Startup Strategies for Mainstream Anammox in Moving Bed Biofilm Reactors (MBBRs)

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Abstract

Partial denitrification/anammox (PdNA) is a biological nitrogen removal technology with significant carbon and aeration savings when compared with conventional nitrification/denitrification. Yet despite these benefits, the use of PdNA in mainstream wastewater treatment remains limited. One of the main barriers to implementation of anammox-based technologies is the slow growth rate of anammox (AMX), which results in a long startup time. To accelerate startup, the typical approach to commissioning AMX-based processes, specifically used for sidestream partial nitritation/AMX, is with biomass augmentation, which is practically unrealistic for full-scale mainstream applications. Thus, this study evaluated startup strategies for mainstream PdNA without AMX inoculation in moving bed biofilm reactors (MBBRs) with two simultaneous experiments. In one experiment, an MBBR was started using IFAS carriers with a preliminary biofilm and no external carbon dosing or AMX biomass inoculation. The feed was controlled to 20°C and included mainstream conditions of nitrite and ammonia controlled to the stoichiometric requirements for AMX growth. After only 84 days of operation, AMX activity was confirmed in the reactor with evidence of activity a few weeks before testing. In the second experiment, four reactors were started with either virgin carriers or integrated fixed-film activated sludge (IFAS) carriers with a preliminary biofilm of heterotrophs and nitrifiers. The reactors were fed mainstream levels of ammonia and nitrate with a temperature control target of 20°C and one reactor of each carrier type was dosed with carbon in the form of either glycerol or methanol. Carbon dosing was based on a feedback proportional-integrative-derivative (PID) control loop with a nitrate residual of 1-1.5 mgNO3-N/L. Of the four reactors, the preliminary biofilm carrier reactor dosed with glycerol achieved AMX activity first after 224 days of operation, but it was determined this was likely limited by synthetic feeding for the first 184 days. These results, along with other recent PdNA work, suggest that growth of AMX on biofilm carriers can be established in mainstream conditions in 50-100 days, depending on media selection and carbon source. Ultimately, this research will help utilities understand methods for starting up mainstream PdNA MBBRs from scratch and make this technology more accessible.

Startup Strategies for Mainstream Anammox in Moving Bed Biofilm Reactors (MBBRs)

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General Audience Abstract

Intensification is the practice by which operational changes and new technologies are employed to reduce economic, resource, energy, and space requirements of wastewater treatment plants. One area of increasing focus involves the use of anaerobic ammonia oxidizing bacteria, or anammox (AMX), to reduce the aeration and carbon dosing needs for treating wastewater. One of the main barriers to implementation of AMX-based technologies is the slow growth rate of AMX, which results in a long startup time. To accelerate startup, the typical approach to commissioning AMX-based processes, specifically used for sidestream partial-nitritation/AMX, is with augmentation of biomass, which is practically unrealistic for full-scale mainstream applications. Thus, this study evaluated startup strategies for mainstream moving bed biofilm reactors (MBBRs) without AMX biomass inoculation in two simultaneous experiments in an AMX MBBR and a partial denitrification/AMX (PdNA) MBBR. In one experiment, idealized stoichiometric conditions for AMX growth were provided to a mainstream MBBR started with carriers from an aerobic integrated fixed-film activated sludge (IFAS) process to determine how fast AMX could potentially grow. In another experiment, different carrier types, virgin or preliminary biofilm carriers from an IFAS process, and different carbon sources, methanol and glycerol, were tested to determine the best methods for encouraging AMX attachment and growth in a PdNA process. These results, along with other recent PdNA work, suggest that growth of AMX on biofilm carriers can be established in mainstream conditions within 50-100 days, depending on media selection and carbon source. Ultimately, this research will help utilities understand methods for starting up mainstream PdNA MBBRs from scratch and make this technology more accessible.

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List of Abbreviations

A/B: Adsorption/bio-oxidation

AerAOB: Aerobic ammonia oxidizing bacteria

AMX: Anammox

AvN: Ammonia versus NO_x (nitrate plus nitrite)

BNR: Biological nutrient removal

CE: Chesapeake Elizabeth

COD: Chemical Oxygen Demand

C/N: Carbon to nitrogen ratio

CSTR: Continuous stirred tank reactor

DO: Dissolved oxygen

FdN: Full denitrification

FNE: Final effluent

HRAS: High rate activated sludge

HRSD: Hampton Roads Sanitation District

HRT: Hydraulic residence time

IFAS: Integrated fixed-film activated sludge

JRTP: James River Treatment Plant

MBBR: Moving bed biofilm reactor

MLSS: Mixed liquor suspended sludge

N_2: Nitrogen gas

NH4-N: Ammonia as nitrogen

NO: Nitric oxide

NOB: Nitrite oxidizing bacteria

N_2O: Nitrous oxide

NO2-N: Nitrite as nitrogen

NO3-N: Nitrate as nitrogen

OHO: Ordinary heterotrophic organisms

OP: Ortho-phosphate

PdN: Partial denitrification

PdNA: Partial denitrification/anammox

PID: Proportional-integrative-derivative

PNA: Partial nitritation/anammox

PO: Progressive onset

PO4-P: Phosphate as phosphorus

RCO: Rapid complete onset

SBR: Sequencing batch reactor

sCOD: Soluble chemical oxygen demand

tCOD: Total chemical oxygen demand

TIN: Total inorganic nitrogen

TSS: Total suspended solids

VSS: Volatile suspended solids

WWTP: Wastewater treatment plant

Introduction

Anaerobic ammonia oxidation, or anammox (AMX), is a current emphasis of wastewater treatment research due to its substantial aeration and carbon savings. Despite these benefits, it is still uncommonly employed in full-scale mainstream treatment due to difficult operational requirements. Sidestream applications in the form of partial nitritation-anammox (PNA) are successful, but PNA necessitates nitrite oxidizing bacteria (NOB) outselection, which requires high ammonia concentrations and higher temperatures to achieve. These characteristics are uncommon in mainstream municipal wastewater, making PNA difficult to stably control outside of sidestream processes.

A more promising option for mainstream AMX may be partial denitrification/AMX (PdNA). This process combines the first step of denitrification (reduction of nitrate to nitrite) with AMX metabolism (oxidizing ammonia to nitrogen gas with nitrite as an electron acceptor). PdNA has slightly less carbon and aeration savings than PNA (Zhang, Zhang, and Chen 2019, Le et al. 2019_b), but the benefits are still significant when compared with conventional nitrification/denitrification. It also functions well at the lower ammonia concentrations and temperatures of mainstream municipal wastewater and can be used as a polishing step (Campolong et al. 2019), making it easy to incorporate into existing processes. Most importantly, this technology avoids the key crux of mainstream PNA operation: the need for NOB outselection.

One method of implementing PdNA is through a moving bed biofilm reactor (MBBR) in which a biofilm of AMX and heterotrophs (OHO) is established on plastic carriers with a high specific surface area. MBBRs encourage AMX attachment and retention of a slow-growing microbial community, even at lower temperatures (Gilbert et al. 2014, Gilbert et al. 2015, Guo et al. 2016). Previous pilot scale results have illustrated the possibility for implementing PdNA MBBRs at full scale (Campolong et al. 2019), but it is still unknown how best to start AMX in MBBRs while meeting effluent nitrogen limits. Thus, it is increasingly crucial to understand the methods of startup in order to make AMX technologies more widely available.

Biomass retention is a key facet of PdNA MBBR startup due to the slow growth rate of AMX. Previous startup experiments of PNA MBBRs have used seeding through carriers with a pre-existing biofilm of AMX (Klaus et al. 2016, Tian et al. 2020), or AMX biomass inoculation (Regmi et al. 2015, Tian et al. 2020). These startup methods are not widely applicable for most existing systems because seeding is practically unrealistic for full-scale mainstream processes. It is also potentially unnecessary for attachment and growth of AMX biomass (Kanders et al. 2014). Based on results from previous research, using preliminary biofilm carriers was expected to lead to faster startup by increasing the attachment rate of planktonic AMX present in wastewater (Klaus et al. 2016, Kowalski et al. 2017). Results from previous experiments have shown time scales for startup of PNA MBBRs ranging from 50-120 days. In one experiment, AMX activity onset occurred after 50 days in a sidestream PNA MBBR on carriers with a

preliminary biofilm of nitrifiers, but without AMX biomass inoculation (Kanders et al. 2014). A mainstream PNA MBBR with AMX biomass inoculation and internal sludge recycle resulted in 78 days of startup (Regmi et al. 2015). And a sidestream ANITA™ Mox reactor with 10% AMX carrier inoculation reached startup after 120 days, at which point the MBBR reached an acceptable level of influent nitrogen loading removal (Klaus et al. 2017). Yet none of these experiments demonstrated mainstream PNA startup without AMX biomass seeding

This study was designed to understand mainstream startup of AMX-based MBBRs in two different experiments. The first experiment involved the startup of an AMX MBBR under mainstream nutrient loading and temperature conditions. This was operated in a two-stage PNA system wherein an AMX MBBR was fed ammonia, nitrite, and nitrate with no external carbon and no AMX biomass seeding. The reactor was controlled to 20°C and fed nonlimiting nitrite and ammonia from the start at mainstream stoichiometric conditions for AMX growth. The objectives of this experiment were to

1. Understand the time required for AMX to become established on plastic carriers with a preliminary biofilm (from mainstream nitrifying aerobic IFAS) under stoichiometrically controlled ammonia and nitrite feed conditions.
2. Determine the time required for maximum AMX activity to reach levels needed for typical full-scale PdNA process implementation.
3. Perform this AMX startup experiment in a condition where there is limited opportunity for continuous low-level seeding from a sidestream PNA process.

The second experiment was designed to understand startup time without any AMX biomass seeding across four mainstream PdNA MBBRs dosed with methanol or glycerol and started with either virgin or IFAS carriers with an existing biofilm of OHO and nitrifiers. PdNA can be operated using various supplemental carbon sources, and while glycerol is well known to be an effective carbon source for PdN, methanol is newly discovered to be applicable to PdNA (Campolong et al. 2019, Le et al. 2019_a). Methanol is less expensive than other carbon sources, so understanding the mechanisms of startup under methanol will broaden the applications of PdNA in full-scale treatment. The objectives of this study were to:

1. Compare startup time and performance in mainstream MBBRs with either virgin carriers or aerobic IFAS carriers with a preliminary biofilm.
2. Compare startup time and performance between reactors dosed with glycerol and methanol as carbon sources.

The motivation behind this research was to determine if AMX-based MBBRs could be started up using only the planktonic AMX already present in mainstream wastewater without the need for any AMX biomass inoculation. As well, polishing PdNA MBBR technology has never been used before at full scale, so studying mechanisms in a pilot scale application will inform ideal conditions for startup. Understanding startup time under different carbon sources and carrier types will help determine the ideal choices for reactor operation at full-scale.

Literature Review

1. Anaerobic Ammonium Oxidation

Anaerobic ammonium oxidation, or anammox (AMX), is a biological nutrient removal (BNR) process by which nitrogen can be removed without carbon or aeration inputs. AMX oxidize ammonia using nitrite as the electron acceptor and a small inorganic carbon requirement, with production of AMX biomass, dinitrogen gas, water, and a small amount of nitrate. The AMX metabolic reaction is as follows (Strous et al. 1998):

$$NH_4^+ + 1.32\ NO_2^- + 0.066\ HCO_3^- + 0.13\ H^+ \rightarrow 1.02\ N_2 + 0.26\ NO_3^- + 0.066\ CH_2O_{0.5}N_{0.15} + 2.03\ H_2O$$

This reaction is undergone by bacteria in the Planctomycetales order. These bacteria have a very slow doubling time of 11 days, or a maximum specific growth rate of 0.0027 h^{-1} (Strous et al. 1998). Thus, retention of the slow-growing microorganisms is crucial to successful operation of an AMX-based process.

2. Partial Nitritation/AMX

Partial nitritation/AMX (PNA), otherwise known as deammonification, is a BNR process by which ammonia is removed from wastewater using nitrite produced by AerAOB. In this method, part of the influent ammonia is oxidized to nitrite, which is then used by AMX to oxidize ammonia. Because this process requires no external carbon, PNA translates to 60% aeration and 100% external carbon savings over conventional nitrification/denitrification. The PNA process is shown in Figure 1.

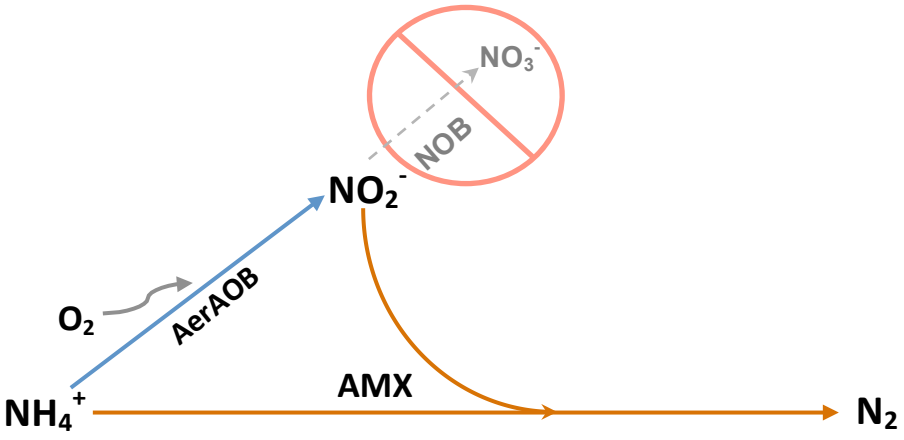

Figure 1: Partial Nitritation/Anammox pathway.

PNA is commonly used in sidestream conditions (treating centrate, etc.) with high free ammonia concentrations, ability to leave an ammonia residual, and high temperatures (Lackner et al. 2014). These conditions result in NOB outselection, which is a requirement for reliable operation of PNA. Though sidestream PNA applications operate stably, PNA does not function well in

mainstream municipal wastewater conditions because NOB outselection is often lost when ammonia concentrations and temperatures fall below those found in sidestream processes. The NOB outselection required of PNA also means that it cannot incorporate removal of the stoichiometric nitrate produced by AMX (Bahtiar et al. 2020). Ultimately, the high ammonia concentrations required of PNA and lack of AMX stoichiometric nitrate removal make it ineffective for mainstream applications.

2a. Process approaches

PNA can be operated in single- or two-stage configurations based on whether partial nitritation and AMX are occurring simultaneously in the same reactor or undergone in separate reactors, respectively. Single-stage sidestream PNA is well proven and relatively easy to implement, while two-stage PNA is less common but still operable. However, single- and two-stage mainstream PNA still remain difficult to implement. Approaches for single-stage mainstream PNA include biofilm or granular processes. Moving bed biofilm reactors (MBBRs) are a common choice for mainstream single-stage biofilm PNA processes (Gilbert et al. 2014, Laureni et al. 2016, Gustavsson, Persson, and Jansen 2014, Gustavsson et al. 2015, Veuillet et al. 2015). MBBRs incorporate plastic or fabric carriers with a high specific surface area for biofilm growth. The carriers are constantly in motion using mixers or aeration, which allows for excess biofilm sloughing and interaction between biofilm and substrate (Ødegaard 1999). The biofilms present in these systems are ideal for AMX by providing resilience to changes due to its presence at the interior of the biofilm (Gilbert et al. 2015) and because of their ability to increase AMX retention and promote the growth of a specialized microbial community (Guo et al. 2016). This allows AMX to survive process changes in concentration and temperature without long-term inhibition. Single-stage PNA can also be operated in granular processes (Lotti et al. 2014, Gao et al. 2015, Winkler, Kleerebezem, and van Loosdrecht 2012, Morales et al. 2016). While biofilm processes are fully attached, granular processes require careful retention of AMX biomass due to its slow growth rate. This is accomplished through the settling of biomass with sequencing batch reactors (SBRs) or clarifiers before decanting, or through screens at reactor effluent points which can be used for granular sludge processes.

Options for two-stage mainstream PNA include a hybrid of granular and suspended growth (Wett et al. 2015, Han et al. 2016, Cao et al. 2013), or a two-phase process in which partial nitritation occurs first in a suspended growth reactor and is then fed to an AMX-based reactor such as an MBBR (Regmi et al. 2015, Ma et al. 2011, Piculell et al. 2016). Hybrid systems have OHO and nitrifiers in suspended growth and AMX in granules within the same reactor. Like granular processes, hybrid systems require careful AMX retention through screens or hydrocyclones with recycling to prevent biomass loss. The two-phase system does not require such recycling due to the presence of AMX on

biofilms, but the partially nitritated feed must be carefully controlled for NOB outselection to provide nitrite for AMX.

3. Partial Denitrification/AMX

Though mainstream PNA may not be easily operable, partial denitrification/AMX (PdNA) has been shown to operate stably in mainstream conditions. To facilitate this process, nitrite must first be produced from ammonia, which requires initial aeration inputs to partially oxidize ammonia by aerobic AOB (AerAOB) to nitrite. Nitrite is then oxidized to nitrate by NOB, and organic carbon addition allows for partial denitrification (PdN) by heterotrophs (OHO). However, because the AMX pathway requires no additional organic carbon and undergoes the remainder of the ammonia oxidation anoxically, the PdNA process ultimately produces 50% aeration savings and 80% external carbon savings over conventional nitrification/denitrification (Strous et al. 1998, Le et al. 2019$_b$, Zhang, Zhang, and Chen 2019). The complete PdNA process is shown in Figure 2.

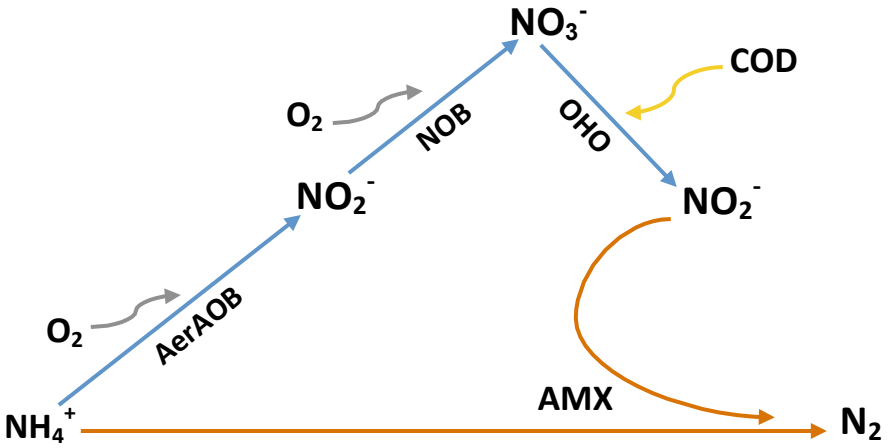

Figure 2: Partial Denitrification/Anammox pathway.

While PNA is theoretically more beneficial in terms of its higher aeration and carbon savings, PdNA still has comparable savings and may be more practical for mainstream applications in municipal wastewater. The main benefit of PdNA is that it doesn't require NOB outselection, instead allowing for the nitrite produced by AerAOB to be oxidized to nitrate. Nitrate is then reduced back to nitrite by OHO with only a small amount of additional carbon required. Though these additional steps increase aeration and carbon demand slightly, they also make mainstream operation much more stable. The lack of NOB outselection also means that PdNA can operate with low ammonia and temperature conditions common of mainstream wastewater, making it a good option as a polishing step and increasing its capability for implementation into existing processes. Also, since AMX remove ammonia anoxically, less ammonia needs to be removed upstream through aeration if PdNA is used as a polishing step.

If desired, PNA and PdNA could even be operated in the same process. For instance, in times where nitrite accumulation occurs in the upstream process, carbon can be withheld to a PdNA reactor and the system can flex to operate as a PNA process. The system can then transition back to PdNA if carbon addition is reinitiated.

3a. Partial Denitrification

Partial denitrification (PdN), otherwise known as denitratation, is the first part of the two-stage denitrification process by which nitrate is reduced to nitrite. This reaction is undergone by OHO using an electron source such as organic carbon. The partial denitrification reaction is as follows:

$$NO_3^- + 2H^+ + 2\ e^- \rightarrow NO_2^- + H_2O$$

Full denitrification (FdN) occurs when OHO continue to reduce nitrite to dinitrogen gas with nitric oxide (NO) and nitrous oxide (N_2O) as intermediates. By halting FdN before nitrite is reduced to nitrogen gas, in situ nitrite accumulation can occur. This can be encouraged if electron source (often supplemental carbon dosing) conditions are limiting enough so that OHO stop reduction after the first step of denitrification.

3b. Process Approaches

While PNA can be operated in mainstream or sidestream conditions, PdNA is rarely operated outside of mainstream. While PdNA can technically be operated in sidestream (Sharp et al. 2017), sidestream PNA would almost always be more favorable due to the lack of a carbon requirement for operation. Like PNA, mainstream PdNA processes can be operated in either single- or two-stage configurations. In a two-stage process, PdN and AMX reactions occur separately with the partially denitrified feed sent to a subsequent AMX process (Cao et al. 2019). One potential benefit of two-stage PdNA is the separation of AMX biomass, which removes competition for nitrite by other microorganisms (Bahtiar et al. 2020, Cao et al. 2017). However, this is not necessarily a benefit as some research in single-stage systems has shown PdN efficiencies unaffected without employing steps to limit nitrite competition (Le et al. 2019_b). In a two-stage process, the AMX reactor can also be controlled at a higher temperature to encourage faster growth of AMX if desired. However, separating the processes is not necessary for proper functioning of PdNA and can lead to higher space and cost requirements associated with operating multiple reactors, so single-stage is a more common choice. As well, for every part ammonium oxidized, AMX produces 0.26 parts of nitrate. This additional nitrate can be incorporated back into the PdN process and reduced to nitrite by OHO in a single-stage PdNA reactor, but cannot be accounted for in a two-stage process where PdN and AMX reactions are isolated from one another.

In a single-stage PdNA process, PdN and AMX reactions occur in the same reactor wherein in situ nitrite accumulation by OHO drives ammonia oxidation by AMX. Single-stage PdNA is more commonly employed in polishing processes, which occur at the end of a BNR process and are characterized by low nitrogen loading. Single-stage PdNA processes are ideally fed with equal parts of ammonia and nitrate to meet AMX stoichiometry after partial denitrification. Common approaches for polishing PdNA include biofilm-based systems such as MBBRs or filters. PdNA MBBRs can be operated anoxically with a biofilm of OHO, nitrifiers, and AMX existing together on the same carriers (Campolong et al. 2019), while filters incorporate OHO, nitrifiers, and AMX biomass growing in media such as sand or activated carbon (Klaus et al. 2020, Fofana et al. 2020, Cui et al 2020). Two-stage polishing systems have also been operated with a hybrid of suspended growth and granular AMX with bioaugmentation from a sidestream process and screened effluent for AMX retention (Le et al. 2019_b). In contrast to polishing processes, integrated processes occur upstream and receive higher nitrogen loading (Forney et al. 2020). These can operate as hybrid systems, such as with suspended growth and IFAS media, and are operated with anoxic zones or periods for AMX activity to occur.

Though PdNA is not yet commonly utilized in full-scale mainstream processes, there is one such application in a deep-bed filter at the Hampton Roads Sanitation District (HRSD) York River Treatment Plant that has been operating since 2018. This is the first application of full-scale mainstream AMX and is the first instance of full-scale PdNA (Klaus et al. 2020).

3c. Carbon Sources for PdNA

In PdN processes, organic carbon sources provide an electron source for heterotrophs to reduce nitrate to nitrite. The uses of methanol, glycerol, acetate, glucose, ethanol, and propionate in denitrification have all been shown to produce nitrite accumulation (van Rijn et al. 1996, Le et al. 2019_a, Bill et al. 2009). Glycerol is well known to be an effective carbon source for PdN, but methanol is newly discovered to be applicable to PdNA (Le et al. 2019_a, Campolong et al. 2019). Methanol has been shown to be inhibitory to AMX in batch experiments on enriched cultures (Isaka et al. 2008, Güven et al. 2005). However, in a pilot scale PdNA MBBR, AMX activity continued when methanol was overdosed, proving that methanol may not be inhibitory to AMX as previously thought (Campolong et al. 2019). This could potentially be due to the presence of AMX at the interior of biofilms in an MBBR process where it is not in contact with methanol at an inhibitory level.

PdN efficiency is a key metric of PdNA operation, representing the percentage of nitrate converted to nitrite. The remainder of the nitrate not accounted for by PdN is presumably removed through FdN, which is undesirable for PdNA. Glycerol and acetate have shown

capabilities for higher PdN efficiencies when compared to methanol (Le et al. 2019a, Campolong et al. 2019). Methanol's lower PdN efficiency could be partially attributed to the utilization of methanol by specialized methylotrophs as opposed to a more general population of OHO that can utilize a carbon source like glycerol (Akunna, Bizeau, and Moletta 1993, Sperl and Hoare 1971). However, when considering methanol's lower bulk cost and lower yield compared to carbon sources like glycerol, it can have a lower cost for PdN (Campolong et al. 2019). While methanol is less expensive than other external carbon sources like acetate or glycerol, it is also dangerous due to its high flammability and requires careful storage and handling.

3d. Controlling for PdN

One way to encourage PdN is by maintaining a low carbon to nitrogen (C/N) ratio (Le et al. 2019_a). This is achieved by dosing carbon at limiting conditions that prevent FdN from occurring. However, this is only possible if upstream carbon diversion is utilized to limit the C/N ratio in wastewater supplied to a PdN process. For instance, high-rate activated sludge (HRAS) processes can be utilized to provide low C/N wastewater for PdN (Ma et al. 2017, Campolong et al. 2019).

Providing a low C/N ratio has previously been used to control for PdN, but recent studies have demonstrated the ability for a nitrate residual to select PdN over FdN (Le at al. 2019_b, Campolong et al. 2019). Controlling PdN with a nitrate residual ensures nitrite accumulates while leaving low levels of nitrate in the reactor effluent. For instance, Le et al. (2019_b) reported a nitrate effluent setpoint of 2-3 mgNO3-N/L to limit nitrite reduction while Campolong et al. (2019) showed 1 mgNO3-N/L is possible.

Using a nitrate residual to select for PdN works because maintaining nitrate concentrations above limiting conditions can prevent the second step of denitrification (reduction of nitrite to dinitrogen gas). For instance, Le et al. (2019_b) showed that nitrite accumulated until nitrate reached limiting conditions, after which a switch to FdN occurred. This switch occurred at two markedly different C/N ratios, indicating that a low C/N ratio is not the only method of controlling for PdN. As well, nitrite reduction is reversible due to the dependency of nitrite reduction on nitrate concentrations; for instance, even if nitrate is depleted to the point that nitrite reduction does occur, subsequently adding more nitrate can halt nitrite reduction until the nitrate is once again depleted (van Rijn et al. 1996). This illustrates that nitrate concentration is an important factor to limiting nitrite reduction, and the mechanism for PdN selection is not based solely on carbon limitation.

3e. Electron Transport in PdN

The prevailing theory behind PdN is that electrons are preferentially transferred to nitrate reductase on the electron transport chain because of its upstream position (Almeida et al. 1995, van Rijn et al. 1996, Baideme 2019). The reduction rate of both nitrate and nitrite depends on nitrate concentration (Almeida et al. 1995, Betlach and Tiedje 1981), so maintaining nitrate at nonlimiting conditions can encourage nitrite accumulation because electrons aren't transferred to nitrite reductase. It is also theorized that different carbon sources have different electron transport routes, which can limit their ability to produce nitrite accumulation (van Rijn et al. 1996). For instance, some carbon sources including acetate and propionate lead to nitrite accumulation due to the differing rates of nitrate and nitrite reduction and competition between nitrite and nitrate reductases. Acetate is also theorized to donate electrons upstream and closer to nitrate reductase, meaning that electrons are utilized by nitrate reductase first. However, some other carbon sources such as butyrate don't lead to nitrite accumulation at all possibly due to the donation of electrons downstream and closer to nitrite reductase (van Rijn et al 1996). Methanol may also have donation of electrons downstream and closer to nitrite reductase, but it still has been proven to produce nitrite accumulation (Van Verseveld and Stouthamer 1978, Le et al. 2019_a, Campolong et al. 2019).

Another potential factor in the amount of nitrite accumulation in PdN is the different pathways utilized for denitrification based on the enzyme onset methods possessed by denitrifiers. Three different pathways are theorized to exist, and each one has differing capabilities for nitrite accumulation (Martienssen and Schöps 1999, Liu et al. 2013, Ma et al. 2020). The first method is through truncated denitrification, in which denitrifiers are only capable of reducing nitrate to nitrite. This is favorable for PdN as only the first step of denitrification occurs and nitrite can accumulate. The second method is rapid complete onset of denitrification (RCO), in which nitrate and nitrite reductase enzymes operate simultaneously and completely denitrify with no nitrite accumulation occurring. Lastly, organisms can undergo progressive onset (PO) of denitrification enzymes; in this instance, organisms are capable of full denitrification, but nitrate reduction occurs first. Then, only after nitrate runs out, nitrite reduction occurs. This pathway leads to nitrite accumulation if a nitrate residual is maintained. Ultimately, of the three enzyme onset methods, truncated and PO denitrifiers are the most favorable types for PdN.

4. Methods of Increasing AMX Attachment Rate

Previous pilot scale results have illustrated the possibility for implementing mainstream PdNA MBBRs at full scale. However, it is still unknown how best to start AMX in mainstream MBBRs without AMX biomass inoculation. In previous experiments, sidestream PNA reactors were started up through the aid of carriers with a pre-existing AMX biofilm or through seeding with AMX biomass (Regmi et al. 2015, Klaus et al. 2017, Tian et al. 2020), which is practically

unrealistic for startup in full-scale systems. However, Kanders et al. (2014) illustrated the possibility of starting up lab-scale sidestream PNA MBBRs with virgin carriers and no AMX biomass seeding whatsoever. They showed that seeding may not be necessary for AMX attachment and growth in an MBBR, but that it may be helpful for a more immediate onset of nitrogen removal (Kanders et al. 2014).

Due to the slow growth rate of AMX, the main limiting factor for startup is the time requirement for growing AMX in a carrier biofilm. Retention is also a hurdle to the startup process as the low yield of AMX at mainstream wastewater temperatures can lead to washout (Hoekstra et al. 2017). The use of carriers with a preliminary biofilm of OHO and nitrifiers has been shown to increase AMX attachment rate in the startup of MBBR processes (Klaus et al. 2016, Kowalski et al. 2017, Tian et al. 2020). For instance, in a sidestream ANITA™ Mox process, the use of carriers with a preliminary biofilm from an aerobic IFAS system resulted in faster AMX attachment when compared with surface modifications such as Fenton's reagent, potassium permanganate, and ozone (Klaus et al. 2016). In SBRs that were either seeded with 10% carriers with a pre-existing biofilm of AMX or dosed with biomass of AMX originating from a biofilm, Tian et al. (2020) discovered that the biofilm-originating AMX biomass led to faster AMX attachment than pre-existing AMX carriers. They also found that carriers with a preliminary biofilm of denitrifiers led to faster AMX attachment than virgin carriers. However, all of those startup instances were conducted with seeding and in sidestream conditions, so it is still unknown if this strategy will succeed in a mainstream reactor with no seeding.

5. Nitrous Oxide Production in PNA/PdNA

As OHO denitrify, they have the possibility of forming intermediate products such as N_2O. N_2O is a potent and persistent greenhouse gas with a global warming potential 300 times higher than carbon dioxide (Montzka et al. 2011), so limiting emissions from wastewater treatment is important. The order of intermediates produced during denitrification is shown in Figure 3.

Figure 3: Order of intermediates produced in the denitrification pathway.

Understanding the mechanisms of N_2O production in wastewater is crucial for controlling emissions. In denitrifying biofilm processes, electron donor limitations (such as a low C/N ratio) can increase N_2O production (Sabba et al. 2018). Thus, PdN occurring in MBBRs is of concern for N_2O accumulation due to nitrite accumulation and electron donor limitations associated with halting full denitrification. However, providing a consistent bulk environment for biofilm interaction, such as in an MBBR operated as a continuous stirred tank reactor (CSTR), can avoid high N_2O emissions (Sabba et al. 2018). For example, one study showed a reduction in N_2O emissions when a nitrification/denitrification SBR was replaced with a PNA MBBR process

(Kanders 2019). Ultimately, microbial composition, biofilm thickness, substrate concentrations, and reactor operation can all impact N_2O emissions (Sabba et al. 2018).

N_2O production is closely related to nitrite reduction and doesn't typically occur until nitrate is fully depleted (Du et al. 2016). As well, in PdN processes with high nitrite accumulation, a high C/N ratio is also related to increased N_2O production (Du et al. 2016). These situations would likely be avoided in a PdN process that employs a nitrate residual carbon control method, as nitrate is never entirely depleted and a low C/N ratio is typically produced to avoid full denitrification. However, the external carbon source choice also has the potential to effect N_2O emissions (Adouani et al. 2010, Hu et al. 2012, Song et al. 2015), but other process operational factors including nitrite accumulation can account for more of an impact on N_2O emissions than the choice of carbon source (Song et al. 2015).

Research on sidestream PNA MBBRs has shown higher N_2O production at low dissolved oxygen and high nitrite concentrations (Ma et al. 2017), and an increase in N_2O production with a higher nitrogen load (Yang et al. 2013). The impacts of a high nitrogen load would not necessarily be of consequence for a polishing process where nitrogen load is expected to be low, but low oxygen concentrations are of concern for N_2O emissions from an anoxic MBBR process. PNA in biofilm processes can also allow for the utilization of AMX as a nitrite sink, potentially decreasing the possibility of N_2O production (Sabba et al. 2018). N_2O production can also be lower in single-stage PNA processes compared to two-stage (de Clippeleir et al. 2013), potentially due to the lack of immediate nitrite consumption by AMX in a two-stage process.

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Manuscript 1: Startup Strategies for Mainstream Anammox Polishing in a Moving Bed Biofilm Reactor (MBBR)

Abstract

Anaerobic ammonia oxidation, or anammox (AMX), is a current emphasis of wastewater treatment research due to its substantial aeration and carbon savings. Yet despite these benefits, the use of AMX-based technologies in mainstream wastewater treatment remains limited. One of the main barriers to implementation is the slow growth rate of AMX, which results in long startup times. Consequently, the typical approach to commissioning an AMX-based process, specifically used for sidestream partial nitritation/AMX, is with biomass augmentation. This approach is practically unrealistic for full-scale mainstream applications, so this study evaluated startup strategies for mainstream AMX in a moving bed biofilm reactor (MBBR) without AMX biomass inoculation. A pilot-scale MBBR was started using carriers from a mainstream aerobic integrated fixed-film activated sludge (IFAS) process with a preliminary biofilm. The feed was controlled to 20°C and included mainstream-relevant concentrations of nitrite and ammonia controlled to meet the stoichiometric requirements for AMX growth. No external carbon or AMX biomass seeding was utilized. After only 84 days of operation, AMX activity was confirmed in the reactor with evidence of activity a few weeks before testing. These results, along with other recent work, suggest that growth of AMX on biofilm carriers can be established in mainstream conditions within 50-100 days, depending on media selection. The aim of this research is to help utilities understand methods for starting up mainstream AMX MBBRs without the barrier of AMX biomass seeding.

Introduction

Anaerobic ammonia oxidation, or anammox (AMX), is a current emphasis of wastewater treatment research due to its substantial aeration and carbon savings. Sidestream applications in the form of partial nitritation-anammox (PNA) are successful, but PNA necessitates nitrite oxidizing bacteria (NOB) outselection, which requires high ammonia concentrations and temperatures to achieve. These characteristics are uncommon in mainstream municipal wastewater, making PNA difficult to stably control outside of sidestream processes (Cao et al. 2017).

PNA can be operated in single- or two-stage configurations, in which partial nitritation and AMX are occurring simultaneously in the same reactor or in separate reactors, respectively. Approaches for single-stage mainstream PNA include biofilm or granular processes. Moving bed biofilm reactors (MBBRs), incorporating plastic or fabric carriers with a high specific surface area for biofilm growth, are a common choice for mainstream single-stage biofilm PNA

processes (Gilbert et al. 2014, Laureni et al. 2016, Gustavsson, Persson, and Jansen 2014, Gustavsson et al. 2015, Veuillet et al. 2015). The carriers are constantly in motion using mixers or aeration, which allows for excess biofilm sloughing and interaction between biofilm and substrate (Ødegaard 1999). The biofilms present in these systems are ideal for AMX by providing resilience to changes due to its presence at the interior of the biofilm (Gilbert et al. 2015) and because of their ability to increase AMX retention and promote the growth of a specialized microbial community (Guo et al. 2016). This allows AMX to survive process changes in concentration and temperature without long-term inhibition. Single-stage PNA can also be operated in granular processes (Lotti et al. 2014, Gao et al. 2015, Winkler, Kleerebezem, and van Loosdrecht 2012, Morales et al. 2016). While biofilm processes are fully attached, granular processes require careful retention of AMX biomass due to its slow growth rate. This is accomplished through the settling of biomass with sequencing batch reactors (SBRs) or clarifiers before decanting, or through screens at reactor effluent points which can be used for granular sludge processes (Han et al. 2016).

Mainstream PNA can also be operated in a two-stage configuration. Options for two-stage PNA include a hybrid of granular and suspended growth (Wett et al. 2015, Han et al. 2016, Cao et al. 2013), or a two-phase process in which partial nitritation occurs first in a suspended growth reactor and is then fed to an AMX reactor such as an MBBR (Regmi et al. 2015, Ma et al. 2011, Piculell et al. 2016). Hybrid systems have nitrifiers in suspended growth and AMX in granules within the same reactor, and like granular processes, hybrid systems require careful AMX retention through screens or hydrocyclones with recycling to prevent biomass loss. The two-phase system does not require such recycling due to the presence of AMX on biofilms, but the partially nitritated feed must be carefully controlled for NOB outselection to provide nitrite for AMX.

A more promising option for mainstream AMX may be partial denitrification/AMX (PdNA). This process combines the first step of denitrification (reduction of nitrate to nitrite) with AMX metabolism (oxidizing ammonia to nitrogen gas with nitrite as an electron acceptor). PdNA has slightly less carbon and aeration savings than PNA (Zhang, Zhang, and Chen 2019, Le et al. 2019_b), but the benefits are still significant when compared with conventional nitrification/denitrification (Strous et al. 1998). Most importantly, this technology avoids the key crux of mainstream PNA operation: the need for NOB outselection. Because of this, PdNA functions well at the lower ammonia concentrations and temperatures of mainstream municipal wastewater and can be used as a polishing step, making it easy to incorporate into existing processes (Campolong et al. 2019). While attempts at full-scale mainstream PNA have not been successful, pilot scale research on mainstream PdNA has been promising. However, research has not yet addressed how to start up a full-scale mainstream PdNA or PNA MBBR process without AMX biomass seeding, so it is crucial to understand the methods of startup in order to make AMX-based technologies more widely available.

Biomass retention is a key facet of AMX MBBR startup due to the slow growth rate of AMX. Previous startup experiments of PNA MBBRs have used seeding through carriers with a pre-existing biofilm of AMX (Klaus et al. 2016, Tian et al. 2020), or AMX biomass inoculation (Regmi et al. 2015, Tian et al. 2020). These startup methods are not widely applicable for most existing systems because seeding would be expensive or even entirely impossible (due to lack of AMX seed availability) to employ for full-scale mainstream processes. It is also potentially unnecessary for attachment and growth of AMX (Kanders et al. 2014).

Results from previous experiments have shown time scales for startup of PNA MBBRs ranging from 50-120 days. In one experiment, AMX activity onset occurred after 50 days in a sidestream PNA MBBR on carriers with a preliminary biofilm of nitrifiers, but without AMX biomass inoculation (Kanders et al. 2014). In another experiment, a mainstream PNA MBBR with AMX biomass inoculation and internal sludge recycle demonstrated 78 days of startup (Regmi et al. 2015). Furthermore, a sidestream ANITA™ Mox reactor with 10% AMX carrier inoculation reached startup after 120 days, at which point the MBBR reached an acceptable level of influent nitrogen loading removal (Klaus et al. 2017). Yet none of these experiments demonstrated mainstream PNA startup without AMX biomass seeding, so the objectives of this study were to determine startup time under such conditions. Because AMX was not seeded to the reactors in this study, increasing the rate of attachment was key for decreasing startup time. Based on results from previous research, the use of preliminary biofilm carriers from aerobic IFAS was expected to increase the attachment rate of planktonic AMX present in wastewater (Klaus et al. 2016, Kowalski et al. 2017). The objectives of this experiment were to

1. Understand the time required for AMX to become established on plastic carriers with a preliminary biofilm (from mainstream nitrifying aerobic IFAS) under stoichiometrically controlled ammonia and nitrite feed conditions.
2. Determine the time required for maximum AMX activity to reach levels needed for typical full-scale PdNA process implementation.
3. Perform this AMX startup experiment in a condition where there is limited opportunity for continuous low-level seeding from a sidestream PNA process.

The motivation behind this research was to determine if mainstream AMX-based MBBRs could be started up using only the planktonic AMX already present in wastewater without the need for any AMX biomass inoculation. Based on an extensive literature review, this has research has not previously been conducted. As well, mainstream AMX has never been used before at full scale in MBBRs, so understanding mechanisms in a pilot scale application will inform ideal conditions for startup.

Materials and Methods

Reactor Operation and Nutrient Feed

A 340L polishing MBBR was started at the Hampton Roads Sanitation District (HRSD) Chesapeake Elizabeth (CE) adsorption/bio-oxidation (A/B) pilot (Figure 4). The pilot operated with carbon diversion in the A-Stage high rate activated sludge (HRAS) process before a B-Stage biological nutrient removal (BNR) process. The B-Stage was designed for both nitrogen removal and biological phosphorus removal and concluded with a secondary clarifier that fed into the polishing MBBR.

The MBBR was started with mature aerobic IFAS carriers from HRSD's James River Treatment Plant (JRTP). This plant had a sidestream ANITA™ Mox process that recycled to a point before the IFAS process. Carriers were supplied to the CE Pilot MBBR at a fill percentage of 55%, and the reactor was fed B-Stage secondary clarifier effluent at a hydraulic residence time (HRT) of 2 hrs. The B-stage BNR process preceding the secondary clarifier was operated under ammonia versus NO_x (AvN) intermittent aeration control where NO_x equaled nitrite plus nitrate. In this process, aeration was cycled on and off to meet a 1:1 ratio of ammonia and NO_x based on feedback from sensors in the fourth continuously stirred tank reactor (CSTR) of the process. The B-Stage process was temperature controlled to 20°C and pH was controlled to 6.8. Temperature and pH were monitored daily with a handheld probe (Hach, Loveland, Colorado).

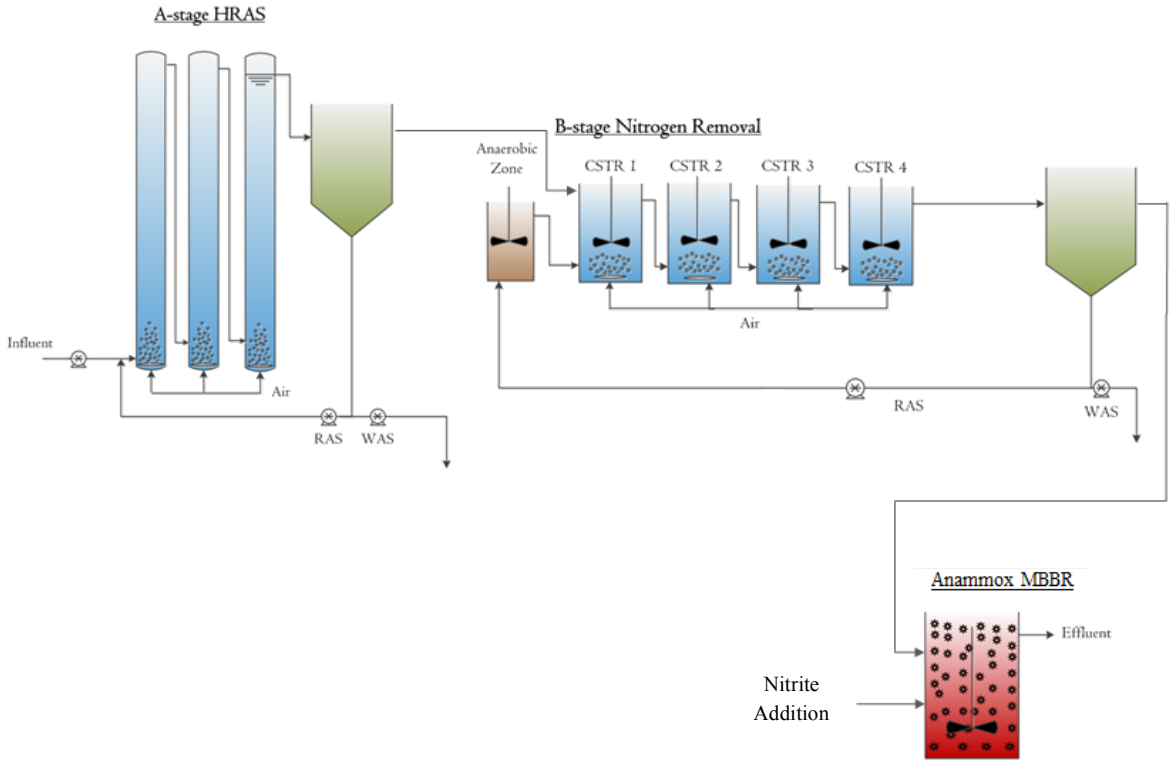

Figure 4: CE Pilot process flow diagram.

The feed from the B-Stage process included ammonia, nitrite, and nitrate, making this a two-stage PNA process with partial nitritation occurring before a separate reactor with AMX biomass. Sodium nitrite was supplemented to the reactor as necessary, and there was no external carbon dosed during startup.

Mixing and Oxygen Transfer Protection

Mixing was applied with a dual-blade vertical shaft mixer at a speed of 45 rpm (Caframo, Ontario, Canada). The reactor was covered with polystyrene to prevent oxygen transfer and generate anoxic conditions.

Nutrient, Total and Volatile Suspended Solids Sampling

Reactor performance was evaluated daily with 0.45 μm-filtered 24 hr composite samples of the influent and effluent NH4-N, NO3-N, NO2-N, PO4-P, and total and soluble chemical oxygen demand (sCOD). Total chemical oxygen demand (tCOD) was also evaluated on unfiltered samples blended for 30 seconds. All nutrients were measured using Hach TNT kits (Hach, Loveland, Colorado) and read with a Hach DR-2800 spectrophotometer. Sulfamic acid was added to samples evaluated for NO3-N and sCOD to eliminate nitrite interference when nitrite was over 1.5 mgNO2-N/L. In such cases, 50 mg of sulfamic acid was added to 5 mL of sample, shaken to dissolve, and left for 10 minutes before being evaluated. Total suspended solids (TSS) and volatile suspended solids (VSS) were measured daily in influent and effluent composite samples following standard methods.

Activity Testing

Activity tests were run by dosing nonlimiting ammonia (ammonium chloride) at 20 mgNH4-N/L and nitrite (sodium nitrite) at 25 mgNO2-N/L after isolating the reactor for one hour prior to testing. Nutrients were evaluated by measuring NH4-N, NO3-N, NO2-N, PO4-P, and sCOD with Hach tubes in 0.45 μm filtered grab samples of reactors at intervals of 15 minutes for a total of 1-2 hours. Sulfamic acid was added to samples evaluated for NO3-N and sCOD to eliminate nitrite interference when nitrite was over 1.5 mgNO2-N/L. In such cases, 50 mg of sulfamic acid was added to 5 mL of sample, shaken to dissolve, and left for 10 minutes before nutrient samples were evaluated for nitrite. DO (Insite, Slidell, Louisiana), pH, and temperature were evaluated with handheld probes throughout batch experiments.

Total Solids Measurements

Total solids measurements were taken fortnightly by sampling 3 carriers from each reactor. The carriers were dried in an oven at 110°C overnight and weighed. The dried carriers were then placed in a 25 g/L solution of disodium EDTA (ethylenediaminetetraacetic acid) and shaken vigorously. Each carrier was scraped with a plastic brush under running water until all biomass was removed, and then carriers were dried again overnight at 110°C. The final carrier weights

were subtracted from the initial weights to determine the biomass total solids based on the known surface area of each carrier.

Results and Discussion

Influent and Effluent Characteristics and Nitrogen Removals

Ammonia and NO_x provided by the B-Stage effluent increased during the research period (Figure 5). However, the nitrite component of NO_x briefly decreased due to a loss in NOB outselection in the B-Stage process. Thus, after 57 days of operation, the MBBR was supplemented with additional nitrite dosed directly to the reactor based on expected requirements for ammonia removal based on AMX stoichiometry (Strous et al. 1998). The influent composition in Figure 5 includes the additional synthetic nitrite dosing.

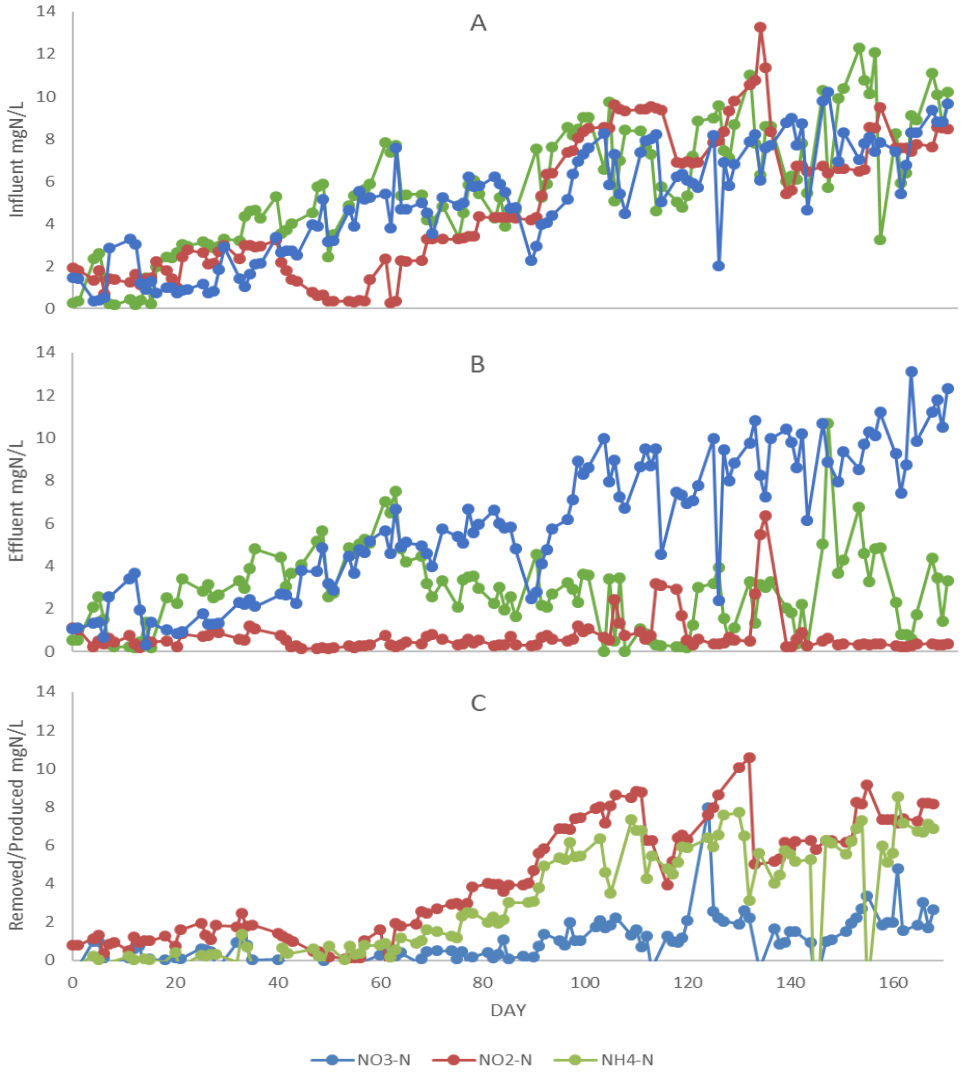

Figure 5: A: Influent nitrogen species, B: Effluent nitrogen species, and C: nitrogen production (nitrate) and removal (ammonia and nitrite).

Influent ammonia was as high as 12.3 mgNH4-L with an average of 5.76 ± 2.87 mgNH4-N/L, while nitrite was as high as 13.3 mgNO2-N/L with an average of 4.90 ± 3.24 mgNO2-N/L. Average effluent ammonia was 2.73 ± 1.69 mgNH4-N/L, nitrite was 0.70 ± 0.90 mgNO2-N/L, and nitrate was 6.04 ± 3.22 mgNO3-N/L.

Average influent OP was 0.37 ± 0.26 mgPO4-P/L, tCOD was 67.3 ± 21.4 mg/L, and sCOD was 31.7 ± 6.1 mg/L. Average influent TSS was 25.7 ± 11.8 mg/L while VSS was 21.2 ± 8.8 mg/L. Temperature of the MBBR throughout operation was 20.7 ± 1.0°C and pH was 6.95 ± 0.15.

On day 84, AMX activity was confirmed when a batch test showed simultaneous ammonia and nitrite removal with nitrate production. However, examination of ammonia removal in the reactor over time indicates that this activity likely began between days 60-70, which was several weeks before the first AMX activity test. Average ammonia removal throughout operation was 3.11 ± 2.73 mgNH4-N/L, with an average of 5.59 ± 1.35 mgNH4-N/L after AMX activity confirmation on day 84. Average nitrite removal was 4.26 ± 2.99 mgNO2-N/L, with an average of 6.84 ± 1.50 mgNO2-N/L after AMX activity confirmation. Average nitrate production was 0.89 ± 1.23 mgNO3-N/L, with an average of 1.59 ± 1.31 mgNO3-N/L after AMX activity confirmation. Ammonia and nitrite removal percentages also increased with increased loading (Figure 6).

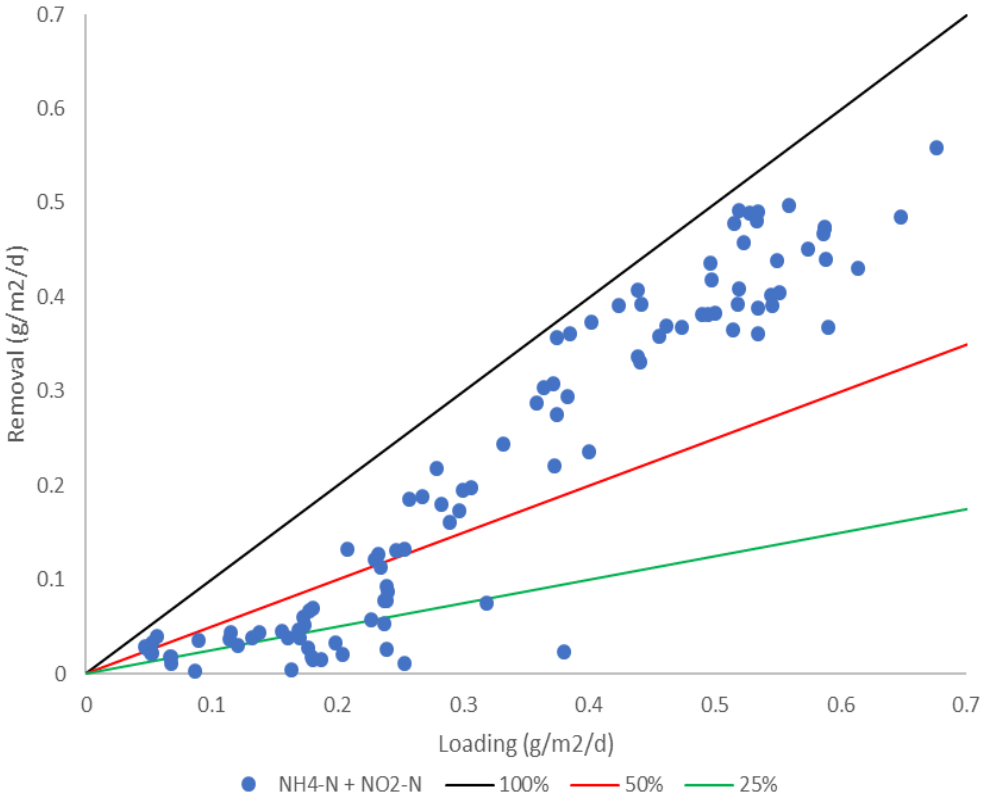

Figure 6: Ammonia and nitrite loading versus removal throughout operation.

After AMX activity was confirmed, the ratio of nitrite removal to ammonia removal was 1.29 ± 0.38 g/g, which was close to the expected value of 1.32 g/g based on AMX stoichiometry (Strous et al. 1998). The ratio of nitrate produced to ammonia removed after AMX activity confirmation, 0.28 ± 0.20 g/g, was also close to the expected stoichiometric value of 0.26 g/g.

Total Solids Measurements

Total solids initially decreased from the IFAS carriers used to start the reactor. The initial IFAS media had a total solids measurement of 7.82 g/m^2 and after the decrease between the first and second measurements, total solids remained relatively stable with an average of 4.75 ± 0.22 g/m^2. The initial decrease in carrier biomass observed in the mainstream AMX MBBR may have been due to sloughing of dead and inactive OHO and nitrifiers after the carriers were moved from an aerobic IFAS system to a fully anoxic MBBR. This was in contrast with biomass measurements in a previous experiment using preliminary biofilm carriers to increase AMX attachment rate in a sidestream ANITA™ Mox process. That experiment showed an increase in carrier biomass over initial IFAS biomass at the end of the experiment, but a decrease in carrier biomass throughout operation (Klaus et al. 2016).

AMX Activity

An AMX activity test was conducted after 84 days of operation. This experiment confirmed AMX activity due to the removal of both ammonia and nitrite with the simultaneous production of nitrate. An example of an AMX activity test is shown in Figure 7.

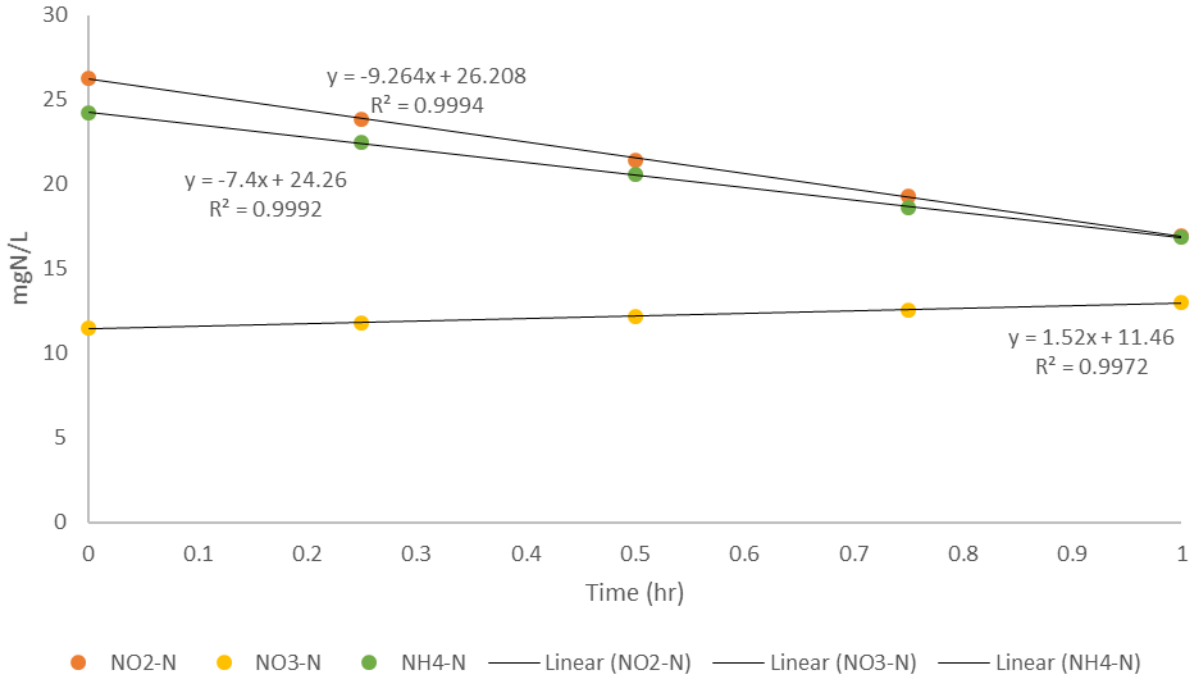

Figure 7: Example AMX activity test on day 167.

After the initial test confirming activity, AMX activity tests were consistently run throughout startup (Figure 8). The maximum TIN and NH4-N removal rates from this experiment were compared with rates from a mature PdNA MBBR previously operated at the CE Pilot as a benchmark to determine when startup in this experiment would be considered complete (Campolong et al. 2019). The maximum ammonia and TIN rates reached the target startup values of 0.7 gNH4-N/m^{2}d and 1.2 gN/m^{2}d after 167 days of operation.

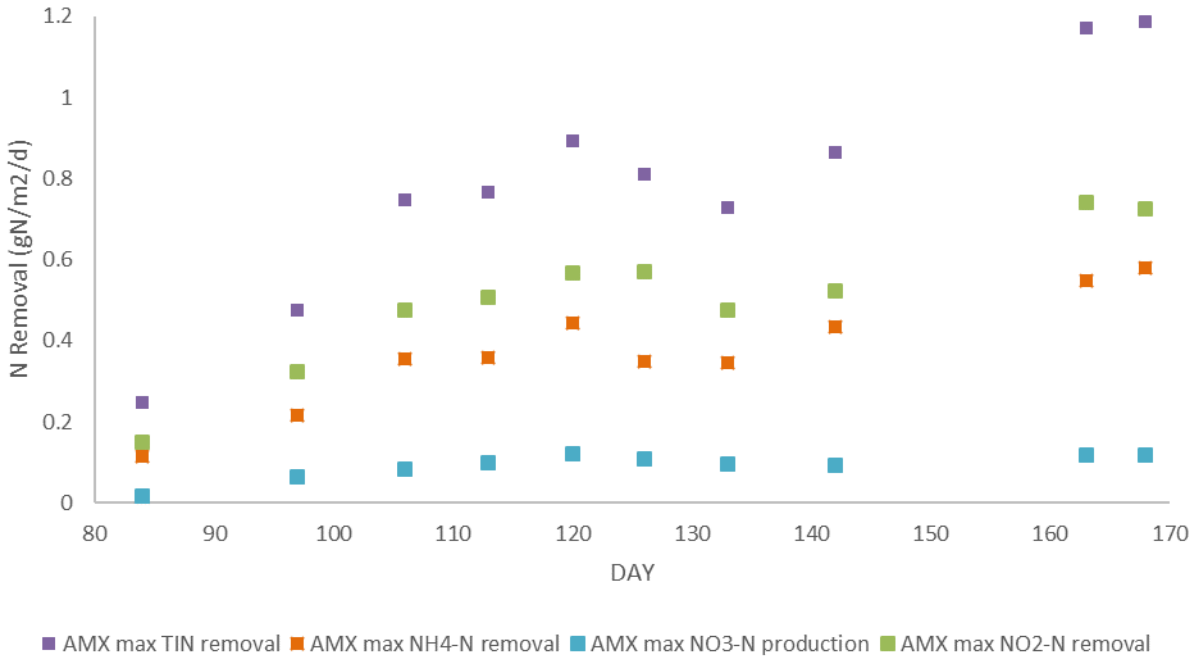

Figure 8: Maximum TIN, ammonia, and nitrite removal rates and nitrate production rates throughout operation.

The performance of this reactor indicates that fast startup of AMX in a mainstream MBBR with no additional AMX biomass seeding is possible. In other experiments involving mainstream and sidestream PNA processes, AMX was inoculated through biomass or carrier seeding (Regmi et al. 2015, Klaus et al. 2017, Kanders et al. 2014), so it was unknown how long it would take for AMX activity to begin in a mainstream reactor without any AMX biomass seeding. For instance, Regmi et al. (2015) operated a startup phase under mainstream conditions in an MBBR for 78 days in which AMX biomass was inoculated and with temporary biomass recycle. Klaus et al. (2017) observed startup within 120 days in a sidestream ANITA™ Mox MBBR process with AMX biomass inoculation, at which point the reactor reached acceptable removal of influent nitrogen loading. Kanders et al. (2014) showed AMX activity in a sidestream PNA MBBR after 50 days on carriers with a preliminary biofilm of nitrifiers without AMX biomass inoculation. Given these results, it was expected that AMX establishment on carriers without any AMX biomass inoculation in a mainstream process would take longer than the observed startup time. And, upon closer inspection of the in situ ammonia removal during startup, this reactor showed evidence of AMX activity between days 60-70 before the first AMX activity test was conducted

illustrating that startup likely began before the first confirmation of activity. To avoid uncertainty in this timeframe, AMX activity tests should be conducted earlier in future experiments to record the first evidence of activity.

It is important to note that although no AMX carriers were seeded to this reactor, the wastewater treatment plant from which the preliminary biofilm carriers were sourced (JRTP mainstream IFAS process) had a sidestream ANITA™ Mox process that recycled effluent to before the IFAS process. This could have potentially provided small amounts of additional AMX to the preliminary biofilm carriers, but was unlikely to have impacted the AMX activity observed in this experiment due to the lack of an AMX-based process at the CE Pilot where the reactor was operated. However, to fully understand if the JRTP sidestream process had an impact on startup time, this experiment should be repeated with preliminary biofilm carriers sourced from a wastewater treatment plant operating without any AMX-based processes.

Ammonia and TIN Removal

Average ammonia loading throughout operation was 0.18 ± 0.09 gNH4-N/m^2d, while removal was 0.10 ± 0.09 gNH4-N/m^2d. After AMX activity confirmation on day 84, average ammonia removal was 0.17 ± 0.07 gNH4-N/m^2d with a removal percentage reaching as high as 96% and an average removal of 72 ± 16%. Average TIN loading throughout operation was 0.50 ± 0.25 gN/m^2d while average removal was 0.16 ± 0.13 gN/m^2d. After AMX activity confirmation, the average TIN removal was 0.27 ± 0.07 gN/m^2d. In situ TIN and ammonia removal never matched maximum removal rates, suggesting that the loading to the reactor could have been further increased (Figure 9).

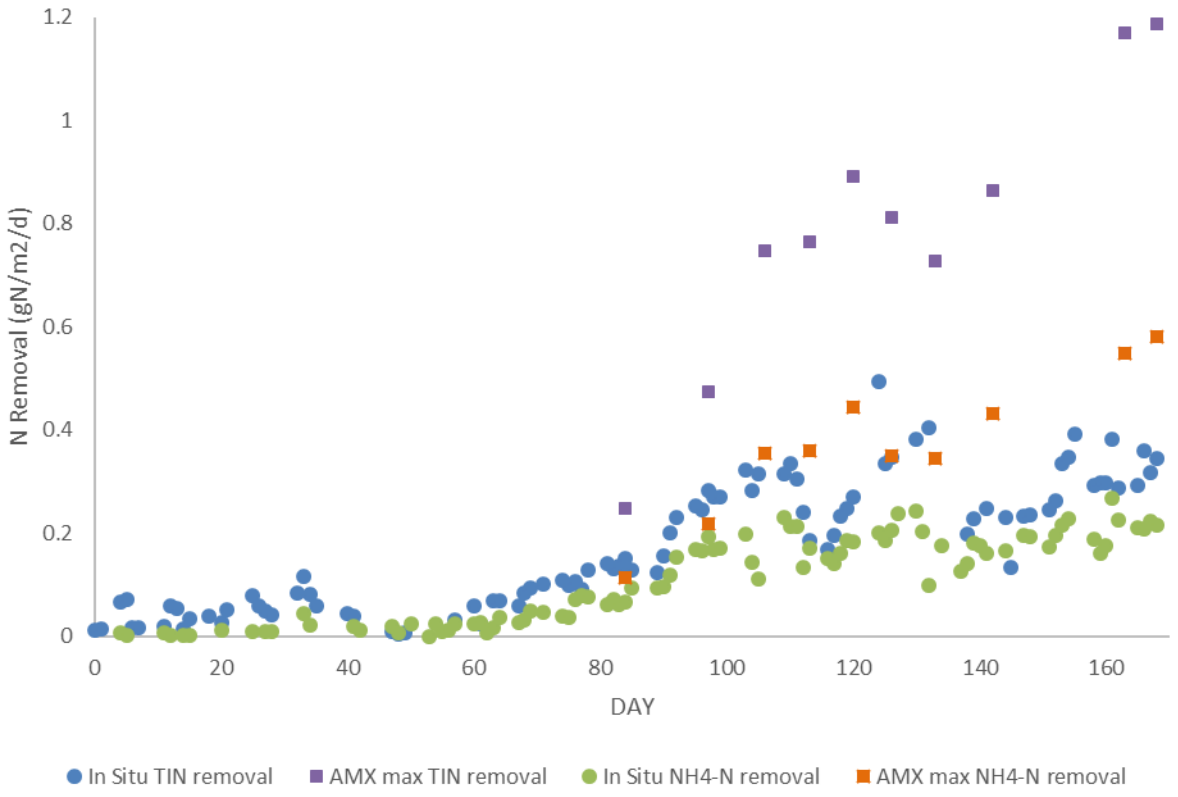

Figure 9: In situ versus maximum TIN and ammonia removal rates throughout operation.

Based on the maximum TIN and ammonia removal rates associated with the final AMX activity test, the reactor theoretically could have removed up to 34 mgTIN/L and up to 17 mgNH4-N/L. However, loading that high is unrealistic for a mainstream polishing process, so the loading rate was not pushed to achieve maximum possible conditions during this experiment. Since AMX removal rates quickly approached rates high enough to remove typical polishing ammonia and nitrite concentrations, these results suggest that removal rates in a full-scale process would be limited by ammonia loading as opposed to AMX activity.

Although the in situ removal rates never reached the maximum rates, the maximum removal rates also never plateaued. This suggests that the maximum removal rates also could have continued to increase if the experiment were operated at higher nitrogen loading and over a longer period of time. Ultimately, at the time this experiment was concluded, AMX was substrate-limited and could have undergone higher loading if provided.

Conclusions

This experiment proved that:

1. Startup of a mainstream AMX MBBR is possible within 60-84 days without AMX biomass seeding assuming that ammonia and nitrite are available and nonlimiting.
2. Maximum AMX activity rates were not achieved in situ, suggesting higher ammonia and nitrite loading rates are possible.

Ultimately, this experiment proved that a mainstream AMX MBBR can startup quickly without any biomass seeding, which has never been observed to the best of the author's knowledge. As well, when the reactor was loaded with increasing ammonia and nitrite throughout startup, removal percentages also increased. The in situ TIN and ammonia removal rates also never matched those of maximum AMX activity tests, so higher loading to the reactor could be achieved. And the maximum removal rates never plateaued, suggesting that they could have continued to increase with increased nitrogen loading. In future experiments, regular AMX activity tests should be conducted earlier in operation to accurately determine the time of the first onset of activity. This experiment should also be repeated with preliminary biofilm carriers sourced from a wastewater treatment plant without any AMX-based processes to determine that all AMX activity observed was a result of only planktonic AMX found in mainstream wastewater.

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Manuscript 2: Startup Strategies for Partial Denitrification with Anammox in a Mainstream Moving Bed Biofilm Reactor (MBBR)

Abstract

Partial denitrification/anammox (PdNA) is a novel technology for biological nitrogen removal with significant carbon and aeration savings when compared to conventional nitrification/denitrification. Yet despite these benefits, the use of PdNA in mainstream wastewater treatment remains limited. One of the main barriers to implementation is the slow growth rate of AMX, which results in a long startup time. To accelerate this process, the typical approach to commissioning a PdNA reactor is with augmentation of AMX biomass, specifically for sidestream partial nitritation/AMX applications, which is practically unrealistic for full-scale mainstream applications. Thus, this study was designed to determine startup strategies for mainstream PdNA without biomass seeding in moving bed biofilm reactors (MBBRs). Four reactors were started with either virgin carriers or carriers from an integrated fixed-film activated sludge (IFAS) process with a preliminary biofilm of heterotrophs and nitrifiers. The reactors were not inoculated with any additional AMX biomass throughout startup. One reactor of each carrier type was dosed with carbon in the form of either glycerol or methanol based on a proportional-integrative-derivative (PID) control loop with a nitrate residual. The reactors were initially fed with a semi-synthetic wastewater including a tap water dilution. This feed was chemically controlled for dechlorination, which was suspected to have caused inhibition due to the lack of AMX activity after 184 days of operation. The feed was switched to B-Stage secondary clarifier effluent, after which AMX activity began. The preliminary biofilm carrier reactor dosed with glycerol was the first reactor to show signs of AMX activity, which occurred shortly after switching the influent source. These results, along with other recent PdNA work, suggest that growth of AMX on biofilm carriers can be established in mainstream conditions with 50-100 days, depending on media selection and carbon source. Ultimately, this research will help utilities understand methods for starting up mainstream PdNA MBBRs from scratch and make this technology more accessible.

Introduction

Anaerobic ammonia oxidation, or anammox (AMX), is a current emphasis of wastewater treatment research due to its substantial aeration and carbon savings. Despite these benefits, it is still uncommonly employed in full-scale mainstream treatment due to difficult operational requirements. Sidestream applications in the form of partial nitritation anammox (PNA) are successful, but PNA necessitates nitrite oxidizing bacteria (NOB) outselection, which requires high ammonia concentrations, and higher temperatures to achieve. These characteristics are

uncommon in mainstream municipal wastewater, making PNA difficult to stably control outside of sidestream processes.

A more promising option for mainstream AMX may be partial denitrification/AMX (PdNA). This process combines the first step of denitrification (reduction of nitrate to nitrite) with AMX metabolism (oxidizing ammonia to nitrogen gas with nitrite as an electron acceptor). PdNA has slightly less carbon and aeration savings than PNA (Zhang, Zhang, and Chen 2019, Le et al. 2019_b), but the benefits are still significant when compared with conventional nitrification/denitrification (Strous et al. 1998). Most importantly, this technology avoids the key crux of mainstream PNA operation: the need for NOB outselection. Because of this, PdNA functions well at the lower ammonia concentrations and temperatures of mainstream municipal wastewater and can be used as a polishing step, making it easy to incorporate into existing processes (Campolong et al. 2019).

While PNA can be operated in mainstream or sidestream conditions, PdNA is rarely operated outside of mainstream. While PdNA can technically be operated in sidestream (Sharp et al. 2017), sidestream PNA would almost always be more favorable due to the lack of a carbon requirement for operation. Like PNA, mainstream PdNA processes can be operated in either single- or two-stage configurations. In a two-stage process, PdN and AMX reactions occur separately with the partially denitrified feed sent to a subsequent AMX process (Cao et al. 2019). One potential benefit of two-stage PdNA is the separation of AMX biomass, which removes competition for nitrite by other microorganisms (Bahtiar et al. 2020, Cao et al. 2017). However, this is not necessarily a benefit as some research in single-stage systems has shown PdN efficiencies unaffected without employing steps to limit nitrite competition (Le et al. 2019_b). In a two-stage process, the AMX reactor can also be controlled at a higher temperature to encourage faster growth of AMX if desired. However, separating the processes is not necessary for proper functioning of PdNA and can lead to higher space and cost requirements associated with operating multiple reactors, so single-stage is a more common choice. As well, for every part ammonium oxidized, AMX produces 0.26 parts of nitrate. This additional nitrate can be incorporated back into the PdN process and reduced to nitrite by OHO in a single-stage PdNA reactor, but cannot be accounted for in a two-stage process where PdN and AMX reactions are isolated from one another.

In a single-stage PdNA process, PdN and AMX reactions occur in the same reactor wherein in situ nitrite accumulation by OHO drives ammonia oxidation by AMX. Single-stage PdNA is more commonly employed in polishing processes, which occur at the end of a BNR process and are characterized by low nitrogen loading. Single-stage PdNA processes are ideally fed with equal parts of ammonia and nitrate to meet AMX stoichiometry after partial denitrification. Common approaches for polishing PdNA include biofilm-based systems such as MBBRs or filters. PdNA MBBRs can be operated anoxically with a biofilm of OHO, nitrifiers, and AMX existing together on the same carriers (Campolong et al. 2019), while filters incorporate OHO, nitrifiers, and AMX biomass growing in media such as sand or activated carbon (Klaus et al.

2020, Fofana et al. 2020, Cui et al 2020). Two-stage polishing systems have also been operated with a hybrid of suspended growth and granular AMX with bioaugmentation from a sidestream process and screened effluent for AMX retention (Le et al. 2019_b). In contrast to polishing processes, integrated processes occur upstream and receive higher nitrogen loading (Forney et al. 2020). These can operate as hybrid systems, such as with suspended growth and IFAS media, and are operated with anoxic zones or periods for AMX activity to occur.

Previous pilot scale results have illustrated the possibility for implementing mainstream PdNA MBBRs at full scale (Campolong et al. 2019). However, it is still unknown how best to establish AMX in MBBRs without biomass seeding. Thus, it is increasingly crucial to understand the methods of startup in order to make AMX-based technologies more widely available. Biomass retention is a key facet of PdNA MBBR startup due to the slow growth rate of AMX. Previous startup experiments of PNA MBBRs have used seeding through carriers with a pre-existing biofilm of AMX (Klaus et al. 2016, Tian et al. 2020), or AMX biomass inoculation (Regmi et al. 2015, Tian et al. 2020). These startup methods are not widely applicable for most existing systems because seeding is practically unrealistic for full-scale mainstream processes. It is also potentially unnecessary for attachment and growth of AMX biomass (Kanders et al. 2014).

PdNA can be operated using various supplemental carbon sources (e.g. glycerol, acetate, methanol). Glycerol is well known to be an effective carbon source for PdN, but methanol is newly discovered to be applicable to PdNA (Le et al. 2019_a, Campolong et al. 2019). Methanol has been shown to be inhibitory to AMX in batch experiments on enriched cultures (Isaka et al. 2008, Güven et al. 2005). However, in a pilot scale PdNA MBBR, AMX activity continued when methanol was overdosed, proving that methanol may not be inhibitory to AMX as previously thought (Campolong et al. 2019). This could potentially be due to the presence of AMX at the interior of biofilms in an MBBR process where it is not in contact with methanol at an inhibitory level.

Glycerol and acetate have shown capabilities for higher PdN efficiencies when compared to methanol (Le et al. 2019_a, Campolong et al. 2019). Methanol's lower PdN efficiency could be partially attributed to the utilization of methanol by specialized methylotrophs as opposed to a more general population of OHO that can utilize a carbon source like glycerol (Akunna, Bizeau, and Moletta 1993, Sperl and Hoare 1971). However, when considering methanol's lower bulk cost and lower yield compared to carbon sources like glycerol, it has a lower cost for PdN (Campolong et al. 2019). Due to methanol's lower cost, understanding the mechanisms of startup under methanol will broaden the applications of the PdNA process in full-scale mainstream treatment.

One way to encourage PdN is by maintaining a low influent carbon to nitrogen (C/N) ratio (Le et al. 2019_a). This is achieved by dosing carbon at limiting conditions that prevent FdN from occurring. A low C/N ratio was previously used to control for PdN, but recent studies have demonstrated the ability for a nitrate residual to select for PdN over FdN (Le at al. 2019_b,

Campolong et al. 2019). Controlling PdN with a nitrate residual ensures nitrite accumulates while leaving low levels of nitrate in the reactor effluent. For instance, Le et al. (2019_b) reported a nitrate effluent setpoint of 2-3 mgNO3-N/L to limit nitrite reduction while Campolong et al. (2019) showed 1 mgNO3-N/L is possible.

The prevailing theory behind PdN is that electrons are preferentially transferred to nitrate reductase on the electron transport chain because of its upstream position (Almeida et al. 1995, van Rijn et al. 1996, Baideme 2019). The reduction rate of both nitrate and nitrite depends on nitrate concentration (Almeida et al. 1995, Betlach and Tiedje 1981), so maintaining nitrate at nonlimiting conditions can encourage nitrite accumulation because electrons aren't transferred to nitrite reductase. It is also theorized that different carbon sources have different electron transport routes, which can limit their ability to produce nitrite accumulation (van Rijn et al. 1996). For instance, some carbon sources including acetate and propionate lead to nitrite accumulation due to the differing rates of nitrate and nitrite reduction and competition between nitrite and nitrate reductases. Acetate is also theorized to donate electrons upstream and closer to nitrate reductase, meaning that electrons are utilized by nitrate reductase first. However, some other carbon sources such as butyrate don't lead to nitrite accumulation at all possibly due to the donation of electrons downstream and closer to nitrite reductase (van Rijn et al 1996). Methanol may also have donation of electrons downstream and closer to nitrite reductase, but it still has been proven to produce nitrite accumulation (Van Verseveld and Stouthamer 1978, Le et al. 2019_a, Campolong et al. 2019).

As OHO denitrify, they have the possibility of forming intermediate products such as nitrous oxide (N_2O). N_2O is a potent and persistent greenhouse gas with a global warming potential 300 times higher than carbon dioxide, so limiting N_2O emissions from wastewater treatment is important (Montzka et al. 2011). In denitrifying biofilm processes, electron donor limitations (such as a low C/N ratio) can increase N_2O production (Sabba et al. 2018). Thus, PdN occurring in a biofilm process such MBBRs is of concern for N_2O accumulation due to nitrite accumulation and electron donor limitations associated with halting full denitrification. However, providing a consistent bulk environment for biofilm interaction, such as in an MBBR operated as a continuous stirred tank reactor (CSTR), can avoid high N_2O emissions (Sabba et al. 2018). Ultimately, microbial composition, biofilm thickness, substrate concentrations and variability can all impact N_2O production (Sabba et al. 2018).

N_2O production is closely related to nitrite reduction and doesn't typically occur until nitrate is fully depleted (Du et al. 2016). As well, in PdN processes with high nitrite accumulation, a high C/N ratio is also related to increased N_2O production (Du et al. 2016). These situations would likely be avoided in a PdN process that employs a carbon dosing control based on a nitrate residual, as nitrate is never entirely removed and a low C/N ratio is typically produced to avoid full denitrification.

To determine N_2O production during startup of a PdNA MBBR, in situ N_2O accumulation was measured in each reactor with a liquid phase probe. It was hypothesized that continuously low nitrite concentrations in the reactor under PdNA conditions would prevent significant N_2O generation by OHO partial denitrification. However, the nitrite conditions before AMX activity was discovered were expected to be higher due to the lack of a nitrite sink provided by AMX. Thus, it was important to understand N_2O emissions during startup wherein nitrite accumulation was possible. N_2O measurements were also taken during maximum AMX activity tests where higher concentrations of nitrite were dosed in comparison to typical in situ levels.

This experiment was designed to compare startup time without any AMX biomass seeding across four PdNA MBBRs dosed with methanol or glycerol and started with either virgin or IFAS carriers with an existing biofilm of OHO and nitrifiers. Using preliminary biofilm carriers (from aerobic IFAS) was expected to lead to faster startup by increasing AMX attachment rate (Klaus et al. 2016, Kowalski et al. 2017). AMX biomass was not seeded to the reactors, so increasing the rate of attachment was key for decreasing startup time. The objectives of this study were:

1. To compare startup time and performance in MBBRs with either virgin carriers or aerobic IFAS carriers with a preliminary biofilm.

2. Compare startup time and performance between reactors dosed with glycerol and methanol as carbon sources.

The motivation behind this research was to determine if AMX-based MBBRs could be started up using only the planktonic AMX already present in mainstream wastewater without the need for any AMX biomass inoculation. As well, polishing PdNA MBBR technology has never been used before at full scale, so studying mechanisms in a pilot scale application will inform ideal conditions for startup. For instance, this research will be used to inform startup choices for a full-scale implementation of a mainstream polishing PdNA MBBR system at James River Treatment Plant (JRTP) in Newport News, Virginia.

Materials and Methods

Startup of PdNA MBBRs

This study was conducted on four PdNA MBBRs at the Hampton Roads Sanitation District (HRSD) Chesapeake-Elizabeth (CE) Treatment Plant biological nutrient removal (BNR) pilot facility in Virginia Beach, VA. Figure 10 shows the uncovered reactors during operation.

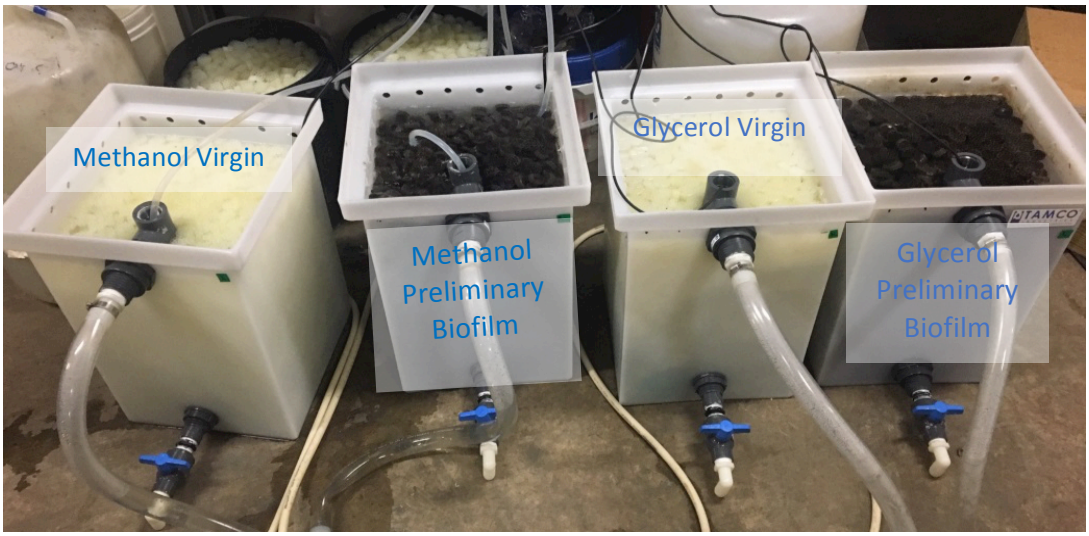


Figure 10: Uncovered PdNA MBBRs in operation at the CE Pilot. Styrofoam covers were utilized during operation to prevent oxygen transfer.

For the first 184 days of operation, the MBBRs were fed a semi-synthetic mix of CE Treatment Plant post-chlorination final effluent (FNE) diluted with tap water and chemically dosed to meet a 1:1 ratio of ammonia to nitrate (5 mgNH4-N/L and 5 mgNO3-N/L) (Figure 11). Ammonia was provided by FNE and nitrate was provided by dosing sodium nitrate. Phosphorus was briefly supplemented from day 116-119 using monopotassium phosphate. Dechlorination was conducted using sodium bisulfite at first, followed by sodium thiosulfate after 2 months of operation with a target chlorine residual of 0.02 mgCl_2/L. Sodium bicarbonate was dosed with a target alkalinity of 100 mgCaCO3/L in the influent. Dissolved oxygen (DO) in the influent was 7.2 ± 1.3 mg/L, pH was 7.4 ± 0.3, and temperature was 20.2 ± 2.3°C, controlled to 20°C by a chiller during warmer months. Each 33 L reactor had an AnoxKaldnes© K3 (Veolia, Aubervilliers, France) carrier fill of 43%, hydraulic residence time (HRT) of 1 hour, and a designed total inorganic nitrogen (TIN) loading rate of 1 gN/m^2d.

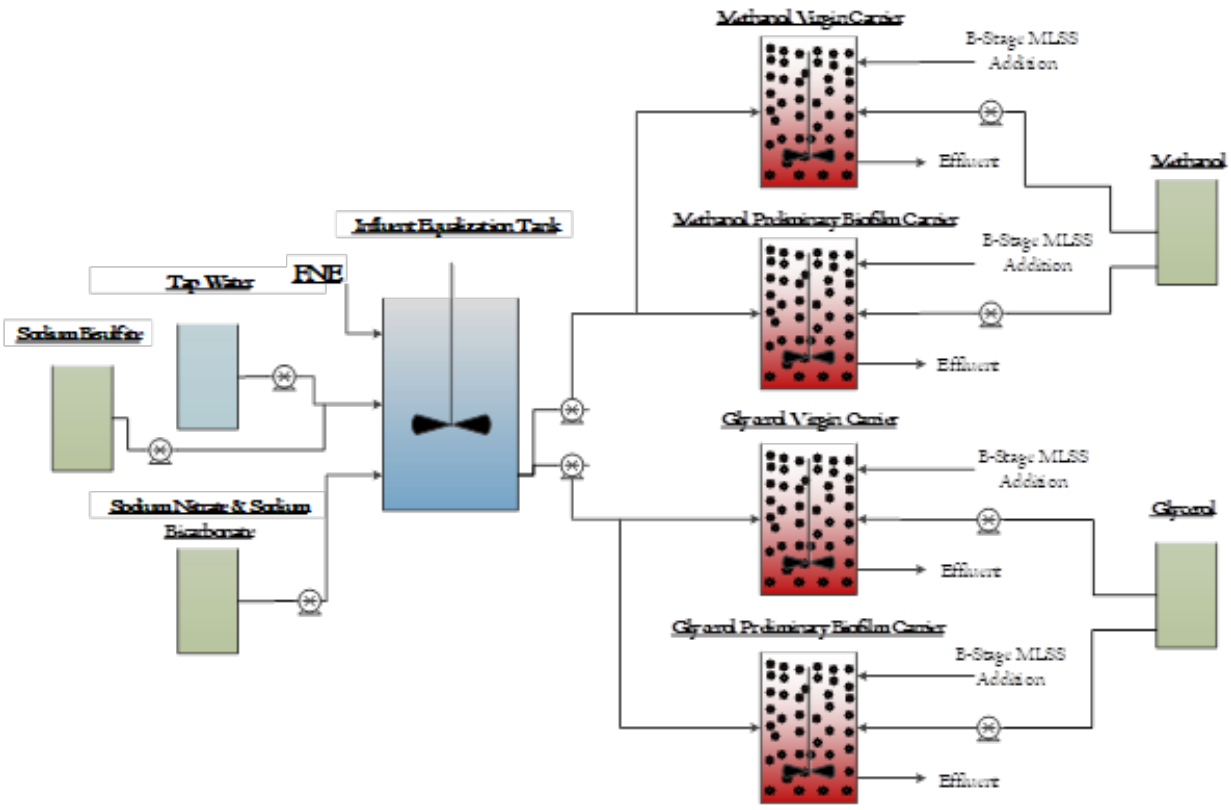


Figure 11: PdNA MBBR process flow diagram.

After 184 days of operation, the influent feed was switched from the semi-synthetic wastewater to effluent from the secondary clarifier of an adsorption/bio-oxidation (A/B) process at the CE Pilot (Figure 12). The A/B process operated with carbon diversion in the A-Stage high rate activated sludge (HRAS) process before BNR in the B-Stage. The B-Stage was designed for both nitrogen removal and biological phosphorus removal and concluded with a secondary clarifier that fed into the polishing MBBRs.

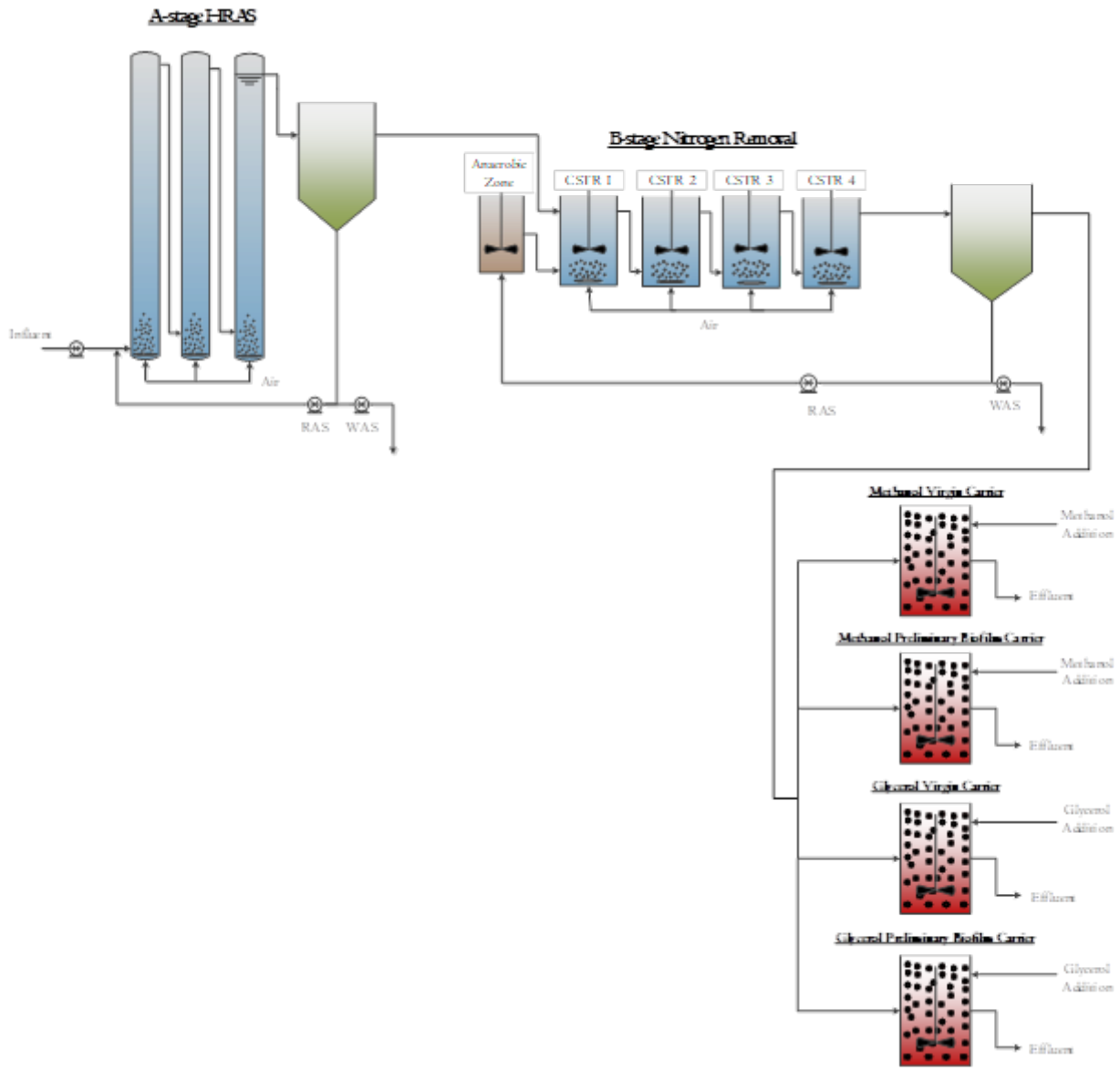


Figure 12: A/B process and PdNA MBBR process flow diagram.

The B-stage BNR process was operated under ammonia versus NO_x (AvN) intermittent aeration control. In this process, aeration was cycled on and off to meet a 1:1 ratio of ammonia and NO_x (nitrite plus nitrate). This was based on feedback from sensors in the fourth CSTR of the process. The B-Stage process was temperature controlled to 20°C and pH was controlled to 6.8. The feed from the B-Stage process included ammonia, nitrate, and a small amount of nitrite. The second feed source had a DO of 0.15 ± 0.11 mg/L, pH of 7.11 ± 0.05 and a temperature of 21.3 ± 0.65°C.

Carrier Choice

Two reactors were started with virgin MBBR K3 carriers and two were started with K3 carriers containing a preliminary biofilm of heterotrophs (Figure 13).

Figure 13: Virgin carrier (left) versus preliminary biofilm carrier (right).

The virgin carriers were pre-wetted for several weeks in tap water prior to starting the reactors. The preliminary biofilm carriers were sourced from the HRSD James River Treatment Plant aerobic IFAS process. This plant had a sidestream ANITA™ Mox process that recycled to a point before which the IFAS process operated.

Mixed Liquor Dosing

A small amount of mixed liquor suspended solids (MLSS) was dosed from the final CSTR of the B-Stage at a target of 5 mgTSS/L for the first 184 days of operation. This was done to provide a community of microorganisms and augment the post-chlorination FNE provided in the influent, consistent with what would be expected in secondary clarifier effluent. The MLSS dosing was suspended after 184 days when the influent was switched to B-Stage clarifier effluent.

Mixing and Oxygen Transfer Prevention

The reactors were operated with vertical shaft mixers (Caframo, Ontario, Canada) initially mixed at speeds of 100 rpm for preliminary biofilm carrier reactors and 150 rpm for virgin carrier reactors. Mixing was then briefly increased to 175 rpm for all reactors after 49 days of operation, and returned to 150 rpm after 7 more days to ensure consistent conditions were applied to each reactor. All reactors were covered with polystyrene to prevent oxygen transfer and generate anoxic conditions.

Nutrient and Total Suspended Solids, Volatile Suspended Solids Sampling

Nutrient samples were conducted on 24-hour composite samples for the first 116 days of reactor operation, followed by grab samples for the remainder of operation. All samples were filtered through 0.45 μm filters and evaluated for NH4-N, NO3-N, NO2-N, ortho-phosphate (OP) as PO4-P, Alkalinity as $CaCO_3$, and soluble chemical oxygen demand (sCOD). Total COD (tCOD) was measured on unfiltered samples after blending for 30 seconds. All nutrients were measured

with Hach TNT kits (Loveland, Colorado) and read with a Hach DR-2800 spectrophotometer. Sulfamic acid was added to samples evaluated for NO3-N and sCOD to eliminate nitrite interference when nitrite was over 1.5 mgNO2-N/L. In such cases, 50 mg of sulfamic acid was added to 5 mL of sample, shaken to dissolve, and left for 10 minutes before nutrient samples were evaluated for nitrite. Temperature, pH (Hach, Loveland, Colorado), and dissolved oxygen (DO) (Insite, Slidell, Louisiana) were evaluated in each reactor and the influent source daily using handheld probes.

For the first 116 days of operation, total suspended solids (TSS) and volatile suspended solids (VSS) measurements were evaluated weekly on 24 hr composite samples following standard methods. For the remainder of the experiment, TSS and VSS measurements were conducted on grab samples from each reactor and the influent source.

COD Dosing

Two reactors, one with each carrier type, were dosed with methanol (VWR, Radnor, PA) and two with MicroC 2000© glycerol (EOSi, Bourne, MA). At the beginning of the experiment, reactors were fed a constant COD dose for about one month. The reactors were then dosed COD with feedback control based off a PID loop. COD was dosed to meet a nitrate residual of 1-1.5 mgNO3-N/L and nitrate measurements were taken by a wet chemical analyzer.

C/N Ratio

Carbon to nitrogen ratio (C/N) was evaluated for each reactor throughout startup by comparing sCOD dosed to each reactor minus the sCOD utilized for removal of influent DO, assuming a ratio of 1 gCOD/gDO removed. This was divided by the nitrate removal in each reactor to determine C/N ratio accounting for DO (Equation 1).

Eq 1:

$$\frac{C}{N}\ Ratio\ Accounting\ for\ DO = \frac{COD_{dosed} - DO_{rem}}{{NO_3}^-{}_{rem}}$$

PdN Efficiency

PdN efficiency was also evaluated in each reactor throughout startup. This was measured as the percent of the influent nitrate removed that was converted to nitrite (Equation 2). The stoichiometric utilization of nitrite, 1.32 g/g, and production of nitrate, 0.26 g/g, for each part of ammonia oxidized by AMX was also accounted for in the equation.

Eq 2:

$$PdN\ \% = 100 * \left(\frac{{NO_2^-}_{prod} + 1.32 \cdot {NH_4^+}_{rem}}{{NO_3^-}_{rem} + 0.26 \cdot {NH_4^+}_{rem}}\right)$$

Total Solids Sampling

Total solids measurements were taken once per month for the first 184 days of operation followed by fortnightly for the remainder of the experiment by sampling 3 carriers from each reactor. The carriers were dried in an oven at 110°C overnight and weighed. The dried carriers were then placed in a 25 g/L solution of disodium EDTA (ethylenediaminetetraacetic acid) and shaken vigorously. Each carrier was scraped with a plastic brush under running water until all biomass was removed, and then the carriers were dried again overnight at 110°C. The final carrier weights were recorded and subtracted from the initial weights to determine the biomass total solids based on the known surface area of each carrier. Finally, three virgin carriers per reactor were labeled by punching holes in opposite sides of the carriers and returning them to the reactors to maintain a consistent total surface area.

Activity Testing

In situ batch tests for AMX activity were conducted dosing nonlimiting ammonia at 20 mgNH4-N/L (ammonium chloride) and nitrite at 25 mgNO2-N/L (sodium nitrite) after isolating reactors and suspending COD dosing for one hour prior to testing. NH4-N, NO3-N, NO2-N, and sCOD were evaluated with Hach tubes in 0.45 μm filtered grab samples of reactors at various intervals of time ranging from 30 minutes to 2 hours for periods of 6-24 hrs. Sulfamic acid was added to samples evaluated for NO3-N and sCOD to eliminate nitrite interference when nitrite was over 1.5 mgNO2-N/L. In such cases, 50 mg of sulfamic acid was added to 5 mL of sample, shaken to dissolve, and left for 10 minutes before nutrient samples were evaluated for nitrite. DO, pH, and temperature were evaluated in each reactor with handheld probes throughout the batch experiments at the same time intervals as nutrient sampling.

N_2O Measurements

After 209 days of operation, a liquid phase N_2O sensor was installed to take in situ measurements (Unisense, Denmark). This sensor was rotated daily in each reactor. The sensor was calibrated before installation following instructions outlined by Unisense. N_2O was also measured in the preliminary biofilm carrier reactor dosed with glycerol during in situ AMX activity tests.

Results and Discussion

Influent Characteristics

The influent source for the reactors was switched on day 184 of operation due to the notion that the semi-synthetic wastewater feed was inhibitory to AMX growth. Despite a simultaneous experiment on a mainstream AMX MBBR showing AMX activity 100 days sooner, no AMX activity was observed in any of the PdNA MBBRS in this experiment after 184 days of

operation. The semi-synthetic wastewater was diluted with tap water with a chlorine content of up to 3.6 mgCl_2/L and was dechlorinated with sodium bisulfite followed by sodium thiosulfate. This could have contributed to AMX inhibition due to potential chlorine and inhibitory chemical breakthrough. Thus, the feed was switched to B-Stage effluent at a point when nitrite accumulation was minimal. Ammonia, nitrite, and nitrate loading in the B-Stage effluent were comparable to the semi-synthetic feed, but concentrations were more variable.

Day of Operation	TIN Loading Rate	NH4-N	NO2-N	NO3-N	sCOD	TSS	Alkalinity	OP	pH	DO	Temp
	g/m2d	*mg/L*	*mg/L*	*mg/L*	*mg/L*	*mg/L*	*mgCaCO3/L*	*mgPO4-P/L*		*mg/L*	*°C*
0-184	1.19 ± 0.09	6.17 ± 0.71	0.26 ± 0.16	5.38 ± 0.26	20.7 ± 7.4	3.1 ± 1.5	92 ± 15	0.40 ± 0.10	7.4 ± 0.26	7.2 ± 1.3	20.2 ± 2.3
184-252	1.09 ± 0.26	6.04 ± 1.94	0.74 ± 0.10	6.17 ± 1.51	26.3 ± 1.5	31.7 ± 4.5	222 ± 36	0.83 ± 1.01	7.11 ± 0.05	0.15 ± 0.11	21.4 ± 0.6

Table 1: Characteristics of both influent sources to reactors throughout operation.

The TIN loading rate of both influent sources was similar but slightly higher in the semi-synthetic wastewater than the B-Stage effluent (Table 1). Average ammonia was slightly higher in the semi-synthetic wastewater and less variable than B-Stage effluent. Nitrite was lower in the semi-synthetic wastewater than in the B-Stage effluent, but the latter was still relatively low especially compared to nitrite provided by in situ PdN. Nitrate was lower in the semi-synthetic wastewater and less variable than the B-Stage effluent. Figure 14 shows the slight increase in average influent nitrite after the switch to the B-Stage effluent wastewater source as well as the increased variation in ammonia and nitrate loading.

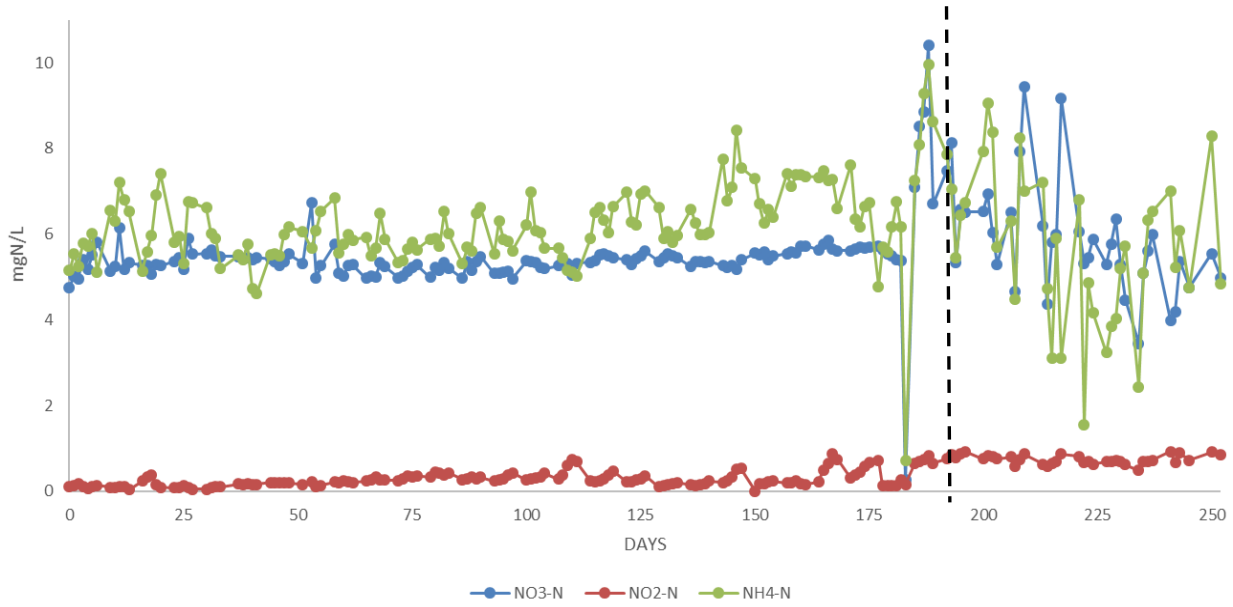


Figure 14: Influent Characteristics. Influent source switch on day 184 represented by vertical dashed line.

Phosphorus limitation has been shown to negatively impact PdNA operation in a filter process (Fofana et al. 2020). Thus, OP was monitored and kept above limiting conditions determined by yield calculations of OHO based on typical carbon dosing and an assumed phosphorus requirement of 0.017 gPO4-P/gBiomass as COD. Phosphorus addition was only required at one time during operation for a period of 4 days.

TSS was much lower in the semi-synthetic wastewater than B-Stage effluent, while DO was much higher in the semi-synthetic source. Chlorine was only measured in the semi-synthetic wastewater, resulting in an average of 0.12 ± 0.27 $mgCl_2/L$ and a median value of 0.03 $mgCl_2/L$.

Effluent Characteristics and Nitrogen Removal and Accumulation

Reactor	Day of Operation	Effluent NH4-N	Removed NH4-N	Effluent NO2-N	Accumulated NO2-N	Effluent NO3-N	Removed NO3-N	Effluent sCOD	Effluent TSS	Effluent OP	Effluent pH	Effluent DO	Effluent Temp
		mg/L	*mg/L*	*mg/L*	*mg/L*	*mg/L*	*mg/L*	*mg/L*	*mg/L*	*mgPO4-P/L*		*mg/L*	*°C*
Methanol Virgin	0-184	5.77 ± 0.61	0.40 ± 0.46	1.76 ± 1.10	1.51 ± 1.06	1.81 ± 1.44	3.58 ± 1.51	17.9 ± 6.13	12.1 ± 5.85	0.35 ± 0.09	7.12 ± 0.23	0.42 ± 1.24	20.3 ± 2.07
	184-252	5.77 ± 1.98	0.36 ± 0.64	3.19 ± 0.93	2.45 ± 0.88	1.45 ± 0.51	4.72 ± 1.43	26.1 ± 2.32	32.8 ± 5.98	0.69 ± 0.68	6.96 ± 0.10	0.06 ± 0.12	21.3 ± 0.64
Methanol Preliminary Biofilm	0-184	5.75 ± 0.62	0.42 ± 0.47	1.45 ± 0.86	1.19 ± 0.84	1.82 ± 1.25	3.56 ± 1.32	17.2 ± 5.29	10.6 ± 2.20	0.35 ± 0.10	7.01 ± 0.20	0.11 ± 0.08	20.4 ± 2.11
	184-252	5.64 ± 1.99	0.58 ± 0.54	2.47 ± 1.26	1.79 ± 1.20	1.41 ± 0.58	4.74 ± 1.49	26.2 ± 3.13	34.7 ± 8.31	0.69 ± 0.66	7.03 ± 0.08	0.03 ± 0.03	21.4 ± 0.62
Glycerol Virgin	0-184	5.30 ± 0.66	0.87 ± 0.46	1.97 ± 0.98	1.71 ± 0.90	1.83 ± 1.37	3.55 ± 1.41	17.7 ± 4.12	16.5 ± 4.94	0.26 ± 0.11	6.94 ± 0.19	0.24 ± 0.95	20.4 ± 2.10
	184-252	5.58 ± 1.98	0.57 ± 0.64	3.40 ± 1.24	2.66 ± 1.22	1.63 ± 0.78	4.54 ± 1.17	26.6 ± 4.62	38.2 ± 9.70	0.68 ± 0.68	6.99 ± 0.09	0.02 ± 0.03	21.4 ± 0.63
Glycerol Preliminary Biofilm	0-184	5.33 ± 0.62	0.84 ± 0.43	1.87 ± 1.07	1.61 ± 0.99	1.92 ± 1.26	3.45 ± 1.31	18.1 ± 4.79	14.9 ± 2.49	0.28 ± 0.10	6.91 ± 0.16	0.09 ± 0.09	20.4 ± 2.09
	184-252	5.32 ± 2.07	0.93 ± 0.56	3.00 ± 1.24	2.24 ± 1.22	1.72 ± 0.79	4.46 ± 1.20	25.5 ± 1.43	39.9 ± 6.84	0.68 ± 0.65	6.98 ± 0.08	0.01 ± 0.01	21.4 ± 0.61

Table 2: Effluent characteristics and nitrogen removal and accumulation in reactors before and after influent source switch on day 184.

All reactors had higher nitrite accumulation on average after switching the influent source on day 184 as well as higher effluent TSS and sCOD (Table 2). Average temperature and DO remained low throughout operation, with pH remaining neutral in all reactors before and after the influent source switch. Average effluent ammonia remained similar before and after switching effluent sources, with glycerol-dosed reactors exhibiting lower effluent ammonia and higher ammonia removals than methanol-dosed reactors. Despite increased variability of influent nitrate, average effluent nitrate remained similar with both influent sources. However, variability in nitrate removals of all reactors increased after the influent source switch alongside increased influent variability (Figures 15-18). In all reactors, average effluent nitrate was below 2 mgNO3-N/L.

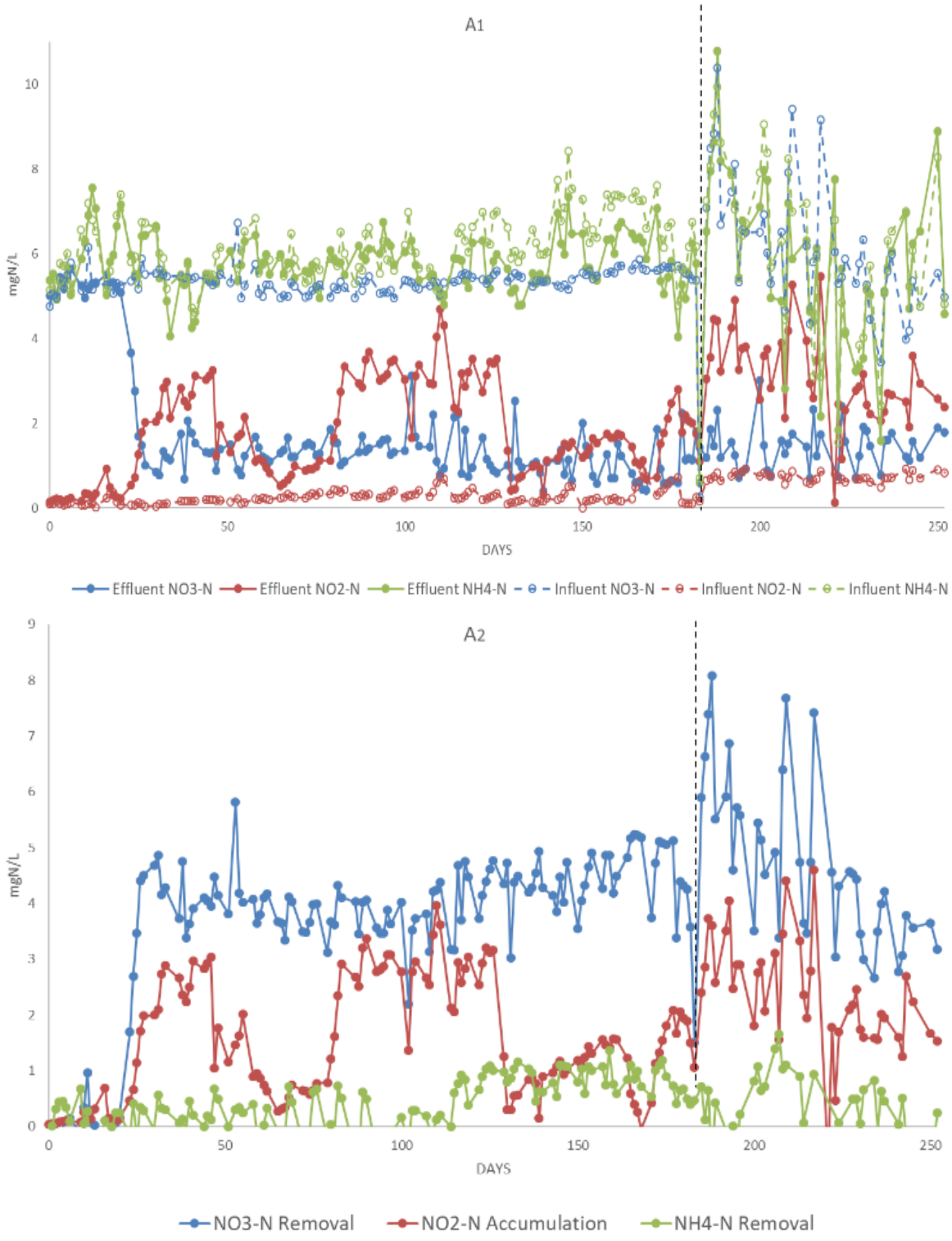


Figure 15: Influent nitrogen species (A_1), and effluent, removal, and accumulation of nitrogen species (A_2) in virgin carrier reactor dosed with methanol. Influent source switch on day 184 represented by vertical dashed line.

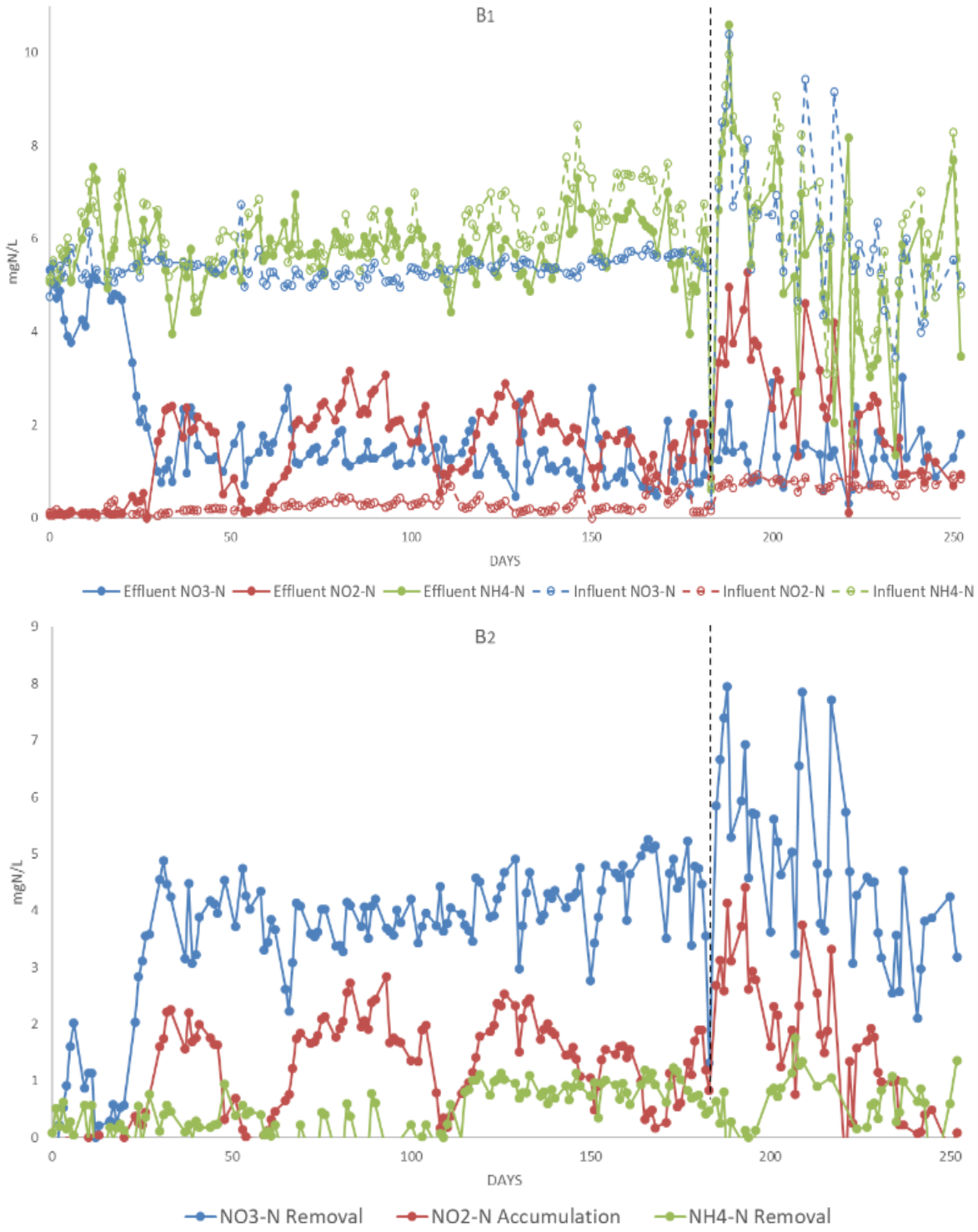


Figure 16: Influent nitrogen species (B_1), and effluent, removal, and accumulation of nitrogen species (B_2) in preliminary biofilm carrier reactor dosed with methanol. Influent source switch on day 184 represented by vertical dashed line.

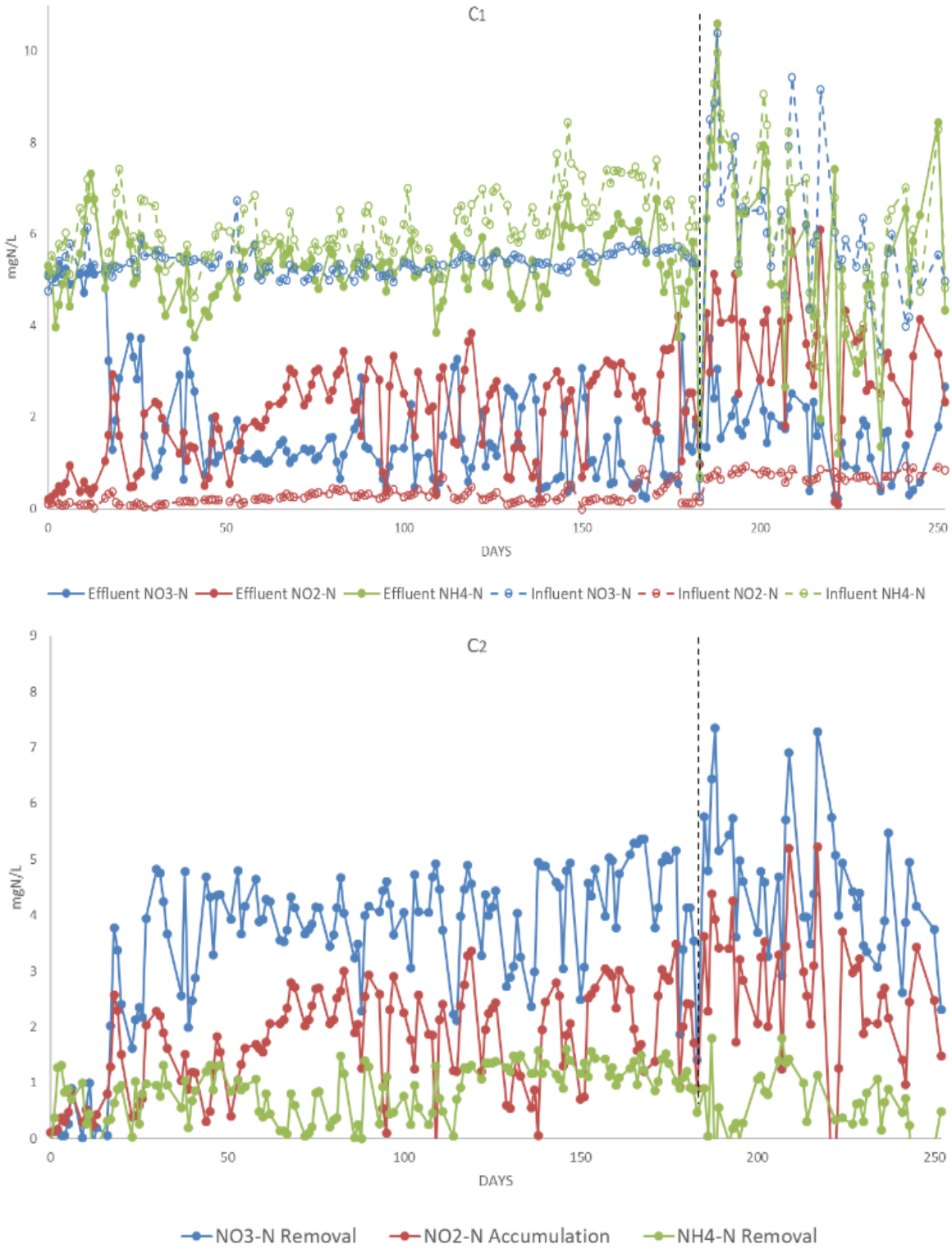


Figure 17: Influent nitrogen species (C_1), and effluent, removal, and accumulation of nitrogen species (C_2) in virgin carrier reactor dosed with glycerol. Influent source switch on day 184 represented by vertical dashed line.

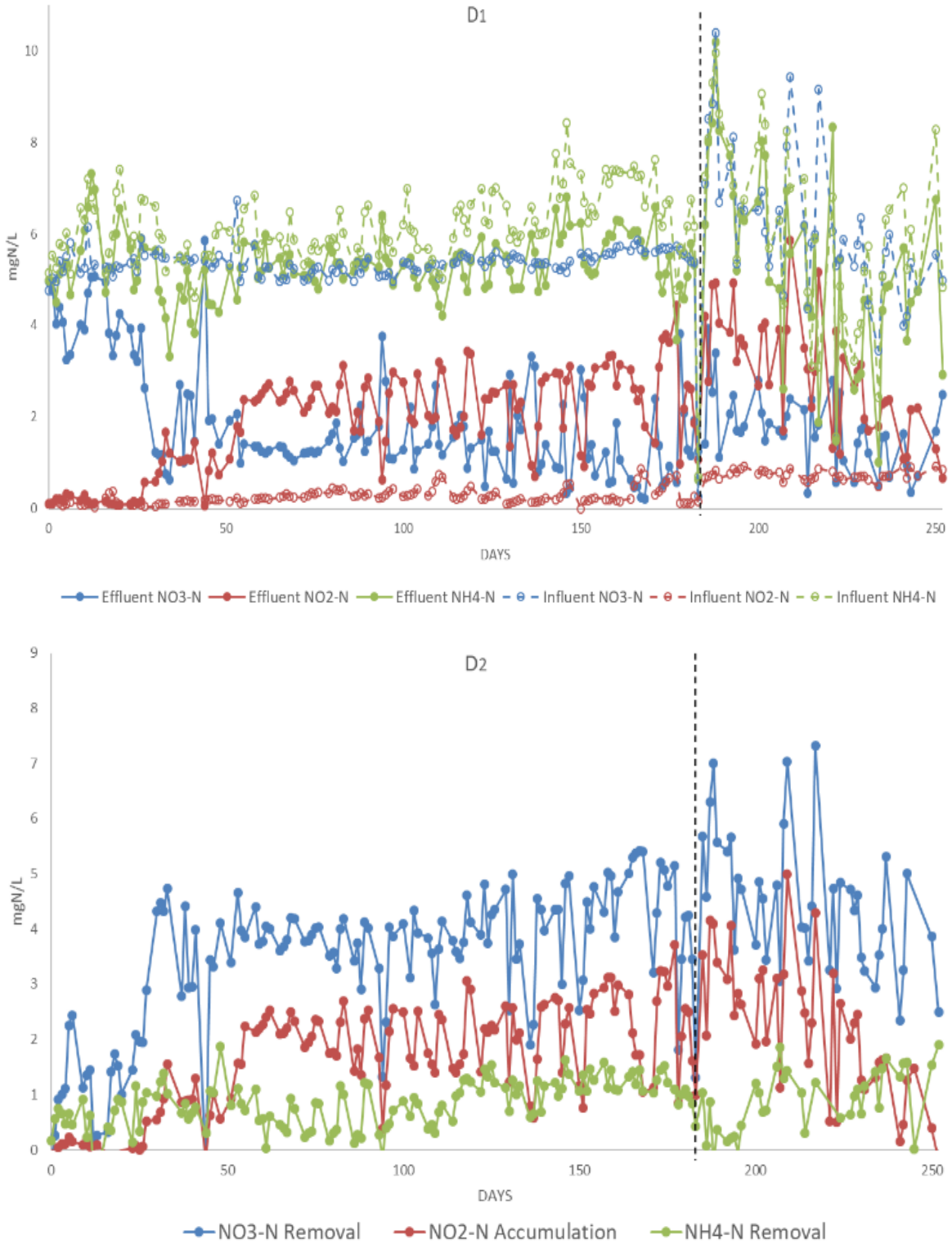


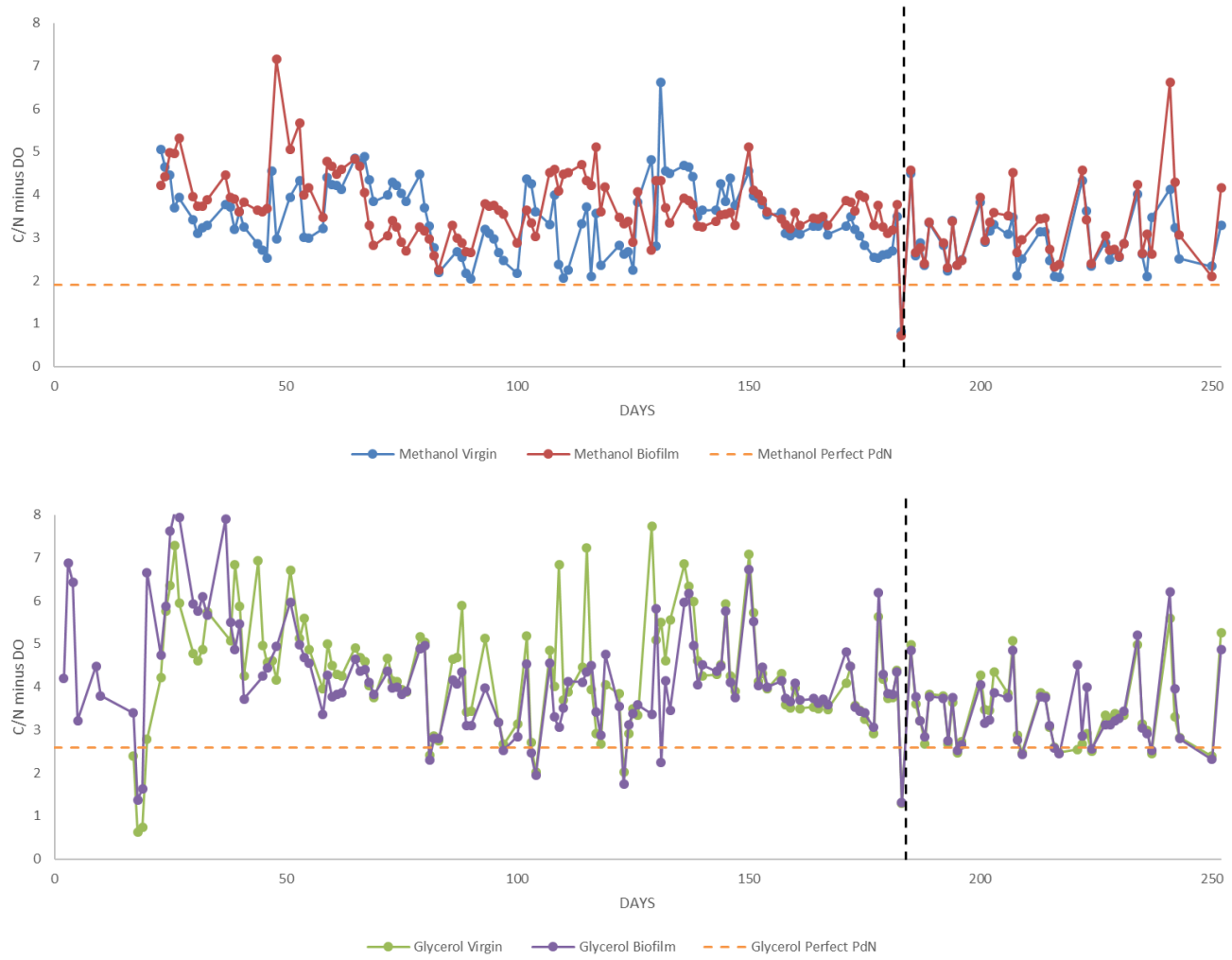
Figure 18: Influent nitrogen species (D_1), and effluent, removal, and accumulation of nitrogen species (D_2) in preliminary biofilm carrier reactor dosed with glycerol. Influent source switch on day 184 represented by vertical dashed line.

C/N Ratio

Reactor	Day of Operation	Adjusted C/N Ratio
		mg/L
Methanol Virgin	0-184	3.50 ± 0.82
	184-252	2.94 ± 0.63
Methanol Preliminary Biofilm	0-184	3.78 ± 0.72
	184-252	3.20 ± 0.87
Glycerol Virgin	0-184	4.38 ± 1.30
	184-252	3.40 ± 0.84
Glycerol Preliminary Biofilm	0-184	4.34 ± 1.31
	184-252	3.45 ± 0.85

Table 3: C/N ratios of reactors before and after influent source switch on day 184.

The glycerol-dosed reactors had higher average C/N ratios than methanol-dosed reactors, possibly due to the higher yield of glycerol (Table 3). C/N values from theoretical perfect PdN calculations were compared to the startup reactor values for each carbon source to see if the C/N ratios approached a similar range (Figure 19). This was also to ensure carbon was being dosed at the proper ratio for PdN as opposed to FdN, which requires more carbon. Though C/N ratio was not directly controlled in this experiment, it was still monitored to inform operational conditions and prevent overdosing of carbon.



Methanol perfect PdN 1.91g/g and glycerol perfect PdN 2.58 g/g.

Figure 19: C/N ratios of PdNA MBBRs adjusted for COD consumption related to influent DO. Abnormally high initial calculations removed. Influent source switch on day 184 represented by vertical dashed line.

After switching influent sources, the C/N ratios for all reactors decreased. Variations in characteristics of the different influent sources could have had an impact on the calculated C/N ratio throughout operation. First is the difference in influent DO, which relates to COD demand. This was accounted for by the C/N ratio calculation, which included the requirement for influent DO removal (assumed to be 1 gCOD/gDO removed). Because the COD was dosed off of a feedback PID control, the dose decreased after the influent switch made on day 184 when influent DO significantly decreased. At this time, influent nitrate also became more variable due to the switch from a chemically dosed and carefully controlled nitrate concentration to nitrate provided from a B-Stage process, which had more variation. The use of a feedback PID control and a calculation which adjusted for the additional COD required for DO removal were both

designed to minimize the impacts of these variations on reactor operation and data analysis but an overall decrease in calculated C/N ratio was still observed after switching influent sources.

PdN Efficiency

Reactor	Day of Operation	PdN Efficiency
		%
Methanol Virgin	0-184	43.6 ± 26.5
	184-252	53.7 ± 16.5
Methanol Preliminary Biofilm	0-184	34.8 ± 22.5
	184-252	42.5 ± 15.1
Glycerol Virgin	0-184	56.4 ± 20.4
	184-252	64.4 ± 17.0
Glycerol Preliminary Biofilm	0-184	55.5 ± 20.6
	184-252	60.5 ± 13.3

Table 4: PdN efficiencies of reactors before and after influent source switch on day 184.

All reactors achieved PdN shortly after the beginning of operation, within the first three weeks. The glycerol-dosed reactors achieved higher efficiencies overall with the virgin carrier reactor dosed with glycerol having the highest PdN efficiency (Table 4). Unexpectedly, both virgin carrier reactors had higher PdN efficiencies than preliminary biofilm carrier reactors dosed with the same carbon source. This could potentially be related to the thinner biofilms present on the virgin carriers having better exposure to bulk conditions, leading to more efficient substrate utilization (Piculell et al. 2016).

After switching influent sources on day 184, the PdN efficiencies increased in all reactors. This is possibly due to the switch from an influent with inhibitory characteristics to a more suitable influent source that allowed for better proliferation and activity of OHO in all reactors.

During the last few months of operation, the glycerol-dosed reactors with both media types remained at higher efficiencies than the methanol-dosed reactors (Figure 20). This result was expected based on previous research in a PdNA MBBR wherein methanol had a lower PdN efficiency than glycerol throughout operation (Campolong et al. 2019). This can be partially attributed to the utilization of methanol by specialized methylotrophs as opposed to a more general population of OHO that can utilize glycerol (Akunna, Bizeau, and Moletta 1993, Sperl and Hoare 1971).

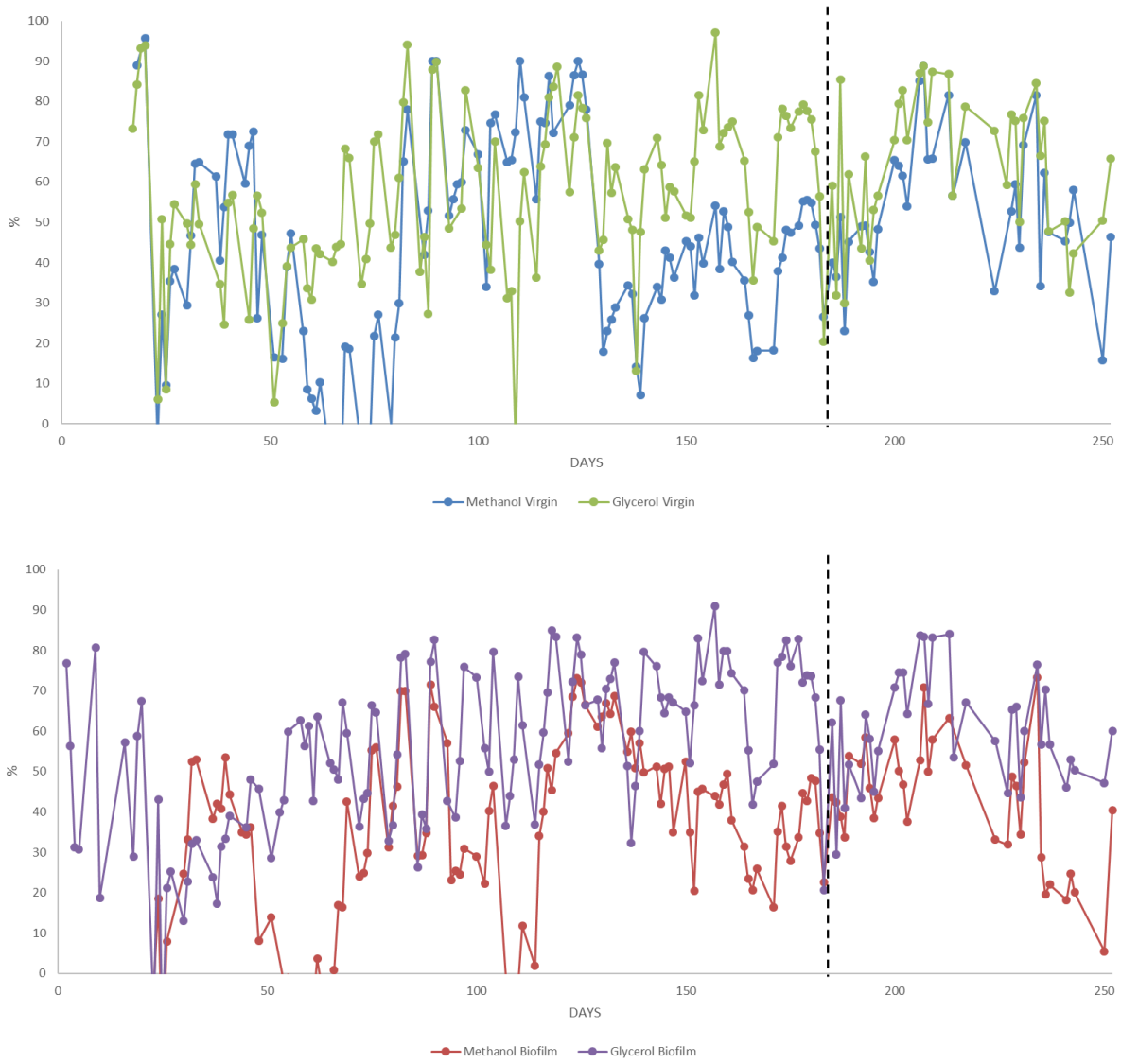


Figure 20: PdN efficiencies of PdNA MBBRs. Initial calculations outside of 0-100 % efficiency were removed. Influent source switch on day 184 represented by vertical dashed line.

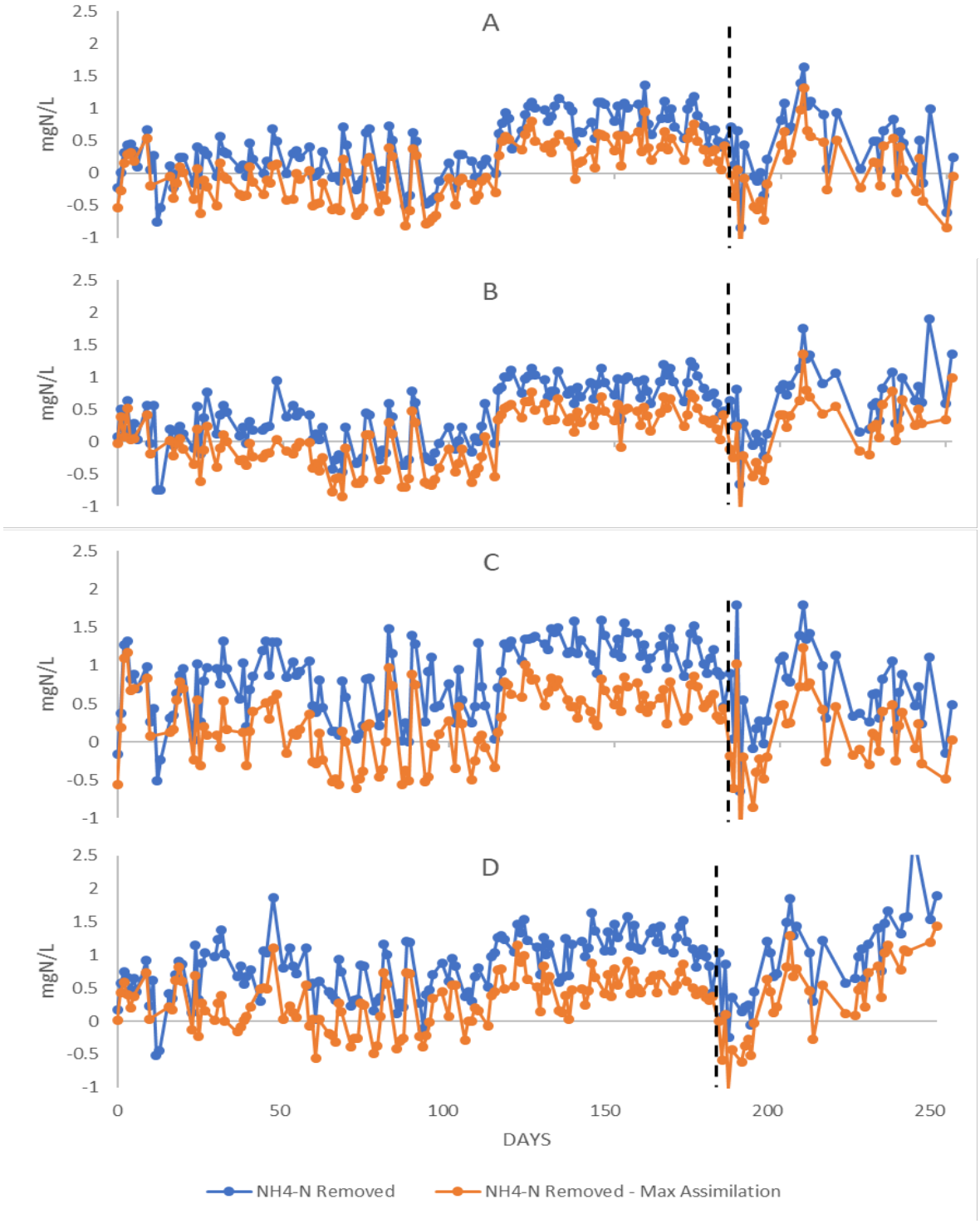
Periods of variation in PdN efficiency throughout operation could be attributed to periodic carbon dosing control issues and an increase in mixing speed. For instance, on day 130-184, carbon dosing was switched from automatic feedback control through a PID loop to a feed-forward manual control based on expected COD demand to reach the targeted nitrate residual in each reactor. PdN efficiency decreased after this change, most likely due to carbon dosing remaining constant and unable to respond to changes in demand. This may have led to conditions of over- or under-dosing carbon, both of which were observed to correspond with poorer PdN efficiencies in the reactors. As well, on day 49, mixing speeds were increased in all reactors, which resulted in a brief decrease in PdN efficiencies of both virgin carrier reactors. This could

have been related to the thinner and less robust biofilm presence on these carriers sloughing off with an increased mixing speed before recovering.

Carbon dosing controls sometimes failed or were inaccurate at points throughout operation, leading to variable nitrate residuals. Through examining the relationship between PdN efficiency and nitrate residual, it was determined that the probability of high PdN efficiencies (above the average PdN efficiency for each carbon source) was highest at residuals between 1-2 mgNO3-N/L. It was also determined that the probability of high PdN efficiencies decreased with conditions of nitrate residuals above 2 mgNO3-N/L and below 1 mg NO3-N/L. This result was expected due to high and low nitrate residuals likely being caused by over- or under-dosing carbon, which would lead to poor PdN efficiencies. However, it is still valuable to understand that despite evidence from other research showing that limiting conditions for nitrate are between 2-3 mgNO3-N/L (Le et al. 2019_a), PdN efficiencies were not adversely affected in this experiment when the reactors were operated at lower concentrations of 1-2 mgNO3-N/L.

Ammonia Removal

Throughout operation, the glycerol-dosed reactors had higher ammonia removal than methanol-dosed reactors, some of which can be attributed to greater assimilation for glycerol based on its higher yield. Ammonia removal was compared with adjusted removals accounting for calculated maximum assimilation based on yields from each carbon source and expected nitrogen content of 0.07 gN/gBiomass as COD (Figure 21). An increase in ammonia removal is visible in the preliminary biofilm carrier reactor dosed with glycerol towards the end of operation, which was consistent with the onset of observable AMX activity.



A: methanol virgin, **B**: methanol preliminary biofilm, **C**: glycerol virgin, **D**: glycerol preliminary biofilm

Figure 21: Ammonia removal and Ammonia removal accounting for maximum assimilation. Methanol theoretical yield: 0.4 g/g, glycerol theoretical yield: 0.54 g/g. Influent source switch on day 184 represented by vertical dashed line.

Ammonia removal was initially analyzed between influent and effluent 24-hr composite samples. After several months of operation, calculations between influent and effluent ammonia showed ammonia production in methanol-dosed reactors. This was suspected to be inaccurate, so testing was completed that showed ammonia production from the composite sampler for the influent wastewater source. Thus, after the first 115 days of operation, a switch was made to grab samples and ammonia removal became more consistently positive in methanol-dosed reactors. After switching influent sources on day 184, ammonia removal briefly decreased and then increased again likely due to OHO adjustment to the new influent source. AMX activity was evaluated immediately before the time of the influent switch and was not confirmed.

Negative ammonia removals were again observed in all reactors between days 215 and 227 due to effluent ammonia being higher than influent ammonia. After a review of ammonia sensor readings from the influent source, this was determined to be due to AvN control issues and variable ammonia sensor readings. Because influent and effluent grab samples were taken simultaneously, the appearance of ammonia production in the MBBRs was suspected to be a relic of the HRTs of the reactors; in all instances, ammonia was higher in the influent source one hour prior to sampling than at the time of sampling, indicating that the 1 hr HRT of each reactor could have resulted in higher ammonia in the MBBRs at the time of sampling. The inaccurate removals observed during this time were removed from Figure 21.

AMX Activity

AMX activity tests were conducted on all four PdNA MBBRs throughout operation, with one such test exemplified by Figure 22. After the first period of 184 days, no activity was detected. Due to the lack of AMX activity during that period, in which reactors were fed semi-synthetic wastewater, it is theorized that the composition of the feed was inhibitory and not conducive to AMX growth.

After 224 days of operation, the glycerol preliminary biofilm carrier reactor showed signs of AMX activity. This activity began only 40 days after switching the influent feed from a semi-synthetic feed to B-Stage effluent, further suggesting that the previous feed was inhibiting AMX growth. Ultimately, this result indicates that preliminary biofilm carriers have a faster AMX attachment rate than virgin carriers and that glycerol's higher PdN efficiency throughout operation could have encouraged better AMX growth by providing more consistent nitrite.

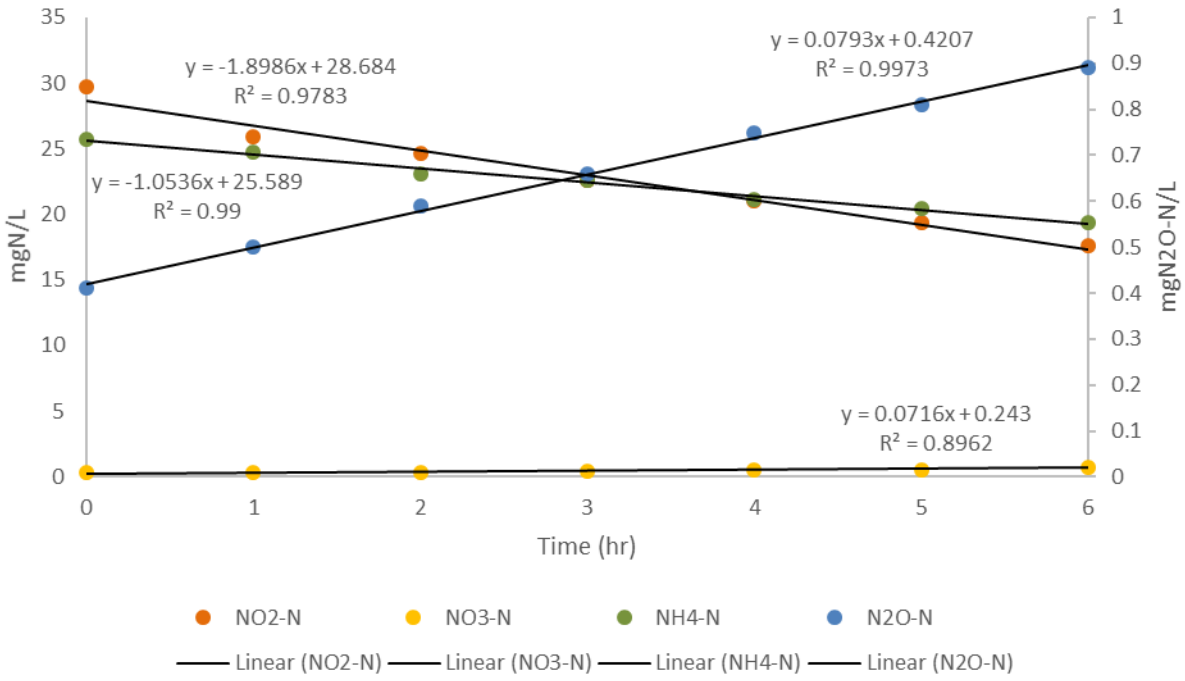


Figure 22: Example AMX activity test on preliminary biofilm carrier reactor dosed with glycerol on day 245.

After the discovery of AMX, maximum ammonia and TIN removal rates increased and N_2O accumulation percentage decreased in subsequent activity tests on the preliminary biofilm carrier reactor dosed with glycerol. The virgin carrier reactor dosed with glycerol and the virgin and preliminary biofilm carrier reactors dosed with methanol still have not exhibited signs of AMX activity after 272 days of operation.

It is important to note that the wastewater treatment plant from which the preliminary biofilm carriers were sourced (mainstream IFAS process) had a sidestream ANITA™ Mox process that recycled to a point before which the IFAS process operated. This could have potentially provided AMX to the carriers, but is unlikely to have impacted the AMX activity observed in this experiment due to the sources providing wastewater to the reactors having no AMX-based processes in operation. However, to fully understand if the ANITA™ Mox process at JRTP had an impact, this experiment should be repeated with preliminary biofilm carriers sourced from a wastewater treatment plant operating without any AMX-based processes.

Total Solids Measurements

In the preliminary biofilm carrier reactors, the total solids decreased throughout operation (Figure 23). The virgin media initially increased in total solids and remained steady for the remainder of operation.

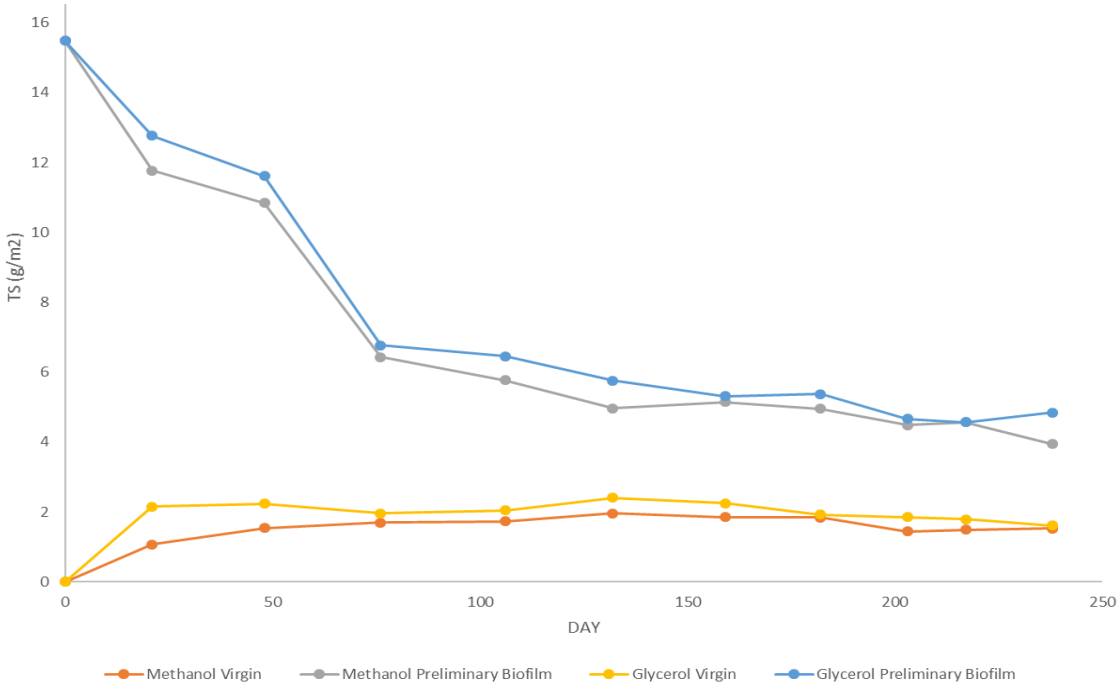


Figure 23: Total solids measurements throughout operation.

The preliminary biofilm carrier reactor results were in contrast with biomass measurements in an experiment using preliminary biofilm carriers to increase AMX attachment rate in a sidestream ANITA™ Mox process. That experiment showed an increase in carrier biomass over initial IFAS biomass at the end of the experiment, but a decrease in carrier biomass throughout operation (Klaus et al. 2016). The initial decrease in carrier biomass observed in the preliminary biofilm carrier MBBRs may have been due to sloughing of some of the dead and inactive OHO and nitrifier population after the carriers were moved from an aerobic IFAS system to a fully anoxic MBBR.

N_2O Production

After 209 days of operation, and 25 days after switching from a semi-synthetic wastewater feed to B-Stage effluent, in situ N_2O measurements were begun. These measurements were initiated before AMX activity was confirmed. As discussed previously, it was hypothesized that continuously low nitrite concentrations in the reactor under PdNA conditions would prevent significant N_2O generation by OHO partial denitrification. However, this hypothesis was only

applicable when AMX activity was high enough to consume nitrite and prevent further denitrification to N_2O.

In situ N_2O accumulation in all reactors was low despite observable AMX activity in only one of the four reactors during the majority of N_2O measurements. The virgin and preliminary biofilm carrier reactors dosed with methanol averaged 0.02 ± 0.03 mgN2O-N/L and 0.01 ± 0.01 mgN2O-N/L, respectively, while the virgin and preliminary biofilm carrier reactors dosed with glycerol averaged 0.06 ± 0.05 mgN2O-N/L and 0.06 ± 0.1 mgN2O-N/L, respectively. Those measurements corresponded to an average of 0.72 ± 0.01%, 0.24 ± 0.00 %, 1.29 ± 0.01%, and 0.70 ± 0.01 % of nitrate removal, respectively.

N_2O measurements were also recorded in the preliminary biofilm carrier reactor dosed with glycerol during AMX activity tests. The N_2O concentrations observed during batch AMX activity tests were higher than in situ production, which was expected due to the higher amounts of nitrite dosed to reactors for batch tests. More nitrite was removed than can be explained by the stoichiometric utilization by AMX, suggesting that some was emitted as N_2O, nitric oxide (NO), or through full denitrification to nitrogen (N_2) gas. However, as AMX activity increased, N_2O production during batch tests decreased. N_2O accumulation during AMX activity tests decreased from 38% to 8% of NO_x removal during tests over the course of one month while AMX activity simultaneously increased.

Conclusions

1. Startup of a mainstream PdNA MBBR is possible without any AMX biomass seeding.
2. Preliminary biofilm carriers led to faster AMX activity than virgin carriers.
3. The use of glycerol as a carbon source led to faster AMX activity than methanol.
4. Glycerol reactors had higher average PdN efficiencies than methanol reactors.
5. Virgin carriers had higher average PdN efficiencies than preliminary biofilm carriers dosed with the same carbon source.
6. PdN efficiencies were not negatively impacted by nitrate residuals as low as 1-2 mgNO3-N/L.
7. PdN was achieved within 3 weeks for all reactors.
8. In situ N_2O accumulation was low.
9. N_2O accumulation decreased during batch AMX activity tests as AMX activity increased.
10. Synthetic wastewater is not conducive to AMX growth, particularly if dechlorination is required.

This experiment showed that preliminary biofilm carriers encouraged faster AMX attachment than virgin carriers and that glycerol encouraged AMX growth before methanol. Though startup was achieved in this experiment without any AMX biomass seeding, this research should be repeated with carriers sourced from a treatment plant without any AMX-based processes and with a non-synthetic wastewater source in order to determine the true startup time for AMX in a mainstream PdNA MBBR.

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Summary and Engineering Significance

This research showed that anammox (AMX) biomass inoculation is not required to quickly start a mainstream AMX-based process in an MBBR, which has never been proven based on an extensive literature review. In two instances, mainstream MBBRs with AMX-based processes were started with carriers sourced from an aerobic integrated fixed-film activated sludge (IFAS) process with a preliminary biofilm of heterotrophs. In one experiment, the reactor was fed mainstream ammonia and nitrite concentrations based on the stoichiometry for AMX growth with no external carbon dosed to the reactor. AMX activity was confirmed in this reactor after 84 days of operation, with evidence that activity began a few weeks before confirmation. AMX activity rates continued to increase throughout operation, but maximum ammonia and total inorganic nitrogen (TIN) removal rates were never reached in situ, indicating that a higher loading rate could have been used.

In another experiment, four mainstream partial denitrification/AMX (PdNA) reactors were started using two different carbon sources, methanol and glycerol, and two different carrier types, virgin and preliminary biofilm carrier reactors from an IFAS process. All four reactors achieved partial denitrification (PdN) right away, and PdN efficiency was not negatively affected by nitrate residuals of 1-2 mg NO3-N/L. Overall, glycerol-dosed reactors had higher PdN efficiencies than methanol-dosed reactors and virgin carrier reactors had higher PdN efficiencies than preliminary biofilm carrier reactors dosed with the same carbon source throughout operation. This experiment resulted in the preliminary biofilm carrier reactor dosed with glycerol achieving AMX activity first, indicating that preliminary biofilm carriers lead to faster AMX attachment and that glycerol's higher PdN efficiency possibly encouraged faster growth. However, this reactor did not show signs of AMX activity until 224 days of operating, 40 days after a non-synthetic wastewater influent source was used. This indicated that the first semi-synthetic influent source used was not conducive to AMX growth and startup could have been achieved faster with a non-synthetic feed. Nitrous oxide (N_2O) was also measured in situ throughout all four reactors resulting in low accumulation and results from maximum AMX activity batch tests showed a decrease in accumulation with an increase in AMX activity.

The discoveries provided by this research suggest that AMX-based technologies such as PdNA could be more accessible to a larger range of municipal wastewater treatment plants (WWTPs) for full-scale utilization. Based on an extensive literature review, full-scale mainstream PdNA in an MBBR has never been practiced at any wastewater treatment plant. In fact, only one mainstream PdNA application has been used at full scale in a deep-bed filter configuration (Klaus et al. 2020). The use of mainstream PdNA processes would be able to significantly decrease aeration and carbon needs of wastewater treatment, while offering cost, energy, and space savings. It is well known that aeration is one of the major energy inputs associated with WWTPs, so expanding this technology will significantly decrease the environmental and economic expenses of WWTPs. By understanding the requirements for startup of MBBRs without AMX biomass seeding, this technology can be more easily implemented at a lower cost and may become more commonly used.