A Molecular - Level Interrogation of Ammonia Oxidizing Bioreactors: Comparing and Contrasting Nitritation and Nitrification

A Thesis

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Abstract

National Pollution Discharge Elimination System (NPDES) permits are becoming increasingly stringent for both ammonia and total nitrogen. While traditional nitrification processes are generally reliable, nitrification's heavy oxygen demand contributes to large electricity budget expenditures. In addition to becoming a detriment to community "wallets," adverse effects are measured in the environment; electricity production is the second leading source of CO_2 emissions globally. Moreover, when total nitrogen removal is required, incomplete denitrification can result in N_2O emissions, depending on carbon availability. Research is being conducted at the University of Idaho in the Department of Civil and Environmental Engineering to investigate alternate biological nitrogen removal strategies that can potentially mitigate both concerns. Specifically, a shortcut nitrogen removal mechanism, known as nitritation has been identified as viable process options to offer aeration savings and potentially reduce N_2O emissions. Applying aeration control strategies, nitrogen removal mechanisms were studied at a macro and molecular level, with the aim to provide critical insight for process implementation.

Sequencing batch reactors (SBRs) aimed at achieving nitritation and nitrification were studied. Nutrients, pH, quantitative polymerase chain reaction (qPCR), real time qPCR, and other metabolic tools were utilized to distinguish between processes and to verify nitrogen removal activity within each SBR. A detailed investigation of structural and functional molecular level differences between nitritation and nitrification was conducted. The aim of these investigations are to provide insight to further specify operational criteria to achieve and sustain nitritation.

Acknowledgements

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Dedication

For my mom, for always leading by example.

Acronyms and Abbreviations

ABAC	Ammonia Based Aeration Control
AMO	Ammonia Monooxygenase
AOB	Ammonia Oxidizing Bacteria
ATP	Adenosine Triphosphate
BNR	Biological Nitrogen Removal
CBC	Calvin Benson Cycle
CWA	Clean Water Act
DO	Dissolved Oxygen
EBPR	Enhanced Biological Phosphorus Removal
EPA	Environmental Protection Agency
GHG	Greenhouse Gas
HAO	Hydroxylamine Oxioreductase
HRT	Hydraulic Retention Time
IR	Internal Recycle
LC	Liquid Chromatography
MLE	Modified Ludzack - Ettinger
MMC	Mixed Microbial Culture
Nap	Periplasmic Nitrate Reductase
Nar	Respiratory Nitrate Reductase
NAR	Nitrite Accumulation Ratio
NF	Nitrification Focused Bioreactor
NiR	Nitrite Reductase

NO	Nitrite Oxidase
NOB	Nitrite Oxidizing Bacteria
NoR	Nitric Oxide Reductase
NoS	Nitrous Oxide Reductase
NO_x	Oxidized Ammonia – present as Nitrate or Nitrite
NR	Nitrate Reductase
NT	Nitritation Focused Bioreactor
NxR	Nitrite Oxioreductase
oTCA	Oxidative Tricarboxylic Acid Cycle
PAO	Phosphorus Accumulating Organism
PHB	Polyhydroxybutanoate
PMF	Proton Motive Force
qPCR	Quantitative Polymerase Chain Reaction
RAS	Return Activated Sludge
RQ	Research Question
rTCA	Reductive Tricarboxylic Acid Cycle
RT	Retention Time
SBR	Sequencing Batch Reactor
SND	Simultaneous Nitrification Denitrification
SRT	Solids Retention Time
TKN	Total Kjeldahl Nitrogen
TS/TSS	Total Solids/Total Suspended Solids

VS/VSS	Volatile Solids/Volatile Suspended Solids
WAS	Waste Activated Sludge

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Chapter 1: Introduction

1.1 Research Incentives

Water resource recovery facilities (WRRFs) are at the forefront in protecting water resources. However, in serving this role WRRFs face significant challenges to treat wastewater under increasingly stringent discharge requirements while concurrently aiming to reduce their environmental footprint and operational expenses. Discharge requirements are tailored to location and WRRF by National Pollutant Discharge Elimination System (NPDES) permits. All WRRFs that discharge to surface water operate under a federal or state issued NPDES permit limiting allowable nutrient discharges. The Environmental Protection Agency (EPA) partners with WRRFs to play an integral part in protecting our nation's water bodies. In seeking water body protections, NPDES permits address point source discharges of potential pollutants to prevent oxygen depletion and eutrophication of rivers, streams, and creeks used as receiving water bodies.

Eutrophication is a particular concern addressed with NPDES permits; it is caused by a surplus of nutrients in a water body, which triggers a dense growth of plant life (mostly commonly algae), leading to the death of animal life due to lack of oxygen and ultimately death of a water body. Nutrient sources may include non-point sources such as fertilizer or agricultural/livestock runoff, or point source pollutants, chiefly WRRF discharges. Dense algae growth, known as algal blooms, deplete oxygen while blocking sunlight in the water body before eventually decomposing. The result is large carbon dioxide (CO_2) emissions, fish kills, and dead zones in our nation's estuaries [1]. Nutrient driven toxicity (e.g., cytotoxins produced by blue-green algae) also poses a threat to humans and livestock that use these water bodies as a drinking source, as well as swimmers and their pets. These effects are seen nationwide, as illustrated by Figure 1.1.

Figure 1.1 Effects of Eutrophication seen in Provo Bay, UT [2], Lake Erie, MI [3], and Lake County, OR [4]

Nitrogen and phosphorus are the two core nutrients of interest for eutrophication prevention, thus ordering the strict limitations of total N and P seen in numerous WRRF discharge permits. Abiding by these permits dictates the success of a project and is ultimately the responsibility of the engineer and WRRF operators. Total Kjeldahl nitrogen (TKN) is the most common form of nitrogen in raw wastewater. Biological nitrogen removal (BNR) treatment schemes that target TKN removal are not new technologies utilized by WRRFs. Due to increasingly stringent nitrogen limitations coupled with the need to reduce WRRF environmental emissions associated with operations, new BNR strategies are required. Indeed, the increasing need for environmental protection of the atmosphere and surface waters calls on engineers to implement effective nitrogen removal methods at WRRFs often burdened with conservative budgets.

In achieving wastewater treatment, one of the largest expenses experienced by any WRRF is aeration, which can account for 40 – 60% of the total operation and maintenance costs [5]. Large aeration demands are not only a budget strain, but a detriment to the environment. Electricity production is the second largest carbon dioxide (CO_2) generator in the U.S., accounting for approximately 27% of all CO_2 emissions [6]. Since 1970 the world has seen a 90% increase in total greenhouse gas emissions, largely composed of CO_2 which remains in the earth's atmosphere for years [6]. Reduction of electricity demands by WRRFs can contribute to an overall commitment to reduce CO_2 production in the U.S.

1.2 Nitrogen Removal Process Operation

Suspended growth BNR processes consume significant energy associated with aeration. Conventional nitrogen removal begins with the biological oxidation of ammonia (NH_3) to nitrate (NO_3), hereafter termed nitrification, which requires dissolved oxygen as the terminal electron acceptor. Nitrate is then denitrified to nitrogen gas in the absence of dissolved oxygen [7] – i.e., in an anoxic environment. Nitrification is a two-step process requiring oxygen, carbon, and alkalinity to drive each process [7]. Influent ammonia is first oxidized to nitrite (NO_2) by ammonia oxidizing bacteria (AOB). Newly formed nitrite is oxidized by nitrate oxidizing bacteria (NOB) to nitrate. The denitrification process takes place anoxically to reduce nitrate to nitrite and nitrite to nitrogen gas [7]; these reactions are performed by heterotrophic organisms.

Full scale operation of a suspended growth nitrification-denitrification process is traditionally accomplished utilizing a two-stage configuration known as the modified Ludzack-Ettinger (MLE) process (see Figure 1.2). This process configuration includes anoxic and aerobic zones equipped with internal recycle (IR) and return activated sludge (RAS), followed by clarification [7]. Nitrification is accomplished in the aerobic reactor, where influent ammonia is oxidized to nitrite via AOB, and nitrite to nitrate via NOB. Lack of dissolved oxygen in the anoxic reactor is vital to this treatment configuration, as this ensures nitrate as the terminal electron acceptor (i.e., the reduction compound). IR and RAS streams are nitrate rich; IR is a mixed liquor solution, while RAS includes concentrated biomass solids. IR and RAS can both be employed to regulate nitrate concentration of the anoxic zone. RAS also plays the role of ensuring a steady inoculum stream is supplied to the influent stream of the WRRF. The remaining sludge pumped from the secondary clarifier is termed waste activated sludge (WAS) and serves to establish the solids residence time (SRT). To further specify, the MLE configuration is termed pre-anoxic denitrification, referencing the sequence of aeration basins.

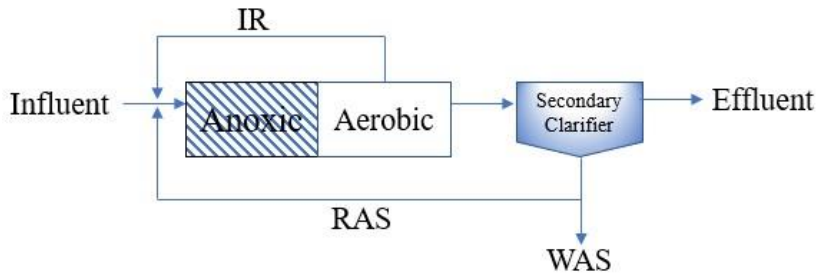

Figure 1.2: MLE Process Schematic

Achieving complete nitrogen removal in a suspended growth system requires establishment of both aerobic and anoxic conditions. Realization at full scale facilities often utilizes a continuous flow design as seen in Figure 1.2, with multiple basins. However, as facilities look to manage their operational budgets more efficiently, reductions in oxygen demand are a logical target. In this regard, nitritation offers a shortcut nitrification process with potential to significantly reduced carbon and oxygen demands. Nitritation works to utilize a common intermediate existing in both nitrification and denitrification processes – nitrite (see Figure 1.3). The novel idea of inhibiting nitrate formation and utilizing nitrite to join these processes is termed nitritation-denitritation.

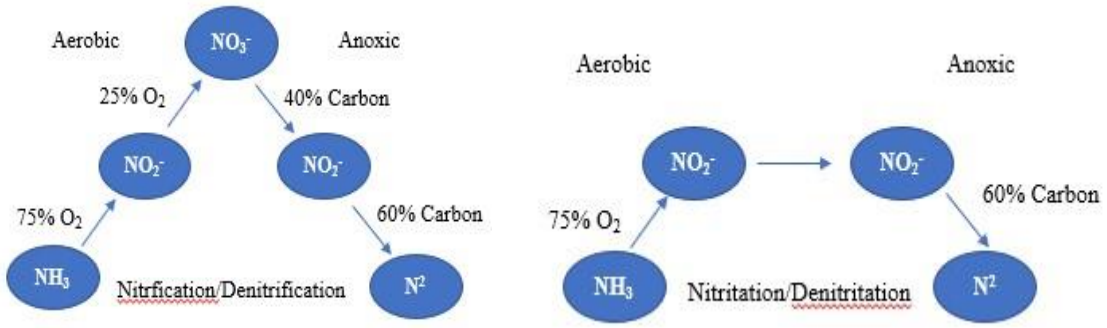

Figure.1.3: Nirification – Denitrification (left) vs. Nitritation – Denitritation (right)

1.3 Nitritation Incentives

Nitritation may offer significant solutions to WRRF environmental and economic issues. Metabolically, less carbon is required to achieve nitritation-denitritation vs. conventional nitrification-denitrification; municipal wastewater is carbon limited, and thus carbon savings can be advantageous to WRRF operations. Nitritation is achieved by oxidizing ammonia to nitrite by AOB while inhibiting oxidation of nitrite to nitrate by NOB. To accomplish nitritation, a differential between AOB and NOB populations must occur. This is accomplished by providing environments to enrich for AOB and wash out NOB [8]. Halting the nitrogen cycle at nitrite as opposed to nitrate can conserve carbon and thus reduce the risk of incomplete denitrification. Incomplete denitrification leads to the production of nitrous oxide (N_2O), which is a potent GHG.

Economically, the nitritation process has been shown to lower aeration costs by as much as 25% [9]. Moreover, research suggests nitritation systems operate at low dissolved oxygen (DO) setpoints [22, 26], resulting in lower operation and maintenance costs and CO_2 production. Research has shown that lower DO setpoints, high temperature, high free ammonia concentrations (a function of temperature and pH), and SRT are effective ways to enrich for AOB over NOB [8]. However, WWRFs do not have the luxury of controlling each of these parameters. Dissolved oxygen setpoints and SRT are common design parameters with good scale up potential.

If in fact nitritation is achieved and sustained at low DO setpoints, this process provides solutions to tighter budgets and permits without compromising treatment quality. A fundamental focus on elimination of extraneous energy demands via stoichiometric oxygen

reductions lays the framework for nitritation research. Insight gained from such research will directly benefit facilities with ammonia permits by allowing them to operate utilizing aerobic nitritation techniques. This operational scheme coupled with ultraviolet disinfection (as chlorination consumes nitrite) may become the normal for small municipalities with even smaller budgets.

Benefits reaped from achievement of an aerobic nitritation system are not strictly monetary. Predicted decreases in oxygen demand result in significant reductions in electricity usage and subsequent CO_2 emissions, while reducing potential risk of incomplete denitrification – a leading cause of nitrous oxide emissions [6]. Achievement of sustainable nitritation systems would also create great opportunities for process expansion to include phosphorus removal and denitritation. Shifting from denitrification to denitritation cuts carbon demands by 40%, reducing costs and benefitting the environment. Elimination of external carbon needs would greatly impact facilities that must utilize sources such as methanol, ethanol, or acetic acid to enhance denitrification. These sources are an additional expense, add to a facilities carbon footprint, and create chemical handling safety considerations. Other carbon sources such as spent sugars or glycerol may be obtained as by-products from beverage or bio diesel manufacturing however, by product purity may be inconsistent, resulting in unpredictable or incomplete denitrification rates [10].

Nitritation demonstrates clear monetary and environmental benefits however, research has shown that nitritation control is no easy task [22, 26]. The process has been observed to occur within specific operational windows, influenced by dissolved oxygen, influent ammonia concentrations, pH and temperature of bulk solution, SRT, and microbial community. Nevertheless, a deeper understanding of the process is lacking. In this regard, a stricter research focus on aerobic metabolisms and active microbial groups is a key first step in determining scale up potential of nitritation. A simplified aerobic study of nitritation eliminates external process variables and allows researchers and operators alike to identify drivers and detriments, and to gain an overall understanding of the oxidation process. Discovery of process parameters to select for desired microbes and regulation of bulk solution oxygen content is a key first step to sustainable BNR systems.

It is clear this BNR strategy promises significant energy and monetary savings, motivating researchers around the world to unlock process fundamentals. However, achievement and sustainability of nitritation remains poorly understood both at a molecular and operational level. To help contribute new insight into nitritation fundamentals, the objective of this thesis is to explore and examine how nitritation differs from conventional nitrification at a molecular level and how this may translate to WRRF operation and design. Investigations were conducted utilizing metabolomics, transcriptomics, quantitative polymerase chain reaction (qPCR), and phylogenetic comparisons to gain insight on the uniqueness of this promising BNR strategy.

1.4 Research Questions and Hypotheses

As nitritation is a process with potential to reduce oxygen demands significantly, it is logical to focus research to gain a greater understanding of this process aerobically. In this regard, gaining an understanding of nitrifying communities from a molecular level will ultimately translate to operational strategies for process achievement and maintenance. Nitritation is achieved when NOB wash out and AOB enrichment is achieved. Thriving AOBs catalyze ammonia oxidation to nitrite, which accumulates due to low populations of NOB. However, in reality complete NOB washout is purely theoretical due to the nature of mixed microbial cultures present at WRRFs. Nevertheless, while NOB washout may be infeasible, targeted enrichment of certain NOBs to sustain partial nitritation may be achievable via operational strategies.

Two species of NOB have been identified by researchers to play vital roles in nitrite oxidation – *Nitrobacter* spp. and *Nitrospira* spp. Importantly, the two species demonstrate small structural and functional differences which may provide answers to nitritation achievement. Specifically, interrogation of two representative NOB genomes from publicly available databases – for *Nitrobacter winogradski* and *Nitrospira defluvii* – indicate unique metabolic pathways that might help explain how nitritation is achieved in a *Nitrobacter* spp. dominated culture. Investigation of these two genomes reveals differences in nitrite oxidoreductase (NxR) orientation, carbon metabolism, and polyhydroxybutanoate (PHB) biosynthesis capabilities. Considering these differences, along with some preliminary data, it has been hypothesized that NOB enrichment of *Nitrobacter* spp. may induce partial nitritation.

NxR is a protein complex responsible for nitrite oxidation that is present in both *Nitrobacter* and *Nitrospira* species. However, the oxidation site is oriented in opposite directions - towards the cytoplasm (inside cell membrane) for *Nitrobacter* spp. and towards the periplasm (outside cell membrane) for *Nitrospira* spp [11]. Due to the difference in orientations, *Nitrobacter* must transport nitrite across the cell membrane prior to oxidation, and nitrate back across upon completion of oxidation. This suggests that *Nitrospira* spp. may be energetically advantaged in terms of nitrite oxidation and subsequent nitrate production – confounding nitritation. Thus, if *Nitrobacter* spp. is the dominant NOB, higher nitrite accumulation ratios (NAR) could be achieved.

Further metabolic distinctions have been realized via utilization of genomic databases, namely between carbon fixation metabolisms. *Nitrospira defluvii* employ the reductive tricarboxylic acid cycle (rTCA) while *Nitrobacter winogradski* employ the Calvin Benson cycle (CBC) [12]. This distinction provides researchers with a means to infer genomic activity of the two via presence of indicative metabolites of either the rTCA or CBC, and to further infer potential involvement with nitritation. Finally, *Nitrobacter winogradski*'s ability to utilize organic acids (namely acetate) and store as an internal carbon reserve (known as PHB) set it apart from *Nitrospira defluvii*. It is suspected this *Nitrobacter winogradski* ability to utilize another carbon source may give this NOB an advantage – yet another potential indication of nitritation.

Uncertainties of nitritation achievement coupled with newfound structural and functional differences between crucial NOB genomes yields the following research questions.

> ***RQ 1:*** Can nitritation be achieved and sustained simply through control of residual dissolved oxygen and SRT?
>
> ***Hypothesis 1:*** Nitritation can be sustained under strict control of residual dissolved oxygen and SRT.
>
> - AOB population will exceed that of the NOBs,
> - Nxr gene activity exhibited by *Nitrobacter* will be greater than *Nitrospira*,
> - Larger quantities of carbon fixation metabolic intermediates associated with the *Nitrobacter winogradski* genome are observed opposed to *Nitrospira defluvii*.

These metrics must demonstrate significant differentials within a bioreactor.

Objective 1: Monitor and assess nitritation/nitrification performance (macro- and molecular level) of two SBRs at different residual dissolved oxygen concentrations.

RQ 2: Nitritation appears to be achieved by enriching for *Nitrobacter* spp. over *Nitrospira* spp. Can significant differences be observed in NOB populations for nitrification and nitritation events?

Hypothesis 2: Nitritating SBRs exhibit *Nitrobacter* spp. dominated NOB populations.

Objective 2: Apply phylogenetic methods (qPCR) to compare and contrast mixed microbial consortia performing nitritation and nitrification.

RQ 3: Differences in carbon fixation pathways reveal unique metabolic intermediates to serve as functional surrogates for the *winogradski* and *defluvii* genomes. Can these indicative metabolites in conjunction with Nxr expressions indicate a significant difference between *Nitrobacter winogradski* and *Nitrospira defluvii* activity in a nitritating SBR?

Hypothesis 3: NOB genomic activity associated with *Nitrobacter winogradski* and *Nitrospira defluvii* can be inferred if significant differences are observed between Calvin cycle and rTCA cycle intermediates and Nxr gene expressions. Nitritation will exhibit higher abundances of Calvin cycle intermediates and cytoplasmic orientation of the Nxr gene, whereas nitrification will exhibit higher abundances of rTCA cycle intermediates and periplasmic Nxr orientations.

Objective 3: Apply metabolomic and transcriptomic techniques to characterize functional performance of nitritating vs. nitrifying mixed microbial consortia. Assess significance of observed relative differences between nitritation and nitrification events to determine what NOB activity can be inferred.

Chapter 2: Literature Review

2.1 Nitrogen and the Nitrogen Cycle

Our understanding of nitrogen and the nitrogen cycle has been evolving for centuries. Nitrogen's importance in agriculture, industry, and the environment has sparked numerous studies yielding both beneficial and detrimental effects of the element's application. The discovery of eutrophication in the mid-1950s called for dramatic cessation of nitrogen additions (previously thought to only be beneficial) and changes in the chemical makeup of treated effluent wastewater. Further changes were realized by the Clean Water Act (CWA) passed in 1977, largely fueling and influencing advances in the wastewater industry [13]. Nutrient removal regulations continue to drive such advances, requiring research for viable and cost-effective nitrogen removal strategies to keep our nation's waterways healthy. Such solutions begin with a thorough functional understanding of nitrification.

Nitrogen exists in multiple oxidation states. Success of a biologic nitrogen removal (BNR) strategy hinges on its ability to navigate what is known as the nitrogen cycle (see Figure 2.1) – specifically the ability to navigate between the different oxidative states when desired. Knowledge of the nitrogen cycle is utilized in engineered process schemes such as those seen at water reclamation and reuse facilities (WRRF). For wastewater treatment processes the cycle begins with ammonia (most commonly present as NH_4-N). Ammonia is present in municipal wastewater in typical concentrations of 20 - 30 mg/L [14] and 35- 40 mg/L present as total kjeldahl nitrogen (TKN) [7].

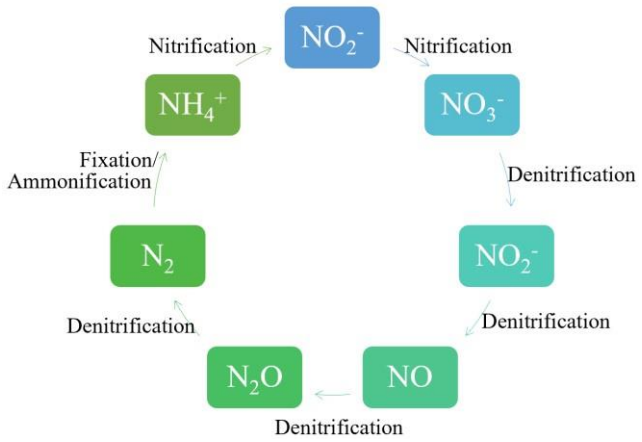

Figure 2.1: The Nitrogen Cycle

The goal of facilities operating under total nitrogen National Pollutant Discharge Elimination System (NPDES) limitations is to biochemically convert as much of this influent ammonia to nitrogen gas as possible. This is achieved through a series of oxidation-reduction (redox) reactions composing two main processes – nitrification and denitrification.

During nitrification two different groups of microorganisms are active in a set of redox reactions. Ammonia is oxidized to nitrite by ammonia oxidizing bacteria (AOB), followed by oxidation of nitrite to nitrate by nitrite oxidizing bacteria (NOB). In both exchanges dissolved oxygen acts as the terminal electron acceptor. In addition to oxygen, alkalinity is needed to drive this process. Ammonia oxidation consumes alkalinity, most commonly in the form of carbon dioxide (CO_2). Such consumption requires sufficient concentrations of this compound or other alkaline sources to drive nitrification [7].

Denitrification is composed of yet another series of redox reactions that when fully accomplished produce nitrogen gas. In this series of exchanges, nitrate and nitrite become electron acceptors, stressing the importance of a lack of oxygen in a designated “anoxic” zone. Denitrification reactions consume organic carbon, utilizing it as an electron source to reduce oxidized ammonia (NO_x). The denitrification process is completed by a diverse population of microbes commonly dominated by ordinary heterotrophic organisms (OHO) in a step wise fashion [7].

A kinetic and metabolic understanding of the processes necessary to oxidize and reduce nitrogen is crucial for continued research and development of BNR strategies. Additionally, kinetic and metabolic insight gained from research is integral to treatment design and process improvement. Such knowledge is leveraged to inform new and existing wastewater treatment technologies, processes and corresponding operations, in addition to troubleshooting.

2.1.1 Nitrogen-based Oxidation Reduction Reactions

Redox reactions serve to generate energy in biological systems. Fundamentally, oxidationreduction reactions are an exchange of electrons. The law of conservation of charge allows us to breakdown this electron exchange to aid in metabolic understanding. Abiding by the law of conservation of charge, a redox electron exchange occurs as a simultaneous loss and gain of electrons in two elements [15]. Oxidation of an element indicates a loss of electrons, making this element the electron donor. The reduced element receives these

electrons, thus reducing its charge. Due to the relatively large electro negativity of oxygen, most redox reactions utilize this element as the electron acceptor. With nitrification as a prime example, in the presence of oxygen ammonia is oxidized by AOBs and transfers its electrons to oxygen. Such exchange reduces oxygen while simultaneously oxidizing ammonia. This same exchange occurs in the case of nitrite and NOBs, as illustrated in Figure 2.2.

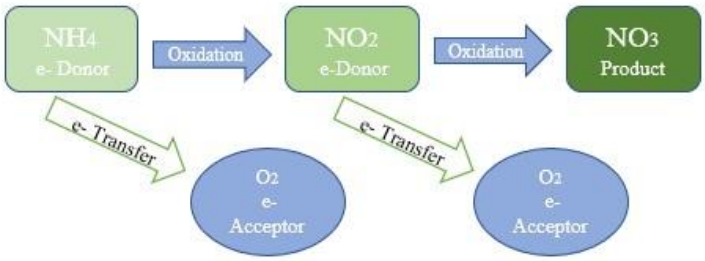

Figure 2.2: Nitrification Redox

Denitrification is accomplished in anoxic environments, or environments lacking oxygen. To navigate the series of redox reactions shown in Figure 2.3, a new electron acceptor must be available. The acceptor is presented in the form of oxidized ammonia - nitrate or nitrite (NO_x). Such compounds are supplied to the anoxic zone following nitrification. To fuel these redox reactions, organic matter can be leveraged as an electron source to reduce supplied NO_x. The source of carbon can be present intracellularly, as a product of endogenous decay, within influent wastewater, or can be added externally [16].

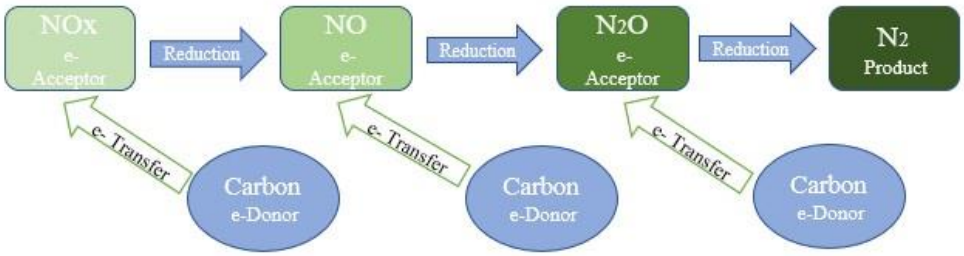

Figure 2.3: Denitrification Redox

2.2 Nitrification

Nitrification is achieved when different types of nitrogen oxidizing bacteria are active, each fueled by electrons and carbon. Electrons are needed for both cell growth and energy (i.e., nitrate reduction) [16], thus when an energy demand is reduced more electrons may be allocated to cell growth efforts. Ammonia and nitrite oxidizing bacteria utilize dissolved oxygen to oxidize ammonia and nitrite in this first phase of the nitrogen removal process.

Nitrification relies heavily on the health of these microorganisms in the two-step oxidation process illustrated by the left side of Figure 2.4.

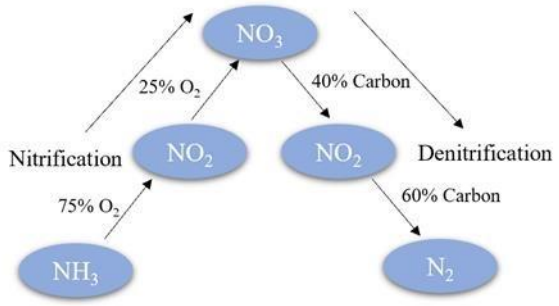

Figure 2.4:Nitrification & Denitrification

Oxygen and carbon demand quantifications illustrated in Figure 2.4 are derived from stoichiometric relationships seen in Reactions I and II.

$$2NH_4^+ + 3O_2 \xrightarrow{AOB} 2NO_2^- + 4H^+ + 2H_2O \qquad \textit{Reaction I}$$

$$2NO_2^- + O_2 \xrightarrow{NOB} 2NO_3^- \qquad \textit{Reaction II}$$

2.2.1 Nitrifying Microbial Community: AOB and NOB

Classifying active microbes involved in biochemically driven systems is essential for identifying active metabolisms and their subsequent necessary substrates. Microorganism classification is largely dictated by the differences in sources or methods employed to obtain energy and carbon. In general, microbial carbon sources may be organic or inorganic, thus classifying *heterotrophs* and *autotrophs*. Heterotrophic bacteria produce new biomass fueled by organic compounds, while autotrophic bacteria utilize inorganic compounds, specifically carbon dioxide. Further microbial classification serves to specify cellular energy sources. *Chemotrophs* generate energy via chemical reactions as opposed to *phototrophs* which utilize light for cell synthesis [7].

AOB present in municipal BNR operations are classified as chemosynthetic autotrophs [17]. These AOBs can derive energy through utilization of reduced inorganic compounds such ammonia, nitrite, ferrous iron, or sulfide as a carbon source [7, 18], and generate energy from electron transfer accomplished by redox reactions. Active NOB are also autotrophs and are

equipped with the same carbon metabolisms as AOB. Electrons and carbon are the building blocks for microbial populations, providing means for cell activity and synthesis respectively.

Nitrifying AOB exist as a subclass of *β-proteobacteria* and include the *Nitrosomonas* and *Nitrospira* genera. *Nitrosomonas* often dominate the AOB populations seen at WRRFs, although a few studies evidence existence of *Nitrospira* dominated environments [7]. The nitrifying NOB population consists of four genera within three subclasses of proteobacteria. The genera include *Nitrobacter, Nitrococcus, Nitrospina,* and *Nitrospira*. *Nitrobacter* and *Nitrospira* hold significant roles in BNR and belong to the respective subclasses of *αProteobacteria* and *δ-Proteobacteria* [18]. In municipal treatment facilities, *Nitrospira* often dominate the NOB population [19], speculated to be kinetically driven, due to typical nitrite and dissolved oxygen concentrations of activated sludge processes.

2.2.2 Nitrification Achievement: Kinetics

Engineering an environment for AOB and NOB enrichment is crucial to the success of a nitrifying system. Ideal concentrations of limiting substrates can be identified utilizing kinetic relationships to inform process decisions. A further kinetic classification amongst both AOB and NOB genera can be established from specific growth rates. This distinction is referred to as “r-strategists” and “K-strategists.” As illustrated in Figure 5, K-strategists (*Nitrospira*) grow faster than r-strategists (*Nitrobacter*) at low substrate concentrations, while r-strategists thrive in high substrate conditions [16].

In the case of NOBs, *Nitrospira* are K-strategists, and *Nitrobacter* r-strategists. This strategist-based classification is illustrated by Monod kinetics (see Figure 2.5), as growth rates are much higher for K-strategists (*Nitrospira*) at low nitrite and dissolved oxygen concentrations. Such relationships help to understand kinetic selection of bacteria. Kinetic relationships also help inform design criteria and operational conditions suitable to engineer environments in which desired bacteria can thrive.

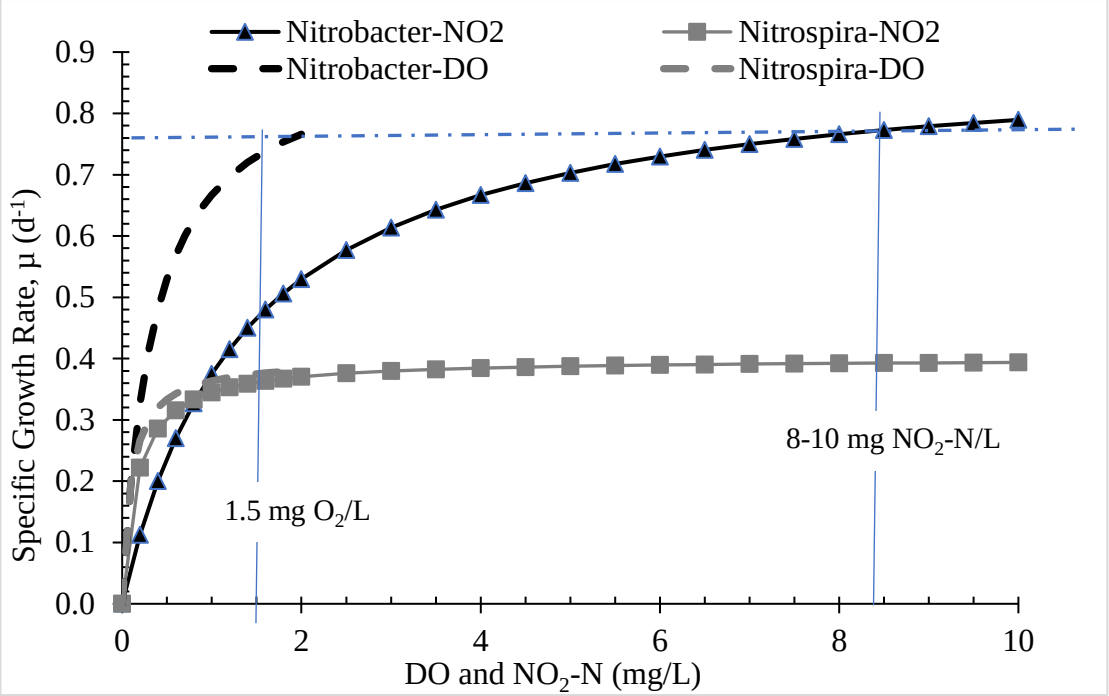

Figure 2.5: Dual Monod Curve; Nitrite & DO Concentrations

2.2.3 Nitrification Insight: Metabolisms

In addition to kinetic selection principles, metabolic-level inquires can be made to understand how to further advantage desired microbes in an engineered environment. Identifying active enzymes and metabolic intermediates can aid in BNR process recognition, which may not always be evidenced by bulk solution concentrations, pH trends, or operational strategies. AOB metabolism are fueled by electrons provided from redox reactions to drive ammonia oxidation. Step one (see Reaction III and Figure 2.6) in ammonia oxidation facilitates the oxidation of ammonia to nitrite via the enzymes ammonia monooxygenase (AMO) and *hydroxylamine oxidoreductase* (HAO). These enzymes function as catalysts in the oxidation process [18]. AMO utilizes electrons provided by the ammonia molecule and present oxygen to form hydroxylamine (NH_2OH), an intermediate, which is in turn oxidized by HAO to produce nitrite. Metabolic activity of AOB (Figure 2.6) illustrates importation of influent ammonia across the cell membrane to complete the two-step oxidation process, yielding nitrite.

$$NH_3^+ + O_2 + 2H^+ + 2e^- \xrightarrow{AMO} NH_2OH + H_2O \xrightarrow{HAO} NO_2^- + 5H^+ + 2e^- \qquad \textit{Reaction III}$$

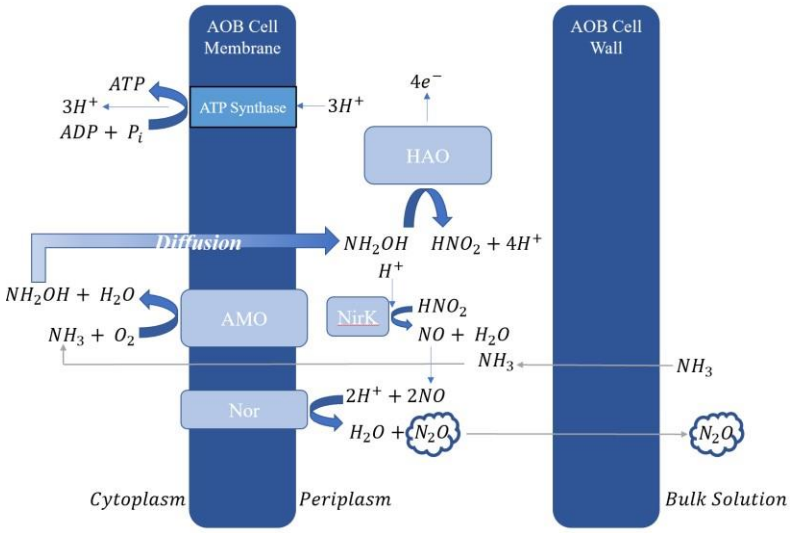

Figure 2.6: Illustration of the AOB Nitrification Metabolism and Key Proteins

Active NOBs utilize the produced nitrite as an electron donor, oxidizing the compound to nitrate and producing electrons which fuel cellular growth and provide energy. These microbes are also autotrophic, equipped with the same carbon metabolisms as AOBs, only utilizing nitrite as their electron source. A nitrite oxidase (NO) enzyme is employed to catalyze Reaction IV, with supplied dissolved oxygen acting as the electron acceptor for nitrate production [20]. Metabolic activity relevant to nitrification of both *Nitrospira* and *Nitrobacter* NOB is shown in Figure 2.7. Nitrite oxidation is completed by the nitrite oxidoreductase (Nxr) protein complex for both NOB species.

$$NO_2^- + O_2 \xrightarrow{NO} NO_3^- + 2H^+ + 2e^- \qquad \textit{Reaction IV}$$

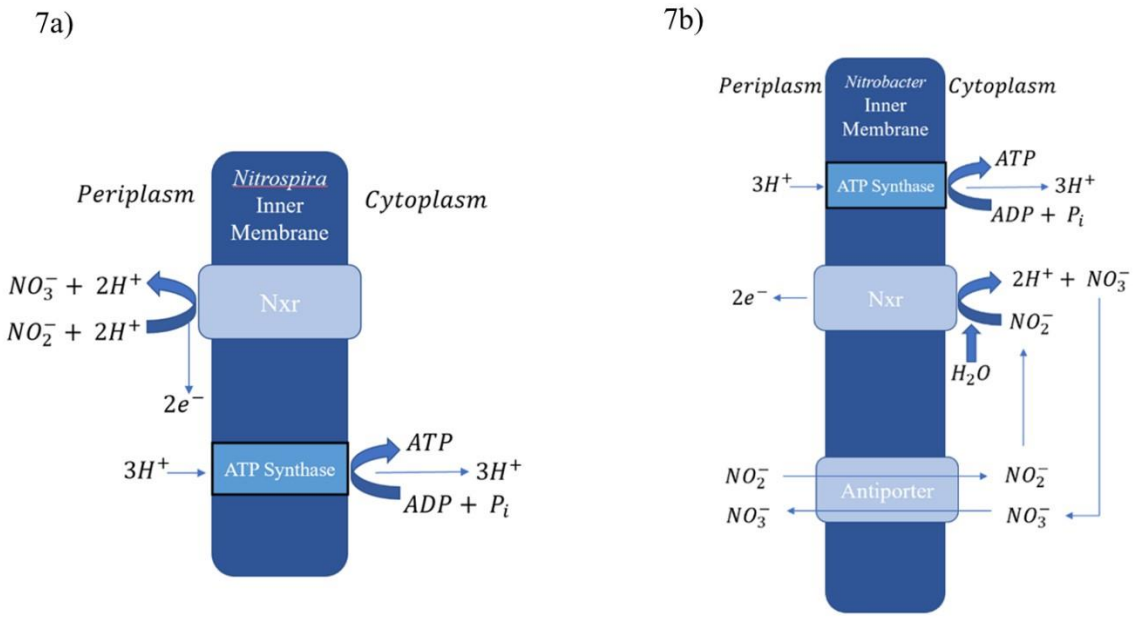

Figure 2.7: Illustrations of Nitrospira (7a) and Nitrobacter (7b) Nitrification Metabolisms and Key Proteins

Evidenced by Reactions III and IV, successful nitrification not only produces nitrate, but also hydrogen ions. This production of hydrogen ions can cause a shift in pH, trending lower (more acidic) with oxidation until nitrification is complete. To mitigate excess pH decreases and ensure nitrification, alkalinity must be available. Overall nitrification stoichiometrically consumes approximately 2 moles of alkalinity (shown as bicarbonate in Reaction V) per 1 mole of ammonia. As calcium carbonate ($CaCO_3$), this is equivalent to 7.14 grams per gram of ammonia. Such demands require a typical minimum concentration of 170 - 180 mg/L $CaCO_3$ in raw wastewater, based on typical raw wastewater influent TKN concentrations, to drive the nitrification process and prevent drastic pH shifts [16].

$$NH_4^+ + 1.98HCO_3^- + 1.83O_2 \xrightarrow{Alkalinity\ Consumed} 0.98NO_3^- + 1.88H_2CO_3 + H_2O + 0.021C_5H_7NO_2$$

Reaction V

2.3 Denitrification

Nitrification is only the first half of total nitrogen removal in a BNR system. While ammonia and nitrite are oxidized, nitrate, which is another EPA regulated nutrient, is produced. NPDES permits with nitrate limits require operation of total nitrification-denitrification systems, achieving the full biochemical process illustrated in Figure 2.4 (previously presented).

The denitrifying microbial population is much more diverse than that of nitrification. Ordinary heterotrophic organisms (OHO) largely make up the denitrifying microbial community. The

process, like nitrification, is achieved via a series of redox reactions. However, from a redox perspective, the aim is reducing the electron acceptor rather than the electron donor. This is only possible in the absence of oxygen, as it is a favored electron acceptor. For such reasons, this reduction process imposes carbon demands on a biological system instead of oxygen. Full denitrification of nitrate to nitrogen is gas is demonstrated in Reaction VI, utilizing acetate (CH_3COO^-) as a carbon source.

$$NO_3^- + H^+ + 0.33NH_4^+ + 1.45CH_3COO^- \xrightarrow{OHO} 0.5N_2 + 0.33C_5H_7O_2N + 1.60H_2O + 1.12HCO_3^- + 0.12CO_2$$

Reaction VI

An absence of oxygen, or anoxic conditions, yields an environment in which NO_x becomes the terminal electron acceptor. This allows biodegradable organic matter present in influent wastewater to be oxidized as the electron donor. Within BNR, the process results in reduction of NO_x, organic matter, and oxygen demand, as well as alkalinity recovery [7, 16]. Anoxic environments are typically created in continuous flow BNR systems by employment of an internal recycle, or nitrate feed seen in the previously listed Figure 1.2. The pre-anoxic configuration is known as the modified Ludzack-Ettinger (MLE) process which utilizes a recycle flow of NO_x rich mixed liquor directed from the aerobic zone to the anoxic zone.

Denitrification depends on the success of the nitrification process to provide an electron acceptor in the form of NO_x. Introduction of influent wastewater containing a carbon substrate provides an adequate electron donor for NO_x reduction. It is critical that the liquid in the recycle stream maintain a low dissolved oxygen concentration to sustain a healthy anoxic environment [21]. If a recycle mechanism is not utilized, a post anoxic denitrification system, in which the anoxic zone follows the aerobic, may also be implemented. In this case, a supplemental carbon source such as methanol or acetate is typically added (due to limited carbon concentrations in influent wastewater) to serve as an electron donor [22].

2.3.1 Denitrifying Microbial Classification

Diversity in the microbial population of denitrifying bacteria is widespread amongst both autotrophs and heterotrophs. Autotrophic bacteria can accomplish nitrogen reduction in anaerobic environments, while heterotrophs require an electron exchange [7, 20]. Denitrifying bacteria employed for nitrogen reduction are facultative aerobes – indicating they may utilize oxygen, nitrate, or nitrite as an electron acceptor. For these reasons, heterotrophs are ideal

candidates for denitrification at BNR facilities. Heterotrophic denitrifiers are classified amid a wide range of genera, including: *Achromobacter, Agrobacterium, Alcaligenes, Arthrobacter, Bacillus, Chromobacterium, Corynebacterium, Flavobacterium, Halobacterium, Hypomicrobium, Methanomonas,Moraxella, Neisseria, Paracoccus, Propionibacterium, Pseudomonas, Rhizobium, Rhodopseudomonas, Spirillum, and Vibrio* [16].

2.3.2 Denitrification Reactions

Active facultative aerobes in the denitrification process require both an electron and carbon source. Carbon sources may come in many forms - dictated by process configuration and availability. Typically, carbon and electrons are supplied in the form of organic matter while nitrate acts as an initial electron acceptor to accomplish this series of redox reactions. Enzymes known to catalyze the redox reactions of denitrification include nitrate reductase (NR), nitrite reductase (NiR), nitric oxide reductase (NoR), and nitrous oxide reductase (NoS) [18]. Collectively these enzymes achieve the four-step process of denitrification, beginning with nitrate reduction. Metabolic activity relevant to denitrification and its active enzymes are shown sequentially from left to right in Figure 2.8, and further represented by Reactions VII – X.

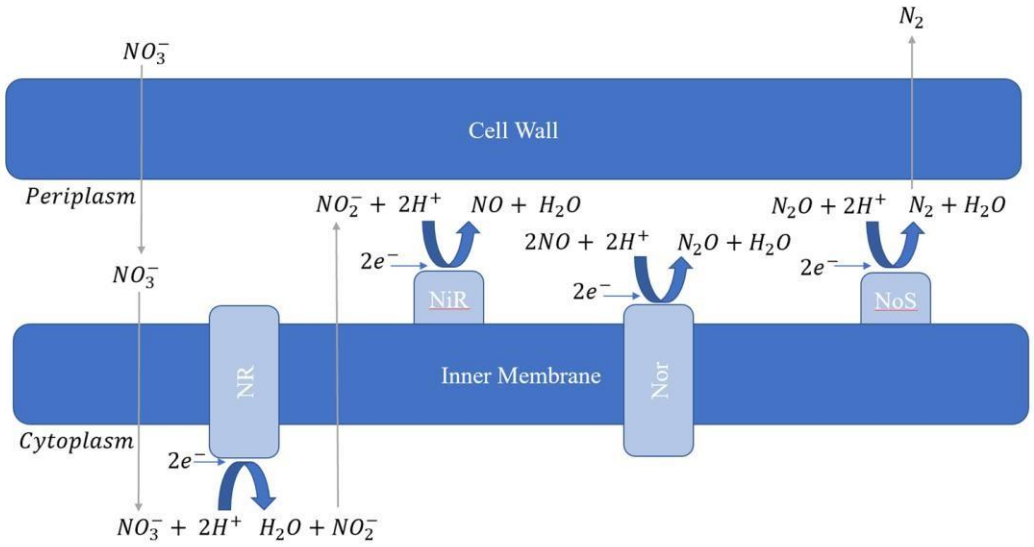

Figure 2.8: Illustration of the Denitrification Metabolism and Key Proteins

$$NO^-_3 + 2H^+ + 2e^- \xrightarrow{NR;Nap,Nar} NO_2 + H_2O \qquad \textit{Reaction VII}$$

Active nitrate reductases (NR; in Figure 2.8 and the arrow in Reaction VII) for nitrate reduction are the periplasmic nitrate reductase (Nap), and the respiratory nitrate reductase

(Nar) [18]. While both enzymes initiate reduction of nitrate, functional diversity is observed for adenosine triphosphate (ATP) generation. It appears Nar couples nitrate respiration (i.e. denitrification) with proton translocation across the cell membrane. This is predicted to create a proton motive force (PMF) utilized by the ATP synthase to drive ATP generation [19]. Due to the location of nitrate respiration of Nap, a net yield of zero protons is generated, thus no PMF. Evidence suggests Nap couples nitrate respiration with formate oxidation to create a PMF and further ATP generation [19].

$$NO^{-}_{2} + 2H^{+} + e^{-} \xrightarrow{NiR} NO + H_2O \qquad \textit{Reaction VIII}$$

Further reduction of nitrite follows either Nap or Nar activity. Nitrite is reduced with the activation of the nitrite reductase (NiR; in Figure 2.8, arrow in Reaction VIII) to produce nitric oxide (NO).

$$2NO + 2H^{+} + 2e^{-} \xrightarrow{NoR} N_2O + H_2O \qquad \textit{Reaction IX}$$

The nitric oxide reductase (NoR; in Figure 2.8, arrow in Reaction IX) enzyme is recruited to reduce NO to nitrous oxide (N_2O) before being reduced yet again to nitrogen gas via the nitrous oxide reductase (NoS; in Figure 2.8, arrow in Reaction X) [18]. Incomplete denitrification, particularly at this stage of denitrification, is risky, as N_2O emissions pose a great threat to the atmosphere. Atmospheric warming impacts of one pound of N_2O is 300 times that of one pound of CO_2 [6].

$$N_2O + 2H^{+} + 2e^{-} \xrightarrow{NoS} N_2 + H_2O \qquad \textit{Reaction X}$$

Inhibition of the nitrous oxide reductase may be caused by introduction of oxygen to the anoxic zone, or an insufficient carbon source. Concentrations as low as 0.2 – 0.25 mg O_2/L may begin to inhibit reductase activity [16], further risking N_2O emissions. N_2O emissions are also risked with low wastewater carbon concentrations, as an electron donor is needed for the final redox reaction. For these reasons it is vital that denitrifying systems have a sufficient carbon source, even if it must be added as an external source. Complete denitrification (Reaction XI) is accomplished with an adequate carbon source, nitrate feed, and anoxic environment. When successful, nitrogen is reduced, nitrogen gas is released, and alkalinity is recovered.

$$C_{10}H_{19}O_3N + 10NO_3^- \xrightarrow{Alkalinity\ Recovered} 5N_2 + 10CO_2 + 3H_2O + NH_3 + 10OH^-$$

Reaction XI

Alkalinity recovery may be measured in the form of hydroxide ions (OH^-) produced. Stoichiometrically, when biodegradable organic matter is utilized as a carbon source, 1 mole of alkalinity is produced per 1 mole of nitrate reduced. As calcium carbonate ($CaCO_3$), this is equivalent to 3.57 grams per gram of nitrate [16]. Alkalinity recovery may be leveraged to supplement nitrification demands if a pre-anoxic denitrification process (MLE) is implemented.

2.4 Nitritation

An ever-increasing public environmental consciousness pressures municipalities to implement more energy efficient systems. Traditional nitrification systems consume a significant portion of a facilities' energy budget [5]. Nitritation is a BNR process that can reduce both the energy and carbon consumption of nitrification facilities. In contrast to complete nitrification, nitritation seeks to utilize a common intermediate in the nitrification denitrification processes – nitrite. Foregoing oxidation of nitrite and subsequent reduction of nitrate reduces both oxygen and carbon demands significantly. Nitrification and nitritation process are contrasted in Figure 2.9.

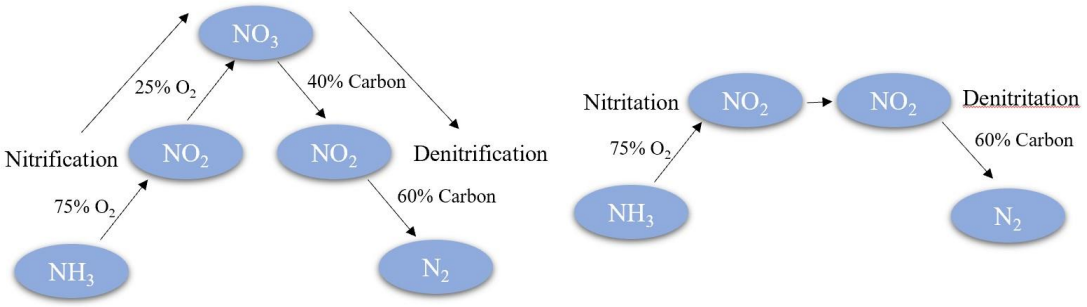

Figure 2.9:Nitrification (left) vs. Nitritation (right)

As illustrated, oxygen and carbon demands can be reduced by 25 and 40% respectively when a full nitritation system is operational. Such reductions are quantified by stoichiometric values observed for oxidation and reduction processes outlined by previously listed nitrification Reactions I and II and denitrification Reaction III. Carbon reductions are computed via comparison of denitritation Reaction XII with the same carbon source (acetate).

$$NO_2^- + H^+ + 0.24NH_4^+ + 0.98CH_3COO^- \xrightarrow{Nir,NoR,NoS} 0.5N_2 + 0.24C_5H_7O_2N + 1.24H_2O + 0.74HCO_3^- + 0.008CO_2$$

Reaction XII

In a completely nitritating system, Reaction II is omitted, and the reduction (denitritation) process begins with Reaction XII.

2.4.1 Nitritation Incentives

Nitrogen regulated WRRFs consume significant amounts of energy to operate in compliance with NPDES permits, principally associated with aeration. Typically, activated sludge processes which include BNR, can account for over half of a facility's total electricity consumption associated with aeration (see Figure 2.10) [23]. Such energy consumption equates to 40 – 60% of typical operational budgets [5].

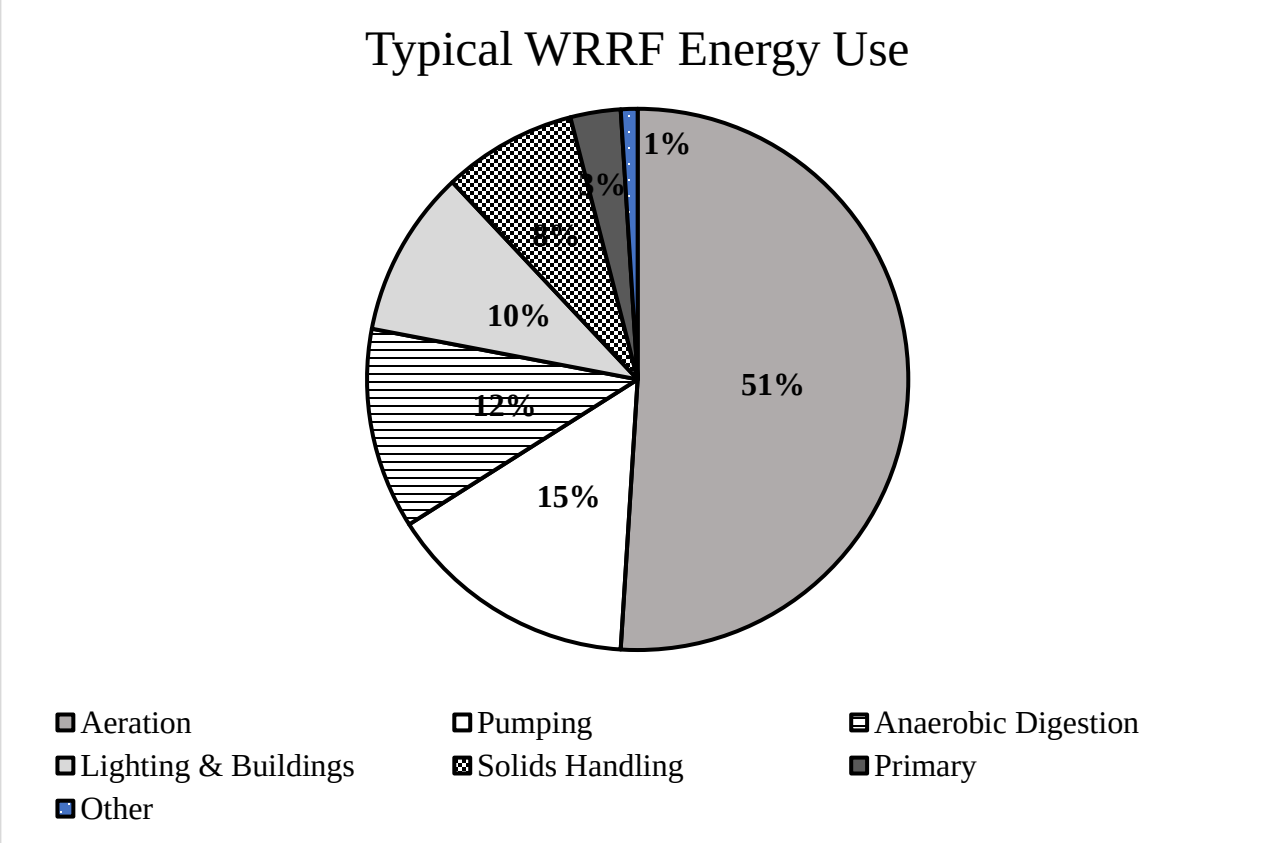

Figure 2.10:Typical Distribution of Energy Consumption at WRRF, Adapted from [23]

Oxygen demand reductions achieved by nitritation systems have the potential to translate to significant budget reductions at facilities operating nitrification systems. Additionally, electricity generation is a leading producer of CO_2, a concerning greenhouse gas [6]. Reduced

electricity consumption begets reduced electricity generation, thus reducing a facilities overall carbon footprint.

Further reductions in carbon footprint are realized with nitritation systems for the denitritation process. When this process begins with a more reduced compound (NO_2 as opposed to NO_3), less carbon overall is needed to drive the denitritation process. This is important, because raw municipal wastewater is carbon limited, and many biological reactions at WRRFs rely on this substrate. If the carbon source is depleted these denitrification reactions cannot be completed. In the case of denitrification, the final reduction step is nitrous oxide (N_2O, a potent greenhouse gas) to nitrogen gas (N_2, a harmless gas abundantly present in the atmosphere). If there is insufficient carbon present to complete this final reduction step of denitrification, inadvertent N_2O emissions could be significant.

Consciousness of N_2O emissions is becoming increasingly critical as more studies indicate wastewater treatment as a significant contributor to global emissions [6, 24, 25]. N_2O emissions seen at WRRFs are classified as anthropogenic sources and make up 6.2% of global N_2O emissions. Wastewater treatment is the main contributor of these anthropogenic emissions, accounting for 4 – 5% of the total 6.2% [24]. Due to difficulties associated with quantifying such emissions for many different facilities, respective configurations, and varying influent carbon concentrations, a conservative approach to ensure complete denitrification should be taken. Reducing denitrification carbon demands surely aids in assuring a complete denitrification process.

Due to the limited carbon quantity seen by influent wastewater it is not uncommon for WRRFs to utilize external carbon sources to drive denitrification reactions. These sources are usually added in the form of ethanol or methanol and can be costly (both from a supply and labor perspective). Conservation of influent carbon realized by denitritation can reduce the amount of this carbon addition, or even eliminate the need. A carbon source reduction/elimination saves WRRFs money and time. Moreover, when denitrification carbon demands are reduced, more carbon is available to drive other biological reactions for nutrient removal. Namely, enhanced biological phosphorus removal (EBPR) also benefits from available system carbon.

2.4.1.1 Nitritation and Oxygen Demand Reduction

Oxygen and carbon savings via the nitrite pathway can be calculated on an electron basis. Nitrification and nitritation reactions shown in Reactions I and II illustrate oxygen demands of both processes, translating to savings of both cellular energy and aeration costs. Reaction II is theoretically not included in the nitritation process, as nitrate production is not desired. Omission of Reaction II reduces the stoichiometric oxygen demand of full nitrification by 1.143 mgO_2/mg N, equivalent to 25% total oxygen reduction [9]. Oxygen savings can also be computed as a valence change in electrons. Full nitrification results in a valence change of 8 total electrons, while nitritation only requires 6. In addition to reduced aeration demands and subsequent lower aeration budgets, reductions seen for nitritation oxidation process translate to significant impacts in energy savings for both the WRRF and denitrifying organisms.

Nitritation systems may also be favored operationally at lower dissolved oxygen setpoints (0.5 – 1.5 mg O_2/L), as illustrated in AOB/NOB Monod kinetics (Figures 2.11 & 2.12). Monitoring of setpoint and airflow rate can greatly impact aeration budgets. One other novel operational control for nitritation aeration systems is known as ammonia-based aeration control (ABAC). Probes monitor bulk solution ammonia concentrations and tailor oxygen supply to only provide the oxygen necessary to achieve ammonia oxidation. Upon completion of oxidation, oxygen supply ceases, thus eliminating excessive aeration.

2.4.1.2 Nitritation and Carbon Demand Reduction

Following the aeration stage in a BNR WRRF, the bulk solution is nitrite rich (i.e., accumulated nitrite). Such large concentrations then begin the nitrite reduction, or denitritation process. Denitritation is similar to denitrification but requires less carbon and electrons. Since reduction begins with nitrite, a lesser valence change is required opposed to nitrate reduction. Like oxidation processes, an oxygen equivalency can be assigned to nitrite and nitrate reduction based on valence changes. As evidenced by omission of denitrification Reaction VII, the two electrons associated with nitrate reduction are no longer needed. This omission results in an oxygen equivalency savings of 1.143 mg O_2 [9].

Carbon savings are also illustrated stoichiometrically via Reactions III and XII. Stoichiometrically, only 0.98 moles of acetate are needed per one mole of nitrite as opposed to 1.45 moles required for reduction per one mole of nitrate. This quantifies as a 38% reduction in carbon demands when denitritation is implemented in place of complete nitrification. Such

a significant carbon conservation can prevent incomplete denitrification and help fuel other carbon-demanding processes such as EBPR.

2.4.1.3 Nitrite Reduction Thermodynamics

In addition to the stoichiometric oxygen and carbon demand reductions associated with nitritation-denitritation, further thermodynamic energy savings can be realized with denitritation as opposed to traditional denitrification. Specifically, utilization of nitrite as opposed to nitrate as an electron acceptor is more energetically favorable to active cells. This is demonstrated by McCarty bioenergetics. Half reactions and their corresponding Gibbs free energy values; ΔG° (kJ/electron mole) are shown in Reactions XIII and IVX for nitrite and nitrate as electron acceptors.

$$\frac{1}{3}NO_2 + \frac{4}{3}H^+ + e^- \xrightarrow{yields} \frac{1}{6}N_2 + \frac{2}{3}H_2O \qquad \Delta G° = -93.23 \qquad \textit{Reaction XIII}$$

$$\frac{1}{5}NO_3 + \frac{6}{5}H^+ + e^- \xrightarrow{yields} \frac{1}{10}N_2 + \frac{3}{5}H_2O \qquad \Delta G° = -71.67 \qquad \textit{Reaction IVX}$$

Negative ΔG° values are associated with exergonic reactions, producing energy. Approximately 60% of produced energy is utilized by the cell for growth and energy [7], while the rest is released as heat. Evidenced by bioenergetics, utilizing nitrite as an electron acceptor over nitrate is energetically favorable, due to the more negative ΔG° value.

A bioenergetic analysis theoretically calculates energy released from redox reactions (ΔG_R) and energy used for cell growth (ΔG_S). This assumes a 60% energy capture to calculate electron moles of substrate oxidized per electron mole of substrate used (f_e) and electron moles of substrate used for cell synthesis of the total electron moles of substrate used (f_s). These values provide further theoretical evidence to support nitrite as the favorable electron acceptor over nitrate. Bioenergetic values are listed for comparison between NO_3 and NO_2 in Table 2.1 when acetate is utilized as an electron donor.

Table 2.1: Bioenergetic Analysis for Nitrate & Nitrite Reduction Utilizing Acetate

Electron Acceptor	ΔG_R (kJ/e mol)	ΔG_S (kJ/e mol)	f_e	f_s
NO_3	-99.35	44.91	0.43	0.57
NO_2	-120.91	44.91	0.38	0.62

Negative ΔG_R values also indicate an energy release. Nitrite's more negative value provides a larger energy release from a redox reaction utilizing acetate as the electron donor and nitrite (as opposed to nitrate) as an acceptor. This is in turn reflected by the larger nitrite f_s value, indicating a larger fraction of that energy being allocated to cell growth.

2.4.2 Nitritation Microbial Community: Kinetics & Metabolisms

2.4.2.1 Kinetic Selection

Engineering a nitritation system begins with considering the process kinetics. Theoretically, complete washout of NOB accomplishes complete nitritation. However, due to the large mixed microbial culture present within mainstream WRRFs, along with multiple other process dynamics, complete nitritation does not appear possible. Due to this impossibility, researchers have shifted their focus to enrichment of AOB and specific NOB species to achieve partial nitritation. Two types of NOB have piqued the interest of researchers hoping to understand nitritation: *Nitrospira* spp. and *Nitrobacter* spp. It has been hypothesized that enrichment of *Nitrobacter* spp. over *Nitrospira* spp. can lead to achievement of sustainable nitritation systems [11]. Researchers utilize both kinetic and metabolic selection techniques to achieve this bacteria enrichment.

The principle of kinetic selection of microbial populations utilizes limiting substrate concentrations to enrich for desired populations. Monod kinetics (Figures 2.11 and 2.12) demonstrate the relationship between these limiting substrates and the maximum specific growth rates of bacteria. In the case of both AOB and NOB these substrates are DO and nitrogen. AOBs utilize ammonia-nitrogen while NOBs rely on nitrite-nitrogen.

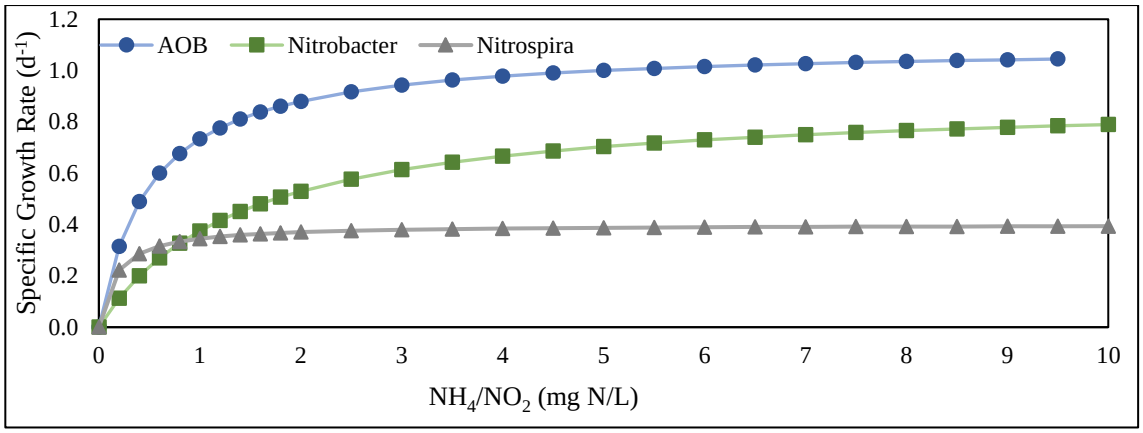

Figure 2.11: DO Based Growth Monod Kinetics of Relevant Nitrifying Bacteria

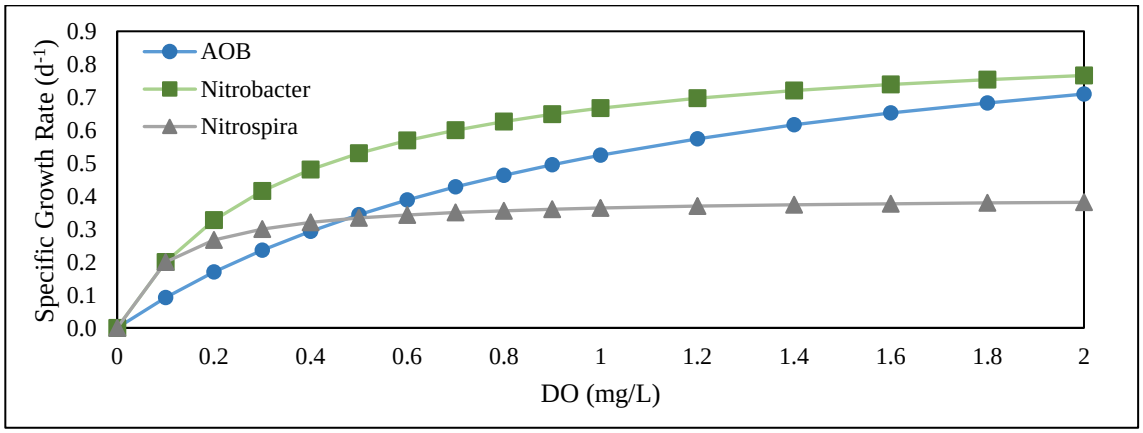

Figure 2.12: Nitrogen Based Growth Monod Kinetics of Relevant Nitrifying Bacteria

As shown, *Nitrobacter* and AOB outcompete *Nitrospira* at DO setpoints of 0.5 mg O_2/L and greater. *Nitrobacter* and AOBs are also advantaged at high nitrogen concentrations. For such kinetic reasons, a high nitrite and sufficient ammonia concentration may be targeted to maintain the health of *Nitrobacter* NOB and AOB populations. This process is known as partial nitritation. Partial nitritation seeks to maintain an ammonia residual typically between 2 – 3 mg NH_3/L [22] to maintain healthy AOB populations. The remaining nitrogen present in the effluent is in the form of nitrite. Such nitrogen speciation provides an environment for both AOB and *Nitrobacter* to thrive. Operationally, partial nitritation is typically achieved utilizing ammonia-based aeration control (ABAC). ABAC monitors ammonia concentration and provides aeration pulses in response to a declining concentration until full ammonia oxidation or ammonia setpoint is reached [22].

An intermittent aeration control strategy is also a technique aimed at NOB suppression. Intermittent aeration aims to provide oxygen sporadically since AOBs outcompete NOBs at low DO concentrations. NOB also experience a lag in metabolic mechanisms as opposed to AOB following transient anoxic periods [26, 27]. This lag is proportional to the length of the anoxic disturbance. A larger metabolic impact may be realized with pre or post anoxic configurations, but intermittent aeration techniques also beget an NOB metabolic lag [27]. ABAC or intermittent aeration control systems are viable methods to reduce aeration costs.

2.4.2.2 Metabolic Identification

NOB metabolic lag may be due to structural differences in the location of the critical NxR protein between *Nitrobacter* and *Nitrospira* (see Figure 2.7). The NxR protein complex is responsible for nitrite oxidation in NOB species. It is membrane bound in both *Nitrobacter* and *Nitrospira*, however the actual nitrite oxidation site is located on different sides of the cell membrane. *Nitrospira* oxidize nitrite in the periplasmic space (closest to bulk solution) while *Nitrobacter* oxidize in the cytoplasmic space [18, 28]. Due to differences in oxidation location, it has been speculated that *Nitrospira* may be energetically favored for nitrite oxidation. However, considering this critical difference in NxR, a *Nitrobacter* enrichment may explain how partial nitritation is achieved. Specifically, the additional movement of nitrite and nitrate across *Nitrobacter* cell membrane as opposed to *Nitrospira*, could lead to nitrite accumulation, thus achieving nitritation [11].

In seeking to develop a better understanding of nitritation, further interrogation of the different NOBs revealed key metabolic differences. NOB genomes were examined in detail for two model NOBs: *Nitrobacter winogradski* and *Nitrospira defluvii*. Metabolic investigations revealed differences in carbon sequestration metabolisms and polyhydroxybutyrate (PHB) biosynthesis capabilities. *N. winogradski* utilizes the Calvin Benson Cycle and codes for PHB biosynthesis while *N. defuvii* is reliant upon the reductive tricarboxylic acid cycle (rTCA) and lacks PHB biosynthesis coding [19, 28].

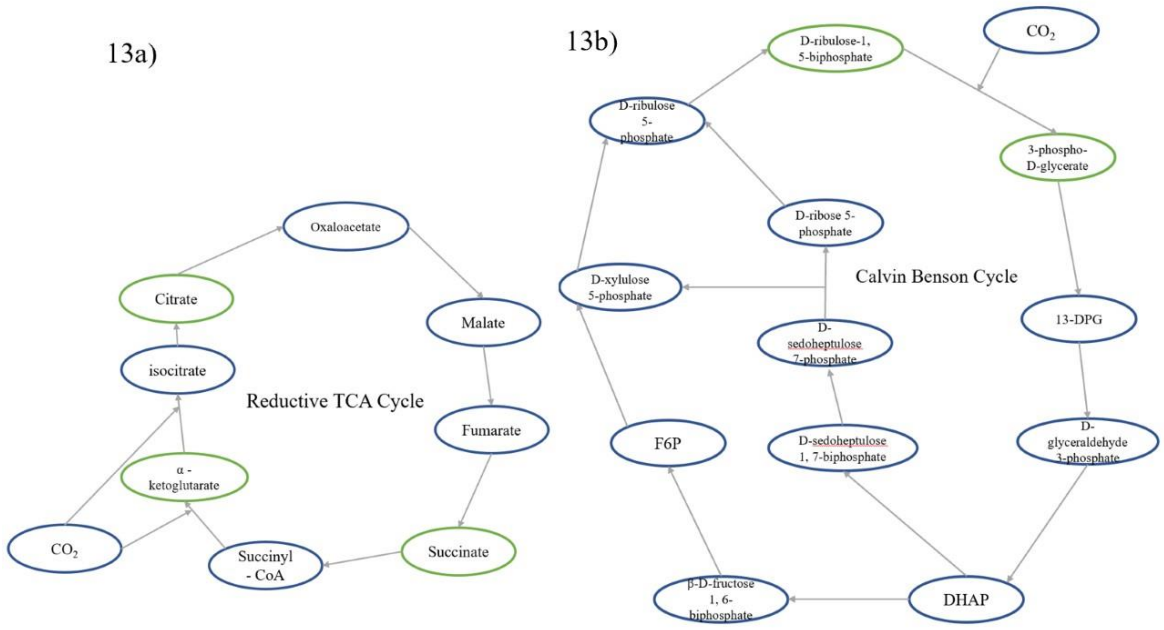

Figure 2.13: Carbon Fixation Metabolisms and Indicative Metabolic Intermediates of Nitrospira Defluvii (13a) and Nitrobacter Winogradski (13b)

These different carbon metabolisms produce different metabolic intermediates, or metabolites, that can potentially be monitored in the study of nitritation. Indicative metabolites of the Calvin cycle include D-Ribulose 1, 5 bi-phosphate, and 3-phosphoglycerate [19], while indicative metabolites of the rTCA cycle are succinate, α - ketoglutarate, and citrate (indicated by green nodes in Figure 2.13). The reductive TCA cycle shares many metabolites with the oxidative TCA (oTCA) cycle, a key energy generating metabolism utilized by many microorganisms. However, enzymatic differences between rTCA and oTCA cycles exists and may aid in distinguishing dominant activity of either cycle. For example, *Nitrospira defluvii* have been shown to code for citrate synthase but not the α – ketoglutarate dehydrogenase [19, 29]. This metabolic difference may provide a means of distinguishing TCA cycles and possible carbon metabolism activity.

Metabolic differences can give bacteria competitive advantages over others, depending on the engineered environment. Utilization of the rTCA cycle allows *Nitrospira defluvii* to consume inorganic substrates for growth in addition to nitrite and carbon dioxide [19]. This is known as mixotrophic growth and may advantage *Nitrospira* NOBs over *Nitrobacter*. Mixotrophic growth capabilities may contribute to the typical *Nitrospira* dominance seen at full scale

facilities [11]. However, *Nitrobacter* also have competitive metabolic capabilities, namely PHB biosynthesis. PHB is an internal carbon storage polymer and can be utilized when substrate is not readily available. Biosynthesis capabilities are often induced by stress or starvation periods experienced by NOBs in the form of anoxic or anaerobic environments [16].

If in fact the unique mechanisms necessary to achieve nitrite oxidation (i.e. requirement of NO_2 transportation inside the cell) on *N.winogradski* contribute to nitrite accumulation, a *N.winogradski* enriched mixed microbial culture (MMC) may induce nitritation. Theoretically a *N.winogradski* enriched NOB community could synthesize and store PHB (perhaps anaerobically in a BPR configuration), advantaging it during anoxic periods. Therefore, enrichment may be realized with proper aeration techniques in conjunction with periodic anoxic disturbances. Such disturbances cause an overall NOB metabolic lag [27] (key for nitritation) and may also induce *N.winogradski's* PHB biosynthesis capabilities.

2.4.3 Measuring Nitritation

Stable nitritation is marked by high accumulations of nitrite relative to NO_x during and following the aerobic period. This is termed a nitrite accumulation ratio (NAR), quantified in Equation I.

$$NAR = \left(\frac{NO_2}{NO_3 + NO_2}\right) * 100 \qquad \textit{Equation I}$$

Reliable operational strategies to achieve and sustain high NAR remain unclear, thus sparking kinetic and metabolic investigations of nitritation. To inform process design criteria, research must be conducted on nitritation achievement, the enriched microbial population, and associated metabolic level activities. Parameters such as SRT, HRT, anoxic disturbances, and aeration control strategies are design variables with good scale up potential from a lab or pilot scale study. In this regard, a literature review of such studies, coupled with kinetic and metabolic knowledge gained, partial nitritation in a mainstream BNR WRRF configuration should be achievable through a combination of the following parameters.

- Low dissolved oxygen concentrations (0.5 – 1.5 mg O_2/L) [8]
- Ammonia residual (2 – 3 mg NH_3/L) [22, 26]
- Non-limited nitrite concentrations [8, 26]
- Anoxic disturbance [26, 27]
- AOB and *Nitrobacter* enrichment

While nitritation achievement has been observed in differing scenarios employing all or some of the listed operational strategies, the process is still largely misunderstood at a molecular level. Additionally, sustaining a nitritating system has proven to be difficult. In contrast, many nitrifying WRRFs operate on all scales throughout the nation, largely without issue. Nitrification is a traditional process in activated sludge that is relatively simple to achieve and sustain. Thw question, why is nitritation achievement and sustainability so complicated in comparison? A molecular contrast of the two processes from a structural and functional view may provide insight to the similarities and differences between the nitrogen removal processes.

Chapter 3: Materials & Methods

3.1 Experimental Setup

3.1.1 Bioreactor Setup

3.1.1.1 Reactors NF & NT

To inform investigations conducted in this thesis, ammonia oxidizing bioreactors NF & NT (shown in Figure 3.1) were operated from May 2020 – September 2021. The two bioreactors, operated with the aim the achieve nitrification and nitritation (NF and NT respectively), were inoculated on May 21, 2020 each with 0.5 L of mixed liquor from the oxidation ditch at Moscow WRRF. The Moscow WRRF is configured as an A2O process (anaerobic, anoxic, aerobic) [7].Reactors were fed raw municipal wastewater, also obtained from the Moscow WRRF to achieve a total working volume of 2 L.

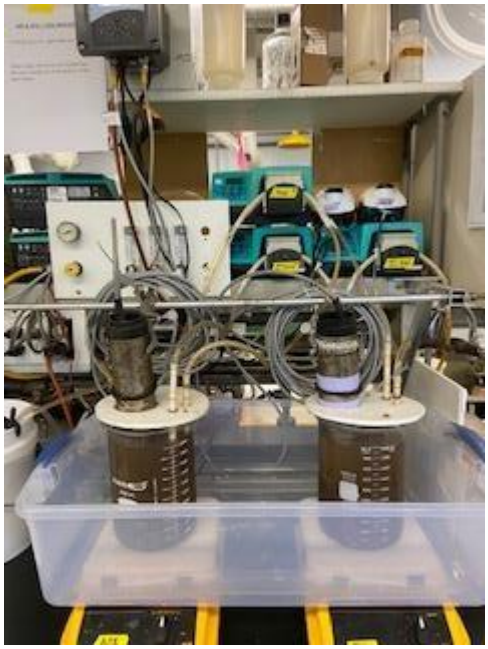

Figure 3.1: Laboratory Bioreactors aimed at Nitrification (NF; left) and Nitritation (NT; right)

Reactors were operated as sequencing batch reactors (SBRs). Each operational cycle consisted of the following relay-controlled periods: feed, treatment (aeration and mixing), wasting, settling, and decant. Operational states were interrogated via manipulation of solids retention time (SRT), hydraulic retention time (HRT), and dissolved oxygen (DO) setpoints. Operational criteria for each reactor are described in Tables 3.1 and 3.2.

Table 3.1: NF Operational Matrix

"NF" Bioreactor Operational Matrix		
SRT (days)	HRT (hours)	DO Setpoint (mg O_2/L)
8	8	0.8
8	18	2.0
12	18	2.0

Table 3.2: NT Operational Matrix

"NΓ" Bioreactor Operational Matrix		
SRT (days)	HRT (hours)	DO Setpoint (mg O_2/L)
16	18	0.5
8	18	0.5
12	18	0.5

Operational parameters of SRT and HRT were controlled via wasting and decanting calculated volumes of mixed liquor and reactor effluent. Feed, waste, and decant mechanisms were controlled using Watson Marlow model 323 peristaltic pumps operating on a PLCcontrolled relay system. Relays triggered beginning and end of cycle periods, totaling to 6 hours. NF and NT were operated under completely aerobic conditions for the duration of their treatment period (i.e., no dedicated anaerobic or anoxic phase). The objective of employing a purely aerobic treatment period was to generate operational conditions that favored ammonia oxidation while excluding other nutrient removal mechanisms.

Feed consisted of raw municipal wastewater obtained from the Moscow WRRF on a weekly basis and stored at 4°C until use. Ammonia concentration of influent was tested three times weekly using grab samples. Air was introduced using Wenshall 1" cylinder air stones supplied by Fusion Quiet Power model 600 aquarium pumps. Dissolved oxygen was controlled at specified DO setpoint (mg O_2/L) using a Hach (Loveland, CO, USA) SC100™

Controller equipped with a Hach LDO™ Dissolved Oxygen Sensor. The reactors were mixed with Teflon™-coated stir bars and magnetic stir plates. The reactors were covered but vented to the atmosphere.

3.1.1.2 BIOPHO – PX N1 & N2 Reactors

Two additional bioreactors were profiled to provide data that would serve as a molecular contrast to the ammonia oxidizing systems. Bioreactors PX-N1 and PX-N2, shown in Figure 3.2, were operated as SBRs, with nine liters of total working volume each. Both reactors target an 8 day SRT and operate under completely mixed conditions provided by overhead propeller type mixers. SRT is controlled through Garett wasting of mixed liquor during the anoxic period, once each cycle. PX-N1 and PX-N2 total SBR cycle is also six hours (like NF and NT), but with operational conditions focused on total nitrogen and phosphorus removal. In general, the core BIOPHO–PX operational scheme that is central to reactors PX-N1 and N2 follows an anaerobic-aerobic-anoxic sequence. House air is introduced via stone diffusers during the aerobic period.

Figure 3.2: BIOPHO Reactors PX-N1 (left) and PX-N2 (right)

The anaerobic period is fixed at one hour, while aerobic treatment utilizes ABAC resulting in a variable aerobic duration. A DO setpoint in each reactor is maintained through use of PID control in Hach SC100 controllers. The DO setpoint is 1.5 mg O_2/L in PX-N1 and 0.5 mg O_2/L in PX-N2. ABAC targets a bulk solution ammonia concentration of 3.0 mg N/L, then stops aeration. The SC100 switches a relay which closes the shutoff valve in the air line.

ABAC results in typical aeration lengths of 2 – 2.5 hours for PX-N1 and 2 – 2.75 hours for PX-N2. The post-anoxic period is designed to achieve NO_x reduction.

Ammonium, nitrate, and nitrite concentrations are continuously monitored in each reactor. Ammonium is monitored using Hach AN-ISE probes via ion-selective electrodes. Nitrate and nitrite are monitored using Hach NX7500 scanning UV probes. Probes are regularly calibrated by using reactor grab samples. DO is monitored via a Hach LDO probe.

3.2 Analytical Techniques

Samples were collected to monitor NO_2, NO_3, NH_3, TSS, and VSS. For soluble constituents, samples were filtered through a 0.22 μm syringe filter (Millipore Corp., Billerica, MA, USA) prior to testing. Soluble NO_3 was determined in accordance with Hach method 10020 and soluble NH_3 testing followed Hach method 10031 (both consistent with Standard Methods) [30] A Spectronic® 20 Genesys™ spectrophotometer (Thermo-Fisher Scientific Corp, Waltham, MA, USA) was utilized to measure the absorbance of the reacted sample at a wavelength of 410 nm for NO_3 and 655 nm for NH_3. NO_3 and NH_3 concentrations were determined utilizing a standard curve (R^2>0.99).

TSS and VSS were measured in accordance with Standard Methods 2540 D and 2540 E [30], respectively. Measurement of pH was accomplished with a Thermo-Fisher Scientific Accumet AP85 Waterproof pH/Conductivity Meter. DO measurements were collected using a Hach HQ30d Meter with a LDO101 DO Probe.

Molecular investigations were completed utilizing the following analytical methods:

- Quantitative Polymerase Chain Reaction (qPCR)
- Targeted transcriptomics
- Targeted metabolomics

3.2.1 Quantitative Polymerase Chain Reaction (qPCR)

qPCR was applied to estimate the relative abundance of ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) in the Enrichment reactors. Bulk genomic DNA from each reactor was extracted using a PowerSoil® DNA Extraction Kit (MO BIO Laboratories

Inc., Carlsbad, CA USA). Genomic DNA yield and purity was quantified using a Synergy H1 Multi-Mode Reader (BioTek, Winooski, VT). qPCR was conducted on a StepOne Plus™ Real-Time PCR system (Applied Biosystems, Foster City, CA) using iTaq™ SYBR® Green Supermix w/ROX (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and a total reaction volume of 25 µl. Eubacteria were amplified using primer sets developed by Muyzer et al. [31]. Amplification of AOBs was based on a primer set for the gene ammonia monooxygenase (*amoA*) [32]. For NOBs, *Nitrobacter* spp. and *Nitrospira* were amplified using 16S rDNA sequences. qPCR settings were in accordance with Winkler et al. [33]. AOB, *Nitrobacter*, and *Nitrospira* abundance relative to eubacteria was estimated using the mean efficiencies for each primer set and the Cq values for the individual samples, assuming average 16S rDNA gene copy numbers of 4.1 for eubacteria, 2.5 for AOB [34], and 1.0 for both *Nitrobacter* and *Nitrospira* [35]. All samples were assessed in triplicate with 5 ng of total genomic DNA per reaction. qPCR melting curves were evaluated to confirm a single melting peak, and agarose gel analysis confirmed a single band for each primer set. Amplification efficiencies were calculated for each primer set using baseline-corrected fluorescence data (StepOne software v2.0), and the LinRegPCR program [36]. The cycle threshold was set at a constant value across all samples based on location within the log-linear region for determination of Cq values (cycle number at which the measured fluorescence exceeds the cycle threshold).

3.2.2 Targeted Transcriptomics

Biomass was centrifuged at 5,000 rcf to recover material for RT-qPCR analysis. Total RNA was extracted from 100 mg of biomass using the RNeasy PowerMicrobiome kit (Qiagen) according to the manufacturer's protocol. The RNA concentration was quantified using a ThermoFisher Nanodrop (ThermoFisher Scientific, Waltham, MA). 5 µg total RNA from each reactor was carried out using ThermoFisher Scientific Verso 1-step RT-qPCR ROX cDNA synthesis kit according to the manufacturer's protocol. Primers targeting nxrB and nxrA genes were used for RT-qPCR cDNA synthesis. Amplification reaction mixtures contained a final primer concentration of 70 µM. Primer sequences were: i) for the nxrA gene of *Nitrobacter* and *Nitrospira* [37], nxrA1F CAG ACC GAC GTG TGC GAA AG and nxrA2R TCY ACA AGG AAC GGA AGG TC; ii) for the nxrB gene for *Nitrobacter* [38], nxrB1F ACG TGG AGA CCA AGC CGG G and nxrB1R CCG TGC TGT TGA YCT CGT

TGA; and iii) for the nxrB gene for *Nitrospira* [39], nxrBqF TGT GGT GGA ACA ACG TGG AA and nxrBqR CCC GGC ATC GAA AAT GGT CA. Reactions were prepared in a total volume of 20 µL. Thermal cycling conditions were as follows: one cycle of cDNA synthesis at 50C for 15 mins followed by one cycle of thermŏ-start activation at 95℃ for 15 mins followed by 40 cycles of initial denaturation at 95 ℃ for 15 s, annealing at 60℃ for 30 s, and extension at 72℃ for 30 s. Fold change in RT-qPCR amplicons was calculated as $2-\Delta\Delta Ct$.

3.2.3 Targeted Metabolomics

3.2.3.1 Metabolic Sample Preparation

Metabolomic methods were applied to target and relatively quantify metabolic intermediates between the bioreactors. Biomass was extracted from each reactor and immediately subjected to "Fast Filtration" methods developed by Link et al. [40, 41]. To avoid degradation of high energy metabolites, glass microfibre filters (VWR, 1.2 µm) were added to petri dishes with 4 mL of an acidic acetonitrile extraction solution [40-42] immediately following filtration. A 40:40:20 solution of acetonitrile:methanol:DDIwater (v/v) with 0.1M formic acid was prepared using high pressure liquid chromatography (HPLC) grade chemicals, and cooled to 20°C. Petri dishes were incubated for 15 minutes at -20°C. Following incubation, filters and 40:40:20 quenching solution were added to 50 mL falcon tubes containing 400µL of 15% ammonium hydroxide (NH_4OH) solution to neutralize the acidic extract. An additional 4 mL of 40:40:20 quenching solution was added to rinse the petri dishes and also added to the designated falcon tube. Falcon tubes were then centrifuged at 12,000xg (Sorvall Super-Lite 600TC 10,000 rpm) for 10 minutes at 4°C (Sorvall RC 6). Duplicate 1 mL supernatant samples were added to 2mL microcentrifuge tubes (Fisher) and completely evaporated in a Savant SpeedVac Vacuum Concentrator. Following complete evaporation, dried metabolome samples were resuspended in 1mL of 5:1 methanol:DDIwater (v/v) solution, prepared using HPLC grade chemicals. Resuspended samples were filtered through a 13 mm syringe filter with 0.2 µm PTFE and stored at -20°C until LC-MS/MS analysis.

3.2.3.2 Hydrophilic Interaction Liquid Chromatography (HILIC)

Metabolomics aqueous normal phase analysis utilized an Atlantis T3 HILIC 150 mm x 2.1 mm column (MicroSolv, Eatontown, NJ) for LC separation with a flow rate of 600 pL min^{-1}. Solvent A consisted of 0.1% formic acid in water, while solvent B consisted of 0.1% formic

acid in acetonitrile. The elution gradient consisted of 95% solvent B for 2 minutes (with the first minute going to waste to avoid contaminating the source with excess salt), to 50% solvent B over 24 minutes, held at 50% for 2 minutes, and then returned to 95% for 2 minutes, with a total run time of 30 minutes using an Agilent 1290 UPLC (Agilent, Santa Clara, CA) system connected to an Agilent 6538 Q-TOF Mass Spectrometer (Agilent, Santa Clara, CA).

Mass spectrometry analysis was conducted in positive and negative ion mode, with a cone voltage of 3500V and a fragmentor voltage of 120V. Drying gas temperature was 350 °C with a flow of 12 L min^{-1} and the nebulizer was set to 60 psig. Spectra were collected at a rate of 2.52 per second with a mass range of 50 to 1000 m/z. The mass analyzer resolution was 18,000 and post calibration tests had a mass accuracy of approximately one ppm.

3.2.3.3 Metabolic Data Analysis

Data files from the LC-MS were converted to *.MZxml format using the Masshunter Qualitative software provided with Agilent instruments (Agilent, Santa Clara, CA). Analysis of LC-MS data was done using Agilent MassHunter Qualitative Analysis Navigator B.08.00. Quantification was done on a per file (sample) basis by extracting chromatograms of specific mass to charge (m/z) ratios. Fragmentation patterns of each targeted metabolite was studied to inform anticipated product ions and subsequent m/z values. Targeted standards were ran with each sample batch to ensure consistency of m/z ratios and retention times (RT). Targeted metabolites, precursor ions, product ions, and approximate retention times are shown in Table 3.3.

Table 3.3:Targeted Metabolites and Corresponding Precursor/Product Ions & Retention Times

Metabolite	**Precursor ion**	**Product ion**	**Approximate RT (min)**
3-phospho-Dglycerate	185.991	167	2.2 – 2.4
Succinate	118.09	73	2.0 – 2.1
2-oxoglutarate	146.11	145	2.2 – 2.4
Citrate	192.124	111	2.4 – 2.8

Metabolic expressions were quantified with integrated areas of extracted chromatograms associated with each product ion.

Chapter 4: Results

4.1 Nitrification Aimed Reactor; NF

Performance of the MMC enriched in reactor NF is represented by a total of five sampling events conducted during the assessment period. Mixed liquor was sampled from the reactor at designated time points over an operational cycle to obtain the following data sets.

- Bulk solution nitrogen speciation
- Biomass microbial characterization
- Targeted NxR expression
- Targeted metabolite expression

4.1.1 Bulk Solution Nitrogen Speciation

Nitrogen speciation within the NF reactor was assessed via sample collection and subsequent nutrient testing. Speciation included quantification of ammonia, nitrate, and nitrite concentrations. Resultant concentrations have been compiled to illustrate time series nitrogen fluxes for the chosen sampling events. Additionally, time series NAR percentages were computed from these concentrations using Equation I. Figures 4.1 – 4.5 illustrate observed bulk solution nitrogen fluxes and subsequent NAR percentages.

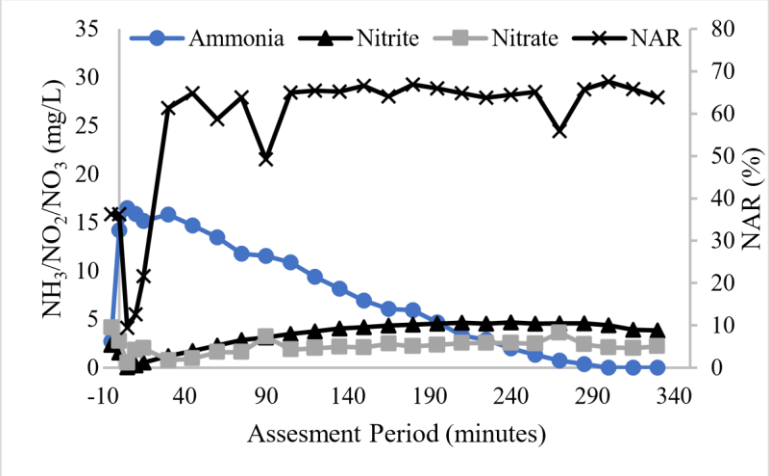

Figure 4.1: May 21st Nitrogen Speciation; NF

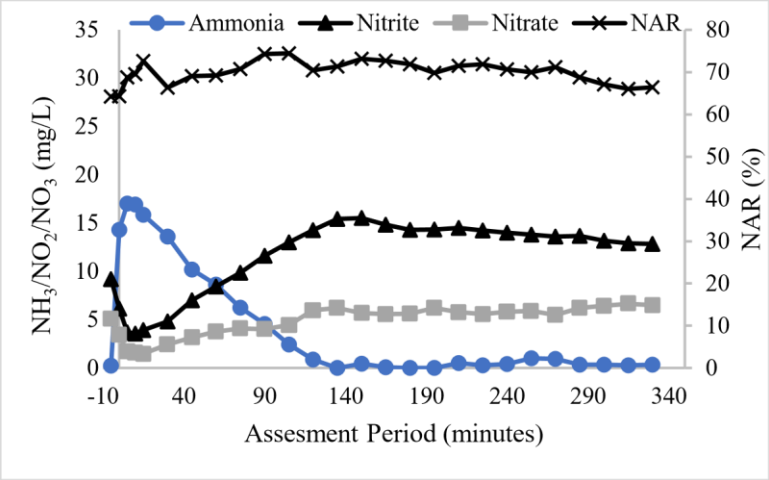

Figure 4.2: May 27th Nitrogen Speciation; NF

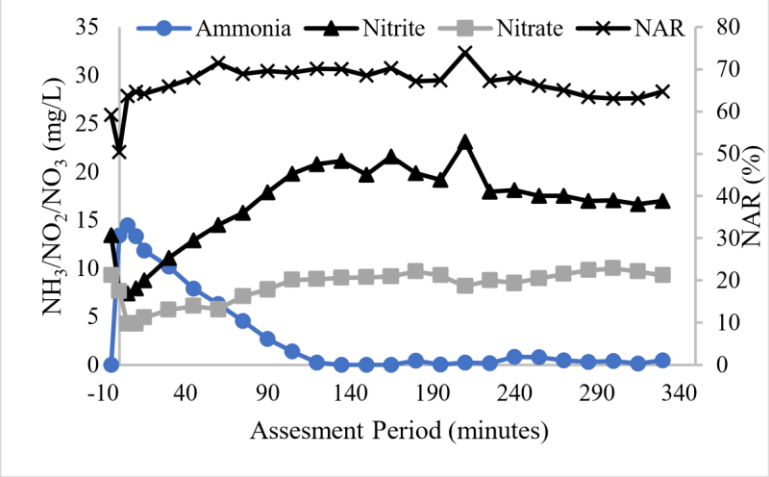

Figure 4.3: June 15th Nitrogen Speciation; NF

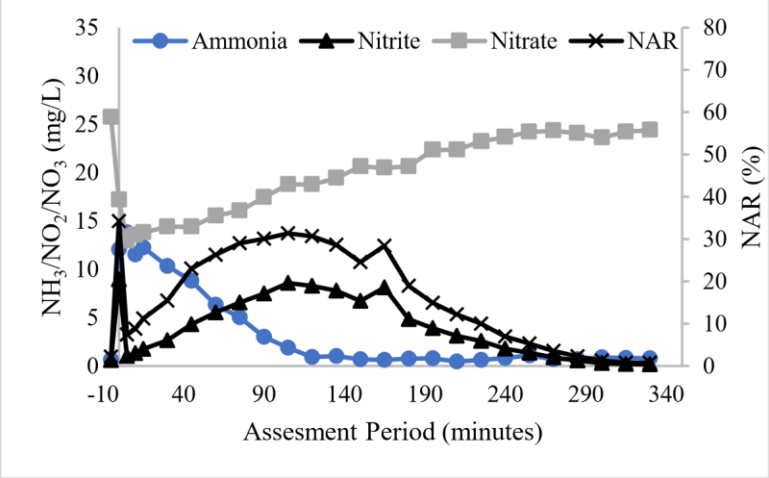

Figure 4.4: June 22nd Nitrogen Speciation; NF

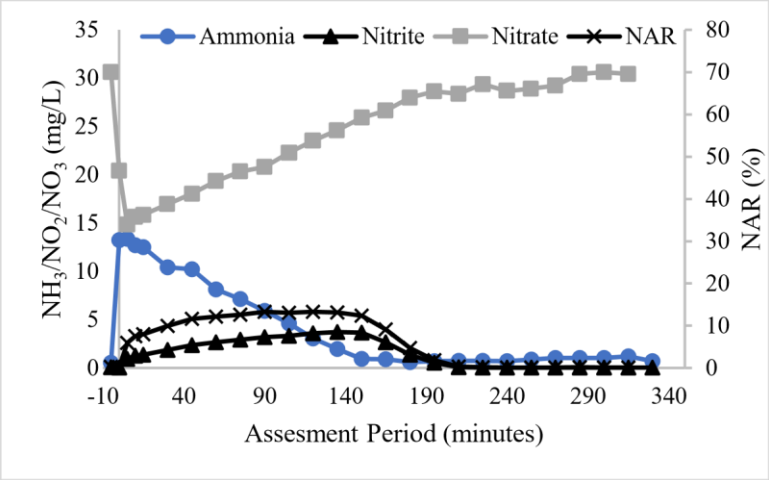

Figure 4.5: July 16th Nitrogen Speciation; NF

SBR time zero nitrogen concentrations were directly affected by previous cycle performance and influent ammonia concentrations: for example, as shown in Figures 4.2, 4.3,4.4, and 4.5. In Figures 4.2 and 4.3, nitrite is dominant (relative to nitrate) at time zero and remains that way throughout the cycle. Figures 4.4 and 4.5 demonstrate this same trend for nitrate concentrations, such dominance is evidenced by resultant NAR percentages for all Figures. Time zero and effluent nitrogen concentrations are presented alongside average NAR percentages in Table 4.1.

Nitritation was observed relatively significantly on May 21, May 27, and June 15 (Figures 4.1, 4.2, & 4.3). Note that on May 27 and June 15 once ammonia was fully oxidized the relative NO_x concentrations remained stable and constant. Thereafter nitritation decreased substantially (Figures 4.4 & 4.5). Additionally, under deteriorated nitritation conditions, significantly greater quantities of nitrate were carried over the previous SBR cycle, leading to incomplete denitrification early in the SBR cycle evaluated.

Table 4.1 summarizes nitrogen concentrations for the cycle assessed and the previous cycle effluent (time zero in Table 4.1) as these concentrations remain in the SBR following the decant period. Nitrogen balances were conducted beginning at the assessed cycle time zero. All nitrogen balance values presented in Chapter 3 are computed utilizing *Equation II*.

Table 4.1: Reactor NF Nitrogen Concentration Summary

Date	Time Zero N Concentrations, mg/L			Effluent N Concentrations, mg/L			Nitrogen Balance (mg/L)	Average NAR
	NH_3	NO_3	NO_2	NH_3	NO_3	NO_2		
May 21st	14.21	2.78	1.58	0.00	2.19	3.86	12.52	55.7 %
May 27th	14.30	3.41	6.13	0.33	6.5	12.83	4.18	69.8 %
June 15th	13.42	6.17	8.95	0.46	9.25	16.98	1.85	66.3 %
June 22nd	12.07	17.18	8.95	0.80	24.41	0.18	12.81	33.5 %
July 16th	13.25	20.41	0.05	0.70	30.63	0.04	2.34	5.9 %

Positive nitrogen balance values indicate higher time zero nitrogen concentrations than nitrogen concentrations in the SBR effluent.

4.1.2 Biomass Microbial Characterization

qPCR techniques were performed on biomass recovered for each operational date presented in Section 4.1.1 to accompany bulk solution data. Targeted populations included AOB using the ammonia monooxygenase gene as a surrogate (amoA), *Nitrobacter* (NITRO), and *Nitrospira* (NSR). Microbial characterization data shown in Table 4.2 are presented as percentages of total Eubacteria quantified from the biomass sample. VSS values are also listed. VSS values in conjunction with qPCR results were utilized to quantify reactor biomass.

Table 4.2: Nitrifying Microbial Populations in Reactor NF

Date	% amoA	% NITRO	% NSR	% Total NOB	VSS (mg/L)	*Nitrobacter* Biomass (mg)	*Nitrospira* Biomass (mg)
May 21st	0.00 (BDL)	0.19	0.27	0.46	1,100	4.18	5.94
May 27th	0.00 (BDL)	0.37	0.34	0.71	1,712	12.69	11.64
June 15th	0.01	0.64	1.60	2.24	1,337	17.11	42.78
June 22nd	0.00 (BDL)	0.62	1.76	2.38	1,200	14.88	42.24
July 16th	0.00 (BDL)	0.32	2.97	3.28	1,363	8.72	80.96

While the AOB population is not represented in Table 4.2 (below detection limit), it is clear AOB were in fact present, evidenced by ammonia oxidation observed in Figures 4.1 – 4.5.

4.1.3 Targeted NxR Expressions

Transcriptomic techniques were utilized to determine NxR expressions associated with *Nitrobacter* and *Nitrospira* nitrite oxidation. Specific subunits of the NxR protein complex were targeted for each NOB species: nxrA and nxrB for *Nitrobacter* (nxrA and nxr B Nitro), and nxrBq (nxrBq NSR) for *Nitrospira*. Individual mRNA subunit expressions were quantified on a relative basis (relative to time zero sample). Samples were taken in five minute intervals for the first 15 minutes of the aerobic period for dates June 15th and June 22nd. Log 2 transform relative expressions of *Nitrobacter* and *Nitrospira* NxR subunits and corresponding bulk solution concentrations of nitrate and nitrite are illustrated in Figures 4.6 and 4.7.

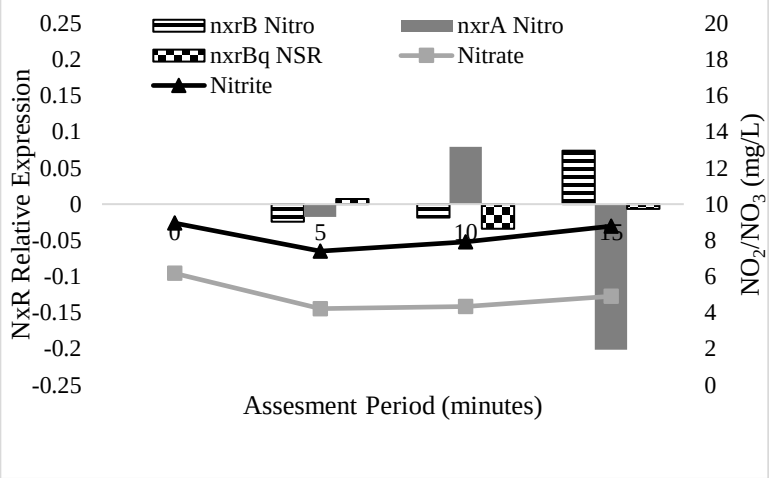

Figure 4.6: June 15th Targeted NxR Expressions

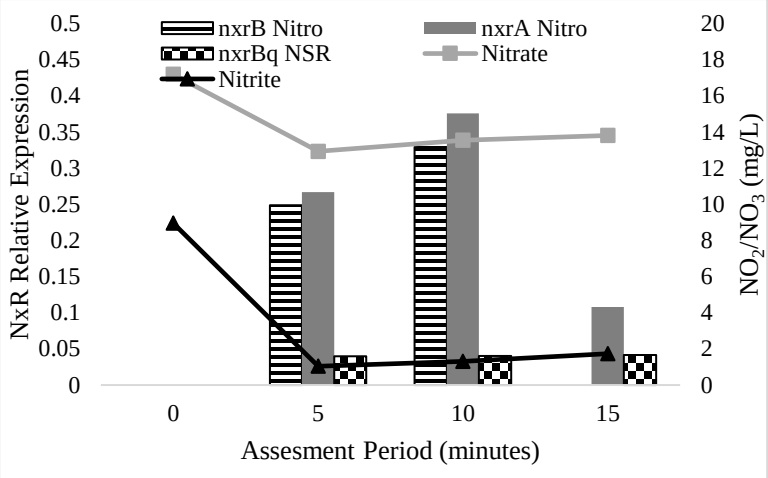

Figure 4.7: June 22nd Targeted NxR Expressions

4.1.4 Targeted Metabolomic Profiling

Metabolite concentrations associated with carbon sequestration mechanisms of the Calvin Benson cycle (CBC) and reductive tricarboxylic acid cycle (rTCA) were identified and quantified from filtered biomass samples obtained from the NF reactor. To interpret the data, metabolite concentrations at each timepoint were first normalized to the concentration in the biomass immediately before the operational cycle began, thus indicating up- or downregulation of a pathway. Normalized relative expressions of 3-phospho-D-glycerate (3PG; CBC metabolite) were then multiplied by observed biomass *Nitrobacter* populations (Table

4.2). rTCA metabolites (succinate, α-ketoglutarate (AKG), and citrate) were multiplied by biomass *Nitrospira* populations (Table 4.2). Finally, all relative expression data at each timepoint summed and presented as a percent of the total mass of the four metabolites quantified (Figures 4.8 – 4.12). With this approach, the relative fraction of the four targeted metabolites across timepoints illustrates the relative flux of carbon within the MMC.

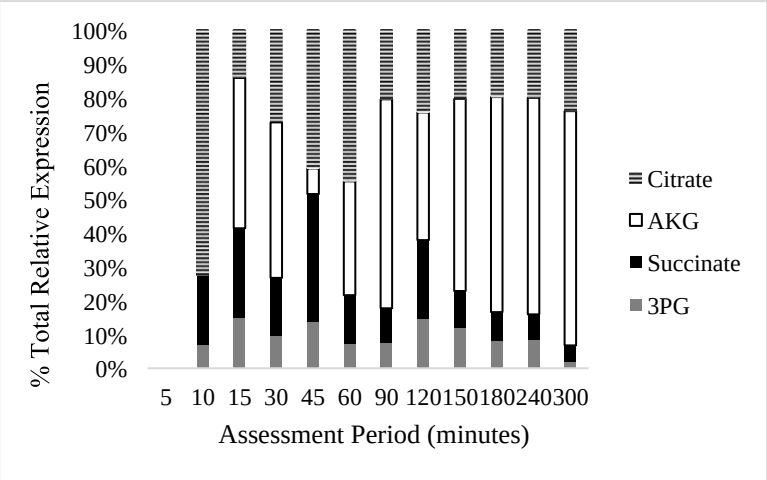

Figure 4.8: May 21st Targeted Metabolite Expressions

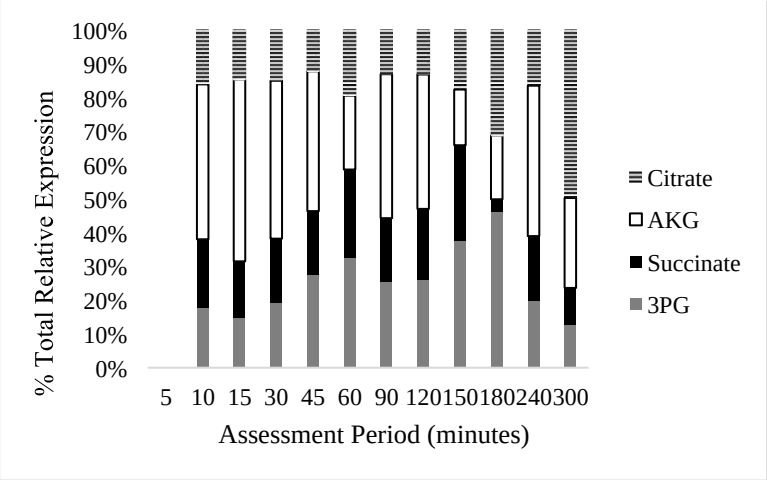

Figure 4.9: May 27th Targeted Metabolite Expressions

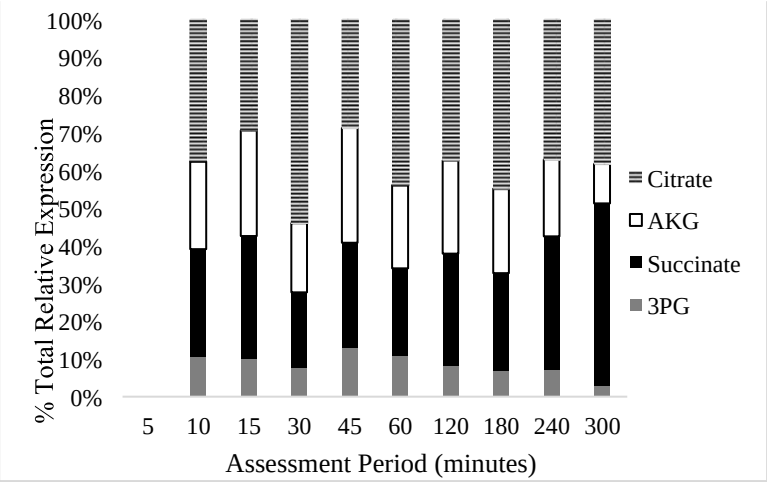

Figure 4.10: June 15th Targeted Metabolite Expressions

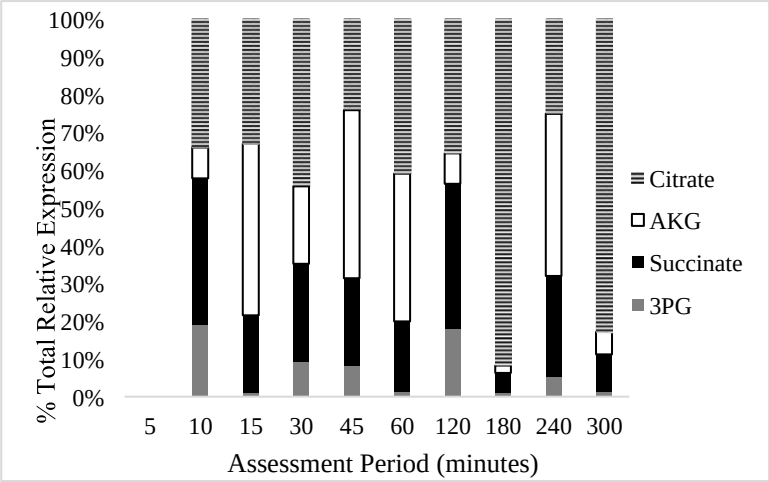

Figure 4.11: June 22nd Targeted Metabolite Expressions

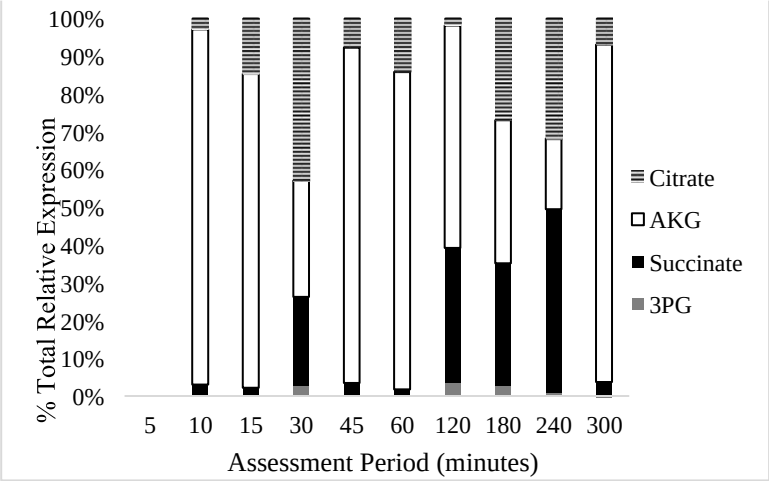

Figure 4.12: July 16th Targeted Metabolite Expressions

4.2 Nitritation Aimed Reactor; NT

Performance of the reactor NT MMC is represented by a total of four sampling events conducted during the assessment period. Mixed liquor was sampled from the reactor at designated time points over an operational cycle to obtain the following data sets.

- Bulk Solution Nitrogen Speciation
- Biomass Microbial Characterization
- Targeted Metabolite Expression

4.2.1 Bulk Solution Nitrogen Speciation

Nitrogen speciation within the NT reactor was assessed via sample collection and subsequent nutrient testing. Speciation included quantification of ammonia, nitrate, and nitrite

concentrations. Resultant concentrations have been compiled to illustrate time series nitrogen fluxes for the chosen sampling events. Additionally, time series NAR percentages were computed from these concentrations using Equation I. Figures 4.13 – 4.16 illustrate observed bulk solution nitrogen fluxes and subsequent NAR percentages.

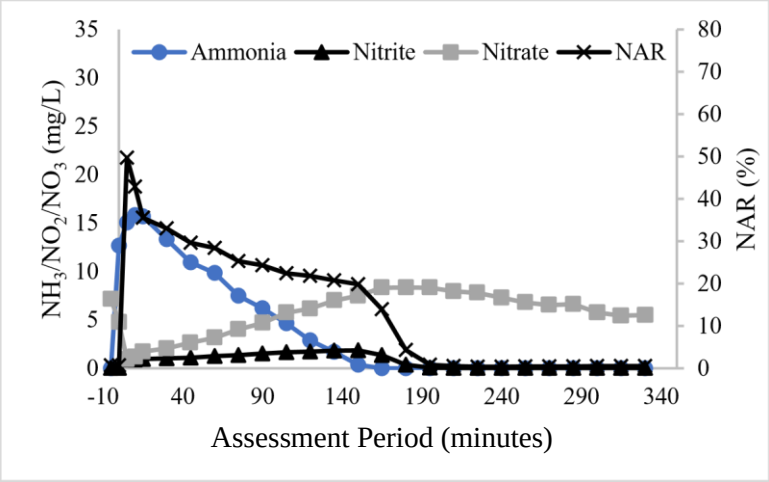

Figure 4.13: May 25th Nitrogen Speciation; NT

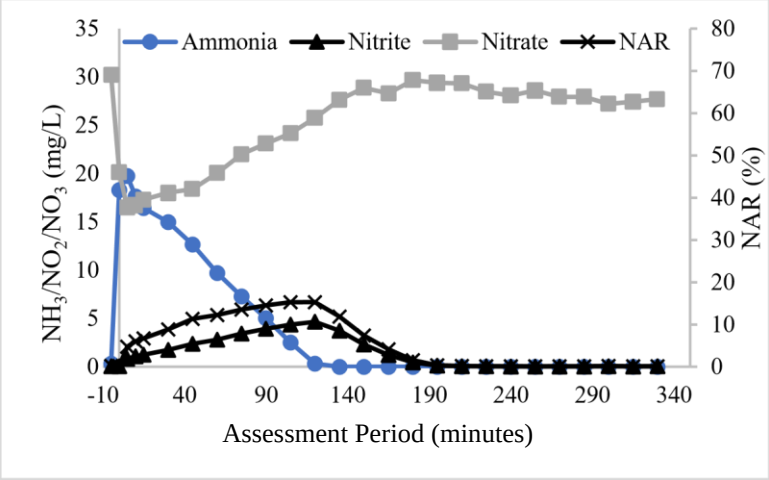

Figure 4.14: June 2nd Nitrogen Speciation; NT

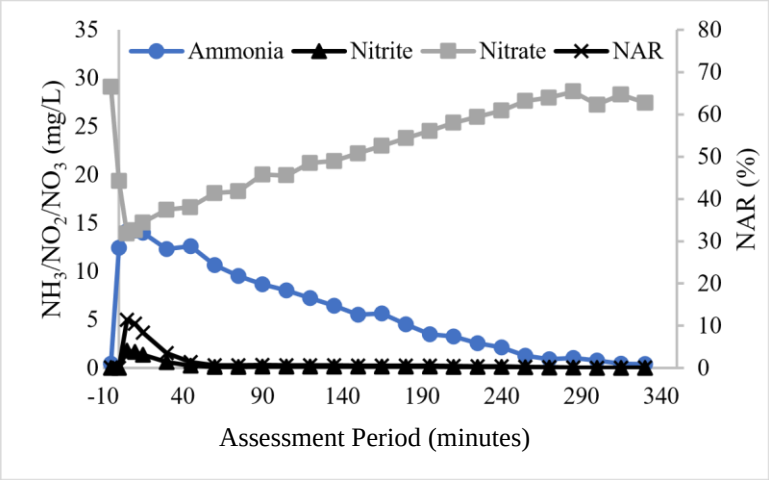

Figure 4.15: July 1st Nitrogen Speciation; NT

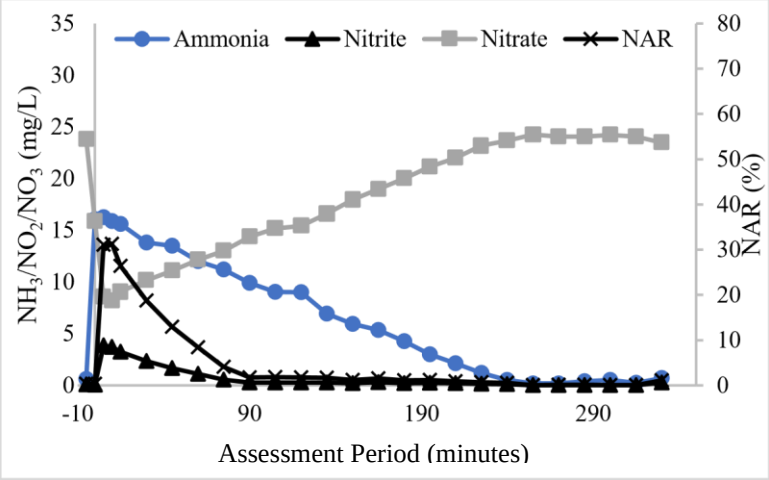

Figure 4.16: July 8th Nitrogen Speciation; NT

As demonstrated by reactor NF, time zero concentrations in NT are directly affected by previous cycle performance and influent ammonia concentrations: for example, see results illustrated in Figures 4.14, 4.15, and 4.16. These three figures demonstrate elevated time zero nitrate concentrations, with increasing concentrations as the assessment period progresses. Nitrite concentrations remain low (below 5 mg/L) in all nitrogen speciation figures for NT (Figures 4.13 – 4.16). Such nitrite and nitrate speciation is reflected in the much lower NAR percentages (as opposed to Figures 4.1, 4.2, and 4.3 for NF) observed in all NT sampling events (Figures 4.13 – 4.16).

Table 4.3: Reactor NT Nitrogen Concentration Summary

Date	Time Zero N Concentrations, mg/L			Effluent N Concentrations, mg/L			Nitrogen Balance (mg/L)	Average NAR
	NH_3	NO_3	NO_2	NH_3	NO_3	NO_2		
May 25th	12.67	4.77	0.03	0.00	5.50	0.03	11.94	15.71 %
June 2nd	18.28	20.13	0.04	0.00	27.69	0.04	10.72	5.63 %
July 1st	12.46	19.38	0.01	0.40	27.46	0.02	3.97	1.80 %
July 8th	16.07	15.87	0.05	0.70	23.48	0.27	7.54	6.19 %

Positive nitrogen balance values indicate higher time zero nitrogen concentrations than nitrogen concentrations in the SBR effluent.

4.2.2 Biomass Microbial Characterization

qPCR techniques were performed on biomass recovered for each operational date presented in Section 4.2.1 to accompany bulk solution data. Targeted populations included AOB (amoA), *Nitrobacter* (NITRO), and *Nitrospira* (NSR). Microbial characterization data shown in Table 4.4 are presented as percentages of total Eubacteria quantified from the biomass sample. VSS values are also listed to quantify reactor biomass.

Table 4.4: Nitrifying Microbial Populations in Reactor NT

Date	% amoA	% NITRO	% NSR	% Total NOB	VSS (mg/L)	*Nitrobacter* Biomass (mg)	*Nitrospira* Biomass (mg)
May 25th	0.00 (BDL)	0.33	0.51	0.84	1,3.50	8.91	13.77
June 2nd	0.00 (BDL)	1.54	1.55	3.09	1,550	47.74	95.79
July 1st	0.00 (BDL)	1.33	7.80	9.14	1,613	42.91	294.63
July 8th	0.00 (BDL)	0.38	2.57	2.95	1,887	14.34	111.33

While the AOB population is not represented in Table 4.4 (below detection limit), it is clear

AOB were in fact present, evidenced by ammonia oxidation observed in Figures 4.13 – 4.16.

4.2.3 Targeted Metabolomic Expressions

Metabolite concentrations associated with carbon sequestration mechanisms of the Calvin Benson cycle (CBC) and reductive tricarboxylic acid cycle (rTCA) were identified and quantified from filtered biomass samples obtained from the NT reactor. To interpret the data, metabolite concentrations at each timepoint were first normalized to the concentration in the biomass immediately before the operational cycle began, thus indicating up- or downregulation of a pathway. Normalized relative expressions of 3-phospho-D-glycerate (3PG; CBC metabolite) were then multiplied by observed biomass *Nitrobacter* populations (Table

4.4). rTCA metabolites (succinate, α-ketoglutarate (AKG), and citrate) were multiplied by biomass *Nitrospira* populations (Table 4.4). Finally, all relative expression data at each timepoint was summed and is presented as a percent of the total mass of the four metabolites quantified (Figures 4.17 – 4.20). With this approach, the relative fraction of the four targeted metabolites across timepoints illustrates the relative flux of carbon within the MMC.

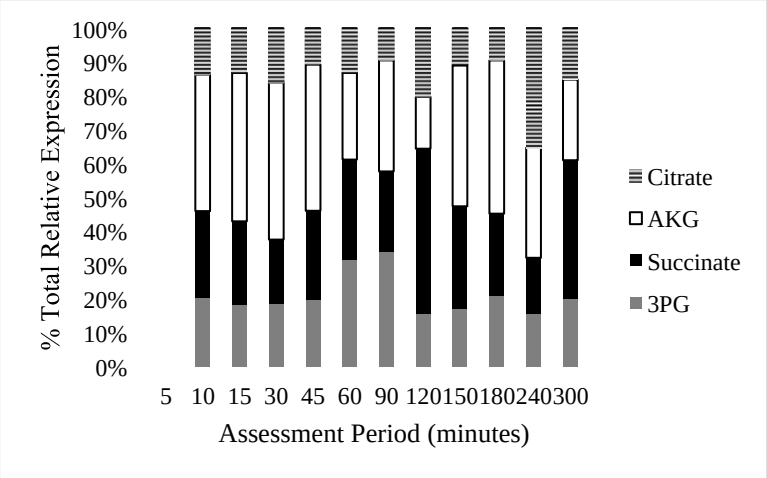

Figure 4.17: May 25th Targeted Metabolite Expressions

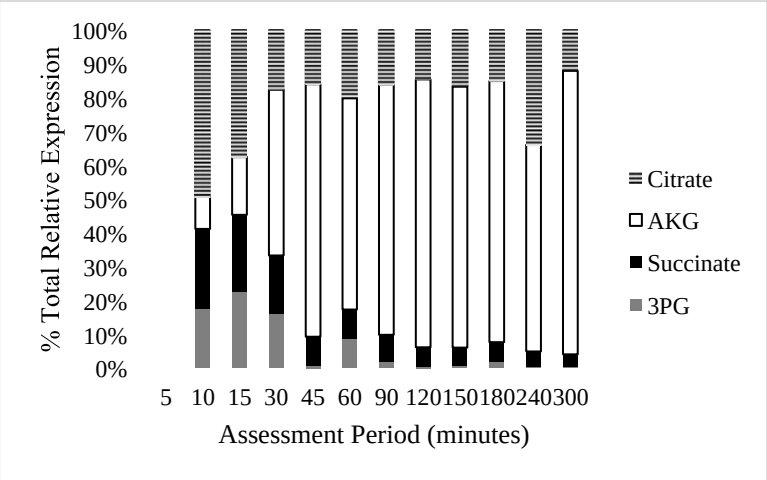

Figure 4.18: June 2nd Targeted Metabolite Expressions

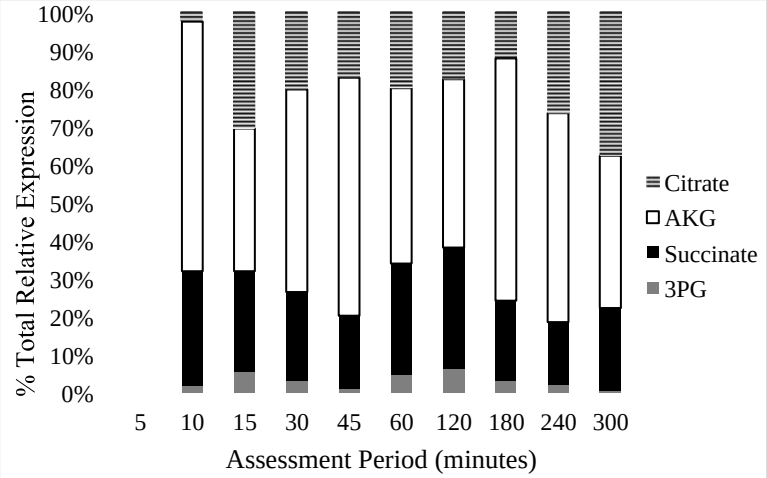

Figure 4.19: July 1st Targeted Metabolite Expressions

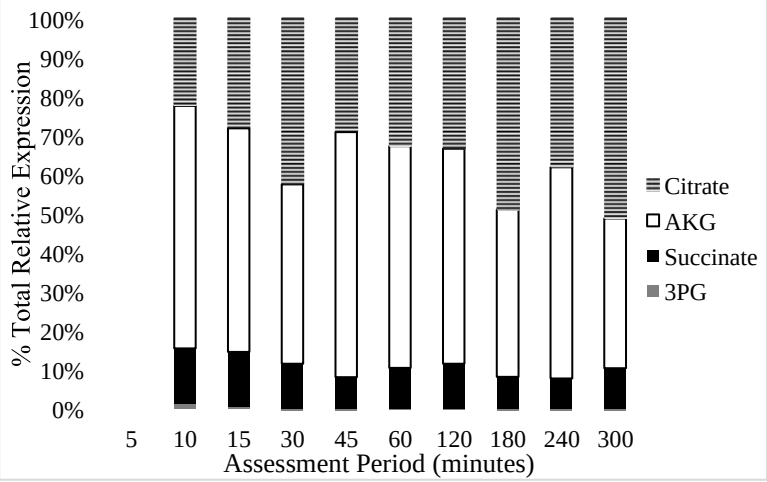

Figure 4.20: July 8th Targeted Metabolite Expressions

4.3 BIOPHO–PX Reactors

To complement data collected on the performance of reactors NF and NT in order to gain further molecular insight into nitritation, BIOPHO–PX Reactors N1 and N2, which more consistently achieve nitritation, are profiled.

4.3.1 BIOPHO-PX N1

Performance of the reactor N1 MMC is represented by a single sampling event conducted during the assessment period. Hach nutrient probes (specified in Chapter 3) were utilized to obtain bulk solution nitrogen speciation. Mixed liquor was sampled from the reactor at designated time points over an operational cycle to obtain the following data sets.

- Biomass microbial characterization
- Targeted NxR expression
- Targeted metabolite expression

4.3.1.1 Bulk Solution Nitrogen Speciation

Nitrogen speciation within the N1 reactor was observed utilizing recorded data files from calibrated Hach probes. Speciation included quantification of ammonia, nitrate, and nitrite concentrations. Resultant concentrations have been compiled to illustrate time series nitrogen fluxes for the chosen sampling events. Additionally, time series NAR percentages were computed from these recorded concentrations using Equation I. Figure 4.21 illustrates observed bulk solution nitrogen fluxes and subsequent NAR percentages.

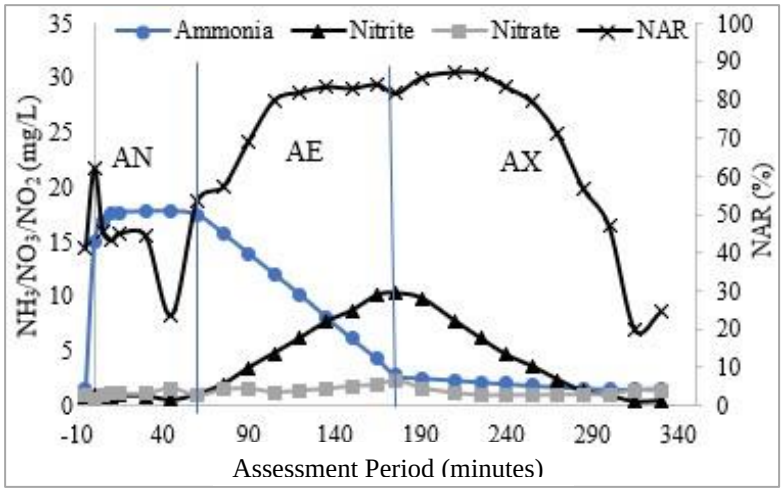

Figure 4.21: N1 Nitrogen Speciation

Nitrogen concentrations remain constant for the anaerobic period (AN), as no electron donor is present to drive nitrification redox reactions (Figure 4.21). Significant nitritation is observed during the N1 aerobic (AE) period. A peak in nitrite and nitrate concentrations is observed 135 minutes into the assessment period. Air is shut off at 176 minutes, as ammonia concentrations reach 3.0 mg/L. Performance during the post-anoxic period (AX) suggests denitritation as a general downtrend of nitrite and subsequent NAR is experienced.

N1 also demonstrates typical SBR trends, as time zero concentrations reflect previous cycle performance. Much smaller concentrations of nitrite and nitrate are observed (as opposed to NF and NT) due to implementation of the post anoxic period. Such conditions create opportunity for total nitrogen removal and allow ammonia to be the dominant initial nitrogen species for the following cycle.

Table 4.5: Reactor N1 Nitrogen Concentration Summary

N1 September 24th	Time Zero N Concentrations, mg/L			Effluent N Concentrations, mg/L			Nitrogen Balance (mg/L)	End Aerobic NAR
	NH_3	NO_3	NO_2	NH_3	NO_3	NO_2		
	15.02	0.63	1.02	1.57	1.03	0.42	13.4	81.68 %

A larger value for nitrogen balance is listed Table 4.5, this value refers to more nitrogen present at time zero than in the SBR effluent. Such an imbalance is due to the post – anoxic configuration of the BIOPHO – PX rector providing a means for SBR denitritation. Denitritation is observed in Figure 4.21 and reflected by the nitrogen balance value in Table 4.5.

4.3.1.2 Biomass Microbial Characterization

qPCR techniques were performed on biomass recovered for each operational date presented in Section 4.3.1 to accompany bulk solution data. Targeted populations included AOB (AmoA), *Nitrobacter* (NITRO), and *Nitrospira* (NSR). Microbial characterization data shown in Table 4.6 are presented as percentages of total Eubacteria quantified from the biomass sample. VSS values are also listed to quantify reactor biomass.

Table 4.6: Nitrifying Microbial Populations in Reactor N1

N1 September 24th	%amoA	%NITRO	%NSR	% Total NOB	VSS (mg/L)	*Nitrobacter* Biomass (mg)	*Nitrospira* Biomass (mg)
	0.00 (BDL)	0.44	0.0019	0.4419	1,640	14.43	0.06

While the AOB population is not represented in Table 4.6 (below detection limit), it is clear AOB were in fact present, evidenced by ammonia oxidation observed in Figure 4.21.

4.3.1.3 Targeted NxR Expressions

Transcriptomic techniques were utilized to determine NxR expressions associated with *Nitrobacter* and *Nitrospira* nitrite oxidation. Specific subunits of the NxR protein complex were targeted for each NOB species: nxrA and nxrB for *Nitrobacter* (nxrA and nxr B Nitro), and nxrBq (nxrBq NSR) for *Nitrospira*. Individual mRNA subunit expressions were quantified on a relative basis (relative to time zero sample). Samples were taken throughout the assessment period for September 24th. Relative expressions of *Nitrobacter* and *Nitrospira* NxR subunits and corresponding bulk solution concentrations of nitrate and nitrite are illustrated in Figures 4.22 and 4.23.

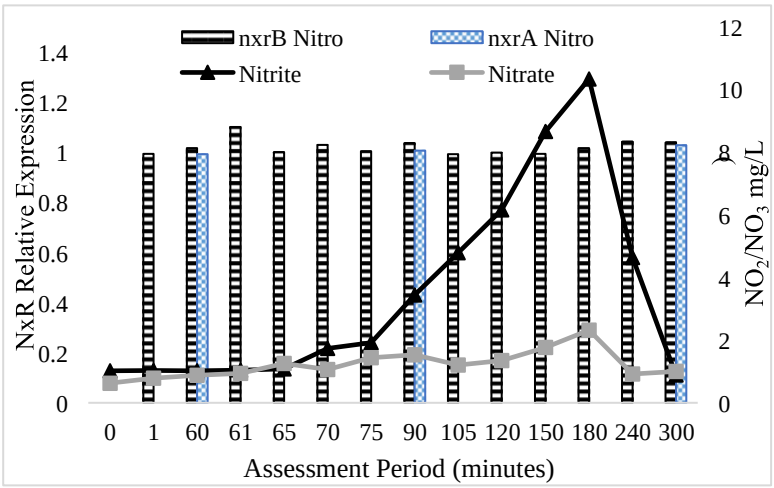

Figure 4.22: Reactor N1 Nitrobacter Targeted NxR Expressions

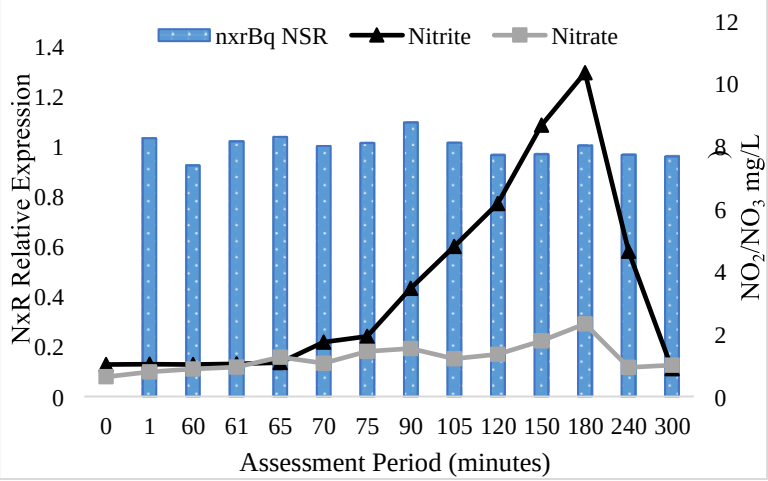

Figure 4.23 N1 Nitrospira Targeted NxR Expressions

Nitrobacter NxR subunit B and *Nitrospira* subunit Bq genes were expressed throughout the cycle, resulting in observed concentrations of nitrate and nitrite. *Nitrobacter* NxR subunit A was not expressed for all timepoints. A notable increase is seen for minute 61 in both *Nitrobacter* and *Nitrospira* NxR expressions, corresponding to the beginning of the aerobic period. *Nitrospira* expression continues to increase at 65 minutes while *Nitrobacter* declines.

4.3.1.4 Targeted Metabolomic Expressions

Metabolite concentrations associated with carbon sequestration mechanisms of the Calvin Benson cycle (CBC) and reductive tricarboxylic acid cycle (rTCA) were identified and quantified from filtered biomass samples obtained from the N1 reactor. To interpret the data, metabolite expressions at each timepoint were normalized to the detected expression immediately before the operational cycle began, thus indicating up- or down-regulation of a pathway. Metabolites were not further normalized to *Nitrobacter* or *Nitrospira* populations (like NF and NT), due to the marginal *Nitrospira* populations detected. Relative expressions of 3-phospho-D-glycerate (3PG; CBC metabolite) and rTCA metabolites (succinate, αketoglutarate (AKG), and citrate) are presented as a percent of the total relative expressions of the four metabolites quantified (Figure 4.24). With this approach, the relative fraction of the four targeted metabolites across timepoints illustrates the relative flux of carbon within the MMC.

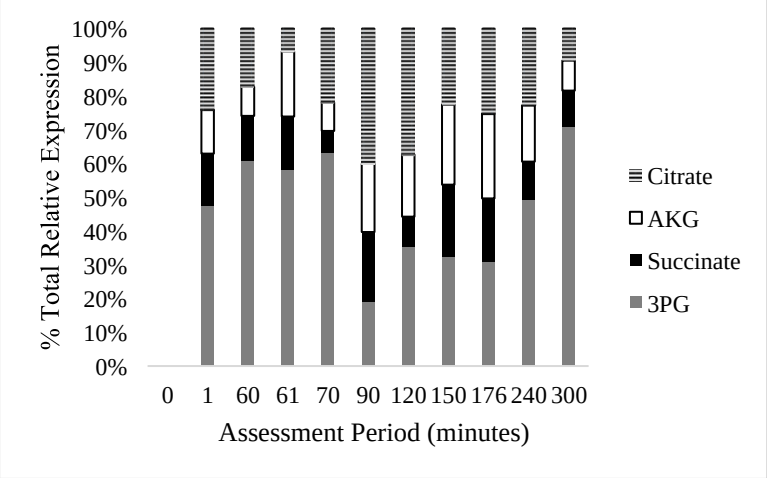

Figure 4.24: N1 Targeted Metabolite Expressions

4.3.2 BIOPHO–PX N2

Performance of the reactor N2 MMC is represented by a single sampling event conducted during the assessment period. Hach nutrient probes (specified in Chapter 3) were utilized to obtain bulk solution nitrogen speciation. Mixed liquor was sampled from the reactor at designated time points over an operational cycle to obtain the following data sets.

- Biomass microbial characterization
- Targeted NxR expression
- Targeted metabolite expression

4.3.2.1 Bulk Solution Nitrogen Speciation

Nitrogen speciation within the N2 reactor was observed utilizing recorded data files from calibrated Hach probes. Speciation included quantification of ammonia, nitrate, and nitrite concentrations. Resultant concentrations have been compiled to illustrate time series nitrogen fluxes for the chosen sampling events. Additionally, time series NAR percentages were computed from these recorded concentrations using Equation I. Figure 4.25 illustrates observed bulk solution nitrogen fluxes and subsequent NAR percentages.

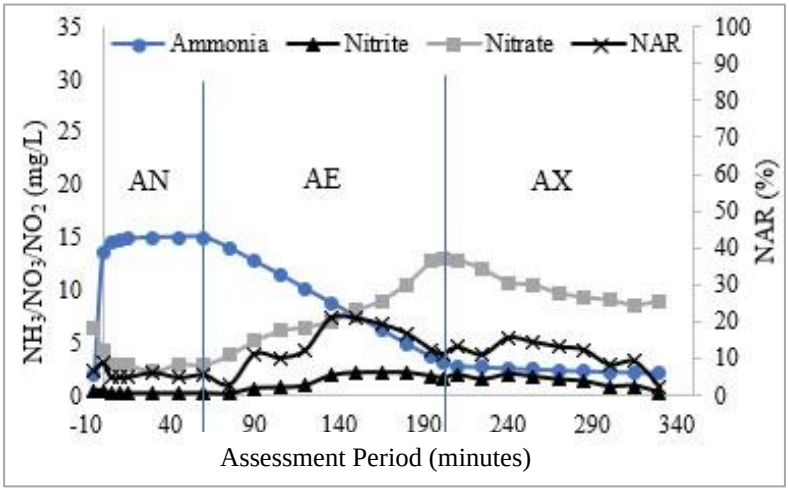

Figure 4.25: N2 Nitrogen Speciation

Consistent with N1, N2 nitrogen concentrations remain constant through the anaerobic period (Figure 4.25). Notable differences are observed particularly for the aerobic period between N1 and N2. N2 demonstrates much higher nitrate concentrations, reflected by the much lower NAR percentages. Nitrate concentration peaks 200 minutes into the aerobic period, and air is shut off at 202 minutes. A decreasing trend in nitrate is observed for the anoxic period, suggesting denitrification. Nitrite concentrations do not exceed 3.1 mg/L for the duration of the assessment period.

During the aerobic period, a lag in nitrite production is observed. Nitrate concentration increases immediately when the aerobic period begins, while nitrite concentrations lag. Nitrite concentrations do not begin to show significant increases until 135 minutes (75 minutes after air is introduced). Downtrends are observed for all nitrogen species during the anoxic period, although incomplete denitrification is experienced. This is also demonstrated by time zero concentrations (Table 4.7 and Figure 4.25) indicating larger nitrate concentrations (as opposed to N1). Elevated nitrate concentrations and incomplete denitrification also contribute to the listed nitrogen balance value for N2 (Table 4.7). This value indicates a larger time zero concentration of nitrogen than in the SBR effluent.

Table 4.7: Reactor N2 Nitrogen Concentration Summary

N2 September 24th	Time Zero N Concentrations, mg/L			Effluent N Concentrations, mg/L			Nitrogen Balance (mg/L)	End Aerobic NAR
	NH_3	NO_3	NO_2	NH_3	NO_3	NO_2		
	13.62	4.22	0.41	2.12	8.98	0.22	6.92	11.22 %

4.3.2.2 Biomass Microbial Characterization qPCR techniques were performed on biomass recovered for each operational date presented in Section 4.1.1 to accompany bulk solution data. Targeted populations included AOB (AmoA), *Nitrobacter* (NITRO), and *Nitrospira* (NSR). Microbial characterization data shown in Table 4.8 are presented as percentages of total Eubacteria quantified from the biomass sample. VSS values are also listed to quantify reactor biomass.

Table 4.8: Nitrifying Microbial Populations in Reactor N2

N2 September 24th	% amoA	% NITRO	% NSR	% Total NOB	VSS (mg/L)	*Nitrobacter* Biomass (mg)	*Nitrospira* Biomass (mg)
	0.00 (BDL)	0.55	0.0025	0.5525	1,180	12.98	0.059

While the AOB population is not represented in Table 4.8 (below detection limit), it is clear AOB were in fact present, evidenced by ammonia oxidation observed in Figure 4.25.

4.3.2.3 Targeted NxR Expressions

Transcriptomic techniques were utilized to determine NxR expressions associated with *Nitrobacter* and *Nitrospira* nitrite oxidation. Specific subunits of the NxR protein complex were targeted for each NOB species: nxrA and nxrB for *Nitrobacter* (nxrA and nxr B Nitro), and nxrBq (nxrBq NSR) for *Nitrospira*. Individual mRNA subunit expressions were quantified on a relative basis (relative to time zero sample). Samples were taken throughout the assessment period for September 24th. Relative expressions of *Nitrobacter* and *Nitrospira*

NxR subunits and corresponding bulk solution concentrations of nitrate and nitrite are illustrated in Figures 4.26 and 4.27.

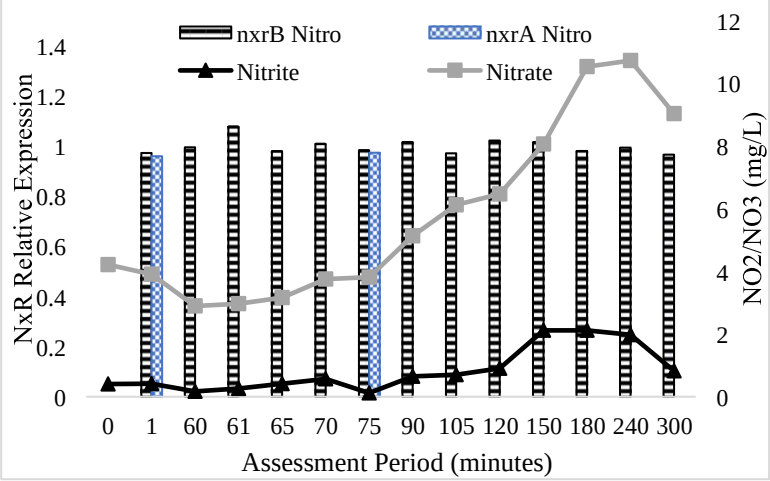

Figure 4.26: N2 Nitrobacter Targeted NxR Subunits

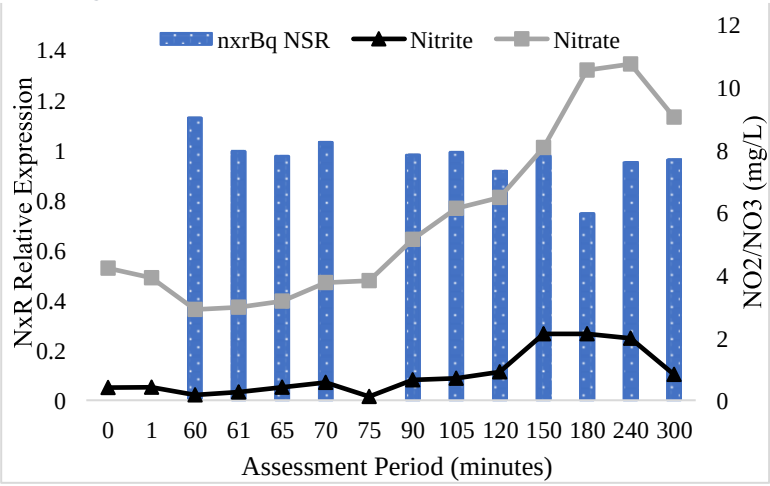

Figure 4.27: N2 Nitrospira Targeted NxR Subunit

Nitrobacter nxrB is the only subunit to be expressed for all timepoints, *Nitrospira* Bq subunit is expressed for all except minute 75. *Nitrobacter* subunit A is only expressed for two separate timepoints, minute 1 and 75. Increases in *Nitrobacter* subunit B and *Nitrospira* Bq are observed at 60 and 61 minutes respectively. Such increases are indicative of the beginning of the aerobic period (minute 60).

4.3.2.4 Targeted Metabolomic Expressions

Metabolite concentrations associated with carbon sequestration mechanisms of the Calvin Benson cycle (CBC) and reductive tricarboxylic acid cycle (rTCA) were identified and quantified from filtered biomass samples obtained from the N2 reactor. To interpret the data,

metabolite expressions at each timepoint were normalized to the detected expression immediately before the operational cycle began, thus indicating up- or down-regulation of a pathway. Metabolites were not further normalized to *Nitrobacter* or *Nitrospira* populations (like NF and NT), due to the marginal *Nitrospira* populations detected. Relative expressions of 3-phospho-D-glycerate (3PG; CBC metabolite) and rTCA metabolites (succinate, αketoglutarate (AKG), and citrate) are presented as a percent of the total relative expressions of the four metabolites quantified (Figure 4.28). With this approach, the relative fraction of the four targeted metabolites across timepoints illustrates the relative flux of carbon within the MMC.

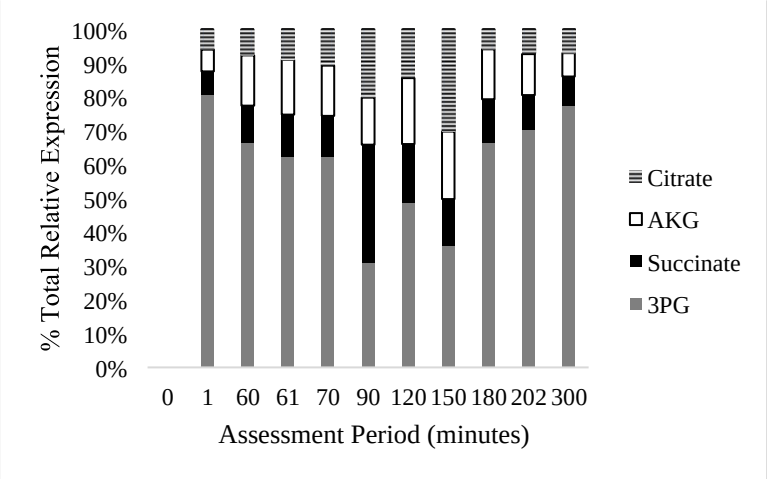

Figure 4.28: N2 Targeted Metabolite Expressions

Chapter 5: Discussion

Research was conducted to establish molecular-level contrasts between nitrifying and nitritating mixed microbial consortia in order to gain new insight into potentially critical aspects involving successful nitritation. Building from the array of molecular-level and bulk solution nitrogen data presented herein that included nitritating and nitrifying bioreactors, nitrogen cycling will first be assessed and classified based on bulk solution time series data. Characterization of event performance based on bulk solution nitrogen speciation can aid in interpreting further metabolic data. Subsequent interpretations intend to explain bioreactor performance utilizing observations of targeted microbial structures and functions, while also considering the aerobic conditions and MMC.

5.1 Bulk Solution Nitrogen Speciation

Interpretations and further performance classification drawn from bulk solution time series nitrogen data considered nitrogen balances for the assessed cycle (i.e., previous effluent not included in calculations) analyzed both graphically and numerically, time zero concentrations, and aerobic NO_x speciation. This section seeks to interpret microbial performance from observed nitrogen concentrations. Specific sampling events will be referred to using the scheme listed in Table 5.1 (Reactor – Event number).

Table 5.1: Reactor - Event Naming Scheme and Corresponding Sampling Run Dates

Reactor – Event	Date
NF – 1	May 21st
NF – 2	May 27th
NF – 3	June 15th
NF – 4	June 22nd
NF – 5	July 16th
NT – 1	May 25th
NT – 2	June 2nd
NT – 3	July 1st
NT – 4	July 8th
N1 – 1	September 24th
N2 – 1	September 24th

5.1.1 Full Nitrification Events

NF – 5 and NT – 3 suggest full nitrification accomplishment by demonstrating complete ammonia oxidation with dominant nitrate speciation of NO_x, reasonable nitrogen balances, and no notable observation of denitrification. Figures 5.1 and 5.2 illustrate ammonia oxidation corresponding to stable NO_x concentrations for the cycles. Additionally, nitrate accounts for 94% and 98% of total NO_x produced for NF – 5 and NT -3 respectively.

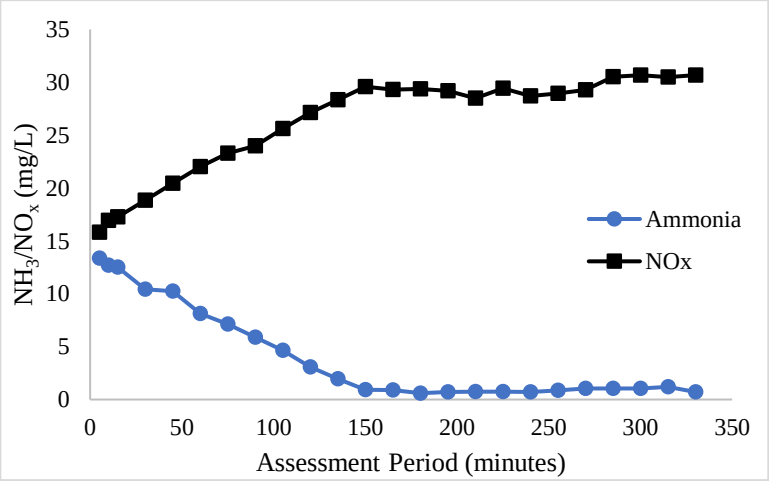

Figure 5.1: NF - 5 Time Series Nitrogen Balance

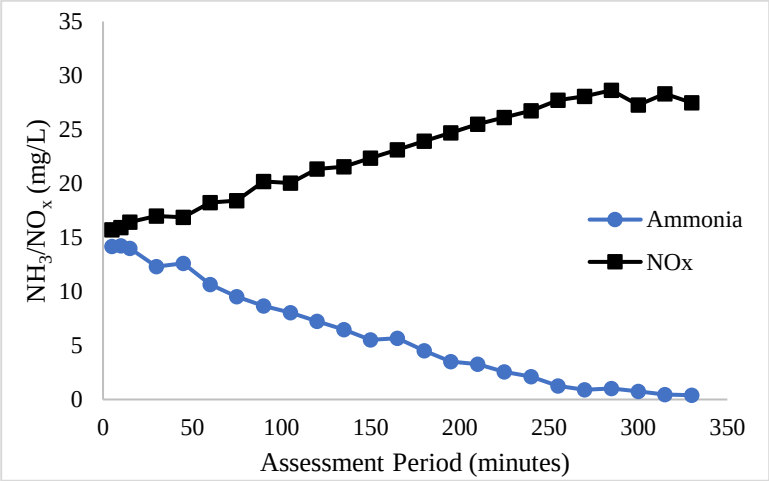

Figure 5.2: NT – 3 Time Series Nitrogen Balance

While time series nitrogen transformations are very clear, nitrogen balances indicate 2.34 and 3.97 mg N/L, or 6.9 and 12.4% of nitrogen, unaccounted for between time zero and end assessment period (for NF – 5 and NT –3). The imbalances are observed in lower effluent ammonia and overall nitrogen present at time zero, and increased nitrite and nitrate concentrations in the effluent (See Tables 4.1 and 4.3).

Inadequate NO_x production may be attributed to lab test accuracy, AOB/NOB growth, SND, and/or AOB N_2O production. Repetitive testing of bulk solution with constant nitrogen concentrations will produce results with some degree of variance, purely out of human error. AOBs allocate some electrons produced to cell growth rather than cell energy (i.e., nitrite production). Additionally, potential N_2O production by AOB (see Figure 2.6) may contribute to the overall nitrogen imbalance. The discrepancies in numerical nitrogen balance may reasonably be attributed to such explanations, considering their relatively small magnitude (compared to total nitrogen present) and graphical analysis of time series nitrogen data.

Comparatively, nitrification events NF – 4 and NT – 4 demonstrate larger nitrogen mass balance discrepancies (Tables 4.1 and 4.3), but similar time series nitrogen cycling characteristics (Figures 5.3 and 5.4) to NF – 5 and NT -3 (Figures 5.1 and 5.2).

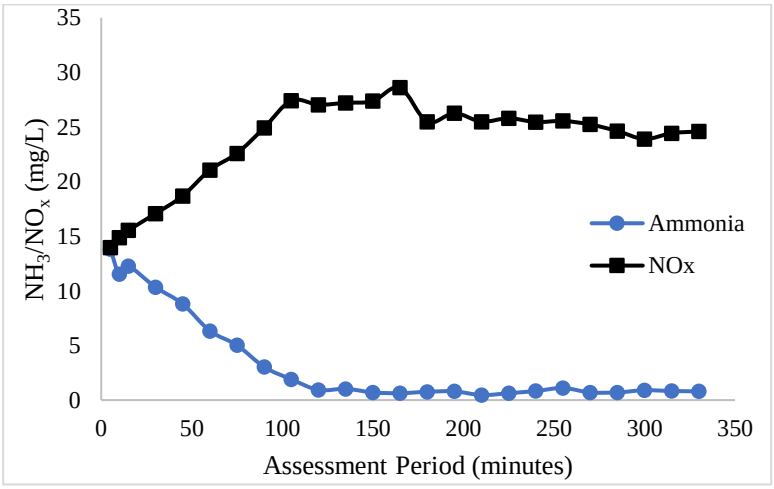

Figure 5.3: NF - 4 Time Series Nitrogen Balance

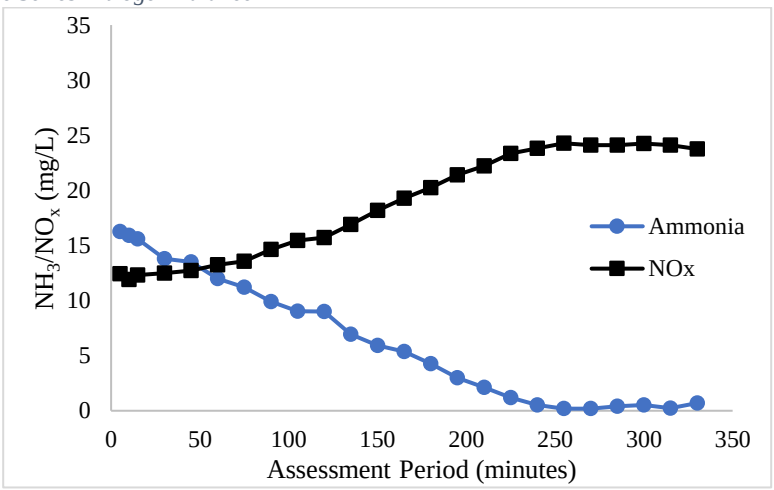

Figure 5.4: NT – 4 Time Series Nitrogen Balance

Nitrogen imbalances indicate 12.81 and 7.54 mg N/L, or 33.5 and 23.5% of unaccounted for nitrogen, between the effluent and time zero concentrations in NF – 4 and NT – 4 respectively. This imbalance refers to a calculated larger quantity of nitrogen present at time zero than nitrogen present in SBR effluent. While ammonia nitrogen present at time zero was nearly fully oxidized (i.e., near zero mg NH_3 – N/L in effluent), this is not reflected in the total NO_x produced. Such imbalances may be attributed to oxidation of residual nitrite (carryover from previous cycle) and/or nitrate reduction. Surely, an environment where both oxidation and reduction could occur was available, especially in the NT reactor, operated under a 0.5 mgO_2/L DO setpoint. At such a setpoint, reactions could occur concurrently (SND) or in phases, either mechanism could be a viable contributor to unaccounted for nitrogen. Additionally, there is possibility for N_2O production from AOBs, as explained previously, and illustrated in Figure 2.6.

NF – 4 results are particularly interesting, as this sampling run was conducted only one week after NF – 3, which indicated nitritation. A performance shift of this magnitude provides a potential explanation of the large nitrogen imbalance reported for NF – 4; elevated time zero concentrations of nitrate (8.95 mg NO_3 – N/L) and nitrite (17.18 mg NO_2 – N/L) were observed, and significantly less nitrite is reflected in the effluent concentrations (opposed to NF – 3). If reductions observed in nitrite concentrations over the operational cycle are assumed to have directly increased nitrate concentrations (i.e., NOB activity), a smaller nitrogen imbalance of 4.04 mg N/L is observed. This reduced number equates to 10.5% of unaccounted for nitrogen between reported time zero and effluent nitrogen concentrations. Nitrite reduction in place of production is observed via speciation of NO_x concentrations for NF – 3 and NF – 4 (Figures 5.5 and 5.6).

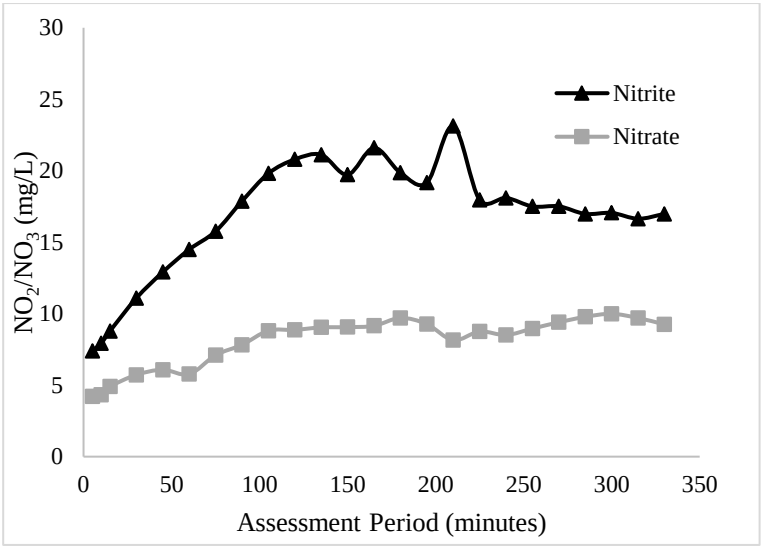

Figure 5.5: NF - 3 NO_x Speciation

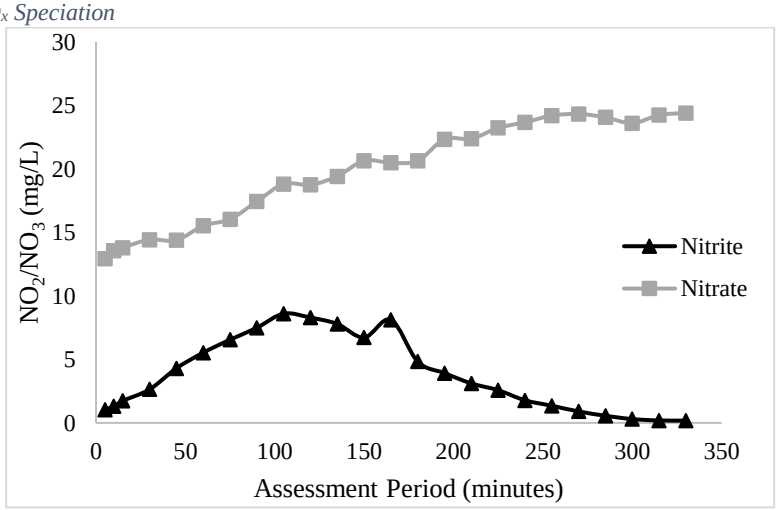

Figure 5.6: NF - 4 NO_x Speciation

Direct comparison of these two events reveals a shift in performance by the MMC in NF from nitritation to nitrification. Performance shift is evidenced by time series nitrogen speciation of NO_x produced (i.e., nitrite dominant to nitrate dominant), as shown in Figures 5.5 and 5.6, and MMC populations reported in Table 4.2 (i.e., *Nitrobacter* to *Nitrospira* dominant). Additionally, differences seen in nitrogen balances between NF – 3 and NF – 4 may be due to larger time zero concentrations of both nitrate and nitrite observed for NF – 4. Overall nitrate concentrations and time zero nitrate concentrations increase by 9.59 and 1.24 mg/L respectively from NF – 3 to NF – 4. As previously discussed, time zero concentrations are directly affected by previous cycle performance, so it is possible that a performance shift led to observed increases. When more nitrate is produced without opportunity for denitrification,

nitrate accumulation may occur within the SBR, resulting in computational nitrogen imbalances seen in both NF – 4 and NT – 4. Additionally, this nitrate carry-over of total NO_x could influence NOB performance. Lemaire et. al. [43] demonstrates this (Figure 5.7), illustrating simulated AOB and NOB populations at the end of the aerobic period variance with a range of NAR percentages (shown as NO_2/NO_x).

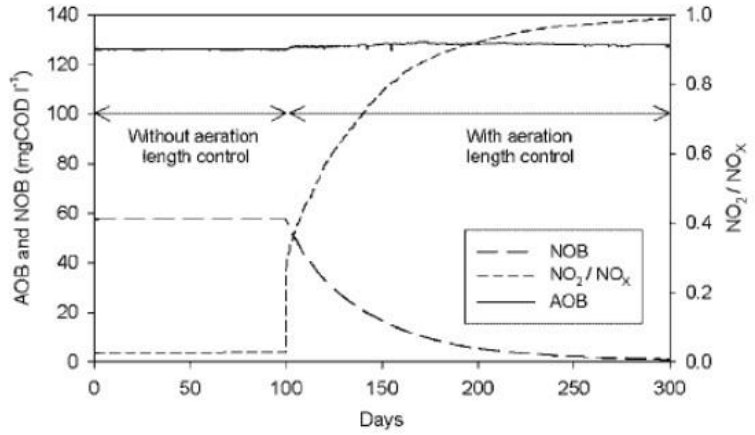

Figure 5.7: Simulated AOB and NOB population with End Aerobic NAR Variance [43]

According to Figure 5.7, total NOB populations are drastically decreased upon nitrite accumulation. This theory indicates that increases in nitrate concentrations (i.e., lower nitrite accumulation) will lead to increases of the NOB population. The larger nitrate concentrations coupled with nitrite reductions (i.e., decreased NAR) observed in NF – 4 may indicate NOB population growth as seen in graphical relationships of Figure 5.7. Lemaire's theory is supported by qPCR data presented in Table 4.2. A larger total NOB population is observed for NF – 4 than NF – 3 (2.24% and 2.38%). Assuming reactor NF was in the process of shifting performance, this NOB increase could surely be attributed to increased nitrate production and was likely still increasing. It is also important to recall that NF – 3 and NF – 4 events were only one week apart, so the observation in NOB population increase is notable for that time frame. Additionally, the population is shown to continue to grow, evidenced by the increased NF – 5 NOB population (3.28%). Stark differences are clearly observed in time series bulk solution data, qPCR results, and average NAR percentages between NF – 3 and NF – 5. NF – 4 results bridge the gap seen between the nitritation and nitrification events.

5.1.2 Full Nitritation Events

Data for NF – 2 and NF – 3 suggest full nitritation achievement by demonstrating full ammonia oxidation with nitrite as the dominant nitrogen speciation, reasonable nitrogen balances, and no notable observation of denitritation. Figures 5.8 and 5.9 illustrate ammonia oxidation corresponding to stable NO_x concentrations for the cycles. Additionally, nitrite accounts for 70% and 68% of total NO_x produced for NF – 2 and NF – 3, respectively.

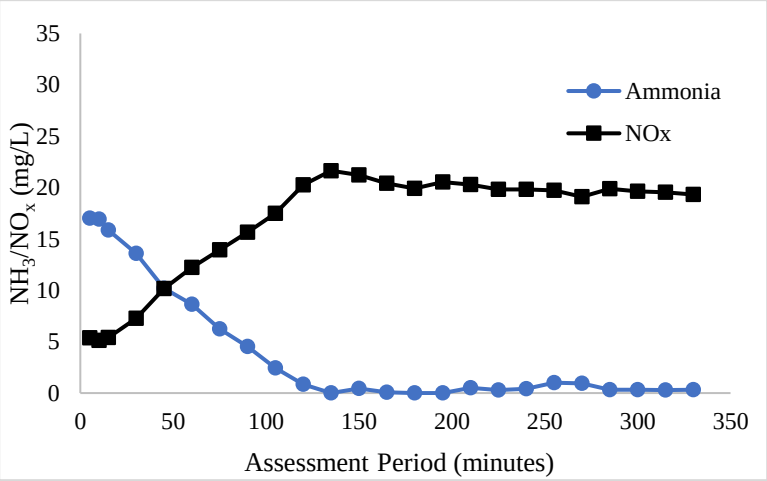

Figure 5.8: NF - 2 Time Series Nitrogen Balance

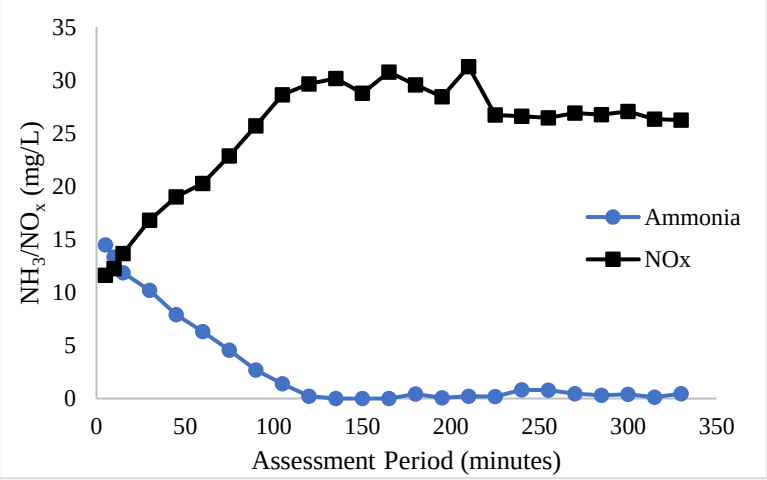

Figure 5.9: NF - 3 Time Series Nitrogen Balance

NF – 2 and NF – 3 events indicate aerobic nitritation achievement, in calculated nitrogen balances, and observed symmetrical ammonia/NO_x concentration trends in Figures 5.8 and 5.9. Observed performance was somewhat surprising, considering the operational DO setpoint (2.0 mg O_2/L). Recalling operational strategies discussed in Chapter 2, a lower DO setpoint appears to be more conducive to nitritation achievement [8, 26]. This outcome may be due to

the poor performance observed and inferred difference in the AOB population in NF's previous operational stage.

As noted in Table 3.1, NF was operated with an 8 – day SRT, 8 – hour HRT, and 0.8 mg O_2/L DO setpoint prior to the sampling events. These operational conditions induced an acidic pH shift (to about 4.6), inhibiting nitrification. Due to the lack of alkalinity present in bulk solution, high ammonia concentrations were experienced for the entirety of the operational cycle. It is suspected that these conditions led to increases in the AOB population, as elevated ammonia concentrations would kinetically favor these microbes (Section 2.2.2, Figure 2.11).

Following this operational scheme, an 8 – day SRT was maintained, while HRT and DO were increased to 18 hours and 2.0 mg O_2/L, respectively. It appears that this operational change allowed a more robust AOB community to establish, as nitritation was observed shortly after in event NF –1. If in fact nitritation achievement can be attributed to an increase in AOB populations, it is not reflected in the qPCR data. However, nitrifying bioreactors have historically shown little to no AmoA detection, suggesting this metric is not an accurate representation of ammonia oxidation.

5.1.3 Nitrification – Denitrification

In contrast to data showing only nitrification or nitritation, data for NT – 1 and NT – 2 suggest nitrification-denitrification achievement by demonstrating full ammonia oxidation corresponding to peak nitrate and nitrite concentrations, followed by NO_x reduction. Nitrate is the dominant NO_x component, contributing 88% (NT – 2) and 95% (NT – 3) of total NO_x produced during the cycle. Further, NO_x concentrations noticeably decline following their peak while ammonia concentrations remain near zero. Figures 5.10 and 5.11 illustrate such trends.

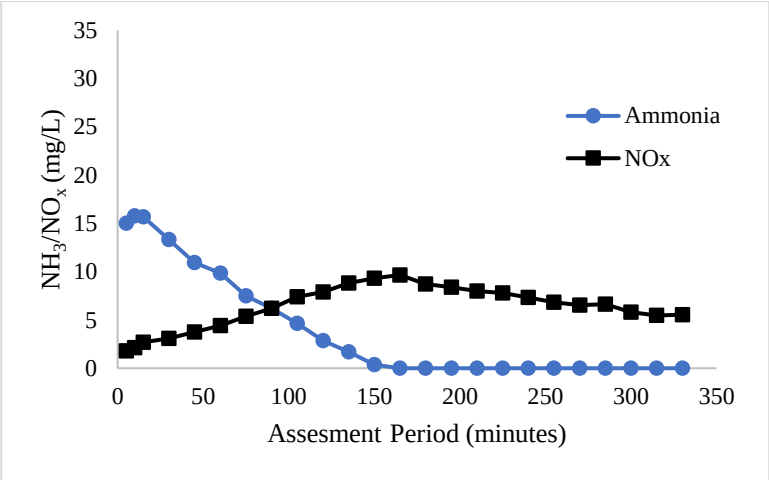

Figure 5.10: NT - 1 Times Series Nitrogen Balance

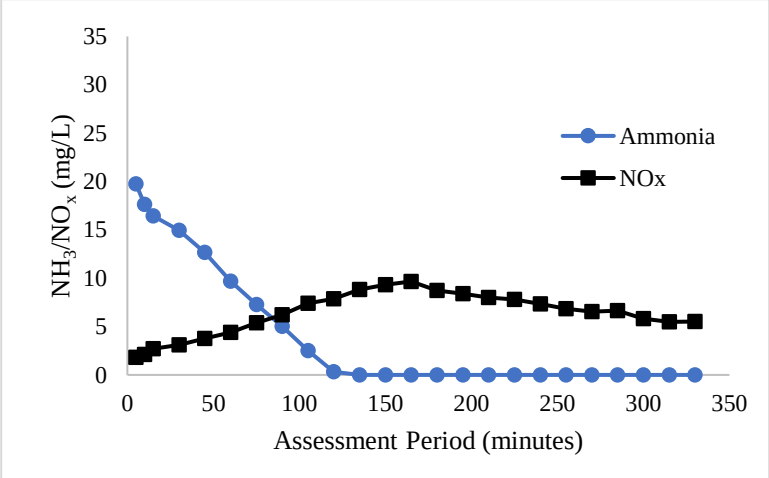

Figure 5.11: NT - 2 Time Series Nitrogen Balance

Observed trends in NO_x concentrations seen in Figures 5.10 and 5.11 indicate that bioreactor operation at 0.5 mg O_2/L allows for simultaneous nitrification and denitrification (i.e., SND). If trends continued, it is assumed that full denitrification could be accomplished with longer cycle lengths. Discrepancies in NT – 1 and NT – 2 nitrogen balances may be attributed to observed incomplete denitrification and potential N_2O production. In the case SND was achieved, N_2O production is likely (as referenced in Chapter 3) considering the low DO setpoint and slow decrease in NO_x observed in Figures 5.10 and 5.11.

N2 – 1 performance also suggests nitrification – denitrification achievement, although under more conventional BNR operational conditions. Ammonia oxidation is achieved during the aerobic period, followed by declining NO_x concentrations in the anoxic period (Figure 5.12).

Complete denitrification is not fully realized, with an effluent nitrate concentration of 8.9 mg/L. Inadequate carbon, insufficient anoxic duration, or time zero nitrogen concentrations could be attributed to this incomplete denitrification.

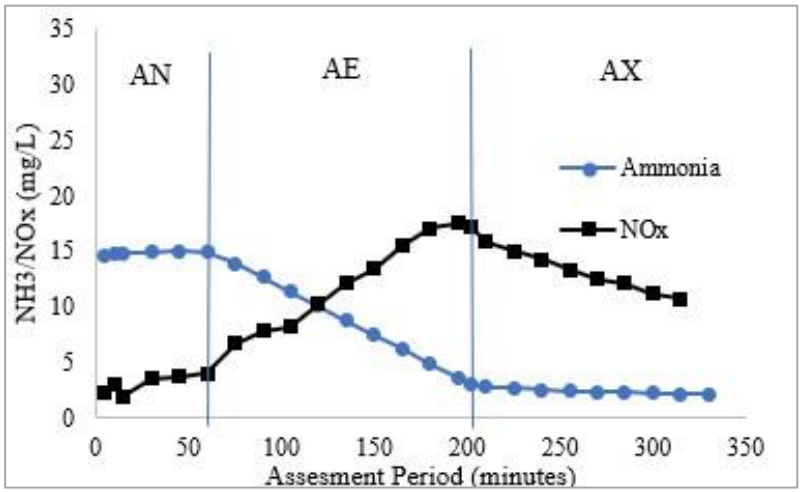

Figure 5.12: N2 - 1 Time Series Nitrogen Balance

5.1.4 Nitritation – Denitritation

NF – 1 and N1 – 1 suggest nitritation – denitritation achievement by demonstrating declining NO_x concentrations prior to full ammonia oxidation (Figures 5.13 and 5.14). Additionally, nitrite dominates total NO_x produced, accounting for 62% and 80% in NF – 1 and N1 -1.

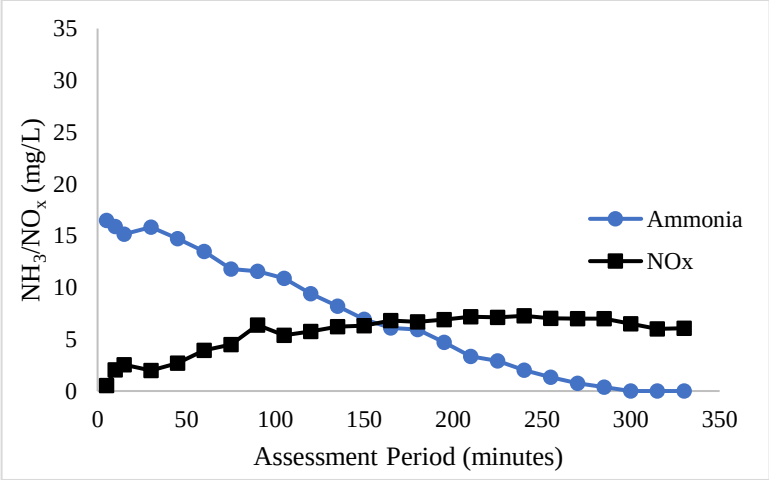

Figure 5.13: NF -1 Time Series Nitrogen Balance

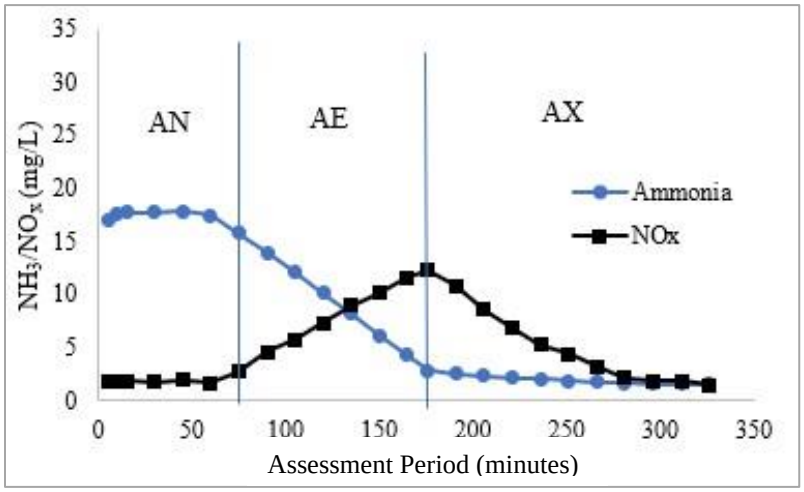

Figure 5.14: N1 - 1 Time Series Nitrogen Balance

A relatively small decline in NO_x concentrations is observed for NF – 1 beginning at 240 minutes. Ammonia concentration reaches zero at 300 minutes. The decline in NO_x concentrations after 240 minutes only sums to 1.39 mg/L. This is much smaller than the total unaccounted-for nitrogen (12.52 mgN/L, 67%), suggesting simultaneous nitritation – denitritation (SNtDt) activity throughout the cycle. SNtDt is achieved when both aerobic and anoxic micro – environments are created within bulk solution [44]. Micro-environments have been shown to occur with low DO setpoints coupled with complex flow patterns [44]. Nitrogen observations of NH_3 and NO_x concentrations suggest that SND can occur at DO setpoints of 2.0 mg O_2/L.

N1 – 1 demonstrates more obvious nitritation (as opposed to NF – 1) in the aerobic period. This activity can further be classified as nitritation – denitritation by speciating the NO_x (Figure 5.15).

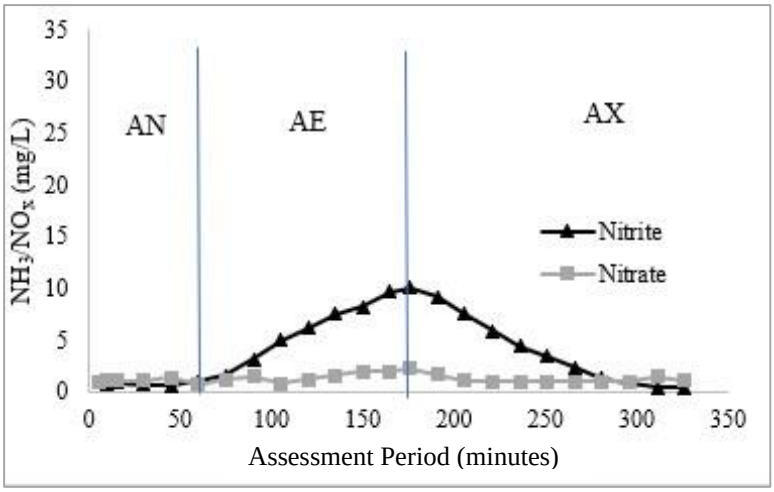

Figure 5.15: N1 - 1 NOx Speciation

Nitrite clearly dominates the aerobic period, matching the total NO_x trend, thus indicating nitritation – denitritation achievement.

5.2 Biomass Microbial Characterization

Biomass microbial characterization interpretations consider performance inferences drawn from bulk solution nitrogen speciation results and the average NAR as an overall performance indicator for the individual sampling events. Table 5.2 summarizes NOB characterization and corresponding average NAR percentages for all sampling events. Note that percent total NOB refers to percentage of total eubacteria detected, while *Nitrobacter* and *Nitrospira* refer to percentage of indicated total NOB population.

Table 5.2: Summarized Biomass NOB Microbial Characterization

Reactor - Event	% Total NOB	% Nitrobacter	% Nitrospira	Average NAR
NF – 1	0.46	41.3	58.7	56.14 %
NF – 2	0.71	52.1	47.9	69.86 %
NF – 3	2.24	28.5	71.4	66.29 %
NF – 4	2.38	26.1	73.9	15.70 %
NF – 5	3.29	9.7	90.3	5.79 %
NT – 1	0.84	39.3	60.7	15.71 %
NT – 2	3.09	49.8	50.2	5.63 %
NT – 3	9.13	14.6	85.4	1.80 %
NT – 4	2.95	12.8	87.2	6.19 %
N1 – 1	0.44	99.6	0.4	62.36 %
N2 – 1	0.55	99.5	0.5	10.52 %

No correlations are observed between populations and average NAR (Figures 5.16 and 5.17).

Note again that for the Figures, percent total NOB refers to percentage of total eubacteria detected, while *Nitrobacter* and *Nitrospira* refer to percentage of indicated total NOB population.

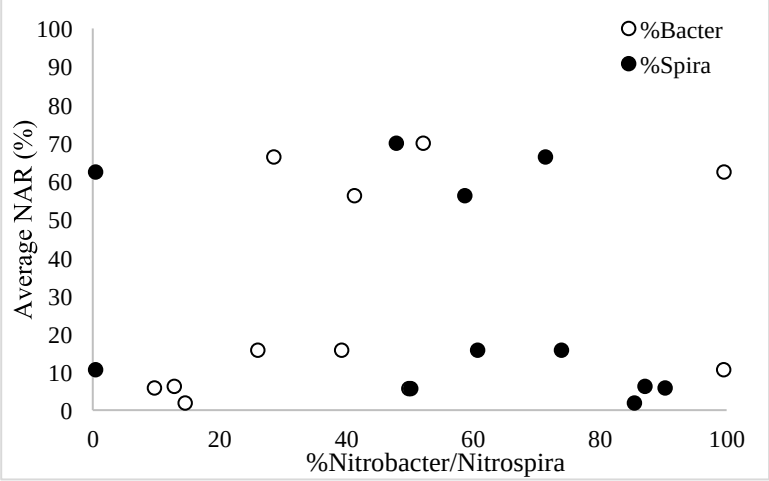

Figure 5.16: Nitrobacter and Nitrospira NOB Percentages vs. Average NAR Percentage

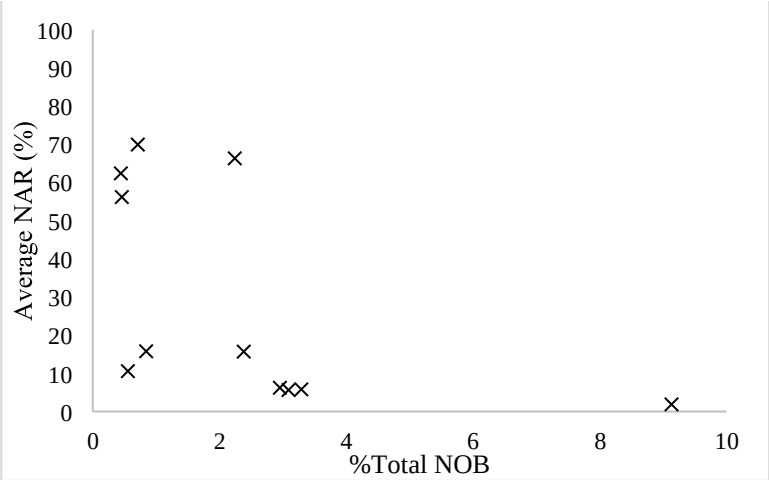

Figure 5.17: Total NOB Percentage vs. Average NAR Percentage

However, notable differences in the total NOB percentage partition the sampling events; Table 5.3 lists the NOB population quantification in descending order for the events.

Table 5.3: Rank of Sampling Event NOB Populations

Reactor – Event	% Total NOB	NAR
NT – 3	9.13	1.80 %
NF – 5	3.29	5.79 %
NT – 2	3.09	5.63 %
NT – 4	2.95	6.19 %
NF – 4	2.38	15.70 %
NF – 3	2.24	66.29 %
NT – 1	0.84	15.71 %
NF – 2	0.71	69.86 %
N2 – 1	0.55	10.52 %
NF – 1	0.46	69.86 %
N1 – 1	0.44	62.36 %

Recalling the bulk solution data performance inferences (Section 5.1), the top five events listed in Table 5.3 align with nitrification. The majority of the lower ranking events in Table 5.3 align with nitritation. N2 – 1 is an exception, demonstrating full nitrification – denitrification (Figure 5.12) this anomaly may be due to the post anoxic configuration of the BIOPHO – PX reactors, as N1 – 1 also demonstrates a very low total NOB population.

Observations of nitrite accumulation and low NOB populations aligns very well with nitritation theory [8, 26]. The BIOPHO – PX reactor results suggest that a post – anoxic configuration may be a viable way to achieve these low percentage of NOBs, thus contributing to nitritation. Utilization of the post-anoxic period as a nitritation strategy is further supported by Kornaros et. al. [27], wherein it was suggested such anoxic periods induce a lag in the NOB metabolisms and subsequent inhibition of growth. If this metabolic lag were induced, NOB washout may be feasible in an SBR operated with a similar configuration to the BIOPHO – PX reactors. Assuming the NOB metabolisms had not recovered from the previous anoxic period, cyclical operation of an SBR could continue to reduce the NOBs via periodic anoxic disturbances.

Additionally, BIOPHO – PX reactors demonstrate obvious *Nitrobacter* dominance (over *Nitrospira*). Such stark differences may be attributed to the post-anoxic configuration. Recalling Chapter 2, *Nitrobacter winogradski* genomically codes for PHB biosynthesis, whereas *Nitrospira defluvii* does not. PHB is a crucial carbon reserve often stored

anaerobically and utilized aerobically by phosphorus accumulating organisms (PAO) in EBPR configurations. Recalling the configuration described in Chapter 3, the BIOPHO – PX cycle begins with an anaerobic period of one hour (for EBPR accomplishment). If *Nitrobacter winogradski* store PHB in the anaerobic period, they would be advantaged over *Nitrospira defluvii* in the cycle's anoxic period. Cyclical operation would thus lead to the observed *Nitrobacter* dominance.

5.3 NxR Expression

As illustrated in Figures 4.22, 4.23, (N1) 4.26, and 4.27 (N2), interpretation of NxR expression amongst the subunits and across the reactors is difficult, as graphs do not provide much contrast. Ultimately NxR expression interpretations were made only for the BIOPHO – PX reactors, due to the small sample size and short time series data available for NF events. Further interpretations of BIOPHO – PX NxR expressions were accomplished by normalizing the 1-minute anaerobic time point for BIOPHO – PX reactors. The decision to normalize to this time point was made based on data for the nxrBq NSR subunit, wherein no expression at time zero was detected. Further, NxR primers target the protein complex responsible for nitrite oxidation amongst NOB species. Consequently, NxR should not be active under anaerobic conditions, and such samples provide an appropriate normalization factor. The percent change between these normalized relative expressions was then calculated to illustrate up- or down-regulation of the NxR gene throughout the operational cycle.

Additionally, only nxrB NITRO and nxrBq NSR subunits have been represented, due to the sporadic and low overall measured nxrA NITRO expression. The NxR regulations of the subunits is depicted as a percent change via scatter plot in which nxrB NITRO represents nitrite oxidation by *Nitrobacter*, and nxrBq represents nitrite oxidation by *Nitrospira*. Figures 5.18 and 5.19 show percent changes for the time series relative expressions.

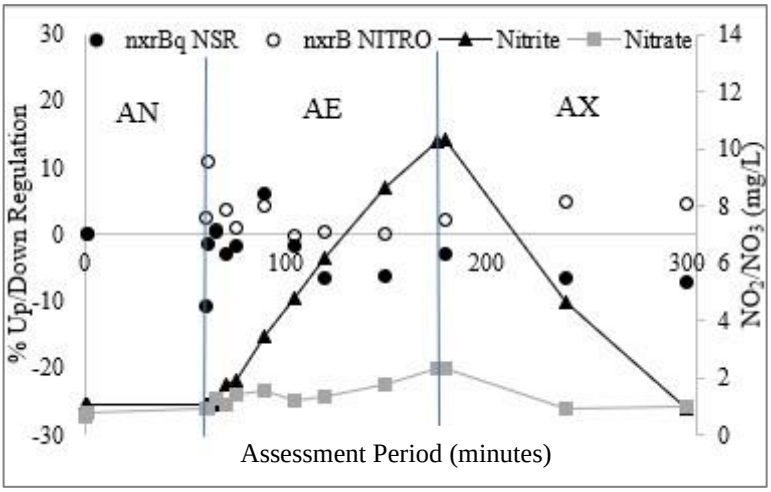

Figure 5.18: N1 – 1 Up and Down Regulation of Nitrobacter and Nitrospira NxR subunits

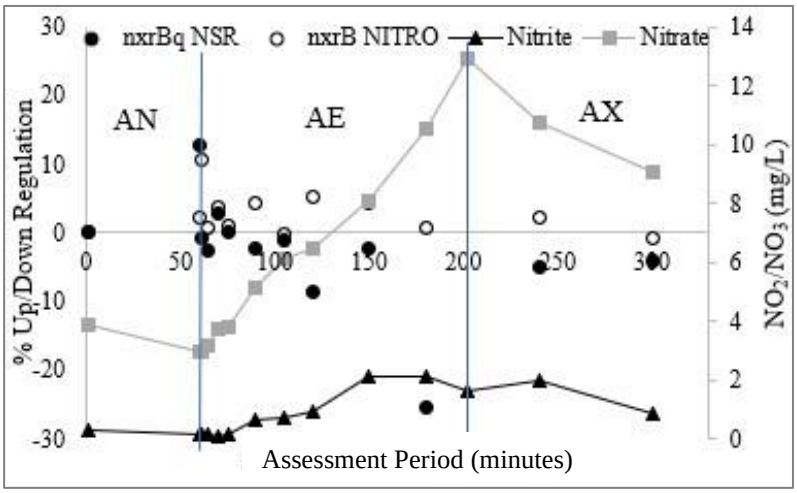

Figure 5.19: N2 – 1 Up and Down Regulation of Nitrobacter and Nitrospira NxR subunits

Nitrospira (nxrBq) regulation is largely negative for both reactors, with the exception of a 12% up-regulation observed at 60 minutes in N2 – 1. This corresponds to the end of the anaerobic period, so this up-regulation may be attributed to the sudden introduction of air and the subsequent growth of *Nitrospira*, with nxrBq expression. N1 – 1 also illustrates a 6% increase in nxrBq NSR at 90 minutes. Increases would be expected during the aerobic period, since nitritation and nitrification are aerobic metabolisms, leading to the small, but still relevant nitrate concentrations observed. Following these spikes, negative or down regulations are observed for the remainder of the operational period for both reactors. This aligns with the observed NOB populations discussed in 5.2, as qPCR for either reactor indicated very small *Nitrospira* communities.

Comparatively, *Nitrobacter* nxrB expression is very different between N1 and N2. Most notably, peaks are observed at different times in the cycle. N1 up regulation is observed during the latter portion of the operational cycle, whereas N2 demonstrates more up regulation in the middle, or aerobic portion of the cycle. Considering bulk solution time series NO_x data, this corresponds to nitrite accumulation (N1) and nitrification (N2).

N1 up-regulation is observed mainly in the anoxic period, making NOB behavior harder to discern. NxR represents nitrite oxidation, however this is not reflected in N1 bulk solution data. Such a contradiction suggests other functions of the nxrB *Nitrobacter* subunit. It has been suggested by Lucker et. al [12] that nitrite oxidation catalyzed by *Nitrobacter* NxR is reversible, meaning nitrate reduction is possible via this protein complex. Nitrate reduction is possible because the *Nitrobacter* NxR and NAR nitrate reductase sequences are closely related [12].

Nitrobacter is clearly active in this anoxic time frame and seems to be more active as the anoxic period continues. As the anoxic period progresses, *Nitrobacter* begin to reduce any residual nitrate and oxygen (via NxR). During this period of increasing nutrient deprivation, *Nitrobacter winogradski* may be prompted to begin utilizing stored PHB. Certainly, in a post–anoxic configuration all available carbon present in the influent wastewater has been consumed, requiring utilization of carbon reserves. These increasingly anaerobic conditions coupled with limited carbon availability would be an appropriate environment to assume *Nitrobacter winogradski* begins metabolizing PHB reserves.

The observed expression of the *Nitrobacter* nxrB subunit may be attributed to the microbes surviving on their PHB reserves. N1 – 1 metabolic expression also supports this hypothesis. Stepwise increases are observed in 3PG expression, much like the nxrB subunit throughout the anoxic period. This may indicate increasing carbon sequestration via *Nitrobacter winogradski* Calvin cycle, aligning with suspected metabolism of PHB reserves.

5.4 Targeted Metabolomic Expressions

Interpretation of metabolomic data considers and builds upon performance inferences drawn from bulk solution nitrogen speciation interpretations. Data in this section will follow the same structure utilized in 5.1 to relate metabolic performance to overall performance observed in bulk solution. Further, in an effort to understand how targeted metabolite relative

expressions (RE) relate to NOB populations and their respective *Nitrobacter* and *Nitrospira* biomass percentages, Calvin Benson and rTCA cycle metabolites were multiplied by either percentages of *Nitrobacter* and *Nitrospira* (of NOB biomass totals) presented in Tables 4.2, 4.4, 4.6, and 4.8 for all reactors. Considering that both NOB species utilize the oTCA cycle, as would other microbes in the MMC (e.g., OHOs, PAOs, GAOs), and the possible redundancy of metabolite presence between the oTCA and rTCA cycles, the TCA metabolites were also normalized to the *Nitrobacter* population. To avoid double counting the TCA metabolites, *Nitobacter* normalized TCA metabolites were subtracted from the *Nitrospira* normalized metabolites. Equation II is provided for clarity.

$$rTCA\ RE = (TCA\ Metabolite\ RE) * (\%Nitrospira\ NOB\ Biomass - \%Nitrobacter\ NOB\ Biomass)$$

Equation II

5.4.1 Full Nitrification Events

NF – 5, NT – 3, and NT – 4 all represent successful nitrification events, indicated by bulk solution results (Figures 4.5, 4.15, and 4.16). Interestingly, normalized metabolite data for these events (Figure 5.20 – 5.22) primarily represent relative expressions of targeted rTCA metabolites, with α – ketoglutarate (AKG) dominating.

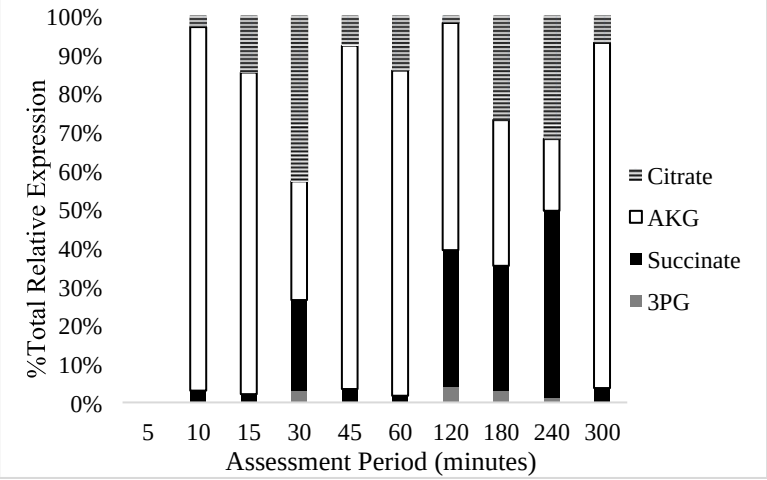

Figure 5.20: NF - 5 NOB Adjusted Metabolite Results

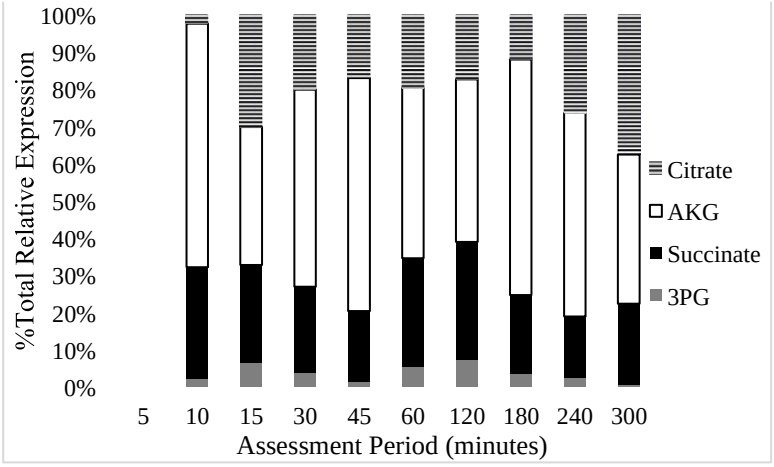

Figure 5.21: NT - 3 NOB Adjusted Metabolite Results

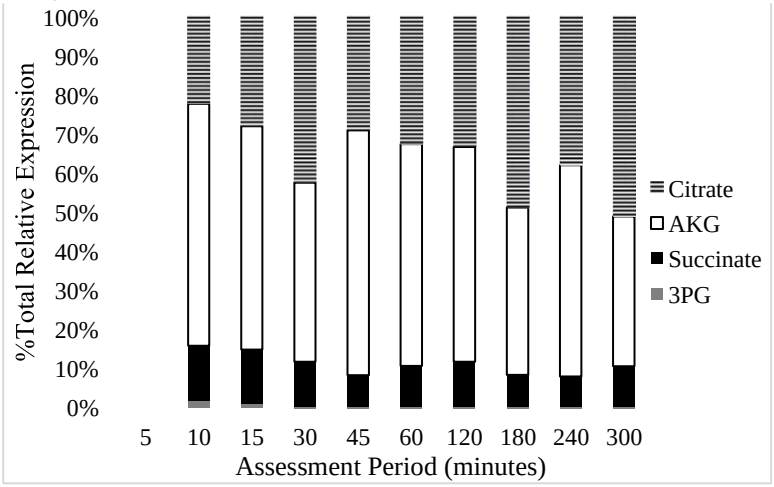

Figure 5.22: NT - 4 NOB Adjusted Metabolite Results

Fairly consistent rTCA RE quantities are seen throughout the cycle, particularly for NT – 3 and NT – 4 events, corresponding to full cycle durations of nitrification. Comparatively, the NF – 4 metabolite data (Figure 5.23) provides a contrast to other nitrification events. In this event, AKG does not dominate; rather, larger overall RE of citrate are observed in addition to more 3PG fluxes (as opposed to Figures 5.19 – 5.22 representing nitrification). This is particularly interesting recalling the bulk solution data (Figure 4.4); specifically, NF – 4 represents a performance shift from nitritation (NF – 3) to nitrification (NF – 4). Additionally, no notable NO_x reduction is observed for NF – 5, NT – 3, or NT – 4, but nitrite reduction is observed in NF – 4 (Figure 5.23).

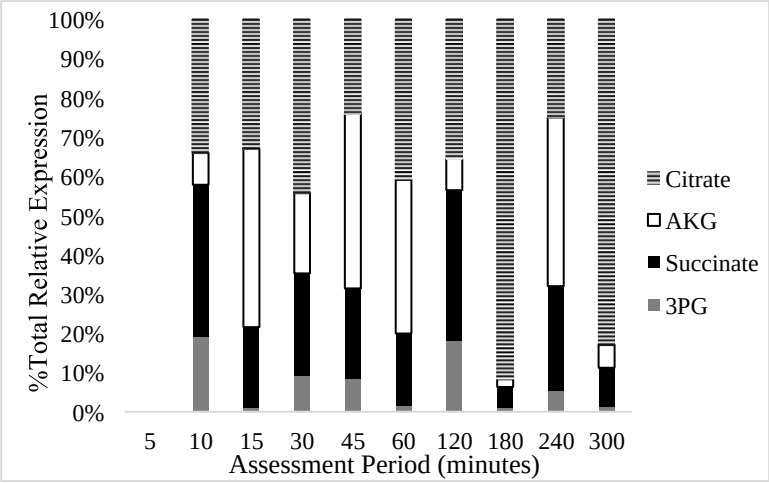

Figure 5.23: NF - 4 NOB Adjusted Metabolite Results

Nitrite reduction begins at 105 minutes (Figure 5.23) in NF – 4, followed by the largest 3PG increase observed for NF – 4 at 120 minutes. While nitrite reduction continues throughout the cycle, 3PG RE does not necessarily reflect this reduction. While inconclusive, such differences in metabolite and bulk solution results between nitrification events may suggest that 3PG production seen for NF – 4 is attributed to denitrifier metabolisms.

5.4.2 Full Nitritation Events

NF – 2 and NF – 3 represent successful nitritation events, indicated by bulk solution data (Figures 4.2 and 4.3). Figures 5.24 and 5.25 represent NOB adjusted metabolite results, which illustrate stark differences between nitritation and nitrification events from a metabolic perspective. NF – 2 (Figure5.24) illustrates large RE of 3PG, and negative RE for all three TCA metabolites. Negative values indicate that *Nitrobacter* population exceeded that of *Nitrospira*. In addition to being negative, the magnitude of TCA metabolites is dwarfed by 3PG magnitude. Recalling bulk solution data (Figure 4.2), NF – 2 was arguably the most successful aerobic nitritation event, with an average overall NAR percentage of 69.86%.

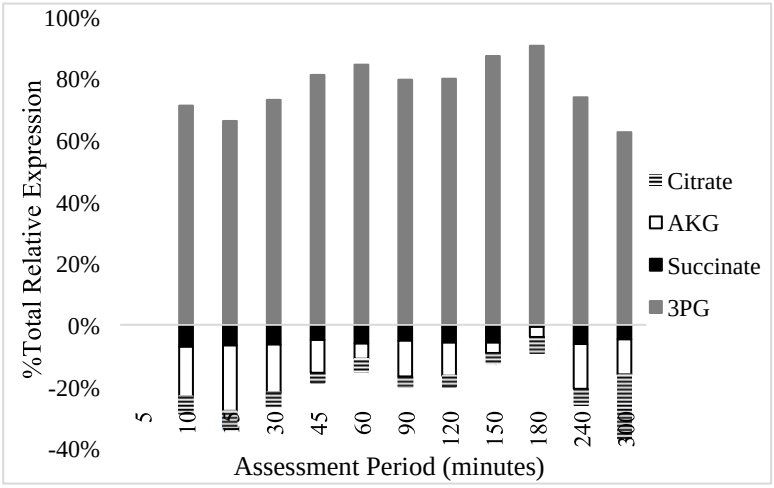

Figure 5.24: NF - 2 NOB Adjusted Metabolite Results

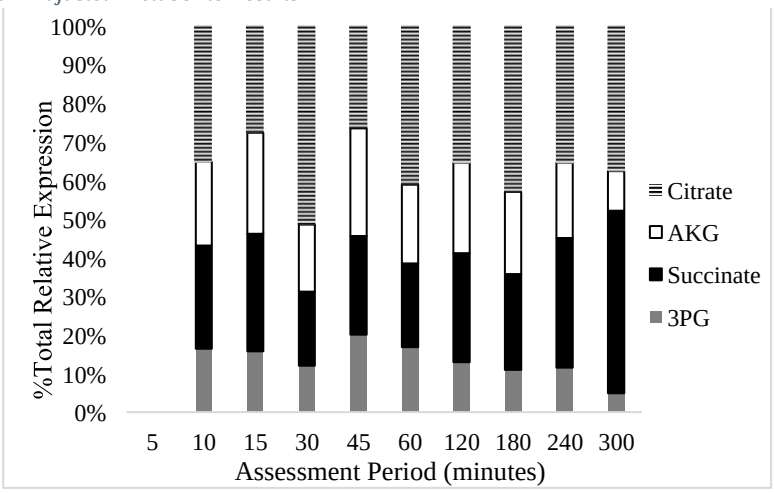

Figure 5.25: NF - 3 NOB Adjusted Metabolite Results

Data presented in NF – 3 (Figure 5.25) is much less clear in terms of metabolite dominance. However, rTCA metabolite RE is much more prevalent throughout the cycle as opposed to the nitritation event experienced in NF – 2. Additionally, it appears that 3PG RE generally declines with time, possibly influencing performance illustrated in NF – 4 (Figure 4.4). If in fact 3PG is indicative of nitritation, increases in rTCA metabolite RE (and subsequent 3PG RE reduction) for NF – 3 could indicate or predict the performance shift observed in NF – 4.

5.4.3 Nitrification – Denitrification

NT – 1 and NT – 2 represent successful nitrification – denitrification events, indicated by bulk solution results (Figures 4.13 and 4.14), and particularly indicated by time series nitrogen balance data (Figures 5.10 and 5.11). Metabolite concentrations illustrate 3PG dominance amongst targeted metabolites for these events (Figures 5.26 and 5.27).

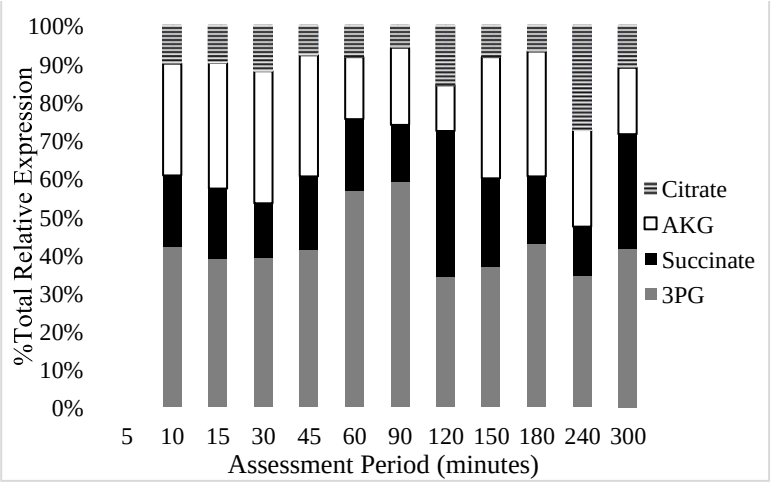

Figure 5.26: NT – 1 NOB Adjusted Metabolite Results

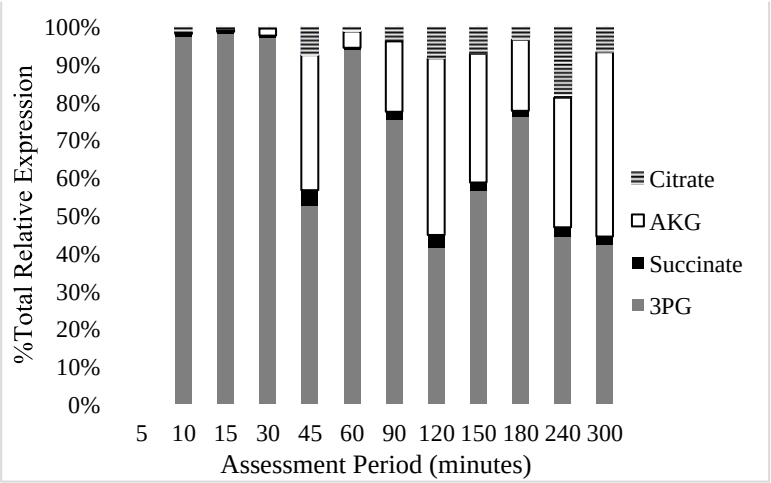

Figure 5.27: NT – 2 NOB Adjusted Metabolite Results

Large quantities of 3PG in Figures 5.26 and 5.27 suggests contributions of 3PG production to be attributed to denitrifying metabolisms. Ammonia oxidation is completed at minutes 150 and 120 for NT – 1 and NT – 2. Lowest RE of 3PG corresponds to these time points for either nitrification event. Further, denitrification begins at minute 165 for both NT – 1 and NT – 2, potentially reflected by an increased flux of 3PG seen between minute 150 and 180 in Figures 5.26 and 5.27.

Recalling denitrification fundamentals, heterotrophic reduction reactions are fueled by carbon (Figure 2.4). If in fact 3PG and TCA metabolites well reflect CBC and rTCA activity, larger 3PG RE *may be* expected for denitrifying events. Utilization of the rTCA cycle is unusual as a

mechanism for carbon sequestration, and employment of the Calvin cycle would be more likely amongst the diverse group of potential denitrifiers.

As noted in Chapter 4, BIOPHO – PX metabolite relative expressions have not been adjusted to NOB populations, due to the marginal presence of *Nitrospira* (and subsequent skewed effect NOB normalization would impart on results). Resultant N2 – 1 metabolite expressions are shown in Figure 5.28.

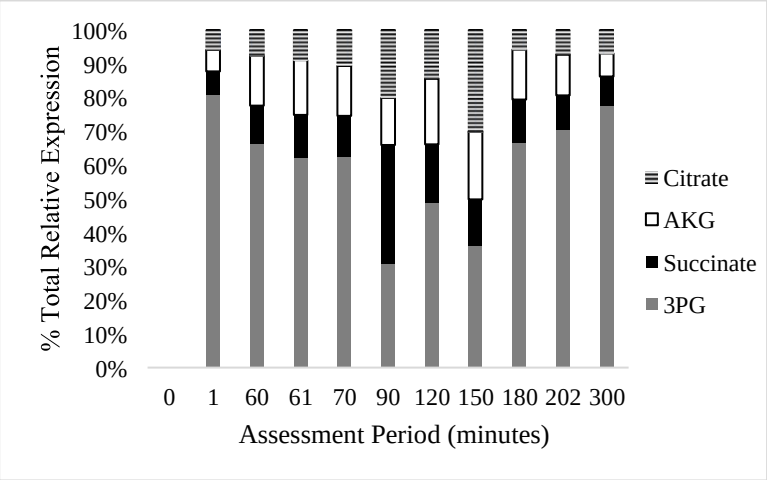

Figure 5.28: N2 - 1 Metabolite Relative Expressions

As shown, the N2 – 1 cycle is largely dominated by the 3PG metabolite. Notable decreases are observed between 90 – 150 minutes, followed by notable increases in between 180 – 300 minutes. Recalling the BIOPHO – PX post – anoxic configuration, such increases later in the N2 – 1 operational cycle (i.e., post-anoxically) may be attributed to denitrifier activity. This inference aligns with denitrification and metabolite observations previously presented (namely NT – 1 and NT – 2). Additionally, a possible carry-over effect is seen in 3PG for the anaerobic period of N2 – 1, suggesting Calvin cycle utilization by PAOs or metabolism of potential PHB reserves by *Nitrobacter winogradski*. Increases in TCA metabolites for the aerobic period may indicate AOB or *Nitrobacter* activity, considering the observed nitrification and NOB population.

5.4.4 Nitritation – Denitritation

NF – 1 bulk solution data (Figure 4.1) suggests nitritation – denitritaion, as well as possible SND, throughout the cycle. Inferences drawn from metabolite RE further support this suspicion. 3PG is well represented throughout the cycle, illustrated by RE in Figure 5.29.

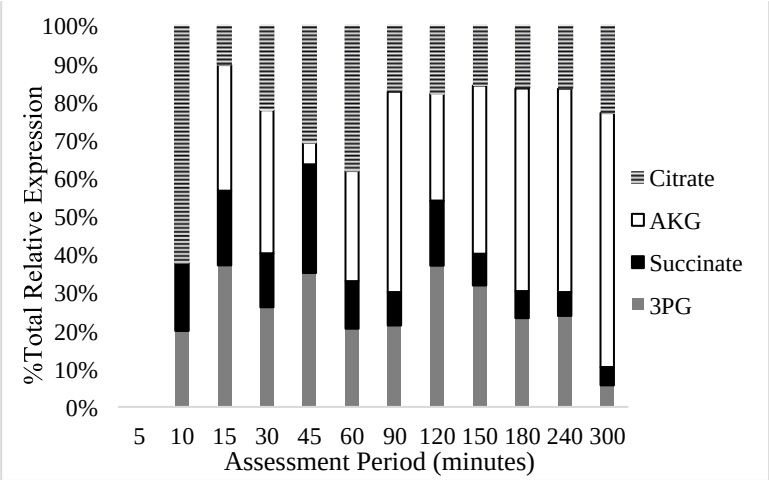

Figure 5.29: NF – 1 NOB Adjusted Metabolite Results

As explained in Section 5.1.4, SND is accomplished in the presence of anoxic microenvironments [44]. Research has shown that NOB metabolisms may also demonstrate a lag following anoxic disturbances [27]. Such evidence suggests utilization of these disturbances as an operational means to achieve nitritation (Chapter 3). If in fact transient anoxic conditions were achieved in NF – 1, increases of AKG RE in Figure 5.27 may evidence such NOB metabolism lag.

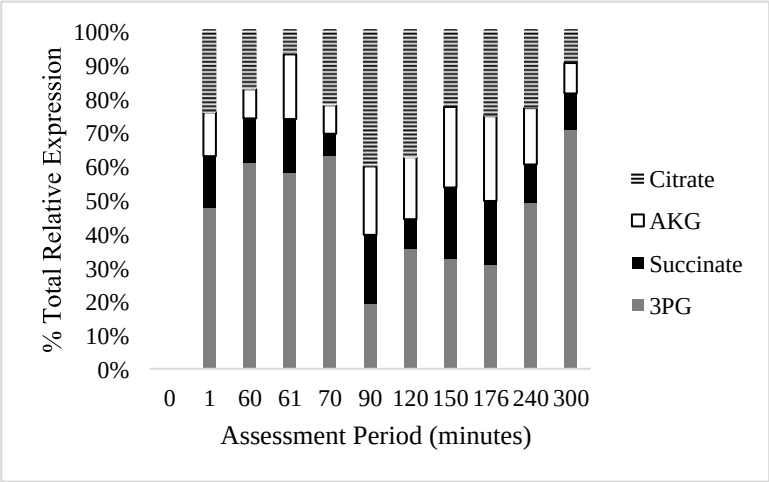

Figure 5.30: N1 – 1 Targeted Metabolite Expressions

Observations in 3PG fluxes of N1 – 1 metabolite relative expressions (Figure 5.30) illustrate a decline during the aerobic period (61 – 176 minutes) and a climb in the anoxic period (176 – 300 minutes). Increases further confirm previous inferences linking 3PG presence to denitrifier activity. Larger TCA metabolite fluxes in the aerobic period likely suggest AOB

and/or OHO activity, considering observed nitrite accumulation and low *Nitrospira* populations. The anaerobic period also illustrates larger fluxes of 3PG, possibly indicating PAO activity or metabolism of potential PHB reserves by *Nitrobacter winogradski*.

Similar trends in metabolite concentrations and NOB populations between the two BIOPHO – PX reactors indicate 3PG production may be attributed to PAO, *Nitrobacter*, and/or denitrifier activity. Similarities between BIOPHO – PX and denitrification events observed in NF and NT, also support denitrifier 3PG contribution. Further comparison between BIOPHO – PX and NF – 1 indicate similar total NOB populations, but more *Nitrospira* is seen in NF – 1. This microbial population difference in conjunction with larger AKG and smaller 3PG metabolite expressions observed in NF – 1 (as opposed to BIOPHO – PX), suggest *Nitrobacter* activity is in fact a contributor to 3PG fluxes.

Chapter 6: Conclusions & Future Work

This chapter revisits research questions, leveraging data results and interpretations presented and discussed herein. The goal of this chapter is to highlight convergence of structural and functional microbial data sets with bulk solution bioreactor performance. Convergence will focus on nitritation events, corresponding microbial communities and their subsequent functional inferences made from the collected data and supporting literature.

6.1 Research Question 1

RQ 1: Can nitritation be achieved and sustained simply through control of residual dissolved oxygen and SRT?

Nitritation was achieved utilizing an 8 – day SRT, 18 – hour HRT, and 2.0 mgO_2/L DO setpoint during four sampling events conducted throughout the research period. Successful nitritation was marked by high nitrite accumulation rates, coupled with low nitrate concentrations. Three of the four events were observed in completely aerobic environments (NF), while another utilized a post – anoxic configuration (N1; BIOPHO – PX). Achievement in two different configurations is advantageous for data interpretation, as relative comparisons provide means to differentiate aerobically and anoxically induced bacterial functions.

Further criteria were hypothesized for nitritation achievement:

- AOB population will exceed that of the NOBs,
- Nxr gene activity exhibited by *Nitrobacter* will be greater than *Nitrospira*,
- Larger quantities of carbon fixation metabolic intermediates associated with the *Nitrobacter winogradski* genome will be observed, as opposed to *Nitrospira defluvii*.

According to qPCR data, AOB population never exceeded NOB population totals; in fact AOBs were only detected in a single sampling event. NF – 3 results indicated 0.01% AOBs as a fraction of eubacteria, corresponding to arguably the second most successful aerobic nitritation event in terms of average NAR. However, this event also demonstrated a substantial NOB population (2.24%) relative to all conducted sampling events. Other nitritation events (NF – 1, NF – 2, and N1 – 1) all demonstrated relatively low NOB population total (less than 1%). A lower overall NOB total aligns with nitritation theory [8, 26] but does not guarantee nitritation achievement. This is indicated by N2 – 1 and NT – 1 nitrification performances coupled with NOB population totals of less than 1.0%.

The low AOB fractions quantified via qPCR do not well align with performance data, wherein ammonia oxidation consistently occurred within all tested reactors. At this point the causes of this discrepancy are not clear. While it could be suggested that amoA might not be the best target for quantitation, it is the standard of AOB assessment. Inhibition could be the cause, since wastewater does contain inhibitors to PCR reactions. Extensive AOB quantitation by the Coats lab across a wide array of bioreactors has revealed a consistently low %AOB population. Ultimately more methodological interrogations are needed to remedy this issue.

Although NxR gene activity data was limited (only four sampling events are transcriptomically represented), nitritation and nitrification are equally represented. N1 – 1 provides NxR data to represent nitritation, while N2 – 1 represents nitrification. Inspection of Figure 5.18 indicates much greater positive aerobic percent changes for nxrB NITRO (*Nitrobacter*) in N1 nitritation event. Figure 5.19 illustrates lesser overall nxrB NITRO activity and more nxrBq NSR (*Nitrospira*) for nitrification events. While qPCR data does not always indicate Nitrobacter dominance for nitritation events, it appears the nxrB subunit associated with *Nitrobacter* nitrite oxidation is more active than the nxrBq subunit of *Nitrospira* for nitritation events.

Interpretations of metabolic data must represent observations in bulk solution and qPCR data, but also consider other functions of microbes present in a MMC. Obviously, AOB and NOB are not the only contributors to the observed quantities of targeted carbon sequestration metabolites. Therefore, interpretations drawn from data analysis can therefore not be conclusive but can utilize all available molecular perspectives to converge on hypothesis stance. NF – 2 produced the highest average NAR, with 3PG largely expressed throughout the cycle, supporting the metabolic portion of Hypothesis 1. Comparatively, NF – 3 (another aerobic nitritation event) metabolite results were much less conclusive; rTCA and Calvin cycle metabolites fluctuate through the cycle, with neither group seemingly more prevalent. Further qPCR difference are notable between the two nitritation events. A NOB population increase of 1.56% is observed between NF – 2 and NF – 3, more critically the *Nitrobacter* fraction is drastically reduced. Nitrobacter accounts for 52.1% of the NOB population in NF – 2 and 28.5% in NF – 3.

Similar metabolic and qPCR results were observed for the other nitritation events, NF – 1 and N1 – 1. NF – 1 was inconclusive, while N1 – 1 produced larger 3PG quantities. Additionally, these two events had very similar total NOB percentages of 0.44 (N1 – 1) and 0.46 (NF – 1) but very different fractions of *Nitrobacter*. *Nitrobacter* accounts for 99.6% of NOB population in N1 – 1, and only 41.3% in NF – 1. Differences observed in *Nitrobacter* fraction and 3PG production for all nitritation events suggest that *Nitrobacter* do in fact contribute to these 3PG concentrations.

6.2 Research Question 2

RQ 2: Nitritation appears to be achieved by enriching for *Nitrobacter* spp. over *Nitrospira* spp. Can significant differences be observed in NOB populations for nitrification and nitritation events?

Three sampling events demonstrated *Nitrobacter* dominated NOB populations; however, only two of these three events suggest nitritation. N2 – 1 qPCR results indicate a *Nitrobacter* fraction of 99.5%, coupled with nitrification. As presented in Chapter 4, and previously discussed in Section 6.1, *Nitrobacter* dominated NOB populations do not guarantee nitritation achievement. Hypothesis 2 is not supported due to inconclusive observations in bulk solution performance and qPCR results of NF – 2, N1 – 1, and N2 – 1.

6.3 Research Question 3

RQ 3: Differences in carbon fixation pathways reveal unique metabolic intermediates to serve as functional surrogates for the *Nitrobacter winogradski* and *Nitrospira defluvii* genomes. Can these indicative metabolites in conjunction with Nxr expressions indicate a significant difference between *Nitrobacter winogradski* and *Nitrospira defluvii* activity in a nitritating SBR?

Per 6.1 metabolic discussion, it does appear that *Nitrobacter* contributes to observed quantities of 3PG. However, alignment of metabolic and bulk solution observations also suggest that PAO, denitrifier, and PHB metabolisms also contribute to 3PG production. BIOPHO – PX 3PG trends are particularly interesting. 3PG is elevated during the anaerobic period, suggesting Calvin cycle activity, possibly by PAOs. This is followed by lower 3PG concentrations in the aerobic period, and step wise increases during the anoxic period. These step wise anoxic 3PG increases are only observed for the BIOPHO – PX reactors. A post-

anoxic configuration and heavy *Nitrobacter* dominance support previously proposed metabolism of *Nitrobacter winogradski* PHB reserves.

Only nitrification (no denitrification) is observed in events NF – 5, NT – 3, and NT – 4, all result in large concentrations of TCA metabolites. Further qPCR results for these results indicate NOB populations dominated by *Nitrospira*, accounting for 90.3, 85.4, and 87.2% of total NOBs. Comparatively, nitrification – denitrification event NF – 4 metabolic results are much less conclusive, although *Nitrospira* population is similar (73.9%). It is suspected that active denitrification metabolisms of NF – 4 influenced the metabolite results. These listed *Nitrospira* dominated events correspond to TCA heavy metabolic profiles, apart from NF – 4. Therefore, TCA metabolic profiles may indicate nitrification, but may not be attributed to *Nitrospira defluvii* specifically.

While bulk solution performance and qPCR data do not fully support Hypothesis 3 transcriptomic data suggests that nitritation is more reliant on increased nxrB NITRO expression (i.e., nitrite oxidation via *Nitrobacter*). Available transcriptomic data indicates more positive percent changes in the nxrB subunit for nitritation (N1 – 1) than nitrification (N2 – 1). Differences observed in the BIOPHO – PX reactors indicate nxrB NITRO (*Nitrobacter* nitrite oxidation) appears to be more significantly expressed during nitritation than nxrBq NSR (*Nitrospira* nitrite oxidation). It is important to note that while the data supports this, the sample size is small.

6.4 Future Work

Reflecting upon the work conducted and presented herein, numerous molecular avenues concerning nitritation and nitrification contrast could be pursued. Coupling qPCR with NxR and metabolic data provide multiple molecular lenses to aid in bulk solution interpretation. Both molecular structure and function can be interpreted, for a variety of configurations. Such a structure could be applied to further nitritation and nitrification events to draw conclusions from larger sample sizes and longer research periods. Larger samples sizes would provide more certainty and longer research periods would provide opportunities to observe microbial populations fluxes and subsequent changes in functional data.

Further, reactor PHB profiles for the operational cycle would aid in anoxic and anaerobic molecular interpretations. Pairing molecular (qPCR, NxR, metabolomics, and PHB) profiles

with bulk solution performance could greatly increase researchers' knowledge of these biological systems. Insight gained from these molecular profiles can be leveraged to optimize system oxygen and carbon requirements, as well as allocated time in designated aerobic states. Optimization would create massive positive impacts on treatment quality and operational costs. Additionally, such metabolic profiles could be utilized to optimize other processes, namely EBPR.

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Appendix

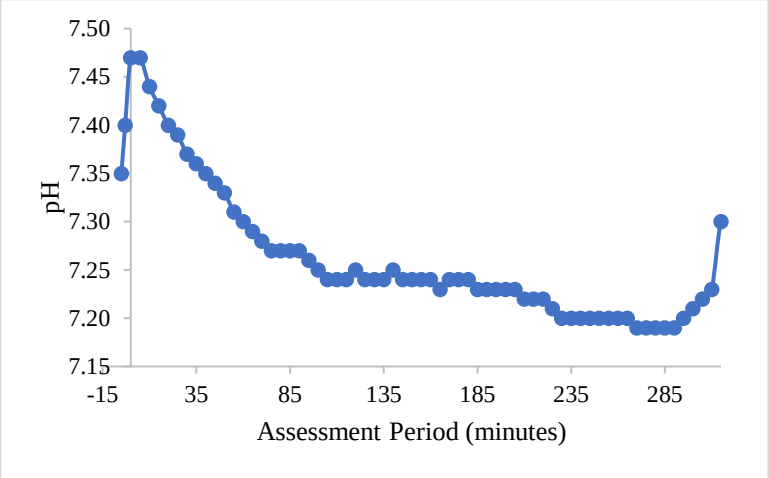

Figure A.1: NF – 1 pH

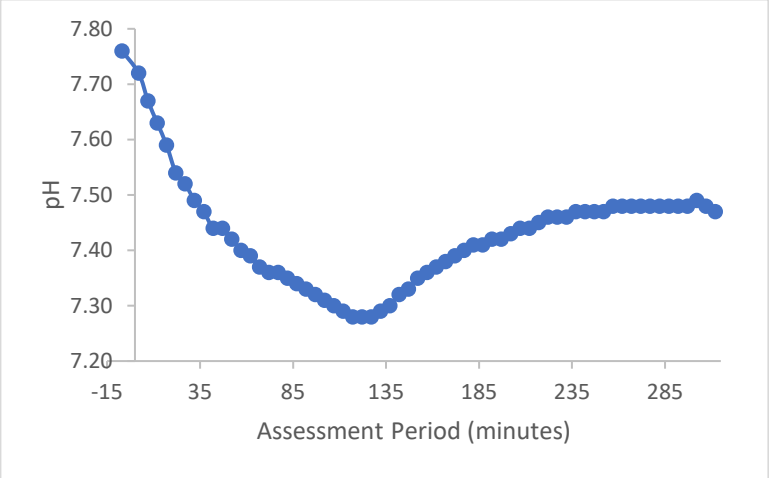

Figure A.2: NF – 2 pH

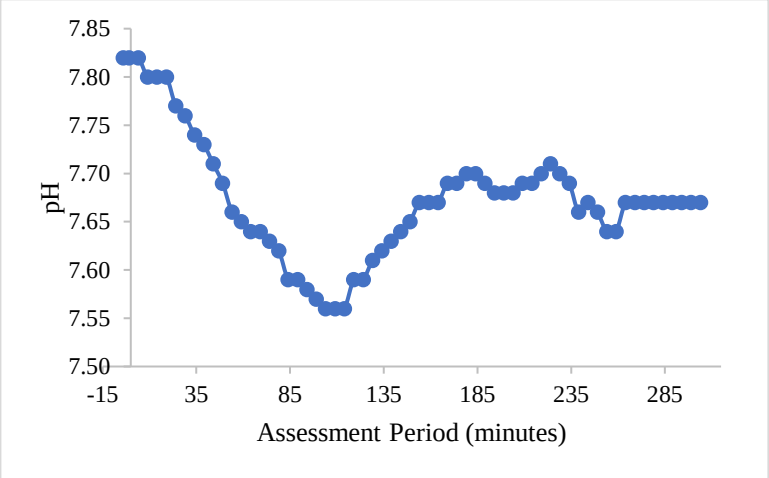

Figure A.3: NF – 3 pH

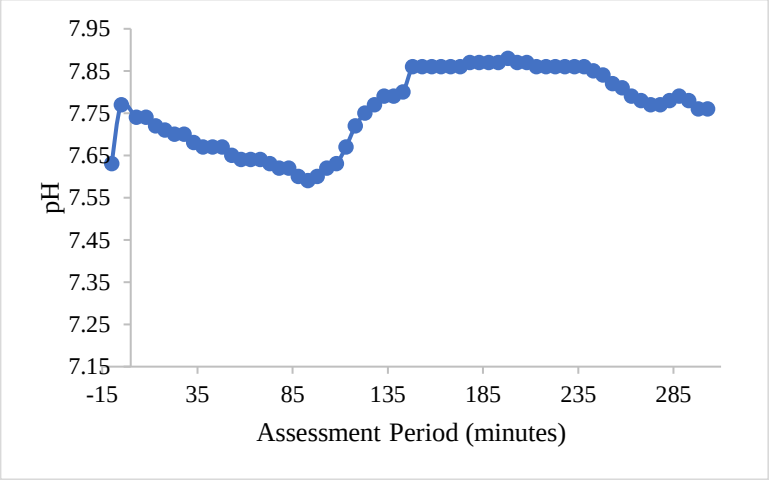

Figure A.4: NF – 4 pH

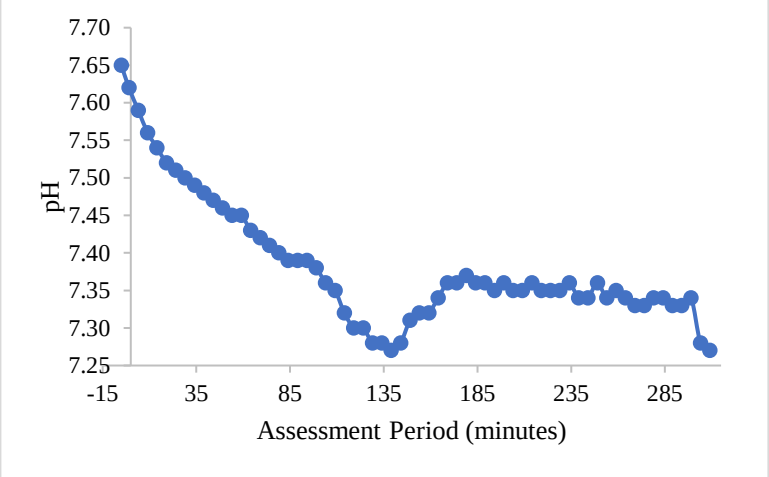

Figure A.5: NF – 5 pH

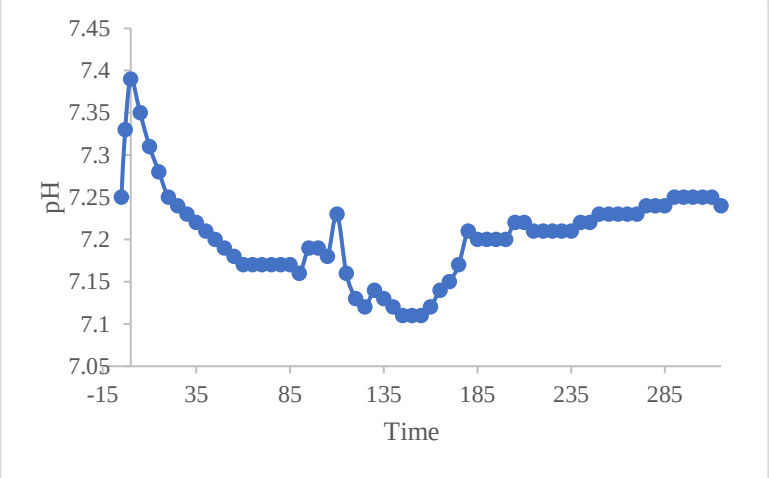

Figure A.6: NT – 1 pH

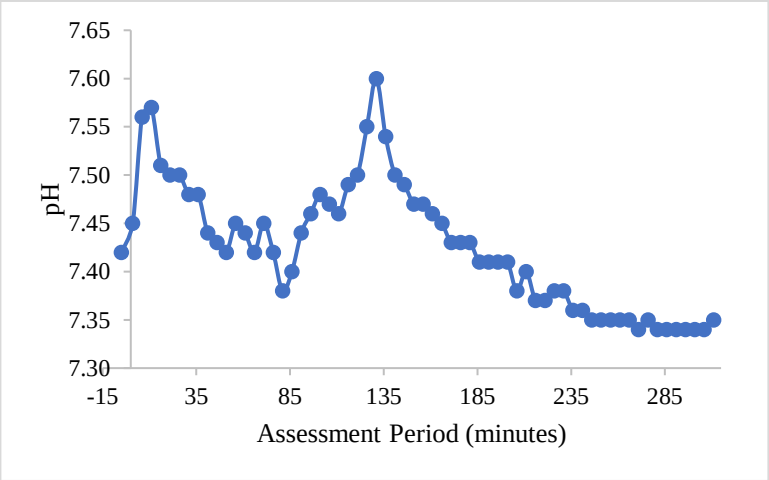

Figure A.7: NT – 2 pH

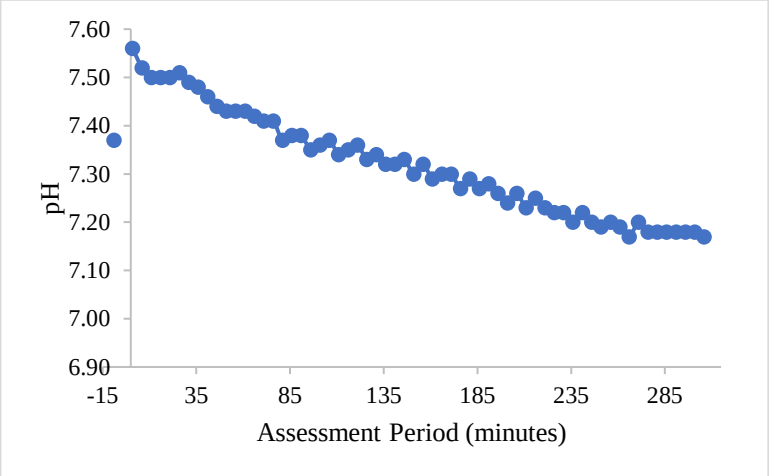

Figure A.8: NT – 3 pH

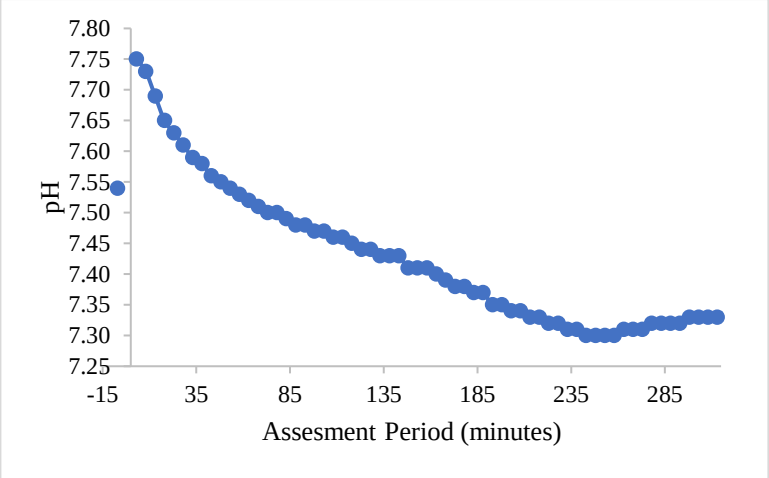

Figure A.9: NT – 4 pH