Bioaugmentation and Retention of Anammox Granules to a Deammonification Bio-Oxidation Process Pilot with an Anoxic Partial Denitrification/Anammox Polishing Moving Bed Biofilm Reactor

Cody James Campolong

Thesis submitted to the faculty of the

Virginia Polytechnic Institute and State University

In partial fulfillment of the requirements for the degree of

Master of Science

In

Environmental Engineering

Charles B. Bott

John T. Novak

Jason He

2/14/2019

Keywords: Mainstream deammonification, BNR, Anammox retention, anammox, PdN/A

Bioaugmentation and Retention of Anammox Granules to a Deammonification Bio-Oxidation Process Pilot with an Anoxic Partial Denitrification/Anammox Polishing Moving Bed Biofilm Reactor

Cody James Campolong

Abstract

The Chesapeake Bay watershed has seen an increase in population, nutrient loading, and stringent effluent limits; therefore, cost-effective technologies must be explored and implemented to intensify the treatment of regional wastewater.

This work describes the bioaugmentation and retention of anammox (AMX) granules in a continuous adsorption/bio-oxidation (A/B) mainstream deammonification pilot-scale process treating domestic wastewater. The AMX granules were collected from the underflow of a sidestream DEMON® process. The bioaugmentation rate was based on several factors including full-scale sidestream DEMON® wasting rate and sidestream vs mainstream AMX activity. The retention of bioaugmented AMX granules required a novel settling column at the end of the deammonification step. The settling column was designed to provide a surface overflow rate (SOR) that allowed dense AMX granules to settle into the underflow and less dense floccular biomass to outselect into the overflow. B-Stage was operated to out-select nitrite oxidizing bacteria (NOB) by maintaining an ammonia residual (>2 mg NH_4-N/L), a relatively high dissolved oxygen (DO) (>1.5 mg O_2/L) concentration, an aggressive solids retention time (SRT) for NOB washout, and intermittent aeration for transient anoxia. AMX activity was not detected in the mainstream at any time. The settling column AMX retention quantification suggested but did not confirm AMX were maintained in the mainstream. NOB were not suppressed during this study and no nitrite accumulation was present in the mainstream process. It was theorized that AMX granules were successfully settled into the settling column underflow and accumulated in the intermittently mixed sidestream biological phosphorus reactor (SBPR) where they disintegrated.

This work also describes optimization of carbon addition to an anoxic partial denitrification anammox (PdN/A) moving bed biofilm reactor (MBBR) testing glycerol, acetate, and methanol as carbon sources to maximize total inorganic nitrogen (TIN) removal through the anammox pathway and to minimize effluent TIN. A carbon feeding strategy was developed and was evaluated by the extent of partial denitrification vs full denitrification (partial denitrification efficiency, PdN efficiency). All three carbon sources were capable of high TIN removal, low effluent TIN, and moderate to high PdN efficiency. Average TIN removal for glycerol was 10.0 ± 3.6 mg TIN/L, for acetate it was 8.7 ± 2.9 mg TIN/L, and for methanol it was 11.5 ± 5.6 mg TIN/L. Average effluent TIN for glycerol was 6.0 ± 4.0 mg TIN/L, for acetate it was 5.0 ± 1.1 mg TIN/L, and for methanol it was 4.3 ± 1.5 mg TIN/L. Average PdN efficiency for glycerol was 91.0 ± 9.0%, for acetate it was 88.0 ± 7.7%, and for methanol it was 74.0 ± 8.5%. When PdN efficiency was factored into the cost of each carbon source, methanol was 5.83% cheaper than glycerol per mass TIN removed and 59.0% cheaper than acetate per mass TIN-N removed.

Bioaugmentation and Retention of Anammox Granules to a Deammonification Bio-Oxidation Process Pilot with an Anoxic Partial Denitrification/Anammox Polishing Moving Bed Biofilm Reactor

Cody James Campolong

General Audience Abstract

The Chesapeake Bay watershed has seen an increase in population, nutrient loading, and stringent effluent limits; therefore, cost-effective technologies must be explored and implemented to intensify the treatment of regional wastewater. This work involves removing nitrogen from wastewater in a pilot sized modeled from a real wastewater treatment plant. The removal of nitrogen from wastewater can become costly. This cost is due to aeration and chemical demands to remove the nitrogen. This masters work uses a type of microorganism that can remove nitrogen without the need for aeration or chemicals through anaerobic ammonia oxidation (AMX bacteria).

A specific environment has been created for AMX bacteria during this study to ensure they perform nitrogen removal optimally. Often times, communities of bacteria can help remove nitrogen more effectively when they work together. Therefore, communities of bacteria were encouraged to grow during this study. We were able to see that nitrogen removal was indeed occurring at high rates and producing high effluent water quality. We used several different metrics to prove this nitrogen removal technology worked well. This research was important because it showed the capabilities of a highly intensified process of successful nitrogen removal at a pilot-scale facility. It is the hope that these findings can be improved upon and implemented at full-scale facilities. These full-scale facilities would be able to achieve low levels of nitrogen in their effluent while saving millions of dollars on operational costs.

Acknowledgements

I would like to thank Dr. Charles Bott for the amazing opportunity to grow and learn as a part of his water technology and research team. I admire your expertise in environmental engineering and your relentless work ethic. I would like to thank my other committee members, Dr. John Novak and Dr. Jason He for their guidance and support.

I would like to thank Stephanie Klaus for literally everything. Words cannot express how grateful I am for your endless guidance and support. I consider myself extremely fortunate to have you as my mentor. I especially want to thank you for answering my on call questions in the middle of the night, articulating the answers to my millions of questions at the pilot, and for possessing the patience to hear out my off-the-wall ideas and theories.

I would like to thank Lindsey Ferguson for her collaboration throughout our Masters journey. I thoroughly enjoyed our creative brainstorms in the box and working side by side with you every day.

Also, I would like to thank Kathryn Printz, Kester McCullough, Sarah Schoepflin, and the undergraduate interns for their help and hard work at the pilot.

Thank you to Hampton Roads Sanitation District for funding my Masters work. Also, thank you to all the employees at the Chesapeake-Elizabeth Wastewater Treatment Plant who aided with the construction, maintenance, and instrumentation during my tenure at the pilot.

Finally, I would like to acknowledge the love and support of my parents, my sister, and The Crew. Everything I have done is dedicated to you!

Contents

List of Tables viii

List of Figures ix

1. Introduction 1

2. Literature Review 5

Nitrification and Denitrification 5

Adsorption/Bio-oxidation Process 8

Deammonification 9

Partial Denitrification/Anammox 11

Mainstream Deammonification Approaches 13

Suspended Growth Granular System 13

Attached Growth Biofilm System 14

Hybrid System 14

Two-Stage System 15

Mainstream NOB Suppression 15

Free Ammonia 15

Ammonia Residual 15

Temperature condition 16

Inorganic Carbon Limitation 16

Bioaugmentation 16

Transient Anoxia 17

Target DO 17

Influent COD Fractionation 18

Greenhouse Gas Emissions 18

Comammox 18

Aeration Control Strategies 19

References 20

3. Manuscript 1: AMX Bioaugmentation and Retention 32

Abstract 32

Introduction 32

Materials and Methods 33

Intermittent Ammonia vs NOx Control 35
AMX Granules Origin and Storage 36
Column Design 36
General Sample Monitoring 38
AOB/NOB Activity Rate Measurement 38
Denitrification Rate Measurement 38
Anammox Activity Rate Measurement 38
Heme Quantification Measurement 39
Initial Settling Velocity (ISV) and Sludge Volume Index (SVI) Measurements 39
Column TSS Gradient Measurements 40
Results and Discussion 40
NOB Suppression 40
Column AMX Retention 41
Settling Column Monitoring 45
AMX Stock and Mainstream Act 46
Conclusions 49
References 49
4. Manuscript 2: Anoxic PdN/AMX Polishing MBBR 51
Abstract 52
Introduction 52
Materials and Methods 54
General Sample Monitoring 56
Carbon Dosing Control Strategy 56
Ex Situ Denitrification Rate Measurement 56
Anoxic PdN/A MBBR in situ Denitrification Rate Analysis 56
Anoxic PdN/A MBBR in situ AMX Activity Analysis 57
Fluorescent in situ hybridization (FISH) Analysis 57
Microbial Quantification 58
Carbon Sources Used, Origin, and Stock Generation 58
Methanol Dosing Transition Phase 58
Methanol Overdose Experiment 59
Methanol TIN Loading Increase 59

Results 59
PdN/A Performance 59
PdN/A Denitrification Activity 63
AMX Activity Monitoring 65
Microbial Quantification 67
FISH Microscopy Analysis 69
Discussion 70
Optimize carbon addition to maximize TIN removal and minimize TIN effluent levels. 70
Compare glycerol, acetate, and methanol performance as carbon sources. 71
AMX biomass went down but activity remained maxed out and the magnitude of removal increased. 73
PdN may have been a result of a specifically cultured species of partial denitrifier. 73
Conclusions 74
Acknowledgments 75
References 75
Appendix A: Supplementary Material 79
A.1. Engineering Significance 84

List of Tables

Table 1: Adapted characteristics of sludge samples from the Strass WWTP (Marie et al, 2015). SL = sludge liquor from the DEMON tank (Sidestream). B = Biological tank (mainstream). OF = overflow of the cyclone. UF = underflow of the cyclone. 48
Table 2: Carbon source yield values. 70
Table 3: Carbon source performance statistics. 71
Table 4: Carbon source cost comparison based on yield values. 72
Table 5: 16s rRNA-targeted oligonucleotide probes used in this study. 80

List of Figures

Figure 1: A map of the HRSD service area and WWTP locations (https://www.hrsd.com/about-us)..... 2
Figure 2: An aerial photo the Hampton Roads Sanitation District's York River Wastewater Treatment Plant with process annotations. 3
Figure 3: Conventional nitrification and denitrification pathways for nitrogen removal with requirements annotated. 5
Figure 4: Specific nomenclature for the pathways of nitrification and denitrification. 6
Figure 5: Deammonification = Partial Nitritation + Anammox 10
Figure 6: A schematic illustrating the PdN/A process pathways as well as the nitrate recycle factor. 12
Figure 7: CE Pilot process flow diagram. 34
Figure 8: B-Stage AvN control schematic that shows the relationship between AvN ratio setpoint and aerobic fraction. 35
Figure 9: Column design schematic shows inflows, outflows, and relevant dimensions. 37
Figure 10: Column photograph shows influent flow combining with internal recycle flow. 37
Figure 11: NOB/AOB Rate ratio vs NO_2^- in B-Stage. 40
Figure 12: NOB/AOB Rate ratio vs NAR in B-Stage. 41
Figure 13: ISV measurements over time for UF, B-Stage MLSS, and OF with respect to a change in SOR. 42
Figure 14: Heme AU/g VSS over time for UF, B-Stage MLSS, OF, and A-Stage MLSS. Note: JR MLSS shows the change in heme content over time from one sample. 43
Figure 15: SVI5 over time for UF, B-Stage MLSS, and OF. 44
Figure 16: SVI30/SVI5 ratio over time for UF, B-Stage MLSS, and OF. 45
Figure 17: TSS over time for UF, B-Stage MLSS, and OF 46
Figure 18: AMX Stock granule activity test showed changes in ammonia, nitrite, nitrate, and TIN over time. 47
Figure 19: Mainstream AMX activity test showed changes in ammonia, nitrite, nitrate and TIN over time. 48
Figure 20: CE Pilot process flow diagram. 55
Figure 21: MBBR influent and effluent nitrogen species concentrations for glycerol, acetate, and methanol. Top Left – nitrite orange circles, Top Right – ammonia blue squares, Bottom Left – nitrate green triangle, and Bottom Right – TIN black diamonds. Vertical dotted lines indicate start and end days for each carbon phase. 60
Figure 22: PdN efficiency values for glycerol, acetate, and methanol are shown with purple circles. Vertical dotted lines indicate start and end days for each carbon phase. 62
Figure 23: Theoretical NOx removed/NH_4-N removed was 1.06 g/g and was compared to the composite NOx removed/NH_4-N removed. Vertical dotted lines indicate the start and end days for each carbon phase 63
Figure 24: MBBR theoretical sCOD used for PdN (green dot dash line) and FdN (red solid line), and of actual composite data sCOD used for NO_3-N. Top Left – composite data for whole study, Top Right – glycerol phase data, Bottom Left – acetate phase data, and Bottom Right – methanol phase data. Vertical dotted lines indicate start and end days for each carbon phase. 64

Figure 25: MBBR TIN removal rates from maximum AMX activity experiments (black rectangles) and MBBR TIN removal rates from in situ performance measurements (black diamonds). MBBR NH_4-N removal rates from maximum AMX activity experiments (blue rectangles) and MBBR NH_4-N removal rates from in situ performance measurements (black diamonds). Vertical dotted lines indicate start and end days for each carbon phase. 66
Figure 26: Top Left- AMX relative abundance of the total microbial community. Top Right- AMX relative abundance of the total microbial community per g/m^2. Bottom Left- AMX bacteria copies per g/m^2. Bottom Right- AMX bacteria copies per mg/tube. 68
Figure 27: Left- A taxonomic distribution of specific microbial population count on the MBBR media. Right- A taxonomic distribution of specific microbial populations on the MBBR media as a percentage of the total copies in the microbial community. 68
Figure 28: FISH imaging of the PdN/A MBBR media. Red is AMX bacteria, white is DAPI staining for overall cell abundance detection, green is AOB, and blue is *nitrospira*. 69
Figure 29: Denitrification activity experiment with all three carbon sources. No ammonia was present during these tests, and there was no AMX activity. Several iterations of this test were performed to obtain theoretical sCOD used/PdN ratios. Top Left-In situ MBBR using acetate. Top Right-In situ MBBR using glycerol. Bottom Left-In situ MBBR using methanol. Bottom Right-Ex situ MBBR bulk liquid using glycerol. 81
Figure 30: Known theoretical AMX metabolic stoichiometric ratios and actual AMX metabolic stoichiometric ratios from MBBR maximum AMX activity experiments for glycerol, acetate, and methanol. Vertical blue lines indicate start and end dates for each carbon phase. 82
Figure 31: Nitrogen removal rates for NH_4-N, NO_2-N, TIN-N, and production rates for NO_3-N from MBBR maximum AMX activity experiments for glycerol, acetate, and methanol. Vertical blue lines indicate start and end dates for each carbon phase. 83
Figure 32: MBBR effluent NO_3-N composite values and MBBR effluent NOx setpoint. The blue line denotes the start of the carbon dosing control strategy. If no NOx setpoint is shown, then the carbon dosing pump was in manual control at a constant rate. 84

1. Introduction

Overall population and city density has increased and has elevated wastewater treatment plant (WWTP) load, and this combined with stricter nutrient effluent limits has generated a need for process intensification to treat both point sources (WWTP and storm water discharge) and non-point sources (urban and agriculture runoff) to protect our most precious resource, water. Although a global issue, regional municipalities have a responsibility to protect local waterways such as the Chesapeake Bay Watershed.

The Chesapeake Bay Watershed spans Virginia, West Virginia, Maryland, Delaware, New York, and Pennsylvania. Over the past few decades, the environmental condition of the bay has declined. The principle concern is nutrient pollution. A lack of adequate mixing in the bay from tidal and ocean current movement has allowed nutrients like nitrogen and phosphorus to accumulate and cause eutrophication. This excess of nutrients causes algal blooms which inhibit sunlight penetration for aquatic vegetation, and decaying algal biomass elevates dissolved oxygen demand and limits or eliminates marine life.

Regulations have forced entities like Hampton Roads Sanitation District (HRSD) to take action to lower the negative environmental impact on the bay. The EPA implemented a Chesapeake Bay Total Maximum Daily Load (TMDL) which limits the nitrogen discharge to 185.9 million pounds, phosphorus to 12.5 million pounds, and sediment to 6.45 billion pounds per year (EPA, 2010). The TMDL forced the local environmental industry to develop useful technologies to mitigate the threat to the bay. HRSD is the wastewater municipality for Southeastern Virginia. HRSD is comprised of 9 major and 7 minor treatment plants with a combined flow capacity of 250 million gallons per day (MGD) (Figure 1). The processed wastewater discharges into the Atlantic Ocean along with the Rappahannock, York, and James Rivers.

Figure 1: A map of the HRSD service area and WWTP locations (https://www.hrsd.com/about-us).

Each river basin has an EPA mandated waste load allocation; therefore, each WWTP is assigned an annual average waste load allocation for total nitrogen (TN) and total phosphorus (TP). HRSD determined that upgrading the current 15 MGD York River (YR) WWTP was the most cost effective solution to continue to meet more stringent nutrient levels (Figure 2).

Figure 2: An aerial photo the Hampton Roads Sanitation District's York River Wastewater Treatment Plant with process annotations.

As a result of the limitations on both cost and footprint, a concept called intensification must be applied to treatment processes. Intensification is the combination of reducing cost and footprint via decreased operation and maintenance cost (O&M) to achieve better results. Another way to look at intensification is achieving better performance without sacrificing efficiency or achieving better efficiency without sacrificing performance. In general, a smaller footprint technology has a higher cost because of additional energy and chemical requirements, vice versa. For example, a lagoon occupies a larger footprint than a moving bed biofilm reactor (MBBR), but a MBBR produces better effluent quality with a higher cost due to added instrumentation, monitoring, sensors, and media.

There is a strong need to remove nitrogen and phosphorus from WWTP discharge because of eutrophication and algal blooms. A conventional biological nutrient removal (BNR) process called nitrification-denitrification is adequate at removing nitrogen but other BNR processes can intensify the nitrogen removal process. Anaerobic ammonia oxidation or anammox (AMX) converts ammonia and nitrite to nitrogen gas without using carbon or aeration. Utilizing AMX for BNR has the potential to highly intensify nitrogen removal from wastewater.

Deammonification is a highly intensified BNR process that combines AMX and partial nitritation. Establishing and maintaining an AMX community in a temperate climate mainstream WWTP BNR process is extremely difficult. Also, the slow doubling time of AMX bacteria results in a very long startup period before the treatment process can be fully operational. A solution to these obstacles is to bioaugment AMX biomass from an established AMX process. The newly seeded AMX biomass would require a retention mechanism to ensure it has a high enough solids retention time (SRT) to proliferate. AMX can be bioaugmented to the B-Stage process from the sidestream DEMON® process at YR. This DEMON® process cultivates and accumulates AMX granules that can be collected for bioaugmentation. There are two main objectives for this research component:

1. Calculate and use an appropriate AMX granule bioaugmentation rate from the sidestream DEMON® process at YR to the CE Pilot B-Stage mainstream deammonification process.
2. Achieve high AMX granule retention percentage in the mainstream via a selection mechanism.

The mainstream deammonification strategy employed in this study used intermittently aerated ammonia vs NOx (AvN) control which was dictated by an AvN ratio setpoint. This means that the ratio of NOx/NH_4 was controlled and not the effluent magnitude of NOx or NH_4. To meet stringent effluent limits a polishing unit would be needed. Using AMX for the polishing unit would be a low cost option, but nitrate would be present from the mainstream deammonification effluent and the small amount produced by AMX metabolism. Therefore, adding a restrictive amount of carbon to the AMX polishing unit would allow denitrifying bacteria to convert nitrate to nitrite (denitratation) and AMX would have the ability to use the nitrite for further TIN removal through the AMX pathway. The partial denitrification and AMX (PdN/A) polishing MBBR would also be a safeguard against disturbances in the deammonification process because it has the ability to remove ammonia, nitrite, and nitrate in a single reactor prior to discharge. The key is to optimize the carbon added for maximum TIN removal across the polishing process to obtain extremely low TIN concentrations. Traditionally, methanol was the carbon source used for full denitrification polishing processes, but other carbon sources such as glycerol and acetate may perform denitratation more effectively. There are two main objectives for this research component:

1. Optimize carbon addition to an anoxic PdN/A MBBR polishing unit to maximize TIN removal and minimize TIN effluent levels.
2. Compare glycerol, acetate, and methanol as carbon sources for the PdN/A MBBR polishing process.

Research was conducted at the Chesapeake-Elizabeth WWTP pilot (CE Pilot) in Virginia Beach, Virginia operated as an adsorption/bio-oxidation (A/B) process at 20°C. The A-Stage was a high-rate activated sludge (HRAS) process that focused on carbon capture for carbon diversion. The B-stage implemented mainstream deammonification with AMX bioaugmentation and retention combined with biological phosphorus removal (Bio-P). Previous research at the CE Pilot achieved partial nitritation through AvN intermittent aeration control and subsequent nitrite oxidizing bacteria (NOB) outselection under the operational conditions of this study. A fully anoxic polishing PdN/A MBBR removed the remaining nitrate through partial denitrification and residual ammonia along with nitrite through AMX. The main

goal of the CE Pilot research was to model HRSD's YR WWTP at pilot-scale with the intention to upgrade and retrofit YR based on the results of the CE Pilot.

2. Literature Review

Nitrification and Denitrification

Since ammonia is toxic to aquatic life (increase of pH and oxygen demand) and is the predominant form of nitrogen in WWTP influent, a widely known and utilized biological nutrient removal (BNR) process to remove nitrogen from wastewater called nitrification and denitrification was developed (Bitton, 2011) (Figure 3). If nitrate is not removed from wastewater, suffocation due to methemoglobinemia or "blue baby syndrome" can occur from residual NO_3^- consumption by infants who are not able to bind oxygen to hemoglobin because of the elevated levels of nitrite in their gastrointestinal tract. Also, if high concentrations of nitrogen and phosphorus are discharged into waterways, eutrophication will ensue. Eutrophication causes algal blooms which inhibit sunlight penetration for aquatic vegetation, and decaying algal biomass elevates dissolved oxygen demand and limits or eliminates marine life.

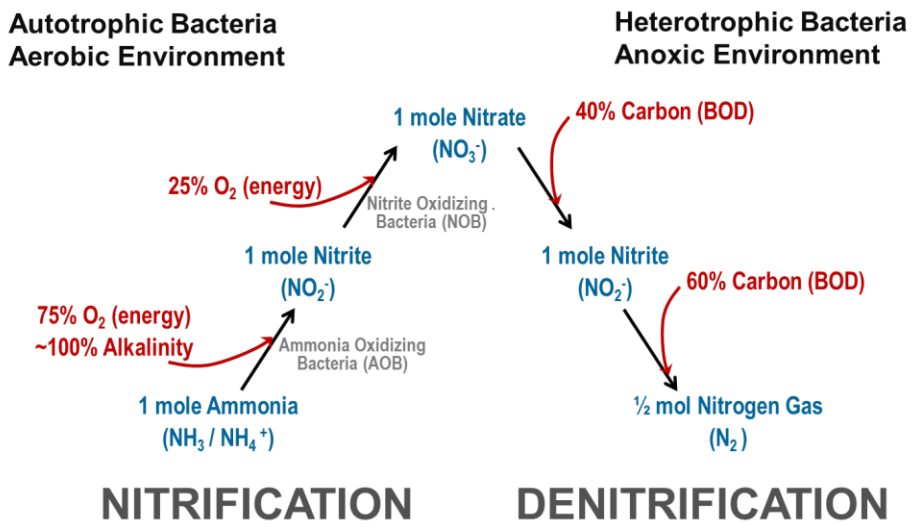

Figure 3: Conventional nitrification and denitrification pathways for nitrogen removal with requirements annotated.

Nitrification occurs aerobically by two main types of autotrophic bacteria. Ammonia oxidizing bacteria (AOB) oxidize ammonia to nitrite during nitritation, and nitrite oxidizing bacteria (NOB) oxidize nitrite to nitrate during nitratation. Denitrification occurs anoxically by heterotrophic bacteria in five steps (oxidation state): NO_3^- (+5) to NO_2^- (+3) to NO (+2) to N_2O (+1) to N_2 (0). Some autotrophic microorganisms are capable of denitrification, but heterotrophs called *Pseudomonas* are the dominant species in wastewater denitrification treatment. The microorganisms involved in denitrification are

mostly facultative heterotrophs called denitrifying bacteria, and true denitrifying bacteria reduce nitrate to nitrite during denitratation, then reduce nitrite to nitrogen gas during denitritation while partial denitrifying bacteria only perform a portion of the denitrification steps (Drysdale et al, 2001; Gardner, 2008) (Figure 4). Since the nature of denitrifying bacteria is facultative, then it is possible that a single species of denitrifying bacteria is capable of performing partial or full denitrification. But, there are certain species that tend to be more prevalent when more partial denitrification is occurring. *Thauera* seem to dominate in partial denitrification systems and could be specifically suited for partial denitrification (Liu et al, 2013). More specifically, incomplete denitrifiers and incomplete nitrite reducers make up the majority of the denitratation microbial community (Drysdale et al, 2001). Incomplete denitrifiers lack nitrite reductase enzymes (Robertson and Kuenen, 1992). Nitrite accumulation in denitrification systems is a result of the predominant presence of incomplete denitrifiers (Rheinheimer, 1985; Robertson and Kuenen, 1992). Incomplete nitrite reducers may have a nitrite reductase enzyme that is experiencing inhibition due to large quantities of nitrate, but a more likely scenario is that nitrates are favored over nitrites because of higher electron accepting capacity (Drysdale et al, 2001).

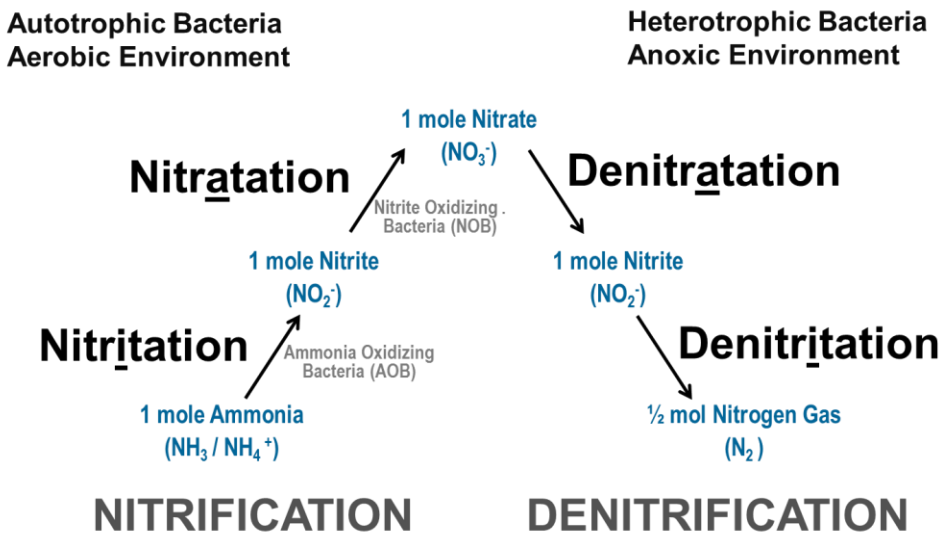

Figure 4: Specific nomenclature for the pathways of nitrification and denitrification.

The stoichiometry involved with the various steps in nitrification and denitrification is well documented and includes biomass growth considerations (Eq. 1 – Eq. 6) (Grady, 2011). Since full nitrification requires 7.14 g $CaCO_3$/g NH_4^+, alkalinity addition is needed to maintain an optimal pH range of 7.5-8.0 (Grady, 2011; Burton et al, 2013). Compared to ordinary heterotrophic organisms (OHO), these slow growing nitrifiers require a solids retention time (SRT) typically in the range of 4 to 20 days depending on influent temperature and composition (Grady 2011). Denitrifiers require carbon for their metabolism, and although influent carbon is available, external carbon sources such as glycerol, acetate, and methanol may be needed to supplement the denitrification step.

Nitritation by AOB:

$NH_4^+ + 1.5O_2 \rightarrow NO_2^- + 2H^+ + H_2O$ (Eq. 1)

Nitratation by NOB:

$NO_2^- + 0.5O_2 \rightarrow NO_3^-$ (Eq. 2)

Nitrification:

$NH_4^+ + 2O_2 \rightarrow 2NO_3^- + 2H^+ + H_2O$ (Eq. 3)

Nitrification Considering Biomass Growth:

$NH_4^+ + 6.708HCO_3^- + 3.300O_2 \rightarrow 3.373NO_3^- + 1.041H_2O + 6.463H_2CO_3 + 0.129C_5H_7O_2N$ (Eq. 4)

Denitrification pathway:

$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$ (Eq. 5)

Denitrification with Domestic Wastewater:

$C_{10}H_{19}O_3N + 10NO_3^- \rightarrow 5N_2 + 10CO_2 + 3H_2O + NH_3 + 10OH^-$ (Eq. 6)

The growth and subsequent competition among microbial communities can be modeled with Monod kinetic equations that have many parameters like maximum specific growth rate and various substrate concentrations (Eq. 7). The parameters are temperature dependent and several values are reported in literature and used in BNR design (Burton et al, 2013; Sedlak, 1991; Randall, 1998; Wett et al, 2003; Sin et al, 2008; Kaelin et al, 2009; Manser et al, 2006; Salem et al, 2006). Furthermore in nitrification, several factors such as temperature, pH, and availability of substrates influence the maximum growth rate of various microorganisms (3.8 d^{-1} for heterotrophs, 0.9 d^{-1} for AOB and 0.7d^{-1} for NOB) (Kayombo et al, 2003; Knowles et al, 1965). It is important to control the parameters that maximize the growth rate of the desired microorganisms in a given microbial community. For example, non-limiting carbon must be added to a full denitrification system to ensure maximum growth rate for denitrifiers and to reduce all nitrate and nitrite to nitrogen gas. Similarly parameters can be minimized to help lower the growth rate of unwanted microorganisms.

The basic form of the Monod equation is:

Monod Equation: $$\mu = \mu_{max}\left(\frac{S}{K_s+S}\right)$$ (Eq. 7)

where: μ = the specific growth rate (hr^{-1})

μ_{max} = the maximum specific growth rate (hr^{-1})

S = the limiting substrate concentration (mg/L)

K_s = the half saturation coefficient and the value of S when $\mu/\mu_{max} = 0.5$

Another important factor for conventional nitrification-denitrification processes is the influent fractionation of carbon. For instance, the denitrification rate is highly dependent on both the quantity and type of readily biodegradable chemical oxygen demand (rbCOD) but is not as dependent on temperature because of the rapidly adaptive nature of denitrifiers (Halling-Sørensen et al, 1993; Sutton et al, 1975).

Adsorption/Bio-oxidation Process

A one-stage treatment system creates competition among nitrifiers and heterotrophs for substrate, and tends to exhibit relatively high SRT, O_2 demand, and volume usage. Alternatively, a two-stage treatment system allows for COD oxidation in the first stage and NH_4^+ oxidation in the second stage resulting in less competition for substrates among microbial communities in either stage (Imhoff, 1955; Versprille et al, 1984). An adsorption/bio-oxidation (A/B) process allows for two-stage treatment of influent wastewater and involves two independent microbial communities to remove carbon and nitrogen. The A-Stage focuses on soluble carbon removal via aerobic heterotrophic assimilation and particulate carbon removal through absorption into the biomass extra polymeric substance (EPS) matrix. The physically and biologically accumulated carbon is ultimately removed from A-Stage through the waste activated sludge (WAS).

The target conditions for an A-Stage process are HRT ≤ 30 minutes, SRT = 3-12 hours, DO ≤ 0.5 mg/L, and a very high food to microorganism ratio (F/M) to grow highly adaptive bacteria with short doubling-times (Boehnke et al, 1998). When A-Stage is operated at very low HRTs, it could be considered a high rate activated sludge (HRAS) process. As an HRAS, A-Stage can produce large quantities of WAS to be diverted to an energy reclamation process such as an anaerobic digester (Schulze-Rettmer et al, 1998). High carbon removal in A-stage would allow for a much smaller B-Stage footprint to treat the nitrogen constituents. For retrofitting, the most notable advantage of a two-stage process would be the increase in B-Stage tank capacity due to the increased carbon diversion in A-stage to an energy recovery process. The adaptive and fast-growing microorganisms in A-Stage may be the key to high carbon removal and process stability. The A-Stage biomass is thought to be comprised of bacteria that effectively remove COD in a relatively small volume, and since the biomass is highly adaptive, shock loads of toxic compounds generate less of a disturbance to the downstream BNR process (Boehnke et al, 1998). The high removal of COD can be attributed to rbCOD taken up and assimilated into biomass while the colloidal and particulate COD is removed by EPS enmeshment (Miller et al, 2012). Since the majority of COD is removed in A-Stage, denitrification potential is limited in B-Stage, but also allows the B-stage BNR process to take on more specialized processes like deammonification to achieve low effluent TN (Brandt

et al, 2012). Although the overall COD removal in A-Stage is important, carbon diversion for energy recovery should be the main focus as opposed to heterotrophic metabolism that converts influent carbon to CO_2 that dissipates into the atmosphere.

Deammonification

The state of the practice for TN removal is biological nitrification-denitrification, but more cost effective technologies like deammonification are being investigated. The drivers for mainstream deammonification are the reduction of external carbon addition, decreased aeration demand for nitrogen removal, decreased need for carbon which allows for more carbon to be diverted for energy recovery, and an overall increase in tank capacity. A preceding carbon capture process is required to realize these benefits, as deammonification alone does not lead to process intensification (Cao et al, 2017; Jetten et al, 1997; Mulder, 2003; Siegrist et al, 2008).

Deammonification consists of partial nitritation and anaerobic ammonia oxidation (AMX) (Figure 5). Ammonia oxidizing bacteria (AOB) oxidize a portion of the available ammonia to nitrite, and AMX converts the residual ammonia and nitrite to nitrogen gas according to the following two equations (Strous et al, 1998). The optimal amount of NH_4^+ to oxidize to NO_2^- during partial nitritation is 55-60% of the influent ammonia (Yamamoto et al, 2008; Zheng et al, 2011). The AMX pathway was theorized in 1977, proven in 1995, and stoichiometrically documented in 1998 (Broda, 1977; Mulder et al. 1995; Strous et al, 1998). The stoichiometric equation for AMX metabolism is still widely recognized as the standard for AMX bacteria (Eq. 8).

Partial Nitritation:

$$2.34NH_4^+ + 1.87O_2 + 2.66HCO_3^- \rightarrow NH_4^+ + 1.32NO_2^- + 2.55CO_2 + 0.02C_5H_7O_2N + 3.94H_2O$$

Anammox Metabolism:

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

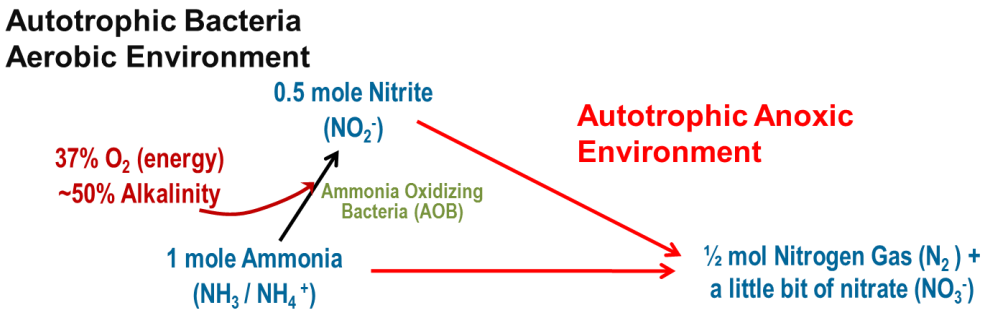

Figure 5: Deammonification = Partial Nitritation + Anammox

AMX bacteria are Planctomycetes, autotrophic, obligate anaerobes, and visibly red when active. To confirm AMX metabolism, the stoichiometric ratios of 1.32 mg/L NO_2–N removed to 1 mg/L NH_4–N removed (catabolism) and 0.26 mg/L NO_3–N produced to 1 mg/L NH_4–N removed (anabolism) are referenced (Arp et al, 2011). AMX bacteria have a very slow growth and decay rate but grow optimally at a pH of 7.8 and temperature of 37°C (Dosta et al, 2008). AMX also have an irregular metabolic pathway to produce their own substrate (NH_4^+) through dissimilatory nitrate reduction, and this consists of oxidizing organic acids (acetate and propionate) and reducing nitrite or nitrate (Guven et al, 2005; Kartal et al, 2007).

For clarification, in a WWTP, the main wastewater treatment flow is known as the mainstream process and any flow that is diverted from the mainstream flow to be treated separately is known as a sidestream process. In sidestream processes, deammonification has become a successful technology with over 200 installations worldwide (Lackner et al, 2014). Most sidestream deammonification systems focus on treating the supernatant of anaerobic digesters, and the supernatant contains high temperature, high ammonia, and low carbon (Lackner et al, 2014). These sidestream conditions allow for highly successful NOB outselection and accumulation of AMX activity. Maintaining a high reservoir of AOB and AMX activity in sidestream deammonification is crucial to long term system performance. The most notable sidestream deammonification technologies include: ANITA™Mox, ANAMMOX®, SHARON®-ANAMMOX®, and DEMON®.

DEMON® is a pH-based aeration control SBR plus hydrocyclone granular sludge process that bioaugments AOB and AMX for mainstream deammonification. DEMON® aeration time is based on a low pH setpoint, and anoxic/feed time is based on a timer or high pH setpoint value, resulting in easy outselection of NOB (Burton et al, 2013). Hydrocyclones separate granular AMX SRT due to biomass density differential. The lighter flocculent biomass separates into the overflow of the cyclone, and the denser AMX granules partition into the underflow (Nifong, 2013; Wett et al, 2010). Another sidestream process called ANITA™Mox is a one stage deammonification MBBR. ANITA™Mox has been shown to produce low N_2O emissions (<1% N reduced), consume low energy (1.5 kW/NH4-N removed), achieve high ammonia removal (1.2 kg N/m3.d), while remaining stable and robust with variations in process and loading conditions (Christensson et al, 2013).

To date, deammonification has been successful in sidestream applications, but mainstream deammonification has remained quite challenging. Mainstream deammonification is difficult because nitrite oxidizing bacteria (NOB) are harder to suppress than in sidestream. Lower N concentrations (< 100 mg N/L) and lower temperatures (< 30 °C) typical of mainstream wastewater do not favor NOB outselection (Regmi et al, 2016), but as long as the slow growth of AMX can be accommodated through biofilm growth or selective retention, wastewater temperatures ≤ 20°C appear to be achievable for AMX (Dosta et al., 2008; Vazquez-Padin et al, 2011; Cema et al, 2007; Hendrickx et al, 2012; Isaka et al, 2008). The two main challenges for mainstream deammonification are maintaining AMX activity in the mainstream and inhibiting NOB activity. For successful long term deammonification, it is crucial to

retain a mainstream biomass with a very high population of AOB to convert a portion of the influent NH_4^+ to NO_2^-, and a high reservoir of active AMX bacteria to perform anaerobic ammonia oxidation.

Partial Denitrification/Anammox

In mainstream deammonification, residual total nitrogen (TN) is typically present and a low-cost polishing step would be needed to ensure low TN effluent limits (Regmi et al, 2014; Regmi et al, 2016; Le et al, 2016). Some pilot studies were successful at shortcut nitrogen removal but unable to reach low effluent total inorganic nitrogen (TIN) (≤ 5 mg TIN-N/L) (Han et al, 2016; Laureni et al, 2016; Lotti et al, 2014; Regmi et al, 2014). To comply with lower effluent discharge limits a nitrogen polishing step may be required following the nitrogen removal process. Commonly, nitrate is the dominant effluent nitrogen species and is polished with a carbon source (electron donor) for full denitrification of nitrate to nitrogen gas. To achieve partial nitritation, for deammonification, it has been shown that a residual ammonia concentration is necessary (Regmi et al, 2014). Also, upsets with AMX activity in the deammonification nitrogen removal process may result in nitrite bleeding into the effluent. Nitrite discharge can be toxic to receiving waterways and can cause a much higher chlorine demand for disinfection. AMX polishing could be considered because AMX can remove ammonia and nitrite without an external carbon source. To remove residual nitrate, a limited amount of carbon could be added to induce partial denitrification (PdN) from nitrate to nitrite, and if a stoichiometrically relevant ammonia concentration is maintained for AMX. The main advantage of utilizing PdN combined with AMX (PdN/A) over a full denitrification process is that only a fraction of the COD is required to reduce nitrate to nitrite. Another benefit is that PdN/A can remove ammonia anoxically, reducing the amount of ammonia that needs to be oxidized in the preceding BNR process. Many types of attached growth reactor configurations such as fluidized bed biofilm reactors, biologically active filters, and rotating biological contactors, and MBBRs could facilitate a PdN/A polishing process, but the critical aspect is maintaining a high population of AMX bacteria in a biofilm process to accommodate the slow growth rate. Partial denitrification (denitratation) combined with anammox (PdN/A) is a beneficial complimentary process to mainstream deammonification (Figure 6).

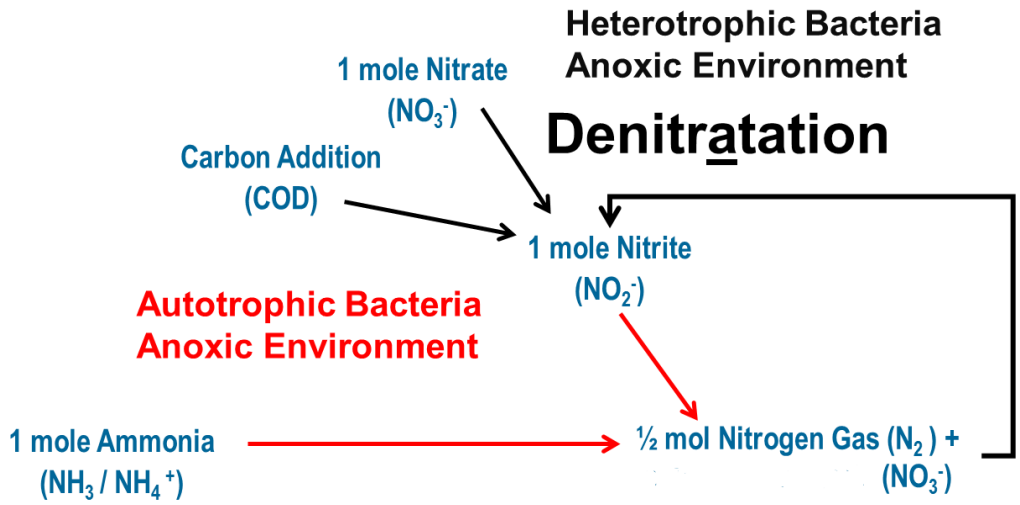

Figure 6: A schematic illustrating the PdN/A process pathways as well as the nitrate recycle factor.

With minimal COD addition, denitrifiers reduce the available NO_3^- to NO_2^-, and then the newly reduced NO_2^- can be used along with residual NH_4^+ by AMX bacteria to produce nitrogen gas and a small portion of NO_3^- . The NO_3^- byproduct of AMX can be used by denitrifiers for further PdN/A.

The main two challenges for PdN/A are retaining AMX activity and limiting full denitrification or denitritation. Carbon addition optimization would limit the carbon available for denitritation. Limiting COD added per NO_3^- present (COD/NO_3^-) would cause an electron shortage for nitrite reductase which can allow nitrite to accumulate (Almeida et al, 1995b). Several studies have confirmed that with inadequate carbon dosing ($COD/NO_3^- \leq 1$) significant nitrite accumulation was observed (Her and Huang, 1995; Oh and Silverstein, 1999).

Also, carbon addition optimization and type of carbon may help select for specialized denitrifiers that prefer to only use NO_3^- for reduction instead of reducing either NO_3^- or NO_2^-. Three common sources of carbon for denitrification are methanol, glycerol, and acetate (Eq. 10-12). The specific population and nitrite accumulation of denitrifiers is influenced by the type of carbon source utilized in the PdN/A process (Akunna et al, 1993). Studies have shown the ability of glycerol and acetate to induce significant NO_2^- accumulation (Bill et al, 2009; Ledwell et al, 2011; van Rijn et al, 1996). In fact, methanol, acetic acid, and glucose showed 17%, 21%, and 23% NO_2^- accumulation when limited carbon was added (Her and Huang, 1995). It is promising for PdN/A that methanol, acetate, and glycerol all showed the capability to accumulate nitrite. Another possible explanation to the mechanism behind partial denitrification is rapid complete onset of all denitrification genes vs progressive onset (Liu et al, 2013). Progressive onset is when electrons flow to nitrate reductase first, and then to nitrite reductase after most of the nitrate has been reduced (Liu et al, 2013). Therefore, the progressive onset of reductase upregulation would inherently accumulate nitrite.

Denitrification with Methanol:

$5CH_3OH + 6NO_3^- \rightarrow 3N_2 + 5CO_2 + 7H_2O + 6OH^-$ (Eq. 10)

Denitrification with Glycerol:

$5C_3H_8O_3 + 14NO_3^- \rightarrow 7N_2 + 15CO_2 + 13H_2O + 14OH^-$ (Eq. 11)

Denitrification with Acetate:

$5CH_3COOH + 8NO_3^- \rightarrow 4N_2 + 10CO_2 + 6H_2O + 8OH^-$ (Eq. 12)

In the United States of America (USA), glycerol and acetate are more expensive than methanol for full-scale full denitrification (Uprety, 2012). Methanol is the most widely used supplemental carbon source for full-scale in the USA because of low cost and favorable kinetics (Bill et al, 2009). Methanol may be well suited for full denitrification, but the success of PdN/A processes hinges on the efficiency of denitratation (NO_3^- to NO_2^-) and inhibition of denitritation (NO_2^- to N_2). Therefore, it is worth considering carbon sources other than methanol for PdN/A such as glycerol and acetate.

Mainstream Deammonification Approaches

Suspended Growth Granular System

Granules are a type of biofilm and in many cases they are suspended in the wastewater treatment process. Nitrifying granules have achieved high (>90%) ammonia removal efficiency (Li et al, 2014; Li et al, 2015; Jin et al, 2008; Wan et al, 2014; Hou et al, 2017). If these granules were to contain an AMX core, it would be advantageous to utilize granules for deammonification systems. Granule formation maturation is possible in the reactors of a WWTP process. But, it is possible that settling velocity is not a selection pressure for the formation of AMX granules because successful formation was seen in a MBR study that did not apply settling velocity (Triago et al, 2006). Another study showed that AMX granules existed with or without hydrocyclones in a DEMON® process which again indicated that settling velocity may not be a selection pressure for AMX granule formation (Shi et al, 2016). On the contrary, it makes logical sense that settling velocity as a selection pressure would be a precursor to granule formation. Since flocs have a higher specific surface area than granules, they have a lower resistance to substrate diffusion, resulting in a competitive advantage (Liu et al, 2015). This is not advantageous for the attachment of AOB to AMX granules for mature granule formation. It is reasonable to suspect that it would be helpful to wash out AOB flocs via a settling velocity selection pressure to promote mature AOB/AMX granule formation. Another way to incorporate granules into a WWTP process is to bioaugment them. Granules could be formed and matured in an external WWTP process such as a DEMON® sidestream process, then bioaugmented to the main deammonification process. In either case, since granules are not a type of attached growth, they require a retention mechanism to keep them in the treatment process. Retention mechanisms include but are not limited to SBRs, clarifiers, screens, and hydrocyclones.

Attached Growth Biofilm System

Typical biofilms in a single stage deammonification system are comprised of AOB and OHO on the outer layer, a miniscule to nonexistent NOB layer in the middle, and an AMX inner layer (Helmer-Madhok et al, 2002). These means that for optimal N removal, the biofilm would be structured so half of the ammonia is oxidized to nitrite and the DO is depleted by the AOB. Then, the AMX can take up the ammonia and nitrite without activity inhibition from DO. Biofilms may be ideal for AMX bacteria because biofilms provide a substrate concentration gradient, a buffer from inhibitory substances, and a SRT dissociation. For example, AMX biofilms in MBBRs are very common and well established for continuous flow systems (Christensson et al, 2013; Rosenwinkel and Cornelius, 2005). Several other types of biofilm systems include rotating biological contactors (RBCs), airlift reactors, sequencing batch biofilm reactors (SBBRs), and fixed bed reactors. Some of the technologies that utilize these different configurations include ANITA™Mox, completely autotrophic nitrogen removal over nitrite (CANON), and single-stage nitrogen removal using anammox and partial nitritation (SNAP).

Hybrid System

For one stage mainstream deammonification, a hybrid system could be comprised of suspended granules plus flocs or of attached growth plus flocs. The important distinction in a hybrid system is that there are separate SRTs for the AMX biomass and the suspended flocs. The floccular biomass would contain OHO, AOB, and minute amounts of NOB and would be responsible for partial nitritation and degradation of COD (Juretschko et al, 2002). The suspended granules contain a thin AOB layer on the surface with a dense AMX core and would perform partial nitritation, utilize the AMX pathway, and stay retained with a SRT separation mechanism. The key element to the suspended granule plus flocs hybrid system would be the granule retention efficiency of the SRT separation mechanism. If the granule formation rate is less than the granules lost in the system, then granule bioaugmentation would have to be supplemented to ensure sufficient and stable deammonification.

The attached growth biofilm contains an inner layer of AMX with an outer layer of AOB (Helmer-Madhok et al, 2002). The attached growth biofilm would perform partial nitritation, use the AMX pathway, and remain retained either on a fixed surface or on a moving media carrier. Unlike the suspended granules/flocs option, the attached growth/flocs would not require an additional retention mechanism or supplementation with bioaugmentation. The key element to the attached growth/flocs hybrid system would be to ensure enough mixing for nutrient availability while minimalizing biofilm shear.

Compared to a biofilm growth system, a suspended growth system may allow certain microorganisms a competitive advantage for available substrate because of its' higher relative diffusivity rate (Stewart, 2003) If suspended growth systems have higher diffusivity rates, they may be more susceptible to process disturbances due to changes in temperature, DO, or presence of inhibitory substances. Overall, to combine resilience and effectiveness, a hybrid system of suspended granules/flocs or attached growth/flocs would be a viable full-scale option.

Two-Stage System

Similar to hybrid systems, a two stage deammonification system must maintain separate SRTs between the AMX population and the rest of the system to help facilitate the presence and proliferation of AMX biomass. Nitrite accumulation via partial nitritation or partial denitrification can be optimized in the first stage, and the second stage can be devoted to the AMX pathway. Two-stage deammonification is a newer nitrogen treatment concept. But, there are many different possible configurations for two stage systems and a few notable ones include SHARON®-ANAMMOX®, two-stage OLAND, DEAMOX, and denammox (van der Star et al, 2007). Separating deammonification into two separate stages may require larger volumes and more instrumentation, but each individual process can be better controlled. The process configurations for two stage deammonification systems can become complex, but they still are capable of achieving up to 90% total nitrogen removal (Cema, 2009). Overall, two-stage deammonification is an advanced approach to mainstream deammonification and may provide a very high quality effluent at a competitive cost.

Mainstream NOB Suppression

Sidestream NOB suppression has been successfully implemented at hundreds of WWTPs, but mainstream NOB out-selection has proven to be elusive and not well established (Lackner et al, 2014). A mainstream full-scale facility (Changi Water Reclamation Plant) saw partial nitritation at an aggressive aerobic SRT of 2.5 days, relatively high temperature between 28°C and 32°C, while performing intermittent aeration through a step feed process (Cao et al, 2013). At moderate influent temperature (25°C) a mainstream pilot-scale study attributed their NOB suppression to a combination of high DO, intermittent aeration, heterotrophic denitrification competition, residual ammonia, and aggressive SRT conditions (Regmi et al, 2014). The same mainstream pilot observed NOB suppression at 20°C with intermittent AvN, high DO, residual ammonia, and aggressive aerobic SRT (Sadowski, 2015). It is clear that mainstream NOB suppression is possible and factors that should be explored regarding NOB outselection include: free ammonia concentration, residual ammonia concentration, temperature, bioaugmentation, inorganic carbon limitation, intermittent aeration for transient anoxia and nitrite reduction, high DO, influent COD fractionation, greenhouse gas emission, and comammox.

Free Ammonia

Many sidestream processes that aim to accumulate nitrite have high free ammonia concentration that has been attributed to NOB suppression (Anthonisen et al, 1976; Bae et al, 2002). Virtually no free ammonia concentration is present in the mainstream compared to the sidestream; therefore, free ammonia cannot help inhibit NOB for mainstream NOB suppression.

Ammonia Residual

A mainstream NH_4^+ residual is vital to achieve NOB outselection (Knowles et al, 1965; Chandran et al, 2000, Regmi et al, 2014). Process NH_4^+ residual helps ensure ammonia is never limited and AOB are

always at μ_{max}. The ammonia concentration has been shown to decrease AOB rates at effluent concentrations below 1.5 mg N/L due to substrate limitation (Stinson et al, 2013). Therefore, a mainstream process that focuses on NOB outselection such as deammonification should be operated with an effluent ammonia concentration greater than 1.5 mg/L to ensure high AOB rates. Maintaining an ammonia residual could also lead to accumulation of nitrite by limiting the aeration length of time in the mainstream via partial nitrification (Peng et al, 2004; Yang et al, 2007; Lemaire et al, 2008). If NOB are not allowed enough aeration time to oxidize the produced ammonia, nitrite should be leftover. Although the exact mechanism was inconclusive, it was theorized that ammonia oxidation limits oxygen abundance to NOB when a residual ammonium was maintained (Ma et al, 2009).

Temperature condition

With respect to nitrogen removal, the temperatures in sidestream processes that utilize digester supernatant have a higher temperature than mainstream. This makes it easier to out-select NOB based on SRT kinetics in the sidestream. In temperate climates, mainstream typical temperatures are 20° C, and NOB SRT becomes more similar to AOB SRT (van Dongen et al, 2001). This makes it more difficult to rely solely on an aggressive SRT for NOB outselection. Seasonal temperature variations (12 to 30°C at HRSD facilities) of mainstream influent do not favor AOB growth rate over NOB. NOB suppression has been successful at moderate temperatures (Regmi et al, 2014; Sadowski, 2015). It is likely that other operational parameters suppressed NOB rather than the SRT disparity due to temperature.

Inorganic Carbon Limitation

Inorganic carbon limitations occur and inhibit AOB and AMX activity in sidestream processes (Guisasola et al, 2007; Ma et al, 2015). Even with a relatively higher inorganic carbon/NH_4^+ ratio, mainstream influent conditions could still limit AOB and NOB activity. Since AOB are more susceptible to inorganic carbon limitations, external inorganic carbon may be added to ensure maximum AOB rates.

Bioaugmentation

Bioaugmentation in WWTPs typically involved transferring nitrifiers from sidestream to mainstream to enhance mainstream nitrification performance during colder influent temperature conditions (Tang and Chen, 2015). In theory, bioaugmentation of biomass comprised of mainly AOB and AMX would be ideal to accumulate nitrite in the mainstream. The inclusion of AOB would ensure the ammonia oxidation rate remains high, and the AMX would compete with NOB for nitrite, resulting in NOB suppression over time. With a selective retention mechanism, AMX could grow in mainstream conditions, but bioaugmenting AMX could establish their presence more quickly. The Strass WWTP continuously bioaugments their mainstream from a sidestream DEMON® process combined with mainstream cyclones to retain AMX granules (Wett et al, 2013). This bioaugmentation strategy may be promising for full-scale deammonification because the Strass WWTP has had long-term success at enriching mainstream treatment process with sidestream AMX granules. It is important to note that drastic

temperature change in microbial environments causes a shock that reversibly decreases the nitrification rates of the biomass (Head et al, 2004; Hwang et al, 2007; Lee et al, 2011; Munz et al, 2012). Cold temperature shock from sidestream to mainstream would also negatively affect AMX activity temporarily.

Transient Anoxia

Intermittent aeration generates periods of transient anoxia. Transient anoxia creates AOB and NOB enzymatic lag, substrate limitation during transition to aerobic, and substrate competition during transition to anoxic (Kornaros et al, 2010; Malovanyy et al, 2015; Regmi et al, 2014). AOB will compete against NOB for oxygen during aeration times, and AMX along with denitrifying bacteria can take up NO_2^- during anoxic times (Lemaire et al, 2008; De Clippeleir et al, 2013). The limited substrates for NOB would restrict their activity and growth rate and allow more NO_2^- removal through more efficient pathways like AMX. The lack of nitrite during aeration times due to nitrite reduction during anoxic times could limit NOB growth; therefore, it would be advantageous to minimize aeration time to only allow oxidation of NH_4^+ to NO_2^- but allow sufficient air off time for full reduction of the available NO_2^- (Chandran et al, 2000; Malovanyy et al, 2014; Al-Omari et al, 2013).

In addition to competitive factors, transient anoxia has been shown to inhibit NOB activity via enzymatic lag because it takes more time for normal NOB activity to continue compared to AOB (Tappe et al, 1999; Kornaros et al, 2010; Gilbert et al, 2014). Enzymatic lag would allow AOB rates to be higher than NOB rates and lead to nitrite accumulation. To ensure transient anoxia is achieved, high oxygen uptake rates (OUR) must be maintained by either having more microorganisms in the mainstream (higher MLSS) or feeding the influent wastewater at the conclusion of the aerobic cycle (Regmi et al, 2014). A higher MLSS would ensure the DO would go to zero quickly, and influent step feed at the end of an aerobic cycle allows for adequate COD availability for efficient oxygen consumption during the anoxic period.

Target DO

Nitrite accumulation was seen in low DO operations (< 1 mg/L) containing largely *Nitrobacter* with significant nitrite accumulation ratios (NAR= NO_2^-/NOx), and is most likely due to the competition of AOB and NOB for the limited amount of oxygen (Blackburne et al, 2008; Jayamohan et al, 1988; Sin et al, 2008). The low DO condition created competition for AOB and NOB and was most likely the cause of nitrite accumulation. The relatively lower affinity of oxygen for NOB enabled AOB to out-compete NOB in a variety of reactors operated at low DO concentrations (Bagchi et al, 2012; Bernet et al, 2001; Hanaki et al, 1990; Joss et al, 2011; Wyffels et al, 2004). An issue with low DO is the possible growth of filamentous bacteria which are known to cause bulking and settling problems (Palm et al, 1980). A higher DO setpoint (1.5 mg/L) may alleviate the bulking and settling problems associated with low DO (1.0 mg/L). Several more studies were able to obtain nitrite accumulation at higher DO concentrations (1.5 mg/L) where *Nitrospira* was the dominant NOB species (Manser et al, 2005; Lemaire et al, 2008; Ge

et al, 2014; Regmi et al, 2014). Overall, it is important to have a DO setpoint high enough to keep AOB rates high and to prevent bulking issues but low enough to facilitate nitrite accumulation.

Influent COD Fractionation

Influent COD fractionation plays an important role for microbial competition. Readily biodegradable COD (rbCOD) favors heterotrophic competition between nitrifiers and heterotrophic bacteria for nutrients, oxygen, and area (Sharma et al, 1977). Heterotrophic bacteria have higher maximum growth rates and yields than nitrifiers and can outcompete nitrifiers for shared substrates when organic carbon is abundant (Okabe et al, 1995; Satoh et al, 2000). It is clear that rbCOD must be maintained at a low level to allow nitrifiers like AOB to thrive. Particulate COD (pCOD) may also have a critical role for NOB suppression. rbCOD is used very quickly but pCOD must be hydrolyzed before it can be used. This extended time for pCOD to be hydrolyzed may give denitrifiers a chance to utilize the carbon along with any accumulated nitrite during anoxic periods of an intermittently aerated process (Kinyua et al, 2018). This reduction of nitrite during an anoxic period would significantly low NOB rates during the aeration period.

Greenhouse Gas Emissions

The conditions that allow mainstream deammonification to succeed, low DO, high NH_4^+ oxidation rate, transient anoxia, and nitrite accumulation, are known to enhance nitrous oxide (N_2O) production (Kampschreur et al, 2009a; Kampschreur et al, 2009b; Wunderlin et al, 2012). Nitrous oxide is 300 times more harmful than CO_2, is the product of nitric oxide (NO) reduction in WWTPs, and is a precursor to the formation of nitrogen dioxide which induces smog production (Carr et al, 1990). Therefore, emphasis on greenhouse gas emissions like carbon dioxide (CO_2), methane (CH_4), and nitrous oxide from WWTPs need to be understood and quantified. An increase in concentrations has been seen in processes with low DO, high nitrite, high ammonia load, and low C/N influent ratios (Kampschreur et al, 2009a; Kampschreuer et al, 2009b). In fact, when systems were operated at DO < 1 mg/L, the generated N_2O contributed 10% of the total nitrogen loading to a WWTP (Goreau et al, 1980). During steady state, little to no nitrous oxide should be present because it is reduced 4 times faster than nitrite or nitrate (Wicht, 1996). But, transient conditions induced by process control strategies like intermittent aeration can cause NO and N_2O to accumulate (Holtan-Hartwig et al, 2000; Yu et al, 2010; Wett et al, 2013). Due to the similarity in operational conditions, it is unclear whether the observed accumulation of nitrite when NO and N_2O emissions increased is the cause or effect of NOB outselection (Chandran et al, 2011; De Clippeleir et al, 2012; De Clippeleir et al, 2013).

Comammox

Another reason why NOB outselection for partial nitritation is difficult is because of a recently discovered pathway that oxidizes ammonia directly to nitrate (Daims et al, 2016; van Kessel et al, 2015). If ammonia can be oxidized directly to nitrate, then nitrite accumulation would not occur from partial

nitritation for deammonification. But, it is possible that nitrate could be partially denitrified to nitrite for the AMX pathway. This would be a PdN/A process that is still more efficient at nitrogen removal than traditional nitrification-denitrification but not as efficient as deammonification. The complete ammonia oxidation or comammox metabolism has been identified in several varying WWTP processes which adds another piece of complexity to mainstream NOB outselection (Annavajhala et al, 2018). The development of comammox bacteria in various processes is a concern for deammonification, but further comammox research must be done to fully understand the long-term implications on deammonification.

Aeration Control Strategies

Aeration control optimization is important because it is the largest use of energy at WWTPs besides influent pumping (Åmand et al, 2013). In fact, up to 75% of the total WWTP power usage comes from aeration energy (Rosso et al, 2008). Since oxygen demand (OD) is an important criterion for cost-effective wastewater treatment, aeration should be optimized. Optimizing aeration through process control systems could make ammonia oxidation more cost-effective for BNR.

At state of the practice BNR WWTPs, the simplest form of aeration control was holding a constant airflow rate, but this did not account for influent variation and was extremely wasteful from an energy standpoint. In fact, nitrifier growth rates are hardly affected by DO concentrations above 2 mg/L (Stenstrom et al, 1980). Therefore, any aeration above 2 mg/L would be wasteful in a BNR process and it would be advantageous to perform BNR with a control based on DO rather than an airflow rate. Even though airflow control still occurs in modern systems, introduction of online DO sensors in the 1970s allowed for DO setpoint control. DO setpoint control relies on valves that control the degree of airflow, depending on the DO sensor value, to meet a desired DO setpoint. DO setpoint control can reduce wasted energy, but influent load variation still presented an obstacle for aeration optimization.

DO setpoint control can be upgraded to ammonia-based aeration control (ABAC) with the inclusion of an online ammonia sensor. If the ammonia sensor is reading higher than the ammonia setpoint, then the PLC will coordinate with the process to aerate less, vice versa. ABAC reduces energy consumption by converting less NH_4, using a lower DO setpoint, and less carbon oxidation (Sadowski et al, 2015). It also can be used to reduce ammonia effluent peaks as well as decrease alkalinity and chlorine demand (Sadowski et al, 2015). ABAC reduces external carbon requirements due to improved usage of readily biodegradable COD, and it may allow for some simultaneous nitrification-denitrification. ABAC is a state of the art control strategy that optimizes aeration for conventional nitrification-denitrification systems, but research into more cost-effective BNR technologies such as shortcut nitrogen removal may require more advanced control schemes.

ABAC can be upgraded to AvN control with the addition of nitrite and nitrate sensors. AvN control balances the oxidation of ammonia with the reduction of NOx based on a user setpoint (Regmi et al, 2014). The aerobic fraction is manipulated to meet the NH_4^+:NOx setpoint by controlling either the length of aerobic time in intermittent aeration mode, or the DO setpoint in continuous aeration mode (Regmi et al, 2014). AvN can be controlled with continuous aeration or intermittent aeration.

Continuously aerated AvN is simple, easy, may provide more simultaneous nitrification-denitrification, and most WWTPs cannot quickly switch to intermittent aeration (Regmi, et al 2014). But, intermittent aeration can out-select NOB and provide an anoxic period for denitrification or AMX utilization (Regmi et al, 2014). Intermittently aerated AvN may be an ideal process control strategy for mainstream deammonification. The outselection of NOB was verified in an AvN operated study by AOB/NOB activity ratios well above 1.0 (Regmi et al, 2014). This indicated nitrite was produced faster than it could be consumed. The same study saw NOB outselection corresponding to an elevated nitrite accumulation ratio (NAR) = 0.43. This accumulated nitrite could only be from the suppression of NOB activity. The WAS rate in AvN control can be increased to provide a more aggressive SRT for NOB washout, resulting in a decrease in process MLSS. This causes the aerobic fraction to increase in an attempt to maintain the nitrification rate necessary to meet the AvN setpoint. Besides the ability to outselect NOB, AvN control may be the most efficient N removal strategy with available carbon. AvN control minimizes aeration requirements for N removal, ensures acceptable alkalinity management, and enables the inclusion of deammonification.

References

About Us. *HRSD*, www.hrsd.com/about-us. Site accessed on November 2018.

Akunna, J.C., Bizeau, C., Moletta, R., 1993. Nitrate and nitrite reductions with anaerobic sludge using various carbon sources: glucose, glycerol, acetic acid, lactic acid and methanol. *Water Research.* 27(8):1303-1312.

Al-Omari, A., Wett, B., Han, M., De Clippeleir, H., Bott, C.B., Nopens, I., Murthy, S., 2013. Competition over Nitrite in Single Sludge Mainstream Deammonification Process. Proceedings of the Water Environment Federation.

Almeida, J.S., Júlio, S.M., Reis, M.A., Carrondo, M.J., 1995a. Nitrite inhibition of denitrification by Pseudomonas fluorescens. *Biotechnology and Bioengineering.* 46(3):194-201.

Almeida, J.S., Reis, M.A., Carrondo, M.J., 1995b. Competition between nitrate and nitrite reduction in denitrification by Pseudomonas fluorescens. *Biotechnology and Bioengineering*. 46(5):476-84.

Åmand, L., Olsson, G., Carlsson, B., 2013. Aeration Control - a Review. *Water Science & Technology*. 67(11):2374-2398.

Annavajhala, M.K., Kapoor, V., Santo-Domingo, J., Chandran, K., 2018. Comammox Functionality Identified in Diverse Engineered Biological Wastewater Treatment Systems. *Environmental Science and Technology Letters.* 5:110-116.

Anthonisen, A.C., Loehr, R.C., Prakasam, T.B.S., Srinath, E.G., 1976. Inhibition of Nitrification by Ammonia and Nitrous Acid. *Journal (Water Pollution Control Federation).* 48(5):835-852.

Arp, D. J., Klotz, M.G., Ward, B.B., 2011. Nitrification, ASM Press.

Bae, W., Baek, S., Chung, J., Lee, Y., 2002. Optimal operational factors for nitrite accumulation in batch reactors. *Biodegradation*. 12:359-366.

Bagchi, S., Biswas, R., Vlaeminck, S.E., Roychoudhury, K., Nandy, T., 2012. Stable performance of non-aerated two-stage partial nitritation/anammox (PANAM) with minimal process control. *Microbiology and Biotechnology*. 5(3):425-432.

Bernet, N., Dangcong, P., Delgènes, J.P., Moletta, R., 2001. Nitrification at low oxygen concentration in biofilm reactor. *Journal of Environmental Engineering.* 127: 266-271.

Bill, K.A., Bott, C.B., Murthy, S., 2009. Evaluation of alternative electron donors for denitrifying moving bed biofilm reactors (MBBRs). *Water Science & Technology.* 60(10):2647-2657.

Bitton, G., 2011. *Wastewater Microbiology*. Hoboken, N.J, Wiley-Blackwell.

Blackburne, R., Yuan, Z., Keller, J., 2008. Partial Nitrification to Nitrite Using Low Dissolved Oxygen Concentration as the Main Selection Factor. Biodegradation. 19(2):303-312.

Boehnke, B., Schulze-Rettmer, R., Zuckut, S.W., 1998. Cost-Effective Reduction of High-Strength Wastewater by Adsorption-Based Activated Sludge Technology. Water/Engineering & Management [H.W.Wilson - AST]. 145(12):31.

Brandt, M., Middleton, R., Wang, S., 2012. Energy Efficiency in the Water Industry: A Compendium of Best Practices and Case Studies-Global Report, IWA Publishing.

Broda, E., 1977. Two Kinds of Lithotrophs Missing in Nature. Zeitschrift für allgemeine Mikrobiologie. 17(6):491.

Burton, F., Tchobanoglous, G., Tsuchihashi, R., Stensel, H.D., 2013. Wastewater Engineering: Treatment and Resource Recovery, McGraw-Hill Education.

Cao, Y., Kwok, B.H., Yong, W.H., Chua, S.C., Wah, Y.L., Ghani, Y., 2013. Mainstream Partial Nitritation—Anammox Nitrogen Removal in the Largest Full-Scale Activated Sludge Process in Singapore: Process Analysis. WEF/IWA Nutrient Removal and Recovery. WEF/IWA Nutrient Removal and Recovery, Canada.

Cao, Y., van Loosdrecht, M.C.M, Daigger, G.T., 2017. Mainstream partial nitritation–anammox in municipal wastewater treatment: status, bottlenecks, and further studies. Applied Microbiology and Biotechnology. 101(4):1365-83.

Carr, G.J., Ferguson, S.J., 1990. Nitric-Oxide Formed by Nitrite Reductase of Paracoccus Denitrificans Is Sufficiently Stable to Inhibit Cytochrome-Oxidase Activity and Is Reduced by Its Reductase under Aerobic Conditions. Biochimica et biophysica acta. 1017(1):57-62.

Cema, G., 2009. Comparative study on different Anammox systems. Dissertation/Thesis, KTH Stockholm.

Cema, G., Wiszniowski, J., Zabczynski, S., Zablocka-Godlewska, E., Raszka, A., Surmacz-Gorska, J., 2007. Biological nitrogen removal from landfill leachate by deammonification assisted by heterotrophic denitrification in a rotating biological contactor (RBC). Water Science & Technology. 55(8-9):35-42.

Chandran, K., Smets, B.F., 2000. Single-Step Nitrification Models Erroneously Describe Batch Ammonia Oxidation Profiles When Nitrite Oxidation Becomes Rate Limiting. Biotechnology and bioengineering 68(4):396-406.

Chandran, K., Stein, L.Y., Klotz, M.G., van Loosdrecht, M.C.M., 2011. Nitrous Oxide Production by Lithotrophic Ammonia-Oxidizing Bacteria and Implications for Engineered Nitrogen-Removal Systems. Biochemical Society transactions. 39(6):1832-1837.

Christensson, M., Ekstrom, S., Andersson Chan, A., Le Vaillant, E., Lemaire, R., 2013. Experience from the start-ups of the first AnitaMox plants. Water Science and Technology. 67(12):2677-2684.

Daims, H., Lucker, S., Wagner, M., 2016. A New Perspective on Microbes Formerly Known as Nitrite-Oxidizing Bacteria. TRENDS IN MICROBIOLOGY. 24:699-712.

De Clippeleir, H., Vlaeminck, S.E., De Wilde, F., Daeninck, K., Mosquera, M., Boeckx, P., Verstraete, W., Boon, N., 2013. One-Stage Partial Nitritation/Anammox at 15 °C on Pretreated Sewage: Feasibility Demonstration at Lab-Scale. Applied Microbiology and Biotechnology. 97(23):10199-10210.

De Clippeleir, H., Weissenbacher, N., Schaubroeck, T., Hell, M., Boeckx, P., Boon, N., Wett, B., 2012. Mainstream Partial Nitritation/Anammox: Balancing Overall Sustainability with Energy Savings."Proceedings of the Water Environment Federation. (10):5761-5770.

Dosta, J., Fernandez, I., Vazquez-Padin, J.R., Mosquera-Corral, A., Campos, J.L., Mata-Alvarez, J., Mendez, R., 2008. Short- and long-term effects of temperature on the Anammox process. *Journal of Hazardous Materials*. 154:688-693.

Du, R., Cao, S., Li, B., Niu, M., Wang, S., Peng, Y., 2017. Performance and microbial community analysis of a novel DEAMOX based on partial-denitrification and anammox treating ammonia and nitrate wastewaters. Water Res. 108:46-56.

Drysdale, G.D., Kasan, H.C., Bux, F., 2001. Assessment of denitrification by the ordinary heterotrophic organisms in an NDBEPR activated sludge system. Water Science and Technology. 43(1):147-154.

Gardner, L.M., 2008. Denitrification Enzyme Activity as an Indicator of Nitrate Loading in a Wetland Receiving Diverted Mississippi River Water. Dissertation/Thesis. Louisiana State University.

Ge, S., Peng, Y., Qiu, S., Zhu, A., Ren, N., 2014. Complete Nitrogen Removal from Municipal Wastewater Via Partial Nitrification by Appropriately Alternating Anoxic/Aerobic Conditions in a Continuous Plug-Flow Step Feed Process. Water research. 55:95-105.

Gilbert, E.M., Agrawal, S., Karst, S.M., Horn, H., Nielsen, P.H., Lackner, S., 2014. Low Temperature Partial Nitritation/Anammox in a Moving Bed Biofilm Reactor Treating Low Strength Wastewater. *Environmental Science and Technology*. 48(15):8784-8792.

Goreau, T.J., Kaplan, W.A., Wofsy, S.C, McElroy, M.B., Valois, F.W., Watson, S.W., 1980. Production of NO2- and N2o by Nitrifying Bacteria at Reduced Concentrations of Oxygen. Applied and Environmental Microbiology. 40(3):526-532.

Grady Jr, C.L., Daigger, G.T., Love, N.G., Filipe, C.D., Leslie Grady, C., 2011. *Biological Wastewater Treatment*: IWA Publishing.

Guisasola, A., Petzet, S., Baeza, J.A., Carrera, J., Lafuente, J., 2007. Inorganic Carbon Limitations on Nitrification: Experimental Assessment and Modelling. Water research. 41(2):277-286.

Guven, D., Dapena, A., Kartal, B., Schmid, M.C., Maas, B., van de Pas-Schoonen, K., Sozen, S., Mendez, R., Op den Camp, H.J.M., Jetten, M.S.M., Strous, M., Schmidt, I., 2005. Propionate Oxidation by and Methanol Inhibition of Anaerobic Ammonium-Oxidizing Bacteria. Applied and Environmental Microbiology 71(2):1066-1071.

Halling-Sørensen, B., Jorgensen, S.E., 1993. The Removal of Nitrogen Compounds from Wastewater, Elsevier Science.

Han, M., Vlaeminck, S.E., Al-Omari, A., Wett, B., Bott, C., Murthy, S., De Clippeleir, H., 2016. Uncoupling the solids retention times of flocs and granules in mainstream deammonification: A screen as effective out-selection tool for nitrite oxidizing bacteria. Bioresource Technology. 221:195-204.

Hanaki, K., Wantawin, C., Ohgaki, S., 1990. Nitrification at Low Levels of Dissolved Oxygen with and without Organic Loading in a Suspended-Growth Reactor. Water research. 24(3):297-302.

Head, M.A., Oleszkiewicz, J.A., 2004. Bioaugmentation for Nitrification at Cold Temperatures. Water research. 38(3):523-530.

Helmer-Madhok, C., Schmid, M., Filipov, E., Gaul, T., Hippen, A., Rosenwinkel, K., Seyfriend, C.F., Wagner, M., Kunst, S., 2002. Deammonification in biofilm systems: population structure and function. Water Science and Technology. 46(1-2):223-231.

Hendrickx, T.L.G., Wang, Y., Kampman, C., Zeeman, G., Temmink, H., Buisman, C.J.N., 2012. Autotrophic nitrogen removal from low strength waste water at low temperature. *Water Research*. 46:2187-2193.

Her J.J., Huang, J.S., 1995. Influences of carbon source and C/N ratio on nitrate/nitrite denitrification and carbon breakthrough. Bioresour Technol. 54(1):45-51.

Holtan-Hartwig, L., Dorsch, P., Bakken, L.R., 2000. Comparison of Denitrifying Communities in Organic Soils: Kinetics of No3- and N2o Reduction. SOIL BIOLOGY & BIOCHEMISTRY. 32(6):833-843.

Hou, C., Shen, J.Y., Zhang, D.J., Han, Y., Ma, D.H., Sun, X.Y., Li, J.S., Han, W.Q., Wang, L.J., Liu, X.D., 2017. Bioaugmentation of a continuous- flow self- forming dynamic membrane bioreactor for the treatment of wastewater containing high-strength pyridine. *Environmental Science and Pollution Research*. 24(4):3437-3447.

Hwang, J. H., Oleszkiewicz, J.A., 2007. Effect of Cold-Temperature Shock on Nitrification. Water Environ Res. 79(9):964-968.

Imhoff, K., 1955. Two-Stage Operation of Activated Sludge Plants. Sewage and Industrial Wastes. 27(4): 431-433.

Isaka, K., Date, Y., Kimura, Y., Sumino, T., Tsuneda, S., 2008. Nitrogen removal performance using anaerobic ammonium oxidation at low temperatures. FEMS Microbiology Letter. 282:32-38.

Jayamohan, S., Ohgaki, S., Hanaki, K., 1988. Effect of Do on Kinetics of Nitrification. Water Supply. 6(3):141-150.

Jetten, M.S.M., Horn, S.J., van Loosdrecht, M.C.M., 1997. Towards a more sustainable municipal wastewater treatment system. Wat Sci Tech. 35(9):171–180.

Jin, R.C., Zheng, P., Mahmood, Q., Zhang, L., 2008. Performance of a nitrifying airlift reactor using granular sludge. *Separation and Purification Technology*. 63(3):670-675.

Joss, A., Derlon, N., Cyprien, C., Burger, S., Szivak, I., Traber, J., Siegrist, H., Morgenroth, E., 2011. Combined Nitritation-Anammox: Advanced in Understanding Process Stability. *Environmental Science Technology*. 45:9735-9742.

Juretschko, S., Loy, A., Lehner, A., Wagner, M., 2002. The Microbial Community Composition of a Nitrifying-Denitrifying Activated Sludge from an Industrial Sewage Treatment Plant Analyzed by the Full-Cycle rRNA Approach. Systematic and Applied Microbiology. 25:84-99.

Kaelin, D., Manser, R., Rieger, L., Eugster, J., Rottermann, K., Siegrist, H., 2009. Extension of Asm3 for Two-Step Nitrification and Denitrification and Its Calibration and Validation with Batch Tests and Pilot Scale Data. Water research. 43(6):1680-1692.

Kampschreur, M.J., Poldermans, R., Kleerebezem, R., van der Star, W.R.L., Haarhuis, R., Abma, W.R., Jetten, M.S.M., van Loosdrecht, M.C.M., 2009. Emission of Nitrous Oxide and Nitric Oxide from a FullScale Single-Stage Nitritation-Anammox Reactor. Water Science and Technology 60(12): 3211-3217

Kampschreur, M.J., Temmink, H., Kleerebezem, R., Jetten, M.S.M., van Loosdrecht, M.C.M., 2009. Nitrous Oxide Emission During Wastewater Treatment. Water research. 43(17):4093-4103.

Kartal, B., Kuypers, M.M.M., Lavik, G., Schalk, J., Op den Camp, H.J.M., Jetten, M.S.M., Strous, M., 2007. Anammox Bacteria Disguised as Denitrifiers: Nitrate Reduction to Dinitrogen Gas Via Nitrite and Ammonium. Environmental microbiology. 9(3): 635-642.

Kayombo, S., Mbwette, T.S.A., Katima, J.H.Y., Jorgensen, S.E., 2003. Effects of substrate concentration on the growth of heterotrophic bacteria and algae in secondary facultative ponds. Water Research 37:2937-2943.

Kinyua, M., Klaus, S., Sadowski, M., Regmi, P., Wett, B., De Clippeleir, H., Chandran, K., Bott, C.B., 2018. Effect of influent carbon fractionation and reactor configuration on nitrogen removal and NOB out selection during intermittent aeration. IWA Nutrient Removal and Recovery, Brisbane, Australia.

Knowles, G., Downing, A.L., Barrett, M.J., 1965. Determination of Kinetic Constants for Nitrifying Bacteria in Mixed Culture, with the Aid of an Electronic Computer. Journal of general Microbiology. 38(2):263.

Kornaros, M., Dokianakis, S.N., Lyberatos, G., 2010. Partial Nitrification/Denitrification Can BeAttributed to the Slow Response of Nitrite Oxidizing Bacteria to Periodic Anoxic Disturbances. Environmental science & technology. 44(19):7245-7253.

Lackner, S., Gilbert, E.M., Vlaeminck, S.E., Joss, A., Horn, H., van Loosdrecht, M.C.M., 2014. FullScalePartial Nitritation/Anammox Experiences--an Application Survey. Water research. 55:292-303.

Laureni, M., Falas, P., Robin, O., Wick, A., Weissbrodt, D.G., Nielsen, J.L., Ternes, T.A., Morgenroth, E., Joss, A., 2016. Mainstream partial nitritation and anammox: long-term process stability and effluent quality at low temperatures. *Water Research*. 101:628-639.

Le, T., Massoudieh, A., Al-Omari, A., Murthy, S., Wett, B., Bott, C., De Clippeleir, H., 2016. Obtaining discharge limit by controlling competition between anammox bacteria and denitrifiers for nitrite after mainstream deammonification. IWA – Nutrient Removal & Recovery, Denver, USA.

Ledwell, S.A., Fabiyi, M., Farmer, G., 2011. Optimizing denitrification with non-methanol carbon sources in deep-bed denitrification filter technologies. Proceedings of the Water Environment Federation. (1):406-419.

Lee, S., Cho, K., Lim, L., Kim, W., Hwang, S., 2011. Acclimation and Activity of Ammonia-Oxidizing Bacteria with Respect to Variations in Zinc Concentration, Temperature, and Microbial Population. Bioresource technology. 102(5):4196-4203.

Lemaire, R., Marcelino, M., Yuan, Z., 2008. Achieving the Nitrite Pathway Using Aeration Phase Length Control and Step-Feed in an Sbr Removing Nutrients from Abattoir Wastewater. Biotechnology and bioengineering. 100(6):1228-1236.

Li, J., Cai, A., Wang, M., Ding, L.B., Ni, Y.J., 2014. Aerobic granulation in a modified oxidation ditch with an adjustable volume intraclarifier. *Bioresource Technology*. 157:351-354.

Li, J., Cai, A., Ding, L.B., Sellamuthu, B., Perreault, J., 2015. Aerobic sludge granulation in a reverse flow baffled reactor (RFBR) operated in continuous- flow mode for wastewater treatment. *Separation and Purification Technology*. 149:437-444.

Liu, B., Mao, Y., Bergaust, L., Bakken, L.R., Frostegard, A., 2013. Strains in the genus Thauera exhibit remarkably different denitrification regulatory phenotypes. Environmental Microbiology. 15(10):2816-2828.

Liu, Y.Q., Lan, G.H., Zeng, P., 2015. Excessive precipitation of CaCO3 as aragonite in a continuous aerobic granular sludge reactor. Appl. Microbiol. Biotechnol. 99(19),8225-8234.

Lotti, T., Kleerebezem, R., van Erp Taalman Kip, C., Hendrickx, T.L.G., Kruit, J., Hoekstra, M., van Loosdrecht, M.C.M., 2014. Anammox Growth on Pretreated Municipal Wastewater. Environmental Science & Technology. 48:7874-7880.

Ma, Y., Peng, Y., Wang, S., Wang, X., Yuan, Z., 2009. Achieving Nitrogen Removal Via Nitrite in a Pilot-Scale Continuous Pre-Denitrification Plant. Water research. 43(3):563-572.

Ma, Y., Sundar, S., Park, H., Chandran, K., 2015. The Effect of Inorganic Carbon on Microbial Interactions in a Biofilm Nitritation–Anammox Process. Water research. 70:246-254.

Malovanyy, A., Yang, J., Trela, J., Plaza, E., 2015. Combination of upflow anaerobic sludge blanket (UASB) reactor and partial nitritation/anammox moving bed biofilm reactor (MBBR) for municipal wastewater treatment. Bioresource Technology. 180:144-153.

Malovanyy, A., Trela, J., Plaza, E., 2014. Competition between Aob and Nob in Deammonification Mbbr Treating Mainstream Wastewater. Proceedings of IWA Specialist Conference on Global Challenges for Sustainable Wastewater Treatment and Resource Recovery.

Manser, R., Gujer, W., Siegrist, H., 2005. Consequences of Mass Transfer Effects on the Kinetics of Nitrifiers. Water research. 39(19):4633-4642.

Manser, R., Gujer, W., Siegrist, H., 2006. Decay Processes of Nitrifying Bacteria in Biological Wastewater Treatment Systems. Water research. 40(12):2416-2426.

Miller, M.W., Bunce, R., Regmi, P., Hingley, D.M., Kinnear, D., Murthy, S., Wett, B., Bott, C.B., 2012. A/B Process Pilot Optimized for Nitrite Shunt: High Rate Carbon Removal Followed by Bnr with Ammonia-Based Cyclic Aeration Control. Proceedings of the Water Environment Federation. (10): 5808-5825.

Mulder, A., 2003. The quest for sustainable nitrogen removal technologies. Water Science and Technology. 48:67-75.

Mulder, A., van de Graaf, A.A., Robertson, L.A., Kuenen, J.G., 1995. Anaerobic Ammonium Oxidation Discovered in a Denitrifying Fluidized Bed Reactor. FEMS microbiology ecology. 16(3):177-183.

Munz, G., Szoke, N., Oleszkiewicz, J.A., 2012. Effect of Ammonia Oxidizing Bacteria (Aob) Kinetics on Bioaugmentation. Bioresource technology. 125:88-96.

Nifong, A., Nelson, A., Johnson, C., Bott, C.B., 2013. Performance of a Full-Scale Sidestream Demon Deammonification Installation. Vancouver, Bc. WEF/IWA Nutrient Removal and Recovery.

Oh, J., Silverstein, J., 1999. Acetate Limitation and Nitrite Accumulation During Denitrification. *Journal of Environmental Engineering*. 125(3), 234-242.

Okabe, S., Hirata, K., Watanabe, Y., 1995. Dynamic Changes in Spatial Microbial Distribution in Mixed-Population Biofilms: Experimental Results and Model Simulation. WATER SCIENCE AND TECHNOLOGY. 32(8):67-74.

Palm, J.C., Jenkins, D., Parker, D.S., 1980. Relationship between organic loading, dissolved oxygen concentration and sludge settleability in the completely-mixed activated sludge process. Journal (Water Pollution Control Federation). 52(10):2484-2506.

Peng, Y.Z., Chen, Y., Peng, C.Y., Liu, M., Wang, S.Y., Song, X.Q., Cu, Y.W., 2004. Nitrite Accumulation by Aeration Controlled in Sequencing Batch Reactors Treating Domestic Wastewater. Water science and technology: a journal of the International Association on Water Pollution Research. 50(10):35-43.

Randall, C.W., Barnard, J.L., Stensel, H.D., 1998. Design and Retrofit of Wastewater Treatment Plants for Biological Nutrient Removal.

Regmi, P., Miller, M.W., Holgate, B., Bunce, R., Park, H., Chandran, K., Wett, B., Murthy, S., Bott, C.B., 2014. Control of aeration, aerobic SRT and COD input for mainstream nitrititation/denitritation. *Water Research.* 57: 162-171.

Regmi, P., Holgate, B., Miller, M.W., Park, H., Chandran, K., Wett, B., Murthy, S., Bott, C.B., 2016. Nitrogen Polishing in a Fully Anoxic Anammox MBBR Treating Mainstream Nitritation-Denitritation Effluent. *Biotechnology and Bioengineering*. 113(3): 635-642.

Rheinheimer, G., 1985. Aquatic Microbiology. (3rd edn). VEB Gustav Fischer Verlag: Jena.

Robertson, L.A., Kuenen, G., 1992. Nitrogen removal from water and waste. Microbial Control of Pollution. 227–267.

Rosenwinkel, K., Cornelius, A., 2005. Deammonification in the moving-bed process for the treatment of wastewater with high ammonia content. Chemical engineering and technology. 28(1):49-52.

Rosso, D., Larson, L.E., Stenstrom, M.K., 2008. Aeration of Large-Scale Municipal Wastewater Treatment Plants: State of the Art. WATER SCIENCE AND TECHNOLOGY. 57(7):973-978.

Sadowski, M.S., 2014. Comparison of aeration strategies for optimization of nitrogen removal in an adsorption/bio-oxidation process with an emphasis on ammonia vs. NOx control. Dissertation/Thesis, Virginia Polytechnic Institute and State University.

Salem, S., Moussa, M.S., van Loosdrecht, M.C.M., 2006. Determination of the Decay Rate of Nitrifying Bacteria. Biotechnology and bioengineering. 94(2):252-262.

Satoh, H., Okabe, S., Norimatsu, N., Watanabe, Y., 2000. Significance of Substrate C/N Ratio on Structure and Activity of Nitrifying Biofilms Determined by in Situ Hybridization and the Use of Microelectrodes. Water Science and Technology. 41(4-5):317-321.

Schulze-Rettmer, R., Zuckut, S.W., 1998. Treatment of Textile Dyeing Wastewater by

Adsorption/Bio-Oxidation Process. Textile chemist and colorist. 30(5): 19-23.

Sedlak, R., 1991. Phosphorus and Nitrogen Removal from Municipal Wastewater: Principles and Practice. Chelsea, Mich., Lewis Publishers.

Sharma, B., Ahlert, R.C., 1977. Nitrification and Nitrogen Removal. Water research. 11(10):897-925.

Shi, Y.J., Wells, G., Morgenroth, E., 2016. Microbial activity balance in size fractionated suspended growth biomass from full-scale sidestream combined nitritation-anammox reactors. Bioresour. Technol. 218, 38-45.

Siegrist, H., Salzgeber, D., Eugster, J., Joss, A., 2008. Anammox Brings Wwtp Closer to Energy Autarky Due to Increased Biogas Production and Reduced Aeration Energy for N-Removal. Water science and technology : a journal of the International Association on Water Pollution Research. 57(3):383-388.

Sin, G., Kaelin, D., Kampschreur, M.J., Takács, I., Wett, B., Gernaey, K.V., Rieger, L., Siegrist, H., van Loosdrecht, M.C.M., 2008. Modelling Nitrite in Wastewater Treatment Systems: A Discussion of Different Modelling Concepts. Water science and technology: a journal of the International Association on Water Pollution Research. 58(6):1155-1171.

Stinson, B., Murthy, S., Bott, C.B., Wett, B., Al-Omari, A., Bowden, G., Mokhyerie, Y., De Clippeleir, H., 2013. Roadmap toward Energy Neutrality & Chemical Optimization at Enhanced Nutrient Removal Facilities. Proceedings of the Water Environment Federation. (4):702-731.

Stenstrom, M. K., Poduska, R.A., 1980. The Effect of Dissolved Oxygen Concentration on Nitrification. Water research. 14(6):643-649.

Stewart, P.S., 2003. Diffusion in Biofilms. Journal of Bacteriology. 185(5):1485-1491.

Strous, M., Fuerst, J.A., Kramer, E.H., Logemann, S., Muyzer, G., van de Pas-Schoonen, K.T., Webb, R., Kuenen, J.G., Jetten, M.S.M., 1999. Missing lithotroph identified as new planctomycete. *Nature.* 400:446–449.

Strous, M., Heijnen, J.J., Kuenen, J.G., Jetten, M.S.M., 1998. The Sequencing Batch Reactor as a Powerful Tool for the Study of Slowly Growing Anaerobic Ammonium-Oxidizing Microorganisms. Applied Microbiology and Biotechnology. 50(5):589-596.

Sutton, P.M., Murphy, K.L., Dawson, R.N., 1975. Low-Temperature Biological Denitrification of Wastewater. Journal (Water Pollution Control Federation). 47(1):122-134.

Tang, H.L., Chen, H., 2015. Nitrification at full-scale municipal wastewater treatment plants: Evaluation of inhibition and bioaugmentation of nitrifiers. Bioresource Technology. 190:76-81.

Tappe, W., Laverman, A., Bohland, M., Braster, M., Rittershaus, S., Groeneweg, J., Verseveld, H.W., 1999. Maintenance Energy Demand and Starvation Recovery Dynamics of Nitrosomonas Europaea and Nitrobacter Winogradskyi Cultivated in a Retentostat with Complete Biomass Retention. Applied and Environmental Microbiology. 65(6):2471-2477.

Trigo, C., Campos, J.L., Garrido, J.M., Mendez, R., 2006. Start-up of the anammox process in a membrane bioreactor. J. Biotechnol. 126(4):475-487.

Uprety, K. (2012). Evaluation of Glycerol and Waste Alcohol as Supplemental Carbon Sources for Denitrification. Dissertation/Thesis, Virginia Polytechnic Institute and State University.

van der Star, W.R.L, Abma, W.R., Blommers, D., Mulder, J., Tokutomi, T., Strous, M., Picioreanu, C., van Loosdrecht, M.C.M., 2007. Startup of reactors for anoxic ammonium oxidation: Experiences from the first full-scale anammox reactor in Rotterdam. Water Research. 41:4149-4163.

van Dongen, U., Jetten, M.S.M., van Loosdrecht, M.C.M., 2001. The SHARON®-Anammox® process for treatment of ammonium rich wastewater. Water Science and Technology. 44(1):153-160.

van Kessel, M.A.H.J., Speth, D.R., Albertsen, M., Nielsen, P.H., Op den Camp, H.J.M., Kartal, B., Jetten, M.S.M., Lucker, S., 2015. Complete nitrification by a single microorganism. Nature. 528(7583):555-599.

van Rijn J, Tal Y, Barak Y, 1996. Influence of volatile fatty acids on nitrite accumulation by a Pseudomonas stutzeri strain isolated from a denitrifying fluidized Bed reactor. Appl Environ Microbiol. 62(7):2615-2620.

Vazquez-Padin, J.R., Fernandez, I., Morales, N., Campos, J.L., Mosquera-Corral, A., and Mendez, R., 2011. Autotrophic nitrogen removal at low temperature. *Water Science & Technology*. 63(6):1282-1288.

Versprille, A.I., Zuurveen, B., Stein, T., 1984. The A-B Process: A Novel Two Stage Wastewater Treatment System. Water Science and Technology. 17:235-246.

Wan, C.L., Yang, X., Lee, D.J., Liu, X., Sun, S.P., 2014. Partial nitrification using aerobic granule continuous-flow reactor: Operations and microbial community. *Journal of the Taiwan Institute of Chemical Engineers.* 45(5):2681-2687.

Wett, B., Hell, M., Nyhuis, G., Puempel, T., Takacs, I., Murthy, S., 2010. Syntrophy of Aerobic and Anaerobic Ammonia Oxidisers. Water science and technology: a journal of the International

Association on Water Pollution Research. 61(8):1915-1922.

Wett, B., Al-Omari, A., Podmirseg, S.M., Han, N., Akintayo, O., Gómez Brandón, M., Murthy, S., Bott, C.B., Hell, M., Takács, I., Nyhuis, G., O'Shaughnessy, M., 2013. Going for Mainstream Deammonification from Bench to Full Scale for Maximized Resource Efficiency. Water science and technology: a journal of the International Association on Water Pollution Research. 68(2):283-289.

Wett, B. Rauch, W., 2003. The Role of Inorganic Carbon Limitation in Biological Nitrogen Removal of Extremely Ammonia Concentrated Wastewater. Water research. 37(5):1100-1110.

Wicht, H., 1996. A Model for Predicting Nitrous Oxide Production During Denitrification in Activated Sludge.

Wunderlin, P., Mohn, J., Joss, A., Emmenegger, L., Siegrist, H., 2012. Mechanisms of N2O production in biological wastewater treatment under nitrifying and denitrifying conditions. Water Research. 46:1027-1037.

Wyffels, S., Boeckx, P., Pynaert, K., Zhang, D., Van Cleemput, O., Chen, G., Verstraete W., 2004. Nitrogen removal from sludge reject water by a two-stage oxygen-limited autotrophic nitrification denitrification process. *Water Science and Technology*. 49 (5-6):57-64.

Yamamoto, T., Takaki, K., Koyama, T., Furukawa, K., 2008. Long-term stability of partial nitritation of swine wastewater digester liquor and its subsequent treatment by Anammox. *Bioresource Technology*. 99:6419-6425.

Yang, Q., Peng, Y.Z., Liu, X.H., Zeng, W., Mino, T., Satoh, H., 2007. Nitrogen Removal Via Nitrite from Municipal Wastewater at Low Temperatures Using Real-Time Control to Optimize Nitrifying Communities. Environmental science & technology. 41(23):8159-8164.

Yu, R., Kampschreur, M.J., van Loosdrecht, M.C.M., Chandran, K., 2010. Mechanisms and Specific Directionality of Autotrophic Nitrous Oxide and Nitric Oxide Generation During Transient Anoxia. Environmental science & technology. 44(4):1313-1319.

Zheng, D., Deng, L.W., Fan, Z.H., Liu, G.J., Chen, C., Yang, H., Liu, Yi., 2011. Influence of sand layer depth on partial nitritation as pretreatment of anaerobically digested swine wastewater prior to anammox. *Bioresource Technology*. 99:6419-6425.

3. Manuscript 1: AMX Bioaugmentation and Retention

Abstract

This work describes the bioaugmentation and retention of anammox (AMX) granules in a continuous adsorption/bio-oxidation (A/B) mainstream deammonification pilot-scale process treating domestic wastewater. The AMX granules were collected from the underflow of a sidestream DEMON® process. The bioaugmentation rate was based on several factors including full-scale sidestream DEMON® wasting rate and sidestream vs mainstream AMX activity. The retention of bioaugmented AMX granules required a novel settling column at the end of the deammonification step. The settling column was designed to provide a surface overflow rate (SOR) that allowed dense AMX granules to settle into the underflow and less dense floccular biomass to outselect into the overflow. B-Stage was operated to out-select nitrite oxidizing bacteria (NOB) by maintaining an ammonia residual (>2 mg NH4-N/L), a relatively high dissolved oxygen (DO) (>1.5 mg O_2/L) concentration, an aggressive solids retention time (SRT) for NOB washout, and intermittent aeration for transient anoxia. AMX activity was not detected in the mainstream at any time. The settling column AMX retention quantification suggested but did not confirm AMX were maintained in the mainstream. NOB were not suppressed during this study and no nitrite accumulation was present in the mainstream process. It was theorized that AMX granules were successfully settled into the settling column underflow and accumulated in the intermittently mixed sidestream biological phosphorus reactor (SBPR) where they disintegrated.

Introduction

Total nitrogen (N) effluent standards have become more stringent across the globe, and the wastewater industry needs more inexpensive and efficient technologies to comply. The state of the practice for N removal is biological nitrification-denitrification, but more cost effective technologies like deammonification are being investigated. Deammonification consists of partial nitritation and anaerobic ammonia oxidation (AMX). Ammonia oxidizing bacteria (AOB) convert a portion of the available ammonia to nitrite, and AMX converts the residual ammonia and nitrite to nitrogen gas according to the following two equations (Strous et al, 1999).

Partial Nitritation:

$$2.34NH_4^+ + 1.87O_2 + 2.66HCO_3^- \rightarrow NH_4^+ + 1.32NO_2^- + 2.55CO_2 + 0.02C_5H_7O_2N + 3.94H_2O$$

Anammox Metabolism:

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

Deammonification has been successful in sidestream applications, but mainstream deammonification has generated interest for full-scale applications. Full-scale mainstream deammonification is difficult because nitrite oxidizing bacteria (NOB) are harder to suppress than in sidestream. Lower N concentrations (< 100 mg N/L) and lower temperatures (< 30 °C) typical of mainstream wastewater do not favor NOB outselection (Regmi et al, 2016). AMX has been successful in lab-scale treating low

COD/N wastewater at temperatures ≤ 20°C (Dosta et al., 2008; Vazquez-Padin et al, 2011; Cema et al, 2007; Hendrickx et al, 2012; Isaka et al, 2008).

To comply with lower effluent discharge limits (≤ 5 mg TIN-N/L) a nitrogen polishing step may be required following the nitrogen removal process. Commonly, nitrate is the nitrogen species in the residual and is removed with a carbon source (electron donor) for full denitrification of nitrate to nitrogen gas. To achieve partial nitritation, for deammonification, it has been shown that a residual ammonia concentration is necessary (Regmi et al, 2014). Also, upsets with AMX activity in the deammonification nitrogen removal process may result in nitrite bleeding into the effluent. Nitrite discharge can be toxic to receiving waterways and removal causes a much higher chlorine demand for disinfection. Following mainstream deammonification, AMX polishing could be considered because AMX can remove ammonia and nitrite without an external carbon source. Also, AMX produces less biomass that would need solids handling, but it does produce nitrate during metabolic activity. To remove residual nitrate, a limited amount of carbon could be added to induce partial denitrification and AMX could utilize the additional nitrite generated. Many types of reactor configurations such as fluidized bed biofilm reactors, biologically active filters, and rotating biological contactors could facilitate a partial denitrification and AMX (PdN/A) polishing process, but a moving bed biofilm reactor (MBBR) may be the most promising because of simplicity and historically reliable operation.

In this paper, we investigated AMX bioaugmentation and retention for mainstream deammonification using sidestream DEMON® AMX granules and a novel AMX granule retention mechanism. We also presented results of a pilot-scale mainstream deammonification process that sheds light on the complexities of mainstream deammonification.

Materials and Methods

This study occurred in a continuous domestic wastewater A/B process located at the Hampton Roads Sanitation District (HRSD) Chesapeake-Elizabeth pilot (CE Pilot) in Virginia Beach, VA (Figure 7). A-Stage was a high rate activated sludge (HRAS) process designed to maximize carbon capture. B-Stage was a biological nutrient removal (BNR) process designed to remove nitrogen and phosphorus. An anoxic partial denitrification anammox (PdN/A) moving bed biofilm reactor (MBBR) after B-Stage enabled additional nitrogen removal prior to final discharge. A fermenter and thickener process produced fermentate from A-Stage waste activated sludge (WAS). A sidestream biological phosphorus reactor (SBPR) was included to enhance biological phosphorus removal (Bio-P) and accepted a portion of the secondary clarifier return activated sludge (RAS), the fermentate, and the settling column underflow.

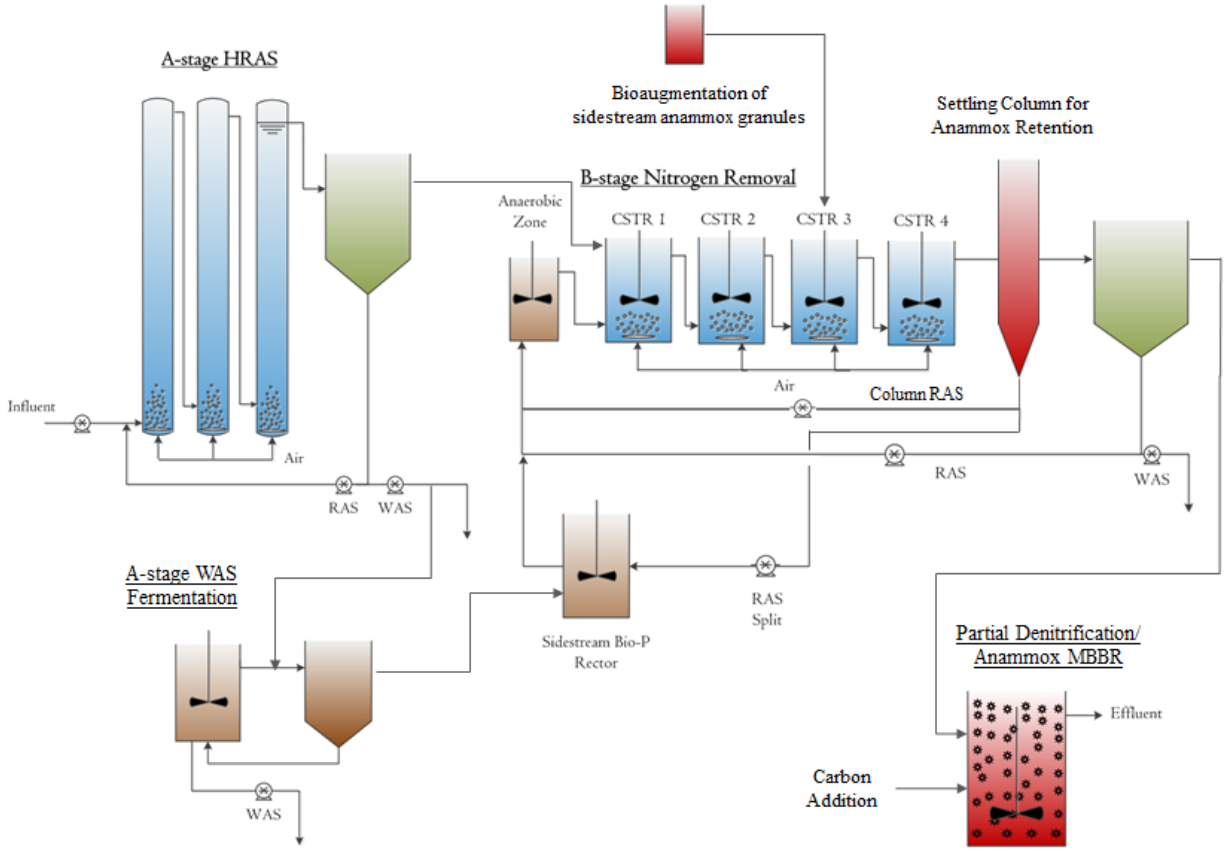

Figure 7: CE Pilot process flow diagram.

Screened and degritted Chesapeake-Elizabeth WWTP influent was fed to an additional grit and scum removal tank in the pilot. After screening and degritting, the influent was sent to a 20°C temperature controlled tank, then to the A-Stage HRAS process. A-Stage had low dissolved oxygen (DO) concentrations (<0.5 mg O_2/L) supplied by motor operated valves (MOVs) and membrane disc diffusers, a hydraulic retention time (HRT) of 30-45 minutes, and a solids retention time (SRT) of 6-12 hours. The A-Stage clarifier sent effluent to the B-Stage nitrogen removal step which was comprised of five continuously stirred tank reactors (CSTRs) with a total volume of 664 L and a 4-5 hour HRT. Each CSTR contained a variable speed mixer and was supplied oxygen via a MOV and a fine-bubble ethylene propylene diene monomer (EPDM) membrane diffuser disc. The third CSTR was fed a stock solution of sodium bicarbonate to maintain a 6.8 effluent pH. B-stage included DO (Hach LDO, CO, USA), pH (Foxboro/Invensys, UK) ammonium (WTW VARiON, Germany), nitrate and nitrite (s::can Spectro::lyser, Austria), TSS (s::can Soli::lyser), and nitrous oxide (Unisense, Denmark) sensors. A bench scale nitric oxide probe (Unisense, Denmark) was used for short term monitoring. A portable oxidation-reduction potential (ORP) probe (Hach, USA) was used for several reactors.

B-Stage effluent flowed into a settling column (Column) that selected for anammox (AMX) granules via surface overflow rate (SOR = 4-10 m/hr). The AMX granules in the Column underflow (UF) were returned to either the first reactor in B-stage or to the SBPR. The SBPR volume was 174 L, and the HRT was determined by the percentage of RAS flow that was pumped to the SBPR. A portion of A-Stage WAS

flowed to a fermenter and thickener process, and fermentate was sent to the SBPR for volatile fatty acid (VFA) addition. After the Column, secondary clarifier RAS went to the beginning of B-Stage and the overflow fed a 340 L anoxic PdN/A polishing MBBR. Following the PdN/A MBBR, final effluent was discharged.

Intermittent Ammonia vs NOx Control

B-Stage was operated under Ammonia vs NOx (AvN) Intermittent Aeration Control (Figure 8). A programmable logic control (PLC) carried out the AvN intermittent aeration control strategy. A user specified AvN (NOx/NH_4) setpoint dictated ammonia oxidation to nitrite and nitrate. Intermittent AvN used two independent proportional-integral-derivative (PID) loops: one to control aerobic fraction based on AvN setpoint and one to control DO to a fixed setpoint. Ammonia, nitrite, nitrate, and DO sensors fed back a NOx/NH_4 ratio and each CSTR DO concentration. The aerobic fraction increased if more ammonia needed oxidation to satisfy the AvN ratio, vice versa. During aerated periods, the MOVs opened until the user specified DO setpoint was met. During the air off times, the MOVs were completely closed to allow DO to reach zero in the reactors.

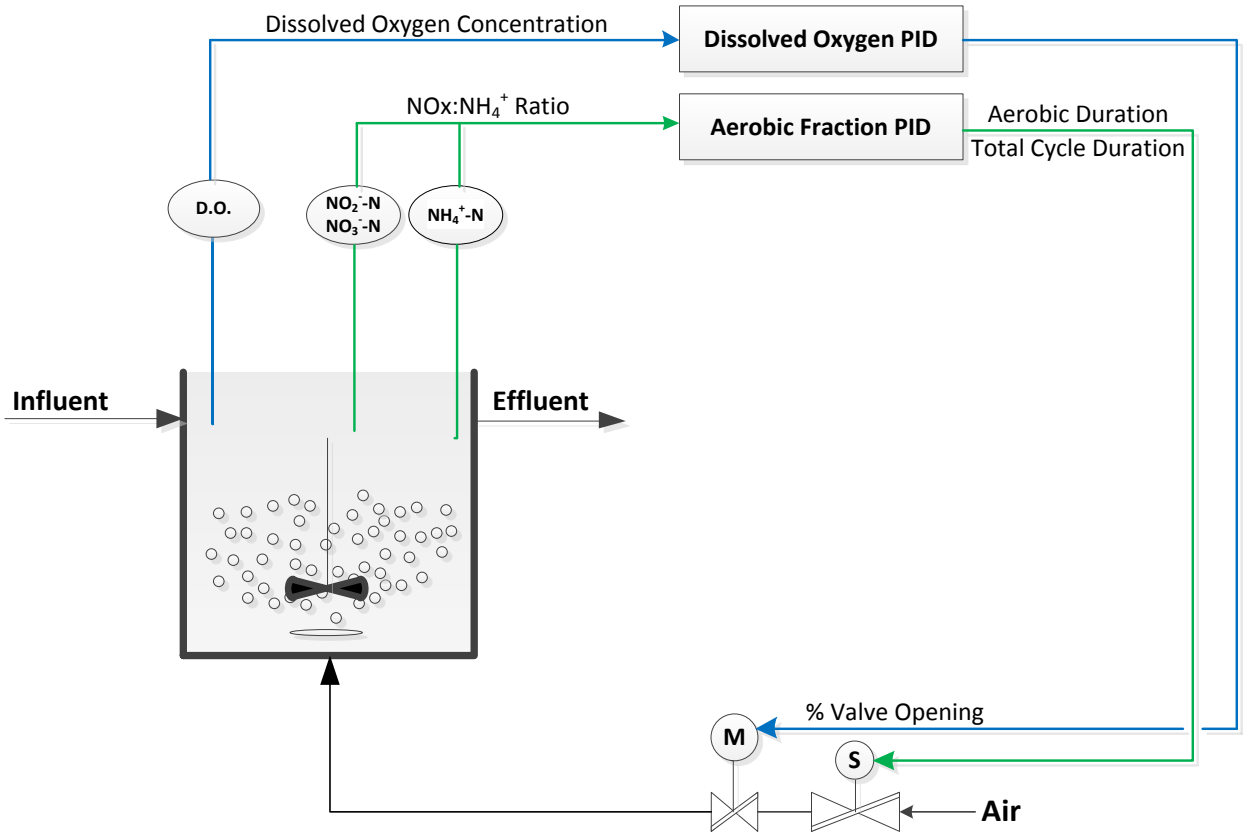

Figure 8: B-Stage AvN control schematic that shows the relationship between AvN ratio setpoint and aerobic fraction.

AMX Granules Origin and Storage

AMX granules were obtained from DEMON® sidestream process underflows at two different WWTPs. The first source was York River Treatment Plant (YR), HRSD and the second was Blue Plains Treatment Plant, DC Water. DEMON® underflow was chosen because it contained the highest concentration of AMX biomass which decreased transportation and storage volume. The DEMON® underflow was further concentrated via settling and decanting. The AMX granules were spiked with an electron acceptor (NO_3) to ensure the stock did not become septic. The AMX granules were stored in an onsite refrigerator at 4 °C. Upon arrival at the CE Pilot, an AMX activity test was conducted to ensure the stock of AMX granules was satisfactory. AMX granules were added to B-Stage weekly.

Column Design

The Column was designed to allow denser AMX granules to settle into the underflow (UF) and lighter flocs into the overflow (OF) effluent. The various heights for the column were chosen to facilitate gravity flow conditions (Figure 9). B-Stage effluent flowed by gravity into the two 1" inlets near the Column bottom. This Column influent collided with the center wall of a 1' long, 8.5" diameter cylindrical PVC pipe (dissipater) to dissipate influent energy to minimize turbulent conditions. The dissipater was suspended by a metal frame secured on top of the column rim, and would allow a quiescent settling zone in the center. Column OF traveled by gravity out of the three 1" outlets near the Column top. The OF entered a secondary clarifier for downstream treatment. An I/P pump was installed to return a portion of the OF to the Column influent (Figure 10). This increased Column flow and raised the SOR. The 12" PVC pipe Column diameter was based on B-Stage flow and preliminary instantaneous settling velocity (ISV) tests performed on YR AMX granules. This ISV analysis revealed a 4 m/hr settling velocity and was used as the SOR design parameter. A solid cylindrical block of 10" long, 12" diameter PVC had an inner cone cut 9" deep. The cone was cut at a 60° angle from 12" top diameter to 2" bottom diameter. The cone was designed to funnel condensed solids to the 2" diameter opening at the bottom. A 1" long through hole was cut at the bottom of the cone to return Column UF to the beginning of B-Stage. The 12" outer diameter cone piece was chemically welded to the inside of the 12" inner diameter column piece. Then, the assembly was chemically welded into a van stone slip flange. The assembly rested on top of a gasket and blind flange with a 2" through hole on center. The whole assembly was bolted into a metal square frame support system.

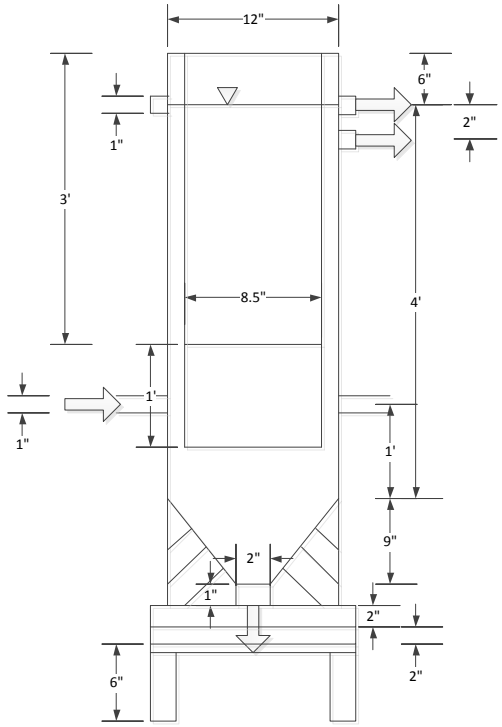

Figure 9: Column design schematic shows inflows, outflows, and relevant dimensions.

Figure 10: Column photograph shows influent flow combining with internal recycle flow.

General Sample Monitoring

Automated samplers provided 24 hour composite samples (ISCO, Lincoln, NE). This allowed average daily influent and effluent characteristics to be measured. Total suspended solids (TSS) and volatile suspended solids (VSS) (gravimetric analysis) were analyzed using 2540D and 2540E in standard methods. NH_4-N, NO_3-N, NO_2-N, orthophosphate (OP), total chemical oxygen demand (COD), and soluble COD were measured with HACH TNTplus kits and a HACH DR2800 spectrophotometer (HACH Loveland, CO). The HRSD central environmental laboratory (CEL) measured total Kjeldahl nitrogen (TKN) (EPA 351.2), total phosphorus (TP) (EPA 365.1), and alkalinity (EPA 310.2) twice a week. A-Stage and B-Stage mixed liquor suspended solids (MLSS) was tested daily for sludge volume index (SVI) via standard methods 2710C. Daily DO was recorded using a HACH handheld luminescent DO sensor (HACH Loveland, CO). Daily pH and temperature was recorded using a HACH handheld pH and temperature meter (HACH Loveland, CO).

AOB/NOB Activity Rate Measurement

A sample of B-Stage MLSS was well mixed and aerated to maintain a DO concentration between 2.5 - 4.0 mg/L and a pH between 6.8 – 7.2. NH_4^+ and NO_2^- were added to the sample to obtain a final concentration of 20 mg N/L of both NH_4^+ and NO_2^- to avoid substrate limitation. The batch reactor was operated for 2 hours and samples were collected at 15-minute intervals. All samples were analyzed for NO_3^-, NO_2^-, and NH_4^+. AOB rates (mg NOx-N/hr) were calculated as the slope of the NOx-N production vs time and NOB rates (mg NO_3-N/hr) were calculated as the slope of the NO_3-N production vs time.

Denitrification Rate Measurement

Batch testing was performed with 4 L of B-Stage MLSS continuously mixed with a magnetic stir bar and plate (Corning, Corning, NY). A floating Styrofoam cover was used to prevent oxygen transfer. The pH was maintained between 6.8 and 7.2 with diluted hydrochloric acid or sodium bicarbonate addition. When DO was stable at 0 mg O_2/L, 20-30 mg N/L of dissolved potassium nitrate and 1-3 mg N/L of dissolved sodium nitrite were spiked to the reactor. Samples were collected every 15 minutes and were immediately filtered with 1.5 µm glass fiber filters and analyzed for sCOD, NH_4-N, NO_3-N, and NO_2-N. The test was concluded after 1.5 hours and denitrification rates were based on the slope of the NOx-N concentration over time.

Anammox Activity Rate Measurement

A sample of B-Stage MLSS was well mixed with a magnetic stir bar and plate (Corning, Corning, NY). A Styrofoam cover helped limit oxygen transfer from ambient air to the batch reactor. If needed, sparging with nitrogen gas was used to ensure the DO remained at 0 mg/L throughout the 2-hr activity test. NH_4^+ and NO_2^- were added to the sample to obtain a final concentration of 20 mg N/L of both NH_4-N and

NO_2-N to avoid substrate limitation. All of the collected samples were analyzed for NO_3-N, NO_2-N, and NH_4-N. AMX rates were calculated from the NH_4-N and NO_2-N consumption versus time, and NO_3-N production versus time.

Heme Quantification Measurement

A reliable method for quantifying metabolically active anammox was recently developed (Marie et al, 2015). This new method measures heme content of a known sample volume of MLSS. The correlation between anammox activity and their heme-proteins have been confirmed by statistical analysis (Marie et al, 2015). The new method of heme quantification was adapted from the basic protocol for heme synthesis pathway evaluation (Sinclair et al, 1999). In general, hot alkaline extraction was performed on the MLSS sample, and was analyzed at different wavelengths in a spectrophotometer to obtain a measurement of the reduced hemochrome. At the CE Pilot, a version of this method was used. This method allowed quick and reliable AMX activity monitoring.

A 10 mL sample was extracted and placed into a 15 mL centrifuge vial. The sample was centrifuged for 3 minutes, at 10,000 RPM, and at 0 C°. The supernatant was decanted so all solids remained in the centrifuge vial. 10 mL of 1 M sodium hydroxide solution and 0.2 g of sodium dithionite was placed into the sample centrifuge vial. The sample centrifuge vial was vortexed vigorously to suspend the biomass and inserted into a heating block controlled to 70 C° for 10 min. The sample centrifuge vial was inverted occasionally during this incubation period. The sample centrifuge vial cooled at room temperature for 15 min, then centrifuged for 3 minutes, at 10,000 RPM, and at 0 C°. The supernatant was filtered with 0.45 µm glass fiber filters, and then poured into a clear glass vial. A spectrophotometer was zeroed with DI water and the sample absorbance unit (AU) was analyzed for nm_{535}, nm_{550} and nm_{570} wavelengths. The heme AU content was calculated from the wavelength measurements recorded (Eq 1.) The heme AU content was normalized to sample TSS and/or VSS.

Eq 1.

$$Heme\ (AU) = nm_{550} - nm_{535} - \left[\frac{nm_{570} - nm_{535}}{(570 - 535) * (550 - 535)}\right]$$

Initial Settling Velocity (ISV) and Sludge Volume Index (SVI) Measurements

A two liter sample of Column OF, Column influent (B-Stage MLSS), and Column UF were collected into separate containers. Each sample was diluted (with secondary clarifier effluent) or concentrated to obtain 2300 ± 300 mg/L using a TSS probe. Each sample was poured into a 2 L glass graduated cylinder and well mixed. Samples for TSS and VSS measurements were obtained and recorded. The distance the sludge settled was recorded at specified time intervals. During the ISV tests, SSV measurements were recorded at 5 and 30 minutes. SVI5 was indicative of sludge settling velocity, and SVI30 was a measure of sludge compaction.

Column TSS Gradient Measurements

The Column top was stirred to dissipate floating solids and flow rates were recorded. A TSS probe was manually lowered into the Column in 0.5 ft increments and TSS was recorded at each increment. The TSS was plotted vs column depth to produce a gradient profile.

Results and Discussion

NOB Suppression

Nitrite concentration in B-Stage did not significantly accumulate (Figure 11). NOB/AOB rate ratios did not fall below one. The nitrite accumulation ratio (NAR) did not significantly increase (Figure 12). Since NOB activity rate was ≥ AOB activity rate, nitrite did not have an opportunity to accumulate and NAR could not increase.

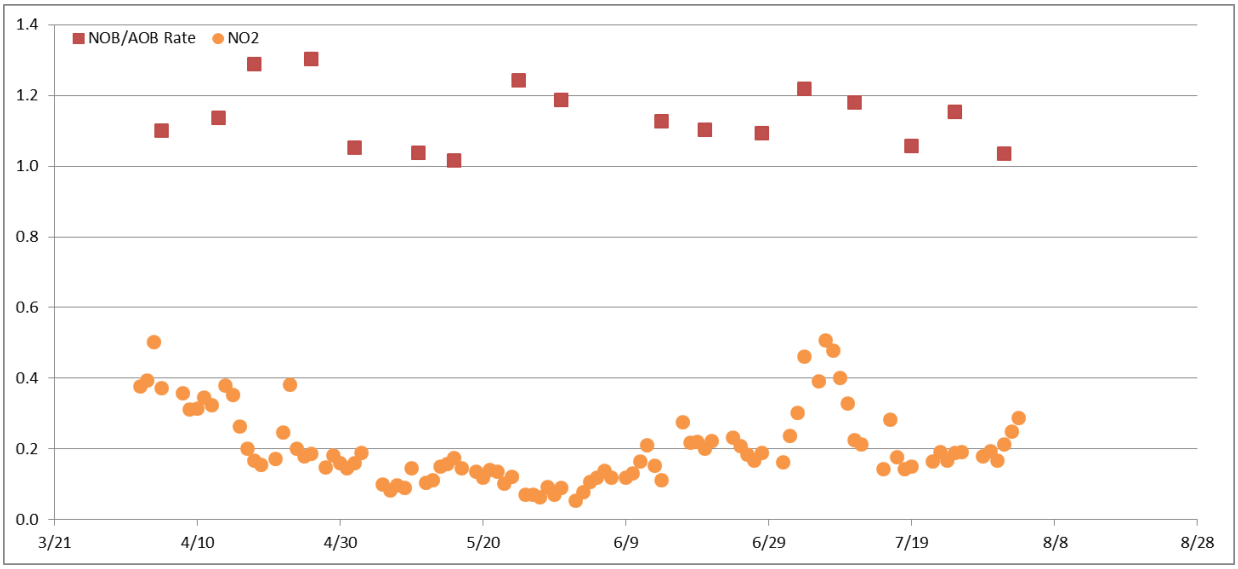

Figure 11: NOB/AOB Rate ratio vs NO_2^- in B-Stage.

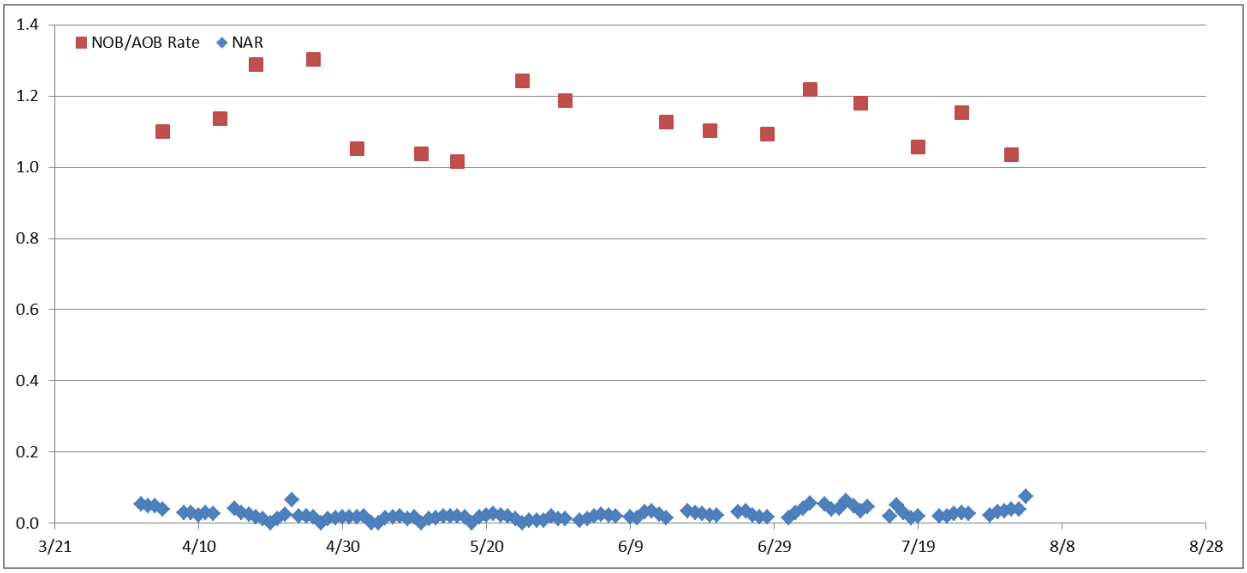

Figure 12: NOB/AOB Rate ratio vs NAR in B-Stage.

B-Stage was intermittently aerated with a 1.5 mg O_2/L setpoint, a NH4-N ≥ 1.5 mg/L effluent, and a SRT close to AOB washout to produce nitrite accumulation. The B-Stage operational conditions used in this study were previously proven to induce NOB outselection in other studies (Regmi et al, 2014; Cao et al, 2017). Previous work at the CE Pilot at 20°C produced NOB outselection and nitrite accumulation (Sadowski, 2015). One reason for failed NOB suppression could be the observed increase in biofilm slime growth in the reactors. The biofilm may have harbored NOB from outselection conditions. NOB could have been sheared or detached from the biofilm. Then, they would become suspended in the mainstream MLSS and in the batch NOB/AOB rate measurement tests. Another factor could have been the large MLSS inventory exposed to anaerobic and anoxic conditions in the sidestream process. In the sidestream, it is possible that NOB could survive the lower DO conditions and AOB could not. The exact reason for this study's failure to produce nitrite accumulation is unclear, but further research into this mystery is ongoing at the CE Pilot.

Column AMX Retention

The ISV analysis produced settling velocities that were used to distinguish between Column UF, B-Stage MLSS, and Column OF (Figure 13). If the Column selected for denser biomass in the UF, the following relationship would appear: UF ISV > B-Stage MLSS ISV > OF ISV. If Column SOR was increased, ISV magnitudes should increase over time.

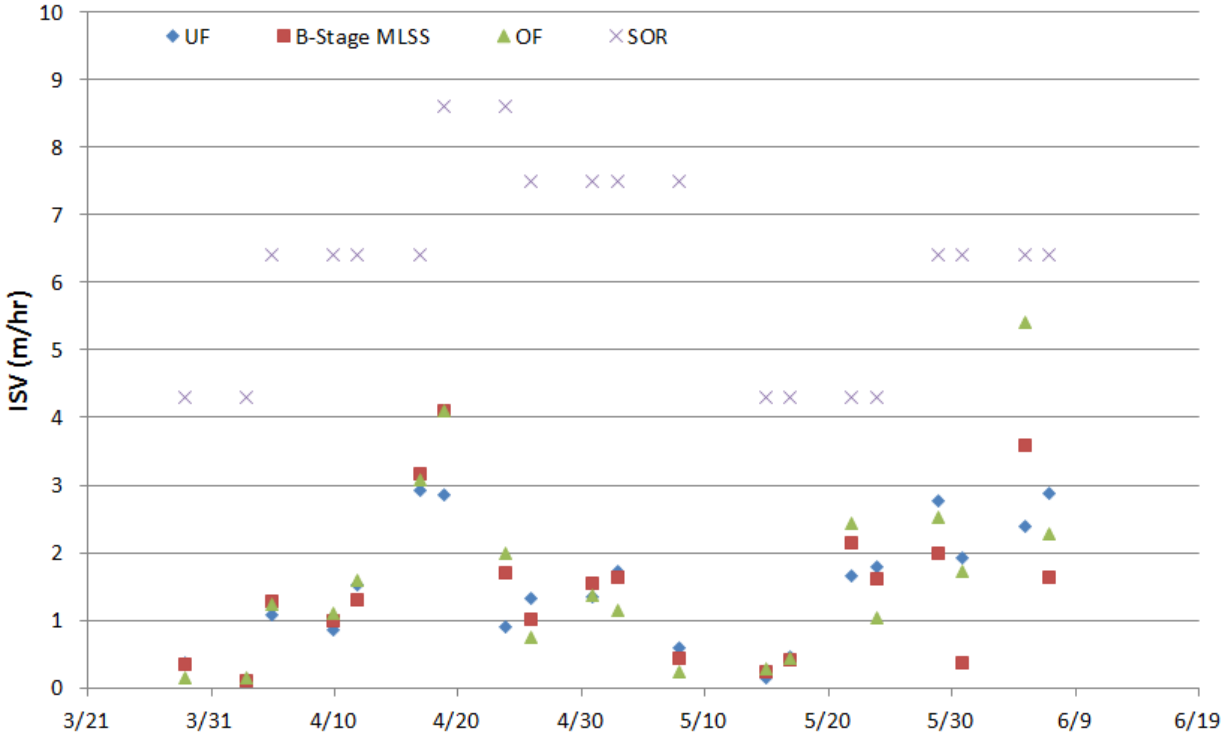

Figure 13: ISV measurements over time for UF, B-Stage MLSS, and OF with respect to a change in SOR.

There does not appear to be a clear trend of UF ISV > B-Stage MLSS ISV > OF ISV throughout the study. The relative difference between the velocities seems insignificant until 5/20. After 5/20, the relative difference in velocity between samples increased. After 5/20, UF ISV was consistently greater than OF ISV and B-Stage MLSS ISV. This shows a change in the column operation around 5/20 could have triggered better outselection. The ISV magnitude loosely trended with SOR fluctuations. This indicates that SOR can influence the magnitude of the settling velocity. Since settling velocity can help select denser biomass, SOR may be a key factor in dense AMX granule retention.

Heme quantification in relatively high amounts can illuminate the presence of AMX. If the column selected for AMX granules in the UF, the following relationship should occur: UF heme > B-Stage heme > OF heme. Two other heme measurements were collected to establish a baseline for comparison. These two other measurements came from samples that do not contain AMX. A-Stage MLSS showed the lowest heme content (Figure 14). MLSS from James River (JR) treatment plant nitrogen removal process was slightly higher than A-Stage MLSS but remained lower than UF, B-Stage MLSS, and OF samples.

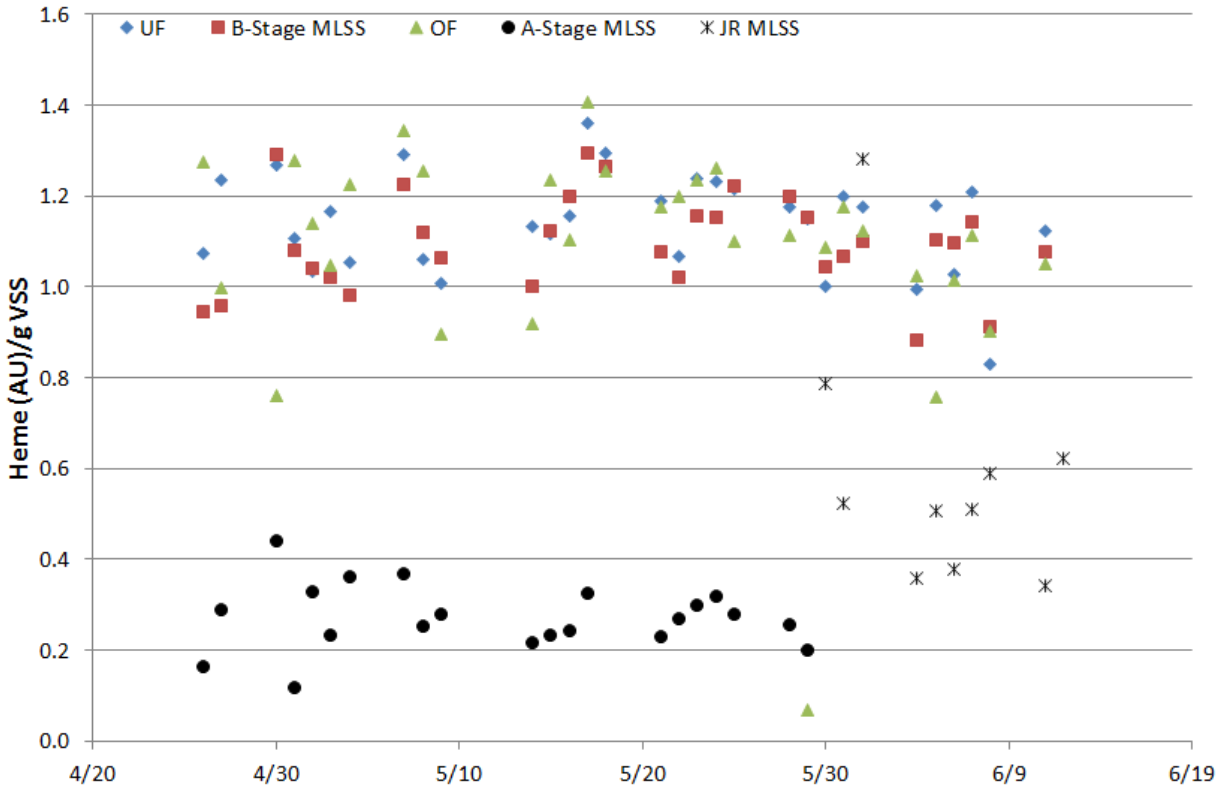

Figure 14: Heme AU/g VSS over time for UF, B-Stage MLSS, OF, and A-Stage MLSS. Note: JR MLSS shows the change in heme content over time from one sample.

The higher magnitude of UF, B-Stage MLSS, and OF heme than the baseline heme content would suggest an elevated amount of AMX was present in the B-Stage process. There does not appear to be a consistent UF heme > B-Stage MLSS heme > OF heme pattern until the later part of the study. This would indicate that the AMX biomass did not settle into the UF until the last month of the study. B-Stage MLSS and OF heme was higher than the baseline samples. This could indicate AMX were retained in the B-Stage process but some AMX were out-selected in the Column OF.

A sludge volume index was determined after five minutes (SVI5) for the UF, B-Stage MLSS, and UF samples (Figure 15). Since SVI5 is a measure of ISV, the UF SVI5 should be > B-Stage MLSS SVI5 > OF SVI5.

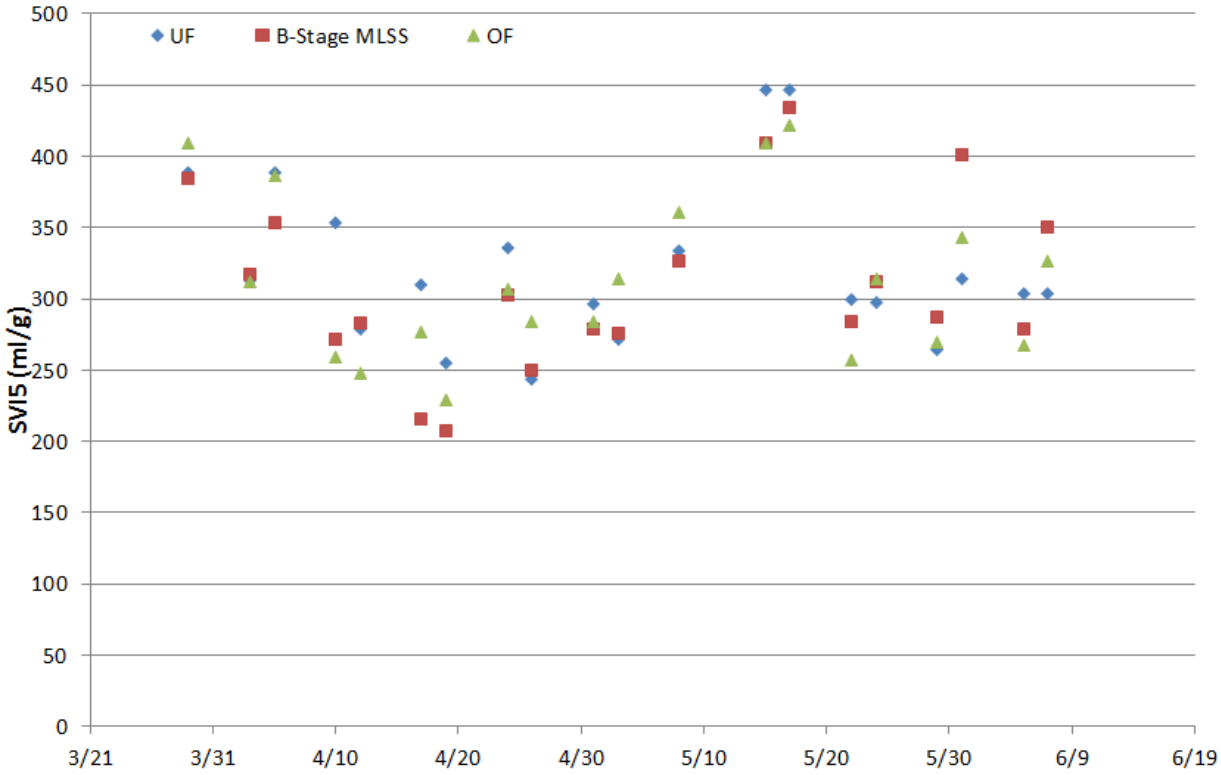

Figure 15: SVI5 over time for UF, B-Stage MLSS, and OF.

Throughout this study, there was not a consistent trend of UF SVI5 > B-Stage MLSS SVI5 > OF SVI5. This could mean the Column did not effectively settle out denser, faster settling biomass in the UF. The magnitude of the SVI5 data was dependent on the performance of B-Stage.

The SVI30 to SVI5 ratio is an indicator of granular to floccular sludge composition. The closer SVI30/SVI5 is to one, the more granular a sludge sample. If the Column selected for AMX granules in the UF, the UF SVI30/SVI5 should be > B-Stage MLSS SVI30/SVI5 > OF SVI30/SVI5 (Figure 16).

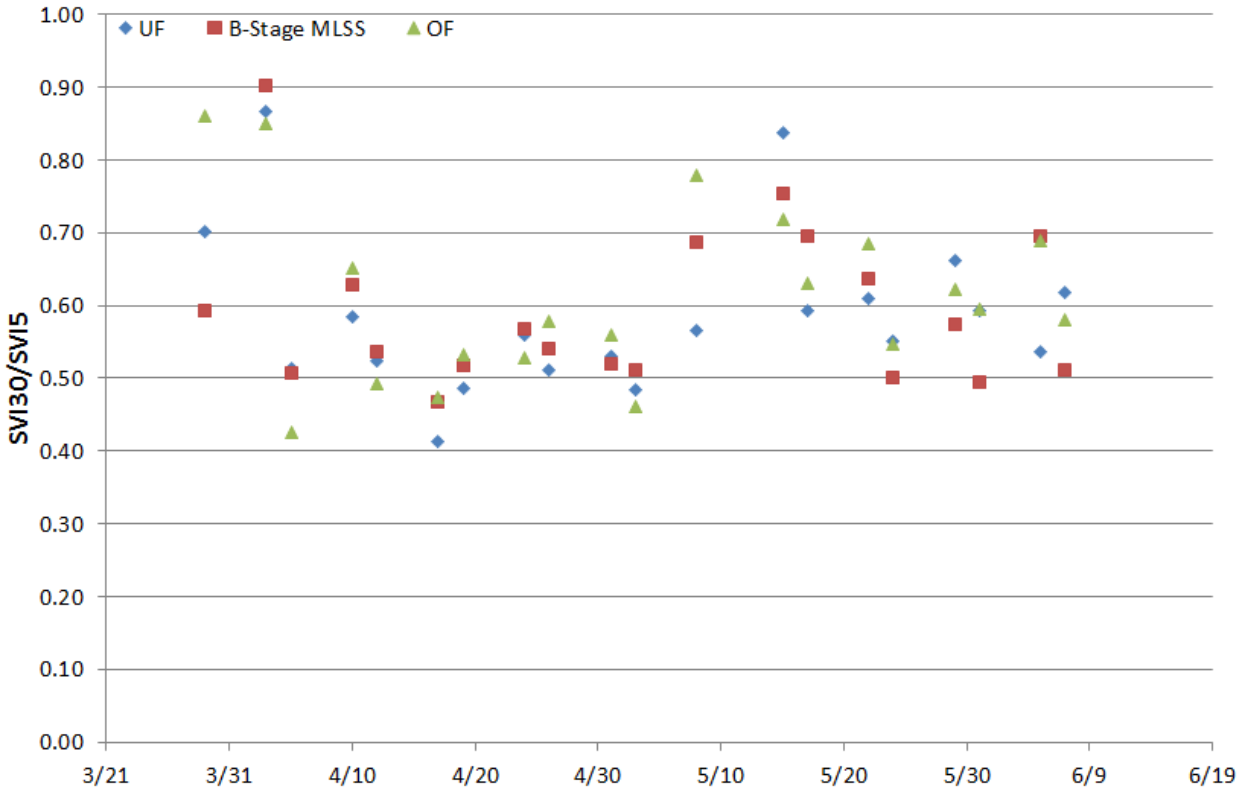

Figure 16: SVI30/SVI5 ratio over time for UF, B-Stage MLSS, and OF.

The SVI30/SVI5 ratio for UF, B-Stage MLSS, and OF exhibit a slight increase, but the increase was not consistent enough to suggest the overall retention of AMX in B-Stage was successful. It is interesting to note that the UF ratio had a steady increase throughout the study, but the B-Stage MLSS and OF ratios steadily decreased after 5/10. This could suggest that the AMX granules were settling into the UF of the Column but did not return to the B-Stage process. During maintenance of the sidestream process, red tinted sludge was trapped in biofilm slime in the SBPR. The SBPR was intermittently mixed and it is possible the granules were trapped in the SBPR and never made it to B-Stage.

Settling Column Monitoring

Gravimetric analysis was performed on UF, B-Stage MLSS, and OF samples from the Column (Figure 17). Successful settling performance in the Column should produce UF TSS > B-Stage MLSS TSS > OF TSS. It is important to note that the magnitude of the B-Stage MLSS was directly affected by B-Stage performance. A change in B-Stage MLSS would alter the magnitudes of UF TSS and OF TSS.

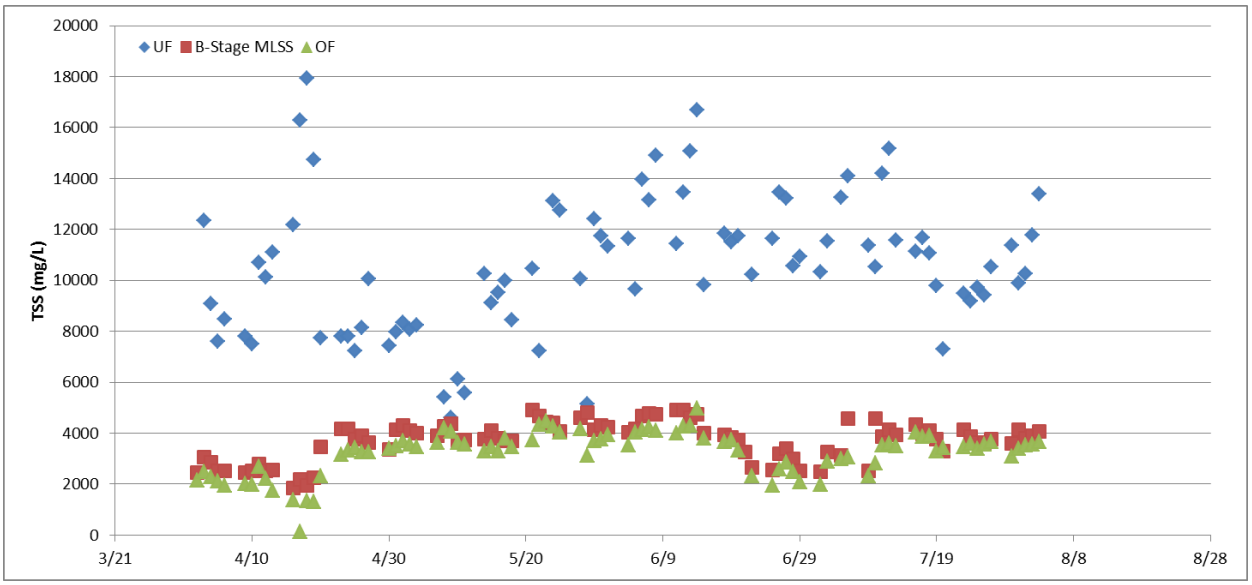

Figure 17: TSS over time for UF, B-Stage MLSS, and OF

Throughout the course of this study, gravimetric analysis has produced the following relationship: UF TSS > B-Stage MLSS TSS > OF TSS values. This is concrete proof that settling occurred in the Column in the duration of this data set. Samples collected from the Column OF were visually less concentrated with solids than the UF. A TSS gradient was obtained several times during this study to confirm the existence of a sludge blanket in the Column bottom. All visual and TSS gradient evidence supports the gravimetric analysis results. Since the UF had a higher TSS than the OF, it can be assumed the more dense AMX granules settled out in the UF.

AMX Stock and Mainstream Act

AMX activity tests were performed on the condensed DEMON® underflow, termed AMX Stock, to ensure highly active AMX granules were added to the mainstream B-Stage (Figure 18). The activity of AMX Stock granules were expected to be very high. This is because the DEMON® sidestream process is exposed to very high nitrogen loading compared to mainstream.

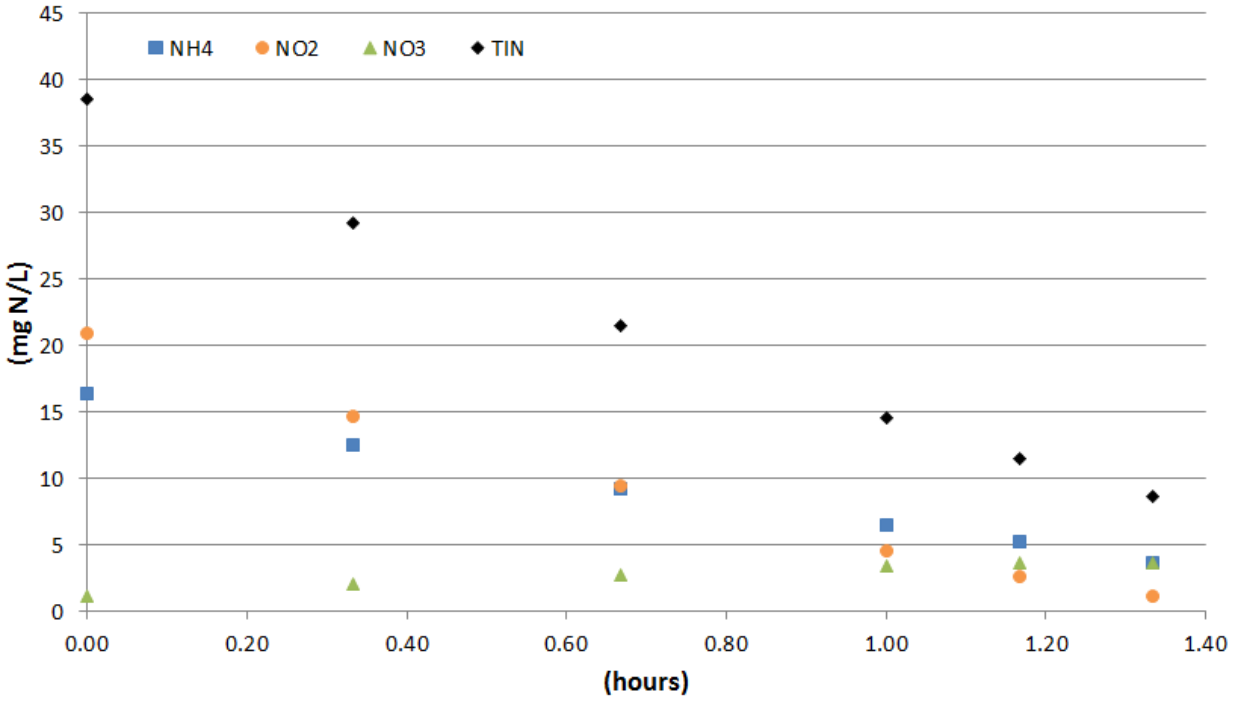

Figure 18: AMX Stock granule activity test showed changes in ammonia, nitrite, nitrate, and TIN over time.

The AMX Stock samples came from sidestream DEMON® processes with high nitrogen loading, and were expected to remove TIN-N at elevated rates. Indeed, AMX Stock showed highly significant TIN removal. Ammonia and nitrite were removed and nitrate was produced throughout the experiment. This is consistent with AMX metabolism. The AMX Stock activity test confirmed significant AMX activity potential was added to B-Stage.

AMX activity tests were conducted on the mainstream B-Stage MLSS to confirm or deny the presence of AMX in the mainstream (Figure 19). Mainstream AMX activity was not expected to be as high as AMX Stock activity. This is because a relatively low volume of AMX Stock granules were diluted into a high volume of mainstream biomass, and mainstream loading conditions were much lower than DEMON® sidestream conditions.

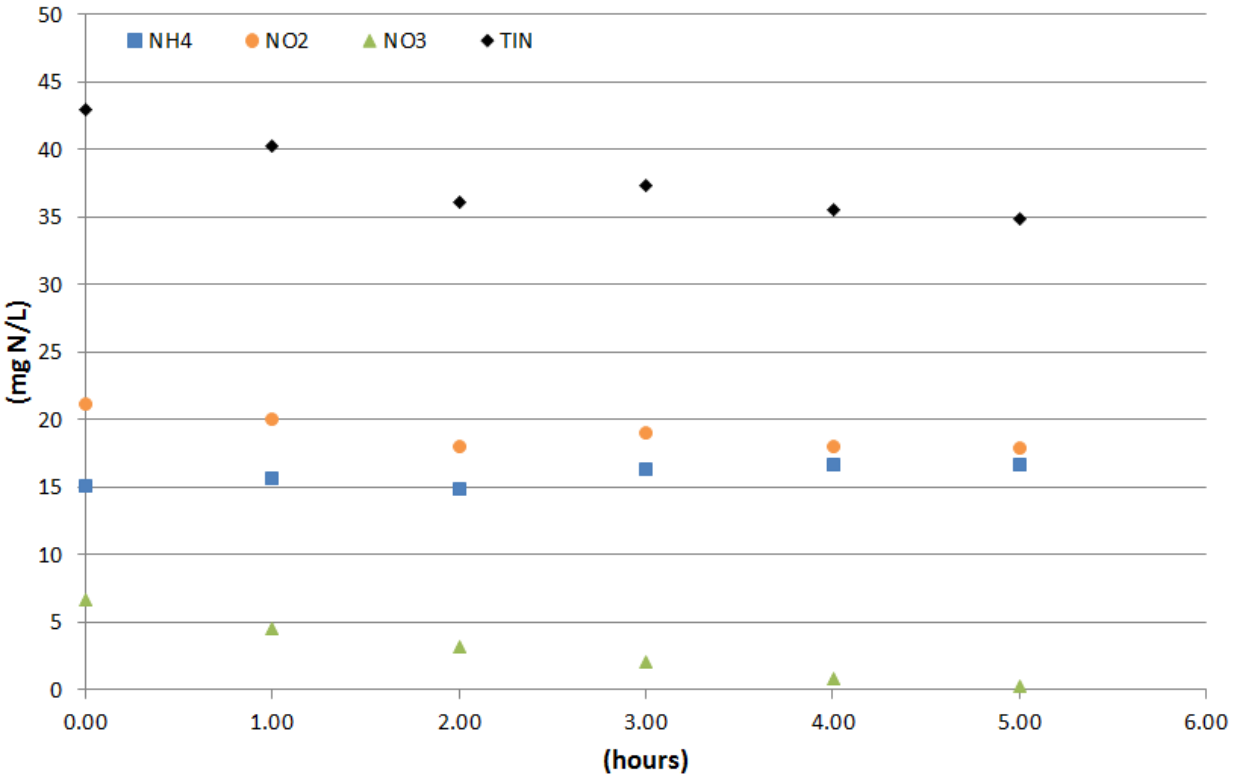

Figure 19: Mainstream AMX activity test showed changes in ammonia, nitrite, nitrate and TIN over time.

Mainstream AMX activity showed a small amount of TIN removal. A higher amount of TIN removal activity was expected but some TIN removal seemed promising. Ammonia had minimal removal over the course of this five hour experiment. Nitrite increased slightly during this test, and nitrate had significant removal. It is clear that TIN removal was due to reduction of nitrate. The nitrate reduction was most likely from denitrification. Overall, there does not appear to be AMX activity in the mainstream. This could mean the Column did not successfully retain AMX granules. The bioaugmentation rate of AMX granules could be another factor affecting apparent mainstream AMX activity. The bioaugmentation rate may not have been high enough to accumulate the AMX biomass necessary to measure AMX activity in the mainstream. The Strass WWTP had similar issues with mainstream AMX detection (Table 1) (Marie et al, 2015).

Table 1: Adapted characteristics of sludge samples from the Strass WWTP (Marie et al, 2015). SL = sludge liquor from the DEMON tank (Sidestream). B = Biological tank (mainstream). OF = overflow of the cyclone. UF = underflow of the cyclone.

	TSS	TSS	VSS	VSS	HQ	HQ	**Heme/VSS**	AM	NH4	**NH4**	NO2	NO3
	(g/L)	(mg/L)	(g/L)	(mg/L)	(mAU/L)	(AU/L)	**(AU/L)/(g/L)**	(mg N/L.hr)	(mg N/L.hr)	**(mg N/mgVSS.d)**	(mg N/L.hr)	(mg N/L.hr)
SL	2.95	2950	2.32	2320	164	0.164	**0.071**	-36.1	-17.2	**-0.178**	-22.7	3.72
SL-OF	2.19	2190	1.77	1770	86.4	0.0864	**0.049**	-23.1	-10.8	**-0.146**	-14.6	2.22
SL-UF	8.95	8950	6.59	6590	1143	1.143	**0.173**	-115	-71.3	**-0.260**	54.7	10.9
B	4.25	4250	3.05	3050	12.3	0.0123	**0.004**	-0.45	3.65	**0.029**	-4.11	0.01
B-OF	4.2	4200	3.03	3030	11.6	0.0116	**0.004**	-1.2	2.57	**0.020**	-3.78	0.01
B-UF	6.71	6710	4.53	4530	25.1	0.0251	**0.006**	-3.23	4	**0.021**	-7.22	-0.01

Since partial nitritation was not established in B-Stage, the AMX granules may not have retained detectable activity. For these reasons, it is difficult to conclude whether AMX granule retention or AMX activity was the issue for mainstream AMX detection.

In the YR full-scale process, DEMON® MLSS would be bioaugmented instead of concentrated underflow. The MLSS would contain AOB and AMX. The addition of supplemental AOB would ensure higher AOB rates than NOB and nitrite should accumulate in the mainstream.

Conclusions

In this study we demonstrated the many challenges to mainstream deammonification. Partial nitritation could not be achieved because, for unconfirmed reasons, NOB suppression did not occur throughout this study. The AMX bioaugmentation process was satisfactory, but the inconclusive retention results paired with disintegration of AMX granules in the SBPR led to zero AMX activity in the mainstream. Therefore, this study presents the numerous obstacles to AMX retention and partial nitritation for mainstream deammonification. Although this study did not illustrate successful deammonification, it has illuminated areas of research for future work.

References

APHA (2012). Standard Methods for the Examination of Water and Wastewater, American Public Health Association.

Åmand, L., G. Olsson and B. Carlsson (2013). "Aeration Control - a Review." Water science and technology : a journal of the International Association on Water Pollution Research 67(11): 2374-2398.

Batchelor, B. (1983). "Simulation of Single-Sludge Nitrogen Removal." Journal of Environmental Engineering 109(1): 1-16.

Boehnke, B., B. Diering and S. W. Zuckut (1997). Ab Process Removes Organics and Nutrients. Alexandria, Water Environment Federation. 9: 23.

Burton, F., G. Tchobanoglous, R. Tsuchihashi, H. D. Stensel, Metcalf and I. Eddy (2013). Wastewater Engineering: Treatment and Resource Recovery, McGraw-Hill Education.

Cao Y, van Loosdrecht, Mark C. M, Daigger GT. Mainstream partial nitritation–anammox in municipal wastewater treatment: status, bottlenecks, and further studies. Applied Microbiology and Biotechnology. 2017;101(4):1365-83.

DeArmond, J. (2015). Adsorption-Style Activated-Sludge: Is It a Practical Treatment Process in North America? , Old Dominion University.

Ferris, M. J., G. Muyzer and D. M. Ward (1996). "Denaturing Gradient Gel Electrophoresis Profiles of 16s Rrna-Defined Populations Inhabiting a Hot Spring Microbial Mat Community." Applied and Environmental Microbiology 62(2): 340-346.

Fredericks, D. K. (2014). Optimization of Intermittent Aeration for Increased Nitrogen Removal Efficiency and Improved Settling. Dissertation/Thesis, Virginia Tech.

Gilbert, E. M., S. Agrawal, F. Brunner, T. Schwartz, H. Horn and S. Lackner (2014). "Response of Different Nitrospira Species to Anoxic Periods Depends on Operational Do." Environmental science & technology 48(5): 2934-2941.

Godwin, J. (2015). Impact of Limited Organic Carbon Addition on Nitrogen Removal in a Mainstream Anammox Moving Bed Biofilm Reactor. Environmental Engineering Master's Thesis, Old Dominion University.

Graham, D. W., C. W. Knapp, E. S. Van Vleck, K. Bloor, T. B. Lane and C. E. Graham (2007). "Experimental Demonstration of Chaotic Instability in Biological Nitrification." The ISME Journal 1(5): 385-393.

Ingildsen, P. (2002). Realising Full-Scale Control in Wastewater Treatment Systems Using in Situ Nutrient Sensors PhD Thesis, Lund University.

Jimenez, J., C. Bott, P. Regmi and L. Rieger (2013). "Process Control Strategies for Simultaneous Nitrogen Removal Systems." Proceedings of the Water Environment Federation 2013(4): 492505.

Kindaichi, T., I. Tsushima, Y. Ogasawara, M. Shimokawa, N. Ozaki, H. Satoh and S. Okabe (2007). "In Situ Activity and Spatial Organization of Anaerobic Ammonium-Oxidizing (Anammox) Bacteria in Biofilms." Applied and Environmental Microbiology 73(15): 4931-4939.

Ma, Y., S. Sundar, H. Park and K. Chandran (2015). "The Effect of Inorganic Carbon on Microbial Interactions in a Biofilm Nitritation–Anammox Process." Water research 70: 246-254.

Malovanyy, A., J. Trela and E. Plaza (2014). "Competition between Aob and Nob in Deammonification Mbbr Treating Mainstream Wastewater." Proceedings of IWA Specialist Conference on Global Challenges for Sustainable Wastewater Treatment and Resource Recovery.

Miller, M. W., R. Bunce, P. Regmi, D. M. Hingley, D. Kinnear, S. Murthy, B. Wett and C. B. Bott (2012). "A/B Process Pilot Optimized for Nitrite Shunt: High Rate Carbon Removal Followed by Bnr with Ammonia-Based Cyclic Aeration Control." Proceedings of the Water Environment Federation 2012(10): 5808-5825.

Park, H., S. Sundar, Y. Ma and K. Chandran (2015). "Differentiation in the Microbial Ecology and Activity of Suspended and Attached Bacteria in a Nitritation-Anammox Process." Biotechnology and bioengineering 112(2): 272-279.

Regmi, P., Holgate, B., Miller, M.W., Park, H., Chandran, K., Wett, B., Murthy, S., and Bott, C.B. (2016). “Nitrogen Polishing in a Fully Anoxic Anammox MBBR Treating Mainstream Nitritation-Denitritation Effluent.” *Biotechnology and Bioengineering*. 113(3):635-642.

Regmi, P., Miller M.W., Holgate, B., Bunce, R., Park, H., Chandran, K., Wett, B., Murthy S., and Bott, C.B. (2014). "Control of aeration, aerobic SRT and COD input for mainstream nitritation/denitritation." *Water Research*. 57:162-171.

Rieger, L., R. M. Jones, P. L. Dold and C. B. Bott (2014). "Ammonia-Based Feedforward and Feedback Aeration Control in Activated Sludge Processes." Water environment research : a research publication of the Water Environment Federation 86(1): 63-73.

Rotthauwe, J. H., K. P. Witzel and W. Liesack (1997). "The Ammonia Monooxygenase Structural Gene Amoa as a Functional Marker: Molecular Fine-Scale Analysis of Natural Ammonia-Oxidizing Populations." Applied and Environmental Microbiology 63(12): 4704-4712.

Strous, M., Fuerst, J.A., Kramer, E.H., Logemann, S., Muyzer, G., van de Pas-Schoonen, K.T., Webb, R., Kuenen, J.G., and Jetten, M.S.M. (1999). "Missing lithotroph identified as new planctomycete." *Nature.* 400:446–449.

Stenstrom, M. K. and R. A. Poduska (1980). "The Effect of Dissolved Oxygen Concentration on Nitrification." Water research 14(6): 643-649.

Stinson, B., S. Murthy, C. Bott, B. Wett, A. Al-Omari, G. Bowden, Y. Mokhyerie and H. De Clippeleir (2013). "Roadmap toward Energy Neutrality & Chemical Optimization at Enhanced Nutrient Removal Facilities." Proceedings of the Water Environment Federation 2013(4): 702-731.

van Dongen, L. G. J. M., S. M. Jetten, M. C. M. van Loosdrecht and C. M. van Loosdrecht (2001). The Combined Sharon/Anammox Process: A Sustainable Method for N-Removal from Sludge Water, IWA Publishing.

Hendrickx, T.L.G., Wang, Y., Kampman, C., Zeeman, G., Temmink, H., and Buisman, C.J.N. (2012). "Autotrophic nitrogen removal from low strength waste water at low temperature." *Water Research*. 46:2187-2193.

Dosta, J., Fernandez, I., Vazquez-Padin, J.R., Mosquera-Corral, A., Campos, J.L., Mata-Alvarez, J., and Mendez, R. (2008). "Short- and long-term effects of temperature on the Anammox process." *Journal of Hazardous Materials*. 154:688-693.

Vazquez-Padin, J.R., Fernandez, I., Morales, N., Campos, J.L., Mosquera-Corral, A., and Mendez, R. (2011). "Autotrophic nitrogen removal at low temperature." *Water Science & Technology*. 63(6):1282-1288.

Cema, G., Wiszniowski, J., Zabczynski, S., Zablocka-Godlewska, E., Raszka, A., and Surmacz-Gorska, J. (2007). "Biological nitrogen removal from landfill leachate by deammonification assisted by heterotrophic denitrification in a rotating biological contactor (RBC)." Water Science & Technology. 55(8-9):35-42.

Isaka, K., Date, Y., Kimura, Y., Sumino, T., and Tsuneda, S. (2008). "Nitrogen removal performance using anaerobic ammonium oxidation at low temperatures." FEMS Microbiology Letter. 282:32-38.

4. Manuscript 2: Anoxic PdN/AMX Polishing MBBR

Abstract

This work describes optimization of carbon addition to an anoxic partial denitrification anammox (PdN/A) moving bed biofilm reactor (MBBR) testing glycerol, acetate, and methanol as carbon sources to maximize total inorganic nitrogen (TIN) removal through the anammox pathway and to minimize effluent TIN. A carbon feeding strategy was developed and was evaluated by the extent of partial denitrification vs full denitrification (partial denitrification efficiency, PdN efficiency). All three carbon sources were capable of high TIN removal, low effluent TIN, and moderate to high PdN efficiency. Average TIN removal for glycerol was 10.0 ± 3.6 mg TIN/L, for acetate it was 8.7 ± 2.9 mg TIN/L, and for methanol it was 11.5 ± 5.6 mg TIN/L. Average effluent TIN for glycerol was 6.0 ± 4.0 mg TIN/L, for acetate it was 5.0 ± 1.1 mg TIN/L, and for methanol it was 4.3 ± 1.5 mg TIN/L. Average PdN efficiency for glycerol was 91.0 ± 9.0%, for acetate it was 88.0 ± 7.7%, and for methanol it was 74.0 ± 8.5%. When PdN efficiency was factored into the cost of each carbon source, methanol was 5.83% cheaper than glycerol per mass TIN removed and 59.0% cheaper than acetate per mass TIN-N removed.

Introduction

Total nitrogen (TN) effluent standards have become more stringent across the globe, and the wastewater industry needs less expensive and more resource efficient technologies to comply. The state of the practice for TN removal is biological nitrification-denitrification, but more cost effective technologies like deammonification are being investigated. The drivers for mainstream deammonification are the reduction of external carbon addition, decreased aeration demand for nitrogen removal, and decreased need for carbon which allows for more carbon to be diverted for energy recovery. Deammonification consists of partial nitritation and anaerobic ammonia oxidation (AMX). Ammonia oxidizing bacteria (AOB) convert a portion of the available ammonia to nitrite, and AMX converts the residual ammonia and nitrite to nitrogen gas according to the following equation (Strous et al, 1998).

Anammox Metabolism:

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

Deammonification has been successful in sidestream applications, but mainstream deammonification has remained challenging. Mainstream deammonification is difficult because nitrite oxidizing bacteria (NOB) are harder to suppress than in sidestream. Lower ammonia concentrations (< 100 mg N/L) and lower temperatures (< 30 °C) typical of mainstream wastewater do not favor NOB outselection (Regmi et al, 2016), but as long as the slow growth of AMX can be accommodated through biofilm growth or selective retention, wastewater temperatures ≤ 20°C appear to be achievable for AMX (Dosta et al., 2008; Vazquez-Padin et al, 2011; Cema et al, 2007; Hendrickx et al, 2012; Isaka et al, 2008).

Some studies have been successful at shortcut nitrogen removal but were unable to reach low effluent total inorganic nitrogen (TIN) (≤ 5 mg TIN-N/L) (Han et al, 2016; Laureni et al, 2016; Lotti et al, 2014; Regmi et al, 2014). To comply with lower effluent discharge limits a nitrogen polishing step may be

required following the nitrogen removal process. Also, to achieve partial nitritation for deammonification, it has been shown that a residual ammonia concentration (1-2 mg/L) is necessary (Regmi et al, 2014; Al-Omari et al, 2015; Han et al, 2015). Also, upsets with AMX activity in the deammonification process may result in nitrite bleeding into the effluent. Nitrite discharge can be toxic to receiving waterways and can cause a much higher chlorine demand for disinfection. Nitrate can be polished with a carbon source (electron donor) for full denitrification of nitrate to nitrogen gas. AMX polishing can remove ammonia and nitrite without an external carbon source. It is possible to remove ammonia, nitrite, and nitrate effectively in a single polishing reactor. To remove residual nitrate, a carbon source can be added to induce partial denitrification (PdN) from nitrate to nitrite, and residual ammonia and available nitrite can be removed through the AMX pathway. The main advantage of utilizing PdN combined with AMX (PdN/A) over a full denitrification process is that only a fraction of the COD is required to reduce nitrate to nitrite. Another benefit is that since AMX can remove ammonia anoxically, the amount of ammonia that needs to be oxidized in the preceding BNR process is reduced.

The main challenge for efficient PdN/A is limiting full denitrification (denitritation), therefore maximizing PdN for nitrite to be available for AMX. Nitrite can be accumulated by a system with a higher nitrate reduction rate than nitrite reduction rate (Betlach and Tiedje, 1981). It can also be accumulated by creating conditions to proliferate bacteria that can only reduce nitrate to nitrite (Blaszczyk, 1993). PdN can be influenced by several factors such as the, COD/NO_3-N, pH, DO, SRT, and carbon source (Obaja et al, 2005; Beccari et al, 1983; Almeida et al, 1995a; Tiedje, 1988). For a mainstream single reactor that combines PdN and AMX, pH would not be a cost effective parameter to change to increase PdN. Also, AMX require a DO of 0 mg/L and this means DO is not a viable parameter to increase PdN. For an attached growth system like the MBBR discussed in this study, SRT is not a parameter that is easily changed or monitored in order to increase PdN.

Carbon addition optimization would limit the carbon available for denitritation. Low COD/NO_3-N ratios may induce good PdN through limiting the quantity of electrons available to nitrite reductase in OHOs (Almeida et al., 1995b). A study confirmed that with inadequate carbon dosing (COD/NO_3-N $\leq$ 1) significant nitrite accumulation was observed (Her and Huang, 1995). Another study saw FdN and no PdN with a COD/NO_3-N ratio of 2 (g/g), but when the COD/NO_3-N ratio was decreased to 1 (g/g), significant nitrite accumulation occurred (Oh and Silverstein, 1999). Interestingly, the COD/NO_3-N ratio did not directly select for PdN, but did control the rate of nitrate reduction in one study (Le et al, 2019a). Therefore, the COD/NO_3-N ratio may be a good indicator to monitor for a PdN/A system.

The specific bacterial culmination and nitrite accumulation abilities of denitrifiers were influenced by the type of carbon source utilized in the PdN/A process (Akunna et al, 1993). Studies have shown the ability of glycerol and acetate to induce significant nitrite accumulation (Bill et al, 2009; Ledwell et al, 2011; van Rijn et al, 1996). In fact, methanol, acetic acid, and glucose showed 17%, 21%, and 23% NO_2-N accumulation with a low COD/NO_3-N (Her and Huang, 1995). In another study, glycerol, acetate, and methanol were all capable of performing PdN, but glycerol and acetate displayed more efficient PdN than methanol and this was attributed to their different electron transport pathways (Le et al, 2019a). It is promising for PdN/A that methanol, acetate, and glycerol all showed the capability to accumulate nitrite. Methanol is the most widely used supplemental carbon source for full-scale in the USA because

of low cost and favorable kinetics (Bill et al, 2009). Methanol may be well suited for full denitrification, but the success of PdN/A processes hinges on the efficiency of denitratation and inhibition of denitritation. Therefore, it is worth considering carbon sources other than methanol for PdN/A such as glycerol and acetate.

Another possible explanation to the mechanism behind PdN is rapid complete onset of all denitrification genes vs progressive onset (Liu et al, 2013). Progressive onset is when electrons flow to nitrate reductase first, and then to nitrite reductase after most of the nitrate has been reduced (Liu et al, 2013). Therefore, the progressive onset of reductase upregulation would inherently accumulate nitrite.

A few studies have coupled AMX with PdN. PdN and AMX were able to perform PdN/A using sidestream sulfide as an electron donor for PdN (Mulder et al, 1995). DEnitrifying AMmonium OXidation (DEAMOX), functionally synonymous with PdN/A, has also been done with acetate, with a COD/NO3-N ratio of 3.48 (g/g), and resulted in 95% ammonia removal (Kalyuzhnyi et al, 2006). Using acetate, a SBR DEAMOX process was also able to remove 95% of total influent nitrogen with mainstream wastewater properties (Du et al., 2017). Another study concluded that as long as a large enough reservoir of AMX activity was competing for nitrite, the three carbon sources used (glycerol, acetate, and methanol) were capable of performing PdN/A, but glycerol and acetate were more effective at PdN than methanol (Le et al, 2019a).

The objectives of this research were to maximize TIN removal through the AMX pathway, optimize carbon addition, and to minimize effluent TIN using glycerol, acetate, and methanol through a PdN/A MBBR process. It was hypothesized that methanol would not perform as well with PdN as glycerol and acetate, and could potentially even inhibit AMX activity as several studies previously indicated (Guven et al, 2005; Jensen et al, 2007; Isaka et al, 2008; Oshiki et al, 2011). To date, there has not been a successful PdN/A process using methanol, and this research closes that knowledge gap.

In this paper, we investigated glycerol, acetate, and methanol for PdN/A. We also presented results of a pilot-scale fully anoxic PdN/A polishing MBBR process that can be implemented at full-scale facilities to maximize TIN removal and minimize effluent TIN using minimal external carbon.

Materials and Methods

This study occurred in a continuous domestic wastewater adsorption/bio-oxidation (A/B) process pilot system located at the Hampton Roads Sanitation District (HRSD) Chesapeake-Elizabeth treatment plant (CE Pilot) in Virginia Beach, VA (Figure 20). Screened and degritted Chesapeake-Elizabeth WWTP influent was fed to an additional grit and scum removal tank in the CE Pilot. After screening and degritting, the influent went to a 20°C temperature controlled tank, then to the A-Stage high rate activated sludge (HRAS) process. B-Stage was a biological nutrient removal (BNR) process comprised of five CSTRs with a total volume of 660 L and a 4.3 hour HRT. The B-Stage process utilized intermittent NH_4 vs NOx (AvN) aeration control, where air on/off time was controlled via PID to meet a NOx/NH_4 ratio (typically 1-1.3) in the last aerated reactor. A 340 L anoxic PdN/A MBBR following B-Stage enabled additional nitrogen removal prior to final discharge. The MBBR had a Styrofoam cover to limit oxygen transfer, a variable speed mixer (Caframo: Georgian Bluffs, Ontario, CA), a 60% AnoxKaldness K3 media

fill volume, a 2-hr HRT, and a TIN loading rate between 0.5-0.9 g TIN/m^2/day. The carbon dosing control strategy for the MBBR was a feedback PID loop and the carbon dosing rate was controlled to meet a NOx setpoint in the MBBR based on a NOx sensor in the process (Nitratax plus sc, Hach). This NOx probe read a sum of the in situ nitrate and half of the in situ nitrite. Since the MBBR rarely had significant nitrite concentrations, the NOx probe and setpoint were good representations of the in situ/effluent nitrate concentration. The NOx setpoint was adjusted manually to maximize TIN removed in the MBBR while minimizing full denitrification to N_2 gas.

The MBBR began operation 7 years ago. It was initially seeded with AMX granules, but complications washed out the AMX from the reactor. Then, a clarifier was installed to retain AMX biomass and it was fed ammonia, nitrate and some nitrite from the B-Stage effluent process. Some carbon addition studies were previously done at the CE Pilot, but prior to this research the MBBR work was idle for 2 years. It simply was receiving ammonia and nitrate from B-Stage effluent with no particular attention paid to its operation.

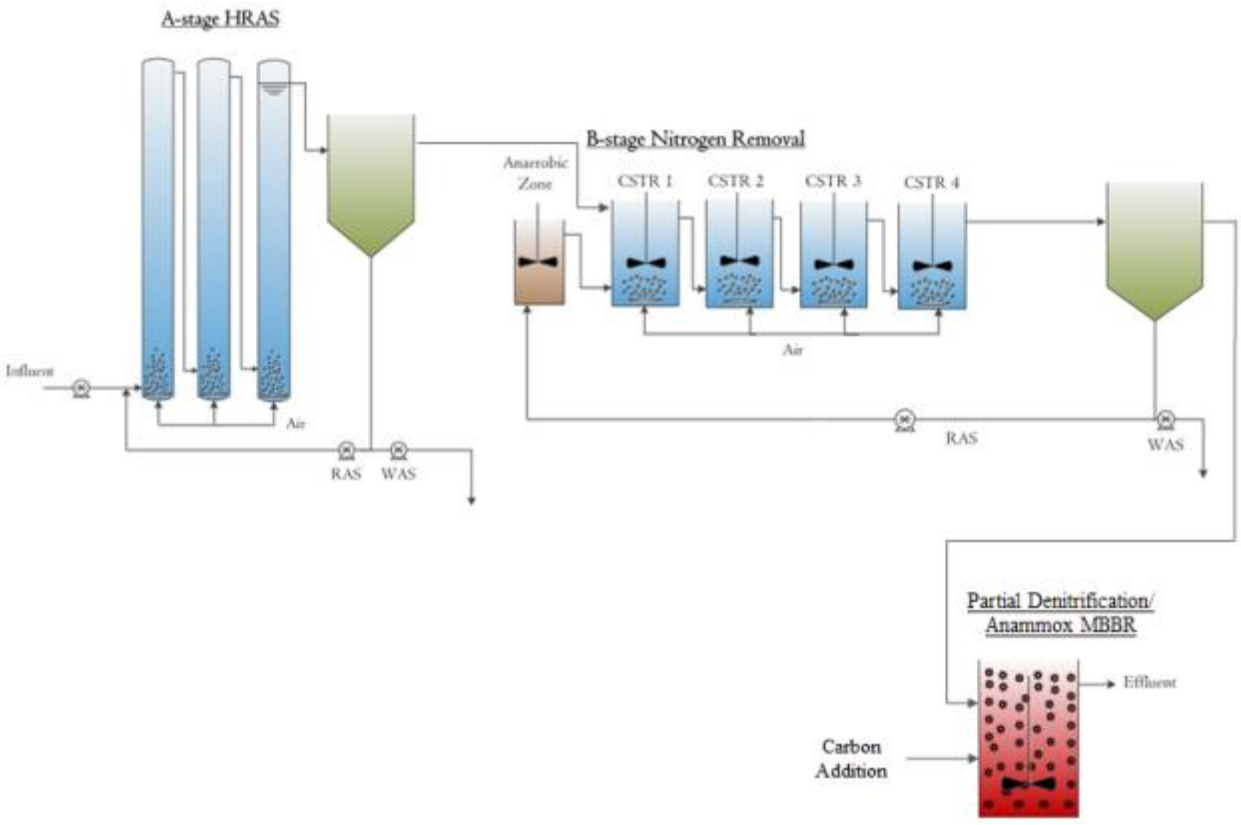

Figure 20: CE Pilot process flow diagram.

General Sample Monitoring

Automated samplers provided 24-hour composite samples (ISCO, Lincoln, NE). This enabled average daily influent and effluent characteristics to be measured. TSS and volatile suspended solids (VSS) were analyzed using 2540D and 2540E in standard methods. NH_4-N, NO_3-N, NO_2-N, orthophosphate (OP), total chemical oxygen demand (COD), and soluble COD (sCOD) were measured with HACH TNTplus kits and a HACH DR2800 spectrophotometer (HACH Loveland, CO). Daily DO was recorded with a HACH handheld luminescent DO sensor (HACH Loveland, CO). Daily pH and temperature was recorded with a HACH handheld pH and temperature meter (HACH Loveland, CO).

Carbon Dosing Control Strategy

A carbon dosing strategy was developed for this study. A NOx setpoint was user specified in the PLC. A NOx sensor in the PdN MBBR returned a signal to the PLC. If NOx in the PdN MBBR was higher than the setpoint in the PLC, the PLC sent a signal to increase the carbon addition flowrate, vice versa. AMX metabolism has the following stoichiometric properties: NO_2-N removed/NH_4-N removed = 1.32 g/g, NO_3-N produced/NH_4-N removed = 0.26 g/g. The nitrate produced by AMX was reduced by denitrifiers to nitrite for AMX and more ammonia can be removed through the AMX pathway. Therefore, the net NOx-N removed/NH_4-N removed = 1.06 g/g. Overdose of carbon facilitated full denitrification (FdN) and less nitrogen available for AMX. More FdN increased the net NOx-N removed/NH_4-N removed ratio. To prevent carbon overdose and maximize partial denitrification, NOx setpoint was increased when the net NOx-N removed/NH_4-N removed rose above 1.16 g/g. A 0.10 g/g tolerance increased the aggressiveness of this strategy. To ensure sufficient carbon was available for PdN, NOx setpoint was decreased when the net NOx-N removed/NH_4-N removed ratio was below 1.06 g/g. The NOx setpoint was recorded daily and was compared to the composite effluent NO_3-N values.

Ex Situ Denitrification Rate Measurement

Batch testing was performed with 4 L of B-Stage MLSS continuously mixed with a magnetic stir bar and plate (Corning, Corning, NY). A floating Styrofoam cover was used to prevent oxygen transfer. The pH was maintained between 6.8 and 7.2 with diluted hydrochloric acid and sodium bicarbonate addition. When DO was stable at 0 mg O_2/L via nitrogen gas sparging, 20-30 mg N/L of dissolved potassium nitrate, 1-3 mg N/L of dissolved sodium nitrite, and non-limiting (100-400 mg/L) sCOD were spiked to the reactor. Samples were collected every 15 minutes and were immediately filtered with 0.45 µm cellulose acetate filters and analyzed for sCOD, NH_4-N, NO_3-N, and NO_2-N. The test was concluded after 1.5 hours and denitrification rates were based on the slope of the NOx-N concentration over time.

Anoxic PdN/A MBBR in situ Denitrification Rate Analysis

The carbon addition was halted, and the PdN/A MBBR was isolated from influent flow for approximately 30 min. DO and pH were recorded and pH was maintained between 6.5 and 7.5 via alkalinity reimbursement from AMX activity. Residual ammonia was removed via AMX with nitrite addition. To

avoid substrate limitation, NO_3^- and NO_2^- were added to obtain an initial concentration of 15 and 5 mg N/L respectively. Also, non-limiting sCOD (100-400 mg/L) was added via glycerol, acetate, or methanol. After spiking, an initial sample was taken. Samples, pH values, and DO concentrations were collected every 15 minutes. All collected samples were immediately filtered with 0.45 µm cellulose acetate filters and analyzed for sCOD, NO_3-N, NO_2-N, and NH_4-N. The sCOD used per nitrite and nitrate reduction was calculated using these rates. Since denitrification rate and sCOD consumption was known for solely nitrite and also for nitrate and nitrite together, the sCOD utilized per nitrite and nitrate reduction could be calculated using a system of equations (See SI for detailed calculations).

Anoxic PdN/A MBBR in situ AMX Activity Analysis

The carbon addition was halted, and the PdN/A MBBR was isolated from influent flow. Approximately one hour was allotted for residual sCOD degradation. Handheld DO and pH probes were installed in the MBBR. DO was maintained at 0 mg/L via a Styrofoam cover and pH remained between 6.5-7.5 via AMX alkalinity reimbursement from activity. To avoid substrate limitation, ammonia chloride and sodium nitrite were added to obtain an initial concentration of 20 mg N/L of both NH_4^+ and NO_2^-. Samples, pH values, and DO concentrations were collected every 15 minutes. All collected samples were filtered with 0.45 µm cellulose acetate filters and analyzed for sCOD, NO_3-N, NO_2-N, and NH_4-N. AMX rates were calculated from the NH_4-N and NO_2-N consumption versus time, and NO_3-N production versus time slopes.

Fluorescent in situ hybridization (FISH) Analysis

FISH was performed to detect and qualitatively assess the relative abundance of nitrospira, AOB, and AMX in the PdN/A MBBR media samples. Biofilm enriched carriers were chemically fixed in a 4% formaldehyde solution for eight hours at 4°C, embedded in optical cutting temperature medium (Tissue-Tek® O.C.T.), and cryosectioned (at -25°C) to yield biofilm sections with a 20 µm thickness (Leica Reichert Jung 2800N Frigocut) on poly-L-lysine coated slides. FISH was performed using an oligonucleotide probe mix targeting a wide diversity of nitrospira (6-FAM fluorophore), AOB (Cy3 fluorophore), and AMX (Cy5 fluorophore) found in wastewater treatment bioreactors. Cryosections were hybridized overnight following protocols adapted from Nielsen et al. (2009) in a 35% formamide v/v hybridization buffer containing nitrospira oligonucleotide probe concentrations of 0.83 µM, AOB oligonucleotide probe concentrations of 0.5 µM, and AMX oligonucleotide probe concentrations of 0.5 µM. All probe mixes included equimolar concentrations of the recommended competitor oligonucleotides. Each sample was separately stained with the nonsense probe (Non-EUB338) tagged with 6-FAM, Cy3, and Cy5 fluorophores as a negative control for non-specific binding. Hybridized cryosections were counterstained with DAPI at a concentration of 1 µg/mL in a glycerol-based antifadent mounting media (Citifluor AF1). Image stacks were acquired on an inverted confocal laser scanning microscope (Model TCS SP5, Leica Microsystems) equipped with an oil immersion 63× (1.44 NA) objective at a lateral resolution of 0.48 µm and an axial step size of 1 µm. DAPI, 6-FAM, Cy3, and Cy5 fluorophores were excited sequentially with 405nm, 488nm, 561nm, and 633nm laser lines, respectively.

Microbial Quantification

PdN/A MBBR media was collected, and placed into 50 mL of Tris-Acetate-EDTA 1x solution. As much attached biomass as possible was scraped (0.1 mL minimum) into a 1.5 mL centrifuge vial. Each vial was centrifuged at 13,000 rpm for 3 minutes. Supernatant was decanted and 1.5 mL of RNA protect (Qiagen, Venlo, NL) was added. Each vial was vortexed and incubated at room temperature for 5 minutes. Each vial was centrifuged again for 3 minutes at 13,000 rpm.

Supernatant was decanted and the pellet was stored in a freezer at -80°C. Frozen samples were shipped to Columbia University Department of Earth and Environmental Engineering Dr. Kartik Chandran's laboratory for analysis. The frozen sample was homogenized with a grinder, and a 1 ml TE buffer solution was added. The sample was re-suspended with a vortex machine and either 0.1 ml (10 fold dilution) or 0.2 ml (5 fold dilution) re-suspended sample was used for DNA extraction.

The AOB and NOB abundance was quantified using TaqMan quantitative polymerase chain reaction (qPCR). AOB were targeted with the NH_4þ mono-oxygenase subunit A (amoA) gene (Rotthauwe et al, 1997), and NOB were targeted with the Nitrobacter 16S rRNA gene (Graham et al, 2007) and the Nitrospira 16S rRNA gene (Kindaichi et al, 2007). Total bacterial abundance was quantified with eubacterial 16S rRNA gene targeted primers (Ferris et al, 1996). qPCR assays were conducted on a iQ5 real-time PCR thermal cycler (BioRad Laboratories, Hercules, CA). Standard curves for qPCR were generated via serial decimal dilutions of plasmid DNA containing specific target gene inserts. qPCR for standard plasmid DNA and sample DNA were conducted with duplication and triplication, respectively. DNA-grade deionized distilled water (Fisher Scientific, MA) was used for non-template control. Primer specificity and the absence of primer-dimers were confirmed via melt curve analysis of each qPCR profile (Ma et al, 2015; Park et al, 2015).

Carbon Sources Used, Origin, and Stock Generation

The carbon sources used in this research were glycerol, acetate, and methanol. Glycerol was a MicroC®2000 product. Acetate was from sodium acetate and glacial acetic acid from Fisher Scientific. Methanol was 99% pure and from Fisher Scientific. The COD stock solution was created in a 50 L carboy. The COD source was diluted to create 50 L of solution and a concentration of approximately 10,000 mg COD/L. The COD stock concentration was confirmed with TNT COD Hach tube test kits.

Methanol Dosing Transition Phase

Methylotroph laden sludge was seeded from the anoxic zone methanol denitrification step at the VIP WWTP at HRSD. The methanol dosing started with 1 mg MeOH/L MBBR in situ concentration and was increased daily. AMX activity tests were conducted to ensure no adverse consequences came to the AMX bacteria. After two weeks, the reactor was capable of normal carbon dosing operation using methanol.

Methanol Overdose Experiment

A baseline AMX activity test was performed. Then, the AMX MBBR was exposed to 24 hours of a toxic concentration of methanol. At the conclusion of the 24 hour overdose, an overnight wash occurred. Then, another AMX activity test was conducted to see if the methanol had affected AMX activity when compared to the baseline test. This experiment simulated a full-scale malfunction that resulted in an overdose of methanol to an AMX MBBR.

A graduated cylinder was used to measure 758 ml of pure methanol. The measured methanol was poured into an empty 50 L carboy. The carboy was filled to the 50 L mark with potable water and this yielded a stock concentration of 18000 mg COD/L. A digital L/S pump provided 203 mg COD/L at a constant 29 ml/min flow rate in the MBBR for 24 hrs. The maximum concentration of COD the MBBR would be exposed to is about 50 mg COD/L so the overdose design of 203 mg COD/L represents a safety factor of greater than 400% for this design. After the 24-hr overdosing, methanol feed was halted, and the MBBR was allowed to wash out excess methanol overnight before another AMX activity test was performed.

Methanol TIN Loading Increase

Toward the of the methanol phase ammonia chloride and potassium nitrate were dissolved into a solution to be added to the MBBR. The intent was to raise the TIN loading to the PdN/A process to explore the limits of the treatment capacity.

Results

PdN/A Performance

B-Stage effluent had an average AvN of 1.3 ± 0.4 NOx/NH_4-N and was comprised of an average 6.6 ± 2.2 mg NH_4-N/L, 0.6 ± 0.7 mg NO_2-N/L, and 7.4 ± 2.2 mg NO_3-N/L. The study was organized into five phases.

Nitrogen Species Removed

Phase I used glycerol and was from day 1 to day 60. Phase II used acetate and was from day 61 to day 108. Phase III used no carbon and was from day 109 to day 116. Phase IV was the methanol startup phase and was from day 117 to day 128. Phase V used methanol and was from day 129 to day 172. At times, effluent NO_2-N was greater than the influent, and this indicated NO_2-N production via partial denitrification was greater than influent NO_2-N plus NO_2-N reduction (Figure 21). This NO_2-N accumulation was due to greater PdN than influent NO_2-N, AMX activity, and FdN combined. High influent TIN loading may have also caused this NO_2-N accumulation when NH_4-N was limiting for AMX activity.

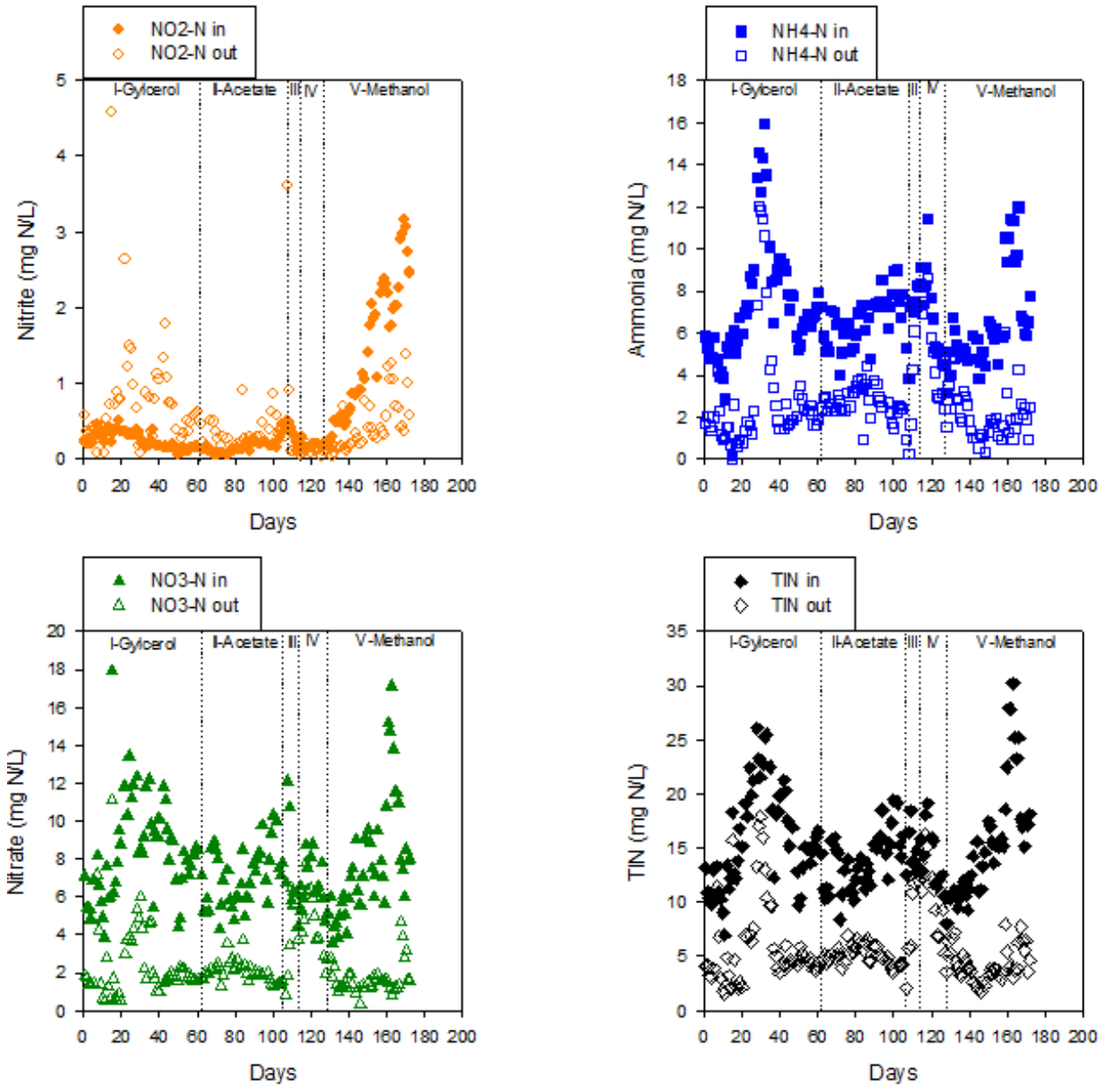

Figure 21: MBBR influent and effluent nitrogen species concentrations for glycerol, acetate, and methanol. Top Left – nitrite orange circles, Top Right – ammonia blue squares, Bottom Left – nitrate green triangle, and Bottom Right – TIN black diamonds. Vertical dotted lines indicate start and end days for each carbon phase.

B-Stage disturbances directly affected TIN loading to the PdN/A MBBR. For example, B-Stage nitrification complications would not supply sufficient nitrate to the MBBR for effective PdN/A. Some nitrite breakthrough was observed as the loading to the MBBR increased. This could indicate the system became limited by AMX activity, and specifically ammonia. Target effluent TIN was 5 mg/L. During the methanol phase, additional NH_4-N and NO_3-N were loaded to the MBBR to synthetically increase TIN loading. This sharp increase in TIN loading resulted in very high TIN removed, and showed the MBBR had a higher treatment capacity than typical B-Stage effluent TIN loading provided.

Glycerol had an average of 10.0 ± 3.6 TIN removed (mg N/L), acetate had an average of 8.7 ± 2.9 TIN removed (mg N/L), and methanol had an average of 11.5 ± 5.6 TIN removed (mg N/L). Glycerol has an average effluent TIN of 6.0 ± 4.0 (mg N/L), acetate had an average effluent TIN of 5.0 ± 1.1 (mg N/L), and methanol had an average effluent TIN of 4.3 ± 1.5. It is also worth noting that the no carbon addition phase had an average of 1.9 ± 0.53 TIN removed (mg N/L).

PdN Efficiency

An important metric in evaluating the PdN/A MBBR was PdN efficiency. PdN efficiency was a measure of NO_3-N reduction to NO_2-N (PdN) vs NO_3-N reduction to NO_2-N and then to gaseous nitrogen for full denitrification (FdN). NO_2-N produced in the MBBR was predicted by applying AMX metabolic stoichiometry to the actual NH_4-N removed (Eq. 1). The most efficient carbon dose would reduce NO_3-N to NO_2-N (PdN) and no NO_2-N would reduce to gaseous nitrogen. This would yield 100% PdN efficiency, and PdN efficiency could vary with carbon source, carbon dosage, biofilm community composition, substrate availability, and AMX activity (Figure 22).

Eq. 1:

$$PdN\ Efficiency\ (\%) = 100 - \left[100 * \left(\frac{NO_{x,rem} - \left\{NH_{4,rem} * \left(\frac{1.32 * NO_{2,rem}}{NH_{4,rem}} - \frac{0.26 * NO_{3,pro}}{NH_{4,rem}}\right)\right\}}{NO_{x,in}}\right)\right]$$

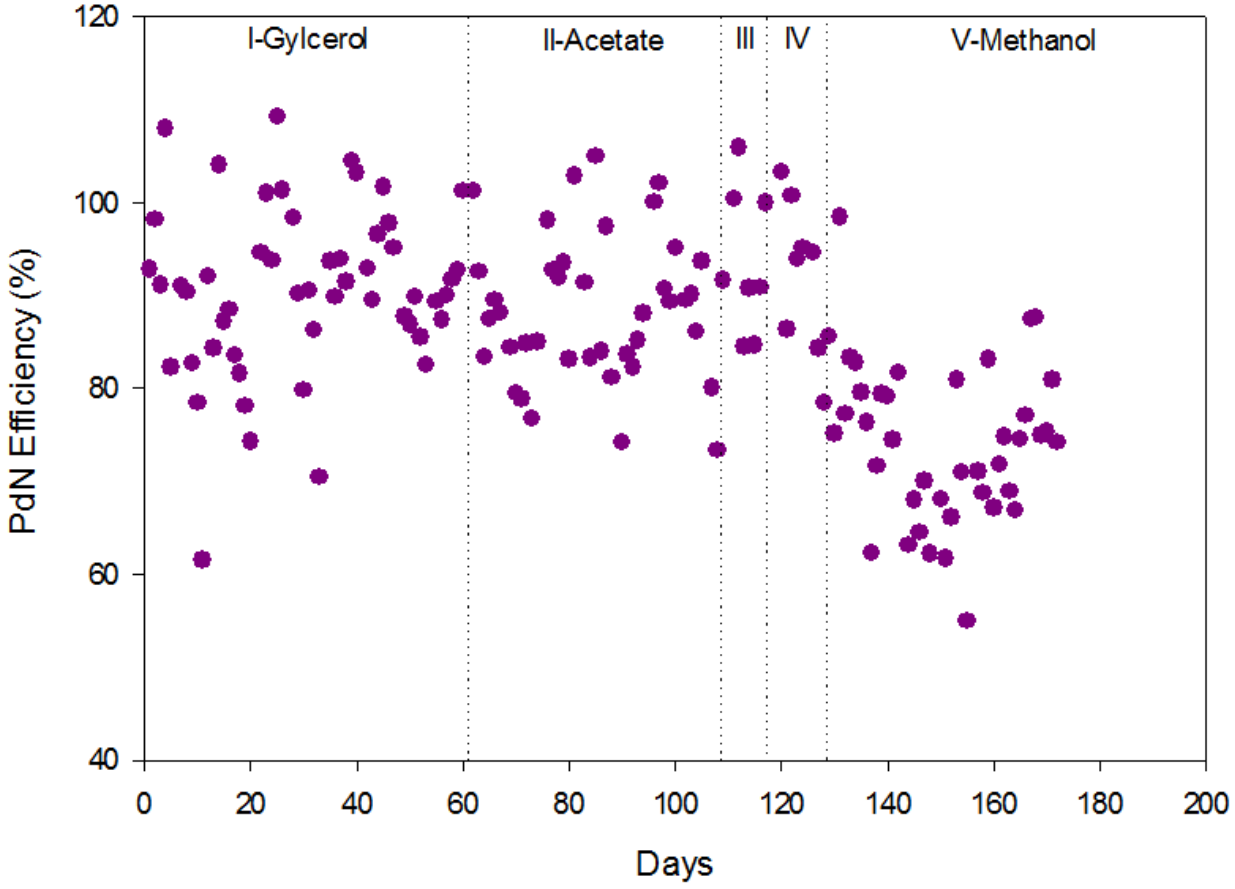

Figure 22: PdN efficiency values for glycerol, acetate, and methanol are shown with purple circles. Vertical dotted lines indicate start and end days for each carbon phase.

Theoretically, the PdN efficiency calculation cannot be over 100%. When removal concentrations were low, small errors in lab measurements were amplified and generated PdN efficiency greater than 100%. Glycerol had an average PdN efficiency of 91.0 ± 9.0%, acetate had an average PdN efficiency of 88.0 ± 7.7%, and methanol had an average PdN efficiency of 74.0 ± 8.5%.

NOx removed /NH_4-N removed (g/g)

Influent and effluent composite NH_4-N, NO_2-N, and NO_3-N concentrations were used to calculate actual NOx removed/NH_4-N removed. The theoretical vs actual NOx removed/NH_4-N removed ratio was a comparison that dictated the NOx setpoint for the carbon dosing control strategy. It also represented the PdN/A performance in the MBBR (Figure 23).

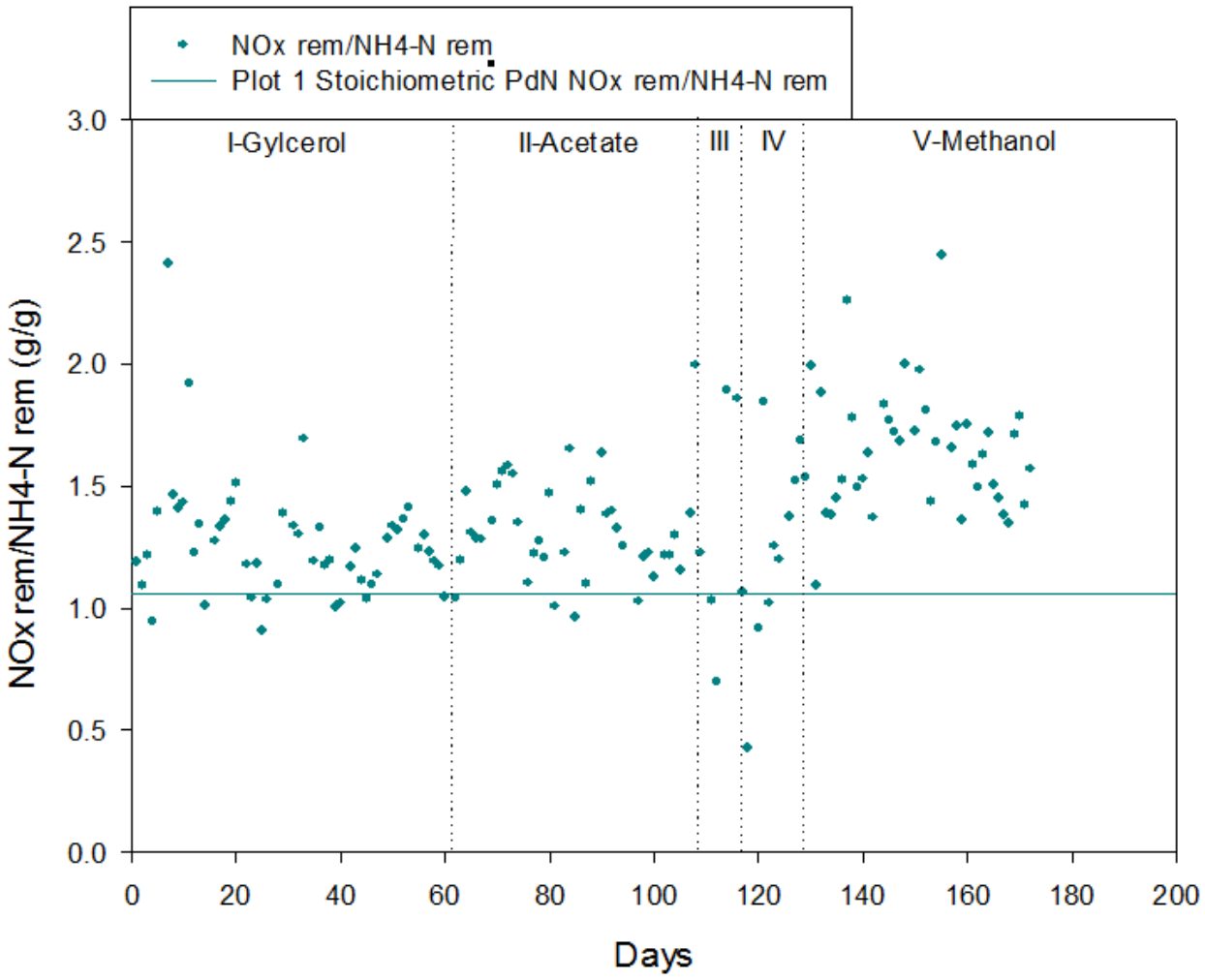

Figure 23: Theoretical NOx removed/NH_4-N removed was 1.06 g/g and was compared to the composite NOx removed/NH_4-N removed. Vertical dotted lines indicate the start and end days for each carbon phase

Glycerol had an average NOx-N removed/NH_4-N removed value of 1.65 ± 2.51 (g/g), acetate had an average NOx-N removed/NH_4-N removed value of 1.32 ± 0.21 (g/g), and methanol had an average NOx-N removed/NH_4-N removed value of 1.60 ± 0.25 (g/g)..

Most of the actual NOx-N removed/NH_4-N removed values are higher than the theoretical 1.06 g/g. For glycerol and acetate, the composites show results close to PdN/A metabolic stoichiometry and this indicates a small degree of FdN occurred. For methanol, the composites show results higher than PdN/A metabolic stoichiometry and this indicates a larger degree of FdN occurred.

PdN/A Denitrification Activity

Equally important as PdN efficiency and NOx removed/NH_4-N removed was the soluble carbon usage rate to nitrogen removal rate ratio. For each carbon source, denitrification activity tests were performed in the MBBR to obtain sCOD used/PdN and sCOD used/FdN ratios (See SI).

The theoretical sCOD used/PdN was directly compared to composite sCOD used/NO_3-N reduced (Figure 24). The theoretical sCOD used/FdN value represented carbon demand for a FdN MBBR, and the composite sCOD used/TIN removed showed carbon demand for a PdN/A MBBR.

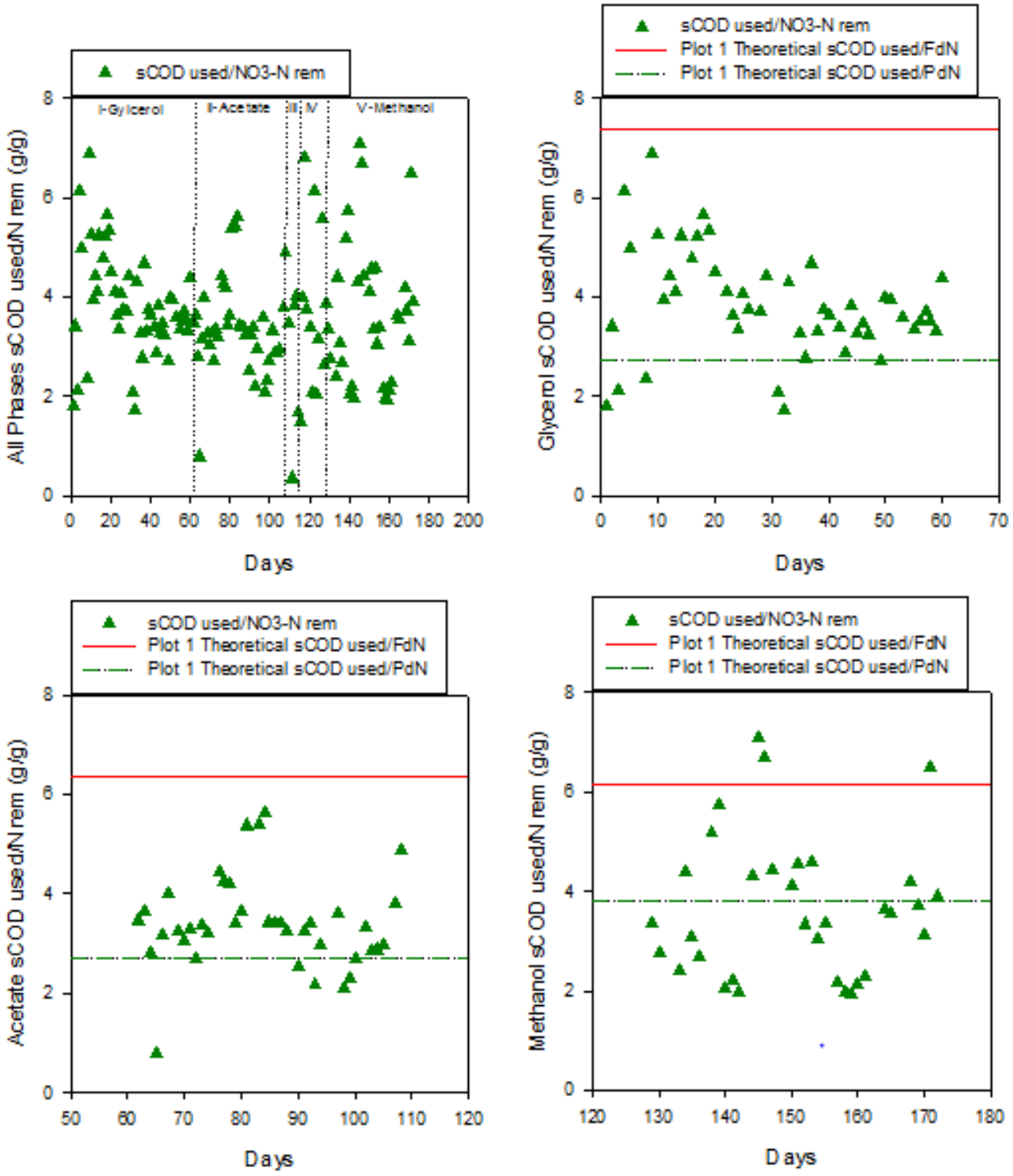

Figure 24: MBBR theoretical sCOD used for PdN (green dot dash line) and FdN (red solid line), and of actual composite data sCOD used for NO_3-N. Top Left – composite data for whole study, Top Right – glycerol phase data, Bottom Left – acetate

phase data, and Bottom Right – methanol phase data. Vertical dotted lines indicate start and end days for each carbon phase.

Glycerol had an average sCOD used/NO_3-N rem of 4.0 ± 2.0 (g/g), acetate had an average sCOD used/NO_3-N rem of 3.4 ± 0.9 (g/g), and methanol had an average sCOD used/NO_3-N rem of 3.7 ± 1.4 (g/g). The red line is the theoretical sCOD used/NO_3-N rem for FdN, and without the presence of AMX, this value represents the sCOD used/TIN rem of a FdN system. The red line can be compared to the actual sCOD used/NO_3-N rem values to show the more efficient use of carbon in the PdN/A system compared to FdN. The green dash dot line is the sCOD used/NO_3-N to NO_2-N for PdN from in situ batch experiments, and this should be compared to the actual sCOD used/NO_3-N rem.

AMX Activity Monitoring

Maximum AMX activity tests were performed in the MBBR to monitor AMX stability (See SI). The theoretical NO_2-N removed/NH_4-N removed and NO_3-N produced/NH_4-N removed based on AMX metabolic stoichiometry were 1.32 and 0.26 g/g respectively. Actual NO_2-N removed/NH_4-N removed and NO_3-N produced/NH_4-N removed values were obtained from MBBR maximum AMX activity experiments. The glycerol average NO_2-N removed/NH_4-N removed was 1.36 ± 0.09 (g/g), average NO_3-N produced/NH_4-N removed was 0.21 ± 0.06 (g/g), and NOx removed/NH_4-N removed was 1.15 ± 0.05 (g/g). The acetate average NO_2-N removed/NH_4-N removed was 1.37 ± 0.04 (g/g), average NO_3-N produced/NH_4-N removed was 0.21 ± 0.03 (g/g), and NOx removed/NH_4-N removed was 1.16 ± 0.05 (g/g). The methanol average NO_2-N removed/NH_4-N removed was 1.44 ± 0.05 (g/g), average NO_3-N produced/NH_4-N removed was 0.23 ± 0.03 (g/g), and NOx removed/NH_4-N removed was 1.21 ± 0.05 (g/g).

The main purpose of the MBBR maximum AMX activity experiments was to ensure AMX maximum activity was not poorly affected throughout the course of this study. The results confirmed that the MBBR AMX population performed very close to expected AMX metabolic stoichiometry. This means that AMX maximum activity remained optimum for all three carbon sources. Maximum AMX activity tests were also performed in the MBBR to track AMX growth (See SI). Comparing maximum AMX activity experiments over time can illuminate changes in microbial biomass. For AMX, if NH_4-N removed rate increases, it can be assumed there was an increase in AMX biomass between activity experiments.

In situ performance TIN and ammonia removal rates were compared to maximum AMX activity TIN and ammonia removal rates (Figure 25). The in situ performance TIN removal rates were calculated from the influent and effluent composite values, and the maximum AMX activity TIN removal rates were from maximum AMX activity tests. It is important to note that in situ performance includes both PdN and AMX activity, but the max activity only involves AMX.

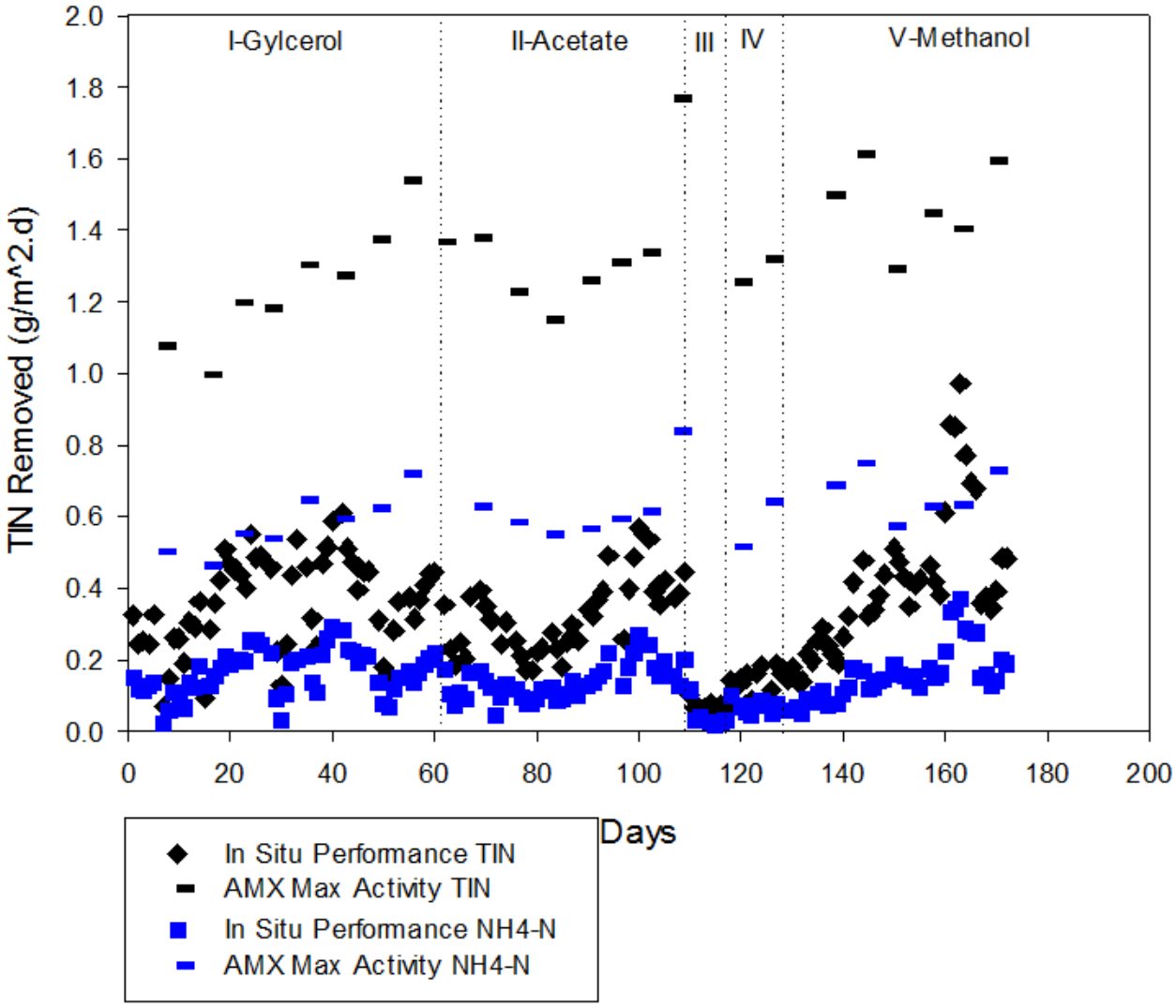

Figure 25: MBBR TIN removal rates from maximum AMX activity experiments (black rectangles) and MBBR TIN removal rates from in situ performance measurements (black diamonds). MBBR NH_4-N removal rates from maximum AMX activity experiments (blue rectangles) and MBBR NH_4-N removal rates from in situ performance measurements (black diamonds). Vertical dotted lines indicate start and end days for each carbon phase.

Glycerol had an average composite TIN removal rate of 0.36 ± 0.13 (g/m^2.d), an average max TIN removal rate of 1.24 ± 0.16 (g/m^2.d), an average composite ammonia removal rate of 0.16 ± 0.07 (g/m^2.d), and an average max ammonia removal rate of 0.58 ± 0.08 (g/m^2.d). Acetate had an average composite TIN removal rate of 0.31 ± 0.10 (g/m^2.d), an average max TIN removal rate of 1.35 ± 0.21 (g/m^2.d), an average composite ammonia removal rate of 0.14 ± 0.05 (g/m^2.d), and an average max ammonia removal rate of 0.62 ± 0.08 (g/m^2.d). Methanol had an average composite TIN removal rate of 0.41 ± 0.20 (g/m^2.d), an average max TIN removal rate of 1.47 ± 0.11 (g/m^2.d), an average composite ammonia removal rate of 0.16 ± 0.08 (g/m^2.d), and an average max ammonia removal rate of 0.66 ± 0.06 (g/m^2.d).

The maximum TIN removal rates increased throughout this study and may indicate that AMX biomass increased. Interestingly, composite TIN removal rates never reached the maximum TIN removal rates.

This shows that the PdN/A MBBR had a much higher TIN removal capacity than the system had experienced. This also indicates that any TIN residual in the effluent may be a result of substrate diffusion limitations. Another reason could be that there was a shortage of a key substrate such as ammonia to achieve further removals.

Microbial Quantification

Quantitative polymerase chain reaction (aPCR) tests were performed on the MBBR media biomass. The results enabled several ratios to be calculated for AMX bacteria to the whole microbial population (Figure 26).

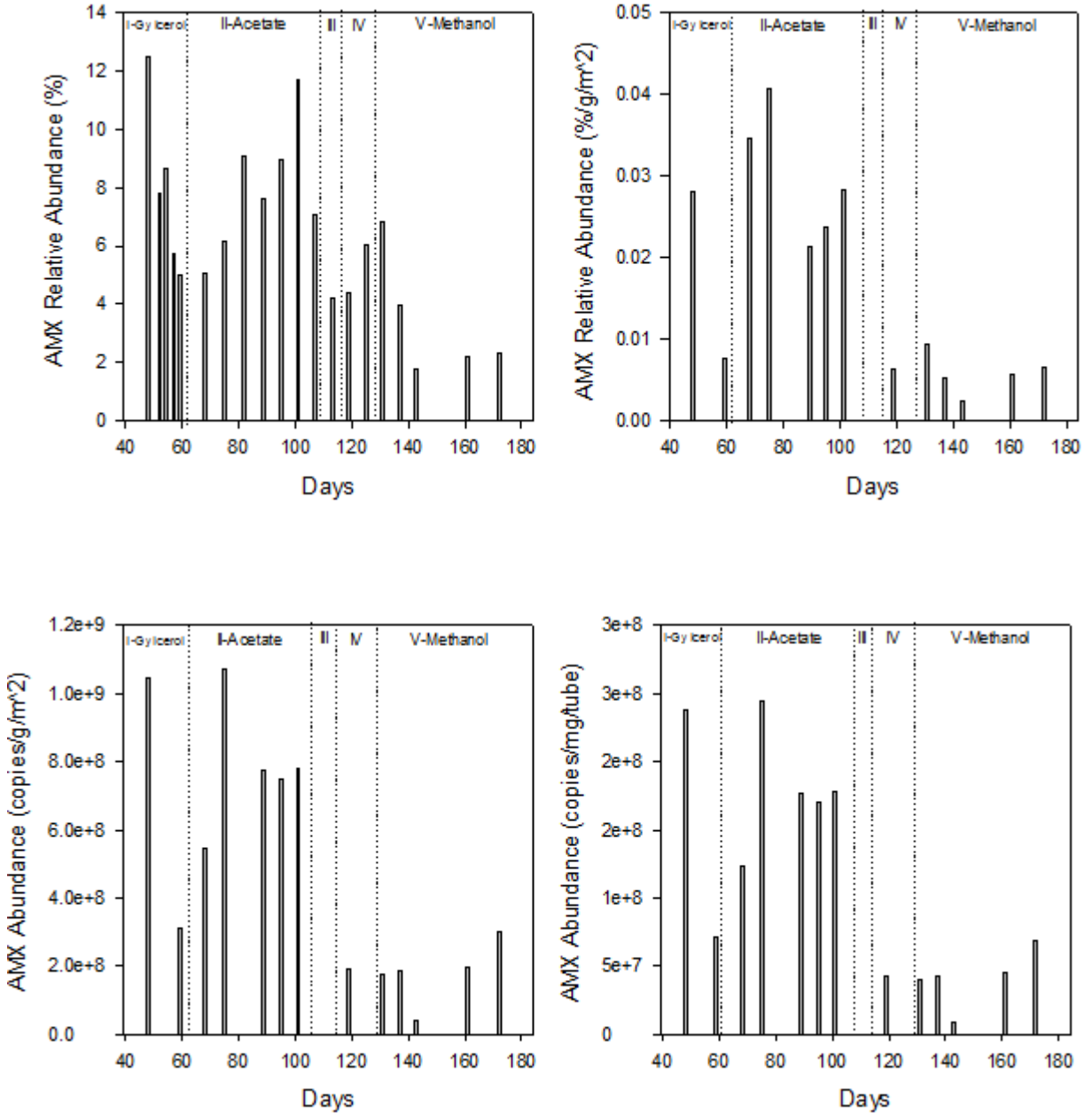

Figure 26: Top Left- AMX relative abundance of the total microbial community. Top Right- AMX relative abundance of the total microbial community per g/m^2. Bottom Left- AMX bacteria copies per g/m^2. Bottom Right- AMX bacteria copies per mg/tube.

There was an insufficient sample size to obtain reliable average and standard deviation statistics for each carbon source, but the graphs clearly show the AMX relative abundance decreased during the glycerol phase, increased during the acetate phase, then decreased during the methanol phase. The graphs also showed that AMX abundance (copies/g/m^2 and copies/mg/tube) were lower during the methanol phase than in the glycerol and acetate phases. Methanol showed a decrease in AMX biomass species count and relative abundance despite the increase in max AMX activity (Figure 27).

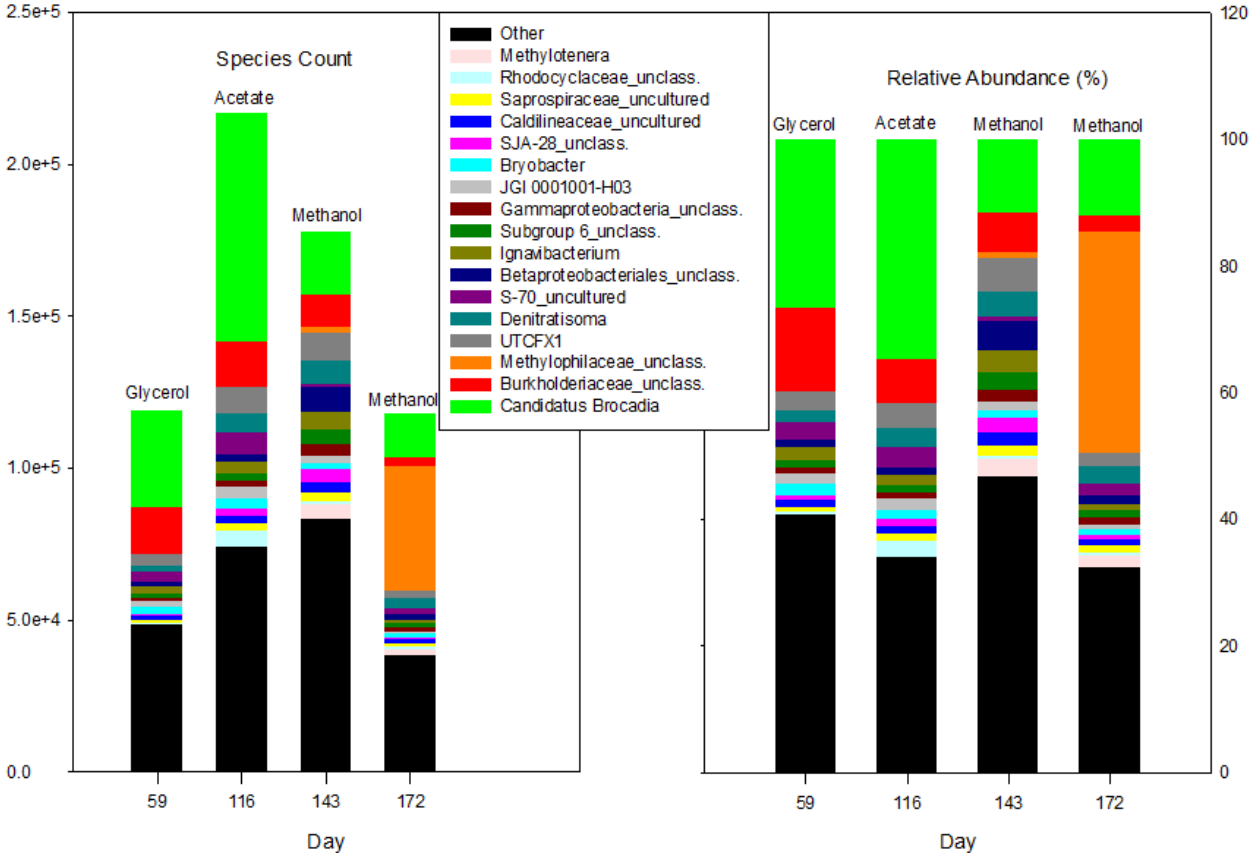

Figure 27: Left- A taxonomic distribution of specific microbial population count on the MBBR media. Right- A taxonomic distribution of specific microbial populations on the MBBR media as a percentage of the total copies in the microbial community.

From the taxonomy bar chart results, it is clear that the addition of methanol shifted the microbial population to include more Methylotrophs. Specifically, Methylophilaceae_unclass proliferated more than *Methylotenera* as the dominant Methylotrophic species. The inclusion of methanol also decreased the abundance of *Candidatus Brocadia*. It is unclear which microbial species contributed to the PdN.

FISH Microscopy Analysis

Thin section FISH microscopy analysis was performed on the PdN/A MBBR media to illuminate AMX microbial spatial composition and relative abundance. The FISH microscopy analysis confirmed high levels of AMX enrichment in all scanned fields of view (Figure 28). Based on visual inspection, AMX abundance was lower in the thinner, lower density regions of biofilms.

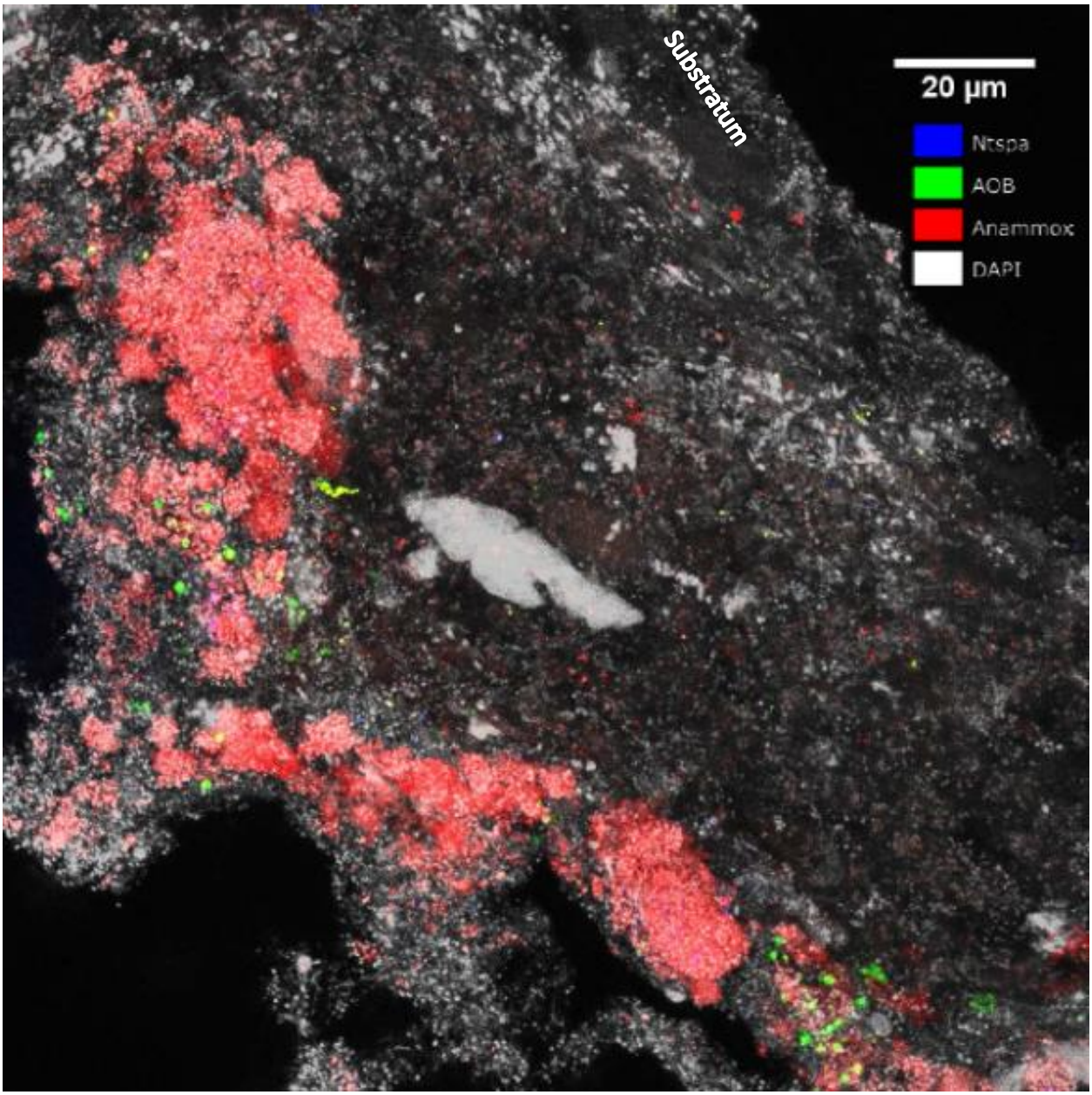

Figure 28: FISH imaging of the PdN/A MBBR media. Red is AMX bacteria, white is DAPI staining for overall cell abundance detection, green is AOB, and blue is *nitrospira*.

FISH imaging showed relatively lower abundance of AMX bacteria (red) in the lower density portions of the biofilm. This makes sense because AMX were highly dependent on partial denitrifiers for nitrite. AMX bacteria were more prevalent near the substrate than the substratum. This was most likely due to

substrate diffusion limitations. Thin section FISH microscopy did not reveal evidence of *nitrospira* enrichment in any scanned fields of view, but showed very bright signals in the AOB channel. This was surprising given that the MBBR was completely anoxic. These signals may be due to non-specific fluorescence binding. Also, the AOB signals may be due to carry-over of AOB from an upstream unit process.

Discussion

Optimize carbon addition to maximize TIN removal and minimize TIN effluent levels.

Glycerol and acetate were expected to and did produce very high PdN efficiency. Methanol was not expected to accumulate NO_2-N or produce significant PdN, but it exhibited good PdN efficiency. It is unclear if PdN efficiency is due to competition between AMX bacteria and heterotrophs, or due to glycerol and acetate preferentially causing PdN in heterotrophs.

As the controlling strategy became optimized, the sCOD used/NO3-N rem ratio decreased. It was then constant throughout the remainder of the study (Table 2).

Table 2: Carbon source yield values.

g COD used/g N removed	Glycerol			Acetate			Methanol		
	Theoretical	Batch	Actual	Theoretical	Batch	Actual	Theoretical	Batch	Actual
Full Denitrification	6.45	7.4±0.3	-	5.08	6.4±1.3	-	4.77	6.2±0.4	-
Partial Denitrification	2.58	2.7±0.2	4.0±2.0	2.03	2.7±0.5	3.4±0.9	1.91	3.8±0.4	3.9±1.4
TIN	1.25	1.3	2.3±1.1	0.99	1.3	2.0±0.8	0.93	1.8	1.9±0.8
TIN – NO2	1.25	1.3	3.6±1.5	0.99	1.3	3.2±0.9	0.93	1.8	2.6±1.1

Actual sCOD used/NO_3-N removed values were lower than the theoretical sCOD used/FdN number for glycerol, acetate, and methanol. This indicates that the PdN/A MBBR utilized carbon more effectively than a FdN system. Actual sCOD used/NO_3-N values were higher than the theoretical sCOD used/PdN number for glycerol and acetate. This may be due to surplus storage of carbon in the biofilm. The lower carbon usage rate for NO_2-N to gaseous nitrogen reduction could be a result of stored carbon in the first part when carbon was used for NO_3-N to NO_2-N reduction. This stored carbon would be used internally for NO_2-N gaseous nitrogen resulting in the lower apparent carbon usage. This would also mean that the sCOD used/PdN ratio had higher apparent carbon usage than was metabolically required.

Methanol exhibited lower sCOD used per nitrogen removal than acetate and glycerol. This was due to the nitrite accumulation in B-Stage. With an increase in influent nitrite to the MBBR, AMX bacteria were able to remove the same amount of TIN without as much of a carbon requirement for PdN. More nitrite accumulation in B-Stage allowed ammonia to be removed downstream of B-Stage with less carbon, and

resulted in less aeration demand, less energy usage, and less alkalinity required in B-Stage because less ammonia needed to be oxidized in B-Stage.

NOx rem/NH_4-N rem ratios can indicate optimization. 100% PdN efficiency gives 1.06 g/g, but since the carbon sources used in this study were about 80%, the NOx rem/NH_4-N rem ratio was higher than 1.06 g/g (Figure 23). Methanol had higher NOx rem/NH_4-N rem ratios than glycerol and acetate. This indicated either more nitrite was removed per ammonia removed (g/g) or less nitrate was produced per ammonia removed (g/g). Nothing was wrong with AMX metabolism throughout the study which means more nitrogen had been removed through FdN.

Compare glycerol, acetate, and methanol performance as carbon sources.

The PdN/A MBBR reactor produced high levels of PdN even at 1 mg N/L effluent nitrate. This would indicate that the system was only limited by the sensor for the dosing strategy and not the 2-3 mg N/L nitrate mark that some suspended growth systems have experienced (Le et al, 2019b).

Methanol had the highest average TIN removed, but this was due to externally increased TIN loading to the PdN/A MBBR. Additional ammonia and nitrate was added to the PdN/A MBBR during a portion of the methanol phase to double the influent TIN concentration in order to explore the performance capacity of the system. Glycerol performed better than acetate with regards to TIN removed under normal loading conditions. It is clear that all three carbon sources can remove most of the TIN under normal loading conditions.

Acetate and methanol were both able to obtain an average effluent TIN concentration less than or equal to 5 mg/L (Table 3). During the glycerol phase, B-Stage experienced major upsets (loss of nitrification) that affected the PdN/A MBBR polishing process performance and resulted in higher average effluent TIN (6 instead of 5 mg/L). At the same time, the optimizing process of the dosing strategy and higher NOx setpoints (6 instead of 1.5) used also influenced the higher average effluent TIN of glycerol.

Glycerol performed better than acetate with average PdN efficiency, and both were well above the target average PdN efficiency of 80%. Methanol did not reach the target average PdN efficiency, but it performed much better than expected. Methanol was thought to send electrons further down the transport chain without showing preference to nitrate reductase over nitrite reductase (Le et al, 2019a). Methanol PdN efficiency was increasing towards the end of the methanol phase. This increase in PdN efficiency could be from the increase in Methylotroph bacteria that was evident (Figure 8). If given enough time to adapt, it may be possible that methanol has the potential to perform PdN as well as glycerol and acetate.

Table 3: Carbon source performance statistics.

Average ± Standard Deviation	Glycerol	Acetate	Methanol
TIN Removed (mg N/L)	10.0 ± 3.6	8.7 ± 2.9	11.5 ± 5.6

TIN Out (mg N/L)	6.0 ± 4.0	5.0 ± 1.1	4.3 ± 1.5
PdN Efficiency (%)	91.0 ± 9.0	88.0 ± 7.7	74.0 ± 8.5

During the methanol phase, when TIN loading was increased by almost double (16 mg/L to 30 mg/L) the normal conditions, effluent TIN only rose slightly above the 5 mg/L target (7 mg/L) for that period. This showed that the PdN/A MBBR was capable of achieving very high TIN removal while maintaining very low effluent TIN and exemplifies this PdN/A MBBR polishing process as truly robust. Also, the AMX max activity removal rates for TIN and NH_4-N vs the actual removal rates for TIN and NH_4-N show that the PdN/A MBBR could have taken on a much higher TIN load than was typical of B-Stage effluent.

Methanol was a risk for PdN/A because it had induced AMX inhibition in several studies (Guven et al, 2005; Jensen et al, 2007; Isaka et al, 2008; Oshiki et al, 2011). A methylotrophic outer layer on the media most likely provided a buffer for AMX bacteria and it is possible that methanol is not toxic to AMX. The symbiotic relationship between Methylotrophs and AMX bacteria allowed PdN/A to thrive, but methanol may not be an ideal source to grow new AMX biomass without a significant methylotrophic presence. Surprisingly, methanol produced moderate PdN efficiency values. It did not inhibit AMX activity during normal carbon dosing operation. Also, methanol did not inhibit AMX activity during ex situ batch experiments, in situ batch denitrification experiments, or in situ 24-hr 150 mg/L methanol exposure experiments.

A carbon source cost analysis was performed based on the budget level chemical costs for HRSD and PdN yield test results (Table 4). This table shows that methanol was the least expensive carbon source per kg TIN removed, closely followed by glycerol, then acetate.

Table 4: Carbon source cost comparison based on yield values.

Carbon Source	COD (mg/L)	Cost ($/L)	Cost ($/kg COD)	PdN Yield (g COD rem/g NO3-N rem)	PdN Cost ($/kg TIN rem)	FdN Cost ($/kg TIN rem)
Glycerol	1100000	0.53	0.48	2.73	1.13	3.10
Acetate	1123500	1.35	1.21	2.72	3.28	6.15
Methanol	1185000	0.38	0.32	3.82	1.23	1.53

When the PdN yield is factored into the cost of each carbon source, glycerol was the least expensive, but methanol had a similarly low cost as well. For all three carbon sources, the PdN cost ($/kg TIN rem) was lower than traditional FdN system cost ($/kg TIN rem). Of course costs will vary from region to region, but this cost analysis forms a basis for carbon comparison for the PdN/A process.

AMX biomass went down but activity remained maxed out and the magnitude of removal increased.

The taxonomy and relative abundance data showed that AMX bacteria counts decreased during the methanol phase. This was surprising because the AMX max activity metabolism ratios did not change throughout the study, and the magnitude of nitrogen removal increased over this time period as well. Also, AMX maximum activity TIN and NH_4-N removal rates increased throughout the study and this would indicate that new AMX biomass was created. There was an upset in nitrogen removal during the middle of the methanol phase and this could explain the inconsistent removed rates. Methanol max activity did recover and still exhibited an overall trend upward during the methanol phase.

From FISH analysis during the glycerol phase, AMX enrichment appeared lower than observed in sidestream enriched AMX biofilms (Christensson et al, 2013; Zhao et al, 2014). AMX enrichment appeared qualitatively similar to mainstream AMX biofilms from EAWAG (Laureni et al, 2016). This was due to higher loading conditions in sidestream vs mainstream. The AMX bacteria decrease may indicate that the PdN/A MBBR process was capable of high TIN removal with a lower reservoir of AMX biomass. This would only be possible if the AMX community in the MBBR adapted to possess faster metabolic reactions over the course of this study. Higher loading conditions can induce this type of adaption, but the increase in loading only occurred during a small portion of the methanol phase.

PdN may have been a result of a specifically cultured species of partial denitrifier.

Since there was non-limiting sCOD present, maintaining a low sCOD/N ratio was not the reason why PdN occurred. Therefore, PdN was not dependent on the sCOD/N ratio as suggested in literature (Almedia et al, 1995b; Her and Huang, 1995; Oh and Silverstein, 1999; Le et al, 2019a). Since there was no ammonia present for AMX, the AMX bacteria present as a nitrite sink was not the reason why PdN occurred as previously suggested (Le et al, 2019a). Bulk liquid denitrification tests prove this further (see SI). The PdN/A MBBR bulk denitrification tests revealed that the bulk liquid contained microorganisms that showed preference for nitrate over nitrite. This allowed nitrite to accumulate throughout the experiment. The MBBR HRT and SRT were most likely too low (2 hours) to accommodate growth of these specialized microorganisms in the bulk liquid. These microorganisms may have detached from the biofilm in search for more ideal conditions or they may have sloughed off the carriers due to mixing shear forces and media overcrowding. It is fair to say that the presence of AMX provided a nitrite “sink” for specialized partial denitrifiers to thrive on the media, and these partial denitrifiers relocated to the bulk liquid. Therefore, PdN was not dependent on the presence of the AMX reaction occurring. PdN was probably not due to the carbon source because all three carbon sources were capable of moderate to high PdN. Even though methanol did not perform PdN as well as glycerol and acetate early in its phase, the PdN efficiency started to increase towards the end and this may be due to the shift in methylotrophic species present in the biofilm. Also, the low amounts of known bacterial families that do truncated denitrification and/or progressive onset denitrification beg the question of where the high levels of PdN is coming from. It is possible that specialized PdN bacteria were present and had high preference for nitrate over nitrite. The AMX bacteria present would take up the nitrite and since it behooves OHOs to use nitrate over nitrite, it is possible that the OHOs had adapted to possess this

progressive onset of preference for nitrate over nitrite. The syntrophic relationship between AMX bacteria and these specialized partial denitrifiers may explain what selection pressures were needed to have high levels of PdN. Also, the nitrate produced by AMX anabolism could be a constant source of nitrate from which these OHOs could always utilize and never need to use nitrite instead. This relationship may translate to Methylotrophs as well. From a denitrification standpoint, it makes sense to let AMX bacteria use the nitrite and produce the nitrate needed for denitrification.

In the taxonomy results, there does not appear to be a significant amount of the family *Ignavibacterium* which encompasses the genus *thauera* and is a known partial denitrifier. It can be postulated that the family *denitratisoma* could contribute to partial denitrification, but since it does not make up a significant portion of the bacterial community it does not seem like the sole cause of great PdN. Overall, it is too difficult to confirm exactly what the source of high levels of PdN was in this study, but it is clear that the combination of selection pressures performed during this study led to a robust PdN/A process.

Conclusions

In this study we demonstrated the performance and feasibility of glycerol, acetate, and methanol dosing to an anoxic PdN/A polishing MBBR. We showed optimization of a carbon dosing control strategy using PdN efficiency as a metric. Methanol and glycerol proved to be highly competitive with regards to a key metric, cost per mass TIN removed. Therefore, this study presented a highly efficient and robust nitrogen removal process via AMX. The main take away messages for this research include the following:

1. The PdN/A MBBR reactor can remove extra ammonia with any influent combination of nitrite or nitrate (Figure 21).
2. Although FdN can achieve very low effluent TIN, the PdN/A MBBR system can achieve very low TIN with less carbon than FdN (Figure 21, Table 2, Table 4).
3. Since biomass is retained in the PdN/A MBBR system, changes in influent loading are not an operational issue (see max AMX activity tests).
4. Methanol is not toxic to AMX, and can produce 74% PdN efficiency (see max AMX activity tests, Figure 22).
5. The PdN/A MBBR reactor produced good PdN even at 1 mg N/L effluent nitrate (Figure 21, Figure 22).
6. The PdN/A MBBR system was very low maintenance and was easy to operate. AMX was easily maintained on the carriers, and good PdN occurred at all points in the study (Figure 22).
7. The PdN/A MBBR system is not only useful for polishing shortcut nitrogen removal processes; it could be implemented at the end of any BNR process.
8. The PdN/A MBBR process is ready for full-scale implementation. The main challenge with starting this system with barren media is influencing AMX bacterial growth. Future research should explore different options to address this issue including: feeding sufficient nitrite and ammonia with very low carbon to the barren media, seeding the reactor with already

established PdN/A MBBR media, seeding the reactor with AMX granules, and seeding the reactor with suspended AMX biomass.

This study has provided significant strides towards full-scale PdN/A, but further research should be devoted to the startup of these systems.

Acknowledgments

We would like to sincerely thank the support of Hampton Roads Sanitation District (HRSD). We also thank DC Water for their scientific discussions, feedback, and collaboration.

References

Adamczyk, J., Hesselsoe, M., Iversen, N., Horn, M., Lehner, A., Nielsen, P.K., Schloter, M., Roslev, P., Wagner, M., 2003. The Isotope Array, a New Tool That Employs Substrate-Mediated Labeling of rRNA for Determination of Microbial Community Structure and Function. *Applied and Environmental Microbiology*. 69 (11), 6875-6887.

Akunna, J.C., Bizeau, C., Moletta, R., 1993. Nitrate and nitrite reductions with anaerobic sludge using various carbon sources: glucose, glycerol, acetic acid, lactic acid and methanol. Water Res., 27, 1303-1312.

Al-Omari, A., Wett, B., Nopens, I., De Clippeleir, H., Han, M., Regmi, P., Bott, C., Murthy, S., 2015. Model-based evaluation of mechanisms and benefits of mainstream shortcut nitrogen removal processes. Water Sci. Technol. ,71, 840-847.

Almeida, J.S., Júlio, S.M., Reis, M.A., Carrondo, M.J, 1995a. Nitrite inhibition of denitrification by Pseudomonas fluorescens. Biotechnol. and Bioeng., 46, 194-201.

Almeida, J.S., Reis, M.A., Carrondo, M.J., 1995b. Competition between nitrate and nitrite reduction in denitrification by Pseudomonas fluorescens. Biotechnol. Bioeng., 46, 476-484.

American Public Health Association, A.W.W.A., Water Environment Federation, 2012. Standard Methods for Examination of Water and Wastewater in: Association, A.P. (Ed.), US.

Beccari, M., Passino, R., Ramadori, R., Tandoi, V., 1983. Kinetics of dissimilatory nitrate and nitrite reduction in suspended growth culture. Journal (Water Pollution Control Federation), 58-64.

Betlach, M.R., Tiedje, J.M, 1981. Kinetic Explanation for Accumulation of Nitrite, Nitric Oxide, and Nitrous Oxide During Bacterial Denitrification. Appl. Environ. Microbiol., 42, 1074-1084.

Bill, K.A., Bott, C.B., Murthy, S.N., 2009. Evaluation of alternative electron donors for denitrifying moving bed biofilm reactors (MBBRs). Water Sci. Technol., 60, 2647-2657.

Blaszczyk, M., 1993. Effect of medium composition on the denitrification of nitrate by Paracoccus denitrificans. Appl. Environ. Microbiol., 59, 3951-3953.

Cema, G., Wiszniowski, J., Zabczynski, S., Zablocka-Godlewska, E., Raszka, A., Surmacz-Gorska, J., 2007. Biological nitrogen removal from landfill leachate by deammonification assisted by heterotrophic denitrification in a rotating biological contactor (RBC). Water Science & Technology. 55 (8-9), 35-42.

Christensson, M., Ekstrom, S., Andersson, A.C., Le Vaillant, E., Lemaire, R., 2013. Experience from strat-ups of the first ANITA Mox Plants. *Water Science and Technology*. 67 (12), 2677-2684.

Daims, H., Nielsen, J.L., Nielsen, P.H., Schleifer, K., Wagner, M., 2001. In Situ Characterization of Nitrospira-Like Nitrite-Oxidizing Bacteria Active in Wastewater Treatment Plants. *Applied and Environmental Microbiology*. 67 (11), 5273-5284.

Dosta, J., Fernandez, I., Vazquez-Padin, J.R., Mosquera-Corral, A., Campos, J.L., Mata-Alvarez, J., Mendez, R., 2008. Short- and long-term effects of temperature on the Anammox process. *Journal of Hazardous Materials*. 154, 688-693.

Du, R., Cao, S., Li, B., Niu, M., Wang, S., Peng, Y., 2017. Performance and microbial community analysis of a novel DEAMOX based on partial-denitrification and anammox treating ammonia and nitrate wastewaters. Water Res., 108, 46-56.

Ferris, M.J., Muyzer, G., Ward, D.M., 1996. Denaturing Gradient Gel Electrophoresis Profiles of 16S rRNA-Defined Populations Inhabiting a Hot Spring Microbial Mat Community. *Applied and Environmental Microbiology*. 62 (2), 340-346.

Graham, D.G., Knapp, C.W., Van Kleck, E.S., Bloor, K., Lane, T.B., Graham, C.E., 2007. Experimental demonstration of chaotic instability in biological nitrification. *The ISME Journal*. 1 (5), 385-393.

Guven, D., Dapena, A., Kartal, B., Schmid, M.C., Maas, B., van de Pas-Schoonen, K., Sozen, S., Mendez, R., Op den Camp, H.J.M., Jetten, M.S.M., Strous, M., Schmidt, I., 2005. Propionate Oxidation by and Methanol Inhibition of Anaerobic Ammonium-Oxidizing Bacteria. *Applied and Environmental Microbiology*. 72 (2), 1066-1071.

Han, M., De Clippeleir, H., Al-Omari, A., Stewart, H., Wett, B., Vlaeminck, S., Bott, C., Murthy, S., 2015. NOB out-selection in mainstream deammonification–A resilience evaluation. Proceedings of the Water Environment Federation, 2015, 2237-2242.

Han, M., Vlaeminck, S.E., Al-Omari, A., Wett, B., Bott, C., Murthy, S., De Clippeleir, H., 2016. Uncoupling the solids retention times of flocs and granules in mainstream deammonification: A screen as effective out-selection tool for nitrite oxidizing bacteria. Bioresource Technology. 221, 195-204.

Hendrickx, T.L.G., Wang, Y., Kampman, C., Zeeman, G., Temmink, H., Buisman, C.J.N., 2012. Autotrophic nitrogen removal from low strength waste water at low temperature. *Water Research*. 46, 2187-2193.

Her, J., Huang, J., 1995. Influences of carbon source and C/N ratio on nitrate/nitrite denitrification and carbon breakthrough. Bioresource Technology. 54, 45-51.

Isaka, K., Date, Y., Kimura, Y., Sumino, T., Tsuneda, S., 2008. Nitrogen removal performance using anaerobic ammonium oxidation at low temperatures. FEMS Microbiology Letter. 282, 32-38.

Isaka, K., Suwa, Y., Kimura, Y., Yamagishi, T., Sumino, T., Tsuneda, S., 2008. Anaerobic ammonium oxidation (anammox) irreversibly inhibited by methanol. *Applied Microbiology & Biotechnology*. 81, 379-385.

Jensen, M.M., Thamdrup, B., Dalsgaard, T., 2007. Effects of Specific Inhibitors on Anammox and Denitrification in Marine Sediments. *Applied and Environmental Microbiology*. 73 (10), 3151-3158.

Kalyuzhnyi, S., Gladchenko, M., Mulder, A., Versprille, B., 2006. DEAMOX—new biological nitrogen removal process based on anaerobic ammonia oxidation coupled to sulphide-driven conversion of nitrate into nitrite. Water Res., 40, 3637-3645.

Kartal, B., van Niftrik, L., Rattray, J., van de Vossenberg, J.L.C.M., Schmid, M.C., Damste, J.S., Jetten, M.S.M., Strous, M., 2008. Candidatus 'Brocadia fulgida': an autofluorescent anaerobic ammonium oxidizing bacterium. *FEMS Microbial Ecology*. 63, 46-55.

Kindaichi, T., Tsushima, I., Ogasawara, Y., Shimokawa, M., Ozaki, N., Satoh, H., Okabe, S., 2007. In Situ Activity and Spatial Organization of Anaerobic Ammonium-Oxidizing (Anammox) Bacteria in Biofilms. *Applied and Environmental Microbiology*. 73 (15), 4931-4939.

Laureni, M., Falas, P., Robin, O., Wick, A., Weissbrodt, D.G., Nielsen, J.L., Ternes, T.A., Morgenroth, E., Joss, A., 2016. Mainstream partial nitritation and anammox: long-term process stability and effluent quality at low temperatures. *Water Research*. 101 (2016), 628-639.

Le, T., Peng, B., Su, C., Massoudieh, A., Torrents, A., Al-Omari, A., Murthy, S., Wett, B., Chandran, K., DeBarbadillo, C., Bott, C., De Clippeleir, H., 2019. Impact of carbon source and COD/N on the concurrent operation of partial denitrification and anammox. Water Environmental Research. Accepted Author Manuscript. doi:10.1002/wer.1016.

Le, T., Peng, B., Su, C., Massoudieh, A., Torrents, A., Al-Omari, A., Murthy, S., Wett, B., Chandran, K., DeBarbadillo, C., Bott, C., De Clippeleir, H., 2019. Nitrate residual as a key parameter to efficiently control partial denitrification coupling with anammox. Water Environmental Research. Submitted.

Ledwell, S.A., Fabiyi, M., Farmer, G., 2011. Optimizing denitrification with non-methanol carbon sources in deep-bed denitrification filter technologies. Proceedings of the Water Environment Federation, 2011, 406-419.

Liu, B., Mao, Y., Bergaust, L., Bakken, L.R., Frostegard, A., 2013. Strains in the genus Thauera exhibit remarkably different denitrification regulatory phenotypes. Environmental Microbiology. 15(10), 2816-2828.

Lotti, T., Kleerebezem, R., van Erp Taalman Kip, C., Hendrickx, T.L.G., Kruit, J., Hoekstra, M., van Loosdrecht, M.C.M., 2014. Anammox Growth on Pretreated Municipal Wastewater. Environmental Science & Technology. 48, 7874-7880.

Ma, Y., Sundar, S., Park, H., Chandran, K., 2015. The effect of inorganic carbon on microbial interactions in a biofilm nitritation-anammox process. *Water Research*. 70, 246-254.

Mobarry, B.K., Wagner, M., Urbain, V., Rittmann, B.E., Stahl, D.A., 1996. Phylogenetic Probes for Analyzing Abundance and Spatial Organization of Nitrifying Bacteria. *Applied and Environmental Microbiology*. 62 (6), 2156-2162.

Mulder, A., Van de Graaf, A.A., Robertson, L., Kuenen, J., 1995. Anaerobic ammonium oxidation discovered in a denitrifying fluidized bed reactor. FEMS microbiology ecology, 16, 177-183.

Nielsen, P.K., Daims, H., Lemmer, H., 2009. FISH Handbook for Biological Wastewater Treatment. *IWA Publishing*.

Oshiki, M., Shimokawa, M., Fujii, N., Satoh, H., Okabe, S., 2011. Physiological characteristics of the anaerobic ammonium-oxidizing bacterium '*Candidatus* Brocadia sinica'. *Microbiology*. 157, 1706-1713.

Obaja, D., Macé, S., Mata-Alvarez, J., 2005. Biological nutrient removal by a sequencing batch reactor (SBR) using an internal organic carbon source in digested piggery wastewater. Bioresour. Technol., 96, 7-14.

Oh, J., Silverstein, J., 1999. Acetate Limitation and Nitrite Accumulation during Denitrification. J. Environ. Eng., 125, 234-242.

Park, H., Sundar, S. Ma, Y., Chandran, K., 2015. Differentiation in the Microbial Ecology and Activity of Suspended and Attached Bacteria in a Nitritation-Anammox Process. *Biotechnology and Bioengineering*. 112 (2), 272-279.

Regmi, P., Holgate, B., Miller, M.W., Park, H., Chandran, K., Wett, B., Murthy, S., Bott, C.B., 2016. Nitrogen Polishing in a Fully Anoxic Anammox MBBR Treating Mainstream Nitritation-Denitritation Effluent. *Biotechnology and Bioengineering*. 113 (3), 635-642.

Regmi, P., Miller M.W., Holgate, B., Bunce, R., Park, H., Chandran, K., Wett, B., Murthy S., Bott, C.B., 2014. Control of aeration, aerobic SRT and COD input for mainstream nitritation/denitritation. *Water Research*. 57, 162-171.

Rotthauwe, J., Witzel, K., Liesack, W., 1997. The Ammonia Monooxygenase Structural Gene amoA as a Functional Marker: Molecular Fine-Scale Analysis of Natural Ammonia-Oxidizing Populations. *Applied and Environmental Microbiology*. 63 (12), 4704-4712.

Schalk, J., de Vries, S., Kuenen, J.G., and Jetten, M.S.M., 2000. Involvement of a Novel Hydroxylamine Oxidoreductase in Anaerobic Ammonium Oxidation. *Biochemistry*. 39 (18), 5405-5412.

Schmid, M., Twachtmann, U., Klein, M., Strous., Juretschko, S., Jetten, M., Metzger., J.W., Schleifer, K., Wagner, M., 2000. Molecular Evidence for Genus Level Diversity of Bacteria Capable of Catalyzing Anaerobic Ammonium Oxidation. *System Applied Microbiology*. 23, 93-106.

Strous, M., Fuerst, J.A., Kramer, E.H., Logemann, S., Muyzer, G., van de Pas-Schoonen, K.T., Webb, R., Kuenen, J.G., Jetten, M.S.M., 1999. Missing lithotroph identified as new planctomycete. *Nature.* 400, 446–449.

Tang, C., Zheng, P., Zhang, L., Chen, J., Mahmood, Q., Chen, X., Hu, B., Wang, C., Yu, Y., 2010. Enrichment feature of anammox consortia from methanogenic granules loaded with high organic and methanol contents. *Chemosphere*. 79, 613-619.

Tiedje, J.M., 1988. Ecology of denitrification and dissimilatory nitrate reduction to ammonium. Biol. Anaerob. Microorg., 717, 179-244.

van Rijn, J., Tal, Y., Barak, Y., 1996. Influence of Volatile Fatty Acids on Nitrite Accumulation by a Pseudomonas stutzeri Strain Isolated from a Denitrifying Fluidized Bed Reactor. Appl. Environ. Microbiol., 62, 2615-2620.

Vazquez-Padin, J.R., Fernandez, I., Morales, N., Campos, J.L., Mosquera-Corral, A., Mendez, R., 2011. Autotrophic nitrogen removal at low temperature. *Water Science & Technology*. 63 (6), 1282-1288.

Wagner, M., Rath, G., Amann, R., Koops, H., Schleifer, K., 1995. In situ Identification of Ammonia-oxidizing Bacteria. *System Applied Microbiology*. 18, 251-264.

Wallner G., Amann, R., Beisker, W., 1993. Optimizing Fluorescent In Situ Hybridization With rRNA-Targeted Oligonucleotide Probes for Flow Cytometric Identification of Microorganisms. *Cytometry*. 14, 136-143.

Zhao, H., Bell, J., Thesing, G., Piveteau, S., Christensson, M., Veuillet, F., Ochoa, J., Lemaire, R., 2014. One-Stage Deammonification Process – MBBR Versus IFAS Configurations – Development Updates and Application Considerations. *Proceedings of the Water Environment Federation.* 1-16 (16).

Appendix A: Supplementary Material

Table 5: 16s rRNA-targeted oligonucleotide probes used in this study.

Probe	Sequence (5' to 3')	FA (%)	Specificity	Reference
AOB Mix				
NEU	CCC CTC TGC TGC ACT CTA	35	Most halophilic and halotolerant Nitrosomonas spp.	Wagner et al. 1995
CTE (NEU Competitor)	TTC CAT CCC CCT CTG CCG	35	Unlabeled together with NEU Comamonas spp., Acidovorax spp., Hydrogenophaga spp., Aquaspirillum spp.	Wagner et al. 1995
Nso1225	CGC CAT TGT ATT ACG TGT GA	35	Ammonia-oxidizing β-proteobacteria	Mobarry et al. 1996
Cluster6a192	CTT TCG ATC CCC TAC TTT CC	35	Nitrosomonas oligotropha lineage (Cluster 6a)	Adamczyk et al. 2003
Cluster6a 192 Competitor	CTT TCG ATC CCC TGC TTT CC	35	Competitor for Cluster6a192	Adamczyk et al. 2003
Nitrospira Mix				
Ntspa662	GGA ATT CCG CGC TCC TCT	35	Genus Nitrospira	Daims et al. 2001
Ntspa662 Competitor	GGA ATT CCG CTC TCC TCT	35	Competitor for Ntspa662	Daims et al. 2001
Ntspa712	CGC CTT CGC CAC CGG CCT TCC	35	Most members of the phylum Nitrospirae	Daims et al. 2001
Ntspa712 Competitor	CGC CTT CGC CAC CGG TGT TCC	35	Competitor for Ntspa712	Daims et al. 2001
Anammox Mix				
Amx820	AAA ACC CCT CTA CTT AGT GCC C	35	Brocadia anammoxidans Kuenenia stuttgartiensis	Schmid et al. 2000
Bfu 613	GGA TGC CGT TCT TCC GTT AAG CGG	35	Brocadia fulgida"	Kartal et al. 2008
NON Eub	ACT CCT ACG GGA GGC AGC	35	Negative control for nonspecific binding	Wallner et al. 1993

sCOD used per nitrogen species removed was calculated from the anoxic PdN/A MBBR in situ denitrification rate (mg/L.hr) experiments. As NO_3-N was reduced, NO_2-N accumulated until NO_3-N was virtually zero, and this portion was defined as the first half. With no NO_3-N left, the sCOD used rate was solely used toward NO_2-N reduction rate, and this portion was defined as the second half. From the second half, sCOD used/NO_2-N reduced was calculated from the sCOD used rate and NO_2-N reduction rate. In the first half, the difference between the NO_3-N reduction rate and NO_2-N production rate was NO_2-N reduction rate. NO_2-N reduction rate was multiplied by sCOD used/NO_2-N reduced to yield sCOD used rate for NO_2-N reduction in the first half. In the first half, the difference between the entire sCOD used rate and sCOD used rate for NO_2-N reduction rate was sCOD used rate for PdN. In the first half, the

sCOD used rate for PdN was divided by NO_2-N production rate to get sCOD used/NO_3-N to NO_2-N (sCOD used/PdN).

The theoretical NOx removed/NH_4-N removed (1.06 g/g) was calculated from AMX metabolic stoichiometry (1.32 NO_2-N rem/NH_4-N rem (g/g); 0.26 NO_3-N pro/NH_4-N rem (g/g)) combined with PdN assuming the 0.26 g NO_3-N produced per g NH_4-N removed was partially denitrified only to NO_2-N (100% PdN efficiency).

No NH_4-N was present during these tests, and every experiment accumulated nitrite. When nitrate was depleted, nitrite accumulation peaked and began to reduce. These denitrification experiments gave theoretical sCOD used/PdN and sCOD used/FdN specific to each carbon source.

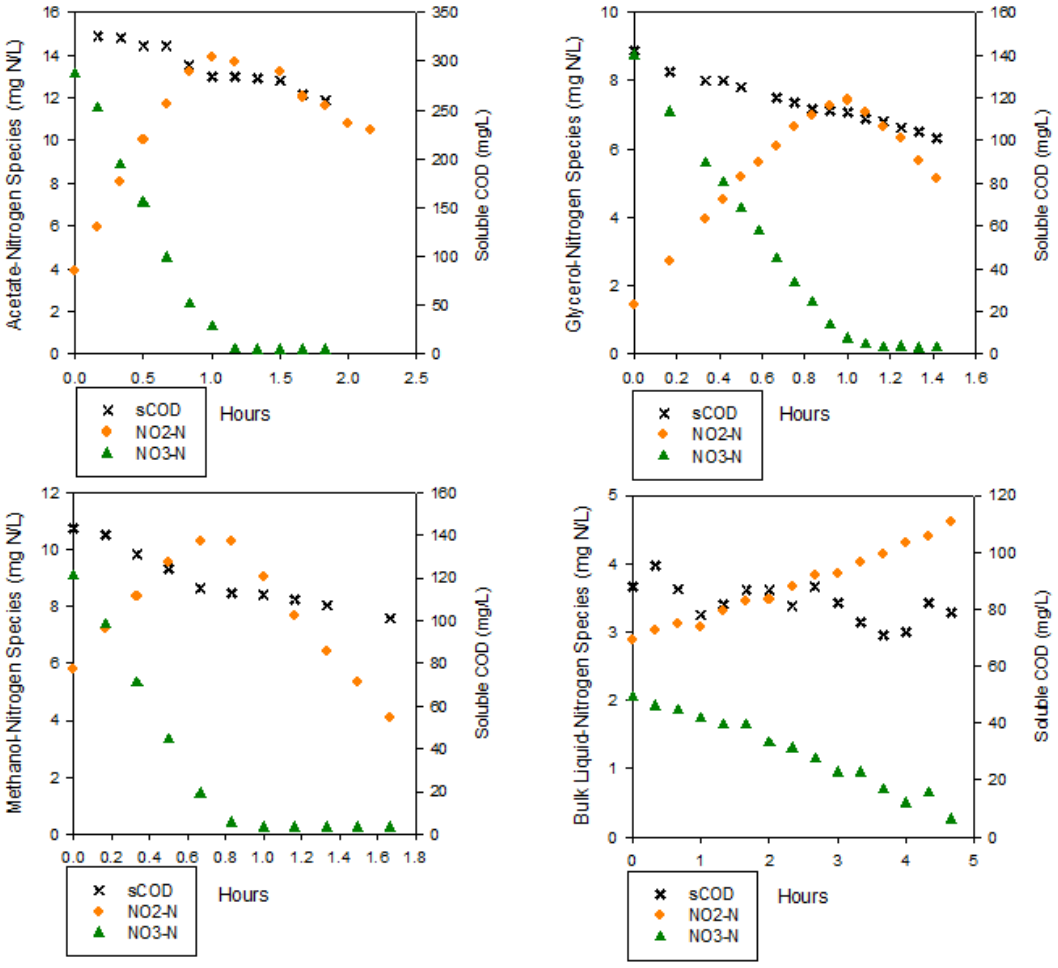

Figure 29: Denitrification activity experiment with all three carbon sources. No ammonia was present during these tests, and there was no AMX activity. Several iterations of this test were performed to obtain theoretical sCOD used/PdN ratios. Top Left-In situ MBBR using acetate. Top Right-In situ MBBR using glycerol. Bottom Left-In situ MBBR using methanol. Bottom Right-Ex situ MBBR bulk liquid using glycerol.

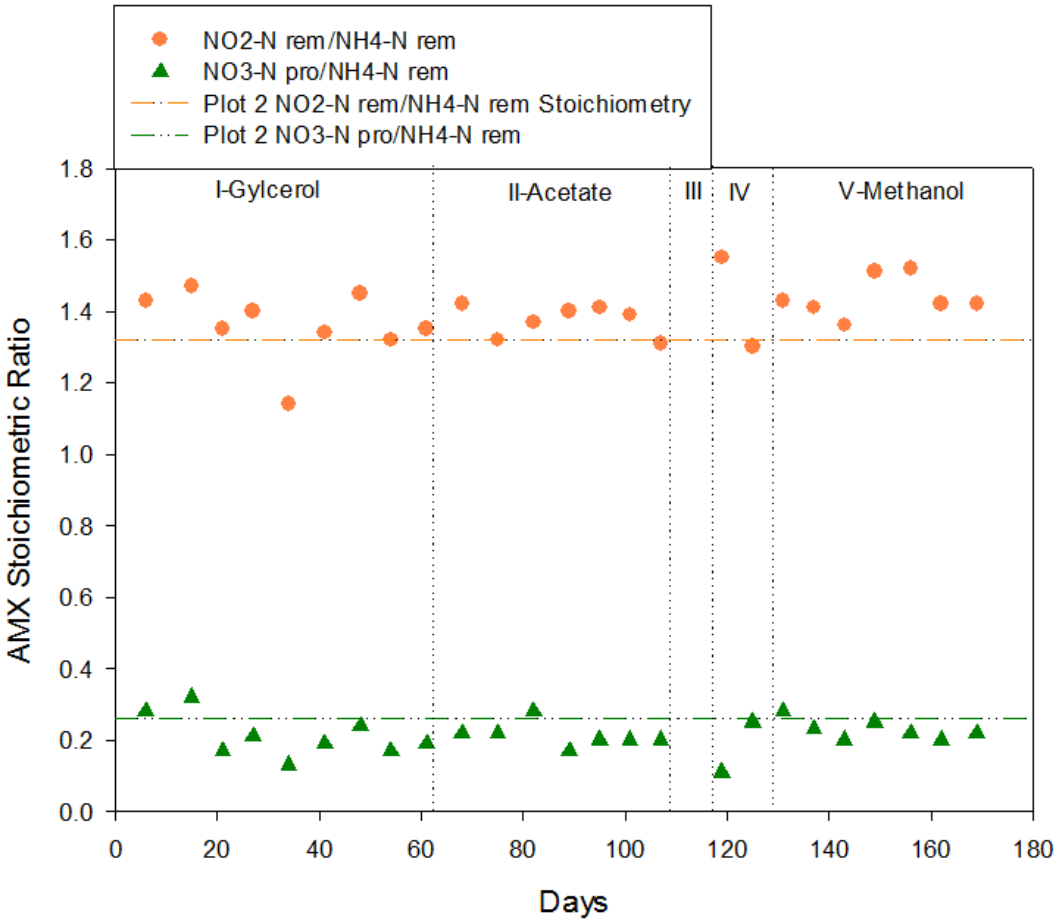

Figure 30: Known theoretical AMX metabolic stoichiometric ratios and actual AMX metabolic stoichiometric ratios from MBBR maximum AMX activity experiments for glycerol, acetate, and methanol. Vertical blue lines indicate start and end dates for each carbon phase.

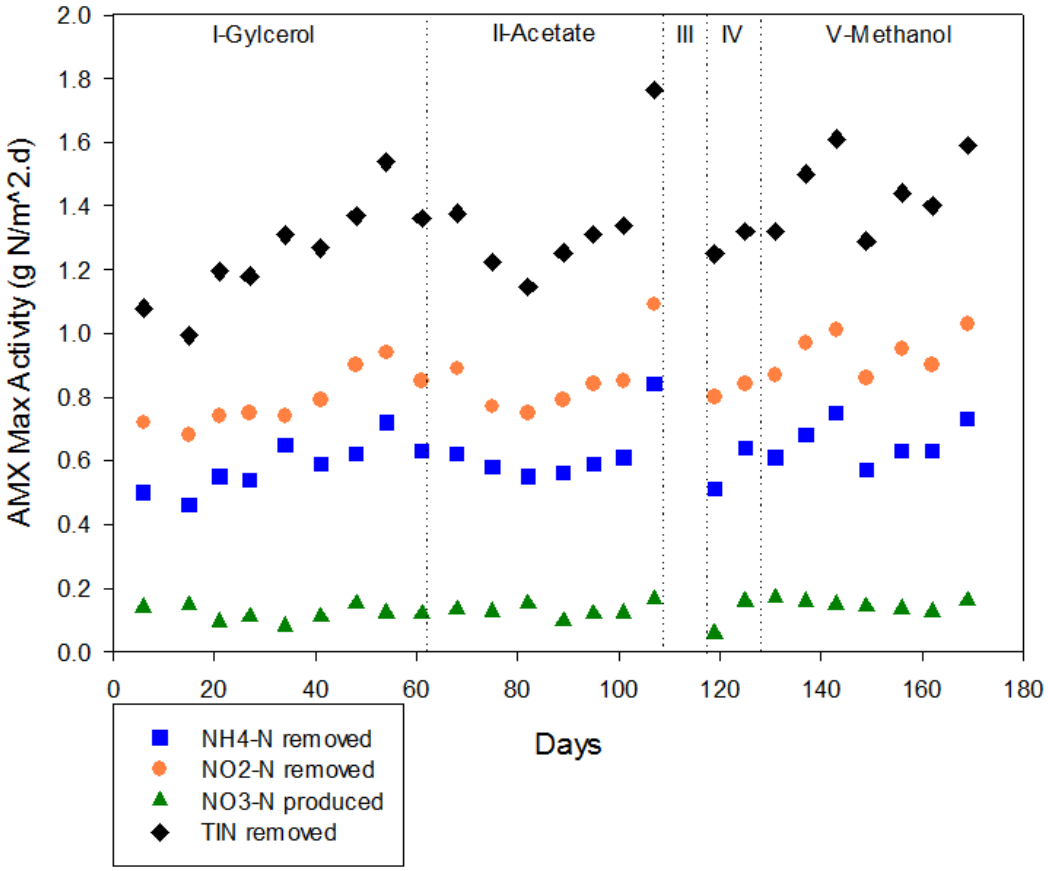

Figure 31: Nitrogen removal rates for NH_4-N, NO_2-N, TIN-N, and production rates for NO_3-N from MBBR maximum AMX activity experiments for glycerol, acetate, and methanol. Vertical blue lines indicate start and end dates for each carbon phase.

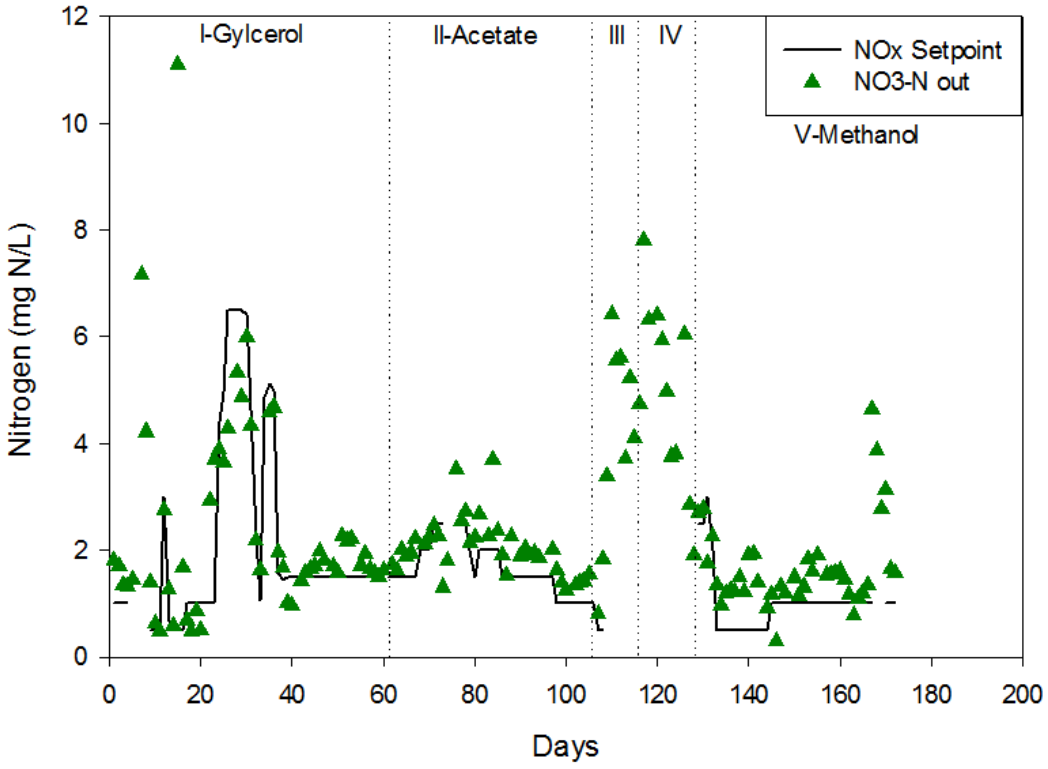

Figure 32: MBBR effluent NO_3-N composite values and MBBR effluent NOx setpoint. The blue line denotes the start of the carbon dosing control strategy. If no NOx setpoint is shown, then the carbon dosing pump was in manual control at a constant rate.

A.1. Engineering Significance

An increase in population, human development, and city densification has endangered our country's waterways. As a response, environmental regulation has increased to help protect waterway quality. Typically focused on solids and oxygen demand removal, wastewater treatment was selected as an area of improvement, and industry authorities such as HRSD must develop strategies to remove or recover nutrients like nitrogen and phosphorus. In order to comply with more stringent regulations, WWTP's must adopt advanced and innovative technologies that focus on improved performance without sacrificing efficiency (intensification).

The pilot-scale mainstream A/B process in this research comprised of a HRAS A-Stage, a BNR B-Stage, and a fully anoxic MBBR tertiary stage. This study implemented two different strategies to incorporate AMX bacteria.

The first is partial nitritation/anammox with partial nitritation occurring in the B-stage, and anammox occurring in both B-stage via bioaugmentation, and as a polishing step in the MBBR. The first was to bioaugment AMX granules from a sidestream deammonification process to B-Stage and retain those

granules with a selection column. B-Stage partial nitritation via intermittent AvN control in combination with AMX is the highly sought after BNR process, deammonification.

The other AMX technology was a polishing PdN/A MBBR. A biofilm formed on the MBBR media with AMX bacteria in an inner layer and OHOs on an outer layer, and together they removed residual NH_4 and NOx. B-Stage effluent nitrate was partially denitrified to nitrite by OHOs using optimal dosing of external carbon, and then AMX removed the nitrite along with B-Stage effluent NH_4. Glycerol, acetate, and methanol were explored and evaluated as carbon sources.

Energy neutrality and process intensification were driving factors for this study. The A-Stage process captured carbon that could be diverted to a digestion process for energy recovery. This upstream carbon removal allowed B-Stage to have a significantly smaller footprint, have reduced energy demand, and reduced HRT and SRT. The B-Stage process operated in a high DO intermittently aerated AvN process controlled by an AvN ratio setpoint. The deammonification process in B-Stage can remove a significant portion of the influent TIN through the AMX pathway, and the PdN/A MBBR polishing unit can remove residual TIN from B-Stage effluent with minimal carbon addition.

Results of this study uncovered complications with mainstream deammonification and a robust process termed PdN/A for full-scale mainstream wastewater treatment. This study also showed that mainstream deammonification should have a complimentary process such as a polishing PdN/A MBBR to safeguard against deammonification disturbances and inefficiencies.

For B-Stage it was found that partial nitrification can be extremely difficult to maintain, and it was not established during the deammonification phase timeline of this study. It was likely but not confirmed that AMX granules were maintained in the B-Stage via the column retention mechanism. Bioaugmentation was dependent on the available WAS AMX biomass from the sidestream DEMON® process, and may not have been significant enough to detect in AMX activity tests. In addition, AMX granule retention could not be confirmed because of inadequate mixing in the sidestream RAS fermenter. As a result of the sidestream RAS complications, the AMX granules were disintegrated and did not have a measureable impact on the mainstream process.

For the PdN/A MBBR it was found that acetate and glycerol performed very high PdN. Despite the inhibition of AMX by methanol in literature, methanol was successful in the PdN/A process. Methanol did not perform as well with PdN as acetate or glycerol but it's low cost made it a competitor with glycerol as the best carbon source to use for PdN/A. AMX metabolic ratios (1.32 NO_2-N rem/NH_4-N rem and 0.26 NO_3-N pro/NH_4-N rem) remained maximized the entire study. In fact, the maximum specific TIN removal from AMX increased throughout the study as well.

B-Stage future work should include explaining why NOB out-selection did not occur during the deammonification portion of this study. A possible area of future work could be step feeding A-Stage effluent into B-Stage to possibly provide more denitrification competition for nitrite to suppress NOB activity. Further testing of the AMX retention column should be done to optimize the retention efficiency of AMX granules. Further exploration and experimentation with AMX quantification techniques should be done to enable cheap, quick, and reliable diagnosis of daily AMX operation.

The PdN/A MBBR future work should include startup optimization of a new MBBR with virgin media. The selection of the right carbon source, optimal dosing, and influent characteristics is paramount for the PdN/A MBBR startup research. Using the PdN/A process, further research will look into high removals of TIN with very little to no external carbon in a mainstream B-Stage process.

There are hundreds of successful sidestream deammonification WWTP's, but mainstream deammonification has been elusive. This research will add knowledge about the complication of NOB out-selection in temperate climate mainstream conditions. This study was not able to induce NOB out-selection using proven techniques such as intermittent aeration, ammonia residual, and an aggressive SRT. Full-scale application is possible but long-term success in colder climates is still a challenge to mainstream AMX activity. Full-scale application of the PdN/A process is highly possible. Although the startup of PdN/A may require specialized knowledge and attention, once it is established the PdN/ process is highly robust and reliable.