutrient profiles, so the presence of dPAOs in the biomass again seems unlikely. Or rather, PAOs may have been involved, but were using NO₃ as an electron acceptor for maintenance, rather than for anoxic OP uptake.

The correlation of SDNR to OP uptake may have resulted from a more dominant PAO population that was also partaking in the internal carbon denitrification for maintenance purposes. However, the quantity of the PAO population would not be the only requirement for internal carbon denitrification, as AB and NP are both capable of good bio-P performance, but do not necessarily show this type of denitrification full-scale. In addition, with a dominant PAO population, the OP release rates may be expected to increase. However, OP release rates are dependent on multiple factors, such as SRT, VFAs, or GAO competition, and this could explain the lack of correlation between the SDNRs and OP release rates shown in Figure S14.

During summer there is more VFA in the WRRF influent, supposedly from high temperatures driving more fermentation within the collection system. If both PAOs and GAOs were capable of internal carbon denitrification at VIP, then more internal carbon denitrification in summer would be expected instead of less, but as indicated in the seasonal graphs (Figure S6), there were actually higher methanol doses required in summer at VIP. The higher methanol doses were probably required to make up for a lack of internal carbon denitrification. So while more VFA could be better for carbon storage, it also might encourage more competition from GAOs, and that competition could potentially be detrimental to post-anoxic internal carbon denitrification if PAOs are really the key organisms.

All batch tests were controlled to 20°C, so it was not over-active biomass at elevated temperatures that would explain the correlation between OP uptake rates and internal carbon SDNRs (Figure 6). This correlation does not warrant any definite conclusions about the type(s) of organisms involved or their metabolisms, but does suggest that OP uptake rate could be used as an indicator of the potential for this internal carbon denitrification to occur during full-scale operation.
CONCLUSION

The following conclusions were obtained from the series of batch tests in this study:

- All nitrifying WRRFs had the capacity for internal carbon denitrification despite full-scale observations, and was not limited to biomass from VIP.
- The shorter aeration time led to higher SDNRs than the long aeration time, likely due to shifting quantity or form of internal carbon.
- Internal carbon denitrifiers appear to need prior exposure to NO2/NO3 in order to denitrify, which seems to distinguish them from OHOs.
- The increase in anaerobic acetate from 20 to 100 mg COD/L did increase the internal carbon SDNR, but by less than expected.
- There appears to be a link between bio-P and internal carbon denitrification that is distinct from dPAOs.

Based on these results, multiple factors seem to affect the occurrence of internal carbon denitrification as well as its rate. It is likely that a combination of conditions such as an active PAO population, a plug flow configuration, and high influent rbCOD concentrations would be best to facilitate post-anoxic internal carbon denitrification. Maintaining stable bio-P while encouraging internal carbon denitrification would maximize use of influent carbon to the WRRF, reducing the need for external carbon.

APPLICATION AND FUTURE STUDY

Cost savings from the reduction in external carbon addition in the anoxic zones is the most tangible benefit from internal carbon denitrification. This type of denitrification is observed full-scale at VIP, and the extent of methanol savings there is exemplified by direct comparisons to the other methanol-adding WRRFs. Table 2 shows the average daily and total yearly methanol mass added and associated costs for treatment in 2020–2021 at VIP, NP, and AB. While handling the highest flow at around 27 MGD at VIP, the average methanol cost is the lowest here by almost $150,000 per year. Alternatively, using the methanol dose rate and cost per lb, the cost of N removal at VIP is only $0.03/lb N, which is 10 times lower than the $0.33/lb N required at AB. These major savings in chemical costs can free up funding for other aspects of WRRF operation. Considering internal carbon denitrification was evident in the batch tests for AB and NP biomass, and not just VIP biomass, the potential exists for this type of denitrification at other WRRFs, where similar external carbon cost savings could thus be realized given the right operating conditions.

A relatively new topic of internal carbon denitrification was investigated through the unique lens of how to tap into this potential at other full-scale facilities, and so the authors do acknowledge the speculative nature of this study, particularly at the batch test-scale. There is much more work to be done to validate the initial theories here as well as explore other factors that could encourage or hinder this type of denitrification. For instance, identification of the organisms responsible for internal carbon denitrification and the type of internal carbon being used would be important steps to better develop selection strategies for these organisms. Pilot-scale studies or process modeling tools could be used to aid in further understanding, and ideally this would culminate in the identification of process configurations, control setpoints, and other modifications in a well-defined strategy for application of internal carbon denitrification in full-scale WRRFs to achieve major cost savings for N removal.

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AUTHOR CONTRIBUTIONS
Kayla T. Bauhs: Conceptualization; data curation; formal analysis; investigation; methodology; project administration; resources; software; validation; visualization. Alexandrina A. Gagnon: Conceptualization; data curation; formal analysis; investigation; methodology; project administration; resources; software; supervision; validation; visualization. Charles B. Bott: Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; project administration; resources; software; supervision; validation; visualization.

DATA AVAILABILITY STATEMENT
Data will be made available through email request to corresponding author.

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